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**Mémoire présenté par**

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**-UMR 8576-**

pour l'obtention de l'

**HABILITATION À DIRIGER DES RECHERCHES**

le 11 octobre 2006

sur le thème

# « Fonctions associées à la *O*-GlcNAc »

**Président du jury:** Pr. René CACAN (USTL)
**Rapporteurs:** Pr. Gerald W. HART (Johns Hopkins University, Baltimore)
Dr. Dominique LEPRINCE (Institut de Biologie de Lille)
Pr. Jean GIRARD (Institut Cochin, Paris)
**Directeur de Recherche:** Dr. Jean-Claude MICHALSKI (USTL)

Fonctions associées à la *O*-GlcNAc.

A Vanessa, Nathan et Suzanne.

# REMERCIEMENTS

*Je ne pourrais clore la rédaction de cette Habilitation à Diriger des Recherches sans exprimer ma reconnaissance envers certaines personnes essentielles et sans qui ce travail n'existerait pas.*

*Mes premiers remerciements iront vers* ***Jean-Claude MICHALSKI****, notre chef de groupe et directeur d'unité. Merci Jean-Claude de m'avoir toujours accordé ta confiance. Je me souviens de ce jour de juin 1995 où tu m'as exposé les thématiques te tenant à cœur. Celles-ci portaient entre autres sur les maladies lysosomales et CDG, les problèmes de xénogreffes. Et puis tu m'as parlé d'un sujet que tu voulais entreprendre en collaboration avec l'unité 422 de l'INSERM (avec Marie-Laure CAILLET-BOUDIN, que j'associe à ces remerciements pour m'avoir initié au métier de chercheur) : « l'étude du dynamisme d'une glycosylation à part, la O-GlcNAc ». Impossible d'imaginer à l'époque que cette HDR porterait sur « ce petit bout de sucre » qui « pousse » sur toutes les protéines essentielles à la vie cellulaire. Grâce à toi, j'ai pu réalisé beaucoup de projets et m'adonner quotidiennement à l'une de mes passions, la recherche. Garde toujours en toi cette curiosité instinctive que tu as su transmettre à bon nombre de tes étudiants. Conserve aussi cette énergie, cette façon de penser qu'il y a toujours quelque chose d'intéressant (peut-être un « scoop ») de caché derrière chaque résultat. Je sais que si j'en suis là aujourd'hui, c'est en grande partie grâce à toi.*

***Professeur René CACAN.*** *René. Vous aviez déjà accepté d'assurer la présidence de mon jury de thèse, et c'est avec plaisir, et une grande fierté pour moi, que vous présiderez à nouveau ce jury d'HDR. Ces dix huit mois passés à vos côtés ont été une source d'inspiration constante et ont été l'occasion de discuter de choses très variés : de la science, mais aussi « de la vie » sous toutes ses formes. Comme vous avez été pour moi une référence dans le cadre de l'enseignement, lorsque j'usais mes fonds de culotte sur les bancs d'amphithéâtres, je voudrais vous dire tout le respect et l'admiration que vous m'inspirez. J'espère qu'un jour vous me direz où vous puisez toute cette concentration et cette capacité à synthétiser les choses. Je sais que vous nous manquerez beaucoup en fin d'année et je sais aussi à quel point le vide que vous laisserez, nous sera difficile à combler.*

***Professor Gerald W. HART.*** *Dear Jerry, it is an immense honour for myself, but also for our laboratory, that you accepted to join this jury. This HDR is for me an essential step in my young career and this must not be consider as an outcome, but rather as a beginning of a new research career. It is why it was essential for me that you were present. I am admiring of your work and I would like to thank you for your discovery in 1984 of this essential post-translational modification: O-GlcNAc. I hope I could work again for many years on different aspects of this glycosylation and I will continue to follow your breakthroughs on O-GlcNAc.*

***Docteur Dominique LEPRINCE.*** *Dominique. Merci d'avoir accepté aussi rapidement d'être rapporteur de cette HDR. Je sais que les sucres ce n'est pas vraiment votre tasse de thé, mais la O-GlcNAc, vous le savez, c'est différent : au point de vue de la structure, on ne pouvait rêver plus simple et en terme de régulation fonctionnelle, je crois (j'en suis même persuadé) qu'il n'y a pas mieux. J'ai beaucoup appris avec vous, non seulement sur le point de vue scientifique mais aussi sur la forme qu'il faut donner aux choses pour qu'elles prennent toute leur importance : la quantité de résultats et de preuves ne suffisent pas, il faut savoir lâcher des choses qui tiennent à cœur pour parvenir à l'essentiel ! Pour un optimiste et un excité comme moi, ce n'est pas toujours facile. J'ai également compris avec vous que toute manip devait être imaginée dans le but d'être publiée. J'ai fait mes premières marques en « bio mol » dans votre labo, mais bon, sur ce coup là c'est moi qui n'aime pas trop : je suis peut-être un peu trop biochimiste dans la tête. J'ai beaucoup apprécier votre sens du dialogue et la confiance que vous m'avez accordée. Par-dessus tout, votre honnêteté, votre souci du détail et votre perpétuelle remise en question des résultats m'ont beaucoup impressionné. Je souhaite continuer le plus possible encore travailler avec vous, car la recherche c'est un travail d'échanges, de contacts de regroupements interdisciplinaires, et je sais qu'avec vous ma quête perpétuelle de mieux faire et d'essayer de tout comprendre sera toujours assouvie. Merci aussi aux personnes de votre groupe avec qui j'ai vraiment apprécier de travailler.*



 

***Professeur Jean GIRARD.*** *Merci à vous également d'avoir accepté d'être rapporteur de cette HDR, c'est un honneur de vous compter parmi les membres de ce jury. Je sais qu'avec vous, et Catherine POSTIC, notre petite Céline sera entre de bonnes mains.*

***Céline GUINEZ.*** *Céline. Tu es la première personne que j'accompagne de bout en bout, du DEA (même si c'est vrai qu'à cette époque je n'ai pas pu être toujours là) à la thèse. Je te remercie pour tout ce que tu as fait : j'ai vraiment eu beaucoup de plaisir à t'encadrer sur ces quelques années, toi, qui je l'estime (je ne suis pas le seul) fait des miracles à la paillasse. Tu es quelqu'un de curieux et qui ne laisse jamais l'opportunité de faire un joli coup lui échapper. Ta gentillesse et ton attention de tous les jours ne seront qu'une arme de plus qui te fera adopter partout où tu iras. Tu as beaucoup de qualité et beaucoup de mérite pour en être arrivée à ce niveau. J'espère que ton expérience post-doctorale te permettra de parfaire ta formation de chercheur et que tu reviendras nous faire profiter de tes nouveaux savoirs.*

***Vanessa DEHENNAUT.*** *Evidemment, Vanessa, pour toi ces remerciements sont un peu particulier puisque tu partages, non seulement un « bout » de labo et de bureau avec moi, mais également ma vie. Merci de me supporter 24 h sur 24, je sais à quel point je peux être pompant et usant. Quand je t'ai vue arriver je me suis dit : « Oh là ! elle doit être prétentieuse ! Vu le CV, çà peut se comprendre ». En fait, tu es tout sauf imbue de toi-même et tu es quelqu'un au contraire très sensible. Tu m'as tout appris sur le cycle cellulaire et sur le développement. Tes ressources sont immenses, ton talent, indéfinissable. La carrière qui s'ouvre à toi est très grande et je sais que tu iras très, très loin. Je crois ne pas me tromper en disant que tu es la personne la plus intelligente que j'ai rencontré. Evidemment ces pages ne seraient pas assez longues pour décrire ce que je ressens pour tout ; je ne pourrais t'écrire un roman d'amour ici, l'endroit ne serait pas bien choisi et je ne suis pas vraiment doué pour çà. En tout cas, sache qu'il me serait impossible aujourd'hui de faire un seul pas sans toi.*

*Il serait incorrect de ma part de ne pas remercier le Pr.* ***Jean-Pierre VILAIN****, dont j'apprécie la bonne humeur et la fraîcheur permanentes. Merci de poursuivre avec nous cette collaboration passionnante qu'est la régulation du cycle cellulaire et merci à vous aussi de la confiance que vous m'accordée.*

*Enfin, merci aux personnes qui m'accompagnent tous les jours et plus particulièrement,* ***Anne-Marie MIR****, notre maman à tous au 020 (et au 017) et qui sait tout faire,* ***Sandrine DUVET****, pour les discussions, sa bonne humeur et son café (…),* ***Anne-Sophie VERCOUTTER-EDOUART*** *et* ***Marie-Christine SLOMIANNY****, pour leur soutien permanent, leur aide, en particulier en terme de protéomique et* ***Yves LEROY*** *pour qui j'éprouve également beaucoup de respect, et sans qui une partie de ces travaux n'auraient pu être réalisés.*

*Merci aussi à ceux qui ont bien voulu relire ce manuscrit, Daniel KMIECIK, Vanessa, Céline, Sandrine et René.*

# PREAMBULE : "LA BIOCHIMIE, LA O-GLCNAC ET MOI"

*Après avoir terminé mon deuxième cycle de cursus universitaire, passionné par la biochimie, très attiré par la recherche et par ce que devait procurer comme émotions la vie en laboratoire, je me suis tout naturellement orienté vers le DEA (juin 1995). J'ai pris pour cela divers contacts avec des responsables de DEA différents et plusieurs laboratoires basés sur Paris (Paris V), Fontenay aux Roses (CEA), Lille (Faculté de pharmacie, Lille II) et Villeneuve d'Ascq (laboratoire de chimie et laboratoire de chimie-biologique, Lille I). Après mîntes réflexions, c'est à Villeneuve d'Ascq que j'ai choisi de rester pour continuer ma formation étudiante.*

*J'ai alors rencontré Jean-Claude Michalski qui m'a présenté trois sujets, dont un sur les xénogreffes et l'autre sur la O-GlcNAc. C'est le premier sujet qui, très honnêtement m'inspirait le plus au départ, mais celui-ci avait déjà été attribué à un autre étudiant. Je me décidais donc à travailler sur la O-GlcNAc. Ce sujet m'effrayait un peu au départ, dans le sens ou la seule fois où j'avais entendu parler de cette glycosylation se limitait à trois lignes dans un cours de biochimie (« les glycoconjugués ») du Professeur René Cacan. J'avais donc l'impression de partir à l'aventure vers une thématique inconnue, ce qui ne manquait pas de me stimuler en même temps.*

*Voici en quelques comment la O-GlcNAc est parvenue entre les quatre murs du C9.*

*En 1995, Jean-Claude MICHALSKI avait été contacté par Marie-Laure CAILLET-BOUDIN (INSERM, U422, Lille) avec qui il avait d'ailleurs publié la modification de la fibre adénovirale par la O-GlcNAc quelques années auparavant (Caillet-Boudin et al., 1989). Marie-Laure CAILLET-BOUDIN étudie les protéines Tau, protéines organisant les microtubules neuronaux et connues pour leur implication dans la dégénérescence neuronale : celles-ci engendrent la désorganisation des microtubules et la formation de « paired helical filaments », PHF, responsables en partie de la mort cellulaire. Tau avait toutes les caractéristiques pour être O-GlcNAc : la localisation cytosolique, le fait d'être une protéine de l'architecture cellulaire et elle est phosphorylée (une des caractéristiques de la maladie Alzheimer est d'ailleurs, l'hyperphosphorylation de celle-ci). De plus, cette glycosylation avait été mise en évidence sur la protéine Tau bovine (Arnold et al., 1996), ce qui supposait que les formes humaines l'étaient également. Donc, pourquoi Tau ne pouvait-elle pas être le siège d'une ou plusieurs réciprocités O-GlcNAc/phosphorylation ? Pour mettre en évidence la glycosylation éventuelle de cette protéine, nous avons testé la WGA (couramment utilisée, à l'époque, pour la mise en évidence de la O-GlcNAc) sur des extraits de cellules exprimant Tau. Il s'est alors avéré que Tau était, d'une part, glycosylée et que, d'autre part, cette glycosylation semblait interférer avec la phosphorylation de celle-ci. De cette observation, je m'attelais donc au problème de cette fameuse « balance phospho/O-GlcNAc ».*

Fonctions associées à la *O*-GlcNAc.

## SOMMAIRE

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## RESUME

Plus de 50 % des protéines sont glycosylées. La *O*-GlcNAc constitue la forme majeure de glycosylation des protéines cytosoliques et nucléaires et représente une des modifications post-traductionnelles les plus abondantes du cytosol et du noyau. Cette glycosylation est réversible et dynamique et modifie de nombreuses protéines structurales et fonctionnelles. Le dynamisme de la *O*-GlcNAc est régulée par deux enzymes uniques, la *O*-GlcNAc transférase et la *O*-GlcNAcase ; les processus de *O*-N-acétylglucosaminylation sont étroitement liés au métabolisme du glucose via la voie de biosynthèse des hexosamines. Nos travaux de recherche ont porté sur l'étude des fonctions régulées par la *O*-GlcNAc : en effet, à ce jour, le(s) rôle(s) précis joué(s) par la *O*-GlcNAc demeure(nt) mal compris. Nos travaux ont permis, en parallèle avec d'autres auteurs, de comprendre l'aspect dynamique de la *O*-GlcNAc et l'existence de son antagonisme avec la phosphorylation. Plusieurs observations tendant à montrer que la *O*-GlcNAc peut servir de signal de transport nucléaire aux protéines cytosoliques, nous ont incité à rechercher des protéines douées d'une activité de liaison à la *O*-GlcNAc et jouant le rôle de transporteur de ces protéines glycosylées vers le noyau. Certains membres des protéines de choc thermique de 70 kDa ont été identifiés. Un rôle possible dans la protection des protéines *O*-GlcNAc, vis-à-vis de la dégradation protéasomale, par ces HSP70 nous a alors intrigué. Nous avons compris que les protéines de stress de 70 kDa (et certainement d'autres) dévoilaient une propriété d'activité lectinique envers la *O*-GlcNAc essentiellement dans les situations de stress. Ces travaux seront poursuivis et approfondis. De même nous avons démontré que la *O*-GlcNAc était également une modification post-traductionnelle régulatrice du cycle cellulaire. L'identification des composants régulateurs du cycle ainsi que des protéines structurales nécessaires à la mise en place du fuseau de division, du cytosquelette ainsi qu'au remodelage de la chromatine, modifiés par la *O*-GlcNAc, nous permettrons de comprendre l'impact de la *O*-GlcNAc dans ce type de processus essentiel à la survie cellulaire. Enfin, l'utilisation du facteur de transcription suppresseur de tumeurs, HIC1, nous permettra d'explorer d'autres fonctions assurées par la *O*-GlcNAc telle que la régulation des mécanismes transcriptionnels.

## SUMMARY

More than 50% of proteins are glycosylated. *O*-GlcNAc is the major glycosylation type, and a widespread post-translational modification, found within the cytosolic and nuclear compartments. *O*-GlcNAc is reversible and dynamic and it modifies numerous structural and functional proteins. *O*-GlcNAc versatility is regulated by two enzymes: the *O*-GlcNAc transferase and the *O*-GlcNAcase ; *O*-linked N-acetylglucosaminylation processes are closely linked to glucose metabolism though the hexosamine biosynthetic pathway. Our work has been focus on the functions regulated by *O*-GlcNAc : indeed, while intensively studied, the exact function(s) played by *O*-GlcNAc remain to be determined. Our studies, in parallel with other groups, allowed understanding of the dynamic aspect of *O*-GlcNAc and of the existence of an interplay between *O*-GlcNAc and phosphorylation. Several observations tending to show that *O*-GlcNAc could serve as a nuclear transport signal for cytosolic proteins incite us to look for proteins endowed of an *O*-GlcNAc-binding property and that may play a transport role for these proteins to the nucleus. Members of the HSP70 have been identified. A putative role of this lectin property in the protection against proteasomal degradation intrigued us. We have understood that this lectin activity was essentially exhibited after cell stress. These works will be deepened. We have also demonstrated that *O*-GlcNAc was a post-translational modification that regulates cell cycle. Identification of *O*-GlcNAc-modified cell cycle regulatory components and structural proteins that permit mitotic spindle to take place and that allow cytoskeleton and chromatin restructuration will help us to understand the impact of *O*-GlcNAc in these processes that are essential for cell survival. Lastly, use of the tumour suppressor HIC1, will permit to explore other functions ensured by *O*-GlcNAc, such as the regulation of transcriptional mechanisms.

Fonctions associées à la *O*-GlcNAc.

## PUBLICATIONS SCIENTIFIQUES

### ➢ ARTICLES SCIENTIFIQUES

- **Tony LEFEBVRE**, Catherine ALONSO, Saïd MAHBOUB, Marie-Joëlle DUPIRE, Jean-Pierre ZANETTA, Marie-Laure CAILLET-BOUDIN and Jean-Claude MICHALSKI *Effect of okadaic acid on O-Linked N-acetylGlucosamine levels in a neuroblastoma cell line.* **Biochim.Biophys. Acta** (1999) 1472, 71-81.

- Yann GUERARDEL, Ossarath KOL, Emmanuel MAES, **Tony LEFEBVRE**, Bénoni BOILLY, Monique DAVRIL and Gérard STRECKER *O-Glycan variability of egg-jelly mucins from Xenopus laevis Characterization of four phenotypes that differ by the terminal glycosylation of their mucins.* **Biochem. J.** (2000) 352, 449-463

- **Tony LEFEBVRE**, Caroline CIENIEWSKI, Jérôme LEMOINE, Yann GUERARDEL, Yves LEROY, Jean-Pierre ZANETTA and Jean-Claude MICHALSKI *Identification of N-acetyl-d-glucosamine-specific lectins from rat liver cytosolic and nuclear compartments as Heat-shock proteins.* **Biochem. J.** (2001) 360, 179-188

- Christelle CEBO, Viviane DURIER, Philippe LAGANT, Emmanuel MAES, Doina FLOREA, **Tony LEFEBVRE**, Gérard STRECKER, Gérard VERGOTEN and Jean-Pierre ZANETTA *Function and molecular modeling of the interaction between human interleukin 6 and its HNK-1 oligosaccharide ligands.* **J. Biol. Chem.** (2002) 277, 12246-52.

- **Tony LEFEBVRE**, Nathalie PLANQUE, Denis LELEU, Marie-Laure CAILLET-BOUDIN, Simon SAULE and Jean-Claude MICHALSKI *O-Glycosylation of the nuclear forms of Pax-6 products in Quail Neuroretina Cells* **J. Cell. Biochem**. (2002) 85, 208-218

- **Tony LEFEBVRE**, Laetitia DUPONT-WALLOIS, Stéphanie FERREIRA, Marie-Joëlle DUPIRE, André DELACOURTE, Jean-Claude MICHALSKI and Marie-Laure CAILLET-BOUDIN *Evidence of a balance between O-GlcNAc glycosylation and phosphorylation on Tau proteins- role in the nuclear localization.* **Biochim. Biophys. Acta**. (2003) 1619, 167-176.

- Caroline CIENIEWSKI-BERNARD, Bruno BASTIDE, **Tony LEFEBVRE,** Jérôme LEMOINE, Yvonne MOUNIER and Jean-Claude MICHALSKI *Identification of O-Linked N-Acetylglucosaminylted proteins in skeletal muscle by two-dimensional electrophoresis.* **Mol. Cell. Proteomics** (2004) 3, 577-585

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- Céline GUINEZ, Jérôme LEMOINE, Jean-Claude MICHALSKI and **Tony LEFEBVRE** *70-kDa Heat Shock Proteins presents an adjustable lectinic activity agaisnt O-GlcNAc residues.* **Biochem. Biophys Res. Com.** (2004) 319, 21-26

- **Tony LEFEBVRE**, Frédéric BAERT, Jean-François BODART, Stéphane FLAMENT, Jean-Claude MICHALSKI and Jean-Pierre VILAIN *Modulation of O-GlcNAc glycosylation during Xenopus oocyte maturation.* **J. Cell. Biochem.** (2004) 93, 999-1010

- **Tony LEFEBVRE**, Sébastien PINTE, Cateline GUERARDEL, Sophie DELTOUR, Nathalie MARTIN-SOUDANT, Marie-Christine SLOMIANNY, Jean-Claude MICHALSKI and Dominique LEPRINCE *The tumor suppressor HIC1 (Hyperrmethylated in cancer 1) is O-GlcNAc glycosylated.* **Eur. J. Biochem.** (2004) 271, 3843-3854

- Céline GUINEZ, Marie-Estelle LOSFELD, René CACAN, Jean-Claude MICHALSKI and **Tony LEFEBVRE** *Modulation of HSP70 GlcNAc-directed lectin activity by glucose availability and utilization.* **Glycobiology** (2006) 16, 22-28

➢ **REVUES SCIENTIFIQUES**

- **Tony LEFEBVRE**, Caroline CIENIEWSKI et Jean-claude MICHALSKI *Dynamique de la O-N-acétylglucosaminylation cytosolique et nucléaire des protéines. Une glycosylation pas comme les autres.* **Regard sur la Biochimie** (2002) 1, 34-46

- **Tony LEFEBVRE**, Marie-Laure CAILLET-BOUDIN, Luc BUEE, André DELACOURTE and Jean-Claude MICHALSKI *O-GlcNAc glycosylation and neurological disorders.* **Adv. Exp. Med. Biol.** (2003) 535, 189-202

- Céline GUINEZ, Willy MORELLE, Jean-Claude MICHALSKI and **Tony LEFEBVRE** *O-GlcNAc glycosylation : a signal for the nuclear transport of cytosolic proteins ?* **Int. J. Biochem. Cell Biol.** (2005) 765-774

- **Tony LEFEBVRE**, Céline GUINEZ, Vanessa DEHENNAUT, Olivia BESEME-DEKEYSER, Willy MORELLE and Jean-Claude MICHALSKI *Does O-GlcNAc play a role in neurodegenerative diseases ?* **Exp. Rev. Proteomics** (2005) 2, 265-275

- **Tony LEFEBVRE, Vanessa DEHENNAUT, Céline GUINEZ and Jean-Claude MICHALSKI** *Involvement of O-linked N-acetylglucosamine in type-2 diabetes, neurological disorders and cancer.* A paraître dans **Research signpost**, "New developments in therapeutic glycomics".

Fonctions associées à la *O*-GlcNAc.

**COMMUNICATIONS ORALES**

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- XXV$^{éme}$ forum des jeunes chercheurs de la Société Française de Biochimie et de Biologie Moléculaire du 22 au 25 juin 1998 à Québec

Tony LEFEBVRE, Saïd MAHBOUB, Catherine ALONSO, Jean-Pierre ZANETTA, André DELACOURTE, Marie-Laure CAILLET-BOUDIN, et Jean-Claude MICHALSKI *Implication de la O-N-acétylglucosaminylation des résidus de sérine et de thréonine dans la translocation des protéines du cytosol vers le noyau*

- 10$^{th}$ Joint Meeting à Nuland (Hollande) les 10 et 11 novembre 1998

Tony LEFEBVRE, Catherine ALONSO, Saïd MAHBOUB, Jean-Pierre ZANETTA, Marie-Laure CAILLET-BOUDIN and Jean-Claude MICHALSKI *Effect of okadaic acid on O-GlcNAc level in a neuroblastoma cell line*

- 1$^{er}$ Glycojeune le 31 mars 1999 à Villeneuve d'Ascq (co-organisateur de ce congrès)

Tony LEFEBVRE, Jean-Pierre ZANETTA et Jean-Claude MICHALSKI *Lectines à N-acétylglucosamine du noyau et du cytosol de foie de rat*

- 11$^{th}$ Joint Meeting à Bonn-Röttgen (Allemagne) les 20 et 21 novembre 1999

Tony LEFEBVRE, Jean-Pierre ZANETTA and Jean-Claude MICHALSKI *GlcNAc specific nuclear and cytosolic lectins - Role in the nuclear transport of cytosolic proteins ?*

- 2$^{ème}$ Glycojeune les 30 et 31 mars 2000 à Villeneuve d'Ascq

Tony LEFEBVRE, Marie-Laure CAILLET-BOUDIN, Jean-Pierre ZANETTA et Jean-Claude MICHALSKI *La O-N-Acetylglucosamine des protéines : un signal de transport des protéines cytosoliques vers le noyau ?*

- Séminaire en anglais sur invitation à l'hôpital universitaire de Gand (Belgique) le 21 septembre 2001 *O-GlcNAc : a new glycosylation type* by Tony LEFEBVRE

- 15$^{th}$ Joint Meeting à Wageningen (Pays-Bas) les 28, 29 et 30 novembre 2004

Céline GUINEZ, Marie-Estelle LOESFELD, René CACAN, Jean-Claude MICHALSKI and Tony LEFEBVRE *The chaperones Hsc70 and Hsp70 specifically bind O-linked N-acetylglucosamine residues. Modulation of their lectinic activities.*

- Séminaire interne à l'UGSF/UMR 8576 le 20 mai 2005 et séminaire à l'Institut de Pharmacologie et de Biologie Structurale, Toulouse le 3 novembre 2005

Fonctions associées à la *O*-GlcNAc.

Céline GUINEZ, Anne-Marie MIR, René CACAN, Jean-Claude MICHALSKI et Tony LEFEBVRE *Activité lectinique et glycosylation des protéines de choc thermique de 70 kDa. Un rôle dans la dégradation protéasomale ?*

- 16th Joint Meeting à Hannover (Allemagne) les 27, 28 et 29 octobre 2005

Céline GUINEZ, Anne-Marie MIR, René CACAN, Jean-Claude MICHALSKI and Tony LEFEBVRE *O-GlcNAc glycosylation of proteins : a protective signal agaisnt proteasomal degradation ? Role of Hsp70 lectin activity.*

**COMMUNICATIONS PAR AFFICHE**

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- 9th Joint Meeting à Lille les 24 et 25 novembre 1997

Tony LEFEBVRE, Catherine ALONSO, Jean-Pierre ZANETTA, Marie-Laure CAILLET-BOUDIN and Jean-Claude MICHALSKI *Evidence that O-N-Acetylglucosaminylation of cytosolic proteins contributes to their further nuclear translocation*

- 12th Joint Meeting à Lille les 6 et 7 novembre 2000

Tony LEFEBVRE and Jean-Claude MICHALSKI/Stéphane FLAMENT, Frédéric BAERT, Juliette DELEHAYE and Jean-Pierre VILAIN *O-GlcNAc modulation during Xenopus oocyte maturation*

- 2ème Réunion du réseau Lillois de Cancérologie à Villeneuve d'Ascq, le 31 mai 2001

Catherine ROBBE, Calliope CAPPON, Tony LEFEBVRE, Christophe FLAHAUT, Jérôme LEMOINE et Jean-Claude MICHALSKI *Etude de la glycosylation dans les mucines normales et cancéreuses : mise au point de nouveaux procédés de cartographie rapide des mucines*

- Glyco XVI à La Haye (Pays-Bas), du 19 au 24 août 2001

Tony LEFEBVRE, Caroline CIENIEWSKI, Jérôme LEMOINE, Yann GUERARDEL, Yves LEROY, Stéphanie FERREIRA, Laetitia DUPONT-WALLOIS, Nathalie PLANQUE, Marie-Joëlle DUPIRE, Manuella BAILLY, André DELACOURTE, Jean-Pierre ZANETTA, Simon SAULE, Marie-Laure CAILLET-BOUDIN and Jean-Claude MICHALSKI *What role for O-GlcNAc ?*

- 3ème Réunion Lilloise de Cancérologie (site CHU de Lille), 29 mai 2002

Fonctions associées à la *O*-GlcNAc.

Sophie DELTOUR, Nicolas STANKOVIC-VALENTIN, Sébastien PINTE, Tony LEFEBVRE, Cateline GUERARDEL et Dominique LEPRINCE. *HIC1 et ses mécanismes de répression transcriptionnelle.*

• 3ème Réunion Lilloise de Cancérologie (site CHU de Lille), 29 mai 2002

Sébastien PINTE, Sophie DELTOUR, Nicolas STANKOVIC-VALENTIN, Tony LEFEBVRE, Cateline GUERARDEL et Dominique LEPRINCE. *Caractérisation de la séquence de liaison à l'ADN du répresseur transcriptionnel HIC1.*

• Journée Lilloise de Cancérologie 2004 (USTL, Villeneuve d'Ascq), 26 mai 2004

Jean-François BODART, Tony LEFEBVRE, Frédéric BAERT, Stéphane FLAMENT, Jean-Claude MICHALSKI and Jean-Pierre VILAIN. *Potential role for O-GlcNAc in cell cycle control during amphibian oocytes meiosis.*

• 4th International Symposium on Glycosyltransferases "GlycoT. 2004" (Le Touquet) du 4-7 novembre 2004

Tony LEFEBVRE, Sébastien PINTE, Cateline GUERARDEL, Sophie DELTOUR, Nathalie MARTIN-SOUDANT, Marie-Christine SLOMIANNY, Jean-Claude MICHALSKI and Dominique LEPRINCE. *The tumor suppressor HIC1 (Hypermethylated In Cancer 1) is O-GlcNAc glycosylated – Role of the glycosylation in DNA-binding.*

• 16th Joint Meeting à Hannover (Allemagne), du 27 au 29 octobre 2005

Vanessa DEHENNAUT, Tony LEFEBVRE, Jean-François BODART, Jean-Claude MICHALSKI and Jean-Pierre VILAIN *O-GlcNAc is a post-translational modification that governs Xenopus oocytes G2/M transition.*

• XVIII International Symposium on Glycoconjugates. Florence (Italie) 4-9 septembre 2005 ; 191st meeting of the Belgian Society of Biochemistry and molecular biology, le 2 décembre 2005 ; 5ème journée André Verbert, Lille (France), 28 septembre 2005

Céline GUINEZ, Marie-Estelle LOESFELD, René CACAN, Jean-Claude MICHALSKI and Tony LEFEBVRE *Hsp70 and Hsc70 GlcNAc-directed lectinic activityare modulated by exrtracellular glucose rate.*

• 23èmes Journées Françaises de Spectrométrie de Masse et 4èmes Journées de la Société Française des Isotopes Stables (Nantes), 11-14 septembre 2006

Agnès HOVASSE, Marie-Pierre BOUSQUET-DUBOUCH, Tony LEFEBVRE, Bernard MONSARRAT et Odile BURLET-SCHILTZ. *Etude par spectrométrie de masse de*

Fonctions associées à la *O*-GlcNAc.

*l'effet de la glycosylation de séquences protéiques sur leur dégradation in vitro par le protéasome 20S.*

## COLLABORATIONS ACTUELLES

• **Pr. Jean-Pierre VILAIN et Dr. Jean-François BODART**, Laboratoire de Biologie du Développement, EA 1033, Université des Sciences et Technologies de Lille, bâtiment SN3, Villeneuve d'Ascq

⇨ Régulation du cycle cellulaire par la *O*-GlcNAc.

• **Dr. Catherine POSTIC et Pr. Jean GIRARD**, Institut Cochin, INSERM U567, UMR 8104 du CNRS, Département d'Endocrinologie, Métabolisme et Cancer, Paris

⇨ Etude de la *O*-GlcNAc du facteur de transcription ChREBP.

• **Dr. Brigitte KAHN-PERLES**, Centre de Recherche en Cancérologie de Marseille, UMR 599, INSERM, 27 boulevard Leï Roure, 13009 Marseille

⇨ Etude de la *O*-GlcNAc de Sp1

• **Dr. Dominique LEPRINCE**, UMR 8526 du CNRS, Institut de Biologie de Lille, Institut Pasteur de Lille, Lille

⇨ Modifications post-traductionnelles de HIC1 et sur les interactions HIC1-partenaires.

• **Dr. Bernard MONSARRAT, Dr. Odile BURLET-SCHILTZ et Dr. Marie-Pierre BOUSQUET-DUBOUCH** Institut de Pharmacologie et de Biologie Structurale, CNRS, Toulouse

⇨ Dégradation protéasomale *in vitro* de peptides glycosylés et sur la glycosylation du protéasome.

• **Dr. Karin SERON et Dr. David MEYRE**, UMR 8090 du CNRS, Institut de Biologie de Lille, Institut Pasteur de Lille, Lille

⇨ Recherche de mutations dans les gènes codant l'OGT et la *O*-GlcNAcase de patients diabétiques et de patients obèses.

• **Pr. Gérard VERGOTEN**, UMR 8576 du CNRS, Laboratoire de chimie biologique, Bâtiment C9, Université des Sciences et Technologies de Lille, Villeneuve d'Ascq

Fonctions associées à la *O*-GlcNAc.

⇨ Collaboration interne : modélisation moléculaire et expérineces de « docking » sur HSP70.

• **Dr. Anne-Sophie VERCOUTTER-EDOUART et Mme Marie-Christine SLOMIANNY,** UMR 8576 du CNRS, Laboratoire de chimie biologique, Bâtiment C9, Université des Sciences et Technologies de Lille, Villeneuve d'Ascq

⇨ Collaboration interne sur l'aspect protéomique de nos diverses thématiques.

Fonctions associées à la *O*-GlcNAc.

**Tableau I- Abréviations conventionnelles et non conventionnelles**

| Abréviation | Signification |
|---|---|
| **AC** | adénylate cyclase |
| **ADN** | Acide désoxyribonucléique |
| **Akt** | RAC (related to the A and C kinases)-alpha serine/threonine-protein kinase or protein kinase B |
| **Allo** | alloxane |
| **Aza** | azasérine |
| **AMPc** | adénosine 3', 5'-monophosphate cyclique |
| **APP** | beta-amyloid precursor protein |
| **ARN** | acide ribonucléique |
| **ARNi** | ARN interférentiel |
| **ATP** | adénosine 5'-tri-phosphate |
| **Aza** | azasérine |
| **BAG1** | Bcl-2-associated athanogene 1 |
| **Bcl2** | B-cell lymphoma 2 |
| **BTB-POZ** | broad complex-tramtrack-bric a brac/pox viruses and zinc fingers |
| **CHIP** | Carboxyl-terminus of Hsc70-Interacting Protein |
| **CHO** | chinese hamster ovary |
| **CK-II** | caséine kinase-II |
| **CtBP** | C-terminal binding protein |
| **DAPI** | di aminido phenyl indol |
| **DBD** | DNA-binding domain |
| **DON** | 6-Diazo-5-oxo-L-norleucine |
| **DTT** | di-thiothréitol |
| **EMSA** | electrophoresis mobility shift assay |
| **eNOS** | endothelial nitric oxide synthase |
| **ERK** | extracellular-regulated kinase |
| **FKHR** | Forkhead homolog 1 (rhabdomyosarcoma) |
| **Fru-6P (F6P)** | fructose-6-phosphate |
| **Fru-1, 6bisP (F1, 6bisP)** | fructose-1, 6-bisphosphate |
| **Glc (G)** | glucose |
| **GlcNAc (GNAc)** | N-acétylglucosamine |

Fonctions associées à la *O*-GlcNAc.

| | |
|---|---|
| **Glc-1P (G1P)** | glucose-1-phosphate |
| **Glc-6P (G6P)** | glucose-6-phosphate |
| **$GlcNH_2$ (GN)** | glucosamine |
| **$GlcNH_2$-6P (GN6P)** | glucosamine-6-phosphate |
| **GlcNAc-6P (GNAc6P)** | N-acétylglucosamine-6-phosphate |
| **GlcNAc-1P (GNAc1P)** | N-acétylglucosamine-1-phosphate |
| **GPI** | glycosyl-phosphatidylinositol |
| **Gln** | glutamine |
| **GLUT** | glucose transporter |
| **Glu** | Glutamate (acide glutamique) |
| **Gln** | glutamine |
| **GFAT** | glutamine : fructose-6-phosphate amido-transférase |
| **$GlcNH_2$-6P-Ac Transférase (GN6PAcT)** | glucosamine-6-phosphate acétyltransférase |
| **GS** | glycogène synthase |
| **GSK-3β** | glycogène synthase kinase-3β |
| **HIC1** | hypermethylated in cancer 1 |
| **Hip** | Hsp70/Hsc70-interacting protein |
| **Hop** | Hsp70/Hsp90 organizing protein |
| **HK** | hexokinase |
| **HPLC** | high performance liquid chromatography |
| **Hsc70** | 70 kDa-Heat shock cognate |
| **Hsp40** | 40 kDa-Heat shock protein |
| **Hsp70** | 70 kDa-Heat shock protein |
| **HSP70** | 70 kDa-Heat shock proteins family |
| **IP** | immunoprécipitation |
| **IRS-1/2** | insulin receptor subtrates-1 and 2 |
| **MALDI-TOF** | matrix assisted laser desorption ionization-time of flight |
| **MAP** | microtubules-associated proteins |
| **MAPK** | mitogen-activated protein kinase |
| **MDM2** | murine double minute 2 |
| **MEK** | mitogen-activated protein kinase kinase |
| **MG132** | N-carbobenzoxyl-Leu-Leu-leucinal |
| **Mos** | mitogen-activated protein kinase kinase kinase |
| **MPF** | M-phase promoting factor |

Fonctions associées à la *O*-GlcNAc.

| | |
|---|---|
| **mTOR** | mammalian target of rapamycin |
| **NFκB** | nuclear factor kappa B |
| **NLS** | nuclear localization signal |
| ***O*-GlcNAc** | *O*-N-acétylglucosaminylation (*O*-linked N-acetylglucosaminylation) |
| ***O*-GlcNAcase** | *O*-N-acétylglucosaminidase |
| **OGT** | *O*-GlcNAc transférase |
| **PDX-1** | pancreatic/duodenal homeobox-1 protein |
| **PHF** | paired helical filament |
| **PIP2** | phosphatidylinositol-4, 5 bisphosphate |
| **PIP3** | phosphatidylinositol-3, 4, 5 triphosphate |
| **PGM** | phospho-glucomutase |
| **PGNAcM** | phospho-N-acétylglucosamine mutase |
| **PUGNAc** | *O*-(2-acétamido-2-déoxy-d-glucopyranosylidène)amino-N-phénylcarbamate |
| **Pyr** | pyruvate |
| **PKA** | protéine kinase A |
| **PKB** | protéine kinase B (Akt) |
| **PKC** | protéine kinase C |
| **PGI** | phosphoglucose isomérase |
| **PI3K** | PI-3(phosphatidylinositol-3) kinase (p85/p110) |
| **PDK-1** | phosphatidylinositol-dependent protein kinase-1 |
| **PGNAcM**, | phospho-N-acétylglucosamine mutase |
| **PTEN** | phosphatases and tensin homolog |
| **RSK** | ribosomal S6 kinase |
| **SDS-PAGE** | sodium dodecylsulphate-polyacrylamide gel electrophoresis |
| **STZ** | streptozotocine |
| **SUMO** | small ubiquitin-related modifier |
| **TAF** | TATA-binding-protein associated factor |
| **Tau** | tubulin-associated unit |
| **UCH-L1** | Ubiquitine carboxyl hydrolase-L1 |
| **UDP** | uridine diphosphate |
| **UDP-Glc (UG)** | uridine diphospho-glucose |
| **UDP-GlcNAc (UGNAc)** | uridine diphospho-N-acétylglucosamine |
| **UDP-GlcNAcPP (UGNAcPP)** | uridine di-phospho-N-acétylglucosamine pyrophosphorylase |

Fonctions associées à la *O*-GlcNAc.

| | |
|---|---|
| **UDP-GlcPP (UGPP)** | uridine diphospho-glucose pyrophosphorylase |
| **UPS** | ubiquitin-proteasome system |
| **Ura** | uracile |
| **WB** | western blot |
| **WGA** | Wheat germ agglutinin |

# Introduction :

# La *O*-N-acétylglucosaminylation ou *O*-GlcNAc

Fonctions associées à la *O*-GlcNAc.

### *1- Les modifications post-traductionnelles confèrent aux protéines une certaine variabilité fonctionnelle.*

Le séquençage du génome humain a révélé que celui-ci contenait moins de 30000 gènes contre les 100000 attendus (Venter et al., 2001). Comment alors un organisme aussi complexe que le nôtre peut-il n'être régit que par si peu d'intervenants, si l'on considère qu'un gène codera pour une seule séquence primaire ? La réponse à cette question réside dans le fait qu'une protéine pourra acquérir plusieurs fonctions biologiques que sa séquence primaire seule ne peut expliquer. Cette variabilité fonctionnelle est en grande partie dû au fait que de nombreuses modifications post-traductionnelles (en plus des mécanismes d'épissage alternatif) qui indépendamment, ou en combinaisons variées, donneront à la protéine l'une ou l'autre de ces fonctions ou sa pleine activité fonctionnelle. Cette régulation d'activité induite par les modifications post-traductionnelles (Fig. 1) est généralement le reflet d'une modification de la structure tridimensionnelle de la protéine pouvant adressée celle-ci dans un compartiment cellulaire différent, d'une part, et surtout lui permettre d'interagir avec de multiples partenaires (on peut supposer qu'à partir de ces 30000 gènes, l'organisme est capable de générer plusieurs centaines de milliers, voire plusieurs millions d'activités protéiques différentes). Prenons l'exemple de HSF1 (Heat Shock Factor-1), facteur de transcription induisant la synthèse d'Hsp70 qui, dans des conditions non stressantes, est complexé avec Hsp70 et qui se dissocie de celle-ci lors d'un stress (Kiang & Tsokos, 1998). Cette dissociation du complexe permettra à HSF1 d'être phosphorylé, de se trimériser et d'être transporté dans le noyau où il jouera son rôle de facteur de transcription en s'associant aux « Heat shock Response Element » (HRE).

On peut dénombrer actuellement plus de 70 modifications post-traductionnelles différentes parmi lesquelles 20 sont les plus communément rencontrées, telles que la formation de ponts disulfures, l'ubiquitination, l'ADP-ribosylation, l'acétylation, la méthylation, la glycosylation et, bien entendu, la phosphorylation qui reste, aux yeux des scientifiques, la modification post-traductionnelle par excellence (Seo & Lee, 2004).

La modification post-traductionnelle à laquelle nous nous intéressons plus particulièrement est la *O*-N-acétylglucosaminylation, désignée plus simplement sous le terme de ***O*-GlcNAc**. Dans notre équipe la *O*-GlcNAc est étudiée sous différents aspects que nous développerons dans les parties II et III de ce mémoire.

Fonctions associées à la *O*-GlcNAc.

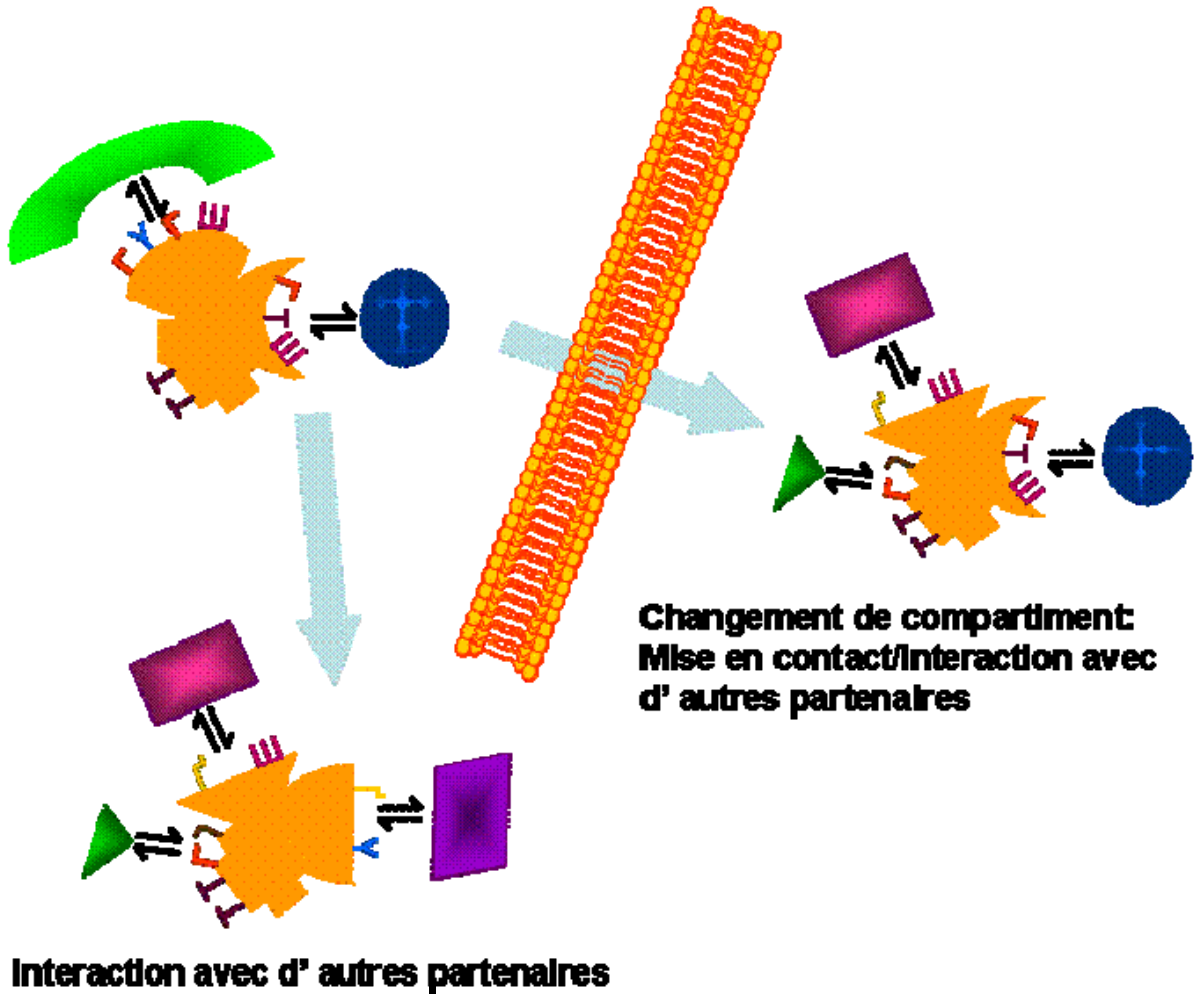



**Figure 1- Les modifications post-traductionnelles modulent les interactions protéine-partenaire.**

Une protéine (jaune-orangé) peut posséder plusieurs domaines d'interaction (représentés par des formes de contact) avec des partenaires (formes de différentes couleurs). Par le biais de modifications post-traductionnelles (Ш, Γ, T, Ґ, Y, ך), seules ou en combinaisons, une déformation locale, qui se traduit par un changement de conformation, est induite créant ainsi une nouvelle surface d'interaction avec un partenaire et abolissant de ce fait la première interaction. Il est évident que ces modifications post-traductionnelles peuvent également affecter les protéines partenaires, multipliant ainsi les combinaisons possibles. Ce changement de partenaire peut se faire également par modification de la localisation sub-cellulaire de la protéine.

## *2- La O-GlcNAc*

### *2.1- La O-GlcNAc se démarque des autres types de glycosylation.*

Les glycosylations, puisqu'il en existe plusieurs types, constituent avec la phosphorylation les modifications post-traductionnelles les plus répandues : on peut

considérer que 50 % des protéines sont glycosylées et que 2 à 4 % du génome humain code des protéines impliquées dans les processus de glycosylation. C'est en 1984, alors qu'il étudiait la distribution des résidus de N-acétylglucosamine terminaux à la surface de lymphocytes activés ou quiescents, que Gerald W. Hart découvrit un nouveau type de glycosylation très original pour l'époque: la *O*-GlcNAc ou *O*-N-acétylglucosaminylation (Torres & Hart, 1984). Tout d'abord cette glycosylation a été localisée sur la surface cellulaire (associée à la membrane plasmique). Un peu plus tard, la *O*-GlcNAc a été associée aux compartiments intracellulaires c'est-à-dire au noyau et à d'autres types d'organites ce qui laissait supposer que la *O*-GlcNAc n'avait pas de localisation particulière (Holt & Hart, 1986). Or, il est rapidement apparu que la majeure partie de la *O*-GlcNAc était cytosolique (Holt et al., 1987a) et nucléaire en association au pore nucléaire (Hanover et al., 1987 ; Holt et al., 1987a).

Structuralement parlant, on ne pouvait envisager de glycosylation beaucoup plus simple que la *O*-GlcNAc puisque celle-ci consiste en l'addition d'un unique monosaccharide de N-acétylglucosamine en anomérie bêta sur des résidus de sérine et de thréonine des protéines cytosoliques et nucléaires. Beaucoup de revues ont été rédigées sur la *O*-GlcNAc, en voici quelques-unes de premier intérêt : Haltiwanger et al., 1997 ; Comer & Hart, 2000 ; Wells et al., 2001 ; O'Donnell, 2002 ; Kamemura & Hart, 2003 ; Slawson & Hart, 2003 ; Wells et al., 2003a ; Wells et al., 2003b ; Whelan & Hart, 2003 ; Zachara & Hart, 2004 ; Love & Hanover, 2005 ; Kudlow, 2006 ; Slawson et al., 2006).

Elle est retrouvée chez de nombreux organismes incluant *Arabidopsis thaliana* et autres plantes supérieures, la drosophile, le nématode *Caenorhabditis elegans*, plusieurs parasites tel que *Plasmodium falciparum* (Dieckmann-Schuppert et al., 1993) (où l'on peut également retrouver de l'α-*O*-GlcNAc à la surface de *Trypanosoma cruzi* ; Previato et al., 1998), plusieurs types de virus et certaines bactéries. Par contre l'existence de la *O*-GlcNAc chez les levures reste un sujet de controverse.

Pour différentes raisons, l'engouement pour l'étude de cette glycosylation s'est très rapidement manifesté. Tout d'abord, et contre toute attente, la *O*-GlcNAc affecte essentiellement des protéines confinées dans les compartiments cytosolique et nucléaire des cellules eucaryotes. Ce fut à l'époque la première glycosylation décrite comme telle, puisque les autres glycosylations du type *N*- et *O*- liées modifient exclusivement des protéines du réticulum endoplasmique, de l'appareil de Golgi, de la membrane plasmique et des voies de sécrétion. Deuxième caractéristique excitante : la *O*-GlcNAc est une modification dynamique

et non pas statique comme la plupart des glycosylations dites « classiques ». Plus tard, il sera même démontré que, sur certaines protéines, la *O*-GlcNAc entre directement en compétition avec la phosphorylation : les deux modifications s'excluant mutuellement sur des mêmes résidus de sérine ou de thréonine, ou sur des résidus voisins (théorie du « Yin-Yang »).

Le tableau I représente vingt dates que nous considérons comme importantes dans l'histoire de la *O*-GlcNAc.

**Tableau II- Vingt faits marquants dans l'histoire de la *O*-GlcNAc**

| Année | Evénement | Référence(s) |
|---|---|---|
| **1984** | La *O*-GlcNAc est découverte de manière fortuite dans des lymphocytes | Torres & Hart, J. Biol. Chem., 1984 |
| **1986-1987** | La *O*-GlcNAc est particuliérement abondante dans les compartiments cytosolique et nucléaire | Holt & Hart, J. Biol. Chem., 1986<br>Hanover et al., J. Biol. Chem. 1987 |
| **1987** | La face cytoplasmique du pore nucléaire est hautement modifiée par la *O*-GlcNAc | Snow et al., J. Cell Biol., 1987<br>Holt et al., J. Cell Biol., 1987a |
| **1988** | Première protéine virale modifiée par la *O*-GlcNAc (cytomégalovirus) | Benko et al., PNAS, 1988 |
| | Sp1 et d'autres facteurs de transcription sont *O*-GlcNAc-Implication de la *O*-GlcNAc dans les mécanismes transcriptionnels | Jackson & Tjian, Cell, 1988 |
| **1989** | Abondance de la *O*-GlcNAc sur les protéines de la chromatine | Kelly & Hart, 1989, Cell |
| **1990** | Identification de l'OGT (*O*-N-acétylglucosaminyltransférase) | Haltiwanger et al., J. Biol. Chem., 1990 |
| **1990-1992** | Mise en évidence du dynamisme de la *O*-GlcNAc sur des lymphocytes activés par des agents mitogènes et sur les cytokératines 8 et 18 | Kearse & Hart, PNAS, 1991<br>Chou et al., J. Biol. Chem., 1992 |
| **1993** | Mise en évidence de la réciprocité entre la *O*-GlcNAc et la phosphorylation sur le domaine C-terminal (CTD) de l'ARN polymérase II | Kelly et al., J. Biol. Chem., 1993 |
| **1994** | Caractérisation de la *O*-GlcNAcase (*O*-N-acétylglucosaminidase) | Dong & Hart, J. Biol. Chem, 1994 |
| **1996** | La *O*-GlcNAc pourrait jouer le rôle d'un signal de translocation nucléaire. | Duverger et al., Glycobiology, 1996 |
| **1997** | Clonage de l'OGT | Kreppel et al., J. Biol. Chem., 1997 |
| | La *O*-GlcNAc jouerait un rôle de protection contre la dégradation protéasomale | Han & Kudlow, Mol. Cell. Biol, 1997 |
| **1998** | Lien entre le métabolisme du glucose et la formation de la *O*-GlcNAc | Yki-Jarvinen et al., Metabolism, 1998 |
| **2000** | L'OGT est essentielle pour la survie de cellules souches embryonnaires | Shafi et al., PNAS, 2000 |
| **2001** | Clonage de la *O*-GlcNAcase | Gao et al., J. Biol. Chem., 2001 |
| | Hsc70 possède un site lectinique de reconnaissance de la *O*-GlcNAc | Lefebvre et al., Biochem. J., 2001 |
| **2004** | Première protéine bactérienne modifiée par la *O*-GlcNAc (flagelline de la Listeria) | Schirm et al., J. Bacteriol, 2004 |
| | La *O*-GlcNAcase possède une activité Histone Acétyltransférase intrinsèque (NCOAT) | Toleman et al., J. Biol. Chem., 2004 |
| **2006** | L'OGT et la *O*-GlcNAcase sont associées sous la forme d'un complexe moléculaire (*O*-GlcNAczyme) | Whisenhunt et al., Glycobiology, 2006 |

### *2.2- La O-GlcNAc modifie une multitude de protéines différentes*

Depuis sa mise en évidence, la liste des protéines modifiées par la *O*-GlcNAc n'a cessé de s'allonger. Comme nous l'avons mentionné, la première mise en évidence de protéines modifiées par la *O*-GlcNAc, fut le cas des protéines associées au pore nucléaire. D'ailleurs ce sont ces protéines (et plus particulièrement huit d'entre-elles, respectivement p210, p180, p145, p100, p63, p58, p54 et p45) qui ont permis l'élaboration du premier anticorps monoclonal dirigé contre le motif *O*-GlcNAc (Snow et al., 1987 ; Holt et al., 1987a). Celui-ci est appelé RL2 en relation avec le clone utilisé pour produire l'anticorps. Par la suite plusieurs protéines structurales ont été décrites comme étant *O*-N-acétylglucosaminylées. Notons parmi celles-ci la « bande 4.1 » érythrocytaire (Holt et al.,

1987b), la taline (Hagmann et al., 1992) et les cytokératines K8 et K18 (Chou et al., 1992). A la même époque a été décrit la *O*-N-acétylglucosaminylation de certaines protéines virales dont la phosphoprotéine basique du cytomégalovirus humain (Benko et al., 1988), la fibre adénovirale de sérotype 2 (Caillet-Boudin et al., 1989) et la protéine NS26 du rotavirus (Gonzalez & Burrone, 1991) ainsi que certains facteurs de transcription tels que Sp1 (Jackson & Tjian, 1988) et le « serum response transcription factor » (Reason et al., 1992). Sur ce point, il est à noter que la richesse en protéines *O*-N-acétylglucosaminylées associées à la chromatine a été également décrite à cette époque (Kelly & Hart, 1989). Depuis ces travaux précurseurs, la liste des protéines modifiées par la *O*-GlcNAc n'a cessé de s'allonger. Ceci est en partie dû à la mise au point de techniques très performantes de détection des résidus *O*-GlcNAc et surtout à l'identification des protéines modifiées. L'élaboration de nouvelles techniques basées sur la modification du résidu *O*-GlcNAc par la technique de BEMAD (bêta-élimination suivie d'une addition de Michael avec le dithiothréitol/ou sa variante où le DTT est remplacé par la biotine pentylamine) (Wells et al., 2002a) ou par l'incorporation d'un dérivé azido du motif *O*-GlcNAc (Sprung et al., 2005) a permis une explosion dans le secteur de l'identification des protéines *O*-N-acétylglucosaminylées. Notons que les premières techniques permettant la mise en évidence de la *O*-GlcNAc (et potentiellement l'identification des protéines modifiées) étaient basées sur l'utilisation de la lectine du germe de blé, WGA (Wheat Germ Agglutinin) et sur le transfert de galactose radiomarqué par la galactosyltranférase bovine. Le tableau II rassemble une liste de plus de 140 protéines *O*-N-acétylglucosaminylées. Dans ce tableau, on retrouve de nombreuses protéines métaboliques, des kinases et des phosphatases, des protéines structurales ou associées au cytosquelette, des protéines chaperonnes et/ou de stress, des facteurs de transcription, des protéines neuronales et autres…

### *2.3- Les fonctions associées à la O-GlcNAc restent mal comprises.*

#### *2.3.1- La O-GlcNAc peut contrecarrer l'effet de la phosphorylation.*

La diversité des protéines modifiées par la *O*-GlcNAc (citées ci-dessus) laisse supposer des rôles aussi riches que variés pour cette glycosylation. Or, actuellement, on ne

Fonctions associées à la *O*-GlcNAc.

**Tableau III- liste des protéines *O*-N-acétylglucosaminylées identifiées.**

## Enzymes

*enzymes métaboliques*

Alcool déshydrogénase
Aldéhyde réductase
Aldolase
Enolase
eNOS
G3P déshydrogénase (GAPDH)
G6P phosphatase
Glycogène synthase
Mannose-1-phosphate guanyl transférase
Myo-inositol-1-phosphate synthase
Phosphoglycérate kinase
Pyruvate kinase
Thymidylate synthase
Triose phosphate isomérase
UDP-Glc pyrophosphorylase

*enzymes de détoxification*

Catalase
Superoxyde dismutase "lente"
Thioredoxine peroxydase 1

*kinases et Phosphatases*

Akt/PKB
CKII
GSK3
PDK1
PH-PTP2 (protéine phosphatase 2C)
PI3 kinase
Protéine S/T phosphatase CG10417
IRS1/2
Tyrosine kinase nucléaire p65

*autres enzymes*

Aminopeptidase
ARN polymérase II
CRMP2
Gluthatione S transférase
OGT
Pepdidylprolylisomérase
Prolyl oligopeptidase
Phospholipase C bêta1

## Protéines de structure ou du cytosquelette

Ankyrine
Annexine I
AP180
AP3
Bande 4.1
Bassoon (cytomatrice des terminaisons nerveuses)*
CAPZB
Cofiline/actine
Cytokératines 8, 13, 18
Dynéine (chaîne légère-1)
E-cadhérine
Moésine
Myosine
Neurofilaments H, M and L *
Piccolo (organisation et trafic des vésicules synaptiques) *
Proteines associées aux microtubules (MAP) *
- MAP1, 2 et 4 (Haute Masse Moléculaire)
- Tau

Synapsine I *
Synaptopodine *
Synucléine bêta *
Taline
Tubuline-α
Vinculine

## Chaperonnes/protéines de stress

Calréguline (ERp60)
Calréticuline
Hsc70
Hsp27
Hsp60
Hsp70 (3/4)
Hsp83
Hsp90

## Protéines du pore nucléaire

Nup153, 214, 358
Nup180
Nup54, 155
p62

## Facteurs de transcription

*facteurs de transcription*

ATF-2 *
béta-caténine
CREB
Elf1
Facteur de transcription humain C1
Facteur de transcription spécifique du pancréas
Hnf1
NF-κB
Octamer binding transcription factor Oct-1/2(?)
P107
Pax6
PDX1
Plakoglobine
Récepteurs des oestrogènes α/β
Sox2 *
Sp1
STAT5
YY1

*oncogènes*

c-Fos
c-Jun
c-Myc
V-erbA

*suppresseurs de tumeurs*

HIC1
p53
pRb

## Traduction

eIF1
eIF2
eIF4
eIF5
EF2
p67 (protéine associée à eIF2)

## Protéines virales

Antigène "Large-T" du SV40
GP41 du baculovirus
Phosphoprotéine basique du cytomégalovirus humain
Protéine capsidaire du Plom Pox virus
Protéine NS26
Protéines fibrillaires 2 et 5 de l'adénovirus

## Ubiquitination et protéasome

Ubiquitin activating enzyme
Sous-unité alpha 4 du protéasome
Sous-unité alpha 6 du protéasome
UCH-L1*
Protéine C2 d'interaction au protéasome

## Protéines liant l'ARN

HnRNP
RBP du sarcome d'Erwin
EIF4A1
EF1a
protéine du ribosome 40S s24

## Protéines non-classées

14.3.3
APP *
Ataxine-10 *
Calpastatine (inhibiteur de la calpaïne)
CRMP2 *
DFF45
Galectine3
GLUT1
Inhibiteur de la phosphatase 2A
MCM6
Nucléophosmine
PDZ-GEF *
Protéine 1 interagissant avec le récepteur au GABA
Ran
Régucalcine
Sec24p *
TRAF2
TRF1

*, protéines exclusivement neuronales.

Fonctions associées à la *O*-GlcNAc.

**Abréviations retrouvées dans le tableau III**

**Akt/PKB**, RAC (related to the A and C kinases) alpha serine/threonine -protein kinase/ serine/threonineprotein kinase B
**AP180**, clathrin coat assembly protein
**AP3**, assembly protein 3
**APP**, amyloid precursor protein (β-amyloid precursor)
**ARN**, acide ribonucléique
**ATF2**, activating transcription factor 2
**CAPZB**, F-actin capping protein beta subunit
**CKII**, casein kinase II
**CREB**, cyclic AMP-responsive element-binding protein
**CRMP2**, Collapsin response mediator protein 2
**DFF45**, DNA fragmenting factor 45
**EF**, elongation factor
**eIF**, eukaryotic translation initiation factor
**eNOS**, endothelial nitric oxide synthase
**ERp60**, endoplasmic reticulum protein 60
**G6P**, glucose-6-phosphate
**G3P**, glyceraldehyde-3-phosphate
**GLUT**, glucose transporter
**GSK3**, glycogen synthase kinase 3
**HIC1**, hypermethylated in cancer1
**Hnf1**, hepatocyte nuclear factor 1
**Hsc**, heat shock cognate protein
**Hsp**, heat shock protein
**IRS1/2**, insulin receptor substrates 1/2
**MAP**, microtubule-associated proteins
**MCM6**, minichromosome maintenance 6 protein
**NF-κB**, nuclear factor-kappaB
**Nup**, nucleoporin
**OGT**, *O* -GlcNAc transferase
**PDX1**, pancreatic/duodenal homeobox-1 protein
**PDZ-GEF**, postsynaptic density-95, dics large and zonula occludens-1-guanine nucleotide exchange factor
**PI3**, phosphatidylinositol 3
**pRb**, retinoblastoma protein
**Sox2**, sry-related high mobility group box 2
**STAT**, signal transducer and activator of transcription
**Tau**, Tubulin-associated unit
**TRAF2**, tumor necrosis factor (TNF) receptor-associated factor 2
**TRF1**, telomeric repeat binding factor 1
**UCH-L1**, Ubiquitin carboxyl hydrolase-L1
**UDP-Glc**, uridine diphosphoglucose
**YY1**, YinYang 1

peut affirmer avec exactitude la fonction réelle jouée par la *O*-GlcNAc. Pourtant nombreux sont les rôles qui lui sont attribués. La mise en évidence de son dynamisme, et plus encore l'existence, d'une relation de réciprocité avec la phosphorylation, appelée « Yin-Yang » (symbole de la conception taoïste qui joint les contraires), augmente plus encore son caractère essentiel dans la cellule.

Cette relation entre la *O*-GlcNAc et la phosphorylation ne peut être considérée comme un rôle à part entière, mais plutôt faut-il le voir comme une caractéristique unique de la *O*-GlcNAc. Cette relation confère au couple *O*-GlcNAc/phosphate une panoplie de combinaisons possibles jouant alors pour chacune d'elle une fonction bien déterminée. Ainsi, comme nous l'avons décrit dans le premier paragraphe, la *O*-GlcNAc à elle seule ou en combinaison avec la phosphorylation, peut, en modifiant des séquences peptiques bien particulières d'une protéine, engendrer une modification conformationnelle localisée offrant à la protéine la possibilité d'interagir avec de multiples partenaires, de changer de localisation sub-cellulaire, d'activer ou d'inactiver une propriété enzymatique…

Cette compétition de site entre la *O*-GlcNAc et la phosphorylation (également appelée « la balance phospho-*O*-GlcNAc ») a été mise en évidence d'une manière très globale par l'utilisation d'agents pharmacologiques bloquant plus généralement les mécanismes phospho-déphospho (Lefebvre et al., 1999 ; Griffith & Schmitz, 1999) ou directement par l'étude de protéines particulières : ARN polymérase II (Kelly et al., 1993 ; Comer & Hart, 2001), proto-oncogène c-Myc (Chou et al., 1995 ; Kamemura et al., 2002), antigène grand-T ou « Large-

T » du SV40 (Medina et al., 1998), récepteur bêta des oestrogènes (Cheng et al., 2000 ; Cheng & Hart, 2001), eNOS (Musicki et al., 2005) et NF-M (medium neurofilament, Ludemann et al., 2005). Cette compétition de site entre les deux modifications post-traductionnelles peut se faire directement sur le même résidu de sérine ou de thréonine ou sur des résidus adjacents ou espacés de quelques acides aminés. On ne peut se représenter, par un schéma simple, la réciprocité entre la *O*-GlcNAc et la phosphorylation, surtout quand plusieurs groupements phosphates ou *O*-GlcNAc sont présents sur la même molécule. Ainsi le nombre de possibilités (de combinaisons) n'augmente pas linéairement, mais exponentiellement, avec le nombre de sites possibles, d'autant plus que la substitution par l'une des modifications post-traductionnelles pourrait favoriser la modification par l'autre d'un résidu voisin et *vice-versa*.

Si l'on compare les sites de *O*-GlcNAc connus, certaines séquences peptidiques modifiées sont proches de séquences reconnues par certaines protéines kinases telles que les caséines kinases I et II, la glycogène synthase kinase-3, les cdk (Cyclin-dependent kinases) ou les MAPK (Mitogen-Actived Protein Kinases). Cela signifie donc que la *O*-GlcNAc n'a pas de séquence consensus propre. Or, actuellement une seule OGT cytosolique et nucléaire (ncOGT et sa forme courte, sOGT), plus une forme mitochondriale (mOGT) ont été décrites : il n'existerait donc pas une OGT par type de séquence modifiée ou une OGT par kinase à contrecarrer. Quoi qu'il en soit, l'OGT nécessite tout de même un environnement peptidique particulier caractérisé par la présence de plusieurs résidus d'acides aminés hydroxylés (sérine ou thréonine) ou la proximité d'un résidu de proline (sites du type PVS/T).

### *2.3.2- La O-GlcNAc intervient à tous les niveaux de régulation de la vie cellulaire.*

Si les rôles attribués à la *O*-GlcNAc restent mal définis et parfois obscurs, il est certain que celle-ci prend une part active à des niveaux très variés dans la cellule. Ainsi cette glycosylation interviendrait dans les processus de transport et/ou de rétention nucléaire (Juang et al., 2002 ; Lefebvre et al., 2002 ; Lefebvre et al., 2003 ; Guinez et al., 2005), le transport vésiculaire (Dudognon et al., 2004), le remodelage de l'architecture cellulaire (Ludemann et al., 2005), le cycle cellulaire (Slawson et al., 2005), les phénomènes d'apoptose (O'Donnell et al., 2004), les processus de transcription (Kudlow, 2006) et de traduction (Datta et al., 2004), la réponse au stress (Zachara et al., 2004), l'activité protéasomale (Zhang et al., 2003), le développement (O'Donnell et al., 2004), dans le métabolisme (Cieniewski-Bernard et al., 2004) ou encore dans les voies de signalisation (Whisenhunt et al., 2006). Aujourd'hui,

aucune observation n'est en mesure de venir contredire ces rôles, mais beaucoup reste à faire pour convaincre la communauté scientifique internationale du caractère crucial de la *O*-GlcNAc dans le déroulement normal de ces différents processus.

On peut supposer que la régulation de ces fonctions puisse se faire via des interactions protéiques modulées par la *O*-GlcNAc en compétition directe ou non avec la phosphorylation (Roos et al., 1997 ; Hiromura et al., 2003 ; Gewinner et al., 2004).

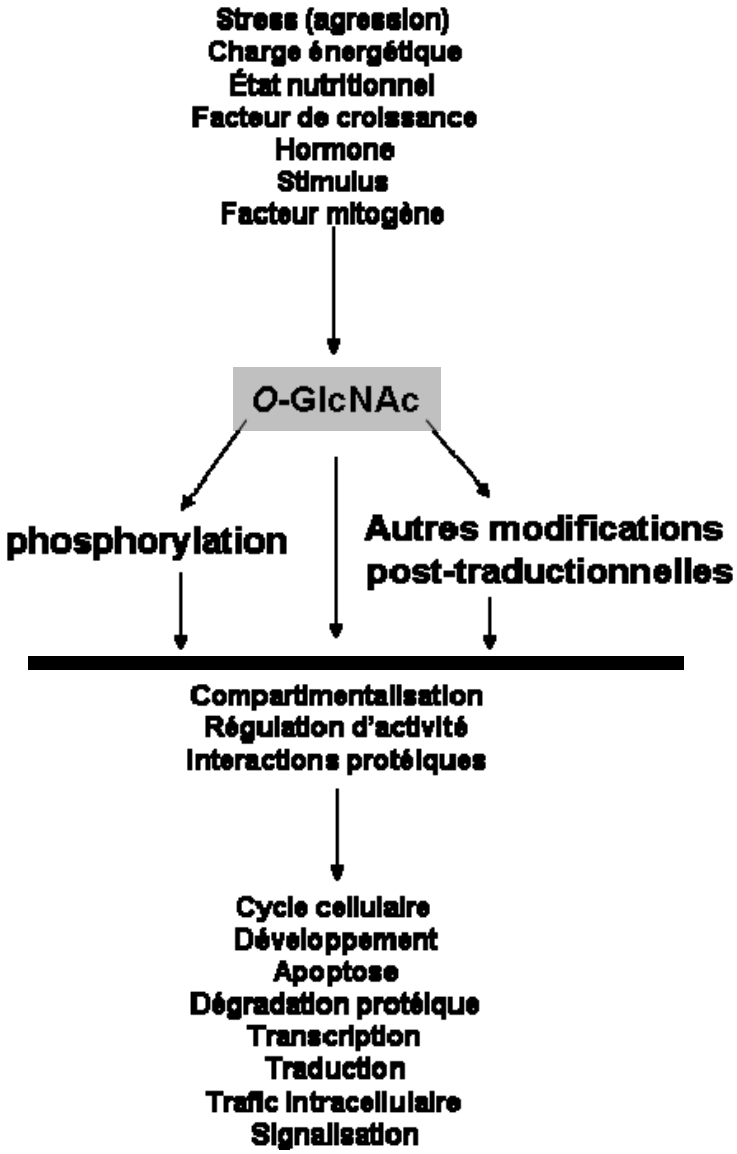



**Figure 2- La *O*-GlcNAc: plaque tournante de l'homéostasie cellulaire ?**

La *O*-GlcNAc, en association avec d'autres modifications post-traductionnelles, se comporterait comme un relais permettant à la cellule de répondre aux évènements extracellulaires et intracellulaires (informations, stress, stimuli…) en adoptant la réponse la plus adaptée.

Fonctions associées à la *O*-GlcNAc.

Dans ce sens, deux études récentes viennent conforter l'hypothèse selon laquelle la *O*-GlcNAc protègerait la cellule après qu'elle a subit une agression ou des dommages. La première étude porte sur l'ischémie du cœur isolé (Liu et al., 2006). L'ischémie entraîne une déplétion des stocks énergétiques et une augmentation du calcium intracellulaire ; lors de la reperfusion, le paradoxe calcique est exacerbé, et la formation de radicaux libres provoque des dommages membranaires entraînant un œdème tissulaire, un syndrome inflammatoire local et général. Un diabète imposé par traitement de cet organe avec de la streptozotocine, ou bien la perfusion de celui-ci avec de la glucosamine, permet au cœur d'accélérer sa récupération après ischémie ou paradoxe du calcium. Le blocage des processus de *O*-N-acétylglucosaminylation par inhibition de la glutamine :fructose-6-phosphate amidotransférase ou GFAT (par l'azasérine) ou de l'OGT (par l'alloxane) ne permet plus cette protection par la glucosamine, démontrant que l'élévation du taux de *O*-GlcNAc est bénéfique à la récupération cardiaque. Dernièrement, le lien entre l'effet protecteur d'une hyperglycémie provoquée par le stress sur des hémorragies sévères a été recherché chez le rat (Yang et al., 2006). L'administration de glucosamine a permit d'établir une relation entre le succès de réanimation cardiaque avec les niveaux de *O*-GlcNAc. Ces deux exemples sont une preuve de la nécessité des processus de *O*-N-acétylglucosaminylation dans la bonne intégrité et le bon fonctionnement cellulaire, et démontrent le rôle de la *O*-GlcNAc en tant qu'intermédiaire d'échanges entre les préjudices subits par la cellule et les événements dont elle est l'actrice, et le type de réponse qu'elle développera pour se sortir d'un mauvais pas (Fig. 2).

Les rôles joués par la *O*-GlcNAc ne seront pas plus développés ici puisque nous en parlerons plus amplement dans la partie dédiée aux pathologies (paragraphe 2.6) ainsi que dans les deuxième et troisième parties de ce mémoire.

### *2.4- Les processus de O-N-acétylglucosaminylation sont régulés par un couple d'enzymes.*

Fonctionnant selon le même principe que le système kinases/phosphatases, le dynamisme de la *O*-GlcNAc est très vraisemblablement régulé par un seul couple d'enzymes antagonistes (alors que plus de 500 kinases et 150 phosphatases ont été répertoriées), la *O*-N-acétylglucosaminyltransférase (*O*-GlcNAc transférase ou OGT, EC 2.4.1.94) qui transfère le résidu GlcNAc sur les protéines cibles et la *O*-N-acétylglucosaminidase (*O*-GlcNAcase, EC 3.2.1.52) qui hydrolyse ce résidu.

Fonctions associées à la *O*-GlcNAc.

### *2.4.1-La O-GlcNAc transférase greffe le résidu de GlcNAc sur les protéines substrats.*

Les deux enzymes responsables du dynamisme de la *O*-GlcNAc ont été hautement conservées au cours de l'évolution (Iyer & Hart, 2003a): des séquences codantes homologues pour l'OGT ont été retrouvées chez l'homme et les archébactéries et plus de 80 % d'identité sont retrouvées chez les eucaryotes (Kreppel et al., 1997). Comme nous l'avons mentionné plus haut, l'OGT semble être absente des levures telles que *Saccharomyces cerevisiae*, tout comme la modification post-traductionnelle correspondante. Cette absence pourrait s'expliquer par le besoin des levures en UDP-GlcNAc pour la synthèse de chitine entrant dans la constitution de la paroi rigide.

Chez les autres organismes, l'organisation de l'OGT a été conservée. La protéine peut être sub-divisée en trois domaines (fig. 3). La partie N-terminale contient des TPR (tetratricopeptide repeat), domaine répété de 34 acides aminés, retrouvé dans une grande variété de protéines, très répandu de la bactérie à l'homme et permettant la modulation des interactions avec les protéines substrats ; on trouve ensuite un domaine intermédiaire de liaison (dit « linker ») de la partie N-terminale avec la partie C-terminale, cette dernière possédant l'activité catalytique. Le nombre de TPR varie de 1 à 16 en fonction de l'organisme mais également en fonction de la localisation sub-cellulaire de l'enzyme. En effet, chez l'homme, l'OGT est codée par un seul gène localisé en Xq13 (Shafi et al., 2000). Cette région est particulièrement intéressante puisque le locus de la dystonie parkinsonienne (DYT3) est également localisé dans cette région (Nolte & Muller, 2002). Le gène codant l'OGT posséde 23 exons et se trouve sous le contrôle de deux promoteurs (P1 et P2). Ainsi plusieurs isoformes issues d'épissages alternatifs sont produites. L'isoforme la plus longue, de 116 kDa, est retrouvée dans le cytosol et le noyau (ncOGT) et contient 11,5 TPR (Iyer & Hart, 2003b) qui lui permettent de s'homotrimériser, alors que l'isoforme mitochondriale (mOGT), issue de la transcription à partir du deuxième promoteur (P2), possède 9 TPR et a une masse de 109 kDa. La mOGT possède une séquence d'adressage à la mitochondrie en N-terminal (Hanover et al., 2003 ; Love et al., 2003). Une isoforme plus petite existe (sOGT, pour « small OGT ») et ne possède plus que 3 TPR pour une masse de 70 kDa. Il semblerait que cette sOGT a une capacité de glycosylation restreinte puisqu'aucun des substrats testés par Lazarus et al (Lazarus et al., 2006) n'est modifié par cette isoforme. En contrepartie, ces auteurs ont démontré une certaine spécificité de substrat liée au nombre de TPR portés par l'OGT. Ainsi, la ncOGT et la mOGT catalysent le transfert de GlcNAc sur Nup62 et la caséine kinase II

alors que la *O*-GlcNAcase et Tau ne sont modifiées que par la ncOGT et que la tyrosine kinase YES n'est glycosylée que par la mOGT (Lazarus et al., 2006).

La partie centrale de l'OGT semble être une région flexible (Love & Hanover, 2005). Chez *C. elegans* cette région contient une séquence potentielle de localisation nucléaire (NLS). Cette séquence NLS ne joue sans doute aucun rôle dans la localisation nucléaire puisqu'elle n'a pas été conservée au cours de l'évolution : la région centrale, en elle-même, est la moins conservée des trois domaines de l'OGT.

Le domaine catalytique de l'OGT, porté par la région C-terminale, contient deux motifs du type Rossman (Wrabl & Grishin, 2001). Ce motif a été retrouvé par comparaison de l'OGT avec des enzymes utilisant l'UDP-GlcNAc. La présence de ce motif classe ainsi l'OGT parmi la superfamille des glycogène phosphorylases/glycosyltransférases (GPGTF).

L'OGT est retrouvée dans tous les tissus mais elle est plus particulièrement enrichie dans les cellules bêta pancréatiques et le cerveau. Chez la plante, elle joue un rôle prédominant dans la voie de la gibbérelline (hormone possédant des fonctions variées dans le développement et la croissance végétale) (Robertson et al., 1998). Deux OGT sont décrites chez *Arabidopsis thaliana* : SPY (Spindly) et SEC (Secret agent) (Hartweck et al., 2006).

Plusieurs partenaires interagissant avec l'OGT ont été décrits. Parmi ceux-ci nous pouvons citer GRIF1 ($GABA_A$ receptor-interacting factor R1) et son homologue OIP106 (OGT-interacting protein-106) (Iyer et al., 2003), l'ARN polymérase II, mSin3A (qui recruterait l'OGT vers la machinerie transcriptionnelle permettant l'inhibition de la transcription par glycosylation) (Yang et al., 2002) et dernièrement une interaction de l'OGT avec la sérine/thréonine phosphatase 1 (sous-unités β et γ) a été décrite (Wells et al., 2004). Cette interaction OGT/PP1β/γ permettrait la déphosphorylation préalable du substrat avant sa modification par la *O*-GlcNAc.

L'OGT possède un Km (constante de Michaelis) pour l'UDP-GlcNAc très bas. Celui-ci a été estimé à 545 nM lors de la purification de l'OGT (Haltiwanger et al., 1992). Cette très haute affinité pouvait paraître très inhabituelle à l'époque, mais celle-ci s'explique par le fait de la compétition pour l'UDP-GlcNAc avec les transporteurs du nucléotide-sucre de l'appareil de Golgi et du réticulum endoplasmique. Des études plus récentes (Kreppel & Hart, 1999) ont montré que l'OGT possédait en fait un Km variable pour l'UDP-GlcNAc (compris entre 0,05 µM et 4,8 mM) capable de s'adapter aux concentrations variables d'UDP-GlcNAc cellulaire (du µM au mM) dépendant principalement des conditions de nutrition (l'UDP-GlcNAc

provient de diverses origines métaboliques telles que celles du glucose, des acides gras, des nucléotides…). Nous développerons cet aspect dans le paragraphe suivant.

### *2.4.2-La O-GlcNAcase hydrolyse le résidu de GlcNAc des protéines modifiées.*

La *O*-N-acétylglucosaminidase avait été identifiée à l'origine comme une hyaluronidase associée aux méningiomes et, pour cette raison, avait été appelée MGEA5 pour *Meningioma expressed antigen-5* (Heckel et al., 1998). Le gène *MGEA5* est localisé sur le chromosome 10 dans la région 10q24.1-q24.3, région associée à de nombreux troubles neurodégénératifs dont la maladie d'Alzheimer (Bertram et al., 2000 ; Myers et al., 2000) et code au moins deux transcrits d'épissages alternatifs très largement répandus dans tous les tissus des mammifères. L'appellation hyaluronidase lui provient de l'analogie de sa partie N-terminale avec la hyaluronidase de *C. elegans* (Heckel et al., 1998). La structure tertiaire de ce domaine adopte un repli en tonneau du type TIM (Triose Phosphate Isomerase)-barrel (alternance d'hélices alpha et de feuillets bêta) très répandu et commun aux enzymes de cette famille.

La région C-terminale de l'enzyme porte des caractéristiques communes à la famille GCN5 des acétyltransférases (Schultz & Pils, 2002). Ainsi le domaine C-terminal de la *O*-GlcNAcase possède une activité histone acétyltransférase (HAT) intrinsèque (Toleman et al., 2004) qui serait incomplète et qui nécessiterait des protéines accessoires pour fonctionner pleinement. La *O*-GlcNAcase a été renommée NCOAT pour « Nuclear and Cytoplasmic *O*-GlcNAcase and AcetylTransferase » (fig. 2).

La mise en évidence de cette activité HAT apparaît de toute première importance puisqu'il a avait été démontré que la *O*-GlcNAc pouvait réprimer la transcription en interagissant avec des histones désacétylases (HDAC) par le biais du co-répresseur mSin3A (Yang et al., 2002). Ainsi l'OGT et la *O*-GlcNAcase seraient toutes les deux impliquées dans l'activité transcriptionnelle : d'une part l'activité *O*-GlcNAcase de NCOAT viendrait contrecarrer l'effet inhibiteur de l'OGT sur les composants de la machinerie transcriptionnelle (et ainsi l'activer) et d'autre part, l'activité HDAC associée à l'OGT où l'activité HAT intrinsèque à NCOAT permettrait le remodelage de la chromatine, se traduisant par une répression transcriptionnelle dans le premier cas ou par une activation dans l'autre.

Il a été décrit très récemment que l'OGT et NCOAT pouvaient faire partie d'un complexe unique appelé *O*-GlcNAczyme (Whisenhunt et al., Glycobiology, 2006). L'interaction entre

les deux enzymes antagonistes implique l'extrémité N-terminale et les six premiers TPR pour l'OGT et le domaine séparant l'activité *O*-GlcNAcase et l'activité HAT de NCOAT (Fig. 3). Ceci permettrait de renforcer encore l'effet coopératif des activités HDAC/OGT et des activités *O*-GlcNAcase/HAT.

Plusieurs partenaires de la *O*-GlcNAcase ont pu être identifiés (Wells et al., 2002b). Parmi ceux-ci nous pouvons citer Hsp110, Hsc70, DRP-2, l'amphiphysine et la calcineurine.

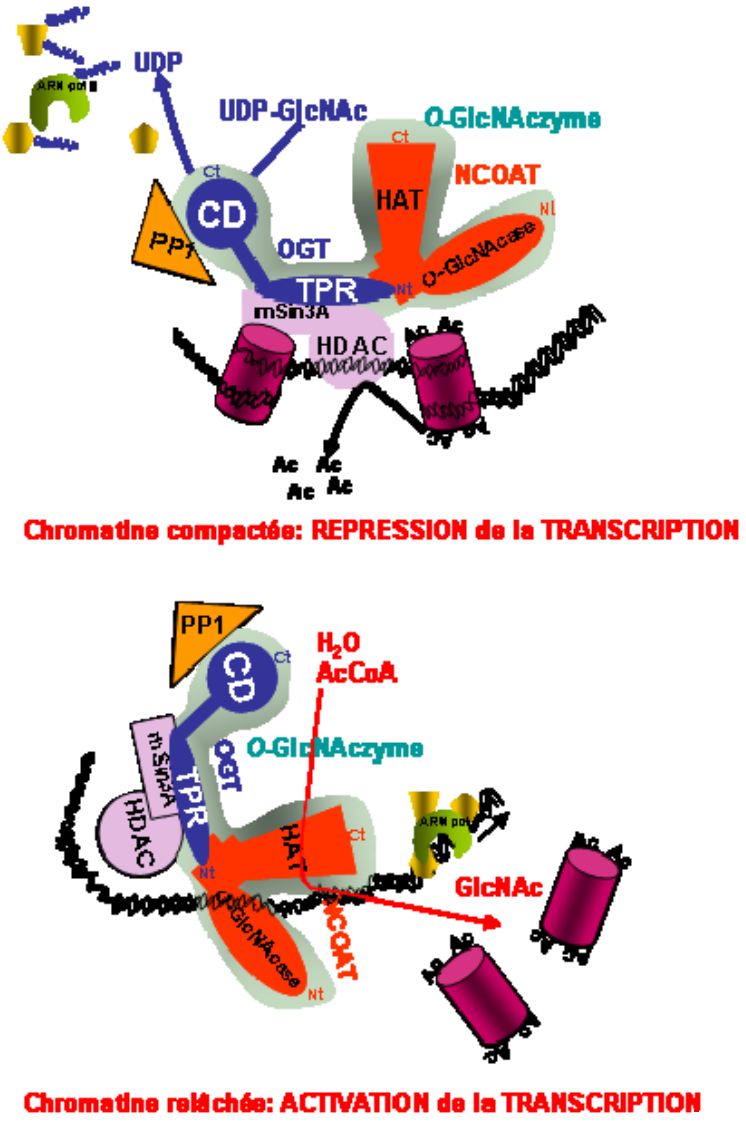



**Figure 3- OGT et NCOAT font partie d'un complexe multiprotéique appelé *O*-GlcNAczyme.** NCOAT est une enzyme bi-fonctionnelle capable d'hydrolyser les résidus de *O*-GlcNAc (domaine *O*-GlcNAcase) et de catalyser le transfert de groupements acétyles sur les histones (domaine HAT). L'OGT transfère (domaine catalytique, CD) les résidus de GlcNAc sur les protéines substrats via des interactions avec son domaine TPR. L'OGT interagit par le biais du répresseur mSin3A avec une HDAC, enzyme hydrolysant les groupements acétyle des histones, et avec PP1a/g. OGT et NCOAT interagissent (par le domaine N-terminal et les six

premiers TPR pour l'OGT et par la région intermédiaire aux deux activités enzymatiques pour NCOAT. L'association PP1/OGT permettrait la déphosphorylation des sites et leur glycosylation immédiate. L'*O*-GlcNAczyme et son partenaire indirect HDAC réguleraient les mécanismes transcriptionnels par deux voies parallèles et très vraisemblablement complémentaires.

### *2.5- Le résidu de GlcNAc provient de la voie métabolique des hexosamines et en partie du glucose extracellulaire.*

Deux à cinq pour cent du glucose extracellulaire peut être utilisé par la cellule pour les processus de *O*-N-acétylglucosaminylation : ainsi la culture de cellules en milieu hyperglucosé permet d'augmenter les niveaux de *O*-GlcNAc (Han et al., 2000 ; Walgren et al., 2003 ; Guinez et al., 2006). La formation de la *O*-GlcNAc peut être considérée comme un des points finaux de la voie de biosynthèse des hexosamines (HBP pour « hexosamine biosynthetic pathway ») (Wells et al., 2003a), même si le produit final de cette voie est l'UDP-GlcNAc, donneur du motif *O*-GlcNAc (Fig. 4). Il est intéressant de noter d'ailleurs que plusieurs protéines participant au métabolisme du glucose sont elles-mêmes modifiées par la *O*-GlcNAc, ce qui suppose une régulation interne de la voie des hexosamines mais également de la voie de signalisation de l'insuline par la *O*-GlcNAc (Fig. 4). Ces protéines sont entre autres la caséine-kinase II, la glycogène synthase kinase-3, les substrats 1 et 2 du récepteur de l'insuline, Akt (Protéine kinase B), Glut-4, la glycogène synthase…

La voie des hexosamines est finement régulée par la GFAT. Cette enzyme permet la conversion du frucose-6-phosphate en glucosamine-6-phosphate, premier produit de la voie des hexosamines. L'activité de la GFAT est régulée par phosphorylation de la sérine 205 par la protéine kinase A (PKA)-AMP cyclique dépendante : cette modification résulte en une inhibition de la GFAT1 (Chang et al., 2001) alors que la phosphorylation du même résidu de la GFAT2 (qui présente 75% d'homologie avec la GFAT1) provoque une augmentation de son activité (Hu et al., 2004). Ainsi les deux isoenzymes ont-elles une activité opposée en réponse à la modification par la PKA. Il serait intéressant de comparer le patron d'expression tissulaire de ces deux enzymes, la GFAT1 et la GFAT2 ne devant pas se retrouver en co-expression dans le même tissu puisque, suivant l'activité de la PKA, l'une serait activée,

Fonctions associées à la *O*-GlcNAc.

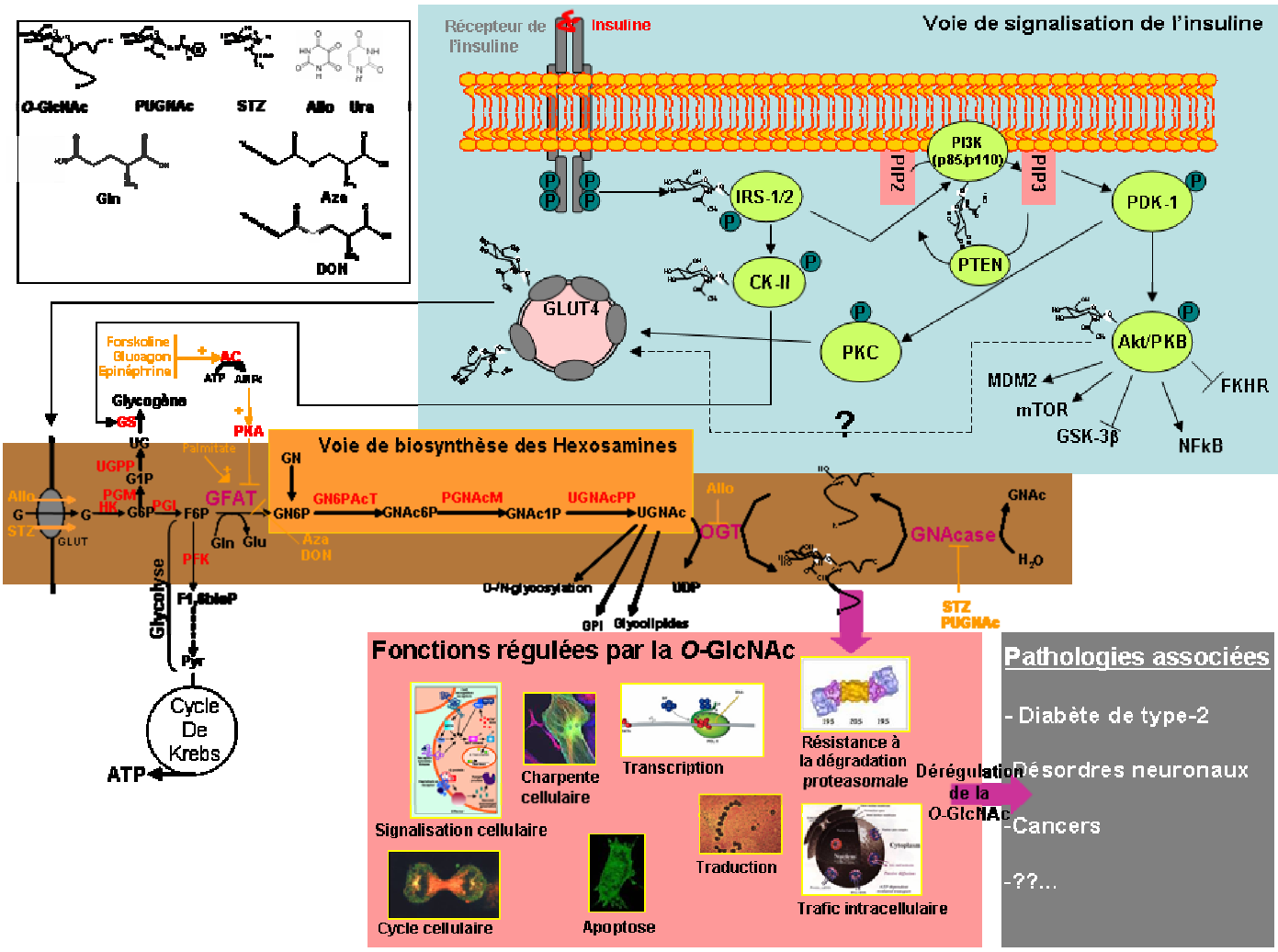



**Figure 4- Du glucose extracellulaire aux fonctions associées à la *O*-GlcNAc.**

Les niveaux de *O*-GlcNAc sont dépendants des concentrations en glucose extracellulaire (encadré marron). La formation de l'UDP-GlcNAc passe par la voie de biosynthèse des hexosamines (encadré orange) : le blocage de cette voie peut se faire par l'inhibition de la GFAT (aza et DON). Des connexions entre la voie de signalisation de l'insuline (encadré bleu), de sa régulation par la *O*-GlcNAc, du transport du glucose et de la synthèse du glycogène sont représentées. Les fonctions les plus représentatives associées à la *O*-GlcNAc sont indiquées dans l'encadré rose, et les dysfonctions du système *O*-GlcNAc/dé-*O*-GlcNAc sont inscrites dans l'encadré gris. Toutes les abréviations de la figure ont été listées dans le tableau I de ce mémoire.

**Encadré blanc**- Structure comparée de la *O*-GlcNAc, de la PUGNAc et de la streptozotocine (inhibiteurs de la *O*-GlcNAcase) ; structure de l'alloxane (inhibiteur de l'OGT) ; structure comparée de la glutamine, de l'azasérine et du DON (inhibiteurs de la GFAT).

**Encadré marron** - Voie de biosynthèse de la *O*-GlcNAc à partir du glucose extracellulaire en passant par la voie de biosynthèse des hexosamines (**encadré orange**).

**Encadré rose**- Fonctions modulées par la *O*-GlcNAc.

**Encadré bleu**- Voie de signalisation de l'insuline et ses composants modifiés par la *O*-GlcNAc.

Fonctions associées à la *O*-GlcNAc.

**Encadré gris**- Pathologies potentiellement générées par un dysfonctionnement de la dynamique de la *O*-GlcNAc.

Fonctions associées à la *O*-GlcNAc.

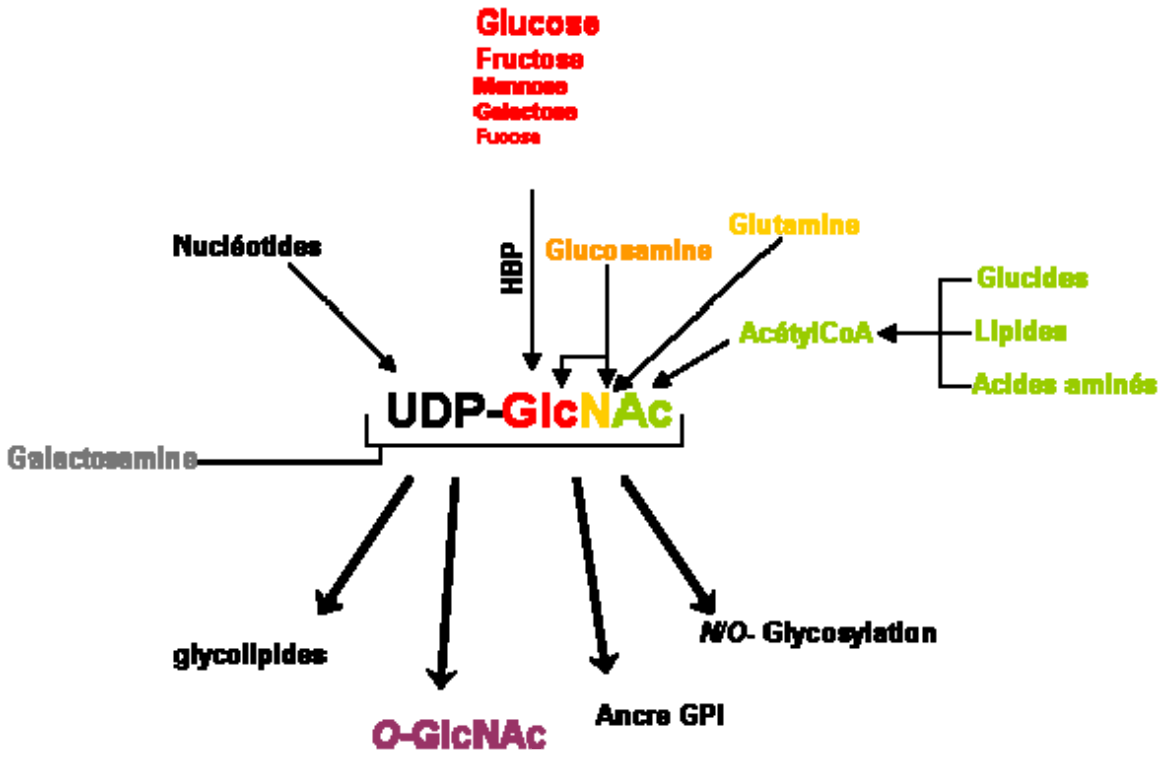



**Figure 5- « Tous les chemins mènent » à l'UDP-GlcNAc ou l'UDP-GlcNAc, « à la croisée des chemins ».**

L'UDP-GlcNAc se situe à un carrefour des métabolismes cellulaires. Chacun de ces groupements est originaire d'un type de métabolisme au moins. Le groupement acétyl, donné par l'acétyl-coenzymeA provient du métabolisme des glucides via la voie de la glycolyse où le produit final, l'acide pyruvique, subit l'action de la pyruvate déshydrogènase pour former l'acétylCoA, forme activée du groupement acétyl. Cet acétylCoA provient également de la dégradation des acides gras par la voie de la bêta-oxydation (hélice de Lynen) et des acides aminés cétogènes. La glucosamine peut être phosphorylée pour donner la $GlcNH_2$-6-phosphate (« by-pass » de la GFAT) et être incorporée en UDP-GlcNAc. La glutamine est le donneur du groupement amine. La partie glucosyl- peut provenir de diverses sources glucidiques grâce aux mécanismes d'interconversions des sucres. La galactosamine activée sous la forme d'UDP-N-acétylgalactosamine (après acétylation de la galactosamine en N-acétylgalactosamine) peut être épimérisée en UDP-GlcNAc par la 4-épimérase. Enfin la partie UDP provient du métabolisme des nucléotides.

l'autre inhibée et *vice-versa*, alors que les deux isoenzymes ont le même substrat. Une autre hypothèse serait que l'une des isoformes est plus exprimée que l'autre, ce qui permettrait à la cellule, quel que soit le niveau d'activité de la PKA, de conserver un niveau minimal de *O*-N-acétylglucosaminylation. On peut très légitimement supposer que, dans la majorité des tissus et des cellules utilisées en culture cellulaire, la GFAT1 soit plus exprimée que la GFAT2, puisque l'activation de l'adénylate cyclase (Fig. 4), l'enzyme convertissant l'ATP en AMPc (activateur de PKA), par des agents tels que la forskoline ou l'épinéphrine, diminue fortement les niveaux de *O*-GlcNAc. Par ailleurs deux inhibiteurs de la GFAT sont couramment utilisés pour diminuer le niveau de *O*-GlcNAc ; il s'agit de l'azasérine et du 6-diazo-5-oxo-norleucine (DON).

### *2.6- Une dérégulation des processus de O-N-acétylglucosaminylation serait impliquée dans plusieurs types de pathologies.*

La pléthore de protéines modifiées par la *O*-GlcNAc et le nombre de processus moléculaires auxquels semble prendre part celle-ci, laisse imaginer qu'un dysfonctionnement de la labilité de la *O*-GlcNAc doit entraîner des dysfonctionnements au sein de la cellule, plus globalement au niveau du tissu et, par extension, au sein de l'organisme tout entier. Au point de vue des pathologies qui pourraient être associées à un « mauvais réglage » du dynamisme de la *O*-GlcNAc, peu d'études ont été publiées jusqu'à présent. Malgré tout, il apparaît qu'une dérégulation des processus *O*-GlcNAc/dé-*O*-GlcNAc interviendrait dans plusieurs types de désordres dont les plus communs sont :

- le diabète de type-2,
- les processus de cancérisation,
- et les troubles neurodégénératifs.

L'implication potentielle de la *O*-GlcNAc dans ce type de pathologies ne doit pas étonner puisque, comme nous l'avons vu plus haut, la formation du motif *O*-GlcNAc provient de diverses sources nutritionnelles et des différents métabolismes (Fig. 5) comme celui des glucides (dans la synthèse du résidu GlcNAc via la voie HBP), des acides aminés (en particulier de la glutamine pour le transfert du groupement amine par la GFAT), des nucléotides (pour la synthèse de la partie UDP- du nucléotide-sucre donneur, l'UDP-GlcNAc)

et enfin du métabolisme des lipides (formation d'acétylcoenzyme A, donneur du groupement acétyl). Toutes ces origines métaboliques font de la *O*-GlcNAc un « senseur nutritionnel » idéal et il apparaît que des problèmes d'alimentation, telle que la malnutrition et les troubles du métabolisme, provoqueront des dysfonctionnements dans la dynamique de la *O*-GlcNAc et de ses fonctions associées. Une augmentation des niveaux de *O*-GlcNAc a même été associée à des troubles de l'érection via la modification de eNOS (Musicki et al., 2005).

**N.B. Cette partie nous servira également à détailler certaines fonctions régulées par la *O*-GlcNAc : régulation transcriptionnelle, dégradation protéasomale, voies de régulation…**

En avant propos, nous insistons sur le fait que le recul sur nos connaissances actuelles de la *O*-GlcNAc n'est pas assez grand pour corréler une déficience dans cette glycosylation avec des pathologies de quelque nature que se soit. Ce paragraphe se veut plus comme un « regroupement de faits », de constatations, de recoupements d'idées et de caractères qui doivent servir de pistes de travail, plutôt que la démonstration, en soit, que la *O*-GlcNAc est l'élément fondamental dans ces désordres divers. Ainsi, il semble aujourd'hui que la *O*-GlcNAc puisse jouer un rôle essentiel dans le développement de neuropathologies, et il semble que celle-ci soit réellement corrélée au diabète, même si beaucoup d'éléments permettant de faire le lien manquent encore à ce jour. Les choses sont un peu différentes dans les diverses formes de cancer. Nous le verrons, les éléments reliant les processus de cancérisation et la *O*-GlcNAc sont plus évidents, puisque certains oncogènes et certains suppresseurs de tumeurs sont modifiés par cette glycosylation. Malgré tout, le chemin permettant de relier cancer et *O*-GlcNAc reste encore long.

Nous ne prétendons donc pas faire de rapprochement qui s'avérerait inapproprié entre ces diverses pathologies et l'influence de la *O*-GlcNAc.

### *2.6.1- La O-GlcNAc peut-elle être le lien entre le métabolisme du glucose et le développement du diabète de type-2 ?*

#### *2.6.1.1- La résistance à l'insuline est une des caractéristiques du diabète de type-2.*

Bien qu'intensément étudié, le développement des diabètes (dont une des caractéristiques majeures est l'hyperglycémie chronique) et, plus particulièrement celui du diabète de type-2, reste mal compris. L'excès nutritionnel chronique et le manque d'activité

physique sont deux des facteurs de prédisposition au diabète de type-2. La résistance à l'insuline, retrouvée dans les cellules bêta pancréatiques, le foie, les adipocytes et le muscle squelettique, couplée à la toxicité du glucose, est responsable des complications tels que les problèmes vasculaires. La résistance à l'insuline inhibe le transport du glucose et la synthèse du glycogène dans le muscle squelettique et le foie. Cette résistance à l'insuline pourrait être, pour l'organisme, une manière de répondre à l'excès alimentaire. Au début des années 90, Marshall et Traxinger, que l'on peut considérer comme les pionniers dans l'étude de la voie de synthèse des hexosamines, observent que la résistance à l'insuline est liée à trois composants : le glucose, l'insuline et la glutamine (Marshall et al., 1991). Le rôle du glucose et de l'insuline apparaît évident dans ce processus. La fonction de la glutamine est à rechercher dans la régulation de la GFAT, l'enzyme dite limitante et déterminante dans la voie de synthèse des hexosamines, comme nous l'avons vu plus haut.

#### *2.6.1.2- La voie de biosynthèse des hexosamines joue un rôle prédominant dans la résistance à l'insuline.*

Puisque la modification des protéines par la *O*-GlcNAc est intimement liée au métabolisme du glucose, il a été postulé que la *O*-GlcNAc puisse être un acteur essentiel dans le phénomène de résistance à l'insuline et dans la diminution du transport du glucose. L'hypothèse d'un rétrocontrôle négatif de la régulation du transport du glucose par l'utilisation d'une partie du glucose dans la voie de biosynthèse des hexosamines a d'abord été avancée dans les cellules cibles de l'insuline (Wells et al., 2003a). Puis, il a été montré que lorsque des hémidiaphragmes de rat étaient incubés en présence de glucosamine ou en milieu hyperglucosé, le transport du glucose était diminué (Robinson et al., 1993). Plus tard, Rossetti et al ont mis en évidence que la résistance à l'insuline induite par la glucosamine était accompagnée d'une augmentation du « pool » d'UDP-GlcNAc (Rossetti et al., 1995). En provoquant un diabète de type-2 par la sur-expression de l'OGT, McClain et al. ont relié le phénomène de la résistance à l'insuline avec les processus de *O*-GlcNAc (McClain et al., 2002). L'incubation de muscles epitrochlearis de rat avec du PUGNAc, un inhibiteur de la *O*-GlcNAcase (Haltiwanger et al., 1998), induit une augmentation des niveaux de *O*-GlcNAc et une diminution du transport de glucose, suggérant que la modification des protéines par la *O*-GlcNAc puisse induire la résistance à l'insuline (Arias et al., 2004).

Fonctions associées à la *O*-GlcNAc.

Ces différentes observations sont en accord avec un rôle pivot de la *O*-GlcNAc dans la diminution du transport du glucose et la résistance à l'insuline au travers de la voie de biosynthèse des hexosamines. Les mécanismes de conversion du glucose en UDP-GlcNAc sont bien connus (Fig. 4). Une fois le glucose entré dans la cellule, il est immédiatement phosphorylé en glucose-6-phosphate par l'hexokinase. Cette phosphorylation a pour but de piéger le glucose dans la cellule. Le glucose-6-phosphate peut alors être épimérisé en glucose-1-phosphate par la phosphoglucomutase, qui sera activé en UDP-glucose pour la synthèse du glycogène ou convertit en fructose-6-phosphate par la phosphoglucose(hexose) isomérase. Une petite partie de ce fructose-6-phosphate est convertie en glucosamine-6-phosphate par la GFAT alors que la majorité de ce fructose-6-phosphate est dirigée vers la glycolyse. La glucosamine-6-phosphate, qui est le premier produit de la voie de synthèse des hexosamines, est acétylée par la N-acétyl-glucosamine N-acétyltransférase en N-acétyl-glucosamine-6-phosphate qui est épimérisée en N-acétyl-glucosamine-1-phosphate par la phospho-N-acétylglucosamine mutase. Cette N-acétyl-glucosamine-1-phosphate est activée en UDP-GlcNAc par l'UDP-GlcNAc pyrophosphorylase. L'UDP-GlcNAc est le produit final de la voie des hexosamines et entre dans les processus de glycosylation des *N*- et *O*-glycosylations, de la *O*-GlcNAc, des ancres GPI (glycosylphosphatidylinositol) et des glycolipides (Fig. 4). Il est intéressant de noter que la glucosamine est capable de provoquer à elle seule la résistance à l'insuline par le fait qu'elle peut court-circuiter la GFAT, pour former la glucosamine-6-phosphate.

#### *2.6.1.3- La O-GlcNAc participe à la voie de signalisation de l'insuline.*

La résistance à l'insuline, et donc une diminution du transport du glucose, a été corrélée à une diminution de l'adressage du nombre de transporteurs de glucose, GLUT4, vers la membrane plasmique (Cooksey et al., 1999). Dans des conditions physiologiques normales, les vésicules contenant les transporteurs GLUT4 sont transportées et intégrées à la membrane plasmique en réponse à la stimulation par le glucose, ce qui permet au glucose de pénétrer dans les adipocytes et les cellules musculaires. Le mécanisme par lequel les vésicules GLUT4 n'intègrent plus la membrane plasmique est inconnu, mais il semblerait que la voie Akt puisse être à l'origine de cette défaillance (Cho et al., 2001). Lorsque l'insuline se fixe sur son récepteur membranaire, une activité tyrosine kinase intrinsèque et intracellulaire se met en place. Les substrats du récepteur de l'insuline (IRS) sont alors recrutés vers celui-ci et sont à

leur tour phosphorylés sur des résidus de tyrosine. La phosphoinositide-3 (PI-3) kinase (p85/p110) migre vers la membrane plasmique pour phosphoryler le phosphatidylinositol-4, 5-bisphosphate (PIP2) en phosphatidylinositol-3, 4, 5-triphosphate (PIP3). La protéine kinase dépendante du phosphatidylinositol (PDK-1) est alors activée et phosphoryle la protéine kinase B (PKB)/Akt laquelle, en retour, phosphoryle différents substrats dont GSK-3β (Fig. 4). En fait le lien entre Akt et GLUT4 n'est pas réellement établi : on ne sait pas aujourd'hui comment la résistance à l'insuline se produit à ce niveau. Or, on sait que plusieurs composants intervenant dans cette voie de signalisation ou impliqués dans le métabolisme du glucose sont modifiés par la *O*-GlcNAc. C'est le cas d'Akt, de GLUT4, de la caséine kinase II, de GSK-3 (Lubas & Hanover, 2000) et de IRS-1 et 2 (Patti et al., 1999) par exemple. De ce constat on ne peut imaginer que la *O*-GlcNAc prenne une part active dans la régulation du transport du glucose par le biais de la voie de l'insuline, et qu'une défection de la glycosylation de certains de ces composants soit à l'origine de la résistance à l'insuline et donc d'un transport diminué du glucose.

#### *2.6.1.4- Plusieurs drogues perturbant la dynamique de la O-GlcNAc sont diabétogènes.*

Trois inhibiteurs du cycle *O*-GlcNAc/dé-*O*-GlcNAc sont couramment utilisés. La *O*-GlcNAcase peut être inhibée par deux drogues : la streptozotocine (STZ, 2-déoxy-2-(3-5-méthyl-3-nitrosouréido)-D-glucopyranose) et le PUGNAc (*O*-(2-acétamido-2-déoxy-D-glucopyranosylidène)amino-N-phénylcarbamate). L'OGT est inhibée par l'alloxane (Konrad et al., 2002), un analogue de l'uracile (Fig. 4).

La streptozotocine et l'alloxane sont très largement utilisés pour produire des modèles animaux diabétiques (Szkudelski, 2001). Leur action est ciblée sur les cellules bêta pancréatiques et leur mode d'action est bien connu. Les deux drogues sont rapidement transportées dans la cellule par les transporteurs GLUT2, et leur effet diabétogène résulte de la production de radicaux libres par ces deux produits. Par contre le mécanisme de production de radicaux libres est différent entre les deux drogues.

En solution, l'alloxane (hydrophile et instable) est en équilibre rédox avec son produit de réduction, l'acide dialurique, ce qui génère des radicaux superoxyde ($O_2^{\cdot}$). Sous l'action de la superoxyde dismutase, les radicaux superoxyde forment du peroxyde d'hydrogène ($H_2O_2$). Le peroxyde d'hydrogène subit la réaction de Fenton et est scindé en deux radicaux hydroxyles ($OH^{\cdot}$) hautement réactifs et toxiques pour la cellule. En conjonction avec un flux calcique

accru, ces radicaux provoquent la mort des cellules bêta et, par conséquent, entraînent une diminution de la sécrétion d'insuline.

La streptozotocine provoque une alkylation de l'ADN, plus particulièrement sur l'$O^6$ de la guanine, par l'intermédiaire de son groupement nitroso-urée. L'ADN endommagé induit une activation de l'ADP-ribosylation et la poly-ADP-ribosylation résultant en une consommation accrue de $NAD^+$ et d'ATP : les deux molécules sont alors « déplétées » de la cellule. Le groupement nitroso-urée est également une source de NO lorsque la STZ est métabolisée dans la cellule : ce NO est un producteur indirect de radicaux libres (augmentation de l'activité xanthine oxydase) qui infligent à leur tour des dommages à l'ADN.

Partant du fait que l'alloxane et la STZ sont des « producteurs » de radicaux libres, et que ces drogues sont inhibitrices des enzymes responsables de la dynamique de la *O*-GlcNAc, nous pouvons supposer que les effets de ceux-ci sur les cellules bêta, soient le résultat d'une synergie des deux phénomènes induisant l'effet diabétogène. Il semble donc que la *O*-GlcNAc prenne une part active dans le développement de la résistance à l'insuline et dans la toxicité du glucose. Cet effet doit être plus particulièrement amplifié par le fait que l'OGT est enrichie au niveau du pancréas (Hanover et al., 1999). Ceci expliquerait pourquoi la STZ est aussi toxique pour les cellules bêta : une forte expression de l'OGT dans ces cellules résulterait en une glycosylation abondante des protéines par la *O*-GlcNAc qui ne pourrait plus être contrecarrée par la *O*-GlcNAcase (puisque inhibée).

### *2.6.2- Les niveaux de O-GlcNAc sont modifiés dans certaines maladies neurodégénératives.*

#### *2.6.2.1- La O-GlcNAc est retrouvée en abondance dans le système nerveux central.*

Même s'il est difficile aujourd'hui de dresser la liste complète des protéines *O*-GlcNAc identifiées (Tableau II), bon nombre d'entre elles ont une localisation spécifiquement neuronale. La plupart de ces protéines neuronales *O*-N-acétylglucosaminylées sont impliquées dans le développement de maladies neurodégénératives, telle que la protéine Tau ou le précurseur du peptide amyloïde dans la maladie d'Alzheimer ou encore les neurofilaments dans la sclérose amyotrophique latérale.

Les trois points suivants soulignent l'importance de la *O*-GlcNAc dans la physiologie des neurones :

Fonctions associées à la *O*-GlcNAc.

- l'étude de l'expression de la *O*-GlcNAc dans des neurones, de souris saines de différents âges, a démontré que cette glycosylation était ubiquitairement exprimée de l'embryon (10 jours) à l'adulte, sans différence significative d'expression selon la fraction subcellulaire examinée (Rex-Mathes et al., 2001) ;

- le synaptosome (synapse isolée du neurone) est riche en protéines *O*-N-acétylglucosaminylées (Cole & Hart, 2001) et la terminaison nerveuse est enrichie en OGT et *O*-GlcNAcase (Akimoto et al., 2003) ;

- des cellules neuronales en culture (Kelly) sont plus sensibles à la dérégulation de la balance phosphorylation/*O*-GlcNAc provoquée par l'utilisation de l'acide okadaïque (inhibiteur de phosphatases à spectre large, voir chapitre suivant) que ne le sont les cellules HeLa ou Cos (Lefebvre et al., 1999).

Les premiers travaux portant sur le système nerveux et la *O*-GlcNAc ont été effectués sur des cerveaux humains autopsiés non pathologiques et de patients atteints de la maladie d'Alzheimer (Griffith & Schmitz, 1995). On a ainsi établit, à cette époque une augmentation des niveaux de *O*-GlcNAc chez les personnes atteintes de la maladie d'Alzheimer. Par contre, d'autres études effectuées un peu plus tard n'ont pas permis de mettre en évidence de différences notables dans les niveaux de *O*-GlcNAc chez les patients Alzheimer comparés aux personnes saines, hormis sur une protéine de 160 kDa identifiée comme AP-3 (Yao & Coleman, 1998). Plus récemment, Robertson et al. ont montré que la *O*-GlcNAc était réduite dans des préparations enrichies en « heat-stable Tau » de patients Alzheimer ou souffrant de démence fronto-temporale (Robertson et al., 2004). Ces derniers auteurs imputent les différences trouvées dans les niveaux de *O*-GlcNAc par ces trois équipes, aux méthodes de préparation des échantillons et d'analyses utilisées pour mettre en évidence cette glycosylation.

#### *2.6.2.2- De nombreuses protéines spécifiques du neurone sont O-N-acétylglucosaminylées.*

Nous ne détaillerons pas la liste des protéines neuronales modifiées par la *O*-GlcNAc. Celles-ci sont pour la plupart répertoriées dans le tableau II. Signalons ici simplement que deux protéines directement impliquées dans la maladie d'Alzheimer sont *O*-N-acétylglucosaminylées. Il s'agit de Tau (Arnold et al., 1996) qui permet l'assemblage, la

stabilisation et l'orientation des microtubules, et de la protéine précurseur du peptide amyloïde (Griffith et al., 1995). Tout semble supposer qu'une déficience en *O*-GlcNAc sur Tau associée à une hyperphosphorylation (Lefebvre et al., 1999 ; Robertson et al., 2004 ; Liu et al., 2004) joue un rôle essentiel dans le dysfonctionnement de la protéine Tau et dans le développement de la pathologie neurodégénérative (Lefebvre et al., 2005).

#### *2.6.2.3- Les neuropathies se caractérisent par une agrégation protéique.*

L'agrégation protéique et le dysfonctionnement du système ubiquitine-protéasome (UPS, « Ubiquitin-Proteasome System ») sont deux caractéristiques fondamentales des maladies neurodégénératives (Bence et al., 2001). La mauvaise conformation, ainsi que l'agrégation protéique, peut se produire aussi bien dans la cellule elle-même (formation de corps d'inclusion) que dans l'espace intracellulaire.

Les agrégats consistent en la formation de fibres, constituées de protéines mal repliées ou « dénaturées », adoptant une conformation en feuillets bêta (« beta-sheets »), appelées amyloïdes. En voici quelques exemples :

- dans la maladie de Huntington, l'huntingtine est le principal composant des dépôts. Cette agrégation est le résultat de répétitions CAG codant des séquences de glutamine (polyglutamine).

- dans la maladie de Parkinson, c'est l'alpha-synucléine qui forme les agrégats.

- dans la sclérose amyotrophique latérale, le facteur s'agrégeant reste inconnu à ce jour.

- dans les maladies à Prions, c'est la protéine Prion qui forme des agrégats.

L'agrégation peut être le résultat d'une déficience de repliement de la protéine impliquée, ce qui augmente son insolubilité ; le résultat d'une accélération de sa synthèse ou le résultat d'une diminution de la dégradation de celle-ci. Cette dernière idée a été largement renforcée par le fait que l'ubiquitine est retrouvée en très grande quantité dans les agrégats protéiques et les cellules nerveuses mortes. Ceci amène la conclusion que l'UPS est déréglé et que le protéasome est très certainement inhibé. Par exemple les agrégats d'huntingtines peuvent être directement marqués par des anticorps dirigés contre l'ubiquitine : ceci peut même servir de marqueur de l'agrégation protéique et du dysfonctionnement protéasomal.

Fonctions associées à la *O*-GlcNAc.

Les marqueurs pathologiques de la maladie de Parkinson sont les corps de Lewy, inclusions de fibrilles et de protéines mal conformées. Comme pour la maladie de Huntington, les corps de Lewy peuvent être marqués par des anticorps anti-ubiquitine mais également anti-synucléine, anti-parkine, anti-syphiline, anti-neurofilaments et anti-« protéines » associées aux vésicules synaptiques. Dans la sclérose amyotrophique latérale, des agrégats ubiquitinés sont également retrouvés dans des cerveaux de malades. Enfin, les maladies à Prions sont causées par un repliement anormal de la protéine Prion à l'intérieur et à l'extérieur du neurone.

L'exemple le plus connu de processus d'agrégation est celui représenté par les plaques neurales ou plaques séniles, retrouvées dans la maladie d'Alzheimer, et dont le composant majeur est le peptide amyloïde bêta. Ce peptide de 42 acides-aminés provient du clivage séquentiel du précurseur du bêta-amyloïde (APP, beta-amyloid precursor protein). Le clivage d'APP (protéine membranaire de type I) est produit, soit par une voie dite non-amyloïdogénique (qui génère un fragment soluble APPα et un fragment Aβ) ou une voie amyloïdogénique (générant le fragment Aβ et un fragment soluble APPβ). Il a été démontré que APP était modifiée par la *O*-GlcNAc (Griffith et al., 1995). Le rapport entre la glycosylation d'APP et son clivage n'est pas connu.

#### *2.6.2.4- La dégradation protéasomale est défaillante dans les maladies neurodégénératives.*

Il a été reporté très récemment que la machinerie protéasomale était modifiée par la *O*-GlcNAc (Sumegi et al., 2003 ; Zhang et al., 2003) et pouvait même être régulée par cette glycosylation puisque le protéasome était inhibé après modification par l'OGT (Zhang et al., 2003). On a proposé que, puisque la *O*-GlcNAcase est localisée sur le locus 10q24.1-24.3, région chromosomique fréquemment mutée chez les patients Alzheimer (Bertram et al., 2000 ; Myers et al., 2000), des altérations génétiques du gène de la *O*-GlcNAcase résulteraient en une altération de l'activité protéasomale : la *O*-GlcNAc n'étant plus hydrolysée de la sous-unité 19S du protéasome, celui-ci serait inhibé. Dans ces conditions, le protéasome ne pourrait plus dégrader ses substrats, la seule alternative étant la voie de l'autophagie. Pour renforcer cette idée, il semble que les protéines substrats du protéasome modifiées par la *O*-GlcNAc seraient résistantes à cette dégradation (Han & Kudlow, 1997 ; Cheng et al., 2000 ; Hatsell et al., 2003). Ainsi, si la *O*-GlcNAc protége les protéines de la dégradation et que cette même

glycosylation inhibe le protéasome, les deux phénomènes agiraient en synergie : la dégradation protéique s'en retrouverait donc très largement réduite.

L'ubiquitine carboxyl hydrolase-L1 (UCH-L1), une enzyme de dé-ubiquinitination synaptosomale, est également modifiée par la *O*-GlcNAc (Cole & Hart, 2001). Même si le rôle de la *O*-GlcNAc sur UCH-L1 est inconnu, nous pouvons supposer que la glycosylation de l'enzyme entrave plus encore le processus de dégradation. Dans ce domaine il serait très intéressant de regarder si une telle glycosylation existe sur parkine, une enzyme E3 ligase impliquée dans la maladie de Parkinson et catalysant l'addition de l'ubiquitine sur les substrats à dégrader par la voie de l'UPS. Il faut noter que des mutations du gène codant UCH-L1 et du gène codant parkine seraient à l'origine de la pathologie.

Enfin il est important de souligner ici que certaines protéines chaperonnes et certains composants du protéasome sont également retrouvés dans les corps d'inclusion : ceci renforce le problème d'une dérégulation de l'UPS dans des désordres neuronaux.

#### *2.6.2.5- Le métabolisme du glucose joue un rôle essentiel dans le bon fonctionnement neuronal.*

Le glucose permet le contrôle de beaucoup de processus cellulaires allant de la production d'ATP à la synthèse de neurotransmetteurs. Le métabolisme du glucose et, par conséquent, la charge énergétique sont modifiés dans le système nerveux central des personnes âgées et ce, aussi bien dans la voie de signalisation de l'insuline que de l'acétylcholine. De nombreuses preuves suggèrent qu'au cours du vieillissement, ou au cours du développement de la maladie d'Alzheimer, le cerveau adapte son utilisation du glucose. Des changements dans ce métabolisme ont été montrés dans de nombreuses maladies neurodégénératives et ces modifications seraient impliquées dans le processus pathologique (Hoyez, 1998). Une hypothèse envisage que le cerveau diminue le métabolisme de son glucose pour favoriser les processus de *O*-N-acétylglucosaminylation. La consommation du glucose serait également préservée pour les réactions anaboliques et, dans ce cas, l'utilisation des corps cétoniques est augmentée (Heininger, 2000).

Il est intéressant de noter que les neuropathies diabétiques sont en fait des complications des diabètes, et sont liées à un mauvais contrôle de la glycémie. Ainsi le diabète doublerait le risque de développer une maladie neurodégénérative (Craft et al., 1996). Il est connu que le transport et l'utilisation du glucose chez les patients atteints de la maladie d'Alzheimer sont

défaillants et il semble que cette déficience soit due à la neurodégénérescence. Des expériences menées sur des primates ont démontrées une corrélation entre la diminution de la consommation du glucose cérébral et la perte de mémoire (Blaizot et al., 2002). Bien qu'il apparaisse que la *O*-GlcNAc soit déficiente dans la maladie d'Alzheimer, la corrélation entre l'utilisation du glucose et les problèmes mémoriels n'a pas été faite. Pour tenter de répondre en partie à cette question, la *O*-N-acétylglucosaminylation de protéines de 19 cerveaux de personnes ayant souffert d'Alzheimer et de 15 cerveaux sains a été déterminée en fonction du délai post-mortem (Liu et al., 2004). L'analyse des résultats a démontré que la *O*-GlcNAc était de 22% plus faible dans les cerveaux pathologiques, démontrant une implication de cette glycosylation dans cette maladie.

### *2.6.3- Des dérégulations dans les niveaux de O-GlcNAc pourraient influer sur les processus de tumorisation/Relation O-GlcNAc et activité transcriptionnelle.*

Les cancers se caractérisent par une prolifération cellulaire incontrôlée pouvant résulter de la mutation d'oncogènes ou de gènes codant des suppresseurs de tumeurs et par la transcription aberrante de gènes dits cancer-spécifiques. Parmi toutes les protéines décrites comme étant modifiées par la *O*-GlcNAc plusieurs sont impliquées dans les processus de cancérisation. Ces protéines peuvent être des oncogènes comme c-Myc (Chou et al., 1995) et v-erb-A (Privalsky, 1990), des suppresseurs de tumeurs comme p53 (Shaw et al., 1996) ou HIC1 (Lefebvre et al., 2004a). A ceux-ci nous pouvons ajouter que beaucoup de facteurs impliqués dans les processus transcriptionnels sont eux-mêmes *O*-N-acétylglucosaminylés (voir ci-dessous).

#### *2.6.3.1- L'activité transcriptionnelle est en partie contrôlée par les mécanismes de O-GlcNAc/dé-O-GlcNAc.*

Le rôle de la *O*-GlcNAc dans les mécanismes transcriptionnels a été décrit pour plusieurs facteurs de transcription, dont le facteur de transcription, dit « de ménage ou domestique », Sp1 (Jackson & Tjian, 1988 ; Han & Kudlow, 1997 ; Weigert et al., 2003). Cette glycosylation peut avoir des conséquences fonctionnelles diverses sur l'activité transcriptionnelle de la protéine.

Fonctions associées à la *O*-GlcNAc.

***2.6.3.1.1- La O-GlcNAc modifie la sensibilité protéasomale des protéines.***

Comme nous l'avons décrit dans la partie dédiée aux maladies neurodégénératives, il a été signalé une relation entre la *O*-GlcNAc et la dégradation des protéines intracellulaires par le protéasome. C'est le cas de Sp1 qui, sous sa forme hyperglycosylée (traitement de cellules en culture avec de la glucosamine), voit sa demi-vie augmentée alors que dans des conditions de déprivation en glucose (état hypoglycosylé) Sp1 est moins stable, conséquence d'une protéolyse augmentée (Han & Kudlow, 1997). Cette modulation de glycosylation en fonction des conditions de culture est le reflet d'une activité de la voie de biosynthèse des hexosamines accrue ou diminuée. Autre exemple, celui du récepteur bêta des oestrogènes glycosylé sur la sérine 16, site de phosphorylation localisé à proximité d'une séquence PEST (Cheng et al., 2000). La mutation du site de *O*-GlcNAc sur la Ser16 par un acide glutamique mimant une phosphorylation constitutive, montre une dégradation plus rapide de la protéine mutante. Des études sur le récepteur alpha des oestrogènes ont également démontré que certains sites de glycosylation étaient proches de séquences PEST (Cheng & Hart, 2000). Ces données confortent l'hypothèse selon laquelle la *O*-GlcNAc freine la dégradation protéique et suggère une régulation du « turnover » des protéines par la réciprocité *O*-GlcNAc/phosphorylation.

***2.6.3.1.2- La O-GlcNAc régule les interactions protéiques.***

La régulation de la transcription par la *O*-GlcNAc peut également s'effectuer par l'intermédiaire de la modulation d'interactions protéiques. C'est ainsi que la présence d'un résidu *O*-GlcNAc dans le domaine de transactivation riche en glutamine de Sp1 (B-c) modifie les interactions de type hydrophobe avec deux de ces partenaires, TAF110 et holo-Sp1 : la glycosylation bloque en effet ces interactions (Roos et al., 1997 ; Yang et al., 2001). Plus récemment, il a été décrit que la glycosylation de CREB sur deux sites dans le domaine Q2 rompait l'interaction entre celui-ci et TAFII (Lamarre-Vincent et al., 2003), inhibant l'activité transcriptionnelle de CREB. En 2002, un travail particulièrement intéressant indiquait directement le lien entre la *O*-GlcNAc et la répression transcriptionnelle (Yang et al., 2002). L'OGT interagirait avec le co-répresseur mSin3A pour être ciblée vers les promoteurs de gènes à inactiver : cette enzyme réprimerait ainsi la transcription par glycosylation de l'ARN polymérase II et des facteurs de transcription. Ce mécanisme HDAC-indépendant agirait de concert avec la déacétylation des histones dans la répression transcriptionnelle (Fig. 3).

Fonctions associées à la *O*-GlcNAc.

### *2.6.3.1.3- L'interaction de certains facteurs de transcription avec leur cible nucléotidique pourrait être modulée par la O-GlcNAc.*

La modulation des interactions protéine-cible nucléotidique pourrait être un autre niveau de régulation de l'activité transcriptionnelle par la *O*-GlcNAc. Par exemple, le suppresseur de tumeurs p53 contient une région C-terminale riche en acides aminés basiques jouant un rôle très actif dans la répression transcriptionnelle. Il a été ensuite démontré une corrélation entre la modification de Sp1 par la *O*-GlcNAc et sa capacité à lier l'ADN : en effet la glycosylation de la région C-terminale par la *O*-GlcNAc abroge la répression (Shaw et al., 1996). Sur le facteur de transcription Sp1, une corrélation entre l'activité de fixation sur l'ADN et la glycosylation a été établie. Cette activité de liaison à l'ADN est augmentée par le palmitate, lequel augmente l'expression de la GFAT et donc favorise la synthèse d'UDP-GlcNAc via la voie de biosynthèse des hexosamines (Fig. 4). A l'inverse, cette activité de liaison à l'ADN est diminuée quand Sp1 est déglycosylé enzymatiquement (Weigert et al., 2003). Pour notre part nous avons étudié l'effet de la glycosylation de HIC1 sur sa capacité à lier l'ADN (Lefebvre et al., 2004a). La glycosylation de HIC1 est plus particulièrement localisée au niveau du domaine de liaison à l'ADN (DBD, DNA-binding domain). Or les formes glycosylées et non-glycosylées de HIC1 fixent de la même manière une sonde oligonucléotidique suggérant que la glycosylation n'intervient pas dans l'activité de reconnaissance. Par contre, des formes tronquées de HIC1 en N-terminal ne sont capables de se lier à l'ADN que sous leur forme non glycosylée. L'importance biologique et fonctionnelle de cette particularité reste à découvrir, d'autant plus que ces formes courtes de HIC1 ne sont pas glycosylées dans leur DBD mais au niveau de l'extrémité C-terminale. Enfin, le facteur de transcription PDX-1 possède deux sites majeurs de *O*-GlcNAc et la glycosylation favorise la reconnaissance nucléotidique (Gao et al., 2003).

### *2.6.3.2- Le déroulement du cycle cellulaire est régulé en partie par la O-GlcNAc.*

Les mécanismes de la progression tumorale sont caractérisés par une prolifération cellulaire incontrôlée. Cette prolifération anarchique est, en général, le résultat de dysfonctionnements du cycle cellulaire (Golias et al., 2004). Plusieurs études tendent à montrer que la régulation du cycle cellulaire et les processus de *O*-N-acétylglucosaminylation

sont intimement liés et confortent même l'hypothèse selon laquelle la *O*-GlcNAc, en tant que telle, puisse réguler ce cycle :

- il a été observé, par exemple, des changements dans les degrés de *O*-GlcNAc lymphocytaires lorsque ceux-ci étaient activés par des agents mitogènes,

- la déstabilisation des microtubules chez les cellules HT29 provoque une augmentation de la *O*-GlcNAc sur des cytokératines (Haltiwanger & Philipsberg, 1997),

- la modification des résidus de *O*-GlcNAc par transfert de galactose à l'aide de la galactosyltransférase bovine inhibe l'entrée en phase M des ovocytes de Xénope et bloque la transition S/M (Fang & Miller, 2001),

- sur le même modèle, on a démontré que la transition G2/M s'accompagnait d'une augmentation globale de *O*-GlcNAc (Lefebvre et al., 2004b).

Dernièrement, l'utilisation de PUGNAc dans l'inhibition de la *O*-GlcNAcase a montré que le blocage des processus de déglycosylation ralentissait la progression du cycle cellulaire de cellules en culture (Slawson et al., 2005). Ce retard est plus important au cours de la phase S et lors de la transition G2/M, ce qui corrobore nos études effectuées sur l'ovocyte de Xénope (Lefebvre et al., 2004b). A l'inverse, l'inhibition de la GFAT avec le DON diminue, d'un côté, les niveaux de *O*-GlcNAc et, de l'autre, accélère la phase M et la transition G2/M. Cette inhibition de la GFAT retarde en contrepartie la progression des cellules en G1, en comparaison avec les cellules contrôles ou traitées par le PUGNAc.

Indubitablement, ces dernières expériences démontrent que le dynamisme de la *O*-GlcNAc est un élément-clé dans la progression du cycle cellulaire. On suspecte fortement que des acteurs impliqués dans la régulation du cycle (CDK, cyclines, MAPK…) sont eux-mêmes modifiés par la *O*-GlcNAc et que cette glycosylation, qui peut entrer en compétition avec la phosphorylation, agisse comme un régulateur de leur activité. Nous rediscuterons de ce dernier point dans la troisième partie de notre mémoire.

Fonctions associées à la *O*-GlcNAc.

# Travaux personnels sur la *O*-GlcNAc

Fonctions associées à la *O*-GlcNAc.

Dans cette partie, j'ai choisi de résumer le travail présenté dans huit articles (sept publiés et un en cours de publication) et d'y ajouter deux revues. Ces documents sont, selon mon sentiment, les plus représentatifs de nos travaux sur la *O*-GlcNAc. Certains de ces travaux (que je continuerai à développer dans notre projet, troisième partie) ont été réalisés sous ma tutelle, par deux étudiantes que j'ai encadrées au cours de leur DEA/Master-Recherche et de leur thèse. Il s'agit de **Céline GUINEZ** pour la partie « activité lectinique des HSP70 » et de **Vanessa DEHENNAUT** pour la partie « régulation du cycle cellulaire ».

Les travaux concernant les variations de *O*-GlcNAc dans un modèle d'hypokinésie-hypodynamie réalisés par Caroline CIENIEWSKI au cours de son DEA, et en partie sous ma tutelle, ne seront pas exposés ici. Chacune de ces trois étudiantes a obtenu une allocation de recherche du Ministère de l'Education Nationale et de la Recherche.

### *3- Etude de la « balance » phospho-O-GlcNAc sur un système neuronal (Articles I, II et III).*

Afin d'appréhender le problème de réciprocité entre la *O*-GlcNAc et la phosphorylation, il nous fallait un système simple permettant de bloquer l'une ou l'autre modification post-traductionnelle et d'observer les changements espérés sur l'autre. Marie-Laure CAILLET-BOUDIN avait coutume d'utiliser un inhibiteur de phosphatases à large spectre, l'acide okadaïque, permettant de mimer l'hyperphosphorylation de Tau caractéristique de la maladie d'Alzheimer : en bloquant les processus de déphosphorylation, nous devions diminuer les processus de *O*-N-acétylglucosaminylation.

L'acide okadaïque est extrait de glandes digestives d'éponges marines colonisées par des dinoflagellés. C'est un inhibiteur des phosphatases PP1 ($IC_{50}$, 10-60 nM), PP2A ($IC_{50,}$ 0.1-1 nM) et PP2B ($IC_{50,}$ 5-10 µM).

Dans notre premier article (**Article I**), nous ne nous sommes pas intéressés à la glycosylation de Tau en particulier mais à la glycosylation des protéines globales cytosoliques et nucléaires. L'étude de l'effet de l'acide okadaïque sur trois lignées cellulaires, dont une d'origine neuronale (Kelly), nous a indiqué que cette lignée était plus « sensible » à cet inhibiteur, du fait que plusieurs bandes observées en SDS-PAGE avaient une masse moléculaire apparente supérieure (« shift » de masse) après traitement par l'acide okadaïque en comparaison aux

cellules contrôles (Article I, Fig. 1). Ces mêmes bandes étaient mises en évidence par la WGA (après N-déglycosylation et désialylation préalables) indiquant la présence du motif *O*-GlcNAc sur des protéines phosphorylées. Il était intéressant de constater que certaines protéines « shiftées » voyaient leur détection par la WGA diminuer, indiquant que leur contenu en *O*-GlcNAc était par conséquent plus faible. Ainsi l'inhibition des processus de déphosphosphorylation bloquait-il les processus de *O*-N-acétylglucosaminylation : les deux phénomènes pouvant donc être liés. Des résultats similaires ont été obtenus pour d'autres cellules neuronales telles que les cellules SY-5Y et les cellules CCF astrocytaires (résultats non publiés). Alors que ce premier article était sous presse, une équipe allemande publiait des résultats similaires en utilisant des activateurs et des inhibiteurs de kinases sur un système neuronal également (Griffith & Schmitz, 1999).

Dans ce travail nous avons également entrepris l'étude de l'effet de l'acide okadaïque sur la *O*-N-acétylglucosaminylation des protéines cytosoliques et nucléaires. Pour cela l'utilisation de la WGA (Article I, Fig. 2) a été renforcée par des marquages métaboliques en [$^{33}$P]-orthophosphate et [$^{14}$C]-glucosamine (Article I, Fig. 4-6) et par un marquage *in vitro* en utilisant la galactosyltransférase bovine et l'UDP-[$^{3}$H]-galactose (Article I, Fig. 7). Ces expériences nous ont démontré que l'effet de l'acide okadaïque était plus marqué dans la fraction nucléaire enrichie que dans la fraction cytosolique. Nous avons alors émis l'hypothèse que l'inhibition indirecte des processus de *O*-N-acétylglucosaminylation, par le blocage des protéines phosphatases, influençait le transport nucléaire a alors été émise.

L'étude de l'effet de l'acide okadaïque, sur la glycosylation globale des protéines neuronales, a été limitée à une protéine particulière, la protéine Tau (**Article II**). Pour cela nous avons utilisé une souche de cellules Kelly exprimant de manière stable (Kelly Cl.16) l'isoforme la plus longue de Tau (sur les six cytoplasmiques connues) : Tau441. Cette protéine possède 441 acides aminés. Comme nous l'avons indiqué plus haut, Tau est une protéine impliquée dans la polymérisation et la stabilité des microtubules neuronaux : elle fait partie des « Microtubule-associated proteins ». Tau est connue pour son haut potentiel de phosphorylation, et l'hyperphosphorylation de cette protéine est en partie corrélée à des dysfonctionnements neuronaux. De nombreux autres types de modifications post-traductionnelles sont retrouvées sur Tau et parmi celles-ci, la *O*-GlcNAc (Arnold et al., 1996).

Dans cette étude, nous avons tout d'abord démontré que Tau441 était *O*-GlcNAc (Article II, Fig. 1), comme la protéine Tau endogène, corroborant les travaux d'Arnold et al. (Arnold et

al., 1996). Sur le même principe de base que celui mis en place dans l'article I, c'est-à-dire en utilisant l'acide okadaïque, nous avons montré que l'hyperphosphorylation de Tau était associé à une diminution de son niveau de *O*-GlcNAc, ainsi que la localisation de sa forme nucléaire, corrélant ainsi phosphorylation/*O*-GlcNAc et transport nucléaire (Article II, Fig. 3).

Des études de Tau441 par électrophorèse bidimensionnelle nous ont permis de montrer que les isoformes cytosoliques les plus acides, c'est-à-dire les isoformes les plus phosphorylées de Tau441, sont les moins glycosylées (Article II, Fig. 4). Des électrophorèses bidimensionnelles de Tau441 nucléaires ont indiqué que toutes les isoformes nucléaires étaient glycosylées (Article II, Fig. 6). Ainsi comme cela avait déjà été suggéré, la *O*-GlcNAc serait un signal de transport et/ou de rétention des protéines cytosoliques vers le noyau. Une telle éventualité avait déjà été soulevée par d'autres auteurs (Duverger et al., 1996).

Récemment, des études ont permis d'établir que certains sites de phosphorylation de Tau étaient en compétition directe avec la *O*-GlcNAc (Fig. 6). Ceci a pu être établi grâce à l'utilisation d'anticorps phosphodépendants très précis (Liu et al., 2004 ; Robertson et al., 2004). Une synthèse de ces différentes données est présentée dans une de nos revues (**Article III**).

### *4- Recherche de protéines « navettes » assurant le transport de protéines cytosoliques modifiées par la O-GlcNAc vers le noyau (Article IV et V).*

Fort de nos précédents résultats incluant une étude sur les facteurs de transcription Pax-6 qui ne sera pas présentée ici (Lefebvre et al., 2002 ; collaboration Dr. Simon Saule, Institut de Biologie de Lille, Lille) et en nous appuyant sur diverses études réalisées par d'autres auteurs (Hubert et al., 1989 ; Duverger et al., 1993, 1995, 1996 ; Rondanino et al., 2003), nous avons émis l'hypothèse suivante : **si la *O*-GlcNAc peut servir de signal de transport de protéines cytosoliques vers le noyau, alors il doit exister des protéines dotées d'une activité lectinique envers ce résidu *O*-GlcNAc et capables de faire la navette entre le cytosol et le noyau**.

Fonctions associées à la *O*-GlcNAc.

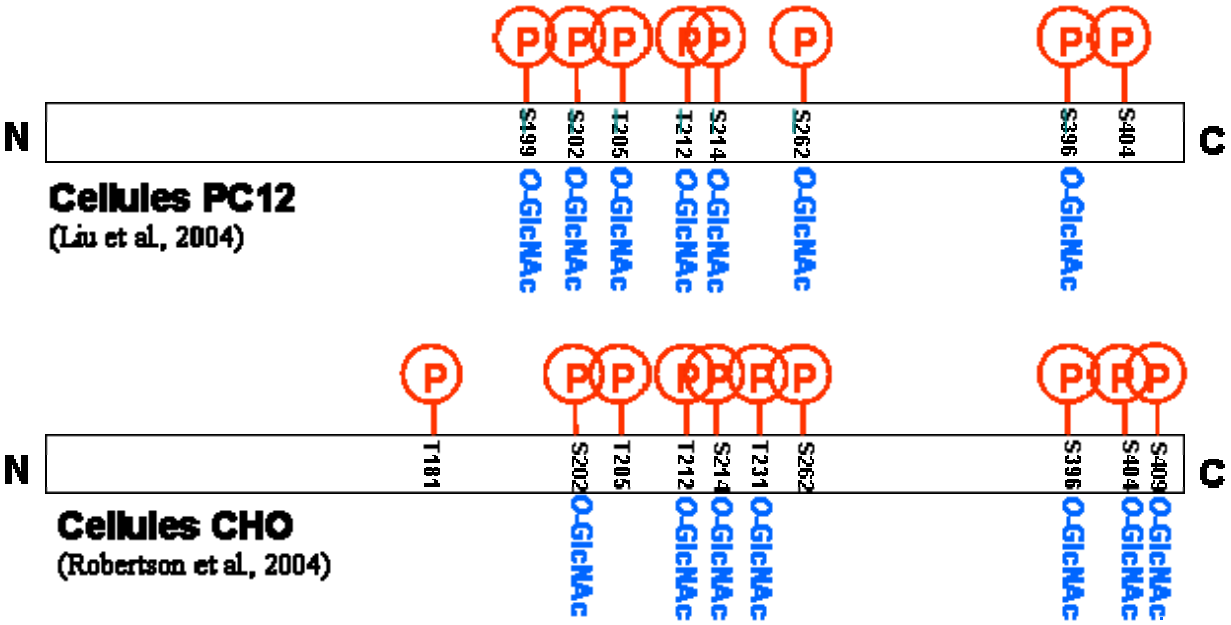



**Figure 6- Sites décrits de phosphorylation et de *O*-GlcNAc sur la protéine Tau.**

La différence de localisation de sites et de relation éventuelle entre les deux modifications post-traductionnelles peut s'expliquer par les systèmes utilisés : PC12 pour la première et système d'expression en CHO pour la seconde.

Pour rechercher ces éventuelles « navettes », nous avons entrepris la caractérisation et l'identification de « GlcNAc-binding proteins » cytosoliques et nucléaires par chromatographie d'affinité sur GlcNAc immobilisée de fractions cytosolique et nucléaire de foie de rat. Des études en SDS-PAGE et en électrophorèse bidimensionnelle des protéines enrichies nous ont permis de mettre en évidence trois protéines majeures cytosoliques et nucléaires (Article IV, Fig. 1) de 70, 65 et 55 kDa. Ces protéines, qu'elles soient cytosoliques ou nucléaires, diffèrent selon leur pHi. Ces protéines sont phosphorylées essentiellement sur des résidus de sérine (Article IV, Fig. 2A, B) et sont elles-mêmes *O*-N-acétylglucosaminylées (Article IV, Fig. 2C, D). Ces lectines, spécifiques des résidus de GlcNAc, existent sous la forme de complexes multimoléculaires comme l'ont attesté des électrophorèses en gel d'acrylamide en mode non dénaturant (Article IV, Fig. 3) et des expériences de gel-filtration (Article IV, Fig. 4). L'identité d'une de ces protéines a pu être déterminée grâce à la technique dite d'« approche protéomique ». Il s'agit d'Hsc70 (70 kDa-Heat shock cognate protein), première protéine identifiée comme capable de reconnaître certaines protéines par leur motif

Fonctions associées à la *O*-GlcNAc.

*O*-GlcNAc (Article IV, Fig. 5, 6 et 7), et première protéine de choc thermique connue comme étant *O*-GlcNAc.

Un parallèle entre Hsc70 et CBP70 (70 kDa-Carbohydrate Binding Protein, Felin et al., 1994) peut être établi, sans prouver qu'il s'agisse réellement de la même protéine. Les deux protéines ont la même masse moléculaire, la même localisation sub-cellulaire (CBP70 a été également retrouvée dans le réticulum endoplasmique et l'appareil de Golgi comme certaines isoformes de Hsp70, i.e. BiP) (Rousseau et al., 2000a) et ces deux protéines ont une activité lectinique persistante au stress thermique (Rousseau et al., 2000b). Malheureusement, les études menées sur CBP70 ont été abandonnées (Botti, J., communication personnelle) et la confirmation ou l'infirmation de la double identité ne pourra être effectuée pour le moment.

Le rôle d'Hsc70 (Okuno et al., 1993), et plus largement d'Hsp70 (Shi & Thomas, 1992), dans la translocation nucléaire avait déjà été soulevé. Or, il ne semble pas que ces études aient été poursuivies.

Pour notre part, nous avions proposé un modèle de mécanisme de transport nucléaire joué par ces protéines de choc thermique (article V, Fig. 2). Ce modèle fait intervenir le complexe multimoléculaire lectinique comportant Hsc70 et les protéines du pore nucléaire elles-mêmes *O*-N-acétylglucosaminylées sur leur face cytoplasmique (Snow et al., 1987 ; Holt et al., 1987a). De cette manière la protéine à transporter se rapprocherait du pore nucléaire ; le complexe lectinique pourrait « ponter » cette protéine avec le pore nucléaire par l'intermédiaire des résidus *O*-GlcNAc. Il n'est pas exclu que le NLS participe à ce transport *O*-GlcNAc-dépendant et que plutôt que d'avoir deux systèmes de transport parallèles et autonomes, nous ayons en fait une complémentarité des deux mécanismes. **Ceci reste bien évidemment hypothétique**. Plus récemment, nous avons tenté de mettre en évidence une interaction physique entre le pore nucléaire et Hsc70, mais ces expériences sont restées infructueuses pour le moment et aucun contact n'a pu être mis formellement en évidence entre ces deux partenaires potentiels.

Cet aspect du transport *O*-GlcNAc-dépendant, des protéines cytosoliques vers le noyau, a été laissé un peu de côté pour privilégier la propriété lectinique des HSP70 dans la protection potentielle des protéines intracellulaires. Cette partie a fait l'objet du sujet de thèse de Céline GUINEZ et a permis la publication de deux articles scientifiques (Guinez et al., 2004 - article VI - ; Guinez et al., 2005 - article VIII). Un article sera soumis pour publication (article VII) et un autre est en cours de rédaction.

Fonctions associées à la *O*-GlcNAc.

### *5- Etude de l'activité lectinique des HSP70 envers les résidus de O-GlcNAc (Articles VI, VII et VIII).*

Après avoir mis en évidence l'existence d'une activité lectinique envers les résidus de *O*-GlcNAc de la protéine Hsc70 (Lefebvre et al., 2001), nous avons orienté une partie de nos activités de recherche sur cette nouvelle propriété des protéines de choc thermique. Ce choix de thématique a été motivé par plusieurs observations effectuées par différents groupes. Des éléments sur la relation entre la *O*-GlcNAc et l'activité protéasomale a été en partie décrite dans le paragraphe 2.3.2.1.1.

Han et Kudlow ont été les premiers à établir une relation entre la glycosylation de Sp1 et sa « sensibilité » protéasomale (Han & Kudlow, 1997). D'autres exemples semblent indiquer que la *O*-N-acétylglucosaminylation stabilise certaines protéines par une diminution de leur dégradation protéasomale. C'est le cas des récepteurs aux oestrogènes (Cheng et al., 2000 ; Cheng et al., 2001) et de la plakoglobine (Hatsell et al., 2003).

La question posée ici était simple : **quel élément permet aux protéines *O*-GlcNAc d'être plus stables ?** La réponse semblait, pour nous, résider dans le fait que certaines protéines de choc thermique possédaient une activité lectinique dirigée contre le résidu *O*-GlcNAc. Nous l'avions démontré pour Hsc70, qu'en était-il de la forme induite, Hsp70 ?

Notre premier travail sur ce thème a été de regarder si Hsp70 était douée d'une activité lectinique comme la forme constitutive Hsc70. Ceci a été réalisé dans des conditions normales de température (37°C) et dans des conditions de stress thermique (hyperthermie à 42°C), puisque Hsp70 est majoritairement induite après un stress. Ainsi nous avons pu démontrer qu'Hsp70 était bien capable de fixer des résidus de GlcNAc tout comme Hsc70 (Article VI, Fig. 1), surtout après qu'un stress thermique soit survenu : ceci est dû en partie à la synthèse même, c'est-à-dire à une expression d'Hsp70. La question suivante était donc de savoir si une telle activité lectinique pouvait être induite uniquement par le stress thermique. Pour répondre à cette question, nous avons induit un stress du type nutritionnel : la déplétion en glucose (Article VI, Fig. 2). Tout comme le stress thermique, le stress nutritionnel induit une activité lectinique d'Hsp70. D'autres types de stress ont également été étudiés : stress oxydant (peroxyde d'hydrogène), stress osmotique (NaCl), stress aux métaux lourds ($HgCl_2$), stress à l'arsenic (Fig. 7). Pratiquement tous les types de stress se sont avérés déclencheurs d'activité

lectinique sur Hsp70. Des études de stress éthanolique et d'exposition aux rayons ultraviolets ont également été effectués : ils ont également déclenché l'activité lectinique (non montré). Ceci suggère que le stress cellulaire provoque d'une part la synthèse d'Hsp70 mais également « dévoile » sa propriété de lectine envers les résidus de *O*-GlcNAc.

**A quoi cela peut-il bien servir ?**

L'équipe de G.W. Hart a décrit en 2004 un phénomène mettant en jeu la *O*-GlcNAc après qu'un stress cellulaire ait eu lieu (Zachara et al., 2004). Selon ces auteurs la *O*-GlcNAc pourrait permettre à la cellule de répondre à un stress et surtout de surmonter le choc occasionné. Nous sommes en effet partis de cette observation pour mettre au point l'expérience décrite dans la figure 7. Il semble que l'on n'ait pas de réelle corrélation entre **le stress cellulaire, l'augmentation de *O*-GlcNAc et l'activité lectinique déclenchées par ces stress.**

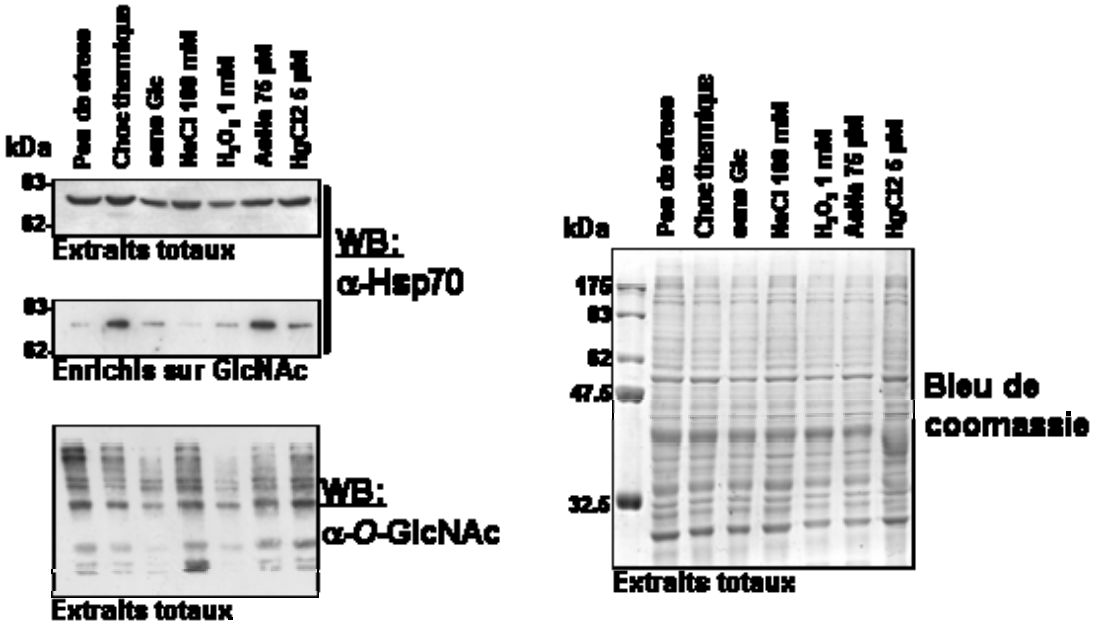



**Figure 7- Effet de stress de natures diverses sur l'activité lectinique d'Hsp70 envers la *O*-GlcNAc.**

Pour cette expérience des cellules HepG2 ont été soumises à des stress variés pendant 24h. Après lyse des cellules et passage des extraits cellulaires sur billes d'agarose couplées à la N-acétylglucosamine, l'expression et l'activité lectinique d'Hsp70 ont été « mesurées ». Une détection de la *O*-GlcNAc a été effectuée avec un anticorps monoclonal (RL2) et la charge des dépôts a été vérifiée par coloration au bleu de coomassie d'une électrophorèse en SDS-PAGE des différents extraits cellulaires utilisés.

**Que ce passe t-il au sein de la cellule après que celle-ci a subi une agression ?**

Lorsqu'une cellule subit un stress, le taux de protéines de choc thermique, dont fait partie Hsp70, augmente. Cette activation de l'expression des HSP s'effectue en partie par la dissociation d'un complexe Hsp70/HSF (Heat Shock Factor), qui se trouve dans le cytosol. Ceci permet à HSF, après que celui-ci soit phosphorylé et qu'il se soit trimérisé, de migrer vers le noyau, pour s'associer au « Heat Shock Response Element » et induire l'expression de Hsp70. Le premier rôle d'Hsp70 est d'empêcher l'agrégation des protéines endommagées. Cette agrégation est souvent le fruit d'une association (« empilement ») de protéines ayant une partie hydrophobe normalement enfouie à l'intérieur de leur structure tridimensionnelle, qui se retrouve alors exposée au milieu extérieur. Le second rôle d'Hsp70 est de tenter de remettre en bonne conformation la protéine endommagée et de lui rendre sa fonction originelle. Ceci s'effectue par une association avec des protéines co-chaperonnes telles que CHIP ou Bag-1 et par une dépense énergétique (hydrolyse d'ATP). Or, comme nous l'avons décrit dans la partie introductive de ce mémoire, l'OGT greffe un groupement *O*-GlcNAc sur des séquences similaires aux segments PEST. En 2003, une équipe coréenne a émis l'hypothèse selon laquelle l'OGT pouvait elle-même se comporter comme une protéine chaperonne (Sohn et al., 2003). Après endommagement d'une protéine, des segments hydrophobes exposés au milieu environnant aqueux pourraient contenir des séquences PEST. Ainsi nous supposons que l'OGT augmente transitoirement la solubilisation de la protéine par « greffage » de GlcNAc. Ce résidu serait par la suite reconnu par Hsp70 via une interaction lectinique. Hsp70 empêcherait alors l'agrégation de la protéine puis tenterait, par un mécanisme proche du modèle gluco-dégluco du réticulum endoplasmique, de remettre la protéine en bonne conformation. Si la remise en conformation échoue, c'est la voie de dégradation par le protéasome qui est ouverte via l'ubiquitination (Ubiquitin-proteasome system ou UPS).

Ceci nous a amené à rechercher une relation entre l'ubiquitinylation des protéines et leur *O*-N-acétylglucosaminylation (Article VII, à soumettre pour publication).

La question posée est la suivante : « **Si la *O*-GlcNAc et l'ubiquitine sont deux modifications post-traductionnelles possédant un effet opposé sur la stabilité des protéines, alors ces deux modifications s'excluent-elles mutuellement ?** »

Fonctions associées à la *O*-GlcNAc.

Le premier travail a été de rechercher une co-existence possible de l'ubiquitine et de la *O*-GlcNAc sur les mêmes protéines. Ceci a pu être réalisé par immunoprécipitation avec un anticorps anti-*O*-GlcNAc (RL2) suivie d'un « western blot » anti-ubiquitine (Article VII, Fig. 1C). De cette manière, nous avons pu montrer que certaines protéines peuvent être modifiées simultanément par les deux types de modification. Nous pouvons donc admettre que l'ubiquitinylation et la *O*-N-acétylglucosaminylation ne sont pas des modifications s'excluant mutuellement ou, tout au moins, pas de manière globale. Des expériences de choc thermique, réalisées à une température sub-létale de 42°C, nous ont révélé qu'après le stress, la *O*-GlcNAc et l'ubiquitinylation des protéines augmentaient toutes deux, mais que leur profil d'expression était différent en fonction du délai post-stress (Article VII, Fig. 2). Il était intéressant de noter que contrairement à l'ubiquitinylation, la *O*-N-acétylglucosaminylation ne semblait pas stabiliser par l'inhibition protéasomale (utilisation du MG132), ce qui suggérerait que la *O*-GlcNAc puisse protèger les protéines intracellulaires de la dégradation protéasomale. Dans la même expérience, l'activité lectinique d'Hsp70 a été mesurée : celle-ci augmente très rapidement, c'est-à-dire aussitôt le stress infligé aux cellules (premier point mesuré à 2 min 30 s). Il est également très intéressant de noter que l'activité lectinique d'Hsp70 est activée par l'inhibition protéasomale (Article VII, Fig. 2, comparer les pistes 1 et 2 ; Fig. 3A). Les mêmes conclusions ont été tirées par inhibition du protéasome avec l'époxomicine et la lactacystine (Article VII, Fig. 3B). On peut suggérer que certaines protéines de choc thermique exhibent leur propriété lectinique lors d'une inhibition protéasomale pour empêcher les protéines dont la conformation a été modifiée de s'agglomérer et de former des agrégats toxiques pour la cellule. Il s'agirait d'une réaction de première urgence, avant même le processus de remise en conformation des protéines. Malgré ces observations, il nous est apparu difficile de corréler directement une augmentation d'activité lectinique avec une augmentation concomitante des niveaux de *O*-GlcNAc. **L'activité lectinique des Hsp70 augmente t-elle pour s'adapter au flux, important et soudain, de protéines dénaturées et potentiellement *O*-N-acétylglucosaminylées, ou cette activité s'adapte t-elle comme certaines enzymes modulent leur affinité pour de faibles concentrations en substrat ? S'agit-il ainsi d'une régulation constitutive ou adaptative ?** Aussi surprenant que cela puisse paraître, nous ne pouvons pas répondre à cette question pour le moment.

Fonctions associées à la *O*-GlcNAc.

Puisque, comme nous venons de le voir, *O*-GlcNAc et ubiquitine peuvent faire « bon ménage » sur certaines protéines, mais que leur influence sur la destinée de la protéine pourrait être opposée (observations personnelles et données bibliographiques), il doit y avoir un centre commun de régulation pour les deux modifications post-traductionnelles. Tout naturellement, nous avons recherché celui-ci sur l'enzyme E1, enzyme activant l'ubiquitine. Très rapidement il nous est apparu que E1 était elle-même modifiée par la *O*-GlcNAc et que cette glycosylation était, comme la majorité des protéines *O*-N-acétylglucosaminylées, dépendante des conditions de culture et/ou de stress (Article VII, Fig. 4A). Il est très intéressant de noter qu'une interaction entre E1 et Hsp70 a pu être mise en évidence (Article VII, Fig. 4B) et que cette interaction semble être *O*-GlcNAc dépendante. Il est également intéressant de supposer que dans la cellule, E1 est une protéine subissant les agressions comme toutes les protéines cellulaires et qu'elle soit également un substrat de l'OGT comme beaucoup d'autres facteurs. De cette manière une régulation des processus d'ubiquitinylation par E1 se ferait de « manière naturelle » puisque celle-ci subirait les mêmes dommages et les mêmes modifications que tout autre protéine. Comme le reste des protéines malmenées, elle pourrait interagir avec Hsp70 ; dans le but de la préserver de l'agrégation ou de la destruction (?) : ceci reste encore à élucider.

L'avant-dernier point, présenté dans l'article VII, concerne la mise au point de la technique d'ARN interférentiel (ARNi) sur l'OGT humaine (Article VII, Fig. 5). L'efficacité de l'ARNi sur la sous-expression de l'OGT a été vérifiée par la mise en évidence de la diminution de l'expression de l'OGT elle-même et du produit de la réaction enzymatique, à savoir de l'expression de la *O*-N-acétylglucosaminylation (Article VII, Fig. 5 A et B). La diminution très prononcée de l'OGT se répercute directement sur les niveaux d'ubiquitinylation (Article VII, Fig. 5C) et sur la viabilité cellulaire (Article VII, Fig. 5D) comme l'avaient précédemment démontré l'équipe de Gerald Hart (Zachara et al., 2005).

Enfin dans cet article nous avons montré que l'activité lectinique d'Hsp70 était induite par la mauvaise conformation des protéines. Pour provoquer une mauvaise conformation nous avons utilisé un analogue de la proline, l'azétidine (Article VII, Fig. 6). La culture des cellules en présence de cet analogue d'acide aminé provoque une augmentation de la propriété lectinique d'Hsp70. Ceci peut en partie expliquer pourquoi le déclenchement d'activité lectinique est aussi rapide lors du stress, puisque la dénaturation protéique lors d'un stress thermique est quasiment instantanée.

Fonctions associées à la *O*-GlcNAc.

L'intervention d'Hsp70 dans une tentative de « remise en forme » des protéines malmenées par le stress et par intervention de sa propriété lectinique pourrait s'expliquer selon le schéma hypothétique présenté à la fin de l'article VII (Article VII, Fig. 7). Après un stress, des segments « masqués » de la protéine, présentant peut-être des séquences PEST, se retrouveraient exposés à la surface de la protéine. Ces séquences PEST seraient reconnues par l'OGT qui les modifieraient par la *O*-GlcNAc. La protéine ainsi modifiée serait présentée au système Hsp70-co-chaperonnes dans un souci d'empêcher, tout d'abord, son agrégation, puis dans une tentative de remise en « bon état ». Dans ce dernier cas, Hsp70 serait assistée d'Hsp40. Un peu comme on le retrouve dans le cycle gluco-dégluco du réticulum endoplasmique, il y aurait un cycle *O*-GlcNAc/dé-*O*-GlcNAc en parallèle à une succession d'hydrolyses d'ATP permettant le relargage et la re-fixation de la protéine à remettre en conformation. Si, au bout de quelques cycles, la protéine n'a pas réussi à ré-adopter une conformation qui lui permette d'être à nouveau opérationnelle dans la cellule, un échange de co-chaperonne s'effectue (CHIP, BAG-1). L'interaction entre E1 (qui est elle-même *O*-N-acétylglucosaminylée) et Hsp70 déclencherait le processus d'ubiquitinylation de la protéine en vue de son adressage au protéasome. La protéine à dégrader est finalement modifiée par la *O*-GlcNAc (étape 1) et par l'ubiquitine (étape 2). On peut également penser que la modification par la *O*-GlcNAc de la sous-unité régulatrice 19S prenne part également au phénomène prévention-activation de la dégradation protéasomale (Zhang et al., 2003).

Nous nous sommes intéressés à l'impact d'une carence en glucose sur l'activité lectinique d'Hsc70 et d'Hsp70 (Article VIII). Cet article fait suite aux observations publiées dans Biochemical and Biophysical Research Communications (Article VI). Dans ce travail nous avons reporté l'observation selon laquelle une carence en glucose, tout comme l'hyperthermie, était capable de provoquer cette activité. Le rôle du glucose sur sa capacité à réguler l'activité lectinique des HSP70 nous est apparu particulièrement important puisque le glucose lui-même prend une part importante dans la synthèse de l'UDP-GlcNAc donc dans les processus de *O*-N-acétylglucosaminylation (Fig. 4). Nous nous sommes donc posés la question suivante : **« L'activité lectinique des HSP70 est-elle sous la dépendance de la concentration en glucose, comme c'est le cas pour la *O*-GlcNAc ? »**

La première chose que nous avons étudiée, a été l'activité lectinique d'Hsp70 et d'Hsc70 en fonction de la concentration en glucose du milieu de culture. Deux gammes de concentrations ont été effectuées : de 0 à 100 mM (Article VIII, Fig. 1A) puis de 0 à 12,5 mM (Article VIII,

Fig. 1B). Pour ces deux gammes les comportements d'Hsp70 et d'Hsc70, en terme d'activité lectinique sont différents : Hsp70 possède une activité lectinique très forte entre 0 et 5 mM de glucose, alors qu'Hsc70 a une activité lectinique entre ces deux valeurs mais également pour des concentrations en glucose supérieures à 25 mM. On peut penser que ces deux protéines de stress travaillent de manière complémentaire pour des concentrations en glucose différentes. Nous avons recherché cette activité lectinique lorsque l'utilisation du glucose était bloquée par le 2-déoxyglucose (Article VIII, Fig. 2A) et lorsque le transport du glucose était inhibé par la cytochalasine B (Article VIII, Fig. 2B). Dans les deux cas les activités lectiniques d'Hsp70 et d'Hsc70 ont été activée. Ainsi ces données indiquent que l'activité lectinique des HSP70 est intimement liée à la concentration, au transport et à l'utilisation du glucose.

Dans deux articles précédents (Articles III et VI) nous avions démontré qu'en plus d'avoir une activité lectinique, Hsc70 et Hsp70 étaient elles-mêmes modifiées par la *O*-GlcNAc. Il nous est apparu intéressant de répondre à la question suivante : **« Puisque ces deux protéines de choc thermique sont dotées d'une activité lectinique envers la *O*-GlcNAc et qu'elles sont elles-mêmes modifiées par cette glycosylation, peut-on envisager que la première propriété soit régulée par une auto-reconnaissance d'un propre résidu de *O*-GlcNAc dans le site lectinique ? »** Trois expériences nous ont permis d'infirmer cette hypothèse.

Nous avons cultivé des cellules HepG2 dans des conditions de déprivation en glucose différentes (Article VIII, Fig. 3A). Dans des conditions de déprivation en glucose « seules », Hsp70 et Hsc70 ont une activité lectinique augmentée alors que leur *O*-N-acétylglucosaminylation diminue. Ces propriétés sont exactement inversées dans des conditions normales de température et de « concentration en glucose ». Il apparaissait logique ici de supposer que la modification par la *O*-GlcNAc des HSP70 bloque leur activité lectinique peut-être en masquant le site de reconnaissance de la *O*-GlcNAc. Or si on se place à 42°C, ou que l'on ajoute, dans un milieu hypoglucosé, de la glucosamine de manière à former l'UDP-GlcNAc en « by-passant » la GFAT (Fig. 4), alors les HSP70 peuvent être à la fois porteuses de résidus *O*-GlcNAc mais également dotées d'une activité lectinique. La seconde expérience a permis d'aboutir à la même conclusion : une cinétique de stress thermique a permis de voir que le profil d'expression de la *O*-GlcNAc sur Hsc70 et Hsp70 était similaire à celui de l'activité lectinique (Article VIII, Fig. 3B). Enfin la déglycosylation préalable d'Hsp70 par une bêta-hexosaminidase n'augmente en rien son pouvoir de reconnaissance de la *O*-GlcNAc (Article VIII, Fig. 4). Nous en avons conclu que la *O*-GlcNAc des HSP70 ne jouait en rien un rôle de régulateur (« switch ON/OFF ») de leur activité lectinique.

Fonctions associées à la *O*-GlcNAc.

**Dans ces trois derniers articles nous avons démontré l'existence de propriétés lectiniques des HSP70 vis-à-vis du motif *O*-GlcNAc. Cette activité est déclenchée plus particulièrement lors d'un stress et pourrait intervenir dans la protection des protéines vis-à-vis de la dégradation protéasomale. Nous n'avons pas réellement pu corréler une augmentation ou une diminution de cette activité lectinique avec les niveaux de *O*-GlcNAc retrouvés lors des différentes conditions de stress et/ou de culture. La relation entre la *O*-GlcNAc des protéines cytosoliques et nucléaires et de cette activité reste une énigme à part entière pour le moment.**

***6- Régulation du cycle cellulaire par les processus de O-N-acétylglucosaminylation (Article IX).***

Ces travaux (initiés tout d'abord avec le Pr. Stéphane FLAMENT) sont réalisés en étroite collaboration avec le Pr. Jean-Pierre VILAIN et le Dr. Jean-François BODART du laboratoire de biologie du développement (EA 4020, Laboratoire de régulation des signaux de division, Bâtiment SN3, Université des Sciences et Technologies de Lille à Villeneuve d'Ascq). Cette thématique fait actuellement l'objet du sujet de thèse de Melle Vanessa DEHENNAUT. Les résultats obtenus par Vanessa DEHENNAUT au cours de sa première année de thèse ne seront discutés en partie que dans la troisième section de ce mémoire.

L'ovocyte de Xénope est un modèle que nous avons commencé à étudier en 2000, c'est-à-dire en fin de thèse de doctorat (soutenue le 24 novembre 2000). Ce modèle de cellule unique présente une caractéristique très particulière : à la fin de l'ovogénèse, l'ovocyte de stade VI immature (Dumont, 1972) est bloqué physiologiquement en prophase de première division de méiose (prophase I) dans un état appelé « G2-like », en attente d'une stimulation hormonale (Hausen & Riebesell, 1991 ; Ferrel, 1999 ; Nebreda & Ferby, 2000). La reprise du cycle cellulaire, ou reprise méiotique, peut-être provoquée par la progestérone *in vivo* et *in vitro* (voie Ras-indépendante) (Maller & Krebs, 1977) ou par l'insuline *in vitro* (voie Ras-dépendante) (Deshpande & Kung, 1987): c'est le phénomène de maturation ovocytaire. L'ovocyte reprend alors une activité aboutissant à la rupture de l'enveloppe nucléaire, d'une part, à la condensation chromosomique et à la formation du fuseau, d'autre part, pour à nouveau être bloqué physiologiquement en métaphase de deuxième division (métaphase II).

Cette transition G2/M est accompagnée d'une forte activité kinasique résultant d'une augmentation du niveau global de phosphorylation dans l'ovocyte.

C'est cette caractéristique qui nous a plus particulièrement amenés à nous poser la question suivante : **puisque la reprise méiotique se caractérise par une augmentation de phosphorylation et puisqu'il a été démontré une relation de réciprocité entre la phosphorylation et la *O*-GlcNAc, alors qu'advenait-il de cette dernière après stimulation par la progestérone ?**

Naturellement, nos premiers travaux se sont portés sur l'analyse du contenu en *O*-GlcNAc d'ovocytes immatures et matures (après stimulation par la progestérone). Nous nous attendions, au départ, à observer une diminution du niveau de *O*-GlcNAc lors de l'entrée en phase M, au vu de l'antagonisme pouvant exister entre les deux modifications post-traductionnelles. Or, nous avons observé le contraire, c'est-à-dire que la maturation de l'ovocyte de Xénope s'accompagnait d'une augmentation de la *O*-GlcNAc (les mêmes observations ont pu être faites par stimulation des ovocytes avec l'insuline, résultats non montrés). Ceci à pu être mis en évidence par des expériences de western blot utilisant la WGA, en conjonction avec une désialylation et une dé-N-glycosylation préalables (Article IX, Fig. 1 A, C) et par analyse des produits de bêta-élimination (dérivés itols-acétates) par chromatographie en phase gazeuse (Article IX, Fig. 1 B). L'analyse des ovocytes énucléés a permis de démontrer que cette augmentation de *O*-GlcNAc s'adressait plus particulièrement aux protéines cytosoliques (Article IX, Fig. 2), quoiqu'il faille admettre la faible contenance protéique du noyau en comparaison à l'ovocyte lui-même (1.3 mm de diamètre) ne permet pas de détecter de réels changements de glycosylation dans cet organite. Des expériences de cinétique de maturation nous ont permis de montrer que l'augmentation des niveaux de *O*-GlcNAc ne suivait pas un schéma simple du type « peu de *O*-GlcNAc au départ, beaucoup à la fin » (Article IX, Fig. 3). En fait, plusieurs protéines ont des niveaux de *O*-GlcNAc suivant des profils d'expression très différents. Une de ces protéines a pu être identifiée : il s'agit de la bêta-caténine (Article IX, Fig. 6). La bêta-caténine est un facteur de transcription faisant partie de la voie de signalisation Wnt et qui intervient dans le développement embryonnaire (dorsalisation) (Larabell et al., 1997). Or, dans des ovocytes immatures, la bêta-caténine est déstabilisée par phosphorylation par la GSK3-bêta, ce qui engendre une dégradation protéasomale de la protéine (Fisher et al., 1999). La reprise méiotique induit une inhibition de GSK3-bêta grâce à la phosphorylation par RSK (via la voie MAPK). On peut supposer que

cette diminution de phosphorylation profite à la *O*-N-acétylglucosaminylation de la bêta-caténine, ce qui lui permettrait alors d'échapper à l'action du protéasome. D'autres protéines subissant cette augmentation de *O*-GlcNAc sont actuellement en cours d'identification (collaboration avec le Dr. Anne-Sophie VERCOUTTER-EDOUART). Nous rediscuterons de ce point dans la dernière partie de notre mémoire.

Enfin dans cet article, nous avons démontré que la microinjection de N-acétylglucosamine « libre » dans le cytoplasme de l'ovocyte retardait la reprise méiotique (Article IX, Fig. 4). Nous pensons que ce délai de maturation peut être le fait que la GlcNAc gêne les interactions entre les protéines modifiées par la *O*-GlcNAc et les protéines possédant une activité lectinique envers ce résidu, indispensables au bon déroulement de la reprise méiotique. C'est plus particulièrement aux protéines du cytosquelette et du fuseau dont que nous faisons allusion. Ceci sera également discuté dans la dernière partie.

### *7- Etude de la O-N-acétylglucosaminylation du suppresseur de tumeurs HIC1 (Article X).*

Au cours de mon stage post-doctoral effectué de février 2002 à août 2003, à l'Institut de Biologie de Lille (IBL), au sein de l'UMR 8526 dirigée par le Pr. Dominique STEHELIN et dans le groupe du Dr. Dominique LEPRINCE, je me suis plus particulièrement intéressé à l'étude de HIC1 (Hypermethylated in cancer 1), facteur suppresseur de tumeurs impliqué dans plusieurs déficiences tel que le syndrome de Miller-Diecker (Carter et al., 2000) et la tumorisation des tissus (Chen et al., 2003). Mon travail a été centré sur la glycosylation de HIC1, d'une part, et sur la mise en évidence de la protéine endogène HIC1, d'autre part. Cette deuxième partie ne sera pas discutée dans ce mémoire.

HIC1 tire son nom du fait qu'on retrouve le gène codant cette protéine, *HIC1*, dans la région 13.3 du bras court du chromosome 17 (Wales et al., 1995), région fréquemment hyperméthylée ou délétée dans bon nombre de cancers. Le fait que le gène *HIC1* soit dans une région fortement méthylée (îlots CpG) explique pourquoi celui-ci est très peu exprimé puisque la méthylation de régions promotrices favorise la répression de l'expression génique (« gene silencing ») (Baylin & Herman, 2001 ; Herman & Baylin, 2003) : elle peut concerner des gènes codant des suppresseurs de tumeurs et favoriser ainsi le déroulement des processus de cancérisation.

HIC1 est une protéine de 714 acides aminés qui présente trois domaines fonctionnels. Le premier domaine, situé dans la région N-terminale, est en fait un domaine BTB-POZ de 120

acides aminés. Ce type de domaine est connu pour sa capacité à se dimériser et à réguler certaines interactions protéiques. C'est également un domaine autonome de répression transcriptionnelle. La région centrale de HIC1 est peu conservée entre les espèces mais celle-ci possède par contre un motif conservé du type GLDLSKK proche de la séquence PxDLSxK permettant le recrutement du co-represseur CtBP (Deltour et al., 2002). Enfin la région C-terminale de HIC1 possède cinq doigts de zinc du type Krüppel (C2H2) permettant la reconnaissance nucléotidique, et une extrémité sans domaine fonctionnel apparent mais qui est, par contre, très conservée.

Notre premier travail sur HIC1 a d'abord été de mettre en évidence l'existence de la *O*-GlcNAc. Pour cela différents systèmes cellulaires ou d'expression ont été utilisés, ainsi que plusieurs outils de mise en évidence de cette glycosylation. Nous avons ainsi pu mettre en évidence la *O*-GlcNAc sur HIC1 par traduction de celui-ci dans un lysat de réticulocyte (construction pcDNA3-FlagHIC1), soit par enrichissement des formes glycosylées sur billes couplées à la WGA (Article X, Fig.1A), ou par immunoprécipitation avec un anticorps anti-*O*-GlcNAc (RL2) (Article X, Fig. 1B). La glycosylation a pu également être détectée dans une lignée de cellules CHO exprimant de manière stable HIC1 (Article X, Fig.1C). Enfin des transfections transitoires dans des cellules Cos-7 ont permis, d'une part, d'utiliser l'anticorps anti-*O*-GlcNAc par immunoprécipitation (Article X, Fig.1D), d'autre part, d'enrichir les formes glycosylées sur WGA immobilisée ou de prévenir cet enrichissement par traitement préalable par la bêta-hexosaminidase (Article X, Fig.1E). Enfin, cela nous a permis de confirmer définitivement la présence de cette modification post-traductionnelle par transfert de galactose tritié sur les résidus de GlcNAc (Article X, Fig.1E). Ce résultat nous était apparu très important puisque HIC1 n'était que le second facteur suppresseur de tumeurs à avoir été démontré comme modifié par la *O*-GlcNAc, le premier étant p53 (Shaw et al., 1996).

Des enrichissements en protéine HIC1 (facilités par la présence d'une séquence « Flag » en position N-terminale) marquée au galactose tritié, suivi d'une hydrolyse trypsique, a montré que HIC1 était modifié par la *O*-GlcNAc au moins sur trois sites (Article X, Fig.2). L'identification de ces sites fait l'objet en partie de notre projet (paragraphe 10).

Connaissant un des partenaires de HIC1, CtBP, et sachant que la *O*-GlcNAc est un des produits finaux de la voie de biosynthèse des hexosamines, nous avons voulu savoir si l'interaction HIC1-CtBP pouvait être sous l'influence de la glycosylation de HIC1. Pour cela des cellules Cos-7 exprimant transitoirement HIC1 dépourvu de domaine BTB-POZ ont été cultivées en présence de glucosamine de sorte à augmenter « artificiellement » le niveau de *O*-

Fonctions associées à la *O*-GlcNAc.

GlcNAc de HIC1 (Article X, Fig.3). Des expériences de double hybride mammifère effectuées dans ces conditions ont démontré que l'interaction entre les deux partenaires ne semblait pas sous l'influence de la glycosylation de HIC1 puisqu'en présence de glucosamine cette interaction n'était pas modifiée (Fig. 8). Le rôle de cette glycosylation était donc à rechercher ailleurs, soit dans la modulation d'une interaction avec un autre partenaire, soit dans une toute autre fonction.

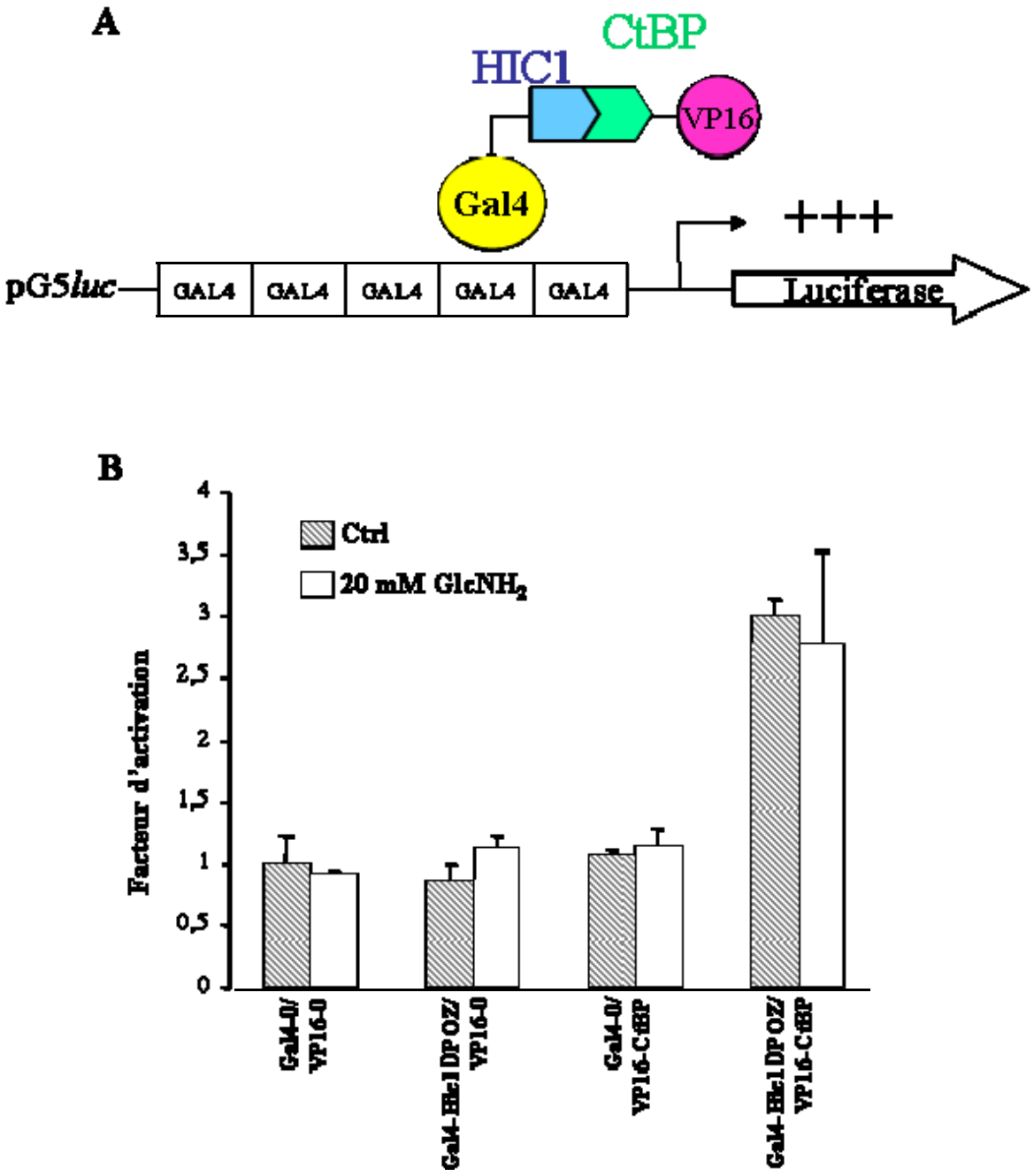



**Figure 8- La glycosylation de HIC1 ne module pas son interaction avec le co-répresseur CtBP**

Fonctions associées à la *O*-GlcNAc.

Des constructions HIC1ΔPOZ-Gal4 et CtBP-VP16 ont été utilisées pour évaluer l'influence de la *O*-GlcNAc de HIC1 sur l'interaction avec CtBP (Fig. 8A). Les expériences ont été réalisées en absence (Ctrl) ou en présence de glucosamine à 20 mM, de sorte à augmenter le niveau de glycosylation de HIC1 (Fig. 8B). La mesure de l'activité luciférase a été effectuée par luminométrie.

L'utilisation de mutants de délétions, nous a permis de localiser « grossièrement » les sites de *O*-GlcNAc (Article X, Fig. 4) dans le domaine de liaison de l'ADN (DBD) de HIC1 (entre les résidus 400 et 616). Nous avons donc voulu vérifier l'hypothèse selon laquelle la glycosylation de HIC1 dans le DBD peut influer sur la reconnaissance oligonucléotidique. HIC1 a donc été produit par traduction *in vitro* en utilisant le système du lysat de réticulocyte. Les protéines HIC1 glycosylées et non-glycosylées ont été fractionnées par enrichissement sur WGA immobilisée (Article X, Fig. 5A). Chacune des fractions a alors été testée en « gel-shift », ou EMSA, pour sa capacité à lier l'ADN (Article X, Fig. 5B). Contre toute attente nous n'avons pas vu de différence de fixation entre les formes glycosylées de HIC1 et celles non-glycosylées. Par contre des complexes de petite taille mettant en jeu des formes tronquées de HIC1 dans la région N-terminale permettent d'observer un différentiel dans la fixation de la sonde oligonucléotidique par ces formes : les formes tronquées et glycosylées de HIC1 ne sont pas capables de fixer la sonde. Nous avons donc supposé que la glycosylation des formes tronquées de HIC1 n'était pas localisée au même endroit de la protéine que sur la forme de pleine longueur. En suivant la même stratégie que pour la protéine de pleine longueur, des mutants de délétion ont permis de localiser la *O*-GlcNAc de la forme tronquée de HIC1 dans une séquence située entre les acides aminés 339 et 669 (Article X, Fig. 6). De nouvelles expériences de « gel-shift » sont venues confirmer que la forme glycosylée de HIC1 tronquée ne se fixait pas l'ADN (Article X, Fig. 7).

L'utilisation d'anticorps anti-phosphosérine, anti-phosphothréonine et anti-phosphotyrosine sur les différentes constructions de HIC1 a permis de localiser « grossièrement » certains sites de phosphorylation en plus des sites *O*-GlcNAc (Fig. 9).

Fonctions associées à la *O*-GlcNAc.

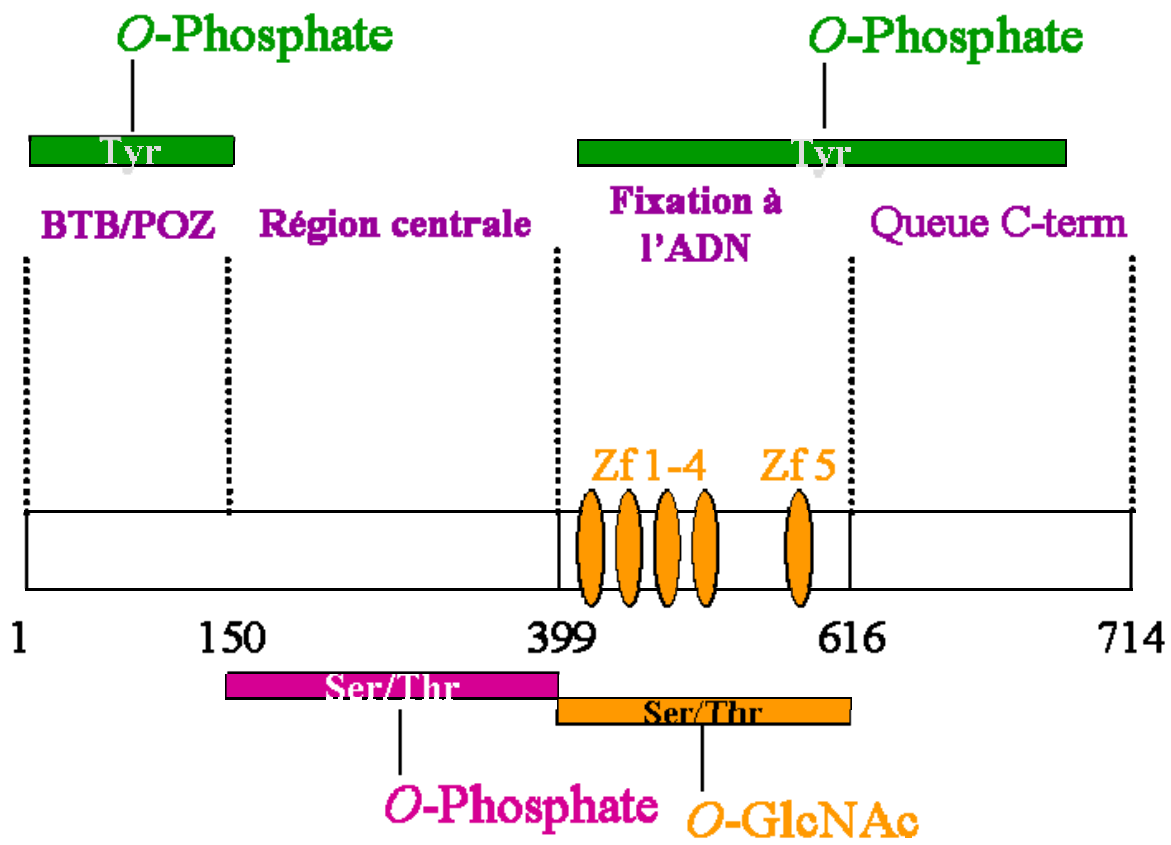



**Figure 9- Localisation « grossière » des sites de *O*-N-acétylglucosaminylation et de phosphorylation de HIC1.**

La figure 10 présente la glycosylation de HIC1 sur les deux formes de HIC1, longue et tronquée, et propose que les gènes cibles des deux formes puissent être, d'une part, de nature différente et que l'effet de la glycosylation puisse, d'autre part, influencer la fixation sur la cible nucléotidique. Si l'on considère comme indiqué dans la figure 5, que l'UDP-GlcNAc est sous l'influence directe de l'état nutritionnel de la cellule, alors la fixation à l'ADN de la forme de pleine longueur de HIC1 ne sera pas dépendante des conditions physiologiques modulant les niveaux de *O*-GlcNAc contrairement à la forme tronquée : plus le niveau de glycosylation de cette forme de HIC1 est élevé, moins cette forme se fixerait à l'ADN (Fig. 10).

Fonctions associées à la *O*-GlcNAc.

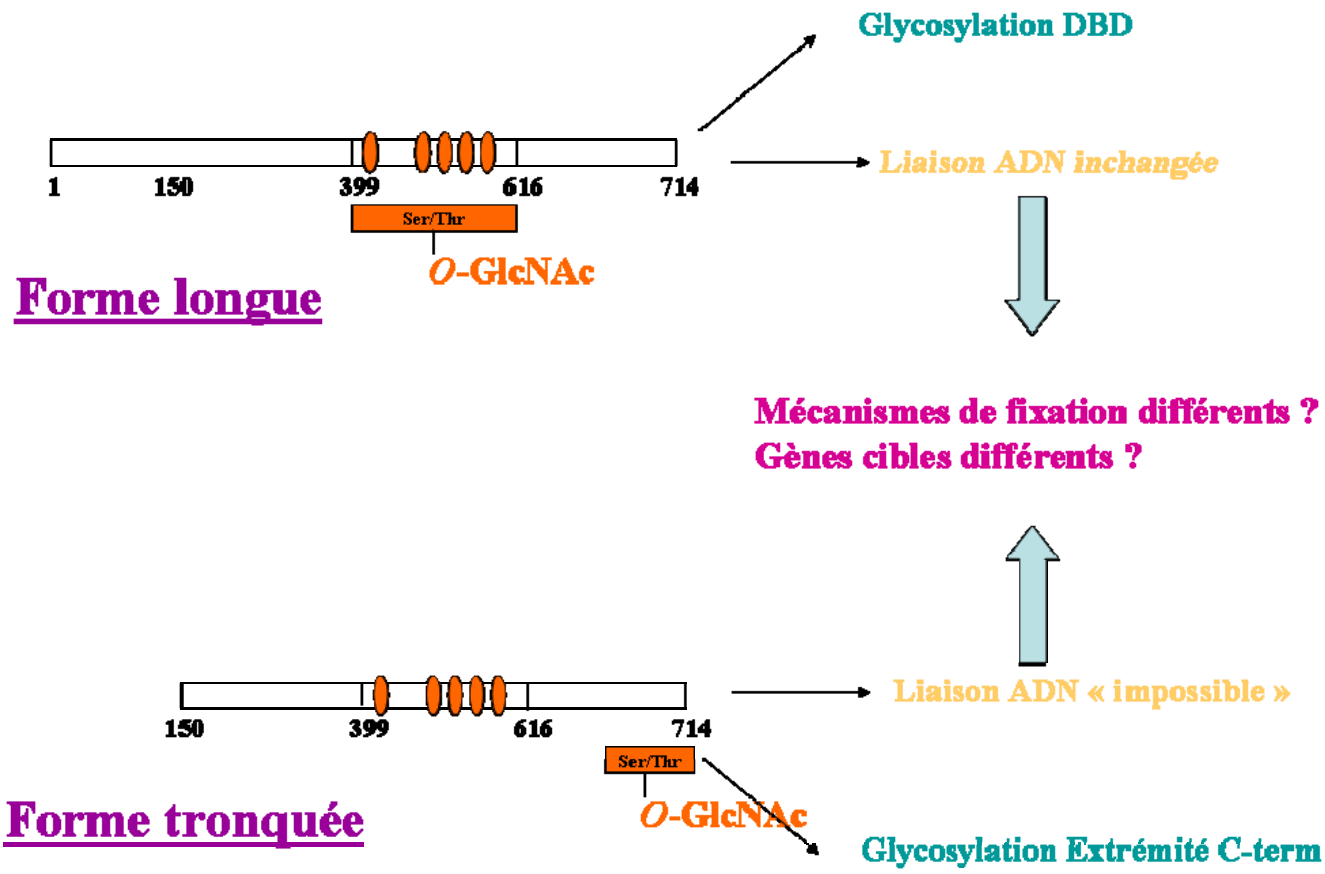



**Figure 10- Localisation des sites de *O*-GlcNAc sur les deux formes de HIC1 et l'influence de la glycosylation sur l'activité transcriptionnelle.**

Fonctions associées à la *O*-GlcNAc.

# Projet de Recherche

Fonctions associées à la *O*-GlcNAc.

L'essentiel de nos activités de recherche sera orienté sur les **fonctions régulées par la *O*-GlcNAc**. Ces activités seront plus particulièrement ciblées sur **la résistance protéique à la dégradation protéasomale** (paragraphe 8) et sur **la régulation du cycle cellulaire** (paragraphe 9). Nous avons également entrepris un projet sur la localisation des sites de modifications post-traductionnelles de HIC1 (paragraphe 10).

Ces trois lignes de recherche seront détaillées une à une.

### *8- La O-GlcNAc dans la résistance à la dégradation protéasomale*

La poursuite et l'approfondissement de nos travaux sur le rôle de la *O*-GlcNAc dans la stabilité protéique (débutés après ma thèse de 3ème cycle et qui ont fait en partie l'objet du sujet de thèse de Céline GUINEZ ) sera une de nos priorités de recherche dans les prochaines années.

#### Les objectifs

Cette thématique de recherche tentera de répondre aux points suivants :

- quelle est la fonction de l'activité lectinique des HSP70 envers le résidu *O*-GlcNAc ;

- que peut-on apprendre de la relation entre l'ubiquitinylation et la *O*-N-acétylglucosaminylation des protéines en terme de régulation et de conséquence sur la destinée protéique ;

- comment le motif *O*-GlcNAc peut-il protéger une protéine de la dégradation, aussi bien par la modification de la protéine adressée au protéasome, que par la modification du protéasome lui-même.

### *8.1- Mutation du site d'activité lectinique d'Hsc70 et étude des interactions Hsp70/Hsc70 wt/Hsc70 mut-partenaires*

Comme nous l'avons décrit dans la deuxième partie de ce mémoire, ces travaux ont permis de définir que la forme nucléocytoplasmique constitutive d'HSP70, c'est-à-dire Hsc70 et sa forme induite, Hsp70, étaient dotées d'une activité de reconnaissance lectinique vis-à-vis

du motif *O*-GlcNAc (Articles IV, VI, VII et VIII). Au cours de sa thèse Céline GUINEZ a entrepris la mutation d'Hsc70-flag (étiquetée en position N-terminale) dans un site très probablement impliqué dans la reconnaissance du motif *O*-GlcNAc. La localisation de ce site a pu être réalisée par des expériences du type « docking » - glucide-protéine - en collaboration avec le Pr. Gérard VERGOTEN (UMR 8576/UGSF). Hsc70 est une protéine de 647 acides aminés, composée de trois domaines. La partie N-terminale de 44 kDa, et longue de 386 acides aminés, porte l'activité ATPasique (ATP-binding domain) ; la partie centrale de 18 kDa (acides aminés 384-543) représente le domaine de fixation des peptides (peptide-binding domain) et la partie C-terminale de 10 kDa se termine par la séquence conservée EEVD. La mutation effectuée par Céline GUINEZ concerne l'arginine en position 469, c'est à dire un résidu placé dans la région de fixation des peptides. Celle-ci a été mutée en alanine (collaboration avec le Dr. Dominique LEPRINCE). La production de la protéine mutée, réalisée par transfection en cellules Cos-7, et la mesure d'activité lectinique sur bille de GlcNAc immobilisée a permis d'établir que la mutation R469A diminuait de 70% l'activité lectinique d'Hsc70. Ces résultats préliminaires méritent d'être confirmés. De plus, Gérard VERGOTEN a défini qu'une tyrosine en position 431 devait être également impliquée dans la reconnaissance de la *O*-GlcNAc. Malheureusement, nous avons rencontré des problèmes au cours du clonage du double mutant d'Hsc70 Y431A/R469A. Ce travail sera repris et mené à terme dès que possible. Nous espérons ainsi avoir localisé le site lectinique d'Hsc70. Cette protéine mutée nous permettra alors de mettre en place des expériences de dégradation protéasomale *in vitro*.

Cette observation selon laquelle le site lectinique d'Hsc70 se retrouverait dans le domaine de fixation des peptides est en adéquation avec notre schéma hypothétique présentant la reconnaissance de segments peptidiques « masqués » ou « enfouis » dans la protéine native et exposés après dénaturation (Fig. 11). Ceux-ci entreraient en interaction avec l'OGT (Sohn et al., 2003) pour être modifiés par la *O*-GlcNAc. Ces protéines mal conformées seraient ensuite prises en charge par les HSP70, qui en fonction de « l'état de gravité » de la protéine, interagirait plus avec l'une ou l'autre co-chaperonne, pour fixer la destinée de cette protéine (Fig. 11).

Nous pensons qu'en fait dans la cellule plusieurs éléments, intervenant dans la dégradation protéique, sont régulés par la *O*-GlcNAc. C'est le cas, de la protéine à dégrader elle-même, du protéasome (Zhang et al., 2003) et de l'enzyme d'activation de l'ubiquitine E1 (Article VIII). Comment la *O*-GlcNAc pourrait-elle réguler la dégradation des protéines cytosoliques et

nucléaires en modifiant des facteurs intervenant à divers niveaux de ce processus ? Tout se passerait selon ce qu'on pourrait appeler, comme pour le transport vésiculaire, un « bulk flow »: l'augmentation ou la diminution de *O*-GlcNAc affecterait toutes les protéines *O*-N-acétylglucosaminylées. Chacune d'elles étant alors régulée positivement ou négativement par cette glycosylation, elles seraient de ce fait activées ou inactivées selon l'activité de l'OGT. Il apparaît évident que cette régulation « de masse » serait perfectionnée au cas par cas : faut-il, en effet, que le site de glycosylation soit accessible. Nous ne devons pas oublier non plus que l'OGT a un Km qui se régule en fonction des niveaux d'UDP-GlcNAc (Kreppel & Hart, 1999) : cela signifie t-il que l'OGT conserve toujours une activité de transfert variable et que seule la mise en évidence d'une séquence peptidique permettrait la modification par la *O*-GlcNAc ?

Des expériences de « biosensor» (Biacore) nous permettront de définir les paramètres d'interaction (K ON et K OFF) d'Hsc70 wt et mutée sur des substrats *O*-GlcNAc purifiés. Cette partie sera réalisée en collaboration avec le Dr. Dominique LEGRAND (UMR 8576/UGSF). Des expériences préliminaires ont indiqué qu'il sera préférable de fixer les substrats *O*-GlcNAc sur le « sensor chip », plutôt que la protéine de choc thermique elle-même, et d'étudier l'interaction Hsc70-substrat. Pour cela, Hsc70 sera produite en cellule Cos-7 puis purifiée sur colonne de M2 (anti-Flag).

Dans cette partie du projet, une collaboration avec les Drs. Guy LIPPENS et Isabelle LANDRIEU (UMR 8576/UGSF) permettra d'étudier l'interaction lectinique Hsc70/Hsp70 avec la protéine Tau par RMN. La glycosylation de Tau s'effectuera par incubation de cette protéine avec l'OGT recombinante. Ces expériences permettront de comprendre si la glycosylation de Tau favorise son interaction avec les protéines de choc thermique. L'utilisation de la protéine Hsc70 mutée dans le site de reconnaissance du motif *O*-GlcNAc, servira ici de très bon témoin négatif.

Enfin de la même façon que l'OGT interagit avec PP1 (Wells et al., 2004), nous chercherons une éventuelle interaction entre l'OGT et Hsp70. Cette recherche se fera avant tout par des expériences de co-immunoprécipitation. Nous supposons qu'une telle interaction existe au sein de la cellule, et que cette association permettrait le « sauvetage » de protéines endommagées, par leur modification par l'OGT et leur reconnaissance subséquente par le

domaine lectinique d'Hsp70. Cette interaction potentielle serait augmentée par le stress cellulaire : des expériences de co-immunoprécipitations seront également réalisées à 42°C et dans diverses conditions de stress.

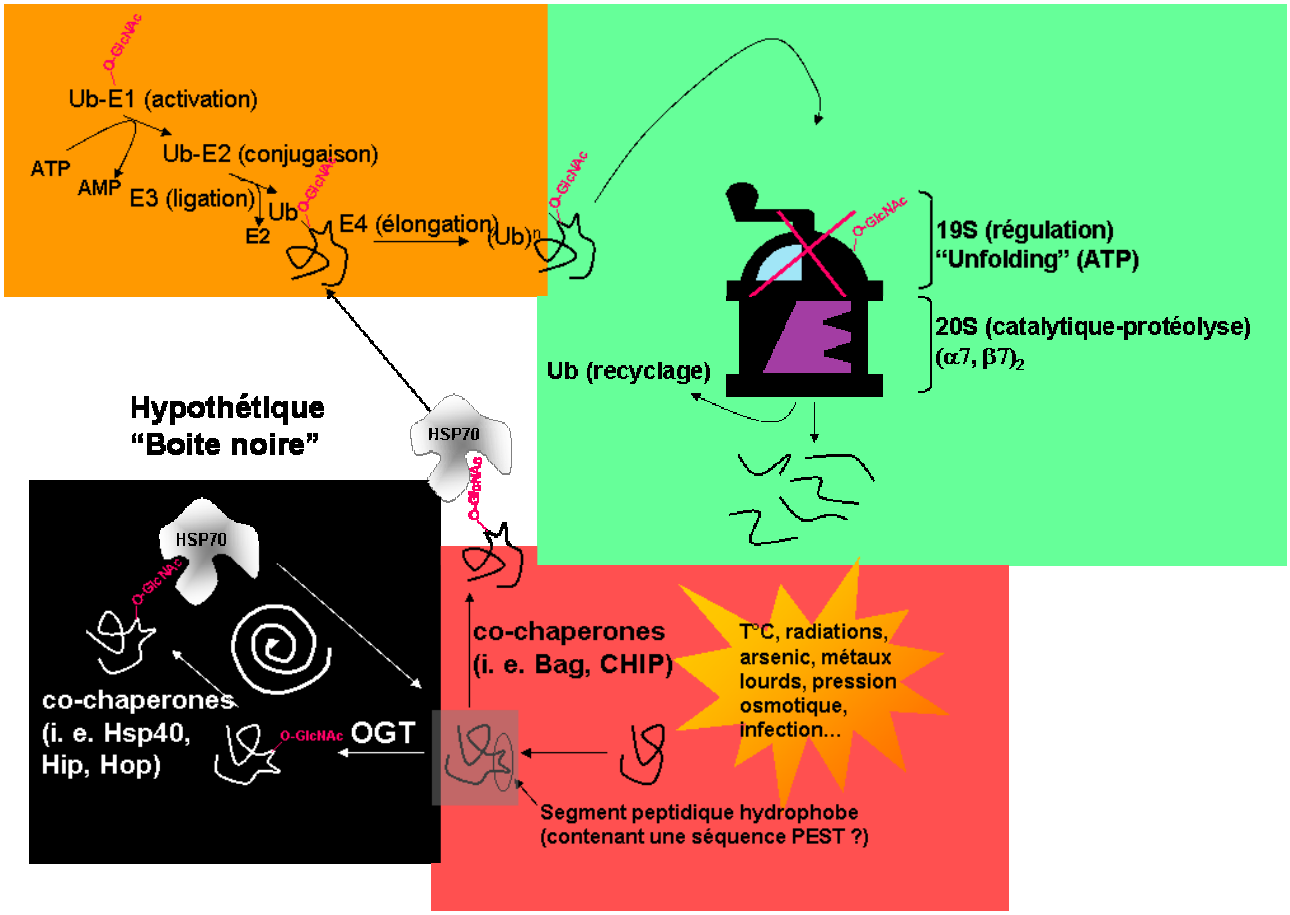



**Figure 11- Rôles de la *O*-GlcNAc et de l'ubiquitine dans la stabilité protéique : des effets antagonistes ?**

Cette figure est une conception, plus ou moins personnelle, du rôle qu'aurait la *O*-GlcNAc sur la stabilisation protéique. La *O*-GlcNAc et l'ubiquitine pourraient être un couple de modifications post-traductionnelles responsables de la destinée des protéines intracellulaires. Nous avons représenté sur cette figure des éléments clairement établis, il s'agit des encadrés rose, vert et orange, et des éléments nouveaux restant à ce jour hypothétiques (encadré noir et gris-rose).

Lorsqu'un stress est infligé à une cellule, beaucoup de protéines cellulaires subissent un changement de conformation local ou global qui peut être soit réversible ou au contraire irréversible (encadré rose). Des séquences hydrophobes, masquées au sein de la protéine, sont alors exposées au milieu extérieur en contact avec d'autres facteurs protéiques. Certains de ces segments hydrophobes contiennent des séquences PEST qui pourraient être modifiées par la *O*-GlcNAc (encadré gris-rose). Les protéines de choc thermique (du type HSP70) interviennent alors pour tenter la remise en conformation des protéines malmenées, en association avec

des protéines co-chaperonnes telles que Hsp40, Hip ou Hop (encadré noir). Plusieurs cycles de fixation-dissociation de la protéine mal conformée peuvent être nécessaires à la bonne remise en forme de celles-ci (cycles d'hydrolyse d'ATP). Si cette remise en conformation échoue (trop longue et trop coûteuse en énergie), les HSP70 changent alors de co-chaperonne (BAG et CHIP) ce qui va engendrer la polyubiquitination de la protéine mal conformée. Cette étape nécessite une activation de l'ubiquitine (encadré orange) qui pourrait être elle-même sous le contrôle de la *O*-GlcNAc (via la modification de E1). La protéine est alors adressée au protéasome, également sous le contrôle de la *O*-GlcNAc, qui se chargera de la dégrader (encadré vert).

### *8.2- Etude de l'implication de la O-GlcNAc dans la protection protéique par des expériences de dégradation protéasomale.*

Comme annoncé plus haut, des expériences de dégradation protéasomale *in cell* ou *in vitro* pourraient nous aider à comprendre l'impact de la *O*-GlcNAc sur la stabilité protéique. Pour cela nous avons prévu de faire du marquage métabolique, soit sur l'ensemble des protéines cellulaires, soit sur une protéine particulière.

Dans le premier cas il s'agira de marquer les cellules à la [$^{35}$S]-méthionine (« pulse ») puis d'effectuer une chasse en milieu de culture froid (« chase ») et de suivre par autoradiographie (SDS-PAGE) ou par comptage à scintillation le devenir de la radioactivité globale incorporée. Dans le second cas il s'agira d'isoler par immunoprécipitation une protéine désignée, présentant les caractéristiques suivantes : être une protéine cytosolique et/ou nucléaire, être *O*-N-acétylglucosaminylée, avoir une demi-vie relativement courte et être un substrat du protéasome. La bêta-caténine semble tout à fait répondre à ce type de profil. Elle sera donc prise comme protéine candidate dans ces études puisqu'elle est *O*-N-acétylglucosaminylée (Zhu et al., 2001 ; Lefebvre et al., 2004b), que sa demi-vie est d'environ 20-30 min (Kang et al., 1999) et qu'elle peut être ubiquitinylée en vue de son adressage au protéasome (Aberle et al., 1997 ; Orford et al., 1997 ; Serban al., 2005). Un autre candidat pour ce type d'étude, nous semble également très intéressant : il s'agit de HIC1. Dans le cas de cette protéine, la détermination de sa demi-vie sera nécessaire avant d'envisager des suivis de stabilité protéique. De plus, sa quasi-absence des cellules en culture (puisqu'immortalisées) nous conduira à travailler avec un système de transfection (Cos-7 ou HeLa). La construction utilisée contient une séquence « Flag » permettant sa purification et sa mise en évidence.

Dans cette série de manipulations nous modulerons les niveaux de *O*-GlcNAc des protéines pour comprendre l'impact de cette glycosylation sur la stabilité de la protéine étudiée. Pour cela plusieurs approches s'offrent à nous. La première sera l'utilisation d'inhibiteurs des

enzymes responsables du dynamisme de la *O*-GlcNAc, à savoir la streptozotocine et le PUGNAc, inhibiteurs de la *O*-GlcNAcase, et l'alloxane, inhibiteur de l'OGT. La seconde se base sur l'inhibition de la synthèse de l'UDP-GlcNAc en ciblant la GFAT. Dans ce cas le DON et l'azasérine pourront être utilisés. Dans la même optique la synthèse d'UDP-GlcNAc pourra être modulée soit en utilisant un milieu de culture hyperglucosé ou contenant de la glucosamine (5 à 20 mM) pour augmenter les niveaux de *O*-GlcNAc, ou d'utiliser un milieu de culture soit appauvri (hypoglucosé), soit déplété en glucose, de sorte à réduire cette glycosylation. Enfin, la technique d'ARN interférentiel, récemment utilisé dans notre laboratoire, pourra également permettre la réduction des niveaux de *O*-GlcNAc. L'utilisation d'inhibiteurs du protéasome, tels que le MG132, la lactacystine ou l'époxomicine, permettra d'établir le lien entre la stabilité/dégradation de ces protéines et l'activité protéasomale.

L'identification des sites de *O*-GlcNAc sur nos protéines candidats, c'est-à-dire la bêta-caténine et HIC1 (cet aspect est exposé dans le paragraphe 10 de ce chapitre) permettra la mutation des sites de glycosylation et de définir l'influence de la *O*-GlcNAc sur la stabilité de ces protéines.

Des expériences a plus longue échéance, nous permettrons d'étudier comment l'activité lectinique des HSP70 protége les protéines intracellulaires de la dégradation protéasomale. Il s'agira ici d'imaginer et de mettre au point un protocole dans lequel on induira la dégradation protéasomale d'une protéine (en utilisant la forskoline par exemple) et où on essaiera de contrecarrer ou de diminuer cette dégradation par la transfection de la protéine Hsc70 sauvage ou de la protéine mutée dans son site de fixation de la *O*-GlcNAc. La nécessité de terminer le clonage sur cette protéine et d'en vérifier l'activité lectinique s'avère ici nécessaire.

Le troisième type d'étude de la *O*-GlcNAc sur la dégradation protéasomale sera réalisé en collaboration avec les Drs. Bernard Monsarrat et Odile Burlet-Schiltz (Institut de Pharmacologie et de Biologie Structurale, CNRS UMR 5089, Toulouse). Cette partie a d'ores et déjà été commencée. Elle concerne l'utilisation de peptides issus de séquences PEST connues que l'on aura, soit modifiés par la *O*-GlcNAc, soit par la *O*-GalNAc, soit par un groupement phosphate. La séquence des peptides utilisés est la suivante :

- MTFYSPAVMNYSVPSS(*O*-GlcNAc)TGNLEGGPVRQTASPNV, récepteur bêta des oestrogènes murin,
- PAPSEDIWKKFELLPT(*O*-GlcNAc)PPLSPSRRSGLCSPSY, proto-oncogène c-Myc.

Fonctions associées à la *O*-GlcNAc.

Ces peptides seront soumis à l'activité du protéasome 20S purifié *in vitro*. L'analyse par spectrométrie de masse permettra d'établir le(s) site(s) de coupure de chaque peptide sous chacune de ses formes (glycosylé, phosphorylé ou non-modifié). Des analyses cinétiques permettront de déterminer si l'une ou l'autre modification post-traductionnelle accentue, réduit ou ne change pas la dégradation peptidique.

***9- La régulation du cycle cellulaire par la O-GlcNAc.***

La partie concernant la régulation du cycle cellulaire sera l'une de nos priorités dans les années à venir. Cette partie fait actuellement l'objet de la thèse de Melle Vanessa DEHENNAUT. Cette thèse est la suite logique des travaux réalisés en collaboration avec le « laboratoire de régulation des signaux de division » dirigé par le Pr. Jean-Pierre Vilain, avec qui nous co-encadrons d'ailleurs Vanessa DEHENNAUT.

Le projet sur la régulation du cycle cellulaire s'articule autour de quatre questions :

- quelle est l'influence de la *O*-GlcNAc et de l'OGT sur le déroulement du cycle cellulaire et en particulier sur la reprise méiotique de l'ovocyte de Xénope ?

- quelles sont les protéines *O*-N-acétylglucosaminylées impliquées dans la progression du cycle : les protéines de régulation du cycle telles que les cyclines, les composants de la voie MAPK et les protéines cytosquelettiques et/ou du fuseau de division (tubulines) feront plus particulièrement l'objet de notre attention ?

- Hsp70 interagit avec le MPF, facteur universel d'entrée en phase M (Zhu et al., 1997) : cette interaction est-elle *O*-GlcNAc dépendante et l'activité lectinique d'Hsp70 intervient-elle dans cette association protéique ?

- Qu'en est-il des événements postérieurs à la maturation : la fécondation et l'embryogenèse sont-elles également en partie régulées par les processus de *O*-N-acétylglucosaminylation ?

Ces travaux seront réalisés sur deux modèles : le premier sera l'ovocyte de Xénope (cellule sexuelle) et les cellules somatiques du type HeLa.

### *9.1- La O-GlcNAc et l'OGT sont-elles indispensables au bon déroulement du cycle cellulaire ? La O-GlcNAc permet-elle le remodelage de la chromatine ?*

Les premiers résultats obtenus par Vanessa DEHENNAUT sur ces études sont actuellement soumis pour publication (Dehennaut et al., J. Biol. Chem.). En effet, nous avons démontré que l'inhibition de l'OGT par l'alloxane, bloquait la reprise méiotique induite par la progestérone (Fig. 12).

Ces données permettent d'affirmer que les processus de *O*-N-acétylglucosaminylation sont nécessaires et indispensables pour la transition G2/M de l'ovocyte (processus pré-requis pour la maturation ovocytaire). Ces travaux seront étendus aux cellules HeLa synchronisées par déprivation en sérum ou par l'utilisation de drogues dites cycle-dépendantes (bléomycine, vinblastine, aphidicoline, hydroxyurée...). Dans ces dernières conditions, nous espérons retrouver le phénomène d'inhibition de reprise du cycle (induite par l'alloxane sur les ovocytes de Xénope) sur les cellules HeLa synchronisées.

Des inhibiteurs des deux enzymes de la *O*-GlcNAc seront utilisés (PUGNAc et streptozotocine pour la *O*-GlcNAcase et alloxane pour l'OGT), et nous étudierons également l'effet d'une « extinction » de l'OGT par la technique d'ARN interférentiel. La position des cellules somatiques dans le cycle cellulaire sera déterminée par un marquage de l'ADN à l'iodure de propidium suivi d'une analyse par cytométrie en flux (collaboration avec le Pr. Fabrice ALLAIN, UMR 8576/UGSF). Dans ce même type d'expérience, la correspondance quantité d'ADN (c'est-à-dire phase du cycle dans lequel se trouvent les cellules) et niveaux de *O*-GlcNAc, sera établie par un marquage des cellules perméabilisées avec un anticorps anti-*O*-GlcNAc suivi d'un anticorps secondaire marqué à la fluorescéine.

Fonctions associées à la *O*-GlcNAc.

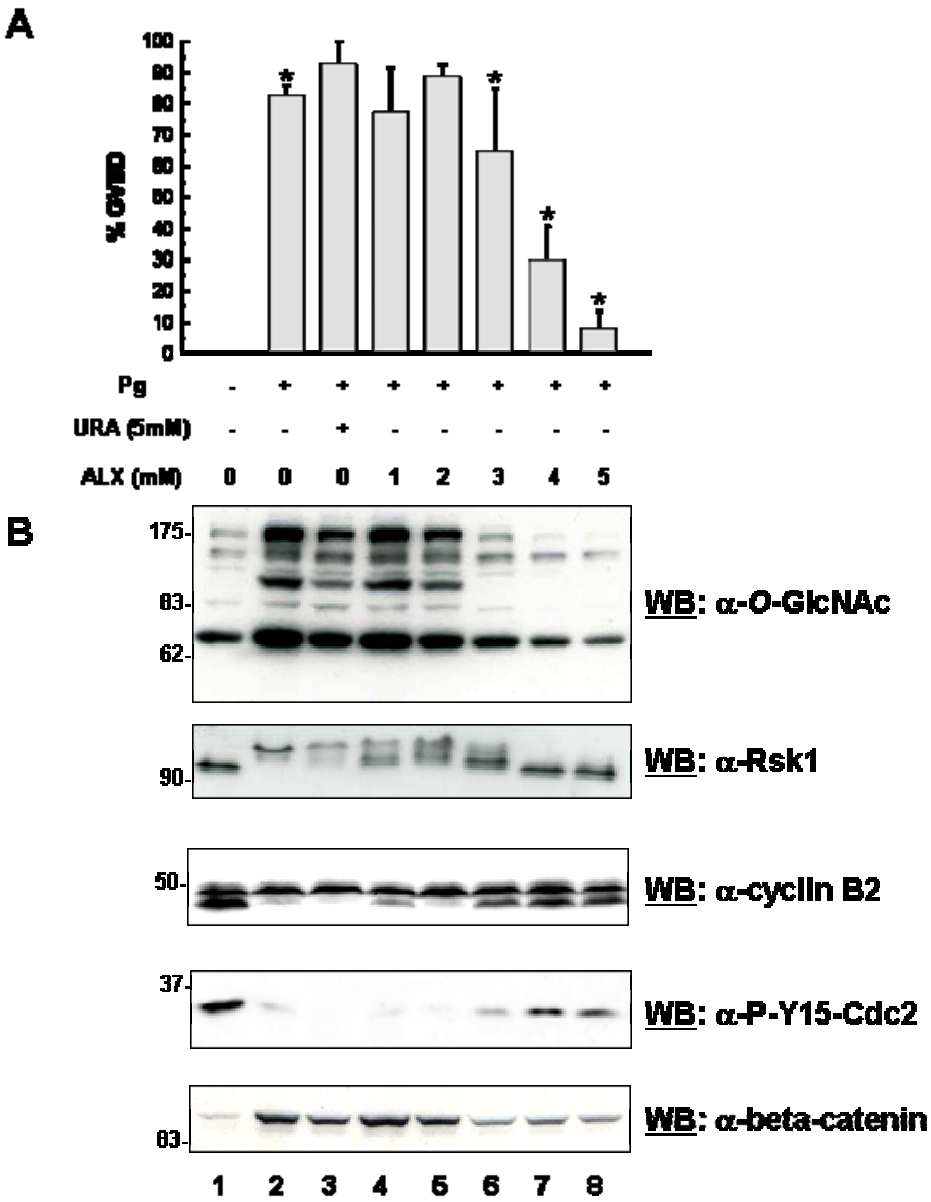



**Figure 12- Effet d'une inhibition de l'OGT sur la reprise méiotique (extrait d'un article en cours de publication, Dehennaut et al.).**

Des ovocytes de Xénope immatures ont été incubés avec des quantités croissantes d'alloxane (0 à 5 mM), puis avec la progestérone afin de déclencher la maturation. Après maturation des ovocytes contrôles, les tâches de maturation ont été comptées pour chaque condition et ramenées à un pourcentage d'ovocytes matures (Fig. 12A). On observe ainsi que l'inhibition de la reprise méiotique provoquée par l'alloxane est dose-dépendante. L'uracile a été utilisé en contrôle puisque celui-ci et l'alloxane sont des analogues structuraux. Des expériences de western blot permettent de corréler une observation morphologique de l'inhibition de la reprise méiotique par l'alloxane, à des observations biochimiques (Fig. 12B). L'alloxane bloque d'une part la voie MAPK (Rsk1, une des cibles de cette voie, n'est plus activé en présence d'alloxane) et l'activation du pré-MPF d'autre part, puisque ni la cycline B, ni cdc2 ne sont plus actifs à partir d'une certaine concentration en alloxane. L'alloxane bloque également l'accumulation de la bêta-caténine.

Nous pensons que certaines protéines impliquées dans la compaction-décompaction de la chromatine peuvent être de bonnes candidates *O*-GlcNAc. En effet, une observation fortuite de cellules Cos-7 en immunofluorescence (Tony LEFEBVRE, observation personnelle) confirmées par des expériences de Vanessa DEHENNAUT, indiquent que la chromatine relâchée (cellules en interphase) et surtout les chromosomes (cellules en mitose) sont très fortement marqués par un anticorps anti-*O*-GlcNAc (Fig. 13). Une telle observation avait déjà été effectuée par un marquage de la chromatine de Drosophile avec de la WGA couplée au FITC (Kelly & Hart, 1989). Ces auteurs ont montré que la chromatine condensée était plus marquée que la chromatine relâchée. Ceci suggère une glycosylation des protéines entrant dans la formation du nucléosome (histones) et un rôle de la *O*-GlcNAc dans la compaction-décompaction de la chromatine (en association avec l'acétylation des histones).

La première expérience sera de déterminer si les histones, et plus particulièrement l'histone H1, sont modifiées par la *O*-GlcNAc. Nous chercherons à voir si cette glycosylation varie en fonction du cycle et si elle est impliquée dans le remodelage de la chromatine (via également des interactions protéiques). Sur ce point une relation entre la *O*-GlcNAc et l'acétylation des histones pourrait exister puisqu'il vient récemment d'être démontré que l'OGT et NCOAT interagissaient pour former la *O*-GlcNAczyme (Whisenhunt et al., 2006). Ce point a été détaillé au paragraphe 2.4 (voir Fig. 3).

### *9.2- Identification des protéines O-N-acétylglucosaminylées nécessaires au cycle cellulaire.*

Les travaux de Vanessa DEHENNAUT montrent que plusieurs protéines subissent une *O*-N-acétylglucosaminylation au cours de la reprise méiotique (Fig. 12B). Une partie de ce projet sera d'identifier celles-ci. Deux groupes de protéines attirent plus particulièrement notre attention :

- les protéines impliquées directement dans le déclenchement de la reprise méiotique et plus largement dans la progression du cycle ;

- des protéines structurales nécessaires au remodelage de l'architecture cellulaire et à la formation du fuseau de division (tubulines).

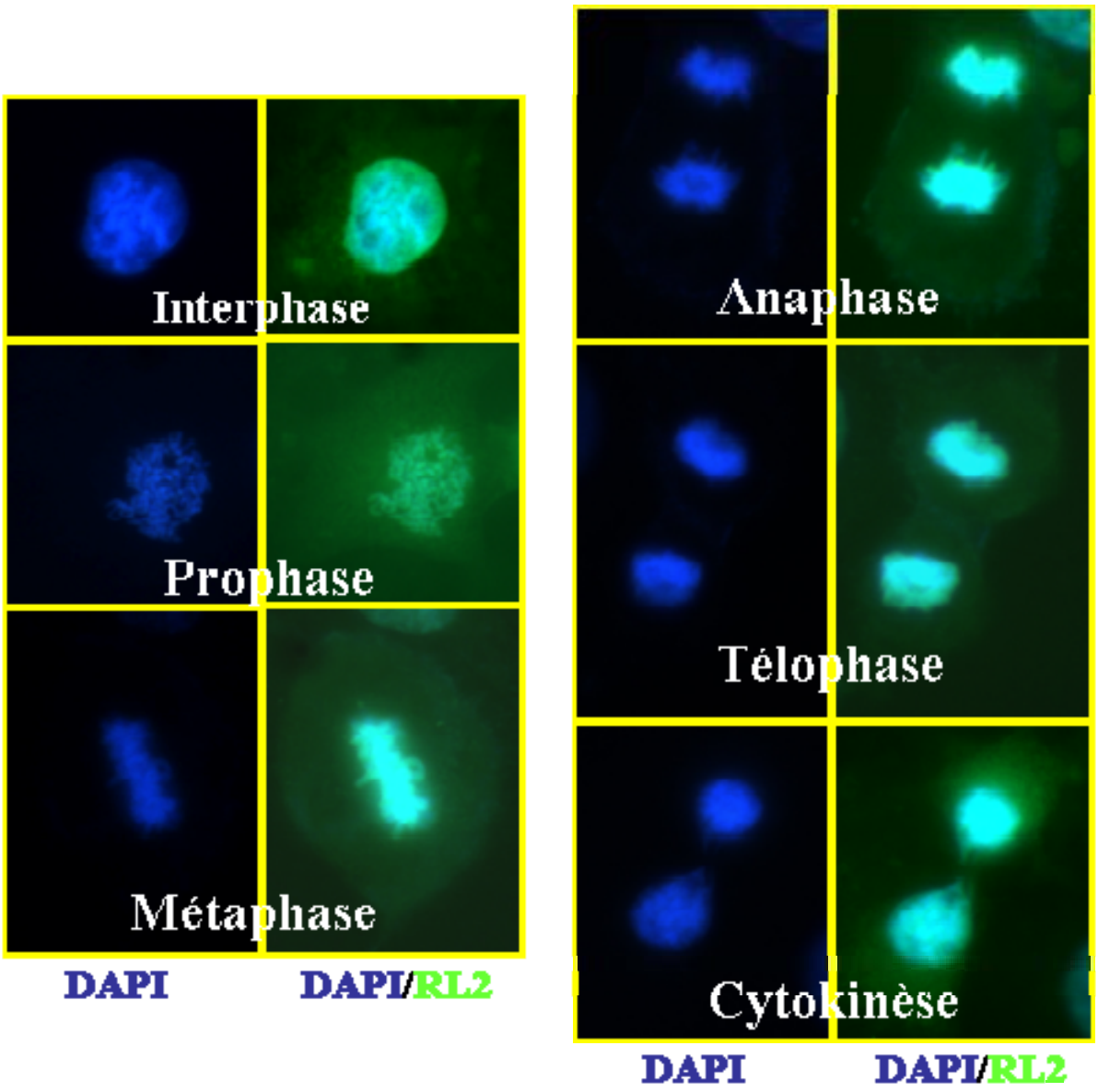



**Figure 14- Utilisation de l'anticorps anti-*O*-GlcNAc sur des cellules en division (microscopie par immunofluorescence).**

Des cellules HeLa ont été marquées avec un anticorps anti-*O*-GlcNAc (RL2) (vert) et avec le DAPI (bleu). Un marquage intense des chromosomes avec l'anti-*O*-GlcNAc est visible de la métaphase à la cytokinèse. Ces expériences rappellent celles effectuées par Kelly et Hart (Kelly & Hart, 1989) et laissent supposer une glycosylation très forte des protéines de compaction chromatinienne.

Fonctions associées à la *O*-GlcNAc.

Dans le premier groupe de protéines, nous supposons que certaines protéines impliquées dans la régulation du cycle sont *O*-N-acétylglucosaminylées : il s'agit de la cycline B, de cdc2 (les deux sous-unités du MPF, Masui, 2001), de certains composants de la voie MAPK, à savoir Mos, MEK et erk1/2 et certains cibles de cette voie tel que Rsk. La phosphatase cdc25C est également suspectée d'être *O*-N-acétylglucosaminylée.

Dans les protéines de structure, plusieurs sont connues pour porter des résidus de *O*-GlcNAc (voir tableau III). Celles qui nous intéressent le plus sont impliquées dans la formation des microtubules formant le fuseau de division et impliquées dans la ségrégation chromosomique. Sur ce point il faut noter que l'α-tubuline a été décrite comme étant modifiée par la *O*-GlcNAc (Walgren et al., 2003). Nous rechercherons la présence potentielle de *O*-GlcNAc sur les α-, β et γ-tubulines humaines et de Xénope. Des analyses préliminaires *in silico* nous ont montré que l'α-tubuline présentait 8 sites potentiels de *O*-GlcNAc ; la β-tubuline, 6 et la γ-tubuline, 3. Cette différence peut s'expliquer par les identités de séquences entre les trois tubulines : l'α- et la β-tubuline présentent 40.4% d'identité ; l'α- et la γ-, 31.4% et la β- la γ-, 33.8%.

Nous regarderons si Hsc70 ou Hsp70 interagit de manière *O*-GlcNAc dépendante avec les microtubules puisque l'interaction de cette protéine de choc thermique avec les fuseaux et asters de cellules en métaphase a été démontré (Agueli et al., 2001). En effet, les protéines de choc thermique sont connues pour promouvoir l'association de protéines en multimères (complexes homo- ou hétéro-multiprotéiques) : la *O*-GlcNAc des tubulines est-elle indispensable à leur polymérisation et cette association fait-elle intervenir la propriété lectinique des HSP70 ?

Le fait que plusieurs protéines structurales nécessaires à la progression du cycle cellulaire et à la formation du fuseau lors de phase M sont *O*-GlcNAc semble très probable. En effet comme nous l'avons mentionné dans la première partie de ce mémoire, la *O*-GlcNAc a déjà été, à plusieurs reprises, décrite comme régulant certaines interactions protéiques (Roos et al., 1997 ; Hiromura et al., 2003 ; Gewinner et al., 2004) : l'effet coopératif « *O*-GlcNAc des protéines à assembler » et activité lectinique des HSP70 nous apparaît alors très plausible.

L'identification de ces protéines *O*-GlcNAc se fera soit par la technique dite d'« approche protéomique », c'est-à-dire par digestion trypsique des protéines d'intérêt et identification *in silico* de celles-ci par reconstitution de leur carte peptique après analyse MALDI-TOF (collaboration interne avec le Dr. Anne-Sophie Vercoutter-Edouart) ou par l'utilisation d'anticorps ciblés contre les protéines citées plus haut.

### *9.3- L'interaction Hsp70-MPF est-elle dépendante de la O-GlcNAc ? L'activité d'Hsp70 envers la O-GlcNAc intervient-elle dans cette interaction ?*

Il a été décrit qu'Hsp70 pouvait interagir avec le MPF dans des spermatocytes de souris (Zhu et al., 1997). Hsp70 serait nécessaire à l'activité cdc2 kinasique puisque le « knock-out » du gène *Hsp70-2* ne permet plus aux spermatocytes de terminer leur première division de méiose. Sachant qu'Hsp70 intervient dans la formation de multimères, nous supposons que celle-ci puisse se lier au MPF en vue de sa stabilisation. Cette stabilisation permettrait l'activation de cdc2. Récemment Vanessa DEHENNAUT a démontré que la cycline B2 était modifiée par la *O*-GlcNAc, mais que, de façon surprenante, cette glycosylation était constante au cours du phénomène de maturation (Dehennaut et al., article en cours de publication). Nous ne pensons pas que la glycosylation de la cycline B2 soit directement impliquée dans son activité, puisque les deux formes de celle-ci, inactive et active, portent des résidus de *O*-GlcNAc. Le rôle de la *O*-GlcNAc dans la stabilisation de la cycline B2 nous apparaît peu plausible ici (même si l'on sait que l'activité des cyclines est en partie régulée par leur synthèse et leur dégradation par le protéasome). Malgré tout, nous avons démontré que le niveau de la cycline B2 était constant lors de la transition G2/M et qu'il y avait une corrélation directe entre l'expression de celle-ci et sa glycosylation (Dehennaut et al., manuscrit soumis pour publication). Il est donc tout de même permis de supposer que la *O*-GlcNAc empêche la dégradation de la cycline B2 : pour confirmer ou infirmer cette hypothèse, des expériences d'inhibition des processus de *O*-GlcNAc devront être faites pour savoir si la forme non-glycosylée de cette protéine lui permet d'être exprimée. Dans ce cas il nous faudra travailler sur l'ovogénèse puisque la cycline B2 s'associe à cdc2 pour former le pré-MPF avant que le premier arrêt en prophase I n'ait lieu (Ferrel, 1999) et nous supposons donc que sa glycosylation a lieu avant son assemblage avec cdc2. Il sera alors intéressant de voir si la *O*-GlcNAc n'est pas indispensable à l'association du pré-MPF : cette glycosylation empêche peut-être la dissociation du complexe sous sa forme pré-MPF (inactif) ou sous sa forme MPF (actif). Dans ce modèle Hsp70 pourrait alors interagir avec le MPF, voire le pré-MPF, soit via la cycline, soit via cdc2 lui-même (Zhu et al. 1997), même si pour le moment nous n'avons pas pu mettre en évidence la glycosylation de celui-ci. Nous pourrons également regarder l'activité histone H1 kinase de cdc2 : y a t-il une corrélation entre l'activité kinasique de cdc2 et la fixation du MPF par HSP70 comme le laisse pense Zhu et al. (Zhu et al., 1997) ?

Fonctions associées à la *O*-GlcNAc.

### *9.4- La O-GlcNAc intervient-elle dans le processus de fécondation et dans l'embryogénèse ?*

En marge de nos travaux portant sur la régulation du cycle cellulaire et de la reprise méiotique, nous étendrons l'étude de l'implication de la *O*-GlcNAc dans la fécondation de l'ovocyte et dans les premiers stades du développement embryonnaire. Nous savons d'ores et déjà que l'OGT, et par conséquent la *O*-GlcNAc, sont indispensables et essentiels à la survie des cellules souches embryonnaires (Shafi et al., 2000) et au développement embryonnaire (O'Donnell et al., 2004). Pour ces études, nous effectuerons des fécondations *in vitro* d'ovocytes matures par des spermatozoïdes de Xénopes mâles sacrifiés. Comme nous l'avons réalisé pour la maturation ovocytaire, l'alloxane sera utilisé en vue d'une inhibition de l'OGT : dans ces conditions qu'en sera-t-il du phénomène de fécondation (réaction/rotation corticale) et que ce passera t-il au niveau embryogénèse ? Pour étudier cela des études en « western blot » seront réalisées pour mettre en évidence d'une part les niveaux de *O*-GlcNAc et d'autre part les intervenants essentiels à la progression dans ces stades. Pour cela, la bêta-caténine sera l'objet de notre plus grande attention puisqu'elle s'accumule au cours de la maturation en vue de la mise en place de l'axe dorso-ventral embryonnaire. Si certaines protéines révélées par l'anticorps anti-*O*-GlcNAc nous semblent intéressantes et inconnues, nous tacherons de les identifier par protéomique.

Enfin, nous pourrons également nous intéresser aux différents stades de l'ovogénèse en étudiant la distribution de la *O*-GlcNAc sur les six stades de Dumont : des expériences préliminaires effectuées par révélation des protéines *O*-GlcNAc avec la WGA nous ont indiqué que contrairement à la maturation, l'ovogénèse tend à avoir un « pool » de *O*-GlcNAc décroissant. De plus les protéines *O*-GlcNAc semblaient ne pas toutes être les mêmes entre la maturation et l'ovogénèse. Leur identification pourra également être réalisée.

Au final, ces études devraient nous permettre de montrer que la *O*-N-acétylglucosaminylation est indispensable à tous les stades du développement de l'ovogénèse à l'embryogénèse, mais que les protéines régulatrices et structurales *O*-N-acétylglucosaminylées et impliquées dans ces processus sont très certainement différentes d'un niveau à l'autre du développement.

Fonctions associées à la *O*-GlcNAc.

### *10- Etude des modifications post-traductionnelles de HIC1.*

L'**objectif** de ce projet est de localiser précisément certaines modifications post-traductionnelles telles que la phosphorylation, l'acétylation et la *O*-GlcNAc du facteur de transcription suppresseur de tumeur, HIC1. Nous rechercherons l'impact de ces modifications sur les mécanismes de répression transcriptionnelle de HIC1 et sur l'interaction avec ces partenaires.

Ce projet se compose de quatre parties faisant appel à des techniques et des savoir-faire très différents : techniques de biochimie pour la production et la purification protéique, approche protéomique (électrophorèse 1D et 2D, spectrométrie de masse) et techniques de biologie moléculaire pour la construction de mutants et l'expression de protéines par transfection.

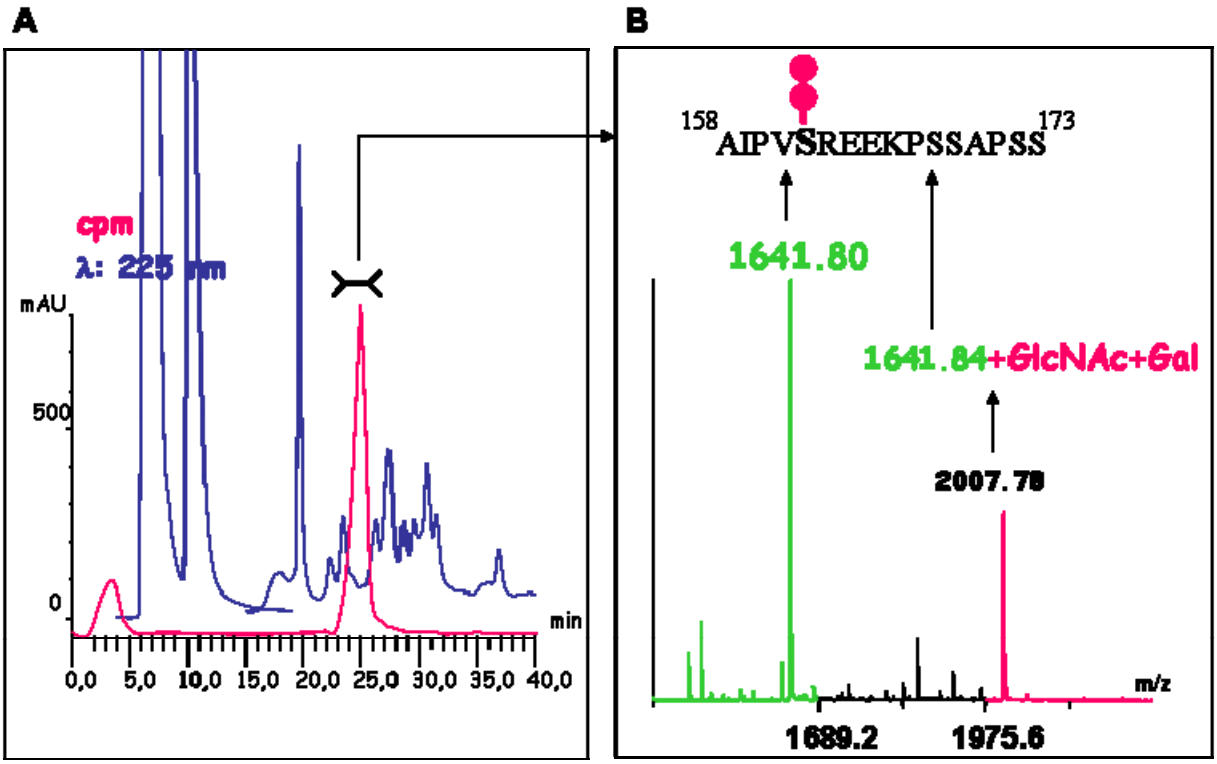
### *10.1-Identification des sites de phosphorylation, d'acétylation et de O-GlcNAc majeurs de HIC1.*

Le premier travail réalisé dans ce projet sera de localiser précisément les sites de phosphorylation, d'acétylation et de *O*-N-acétylglucosaminylation de HIC1.

Nous avons récemment développé au laboratoire avec Marie-Christine Slomianny, une technique de « mapping »/localisation des sites de modifications post-traductionnelles en faisant intervenir la technologie HPLC (High Performance Liquid Chromatography). La protéine étudiée peut être d'origine endogène, si la quantité synthétisée par la cellule est suffisante pour la détection, ou cette protéine peut être produite par la cellule après transfection d'un vecteur l'exprimant.

Le principe de cette approche est le suivant : des cellules (Cos-7 ou HeLa) sont cultivées en présence d'un précurseur radioactif permettant de modifier post-traductionnellement la protéine d'intérêt. Ce précurseur diffère en fonction de la modification étudiée : il s'agit de [$^{3}$H]-Glucosamine pour la *O*-GlcNAc, d' [$^{33}$P]-orthophosphate pour la phosphorylation et d' [$^{14}$C]-acétylCoA pour l'acétylation. La protéine étudiée est ensuite purifiée de l'extrait cellulaire. Cette purification est facilitée si la protéine est étiquetée à l'aide d'un court peptide ; dans ce cas celle-ci doit être transfectée. C'est le cas de HIC1 qui a été étiquetée en N-terminal par un octaptide du type « Flag » permettant une chromatographie d'affinité sur

un anticorps immobilisé (M2) sur billes de sépharose. Alternativement, pour la localisation des sites *O*-GlcNAc, un marquage peut être réalisé *in vitro*, après purification de la protéine d'intérêt, sur des résidus de *O*-GlcNAc par la galactosyltranférase bovine. Cette enzyme permet de greffer un résidu de galactose tritié sur les résidus de *O*-GlcNAc via l'UDP-[$^3$H]Galactose, le donneur de sucre. On obtient ainsi un disaccharide radiomarqué. La protéine est ensuite soumise à une électrophorèse du type SDS-PAGE, le gel est coloré (Coomassie ou nitrate d'argent), séché sous vide, et exposé à un film ou la révélation peut être effectuée par un Phosphorimager (Biorad). La bande d'intérêt est repérée, découpée et incubée avec de la trypsine toute la nuit. Les peptides de digestion sont extraits du gel et séparés par HPLC. Un comptage radioactif (scintillation du type bêta) est réalisé afin de repérer les peptides radiomarqués, le peptide « froid », cette fois-ci, de temps de rétention identique au peptide « chaud », est analysé par spectrométrie de masse du type MALDI-TOF afin d'en connaître la masse précise. Ceci permet à partir de la séquence primaire de localiser finement les sites de modifications post-traductionnelles. Cette technique a été mise en place au laboratoire sur l'alpha-cristalline A, protéine dont le site de glycosylation (sérine 162) est connu (Roquemore et al., 1992). Nous avons grâce à cette technique, retrouvé ce site sans difficulté (Fig. 15).



Fonctions associées à la *O*-GlcNAc.

**Figure 15- Localisation du site de *O*-N-acétylglucosaminylation de l'alpha-cristalline A**

L'alpha-cristalline a été marquée par l'ajout d'un résidu de galactose tritié *in vitro* sur les résidus de *O*-GlcNAc. Après digestion trypsique, les peptides sont séparés par HPLC (A) et les fractions radioactives sont repérées par comptage à scintillation. La masse du même peptide, mais marqué cette fois-ci par du galactose froid, est alors mesurée par spectrométrie de masse du type MALDI-TOF permettant son identification (B).

### *10.2- Impact fonctionnel des mutants de modification post-traductionnelle de HIC1.*

Lorsque les sites de modifications post-traductionnelles majeurs de HIC1 auront été localisés, nous réaliserons des constructions de HIC1 muté sur certains acides aminés porteurs de ces modifications post-traductionnelles. Ceci sera réalisé grâce à la technique de mutagénèse dirigée par double PCR.

#### *10.2.1- Rôle des modifications post-traductionnelles sur les interactions protéiques entre HIC1 et ses partenaires.*

Cette partie sera centrée sur l'influence des modifications post-traductionnelles de HIC1 sur les interactions de celui-ci avec ses partenaires protéiques. Ce type d'analyse est actuellement développée dans le groupe de Dominique LEPRINCE et concerne notamment le rôle de l'acétylation et de la SUMOylation (travail de thèse de Nicolas STANKOVIC-VALENTIN). Nous proposons donc d'étendre cette étude au rôle de la phosphorylation et de la *O*-GlcNAc dans les interactions protéiques.

Différents facteurs interagissant avec HIC1 ont été identifiés. Le premier a avoir été mis en évidence est CtBP (C-terminal binding protein) (Deltour et al., 2002), un co-répresseur transcriptionnel, c'est à dire une protéine qui ne lie pas l'ADN mais qui interagit directement avec certains facteurs de transcription afin de réprimer la transcription. Nous proposons d'étudier l'influence des modifications post-traductionnelles sur l'interaction CtBP-HIC1. Pour cela, deux stratégies expérimentales pourront être envisagées. La première stratégie fait appel à la technique de co-immunoprécipitation ; la deuxième stratégie fait appel à la technique du double-hybride en cellule mammifère. Dans cette technique, un des partenaires est produit sous la forme d'une protéine chimère avec le domaine de liaison à l'ADN de Gal4, le second partenaire, sous la forme d'une protéine chimère avec le domaine d'activation de la transcription de VP16 (voir exemple en Fig. 8). De cette manière, si les deux partenaires

interagissent, le rapprochement spatial du domaine transactivateur et du domaine de liaison à l'ADN « re-crée » un facteur de transcription qui induit la transcription d'un gène rapporteur (la luciférase) placé sous le contrôle d'éléments de réponse à Gal4.

Récemment, Nicolas STANKOVIC-VALENTIN a isolé un nouveau partenaire de HIC1, Hsp60 ou chaperonine, en l'immunoprécipitant avec la protéine HIC1 endogène. L'identification d'Hsp60 a été effectuée par analyse protéomique (Marie-Christine SLOMIANNY) puis confirmée par western blot à l'aide d'un anticorps anti-Hsp60. L'expérience inverse a été réalisée par transfection d'un vecteur exprimant Flag-HIC1 (pcDNA3-Flag-HIC1) et par co-immunoprécipitation du lysat cellulaire avec l'anticorps anti-Hsp60 (Fig. 16 A). Pour aller plus loin, nous avons incubé de la N-acétylglucosamine lors de l'étape de co-immunoprécipitation de HIC1 et des lavages des billes de protéine G. Cette expérience nous a révélé que l'interaction HIC1-Hsp60 semblait être *O*-GlcNAc-dépendante (Fig. 16A). Nous avons pu également mettre en évidence l'activité lectinique de Hsp60 (Fig. 16B). Notons que Hsp60 a été très récemment décrite comme porteur de motifs *O*-GlcNAc (Kim et al., 2006). L'importance biologique de l'interaction physique HIC1-Hsp60 reste à démontrer. Un manuscrit sur ces résultats sera prochainement rédigé (STANKOVIC-VALENTIN, GUINEZ et al.).

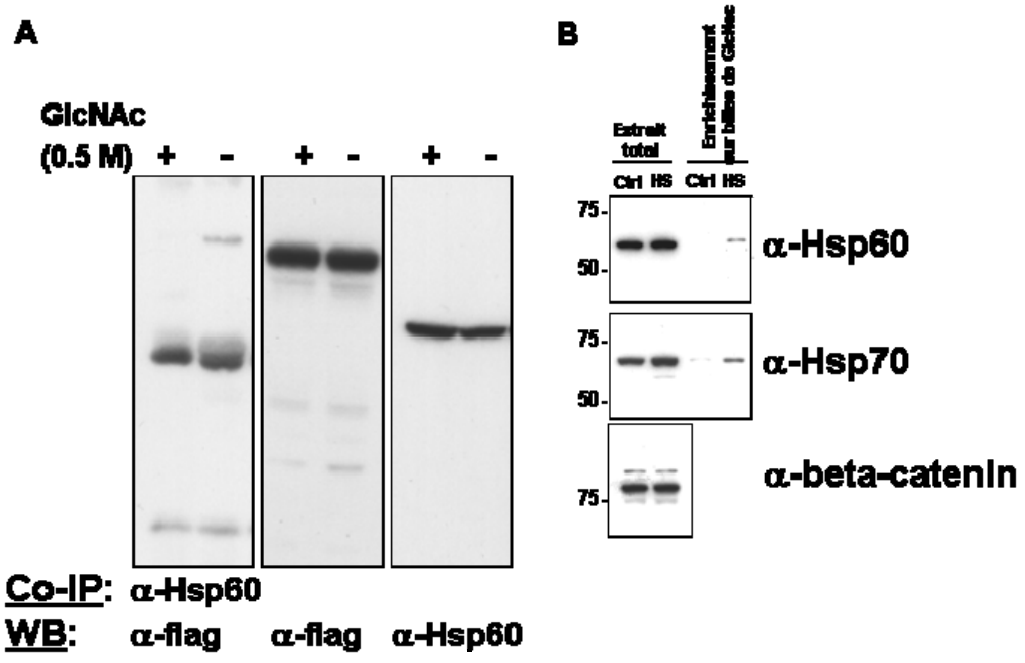



**Fig. 16- HIC1 et Hsp60 interagissent de manière *O*-GlcNAc-dépendante**

Des cellules HeLa ont été transfectées avec un vecteur exprimant la protéine HIC1 « flaggée » sur sa partie N-terminale (48h). Après lyse des cellules dans un tampon doux, des co-immunoprécipitations ont été effectuées

avec un anticorps anti-Hsp60 en présence (+) ou en absence (-) de N-acétylglucosamine (GlcNAc). Les immunoprécipités ont été soumis à une électrophorèse en mode SDS-PAGE, transférés sur membrane de nitrocellulose, puis une révélation a été réalisée avec un anticorps dirigé contre le peptide Flag ou avec un anti-Hsp60 (A). L'activité lectinique d'Hsp60 a été mise en évidence par enrichissement de celle-ci sur billes couplées à la GlcNAc (B). Hsp70 nous a servis de témoin positif, et la bêta-caténine de témoin de charge. L'expérience a été effectuée à 37°C (Ctrl) et à 42°C (HS, heat shock).

### *10.2.2- Rôle des modifications post-traductionnelles sur la répression transcriptionnelle de HIC1.*

La dernière partie de ce projet sera consacrée à l'influence des modifications post-traductionnelles sur l'activité transcriptionnelle de HIC1. Pour cela les mutants de modifications post-traductionnelles seront étudiés sur la capacité qu'aura HIC1 à inhiber la transcription d'un gène rapporteur placé en aval d'un concatémère de 5 sites consensus de liaison à l'ADN de HIC1 (Pinte et al., 2004).

# REFERENCES BIBLIOGRAPHIQUES

Aberle H, Bauer A, Stappert J, Kispert A and Kemler R (1997) Beta-catenin is a target for the ubiquitin-proteasome pathway. EMBO J. 16, 3797-3804

Agueli C, Geraci F, Giudice, G, Chimenti L, Cascino D and Sconzo G (2001) A constitutive 70 kDa heat-shock protein is localized on the fibres of spindles and asters at metaphase in an ATP-dependent manner: a new chaperone role is proposed. Biochem. J. 360, 413-419

Akimoto Y, Comer FI, Cole RN, Kudo A, Kawakami H, Hirano H and Hart GW (2003) Localization of the *O*-GlcNAc transferase and *O*-GlcNAc-modified proteins in rat cerebellar cortex. Brain Res. 966, 194-205

Arias EB, Kim J and Cartee GD (2004) Prolonged incubation in PUGNAc results in increased protein *O*-Linked glycosylation and insulin resistance in rat skeletal muscle. Diabetes 53, 921-930

Arnold CS, Johnson GV, Cole RN, Dong DL, Lee M and Hart GW (1996) The microtubule-associated protein Tau is extensively modified with *O*-linked N-acetylglucosamine. J. Biol. Chem. 271, 28741-28744

Baylin SB and Herman JG (2001) Promoter hypermethylation : can this change alone ever designate true tumor suppressor gene function? J. Natl. Cancer Inst. (2001) 93, 664-665

Bence NF, Sampat RM and Kopito RR (2001) Impairment of the ubiquitin-proteasome system by protein aggregation. Science, 292, 1552-1555

Benko DM, Haltiwanger RS, Hart GW and Gibson W (1988) Virion basic phosphoprotein from human cytomegalovirus contains *O*-linked N-acetylglucosamine. Proc. Natl. Acad. Sci. USA 85, 2573-2577

Bertram L, Blacker D, Mullin K, Keeney D, Jones J, Basu S, Yhu S, McInnis MG, Go RC, Vekrellis K, Selkoe DJ, Saunders AJ and Tanzi RE (2000) Evidence for genetic linkage of Alzheimer's disease to chromosome 10q. Science. 290, 2302-2303

Blaizot X, Meguro K, Millien I, Baron JC and Chavoix C (2002) Correlations between visual recognition memory and neocortical and hippocampal glucose metabolism after bilateral rhinal cortex lesions in the baboon: implications for Alzheimer's disease. J. Neurosc. 21, 9166-9170

Caillet-Boudin ML, Strecker G and Michalski JC. (1989) O-linked GlcNAc in serotype-2 adenovirus fibre. Eur. J. Biochem. 184, 205-221

Carter MG, Johns MA, Zeng X., Zhou L, Zink MC, Mankowski JL, Donovan DM and Baylin SB (2000) Mice deficient in the candidate tumor suppressor gene Hic1 exhibit developmental defects of structures affected in the Miller-Dieker syndrome. Hum. Mol. Genet. 9, 413-419

Chang Q, Su K, Baker JR, Yang X, Paterson AJ and Kudlow JE (2001) Phosphorylation of human glutamine:fructose-6-phosphate amidotransferase by cAMP-dependent protein kinase at serine 205 blocks the enzyme activity. J. Biol. Chem. 275, 21981-21987

Chen WY, Zeng X, Carter MG, Morrell CN, Chiu Yen RW, Esteller M, Watkins DN, Herman JG, Mankowski JL and Baylin SB (2003) Heterozygous disruption of Hic1 predisposes mice to a gender-dependent spectrum of malignant tumors. Nat. Genet. 33, 197-202

Cheng X and Hart GW (2000) Glycosylation of the murine estrogen receptor-alpha. J. Steroid. Biochem. Mol. Biol. 75, 147-158

Cheng X, Cole RN, Zaia J and Hart GW (2000) Alternative *O*-glycosylation/*O*-phosphorylation of the murine estrogen receptor beta. Biochemistry 39, 11609-11620

Cheng X and Hart GW (2001) Alternate *O*-Glycosylation /*O*-phosphorylation of Serine 16 in the murine estrogen receptor-Beta. J. Biol. Chem. 276, 10570-10575

Cho H, Mu J, Kim JK, Thorvaldsen JL, Chu Q, Crenshaw EB 3rd, Kaestner KH, Bartolomei MS, Shulman GI and Birnbaum MJ (2001) Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). Science, 292, 1728-1731

Chou CF, Smith AJ and Omary MB (1992) Characterization and dynamics of *O*-linked glycosylation of human cytokeratin 8 and 18. J. Biol. Chem. 267, 3901-3906

Chou TY, Dang CV and Hart GW (1995) Glycosylation of the c-Myc transactivation domain. Proc. Natl. Acad. Sci. USA 92, 4417-4421

Cieniewski-Bernard C, Bastide B, Lefebvre T, Lemoine J, Mounier Y and Michalski JC (2004) Identification of *O*-linked N-acetylglucosamine proteins in rat skeletal muscle using two-dimensional gel electrophoresis and mass spectrometry. Mol. Cell. Proteomics 3, 577-585

Cole RN and Hart GW (2001) Cytosolic *O*-glycosylation is abundant in nerve terminals. J. Neurochem. 79, 1080-1089

Comer FI and Hart GW (2000) *O*-Glycosylation of nuclear and cytosolic proteins. Dynamic interplay between *O*-GlcNAc and *O*-Phosphate. J. Biol. Chem. 275, 29179-29182

Comer FI and Hart G W (2001) Reciprocity between *O*-GlcNAc and *O*-phosphate on the carboxyl terminal domain of RNA polymerase II. Biochemistry, 40, 7845-7852

Cooksey RC, Hebert LF Jr, Zhu JH, Wofford P., Garvey WT and McClain DA (1999) Mechanism of hexosamine-induced insulin resistance in transgenic mice overexpressing glutamine:fructose-6-phosphate amidotransferase: decreased glucose transporter GLUT4 translocation and reversal by treatment with thiazolidinedione. Endocrinology 140, 1151-1157

Craft S, Newcomer J, Kanne S, Dagogo-Jack S, Cryer P, Sheline Y, Luby J, Dagogo-Jack A and Alderson A (1996) Memory improvement following induced hyperinsulinemia in Alzheimer's disease. Neurobiol. Aging 17, 123-130

Datta B, Datta R, Ghosh A and Majumdar A (2004) Eukaryotic initiation factor 2-associated glycoprotein, p67, shows differential effects on the activity of certain kinases during serum-starved conditions. Arch. Biochem. Biophys. 427, 68-78

Delacourte A and Buee L (2000) Tau pathology: a marker of neurodegenerative disorders. Curr. Opin. Neurol. 13, 371-376

Deltour S, Pinte S, Guerardel C, Wasylyk B and Leprince D (2002) The human candidate tumor suppressor gene HIC1 recruits CtBP through a degenerate GLDLSKK motif. Mol. Cell. Biol. 22, 4890-4901

Deshpande AK and Kung HF (1987) Insulin induction of Xenopus laevis oocyte maturation is inhibited by monoclonal antibody against p21 ras proteins. Mol. Cell. Biol. 7, 1285-1288

Dieckmann-Schuppert A, Bause E and Schwarz RT (1993) Studies on *O*-glycans of Plasmodium-falciparum-infected human erythrocytes. Evidence for *O*-GlcNAc and *O*-GlcNAc-transferase in malaria parasites. Eur. J. Biochem. 216, 779-788

Dong DL and Hart GW (1994) Purification and characterization of an *O*-GlcNAc selective N-acetyl-beta-D-glucosaminidase from rat spleen cytosol. J. Biol. Chem. 269, 19321-19330

Dudognon P, Maeder-Garavaglia C, Carpentier JL and Paccaud JP (2004) Regulation of a COPII component by cytosolic *O*-glycosylation during mitosis. FEBS Lett. 561, 44-50

Dumont JN (1972) Oogenesis in *Xenopus laevis* (Daudin). I. Stages of oocyte development in laboratory maintained animals. J. Morphol. 136, 153-179

Duverger E, Roche AC and Monsigny M (1996) N-acetylglucosamine-dependent nuclear import of neoglycoproteins. Glycobiology 6, 381-386

Duverger E, Carpentier V, Roche AC and Monsigny M (1993) Sugar-dependent nuclear import of glycoconjugates from the cytosol. Exp. Cell Res. 207, 197-201

Duverger E, Pellerin-Mendes C, Mayer R, Roche AC and Monsigny M (1995) Nuclear import of glycoconjugates is distinct from the classical NLS pathway. J. Cell Sci. 108, 1325-1332

Fang B and Miller MW (2001) Use of galactosyltransferase to assess the biological function of *O*-linked N-Acetyl-D-glucosamine : a potential role for *O*-GlcNAc during cell division. Exp. Cell. Res. 263, 243-253

Ferrel Jr JE (1999) Xenopus oocyte maturation: new lessons from a good egg. BioEssays 21, 833-842

Fisher DL, Morin N and Doree M (1999) A novel role for glycogen synthase kinase-3 in Xenopus developmemt : maintenance of oocyte cell cycle arrest by a beta-catenin-independent mechanism. Development 126, 567-576

Gao Y, Wells L, Comer FI, Parker GJ and Hart GW (2001) Dynamic *O*-glycosylation of nuclear and cytosolic proteins: cloning and characterization of a neutral, cytosolic beta-N-acetylglucosaminidase from human brain. J. Biol. Chem. 276, 9838-9845

Gao Y, Miyazaki J and Hart GW (2003) The transcription factor PDX-1 is post-translationally modified by *O*-linked N-acetylglucosamine and this modification is correlated with its DNA binding activity and insulin secretion in min6 beta-cells. Arch Biochem Biophys. 415, 155-163

Gewinner C, Hart G, Zachara N, Cole R, Beisenherz-Huss C and Groner B (2004) The coactivator of transcription CREB binding protein interacts preferentially with the glycosylated form of Stat5. J. Biol. Chem. 279, 3563-3572

Golias CH, Charalabopoulos A and Charalabopoulos K (2004) Cell proliferation and cell cycle control: a mini review. Int. J. Clin. Pract. 58, 1134-1141

Gonzalez SA and Burrone OR (1991) Rotavirus NS26 is modified by addition of single *O*-linked residues of N-acetylglucosamine. Virology. 182, 8-16

Griffith LS and Schmitz B (1999) *O*-linked N-acetylglucosamine levels is upregulated in Alzheimer brains. Eur. J. Biochem. 262, 824-831

Griffith LS and Schmitz B (1995) *O*-linked N-acetylglucosamine is upregulated in Alzheimer brains. Biochem. Biophys. Res. Commun. 213, 424-431

Griffith LS, Mathes M and Schmitz B (1995) Beta-amyloid precursor protein is modified with *O*-linked N-acetylglucosamine. J. Neurosc. Res. 41, 270-278

Guinez C, Lemoine J, Michalski JC, Lefebvre T (2004) 70-kDa Heat Shock Proteins presents an adjustable lectinic activity agaisnt O-GlcNAc residues. Biochem. Biophys Res. Com. 319, 21-26

Guinez C, Morelle W, Michalski JC and Lefebvre T (2005) *O*-GlcNAc glycosylation: a signal for the nuclear transport of cytosolic proteins? Int. J. Biochem. Cell. Biol. 37, 765-774

Guinez C, Losfeld ME, Cacan R, Michalski JC and Lefebvre T (2006) Modulation of HSP70 GlcNAc-directed lectin activity by glucose availability and utilization. Glycobiology 16, 22-28

Hagmann J, Grob M and Burger MM (1992) The cytoskeletal protein talin is *O*-Glycosylated. J. Biol. Chem. 267, 14424-14428

Haltiwanger RS, Holt GD and Hart GW (1990) Enzymatic addition of *O*-GlcNAc to nuclear and cytoplasmic proteins, identification of a uridine diphospho-N-Acetylglucosamine: peptide β-N-Acetylglucosaminyltransferase. J. Biol. Chem. 265, 2563-2568

Haltiwanger RS, Blomberg MA and Hart GW (1992) Glycosylation of nuclear and cytoplasmic proteins. Purification and characterization of a uridine diphospho-N-acetylglucosamine:polypeptide beta-N-acetylglucosaminyltransferase. J. Biol. Chem. 267, 9005-9013

Haltiwanger RS and Philipsberg GA (1997) Mitotic arrest with nocodazole induces selective changes in the level of O-linked N-acetylglucosamine and accumulation of incompletely processed N-glycans on proteins from HT29 cells. J. Biol. Chem. 272, 8752-8758

Haltiwanger RS, Busby S, Grove K, Li S, Mason D, Medina L, Moloney D, Philipsberg, G and Scartozzi R. (1997) *O*-glycosylation of nuclear and cytoplasmic proteins: regulation analogous to phosphorylation ? Biochem. Biophys. Res. Commun. 231, 237-242

Haltiwanger RS, Grove K and Philipsberg GA (1998) Modulation of O-linked N-acetylglucosamine levels on nuclear and cytoplasmic proteins in vivo using the peptide *O*-GlcNAc-b-N-acetylglucosaminidase inhibitor *O*-(2-acetamido-2-deoxy-D glucosaminopyranossylidene) amino-N-phenylcarbamate. J. Biol. Chem. 273, 3611-3617

Han I and Kudlow JE (1997) Reduced *O*-glycosylation of Sp1 is associated with increased proteasome susceptibility. Mol. Cell. Biol. 17, 2550-2558

Han I, Oh ES and Kudlow JE (2000) Responsiveness of the state of *O*-linked N-acetylglucosamine modification of nuclear pore protein p62 to the extracellular glucose concentration. Biochem. J. 350, 109-114

Hanover JA, Cohen CK, Willingham MC and Park MK (1987) *O*-linked N-acetylglucosamine is attached to proteins of the nuclear pore. Evidence for cytoplasmic and nucleoplasmic glycoproteins. J Biol Chem. 262, 9887-94.

Hanover JA, Lai Z, Lee G, Lubas WA and Sato SM (1999) Elevated *O*-linked N-acetylglucosamine metabolism in pancreatic beta-cells. Arch. Biochem. Biophys. 362, 38-45

Hanover JA, Yu S, Lubas WB, Shin SH, Ragano-Caracciola M, Kochran J and Love DC (2003) Mitochondrial and nucleocytoplasmic isoforms of *O*-linked GlcNAc transferase encoded by a single mammalian gene. Arch. Biochem. Biophys. 409, 287-297

Hartweck LM, Genger RK, Grey WM and Olszewski NE (2006) SECRET AGENT and SPINDLY have overlapping roles in the development of Arabidopsis thaliana L. Heyn. J. Exp. Bot. 57, 865-875

Hatsell S, Medina L, Merola J, Haltiwanger R and Cowin P (2003) Plakoglobin is *O*-glycosylated close to the N-terminal destruction box. J. Biol. Chem. 278, 37745-37752

Hausen P and Riebesell M (1991) The Early Development of Xenopus laevis. An Atlas of Histology. Springer-Verlag, Berlin.

Heckel D, Comtesse N, Brass N, Blin N, Zang KD and Meese E (1998) Novel immunogenic antigen homologous to hyaluronidase in meningioma. Hum. Mol. Genet. 7, 1859-1872

Heininger K (2000) A unifying hypothesis of Alzheimer's disease. IV. Causation and sequence of events. Rev. Neurosci. 11, 213-328

Herman JG and Baylin SB (2003) Gene silencing in cancer in association with promoter hypermethylation. N. Engl. J. Med., 349, 2042-2054

Hiromura M, Choi CH, Sabourin NA, Jones H, Bachvarov D and Usheva A (2003) YY1 is regulated by *O*-linked N-acetylglucosaminylation (*O*-GlcNAcylation). J. Biol. Chem. 278, 14046-14052

Holt GD and Hart GW (1986) The subcellular distribution of terminal N-acetylglucosamine moieties. Localization of a novel protein-saccharide linkage, *O*-linked GlcNAc. J. Biol. Chem. 261, 8049-8057

Holt GD, Snow CM, Senior A, Haltiwanger RS, Gerace L and Hart GW (1987a) Nuclear pore complex glycoproteins contain cytoplasmically disposed *O*-linked N-acetylglucosamine. J. Cell. Biol. 104, 1157-1164

Holt GD, Haltiwanger RS, Torres CR and Hart GW (1987b) Erythrocytes contain cytoplasmic glycoproteins. *O*-linked GlcNAc on Band 4.1. J. Biol. Chem. 262, 14847-14850

Hoyez S (1998) Risk factors for Alzheimer's disease during aging. Impacts of glucose/energy metabolism. J. Neural Transm. Suppl. 54, 187-194

Hu Y, Riesland L, Paterson AJ and Kudlow JE (2004) Phosphorylation of mouse glutamine-fructose-6-phosphate amidotransferase 2 (GFAT2) by cAMP-dependent protein kinase increases the enzyme activity. J. Biol. Chem. 279, 29988-29993

Hubert J, Seve AP, Facy P and Monsigny M (1989) Are nuclear lectins and nuclear glycoproteins involved in the modulation of nuclear functions? Cell Differentiation and Development 27, 69-81

Iyer SP and Hart GW (2003a) Dynamic nuclear and cytoplasmic glycosylation: enzymes of *O*-GlcNAc cycling. Biochemistry, 42, 2493-2499

Iyer SP and Hart GW (2003b) Roles of the tetratricopeptide repeat domain in *O*-GlcNAc transferase targeting and protein substrate specificity. J. Biol. Chem. 278, 24608-24616

Iyer SP, Akimoto Y and Hart GW (2003) Identification and cloning of a novel family of coiled-coil domain proteins that interact with *O*-GlcNAc transferase. J. Biol. Chem. 278, 5399-5409

Jackson SP and Tjian R (1988) *O*-glycosylation of eukaryotic transcription factors: implications for mechanisms of transcriptional regulation. 55, 125-133

Juang YT, Solomou EE, Rellahan B and Tsokos GC (2002) Phosphorylation and *O*-linked glycosylation of Elf-1 leads to its translocation to the nucleus and binding to the promoter of the TCR zeta-chain. J. Immunol. 168, 2865-2871

Kang DE, Soriano S, Frosch MP, Collins T, Naruse S, Sisodia SS, Leibowitz G, Levine F and Koo EH (1999) Presenilin 1 facilitates the constitutive turnover of beta-catenin: differential activity of Alzheimer's disease-linked PS1 mutants in the beta-catenin-signaling pathway. J. Neurosci. 19, 4229-4237

Kamemura K, Hayes BK, Comer FI and Hart GW (2002) Dynamic interplay between *O*-glycosylation and *O*-phosphorylation of nucleocytoplasmic proteins: alternative glycosylation/phosphorylation of THR-58, a known mutational hot spot of c-Myc in lymphomas, is regulated by mitogens. J. Biol. Chem. 277, 19229-19235.

Kamemura K and Hart GW (2003) Dynamic interplay between *O*-glycosylation and *O*-phosphorylation of nucleocytoplasmic proteins: a new paradigm for metabolic control of signal transduction and transcription. Prog. Nucleic Acid Res. Mol. Biol. 73,107-136

Kearse KP and Hart GW (1991) Lymphocyte activation induces rapid changes in nuclear and cytoplasmic glycoproteins. Proc. Natl. Acad. Sci. USA 88, 1701-1717

Kelly WG and Hart GW (1989) Glycosylation of chromosomal proteins: localization of *O*-linked N-acetylglucosamine in Drosophila chromatin. 57, 243-251

Kelly WG, Dahmus ME and Hart GW (1993) RNA polymerase II is a glycoprotein. Modification of the COOH-terminal domain by *O*-GlcNAc. J. Biol. Chem. 268, 10416-10424

Kiang JG and Tsokos GC (1998) Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. Pharmacol. Ther. 80, 183-201

Kim HS, Kim EM, Lee J, Yang WH, Park TY, Kim YM and Cho JW (2006) Heat shock protein 60 modified with *O*-linked N-acetylglucosamine is involved in pancreatic beta-cell death under hyperglycemic conditions. FEBS Lett. 580, 2311-2316

Konrad RJ, Zhang F, Hale JE, Knierman MD, Becker GW and Kudlow JE (2002) Alloxan is an inhibitor of the enzyme *O*-linked N-acetylglucosamine transferase. Biochem. Biophys. Res. Com. 293, 207-212

Kreppel LK, Blomberg MA and Hart GW (1997) Dynamic glycosylation of nuclear and cytosolic proteins. Cloning and characterization of a unique *O*-GlcNAc transferase with multiple tetratricopeptide repeats. J. Biol. Chem. 272, 9308-9315

Kreppel LK and Hart GW (1999) Regulation of a cytosolic and nuclear *O*-GlcNAc transferase. Role of the tetratricopeptide repeats. J. Biol. Chem. 274, 32015-32022

Kudlow JE (2006) Post-translational modification by *O*-GlcNAc: Another way to change protein function. J. Cell. Biochem. [Epub ahead of print]

Lamarre-Vincent N and Hsieh-Wilson LC (2003) Dynamic Glycosylation of the Transcription Factor CREB: A Potential Role in Gene Regulation. J. Am. Chem. Soc. 125, 6612-6613

Larabell CA, Torres M., Rowning BA, Yost C, Miller JR, Wu M, Kimelman D and Moon RT (1997) Establishment of the dorso-ventral axis in Xenopus embryos is presaged by early asymmetries in β-catenin that are modulated by the Wnt signaling pathway. J. Cell. Biol. 136, 1123-1136

Lazarus BD, Love DC and Hanover JA (2006) Recombinant *O*-GlcNAc transferase isoforms: identification of O-GlcNAcase, yes tyrosine kinase, and tau as isoform-specific substrates. Glycobiology 16, 415-421

Lefebvre T, Alonso C, Mahboub S, Dupire MJ, Zanetta JP, Caillet-Boudin ML and Michalski JC (1999) Effect of okadaic acid on *O*-linked N-acetylglucosamine levels in a neuroblastoma cell line. Biochim. Biophys. Acta. 1472, 71-81

Lefebvre T, Cieniewski C, Lemoine J, Guerardel Y, Leroy Y, Zanetta JP and Michalski JC (2001) Identification of N-acetyl-d-glucosamine-specific lectins from rat liver cytosolic and nuclear compartments as Heat-shock proteins. Biochem. J. 360, 179-188

Lefebvre,T, Planque N, Leleu D, Bailly M, Caillet-Boudin ML, Saule S and Michalski JC (2002) *O*-glycosylation of the nuclear forms of Pax-6 products in quail neuroretina cells. J. Cell. Biochem., 85, 208-218

Lefebvre T, Ferreira S, Dupont-Wallois L, Bussiere T, Dupire MJ, Delacourte A, Michalski JC. and Caillet-Boudin ML (2003) Evidence of a balance between phosphorylation and *O*-GlcNAc glycosylation of Tau proteins-a role in nuclear localization. Biochim. Biophys. Acta. 1619, 167-176

Lefebvre T, Pinte S, Guerardel C, Deltour S, Martin-Soudant, N, Slomianny MC, Michalski JC and Leprince D (2004a) The tumor suppressor HIC1 (Hypermethylated in cancer 1) is *O*-GlcNAc glycosylated. Eur. J. Biochem. 271, 3843-3854

Lefebvre T, Baert F, Bodart JF, Flament, S, Michalski, JC and Vilain JP (2004b) Modulation of *O*-GlcNAc glycosylation during Xenopus oocyte maturation. J. Cell. Biochem. 93, 999-1010

Lefebvre T, Guinez C, Dehennaut V, Beseme-Dekeyser O, Morelle W and Michalski JC (2005) Does *O*-GlcNAc play a role in neurodegenerative diseases? Expert Rev Proteomics. 2, 265-275

Liu F, Iqbal K, Grundke-Iqbal I, Hart GW and Gong CX (2004) *O*-GlcNAcylation regulates phosphorylation of tau: a mechanism involved in Alzheimer's disease. Proc. Nat. Acad. Sci. USA. 101, 10804-10809

Liu J, Pang Y, Chang T, Bounelis P, Chatham JC and Marchase RB (2006) Increased hexosamine biosynthesis and protein *O*-GlcNAc levels associated with myocardial protection against calcium paradox and ischemia. J. Mol. Cell. Cardiol. 40, 303-312.

Love DC, Kochan J, Cathey RL, Shin SH and Hanover JA (2003) Mitochondrial and nucleocytoplasmic targeting of *O*-linked GlcNAc transferase. J. Cell Sci. 116, 647-654

Love DC and Hanover JA (2005) The hexosamine signalling pathway: deciphering the "*O*-GlcNAc code". Sci. STKE. 312, re13

Lubas WA. and Hanover JA. (2000) Functional expression of *O*-linked GlcNAc transferase. Domain structure and substrate specificity. J. Biol. Chem. 275, 10983-10988

Ludemann N, Clement A, Hans VH, Leschik J, Behl C and Brandt R (2005) *O*-glycosylation of the tail domain of neurofilament protein M in human neurons and in spinal cord tissue of a rat model of amyotrophic lateral sclerosis (ALS). J. Biol. Chem. 280, 31648-31658

Maller JL and Krebs EG (1977) Progesterone-stimulated meiotic cell division in Xenopus oocytes. Induction by regulation subunit and inhibition by catalytic subunit 3', 5'-monophosphate dependent protein kinase. J. Biol. Chem. 252, 1712-1718

Marshall S, Bacote V and Traxinger RR (1991) Discovery of a metabolic pathway mediating glucose-induced desensitization of the glucose transport system. Role of

hexosamine biosynthesis in the induction of insulin resistance. J. Biol. Chem. 266, 4706-4712

Masui Y (2001) From oocytes maturation to the *in vitro* cell cycle: the history of discoveries of Maturation-Promoting Factor (MPF) and Cytostatic Factor (CSF). Differentiation 69, 1-17

McClain DA, Lubas WA, Cooksey RC, Hazel M, Parker GJ, Love DC and Hanover JA (2002) Altered glycan-dependent signaling induces insulin resistance and hyperleptinemia. Proc. Natl. Acad. Sci. USA 99, 10695-10699

Medina L, Grove K and Haltiwanger RS (1998) SV40 large T antigen is modified with *O*-linked N-acetylglucosamine but not with other forms of glycosylation. Glycobiology 8, 383-391

Musicki B, Kramer MF, Becker RE and Burnett AL (2005) Inactivation of phosphorylated endothelial nitric oxide synthase (Ser-1177) by *O*-GlcNAc in diabetes-associated erectile dysfunction. Proc. Natl. Acad. Sci. USA 102, 11870-11875

Myers A, Holmans P, Marshall H, Kwon J, Meyer D, Ramic D, Shears S, Booth J, DeVrieze FW, Crook R, Hamshere M, Abraham R, Tunstall N, Rice F, Carty S, Lillystone S, Kehoe P, Rudrasingham V, Jones L, Lovestone S, Perez-Tur J, Williams J, Owen MJ, Hardy J and Goate AM (2000) Susceptibility locus for Alzheimer's disease on chromosome 10. Science 290, 2304-2305

Nebreda AR and Ferby I (2000) Regulation of the meiotic cell cycle in oocytes. Curr. Opin. Cell. Biol. 12, 666-675

Nolte D and Muller U (2002) Human *O*-GlcNAc transferase (OGT): genomic structure, analysis of splice variants, fine mapping in Xq13.1. Mamm. Genome 13, 62-64

O'Donnell N (2002) Intracellular glycosylation and development. Biochim. Biophys. Acta. 1573, 336-345

O'Donnell N, Zachara NE, Hart GW and Marth JD (2004) Ogt-dependent X-chromosome-linked protein glycosylation is a requisite modification in somatic cell function and embryo viability. Mol. Cell. Biol. 24, 1680-1690

Okuno Y, Imamoto N and Yoneda Y (1993) 70-kDa Heat-shock cognate protein colocalizes with karyophilic proteins into the nucleus during their transport in vitro. Exp. Cell Res. 206, 134-142

Orford K, Crockett C, Jensen JP, Weissman A and Byers SW (1997) Serine phosphorylation-regulated ubiquitination and degradation of beta-catenin. J. Biol. Chem. 272, 24735-24738

Patti ME, Virkamäki A, Landaker EJ, Kahn CR and Yki-Järvinen H (1999) Activation of the hexosamine pathway by glucosamine in vivo induces insulin resistance of early postreceptor insulin signaling events in skeletal muscle. Diabetes 48, 1562-1571

Pinte S, Stankovic-Valentin N, Deltour S, Rood BR, Guerardel C and Leprince D (2004) The tumor suppressor gene HIC1 (hypermethylated in cancer 1) is a sequence-specific transcriptional repressor: definition of its consensus binding sequence and analysis of its DNA binding and repressive properties. J. Biol. Chem. 279, 38313-38324

Previato JO, Sola-Penna M, Agrellos OA, Jones C, Oeltmann T, Travassos LR, Mendonca-Previato L. (1998) Biosynthesis of *O*-N-acetylglucosamine-linked glycans in Trypanosoma cruzi. Characterization of the novel uridine diphospho-N-acetylglucosamine:polypeptide N-acetylglucosaminyltransferase-catalyzing formation of N-acetylglucosamine alpha1-->*O*-threonine. J. Biol. Chem. 273, 14982-14988

Privalsky ML (1990) A subpopulation of the avian erythroblastosis virus v-erbA protein, a member of the nuclear hormone receptor family, is glycosylated. J. Virol. 64, 463-466

Reason AJ, Morris HR, Panico M, Marais R, Treisman RH, Haltiwanger RS, Hart GW, Kelly WG and Dell A. (1992) Localization of *O*-GlcNAc modification on the serum response transcription factor. J. Biol. Chem. 267, 16911-16921.

Rex-Mathes M, Werner S, Strutas D, Griffith LS, Viebahn C, Thelen K and Schmitz B (2001) *O*-GlcNAc expression in developing and ageing mouse brain. Biochimie, 83, 583-590

Robertson LA, Moya KL and Breen KC (2004) The potential role of tau protein *O*-glycosylation in Alzheimer's disease. J. Alzheimers Dis. 6, 489-495

Robertson M, Swain SM, Chandler PM and Olszewski NE (1998) Identification of a negative regulator of gibberellin action, HvSPY, in barley. Plant Cell. 10, 995-1007

Robinson KA, Sens DA and Buse MG (1993) Pre-exposure to glucosamine induces insulin resistance of glucose transport and glycogen synthesis in isolated rat skeletal muscles. Study of mechanisms in muscle and in rat-1 fibroblasts overexpressing the human insulin receptor. Diabetes 42, 1333-1346

Rondanino C, Bousser MT, Monsigny, M and Roche AC (2003) Sugar-dependent nuclear import of glycosylated proteins in living cells. Glycobiology 13, 509-519

Roos MD, Su K, Baker JR and Kudlow JE (1997) *O*-glycosylation of an Sp1-derived peptide blocks known Sp1 protein interactions. Mol. Cell. Biol. 17, 6472-6480

Roquemore EP, Dell A, Morris HR, Panico M, Reason AJ, Savoy LA, Wistow GJ, Zigler JS Jr, Earles BJ and Hart GW (1992) Vertebrate lens alpha-crystallins are modified by *O*-linked N-acetylglucosamine. J. Biol. Chem. 267, 555-563

Rossetti L, Hawkins M, Chen W, Gindi J and Barzilai N (1995) In vivo glucosamine infusion induces insulin resistance in normoglycemic but not in hyperglycemic conscious rats. J. Clin. Invest. 96, 132-140

Rousseau C, Muriel MP, Musset M, Botti J and Seve AP (2000a) Glycosylated nuclear lectin CBP70 also associated with endoplasmic reticulum and the Golgi apparatus: does the "classic pathway" of glycosylation also apply to nuclear glycoproteins? J. Cell. Biochem. 78, 638-649

Rousseau C, Felin M and Seve AP (2000b) Stable expression of functional CBP70 lectin during heat shock. J Cell Biochem. 77, 615-623

Schirm M, Kalmokoff M, Aubry A, Thibault P, Sandoz M and Logan SM (2004) Flagellin from Listeria monocytogenes is glycosylated with beta-*O*-linked N-acetylglucosamine. J. Bacteriol. 186, 6721-6727

Schultz J and Pils B (2002) Prediction of structure and functional residues for *O*-GlcNAcase, a divergent homologue of acetyltransferases. FEBS Lett. 529,179-582

Seo J and Lee KJ (2004) Post-translational modifications and their biological functions: proteomic analysis and systematic approaches. J. Biochem. Mol. Biol. 37, 35-44

Serban G, Kouchi Z, Baki L, Georgakopoulos A, Litterst CM, Shioi J and Robakis NK (2005) Cadherins mediate both the association between PS1 and beta-catenin and the effects of PS1 on beta-catenin stability. J Biol. Chem. 280, 36007-36012

Shafi R, Iyer SP, Ellies LG, O'Donnell N, Marek KW, Chui D, Hart GW and Marth JD (2000) The *O*-GlcNAc transferase gene resides on the X chromosome and is essential for embryonic stem cell viability and mouse ontogeny. Proc. Natl. Sci. Acad. USA 97, 5735-5739

Shaw P, Freeman J, Bovey R and Iggo R (1996) Regulation of specific DNA binding by p53: evidence for a role for *O*-glycosylation and charged residues at the carboxy-terminus. Oncogene 12, 921-930

Shi Y and Thomas JO (1992) The transport of proteins into the nucleus requires the 70-Kilodalton Heat shock protein or its cytosolic cognate. Mol. Cell. Biol. 12, 2186-2192

Slawson C and Hart GW (2003) Dynamic interplay between *O*-GlcNAc and *O*-phosphate: the sweet side of protein regulation. Curr. Opin. Struct. Biol. 13, 631-636

Slawson S, Zachara NE, Vosseler K, Cheung WD, Lane MD and Hart GW (2005) Perturbations in *O*-linked beta-N-acetylglucosamine protein modification cause severe defects in mitotic progression and cytokinesis. J. Biol. Chem. 280, 32944-32956

Slawson C, Housley MP and Hart GW (2006) *O*-GlcNAc cycling: how a single sugar post-translational modification is changing the way we think about signaling networks. J. Cell. Biochem. 97, 71-83

Snow CM, Senior A and Gerace L (1987) Nuclear pore complex glycoproteins contain cytoplasmically disposed *O*-linked N-acetylglucosamine. J. Cell. Biol. 104, 1157-1164

Sohn KC, Lee KY, Park JE and Do SI (2003) OGT functions as a catalytic chaperone under heat stress response: a unique defense role of OGT in hyperthermia. Biochem. Biophys. Res. Commun. 322, 1045-1051

Sprung R, Nandi A, Chen Y, Kim SC, Barma D, Falck JR and Zhao Y (2005) Tagging-via-substrate strategy for probing *O*-GlcNAc modified proteins. J. Proteome Res. 4, 950-957

Sumegi M, Hunyadi-Gulyas E, Medzihradszky KF and Udvardy A (2003) 26S proteasome subunits are *O*-linked N-acetylglucosamine-modified in Drosophila melanogaster. Biochem. Biophys. Res. Commun. 312, 1284-1289

Szkudelski, T (2001) The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. Physiol. Res. 50, 537-546

Toleman C, Paterson AJ, Whisenhunt TR and Kudlow JE (2004) Characterization of the histone acetyltransferase (HAT) domain of a bifunctional protein with activable *O*-GlcNAcase and HAT activities. J. Biol. Chem. 279, 53665-53673

Torres CR and Hart GW (1984) Topography and polypeptide distribution of terminal N-acetylglucosamine residues on the surfaces of intact lymphocytes. Evidence for O-linked GlcNAc. J Biol Chem. 259, 3308-3317

Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG, Smith HO, Yandell M, Evans CA, Holt RA, Gocayne JD, Amanatides P, Ballew RM, Huson DH, Wortman JR, Zhang Q, Kodira CD, Zheng XH, Chen L, Skupski M, Subramanian G, Thomas PD, Zhang J, Gabor Miklos GL, Nelson C, Broder S, Clark AG, Nadeau J, McKusick VA, Zinder N, Levine AJ, Roberts RJ, Simon M, Slayman C, Hunkapiller M, Bolanos R, Delcher A, Dew I, Fasulo D, Flanigan M, Florea L, Halpern A, Hannenhalli S, Kravitz S, Levy S, Mobarry C, Reinert K, Remington K, Abu-Threideh J, Beasley E, Biddick K, Bonazzi V, Brandon R, Cargill M, Chandramouliswaran I, Charlab R, Chaturvedi K, Deng Z, Di Francesco V, Dunn P, Eilbeck K, Evangelista C, Gabrielian AE, Gan W, Ge W, Gong F, Gu Z, Guan P, Heiman TJ, Higgins ME, Ji RR, Ke Z, Ketchum KA, Lai Z, Lei Y, Li Z, Li J, Liang Y, Lin X, Lu F, Merkulov GV, Milshina N, Moore HM, Naik AK, Narayan VA, Neelam B, Nusskern D, Rusch DB, Salzberg S, Shao W, Shue B, Sun J, Wang Z, Wang A, Wang X, Wang J, Wei M, Wides R, Xiao C, Yan C, Yao A, Ye J, Zhan M, Zhang W, Zhang H, Zhao Q, Zheng L, Zhong F, Zhong W, Zhu S, Zhao S, Gilbert D, Baumhueter S, Spier G, Carter C, Cravchik A, Woodage T, Ali F, An H, Awe A, Baldwin D, Baden H, Barnstead M, Barrow I, Beeson K, Busam D, Carver A, Center A, Cheng ML, Curry L, Danaher S, Davenport L, Desilets R, Dietz S, Dodson K, Doup L, Ferriera S, Garg N, Gluecksmann A, Hart B, Haynes J, Haynes C, Heiner C, Hladun S, Hostin D, Houck J, Howland T, Ibegwam C, Johnson J, Kalush F, Kline L, Koduru S, Love A, Mann F, May D, McCawley S, McIntosh T, McMullen I, Moy M, Moy L, Murphy B, Nelson K, Pfannkoch C, Pratts E, Puri V, Qureshi H, Reardon M, Rodriguez R, Rogers YH, Romblad D, Ruhfel B, Scott R, Sitter C, Smallwood M, Stewart E, Strong R, Suh E, Thomas R, Tint NN, Tse S, Vech C, Wang G, Wetter J, Williams S, Williams M, Windsor S, Winn-Deen E, Wolfe K, Zaveri J, Zaveri K, Abril JF, Guigo R, Campbell MJ, Sjolander KV, Karlak B, Kejariwal A, Mi H, Lazareva B, Hatton T, Narechania A, Diemer K, Muruganujan A, Guo N, Sato S, Bafna V, Istrail S, Lippert R, Schwartz R, Walenz B, Yooseph S, Allen D, Basu A, Baxendale J, Blick L, Caminha M, Carnes-Stine J, Caulk P, Chiang YH, Coyne M, Dahlke C, Mays A, Dombroski M, Donnelly M, Ely D, Esparham S, Fosler C, Gire H, Glanowski S, Glasser K, Glodek A, Gorokhov M, Graham K, Gropman B, Harris M, Heil J, Henderson S, Hoover J, Jennings D, Jordan C, Jordan J, Kasha J, Kagan L, Kraft C, Levitsky A, Lewis M, Liu X, Lopez J, Ma D, Majoros

W, McDaniel J, Murphy S, Newman M, Nguyen T, Nguyen N, Nodell M, Pan S, Peck J, Peterson M, Rowe W, Sanders R, Scott J, Simpson M, Smith T, Sprague A, Stockwell T, Turner R, Venter E, Wang M, Wen M, Wu D, Wu M, Xia A, Zandieh A and Zhu X (2001) The sequence of the human genome. Science 291, 1304-1351

Wales MM, Biel MA, el Deiry W, Nelkin BD, Issa JP, Cavenee WK, Kuerbitz SJ and Baylin SB. (1995) p53 activates expression of HIC1, a new candidate tumour suppressor gene on 17p13.3. Nat. Med. 1, 570-577

Walgren JL, Vincent TS, Schey KL and Buse MG. (2003) High glucose and insulin promote *O*-GlcNAc modification of proteins, including alpha-tubulin. Am. J. Physiol. Endocrinol. Metab. 284, E424-E434

Weigert C, Klopfer K, Kausch C, Brodbeck K, Stumvoll M, Haring HU and Schleicher ED (2003) Palmitate-induced activation of the hexosamine pathway in human myotubes: increased expression of glutamine:fructose-6-phosphate aminotransferase. Diabetes 52, 650-656

Wells L, Vosseler K and Hart GW (2001) Glycosylation of nucleocytoplasmic proteins: signal transduction and *O*-GlcNAc. Science 291, 2376-2378

Wells L, Vosseller K, Cole RN, Cronshaw JM, Matunis MJ and Hart GW (2002a) Mapping sites of *O*-GlcNAc modification using affinity tags for serine and threonine post-translational modifications. Mol. Cell. Proteomics 1, 791-804.

Wells L, Gao Y, Mahoney JA, Vosseller K, Chen C, Rosen A and Hart GW (2002b) Dynamic *O*-glycosylation of nuclear and cytosolic proteins: further characterization of the nucleocytoplasmic beta-N-acetylglucosaminidase, *O*-GlcNAcase. J. Biol. Chem., 277, 1755-1761

Wells L, Vosseler K and Hart GW (2003a) A role for N-acetylglucosamine as a nutrient sensor and mediator of insulin resistance. Cell. Mol. Life Sci. 60, 222-228

Wells L, Whelan SA and Hart GW (2003b) *O*-GlcNAc: a regulatory post-translational modification. Biochem. Biophys. Res. Commun. 302, 435-441.

Wells L, Kreppel LK, Comer FI, Wadzinski BE and Hart GW (2004) *O*-GlcNAc transferase is in a functional complex with protein phosphatase 1 catalytic subunits. J. Biol. Chem. 279, 38466-38470

Whelan SA and Hart GW (2003) Proteomic approaches to analyze the dynamic relationships between nucleocytoplasmic protein glycosylation and phosphorylation. Circ. Res. 93, 1047-1058

Whisenhunt TR, Yang X, Bowe DB, Paterson AJ, Van Tine BA, Kudlow JE. (2006) Disrupting the enzyme complex regulating *O*-GlcNAcylation blocks signalling and development. Glycobiology 16, 551-563

Wrabl JO and Grshin NV (2001) Homology between *O*-linked GlcNAc transferases and proteins of the glycogen phosphorylase superfamily. J. Mol. Biol. 314, 365-374

Yang S, Zou LY, Bounelis P, Chaudry I, Chatham JC and Marchase RB (2006) Glucosamine administration during resuscitation improves organ function after trauma hemorraghe. Shock 25, 600-607

Yang X, Zhang F, Kudlow JE. (2002) Recruitment of *O*-GlcNAc transferase to promoters by corepressor mSin3A: coupling protein *O*-GlcNAcylation to transcriptional repression. Cell. 110, 69-80

Yang X, Su K, Roos MD, Chang Q, Paterson AJ and Kudlow JE (2001) *O*-linkage of N-acetylglucosamine to Sp1 activation domain inhibits its transcriptional capability. Proc. Natl. Acad. Sci. USA 98, 6611-6616

Yao PJ and Coleman PD (1998) Reduction of *O*-linked N-acetylglucosamine-modified assembly protein-3 in Alzheimer's disease. J. Neurosci. 18, 2399-2411

Yki-Jarvinen H, Virkamaki A, Daniels MC, McClain D and Gottschalk WK (1998) Insulin and glucosamine infusions increase O-linked N-acetyl-glucosamine in skeletal muscle proteins in vivo. Metabolism 47, 449-55

Zachara NE and Hart GW (2004) *O*-GlcNAc modification: a nutritional sensor that modulates proteasome function. Trends Cell. Biol. 14, 218-221

Zachara NE, O'Donnell N, Cheung WD, Mercer J J, Marth JD and Hart GW (2004) Dynamic *O*-GlcNAc modification of nucleocytoplasmic proteins in response to stress. A survival response of mammalian cells. J. Biol. Chem. 279, 30133-30142

Zhang F, Su K, Yang X, Bowe DB, Paterson AJ and Kudlow JE (2003) *O*-GlcNAc modification is an endogenous inhibitor of the proteasome. Cell 115, 715-725

Zhu D, Dix DJ and Eddy EM (1997) HSP70-2 is for CDC2 kinase activity in meiosis I of mouse spermatocytes. Development 124, 3007-3014

Zhu W, Leber B and Andrews DW (2001) Cytoplasmic *O*-Glycosylation prevents cell surface transport of E-cadherin during apoptosis. EMBO J. 20, 5999-6007

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# Effect of okadaic acid on O-linked *N*-acetylglucosamine levels in a neuroblastoma cell line

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**Abstract**

O-Linked *N*-Acetylglucosamine (*O*-GlcNAc) is a major form of post-translational modification found in nuclear and cytoplasmic proteins. Several authors have advanced the hypothesis according to which phosphorylation and *O*-GlcNAc glycosylation are reciprocally related to one another [1,2]. In order to test this hypothesis we have investigated the effect of a broad spectrum phosphatase inhibitor, okadaic acid (OA), generally used to induce protein hyperphosphorylation, on the GlcNAc content of cellular glycoproteins. We demonstrate that in neuronal cells lines OA decreases the level of *O*-GlcNAc in both nuclear and cytoplasmic proteins with a greater effect in the nuclear fraction. This phenomenon was demonstrated by the use of three different procedures for the detection of *O*-GlcNAc in conjunction with a systematic treatment with PNGase F. O-Linked GlcNAc was characterized using respectively lectin staining with WGA, galactosyltransferase labeling and metabolic labeling of cultured cells with [$^3$H]glucosamine. Although the effects on individual proteins varied, a less pronounced effect was observed on HeLa or COS cell total homogenates. When Kelly cells were treated with OA, the major observation was a decrease in *O*-GlcNAc content of nuclear proteins. The measurement of the UDP-GlcNAc level clearly demonstrates that the decrease on the *O*-GlcNAc level in the neuroblastoma cell line after treatment with okadaic acid is not a consequence of the modification of the UDP-GlcNAc pool. 

*Keywords:* Cytosolic glycoprotein; Nuclear glycoprotein; *O*-*N*-Acetylglucosamine; Phosphorylation; Okadaic acid; Nuclear translocation

## 1. Introduction

It is now well established that a number of cytosolic and nuclear proteins undergo post-translational modification by addition of a single GlcNAc residue *O*-glycosidically linked to the side chain hydroxyl of serine and threonine residues [3–5]. Although the exact functions of this modification remain to be established, many lines of evidence suggest that this type of glycosylation is dynamic and in particular it has been speculated to function as an alternative cellular

Abbreviations: BSA, bovine serum albumin; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; PNGase F, peptide-*N*-glycosidase F; WGA, wheat germ agglutinin; PNA, peanut agglutinin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; AMP, adenosine 5′-monophosphate; UDP-GlcNAc, uridine diphospho-*N*-acetylglucosamine

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signaling pathway to phosphorylation [1,2]. This hypothesis is supported by the finding that *O*-GlcNAc bearing domains on certain proteins have been demonstrated to coincide with known sites of phosphorylation [6–8]. Direct evidence for competition between *O*-GlcNAc and phosphorylation was recently demonstrated for threonine-58 of c-*myc*, a known glycogen synthase kinase-3 phosphorylation site, which is also modified with *O*-GlcNAc [9]. Among proteins undergoing *O*-GlcNAc modification are several neuronal proteins such as neurofilaments [8], microtubule-associated protein Tau [10], or β-amyloid precursor [11]. These proteins are also known to be phosphorylated. Selective inhibitors of protein kinases and phosphatases have been extensively used to modulate the level of phosphate on proteins. In particular, okadaic acid (OA), a well-known phosphatase inhibitor, has been used to increase the phosphate level on proteins [12], but its effect on *O*-GlcNAc protein modification has not yet been investigated. Recently, it has been demonstrated that an inhibitor of *O*-GlcNAc-β-*N*-acetylglucosaminidase, *O*-(2-acetamido-2-deoxy-D-glucosaminopyranosylidene)amino-*N*-phenylcarbamate (PUGNAc), was able to induce a 2-fold increase of *O*-glycosylation in the human colon cancer cells HT29, resulting in a reciprocal decrease of phosphorylation. Other cell lines were tested (NIH 3T3, CV-1 and HeLa) and were also affected by PUGNAc although the effects on HeLa cells were minimal [13]. In the present study we address the question of what would be the effect of a physiological drug affecting phosphorylation on the *O*-GlcNAc level of cytosolic and nuclear proteins. OA [14–16] was found to decrease *O*-GlcNAc glycosylation in Kelly cells, a neuroblastoma cell line; in contrast no apparent effect was appreciable with a human uterus cervix cancer cell line (HeLa) or SV40 virus transformed CV-1 cells (COS).

## 2. Materials and methods

### 2.1. Cell culture

Kelly, human neuroblastoma cells, were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (Boehringer), 50 IU/ml penicillin, 50 μg/ml streptomycin (Sigma), 2 mM glutamine (Life Technologies).

HeLa (human uterus cervix cell line) and COS cells (SV40 virus transformed CV-1 cells) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 50 IU/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine.

Cells were grown to 80% confluence and treated with 0.25 μM okadaic acid (Sigma) for 4 h as described by Dupont-Wallois et al. [16].

### 2.2. Cell fractionation

The cell pellet was washed in phosphate buffered saline (20 mM phosphate, 150 mM NaCl pH 7.5) and then resuspended in PBS containing 500 μg/ml digitonin and protease inhibitors (1 mM PMSF and 5 μg/ml of leupeptin and pepstatin; Sigma) and incubated for 10 min at room temperature as described by Kearse and Hart [1]. After centrifugation at $1000\times g$ for 10 min at 4°C, the supernatant corresponding to the cytosoluble material and the pellet containing nucleus and membranous fractions were separated. According to Bronfman et al. [17], at the digitonin concentration used in this study (0.4 mM final concentration) the cytosoluble fraction is free of membrane fragments. The pellet was washed twice in PBS and membranes were solubilized by incubation in 0.5% (v/v) Triton X-100 diluted in PBS. Nuclei were collected by centrifugation at 2000 rpm for 10 min, washed twice in PBS and solubilized in buffer H (50 mM HEPES, 500 mM NaCl, 2% (v/v) Triton X-100 and protease inhibitors).

### 2.3. Radioactive labeling

To label proteins with [$^{33}$P]orthophosphate or [$^{35}$S]Met/Cys, cells were washed and then maintained 15 min in phosphate or Met/Cys depleted medium before adding 100 μCi/ml [$^{33}$P]orthophosphate (Amersham) or 50 μCi/ml [$^{35}$S]Met/Cys (Pro-Mix in vivo cell labeling mixture, Amersham) respectively. Glucosamine radioactive labeling was done by addition of 5 μCi/ml [$^{14}$C]glucosamine added in fresh medium.

The prelabeling experiment was performed to label the cells during 1 h just before okadaic acid treatment. The cells were then washed with fresh medium

free of radioactivity before adding 250 nM OA during 4 h.

### 2.4. Labeled galactose transfer on GlcNAc residues with galactosyltransferase

Galactosyltransferase is a specific and sensitive probe frequently used in the detection of *O*-GlcNAc on cytosolic and nuclear proteins [1,18]. Cytosolic and nuclear fractions were added to an equal volume of sample buffer (56.25 mM HEPES, 11.25 mM $MnCl_2$, 250 mM galactose and 12.5 mM AMP) with protease inhibitor (1 mM PMSF and 5 μg/ml of leupeptin and pepstatin; Sigma). 0.025 U of bovine milk GlcNAc β-1,4-galactosyltransferase (Sigma) and 5 μCi of UDP-[6-$^3$H]Gal (Amersham) were finally added to initiate the reaction. The samples were incubated at 37°C for 2 h.

### 2.5. Radioactivity counting

Radioactivity was measured on 10 μl samples, precipitated by 10% trichloroacetic acid with 1 mg/ml BSA at 4°C. Before counting [$^{14}$C]glucosamine and [$^3$H]galactose, the samples were treated with PNGase F (BioLabs) according to the manufacturer's kit recommendation.

For each sample, the cpm numbers were calculated per mg of proteins. Proteins were assayed with the Bio-Rad microassay procedure according to the manufacturer's protocol. Radioactive labeling of okadaic acid treated samples was compared to the control ones.

### 2.6. Polyacrylamide gel electrophoresis, Western blotting and PNGase F treatment

The radiolabeled proteins were separated on SDS-PAGE [19], Coomassie blue stained, dried under vacuum and then exposed on Biomax film. Before electrophoresis, the radiolabeled samples with [$^{14}$C]glucosamine were treated with PNGase F as described above.

The non-radioactive samples were resolved on 10% SDS-PAGE. Then, proteins were electrophoretically transferred to nitrocellulose membrane (Schleicher and Schuell). Blots were saturated in 3% BSA in TBS-Tween (15 mM Tris, 140 mM NaCl, 0.05% (v/v) Tween) for 45 min and washed three times for 15 min with TBS-Tween [20]. PNGase F treatment which specifically releases N-linked oligosaccharides was directly performed by incubation of the nitrocellulose sheets in 5 ml phosphate buffer (pH 7.5–50 mM) containing 2500 units of PNGase F overnight. Horseradish peroxidase labeled wheat germ agglutinin was incubated in TBS-Tween (1/10 000) for 1 h. Blots were washed 3 times in TBS-Tween for 15 min and detection was done with the ECL Western blotting detection reagents (Amersham).

To control wheat germ agglutinin specificity and avoid non-specific lectin binding, experiments were performed in which 0.5 M free *N*-acetylglucosamine was added together with WGA for 1 h. Moreover, since WGA is also known to recognise sialic acid residues, control experiments were done on glycoprotein samples previously chemically desialylated with pH 2.0 formic acid solution.

### 2.7. UDP-GlcNAc level assay

Cytosolic fractions were passed over a 1 ml Dowex AG 1-X2 ($HCOO^-$ form) column. The column was washed with 5 ml $H_2O$, and then acidic material including UDP-GlcNAc was eluted with 600 mM ammonium formate pH 4.0. The acidic fraction was evaporated under vacuum and then analyzed for its UDP-GlcNAc content by anion-exchange HPLC using a Spectra Physics HPLC system consisting of a Supelcosyl LC-SAX column 250×4.6 mm (Supelco, Bellefonte, USA), a SP8800 pump, a Spectra 100 detector and a Chromjet integrator. 100% $H_2O$ (solvent A) was passed over the column for 15 min followed by a linear gradient for 30 min to 5% $KH_2PO_4$ (of a 500 mM solvent B stock solution), increased to 40% $KH_2PO_4$ for 10 min and returned to the initial conditions in 5 min (100% $H_2O$). Peaks were identified by comparison of their retention times to authentic UDP-GlcNAc sample and by co-injection of UDP-GlcNAc standard. Quantification was made by peak integration after calibration with increasing UDP-GlcNAc concentrations.

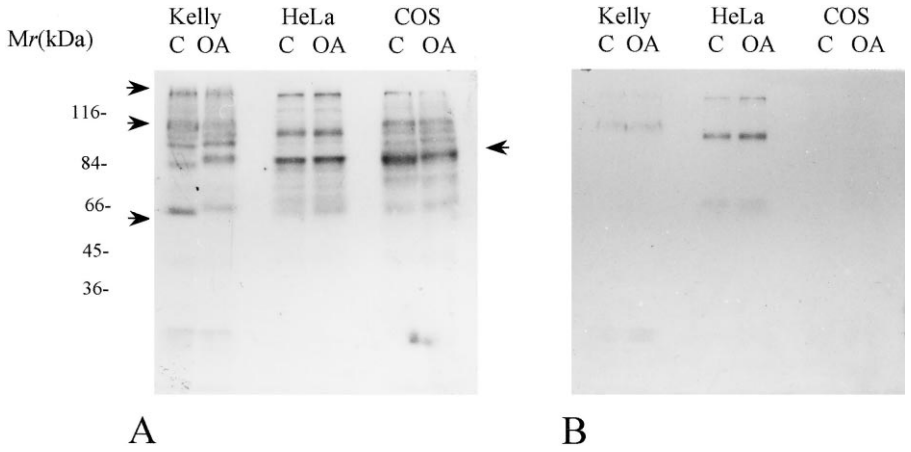


Fig. 1. Okadaic acid effect on total homogenate. Three cell lines, respectively neuroblastoma cells (Kelly), HeLa and COS (CV-1 cells transformed by SV40), were treated by okadaic acid as described in Section 2. After treatment, cells were homogenized and proteins analyzed by SDS-PAGE (10%) and electroblotted on nitrocellulose sheet. After de-*N*-glycosylation by PNGase F and desialylation with formic acid, glycoproteins were stained according to their *O*-glycosylated terminal GlcNAc content using WGA-peroxidase (A) or their *O*-glycosylated terminal GalNAc content using PNA-digoxigenin (B). C, control; OA, treatment with okadaic acid for 4 h.

## 3. Results

### 3.1. Okadaic acid effect on total homogenate

Three cell lines were used to test the effect of OA on cellular proteins: human neuroblastoma cells (Kelly), human epithelial cells (HeLa) and monkey transformed kidney cells (COS). Treatment with OA was performed as described in Section 2. After treatment, cells were homogenized and proteins analyzed by SDS-PAGE. After de-*N*-glycosylation by PNGase F, glycoproteins were stained according to their terminal GlcNAc content using WGA-peroxidase conjugate (Fig. 1A). WGA is known to recognise terminal GlcNAc, clusters borne by glycoproteins. In order to assess the eventual presence of GlcNAc containing *O*-glycans other than *O*-GlcNAc-serine/threonine a PNA staining was performed as a control. PNA lectin reacts specifically with the GalNAc-serine sequence present in most *O*-glycans (Fig. 1B).

While no modification in the electrophoretic pattern was observed after PNA staining, two types of changes in electrophoretic pattern of glycoproteins were observed for the neuronal cell line after WGA staining (arrows), but no significant modification in the electrophoretic pattern was detected for the epithelial cell lines (HeLa) and COS cells except for an approx. 90 kDa protein in the COS cells (arrow). A migration shift was observed in the higher molecular weight for some glycoproteins in Kelly cells which may certainly be related with a hyperphosphorylation content of those proteins after phosphatase inhibition by okadaic acid [15,16,21]. Moreover, a decrease in WGA staining is also remarkable for some other proteins, mostly glycoproteins with an apparent molecular mass over 55 kDa.

This study indicates that: (i) in neuronal cells phosphorylation and O-linked terminal GlcNAc glycoprotein could occur on the same proteins; (ii) for some of these glycoproteins okadaic acid treatment affects both phosphorylation and O-linked terminal GlcNAc content; (iii) this phenomenon is less pronounced in epithelium derived cells (COS, HeLa); (iv) this phenomenon does not seem to affect GalNAc containing *O*-glycans since no modification is observed for PNA stained glycoproteins.

### 3.2. Subcellular location of O-linked terminal GlcNAc proteins in Kelly cells

Kelly cells were further used to more clearly establish the change in O-linked terminal GlcNAc content induced by okadaic acid treatment. In a first experiment, after homogenization, subcellular fractionation was performed in order to obtain, respectively, a cytosoluble, a nuclear and a membrane fraction. Nu-

clear and cytosolic fractions were separately analyzed for O-linked terminal GlcNAc containing glycoproteins as previously described (Fig. 2).

Differences in the migration profile similar to that described before in the case of the crude homogenate are observed in the two subcellular compartments. Three major differences were found. (1) Some proteins just present a lower electrophoretic migration corresponding to an apparent higher molecular weight; as previously reported for total homogenate this phenomenon may be derived from an increase in the phosphorylation status of the protein. *O*-GlcNAc and phosphorylation could occur on different sites of proteins and may not always be reciprocal.

More interestingly, some other proteins (2) are stained less intensively or (3) not stained at all by the WGA upon okadaic acid treatment. This last observation supports the idea [1,2] that *O*-GlcNAc glycosylation and phosphorylation occur on the same or nearby serine/threonine residues in some glycoproteins. Dephosphorylation blockage by okadaic acid seems to inhibit the further addition of GlcNAc.

Since *O*-GlcNAc glycosylation is known to occur in the cytosol, the absence of a particular glycoprotein containing *O*-GlcNAc in the nucleus after okadaic acid treatment may account for a specific role of *O*-GlcNAc in nucleus import of cytosolic proteins as previously suggested by Duverger et al. [22–24].

### 3.3. *Study of GlcNAc incorporation on glycoproteins after okadaic acid treatment*

In order to follow the time course and the extent of GlcNAc incorporation in cytosolic and nuclear glycoproteins, three types of labeling were performed. [$^{14}$C]GlcNH$_2$ was used to follow *O*-*N*-acetylglucosaminylation, [$^{33}$P]orthophosphate to follow the phosphorylation process and [$^{35}$S]Cys/Met was used to follow protein synthesis. This strategy was used to know (Fig. 3) the influence of okadaic acid on the incorporation of [$^{14}$C]GlcNH$_2$, [$^{33}$P]orthophosphate and [$^{35}$S]Cys/Met.

In order to improve the formation of *O*-GlcNAc after okadaic acid treatment, cells were labeled for 1 h with the different radioactive precursors. After washing, okadaic acid was introduced into the cul-

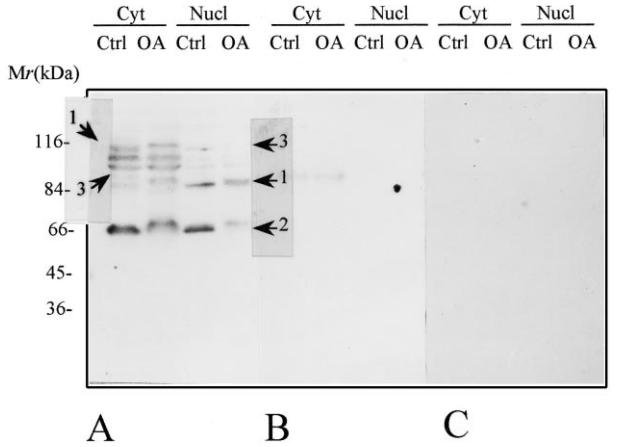


Fig. 2. Subcellular location of *O*-GlcNAc-linked proteins in Kelly cells. Kelly cells were further used to more clearly establish the change in *O*-GlcNAc content induced by okadaic acid treatment. In a first experiment, after homogenization, subcellular fractionation was performed in order to obtain a cytosoluble, a nuclear and a membrane fraction respectively. Nuclear and cytosolic fractions were separately analyzed for *O*-GlcNAc containing glycoproteins (A) and according to their GalNAc containing *O*-glycans (B) as previously described in Fig. 1. The protein loading was normalized in each lane after Ponceau red staining. To verify the specificity of the wheat germ agglutinin, control was done with 0.5 M free GlcNAc (C). Ctrl, control, OA, treatment with okadaic acid for 4 h.

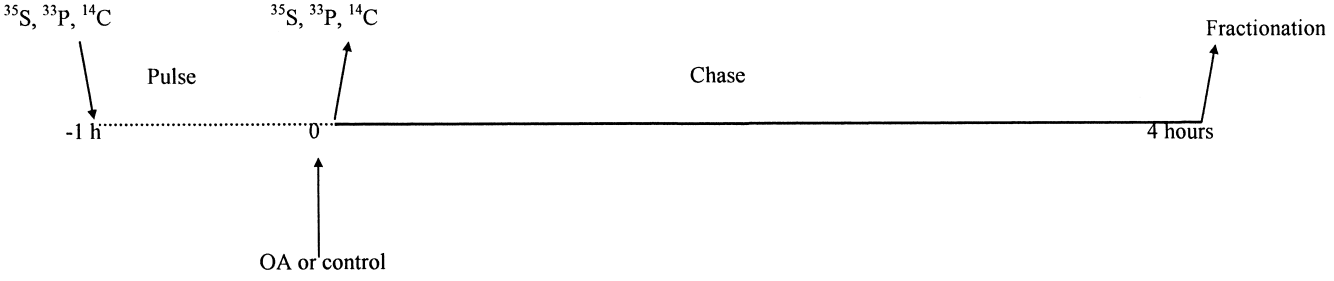


Fig. 3. Prelabeling experiment. Before treatment with okadaic acid for 4 h, Kelly cells were radiolabeled separately with [$^{33}$P]orthophosphate, [$^{14}$C]glucosamine and [$^{35}$S]Cys/Met for 1 h.

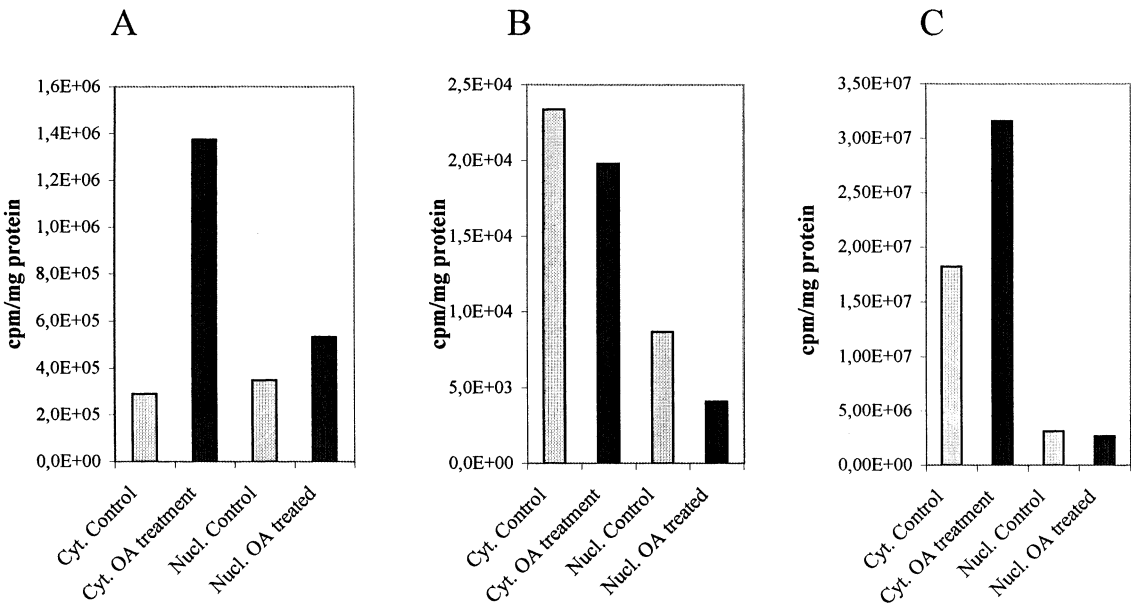


Fig. 4. Prelabeling experiment. Kelly cells were labeled for 1 h with the different radioactive precursors. The [$^{33}$P]orthophosphate was used to follow the phosphorylation (A), [$^{14}$C]GlcNH$_2$ was used to follow *O*-*N*-acetylglucosaminylation (B) and [$^{35}$S]cysteine/methionine to follow the protein synthesis process (C). After labeling, cells were washed with fresh medium and okadaic acid was introduced into the culture medium and cells further cultivated for 4 h. Then they were fractionated in cytosolic and nuclear compartments. Proteins were precipitated with trichloroacetic acid 10% and filtrated under vacuum. Grey, control and black, OA treatment.

ture medium and cells further cultivated for 4 h. In this type of experiment we can determine the fate of the prelabeled proteins (*O*-*N*-acetylglucosaminylated proteins, phosphorylated proteins) upon okadaic acid treatment.

Fig. 4 illustrates the incorporation of the different isotopes. Okadaic acid treatment induces an increase in the phosphate level (Fig. 4A) of both cytosolic and nuclear proteins consistent with a phosphatase inhibition. As a result of OA treatment, proteins were not dephosphorylated and phosphorylated proteins accumulate in the different cellular compartments. In

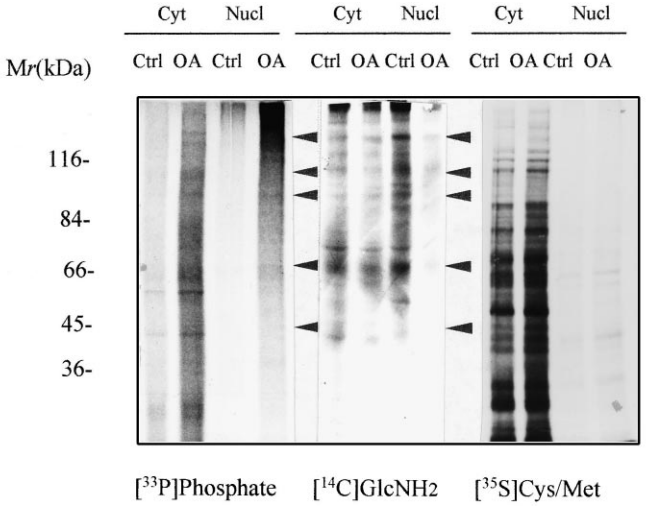


Fig. 5. Autoradiograms of the different prelabeling experiments. For the [$^{14}$C]glucosamine prelabeled proteins were treated with PNGase F. The different prelabeled proteins were separated on a 10% SDS-PAGE gel, Coomassie blue stained, dried under vacuum and exposed to Kodak film.

contrast, the content of *O*-GlcNAc residues decreases following OA treatment (Fig. 4B). Blocking dephosphorylation seems to favor the elimination of *O*-GlcNAc residues. Furthermore the decrease of *O*-GlcNAc content was more pronounced in the nucleus than in the cytoplasm (53% decrease for *O*-GlcNAc in the nucleus against just 15% in the cytosol). This increase in phosphate content is correlated with a decrease in glucosamine incorporation, which is predominant in the nucleus. On the basis of [$^{35}$S]Cys/Met labeling, it may be deduced that okadaic acid increases protein stability. Mainly, the cytosolic level is twice the control value but no change in the protein content is observed for nuclear proteins. This result may be interpreted in two ways: (i) an increase in cytosolic protein half-life induced by okadaic acid, in this respect one may speculate that hyperphosphorylation increases protein stability or *O*-GlcNAc may also serve as a degradation signal for cytosolic proteins; (ii) the second interpretation is that after OA treatment, proteins can no longer reach the nucleus and are retained in the cytosol.

Fig. 5 shows the autoradiograms corresponding to labeling experiments. The results corroborate the quantification values presented in Fig. 4. Briefly, an increase in phosphate incorporation is observed in cytosol and nucleus in okadaic acid treated extracts. For some individual proteins (arrowed in Fig. 5) we can observe a decrease in [$^{14}$C]GlcNH$_2$ incorporation after OA treatment, matched by an opposite increase in [$^{33}$P]orthophosphate labeling, reinforcing the idea of a balance between *O*-*N*-acetylglucosaminylation and phosphorylation. The difference in [$^{14}$C]GlcNH$_2$ incorporation after OA treatment is confirmed; this phenomenon is particularly intense for nucleus proteins. Moreover, the difference in [$^{14}$C]GlcNH$_2$ incorporation is restricted to high molecular mass proteins, no difference is observed for proteins with an apparent molecular mass under 50 kDa.

An increase in [$^{35}$S]Cys/Met incorporation is observed after OA treatment in the cytosolic compartment, which confirms the absence of protein synthesis level modification during the treatment.

To consider changes in the protein level in Fig. 4C, the labeling experiment values were reexpressed as $^{33}$P/$^{35}$S, $^{14}$C/$^{35}$S and $^{33}$P/$^{14}$C ratios in Fig. 6. This presentation of the data confirmed the increase in phosphorylation level and the decrease in *O*-GlcNAc content in both cytosolic and nuclear compartments. The ratio of $^{33}$P/$^{35}$S (Fig. 6A) to $^{14}$C/$^{35}$S (Fig. 6B) is in accordance with the ratio $^{33}$P/$^{14}$C (Fig. 6C) for a determinate amount of proteins. In this way, we showed a clear increase of phosphorylated proteins when compared to *O*-GlcNAc content in both cytosol and nucleus. We could also add that for newly synthesized proteins we have approximately the same decrease in *O*-GlcNAc content for the cytosolic proteins (62% decrease) as for the nuclear proteins (47%) after okadaic acid treatment.

The decrease in GlcNAc content is a reflection of

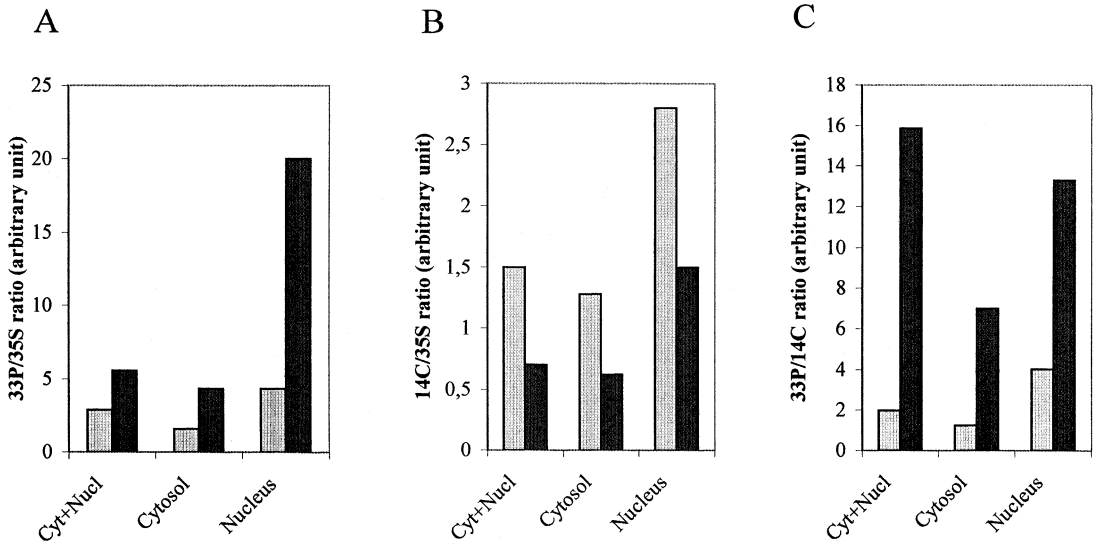


Fig. 6. $^{33}$P/$^{35}$S, $^{14}$C/$^{35}$S and $^{33}$P/$^{14}$C ratios (arbitrary units). Grey, control and black, OA treatment.

the decrease in acceptor sites for *O*-GlcNAc glycosylation. Both results are in favor of a 'balance' between phosphorylation and glycosylation, the same serine/threonine residues being alternatively substituted either by phosphate or GlcNAc. Alternatively the presence of phosphate may impair the glycosylation of Ser/Thr nearby. Moreover, the decrease of GlcNAc content in the nucleus after okadaic acid treatment could suggest that *O*-GlcNAc addition in cytosol may serve as a signal for nuclear translocation of specific proteins.

### 3.4. [$^3$H]Galactose labeling with galactosyltransferase

In order to verify that the decrease in [$^{14}$C]glucosamine incorporation after treatment with okadaic acid corresponds to a decrease in GlcNAc content and not in GalNAc or sialic acid, we realized a galactose transfer on the cytosolic and the nuclear fractions using galactosyltransferase as described in Section 2 according to Kearse and Hart [1].

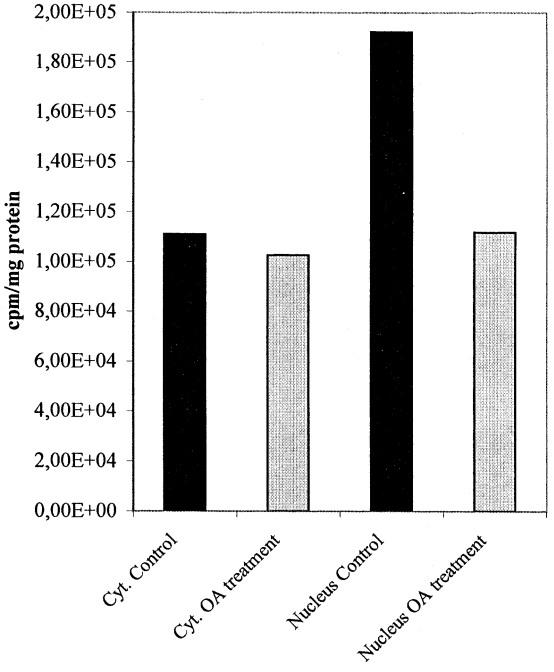


Fig. 7. Labeling with galactosyltransferase. Okadaic acid was introduced into the culture medium and Kelly cells were cultivated for 4 h. Then they were fractionated into cytosolic and nuclear compartments, proteins were labeled with galactosyltransferase and treated with PNGase F. Proteins were precipitated with trichloroacetic acid 10% and filtrated under vacuum. Grey, control and black, OA treatment.

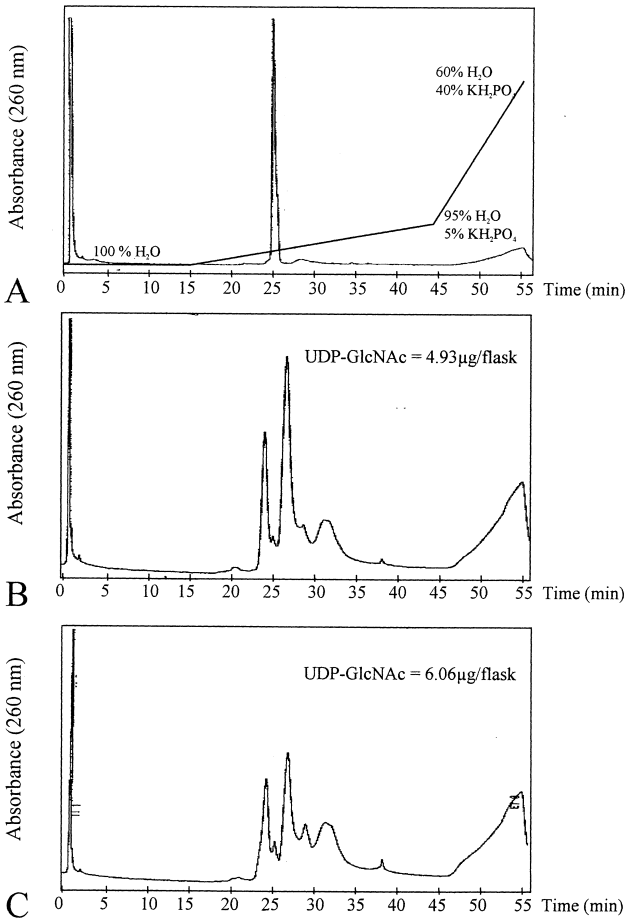


Fig. 8. UDP-GlcNAc precursor pool measurement. Cytosolic fractions of Kelly cells were previously passed over a 1 ml Dowex AG 1-X2 ($HCOO^-$ form) column, washed with 5 ml $H_2O$, and then UDP-GlcNAc was eluted with 600 mM ammonium formate pH 4.0. The acidic fraction was evaporated under vacuum and then analyzed for its UDP-GlcNAc content using a SAX analytical anion-exchange column on a Spectra Physics HPLC system. (A) UDP-GlcNAc; (B) control cells; (C) cells treated with OA.

This experiment confirms the results of the first and the second experiments (Fig. 7). [$^3$H]Galactose labeling decreased both in the cytosolic and the nuclear compartments upon OA treatment. These results corroborate the prelabeling experiment: we saw a decrease of about 10% for the cytosol and a greater decrease for the nuclear compartment, 47%, and a total decrease of about 25% after okadaic acid treatment.

### 3.5. UDP-GlcNAc level assay

In order to verify that the decrease of the *O*-GlcNAc level after treatment with okadaic acid is not a consequence of a decrease in the cytosolic UDP-GlcNAc precursor pool, the soluble UDP-GlcNAc level was measured before and after OA treatment as described in Section 2. Fig. 8 illustrates the HPLC diagrams obtained. UDP-GlcNAc concentrations were estimated to be 4.94 μg per flask for control cells (Fig. 8B) and 6.06 μg per flask after okadaic acid treatment (Fig. 8C). As a consequence the difference in the UDP-GlcNAc pool level is not significant enough to explain a modification in the glucosaminyltransferase activity. Nevertheless a modification in glucosaminyltransferase catalytic parameters induced by OA and independent from phosphorylation cannot be completely excluded.

## 4. Discussion

The best characterized example of nuclear and cytoplasmic glycosylation to date is that of O-linked *N*-acetylglucosamine (*O*-GlcNAc) [25]. Since both GlcNAc and phosphate modify the hydroxyl of serine and threonine, it has been suggested that a competition occurs between these two post-translational modifications, as supported by the observation that most *O*-GlcNAc modified proteins are also phosphoproteins [26]. Both phenomena are dynamic and controlled by the relative activity of the enzymes responsible for addition (kinases and *N*-acetylglucosaminyltransferase) and removal (phosphatases and *N*-acetylglucosaminidase) of the phosphate and GlcNAc residues respectively. In the present report, in order to assess the hypothesis that protein modifications by *O*-GlcNAc and phosphate are reciprocally related, we investigated the effect of a broad phosphatase inhibitor (OA) on the *O*-GlcNAc content of cellular glycoproteins.

The first observation was changes in the electrophoretic pattern of proteins induced by okadaic acid treatment in the neuroblastoma cell line (Kelly). These migration shifts may certainly be related with changes in the phosphorylation content of those proteins after phosphatases inhibition by okadaic acid [15,16,21]. Then we could observe that OA induced a severe decrease in *O*-GlcNAc content of both cytosolic and nuclear proteins in neuronal cells supporting the hypothesis according to which *O*-GlcNAc and phosphate are reciprocally related to one another. However, the relationship of these two types of modification very likely depends on the biologic context and the nature of the protein. In a previous report, Chou and Omary [27] described a reciprocal increase in terminal GlcNAc and phosphorylation of intermediate filament after treatment of cells with okadaic acid at a concentration which induces mitotic arrest. However, no appreciable effect on the overall O-linked terminal-GlcNAc pattern in HeLa and COS cells was observed except for a 90 kDa protein in COS cells. This protein could correspond to the Large T-antigen previously described as containing *O*-GlcNAc in a region known to be phosphorylated [7]. These results suggest that the major proteins in HeLa and COS cells lines are not affected by changes in *O*-GlcNAc levels. Similar results were previously obtained by Haltiwanger et al. using a β-*N*-acetylglucosaminidase inhibitor (PUGNAc) [13], indicating that PUGNAc effects were minimal on HeLa cells. Other studies using streptozotocin, another analogue of *N*-acetylglucosamine, demonstrated a blockage of the removal of *O*-GlcNAc from intracellular proteins in an early pancreatic β cell [28].

When we looked specifically at the individual protein level, we observed that *O*-*N*-acetylglucosaminylation of some proteins remained unchanged after OA treatment both in total homogenate and subcellular fractions. For that reason it is likely that there is no complete overlap between the glycosylation and the phosphorylation sites.

The phenomenon was further quantified using pulse/chase labeling experiments with [$^{14}$C]GlcNH$_2$, [$^{33}$P]Phosphate and [$^{35}$S]cysteine/methionine precursors. The major finding was a decrease of the GlcNAc labeling in the nucleus. This observation reinforces previous work [22–24] demonstrating that GlcNAc acts as a signal for nuclear translocation of cytosolic glycoproteins independent of the NLS peptidic signal. As a consequence the nuclear translocation or at least the cytosolic retention of specific glycoproteins could be directly related to the *O*-GlcNAc/phosphate balance.

In opposition to OA which presents a broad inhib-

ition spectrum towards phosphatases, kinases are rather specific and their inhibition also requires specific inhibitors. Nevertheless we speculate that pharmacological manipulation of cells with kinase inhibitors may provide opposite effects and mainly an increase in nuclear routing of cytosolic glycoproteins. In this respect in a number of cases such as for the transcription factor c-*myc* [29] the site modified by *O*-GlcNAc, threonine-58, was found to closely resemble those of glycogen synthase kinases (GSK3). For that reason it has been suggested that O-linked GlcNAc addition and phosphorylation by kinases such as GSK-3, may have a common denominator: their involvement in transcriptional regulation of glucose metabolism [30]. Curiously, in addition to its inhibition towards phosphatases 1 and 2A, OA was demonstrated to act as an inducer of the 78 kDa glucose-regulated protein in brain tumor cells [31]. For that reason in addition to the phosphatase inhibition, modification of glucose metabolism in neuronal cells may contribute to the protein modification observed. The type of alteration induced by OA in brain cells may contribute to three different *O*-GlcNAc patterns observed in neuronal cells: (1) some proteins just present a migration shift in the higher molecular weight and, more interestingly, other proteins are (2) understained or (3) not stained by the WGA after okadaic acid treatment.

It should also be mentioned that brain proteins such as Tau proteins [10], neurofilaments [8] or neuronal proteins [11] have been demonstrated to be extensively modified by *O*-GlcNAc. An effect similar to that of OA has previously been described after mitotic arrest with microtubule destabilizing agents such as nocodazole [32].

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## References

[1] K.P. Kearse, G.W. Hart, Lymphocyte activation induces rapid changes in nuclear and cytoplasmic glycoproteins, Proc. Natl. Acad. Sci. USA 88 (1991) 1701–1705.
[2] C.F. Chou, A.J. Smith, M.B. Omary, Characterization and dynamics of O-linked glycosylation of human cytokeratin 8 and 18, J. Biol. Chem. 267 (1992) 3901–3906.
[3] C.-R. Torres, G.W. Hart, Topography and polypeptide distribution of terminal N-acetylglucosamine residues on the surface of intact lymphocytes. Evidence for O-linked GlcNAc, J. Biol. Chem. 259 (1984) 3308–3317.
[4] G.W. Hart, R.S. Haltiwanger, G.D. Holt, W.G. Kelly, Nucleoplasmic and cytoplasmic glycoproteins, Ciba Found. Symp. 145 (1989) 102–112.
[5] G.W. Hart, R.S. Haltiwanger, G.D. Holt, W.G. Kelly, Glycosylation in the nucleus and cytoplasm, Annu. Rev. Biochem. 58 (1989) 841–874.
[6] W.G. Kelly, M.E. Dahmus, G.W. Hart, RNA polymerase II is a glycoprotein, modification of the COOH-terminal domain by O-GlcNAc, J. Biol. Chem. 268 (1993) 10416–10424.
[7] L. Medina, K. Grove, R.S. Haltiwanger, SV40 large T antigen is modified with O-linked N-acetylglucosamine but not with other forms of glycosylation, Glycobiology 8 (1998) 383–391.
[8] D.L.-Y. Dong, Z.-S. Xu, M.R. Chevrier, R.J. Cotter, D.W. Cleveland, G.W. Hart, Glycosylation of mammalian neurofilaments. Localization of multiple O-linked N-acetylglucosamine moieties on neurofilament polypeptides L and M, J. Biol. Chem. 268 (1993) 16679–16687.
[9] T.-Y. Chou, G.W. Hart, C.-V. Dang, c-Myc is glycosylated at threonine 58, a known phosphorylation site and a mutational hot spot in lymphomas, J. Biol. Chem. 270 (1995) 18961–18965.
[10] C. Shane Arnold, G.V.W. Johnson, R.N. Cole, D.L.-Y. Dong, M. Lee, G.W. Hart, The microtubule-associated protein Tau is extensively modified with O-linked N-acetylglucosamine, J. Biol. Chem. 271 (1996) 28741–28744.
[11] L.S. Griffith, M. Mathes, B. Schmitz, Beta-amyloid precursor protein is modified with O-linked N-acetylglucosamine, J. Neurosci. Res. 41 (1995) 270–278.
[12] P. Cohen, The structure and regulation of protein phosphatases, Annu. Rev. Biochem. 58 (1989) 453–508.
[13] R.S. Haltiwanger, K. Grove, G.A. Philipsberg, Modulation of O-linked N-acetylglucosamine levels on nuclear and cytoplasmic proteins in vivo using the peptide O-GlcNAc-β-N-acetylglucosaminidase inhibitor O-(2-acetamido-2-deoxy-D-glucosaminopyranossylidene)amino-N-phenylcarbamate, J. Biol. Chem. 273 (1998) 3611–3617.
[14] P. Cohen, C.F.B. Holmes, Y. Tsukitani, Okadaic acid: a new probe for the study of cellular regulation, Trends Biochem. Sci. 15 (1990) 98–102.
[15] P.-S. Sautière, M.-L. Caillet-Boudin, A. Wattez, A. Delacourte, Detection of Alzheimer-type Tau proteins in okadaic acid-treated SKSNSH-SY 5Y neuroblastoma cells, Neurodegeneration 3 (1994) 53–60.

[16] L. Dupont-Wallois, P.E. Sautière, C. Cocquerelle, B. Bailleul, A. Delacourte, M.-L. Caillet-Boudin, Shift from fetal-type to Alzheimer-type phosphorylated Tau proteins in SKNSH-SY 5Y cells treated with okadaic acid, FEBS Lett. 357 (1995) 197–201.
[17] M. Bronfman, G. Loyola, C.S. Koening, Isolation of intact organelles by differential centrifugation of digitonin-treated hepatocytes using a table Eppendorf centrifuge, Anal. Biochem. 255 (1998) 252–256.
[18] S.W. Whiteheart, A. Passaniti, J.S. Reichner, G.D. Holt, R.S. Haltiwanger, G.W. Hart, Glycosyltransferase probes, Methods Enzymol. 179 (1989) 82–95.
[19] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 270 (1970) 680–685.
[20] B. Datta, M.K. Ray, D. Chakrabarti, D.E. Wylie, N.K. Gupta, Glycosylation of eukaryotic peptide chain initiation factor 2 (eIF2)-associated 67-kDa polypeptide ($p^{67}$) and its possible role in the inhibition of eIF-2 kinase-catalysed phosphorylation of the eIF-2 α-subunit, J. Biol. Chem. 34 (1989) 20620–20624.
[21] Y. Furiya, N. Sahara, H. Mori, Okadaic acid enhances abnormal phosphorylation on Tau proteins, Neurosci. Lett. 156 (1993) 67–69.
[22] E. Duverger, V. Carpentier, A.-C. Roche, M. Monsigny, Sugar-dependent nuclear import of glycoconjugates from the cytosol, Exp. Cell Res. 207 (1993) 197–201.
[23] E. Duverger, C. Pellerin-Mendes, R. Mayer, A.-C. Roche, M. Monsigny, Nuclear import of glycoconjugates is distinct from the classical NLS pathway, J. Cell Sci. 108 (1995) 1325–1332.
[24] E. Duverger, A.-C. Roche, M. Monsigny, N-Acetylglucosamine-dependent nuclear import of neoglycoproteins, Glycobiology 6 (1996) 381–386.
[25] R.S. Haltiwanger, W.G. Kelly, E.P. Roquemore, M.A. Blomberg, L.-Y. Dennis Dong, L. Kreppel, T.-Y. Chou, G.W. Hart, Glycosylation of nuclear and cytoplasmic proteins is ubiquitous and dynamic, Biochem. Soc. Trans. 20 (1992) 264–269.
[26] R.S. Haltiwanger, S. Busby, K. Grove, S. Li, D. Mason, L. Medina, D. Moloney, G. Philipsberg, R. Scartozzi, O-Glycosylation of nuclear and cytoplasmic proteins: regulation analogous to phosphorylation?, Biochem. Biophys. Res. Commun. 231 (1997) 237–242.
[27] C.F. Chou, M.B. Omary, Mitotic arrest with anti-microtubule agents or okadaic acid is associated with increased glycoprotein terminal GlcNA's, J. Cell Sci. 107 (1994) 1833–1843.
[28] M.D. Ross, W. Xie, K. Su, J.A. Clark, X. Yang, E. Chin, A.J. Paterson, J.E. Kudlow, Streptozotocin, an analog of N-acetylglucosamine, blocks the removal of O-GlcNAc from intracellular proteins, Proc. Assoc. Am. Phys. 110 (1998) 422–432.
[29] T.-Y. Chou, C.-V. Dang, G.W. Hart, Glycosylation of the c-Myc transactivation domain, Proc. Natl. Acad. Sci. USA 92 (1995) 4417–4421.
[30] H. Yki-Jarvinen, A. Virkamaki, M.-C. Daniels, D. McClain, W.-K. Gottschalk, Insulin and glucosamine infusions increase O-linked N-acetylglucosamine in skeletal muscle proteins in vivo, Metabolism 47 (1998) 449–455.
[31] M.-C. Hou, C.-H. Shen, W.-C. Lee, Y.-K. Lai, Okadaic acid as an inducer of the 78-kDa glucose-regulated protein in 9L rat brain tumor cells, J. Cell. Biochem. 51 (1993) 91–101.
[32] R.S. Haltiwanger, G.A. Philipsberg, Mitotic arrest with nocodazole induces selective changes in the level of O-linked N-acetylglucosamine and accumulation of incompletely processed N-glycans on proteins from HT29 cells, J. Biol. Chem. 272 (1997) 8752–8758.

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# Evidence of a balance between phosphorylation and *O*-GlcNAc glycosylation of Tau proteins—a role in nuclear localization

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**Abstract**

Both phosphorylation and *O*-GlcNAc glycosylation posttranslationally modify microtubule-associated Tau proteins. Whereas the hyperphosphorylation of these proteins that occurs in Alzheimer's disease is well characterized, little is known about the *O*-GlcNAc glycosylation. The present study demonstrates that a balance exists between phosphorylation and *O*-GlcNAc glycosylation of Tau proteins, and furthermore that a dysfunction of this balance correlates with reduced nuclear localization.

The affinity of Tau proteins for WGA lectin, together with evidence from [$^3$H]-galactose transfer and analysis of beta-eliminated products, demonstrated the presence of *O*-GlcNAc residues on both cytosolic and nuclear Tau proteins. In addition, our data indicated the existence of a balance between phosphorylation and *O*-GlcNAc glycosylation events. Indeed, as demonstrated by 2D-electrophoresis and Western blotting, *O*-GlcNAc residues were mainly located on the less phosphorylated Tau 441 variants, whereas the more phosphorylated forms were devoid of *O*-GlcNAc residues. Furthermore, the Tau protein hyperphosphorylation induced by cellular okadaic acid treatment was correlated with reduced incorporation of *O*-GlcNAc residues into Tau proteins and with diminished Tau transfer into the nucleus. Hence, this paper establishes a direct relationship between *O*-GlcNAc glycosylation, phosphorylation and cellular localization of Tau proteins.


*Keywords:* *O*-*N*-acetylglucosamine; Phosphorylation; Tau protein; Nuclear transport; Alzheimer's disease

## 1. Introduction

Tau proteins belong to the family of brain microtubule-associated proteins involved in polymerisation and stability of neuronal microtubules. In adult brain, six different Tau isoforms (ranging from 352 to 441 amino acids in length) are present and arise from alternative splicing of a common, primary transcript [1]. A long Tau isoform (named big Tau), containing an additional insert in the middle part of the protein, is found only in the peripheral nervous tissue [2]. Another Tau protein, named small Tau because of its 26–30 kDa apparent molecular mass, has only been observed in the nuclei of neuroblastoma cells [3]. In contrast, the six adult Tau isoforms were predominantly found in the cytoplasmic compartment, although some of these isoforms were also found within the nucleus [4].

Tau proteins can be posttranslationally modified by events such as phosphorylation, *N*- and *O*-linked glycosylation, ubiquitination, glycation, proteolysis, etc. (reviewed in Ref. [5]). Under normal circumstances, phosphorylation of Tau proteins controls microtubule polymerisation, whereas abnormal phosphorylation of Tau proteins occurs during neurodegenerative diseases such as Alzheimer's disease, progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD) (reviewed in Ref. [5]). These abnormally phosphorylated Tau proteins are the main components of filaments that accumulate in degenerating neurons. A cellular model of Alzheimer-type Tau protein phosphorylation can be obtained by treating human neuro-

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blastoma cells with okadaic acid (OA), an inhibitor of protein phosphatases 1 and 2A [6–8].

In addition to this hyperphosphorylation phenomenon, Tau proteins from Alzheimer brains (but not from normal ones) are glycosylated with *N*- and *O*-linked saccharides [9,10]. In contrast, another type of glycosylation-*O*-GlcNAc glycosylation-was reported on normal Tau proteins [11], albeit in a bovine system. Despite a high degree of sequence homology, bovine Tau proteins differ from human ones in terms of (i) the number of major Tau isoforms (four and six, respectively) [12], (ii) a possible insert due to alternative splicing of exon 8 [13], (iii) their immunoreactivity [14] and (iv) their conformation [15]. As Tau proteins are involved in human neurodegenerative diseases such as Alzheimer's disease, we felt that it was important to determine whether human Tau proteins were modified by *O*-GlcNAc glycosylation.

*O*-GlcNAc glycosylation occurs on numerous cytoplasmic and nuclear proteins, such as cytoskeletal proteins [16–18], nuclear pore proteins [19], transcription factors [20,21] and viral proteins [22,23]. It has been suggested that this type of glycosylation shares certain features with protein phosphorylation [24], mainly by occupying the same or neighbouring sites on the peptide backbone. A global relationship between *O*-GlcNAc and *O*-phosphate has been demonstrated using different inhibitors. We have previously demonstrated that the *O*-GlcNAc level decreased in several Kelly cell neuroblastoma proteins following their hyperphosphorylation induced by OA treatment [25]. On the contrary, kinase inhibitors induced an increase in staining with an *O*-GlcNAc antibody following treatment of neuroblastoma cells [26]. Furthermore, a potent peptide *O*-GlcNAc-β-*N*-acetylglucosaminidase inhibitor increases *O*-GlcNAc levels and decreases incorporation of phosphate into the Sp1 transcription factor [27]. Thus, *O*-GlcNAc is reciprocal with phosphorylation on some sites. An increasing number of well-studied proteins are now identified to be submitted to such a balance *O*-GlcNAc glycosylation/phosphorylation (reviewed in Ref. [28]).

The occurrence of an *O*-GlcNAc/phosphorylation balance raises the question of its biological significance. While the role of phosphorylation is well documented, the role of *O*-GlcNAc glycosylation is still poorly understood. Some authors have suggested a role of *O*-GlcNAc residues in the nuclear transport of cytosolic proteins [29,30]. In support of this hypothesis, we recently showed that the balance between phosphorylation and *O*-GlcNAc glycosylation in Kelly cells was strongly involved in the control of protein transfer to the nucleus [25]. We therefore decided to use this model to determine whether such a phosphorylation/*O*-GlcNAc glycosylation balance occurs for human Tau proteins, and whether this balance interferes with their transfer into the nucleus. To ensure a high level of Tau expression (and thus better detection of *O*-GlcNAc modified Tau proteins), the long Tau isoform (Tau 441) was overexpressed in human neuroblastoma Kelly cells. A stable clone (Kelly clone 16, K Cl16) was selected and used to study the effect of phosphorylation and *O*-GlcNAc levels on the cellular localization of Tau proteins.

## 2. Materials and methods

### 2.1. Materials

The PcDNA3 vector was obtained from Invitrogen (Carlsbad, CA, USA) and the PRK172 plasmid was a kind gift from Dr. M. Goedert (Cambridge University, UK). Penicillin, streptomycin, okadaic acid, digitonin, leupeptin, pepstatin, DTT, CHAPS, nonidet P40, horseradish peroxidase-labelled wheat germ agglutinin (WGA), WGA immobilized on cross-linked 4% beaded agarose and bovine galactosyltransferase were all purchased from Sigma-Aldrich Chimie (St. Quentin-Fallavier, France). RPMI 1640, glutamine and fetal calf serum were purchased from Life Technology (Cergy Pontoise, France). TFx™-50 reagent was purchased from Promega France (Charbonnières, France). PNGase F was purchased from Ozyme (Montigny Le Bretonneux, France). The enhanced chemiluminescence kit and UDP-[$^3$H] galactose were bought from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). Tau-1 was purchased from Chemicon (Temecula, USA). Vectashield was supplied by Vector Laboratories (Burlingame, USA). HPAEC apparatus was from Dionex (Sunnyvale, CA, USA). Monoclonal antibodies directed against Lamin B2 were purchased from Novocastra.

### 2.2. Expression vector construction

Tau 441 cDNA (hTau40) was cloned into the *Nde*I/*Eco*RI sites of the pRK 172 plasmid. For direct subcloning of Tau 441 cDNA into the pcDNA3 eukaryotic expression vector, a *Bam*HI site was introduced upstream of its initiator site, eliminating the initial *Nde*I site. Sequencing of the resulting pcDNA-Tau 441 plasmid showed that the subcloned cDNA sequence was identical to that previously published by Goedert et al. [1]. The plasmid was propagated in *Escherichia coli* cells and CsCl-purified according to standard protocols, prior to the transfection experiments.

### 2.3. Cell culture and transfection

Human neuroblastoma Kelly cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 50 IU/ml penicillin, 50 μg/ml streptomycin and 2 mM glutamine. Transfection experiments were performed by adding 5 μg of pcDNA3-Tau 40 and 39.4 μg of Tfx™-50 Reagent to 80% confluency Kelly cells grown in 60-mm dishes and in serum-free medium. After incubation for 1 h, additional medium (supplemented with 10% serum) was

added. After 48 h, the cells were transferred onto four Petri dishes. Stable clones were selected by growth in Geneticin®-supplemented medium. Kelly clone 16 was chosen for its high Tau 441 expression level, as determined by Western blotting of cell lysates.

To induce Tau protein hyperphosphorylation, cells were grown to 80% confluency and treated with 0.25 μM OA for 4 h, as described by Dupont-Wallois et al. [8].

### 2.4. Cell fractionation

Cell pellets were washed in phosphate buffered saline (20 mM phosphate, 150 mM NaCl, pH 7.5), resuspended in PBS containing protease inhibitors (1 mM PMSF, 5 μg/ml of leupeptin and 5 μg/ml pepstatin) and 500 μg/ml digitonin, and incubated for 10 min at room temperature, as described by Kearse and Hart [31]. After centrifugation at 200 × *g* for 10 min at 4 °C, the supernatant (corresponding to the cytosolic material) was separated from the pellet (containing nuclear and membrane fractions). According to Bronfman et al. [32], the digitonin concentration used in this study (0.4 mM final concentration) ensures the absence of membrane fragments in the cytosolic fraction. The pellet was washed twice in PBS, and membranes were solubilized by incubation in PBS containing 0.5% Triton X100. Nuclei were collected by centrifugation at 800 × *g* for 10 min, washed twice in PBS and solubilized in buffer H (50 mM Hepes, 500 mM NaCl, 2% Triton X100 plus protease inhibitors).

### 2.5. Polyacrylamide gel electrophoresis

#### 2.5.1. 1-D electrophoresis

One-dimensional analysis was performed after migration of cellular proteins through a 10% polyacrylamide gel. Prior total protein determination using the BCA kit (Pierce) ensured that the same quantity of proteinaceous material was loaded onto each gel, thus facilitating comparison between treated and untreated cells.

#### 2.5.2. 2-D electrophoresis

For 2-D electrophoresis, samples were diluted in 20% (v/v) IsoA (0.34 M sodium dodecyl sulfate (SDS), 0.15 M DTT) and 80% (v/v) IsoE (0.108 M DTT, 0.108 M CHAPS, 15 M urea, 83.3 ml/L pI 3–10 range ampholytes). The samples were run on a 4% gel (containing 9.5 M urea, 1% (v/v) 5–7 range ampholytes, 4% (v/v) 3–10 range ampholytes, 5% (v/v) detergent solution—0.3 g CHAPS, 100 μl Nonidet P40 to 900 μl dd$H_2O$) at 200 V for 1 h, then at 500 V for 1 h and finally at 800 V for 16 h. The lower and upper buffers were 20 mM phosphoric acid and 20 mM NaOH, respectively. After isoelectrofocusing, gels were extracted from their capillary and placed in transfer solution (0.5 M Tris/HCl, 10% SDS, 0.05% bromophenol blue, pH 8.8) for 10 min: proteins were then resolved on 10% SDS/PAGE, as described by Laemmli [33].

### 2.6. Western blotting

Proteins were electrophoretically transferred onto nitrocellulose membrane. Ponceau red staining confirmed that the same quantity of total proteins have been loaded and transferred onto nitrocellulose membrane (not shown). Then, blots were saturated in TBS–Tween (15 mM Tris, 140 mM NaCl, 0.5 ml/l Tween 20) containing 30 g/l BSA for 45 min, and then washed three times in TBS–Tween for 15 min [34]. Proteins were analysed by Western blotting, using horseradish peroxidase-WGA for *O*-GlcNAc detection and a range of appropriate antibodies for Tau protein detection.

#### 2.6.1. O-GlcNAc residue detection

WGA is a lectin able to bind *O*-GlcNAc residues [35]. WGA detection was performed as described in Lefebvre et al. [25]. Briefly, the N-linked oligosaccharides were released from the peptide backbone by hydrolysis with PNGase F: the nitrocellulose sheets were incubated overnight in 5 to 20 ml phosphate buffer (50 mM; pH 7.5) containing 2500 to 10,000 units PNGase F. The next day, the membrane was incubated in TBS–Tween containing horseradish peroxidase-labelled WGA (1:10,000) for 1 h. To assess WGA detection specificity, control chase experiments were performed in which 0.2 M free *N*-acetylglucosamine was added together with WGA for 1 h.

#### 2.6.2. Tau protein detection

Tau proteins were detected by both phospho-independent and -dependent antibodies. M19G, a polyclonal antibody specific for the first 19 amino acids of Tau proteins (1:2,500; 1 h) was used to detect total Tau isoforms [8]. The phospho-dependent Tau antibodies used in this study were as follows: Tau-1 (1:2000) (Chemicon), a monoclonal antibody specific for Tau sequence 192–204 in the absence of phosphate residues; AD2 (1:10,000), specific for phosphorylated Ser-396, 404 [36]; AT 180, specific for phosphorylated Thr-231; ADI 294 (1:1000), a monoclonal antibody specific for Tau proteins in their phosphorylated form, generated via collaboration with the Immunotech. This antibody was obtained by immunizing mice with purified PHF-Tau proteins: it recognizes Tau in a phospho-dependent manner as demonstrated by (i) its detection of normally phosphorylated Tau from brain biopsy samples and its lack of detection of dephosphorylated Tau from brain autopsy samples in Western blotting experiments, (ii) its affinity for Tau molecules with an acidic p*I* (this paper). In Alzheimer brain slices, this antibody specifically detected tangles, dystrophic neurites and neuritic plaques.

After incubation with primary antibodies, blots were washed three times in TBS–Tween for 15 min, and secondary antibodies were incubated at a dilution of 1:3000 for the antibodies directed against rabbit immunoglobulins and at a dilution of 1:2000 for the antibodies directed against

mouse immunoglobulins. Blots were washed three times in TBS-Tween for 15 min, and detection was performed using ECL Western blotting detection reagents.

*2.6.3. Neurofilaments and Lamin B detection*

Neurofilaments are known as neuronal cytoskeleton proteins. We used a polyclonal serum made in the laboratory. It mainly detects NF-L (70 kDa) and more weakly NF-M and NF-H (respectively 160 and 200 kDa). Lamin B2, an inner nuclear membrane protein of 66 kDa, was detected with a monoclonal antibody purchased in Novo-Castra.

*2.6.4. Densitometric analysis*

Densitometric analysis of the films was performed after detection of Tau by WGA and M19G. The values were normalized to an equal quantity of transferred proteins after scanning of Ponceau red staining. For this analysis, we used a Hewlett Packard ScanJet 4C scanner and the Quantiscan 1.5 program.

*2.7. Precipitation of O-GlcNAc bearing proteins by WGA immobilized on agarose beads*

Cytosolic extracts were incubated with 20 μl of WGA–agarose beads in 500 μl of phosphate buffer for 1 h at 4 °C. Proteins bound to WGA–agarose were collected by centrifugation, washed five times with phosphate buffer and resuspended in Laemmli buffer.

*2.8. Labelled galactose transfer on O-GlcNAc residues with galactosyl transferase: β-elimination and saccharide analysis*

Galactosyl transfer is a specific and sensitive method frequently used for the detection of *O*-GlcNAc residues on cytosolic and nuclear proteins [31]. Cytosolic fractions (control and OA-treated) were added to an equal volume of sample buffer (56.25 mM Hepes, 11.25 mM $MnCl_2$, 250 mM galactose and 12.5 mM AMP) containing protease inhibitors (1 mM PMSF, 5 μg/ml leupeptin and 5 μg/ml pepstatin), 0.025 U of bovine milk GlcNAc β-1,4-galactosyltransferase and 5 μCi of UDP-[6-$^3$H] Gal. The samples were incubated at 37 °C for 2 h β-elimination experiments were then performed by treating the samples with 1 M sodium borohydride and 0.1 M NaOH at 37 °C for 72 h. The reaction was stopped with dropwise addition of ice-cold acetic acid under vigorous stirring until pH 5.0 was reached. The β-eliminated material was dried several times under vacuum with anhydrous methanol in order to remove borate as its methyl ester, and then desalted by descending paper chromatography on Whatman 3 MM paper using *n*-butanol/ethanol/water (4:1:1 v/v/v) as solvent. Radioactivity was detected after cutting the lanes into 1-cm pieces and counting in a LS6000TA scintillation counter (Beck-

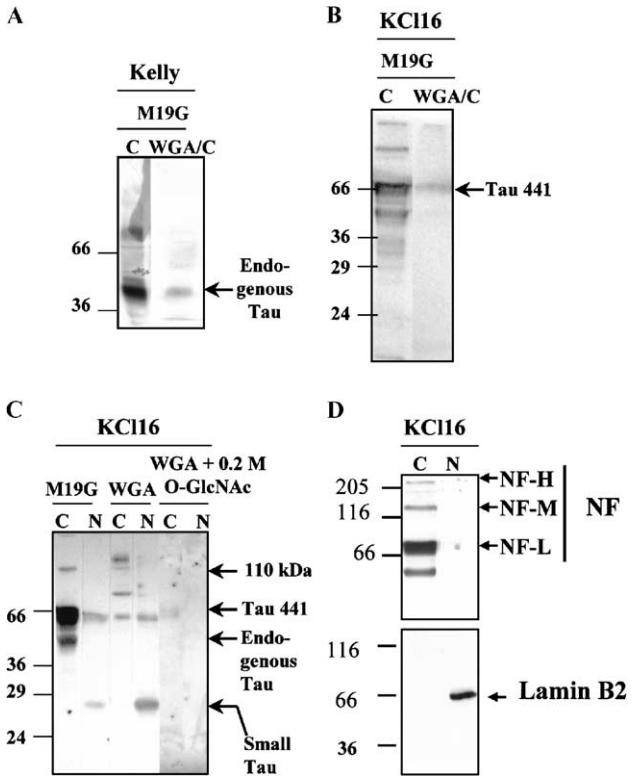


Fig. 1. WGA binding to cytosolic and nuclear Tau proteins. (A) Analysis of WGA-precipitated proteins from cytosolic Kelly extracts. The cytosolic fraction of human neuroblastoma Kelly cells was incubated with WGA–agarose beads. The proteins bound to WGA were analysed by Western blotting. A 52 kDa band was detected with Tau M19G antibodies. The molecular mass of each protein marker is indicated on the left of the panel. C: whole cytosolic extract; WGA/C: WGA-precipitated cytosolic proteins. (B) Analysis of WGA-precipitated proteins from cytosolic K CL16 extracts. K Cl16 is a Kelly clone stably transfected with Tau 441 cDNA. Using M19G, Tau 441 was detected among the WGA-precipitated K CL16 proteins. The absence of endogenous Tau in the precipitate probably results from the low level of endogenous Tau when compared to Tau 441. The molecular mass of each protein marker is indicated on the left of the panel. C: whole cytosolic extract; WGA/C: WGA-precipitated cytosolic proteins. (C) Analysis of cytosolic and nuclear proteins from K Cl16 cells. Neuroblastoma K Cl16 cells were fractionated into cytosolic and nuclear extracts, as described in Materials and methods. Cytosolic (C) and nuclear (N) proteins were analysed with M19G or peroxidase–WGA. To avoid binding of WGA by contaminant N-linked glycans, the nitrocellulose membrane was pre-treated by PNGase F before incubation with the lectin. The specificity of WGA binding was checked by co-incubation of WGA and 0.2 M GlcNAc. Bands migrating at 52 and 70 kDa were stained with M19G Tau antibodies, and corresponded to endogenous and transfected Tau 441 isoforms, respectively (Fig. 2). A minor 110 kDa band was also detected: this might correspond to the Tau isoform found in peripheral nervous tissue. Peroxidase–WGA bound to several bands (180, 90–100 and 70 kDa). The 70 kDa WGA-detected band co-migrated with M19G-detected Tau 441. We also noted a nuclear small band of 26 kDa revealed by both M19G and peroxidase–WGA. The molecular mass of each protein marker is indicated on the left of the panel. (D) Control of the purity of subcellular fraction by analysing the presence of Neurofilaments (NF) and Lamin B2 proteins. The three NF proteins (NF-H, NF-M and NF-L) were detected in cytosol fraction. The serum used here is more specific of the NF-L (70 kDa) than NF-M (160 kDa) and NF-H (200 kDa). Lamin B2 migrates as a 66 kDa protein and was specifically detected in nuclear fraction.

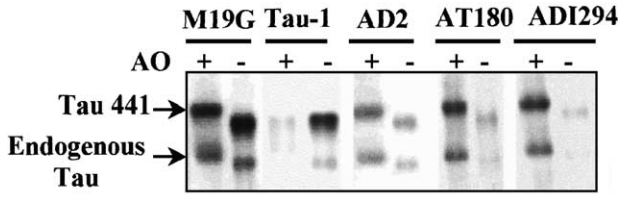


Fig. 2. Effect of okadaic acid on Tau protein phosphorylation. K Cl16 Cells were treated (+) or not (−) with OA for 4 h. Tau phosphorylation was tested by using several phospho-dependent antibodies: Tau-1, which recognizes an unphosphorylated epitope; AD2, specific for phosphorylated Ser-396/Ser-404; AT180, specific for phosphorylated Thr-231; and ADI 294, a new antibody specific for phosphorylated Tau.

man). Radioactive material was eluted from chromatography paper with water, and then analysed using High pH Anion Exchange Chromatography (HPAEC) [37] on a Dionex HPLC system equipped with a model PAD2 pulsed amperometric detector. The column was a Carbopac PA-1 (4 × 250 mm) pellicular anion exchange column with a PA-1 guard column (4 × 50 mm). The column was eluted at a flow rate of 1 ml/min with 15 mM NaOH. Fractions were collected and assayed for radioactivity.

## 3. Results

### 3.1. WGA staining of cytosolic and nuclear Tau proteins

The first stage of this work was to check the presence of *O*-GlcNAc residues on human Tau proteins and to determine whether glycosylated Tau proteins were present both in the cytosolic and nuclear compartments. First, the *O*-GlcNac glycosylated proteins in a cytosolic Kelly extract were selected by WGA–agarose precipitation. Next, the presence of Tau proteins in the precipitate was analysed by Western blotting (Fig. 1A). Anti Tau antibodies (Fig. 1A) recognized a faint band, which migrated at about 52 kDa. This argued in favour of *O*-GlcNAc glycosylation of Tau proteins. However, with the goal of improving detection of *O*-GlcNAc in nuclear extracts and to allow analysis via 2D-electrophoresis, the following experiments were performed with stably transfected Kelly cells (K Cl16) overexpressing Tau 441, the longer Tau isoform. When K Cl16 cytosolic extract was incubated with WGA–agarose, Tau 441 protein was identified in the precipitate, and migrated as a 70 kDa band (Fig. 1B). To confirm the specificity of this detection, we checked that Tau 441 revealed by M19G co-migrated with the 70 kDa band detected by WGA (Fig. 1C). The specificity of WGA binding was checked by chase experiments in the presence of 0.2 M GlcNAc (data not shown). When analysing the nuclear extracts, both M19G and WGA bound to the nuclear Tau 441 band. An additional 26 kDa band was also detected (Fig. 1C). The purity level of each cellular subfraction was tested by analysing the presence of Neurofilaments and Lamin B2 in each subfraction (Fig. 1D). These proteins are known to be respectively located in cytoplasmic and nuclear subfractions.

All these results argue in favour of the *O*-GlcNAc glycosylation of Tau proteins.

### 3.2. Effect of okadaic acid on Tau protein glycosylation

To determine whether the phosphorylation, *O*-GlcNAc glycosylation and cellular location of Tau proteins were interdependent phenomena or not, *O*-GlcNAc glycosylation analysis was performed on hyperphosphorylated Tau

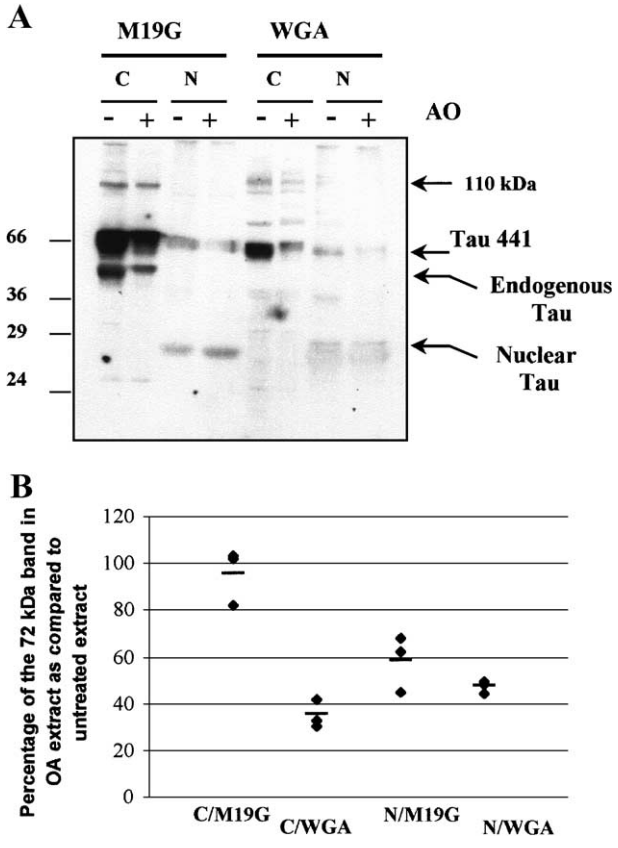


Fig. 3. Effect of okadaic acid on *O*-GlcNAc glycosylation of Tau proteins. (A) K Cl16 Cells were treated (+) or not (−) with OA for 4 h and then fractionated into cytosolic (C) and nuclear (N) fractions. M19G serum and peroxidase–WGA were used for detection of Tau and *O*-GlcNAc bearing proteins, respectively. M19G staining was quite stable in the cytosol after cellular OA treatment but decreased in the nuclei. In contrast, OA treatment decreased WGA-mediated detection of Tau 441 in both cytosolic and nuclear extracts. Ponceau red staining showed that equivalent quantity of transferred proteins was analysed in untreated and treated samples (not shown). (B) Representation of the percentage of the 72 kDa band measured either by M19G or WGA affinity in cytosolic extracts from OA-treated cells, compared to the untreated extract. Results correspond to three independent experiments. The short lines represent the average of the values.

proteins. To achieve this, cells were treated with okadaic acid (OA), a phosphatase inhibitor known to induce Tau protein hyperphosphorylation [6–8]. This OA-induced hyperphosphorylation resulted in (i) less diffusely migrating bands and a difference in electrophoretic mobility, (ii) a clear decrease of Tau detection by the Tau-1 antibody specific for an unphosphorylated epitope, and (iii) a clear increase of Tau detection by phosphorylation-dependent antibodies such as AD2, AT180 and ADI 294, i.e. in agreement with previous publications on Tau from OA-treated cells [8,39] (Fig. 2). OA-induced hyperphosphorylation did not perturb recovery of Tau proteins in the cytosolic fraction as revealed by M19G, an antibody that detects Tau whatever its phosphorylation state (82–100%), whereas it did decrease Tau protein detection in the nuclear fraction (30–42% compared to the control) (Fig. 3A,B). The low level of nuclear Tau (5–10% of total Tau from both untreated and OA-treated cells) did not allow to detect an increase of Tau in cytoplasmic Tau occurring simultaneously to the nuclear Tau decrease. Furthermore, we also observed that OA treatment induced a decrease in the binding of WGA to Tau proteins to a similar degree in both the cytosol (45–68%) and nuclei (45–50%). Hence, these two observations mean that OA treatment induced a decrease in both transfer into the nucleus and *O*-GlcNAc glycosylation of Tau proteins. We also note that the respective decreases in M19G- or WGA-detection of Tau were similar in the nuclear fraction (both around 50%) but not in the cytosol (around 100% for M19G detection and 40% for WGA detection). OA treatment thus induced a variation of the WGA/M19G ratio in cytosolic Tau but not in nuclear Tau. Consequently, the data as a whole suggest that there is a relationship between glycosylation, phosphorylation and nuclear transport of cytosolic Tau proteins.

In contrast to Tau 441 proteins, the nuclear small Tau proteins from untreated or OA-treated cells were detected to the same extent with both M19G and WGA (Fig. 3A).

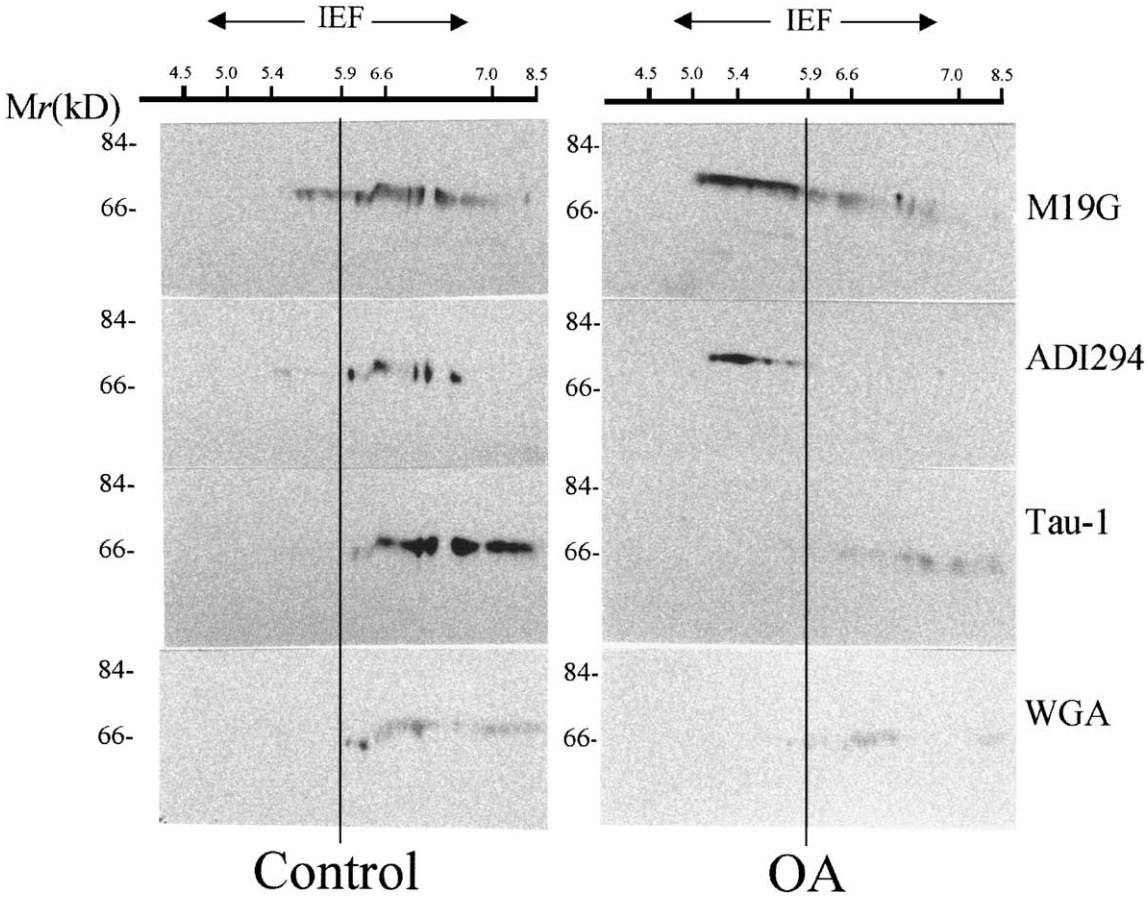


Fig. 4. Two-dimensional electrophoretic analysis of cytosolic Tau 441 variants. To investigate the balance between phosphorylation and *O*-GlcNAc glycosylation, cytosolic extracts were analysed using two-dimensional electrophoresis, as described in Materials and methods. Tau proteins from control and OA-treated cells were specifically detected with M19G (which recognizes Tau independently of its phosphorylation level), ADI 294 mAb (which specifically recognizes an epitope in its phosphorylated form), and Tau-1 (which specifically binds an unphosphorylated epitope). WGA was used to detect *O*-GlcNAc bearing Tau proteins. Ctrl: untreated K Cl16 cells; OA: treatment with OA for 4 h. One should note that WGA and Tau-1 antibodies bound to the less phosphorylated Tau isoforms, whereas ADI294 recognized the most acidic Tau isoforms.

### 3.3. Two-dimensional electrophoretic analysis of cytosolic Tau 441 proteins: evidence for a balance between phosphorylation and O-GLcNAc glycosylation

To further check the existence of a balance between phosphorylation and glycosylation, cytosolic extracts were analysed by two-dimensional electrophoresis, as described in Materials and methods. The phosphorylation of Tau proteins from control and OA-treated cells was investigated using various antibodies: M19G, ADI 294 and Tau-1. Variants bearing *O*-GlcNAc residues were detected by WGA-binding.

#### 3.3.1. Western blot analysis

As shown by M19G staining, OA treatment resulted in a decrease in Tau 441 p*I* (Fig. 4). As reported in previous papers [8,39], this decrease in p*I* suggested an increase in Tau phosphorylation. This was confirmed using the ADI 294 antibody, which only recognizes a phosphorylated epitope on Tau proteins. In control cell lysates, ADI 294 only bound to the more acidic Tau isoforms. After OA treatment, both the p*I* and the number of Tau variants detected by the ADI 294 antibody increased, confirming that the more acidic isoforms corresponded to the more phosphorylated isoforms. In contrast to the ADI 294 antibody staining, Tau-1 (an antibody specific for a non-phosphorylated Tau epitope) recognized a higher number of variants in the control cell samples than in the OA-treated samples. Tau-1 detected little Tau in cytosolic fractions from OA-treated cells. The Tau-1 immunodetected variants had a more basic p*I*. In fact, the overall Tau pattern generated using both Tau-1 and ADI 294 could be superposed on that observed with M19G.

Interestingly, the WGA-detected spots were similar to Tau-1-detected spots. Thus, *O*-GlcNAc residues seemed to be present on the less phosphorylated Tau isoforms (Fig. 4). This result confirmed the existence of a balance between *O*-GlcNAc glycosylation and phosphorylation on Tau proteins.

#### 3.3.2. Analysis using the galactose transfer method

To ensure that the decrease in WGA staining after OA treatment actually corresponded to a decrease in *O*-GlcNAc glycosylation, and to more precisely confirm the nature of Tau glycosylation, radioactive galactose was transferred by galactosyltransferase onto *O*-GlcNAc-bearing proteins in cytosolic extracts from both control and OA-treated cells. The saccharide released by subsequent beta-elimination was identified as lactosaminitol. This confirmed that the cytosolic proteins were indeed glycosylated with *O*-GlcNAc residues. A lower level of [$^3$H]-Gal was incorporated into OA-treated proteins (Fig. 5A). By performing 2D-electrophoresis on the two cytosolic extracts labelled by galactose transfer, we demonstrated that the incorporation of [$^3$H]-Gal into OA-modified Tau proteins was less than for control Tau (Fig. 5B).

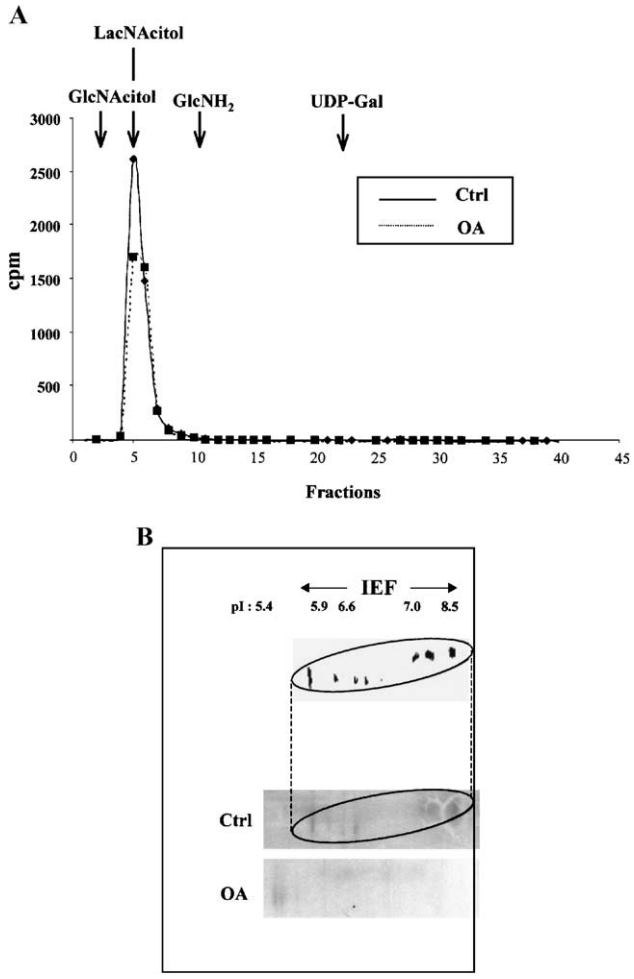


Fig. 5. Radiolabelled galactose transfer onto Tau 441. (A) Radioactive [$^3$H]-galactose labelling. Radioactive [$^3$H]-galactose was transferred by galactosyltransferase reaction onto the *O*-GlcNAc bearing cytosolic proteins of both control and OA-treated cells. Unincorporated UDP-6 [$^3$H]-galactose and salts were separated from the released glycans by descending paper chromatography. The glycans were then analysed by HPAEC. The radioactive peak released from proteins by beta-elimination was co-eluted with excess, nonradioactive LacNAcitol. Peaks corresponding to the elution of GlcNAcitol, LacNAcitol, $GlcNH_2$ and UDP-Gal are indicated. (B) Two-dimensional electrophoretic analysis of cytosolic labelled extracts. Only the radioactive spots detected in the region of Tau 441 are shown.

### 3.4. Two-dimensional electrophoretic analysis of nuclear Tau 441 proteins: influence of the phosphorylation/O-GlcNAc balance on nuclear localization

Nuclear Tau 441 isoforms were analysed by Western blotting following 2-D electrophoresis (Fig. 6). We were able to make four key observations: (i) Tau-70 kDa spots were less numerous in the nuclear fraction when compared to the cytosolic fraction; (ii) the less phosphorylated

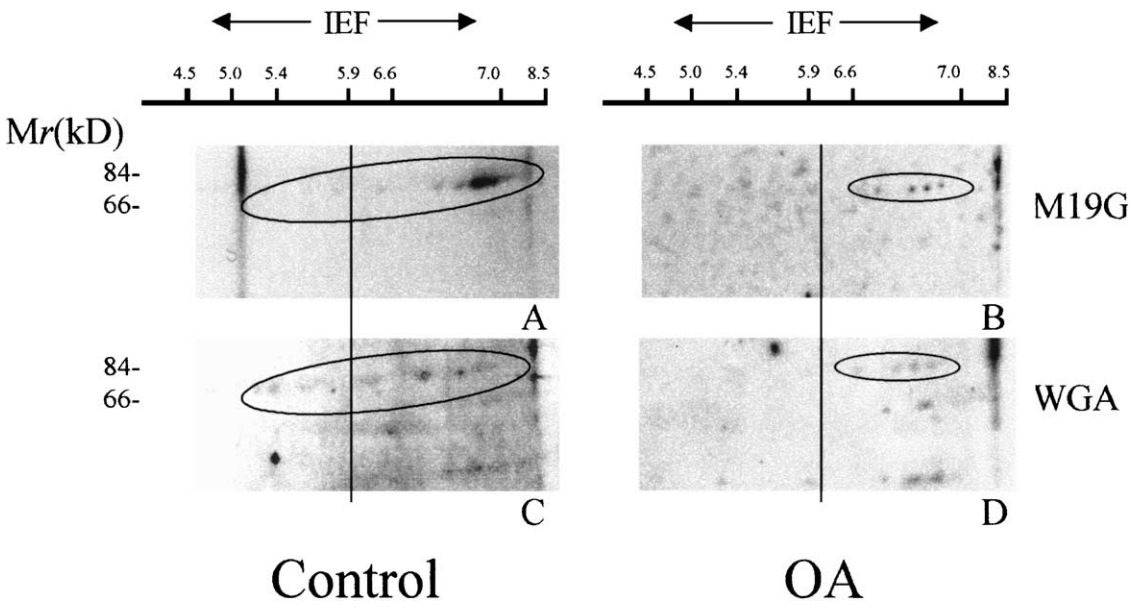


Fig. 6. 2D analysis of nuclear Tau proteins. Nuclear Tau isoforms were analysed by two-dimensional analysis. Nuclear Tau 441 isoforms were detected with both M19G and WGA.

variants were the major forms of Tau found in nuclei; (iii) M19G and WGA both detected nuclear Tau 441 in the same gel region (similar p*I* and MW); (iv) M19G and WGA detection of Tau 441 spots was weaker in OA-treated nuclear fraction than in the control fraction. These observations suggested that transfer of hyperphosphorylated Tau proteins into the nuclei was weak in general but that the less-phosphorylated variants bearing *O*-GlcNAc residues were preferentially transferred.

## 4. Discussion

A dysfunction of Tau protein phosphorylation occurs in numerous neurodegenerative diseases. Over the last 10 years, a number of laboratories have studied Tau protein phosphorylation and have identified the phosphorylated sites and kinases involved (reviewed in Ref. [5]). In contrast, only one group has reported the presence of *O*-GlcNAc residues on normal Tau proteins [11], albeit of bovine origin. Our present work shows that Tau proteins of human origin are posttranslationally modified by *O*-GlcNAc glycosylation, and that a balance between phosphorylation and *O*-GlcNAc glycosylation exists. The presence of *O*-GlcNAc residues on Tau was detected in both untransfected and stably transfected human neuroblastoma cells. Overexpression of Tau in transfected cells made detection of *O*-GlcNAc-bearing Tau proteins easier, especially after 2D-electrophoresis and cell fractionation. It was for this reason that the majority of our experiments were performed on K CL16 cells. The *O*-GlcNAc glycosylation of Tau proteins was demonstrated by their affinity for WGA lectin, by co-migration of M19G-immunoreactive and WGA-binding bands, and by precipitation of Tau proteins with WGA–agarose beads. We verified that the Tau recognition by WGA was indeed specific for *O*-linked GlcNAc residues by: (i) PNGase F pre-treatment of the samples in order to release potential, contaminating *N*-glycans, likely to bind WGA; (ii) suppression of WGA-mediated Tau detection in the presence of free GlcNAc in the incubation buffer and (iii) labelling Tau by [$^{3}$H]-galactose transfer. Beta-elimination and HPAEC techniques confirmed that human Tau proteins were glycosylated with *O*-GlcNAc residues only.

*O*-GlcNAc glycosylation shares certain features with protein phosphorylation [24,28]. These two posttranslational modifications may occur at the same or neighbouring amino acid residues, and could thus be mutually exclusive. Consequently, a balance between phosphorylation and *O*-GlcNAc glycosylation could occur. In a precedent work, [$^{14}$C]-GlcNH2 labelling, [$^{3}$H]-Galactose transfer and WGA detection allowed to detect the numerous proteins modified by *O*-GlcNAc glycosylation but the proteins in question were not identified [25]. This result was confirmed in Ref. [38]. Our present work allows to identify one of these proteins: the protein Tau. Firstly, 2D-electrophoretic analysis of control cell Tau proteins showed that *O*-GlcNAc residues were mainly located on the less phosphorylated Tau 441 variants, whereas the more phosphorylated forms were devoid of *O*-GlcNAc residues. Secondly, an increase in the Tau phosphorylation level resulted in a decrease in the *O*-GlcNAc glycosylation level. Indeed, OA is known to induce hyperphosphorylation of Tau proteins in different cellular models: neuroblastoma cells (SY 5Y cells [8,40], Kelly [39] and LA-*N*-5 [41]), cultured rat neurons [42] and

COS cells [43]. Here, hyperphosphorylation of Tau proteins was demonstrated by a decrease in their p*I*, an increase in their immunoreactivity with phospho-positive (ADI294) dependent antibodies and a decrease in their immunoreactivity with Tau-1, an antibody specific for an unphosphorylated epitope. We also noted that in transfected K Cl16 cells, Tau-1 immunoreactivity did not totally disappear, as was reported in untransfected models [6–8]. This was probably due to the high expression of Tau 441, and consequently to saturation of phosphorylation/dephosphorylation mechanisms. Hyperphosphorylation of cytosolic Tau 441 occurred in parallel with a decrease in their *O*-GlcNAc glycosylation level, as shown by weaker detection with peroxidase-WGA and lower incorporation of [$^3$H]-Gal. Thus, hyperphosphorylation inhibited the *O*-GlcNAc glycosylation of Tau proteins.

Some authors have suggested that both phosphorylation and *O*-GlcNAc glycosylation processes are involved in the nuclear transport of proteins. On one hand, a relationship between the phosphorylation level and nuclear localization has been reported for a variety of proteins, as reviewed in Ref. [44]. However, the correlation between phosphorylation, *O*-GlcNAc glycosylation and nuclear transport has not yet been investigated in these different models. On the other hand, the hypothesis suggesting a role for *O*-GlcNAc glycosylation in nuclear import stems from experiments demonstrating the nuclear internalisation of serum albumin when it was derivatized with β-di-*N*-acetylchitobioside (GlcNAc β-1, 4 GlcNAc) or an α-glucosyl motif [29,30]. The hypothesis that *O*-GlcNAc is a signal for nuclear transport of cytosolic glycoproteins is supported by the presence of GlcNAc-specific lectins in cytosol and nuclei fractions [45–47]. These GlcNAc-specific lectins would act in the shuttling of *O*-*N*-acetylglucosaminylated glycoproteins between the cytosol and the nucleus.

In this paper, we show that the phosphorylation/*O*-GlcNAc balance influences its nuclear localization. Indeed, Tau 441 was present in control cell nuclei, as previously reported by several authors [4,48,49]. In agreement with Tanaka et al. [50], we observed preferential accumulation of the less phosphorylated variants of Tau in the nucleus (Tau-1 immuno-positive variants). Interestingly, Tau 441 was more weakly detected in the nucleus after OA treatment. This probably means that Tau 441 is less efficiently translocated to the nucleus when hyperphosphorylated-although the transfer of the majority of nuclear proteins into the nucleus was not disturbed, as indicated by Ponceau red staining (data not shown). Thus, we hypothesize that hyperphosphorylation blocked (at least partially) incorporation of *O*-GlcNAc into cytosolic Tau proteins, resulting in poor transfer of these proteins to the nucleus. Hence, the balance between phosphorylation and *O*-GlcNAc glycosylation could control the nuclear transfer of Tau proteins.

Our results also confirmed the existence of a small, nuclear form of Tau protein: in addition to Tau 441, a 26 kDa band was detected in the nuclear fraction by both M19G antibody and lectin. According to its apparent molecular mass and its nuclear localization, this small Tau variant probably corresponds to that described by Shea and Cressman [3]. Interestingly, this small nuclear Tau was also *O*-GlcNAc modified but—in contrast to Tau 441 isoforms—OA treatment did not disturb its nuclear localization (Fig. 3A). Nuclear transfer of the small Tau, despite its glycosylation and phosphorylation patterns, could be attributed to its small molecular mass. Indeed, it is known that proteins with a molecular mass lower than 40 kDa could reach the nucleus by direct, passive diffusion. In this way, and in contrast to larger proteins, they would not need to transverse the nuclear pore complex [29,30]. We suggest that nuclear transport of the various Tau proteins is directed by two different processes: the first may depend on the phosphorylation/*O*-GlcNAc glycosylation balance and would control the transport of adult Tau isoforms, and the second may promote nuclear transfer of the small Tau, probably in a manner independent of the phosphorylation process. The specific function of these different nuclear Tau variants has not yet been elucidated, and must be investigated.

In conclusion, our results clearly demonstrated that human adult Tau isoforms are *O*-GlcNAc modified, and that a balance occurred between phosphorylation and *O*-GlcNAc glycosylation. As Tau proteins are hyperphosphorylated in many neurodegenerative diseases, we hypothesize that this balance is disrupted in the course of such pathologies. The existence of a balance between phosphorylation and *O*-GlcNAc glycosylation could explain why, in vivo, the hyperphosphorylated Tau proteins that aggregate in paired helical filaments during neurodegenerative diseases are devoid of *O*-GlcNAc residues [51]. Hence, *O*-GlcNAc glycosylation may be considered to be a marker of healthy brain Tau. Interestingly, *O*-GlcNAcase, the enzyme which removes *O*-GlcNAc from proteins, maps to chromosomal location 10q24, a region implicated in Alzheimer's disease and other neurological disorders [52,53]. Furthermore, this article establishes a direct relationship between phosphorylation, *O*-GlcNAc glycosylation and nuclear localization of Tau proteins.

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## References


[1] M. Goedert, M.G. Spillantini, M.C. Potier, J. Ulrich, R.A. Crowther, EMBO J. 8 (1989) 393–399.
[2] D. Couchie, C. Mavillia, I.S. Georgieff, R.K. Liem, M.L. Shelanski, J. Nunez, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 4378–4381.
[3] T.B. Shea, M. Cressman, Int. J. Dev. Neurosci. 16 (1998) 41–48.
[4] J.A. Greenwood, G.V. Johnson, Exp. Cell Res. 220 (1995) 332–337.
[5] L. Buée, T. Bussière, V. Buée-Scherrer, A. Delacourte, P.R. Hof, Brain Res. Rev. 33 (2000) 95–130.
[6] P.S. Sautière, M.L. Caillet-Boudin, A. Wattez, A. Delacourte, Neurodegeneration 3 (1994) 53–60.
[7] Y. Furiya, N. Sahara, H. Mori, Neurosci. Lett. 15 (1993) 67–69.
[8] L. Dupont-Wallois, P.E. Sautière, C. Cocquerelle, B. Bailleul, A. Delacourte, M.L. Caillet-Boudin, FEBS Lett. 357 (1995) 197–201.
[9] J.Z. Wang, I. Grundke-Iqbal, K. Iqbal, Nat. Med. 2 (1996) 871–875.
[10] M. Takahashi, Y. Tsujioka, T. Yamada, Y. Tsuboi, H. Okada, T. Yamamoto, Z. Liposits, Acta Neuropathol. 97 (1999) 635–641.
[11] S.C. Arnold, G.V.W. Johnson, R.N. Cole, D.L.Y. Dong, M. Lee, G.W. Hart, J. Biol. Chem. 271 (1996) 28741–28744.
[12] A. Himmler, Mol. Cell. Biol. 9 (1989) 1389–1396.
[13] W.T. Chen, W.K. Liu, S.H. Yen, Neurosci. Lett. 172 (1994) 167–170.
[14] A.D. Alonso, I. Grundke-Iqbal, K. Iqbal, Mol. Brain Res. 31 (1995) 194–200.
[15] N. Watanabe, K. Takio, M. Hasegawa, T. Arai, K. Titani, Y. Ihara, J. Neurochem. 58 (1992) 960–966.
[16] C.F. Chou, A.J. Smith, M.B. Omary, J. Biol. Chem. 267 (1992) 3901–3906.
[17] D.L.Y. Dong, Z.S. Xu, M.R. Chevrier, R.J. Cotter, D.W. Cleveland, G.W. Hart, J. Biol. Chem. 268 (1993) 16679–16687.
[18] M. Ding, D.D. Vandre, J. Biol. Chem. 271 (1996) 12555–12561.
[19] G.W. Holt, C.M. Snow, A. Senior, R.S. Haltiwanger, L. Gerace, G.W. Hart, J. Cell Biol. 104 (1987) 1157–1164.
[20] S.P. Jackson, R. Tjian, Cell 55 (1988) 125–133.
[21] T.Y. Chou, C.V. Dang, G.W. Hart, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 4417–4421.
[22] M.L. Caillet-Boudin, G. Strecker, J.C. Michalski, Eur. J. Biochem. 184 (1989) 205–211.
[23] L. Medina, K. Grove, R.S. Haltiwanger, Glycobiology 8 (1998) 383–391.
[24] F.I. Comer, G.W. Hart, J. Biol. Chem. 275 (2000) 29179–29212.
[25] T. Lefebvre, C. Alonso, S. Mahboub, M.J. Dupire, J.P. Zanetta, M.L. Caillet-Boudin, J.C. Michalski, Biochim. Biophys. Acta 1472 (1999) 71–81.
[26] L.S. Griffith, B. Schmitz, Eur. J. Biochem. 262 (1999) 824–831.
[27] R.S. Haltiwanger, K. Grove, G.A. Philipsberg, J. Biol. Chem. 273 (1998) 3611–3617.
[28] N.E. Zachara, G.W. Hart, Chem. Rev. 102 (2002) 431–438.
[29] E. Duverger, C. Pellerin-Mendes, R. Mayer, A.C. Roche, M. Monsigny, J. Cell Sci. 108 (1995) 1325–1332.
[30] E. Duverger, A.C. Roche, M. Monsigny, Glycobiology 6 (1996) 381–386.
[31] K.P. Kearse, G.W. Hart, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 1701–1705.
[32] M. Bronfman, G. Loyola, C.S. Koening, Anal. Biochem. 255 (1998) 252–256.
[33] U.K. Laemmli, Nature 270 (1970) 680–685.
[34] B. Datta, M.K. Ray, C.D. Hakrabarti, D.E. Wylie, N.K. Gupta, J. Biol. Chem. 34 (1989) 20620–20624.
[35] E.P. Roquemore, T.Y. Chou, G.W. Hart, Methods Enzymol. 230 (1994) 443–462.
[36] V. Buée-Scherrer, O. Condamines, C. Mourton-Gilles, R. Jakes, M. Goedert, B. Pau, A. Delacourte, Brain Res. Mol. Brain Res. 91 (1996) 351–359.
[37] A. Diekmann-Schuppert, E. Bause, R.T. Schwarz, Eur. J. Biochem. 216 (1993) 779–788.
[38] R.N. Cole, G.W. Hart, J. Neurochem. 79 (2001) 1080–1089.
[39] M.L. Caillet-Boudin, L. Dupont-Wallois, C. Soulié, A. Delacourte, Neurosci. Lett. 250 (1998) 83–86.
[40] M.L. Caillet-Boudin, A. Delacourte, NeuroReport 8 (1996) 307–310.
[41] M. Vandermeeren, U. Lubke, J. Six, P. Cras, Neurosci. Lett. 153 (1993) 57–60.
[42] D. Kim, J. Su, C.W. Cotman, Brain Res. 839 (1999) 253–262.
[43] C. Mailliot, T. Bussiere, M.L. Caillet-Boudin, A. Delacourte, L. Buée, Neurosci. Lett. 255 (1998) 13–16.
[44] A. Kaffman, E.K. O'Shea, Annu. Rev. Cell Dev. Biol. 15 (1999) 291–333.
[45] T. Lefebvre, C. Cieniewski, J. Lemoine, Y. Guerardel, Y. Leroy, J.P. Zanetta, J.C. Michalski, Biochem. J. 360 (2001) 179–188.
[46] M. Felin, M.-A. Doyenne-Moyne, Y. Hadj-Sahraoui, M. Aubery, J. Hubert, A.-P. Sève, J. Cell. Biochem. 56 (1994) 527–535.
[47] S. Kuchler, J.-P. Zanetta, G. Vincendon, H.-J. Gabius, Detection of binding sites for biotinylated neoglycoproteins and heparin (endogenous lectins) during cerebellar ontogenesis in the rat, Eur. J. Cell Biol. 52 (1990) 87–97.
[48] D.C. Cross, J.P. Munoz, P. Hernandez, R.B. Maccioni, J. Cell Biochem. 78 (2000) 305–317.
[49] P.A. Loomis, T.H. Howard, R.P. Castleberry, L.I. Binder, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 8422–8426.
[50] T. Tanaka, K. Iqbal, E. Trenkner, D.J. Liu, I. Grundke-Iqbal, FEBS Lett. 360 (1995) 5–9.
[51] M.D. Ledesma, P. Bonay, C. Colaco, J. Avila, J. Biol. Chem. 269 (1994) 21614–21619.
[52] A. Myers, P. Holmans, H. Marshall, et al., Science 290 (2000) 2304–20305.
[53] L. Bertram, D. Blacker, K. Mullin, et al., Science 290 (2000) 2302–2303.

# Does *O*-GlcNAc play a role in neurodegenerative diseases?

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**There are several lines of evidence that the modification of proteins by cytosolic- and nuclear-specific *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) glycosylation is closely related to neuropathologies, particularly Alzheimer's disease. Several neuronal proteins have been identified as being modified with *O*-GlcNAc; these proteins could form part of the inclusion bodies found, for example, in the most frequently observed neurologic disorder (i.e., Alzheimer's disease; Tau protein and β-amyloid peptide are the well known aggregated proteins). *O*-GlcNAc proteins are also implicated in synaptosomal transport (e.g., synapsins and clathrin-assembly proteins). Inclusion bodies are partly characterized by a deficiency in the ubiquitin–proteasome system, avoiding the degradation of aggregated proteins. From this perspective, it appears interesting that substrate proteins could be protected against proteasomal degradation by being covalently modified with single N-acetylglucosamine on serine or threonine, and that the proteasome itself is modified and regulated by *O*-GlcNAc (in this case the turnover of neuronal proteins correlates with extracellular glucose). Interestingly, glucose uptake and metabolism are impaired in neuronal disorders, and this phenomenon is linked to increased phosphorylation. In view of the existence of the dynamic interplay between *O*-GlcNAc and phosphorylation, it is tempting to draw a parallel between the use of glucose, *O*-GlcNAc glycosylation and phosphorylation. Lastly, the two enzymes responsible for *O*-GlcNAc dynamism (i.e., *O*-GlcNAc transferase and glucosaminidase) are both enriched in the brain and genes that encode the two enzymes are located in two regions that are found to be frequently mutated in neurologic disorders. The data presented in this review strongly suggest that *O*-GlcNAc could play an active role in neurodegenerative diseases.**

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Glycosylation of serine and threonine residues in cytosolic and nuclear proteins with single *N*-acetylglucosamine (GlcNAc) has been proven crucial for cell life during the past 20 years [1]. The structural simplicity of *O*-GlcNAc, its localization and versatility have put this single monosaccharide on the fringe of classic glycosylation. The most remarkable feature of *O*-GlcNAc is its ability to counteract phosphorylation at the same or neighboring sites on the peptide backbone. This conclusion was drawn by mapping *O*-GlcNAc sites at, or near, known phosphorylation sites [2,3] and by using kinase and phosphatase inhibitors that lead either to an increase or a decrease in the *O*-GlcNAc process [4,5]. One of the consequences of the existing competition between these two post-translational modifications on the same protein is that it results in differences in the behavior, which are mainly due to physical differences induced by these two post-translational modifications: the neutrality of *O*-GlcNAc sugar strikingly contrasts with the acidity of phosphorylation, the first being uncharged regardless of the pH, whereas the second is negatively charged at a physiologic pH (the conformational differences of the protein generate an assembly with different partners, so the activity may be modified (FIGURE 1). It is important to note that not all phosphorylation sites are in competition with *O*-GlcNAc and vice versa. This implicates that the functions of



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*O*-GlcNAc do not all ensue from this dynamic interplay. Beyond this existing competition between *O*-GlcNAc and phosphate, the functions of glycosylation remain unclear. However, it appears obvious that *O*-GlcNAc plays a critical role in:

- Transcriptional processes, with the modification of RNA polymerase II
- Transcription factors (i.e., repressors and histone deacetylase)
- Protein traffic (glycosylation of COPII is implicated in vesicular transport and glycosylation of nucleoporins has a role in nuclear translocation)
- Protein stability (modification of proteins to be protected against proteasomal degradation [6–8] and of the proteasome itself [9,10])

Several lines of evidence suggest that *O*-GlcNAc could be critical in many diseases such as cancers or diabetes and that any dysfunction in the regulation of the glyco–deglyco process could contribute to brain disorders.

This review will focus on the various ways in which modifications in *O*-GlcNAc could be involved in neurologic pathologies.

## OGT & glucosaminidase: the partners that make *O*-GlcNAc versatile

The nucleocytoplasmic enzymes that are responsible for the versatility of *O*-GlcNAc are both well studied: the first transfers GlcNAc groups from UDP-GlcNAc onto serine and threonine residues of the peptidic backbone, and the second hydrolyzes them. They are respectively known as *O*-linked *N*-acetylglucosaminyl transferase (OGT) and *O*-linked *N*-acetylglucosaminidase (GlcNAcase). These enzymes are particularly enriched in the cytosol of synaptosomes [11].

### OGT

#### *Features of* O*-linked* N*-acetylglucosaminyltransferase*

OGT (uridine diphospho-*N*-acetylglucosamine: polypeptide β-*N*-acetylglucosaminyltransferase) was first characterized in rat cytosolic liver and in extracts of rabbit reticulocyte membranes [12,13]. The enzyme is highly conserved, with 80% homology between *Caenorhabditis elegans* and human. OGT migrates as two bands by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE): the first (α) has a molecular weight of 110 kDa and the second (β) a molecular weight of 78 kDa. The 78-kDa unit is thought to be a proteolytic form of the 110 kDa one. Numerous tissues, of which brain is one, only contain the α-subunit (110 kDa). OGT is enriched in the pancreas (β-cells) and brain, however, it is also found in plants, where it is named SPINDLY and is involved in the gibberellin pathway (hormones that have various functions in plant growth and development) [14]. OGT is phosphorylated on a tyrosine residue and is itself *O*-GlcNAc glycosylated, suggesting an autoregulation via the monosaccharide. OGT is reported to bear homology with a large group of various sugar processing enzymes that includes glycogen phosphorylase, UDP-GlcNAc-2-epimerase and MurG, a glycosyl transferase [15]. Proteins homologous to OGT form a large superfamily known as the glycogen phosphorylase/glycogen transferase family.

The gene that encodes OGT is located on the Xq13 chromosomal region [16,17], and Cre-loxP recombination has shown that OGT was necessary for embryonic stem cell viability [17]. Other research efforts (on the molecular mechanism by which growth factors regulate hematopoietic cell viability or apoptosis) have demonstrated that OGT was also involved in the prevention of apoptosis [18].

Recently, alloxan, a β-cell toxin that shares analogy with uracil, was described as an inhibitor of OGT [19]. Alloxan blocked both glucosamine- and streptozotocin-induced protein glycosylation. Nevertheless, this inhibitor is also able to block β-cell glucokinase and may even inhibit other enzymes recognizing uracil moieties.

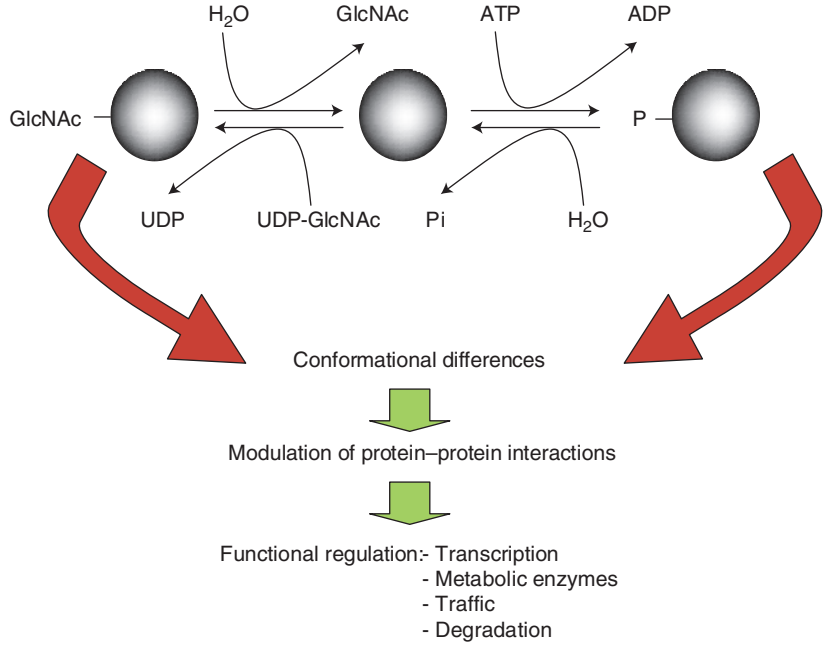


**Figure 1. Hypothetical model by which *O*-GlcNAc regulates protein activity.** *O*-GlcNAc and phosphorylation could be reciprocal on a same serine or threonine residue or on adjacent sites. Since *O*-GlcNAc glycosylation and phosphate differ by their charge (at physiologic pH, phosphate is negatively charged, whereas *O*-GlcNAc is uncharged), this physical difference induces a localized modification in the conformation of the protein (local deformation). The protein conformation is then modified and as a consequence interacting partners differ (since the recognized form is not the same). This finally leads to a regulation of protein functions.
GlcNAc: *N*-acetylglucosamine.

### *OGT partners*

OGT contains 9–13 tetratricopeptides (TPR) [20], a 34-amino acid protein–protein interaction domain found in a plethora of proteins [21], which is localized at the N-terminus and that is involved in interactions with other proteins. Different partners that physically interact with OGT have been described. Using a yeast two-hybrid assay, Iyer and coworkers have shown that GRIF1 (GABAA receptor-interacting factoR1) and its homolog, OIP106, are OGT-interacting proteins [22]. Both proteins, which contain coiled-coil domains, interact with the TPR of OGT. RNA polymerase II also coimmunoprecipitated with OGT and OIP106, suggesting that OIP106 targets OGT to the transcriptional machinery in order to glycosylate many of its components. In the same field, Yang and coworkers have highlighted a new mechanism by which transcription can be repressed [23]. OGT is recruited to promoters by the corepressor mSin3A, where it glycosylates many factors of the transcriptional machinery. This mechanism could act in parallel with histone deacetylation to promote gene silencing. Lastly, OGT has been shown to interact with the serine/threonine protein phosphatase (PP)1-β and PP1-γ [24]. OGT and PP1 could work in co-operation: PP1 should dephosphorylate sites that could be subsequently modified by OGT. This could be one way in which OGT is regulated.

TPRs have been shown to directly interact with *O*-GlcNAc-modified substrates such as OIP106 or p62 [25]. For OIP106, Iyer and Hart showed that it was TPR 2–6 that were important for glycosylation by OGT. Additionally, the isolated TPR domain of OGT competitively inhibited glycosylation of the OGT-interacting protein. These studies demonstrated the pivotal importance of TPR in substrate glycosylation.

### *Importance of OGT in brain*

In several reports, it has been demonstrated that *O*-GlcNAc has important functional roles for physiologic processes in neuronal cells. Recently, it was shown that both OGT and *O*-GlcNAc were enriched in brain and particularly in hippocampal neurons [26] and Purkinje cells [26,27]. Using fluorescent-tagged anti-OGT antibody in Purkinje cells, OGT was shown to be particularly enriched in the nucleus, perikaryon and dentrites. At the subcellular level electron microscopy revealed that OGT was enriched in euchromatin, cytoplasmic matrix, at the nerve terminal and around microtubules in dentrites. In the nerve terminal, OGT was localized with a high density in the presynaptic terminal, rather than in postsynaptic terminals [27]. These results were corroborated using an anti-*O*-GlcNAc antibody. Finally, as mentioned above, OGT is located in the Xq13 region, which is known to contain genes involved in X-linked Parkinson's disease [14].

### GlcNAcase

*O*-GlcNAcase has been purified for the first time from rat spleen [28] and has recently been cloned [29]. *O*-GlcNAcase gene maps to the chromosomal location 10q24, a region associated with Alzheimer's disease (AD) and other neurologic disorders [30,31].The protein encoded by the gene contains 916 amino acids and has a molecular weight of 103 kDa. As OGT, *O*-GlcNAcase is a nucleocytoplasmic enzyme that is distributed in all tissues and is particularly abundant in brain, skeletal muscle and pancreas. *O*-GlcNAcase was originally known as a hyaluronidase, and it was also referred to as hexosaminidase C (with a near-neutral pH optimum in contrast with hexosaminidase A and B, which are lysosomal and have acidic pH optima). Two inhibitors are known for *O*-GlcNAcase: streptozotocin, an irreversible inhibitor [32], and PUGNAc, *O*-(2-acetamido-2-deoxy D-glucopyranosylidene)amino-*N*-phenylcarbamate [33]. *O*-GlcNAcase did not share protein motifs with other components. Heat shock protein (HSP)110 and heat-shock cognate protein (HSC)70 and intracellular signal transducers (amphiphysin, DRP-2 and calcineurin, deregulations of which have all been associated with neurologic diseases) appear to complex with *O*-GlcNAcase [34].

*O*-GlcNAcase possesses an intrinsic histone acetyltransferase (HAT) activity [35]. The authors named the enzyme NCOAT for nuclear cytoplasmic *O*-GlcNAcase and acetyltransferase. It is tempting to consider that via its enzymatic activity, *O*-GlcNAcase could reverse the effect of OGT on the inhibition of the transcriptional machinery and could also act through its HAT activity to allow eukaryotic gene transcription.

### *O*-GlcNAc in brain: autopsy of *O*-GlcNAc glycosylation

Many studies have underlined the importance of *O*-GlcNAc in neuronal tissues. *O*-GlcNAc expression has been studied by enzyme-linked immunosorbent assay measurement in healthy mouse cerebellar neurons at different ages. It has been shown that this glycosylation was ubiquitously expressed from embryo (day 10) until adulthood, with no significant difference in the expression of subcellular fractions from mice brains [36]. Labelling with [$^3$H]-galactose has demonstrated an enrichment of rat synaptosome in *O*-GlcNAc proteins [11] and, using enzymatic activities measurement, the authors showed an enrichment of OGT and *O*-GlcNAcase in the nerve terminal [27].

One of the pioneering studies that pointed to the importance of *O*-GlcNAc in neuropathologies was performed by Griffith and Schmitz, who established that the expression of proteins modified with *O*-GlcNAc was significantly upregulated in AD brains compared with age-matched controls (for this purpose the authors used bovine galactosyltransferase in conjunction with [$^3$H]-galactose for the detection of *O*-GlcNAc residues) [37]. In the same field, and using the same technical approach, *O*-GlcNAc levels have been compared in autopsied normal human brains and in human AD brains [38]. Contrary to Griffith and Schmitz, Yao and Coleman concluded that the number of *O*-GlcNAc-containing proteins and overall *O*-GlcNAc level do not appear to be different between AD and control brains (normal age-matched controls). However, this group found a significant change with a marked reduction on a 160 kDa *O*-GlcNAc-bearing protein in AD that they identified as being AP-3. Recently (using also *O*-GlcNAc residues galactosylation with bovine galactosyltransferase), Robertson and coworkers showed that *O*-GlcNAc was reduced in both heat-stable Tau-enriched preparations from AD and fronto-temporal

dementia [39]. The authors attributed these discrepancies found between research groups to the methods used to generate fractions and to label terminal sugars. Lastly, the level of *O*-GlcNAc was determined in AD and control brains using radioimmuno dot-blots with anti-*O*-GlcNAc antibody [40]. The *O*-GlcNAc level in AD brains was 22% lower than in control brains. To conclude, it appears clear that *O*-GlcNAc levels tend to decrease in neuronal diseases, and more particularly in AD.

To date, it is difficult to draw up a list of *O*-GlcNAc proteins (between 80 and 120 proteins are known) since it is growing almost every day. Nevertheless, numerous neuronal proteins known to be phosphorylated were reported to be *O*-GlcNAc modified. These proteins are itemized in TABLE I. Importantly, the authors note that even if most of these proteins are implicated in neurodegenerative diseases, it cannot currently be considered or asserted whether or not the glycosylation of these proteins is implicated in such disorders. In other words, it is not clearly established that glycosylation has an impact on the function of these proteins.

### *Protein aggregation & the β-amyloid peptide*

Protein aggregation is one of the characteristic features in neuronal disorders [50]. Protein misfolding, and consequently aggregation, could occur either in the cell itself (with inclusion body formation) or in the extracellular space. Aggregates usually consist of fibers that contain misfolded proteins with a β-sheet conformation, known as amyloid. In Huntington's disease, huntingtin is the main protein that deposits (caused by a CAG repeat that encodes polyglutamine repeats, such as in other polyglutamine diseases with deposits of atrophin-1 or ataxins); in Parkinson's disease α-synuclein aggregates; in amyotrophic lateral schlerosis, the aggregate factor remains unknown; and in Prion diseases the prion protein itself aggregates. The well known case of protein aggregation is that of the extracellular aggregates neuritic plaques (senile plaques) found in AD, the major component of which is the β-amyloid peptide (42 amino acids). β-amyloid peptide is derived from the sequential intracellular cleavage of β-amyloid precursor protein (APP) [1]. APP is an ubiquitously expressed Type I membrane protein that contains a large extracellular region, a transmembrane helix and a short cytoplasmic tail. It is generated by alternative splicing that leads to multiple transcripts in which three variants are mainly expressed in brain: APP695 (the most highly expressed isoform), APP751 and APP770. APP is modified with *O*-linked *N*-acetylglucosamine [41]. It was the first reported plasma protein that bears *O*-GlcNAc residues, whereas the glycosylated sites were not mapped. The relationship between APP, *O*-GlcNAc glycosylation, APP processing and AD is unknown.

**Table 1. Identified *O*-GlcNAc neuronal proteins.**

| *O*-GlcNAc neuronal proteins | Function | Ref. |
|---|---|---|
| β-amyloid precursor | Precursor of the β-amyloid that is involved in amyloid plaque formation | [41] |
| Microtubule-associated proteins:<br>-MAP1, -2 and -4 (high-molecular-weight)<br>-Tau protein | Promote microtubule assembly and stability | [5,42,43] |
| Neurofilament H, M and L | Major neuronal intermediate filaments in adult neurons in central and peripheral nervous system. Responsible for maintaining the calibre of axons | [44,45] |
| Synapsin I | Anchors synaptic vesicles to cytoskeleton | [46] |
| Ankyrin (node of Ranvier) | Involved in the coupling of the voltage-dependent sodium channel and in bridging the membrane to the spectrin/actin network | [47] |
| Assembly protein-3 | Clathrin assembly protein | [48] |
| Collapsin response mediator protein-2 | Growth cone collapse | [11] |
| Ubiquitin carboxyl hydrolase-L1 | Deubiquitinating enzyme | [11] |
| β-synuclein | Enriched at the presynaptic membrane and associated with membranes and vesicles | [11] |
| Sox (sry-related high mobility group box)-2 | CNS and embryo transcription factor (belongs to the high-mobility group of minor groove DNA-binding proteins) | [41] |
| Activating transcription factor-2 | Brain-enriched transcription factor that possesses an intrinsic histone acteyltransferase | [49] |
| PDZ-GEF, postsynaptic density-95, discs large, and Zonula occludens-1-guanine nucleotide-exchange factor | Guanine nucleotide-exchange factor for RAP1/2 | [49] |
| Synaptopodin | Dendritic spine formation | [49] |
| Bassoon | Synaptic vesicle cycling | [49] |

## Tau: the best characterized neuronal *O*-GlcNAc protein

### *Structure & function of Tau*

Tubulin associated unit (Tau) proteins are part of a group of low-molecular-weight microtubule-associated proteins [52]. They arise from the alternative splicing of a single gene located on chromosome 17 in the 17q21 region. In neurons, Tau proteins promote the assembly, stability and orientation of tubulin monomers into microtubules to constitute the axonal microtubules network. By SDS-PAGE, Tau proteins appear as phosphorylated isoforms with molecular masses ranging from 45 to 70 kDa. Their degree of phosphorylation is a good marker of cell integrity. Structurally, Tau proteins can be divided into three parts: an acidic N-terminal region that contains a projection domain to cytoskeletal components, a central region that contains numerous proline residues and forms a target for proteins kinases, and a basic C-terminal region containing three (3R) or four (4R) tubulin-binding repeats. Phosphorylation sites are mainly located on each side of tubulin-binding repeats except for a few locations inside this domain. Then, the phosphorylation state of Tau proteins is crucial in microtubule assembly. On this point, Tau proteins are disturbed in numerous neurodegenerative disorders. These Tau dysfunctions (which result from Tau aggregation) lead to the collapse of the microtubule network and the presence of intraneuronal lesions. These abnormalities can differ in terms of structure (paired helical filaments [PHF] or straight filaments) or in terms of Tau isoform composition.

### *Tau* O*-GlcNAc modification studies*

Tau proteins are modified by numerous post-translational modifications (this underlines and reinforces the crucial importance of Tau proteins). These post-translational modifications increase the number of Tau isoforms and may imply numerous different functions for the protein. Among these modifications are phosphorylation, glycation (nonenzymatic glycosylation), ubiquitination, proteolysis, *N*-acetylation, *N*- and *O*-glycosylation and *O*-GlcNAc glycosylation.

*O*-GlcNAc glycosylation of Tau proteins was first demonstrated on bovine Tau, which is modified with 12 or more *O*-GlcNAc at a stoichiometry of 4 moles of *O*-GlcNAc per mole of Tau [43]. Initial site mapping on bovine Tau indicated that one major attachment site for *O*-GlcNAc was localized on the microtubule-binding domain on bovine Tau [43]. Nonpathogenic Tau is modified with two to three moles of phosphate per mole of Tau whereas Tau found in AD could contain up to three to four more *O*-GlcNAc residues. The number of phosphorylated sites can reach 30, both on Tau and PHF-Tau. It appears clear that *O*-GlcNAc could not regulate all phosphorylation sites since it appears that there are more phosphorylation sites than *O*-GlcNAc sites.

### O*-GlcNAc & phosphorylation relationship with Tau*

The putative interaction between *O*-GlcNAc and phosphorylation on Tau proteins was studied in a human neuroblastoma cell line [5]. Okadaic acid (a PP1 and -2A inhibitor extracted from marine sponge) induced Tau hyperphosphorylation and was first accompanied by a decrease in the *O*-GlcNAc content of Tau proteins, and secondly by a decrease in the nuclear transport of these proteins. *O*-GlcNAc glycosylation (detected with a lectin that specifically binds *O*-GlcNAc terminal residues) occurred on the less phosphorylated molecules (evidenced with phospho- and nonphospho-Tau-directed antibodies). These studies only indicated a global correlation between *O*-GlcNAc and phosphorylation on Tau but did not point to the exact location where such a relationship occured.

The regulation of Tau phosphorylation by *O*-GlcNAc was conducted on human brain Tau, using a panel of antiphospho-Tau antibodies [40]. *O*-GlcNAc reduced phosphorylation at S199, S202, T205, T212, S214, S262 and S396 in PC12 cells but not at S404. On rat brain slices, *O*-GlcNAc glycosylation reinforced or had no impact on phosphorylation at S202, S214 and S404. The differences found on the two models could be attributed to the composition in Tau proteins. The authors remarkably suggested that these three phosphorylated sites were near a phosphorylation site that was negatively regulated by *O*-GlcNAc: S202 is near S199, S214 is near S212 and T217, and S404 is near S396. Thus, phosphorylation of Tau could be either negatively regulated on the same site by *O*-GlcNAc and, on a proximal site, by phosphorylation. In the same way, studies using Tau- and OGT-cotransfected cells fully corroborated these experiments since Robertson and coworkers showed a decrease in phosphorylation on S202, T212, S214, T231, S396, S404 and S409 when OGT was overexpressed [39]. On the other hand, they did not see any decrease on T181, T205 and S262. Discrepancies between the work of Liu and coworkers and Robertson and coworkers could be related to the cell line used (PC12 cells for the first study and CHO cells in the latter) or to the treatment performed to modify *O*-GlcNAc (treatment with PUGNAc or streptozotocin in the first report, and transfection of OGT in the second one) or phosphorylation (use of okadaic acid in the first study).

Unfortunately, functions of *O*-GlcNAc on Tau remain unclear: does *O*-GlcNAc just counteract the effect of phosphorylation, leading to interactions with different partners, or are *O*-GlcNAc functions more sophisticated? Part of the answer could lie in the competition between *O*-GlcNAc and phosphorylation on Tau proteins, contributing to their nuclear localization. Exact localization of *O*-GlcNAc sites on Tau proteins could help us to understand how these residues could influence the function of these microtubule-associated unit proteins. Since Tau proteins are hyperphosphorylated in many neurodegenerative diseases, this *O*-GlcNAc/phosphorylation competition may be disrupted in the course of such pathologies [5]. The existence of a balance between phosphorylation and *O*-GlcNAc glycosylation could explain why, *in vivo*, hyperphosphorylated Tau proteins that aggregated in paired-helical filaments are devoid of *O*-GlcNAc residues. Hence, *O*-GlcNAc glycosylation may be a marker of healthy brain Tau. Here again, the authors insist on the point that even on Tau, only the balance between *O*-GlcNAc and phosphorylation could not explain all Tau

functions and all disorders where Tau intervenes. Not all the phosphorylation sites on Tau could be counteracted by *O*-GlcNAc and vice versa. Consequently, some Tau functions could be related only to phosphorylation.

### Numerous vesicular transport proteins are modified with *O*-GlcNAc

#### *CRMP-2, β-synuclein, UCH-L1, synaptodin & bassoon*

The nerve terminal is particularly enriched with *O*-GlcNAc glycosylation [11], and both enzymes of cycling *O*-GlcNAc are abundant in the cytosol of synaptosome. Three neuron-specific proteins have been identified among these *O*-GlcNAc proteins: collapsing mediator protein (CRMP)-2, β-synuclein and ubiquitin carboxyl hydrolase (UCH)-L1 [11]. All three phosphoproteins are involved in signal transduction and are linked to neurodegenerative diseases. CRMP-2 is the major protein identified as containing *O*-GlcNAc. It belongs to a group of developmentally regulated proteins and mediates growth cone collapse. β-synuclein belongs to a group of soluble proteins, the synucleins. They are enriched at the presynaptic terminals of neurons and can reversibly associate with synthetic membranes and vesicles. UCH-L1 belongs to a group of deubiquitinating enzymes and is involved in the turnover of proteins. It is known that phosphorylation is involved in the regulation of proteins and membranes trafficking in pre- and postsynaptic nerve terminal and thus, as proposed by the authors, it could be considered that *O*-GlcNAc can counteract the phosphorylation effect on such proteins via the dynamic interplay between *O*-GlcNAc and phosphate, and that a deregulation of this process can play an active role in neurodegeneration.

Most recently, two proteins that are important for synaptic function were identified using a chemoenzymatic approach that utilizes an engineered galactosyltransferase enzyme to selectively label *O*-GlcNAc proteins with a ketone-biotin tag [49]. Proteins identified include the actin-associated protein synaptopodin, which is essential for dendritic spine formation, and bassoon, a scaffolding protein that plays a critical role in synaptic vesicle cycling [49].

#### *Assembly protein-3*

One of the clathrin assembly proteins (APs), AP-3, has been well studied in terms of glycosylation. Synaptic vesicles assemble at the presynaptic compartment through a clathrin-dependent mechanism that involves one or more APs, one of which is AP-3. AP-3 is a synapse-specific protein (also known as pp155, AP180, NP185 and F1–20) that is involved in promoting clathrin assembly in clathrin-coated vesicles, probably in synaptic vesicle recycling. *In vivo*, phosphorylation of AP-3 weakens both the binding of AP-2 by AP-3 and the co-operative clathrin binding activity of these proteins [53]. Using pulse-chase experiments, it has been shown that the mature form of AP-3 was also *O*-GlcNAc modified but that the phosphorylated form of AP-3 bound to WGA-sepharose [48]. This indicates that both modifications could be present together on the same molecule. These two post-translational modifications (*O*-GlcNAc and phosphorylation) have been mapped to the central 50 kDa structural domain. Capping of *O*-GlcNAc residues by galactosylation did not affect interaction between AP-3 and clathrin, suggesting that *O*-GlcNAc did not interfere with the interaction between AP-3 and clathrin. Yao and Coleman found a marked reduction on AP-3 in AD, which is negatively correlated with the density of neurofibrillary tangles (i.e., intraneuronal aggregates constituted by hyperphosphorylated Tau proteins) [54]. In fact, it was the level of AP-3 proteins that decreased in AD, while the *O*-GlcNAc/AP-3 ratio remained unchanged. Loss of glycosylated AP-3 may be an earlier event in the pathologic cascade of synapses in AD.

#### *Synapsin I*

Synapsin I has also been well characterized in terms of *O*-GlcNAc glycosylation [46]. Synapsin I belongs to a family of five related neuron-specific phosphoproteins (synapsins) associated with synaptic vesicle membranes that are involved in the regulation of neurotransmitter release [55]. They tether synaptic vesicles to actin filaments in a phosphorylation-dependent manner, controlling the number of vesicles available for release at the nerve terminus. A growing body of evidence suggests that synapsins play a significant part in neuronal development. They participate in the formation and maintenance of synaptic contacts among central neurons. Synapsins anchor synaptic vesicles to the cytoskeleton and ensure a steady supply of fusion-competent synaptic vesicles. Synapsins are phosphorylated and it is well known that phosphorylation plays a role in their interactions with synaptic vesicle and the cytoskeleton. Synapsin I is also *O*-GlcNAc glycosylated on seven sites that are present in the B and D domains: S55, T56, T87, S516, T524, T562 and S576 [46]. While these glycosylation sites are located around five phosphorylation sites, it is unlikely that competition between *O*-GlcNAc and phosphate occurs on these residues. With the use of synthetic peptides, the authors demonstrated that *O*-GlcNAc glycosylation at sites T562 and S576 did not strongly modify the Km of the calcium/calmodulin-dependent protein kinase II for phosphorylation at S566.

#### *Sec24p*

A study has demonstrated that Sec24p, a component of COPII, was modified with *O*-GlcNAc during interphase, whereas Sec23p, Sec13p, Sec31p or Sar1p were not [56]. When cells started mitosis the glycosylation was lost and Sec24p was phosphorylated. Nevertheless, the putative competition between *O*-GlcNAc and phosphorylation in the regulation of the mechanism by which the endoplasmic reticulum (ER)-to-Golgi transport could be regulated has not been demonstrated.

### Proteasomal degradation & neuronal diseases

#### *Aggregated proteins found in neurologic diseases & ubiquitin–proteasome system dysfunction*

One of the common characteristics of neurologic diseases is a dysfunction of the ubiquitin–proteasome system (UPS) and the intracellular deposition of aggregated and ubiquitinated proteins [57]. As mentioned above, Parkinson's disease, AD, Huntington's

and other polyglutamine diseases, amyotrophic lateral fibrosis and prion diseases are characterized by protein aggregation and inclusion [50]. Protein aggregation can result from three independent failures:

- A defect in the protein that increases its insolubility
- An acceleration of its synthesis
- A defect in its degradation

This concept is largely reinforced since ubiquitin is found in high concentrations in aggregates and in nerve cell death, leading to the conclusion that UPS is deregulated and that the proteasome is inhibited. For example, huntingtin aggregates can be labeled with antibodies directed against ubiquitin that should be regarded as a marker of misfolded proteins and as a signal for proteasome targeting. The pathologic mark of Parkinson's disease is Lewy body formation, which are inclusions of fibrillar and misfolded proteins found in the cytoplasm of neurons near the nucleus. As for Huntington's disease, Lewy bodies can be labeled with antiubiquitin, anti-α-synuclein, antiparkin, antisynphilin, antineurofilaments and antisynaptic vesicle proteins. In amyotrophic lateral sclerosis, ubiquitinated aggregates are also found in patient brains. Finally in prion diseases, pathologies are caused by an abnormally folded prion protein both intra- and extracellularly.

#### O-GlcNAc glycosylation & proteasomal regulation

Recently, it has been shown that the proteasome machinery was modified with *O*-GlcNAc [9,10] and that it can be regulated by this post-translational modification (OGT modifies the proteasome and inhibits its activity) [10]. Liu and coworkers suggested that since *O*-GlcNAcase was located at the 10q locus [30,31], a chromosomal region frequently mutated during AD, a genetic impairment of *O*-GlcNAcase gene results in a dysfunction of proteasome activity due to the nonhydrolysis of the *O*-GlcNAc inhibitory effect on the 19S regulatory cap. Finally, the proteasome failed to degrade the aggregates (an alternative of this defect is the autophagic pathway). This hypothesis is partly in contradiction with the idea that in neuronal disorders, and more particularly in AD, *O*-GlcNAc tends to decrease. Nevertheless, it appears increasingly likely that when a protein is modified with *O*-GlcNAc, it is less sensitive to proteasomal degradation [6–8]. Consequently, the two phenomena should act in synergy, since if proteins are protected with *O*-GlcNAc, and if the proteasome is inhibited, the degradation rate of proteins will then be greatly reduced.

In another study, Cole and Hart demonstrated that the deubiquitinylation enzyme UCH-L1 was *O*-GlcNAc modified in the synaptosome [11]. It is premature to interpret this result, but it is exciting to put forward that *O*-GlcNAc could also be involved in the regulation of protein targeting at the proteasome by modifying such enzymes: a misregulation of such a process by *O*-GlcNAc could reinforce the effect of *O*-GlcNAc in protein turnover. In this field, it will be very interesting to observe the existence of such a modification on parkin, an E3 ligase catalyzing the addition of ubiquitin to specific substrates targeted to UPS and found in Parkinson's disease. Furthermore, in addition to ubiquitinated substrates found in inclusion, chaperone proteins and proteasome components are also part of these, and this reinforces the problem of UPS dysregulation in neuronal disorders. Recently, the authors' laboratory has shown that chaperones of the HSP70 family share a lectinic activity against GlcNAc residues, and that this biologic function is dramatically enhanced by stress [58]. Such lectinic activity must be studied in neurologic diseases.

### Glucose in *O*-GlcNAc metabolism: importance of glucose in brain

#### Hexosamine biosynthetic pathway

A total of 2–5% of extracellular glucose could be used for the modification of proteins with *O*-GlcNAc through the hexosamine biosynthetic pathway [59]. Interestingly, many proteins involved in the metabolism of glucose are themselves *O*-GlcNAc modified: casein kinase II, glycogen synthase kinase-3 [60] and insulin receptor substrate-1 and -2 [61]. Glutamine:fructose-6-phosphate amido transferase, which catalyzes the conversion of fructose-6-phosphate to glucosamine-6-phosphate, is the rate-limiting enzyme acting in the hexosamine biosynthetic pathway and is considered as the key enzyme [62]. This enzyme can be bypassed by replacement of glucose with glucosamine (directly forming glucosamine-6-phosphate), which allows an enhancement of protein *O*-GlcNAc modification.

#### Glucose & O-GlcNAc in brain

Glucose metabolism is essential for neural function: it influences many normal cellular processes, from neurotransmitter synthesis to ATP production. Glucose metabolism (and consequently energy) changes in aging brain, both in the insulin and in the acetylcholine signal transduction. Cumulative evidences suggest that during aging and AD, brain actively adapts its glucose metabolism. Glucose is preserved for anabolism and the oxidative utilization of ketone bodies is enhanced [63]. It is suggested that in AD, brain may not follow a suicide but a rescue program. Changes in glucose brain levels have been observed in mainly neurodegenerative diseases and so may be involved in pathologic processes [64]. Thus, starting from the link between glucose and *O*-GlcNAc metabolism and since brain is a great glucose consumer, it is likely that brain may be particularly sensitive to glucose metabolism for its *O*-GlcNAc glycosylation processes.

It is well known that in AD brain, glucose uptake and metabolism are impaired, and it appears that this failure could be a cause of neurodegeneration. Experiments performed on nonhuman primates have clearly demonstrated that a decrease in cerebral glucose consumption correlated with an impairment in memory [65]. Nevertheless, although it clearly appears that *O*-GlcNAc in AD is deficient, this impairment is not understood. To tentatively respond in part to this question, cytosolic protein *O*-GlcNAc levels in 19 AD brains and 15 controls was determined depending on the postmortem time [40]. Nonlinear regression of the analyzed results have shown that in AD brain, *O*-GlcNAc was 22% lower than in controls, indicating compromised *O*-GlcNAc glycosylation in the disease.

### *Glucose & phosphorylation in brain*

Several models have described a relationship between glucose level and phosphorylation, involving specific protein kinases. The hypothesis that mitogen-activated protein kinases (MAPKs) form transducers for the damaging effects of high glucose has been tested [66]. MAPKs are involved in the etiology of diabetic neuropathies both via the direct effects of glucose and via glucose-induced oxidative stress.

A link was also established between diabetes and neurofilaments phosphorylation. Changes in the phosphorylation status of neurofilaments could lead to severe impairments in the axon structure and function that may be found in diabetics. Using two animal models of Type 1 diabetes, it was demonstrated that in diabetic animals, an increase in neurofilament phosphorylation occurs via an increase in the phosphorylation of a jun N-terminal kinase (JNK) isoform and of extracellular signal-regulated kinases (ERK), which in turn hyperphosphorylates neurofilaments [67]. In diabetic rat neurons, hyperphosphorylation of neurofilaments may contribute to the distal sensory axonopathy observed in diabetes. It has been suggested that hyperglycemia-induced activation of stress-activated protein kinases (neurofilament kinases such as JNK), may be a primary etiologic event in diabetic neuropathy [68]. Other studies performed on starving mice have shown Tau hyperphosphorylation with a regional selectivity similar to those observed in AD [69]. As for neurofilament, Tau-specific kinases and Ser/Thr phosphatases are involved. During starvation, Tau phosphorylation was accompanied by a decrease in Tau-specific kinase activity: Tau protein kinase I/glycogen synthase kinase-3 and cyclin-dependent kinase-5. In this process of Tau hyperphosphorylation, it is also suggested that inhibition of PP2A is predominant.

To conclude on this section, it is currently not possible to correlate an increase of phosphorylation in neuronal disorders to the decrease of *O*-GlcNAc. Nevertheless, using okadaic acid the authors have shown that a dynamic interplay between *O*-GlcNAc glycosylation and phosphorylation could occur on Tau proteins and on many other nonidentified neuronal proteins [5,70].

### Expert opinion

This review attempts to collect a maximum of links that have been established between *O*-GlcNAc glycosylation and neurologic diseases. Unfortunately, compared with phosphorylation, very little is known about *O*-GlcNAc and brain disorders; phosphorylation has been well studied in numerous neuronal proteins, the best characterized examples being Tau and neurofilaments. In contrast, *O*-GlcNAc is a relatively recently discovered post-translational modification and studies performed on proteins are more a description of the glycosylation than a study of its biologic significance. For the moment, *O*-GlcNAc's impact in pathologies does not allow us to clearly understand its importance in brain disorders, even if there is evidence of its relationship with such diseases. The first parallel is that enzymes regulating *O*-GlcNAc dynamism are located on chromosomal regions linked to these disorders. However, in order to confirm that these enzymes are involved in such diseases, more studies and experiments need to be performed. The function of *O*-GlcNAc in neuronal proteins must be determined. For instance, according to the existence of the *O*-GlcNAc–phosphorylation balance, a protein in its glycosylated form is likely to have a different role than when it is phosphorylated; however, not all *O*-GlcNAc sites are regulated by phosphorylation. Thus, functions of *O*-GlcNAc are not directly correlated to phosphorylation. An exciting finding is that glycosylated Tau could be a marker for healthy brain in contradiction with

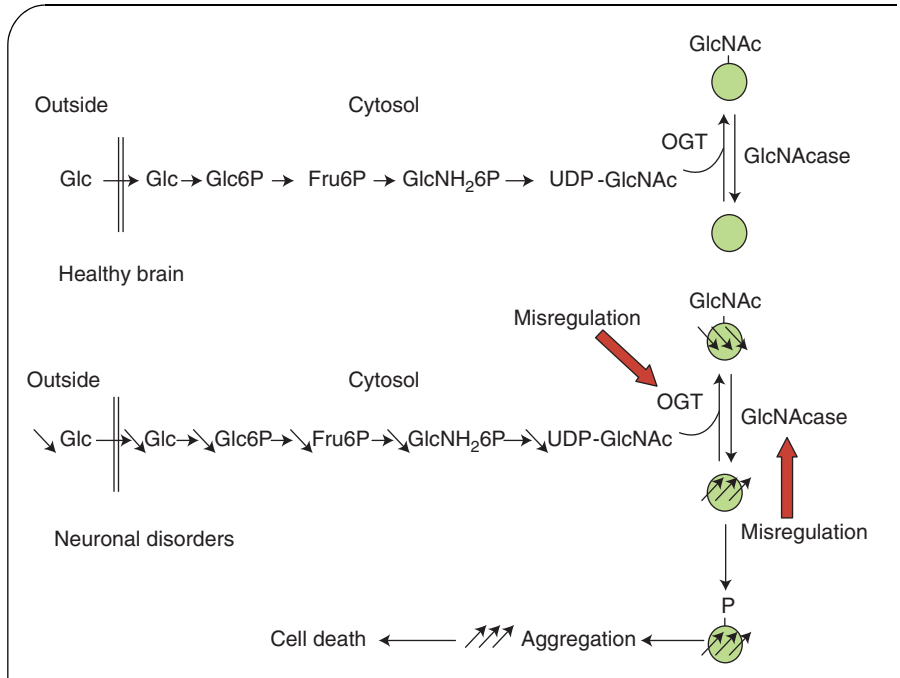


**Figure 2. The importance of *O*-GlcNAc in neuropathologies.** This scheme summarizes the potential involvement of *O*-GlcNAc in neuropathologies. In healthy brains, both OGT and *O*-GlcNAcase are enriched, allowing for efficient regulation of the glyco–deglyco process with *O*-GlcNAc. When neuronal disorders occur, the two enzymes of the cycling *O*-GlcNAc may be misregulated since they are both located on chromosomal regions that are involved in neurodegenerative disorders. This misregulation is all the more important since enzymes are particularly enriched in the brain. As glucose uptake is affected in such pathologies, this contributes to a deregulation in *O*-GlcNAc (which should particularly decrease). In view of the existence of the balance between *O*-GlcNAc and phosphorylation, phosphorylation increases in neurologic diseases (e.g., Tau), and leads to an enhancement of the aggregation that finally induces cell death.
GlcNAc: *N*-acetylglucosamine; GlcNAcase: *O*-linked *N*-acetylglucosaminidase; OGT: *O*-linked *N*-acetylglucosaminyl transferase.

hyperphosphorylated Tau. Finally, the link drawn between glucose metabolism, phosphorylation and *O*-GlcNAc really needs to be further investigated. FIGURE 2 is a recapitulative scheme of the potential role and implication of *O*-GlcNAc in neuronal disorders.

### Five-year view

In the years to come, we should be able to understand the functions of *O*-GlcNAc much better; by locating the exact glycosylation sites of proteins, by knowing the interacting partners in the different protein forms (phosphorylated, unmodified and glycosylated), and by studying conformational changes induced by sugar on neuronal proteins. Research that will be performed on the mechanism by which *O*-GlcNAc processes failed in aging neurons, leading to a perturbation of the phosphorylation status of proteins via the dynamic interplay between *O*-GlcNAc and phosphates, will certainly show one of the key events of neuronal disruption. The authors believe that the answer will be found in glucose metabolism, and those working on gerontology and people working on diabetes will certainly see their research efforts focus on the same point. We hope that these findings will highlight the contribution, and will confirm the critical role of *O*-GlcNAc in neuronal diseases.

### Key issues

- Many neuronal proteins are modified with the cytosolic- and nuclear-specific *O-linked N-acetylglucosamine (O*-GlcNAc) post-translational modification.
- Most of these proteins, if not all, are phosphoproteins, and several are involved in neurologic diseases (Tau, amyloid precursor protein, synapsins and neurofilaments).
- Vesicular transport is mediated by numerous *O*-GlcNAc proteins (e.g., assembly protein-3, synapsin or bassoon).
- Two major mechanisms that are impaired during neuronal disorders can be tightly regulated by *O*-GlcNAc: proteasomal-directed protein degradation (substrate proteins), the proteasome itself and enzymes of deubiquitinylation.
- Numerous components involved in the ubiquitin–proteasome system (chaperones, ubiquitinated ligands, structures of proteasome) are localized on inclusion bodies, which are characteristic of misfolded proteins.
- Enzymes that regulate the versatility of *O*-GlcNAc are enriched in brain and particularly in the cytosol of synaptosomes. They are also located on chromosomal regions that are frequently mutated in neurologic disorders.

### References

Papers of special note have been highlighted as:
• of interest
•• of considerable interest

1 Slawson C, Hart GW. Dynamic interplay between *O*-GlcNAc and *O*-phosphate: the sweet side of protein regulation. *Curr. Opin. Struct. Biol.* 13, 631–636 (2003).

2 Comer FI, Hart GW. Reciprocity between *O*-GlcNAc and *O*-phosphate on the carboxyl terminal domain of RNA polymerase II. *Biochemistry* 40, 7845–7852 (2001).

3 Kamemura K, Hayes BK, Comer FI *et al.* Dynamic interplay between *O*-glycosylation and *O*-phosphorylation of nucleocytoplasmic proteins: alternative glycosylation/phosphorylation of THR-58, a known mutational hot spot of c-Myc in lymphomas, is regulated by mitogens. *J. Biol. Chem.* 277, 19229–19235 (2002).

•• **Using two antibodies, one produced by the authors (one against unmodified Thr58 of c-myc and the other against Thr58 in its glycosylated form) and one commercial antibody (raised against the phosphorylated form of Thr58), the authors indisputably demonstrated the direct relationship between *O*-GlcNAc and phosphorylation.**

4 Griffith LS, Schmitz B. *O*-linked *N*-acetylglucosamine levels in cerebellar neurons respond reciprocally to pertubations of phosphorylation. *Eur. J. Biochem.* 262, 824–831 (1999).

5 Lefebvre T, Ferreira S, Dupond-Wallois L *et al.* Evidence of a balance between phosphorylation and *O*-GlcNAc glycosylation of Tau proteins – A role in nuclear localization. *Biochim. Biophys. Acta* 1619, 167–176 (2003).

6 Han I, Kudlow JE. Reduced *O*-glycosylation of Sp1 is associated with increased proteasome susceptibility. *Mol. Cell. Biol.* 17, 2550–2558 (1997).

•• **First evidence that *O*-GlcNAc could be a protective signal against proteasomal degradation and is the basis of many other studies that demonstrate that one of the functions of glycosylation is to prevent the degradation of proteins.**

7 Cheng X, Cole RN, Zaia J *et al.* Alternative *O*-glycosylation/*O*-phosphorylation of the murine estrogen receptor β. *Biochemistry* 39, 11609–11620 (2000).

8 Hatsell S, Medina L, Merola J *et al.* Plakoglobin is *O*-Glycosylated close to the N-terminal destruction box. *J. Biol. Chem.* 278, 37745–37752 (2003).

9 Sumegi M, Hunyadi-Gulyas E, Medzihradszky KF *et al.* 26S proteasome subunits are *O*-linked *N*-acetylglucosamine-modified in *Drosophila melanogaster. Biochem. Biophys. Res. Commun.* 312, 1284–1289 (2003).

10 Zhang F, Su K, Yang X *et al.* *O*-GlcNAc modification is an endogenous inhibitor of the proteasome. *Cell* 115, 715–725 (2003)

•• **Evidence that *O*-GlcNAc could regulate protein stability not only at the protein level (by modifying the substrate protein), but also in *O*-GlcNAc as an on/off button on the 19S regulatory subunit of 26S proteasome.**

11 Cole RN, Hart GW Cytosolic *O*-glycosylation is abundant in nerve terminals. *J. Neurochem.* 79, 1080–1089 (2001).

12 Haltiwanger RS, Holt GD, Hart GW. Enzymatic addition of *O*-GlcNAc to nuclear and cytoplasmic proteins. Identification of a uridine diphospho-*N*-acetylglucosamine:peptide β-*N*-acetylglucosaminyltransferase. *J. Biol. Chem.* 265, 2563–2568 (1990).

13 Haltiwanger RS, Blomberg MA, Hart GW. Glycosylation of nuclear and cytoplasmic proteins. Purification and characterization of a uridine diphospho-*N*-acetylglucosamine:polypeptide β-*N*-acetylglucosaminyltransferase. *J. Biol. Chem.* 267, 9005–9013 (1992).

14 Swain SM, Tseng TS, Olszewski NE. Altered expression of SPINDLY affects gibberellin response and plant development. *Plant Physiol.* 126, 1174–1185 (2001).

15 Wrabl JO, Grishin NV. Homology between *O*-linked GlcNAc transferases and proteins of the glycogen phosphorylase superfamily. *J. Mol. Biol.* 314, 365–374 (2001).

16 Shafi R, Iyer SP, Ellies LG *et al.* The *O*-GlcNAc transferase gene resides on the X chromosome and is essential for embryonic stem cell viability and mouse ontogeny. *Proc. Natl Acad. Sci. USA* 97, 5735–5739 (2000).

17 Nolte D, Muller U. Human *O*-GlcNAc transferase (OGT): genomic structure, analysis of splice variants, fine mapping in Xq13.1. *Mamm. Genome* 13, 62–64 (2002).

18 Fletcher BS, Dragstedt C, Notterpek L *et al.* Functional cloning of SPIN-2, a nuclear anti-apoptotic protein with roles in cell cycle progression. *Leukemia* 16, 1507–1518 (2002).

19 Konrad RJ, Zhang F, Hale JE *et al.* Alloxan is an inhibitor of the enzyme *O*-linked *N*-acetylglucosamine transferase. *Biochem. Biophys. Res. Commun.* 293, 207–212 (2002).

20 Kreppel LK, Blomberg MA, Hart GW. Dynamic glycosylation of nuclear and cytosolic proteins. Cloning and characterization of a unique *O*-GlcNAc transferase with multiple tetratricopeptide repeats. *J. Biol. Chem.* 272, 9308–9315 (1997).

21 Blatch GL, Lassle M. The tetratricopeptide repeat: a structural motif mediating protein–protein interactions. *Bioessays* 21, 932–939 (1999).

22 Iyer SP, Akimoto Y, Hart GW. Identification and cloning of a novel family of coiled-coil domain proteins that interact with *O*-GlcNAc transferase. *J. Biol. Chem.* 278, 5399–5409 (2003).

23 Yang X, Zhang F, Kudlow JE. Recruitment of *O*-GlcNAc transferase to promoters by corepressor mSin3A: coupling protein *O*-GlcNAcylation to transcriptional repression. *Cell* 110, 69–80 (2002).

24 Wells L, Kreppel LK, Comer FI *et al. O*-GlcNAc transferase is in a functional complex with protein phosphatase 1 catalytic subunits. *J. Biol. Chem.* 38466–38470 (2004).

25 Iyer SP, Hart GW Roles of the tetratricopeptide repeat domain in *O*-GlcNAc transferase targeting and protein substrate specificity. *J. Biol. Chem.* 278, 24608–24616 (2003).

26 Liu K, Paterson AJ, Zhang F *et al.* Accumulation of protein *O*-GlcNAc modification inhibits proteasomes in the brain and coincides with neuronal apoptosis in brain areas with high *O*-GlcNAc metabolism. *J. Neurochem.* 89, 1044–1055 (2004).

27 Akimoto, Y, Comer FI, Cole, RN *et al.* Localization of the *O*-GlcNAc transferase and *O*-GlcNAc-modified proteins in rat cerebellar cortex. *Brain Res.* 966, 194–205 (2003).

•• **Thorough study on the localization of *O*-linked N-acetylglucosaminyl transferase (OGT) (mRNA and protein) using *in situ* hybridization histochemistry, immunofluorescence and electron microscopy.**

28 Dong DL, Hart GW Purification and characterization of an *O*-GlcNAc selective *N*-acetyl-β-D-glucosaminidase from rat spleen cytosol. *J. Biol. Chem.* 269, 19321–19330 (1994).

29 Gao Y, Wells L, Comer FI *et al.* Dynamic *O*-glycosylation of nuclear and cytosolic proteins: cloning and characterization of a neutral, cytosolic β-N-acetyl glucosaminidase from human brain. *J. Biol. Chem.* 276, 9838–9845 (2001).

30 Bertram L, Blacker D, Mullin K *et al.* Evidence for genetic linkage of Alzheimer's disease to chromosome 10q. *Science* 290, 2302–2303 (2000).

31 Myers A, Holmans P, Marshall H *et al.* Susceptibility locus for Alzheimer's disease on chromosome 10. *Science* 290, 2304–2305 (2000).

32 Roos MD, Xie W, Su K *et al.* Streptozotocin, an analog of *N*-acetylglucosamine, blocks the removal of *O*-GlcNAc from intracellular proteins. *Proc. Assoc. Am. Physicians* 110, 422–432 (1998).

33 Haltiwanger RS, Grove K, Philipsberg GA. Modulation of *O*-linked N-acetyl glucosamine levels on nuclear and cytoplasmic proteins *in vivo* using the peptide *O*-GlcNAc-β-N-acetyl glucosaminidase inhibitor *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-N-phenylcarbamate. *J. Biol. Chem.* 273, 3611–3617 (1998).

34 Wells L, Gao Y, Mahoney JA *et al.* Dynamic *O*-glycosylation of nuclear and cytosolic proteins: further characterization of the nucleocytoplasmic β-*N*-acetylglucosaminidase, *O*-GlcNAcase. *J. Biol. Chem.* 277, 1755–1761 (2002).

35 Toleman C, Paterson AJ, Whisenhunt TR, Kudlow JE. Characterization of the histone acetyltransferase (HAT) domain of a bifunctional protein with activable *O*-GlcNAcase and HAT activities. *J. Biol. Chem.* 279, 53665–53673 (2004).

36 Rex-Mathes M, Werner S, Strutas D *et al.* *O*-GlcNAc expression in developing and aging mouse mouse brain. *Biochimie* 83, 583–590 (2001).

37 Griffith LS, Schmitz B. *O*-linked *N*-acetylglucosamine is upregulated in Alzheimer brains. *Biochem. Biophys. Res. Commun.* 213, 424–431 (1995).

38 Yao PJ, Coleman PD. Reduction of *O*-linked *N*-acetylglucosamine-modified assembly protein-3 in Alzheimer's disease. *J. Neurosci.* 18, 2399–2411 (1998).

39 Robertson LA, Moya KL, Breen KC. The potential role of tau protein *O*-glycosylation in Alzheimer's disease. *J. Alzheimers Dis.* 6, 489–495 (2004).

40 Liu F, Iqbal K, Grundke-Iqbal I *et al.* *O*-GlcNAcylation regulates phosphorylation of tau: a mechanism involved in Alzheimer's disease. *Proc. Natl Acad. Sci. USA* 101, 10804–10809 (2004).

41 Griffith LS, Mathes M, Schmitz B. β-amyloid precursor protein is modified with *O*-linked N-acetylglucosamine. *J. Neurosc. Res.* 41, 270–278 (1995).

42 Ding M, Vandre DD. High molecular weight microtubule-associated proteins contain *O*-linked-N-acetylglucosamine. *J. Biol. Chem.* 271, 12555–12561 (1996).

43 Arnold CS, Johnson GV, Cole RN *et al.* The microtubule-associated protein tau is extensively modified with *O*-linked *N*-acetylglucosamine. *J. Biol. Chem.* 271, 28741–28744 (1996).

44 Dong DL, Xu, ZS, Hart GW *et al.* Cytoplasmic *O*-GlcNAc modification of the head domain and the KSP repeat motif of the neurofilament protein neurofilament-H. *J. Biol. Chem.* 271, 20845–20852 (1996).

45 Al-Chalabi A, Miller CC. Neurofilaments and neurological disease. *Bioessays* 4, 346–355 (2003).

46 Cole RN, Hart GW. Glycosylation sites flank phosphorylation sites on synapsin I: *O*-linked N-acetylglucosamine residues are localized within domains mediating synapsin I interactions. *J. Neurochem.* 73, 418–428 (1999).

47 Zhang X, Bennett V. Identification of *O*-linked *N*-acetylglucosamine modification of ankyrinG isoforms targeted to nodes of Ranvier. *J. Biol. Chem.* 271, 31391–31398 (1986).

48 Murphy JE, Hanover JA, Froehlich M *et al.* Clathrin assembly protein AP-3 is phosphorylated and glycosylated on the 50-kDa structural domain. *J. Biol. Chem.* 269, 21346–21352 (1994).

49 Khidekel N, Ficaro SB, Peters EC *et al.* Exploring the *O*-GlcNAc proteome: direct identification of *O*-GlcNAc-modified proteins from the brain. *Proc. Natl Acad. Sci. USA* 101, 13132–13137 (2004).

• **A new strategy for studying the *O*-GlcNAc proteome. This approach utilizes an engineered galactosyltransferase enzyme to selectively label *O*-GlcNAc proteins with a ketone-biotin tag. It permits the identification of 25 new *O*-GlcNAc proteins.**

50 Ross CA, Poirier MA. Protein aggregation and neurodegenerative disease. *Nature Med.* 10, S10–S17 (2004).

•• **Interesting review on the link between protein aggregation and the more common neuronal disorders.**

51 Wolfe MS. Secretase as a target for Alzheimer's disease. *Curr. Top. Med. Chem.* 2, 371–383 (2002).

52 Delacourte A, Buee L. Tau pathology: a marker of neurodegenerative disorders. *Curr. Opin. Neurol.* 13, 371–376 (2000).

•• **Exhaustive review on Tau proteins.**

53 Hao W, Luo Z, Zheng L *et al.* AP180 and AP-2 interact directly in a complex that co-operatively assembles clathrin. *J. Biol. Chem.* 274, 22785–22794 (1999).

54 Yao PJ, Coleman PD. Reduced *O*-glycosylated clathrin assembly protein AP180: implication for synaptic vesicle recycling dysfunction in Alzheimer's disease. *Neurosci. Lett.* 252, 33–36 (1998).

55 Ferreira A, Rapoport M. The synapsins: beyond the regulation of neurotransmitter release. *Cell. Mol. Life Sci.* 59, 589–595 (2002).

56 Dudognon P, Maeder-Garavaglia C, Carpentier JL *et al.* Regulation of a COPII component by cytosolic *O*-glycosylation during mitosis. *FEBS Lett.* 561, 44–50 (2004).

57 Bence NF, Sampat RM, Kopito RR. Impairment of the ubiquitin-proteasome system by protein aggregation. *Science.* 292, 1552–1555 (2001).

58 Guinez C, Lemoine J, Michalski J-C *et al.* 70-kDa heat shock protein presents an adjustable lectinic activity towards *O*-linked N-acetylglucosamine *Biochem. Biophys. Res. Com.* 319, 21–26 (2004).

59 Wells L, Vosseller K, Hart GW. A role for N-acetylglucosamine as a nutrient sensor and mediator of insulin resistance. *Cell. Mol. Life Sci.* 60, 222–228 (2003).

60 Lubas WA, Hanover JA. Functional expression of *O*-linked GlcNAc transferase. Domain structure and substrate specificity. *J. Biol. Chem.* 275, 10983–10988 (2000).

61 Patti ME, Virkamaki A, Landaker EJ *et al.*, Activation of the hexosamine pathway by glucosamine *in vivo* induces insulin resistance of early postreceptor insulin signaling events in skeletal muscle. *Diabetes* 48, 1562–1571 (1999).

62 Sayeski PP, Kudlow JE. Glucose metabolism to glucosamine is necessary for glucose stimulation of transforming growth factor-α gene transcription. *J. Biol. Chem.* 271, 15237–15243 (1996).

63 Heininger K. A unifying hypothesis of Alzheimer's disease. IV. Causation and sequence of events. *Rev. Neurosci.* 11, 213–328 (2000).

• **Interesting view on metabolism in Alzheimer's disease.**

64 Hoyez S. Risk factors for Alzheimer's disease during aging. Impacts of glucose/energy metabolism. *J. Neural. Transm. Suppl.* 54, 187–194 (1998).

65 Blaizot X, Meguro K, Millien I *et al.* Correlations between visual recognition memory and neocortical and hippocampal glucose metabolism after bilateral rhinal cortex lesions in the baboon: implications for Alzheimer's disease. *J. Neurosci.* 21, 9166–9170 (2002).

66 Purves T, Middlemas A, Agthong S *et al.* A role for mitogen-activated protein kinases in the etiology of diabetic neuropathy. *FASEB J.* 15, 2508–2514 (2001).

67 Fernyhough P, Gallagher A, Averill SA. Aberrant neurofilaments phosphorylation in sensory neurons of rats with diabetic neuropathy. *Diabetes* 48, 881–889 (1999).

68 Fernyhough P, Schmidt RE. Neurofilaments in diabetic neuropathy. *Int. Rev. Neurobiol.* 50, 115–144 (2002).

69 Planel E, Yasutake K, Fujita SC *et al.* Inhibition of protein phosphatases 2A overrides tau protein kinase I/glycogen synthase kinase 3-β and cyclin-dependent kinase 5 inhibition and results in tau hyperphosphorylation in the hippocampus of starved mouse. *J. Biol. Chem.* 276, 34298–34306 (2001).

70 Lefebvre T, Alonso C, Mahboub S *et al.* Effect of okadaic acid on *O*-linked N-acetylglucosamine levels in a neuroblastoma cell line. *Biochim. Biophys. Acta* 1472, 71–81 (1999).

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# Identification of *N*-acetyl-D-glucosamine-specific lectins from rat liver cytosolic and nuclear compartments as heat-shock proteins

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Cytosolic and nuclear O-linked N-acetylglucosaminylation has been proposed to be involved in the nuclear transport of cytosolic proteins. We have isolated nuclear and cytosolic *N*-acetyl-D-glucosamine (GlcNAc)-specific lectins from adult rat liver by affinity chromatography on immobilized GlcNAc and identified these lectins, by a proteomic approach, as belonging to the heat-shock protein (HSP)-70 family (one of them being heat-shock cognate 70 stress protein). Two-dimensional electrophoresis indicated that the HSP-70 fraction contained three equally abundant proteins with molecular masses of 70, 65 and 55 kDa. The p70 and p65 proteins are phosphorylated and are themselves O-linked GlcNAc (O-GlcNAc)-modified. The HSP-70 associated into high molecular mass complexes that dissociated in the presence of reductive and chaotropic agents. The lectin(s) present in this complex was (were) able to recognize cytosolic and nuclear ligands, which have been isolated using wheat germ agglutinin affinity chromatography. These ligands are O-GlcNAc glycosylated as demonstrated by [$^3$H]galactose incorporation and analysis of the products released by reductive $\beta$-elimination. The isolated lectins specifically recognized ligands present in both the cytosol and the nucleus of human resting lymphocytes. These results demonstrated the existence of endogenous GlcNAc-specific lectins, identified as HSP-70 proteins, which could act as a shuttle for the nucleo-cytoplasmic transport of O-GlcNAc glycoproteins between the cytosol and the nucleus.

Key words: N-acetylglucosaminylation, cytosol, nucleus, phosphorylation, traffic.

## INTRODUCTION

For a long time, glycosylation was thought to be restricted to proteins confined to the cell surface or within the lumen of intracellular organelles. This view was governed by the general understanding of the biosynthetic pathways of N- and O-linked glycans. But, for fifteen years, the existence of a major form of glycosylation found within the cytosol and the nucleus has been well established, i.e. O-linked N-acetylglucosaminylation [1,2], the addition of a single monosaccharide [*N*-acetyl-D-glucosamine (GlcNAc)] on to serine or threonine residues of the peptide backbone. To date, approximately 100 nuclear and cytoplasmic proteins modified with O-linked GlcNAc (O-GlcNAc) have been identified. They include neurofilament proteins [3], nuclear pore proteins [4,5], and several transcription factors, such as Sp1 [6] and c-Myc [7,8]. Interestingly all of the O-GlcNAc-modified proteins undergo reversible phosphorylation/dephosphorylation and form heteromeric complexes with other proteins, and most of them are translocated between the cytosol and the nucleus [9].

O-linked N-acetylglucosaminylation was suggested to play a role in nucleo-cytoplasmic transport. It is well known that cytosolic proteins with molecular masses exceeding 40 kDa migrate into the nucleus only if they contain a specific nuclear localization sequence. Duverger et al. [10] suggested the existence of sugar-dependent nuclear import of proteins from the cytosol. In digitonin-permeabilized cells, they found that BSA substituted with either di-*N*-acetyl-chitobiose (GlcNAc$\beta$1-4GlcNAc) or $\alpha$-glucose residues was transported into the nucleus by a time- and ATP-dependent mechanism, indicating that GlcNAc could act as a nuclear localization signal. Nuclear pore proteins, being themselves O-linked N-acetylglucosaminylated and facing both sides of the nuclear envelope, are possibly involved in this cytosol-to-nucleus transport. Several reports favoured this possibility. Finlay et al. [11] reported a specific inhibition of the *in vitro* nuclear transport of fluorescein-labelled phycoerythrin by the GlcNAc-specific lectin wheat germ agglutinin (WGA). Miller and Hanover [12] demonstrated in the *Xenopus* egg that functional nuclei can be reconstituted after the addition of nuclear pore proteins from rat liver or *Xenopus* eggs. However, the nuclear transport was not recovered when the nuclear pore proteins were depleted of their WGA-binding glycoproteins.

Six nuclear lectins have been characterized so far: carbohydrate-binding protein (CBP)-35 (galectin-3) and CBP-14 (galectin-1) specific for galactose residues; and CBP-67, CBP-33 and CBP-70 specific for glucose residues. CBP-70 was shown to also be specific for GlcNAc residues, as was CBP-22, which was co-isolated with CBP-70 from HL60 cell nuclei [13]. CBP-70 also localized in the cytosol and one 82 kDa ligand was characterized for the nuclear CBP-70 [14]. Rousseau et al. [15] demonstrated, using electron microscopy, immunofluorescence analysis and subcellular fractionation, that CBP-70 is a pluri-localized lectin that is also found in the endoplasmic reticulum, Golgi apparatus and mitochondria. The nuclear lectins have mainly been found in ribonucleoprotein complexes [16]. The role of these nuclear

Abbreviations used: BiP, immunoglobulin heavy-chain binding protein; CBP, carbohydrate-binding protein; DTT, dithiothreitol; GlcNAc, *N*-acetyl-D-glucosamine; O-GlcNAc, O-linked GlcNAc; GRP78, 78 kDa glucose-regulated protein; HPAEC, high-pH anion-exchange chromatography; HRP, horseradish peroxidase; Hsc-70, heat-shock cognate 70 stress protein; HSP, heat-shock protein; MALDI–TOF, matrix-assisted laser-desorption ionization–time-of-flight; TBS, Tris-buffered saline; WGA, wheat germ agglutinin.

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lectins remains to be determined, but data suggested that CBP-14 and CBP-67 might be involved in the nucleo-cytoplasmic transport of mRNA [17]. Hubert et al. [18] have shown that galectin-3 (CBP-35) is in higher concentration in the nucleus than in the cytoplasm and that it is required for spliceosome formation and mRNA processing. Kuchler et al. [19,20] described the existence of GlcNAc-binding sites during cerebellar ontogenesis in the nucleolus, in specialized regions of the nucleus and in the cytosol using biotinylated neoglycoproteins. Although these previous studies suggest a role for lectins in nuclear transport, their precise function remains to be determined.

The aim of the present study was to characterize GlcNAc-specific lectins in the cytosol and the nucleus of rat liver and to see whether O-GlcNAc could represent a signal for nuclear transport of cytosolic and nuclear glycoprotein ligands. These different lectins were demonstrated to belong to the HSP-70 family and the others were similar to immunoglobulin heavy-chain binding protein (BiP).

## MATERIALS AND METHODS

### Biochemicals and apparatus

WGA immobilized on Sepharose was generously provided by Professor H. Debray (Unité Mixte de Recherches 8576 du CNRS). Centricon cell and exclusion membranes were obtained from Pall Filtron (St Germain-en-Laye, France). Nitrocellulose membranes were from Schleicher & Schuell (CERALABO; Ecquevilly, France). Anti-phosphothreonine and anti-phosphoserine antibodies, horseradish peroxidase (HRP)-labelled WGA, alkaline phosphatase (from bovine intestinal mucosa), $\beta$-*N*-acetylglucosaminidase (from *Diplococcus pneumoniae*), GlcNAc $\beta$-1,4-galactosyltransferase, PMSF, leupeptin, pepstatin, biotin-$\epsilon$-aminocaproic acid *N*-hydrosuccinimide ester, ampholytes and PMA were purchased from Sigma–Aldrich (St Quentin-Fallavier, France). The Superose 6 column, P-500 pump, LCC-500 liquid chromatography controller, dual path monitor UV-2, molecular-mass markers, ECL® Western blotting detection reagents and UDP-[6-$^3$H]galactose (9.70 Ci/mmol) were obtained from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.). CHAPS was from ICN Pharmaceuticals (Orsay, France), silica gel was purchased from Merck (Nogent-sur Marne, France), and silica gel for TLC was also obtained from Merck (Darmstadt, Germany). The gas phase apparatus was a GC-14A from the Shimadzu Corporation (Kyoto, Japan) and the column used was a BP-70 column from SGE International Pty Ltd (Les Ulis, France). The liquid scintillation counter was a 6000 TA from Beckman Instruments Inc. (Palo Alto, CA, U.S.A.). High-pH anion-exchange chromatography (HPAEC) apparatus was purchased from Dionex (Voisins Le Bretonneux, France) and the MS apparatuses [matrix-assisted laser-desorption ionization–time-of-flight (MALDI–TOF) and Q-STAR] were from Applied Biosystems (Les Ulis, France).

### Rat liver nuclei and cytosol preparation

Adult Wistar albino rats were starved overnight prior to decapitation. After perfusion, livers were removed and placed into ice-cold 20 mM Tris/HCl and 0.25 M sucrose (pH 7.4), containing a protease inhibitor cocktail (1 mM PMSF, 1 $\mu$g/ml leupeptin and 1 $\mu$g/ml pepstatin). From this step onwards, all operations were performed between 0 and 4 °C. Livers were ground in a Fischer apparatus and homogenized in 5 vol. of the same medium in a Potter–Elvehjem homogenizer by 3 strokes at 600 rev./min. The homogenate was centrifuged at 1500 ***g*** for 15 min. The supernatant (post-nuclear) was then centrifuged at 100000 ***g*** for 60 min, and the resulting supernatant was considered as the cytosolic fraction. The resulting pellet of the first centrifugation was carefully recovered and resuspended in 2.4 M sucrose, 1 mM $MgCl_2$ and 10 mM potassium phosphate buffer (pH 6.8), containing the protease inhibitor cocktail. The suspension was centrifuged at 100000 ***g*** for 1 h. The pellet corresponding to the nuclear fraction was resuspended in 0.25 M sucrose, 0.5 mM $MgCl_2$ and 20 mM Tris/HCl (pH 7.5) and centrifuged at 1000 ***g*** for 10 min. The washing was repeated twice in order to obtain a white nuclear pellet. The pellet was finally homogenized in 10 vol. of 200 mM KCl, 1 mM dithiothreitol (DTT), 1 mM PMSF, 1 $\mu$g/ml leupeptin, 1 $\mu$g/ml pepstatin, 1 % (v/v) Triton X-100, 0.1 % sodium deoxycholate and 0.2 M GlcNAc in 20 mM Tris/HCl (pH 7.8), and centrifuged at 100000 ***g*** for 1 h. The supernatant corresponding to membrane-depleted nuclei was collected and dialysed extensively against the same buffer without detergent or GlcNAc.

### Affinity chromatography on immobilized GlcNAc and WGA

For the isolation of GlcNAc-binding lectins, the soluble and nuclear extracts were passed through a column (17 cm × 1.4 cm) made of GlcNAc immobilized on 6 % (w/v) agarose through a 6-carbon-atom spacer-arm equilibrated in binding buffer (20 mM Tris/HCl, 200 mM KCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.8). The column was washed with 200 ml of the binding buffer, followed by 50 ml of the same buffer containing 0.1 M GalNAc and, finally, eluted with 50 ml of the buffer containing 0.1 M GlcNAc.

For the isolation of cytosolic and nuclear GlcNAc-containing glycoproteins, the material not adsorbed on to the immobilized GlcNAc column was submitted to chromatography on a column of WGA immobilized on Sepharose at 4 °C. The binding buffer was as described above. After washing overnight, elution was performed with the binding buffer containing 0.2 M GlcNAc. The different fractions were concentrated in a Centricon cell with a 10 kDa exclusion size filter at 4 °C followed by extensive dialysis against the binding buffer.

### PAGE and Western-blot analysis

Proteins were separated by SDS/PAGE (10 % gels) [21] and stained with Coomassie Brilliant Blue and/or silver stain, or electrophoretically transferred on to nitrocellulose. Nitrocellulose membranes were first saturated for 45 min in Tris-buffered saline (TBS)/Tween [15 mM Tris, 140 mM NaCl and 0.05 % Tween (v/v), pH 8.0] containing 3 % (w/v) BSA. Membranes were then incubated in TBS/Tween containing anti-phosphothreonine or anti-phosphoserine antibodies [diluted 1:500 (v/v)] or with HRP-labelled WGA [diluted 1:10000 (v/v)] for 1 h. Blots were washed three times with TBS/Tween (for 10 min each) and detection was carried out using the ECL® Western blotting detection reagents. In order to control the specificity of the WGA binding, experiments were performed in the presence of 0.2 M GlcNAc.

### Isoelectric focusing and PAGE

For two-dimensional electrophoresis, samples were diluted in 20 % (v/v) IsoA (0.34 M SDS/0.15 M DTT) and 80 % (v/v) IsoE [0.108 M DTT, 0.108 M CHAPS, 15 M urea and 8.33 % (v/v) ampholytes (pH 3–10)]. The samples were run on a 4 % (w/v) polyacrylamide gel containing 9.5 M urea, 1 % (v/v) ampholytes (pH 5–7), 4 % (v/v) ampholytes (pH 3–10) and a 5 %

(v/v) detergent solution (0.3 g of CHAPS, 100 $\mu$l of Nonidet P40 and 900 $\mu$l of water) at 200 V for 1 h, followed by 500 V for 1 h and, finally, 800 V for 16 h. Phosphoric acid (20 mM) was used as the lower buffer and NaOH (20 mM) was used as the upper buffer. After isoelectrofocusing, gels were extracted from the capillaries, placed in transfer solution [0.5 M Tris/HCl, 10 % (w/v) SDS and 0.005 % Bromophenol Blue, pH 8.8] for 10 min, and proteins were resolved by SDS/PAGE (10 % gels).

### Electrophoresis under non-denaturing conditions

Samples were placed in 20 mM Tris/HCl, 50 % (v/v) glycerol and 0.005 % Bromophenol Blue (pH 8.8) and were run in Tris/glycine buffer (pH 8.8) on a 5–15 % (w/v) polyacrylamide gradient gel without SDS or 2-mercaptoethanol at 50 V overnight at 4 °C. Proteins were then silver stained.

### Separation of GlcNAc-specific lectins by FPLC

FPLC was performed using a Superose 6 column (29 cm × 1.4 cm) equilibrated with 20 mM Tris/HCl, 200 mM KCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (pH 7.8) and eluted with the same buffer. Absorption was measured at 280 nm. Fractions of 1 ml were collected, pooled according to the absorbance profile and analysed by SDS/PAGE.

### Identification of WGA–Sepharose-bound proteins by MS

The WGA–Sepharose-bound proteins were separated by SDS/PAGE (10 % gels) and stained with Coomassie Brilliant Blue. The different protein bands were excised individually and in-gel digested with modified trypsin as described previously [22,23]. The tryptic peptides from each protein were analysed by MALDI–TOF MS on a Voyager instrument DE STR and/or sequenced by nanospray tandem MS (Q-STAR). Finally, proteins were identified using MS-Fit (http://prospector.ucsf.edu/htmlucsf3.0/msfit.htm).

### Detection of phosphorylation on the GlcNAc-specific lectins

GlcNAc-specific lectins were submitted to acidic hydrolysis in 100 $\mu$l of 1 M HCl for 5 h at 95 °C. Following evaporation, water was added to the sample and the released phosphate groups were analysed by HPAEC, using an AS4A-SC column (Ion Pac®; 4 mm × 250 mm) and an AG4A-SC pre-column. The eluent was 40 mM NaOH at a flow rate of 1.5 ml/min. Phosphate ions were detected by conductimetry with a pulsed electrochemical detector.

### Detection of O-GlcNAc on the GlcNAc-specific lectins

GlcNAc-specific lectins were submitted to reductive $\beta$-elimination in 0.1 M NaOH and 1 M sodium borohydride at 65 °C overnight. The reaction was stopped by drop-wise addition of ice-cold acetic acid under vigorous stirring until a pH value of 5.0 was reached. The $\beta$-eliminated material was dried several times under vacuum with anhydrous methanol in order to remove borate as methyl ester. The released saccharides were peracetylated in acetic anhydride for 4 h at 95 °C, dried and finally extracted in chloroform. After drying under nitrogen, the peracetylated saccharides were taken up in 100 $\mu$l of chloroform and 2 $\mu$l was injected into GLC and analysed on a BP-70 column (30 m × 0.32 mm) at a initial temperature of 150 °C, with a gradient of 3 °C/min to 230 °C, then with a gradient of 5 °C/min to 250 °C and finally with a plateau of 5 min at 250 °C.

### Determination of the nature of the glycosylation of WGA-binding glycoproteins

Galactosyltransferase provides a specific and sensitive probe frequently used in the detection of O-GlcNAc residues on cytosolic and nuclear proteins [24,25]. Samples of glycoproteins isolated by affinity chromatography on WGA–Sepharose were supplemented with an equal volume of sample buffer (56.25 mM Hepes, 11.25 mM $MnCl_2$, 250 mM galactose and 12.5 mM AMP, pH 7.0) containing protease inhibitors (1 mM PMSF, 5 $\mu$g/ml leupeptin and 5 $\mu$g/ml pepstatin). Bovine milk GlcNAc$\beta$1,4-galactosyltransferase (0.025 unit) and 5 $\mu$Ci of UDP-[6-$^3$H]-galactose were finally added to initiate the reaction. The samples were subsequently incubated at 37 °C for 2 h.

Sugar chains were released from the precipitated labelled glycoproteins using reductive alkaline treatment as described above. The $\beta$-eliminated material was dried several times under vacuum with anhydrous methanol and then desalted by descending paper chromatography on Whatman 3 MM paper using 1-butanol/ethanol/water (4:1:1, by vol.) as the solvent. Radioactivity was measured after cutting horizontal strips (1 cm in width). The radioactive fractions were eluted from corresponding regions with water, freeze-dried and finally analysed by silica gel 60 TLC with 1-butanol/acetic acid/water (4:2:3, by vol.) as the solvent. Bands (0.5 cm in width) were recovered and measured for their radioactivity in a liquid scintillation counter.

### Preparation of the biotinylated nuclear GlcNAc-specific lectins

Nuclear GlcNAc-specific lectins were incubated in a potassium phosphate buffer at pH 9.5, with biotin-$\epsilon$-aminocaproic acid *N*-hydrosuccinimide ester and 0.1 M free GlcNAc (in order to protect the carbohydrate-recognition domain) for 2 h at 4 °C. Excess biotin-$\epsilon$-aminocaproic acid *N*-hydrosuccinimide ester and GlcNAc were dialysed and biotinylated nuclear GlcNAc-specific lectins were precipitated with a saturated ammonium sulphate salt solution, centrifuged at 100000 ***g*** for 1 h, and finally dialysed against cold PBS [25 mM sodium phosphate buffer at pH 7.2 containing 150 mM NaCl].

### Staining of WGA-selected proteins with biotinylated nuclear lectins

Blots of the two pools of nuclear and cytoplasmic WGA-binding glycoproteins were saturated in the presence of a 3 % (w/v) solution of periodate-treated BSA [26], supplemented with 5 ng of biotinylated nuclear GlcNAc-specific lectins, followed by HRP-labelled avidin, and revealed with the ECL® kit.

### Binding of biotinylated nuclear lectins to human lymphocytes

Blood was collected, diluted once in buffer [20 mM phosphate, 150 mm NaCl and 1 mM EDTA (pH 7.2)] and loaded on to a 6 % Ficoll-Paque layer in 50 ml plastic centrifuge tubes (Falcon) and centrifuged for 50 min at 800 ***g*** (activation of lymphocytes was performed by incubation in 10 $\mu$g/ml PMA for three days). After washing in PBS (three times), the cells were fixed by addition of a mixture containing 4 % (w/v) paraformaldehyde and 0.1 % glutaraldehyde in PBS for 4 h with intermittent gentle agitation. After elimination of the fixative and washes in PBS by repetitive centrifugations, the fixed cells were incubated with a 3 % (w/v) solution of periodate-treated BSA in PBS for 30 min at 20 °C followed by the addition of biotinylated lectins (5 ng/ml final concentration in 0.01 % Triton X-100). Following incubation for 4 h at 20 °C, the cultures were washed in PBS over a

4 h period, followed by addition of HRP-labelled avidin (1 $\mu$g/ml in PBS), and incubated overnight at 4 °C. After rinsing, as above, the bound HRP-labelled avidin was revealed using the diaminobenzidine method [27]. Controls were made by adding 10 mM GlcNAc during incubation with biotinylated lectins and during the first washing.

### Assays for phosphatase and glucosaminidase activities

Phosphatase activity was assayed by incubation of nuclear and cytosolic GlcNAc-specific lectins with *p*-nitrophenyl phosphate as substrate, in 100 mM Tris/HCl, 50 mM $MgCl_2$ and 100 mM NaCl, pH 9.5. Glucosaminidase activity was assayed using *p*-nitrophenyl-2-acetamido-2-deoxy-$\beta$-D-glucopyranoside in 50 mM acetate, pH 6.0. Positive controls of the reactions were obtained using alkaline phosphatase from bovine intestinal mucosa and $\beta$-*N*-acetylglucosaminidase from *D. pneumoniae*.

## RESULTS

### Cytosolic and nuclear GlcNAc-specific lectins have identical molecular masses, but differ in their pIs

Cytosolic and nuclear GlcNAc-specific lectin fractions were isolated by affinity chromatography on a GlcNAc-immobilized column. The binding of these compounds was considered as specific since the adsorbed compounds could not be eluted with *N*-acetylgalactosamine, but only with 0.2 M GlcNAc. As shown in Figures 1(a) and 1(b), the GlcNAc lectin fractions showed a relatively simple electrophoretic profile with three constituents in equivalent proportions of 70, 65 and 55 kDa. This pattern was identical in the cytosol and in the nucleus.

When the same samples were submitted to two-dimensional electrophoresis (Figures 1c and 1d, for cytosolic and nuclear GlcNAc-specific lectins respectively), differences in the pI and in the number of pI isoforms were found between the cytosolic and nuclear GlcNAc-specific lectins. The 70 kDa cytosolic protein showed three spots, whereas the nuclear protein migrated as six spots. A similar observation was made for the 65 kDa protein, for which only one cytosolic form was observed whereas three nuclear forms occurred. These pI shifts suggested differences in the phosphorylation status of the different proteins. Molecular mass and pI were determined by comparison with standard proteins.

### GlcNAc-specific lectins are phosphoproteins and are modified by O-GlcNAc glycosylation

The phosphorylation status of the 70 and 65 kDa proteins was investigated using anti-phosphoserine and anti-phosphothreonine antibodies. While no binding was observed using the anti-phosphothreonine antibody (Figure 2A), the 70 and 65 kDa proteins were specifically stained using the anti-phosphoserine antibody (Figure 2A) in both the cytosolic and nuclear lectin fractions. The presence of phosphate was also confirmed using acidic hydrolysis of these lectins followed by HPAEC analysis of the released products (Figure 2B). We estimated, using standard phosphate dilutions, the presence of 3–5 mol of phosphate/mol of GlcNAc-specific lectin. When these proteins were stained using the WGA–HRP technique (WGA is a lectin that recognizes all terminal non-reducing GlcNAc residues, especially O-GlcNAc) staining was observed for both cytosolic and nuclear p70 and p65 proteins (Figure 2C). The specificity of the latter binding was verified by incubating the same material in the presence of 0.1 M GlcNAc (Figure 2C). The carbohydrates released from the lectins by $\beta$-elimination were analysed by GLC (Figure 2D). The unique presence of peracetyl glucosaminitol confirmed the O-linked N-acetylglucosaminylation of the nuclear and cytosolic lectins.

### GlcNAc-specific lectins are part of high molecular mass complexes

In order to evaluate the molecular mass of the native GlcNAc-specific lectins, non-denaturing electrophoresis was performed

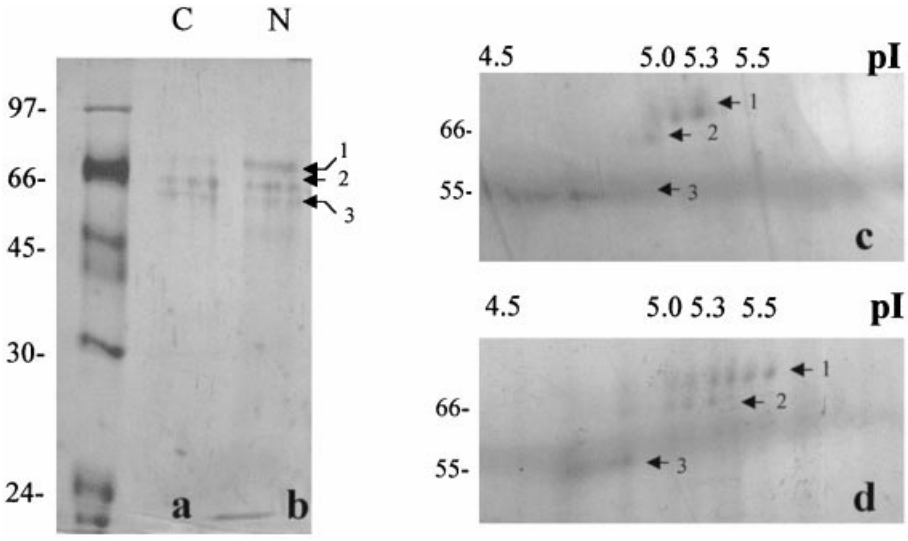


**Figure 1 SDS/PAGE and two-dimensional electrophoresis analysis of the GlcNAc-specific lectin fractions**

Electrophoretic profiles (silver staining) of the GlcNAc-specific lectin fractions isolated from cytosolic (C; **a** and **c**) and nuclear (N; **b** and **d**) fractions on a column of immobilized GlcNAc. (**a** and **b**) SDS/PAGE (10% gels). The quantity of protein loaded was estimated at 0.5 $\mu$g in each well. (**c** and **d**) Two-dimensional electrophoresis. Arrows indicate the position of the 70, 65 and 55 kDa proteins (labelled 1, 2 and 3 respectively). Molecular-mass markers are shown (in kDa) on the left.

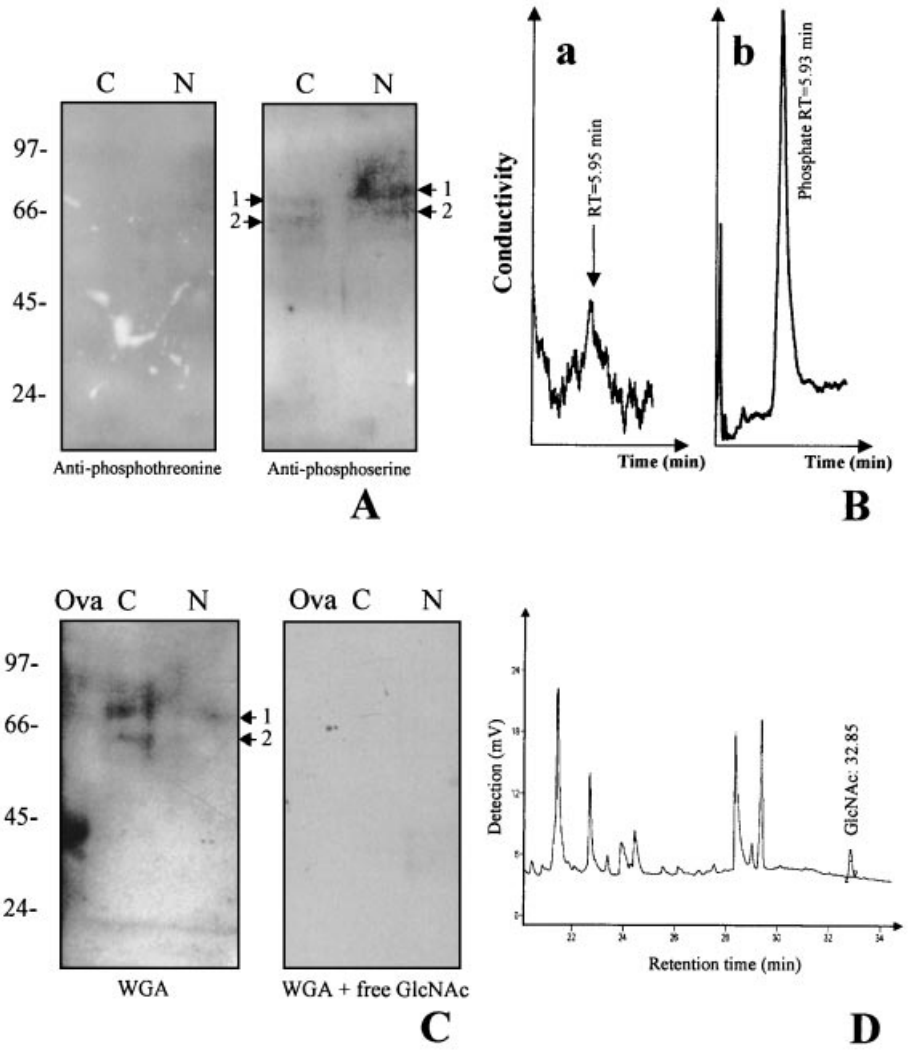


**Figure 2 Studies of the phosphorylation and GlcNAc glycosylation of the 70 and 65 kDa proteins**

(**A**) Phosphorylation of the cytosolic (C) and nuclear (N) GlcNAc-specific lectins was assessed using anti-phosphothreonine and anti-phosphoserine antibodies. The quantity of protein loaded was estimated at 0.5 $\mu$g in each well. Molecular-mass markers are shown (in kDa) on the left. (**B**) Characterization of the lectins phosphate content by mild acid hydrolysis followed by HPAEC analysis. a, GlcNAc lectins; and b, phosphate standard. RT, retention time. (**C**) WGA lectin staining of cytosolic and nuclear lectins. A control chase experiment using 0.1 M GlcNAc in the incubation medium was performed. Ovalbumin (Ova; 45 kDa) was used as a positive control. Molecular-mass markers are shown (in kDa) on the left. (**D**) Characterization of the GlcNAc content of isolated lectins by GLC. Glycans were released by reductive $\beta$-elimination and analysed in the peracetylated form by GLC. The peak at retention time 32.85 min corresponds to peracetylated glucosaminitol. In (**A** and **C**) arrows indicate the reactivity of the positive bands (1, 70 kDa protein; 2, 65 kDa protein).

on both nuclear and cytosolic GlcNAc-specific lectins. As shown in Figure 3, GlcNAc-specific lectins exist as very high molecular mass complexes, some of them having molecular masses that exceed $10^6$ Da. In addition to these major forms, lower molecular mass bands of approx. 600, 800 and 900 kDa could be distinguished.

In order to determine the monomer composition of each native isoform, these high molecular mass complexes were separated by FPLC on a Superose 6 column (Figure 4a) and each collected peak was subjected to SDS/PAGE (10% gels). As shown in Figure 4(b), it could be concluded that each native complex was composed of stoichiometric amounts of the 70, 65 and 55 kDa proteins. Since the three subunits were found in equivalent proportions in all native complexes, it was suggested that the high molecular mass complexes were associations of three (570 kDa), four (760 kDa), five (950 kDa) and six (1140 kDa) triplets.

### Cytosolic and nuclear GlcNAc-specific lectins are associated with a phosphatase activity

The high molecular masses of the complexes found within the cytosolic and nuclear lectin fractions suggested either a co-association between several GlcNAc-specific lectins or an association of one GlcNAc-specific lectin with other proteins.

The hypothesis that the complexes were made of GlcNAc-specific lectins and GlcNAc-containing glycoproteins associated by lectin–ligand interactions (which were dissociated upon SDS/PAGE) was unlikely since the complexes were not dissociated

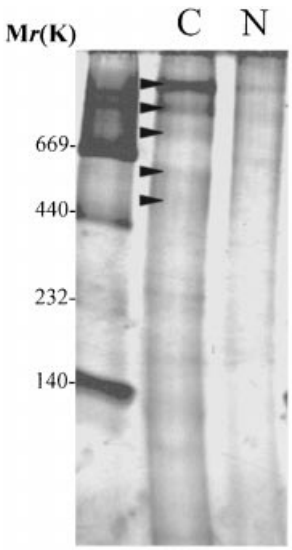


**Figure 3 Non-denaturing PAGE [5–15% (w/v) polyacrylamide gradient] of the GlcNAc-specific lectin fractions**

C, cytosolic fraction; N, nuclear fraction.

upon non-denaturing electrophoresis in the presence of 0.2 M GlcNAc in the sample and in the electrophoretic gel (results not shown).

The alternative hypothesis was that the complex was made of GlcNAc-specific lectins associated with enzymes functionally associated with these lectins. Therefore the cytosolic and nuclear GlcNAc-binding lectin-containing fractions were checked for the presence of phosphatase and *N*-acetylglucosaminidase activities. As shown in Table 1, the cytosolic and nuclear GlcNAc-specific lectin-containing fractions were only associated with phosphatase activity. The phosphatase activity could also be detected using Nitro Blue Tetrazolium and 5-bromo-4-chloroindol-3-yl phosphate after electrophoresis of the cytosolic and nuclear GlcNAc-specific lectin fractions on a non-denaturing gel (results not shown).

**Table 1 Assays for the presence of enzymic activities associated with the cytosolic and nuclear GlcNAc-specific lectin fractions**

Commercially available enzymes (alkaline phosphatase and $\beta$-hexosaminidase) were used as positive controls, and water was used as a negative control.

| Assay | Absorbance (420 nm) |
|---|---|
| Phosphatase activity assay | |
| Positive control (alkaline phosphatase) | 0.269 |
| Negative control (water) | 0.002 |
| *p*-Nitrophenyl phosphate + GlcNAc cytosolic lectin fraction | 0.178 |
| *p*-Nitrophenyl phosphate + GlcNAc nuclear lectin fraction | 0.117 |
| Glucosaminidase activity assay | |
| Positive control ($\beta$-hexosaminidase) | 0.175 |
| Negative control (water) | 0.000 |
| *p*-Nitrophenyl GlcNAc + GlcNAc cytosolic lectin fraction | 0.002 |
| *p*-Nitrophenyl GlcNAc + GlcNAc nuclear lectin fraction | 0.004 |

## Cytosolic and nuclear GlcNAc-specific lectins belong to the HSP-70 family

To establish the identity of the different GlcNAc-specific lectins, these proteins were resolved by 10% (w/v) preparative SDS/PAGE and were stained with Coomassie Brilliant Blue. The three different bands were excised out of the gel, digested with trypsin and analysed by MALDI–TOF-MS. The combination of the molecular masses, the pIs of the proteins and the masses of the peptides allowed us to identify these proteins by the use of algorithms ProteinProspector/MS-FIT or TagIdent, for sequence tags, on the Expasy web site http://www.expasy.ch/tools/ (Table 2, the data presented only refers to the 70 kDa band). This strategy allowed us to identify the GlcNAc-specific proteins as members of the HSP-70 family. p70 was identified as heat-shock cognate 70 stress protein (Hsc-70; pI = 5.38; molecular mass = 70871.6 Da; accession number in SwissProt databank = P08109). The other bands have been identified using

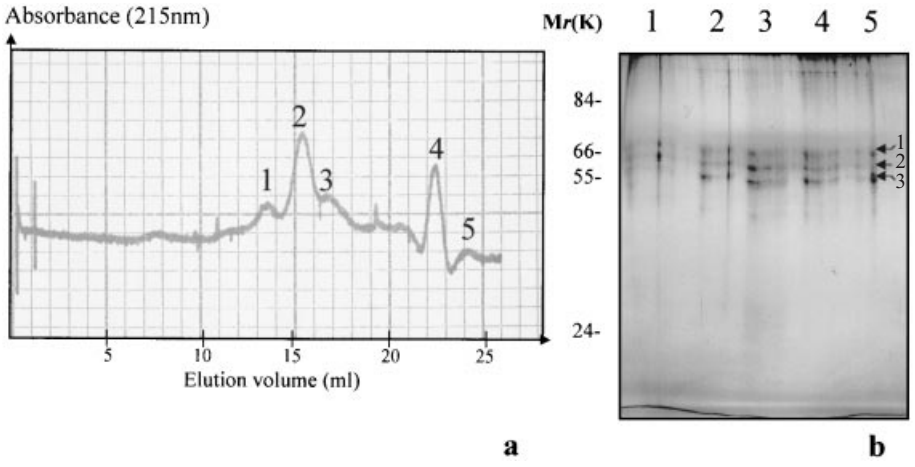


**Figure 4 Evidence for the formation of complexes between the 70, 65 and 55 kDa subunits**

The high molecular mass complexes were separated by FPLC on a Superose 6 column (**a**) and each collected peak was further analysed using SDS/PAGE (10% gels) (**b**). 1; 70 kDa protein, 2; 65 kDa protein; 3, 55 kDa protein. Peaks labelled 1, 2, 3, 4 and 5 correspond to the peaks that we collected by FPLC and that we subjected to SDS/PAGE. Molecular-mass markers are shown (in kDa) on the left.

**Table 2 Identification of the heat-shock cognate 70 stress protein (molecular mass = 70871.6 Da; pI = 5.38; accession number = P08109)**

| Masses submitted (*m/z*) | $M+H^+$ matched | Delta mass (p.p.m.) | Start amino acid | End amino acid | Peptide sequence |
|---|---|---|---|---|---|
| 861.445 | 861.442 | 3.48 | 252 | 258 | DISENKR |
| 1197.673 | 1197.663 | 5.69 | 459 | 469 | FELTGIPPAPR |
| 1199.686 | 1199.674 | 4.32 | 160 | 171 | DAGTIAGLNVLR |
| 1228.641 | 1228.628 | 9.28 | 26 | 36 | VEIIANDQVLR |
| 1253.638 | 1253.616 | 10.66 | 302 | 311 | FEELNADLFR |
| 1481.861 | 1481.807 | 35.35 | 329 | 342 | SQIHDIVLVGGSTR |
| 1487.766 | 1487.701 | 39.11 | 37 | 49 | TTPSYVAFTDTER |
| 1691.680 | 1691.726 | −27.26 | 221 | 236 | STAGDTHLGGEDFDNR |
| 1838.197 | 1838.013 | 98.09 | 326 | 342 | LDKSQIHDIVLVGGSTR |

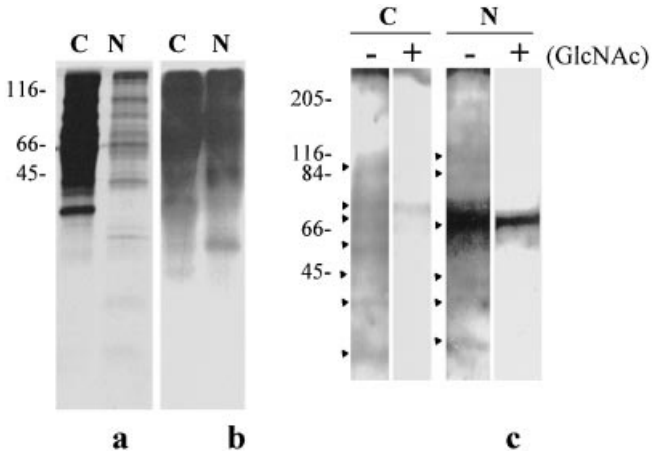


**Figure 5 Identification of the ligands of the GlcNAc-specific lectins**

Cytosolic (C) and nuclear (N) extracts were enriched on a WGA-immobilized column. The retained proteins were resolved by SDS/PAGE (10% gels) and silver stained (**a**) or electrotransferred on to nitrocellulose and tested for their affinity to the biotinylated GlcNAc-specific lectins (**c**). Cytosolic and nuclear extracts were labelled with [$^3$H]galactose, separated by SDS/PAGE (10% gels) and fluorographed (**b**). Molecular-mass markers are shown (in kDa) on the left.

peptide sequences $^{63}$PSYVA$^{67}$ and $^{565}$LES$^{567}$ (where single-letter amino-acid notation has been used). These peptide sequences matched with different members of the HSP-70 family. Nevertheless, in view of the parent peptides masses (IT**PSYVA**FTPEGER, 1566.78 Da; and NE**LES**YAYSLK, 1316.63 Da) they are similar to 78 kDa glucose-regulated protein (GRP78; also termed BiP; pI = 5.07; molecular mass = 72437.6 Da; accession number = P06761) even if the apparent molecular masses of the 65 and 55 kDa proteins were smaller than that of BiP proteins (these proteins may correspond to a truncated BiP). No proteins of similar molecular mass besides HSPs/BiPs matched the two peptide sequences. In addition, this confirms that the proteins characterized in the present study are not contaminating keratin family members.

### Presence of cytosolic and nuclear ligands of GlcNAc-specific cytosolic and nuclear lectins

In order to look for the presence of endogenous ligands of the GlcNAc-specific lectins, cytosolic and nuclear extracts were passed through an immobilized WGA column. The bound proteins were resolved by SDS/PAGE (10% gels) and silver stained (Figure 5a). Cytosolic and nuclear extracts were also labelled in the presence of [$^3$H]galactose in the presence of bovine milk galactosyltranferase, resolved by SDS/PAGE and fluorographed (Figure 5b) to ensure the presence of terminal GlcNAc. The eluted proteins bound on WGA were transferred on to nitrocellulose and tested for their affinity to the biotinylated GlcNAc-specific cytosolic and nuclear lectins (Figure 5c). As shown in Figures 5(a) and 5(b), the electrophoretic profiles of the cytosolic and nuclear WGA-binding glycoproteins were different. The cytosolic fractions showed major high molecular mass constituents, which were not recognized by biotinylated GlcNAc-binding lectins, although they incorporated [$^3$H]galactose radioactivity.

The nature of the glycan ligands of the GlcNAc-binding lectins was confirmed after release of the O-linked glycans, previously labelled with [$^3$H]galactose, using the reductive $\beta$-elimination procedure. The released glycans were further desalted on paper chromatography and then analysed by TLC. As shown in Figure 6, a single peak co-migrating with standard *N*-acetyl-lactosaminitol was detected (no constituents with more than two monosaccharides were found). Consequently, the data demonstrated that in both the cytosolic and nuclear fractions, the unique ligand of the isolated GlcNAc-binding lectin fractions was O-GlcNAc.

### Cytochemical evidence of the presence of nuclear and cytosolic ligands in human lymphocytes

The binding of biotinylated nuclear GlcNAc-specific lectins to human resting and activated (cultured for 3 days after treatment with PMA) lymphocytes fixed with aldehyde mixtures and permeabilized in the presence of 0.01% Triton X-100 was also tested using HRP-labelled avidin and 3,3′-diaminobenzidine detection. As shown in Figure 7, most isolated cells (for resting lymphocytes) and cells at the surface of aggregates (for activated lymphocytes) were stained both in the cytosol and in the nucleus, although the relative intensities of the cytosolic and nuclear staining differed from one cell type to the other. The binding was considered to be specific, since it was completely inhibited using 10 mM GlcNAc during the incubation with biotinylated lectins and in the first washing after incubation.

## DISCUSSION

In the present study, we focused our work on the putative implication of O-GlcNAc in the transport of proteins between the cytosol and the nucleus. Such a role was previously suggested

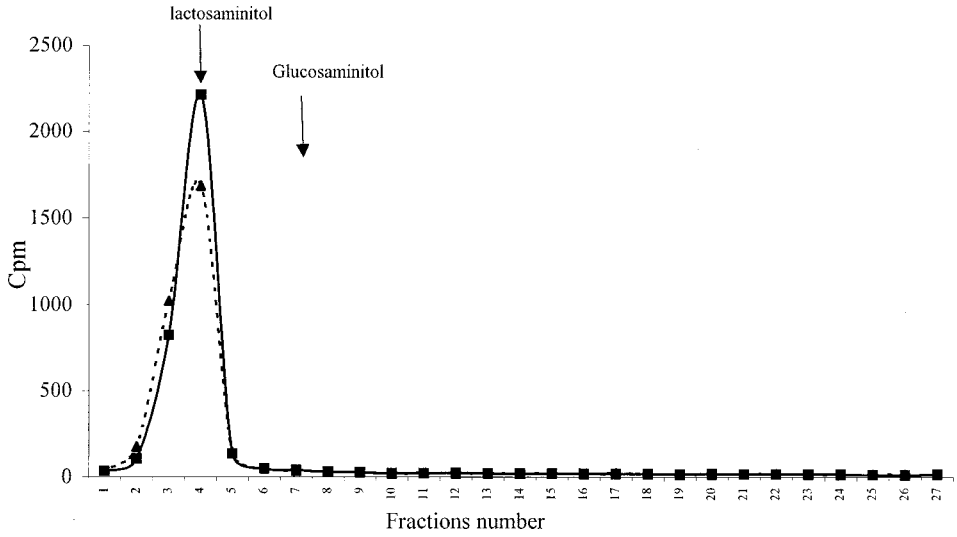


**Figure 6 Nature of the glycans recognized by nuclear GlcNAc-specific lectins**

The [$^3$H]galactose-labelled material was released by reductive $\beta$-elimination. After desalting on paper chromatography, the released glycans were further analysed by TLC (continuous line, rat cytosolic fraction; broken line, nuclear fraction).

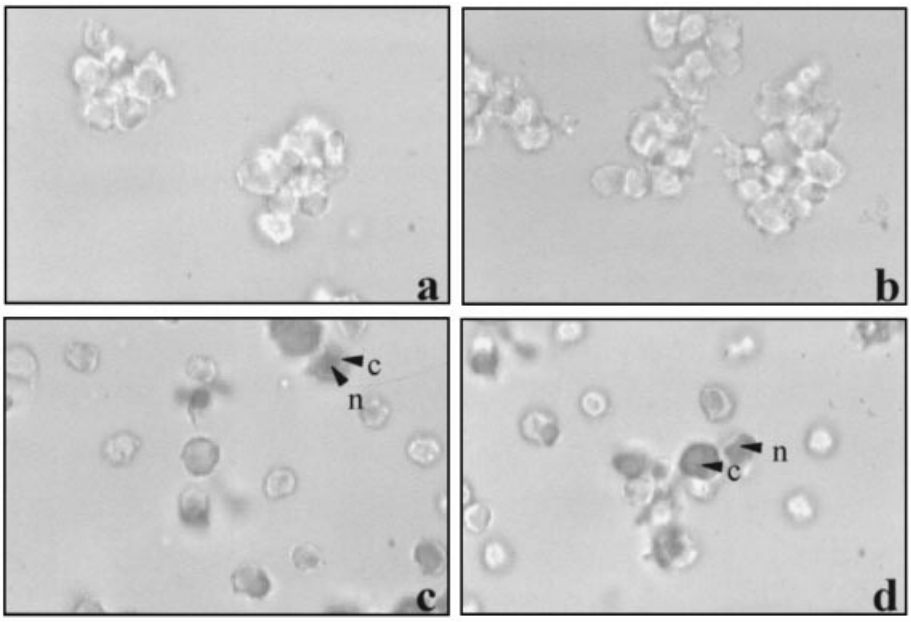


**Figure 7 Localization of the ligands of biotinylated nuclear GlcNAc-specific lectins in human lymphocytes using HRP-labelled avidin**

(**a** and **c**) Resting cells. (**b** and **d**) Activated cells. The specificity of the staining (c, cytosol; n, nucleus) was demonstrated using co-incubation with free 10 mM GlcNAc (**a** and **b**). Magnification, $\times$ 375.

by Duverger et al. [10], demonstrating that BSA substituted with $\beta$-di-*N*-acetyl-chitobiose was transported into the nucleus, whereas BSA was not. Similarly, Finlay et al. [11] showed specific inhibition of nuclear transport by WGA.

If O-GlcNAc is actually a signal for nuclear transport of cytosolic glycoproteins, then GlcNAc-specific lectins should exist. Such lectin activities have already been detected in the developing rat cerebellum by Kuchler et al. [19,20] using neoglycoproteins, and by Felin et al. [13] in the nucleus of HL60 cultured cells.

In the present work, we demonstrated that both the cytosolic and nuclear compartments of rat liver contain a similar pattern of proteins with apparent molecular masses of 70, 65 and 55 kDa. These compounds specifically bind to immobilized GlcNAc. Each of these proteins comprised several isoforms, which may reflect differences in phosphorylation, as demonstrated by two-dimensional electrophoresis. This hypothesis is reinforced by the specific staining of p70 and p65 using anti-phosphoserine antibodies (no staining was detected for p55).

Based on the comparisons of the pIs of these isoforms in each compartment, it was concluded that the nuclear subunits were less phosphorylated than the cytosolic ones. In the same way, we have demonstrated that these GlcNAc-binding lectins were themselves O-linked N-acetylglucosaminylated (it is noteworthy that the GlcNAc-specific lectins that are phosphorylated are also O-linked N-acetylglucosaminylated, i.e. p70 and p65). In addition, we have shown that aggregation into high molecular mass complexes (minimal observable molecular mass of 190 kDa in non-denaturing gels) was independent from GlcNAc self-interactions, or from ligand–lectin interactions, since the complexes between the 70, 65 and 55 kDa subunits were not dissociated using 0.2 M GlcNAc in the non-denaturing polyacrylamide gels.

The constituents of the GlcNAc-specific lectin complexes were identified, using an MS-based approach, as members of the HSP-70 family. p70 was identified as Hsc-70, while p65 and p55 present sequence similarities with GRP78, the endoplasmic-reticulum-localized HSP-70.

The HSP-70s were originally identified in response to stress and to heat shock [28], but they play many other roles, including protein folding, protein translocation, oligomeric assembly and protein degradation. The evidence that an HSP-70-like protein could specifically bind GlcNAc was first demonstrated by Minic et al. [29]. Using affinity chromatography, with either GlcNAc or chitotriose linked to agarose beads, Minic et al. [29] purified an additional protein similar to HSP-70. From a structural point of view, HSP-70 proteins are organized into three different domains among which the 44 kDa N-terminal domain possesses an ATPase activity [30]: the detection of a phosphatase activity associated with the GlcNAc-specific lectins could be related to this ATPase activity of HSPs.

Several parallels can be drawn between the identification of p70, described in the present study as being Hsc-70 and as CBP-70 by Felin et al. [13]. Indeed, Rousseau et al. [15] demonstrated that CBP-70, initially shown to be localized in the cytosol and the nucleus, is also associated with endoplasmic reticulum and the Golgi apparatus. Interestingly HSPs are ubiquitous and are found in numerous cellular compartments, from the cytosolic to the endoplasmic reticulum compartments (Hsc-70, the cytosolic HSP-70 form, and BiP, the endoplasmic reticulum HSP-70 form, have a similarity of 61.2 %). Then, the same authors observed the persistence of CBP-70 and of its GlcNAc binding sites after heat-shock treatments [31]. This observation reinforces the notion that the p70 protein, described in the present study as being Hsc-70, could be CBP-70.

Whether the GlcNAc-specific lectins are actually involved in the shuttling of O-N-acetylglucosaminylated glycoproteins between the cytosol and the nucleus remains unanswered. Yet, HSPs have been shown to play a role in many intracellular protein trafficking systems. Especially, Hsc-70 has been demonstrated to be implicated in nuclear transport. Shi and Thomas [32] examined cytosolic factors involved in nuclear import using ATP-affinity chromatography to remove ATP-binding proteins from a cytosolic extract. This depletion reduced transport of nucleoplasmin by 5–10-fold. The transport was restored by back addition of proteins eluted from the ATP–agarose with free ATP, this fraction containing Hsc-70 and HSP-70. Other studies [33] showed that Hsc-70 depletion of a cytosolic extract using antibodies reduced nuclear transport significantly, whereas the addition of purified Hsc-70 restored this transport. The karyophilic protein-dependent accumulation of Hsc-70 was dependent on the cytosolic extract, the temperature and ATP. Interestingly, this transport was sensitive to WGA, reinforcing the implication of a GlcNAc residue.

Based on our experiments (WGA–Sepharose enrichment, galactosyltranferase specific reaction and carbohydrate nature identification), it seems clear that the major ligand of the GlcNAc-specific lectins concerned O-GlcNAc glycoproteins. The use of the biotinylated lectin fractions for detecting endogenous ligands in human lymphocytes and the demonstration of a dual localization of the ligands both in the nucleus and in the cytosol suggests that the presence of O-GlcNAc glycoproteins is neither tissue- nor cell-specific, as already reported [9].

The finding that these GlcNAc-specific lectins belong to the HSP-70 family provides new insights in the field of HSP function.

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## REFERENCES

1 Torres, C.-R. and Hart, G. W. (1984) Topography and polypeptide distribution of terminal N-acetylglucosamine residues on the surfaces of intact lymphocytes. Evidence for O-linked GlcNAc. J. Biol. Chem. **259**, 3308–3317
2 Holt, G. W. and Hart, G. W. (1986) The subcellular distribution of terminal N-acetylglucosamine moieties. Localization of a novel protein-saccharide linkage, O-linked GlcNAc. J. Biol. Chem. **261**, 8049–8057
3 Dong, D. L.-Y., Xu, Z.-S., Chevrier, M. R., Cotter, R. J., Cleveland, D. W. and Hart, G. W. (1993) Glycosylation of mammalian neurofilaments. Localization of multiple O-linked N-acetylglucosamine moieties on neurofilament polypeptides L and M. J. Biol. Chem. **268**, 16679–16687
4 Holt, G. W., Snow, C. M., Senior, A., Haltiwanger, R. S., Gerace, L. and Hart, G. W. (1987) Nuclear pore complex glycoproteins contain cytoplasmically disposed O-linked N-acetylglucosamine. J. Cell Biol. **104**, 1157–1164
5 Lubas, W. A., Smith, M., Starr, C. M. and Hanover, J. A. (1995) Analysis of nuclear pore protein p62 glycosylation. Biochemistry **34**, 1686–1694
6 Jackson, S. P. and Tjian, R. (1988) O-glycosylation of eukaryotic transcription factors: implications for mechanisms of transcriptional regulation. Cell (Cambridge, Mass.) **55**, 125–133
7 Chou, T.-Y., Dang, C.-V. and Hart, G. W. (1995) Glycosylation of the c-Myc transactivation domain. Proc. Natl. Acad. Sci. U.S.A. **92**, 4417–4421
8 Chou, T.-Y., Hart, G. W. and Dang, C.-V. (1995) c-Myc is glycosylated at threonine 58, a known phosphorylation site and a mutational hot spot in lymphomas. J. Biol. Chem. **270**, 18961–18965
9 Comer, F. I. and Hart, G. W. (2000) O-Glycosylation of nuclear and cytosolic proteins. Dynamic interplay between O-GlcNAc and O-phosphate. J. Biol. Chem. **275**, 29179–29182
10 Duverger, E., Roche, A.-C. and Monsigny, M. (1996) N-acetylglucosamine-dependent nuclear import of neoglycoproteins. Glycobiology **6**, 381–386
11 Finlay, D. R., Newmeyer, D. D., Price, T. M. and Forbes, D. J. (1987) Inhibition of in vitro nuclear transport by a lectin that binds to nuclear pores. J. Cell Biol. **104**, 189–200
12 Miller, M. W. and Hanover, J. A. (1994) Functional nuclear pores reconstituted with $\beta$1-4 galactose-modified O-linked N-acetylglucosamine glycoproteins. J. Biol. Chem. **269**, 9289–9297
13 Felin, M., Doyenne-Moyne, M.-A., Hadj-Sahraoui, Y., Aubery, M., Hubert, J. and Sève, A.-P. (1994) Identification of two nuclear N-acetylglucosamine-binding proteins. J. Cell. Biochem. **56**, 527–535
14 Felin, M., Doyenne-Moyne, M.-A., Rousseau, C., Schröder, H. C. and Sève, A.-P. (1997) Characterization of a putative 82 kDa nuclear ligand for the N-acetylglucosamine-binding protein CBP70. Glycobiology **7**, 23–29
15 Rousseau, C., Muriel, M. P., Musset, M., Botti, J. and Sève, A.-P. (2000) Glycosylated nuclear lectin CBP70 also associated with endoplasmic reticulum and Golgi apparatus: does the "classical pathway" of glycosylation also apply to nuclear glycoproteins? J. Cell. Biochem. **78**, 638–649
16 Sève, A.-P., Hubert, J., Bouvier, D., Bourgeois, C. A., Midoux, P., Roche, A.-C. and Monsigny, M. (1986) Analysis of sugar-binding sites in mammalian cell nuclei by quantitative flow microfluorometry. Proc. Natl. Acad. Sci. U.S.A. **83**, 5997–6001

17 Schröder, H. C., Facy, P., Monsigny, M., Pfeifer, K., Bek, A. and Müller, W. E. G. (1992) Purification of a glucose-binding protein from rat liver nuclei. Evidence for a role in targeting of nuclear mRNP to nuclear pore complex. Eur. J. Biochem. **205**, 1017–1025
18 Hubert, M., Wang, S.-Y., Wang, J. L., Sève, A.-P. and Hubert, J. (1995) Intranuclear distribution of galectin-3 in mouse 3T3 fibroblasts: comparative analyses by immunofluorescence and immunoelectron microscopy. Exp. Cell Res. **220**, 397–406
19 Kuchler, S., Zanetta, J.-P., Vincendon, G. and Gabius, H.-J. (1990) Detection of binding sites for biotinylated neoglycoproteins and heparin (endogenous lectins) during cerebellar ontogenesis in the rat. Eur. J. Cell Biol. **52**, 87–97
20 Kuchler, S., Zanetta, J.-P., Vincendon, G. and Gabius, H.-J. (1992) Detection of binding sites for biotinylated neoglycoproteins and heparin (endogenous lectins) during cerebellar ontogenesis in the rat: an ultrastructural study. Eur. J. Cell Biol. **59**, 373–381
21 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **270**, 680–685
22 Vercoutter-Edouart, A.-S., Lemoine, J., Le Bourhis, X., Louis, H., Boilly, B., Nurcombe, V., Revillion, F., Peyrat, J.-P. and Hondermarck, H. (2001) Proteomic analysis reveals that 14–3–3sigma is down-regulated in human breast cancer cells. Cancer Res. **61**, 76–80
23 Mann, M. and Wilm, M. (1994) Error-tolerant identification of peptides in sequence databases by peptide sequence tags. Anal. Chem. **66**, 4390–4399
24 Kearse, K. P. and Hart, G. W. (1991) Lymphocyte activation induces rapid changes in nuclear and cytoplasmic glycoproteins. Proc. Natl. Acad. Sci. U.S.A. **88**, 1701–1705
25 Whiteheart, S. W., Passaniti, A., Reichner, J. S., Holt, G. D., Haltiwanger, R. S. and Hart, G. W. (1989) Glycosyltransferase probes. Methods Enzymol. **179**, 82–95
26 Glass, II, W. F., Briggs, R. C. and Hnilica, L. S. (1981) Use of lectins for detection of electrophoretically separated glycoproteins transferred onto nitrocellulose sheets. Anal. Biochem. **115**, 219–224
27 Graham, R. C. and Karnovsky, M. J. (1966) The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem. **14**, 291–302
28 Lindquist, S. (1987) The heat-shock response. Annu. Rev. Biochem. **55**, 1151–1191
29 Minic, Z., Leproust-Lecoester, L., Laporte, J., De Kouchkovsky, Y. and Brown, S. C. (2000) Proteins isolated from lucerne roots by affinity chromatography with sugars analogous to Nod factor moieties. Biochem. J. **345**, 255–262
30 Flaherty, K. M., DeLuca-Flaherty, C. and McKay, D. B. (1990) Three-dimensional structure of the ATPase fragment of a 70 K heat shock cognate protein. Nature (London) **346**, 623–628
31 Rousseau, C., Felin, M. and Sève, A.-P. (2000) Stable expression of functional CBP70 lectin during heat shock. J. Cell. Biochem. **77**, 615–623
32 Shi, Y. and Thomas, J. O. (1992) The transport of proteins in to the nucleus requires the 70-kilodalton heat shock protein or its cytosolic cognate. Mol. Cell. Biol. **12**, 2186–2192
33 Okuno, Y., Imamoto, N. and Yoneda, Y. (1993) 70-kDa heat-shock cognate protein colocalizes with karyophilic proteins into the nucleus during their transport in vitro. Exp. Cell Res. **206**, 134–142

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Review

# *O*-GlcNAc glycosylation: a signal for the nuclear transport of cytosolic proteins?

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**Abstract**

Year 2004 marks the 20th anniversary of the discovery of *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) by Gerald W. Hart. Despite interest for *O*-GlcNAc, the functions played by this single monosaccharide remain poorly understood, though numerous roles have been suggested, among which is the involvement of *O*-GlcNAc in the nuclear transport of cytosolic proteins. This idea was first sustained by studies on bovine serum albumin that showed that the protein could be actively carried to the nucleus when it was modified with sugars. In this paper, we will review data on this puzzling problem. We will first describe the well-established nuclear localisation signal (NLS)-dependent nuclear transport by presenting the different factors involved, and then, we will examine where and how *O*-GlcNAc could be involved in nuclear transport. Whereas it has been suggested that *O*-GlcNAc could interfere at two levels in the nuclear transport both by modifying proteins to be translocated to the nucleus and by modifying the nucleoporins of the nuclear pore complex, according to us, this second idea seems unlikely. Part of this study will also be dedicated to a relatively new concept in the nuclear transport: the role of the 70-kDa heat shock proteins (HSP70). The action of the chaperone in nuclear translocation was put forward 10 years ago, but new findings suggest that this mechanism could be linked to *O*-GlcNAc glycosylation.


*Keywords:* Nuclear transport; *O*-GlcNAc; Heat shock proteins; HSP70

**Contents**



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## 1. Introduction

*O*-Linked *N*-acetylglucosamine (*O*-GlcNAc) glycosylation is the modification of proteins by a single residue of *N*-acetylglucosamine on serine and threonine groups. Usually, this monosaccharide is neither epimerized nor elongated. *O*-GlcNAc was first evidenced on the surface of intact lymphocytes using bovine milk galactosyltransferase as an impermeant probe (Torres & Hart, 1984). After much investigation, it appeared that the finding of *O*-GlcNAc constituted a major breakthrough in glycobiology, and that it challenged two dogmas in our understanding of glycosylation: for the first time, a glycosylation type was found to have a high content in the cytosolic and nuclear compartments of eukaryota, whereas, until that date, glycosylation had been confined in the lumen of the endoplasmic reticulum and Golgi apparatus or to membranous proteins and secretory pathways; and secondly, the versatility of *O*-GlcNAc demonstrated that a glycosylation could not be always static (like *N*-glycans or classical *O*-glycans). As was mentioned above, one of the main features of *O*-GlcNAc is its versatility, since it is often compared to phosphorylation (Hart et al., 1995; Whelan & Hart, 2003). Like phosphorylation–dephosphorylation processes, that are regulated by a set of kinases and phosphatases, the glyco–deglyco process is also controlled by two cytosolic and nuclear distributed enzymes (Iyer & Hart, 2003a), i.e. the *O*-GlcNAc transferase (OGT) that transfers the monosaccharide from UDP–GlcNAc to the protein (Iyer & Hart, 2003b) and glucosaminidase that hydrolyses the sugar (Wells et al., 2002). Moreover, *O*-GlcNAc could compete with phosphorylation at the same site or in the vicinity, causing mutual exclusion (Comer & Hart, 2001; Slawson & Hart, 2003). *O*-GlcNAc is found in the cytosolic and nuclear compartments on a high number of proteins that are part of structural proteins (Cieniewski-Bernard et al., 2004); the transcriptional machinery including transcription factors as Sp1 (Yang et al., 2001) and Stat5 (Gewinner et al., 2004), RNA polymerase II (Comer & Hart, 2001); heat shock proteins (Guinez, Lemoine, Michalski, & Lefebvre, 2004; Walgren, Vincent, Schey, & Buse, 2003).

Despite its abundance, functions played by glycosylation remain unclear even though many roles have been put forward. Among them, *O*-GlcNAc could have a crucial importance in the transcriptional activity (Comer & Hart, 2001; Yang et al., 2001), in enzymatic activity (Cieniewski-Bernard et al., 2004), in the protection against proteasome (Guinez et al., 2004; Zachara et al., 2004) and in intracellular transport, especially in nuclear transport, since a few years ago, it was potentially presented as an alternate nuclear transport signal to the traditional nuclear localisation sig-

nal (NLS) controlled by *O*-GlcNAc (Duverger, Roche, & Monsigny, 1996).

## 2. Nuclear transport

### *2.1. Nucleo-cytoplasmic exchanges and nuclear pore complex*

One of the features of eukaryotic cells is the segregation of RNA synthesis and DNA replication – that are nuclear – with respect to the protein synthesis that is cytosolic, via a compartmentation with the double nuclear membrane. This implies precise and efficient bi-directional exchanges (hundreds of molecules are transported through one pore each second) between the cytoplasm and nucleus and vice-versa: nuclear proteins are imported to the nucleus, and RNAs that are synthesised in the nucleus are exported to the cytoplasm. These bi-directional exchanges are ensured by supramolecular structures of $125 \times 10^6$ Da, called nuclear pores complexes (NPC), that are anchored in the nuclear envelope (Allen, Cronshaw, Bagley, Kiseleva, & Goldberg, 2000; Fahrenkrog, Koser, & Aebi, 2004). The nuclear compartment is a structure delineated by a nuclear envelope constituted of a double bi-layer membrane. The outer lipid bi-layer (outer nuclear membrane) follows the endoplasmic reticulum membrane and the inner membrane is mainly covered with lamina, a set of proteins forming a network that ensures the integrity of the nuclear membrane structure (Goldberg & Allen, 1995). The formation of the NPC is ensured by the fusion of the inner and the outer membranes of the nucleus and is composed of 200 proteins. Its architecture is conserved from yeast to higher eukaryotes, with minor differences in the linear dimensions, and it has a diameter of 120 nm and a height of 70 nm. NPC are so large and sophisticated that they are considered as organelles.

### *2.2. The NLS pathway*

In order to enter the nucleus, proteins that share a molecular mass below 40 kDa could cross the NPC via passive diffusion, whereas the transport of proteins with a molecular mass above 40 kDa requires energy and travel must be made through the NPC, by presenting a NLS that consists of sequences with a high content of basic amino-acids (the first described NLS was that of the large-T antigen) (Görlich & Mattaj, 1996; Yoneda, 1997). Many factors are also required for the transport to the nucleus: pore targeting complex (PTAC) 58, PTAC 97, GTPase Ran and p10/NTF2 (nuclear transport factor, NTF) (Moore & Blobel, 1992; Stewart et al., 2001). NLS-dependent nuclear transport is divided into two main steps (Fig. 1). The first step involves NLS and is ATP-dependent. Proteins that translocate to the nucleus anchor on the cytoplasmic face of the NPC. This first stage in the transport, called "docking", could be sub-divided into two parts: PTAC formation (500 kDa) in the cytoplasm with the NLS-containing protein, and the complex anchoring on the NPC. The second step is ATP-independent and consists of the translocation of proteins to the nucleus. This second step, called "translocation", involves GTPase Ran and p10/NTF2, which is combined with Ran–GDP/PTAC 58 and 97 and nucleoporins peptidic sequences. The assumed involvement of *O*-GlcNAc or any other glycosylation type in this NLS-nuclear transport process has never been described.

## 3. Glycosylation: an alternative for the transport of proteins to the nucleus?

### *3.1. Sugar-modified bovine serum albumin is translocated to the nucleus*

Glycosylation-dependent nuclear transport was first supported by studies which assumed that sugar residues could act as a nuclear targeting signal (Hubert, Seve, Facy, & Monsigny, 1989). More precisely, 10 years ago, Duverger et al. described a mechanism in which fluorescein-coupled bovine serum albumin (BSA), which was either electroporated or using digitonin-permeabilized cells, and was substituted to sugars, could reach the nucleus (Duverger, Carpentier, Roche, & Monsigny, 1993; Duverger, Pellerin-Mendes, Mayer, Roche, & Monsigny, 1995; Duverger et al., 1996). As control, un-substituted BSA did not enter the nucleus. This transport was shown to be sugar-, time- and temperature-dependent (Duverger et al., 1995). More interestingly, these authors showed that in digitonin-permeabilized cells, sugar-dependent nuclear transport was NLS-independent, since *N*-ethylmaleimide, an alkylating agent that modifies NLS,

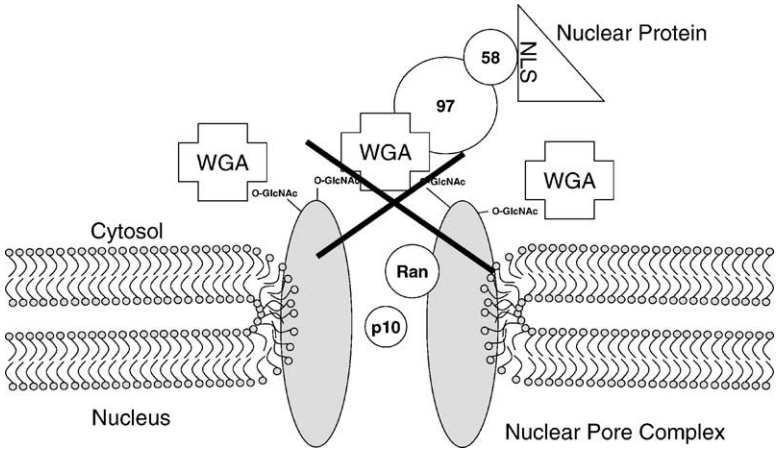


Fig. 1. Traditional nuclear transport. Traditionally, it is well known that a protein to be transported to the nucleus must possess stretches of basic amino-acids; the nuclear localisation signal. NLS is recognized by cytosolic components as forming the pore targeting complex and it docks at the nuclear pore complex and it is translocated to the nucleus where Ran–GTP dissociates the complex. The *O*-GlcNAc residues on the cytoplasmic face of the NPC are represented. WGA certainly inhibited nuclear transport by forming a screen at the surface of the nuclear pore, leading to an obstruction of it.

did not disrupt transport. The absence of nuclear transport when cells were incubated at 4 °C clearly suggested that sugar-dependent nuclear transport was energy-dependent like traditional NLS-dependent nuclear transport. This was confirmed by the destruction of ATP with apyrase. Later, it was shown that β-di-*N*-acetylchitobiose-modified BSA-dependent transport was independent from the addition of a cytosolic extract in contrast with the free-sugar NLS-substituted BSA (Duverger et al., 1996). Oddly enough, *N*-acetylglucosamine-substituted BSA was not carried to the nucleus. The authors argued that contrary to chitobiose, GlcNAc was not accessible for the putative receptor due to a steric constraint. Moreover, di-*N*-acetylchitobiose shares a similar structure with GlcNAc linked to serine or threonine residue. These two points could explain why β-di-*N*-acetylchitobiose-modified BSA was efficiently carried to the nucleus. This last finding was a good argument for a direct involvement of *N*-acetylglucosamine in nuclear transport. Recently, Rondanino, Bousser, Monsigny, and Roche (2003) explored the difference that may exist between NLS-mediated nuclear transport and sugar-dependent nuclear transport. The strategy used was different from that described above, since nuclear transport was tested after a cytosolic microinjection of NLS–BSA or Glc–BSA. The advantage of this technique is its less destructive effect compared to electroporation and digitonin-permeabilization that induce cellular death. The main finding was that sugar-dependent transport of BSA into the nucleus was cell-cycle dependent. Glucose-dependent nuclear transport was more efficient during the G1/S transition and S phases, whereas NLS–BSA transport did not sustain any difference during any stage cycle except a slight decrease for the G2 phase.

It has been assumed that the transport of glycosylated proteins to the nucleus could be achieved using intracellular components that act like a shuttle between the cytosol and nucleus. For this purpose, numerous nuclear lectins have been characterized (Monsigny, Rondanino, Duverger, Fajac, & Roche, 2004). They include CBP-35 (carbohydrate-binding protein, CBP) or galectin-3 (Moutsatsos, Wade, Schindler, & Wang, 1987), CBP-67 (Schröder et al., 1992), CBP-70 (Sève et al., 1993) and HSP70 (Guinez et al., 2004; Lefebvre et al., 2001) (discussed below).

### 3.2. The nuclear pore is O-GlcNAc modified

Numerous nuclear pore proteins located on the periphery of NPC are modified with *O*-GlcNAc. Among them, we find a group of eight proteins with molecular weights of 45, 54, 58, 62, 100, 145, 180 and 210 kDa (Holt et al., 1987). They all contain many degenerated peptides such as FXFG and GLFG, whose function re-

mains unknown. P62, p58 and p54 are crucial for the NPC formation and are directly involved in the nuclear transport process (Finlay, Meier, Bradley, Horecka, & Forbes, 1991). In this way, such protein-depleted NPCs from *Xenopus* eggs are unable to carry proteins across the channel. Nuclear transport experiments performed on fluorescently labelled nucleoplasmin, a protein that is regularly used to study nuclear transport, showed that wheat germ agglutinin (WGA, a lectin extracted from *Triticum vulgaris*) inhibited nuclear transport by binding to the *O*-GlcNAc glycosylation of nucleoporins, whereas no other lectin (i.e. ConA, PNA or UEA) did (Finlay, Newmeyer, Price, & Forbes, 1987).

Nuclear transport inhibition was not observed when the lectin was co-incubated with free GlcNAc, which demonstrates the specificity of inhibition. Other studies performed on *Xenopus* eggs demonstrated the importance of *O*-GlcNAc nucleoporins in nuclear transport. Moreover, WGA inhibited the nuclear transport of NLS-coupled phycoerythrin, but did not alter the transport of dextran (Wollf, Willingham, & Hanover, 1988). One of the characteristics of the NPC of *Xenopus* eggs is that it could be disassembled and reassembled to form functional NPCs in nuclear transport. This phenomenon is met in each cell division, where the NPC completely breaks down and re-forms. Miller and Hanover (1994) have depleted *Xenopus* egg NPC of their *O*-GlcNAc nucleoporins with insolubilised WGA. In this case, nuclear transport was impaired. Transport could be restored with the addition of rat glycosylated nucleoporins again. In the same way, Finlay and Forbes (1990) have demonstrated that nuclei reformed after depletion of their cytosolic WGA-binding proteins impaired the protein import, although the structure of the NPC appeared unchanged. These studies clearly demonstrated that *O*-GlcNAc nucleoporins are indispensable for nuclear transport. Conversely, the modification of the *O*-GlcNAc residues via a capping operation with galactose, using galactosyltransferase, did not change the assembly of the NPC or nuclear transport. On the basis of this finding, Miller and Hanover postulated that the glycosylation of nucleoporins was not required for nuclear transport and argued against the involvement of lectin-like shuttle between the cytosol and nucleus. In addition, it seemed that the mechanism through which WGA blocked nuclear transport consisted in obstructing the channel in a steric manner (Fig. 1). Such a phenomenon has been observed on the inhibition of nucleoplasmin transport when nucleoporins-directed antibodies were injected in *Xenopus* oocytes (Featherstone, Darby, & Gerace, 1988). Interestingly, WGA also inhibited the nuclear export of ribosomal subunits to the cytoplasm (Bataille, Helser, & Fried, 1990); we can suppose this was also done by blocking the aqueous channel.

### 3.3. *O*-GlcNAc and phosphorylation balance: a way to regulate nuclear transport?

#### 3.3.1. Phosphorylation and nuclear transport

Duverger et al. (1996) have suggested that one of the mechanisms through which GlcNAc could act as a nuclear localisation signal was by counteracting the function of phosphorylation. This post-translational modification is known for controlling the regulation of the nuclear transport of cytosolic proteins (Kaffman & O'Shea, 1999). For example, the phosphorylation of five serine residues on yeast transcription factor Pho 4 implies a cytosolic localisation of this protein, whereas its dephosphorylation induces its nuclear localisation (Kaffman, Rank, & O'Shea, 1998). Another example, during *Xenopus laevis* oocytes maturation, nucleolin (the major nucleoli component) is phosphorylated by $p34^{cdc2}$ kinase and is found within the cytosol. After fertilisation, dephosphorylation of the nucleolin occurs and the protein accumulates in the nucleus (Schwab & Dreyer, 1997). Lastly, transcription factor v-Jun accumulates in the nucleus, when NLS-adjacent Ser ($Ser^{248}$) is under-phosphorylated, whereas its phosphorylation inhibits nuclear translocation both in vivo and in vitro (Tagawa, Kuroki, & Chida, 1995). In these three different examples, the role of phosphorylation in nuclear localisation is well defined, but on none of these proteins a relationship between *O*-GlcNAc and phosphorylation in nuclear transport has been described.

#### 3.3.2. The example of Tau

Tau proteins belong to the family of brain microtubule-associated proteins involved in the polymerisation and stability of neuronal microtubules (Delacourte & Buee, 2000). Six different Tau isoforms are present in the adult brain and arise from an alternative splicing of a common primary transcript. In contrast to another Tau protein of 26–30 kDa apparent molecular mass that is found in the nucleus of neurob-

lastoma cells, Tau proteins are exclusively located in the cytosol. Tau protein is abundantly modified with phosphorylation and this post-translational modification is known for controlling microtubules polymerisation. In pathological conditions such as Alzheimer's disease, abnormal phosphorylation of Tau occurs and leads to the accumulation of neurofibrillary tangles. Tau is also *O*-GlcNAc modified (Arnold et al., 1996) but unlike phosphorylation, the function of the glycosylation on Tau is unknown. In a recent study, our group has demonstrated that a competition between phosphorylation and *O*-GlcNAc on Tau could have an impact on its nuclear location (Lefebvre et al., 2003). For this purpose, we used a stable clone expressing the long Tau isoform (Tau 441), so as to ensure a high level expression of the Tau protein. The first finding of the work was used to demonstrate that a competition between phosphorylation and *O*-GlcNAc exists on Tau protein. To generate hyperphosphorylated Tau, okadaic acid, a broad spectrum phosphatase inhibitor that mimics the hyperphosphorylation of Tau in Alzheimer's disease, was used in cultured cells. 2D electrophoresis clearly showed that after okadaic acid treatment, Tau 441 isoforms were shifted to the acidic pH values and that these isoforms were less *O*-GlcNAc modified than in normal conditions. More interestingly, this increase in phosphorylation and consequently the decrease in *O*-GlcNAc glycosylation correlated with a decrease in nuclear transport. Such an observation was previously made by Tanaka, Iqbal, Trenkner, Liu, and Grundke-Iqbal (1995) who showed that un-phosphorylated Tau accumulated to the nucleus. Thus, it appears that the phosphorylation–*O*-GlcNAc relationship could regulate the nuclear localisation of a protein. Unfortunately, the mechanism through which transport is regulated remains unknown.

## 4. *O*-GlcNAc and the nuclear localisation of transcription factors

### 4.1. Elf-1

Elf-1 is a member of the Ets transcription factor family and is expressed in hematopoietic cells. Elf-1 mediates the induction of different groups of genes including genes for the blk and lyn kinases or for surface membrane proteins such as TCR-ζ-chain, IL-2R α-chain (Tsokos, Nambiar, & Juang, 2003). According to its primary sequence, Elf-1 has a molecular mass of 68 kDa, but Juang, Solomou, Rellahan, and Tsokos (2002) have demonstrated that it has a molecular mass of 98 kDa in the nucleus and 80 kDa in the cytoplasm. These authors assumed that post-translational modifications were responsible for this increase in the molecular mass (80 and 98 kDa) and they showed that Elf-1 was both phosphorylated and glycosylated with *O*-GlcNAc, partly excluding any balance between phosphorylation and *O*-GlcNAc. The conversion of the 80 kDa form to the 98 kDa one through a modification with phosphorylation and *O*-GlcNAc makes possible the definition of a location where it binds to the promoter of the TCR-ζ-chain gene.

### 4.2. Pax6

Pax6 is a master control gene for eye morphogenesis in both invertebrates and vertebrates. Its quail homolog encodes five proteins through an alternative splicing and internal initiations. Three proteins of 48, 46 and 43 kDa contain the paired domain (DNA-binding domain) and two proteins of 33 and 32 kDa are devoid of this domain. The *O*-GlcNAc glycosylation of Pax6 proteins rather occurs on the 48 and 46 kDa isoforms that are strictly distributed into the nucleus, whereas glycosylation is not detected, either on the 43 kDa one or on the 33 and 32 kDa forms that are located both in the cytosol and in the nucleus (Lefebvre et al., 2002). This result could be interpreted with the role of *O*-GlcNAc, which is to maintain glycosylated forms of Pax6 in the nucleus, rather than acting as a nuclear localisation signal.

### 4.3. Stat5

Stat5 is part of the signal transducer and activator of transcription (Stat) gene family, that are latent cytoplasmic transcription factors. These transcription factors mediate cellular responses to different cytokines, hormones and growth factors and they have diverse biological functions in cell differentiation, development, proliferation, apoptosis and inflammation. The activation of Stat could be triggered by the binding of extracellular ligands to transmembrane receptors that cause the phosphorylation of Stat via tyrosine kinases, leading to a dimerisation and nuclear translocation of the

transcription factor. Recently, it was shown that Stat5 was modified with *O*-GlcNAc and interestingly, as for Pax6, nuclear forms only are glycosylated (Gewinner et al., 2004). The treatment of HC11 cells with glucose or glucosamine increased the glycosylation of Stat5 but did not have any impact on its nuclear localisation, which strongly suggests that the glycosylation of Stat5 is not involved in its nuclear translocation. However, the authors showed that the glycosylated form of Stat5 only could bind co-activator CBP that is essential for the Stat5-mediated gene transcription.

## 5. Heat shock proteins and nuclear transport

The search for intracellular lectins that could specifically bind *O*-GlcNAc residues showed that 70-kDa heat shock proteins (HSP70) were GlcNAc-binding proteins (Lefebvre et al., 2001). The initial hypothesis was the following: if *O*-GlcNAc was actually a signal for nuclear transport of cytosolic proteins, there should be proteins acting as a shuttle between the nucleus and the cytosol. In addition, even if the precise function of nucleoporins in nuclear transport remains unknown, nucleoporins are key components in this mechanism, since, as was discussed above, (i) WGA inhibits nuclear transport by binding GlcNAc residues of nucleoporins (Finlay et al., 1987), (ii) depletion of the *O*-GlcNAc proteins inhibits transport (Finlay & Forbes, 1990), and (iii) nucleoporins interact with cytosolic factors that are required for nuclear transport. In consideration of this last aspect, it seemed likely that cytosolic lectins acting like a shuttle could play an active part in sugar-dependent nuclear transport. The idea was that multimeric lectins with many lectinic-sites could bridge an *O*-GlcNAc protein that had to be carried to the nucleus with *O*-GlcNAc nucleoporins (Fig. 2). The involvement of heat shock proteins in nuclear transport was still reported. Using a permeabilized cell system, Shi and Thomas (1992) showed that after the depletion of Hsc70 and HSP70, using either ATP-agarose chromatography or antibodies, nuclear transport was lost. This transport is not only performed by heat shock proteins, since other cytosolic components are necessary. The same results were obtained after depletion of Hsc70 on the nuclear transport of the SV40 large-T antigen NLS-conjugated BSA (Okuno, Imamoto, & Yoneda, 1993). This transport is cytosolic extract, energy and temperature-dependent and was inhibited by WGA. This last finding was another solid argument

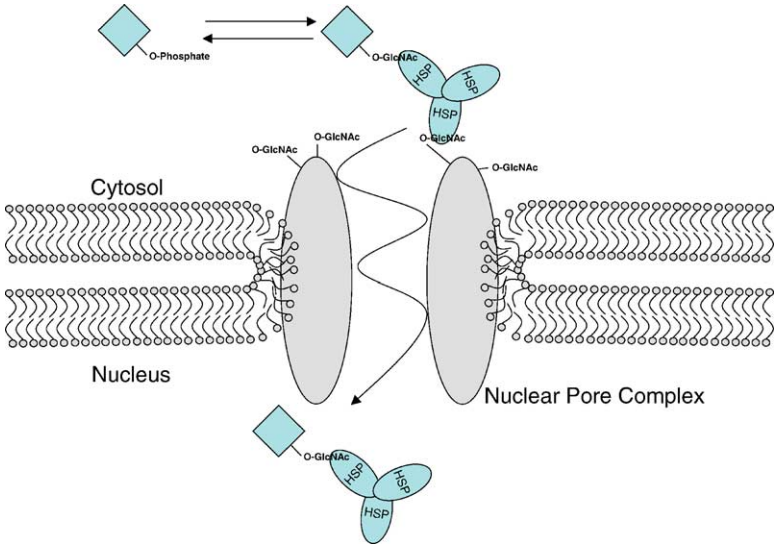


Fig. 2. Hypothetic mechanism by which intracellular lectins and more precisely HSP70 could operate for *O*-GlcNAc-mediated nuclear transport. Lectins could form complexes (Lefebvre et al., 2001): one lectinic site is available to make a possible binding of the protein to be translocated to the nucleus; a second one is used for bridging to the cytosolic face of the NPC via their *O*-GlcNAc residues. This spatial closeness makes it possible for unknown factors to translocate the complex to the nucleus. This phenomenon could be extended to the phospho-*O*-GlcNAc balance, leading the transport of the glycosylated form of the protein only, the phosphorylated form being held in the cytosol. It could be the case of Tau.

supporting the hypothesis of a GlcNAc-dependent heat shock protein nuclear transport. On this basis, we performed co-immunoprecipitation experiments so as to test the putative interaction between Hsc70 or HSP70 with p62 (Guinez, C., Michalski, J. C., & Lefebvre, T., unpublished data). In this way, if HSP70 actually acts as a shuttle between the cytosol and nucleus for the translocation of karyophilic proteins, and if *O*-GlcNAc nucleoporins interfere into this operation, then an interaction between HSP70 or Hsc70 with p62 could exist. Unfortunately, no such interaction was detected, either between Hsc70 and p62 or between HSP70 and p62. Recently, using an Hsc70 inhibitor, it was demonstrated that the glyco-dependent nuclear import of neoglycoproteins was inefficient (Monsigny et al., 2004). So though HSP70 is involved, particularly, though Hsc70 is an interesting candidate for the shuttling of cytosolic proteins to the nuclear compartment, the transport mechanism remains unknown. The function of HSP70 in nuclear transport seems to be indisputable, but either this mechanism does not involve *O*-GlcNAc nucleoporins or perhaps other cytosolic factors exist and need to be identified so as to clarify this NLS-independent nuclear transport.

## 6. Concluding remarks

Despite the intensive study of *O*-GlcNAc, the functions of this monosaccharide remain elusive. Conversely to NLS-dependent nuclear transport that is clearly understood, the putative involvement of *O*-GlcNAc in nuclear translocation is poorly understood. At the moment, it is difficult to draw a parallel between the two systems: do they have components in common (i.e. Ran, PTAC, HSP70, . . .)? And do they operate together or individually? Are they complementary? These questions remain to be answered. Though, if we want concrete examples, it seems rather clear that *O*-GlcNAc is involved in nuclear transport (e.g. Tau protein), especially through the balance between *O*-GlcNAc and phosphorylation, in striking contrast, works performed on other proteins like Stat5 clearly demonstrate that sugar is not a requisite for its nuclear transport. On the basis of these observations, *O*-GlcNAc could rather be considered as a signal for nuclear residence, than as a nuclear localisation signal. However, it is well known that in science, general cases are not the rule, and perhaps each protein needs to be considered as a particular case—the *O*-GlcNAc glycosylation playing a different role according to the protein.

Even if nucleoporins are crucial for nuclear transport, it seems that their *O*-GlcNAc glycosylation did not take an active part in the nuclear translocation process, and that the function of *O*-GlcNAc on nucleoporins certainly is structural (to make sure that there is a contact between nucleoporins). So, *O*-GlcNAc should directly operate, modifying the protein to be translocated to the nucleus. In consideration of this idea, the involvement of HSP70 in the nuclear transport of *O*-GlcNAc proteins had to be seriously considered. Even though no interaction was found between HSP70 and Hsc70 with p62, other studies strongly suggested that Hsc70 was needed for the nuclear transport of glycosylated proteins, and the cooperation of unknown factors is put forward. As a conclusion, the function of these chaperones in nuclear transport is unknown and more investigations need to be performed to understand this nuclear transport mechanism. Beyond this problem of nuclear transport, the lectinic activity of HSP70 could be involved in other processes, the proteasomal protection of substrates by *O*-GlcNAc being a very puzzling possible option.

## References

Allen, T. D., Cronshaw, J. M., Bagley, S., Kiseleva, E., & Goldberg, M. W. (2000). The nuclear pore complex: Mediator of translocation between nucleus and cytoplasm. *Journal of Cell Science*, *113*, 1651–1659.

Arnold, C., Johnson, G. V. W., Cole, R. N., Dong, D. L. Y., Lee, M., & Hart, G. W. (1996). The microtubule-associated protein Tau is extensively modified with *O*-linked *N*-acetylglucosamine. *Journal of Biological Chemistry*, *271*, 28741–28744.

Bataille, N., Helser, T., & Fried, H. M. (1990). Cytoplasmic transport of ribosomal subunits microinjected into the *Xenopus laevis* oocyte nucleus: A generalized, facilitated process. *Journal of Cellular Biology*, *111*, 1571–1582.

Cieniewski-Bernard, C., Bastide, B., Lefebvre, T., Lemoine, J., Mounier, Y., & Michalski, J. C. (2004). Identification of *O*-linked *N*-acetylglucosamine proteins in rat skeletal muscle using two-dimensional gel electrophoresis and mass spectrometry. *Molecular and Cellular Proteomics*, *3*, 577–585.

Comer, F. I., & Hart, G. W. (2001). Reciprocity between *O*-GlcNAc and *O*-phosphate on the carboxyl terminal domain of RNA polymerase II. *Biochemistry*, *40*, 7845–7852.

Delacourte, A., & Buee, L. (2000). Tau pathology: A marker of neurodegenerative disorders. *Current Opinion in Neurology*, *13*, 371–376.

Duverger, E., Carpentier, V., Roche, A. C., & Monsigny, M. (1993). Sugar-dependent nuclear import of glycoconjugates from the cytosol. *Experimental Cell Research*, *207*, 197–201.

Duverger, E., Pellerin-Mendes, C., Mayer, R., Roche, A. C., & Monsigny, M. (1995). Nuclear import of glycoconjugates is distinct from the classical NLS pathway. *Journal of Cell Science*, *108*, 1325–1332.

Duverger, E., Roche, A. C., & Monsigny, M. (1996). *N*-Acetylglucosamine-dependent nuclear import of neoglycoproteins. *Glycobiology*, *6*, 381–386.

Fahrenkrog, B., Koser, J., & Aebi, U. (2004). The nuclear pore complex: A jack of all trades? *Trends in Biochemical Sciences*, *29*, 175–182.

Featherstone, C., Darby, M. K., & Gerace, L. (1988). A monoclonal antibody against the nuclear pore complex inhibits nucleocytoplasmic transport of protein and RNA in vivo. *Journal of Cellular Biology*, *107*, 1289–1297.

Finlay, D. R., & Forbes, D. J. (1990). Reconstitution of biochemically altered nuclear pores: Transport can be eliminated and restored. *Cell*, *60*, 17–29.

Finlay, D. R., Meier, E., Bradley, P., Horecka, J., & Forbes, D. J. (1991). A complex of nuclear pore proteins required for pore function. *Journal of Cellular Biology*, *114*, 169–183.

Finlay, D. R., Newmeyer, D. D., Price, T. M., & Forbes, D. J. (1987). Inhibition of in vitro nuclear transport by a lectin that binds to nuclear pores. *Journal of Cellular Biology*, *104*, 189–200.

Gewinner, C., Hart, G., Zachara, N., Cole, R., Beisenherz-Huss, C., & Groner, B. (2004). The coactivator of transcription CREB-binding protein interacts preferentially with the glycosylated form of Stat5. *Journal of Biological Chemistry*, *279*, 3563–3572.

Goldberg, M. W., & Allen, T. D. (1995). Structural and functional organization of the nuclear envelope. *Current Opinion in Cellular Biology*, *7*, 301–309.

Görlich, D., & Mattaj, L. (1996). Nucleocytoplasmic transport. *Science*, *271*, 1513–1518.

Guinez, C., Lemoine, J., Michalski, J. C., & Lefebvre, T. (2004). 70-kDa-heat shock protein presents an adjustable lectinic activity towards *O*-linked *N*-acetylglucosamine. *Biochemical and Biophysical Research Communications*, *319*, 21–26.

Hart, G. W., Greis, K. D., Dong, L. Y., Blomberg, M. A., Chou, T. Y., Jiang, M. S., et al. (1995). *O*-Linked *N*-acetylglucosamine: The "yin–yang" of Ser/Thr phosphorylation? Nuclear and cytoplasmic glycosylation. *Advances in Experimental Medicine and Biology*, *376*, 115–123.

Holt, G. D., Snow, C. M., Senior, A., Haltiwanger, R. S., Gerace, L., & Hart, G. W. (1987). Nuclear pore complex glycoproteins contain cytoplasmically disposed *O*-linked *N*-acetylglucosamine. *Journal of Cell Biology*, *104*, 1157–1164.

Hubert, J., Seve, A. P., Facy, P., & Monsigny, M. (1989). Are nuclear lectins and nuclear glycoproteins involved in the modulation of nuclear functions? *Cell Differentiation and Development*, *27*, 69–81.

Iyer, S. P., & Hart, G. W. (2003a). Dynamic nuclear and cytoplasmic glycosylation: Enzymes of *O*-GlcNAc cycling. *Biochemistry*, *42*, 2493–2499.

Iyer, S. P., & Hart, G. W. (2003b). Roles of the tetratricopeptide repeat domain in *O*-GlcNAc transferase targeting and protein substrate specificity. *Journal of Biological Chemistry*, *278*, 24608–24616.

Juang, Y. T., Solomou, E. E., Rellahan, B., & Tsokos, G. C. (2002). Phosphorylation and *O*-linked glycosylation of Elf-1 leads to its translocation to the nucleus and binding to the promoter of the TCR zeta-chain. *Journal of Immunology*, *168*, 2865–2871.

Kaffman, A., & O'Shea, E. K. (1999). Regulation of nuclear localization: A key to a door. *Annual Review of Cellular and Developmental Biology*, *15*, 291–339.

Kaffman, A., Rank, N. M., & O'Shea, E. K. (1998). The receptor Msn5 exports the transcription factor Pho4 out of the nucleus. *Nature*, *396*, 482–486.

Lefebvre, T., Cieniewski, C., Lemoine, J., Guerardel, Y., Leroy, Y., Zanetta, J. P., et al. (2001). Identification of *N*-acetyl-D-glucosamine-specific lectins from rat liver cytosolic and nuclear compartments as heat-shock proteins. *Biochemical Journal*, *360*, 179–188.

Lefebvre, T., Ferreira, S., Dupont-Wallois, L., Bussiere, T., Dupire, M. J., Delacourte, A., et al. (2003). Evidence of a balance between phosphorylation and *O*-GlcNAc glycosylation of Tau proteins—A role in nuclear localization. *Biochimica et Biophysica Acta*, *1619*, 167–176.

Lefebvre, T., Planque, N., Leleu, D., Bailly, M., Caillet-Boudin, M. L., Saule, S., et al. (2002). *O*-Glycosylation of the nuclear forms of Pax-6 products in quail neuroretina cells. *Journal of Cellular Biochemistry*, *85*, 208–218.

Miller, D. J., & Hanover, J. A. (1994). Functional nuclear pores reconstituted with beta 1-4 galactose-modified *O*-linked *N*-acetylglucosamine glycoproteins. *Journal of Biological Chemistry*, *269*, 9289–9297.

Monsigny, M., Rondanino, C., Duverger, E., Fajac, I., & Roche, A. C. (2004). Glyco-dependent nuclear import of glycoproteins, glycocomplexes and glycosylated plasmids. *Biochimica et Biophysica Acta*, *1673*, 94–103.

Moore, M. S., & Blobel, G. (1992). The two steps of nuclear import, targeting to the nuclear envelope and translocation through the nuclear pore, require different cytosolic factors. *Cell*, *69*, 939–950.

Moutsatsos, I. K., Wade, M., Schindler, M., & Wang, J. L. (1987). Endogenous lectins from cultured cells: Nuclear localization of carbohydrate-binding protein 35 in proliferating 3T3 fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America*, *84*, 6452–6456.

Okuno, Y., Imamoto, N., & Yoneda, Y. (1993). 70-kDa heat-shock cognate protein colocalizes with karyophilic proteins into the nucleus during their transport in vitro. *Experimental Cell Research*, *206*, 134–142.

Rondanino, C., Bousser, M. T., Monsigny, M., & Roche, A. C. (2003). Sugar-dependent nuclear import of glycosylated proteins in living cells. *Glycobiology*, *13*, 509–519.

Schröder, H. C., Facy, P., Monsigny, M., Pfeifer, K., Bek, A., & Müller, W. E. (1992). Purification of a glucose-binding protein from rat liver nuclei. Evidence for a role in targeting of nuclear

mRNP to nuclear pore complex. *European Journal of Biochemistry*, *205*, 1017–1025.

Schwab, M., & Dreyer, C. (1997). Protein phosphorylation sites regulate the function of the bipartite NLS of nucleolin. *European Journal of Cellular Biology*, *73*, 287–297.

Sève, A. P., Felin, M., Doyennette-Moyne, M. A., Sahraoui, T., Aubery, M., & Hubert, J. (1993). Evidence for a lactose-mediated association between two nuclear carbohydrate binding proteins. *Glycobiology*, *3*, 23–30.

Shi, Y., & Thomas, J. O. (1992). The transport of proteins into the nucleus requires the 70-kilodalton heat shock protein or its cytosolic cognate. *Molecular and Cellular Biology*, *12*, 2186–2192.

Slawson, C., & Hart, G. W. (2003). Dynamic interplay between *O*-GlcNAc and *O*-phosphate: The sweet side of protein regulation. *Current Opinion in Structural Biology*, *13*, 631–636.

Stewart, M., Baker, R. P., Bayliss, R., Clayton, L., Grant, R. P., Littlewood, T., et al. (2001). Molecular mechanism of translocation through nuclear pore complexes during nuclear protein import. *FEBS Letters*, *498*, 145–149.

Tagawa, T., Kuroki, T., & Chida, K. (1995). The cell cycle-dependent nuclear import of v-jun is regulated by phosphorylation of a serine adjacent to the nuclear localization signal. *Journal of Cellular Biology*, *130*, 255–263.

Tanaka, T., Iqbal, K., Trenkner, E., Liu, D. J., & Grundke-Iqbal, I. (1995). Abnormally phosphorylated tau in SY5Y human neuroblastoma cells. *FEBS Letters*, *360*, 5–9.

Torres, C. R., & Hart, G. W. (1984). Topography and polypeptide distribution of terminal *N*-acetylglucosamine residues on the surfaces of intact lymphocytes. Evidence for *O*-linked GlcNAc. *Journal of Biological Chemistry*, *259*, 3308–3317.

Tsokos, G. C., Nambiar, M. P., & Juang, Y. T. (2003). Activation of the Ets transcription factor Elf-1 requires phosphorylation and glycosylation: Defective expression of activated Elf-1 is involved in the decreased TCR zeta chain gene expression in patients with systemic lupus erythematosus. *Annals of the New York Academy of Sciences*, *987*, 240–245.

Walgren, J. L., Vincent, T. S., Schey, K. L., & Buse, M. G. (2003). High glucose and insulin promote *O*-GlcNAc modification of proteins, including alpha-tubulin. *American Journal of Physiology and Endocrinology Metabolism*, *284*, E424–E434.

Wells, L., Gao, Y., Mahoney, J. A., Vosseller, K., Chen, C., Rosen, A., et al. (2002). Dynamic *O*-glycosylation of nuclear and cytosolic proteins: Further characterization of the nucleocytoplasmic beta-*N*-acetylglucosaminidase, *O*-GlcNAcase. *Journal of Biological Chemistry*, *277*, 1755–1761.

Whelan, S. A., & Hart, G. W. (2003). Proteomic approaches to analyze the dynamic relationships between nucleocytoplasmic protein glycosylation and phosphorylation. *Circulation Research*, *93*, 1047–1058.

Wollf, B., Willingham, M. C., & Hanover, J. A. (1988). Nuclear protein import: Specificity for transport across the nuclear pore. *Journal of Biological Chemistry*, *265*, 2563–2568.

Yang, X., Su, K., Roos, M. D., Chang, Q., Paterson, A. J., & Kudlow, J. E. (2001). *O*-Linkage of *N*-acetylglucosamine to Sp1 activation domain inhibits its transcriptional capability. *Proceedings of the National Academy of Sciences of the United States of America*, *98*, 6611–6616.

Yoneda, Y. (1997). How proteins are transported from cytoplasm to the nucleus. *Journal of Biochemistry*, *121*, 811–817.

Zachara, N. E., O'Donnell, N., Cheung, W. D., Mercer, J. J., Marth, J. D., & Hart, G. W. (2004). Dynamic *O*-GlcNAc modification of nucleocytoplasmic proteins in response to stress. A survival response of mammalian cells. *Journal of Biological Chemistry*, *279*, 30133–30142.

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# 70-kDa-heat shock protein presents an adjustable lectinic activity towards O-linked *N*-acetylglucosamine

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**Abstract**

Numerous works demonstrated that the dynamic *O*-GlcNAc glycosylation could protect against the proteasomal degradation by modifying the target proteins and the proteasome itself. Considering that Hsp70 is a crucial component in the quality control of protein conformation in the proteasomal pathway, we investigated the possibility that Hsp70 physically interacts with *O*-GlcNAc proteins through a lectinic activity. First, we demonstrate that in HepG2 cells, Hsp70 can specifically bind to *O*-GlcNAc residues but also is itself modified by *O*-GlcNAc. Second, when cells were deprived of glucose (nutrient stress), Hsp70 lectinic activity markedly increased whereas its glycosylation dramatically decreased. On the other hand, a 42 °C thermic stress did not affect any of these features. Lastly, the nature of *O*-GlcNAc modified proteins co-immunoprecipitating with Hsp70 was similar for cells submitted to the thermic and to nutrient stress. These results strongly suggest that *O*-GlcNAc influences protein stability through specific interaction with 70-kDa-heat shock protein members.


*Keywords:* Heat shock proteins; Hsp70; Chaperone; *O*-GlcNAc; Glucose; Stress

Usually, the proteins targeted to the proteasome present PEST sequences (sequences enriched in proline, glutamic acid, serine, and threonine that direct proteins for rapid degradation [1]) and are modified by poly-ubiquitinylation (for review see reference [2]). Recent reports showed that the dynamic *O*-GlcNAc glycosylation of proteins within the cytosolic and nuclear compartments of eukaryotes [3], such as Sp1 [4], estrogen receptor-β [5], and more recently plakoglobin [6], was less affected by proteasomal degradation. Consequently, it has been proposed that *O*-GlcNAc could protect proteins from the proteasomal degradation. Studies performed on Sp1 have shown that in cells cultured in the presence of 5 mM glucosamine, Sp1 became hyperglycosylated whereas in glucose starvation, the glycosylation decreased and Sp1 was rapidly degraded by the proteasome [4]. Treatment of the cells with glucose or glucosamine protects Sp1 from the degradation. This process seems to be independent of ubiquitinylation process. For the β-estrogen receptor, it has been proposed that the *O*-GlcNAc glycosylation of the site found within the PEST sequence could block protein degradation and would thus played an opposite role to that play by phosphorylation (which activates degradation at PEST sequences) [5]. Recently, it was shown that plakoglobin, which connects cadherins to cytoskeleton, could be modified with *O*-GlcNAc in close proximity to the destruction box [6]. Interestingly, many of the well-characterized *O*-GlcNAc glycosylation sites show high PEST scores, reinforcing the relationship between proteasomal degradation and *O*-GlcNAc. Two recent studies showed that the 26S proteasome is also modified by *O*-GlcNAc both in the regulatory and in the catalytic cores [7,8] and that, the *O*-GlcNAc moiety acts as an inhibitor of the proteolysis function.

In a previous report, we described for the first time the lectinic activity of the Hsc70 protein, a constitutive member of the 70-kDa-heat shock protein family (HSP70) [9]. Heat shock proteins (HSPs) are ubiquitously

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found in all organisms ranging from bacteria to most complex metazoans. They share a conserved structure that suggests a fundamental function in many cellular processes and are distributed in mostly all organelles (for review see [10]). HSP are composed of several families with molecular masses spreading from 20 to 110 kDa. The HSP70 family exhibits 70% base identity among the entire HSP70 eukaryotic family and near 60% with DnaK, its homolog in *Escherichia coli*. HSP70 are mainly localized in the cytosol and the nucleus of eukaryote cells. Their expression is induced by different types of stress (e.g., temperature, irradiation, heavy metals, infection, fever, and inflammation [11]). However, HSP70 are also present at a minimal amount in normal conditions. HSP70 are structured in three main domains [10]: the 44 kDa N-terminal domain involved in ATP hydrolysis, the central domain of 18 kDa that binds hydrophobic peptide sequence, and a 10 kDa glycine–proline rich C-terminal domain with a conserved EEVD tetrapeptide terminal sequence implicated in the mRNA translation. The constitutive form of HSP70, named Hsc70 (Heat shock cognate protein of 70 kDa), is not inducible. Both forms bind and hydrolyse ATP [12]. In conjunction with co-chaperones, heat shock proteins are involved in the protection and in the re-folding of normal and damaged proteins. When the re-folding process is unsuccessful, proteins are directed to the ubiquitin–proteasome system (UPS) for destruction [13].

Here, we have demonstrated the ability of Hsp70 to bind in a specific manner to *O*-GlcNAc residues. These results support a new type of lectinic interaction between *O*-GlcNAc modified proteins and Hsp70, related to protein life and death.

## Materials and methods

*Cell culture and stress treatments*. HepG2 cells were cultured on dishes (diameter 100 mm) treated with 0.1% porcine gelatin (Sigma) in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 5 IU/mL penicillin, and 50 μg/mL streptomycin at 37 °C in a 5%-$CO_2$ enriched atmosphere. For glucose depletion stress cells were rinsed with 10 mL of glucose-free medium before incubation in this culture medium for 24 h. Thermal stress was applied to cells by hyperthermic conditions culturing at 42 °C in a 5%-$CO_2$ enriched atmosphere.

*GlcNAc-beads affinity enrichment, immunoprecipitations, and co-immunoprecipitations*. Before lysis, HepG2 cells were washed with cold phosphate-buffered saline (Gibco).

For lectinic activity studies, cells were lysed in a hypotonic buffer (10 mM Tris/HCl, 10 mM NaCl, 15 mM 2-mercaptoethanol, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and protease inhibitor, pH 7.2) with a Dounce homogenizer on ice [12]. Extracts were centrifuged at 20,000*g* for 30 min at 4 °C, and the supernatant was carefully recovered. Thirty microlitres of GlcNAc-labelled beads (*N*-acetyl-D-glucosamine immobilized on 6% beaded agarose with a spacer of five carbons, Sigma) was added to the supernatant and incubated for 1 h at 4 °C. Beads were then gently centrifuged and washed four times in the buffer containing 20 mM Tris/HCl, 200 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and protease inhibitor, pH 7.4.

For immunoprecipitation, cells were lysed in a lysis buffer (Tris/HCl 10 mM, NaCl 150 mM, Triton X-100 1% (v/v), sodium deoxycholate 0.5% (w/v), sodium dodecyl sulphate 0.1% (w/v), and protease inhibitors, pH 7.4) and centrifugation was performed at 20,000*g* for 30 min at 4 °C. The RL2 anti-*O*-GlcNAc antibody (Affinity BioReagent) was added to a 1:250 final dilution and the lysates were placed at 4 °C overnight. The bound proteins were recovered after addition of protein G–Sepharose (Amersham) for 1 h at 4 °C. Beads were pelleted and washed, respectively, in: lysis buffer; lysis buffer supplemented with 500 mM NaCl; lysis buffer/TNE (Tris/HCl 10 mM, NaCl 150 mM, and EDTA 1 mM) in equal volume, and finally with TNE alone.

For co-immunoprecipitation with the anti-Hsp70 polyclonal antibody (Stressgen BioReagents) the procedure was the same as described for immunoprecipitation except that HepG2 were lysed in Co-IP buffer (Tris/HCl 50 mM, NaCl 150 mM, NP-40 0.5% (v/v), and protease inhibitors, pH 8.0) and anti-Hsp70 was used at a dilution of 1:200. The bound proteins were recovered by incubation with protein A–Sepharose (Amersham) for 1 h. Four washes were performed in the Co-IP buffer with gentle shaking (four times).

*SDS–PAGE and Western blotting*. Proteins were separated on 10% SDS–PAGE and electroblotted onto nitrocellulose sheet. Membranes were saturated for 45 min with 5% non-fatty acid milk in TBS–Tween buffer (Tris/HCl 20 mM, NaCl 150 mM, and Tween 0.05% (v/v), pH 8.0). Anti-Hsp70 antibodies were incubated for 1 h at a dilution of 1:150,000, and RL2 anti-*O*-GlcNAc monoclonal antibodies or anti-β-catenin polyclonal antibodies (Santa Cruz Biotechnologies) were incubated overnight at 4 °C at a dilution of 1:1000. Three washes of 10 min each were performed with TBS–Tween. Anti-rabbit and anti-mouse horseradish peroxidase labelled secondary antibodies were used at a dilution of 1:10,000 for 1 h at room temperature. Three washes of 10 min each were performed with TBS–Tween and the detection was carried out using the Western lightning chemiluminescence reagents plus kit (Perkin–Elmer).

## Results and discussion

### *Hsp70 exhibits a lectinic activity towards the O-GlcNAc motif and is itself O-GlcNAc modified*

In order to test our hypothesis of a putative lectinic property of the 70-kDa-heat shock protein towards the *O*-GlcNAc motif, GlcNAc-labelled agarose beads were incubated with a protein extract obtained from the HepG2 human hepatocarcinoma cell line cultured at 37 or at 42 °C. A lectinic activity of Hsp70 toward GlcNAc is evident in HepG2 both at 37 and at 42 °C (Fig. 1A, lanes 3 and 4). The quantitative differences of Hsp70 expression at 37 and at 42 °C, both in the control lanes and in the GlcNAc-beads enriched ones, are related to its induction after thermic stress. The specificity of the lectinic property of Hsp70 is illustrated by the less intense binding of Hsp70 to chitobiose (GlcNAc β-1,4-GlcNAc) and the lack of interaction with the mannose residue at 37 °C (Fig. 1B).

We have also examined whether Hsp70 itself might be a substrate for the *O*-GlcNAc transferase (OGT). For this purpose, using the extract from HepG2 grown at 37 °C and 42 °C, *O*-GlcNAc modified proteins were

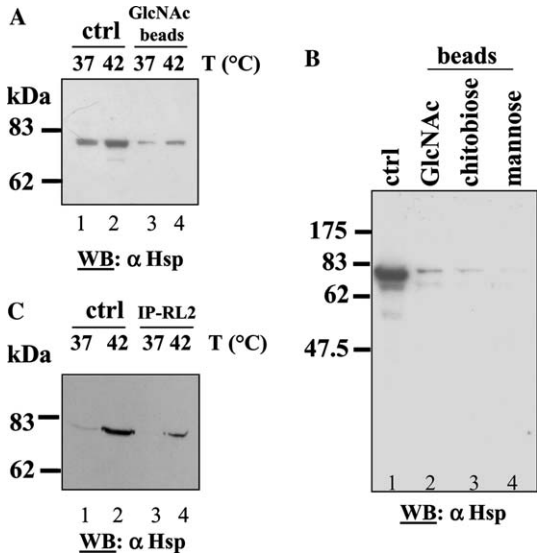


Fig. 1. Hsp70 lectinic activity against *N*-acetylglucosamine residues and *O*-GlcNAc modification of Hsp70. HepG2 cells were cultured either at a temperature of 37 °C or at a temperature of 42 °C as to induce a thermic stress. After lysis of the cells, cellular extracts were incubated either with GlcNAc-agarose beads (A) or with anti-*O*-GlcNAc monoclonal antibodies (C). The bound proteins were submitted to a 10% SDS–PAGE, electroblotted onto nitrocellulose sheet, and stained with an anti-Hsp70 antibody. The lectinic activity of Hsp70 was also tested on chitobiose beads and on mannose beads (B). The glycosylation of Hsp70 by *O*-GlcNAc was checked using the anti-*O*-GlcNAc antibody RL2 in immunoprecipitation experiments and an anti-Hsp70 antibody in Western blot (C). Ctrl, control; WB, Western blot.

enriched by immunoprecipitation with an anti-*O*-GlcNAc antibody (RL2). The bound proteins were separated on a 10% SDS–PAGE and stained with anti-Hsp70 antibodies (Fig. 1C). Indeed, Hsp70 was itself modified with *O*-GlcNAc. Only a very faint band of the glycosylated form of Hsp70 at 37 °C (Fig. 1C, lane 3) was related to the weak expression of Hsp70 at this temperature (Fig. 1C, lane 1). The increase in the GlcNAc binding and in the *O*-GlcNAc glycosylation on Hsp70 at 42 °C is attributed to its induction by the thermic stress; since in both cases the lectinic activity and glycosylation increased following the induction of Hsp70. In conclusion, this set of experiments clearly shows that Hsp70 exerts a lectinic activity towards the *N*-acetylglucosamine residues and that this protein is *O*-GlcNAc modified.

*O-GlcNAc level of Hsp70 is sensitive to glucose starvation and influences its binding activity toward N-acetylglucosamine residues*

UDP-GlcNAc is the sugar donor used by OGT to transfer GlcNAc residues to protein substrates. GlcNAc originates from the conversion of about 3–5% of the glucose entering the cell following the hexosamine biosynthetic pathway (Fig. 2A) [14]. As expected, the level of *O*-GlcNAc modifying proteins in cells cultured in glucose-depleted medium strongly decreased compared to cells growing in normal-culture medium (4.5 g glucose/L, Fig. 2B). Moreover, the modification of the pattern of glycosylation caused by the glucose starvation appears overall not affected by the thermic stress (Fig. 2B, compare lanes 1–3 and 2–4) except for proteins migrating between 50 and 70 kDa (arrowheads). The *O*-GlcNAc glycosylation of Hsp70 exhibits the same pattern with a similar high decrease of anti-*O*-GlcNAc staining both at 37 and at 42 °C (Fig. 2C, lanes 5–8).

Interestingly, the lectinic activity of Hsp70 in glucose-depleted conditions highly increased when cells were cultured in absence of glucose (Fig. 2D, compare lanes 5 and 6 to lanes 1 and 2). As previously shown in Fig. 1, we also observed an increase in the lectinic activity of Hsp70 after thermic stress (42 °C) that is in part related to an induction of Hsp70 (Fig. 2, lanes 1, 3, 5, and 7). This is in contrast to glucose-depleted conditions where the dramatic increase in this lectinic activity of Hsp70 is not due to a protein synthesis (Fig. 2, lanes 1, 2, 5, and 6).

These experiments demonstrate that when HepG2 cells are submitted to glucose starvation, the *O*-GlcNAc glycosylation level of Hsp70 decreases similarly to other extracted proteins but, unexpectedly, this decrease is associated with a noticeable higher lectinic affinity.

The results exposed above led us to evaluate the influence of the thermic stress (37 or 42 °C), combined with glucose starvation on the propensity of Hsp70 to associate with protein partners through interactions with the *O*-GlcNAc motif. For this experiment, anti-Hsp70 antibodies were used to co-immunoprecipitate proteins from a HepG2 cellular lysate. The co-immunoprecipitated proteins were analysed by Western blot using either the anti-*O*-GlcNAc RL2 monoclonal antibodies (Fig. 3A, top panel) or with an anti-β-catenin polyclonal antibody (Fig. 3A, bottom panel). Mainly, we can see that in the different conditions of culture (Fig. 3A, lanes 5–8), the Hsp70 co-immunoprecipitated *O*-GlcNAc protein patterns are rather similar in terms of representation except for a low molecular mass protein of 30 kDa whose co-immunoprecipitation increased in glucose-depleted conditions (Fig. 3, lanes 6 and 8). It may somewhat be intriguous to note that despite the dramatic drop in the *O*-GlcNAc rate affecting the proteins (Fig. 3A, lanes 1–4), many bands corresponding to co-immunoprecipitated *O*-GlcNAc modified proteins with Hsp70 are still detected (Fig. 3A, lanes 5–8). This phenomenon might be related to the increased affinity of the Hsp70 chaperone towards the *O*-GlcNAc, leading to a compensating behaviour with the decrease in glycosylation (Fig. 3B). When free GlcNAc was added, both to the lysate and to the washing buffer, the relative intensity of the bands of the co-immunoprecipitated proteins was lowered suggesting an obvious *O*-GlcNAc interaction between Hsp70 and these proteins

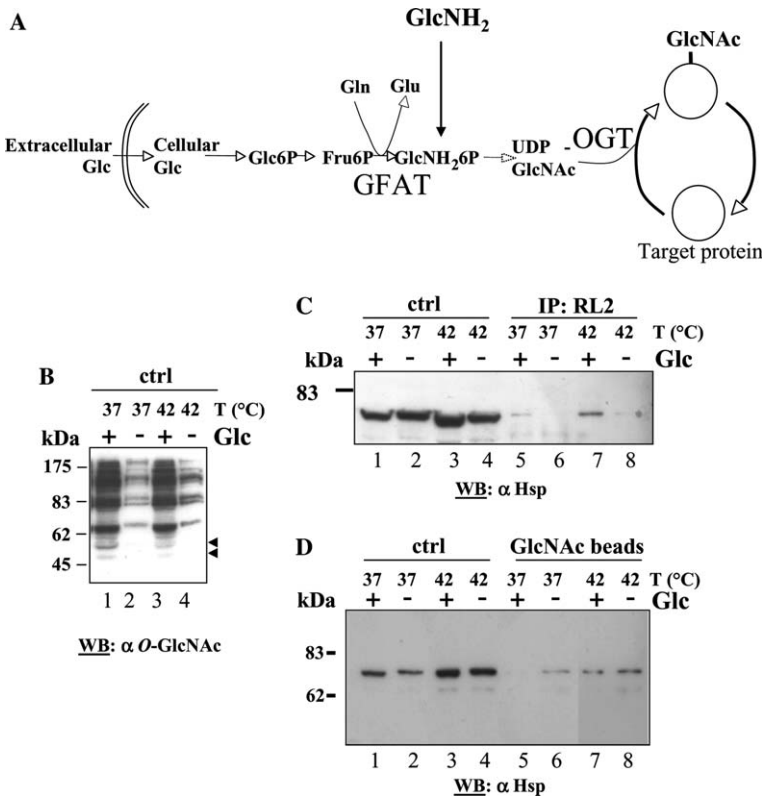


Fig. 2. Hsp70 lectinic activity towards GlcNAc and *O*-GlcNAc level in Hsp70 in HepG2 cells cultured in absence of glucose. (A) The relationship between the *O*-GlcNAc glycosylation and the extracellular glucose is drawn. Glucose enters the cell by a specific transporter, is phosphorylated to glucose 6-phosphate, and is isomerized in fructose 6-phosphate by the phosphoglucose isomerase. At this stage a key-enzyme intervenes: the glutamine-fructose amidotransferase (GFAT). It converts the fructose 6-phosphate to glucosamine 6-phosphate that is in definitive activates as a nucleotide-sugar: the UDP-GlcNAc that is the substrate of the *O*-GlcNAc transferase (OGT). The GFAT could be by-passed by treating cells with glucosamine. (B–D) Two types of stresses were applied to HepG2 cells: a thermic stress and a nutrient stress (glucose deprivation). In both cases, the lectinic activity of Hsp70 (D) and its *O*-GlcNAc glycosylation were studied (C). In the same experiment, the *O*-GlcNAc level of total cellular proteins was looked at (B) as described for Fig. 1. Blots were performed using the anti-Hsp70 antibody after running of the GlcNAc-bound proteins or the *O*-GlcNAc-enriched proteins by a 10% SDS–PAGE. Ctrl, control; T, temperature in degree celsius; IP, immunoprecipitation; and WB, Western blot.

(data not shown). However, this interaction seems to involve also the peptide moiety since free GlcNAc sugar alone is not sufficient to fully abolish this interaction. β-Catenin, that is involved in cell adhesion via interaction with E-cadherin and that plays an essential role in the Wingless/Wnt signalling pathway, has been characterized to be modified by *O*-GlcNAc [15]. Moreover, it has been demonstrated that the turnover of β-catenin is regulated by the ubiquitin–proteasome system [16]. According to the lectinic activity of Hsp70 against *O*-GlcNAc, these observations led us to strongly suppose that this key-protein could interact with Hsp70 via the *O*-GlcNAc moiety. Actually, β-catenin co-immunoprecipitated with Hsp70 (Fig. 3A, bottom panel) and this immunoprecipitation was higher when HepG2 cells were cultured in absence of glucose, i.e., when the lectinic activity of Hsp70 was maximal. In the control samples β-catenin appeared unchanged whatever the stress we applied (thermic or nutrient) and migrated as two bands, but in the co-immunoprecipitated samples, only the upper band was detected suggesting only a protection of this high molecular form (arrowhead). Other proteins that interact with Hsp70 in an *O*-GlcNAc dependent way are in process of identification in the laboratory.

The question we asked at this stage is the relevance of such an activity for Hsp70. The hypothesis of a protection played by *O*-GlcNAc against the proteasomal degradation seems to be well accepted today. First, numerous proteins were described to be protected by the single *O*-GlcNAc glycosylation, i.e., Sp1 [4], β-estrogen receptor [5] or plakoglobin [6]; second, numerous *O*-GlcNAc sites are localized within PEST sequences usually activated by phosphorylation and we can presume that *O*-GlcNAc could protect proteins against destruction by occupying phosphorylated sites (yin–yang relationship); and third, the proteasome appears to be itself modified and regulated by *O*-GlcNAc [7,8]. Nevertheless, the role of chaperone protein in this protection process was never investigated and could appear as the "missing link." We attempt to bring a first response by searching a possible lectinic activity in

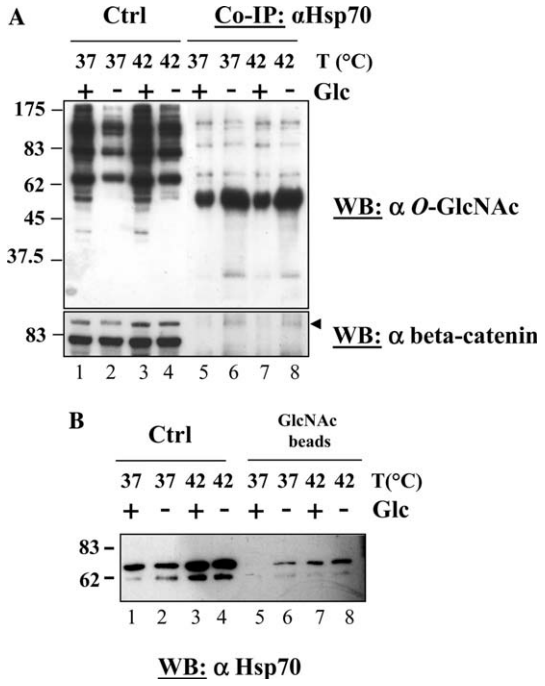


Fig. 3. Different *O*-GlcNAc proteins are associated to Hsp70, but the effect of the thermic and of the nutrient stresses did not profoundly affect their binding. HepG2 cells were cultured in the different conditions of stress (37 or 42 °C with or without glucose). Cells were lysed in a smooth lysate buffer (A, lanes 5–8) and the anti-Hsp70 antibody was added to the extract. After recovering the bound proteins with Sepharose beads-coupled protein A, proteins were run on a 10% SDS–PAGE and stained either with the anti-*O*-GlcNAc RL2 antibody as to detect the *O*-GlcNAc proteins bound to Hsp70 or with anti-β-catenin antibody. (B) The lectinic activity status of Hsp70 (as shown in Fig. 2) during the co-immunoprecipitation experiment. Ctrl, control; T, temperature in degree celsius; Co-IP, co-immunoprecipitation; and WB, Western blot.

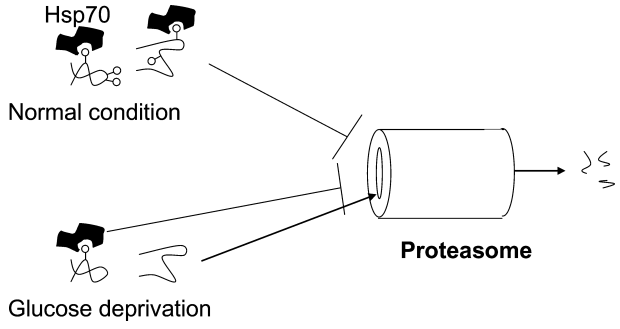


Fig. 4. Proposed mechanism of the protection of *O*-GlcNAc proteins by Hsp70 Using previous data and with the present work we have drawn this hypothesis about the protection of proteins by *O*-GlcNAc. In normal conditions (with glucose) proteins have a certain level of *O*-GlcNAc, and some of these residues are localized within PEST sequences that could be bound by HSP70 via their lectinic domain. When cells are depleted of glucose mainly the *O*-GlcNAc glycosylation decreases and only remnant *O*-GlcNAc residues are preserved, and thus permit the protein to avoid the proteasomal degradation. *O*-GlcNAc residues are represented as a white ball.

Hsp70 and to look at the modulation of this activity during stress. If *O*-GlcNAc is actually a protective signal against proteasomal degradation, chaperone proteins could play their protective role by masking PEST sequences via the glycosylation and thus permit the modified substrate to escape to the proteasomal degradation (Fig. 4).

## Concluding remarks

The present work demonstrates the GlcNAc-binding activity of Hsp70 and its *O*-GlcNAc modification. Modulations of these two features in an opposite way were shown when HepG2 cells were cultured in glucose-depleted medium: the lectinic activity toward *O*-GlcNAc greatly increased in a glucose-deprived stress whereas the *O*-GlcNAc glycosylation dramatically decreased. We propose that *O*-GlcNAc proteins could be protected from proteasomal degradation by counteracting the pro-degradative effect of phosphorylation at PEST sequences by interacting with HSP70, thus reinforcing a role of *O*-GlcNAc in protein stability. This paper presents an emerging new concept in protein protection, by describing the lectinic activity of HSP70, and the new visualization of chaperone lectins by corroborating the hypothesis that *O*-GlcNAc is a glycosylation that may permit proteins to stay in life.

## Acknowledgments

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## References

[1] M. Rechsteiner, S.C. Rogers, PEST sequences and regulation by proteolysis, Trends Biochem. Sci. 21 (1996) 267–276.
[2] M.H. Glickman, A. Ciechanover, The ubiquitin–proteasome proteolytic pathway: destruction for the sake of construction, Physiol. Rev. 82 (2002) 373–482.
[3] S.A. Whelan, G.W. Hart, Proteomic approaches to analyze the dynamic relationships between nucleocytoplasmic protein glycosylation and phosphorylation, Circ. Res. 93 (2003) 1047–1058.
[4] I. Han, J.E. Kudlow, Reduced *O*-glycosylation of Sp1 is associated with increased proteasome susceptibility, Mol. Cell. Biol. 17 (1997) 2550–2558.
[5] X. Cheng, R.N. Cole, J. Zaia, G.W. Hart, Alternative *O*-glycosylation/*O*-phosphorylation of the murine estrogen receptor β, Biochemistry 39 (2000) 11609–11620.

[6] S. Hatsell, L. Medina, J. Merola, R. Haltiwanger, P. Cowin, Plakoglobin is O-Glycosylated close to the N-terminal destruction box, J. Biol. Chem. 278 (2003) 37745–37752.
[7] M. Sumegi, E. Hunyadi-Gulyas, K.F. Medzihradszky, A. Udvardy, 26S proteasome subunits are O-linked *N*-acetylglucosamine-modified in *Drosophila melanogaster*, Biochem. Biophys. Res. Commun. 312 (2003) 1284–1289.
[8] F. Zhang, K. Su, X. Yang, D.B. Bowe, A.J. Paterson, J.E. Kudlow, *O*-GlcNAc modification is an endogenous inhibitor of the proteasome, Cell 115 (2003) 715–725.
[9] T. Lefebvre, C. Cieniewski, J. Lemoine, Y. Guerardel, Y. Leroy, J.P. Zanetta, J.C. Michalski, Identification of *N*-acetyl-D-glucosamine-specific lectins from rat liver cytosolic and nuclear compartments as heat-shock proteins, Biochem. J. 360 (2001) 179–188.
[10] J.C. Kiang, G.C. Tsokos, Heat shock protein 70 kDa: molecular biology, biochemistry and physiology, Pharmacol. Ther. 2 (1998) 183–201.
[11] A.G. Pockley, Heat shock proteins as regulators of the immune response, Lancet 362 (2003) 469–476.
[12] F. Beaulieu, R.M. Tanguay, Members of the *Drosophila* HSP70 family share ATP-binding properties, Eur. J. Biochem. 172 (1988) 341–347.
[13] J. Imai, H. Yashiroda, M. Maruya, I. Yahara, K. Tanaka, Proteasomes and molecular chaperones: cellular machinery responsible for folding and destruction of unfolded proteins, Cell Cycle 2 (2003) 585–590.
[14] L. Wells, K. Vosseler, G.W. Hart, A role for *N*-acetylglucosamine as a nutrient sensor and mediator of insulin resistance, Cell. Mol. Life Sci. 60 (2003) 222–228.
[15] W. Zhu, B. Leber, D.W. Andrews, Cytoplasmic *O*-glycosylation prevents cell surface transport of E-cadherin during apoptosis, EMBO J. 20 (2001) 5999–6007.
[16] H. Aberle, A. Bauer, J. Stappert, A. Kispert, R. Kemler, β-catenin is a target for the ubiquitin–proteasome pathway, EMBO J. 16 (1997) 3797–3804.

# CROSSTALK BETWEEN *O*-GlcNAc GLYCOSYLATION, UBIQUITINATION AND HSP70 GlcNAc-BINDING ACTIVITY DURING CELL INJURY.

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Running title: *O*-GlcNAc and ubiquitin behaviours after cell stress

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**Glycosylation of cytosolic and nuclear proteins by *O*-GlcNAc has been intensively studied over the two last past decades. Nevertheless the exact functions of this post-translational modification (PTM) remain to be determined. It has been recently speculated that *O*-GlcNAc (*O*-linked N-acetylglucosamine) could act as a protective signal against proteasomal degradation of proteins, both by modifying target substrates and by inhibiting proteasome itself. We have investigated the putative relationship between *O*-GlcNAc and ubiquitin pathways. After stress both PTM rapidly increased, although their evolution profiles differed. *O*-GlcNAc proteins were not stabilized by proteasome inhibition. The ubiquitin-activating enzyme E1 is *O*-GlcNAc modified and this glycosylation varies according cell culture conditions and stress. E1 interacts with Hsp70 in an *O*-GlcNAc dependent manner suggesting a cross regulation of response to stress between heat shock proteins and enzymes involved in ubiquitination. *O*-GlcNAc transferase (OGT) silencing speculated a regulation of ubiquitination by *O*-GlcNAc. Moreover *O*-GlcNAc is crucial for cell viability since OGT silencing decreased two-fold cell thermotolerance. Hsp70 GlcNAc-binding activity (HGBA) responded in the first minutes to stress and rapidly decreased thereafter. In addition proteasome inhibition with MG132, epoxomicin or lactacystin led to an activation of HGBA. Finally, this HGBA was also triggered by L-azetidine-2-carboxylic acid-induced protein misfolding demonstrating that change in protein conformation is one of the key-activator of this Hsp70 property. These results show that *O*-GlcNAc and ubiquitin are not strictly opposite PTM and that E1 could be the common denominator of *O*-GlcNAc glycosylation and ubiquitination pathways.**

Proteolysis is essential for cellular function and viability. Nucleocytoplasmic-resident proteins and reticulum endoplasmic newly synthesized proteins that do not find their final conformation and that are retrotranslocated to the cytosol are mainly destroyed by the proteasome pathway (1). This process is observed both in healthy cell and when cells are injured. Proteasome is particularly important in the regulation level of short half-lives proteins like cyclins that intervene at a precise moment of the cell cycle (2) and like transcription factors (3). Proteasome also takes charge the elimination of chemically or metabolically damaged proteins that exhibit hydrophobic peptidic segments and that have the propensity to aggregate. Those proteins are toxic for cell homeostasis requiring their rapid degradation by a cellular mechanism known as the ubiquitin-proteasome system (UPS) (4).

It has been recently speculated that *O*-GlcNAc (*O*-linked N-acetylglucosaminylation) could protect proteins against proteasomal degradation

(5-8). *O*-GlcNAc is the major glycosylation type found within the cytosolic and nuclear compartments of eukaryotic cell (9, 10). This glycosylation is based on the attachment of a single N-acetylglucosamine on serine or threonine residues. While *O*-GlcNAc addition does not require strict consensus sequence, *O*-GlcNAc transferase (OGT) needs a specific peptidic environment. Usually *O*-GlcNAc attachment occurs near a proline residue and in regions enriched in serines and threonines. Very interestingly these sequences are similar to PEST (rich in proline, glutamine, serine, and threonine) sequences (11). According to the existing relationship between *O*-GlcNAc and phosphorylation and since activation of PEST can occur after phosphorylation (12), it can be assumed that *O*-GlcNAc counteracts the effect of phosphorylation to protect proteins. *O*-GlcNAc glycosylation of the transcription factor Sp1 renders it less susceptible to proteasomal destruction as demonstrated by culture in high glucose condition or by culture in a medium supplemented with glucosamine, whereas glucose starvation diminishes its half-life (5). Other example that strongly suggests a protective function of *O*-GlcNAc is that of β-estrogen-receptor (β-ER) that bears *O*-GlcNAc motif on a high PEST sequence score. The glycosylated form of β-ER is more resistant to degradation than the unglycosylated one that can be phosphorylated and consequently sensitive for proteasomal degradation (6). It has been also recently demonstrated that the proteasome could be regulated through the *O*-GlcNAc glycosylation of the 19S-regulatory subunit (7). This inhibition by *O*-GlcNAc adds another regulatory level of protein protection by this glycosylation.

Recently our lab has demonstrated that the constitutive form of the 70kDa-heat shock protein family (Hsc70) and the inducible one (Hsp70) were endowed of a lectin activity toward *O*-GlcNAc residues (13, 14). This activity was enhanced when cells were stressed (14, 15). Once can suppose that OGT and Hsp70 can act in synergy for protecting proteins against aggregation and destruction first by modifying substrates with *O*-GlcNAc and secondly by Hsp70 binding of the glycosylated proteins.

Since it is assumed that one of the principal functions of ubiquitin is to promote protein destruction by proteasome and that *O*-GlcNAc appears to be protective for proteins, we investigated a putative relation between these two PTM. We showed that after a thermal stress both ubiquitin and *O*-GlcNAc pathways were used. The enhancement of these two PTM presented differences in term of evolution profiles. For a short period after the stress *O*-GlcNAc was insensitive to proteasome inhibition, showing that *O*-GlcNAc proteins resist to proteasomal degradation. E1, an ubiquitin-activating enzyme that begin the process of protein ubiquitination is modified with *O*-GlcNAc. This glycosylation of E1 varied with the conditions of culture (and stress) and moreover is correlated with Hsp70 binding. Finally, we showed that a proline analogue-induced misfolding triggered HGBA, demonstrating the link between the induction of lectin property and the protein misfolding.

## EXPERIMENTAL PROCEDURES

***Cell culture and treatments of cells.*** $HepG_2$ and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10 % fetal calf serum (v/v), 2 mM L-glutamine, 5 IU/ml penicillin, 50 µg/ml streptomycin at 37°C on a humidified atmosphere enriched with 5 % $CO_2$. Cultures were carried out on dishes (diameter 100 mm). Dishes were preliminarily treated with 0.1 % porcine gelatine (Sigma) for $HepG_2$ cells.

For glucose deprivation experiments, cells were rinsed with 10 mL of glucose-depleted medium (supplemented with 10 % FCS) and incubated in this medium only, or in this medium supplemented by 5 mM

glucosamine (Sigma) for 24h (12). Thermal stress was induced by placing cells for 24h at 42°C in a 5%-$CO_2$ enriched atmosphere. For the kinetic experiments, $HepG_2$ were grown at 37°C and placed at 42°C for the indicated time periods.

MG132 (N-carbobenzoxyl-Leu-Leu-leucinal ) was used at a concentration of 8µM (8 mM stock solution in DMSO at -80°C). Epoxomicin and lactacystin were used at a concentration of 0.1µM (10 mM stock solution in DMSO) and 1µM (10 mM stock solution in DMSO). L-azetidine-2-carboxylic acid was used at a concentration of 5 mM (50 mM stock solution in PBS, phosphate-buffered saline). Anisomycin and cycloheximide were used at a concentration of 15µg.mL$^{-1}$. Drugs were incubated for 24 h or during indicated time periods.

***Protein enrichment on GlcNAc-beads and immunoprecipitation with the anti-O-GlcNAc antibody (RL-2).*** $HepG_2$ were first washed with 10 mL of cold PBS. Cells were lysed on ice either with a hypotonic buffer (10 mM Tris/HCl, 10 mM NaCl, 15 mM 2-mercaptoethanol, 1 mM $MgCl_2$ and proteases inhibitors, pH 7.2) for GlcNAc-binding studies, either with the lysis buffer (10 mM Tris/HCl, 150 mM NaCl, 1 % Triton X-100 (v/v), 0.5 % sodium deoxycholate (w/v), 0.1 % sodium dodecyl sulphate (w/v) and proteases inhibitors, pH 7.4) for *O*-GlcNAc-bearing proteins enrichment. Cellular extracts were then centrifuged at 20,000 g for 30 min at 4°C. For lectin activity, supernatants were incubated with 30 µL of GlcNAc-labelled beads (N-acetyl-D-glucosamine immobilized on 6% beaded agarose with a spacer of five carbons, Sigma) at 4°C for 1h. The beads were washed four times with the binding buffer (20 mM Tris/HCl, 200 mM KCl, 1 mM $CaCl_2$, 1mM $MgCl_2$, and proteases inhibitors, pH 7.4). For *O*-GlcNAc-bearing proteins enrichment, supernatants were incubated with 2.5 µL of the mouse monoclonal anti-*O*-GlcNAc antibody (RL-2, Affinity Bioreagents) and placed at 4°C overnight. Bound proteins were then recovered after addition of 30 µL of Sepharose-labelled protein G (Amersham Biosciences) for 1h at 4°C. Beads were gently centrifuged for 1 min and washed with the following buffers : lysis buffer, lysis buffer supplemented with 500 mM NaCl, lysis buffer/TNE (10 mM Tris/HCl, 150 mM NaCl, and 1mM EDTA, pH 7.4) in equal volume and finally with TNE alone.

***Co-immunoprecipitation with anti-Hsp70.*** For co-immunoprecipitation, cells were lysed on ice in a smooth lysis buffer (20 mM Tris/HCl, 150 mM NaCl, 0.5% NP-40 (v/v) and proteases inhibitors, pH 8.0). Whole cell extracts were centrifuged at 20,000 g for 30min at 4°C and supernatants were collected. Immunoprecipitation with the anti-Hsp70 antibody was carry out at a final concentration of 1:200 overnight at 4°C, followed by an incubation with Sepharose-labelled protein A (Amersham Biosciences) for 1h at 4°C. Beads were gently centrifuged for 1 min and washed four times for five min each under rotation with the smooth lysis buffer. Bound proteins were analysed by western blot with a monoclonal anti-E1 antibody (Sigma) at a final dilution of 1:1,000.

***SDS-PAGE, western blotting and antibody staining.*** Equal amounts of extracted protein were subjected to western blotting. Samples were analysed by 10 % SDS-PAGE under reducing conditions and proteins were electroblotted onto nitrocellulose sheet (Amersham Biosciences). Equal loading was checked using Ponceau red staining. Membranes were first saturated for 45 min with 5 % non-fatty acid milk in TBS-Tween buffer (20 mM Tris/HCl, 150 mM NaCl and 0.05 % Tween (v/v), pH 8.0). Mouse monoclonal anti-*O*-GlcNAc (RL-2), mouse monoclonal anti-ubiquitin (Sigma), mouse monoclonal anti-E1 (Sigma) antibodies were used at a final dilution of 1:1,000 and rabbit polyclonal anti-Hsp70 antibody at a final dilution of 1:30,000. Rabbit anti-β-catenin was used at a dilution of 1:1,000 ;

rabbit anti-OGT (AL-28) was used at a dilution of 1:2,000 and rabbit anti-β-tubulin antibody was used at a dilution of 1:500. Membranes were incubated overnight at 4°C, then washed three times with TBS-Tween for 10 min and incubated with either an anti-rabbit or an anti-mouse horseradish peroxidase-labelled secondary antibody at a dilution of 1:10,000 for 1h. Finally, three washes of 10min each were performed with TBS-Tween and the detection was carried out with enhanced chemiluminescence (Amersham Biosciences). Primary and secondary antibodies complexes were removed by incubation of the membranes with a stripping buffer (62.5 mM Tris/HCl, 2 % SDS, 100 mM 2-mercaptoethanol, pH 6.5) for 30 min at 50°C, abundantly washed with TBS-Tween and re-incubated with a rat anti-Hsc70 at a dilution of 1:1,000 (Santa Cruz Biotechnologies). Anti-rat horseradish peroxidase labelled secondary antibody was used at a dilution of 1:10,000 for 1 h.

***RNA interference (RNAi).*** RNAi were designed for the human OGT sequence. Oligonucleotides were purchased from Eurogentec. The oligonucleotide (1153) used in this study is the following: GGA-GGC-UAU-UCG-AAU-CAG. 8µL of Dreamfect reagent (OZ biosciences) was diluted with serum-free/antibiotic free Opti-MEM medium (Invitrogen) to a final volume of 100µL. 2 µg of RNAi was diluted in 100µL final volume with Opti-MEM. The 100µL diluted transfection reagent and the 100µL diluted RNAi solution were then mixed and incubated for 20 min. The 200µL RNAi solution was added to HeLa cells maintained in 1.8 mL of Opti-MEM per well in a 6-wells plate. This procedure was repeated every 24 hours for 4 days. For viability tests, the transfection was performed in a 24-well plate (all volumes and quantities were two-fold divided). After 96 hours of incubation with the oligonucleotide, OGT expression and activity were tested by immunoblotting either with rabbit anti-OGT antibodies (AL-28) to test OGT level or with RL-2 antibodies to check *O*-GlcNAc levels. Viabilities of HeLa cells were performed using the trypan blue exclusion method (15).

**Sambucus nigra Agglutinin (SNA) staining**. After OGT silencing, crude cellular extracts were run on a 10% SDS-PAGE, electroblotted and membranes were incubated with the digoxigenin-coupled SNA at a final concentration of 1:1,000 in TBS-Tween for 1h at room temperature. This incubation was followed by three washes of 10 min each with TBS-Tween. The membrane was incubated with an alkaline phosphatase-labelled anti-digoxigenin secondary antibody at a final concentration of 1:1,000 in TBS-Tween for 1h. After three washes of 10 min each with TBS-Tween, revelation was achieved by the addition of 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium.

***Immunofluorescence experiment.*** $HepG_2$ and HeLa cells were grown on coverglasses for 48 h and washed twice with cold PBS. For RNAi experiments, amount of each reactive was adjusted to 6-wells plaque. Cells were fixed in 3% of paraformaldehyde in cold PBS for 15 min and washed with PBS. Excess of paraformaldehyde was destroyed with a solution of 50 mM ammonium chloride for 10 min. After washing with PBS, cells were permeabilised with 0.1% Triton-X100 for 5 min. Non-specific sites were blocked with goat serum. Coverglasses were then incubated with anti-*O*-GlcNAc (RL-2), anti-OGT (AL-28) and with anti-ubiquitin each at a dilution of 1:100 in a 10% goat serum solution (in PBS) for 30 min. After three washes in PBS, FITC and Texas Red-labelled secondary antibodies were incubated on coverglasses (dilution 1:50). Nuclei were specifically stained with DAPI. Cells were visualised using an Axioplan 2 imaging (Zeiss) and an Axio Cam HRc camera (AxioVision).

# RESULTS

To tentatively decipher by which mechanism *O*-GlcNAc glycosylation could protect substrates against proteasomal degradation, we investigated the possibility that *O*-GlcNAc and ubiquitin could antagonise one-to-another in a reciprocal interplay. Indeed, ubiquitination is the first step that prepares a damaged protein to be degraded by the proteasomal machinery. It can be assumed that *O*-GlcNAc could block ubiquitination of proteins, leading to a substrate rescue. As discussed in the introduction section, *O*-GlcNAc sites are frequently similar to PEST sequences (11). We can hypothesise that *O*-GlcNAc blocks phosphorylation at PEST sequences, avoiding their subsequent ubiquitination and finally their degradation.

***O-GlcNAc and ubiquitin are not mutually exclusive***

Proteasomal pathway is the main track by which ubiquitinated-cytosolic and nuclear proteins are destroyed. Evidence of a putative protection of such proteins by *O*-GlcNAc has emerged during the last years. Starting from the role of proteasome targeting played by ubiquitin, we investigated the relation between the two PTM, i.e. *O*-GlcNAc and ubiquitin. The first aim was to visualize the putative relationship/interplay between ubiquitin and *O*-GlcNAc at the cellular and at the molecular levels. *O*-GlcNAc glycosylation was modulated in $HepG_2$ cells after stabilization of ubiquitinated proteins with MG132, a reversible proteasome inhibitor. This was achieved either using glucose deprivation or forskolin (an activator of adenylate cyclase) to down-express *O*-GlcNAc or using glucosamine to enhance *O*-GlcNAc level through the hexosamine biosynthetic pathway. Intriguingly, after hyperthermia, ubiquitin and *O*-GlcNAc evolved in the same way (but not at the same level) suspecting a common regulation of the two PTM (Fig. 1A).

Nuclear and cytosolic distribution of *O*-GlcNAc and ubiquitinated proteins has been examined using a monoclonal anti-*O*-GlcNAc antibody (in conjunction with a FITC-coupled secondary antibody) and a polyclonal anti-ubiquitin antibody (in conjunction with a Texas Red-coupled secondary antibody) by immuno-fluorescence microscopy (Fig. 1B). Ubiquitin (Red) was exclusively present in the cytosolic compartment as demonstrated by the non-superposition of Ub-proteins distribution and DAPI staining (Blue, specific of nucleus). In counterpart, *O*-GlcNAc glycosylation (Green) is more particularly localized in the nucleus (superposition with DAPI staining) and weaker in the cytoplasm (diffuse green). Superposition of ubiquitin staining and *O*-GlcNAc staining showed a co-localisation of the two modifications only in the cytoplasm (orange).

To check the hypothesis that some *O*-GlcNAc proteins could also be ubiquitinated or on the contrary if these two PTM exclude mutually, immunoprecipitation experiments were performed on $HepG_2$ cells extracts with the anti-*O*-GlcNAc antibody and revealed with the anti-*O*-GlcNAc antibody (Fig. 1C, left panel) or the anti-ubiquitin antibody (right panel). We clearly see that proteins, but not all, can be *O*-GlcNAc glycosylated and also modified with ubiquitin: the two PTM may co-exist on the same proteins. Note that for both PTM, the molecular weight range of the most representative modified proteins is the same, i.e. between 50 kDa to the highest molecular weight. Experiments in which ubiquitinated-proteins were enriched using anti-ubiquitin were also done. Western blot of the bound proteins with anti-*O*-GlcNAc antibodies confirmed the co-existence of both PTM on some proteins (data not shown).

***Time dependence of O-GlcNAc and ubiquitin levels after heat shock.*** Since we demonstrated that *O*-GlcNAc and ubiquitin

could be both presents on a same protein, we asked about the evolution scheme of each PTM after heat shock. For that purpose we investigated the modification of proteins with ubiquitin and *O*-GlcNAc after $HepG_2$ cells were exposed to a thermal stress for increasing time periods at 42°C with or without MG132 (Fig. 2). The level of expression of Hsp70 and its lectin activity toward GlcNAc residues were also investigated. For this experiment, whole extract were incubated with GlcNAc-labelled agarose and bound proteins were examined with an anti-Hsp70 antibody by western blot. The content in *O*-GlcNAc and ubiquitin was also followed by western blot. Both ubiquitin and *O*-GlcNAc increased immediately after the stress (near 2.5 to 5 min post-stress) but ubiquitin showed a maximal level at 10 min post-stress (for short times) whereas *O*-GlcNAc increased all along the kinetic (compare lanes 1 to 14). This result is consistent with data published by Zachara *et al.* (16) that showed that *O*-GlcNAc levels rapidly increased in response to a large variety of stress. Intriguingly, *O*-GlcNAc response was insensitive to proteasome inhibition contrary to ubiquitin content. This indicates that *O*-GlcNAc-bearing proteins are not degraded by the proteasome pathway. When the time post-stress was longer (between 60 to 240 min) ubiquitin began to re-enhance, but *O*-GlcNAc-modified proteins were stabilized by MG132 contrary to ubiquitin (lanes 15 to 20). Markedly, when the time post-stress was of 24 or 48 hours, *O*-GlcNAc modified proteins were stabilized by MG132 showing that contrary to shorter times *O*-GlcNAc proteins could be degraded by the proteasome (lanes 21 to 24). The second point of these experiments showed that whereas the 2.5 min time period of stress was too short to induce an increase in Hsp70 level (Hsp70 is induced after a time period of stress of about 2-4 hours, lanes 17 to 20), GlcNAc-directed Hsp70 lectin activity was induced immediately after stress (compare lanes 1 and 3). This GlcNAc-binding activity was rapidly cancelled after about 10 min post-stress. This activity was significantly recovered after 4 to 24 hours post-stress and especially when proteasome was inhibited (compare lane 19 to 20 and lane 21 to 22). These results demonstrate that ubiquitination and *O*-GlcNAc both respond to stress by accumulation but ubiquitination and *O*-GlcNAc are chronologically shifted. We also showed that for a short time after the stress, HGBA intervene before the appearance of newly synthesized Hsp70. Furthermore it is interesting to note that in non-heat shocked control cells, HGBA is triggered by proteasome inhibition (lanes 1 and 2; lanes 21 and 22).

***Hsp70 GlcNAc-binding activity is activated by proteasome inhibition.*** Proteasome inhibition on HGBA has been studied thoroughly. Cells were exposed to different conditions for 24 hours: unstressed conditions (Ctrl), glucose-deprivation with or without glucosamine and thermal stress (Fig. 3A). As expected, MG132 triggered Hsp70 increase whatever the conditions and more interestingly HGBA was greatly induced. Same experiment were realized with Hsc70 (70kDa-Heat shock cognate), but Hsc70 expression (lower panel) and Hsc70 GlcNAc-binding properties were different from that of Hsp70 (data not shown). Control of MG132 proteasomal inhibition was performed using an anti-ubiquitin antibody. As expected, treatment with MG132 stabilized ubiquitinated proteins.

Confirmation of the proteasomal inhibition on Hsp70 lectin activity was achieved using two other proteasome inhibitors, i.e. epoxomicin and lactacystin (Fig. 3B) that are irreversible inhibitors unlike MG132 that is reversible. Like MG132, but at a lower degree, epoxomicin and lactacystin activated HGBA. Thermal stress was used as a positive control of the lectin activity and normal condition (at 37°C without any proteasome inhibitors) was used as a

negative control (compare the expression of Hsp70, Fig.3B upper panel, to the lectin property of Hsp70, lower panel). Control of proteasome inhibition has been performed as in Fig. 3A using anti-ubiquitin staining (not shown).

Taken together, these experiments confirm that HGBA could be triggered by proteasomal inhibition.

***E1/Hsp70 interaction correlates with E1 O-GlcNAc status***

According to the common features existing between ubiquitination and *O*-GlcNAc we hypothesized that ubiquitination process can be regulated by *O*-GlcNAc. We then looked at the putative *O*-GlcNAc glycosylation of E1, an ubiquitin activating enzyme that initiates ubiquitination process. $HepG_2$ cells were cultured either in normal conditions (with 25 mM of glucose, Ctrl), heat-shocked at 42°C, cultured without glucose or without glucose but supplemented with 5 mM glucosamine. After lysis, cellular extracts were immunopurified with the anti-*O*-GlcNAc antibody. E1 is slightly *O*-GlcNAc modified in control cells (Fig. 4A), whereas in stress conditions, i.e. without glucose and at 42°C, the glycosylation was totally abrogated except when cells were cultured with glucosamine. Interestingly, the interaction of E1 with Hsp70 (co-immunoprecipitation experiments exposed in Fig. 4B) correlated with *O*-GlcNAc glycosylation of E1: the interaction was maximal when cells were cultured in presence of glucosamine, i.e. when E1 was highly glycosylated and weaker in normal conditions and in the stress conditions. We can assume that the interaction between E1 and Hsp70 was in part *O*-GlcNAc-dependent. Since E1 *O*-GlcNAc glycosylation and its interaction with Hsp70 are modulated by cell culture conditions and stress, the idea of a control of ubiquitination process by *O*-GlcNAc is reinforced.

***OGT silencing impairs ubiquitination and decreases cell viability.*** OGT level was reduced using RNA interference technology. HeLa cells were tranfected with oligonucleotides (noted 1153) and incubated at 37°C for 96 h. Heat-shock was induced by incubation of HeLa at 42°C for the last 24 h. OGT silencing was controlled by measurement of OGT level and *O*-GlcNAc-bearing proteins level (Fig. 5 A, left panel). Anti-beta-tubulin was used for the loading control. A staining with *Sambucus nigra* Agglutinin (SNA), a lectin that specifically binds sialic acid linked in α(2-6) to a galactose residue demonstrated that OGT silencing did not perturb other glycosylations types (Fig. 5A, right panel). Effectiveness of OGT silencing on OGT expression and *O*-GlcNAc level was also checked using immunofluorescent microscopy (Fig. 5B). The top and middle panels of figure 5B show that OGT silencing (1153) impaired OGT expression, *O*-GlcNAc and ubiquitin levels. The bottom panel shows that ubiquitinated proteins more particularly localised to the cytosolic compartment (as shown for $HepG_2$, Fig. 1B) and that OGT is both distributed to the nuclear and cytosolic compartments as attested by the co-staining with DAPI. According to results exposed above and since *O*-GlcNAc glycosylation of E1 depends on cell culture conditions, E1 glycosylation and ubiquitination rates were examined after OGT silencing (Fig. 5C). OGT silencing was correlated to a decrease in ubiquitinated proteins (as shown in Fig 5B) confirming a regulation of ubiquitination process by *O*-GlcNAc, presumably in part through E1 glycosylation. We also observed a decrease in the β-catenin level, an *O*-GlcNAc protein (17) containing PEST sequences: after heat shock beta-catenin was reduced and OGT silencing completely abolished its expression (Fig. 5C, lower panel). Tolerance of HeLa cells to heat shock was determined using trypan blue exclusion (Fig.5D). Cells transfected with oligonucleotides 1153 were less viable both at 37°C and at 42°C demonstrating that *O*-GlcNAc is an essential PTM for cell viability and is

necessary for cells to resist to stress. Similar results were obtained by Zachara *et al.* (16) since they showed that a reduction or a blockade in *O*-GlcNAc had repercussions on cell viability.

***Protein misfolding induces Hsp70 lectin activity.*** HGBA was tested after $HepG_2$ cells were cultured for 24 hours in presence of L-azetidine-2-carboxylic acid, a proline analogue (Fig. 6A). L-azetidine-2-carboxylic acid induces protein misfolding by enhancing constraints when incorporated during translation. HGBA was greatly enhanced when protein misfolding was provoked by L-azetidine-2-carboxylic acid (Fig. 6B). In order to consolidate this result and to demonstrate that L-azetidine-2-carboxylic acid induced HGBA by incorporation into newly synthesized proteins, we counteracted L-azetidine-2-carboxylic acid effect by inhibiting protein translation. For this purpose, cells were treated with L-azetidine-2-carboxylic acid in conjunction with cycloheximide or anisomycin, two translation inhibitors, at a concentration of $15\mu g.mL^{-1}$ during 24h (Fig 6B). Effect of cycloheximide and anisomycin on cell viability has been checked (data not shown) and results demonstrated that in the conditions used the two drugs did not affect cell viability. Inhibition of protein translation and consequently prevention of L-azetidine-2-carboxylic acid incorporation abrogate the effect of L-azetidine-2-carboxylic acid on HGBA.

## DISCUSSION

*O*-GlcNAc modifies numerous nucleocytoplasmic proteins that belong to diverse families including metabolic enzymes, transcription factors, heat shock proteins, architectural proteins (18). One of the characteristic features of *O*-GlcNAc is that it is highly dynamic and that it can sometimes counteract phosphorylation at the same site or at an adjacent site (19, 20). While the list of proteins modified by this glycosylation does not cease to increase, functions played by *O*-GlcNAc remain to be elucidated.

Although, based on observations made by different groups it has been hypothesized that *O*-GlcNAc could stabilize proteins by preventing proteasomal degradation (5, 6). This protection can be triggered by inhibition of proteasome itself by modifying the 19S-regulatory subunit (7, 8) and by protection of protein-substrates directly. In this last idea, experiments have shown that a wide variety of stress (UV, hyperthermia, $H_2O_2$…) inflicted to cells provoked an increase of *O*-GlcNAc content on a large panel of proteins (18). Similar results were obtained by Sohn *et al*. (21) since they showed that hyperthermia was followed by an increase in *O*-GlcNAc modification. Moreover the same authors demonstrated that cells over-expressing OGT better support stress. They built an attractive hypothesis in which they considered that OGT behave as a chaperone by modifying, and so by protecting, unfolded hydrophobic peptide segment exposed after cell injury (Fig. 7). Finally, our group showed in a previous report the GlcNAc-binding properties of Hsp70 (14). This lectin activity is unmasked after thermal or nutrient stresses (14, 15). The hypothesis is that *O*-GlcNAc glycosylation of damaged proteins and Hsp70-GlcNAc binding properties act together and in synergy to protect target proteins against proteasome activity by binding *O*-GlcNAc-exposed motif to Hsp70 (Fig. 7). Despite these exciting observations, the function of *O*-GlcNAc in protein protection is far to be deciphered.

In the paper, we were interested in the putative relation between *O*-GlcNAc and ubiquitin: the first modification being presumably a signal against protein degradation and the second one being a well-described pro-degradative PTM. Intriguingly, it appeared quickly that in opposition to the reciprocal relationship between *O*-GlcNAc and phosphorylation, no reciprocity between *O*-GlcNAc and ubiquitination exist: when a thermal stress

occurred both PTM increased (Fig. 2). Nevertheless the kinetic of each PTM were quite different. For the fifteen first minutes post-stress the increase in *O*-GlcNAc was continue and regular. For ubiquitination, the progression was stopped near ten minutes and ubiquitination diminished. For longer time periods of stress, ubiquitination was newly triggered with a maximum near 24 hours post-stress. *O*-GlcNAc glycosylation was not stabilized by MG132, unlike ubiquitination, suggesting that *O*-GlcNAc proteins were not degraded by proteasome until four hours post-stress. For longer times post-stress, *O*-GlcNAc proteins were no longer protected against proteasomal degradation and the profile was similar to that of ubiquitin. We hypothesized according to these observations that after stress, proteins could be either modified with *O*-GlcNAc or ubiquitin. The first PTM permit the protection of the modified protein whereas the second one targets the protein to the proteasome. While the stress was continuously applied to cells, proteins that can not recover their native conformation and functionality are both *O*-GlcNAc modified and ubiquitinated, leading to their destruction. Interestingly, ubiquitination seems to be slighter for short time than for longer times suggesting an increased degradation rate for longer periods of stress. Moreover, and reinforcing the capacity of ubiquitin and *O*-GlcNAc to work in concert, immunoprecipitation experiments have shown that *O*-GlcNAc and ubiquitin can co-exist on the same protein (Fig. 1). So it appeared that the couple ubiquitin and *O*-GlcNAc could decide for protein destruction or repairing. We then investigated that ubiquination process could be itself regulated by *O*-GlcNAc. In this idea we showed that the ubiquitin activating enzyme E1 was itself *O*-GlcNAc glycosylated. This modification was modulated by cell culture conditions and stress. In the same field, Cole and Hart have demonstrated that the enzyme of de-ubiquitinylation (ubiquitin carboxyl hydrolase-L1; UCH-L1) was *O*-GlcNAc modified in the synaptosome (22). Effect of *O*-GlcNAc on such enzyme is not known but it can be assumed that it directly regulates its activity.

Hsp70 acts as a molecular chaperone that helps nascent proteins to acquire their correct folding and permits repairing and renaturation of damaged proteins (23). When folding or renaturation failed, Hsp70 targets undesirable proteins to the ubiquitin proteasomal pathway (UPS) (4, 24). Hsp70 (and Hsc70) is endowed of a lectin activity towards GlcNAc residues. According to precedent and current studies, we expect that immediately after cell damage, Hsp70 binds *O*-GlcNAc bearing proteins thus avoiding their aggregation and thereafter to tentatively refold them. In regard of our thermal kinetic experiment and of Zachara' experiments (16), we can see that after a stress occurs the response in *O*-GlcNAc was extremely rapid, and only a few minutes is sufficient for Hsp70 to exhibit its binding toward GlcNAc whereas its rate only increases after several hours. We speculated that after cell shocking, an unknown Hsp70 GlcNAc-binding partner dissociates from Hsp70 unmasking its GlcNAc binding capacities. We have tested the possibility that this factor could be Hsf-1 (Heat shock factor-1) that bind Hsp70 in unstressed conditions and that is released after stress. Unfortunately and unexpectedly Hsf-1 was not shown to be *O*-GlcNAc modified. The identity of this putative interacting factor had to be determined. As also shown in figure 2, HGBA was quickly diminished suggesting a rapid saturation of Hsp70 by *O*-GlcNAc damaged proteins. This activity reappeared after a long period when Hsp70 level was increased.

This paper also demonstrates the activation of HGBA by proteasome inhibition. We speculated that this increase could be considered as a cell response against protein aggregation: since the flux of misfolded proteins (considered as 30% of the newly synthesised proteins, (25)) could

not be destroyed by the 26S proteasome, Hsp70 exhibit lectin property to avoid their aggregation.

The last teaching of this work concerns the triggering of GlcNAc-binding activity of Hsp70 by proline analogue-induced misfolding. The incubation of $HepG_2$ with 2-carboxy L-azetidine indubitably demonstrates that HGBA could be triggered by protein misfolding. These results are in correlation with works performed by Sohn *et al.* (21) suggesting that after cell damage, OGT would modify exposed hydrophobic peptides. These segments could be subsequently recognized by Hsp70 via their lectin domain.

At this stage it appears evident that more work and study had to be done for the comprehension of the exact function of *O*-GlcNAc in proteasomal processing. But at present it seems clear that it has a protective effect in protein stability and the comparison can be drawn between the quality control function played by calreticulin and calnexin in the endoplasmic reticulum via the gluco/degluco cycle on the one hand (26, 27) and Hsp70 via the *O*-GlcNAc/de-*O*-GlcNAc cycle in the other hand (Fig. 7).

## References


1. Pines, J., and Lindon, C. (2005) *Nat. Cell Biol.* **7**, 731-735.
2. Zhang, Z., Wang, H., Li, M., Agrawal, S., Chen, X., and Zhang, R. (2004) *J. Biol. Chem.* **16,** 16000-16006.
3. Minegishi, N., Suzuki, N., Kawatani,Y., Shimizu, R., and Yamamoto, M. (2005) *Genes Cells* **10**, 693-704.
4. Mayer, R. J. & Layfield eds. (2005) *Essays in Biochemistry: The Ubiquitin-Proteasome System*. Portland Press Ltd; London, U.K. + 222 pp.
5. Han, I., and Kudlow, J.I., (1997) *Mol Cell Biol.* **17**, 2550-2558.
6. Cheng, X., and Hart, G.W. (2001) *J. Biol Chem*. **276**, 10570-10575.
7. Zhang, F., Su, K., Yang, X., Bowe, D.B., Paterson, A.J., and Kudlow, J.E. (2003) *Cell* **115,** 715-725.
8. Zachara, N.E., and Hart, G.W. (2004) *Trends Cell. Biol.* **14,** 218-221.
9. Love, D.C., and Hanover, J.A. (2005) *Sci. STKE* **312,** re13.
10. Vosseller, K., Sakabe, K., Wells, L., and Hart, G.W. (2002) *Curr. Opin. Chem. Biol.* **6,** 851-857.
11. Rechsteiner, M., and Rogers, S.C. (1996) *Trends Biochem. Sci.* **21**, 267-276.
12. Martinez, L.O., Agerholm-Larsen, B., Wang, N., Chen, W., and Tall, A.R. (2003) *J. Biol Chem*. **278**, 37368-74.
13. Lefebvre, T. Cieniewski, C. Lemoine, J. Guerardel, Y. Leroy, Y. Zanetta, J.P., and Michalski, J.C. (2001) *Biochem. J.* **360**, 179-188.
14. Guinez, C., Lemoine, J., Michalski, J.C., and Lefebvre T. (2004) *Biochem Biophys Res Commun.* **319**, 21-26.
15. Guinez, C., Losfeld, M.E., Cacan, R., Michalski, J. C., and Lefebvre, T. (2006) Glycobiology **16**, 22-28.
16. Zachara, N.E., O' Donnell, N., Cheung, W.D., Mercer, J.J., Marth, J.D., and Hart, G.W. (2004) *J. Biol. Chem.* **279**, 30133-30142.
17. Zhu W., Leber B., and Andrews D.W. (2001) *EMBO J.* **20**, 5999-6007.
18. Zachara, N.E., and Hart, G.W. (2004) *Biochim. Biophys. Acta*. **1673**, 13-28.
19. Comer, F.I., and Hart, G.W. (2001) *Biochemistry*. **40**, 7845-7852.
20. Kamemura, K., Hayes, B.K., Comer, F.I., and Hart, G.W. (2002) *J. Biol. Chem.* **277,** 19229-19235.
21. Sohn, K.C., Lee, K.Y., Park, J.E., and Do, S.I. (2004) *Biochem. Biophys. Res. Com.* **322,** 1045-1051.
22. Cole, R.N. & Hart, G.W. (2001) *J. Neurochem.* **79,** 1080-1089.
23. Sherman, M.Y., and Goldberg A.M. (2001) *Neuron*. **29**, 15-32.
24. Schwartz, A.L., and Ciechanover, A. (1999) *Annu. Rev. Med.* **50**,57-74.
25. Shubert, U., Anton, L.C., Gibbs, J., Norbury, C.C., Yewdell, J.W., and Bennink, J.R. (2000) *Nature* **404,** 770-774.
26. Parodi, A.J. (2000) *Annu Rev Biochem*. **69,** 69-93.
27. Cacan R., Duvet S., Kmiecik D., Labiau O., Mir A.M., and Verbert A. (1998) *Biochimie* **80**, 59-68.

FOOTNOTES

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**Abbreviations used in the text:** *O*-GlcNAc, *O*-linked N-acetylglucosamine ; OGT, *O*-GlcNAc transferase ; PTM, post-translational modification ; Hsp70, 70-kDa Heat shock protein ; Hsc70, 70-kDa Heat shock cognate ; HGBA, Hsp70 GlcNAc-binding activity ; MG132 (N-carbobenzoxyl-Leu-Leu-leucinal) ; PBS, Phosphate-Buffered Saline ; TBS, Tris-Buffered Saline.

## Figure legends

**Figure 1- Ubiquitin and *O*-GlcNAc are not mutually exclusive.**
**A-** $HepG_2$ cells were cultured without glucose (w/o Glc), without glucose but with glucosamine (w/o Glc + $GlcNH_2$), in presence of forskolin (Forsk.) or in normal condition (Ctrl) to modulate *O*-GlcNAc levels. Crude extracts proteins were analyzed by western blot for their *O*-GlcNAc (left panel) and ubiquitin (right panel) contents. Loading control was performed using an anti-Hsc70 antibody.
**B-** $HepG_2$ cells were analyzed according to their ubiquitinated proteins and *O*-GlcNAc proteins content using indirect immunofluorescent microscopy. Ubiquitin was localized using a polyclonal anti-ubiquitin antibody in conjunction with a Texas Red-labelled secondary antibody (Red staining). *O*-GlcNAc was localized with a monoclonal anti-*O*-GlcNAc antibody in conjunction with a FITC-labelled secondary antibody. DAPI was used for nucleus staining (Blue).
**C-** To check the concomitant presence of *O*-GlcNAc and ubiquitin on the same proteins, immunoprecipitation experiments were performed on $HepG_2$ cells extracts with an anti-*O*-GlcNAc antibody. Bound proteins were analyzed by western blot for their *O*-GlcNAc content (left panel) or their ubiquitin content (right panel).
Protein mass markers are indicated at the left (kDa).
Ub, ubiquitinated proteins ; Ig, immunoglobulins ; WB, Western blot.

**Figure 2- Time course of *O*-GlcNAc and ubiquitin levels after thermal stress.**
$HepG_2$ cells were incubated for increasing time periods at 42°C to induce thermal stress with or without the reversible proteasome inhibitor MG132 (8 µM). The ubiquitin and *O*-GlcNAc contents were analyzed by western blot using an anti-ubiquitin antibody (α-Ub.) or an anti-*O*-GlcNAc antibody. Hsp70 expression was followed using an anti-Hsp70 antibody and HGBA was studied by enrichment of protein crude extract on immobilized GlcNAc-beads and by analysis of the bound proteins with the anti-Hsp70 antibody. Loading charge control was checked using an anti Hsc70 antibody.
Protein mass markers are indicated at the left (kDa).
WB, Western blot ; GlcNAc-enr., proteins enriched on GlcNAc-beads ; min, minutes ; h, hours

**Figure 3- Proteasome inhibition triggers Hsp70 GlcNAc-binding activity**
**A-** $HepG_2$ cells were cultured without glucose (w/o Glc), without glucose but with glucosamine (w/o Glc + $GlcNH_2$, in unstress conditions (Ctrl) or in hyperthermal conditions (42°C). Cells were incubated with MG132 at a concentration of 8 µM to inhibit proteasomal degradation. HGBA was then analyzed by enrichment of crude extract on GlcNAc-beads and by analysis of the bound protein by western blot with an anti-Hsp70 antibody. Proteasome inhibition was checked using an anti-ubiquitin antibody. Loading control was checked using an anti-Hsc70 antibody.
**B-** The procedure was the same as described in A, except that proteasome inhibition was also provoked by two irreversible inhibitors, namely epoxomycin (E) and lactacystin (L).
Protein mass markers are indicated at the left (kDa).
∅, no inhibitor ; M, MG132 ; WB, Western blot ; GlcNAc-enr., proteins enriched on GlcNAc-beads.

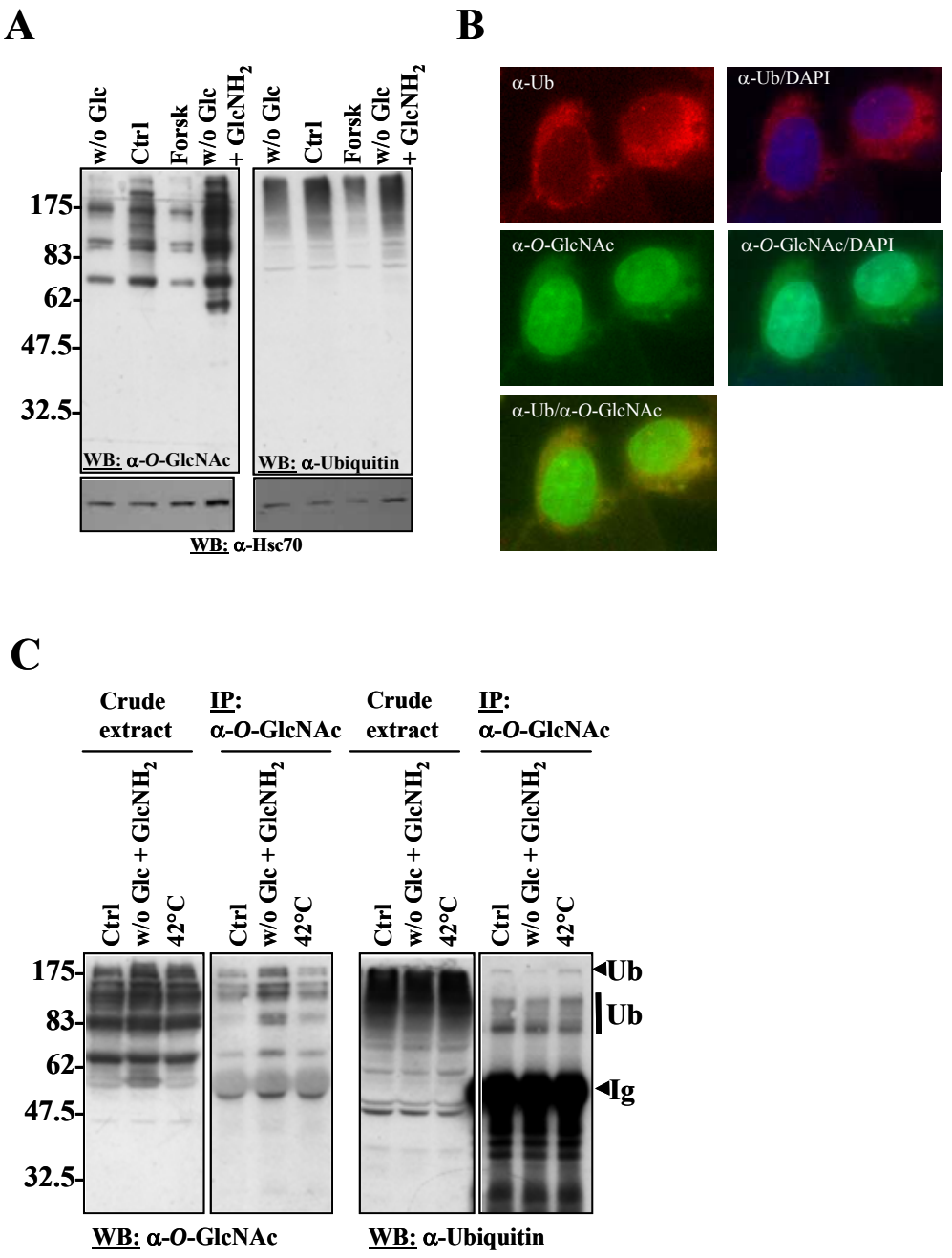
**Figure 4- E1 is *O*-GlcNAc modified and variations in these glycosylation levels correlate with E1/Hsp70 interaction.**

$HepG_2$ were cultured in normal DMEM (Ctrl), in glucose-depleted medium with or without 5 mM glucosamine or at 42°C for 24h. After lysis of the cells, extracts were immuno-precipitated with an anti-*O*-GlcNAc antibody. The immunoprecipitated were run on a 10% SDS-PAGE, electroblotted and stained with an anti-E1 antibody (panel A). An anti-ubiquitin has been performed on crude extracts.
Co-immunoprecipitation experiments were performed with an anti-Hsp70. The presence of E1 in Hsp70-enriched fractions was checked by western blotting using an anti-E1 antibody (panel B).
Equal loading were confirmed using anti-E1 antibody on crude extracts.
Protein mass markers are indicated at the left (kDa).
IP, immunoprecipitation ; Co-IP, co-immunoprecipitation ; WB, Western blot ; Ig, immunoglobulins.

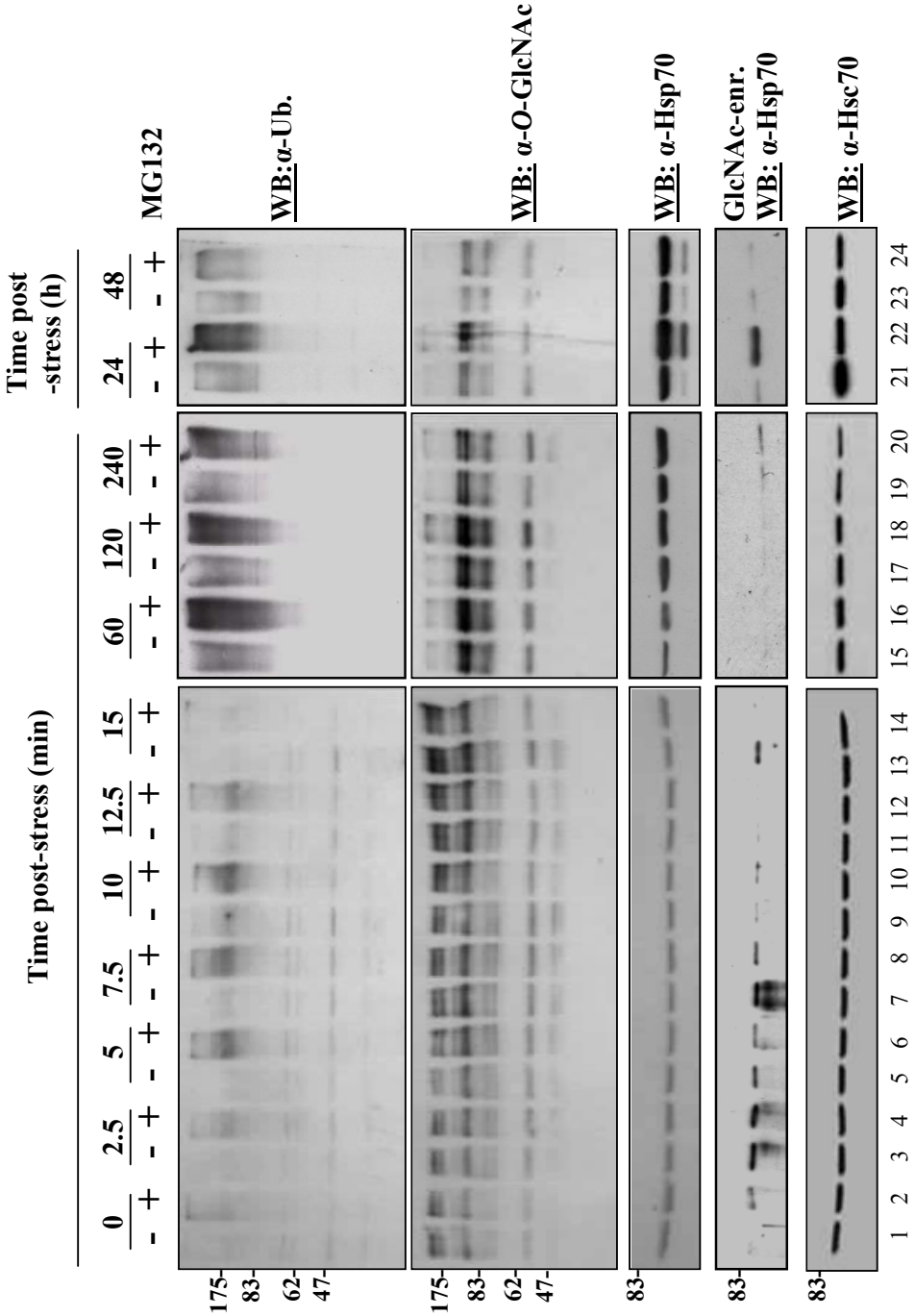
**Figure 5- OGT silencing using RNA interference leads to a decrease in *O*-GlcNAc, ubiquitination and cell viability.**
OGT silencing was carried out using the RNA interference technology. Oligonucleotides were transfected in HeLa cells during 96 h and incubated at 42°C for the last 24 hours to induce thermal stress.
**A-** Effectiveness of RNAi on OGT silencing was checked by looking at OGT and *O*-GlcNAc levels. Control of loading was achieved using anti-β-tubulin antibody. To control that OGT silencing did not affect other type of glycosylation, an SNA staining was performed.
Protein mass markers are indicated at the left (kDa).
**B-** Effectiveness of OGT silencing was also checked using indirect immunofluorescence microscopy.
Top panel: use of an anti-OGT antibody (AL-28) with a secondary antibody coupled to Texas Red. Nuclei were stained with DAPI.
Middle panel: the decrease in *O*-GlcNAc level induced by RNAi was visualized using an anti-*O*-GlcNAc antibody (RL-2) in conjunction with a FITC-coupled anti-mouse secondary antibody. Decrease in ubiquitin content was visualised using an anti-ubiquitin antibody in conjunction with a Texas Red-coupled anti-rabbit secondary antibody.
Bottom panel: localisation of OGT and ubiquitinated proteins using polyclonal AL-28 antibody (anti-OGT) and polyclonal anti-ubiquitin. Nuclei were stained with DAPI.
**C-** E1 glycosylation was checked by immunoprecipitation of crude extracts with anti-*O*-GlcNAc antibody and by staining bound-proteins with an anti-E1 antibody. Ubiquitinated proteins were revealed with an anti-ubiquitin antibody. Level of β-catenin was measured after OGT silencing using an anti-β-catenin antibody. Loading control was performed with the anti-E1-antibody
**D-** Effect of OGT silencing on cell viability after thermal stress was assessed using the blue trypan method.
Ctrl, control cells ; 1153, OGT silencing ; WB, Western blot ; IP, immunoprecipitation.

**Figure 6- Induced-protein misfolding triggers Hsp70-GlcNAc binding activity.**
**A-** Comparative structures of L-azetidine-2-carboxylic acid and L-proline.
**B-** Hsp70 levels and HGBA were studied after culturing $HepG_2$ cells with 5 mM L-azetidine-2-carboxylic acid for 24h. Control of induced-HGBA by L-azetidine-2-carboxylic acid incorporation was checked by translation inhibition using cycloheximide (CHX) or anisomycine (aniso). Loading control was performed using anti-Hsc70 antibody staining.
Protein mass markers are indicated at the left (kDa).
WB, Western blot ; GlcNAc-enr., proteins enriched on GlcNAc-beads.

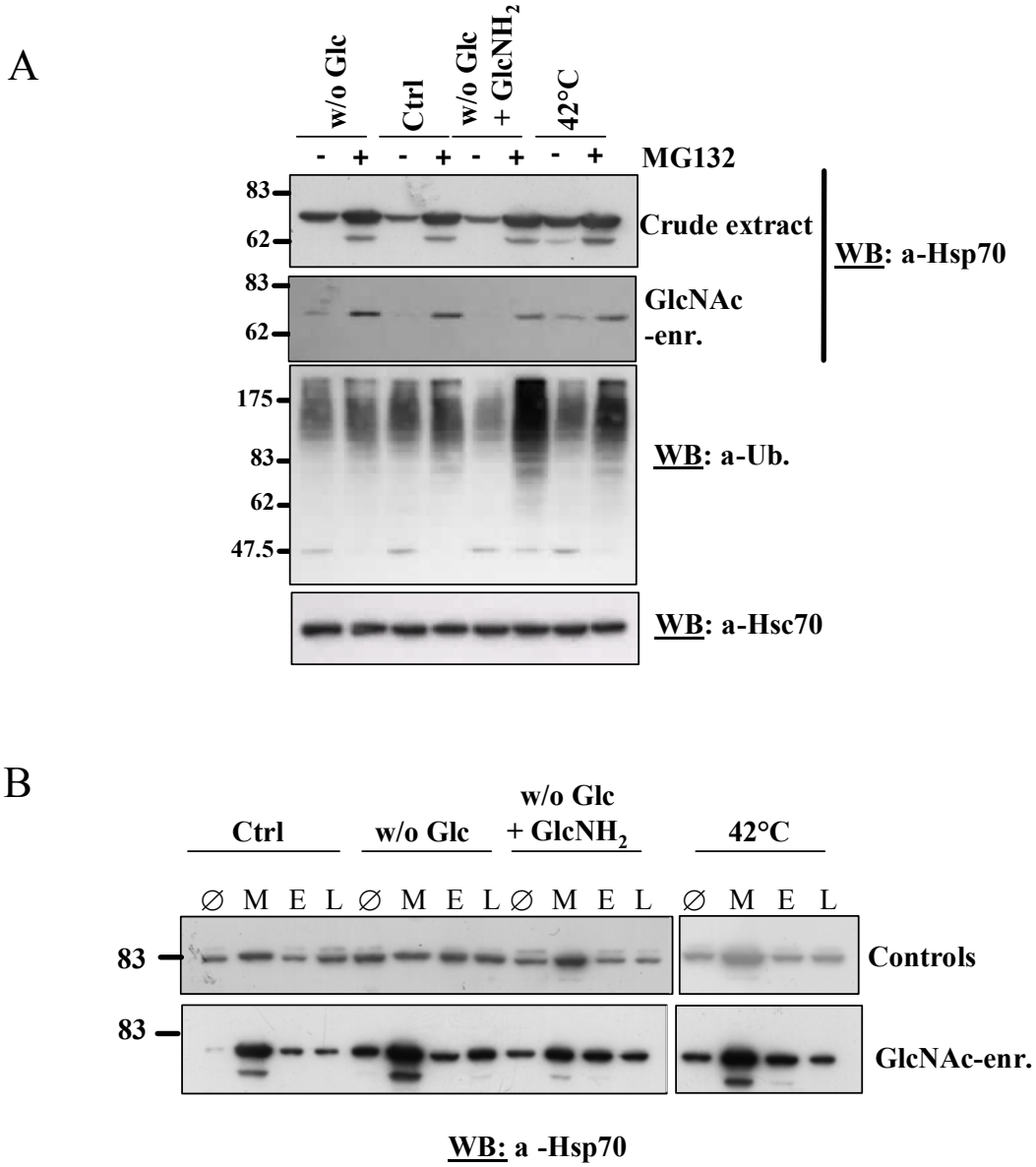
**Figure 7- Hypothetical model by which *O*-GlcNAc could contribute to increase protein stability through lectin interactions with Hsp70.**
Intracellular proteins could be damaged after a stress occurred. Hydrophobic segments including PEST sequences appear at the protein surface and can be subsequently recognized by OGT that modifies them with *O*-GlcNAc residues (21). These *O*-GlcNAc-bearing proteins could be then bound by Hsp70 through a lectin interaction. If the damage caused to the protein can be repaired, Hsp70 and Hsp40 work together to tentatively refold the protein (left part of the scheme). In this case E1 and the proteasome are inhibited by *O*-GlcNAc glycosylation. On the contrary, if the damage can not be reversed by the refolding machinery (right part the scheme), Hsp70 intervenes in cooperation with other co-chaperones like CHIP or Bag-1: E1 is activated, allowing *O*-GlcNAc-bearing proteins to be polyubiquitinated and to be targeted to proteasome (itself activated) for destruction. Proteins are cleaved in small peptides and ubiquitin is recycled.



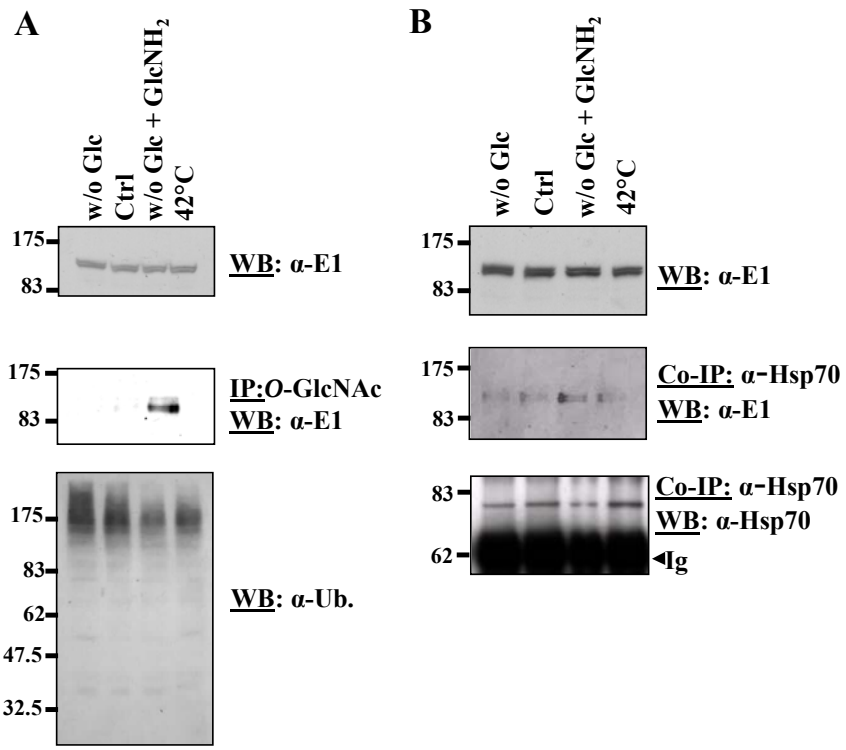

**Guinez et al., 2006 Figure 1**



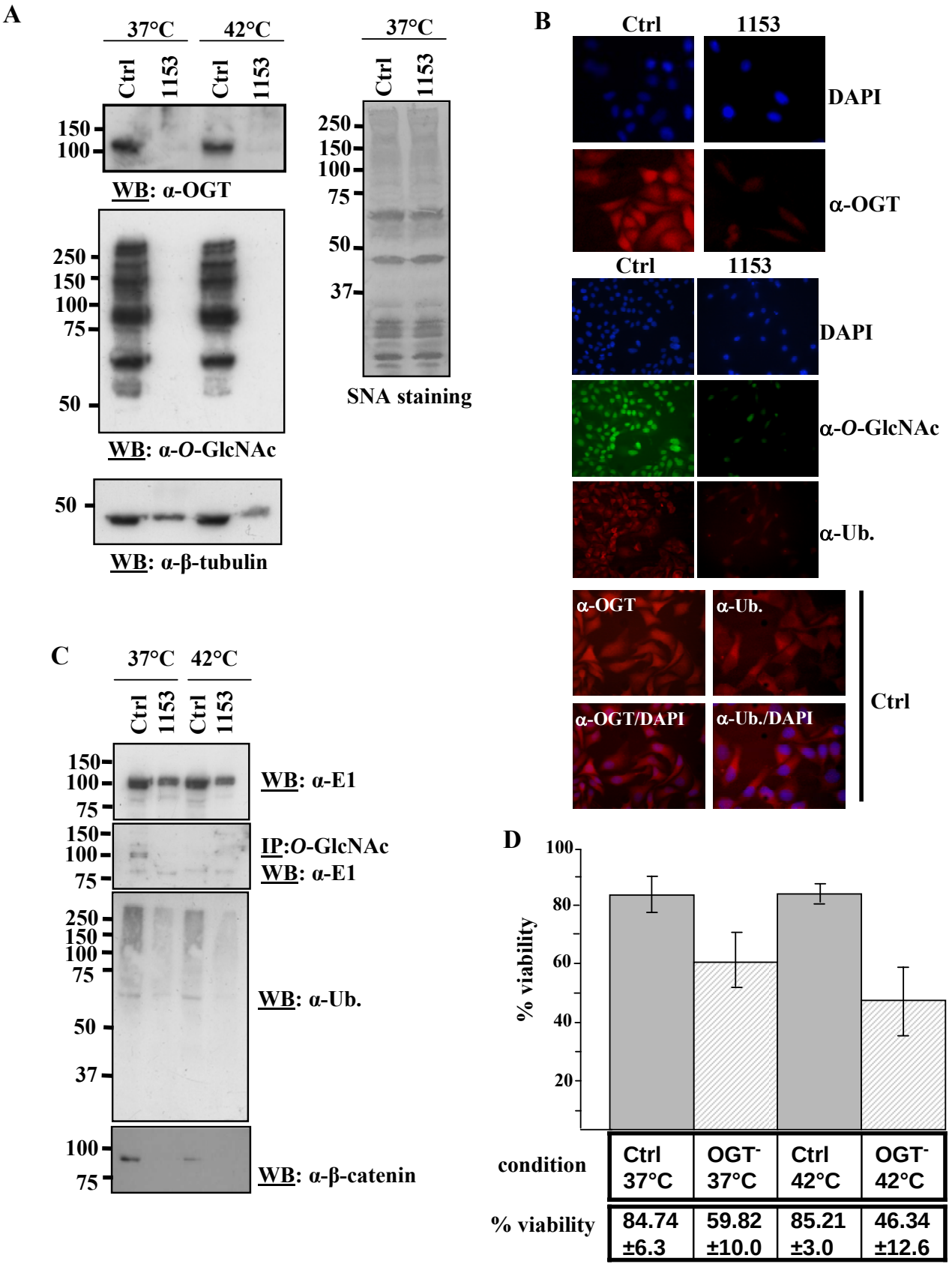

**Guinez et al., 2006 Figure 2**




**Guinez et al., 2006-Figure 3**

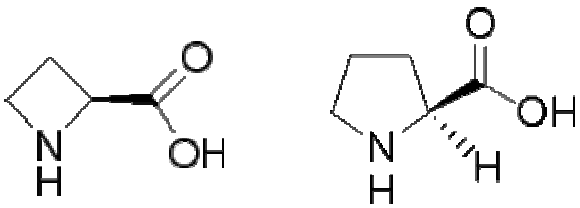



**Guinez et al., 2006 Figure 4**



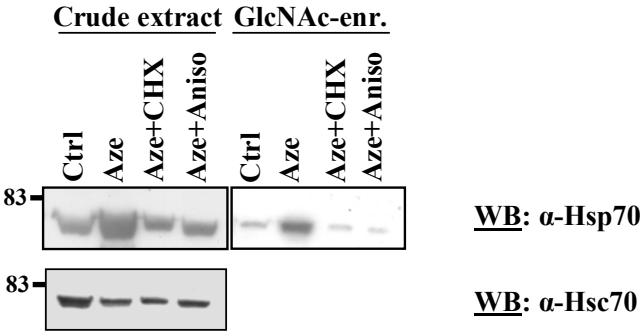

**Guinez et al., 2006 Figure 5**

**A**

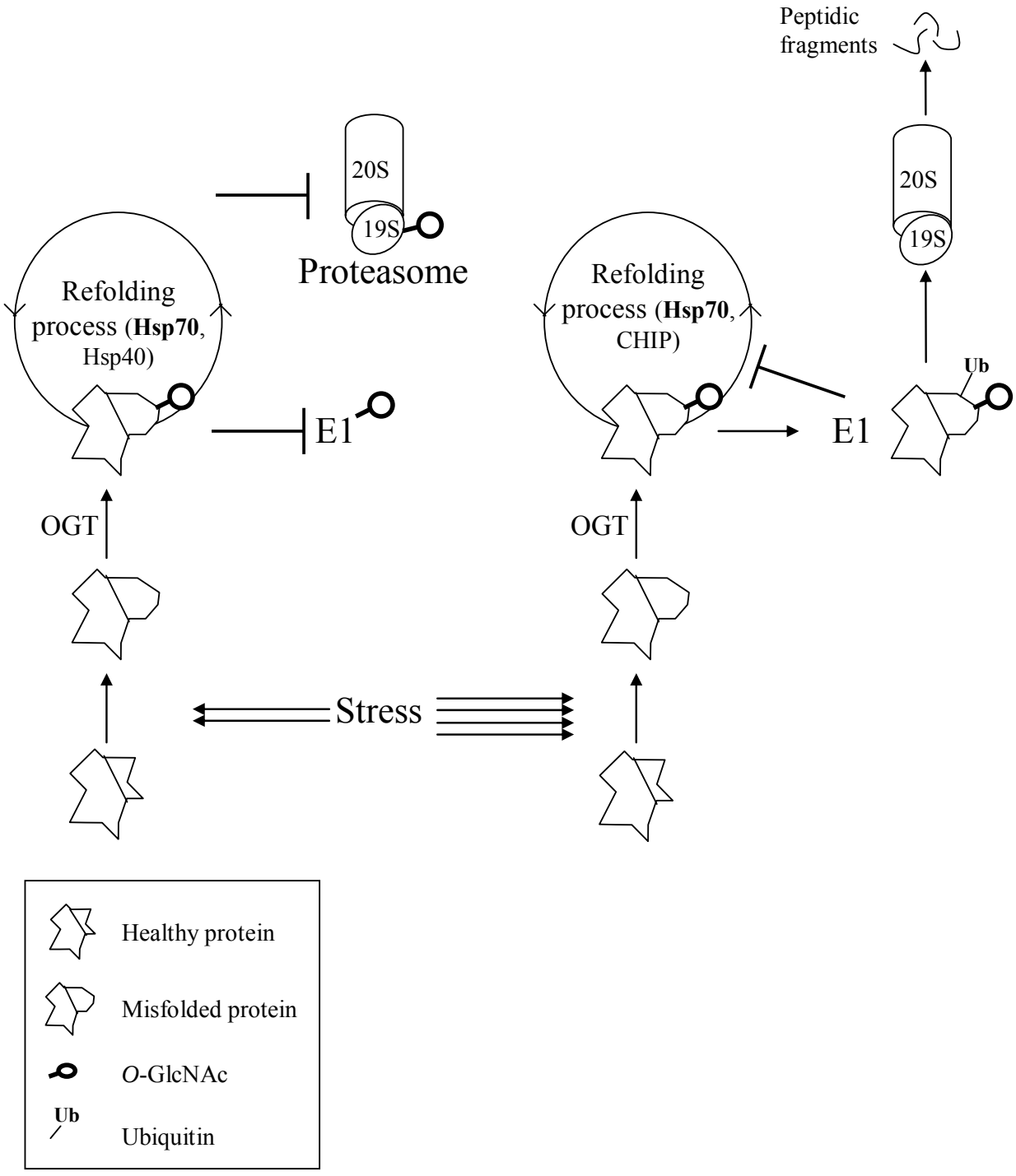



**L-azetidine-2-carboxylic acid** **L-proline**

**B**




**Guinez et al., 2006 Figure 6**




**Guinez et al., 2006 Figure 7**

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# Modulation of HSP70 GlcNAc-directed lectin activity by glucose availability and utilization

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**It is well-accepted that protein quality control (occurring either after protein synthesis or after cell damage) is mainly ensured by HSP, but the mechanism by which HSP decides whether the protein will be degraded or not is poorly understood. Within this framework, it has been hypothesized that *O*-GlcNAc, a cytosolic and nuclear-specific glycosylation whose functions remain unclear, could take a part in the protection of proteins against degradation by modifying both the proteins themselves and the proteasome. Because the synthesis of *O*-GlcNAc is tightly correlated to glucose metabolism and Hsp70 was endowed with GlcNAc-binding property, we studied the relationship between GlcNAc-binding activity of both Hsp70 and Hsc70 (the nucleocytoplasmic forms of HSP70 family) and glucose availability and utilization. We thus demonstrated that low glucose concentration, inhibition of glucose utilization with 2DG, or inhibition of glucose transport with CytB led to an increase of Hsp70 and Hsc70 lectin activities. Interestingly, the response of Hsp70 and Hsc70 lectin activities toward variations of glucose concentration appeared different: Hsp70 lost its lectin activity when glucose concentration was >5 mM (i.e., physiological glucose concentration) in contrast to Hsc70 that exhibited a maximal lectin activity for glucose concentration ~5 mM and at high glucose concentrations. This work also demonstrates that HSP70 does not regulate its GlcNAc-binding properties through its own *O*-GlcNAc glycosylation.**

*Key words:* glucose/heat shock proteins/hexosamine biosynthetic pathway/lectin/*O*-GlcNAc

## Introduction

Members of the 70-kDa heat shock proteins family (HSP70) have been demonstrated to be GlcNAc-binding lectins (Lefebvre *et al*., 2001; Guinez *et al*., 2004). The new concept of lectin-chaperone has arisen from growing evidences that the cytosolic and nuclear-specific glycosylation *O*-linked N-acetylglucosamine (*O*-GlcNAc) could act as a protective signal against proteasomal degradation. Beyond the myriad of proteins that are *O*-GlcNAc glycosylated, proteasome is itself modified with this atypical glycosylation (Sumegi *et al*., 2003; Zhang *et al*., 2003; Zachara and Hart, 2004) and moreover can be regulated by this post-translational modification (Zhang *et al*., 2003; Zachara and Hart, 2004). *O*-GlcNAc was first described by Torres and Hart (1984) and, rapidly, it appeared that this glycosylation was enriched in the cytosolic and nuclear compartments and that it was not static (as classical N- and O-glycosylation) but highly dynamic. Even more, it can counteract the effect of phosphorylation at the same or at adjacent sites in a reciprocal manner (for review, see Kamemura and Hart, 2003). Despite these exciting features, roles of *O*-GlcNAc remain elusive. Nevertheless and as mentioned above, it appears probable that *O*-GlcNAc could act as a protective signal against proteasomal degradation. One of the precursor works in the field was that of Han and Kudlow (1997) showing that when cultured cells were glucose deprived or stimulated by cyclic-adenosine 5′-monophosphate, the transcription factor Sp1 was hypoglycosylated leading to its rapid degradation. This degradation was sensitive to *N*-acetyl-L-leucinyl-L-leucinyl-L-norleucinal (LLnL) and lactacystin suggesting a proteasomal degradation. Treatment of cells with glucosamine or glucose protected Sp1 against degradation. Later, it has been proposed that *O*-GlcNAc glycosylation of a site found within the PEST sequence of the beta-estrogen receptor could block protein degradation and thus play an opposite role to phosphorylation (in this context, it is well known that PEST sequence phosphorylation could activate degradation) (Cheng *et al*., 2000). More recently, the cadherins-to-cytoskeleton connecting protein plakoglobin has been shown to be *O*-GlcNAc modified near a destruction box (Hatsell *et al*., 2003).

Using a wide variety of stresses (hyperthermia, UVB, arsenite, ethanol, etc.), Zachara *et al*. (2004) demonstrated a quick increase of *O*-GlcNAc content in the treated cells. Furthermore, when the level of *O*-GlcNAc was increased by using PUGNAc (an *O*-GlcNAcase inhibitor) or by transfecting COS7 cells with O-linked *N*-acetylglucosamine transferase (OGT), the thermo-tolerance of cells was increased in contrast to a reduction or a blockade of *O*-GlcNAc resulting in an increase of the sensitivity of cells to stress.

A few years ago, it has been established that *O*-GlcNAc was intimately linked to glucose metabolism (for review, see Wells *et al*., 2003). About 2–5% of extracellular glucose could be used for *O*-GlcNAc modification of proteins through the hexosamine biosynthetic pathway (HBP). *O*-GlcNAc has been postulated to be a sensor implicated in insulin resistance and in the decrease of glucose uptake by cells. First, the hypothesis of a negative feedback of glucose

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transport regulation by the flux of glucose through HBP was suggested in insulin target cells (Marshall *et al.*, 1991). Second, Robinson *et al.* (1993) have shown that when rat hemidiaphragms were incubated in glucosamine or in high glucose concentrations, glucose uptake decreased. Insulin-resistance effect and, consequently, glucose uptake decrease were correlated with a defect in GLUT4 plasma membrane glucose transporter (Cooksey *et al.*, 1999). It has also been shown that glucosamine-induced insulin resistance was accompanied by an increase of UDP-GlcNAc concentration (Rossetti *et al.*, 1995). Using transgenic overexpression of OGT, McClain *et al.* (2002) showed a type 2 diabetic phenotype. This observation suggested that insulin resistance and *O*-GlcNAc glycosylation are linked. In the same topic, incubation of rat epitrochlearis muscles with PUGNAc induced an increase in the *O*-GlcNAc level of proteins and a reduced glucose transport, suggesting that *O*-GlcNAc glycosylation of proteins can induce insulin resistance (Arias *et al.*, 2004). Taken together, these observations strongly support the pivotal role of *O*-GlcNAc in reduced glucose transport and insulin resistance through HBP. In this field, it must be noted that numerous proteins involved in the metabolism of glucose are themselves *O*-GlcNAc modified: casein-kinase II, glycogen synthase-kinase-3 (Lubas and Hanover, 2000), and insulin-receptor substrate-1 and 2 (Patti *et al.*, 1999).

Starting from the relation between glucose and *O*-GlcNAc and, since *O*-GlcNAc is a putative protector of proteins against proteasomal degradation, we previously demonstrated the existence of HSP70 lectin properties and we showed that 70-kDa heat shock protein (Hsp70), the cytosolic, and nuclear HSP70 induced form were endowed with a GlcNAc-specific lectin activity (Guinez *et al.*, 2004). Intriguingly, this lectin activity increased with stress. We thus hypothesized that HSP70 lectin activity serves as protection of proteins via *O*-GlcNAc residues.

This article demonstrates the close relationship between HSP70 lectin properties and glucose status. Glucose deprivation, glucose transport inhibition, and inability to use glucose strongly modulate lectin activity. Against all expectations, modulation of the lectin property does not depend on a self-regulation of HSP70 by its own *O*-GlcNAc residues, because the addition of glucosamine in a glucose-depleted medium which restored the *O*-GlcNAc glycosylation of HSP70 did not abrogate its lectin activity. This last point was reinforced by the fact that *O*-GlcNAc deglycosylation of cell extract after beta-hexosaminidase treatment did not enhanced Hsp70 lectin property.

## Results

### *Hsp70 and Hsc70 lectin activities are dependent upon glucose concentration*

To test the hypothesis of a glucose concentration-dependent Hsp70/70-kDa heat shock cognate (Hsc70) lectin activity, HepG2 cells were grown on dishes in medium-containing various glucose concentrations. First, we used a broad range of glucose concentrations ranging from 0 to 100 mM. After cells lysis, proteins were run on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), electroblotted onto nitrocellulose, and stained with an anti-Hsp70 antibody either directly (ctrl) or after enrichment on GlcNAc-beads (GlcNAc-beads enr). After revelation by enhanced chemiluminescence (ECL) (Figure 1A, top panel), nitrocellulose sheet was stripped and stained with an anti-Hsc70 antibody (Figure 1A, bottom panel). The two chaperones possessed a lectin activity when cells were depleted in glucose (Glc, 0 mM), and when glucose concentration was increased to 12.5 mM, this lectin activity strongly decreased for both Hsp70 and Hsc70. On the other hand, when glucose concentration was increased from 12.5 to 100 mM, Hsc70 showed a lectin activity which progressively increased starting from 25 mM to be maximal at 75 mM of glucose, whereas Hsp70 did not show such an enhancement. Right panel of Figure 1A represents the *O*-GlcNAc pattern of the crude cellular protein extract for each glucose concentration condition. It should be pointed out that glycosylation was maximal between 50 and 100 mM of glucose. The same experiment was performed with moderate glucose concentrations, that is, between 0 and 12.5 mM

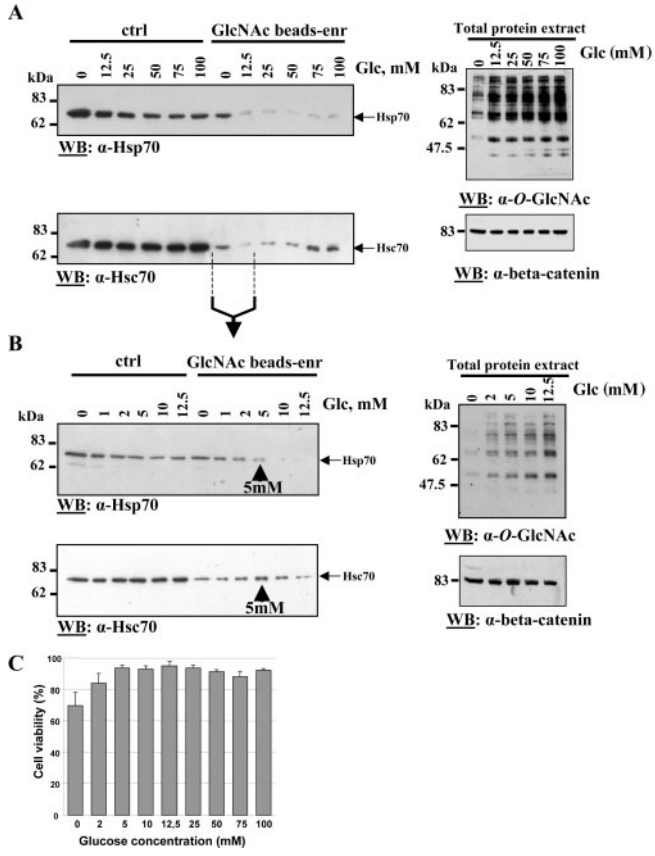


**Fig. 1.** Hsp70 and Hsc70 lectin activities are both dependent upon glucose concentration. HepG2 cells were cultured in a glucose-free medium with increasing glucose concentrations (0–100 mM in panel **A** and 0–12.5 mM in panel **B**). Hsp70 and Hsc70 were tested for their lectin activity against GlcNAc, as described under the *Materials and methods* section. The *O*-GlcNAc status of crude protein extracts is shown at the right of each figure. Anti-beta-catenin staining shows the equality of loading of each lane. Panel **C** represents cell viability after each condition of treatment (performed in triplicate). Ctrl, control of Hsp70 and Hsc70 proteins levels; WB, western blot.

(Figure 1B). Hsp70 showed a progressive decrease in its lectin activity up to 5 mM glucose (top panel) in contrast to Hsc70 (bottom panel) which showed a maximal activity at 5 mM (i.e., physiological glucose concentration). Figure 1B, right panel, represents an anti-*O*-GlcNAc antibody (RL2) staining of control extracts. A progressive increase in *O*-GlcNAc proteins content can be observed with glucose increase. These first experiments clearly correlate glucose concentration to lectin activity of HSP70 and underline that Hsp70 and Hsc70 lectin activities were expressed in a different manner according to the glucose concentration.

*Inhibition of glucose utilization or inhibition of glucose transport leads to an increase in Hsp70 lectin activity*

To extend and reinforce the results described above and to strengthen the hypothesis that lectin activity of Hsp70 and Hsc70 are modulated by the glucose concentration, HepG2 cells were cultured either in presence of 2-deoxyglucose (2DG), a glucose analogue that perturbs utilization of glucose by competing interactions with proteins/enzymes using glucose as a substrate, or in presence of cytochalasin B (CytB), a glucose transporter inhibitor. Control experiments performed on total protein extracts showed a decrease in their *O*-GlcNAc content after staining with the anti-*O*-GlcNAc antibody (Figure 2A, for 2DG treatment, and Figure 2B, for CytB treatment, upper panels). In both cases, whatever the culture conditions (with/without glucose, with/without glucosamine, or at 42°C), Hsp70 and Hsc70 shared an increased GlcNAc-binding properties when 2DG or CytB were added to the culture medium (compare lanes 2 and 6 and lanes 10 and 14). These results showed that when glucose transport is inhibited by CytB or when glucose utilization was decreased by 2DG, GlcNAc lectin activities of HSP70 were enhanced, confirming the results presented in Figure 1 and demonstrating that lectin activities of Hsp70 and Hsc70 depend on glucose availability and utilization.

*Hsp70 and Hsc70 do not regulate their GlcNAc-binding properties with their own* O-*GlcNAc glycosylation*

Previously reported data suggested an opposite relationship between the *O*-GlcNAc modification of Hsp70 and its capability to recognize exogenous *O*-GlcNAc residues (Guinez *et al.*, 2004). In this report, we showed that in a glucose-free medium, Hsp70 was not *O*-GlcNAc modified but exhibited a high lectin activity toward *O*-GlcNAc, and in contrast in a normal culture medium (Dulbecco's modified Eagle's medium [DMEM] with 4.5 g $L^{-1}$ of glucose) Hsp70 was glycosylated but was practically devoid of GlcNAc-binding property. Thus, we hypothesized that Hsp70 *O*-GlcNAc modification could occur on the lectin site avoiding subsequent binding on GlcNAc beads. To tentatively answer this question, we artificially increased *O*-GlcNAc level by incubating cells in a glucose-free medium supplemented with 5 mM glucosamine. As mentioned under *Introduction*, glucosamine can directly enter the HBP without the need of glutamine : fructose 6-phosphate amido-transferase (GFAT), the key- and rate-limiting enzyme of HBP. In these conditions, a lectin activity was induced both for Hsp70 (compared with glucose-deprived conditions but was lower to control, i.e., in presence of glucose) and for Hsc70 (the intensity of binding is more or less the same for the control and for the cell cultured in the presence of glucosamine), whereas the two chaperones were *O*-GlcNAc modified (Figure 3A, left panel for controls and right panel for GlcNAc-enriched and anti-*O*-GlcNAc antibody-enriched samples). This demonstrated that in these conditions, Hsp70 and Hsc70 lectin activities were dependent upon glucose deprivation and not dependent upon their own *O*-GlcNAc level. A second experiment invalidated this later hypothesis. HepG2 cells were exposed to a thermal stress for increasing periods (from 0 to 48 h). After cell lysis, GlcNAc-binding properties and glycosylation of Hsp70/Hsc70 were examined (Figure 3B). Both for Hsp70 and Hsc70, lectin properties and glycosylation reached maximal activities near 20 h: the time progress curves were similar for the lectin activity (left panel) and for the *O*-GlcNAc content (right panel) of Hsp70 and Hsc70. Figure 3C is a control of the *O*-GlcNAc glycosylation of total protein extract. This result reinforces the idea that Hsp70 and Hsc70 lectin properties are not regulated by their own *O*-GlcNAc modification. Finally, treatment of cell extracts with beta-hexosaminidase definitively confirmed these results, because after *O*-GlcNAc hydrolysis with beta-hexosaminidase treatment Hsp70 of cells cultured in normal conditions did not recover GlcNAc-binding property (Figure 4). Taken together, these results confirmed that HSP70 did not self-regulate their GlcNAc-binding properties with their own *O*-GlcNAc glycosylation.

## Discussion

The serine/threonine *O*-GlcNAc modification is widely expressed in cytosolic and nuclear compartments of eukaryotes. It modifies numerous proteins implicated in transcriptional processes (including transcription factors and RNA polymerase II) (Yang *et al.*, 2002), metabolic enzymes (Cieniewski-Bernard *et al.*, 2004), nuclear pore proteins (Guinez *et al.*, 2005), and many other proteins. Despite the great interest brought to the study of *O*-GlcNAc, the exact functions played by this simple glycosylation remain unknown. In this work, we focused our interest on the relationship between utilization of glucose and Hsp70/Hsc70 lectin activities.

It has been demonstrated that a nonnegligible part of glucose that enters the cell was converted into UDP-GlcNAc (the donor of GlcNAc for OGT); the question arose to know whether cellular chaperones such as Hsp70 and Hsc70 were affected, particularly by their GlcNAc-binding activity, when glucose was limiting or in excess. To answer this question, HepG2 cells were cultured with increasing glucose concentrations ranging from 0 to 100 mM (4-fold the glucose concentration present in normal culture medium). Surprisingly, Hsp70 and Hsc70 lectin activities were differently affected (Figure 1A). Indeed, Hsp70 bound GlcNAc when cells were deprived in glucose, but lectin activity decreased after addition of glucose. In contrast, Hsc70 bound GlcNAc beads in glucose-deprived conditions but also for higher glucose concentrations (the optimal activity being between 75 and 100 mM of glucose). To

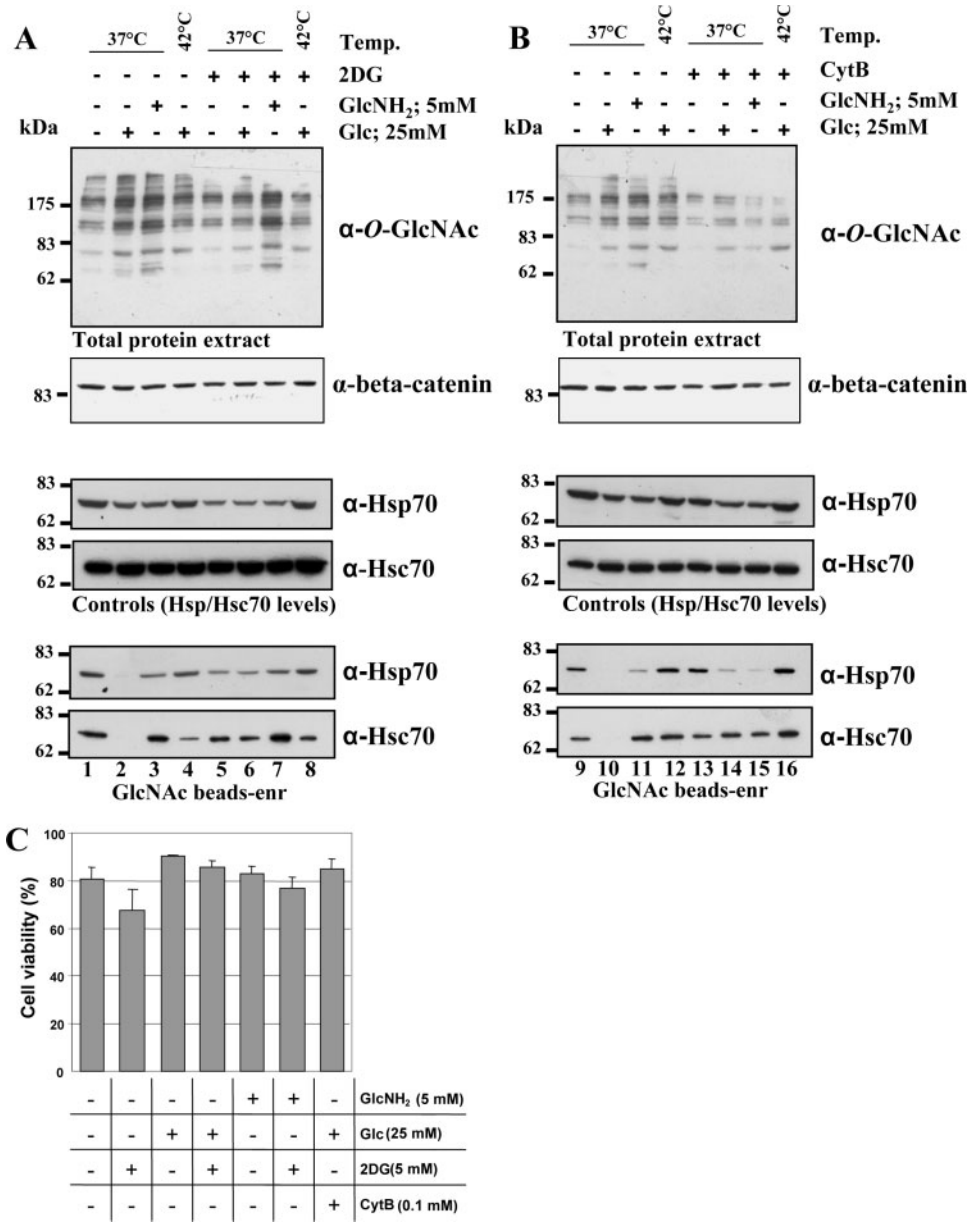


**Fig. 2.** Inhibition of glucose transport or inhibition of glucose use induce an increase of Hsp70/Hsc70 lectin activity. HepG2 cells were cultured in a classical medium (Dulbecco's modified Eagle's medium [DMEM]) with or without 2DG (5 mM), an inhibitor of glucose utilization and with or without CytB (0.1 mM), an inhibitor of glucose transport. **A** and **B** (**top** panels) indicate a decrease of *O*-GlcNAc glycosylation of proteins (controlled with the anti-*O*-GlcNAc antibody) with 2DG and CytB, respectively. In the **middle** panels, controls of Hsp70 and Hsc70 expression are shown. Lectin activities of Hsp70 and Hsc70 toward GlcNAc in the presence of 2DG and CytB are represented on the **bottom** panels. Anti-beta-catenin staining shows the equality of loading of each lane. Panel **C** represents cell viability after each condition of treatment (performed in triplicate). Panel **A** represents the 2DG experiment, and panel **B** represents the CytB experiment.

be more representative of physiological conditions, lower glucose concentrations were used (from 0 to 12.5 mM, i.e., half-fold the glucose concentration of a normal culture medium). In these conditions, the lectin activities of Hsp70 and Hsc70 were different. In contrast to Hsp70 which bound GlcNAc when glucose concentration was <5 mM, Hsc70 reached a maximal binding activity at 5 mM. In both cases, the key glucose concentration was 5 mM, that is, the physiological glucose concentration. We thus demonstrated that the two chaperones did not work similarly, according to the glucose concentration. At low glucose concentration (<5 mM) owing to the stress, Hsp70 level and GlcNAc-binding activity are induced. When glucose concentration reaches a physiological or higher values (>5 mM), Hsp70 level decreases, and the lectin activity disappears in contrast to Hsc70 that shows an increasing lectin activity but at a constant protein expression level. Interestingly, the decrease of glucose concentration induced both a decrease in protein *O*-GlcNAc modification and an enhancement of Hsp70 lectin activity (Figure 1B, right panel); so a decrease

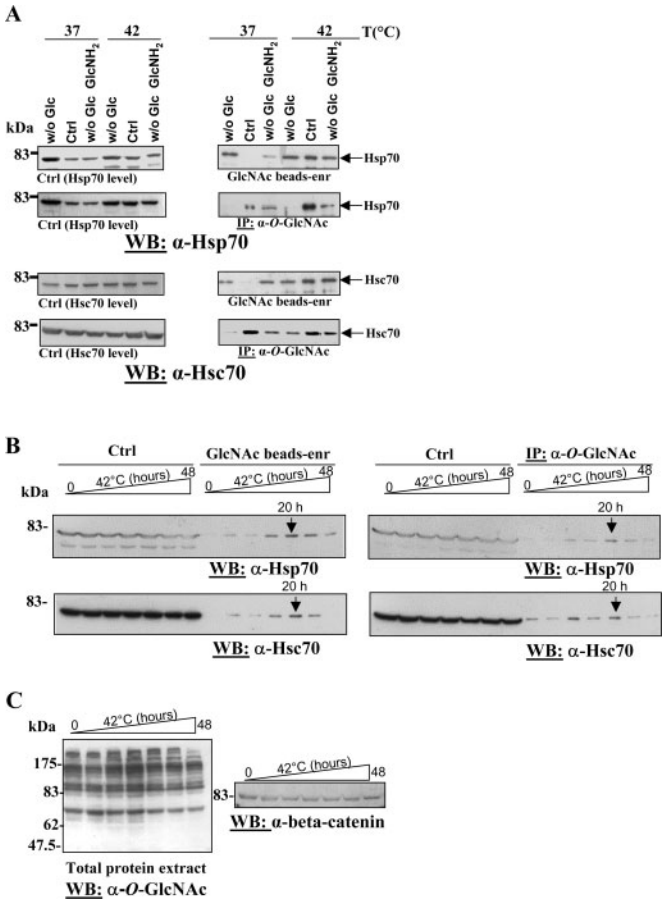


**Fig. 3.** Neither Hsp70 nor Hsc70 self-regulate their GlcNAc-binding property with their own *O*-GlcNAc glycosylation. HepG2 were cultured in presence of glucose (normal condition) or in absence of glucose, with and without 5 mM glucosamine. The lectin activity and the *O*-GlcNAc glycosylation of Hsp70 and Hsc70 were checked in these three conditions (**A**, control expression of Hsp70 and Hsc70 are shown in the **left** panel, and their binding properties and *O*-GlcNAc glycosylation are shown in the **right** panel). The second approach consisted in kinetic experiments: HepG2 cells were incubated at 42°C for increasing periods (from 0 to 48 h) to induce a thermal stress (panel **B**). The GlcNAc-binding properties (**left** panel) and *O*-GlcNAc glycosylation (**right** panel) of Hsp70 and Hsc70 were tested. The *O*-GlcNAc profile of the total extract protein during thermal stress is shown in panel **C**. Control of gel loading is shown with the anti-beta-catenin staining. Ctrl, control of Hsp70 and Hsc70 proteins levels; IP, immunoprecipitation; WB, western blot.

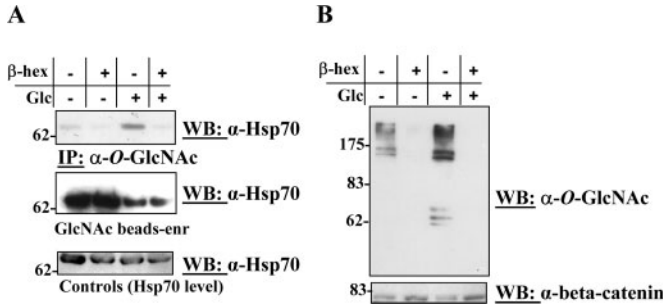


**Fig. 4.** Deglycosylation of Hsp70 did not induce an enhancement of its lectin activity. HepG2 cells extract were previously treated with beta-hexosaminidase before testing the lectin property of Hsp70. Experiments were performed either with cells cultured in normal medium (containing glucose) or in a glucose-depleted medium. After treatment, Hsp70 were enriched either with RL2 antibody to test their glycosylation or on GlcNAc beads to test their lectin property (panel **A**). Efficiency of deglycosylation is shown by staining of the total protein extract with the anti-*O*-GlcNAc antibody. Anti-beta-catenin shows the equality of loading of each lane (panel **B**). β-hex, beta-hexosaminidase; IP, immunoprecipitation; WB, western blot.

in *O*-GlcNAc glycosylation could be compensated by a higher capacity of Hsp70 to recognize *O*-GlcNAc-modified cellular proteins.

To drive further this relationship between glucose and Hsp70/Hsc70 lectin activity, we used two drugs that either enabled glucose utilization or its transport into the cell. In both cases, even in normal conditions, lectin activity of Hsp70 and Hsc70 increased, showing the importance of glucose entry and utilization in the regulation of Hsp70/Hsc70 lectin properties (Figure 2A and B). One explanation for this phenomenon could be that when glucose deprivation occurs, the enhancement of HSP70 lectin activity observed counteracts the decrease of *O*-GlcNAc modification of cellular proteins. This could re-equilibrate the misbalance between *O*-GlcNAc and GlcNAc-binding properties.

The possibility that HSP70 lectin properties are regulated with HSP70 *O*-GlcNAc-glycosylation was tested. It appeared attractive that HSP70 could self-regulate their GlcNAc-binding properties with their own *O*-GlcNAc glycosylation. Three points came to invalidate this hypothesis. First, when HepG2 cells were cultured in absence of glucose but with glucosamine (to by-pass the rate-limiting enzyme of the HBP–GFAT—thus allowing the synthesis of UDP-GlcNAc and the transfer of *O*-GlcNAc residues), Hsp70 and Hsc70 were glycosylated and were endowed with GlcNAc-binding properties. In these conditions, it seems that glucose depletion was critical for activating GlcNAc properties of chaperones independently from their *O*-GlcNAc glycosylation. The second approach was to follow the GlcNAc-binding activity and the glycosylation progression of Hsp70 and Hsc70 during stress. The stress inflected to cells was a thermal one and not a glucose deprivation to maintain the formation of *O*-GlcNAc. In these conditions, we showed that the two features, that is, the *O*-GlcNAc glycosylation and the GlcNAc-binding property of both Hsp70 and Hsc70 evolved similarly along the stress period. This excludes an autoregulation of the chaperones with their own *O*-GlcNAc. Finally, after treatment of the total protein extract with beta-hexosaminidase, Hsp70 did not modify the lectin capacity. These observations indicate that the regulation of HSP70 lectin activity is mediated by something else than *O*-GlcNAc glycosylation. We can suppose that unidentified partners could modulate this property by interacting with chaperones. In this idea, the intervention of co-chaperones must be considered.

This article demonstrates the close relationship between the level of glucose and the lectin property of Hsp70 and Hsc70 toward GlcNAc residue. This phenomenon could be compared with the existing relationship between *O*-GlcNAc level and glucose concentration. The functions of such lectin activities are without any doubt in the protection of proteins against outer attacks. HSP are the guardian

of the cell integrity, and this new function could be an additive weapon to carry their mission through.

## Materials and methods

### *Cell culture and treatments of cells*

HepG2 cells were maintained in DMEM (Gibco, Cergy Pontoise, France) supplemented with 10% fetal calf serum (v/v), 2 mM L-glutamine, 5 IU/mL penicillin, and 50 µg/mL streptomycin at 37°C on a humidified atmosphere enriched with 5% $CO_2$. Cultures were carried out on dishes (diameter 100 mm) preliminarily treated with 0.1% porcine gelatine (Sigma-Aldrich, Lyon, France).

Before stress, cells were washed with 10 mL of glucose-depleted medium and incubated either in this medium (for the starvation condition) or in this medium supplemented with glucose (cell culture tested, Sigma-Aldrich) at low (1–12.5 mM) or at high (25–100 mM) concentrations. Glucose-free medium was also supplemented with 5 mM glucosamine (cell culture tested, Sigma-Aldrich) for 24 h. 2DG (Sigma-Aldrich) was used at a concentration of 5 mM and CytB (Sigma-Aldrich) at a concentration of 0.1 mM. Thermal stress was induced by incubation of the cells for 24 h at 42°C in a 5% $CO_2$-enriched atmosphere. For kinetic experiments, HepG2 were grown at 37°C and placed at 42°C from 0 to 48 h. Cell viability was determined by the Trypan blue exclusion method.

### *GlcNAc-binding proteins and* O-*GlcNAc-bearing proteins enrichment*

HepG2 were first washed with 10 mL of cold phosphate-buffered saline (PBS, Gibco). Cells were lysed with a scrapper on ice either with a hypotonic buffer (10 mM Tris–HCl, 10 mM NaCl, 15 mM 2-mercaptoethanol, 1 mM $MgCl_2$, and proteases inhibitors, pH 7.2) for lectin activity studies or with a detergent-containing buffer (DB) (10 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100 (v/v), 0.5% sodium deoxycholate (w/v), 0.1% sodium dodecyl sulfate (w/v), and proteases inhibitors, pH 7.4) for the *O*-GlcNAc-content studies. Cellular extracts were centrifuged at 20,000 *g* for 30 min at 4°C. To test the lectin activity, supernatants were incubated with 30 µL of GlcNAc-coupled beads (*N*-acetyl-D-glucosamine immobilized on 6% beaded agarose with a spacer of five carbons, Sigma-Aldrich) at 4°C for 1 h. Beads were washed four times with binding buffer (20 mM Tris–HCl, 200 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and proteases inhibitor, pH 7.4). Specificity of binding has been tested with different sugar-coupled beads and with free sugar in excess (Guinez *et al.*, 2004) and data not shown (glucose and GlcNAc). To study the *O*-GlcNAc glycosylation of proteins, immunoprecipitations with the anti-*O*-GlcNAc antibody (RL-2, Affinity Bioreagents, Golden, CO) were performed. RL-2 was added to a 1:250 final dilution, and cellular extracts were incubated at 4°C overnight. The bound proteins were then recovered after addition of protein G-Sepharose (Amersham Biosciences, Orsay, France) for 1 h at 4°C. Beads were gently centrifuged for 1 min and washed with the following buffers: DB; DB supplemented with 500 mM NaCl, DB/TNE (10 mM Tris–HCl, 150 mM NaCl, and 1 mM ethylenediaminetetraacetic acid [EDTA], pH 7.4) in equal volume, and finally with TNE alone.

### *Beta-hexosaminidase treatment*

HepG2 extracts were adjusted to pH 5.2 with 100 mM acetate and incubated with *Escherichia coli* recombinant beta-hexosaminidase (Calbiochem, San Diego, CA) for 24 h at 37°C.

### *SDS–PAGE, western blotting, and antibody staining*

Samples were analyzed by 10% SDS–PAGE under reducing conditions, and proteins were electroblotted onto nitrocellulose sheet (Amersham Biosciences). Membranes were first saturated for 45 min with 5% non-fatty acid milk in Tris-buffered saline (TBS)–Tween buffer (20 mM Tris–HCl, 150 mM NaCl, and 0.05% Tween [v/v], pH 8.0). Rabbit anti-Hsp70 polyclonal antibodies were incubated for 1 h at a dilution of 1:150,000 (Stressgen Bioreagents, Victoria, British Columbia). RL-2 anti-*O*-GlcNAc monoclonal antibodies were incubated overnight at 4°C at a dilution of 1:1000. Membranes were then washed three times with TBS–Tween for 10 min and incubated with either an anti-rabbit or an anti-mouse horseradish peroxidase-labeled secondary antibodies (Amersham Biosciences) at a dilution of 1:10,000 for 1 h. Three washes of 10 min each were performed with TBS–Tween, and the detection was carried out with ECL solution (Amersham Biosciences). Primary and secondary antibodies complexes were removed from the membranes with a stripping buffer (62.5 mM Tris–HCl, 2% SDS, 100 mM 2-mercaptoethanol, pH 6.5) for 30 min at 50°C, abundantly washed with TBS–Tween and reincubated with a rat anti-Hsc70 at a dilution of 1:1000 (Stressgen Bioreagents). Anti-rat secondary antibody labeled with horseradish peroxidase (Amersham Biosciences) was used at a dilution of 1:10,000 for 1 h. Polyclonal anti-beta-catenin was used at a dilution of 1:1000.

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## Abbreviations

2DG, 2-deoxyglucose; CytB, cytochalasin B; HBP, hexosamine biosynthetic pathway; Hsc70, 70-kDa heat shock cognate; Hsp70, 70-kDa heat shock protein; HSP70, members of the 70-kDa heat shock proteins family; *O*-GlcNAc, O-linked *N*-acetylglucosamine; OGT, *O*-linked N-acetylglucosamine transferase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline.

## References

Arias, E.B., Kim, J., and Cartee, G.D. (2004) Prolonged incubation in PUGNAc results in increased protein *O*-linked glycosylation and insulin resistance in rat skeletal muscle. *Diabetes*, **53**, 921–930.

Cheng, X., Cole, R.N., Zaia, J., and Hart, G.W. (2000) Alternative *O*-glycosylation/*O*-phosphorylation of the murine estrogen receptor beta. *Biochemistry*, **39**, 11609–11620.

Cieniewski-Bernard, C., Bastide, B., Lefebvre, T., Lemoine, J., Mounier, Y., and Michalski, J.-C. (2004) Identification of *O*-linked N-acetylglucosamine proteins in rat skeletal muscle using two-dimensional gel electrophoresis and mass spectrometry. *Mol. Cell. Proteomics*, **3**, 577–585.

Cooksey, R.C., Hebert, L.F., Jr., Zhu, J.H., Wofford, P., Garvey, W.T., and McClain, D.A. (1999) Mechanism of hexosamine-induced insulin resistance in transgenic mice overexpressing glutamine: fructose-6-phosphate amidotransferase: decreased glucose transporter GLUT4 translocation and reversal by treatment with thiazolidinedione. *Endocrinology*, **140**, 1151–1157.

Guinez, C., Lemoine, J., Michalski, J.C., and Lefebvre, T. (2004) 70-kDa-heat shock protein presents an adjustable lectinic activity towards *O*-linked N-acetylglucosamine. *Biochem. Biophys. Res. Commun.*, **319**, 21–26.

Guinez, C., Morelle, W., Michalski, J.-C., and Lefebvre, T. (2005) *O*-GlcNAc glycosylation: a signal for the nuclear transport of cytosolic proteins? *Int. J. Biochem. Cell. Biol.*, **37**, 765–774.

Han, I. and Kudlow, J.E. (1997) Reduced *O*-glycosylation of Sp1 is associated with increased proteasome susceptibility. *Mol. Cell. Biol.*, **17**, 2550–2558.

Hatsell, S., Medina, L., Merola, J., Haltiwanger, R., and Cowin, P. (2003) Plakoglobin is *O*-glycosylated close to the N-terminal destruction box. *J. Biol. Chem.*, **278**, 37745–37752.

Kamemura, K. and Hart, G.W. (2003) Dynamic interplay between *O*-glycosylation and *O*-phosphorylation of nucleocytoplasmic proteins: a new paradigm for metabolic control of signal transduction and transcription. *Prog. Nucleic Acid Res. Mol. Biol.*, **73**, 107–136.

Lefebvre, T., Cieniewski, C., Lemoine, J., Guerardel, Y., Leroy, Y., Zanetta, J.P., and Michalski, J.C. (2001) Identification of N-acetyl-d-glucosamine-specific lectins from rat liver cytosolic and nuclear compartments as heat-shock proteins. *Biochem. J.*, **360**, 179–188.

Lubas, W.A. and Hanover, J.A. (2000) Functional expression of *O*-linked GlcNAc transferase. Domain structure and substrate specificity. *J. Biol. Chem.*, **275**, 10983–10988.

Marshall, S., Bacote, V., and Traxinger, R.R. (1991) Discovery of a metabolic pathway mediating glucose-induced desensitization of the glucose transport system. Role of hexosamine biosynthesis in the induction of insulin resistance. *J. Biol. Chem.*, **266**, 4706–4712.

McClain, D.A., Lubas, W.A., Cooksey, R.C., Hazel, M., Parker, G.J., Love, D.C., and Hanover, J.A. (2002) Altered glycan-dependent signaling induces insulin resistance and hyperleptinemia. *Proc. Natl. Acad. Sci. U. S. A.*, **99**, 10695–10699.

Patti, M.E., Virkamäki, A., Landaker, E.J., Kahn, C.R., and Yki-Järvinen, H. (1999) Activation of the hexosamine pathway by glucosamine in vivo induces insulin resistance of early postreceptor insulin signaling events in skeletal muscle. *Diabetes*, **48**, 1562–1571.

Robinson, K.A., Sens, D.A., and Buse, M.G. (1993) Pre-exposure to glucosamine induces insulin resistance of glucose transport and glycogen synthesis in isolated rat skeletal muscles. Study of mechanisms in muscle and in rat-1 fibroblasts overexpressing the human insulin receptor. *Diabetes*, **42**, 1333–1346.

Rossetti, L., Hawkins, M., Chen, W., Gindi, J., and Barzilai, N. (1995) In vivo glucosamine infusion induces insulin resistance in normoglycemic but not in hyperglycemic conscious rats. *J. Clin. Invest.*, **96**, 132–140.

Sumegi, M., Hunyadi-Gulyas, E., Medzihradszky, K.F., and Udvardy, A. (2003) 26S proteasome subunits are *O*-linked N-acetylglucosamine-modified in Drosophila melanogaster. *Biochem. Biophys. Res. Commun.*, **312**, 1284–1289.

Torres, C.R. and Hart, G.W. (1984) Topography and polypeptide distribution of terminal N-acetylglucosamine residues on the surfaces of intact lymphocytes. Evidence for *O*-linked GlcNAc. *J. Biol. Chem.*, **259**, 3308–3317.

Wells, L., Vosseler, K., and Hart, G.W. (2003) A role for N-acetylglucosamine as a nutrient sensor and mediator of insulin resistance. *Cell. Mol. Life Sci.*, **60**, 222–228.

Yang, X., Zhang, F., and Kudlow, J.E. (2002) Recruitment of O-GlcNAc transferase to promoters by corepressor mSin3A: coupling protein O-GlcNAcylation to transcriptional repression. *Cell*, **110**, 69–80.

Zachara, N.E. and Hart, G.W. (2004) *O*-GlcNAc modification: a nutritional sensor that modulates proteasome function. *Trends Cell. Biol.*, **14**, 218–221.

Zachara, N.E., O'Donnell, N., Cheung, W.D., Mercer, J.J., Marth, J.D., and Hart, G.W. (2004) Dynamic *O*-GlcNAc modification of nucleocytoplasmic proteins in response to stress. A survival response of mammalian cells. *J. Biol. Chem.*, **279**, 30133–30142.

Zhang, F., Su, K., Yang, X., Bowe, D.B., Paterson, A.J., and Kudlow, J.E. (2003) *O*-GlcNAc modification is an endogenous inhibitor of the proteasome. *Cell*, **115**, 715–725.

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# Modulation of O-GlcNAc Glycosylation During *Xenopus* Oocyte Maturation

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**Abstract** O-linked *N*-acetylglucosamine (O-GlcNAc) glycosylation is a post-translational modification, which is believed antagonises phosphorylation. We have studied the O-GlcNAc level during *Xenopus* oocyte meiotic resumption, taking advantage of the high synchrony of this model which is dependent upon a burst of phosphorylation. Stimulation of immature stage VI oocytes using progesterone was followed by a $4.51 \pm 0.32$ fold increase in the GlcNAc content, concomitantly to an increase in phosphorylation, notably on two cytoplasmic proteins of 66 and 97 kDa. The increase of O-GlcNAc for the 97 kDa protein, which we identified as β-catenin was partly related to its accumulation during maturation, as was demonstrated by the use of the protein synthesis inhibitor—cycloheximide. Microinjection of free GlcNAc, which inhibits O-glycosylated proteins–lectins interactions, delayed the progesterone-induced maturation without affecting the O-GlcNAc content. Our results suggest that O-GlcNAc glycosylation could regulate protein–protein interactions required for the cell cycle kinetic. J. Cell. Biochem. 93: 999–1010, 2004. 

**Key words:** oocyte; *Xenopus*; maturation; O-GlcNAc; beta-catenin

O-linked *N*-acetylglucosamine (O-GlcNAc) glycosylation is the major glycosylation type found within the nucleus and the cytosolic compartment of eukaryota [for review, see Wells et al., 2001]. O-GlcNAc consists in the addition of a single residue of *N*-acetylglucosamine to the hydroxyl groups of serine and threonine. This type of glycosylation shares similar features with protein phosphorylation [Haltiwanger et al., 1997], and the occurrence of an antagonism between phosphorylation and O-GlcNAc glycosylation was demonstrated. The existence of a direct competition between O-GlcNAc and phosphate to occupy the same sites on proteins was reported [Haltiwanger et al., 1998; Griffith and Schmitz, 1999; Lefebvre et al., 1999]. The emergence of an O-GlcNAc/phosphorylation balance leads to the question of its biological significance: if the role of phosphorylation is well documented and generally contributes to the regulation of the protein activity, the particular role of O-GlcNAc glycosylation is still not well understood. For example, whereas the reciprocity between O-GlcNAc and O-Phosphate on the carboxyl terminal domain (CTD) of RNA polymerase II was studied in detail, the phosphorylation of the CTD is associated with

Abbreviations used: O-GlcNAc, O-linked *N*-acetylglucosamine; GVBD, germinal vesicle breakdown; PNGase F, peptide *N*-glycosidase F; WGA, wheat germ agglutinin; PNA, peanut agglutinin; TBS, tris buffered saline; BSA, bovine serum albumin; AMP, adenosine 5′-monophosphate; MPF, maturation promoting factor; MAPK, mitogen activated protein kinase; CHX, cycloheximide.

Tony Lefebvre and Frédéric Baert are co-authors.

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promoter clearance, stabilization of elongation complexes and with the involvement of the mRNA processing machinery—the function of O-GlcNAc glycosylation of the CTD remains speculative [Comer and Hart, 2001].

Changes in the level of glycosylation in several nuclear and cytoplasmic proteins were observed during the mitogenic activation of both T-lymphocytes and T-cell hybridoma. In T-lymphocytes, O-GlcNAc levels change rapidly after stimulation to enter the cell cycle [Kearse and Hart, 1991]. In the human colon cell line HT 29, mitotic arrest with either okadaic acid or the microtubule destabilizing agent nocodazole, causes an increase in the O-GlcNAc levels of keratins [Haltiwanger and Philipsberg, 1997]. These observations suggest that O-GlcNAc glycosylation might be involved in the cell cycle regulation, while no other data corroborated this assumption. Many of the nuclear pore complexes, which are modified by single series of O-GlcNAc, are phosphorylated in a cell cycle specific manner, though the levels of O-GlcNAc remain constant [Miller et al., 1999]. Other experiments were led to investigate into the role of O-GlcNAc during the cell division process through the microinjection of galactosyltransferase (GalT) into *Xenopus* oocytes arrested in prophase I. However, injected GalT was toxic for oocytes stimulated to resume meiosis [Fang and Miller, 2001].

*Xenopus* oocyte offers opportunities to study the M-phase regulation that is highly controlled by specific kinases and phosphatases. Full-grown *Xenopus* stage VI oocytes are arrested at the prophase of the first division of meiosis in a $G_2$-like state. In response to progesterone, oocytes undergo germinal vesicle breakdown (GVBD), chromosomes condensation, and spindle formation. The end of the first division of meiosis is attested by the extrusion of the first polar body. The second division resumes, but it is arrested at metaphase [Hausen and Riebesell, 1991]. This process, also called maturation, is characterized by the simultaneous activation of two pathways. On one hand, the M-phase Promoting Factor (MPF; p34Cdc2/Cyclin B) that regulates both the entry and exit of the M-phase is activated through the dephosphorylation of its catalytic subunit by a dual-specificity phosphatase Cdc25 [Masui, 2001]. On the other hand, the activation of the Mos/MEK/Xp42Mpk1 Mitogen Activated Protein Kinases (MAPK) cascade depends upon Mos accumulation; and the effects of this pathway are mainly mediated through p90Rsk [Gotoh and Nishida, 1995; Gross et al., 2000].

Since phosphorylation is well known as means to regulate the cell cycle progression and since O-GlcNAc could antagonize phosphorylation, we assumed that O-GlcNAc variations could also occur during the meiosis resumption and that O-glycosylated proteins–lectins interactions might influence the $G_2$/M transition in *Xenopus laevis* oocytes. Thus, we studied the O-GlcNAc level during *Xenopus* oocyte maturation, taking advantage of the high and natural synchrony of this model [Ferrell, 1999]. Moreover, we observed the O-GlcNAc glycosylation of several proteins during maturation. Among them, we identified β-catenin which is phosphorylated by the Glycogen synthase kinase-3 (GSK3) and lead to its degradation. In return, the stabilization of β-catenin through the inactivation of GSK3 is essential for the dorsal determination in *Xenopus* embryos [Heasman et al., 1994]. Moreover, we have shown that the microinjection of free GlcNAc delayed progesterone-induced maturation, thereby suggesting a role for O-GlcNAc mediated interaction in the cell cycle.

## MATERIALS AND METHODS

### Handling of Oocytes

Stage VI *Xenopus* oocytes were isolated and prepared as previously described [Flament et al., 1996]. Briefly, prophase I-arrested oocytes were obtained from animals that were not primed with any gonadotropins. Defolliculated oocytes were kept in ND96 medium (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.5). Progesterone was added at a final concentration of 10 μM. Percentages of maturation were marked by the appearance of a white spot at the animal pole of the oocyte and GVBD was confirmed via microscopically examination after the dissection of heat-fixed oocytes. For biochemical analysis, oocytes were homogenized in β glycerophosphate, 60 mM; paranitrophenylphosphate, 15 mM; MOPS, 25 mM; EGTA, 15 mM; $MgCl_2$, 15 mM; DTT, 2 mM; sodium orthovanadate, 1 mM; NaF, 1 mM and proteases inhibitors; pH 7.2 [Flament et al., 1996] and centrifuged at 10,000 g for 10 min to eliminate yolk and membranous pellet. All the experiments were performed at least in duplicate.

### Enucleation

Immature oocytes were pricked at the animal pole and placed in ND96 diluted four times. Under such conditions, the germinal vesicle that is located in the animal hemisphere went out of the cell. Enucleated oocytes were then replaced in normal ND96 for recovery, at least 1 h prior to treatment.

To check the efficiency of oocytes enucleation, we used an antibody raised against p62 (sc-1916, Santa Cruz Biotechnology, Santa Cruz, CA), a nuclear pore specific protein. After nitrocellulose saturation with milk, the antibody was used at a dilution of 1:1,000 overnight at 4°C, and the horseradish peroxidase-coupled anti-goat secondary antibody was used at a dilution of 1:2,000 for 1 h (Dako, Glostrop, Denmark).

### Cytological Analysis

Oocytes were fixed overnight in Smith's fixative, dehydrated and embedded in paraffin. Sections (7 μm thick) were stained with nuclear red to detect nuclear structures and chromosomes and with picroindigo-carmine, which reveals cytoplasmic and microtubular structures. This method is precise enough to detect spindles and condensed chromosomes, even if they are not located near the plasma membrane [Flament et al., 1996].

### Western Blotting

**Analysis of the O-GlcNAc content.** The proteins were run on a 10% SDS–PAGE and were electrophoretically transferred onto nitrocellulose membrane. Blots were saturated in 3% bovine serum albumin (BSA) in TBS-Tween (15 mM Tris, 140 mM NaCl, 0.05% (v/v) Tween, pH 8.0) for 45 min and washed three times for 15 min with TBS-Tween. Before wheat germ agglutinin (WGA) staining, samples were systematically digested with PNGase F in order to remove N-linked oligosaccharides by direct incubation of the nitrocellulose sheets in 5–20 ml Phosphate Buffer (pH 7.5–50 mM) containing 2,500–10,000 U PNGase F overnight [Lefebvre et al., 1999]. Desialylation was performed with formic acid pH 2.0, for 30 min at 80°C. Proteins were analyzed for their O-GlcNAc status via Western blotting using horseradish peroxidase-coupled WGA (HRP–WGA) was incubated in TBS-Tween (1:10,000) for 1 h, and horseradish peroxidase was detected with ECL (Amersham Biosciences, Saclay, France). In the same way, the occurrence of O-glycans was assessed by the use of peanut agglutinin (PNA).

The anti-O-GlcNAc antibody (RL2, Affinity Bioreagents, Golden, CO) was used at a dilution of 1:1,000 over night at 4°C, after the nitrocellulose sheets were saturated with milk. The horseradish peroxidase-coupled anti-mouse secondary antibody was used for 1 h at a dilution of 1:10,000 (Amersham Biosciences). For immunoprecipitation, we used RL2 at a dilution of 1:200 for 2 h at 4°C, and protein G sepharose was finally added for 1 h at 4°C for the recovery of the O-GlcNAc proteins.

The phosphorylation status of proteins extracted from $G_2$ and M-phase arrested oocytes has been analyzed using an anti-phosphoserine (1:200 dilution, Sigma, Saint Quentin Fallavier, France). Alkaline phosphatase coupled anti-mouse antibody was used as the secondary antibody.

**Analysis of β-catenin content.** Oocyte homogenates were prepared and proteins were submitted to Western blot analysis as previously reported [Bodart et al., 1999]. β-catenin was detected using antibody H102 (Santa Cruz Biotechnology) (1:500). After washes in TBS, horseradish peroxidase-coupled goat anti-rabbit IgG antiserum (1:5,000 in 2.5% of non-fatty milk in TBS) was incubated for 1 h. In order to inhibit GSK-3β, oocytes were transferred to ND96 containing 20 mM LiCl [Fisher et al., 1999] before homogenization or progesterone addition.

**Analysis of the O-GlcNAc content using gas phase chromatography.** Mature and immature oocytes extracts were submitted to a reductive β-elimination in 0.1 M NaOH and 1 M potassium borohydride at 65°C overnight. The reaction was stopped by addition of ice-cold acetic acid under vigorous stirring until the pH value of 5.0 was reached. The β-eliminated material was dried several times under vacuum with anhydrous methanol in order to remove borate in its methyl ester form. The released saccharides were peracetylated in anhydride acetic for 4 h at 95°C, dried and finally extracted in chloroform (the extraction with chloroform was performed twice, and this volume was washed four times with water). After drying under nitrogen and adding 100 μl of chloroform, 2 μl of the peracetyled saccharides were analyzed using gas liquid chromatography

[Lefebvre et al., 2001]. Analysis was performed on a BPX column (30 m × 0.32 mm) at an initial temperature of 150°C, with a gradient of 3°C/min to 230°C, then with a gradient of 5°C/min to 250°C, and finally with a plateau of 5 min at 250°C.

**Oocyte microinjection.** Microinjections were performed in the equatorial region of the oocytes, using a positive displacement digital micropipette (Nichiryo). Free GlcNAc or Gal microinjections were performed in order to obtain a final concentration of 10 nmol per oocyte. To trigger the MPF activation and GVBD, GST-Cdc25A was injected into immature oocytes at a final concentration of 1.2 μM.

## RESULTS

### O-GlcNAc Content in Immature Versus Mature Oocytes

In a first set of experiments, immature oocytes ($G_2$-like phase-arrested) and mature oocytes (metaphase II-arrested) obtained after treatment with 10 μM progesterone during 16 h, were compared for their O-GlcNAc content. After treatments with PNGase F for deglycosylation of the N-linked glycans and formic acid for desialylation, proteins were separated on a 10% SDS–PAGE and checked for the presence of O-GlcNAc using WGA staining (Fig. 1Aa). A competition assay with an excess of free GlcNAc was done in conjunction with WGA (Fig. 1Ab). In this case, staining by WGA disappeared, demonstrating that it was specifically bound to O-GlcNAc proteins. We showed that the progesterone treatment induced an increase in the O-GlcNAc content of several different proteins in the *Xenopus* oocyte. Two proteins of 97 and 66 kDa were mainly concerned (Fig. 1Aa). PNA was used after formic acid desialylation to assess the absence of contaminant O-Glycans (Fig. 1Ac). Finally, the confirmation that the glycosylation borne by the proteins of 97 and 66 kDa was actually O-GlcNAc was done with a specific anti-O-GlcNAc antibody (RL2, Fig. 1Ae). It is to be noted that WGA stained more bands than the anti-O-GlcNAc antibody RL2 (compare panels a and e). This observation is not new and has already been reported [Comer et al., 2001]. The WGA lectin and the RL2 monoclonal antibody did not stained exactly the same motif: the lectin only needs the saccharide moiety for the binding, whereas the RL2 antibody needs not only the saccharide, but also a peptidic backbone. In these conditions, if the peptidic environment of the protein is too different from the peptidic environment against which this antibody was originally raised [Snow et al., 1987], the recognition might not occur.

We performed the characterization and the quantification of the GlcNAc release after the β-elimination of the two extracts and the analysis of the peracetylated released saccharides via gas phase chromatography (Fig. 1B). The integration of the relevant GlcNAc peak areas (Fig. 1Ba) showed a $4.51 \pm 0.32$ fold increase in the O-GlcNAc content in mature oocytes (the calculated values were normalized to an equal protein amount assayed using the Bradford method).

In order to compare the O-GlcNAc profile with the phosphorylation status of immature and matured oocytes, we performed Western blotting using WGA, anti-phosphoserine and anti-O-GlcNAc antibodies. These profiles show that the induction of oocyte maturation with progesterone induces an increase both on the O-GlcNAc (Fig. 1Cc,Cd) and on the phosphorylation (Fig. 1Cb) status of proteins. We must note that interestingly, the two proteins of particular interest, i.e., 66 and 97 kDa, also appear to be phosphorylated and that this phosphorylation was increased by the $G_2$/M transition, especially for the 97 kDa one (arrowheads).

WGA staining on mature enucleated oocytes extracts demonstrated that a majority of proteins exhibiting O-GlcNAc modulation during maturation were localized in the cytoplasm (Fig. 2A). Indeed, we did not observe any difference in the O-GlcNAc proteins profile between the total oocyte and the enucleated oocyte. This result is interesting as many kinases/phosphatases that are involved during meiosis are also cytosolic components. The enucleation efficiency was demonstrated using an antibody raised against a specific nuclear pore protein, p62. The latter was not detected in enucleated oocytes extracts, confirming the absence of any nuclear components (Fig. 2C). Then, O-GlcNAc glycosylation observed in enucleated oocytes as well as in intact cells are representative of cytosolic proteins, and not nuclear proteins.

### O-GlcNAc Modified Proteins During the Maturation Process

In a second set of experiments, modifications of cellular proteins' O-GlcNAc levels during

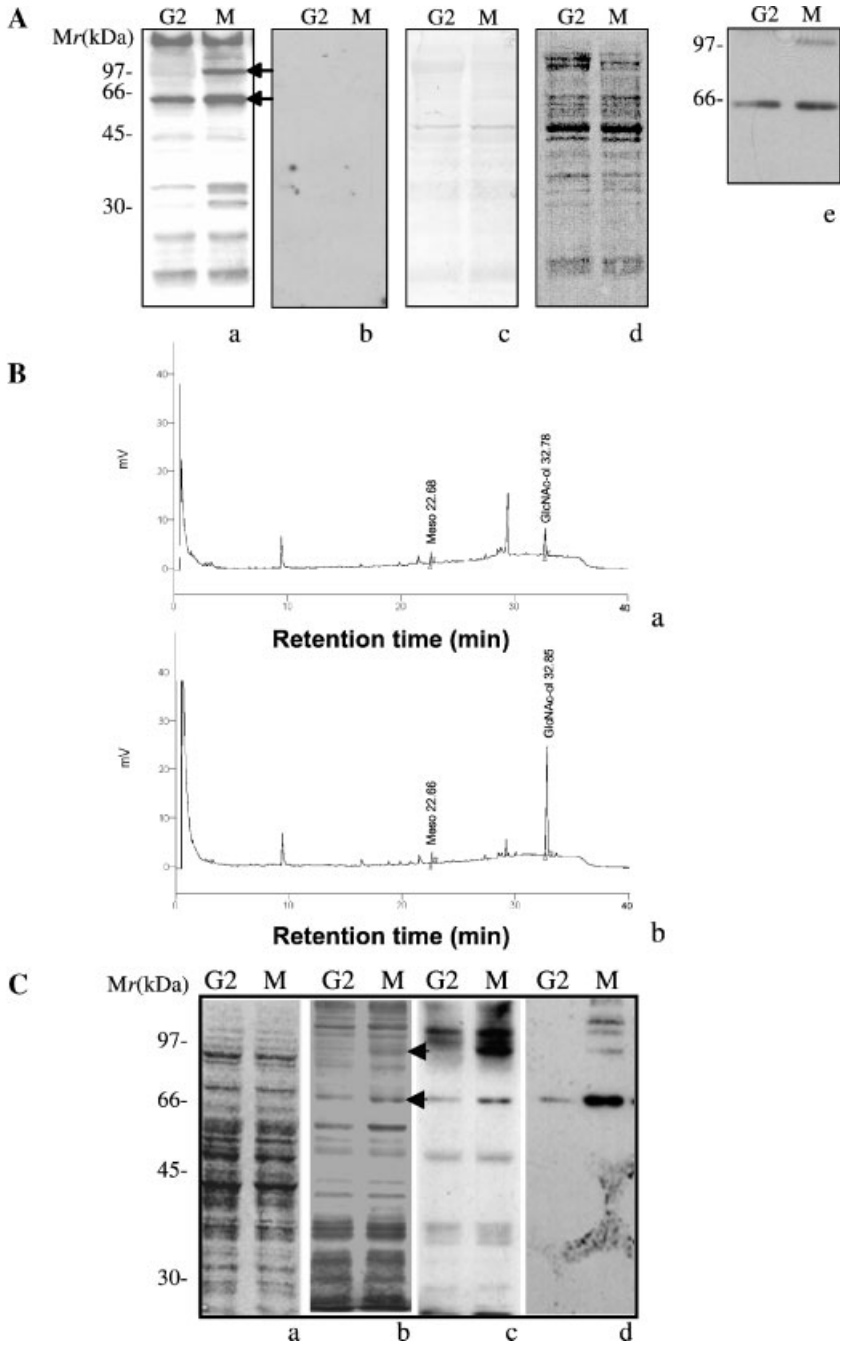


**Fig. 1.** O-linked *N*-acetylglucosamine (O-GlcNAc) content analysis of immature and mature oocytes. **A**: Immature ($G_2$) and mature (M) oocytes were homogenised in lysis buffer. After PNGase F and formic acid treatment, proteins were run on a 10% SDS–PAGE. **Aa**: The blot was then analyzed for the O-GlcNAc content by wheat germ agglutinin (WGA) staining. It revealed an increase in the O-GlcNac content and especially on two proteins of 97 and 66 kDa (Aa, arrows). **Ab**: In the presence of an excess of free GlcNAc, no more staining was observed with WGA, which demonstrated the specificity for O-GlcNAc. **Ac**: Peanut agglutinin (PNA) staining was done to assess the absence of contaminant O-Glycans. **Ad**: Red ponceau staining showing equality of the quantity of proteins in the lanes. **Ae**: To definitely assert the presence of O-GlcNAc glycosylation, we also used RL2, a specific antibody raised against O-GlcNAc. **B**: The same extracts were submitted to a reductive β-elimination and the peracetylated released saccharides were analyzed and quantified by gas phase chromatography (**Ba**, immature oocytes; **Bb**, mature oocytes). The peak corresponding to peracetylated GlcNAc-ol is indicated. Mesoinositol (Meso) was used as an internal standard. After integration, the GlcNAc peak area of mature oocytes appeared to be $4.51 \pm 0.32$ times more important than the peak area of immature oocytes. **C**: The phosphorylation status of immature and mature oocytes was tested using an anti-phosphoserine directed antibody (**Cb**) in conjunction with peroxidase-labeled WGA (**Cc**) and with the anti-O-GlcNAc antibody (**Cd**). The panel Ca shows the ponceau red staining.

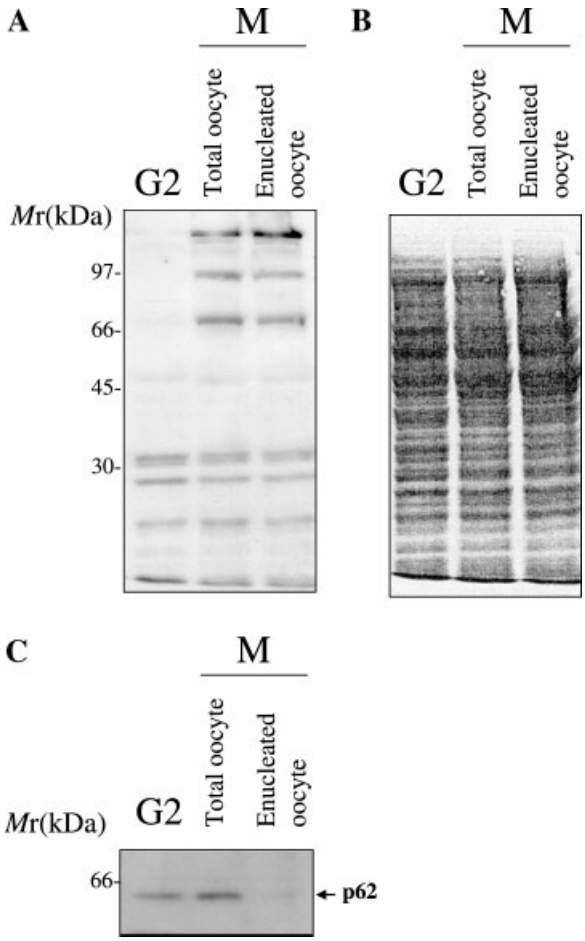


**Fig. 2.** Localization of the proteins that have their O-GlcNAc modulated during maturation. **A**: WGA staining and (**B**) ponceau red staining. Stage VI oocytes were manually enucleated before treatment with progesterone. The lysates were further analyzed for their O-GlcNAc content as described in Figure 1. Following progesterone stimulation, changes in O-GlcNAc glycosylation occurred in these enucleated oocytes (compare with immature oocytes: $G_2$) as was already observed in Figure 1Aa. The profile was not different from those observed in mature nucleated oocytes (total oocyte). **C**: We assessed for correct enucleation of oocytes using an antibody raised against p62, a specific nuclear pore protein. Lack of p62 staining confirmed the absence of any nuclear components.

meiosis were monitored. Kinetic maturation experiments were performed on oocytes after a hormonal stimulation using progesterone: oocytes were taken out at different times and homogenized. Numerous proteins (indicated with arrows in Fig. 3) showed modifications of their O-GlcNAc content during the meiosis resumption. For example, proteins at 150 and at 97 kDa reached a maximum in O-GlcNAc glycosylation 6–8 h after the progesterone addition. Then, their O-GlcNAc level decreased at the end of the maturation. The 66 kDa protein was already O-GlcNAc glycosylated before the progesterone treatment, but this O-glycosylation increased 2 h after the progesterone application and then sharply decreased until the end of meiosis. Lastly, the O-GlcNAc glycosylation of a 30 kDa protein decreased during maturation and increased at the end of the maturation process.

### Free GlcNAc Injection Delays Maturation in *Xenopus* Oocytes

Since oocyte maturation was accompanied by a modulation in O-GlcNAc glycosylation, the potentially inhibiting effect of free GlcNAc on maturation stages was evaluated after the microinjection of this monosaccharide into oocytes (Fig. 4). GlcNAc could not enter the hexosamine biosynthetic pathway, contrary to glucose or glucosamine, and thus could not take part in the synthesis of UDP-GlcNAc for the O-GlcNAc events. Besides, it is well-known that O-GlcNAc transferase is unaffected by GlcNAc [Haltiwanger et al., 1990]. After the microinjection of free GlcNAc, we observed that maturation was delayed. From the three experiments reported in Figure 4, we concluded that $GVBD_{50}$ (the time required for 50% of the oocytes to undergo GVBD) occurred 4 h later than in control oocytes injected with water (Fig. 4A). As a negative control, free galactose was microinjected before the progesterone treatment and it did not delay maturation, and such treated oocytes exhibited the same kinetics of GVBD than water-injected oocytes. As was expected, WGA staining did not shows any significant differences in the O-GlcNAc content among oocytes in the three batches (Fig. 4B).

In order to determine the position in the cell cycle of GlcNAc injected oocytes, a cytological analysis was performed 4 h after the $GVBD_{50}$ of control oocytes injected with galactose. The latter exhibited a metaphase II spindle with condensed chromosomes, located on the plasma membrane, and a polar body (Fig. 5A), as water-injected oocytes did (data not shown). At the same time, cytological sections of GlcNAc-injected oocytes showed either oocytes with a typical metaphase II block (Fig. 5B) or oocytes with a metaphase I spindle and no polar body (Fig. 5C). Thus, though GlcNAc injection delayed progesterone-induced maturation, it did not seem to alter the condensation of chromosomes or the meiotic spindle morphogenesis.

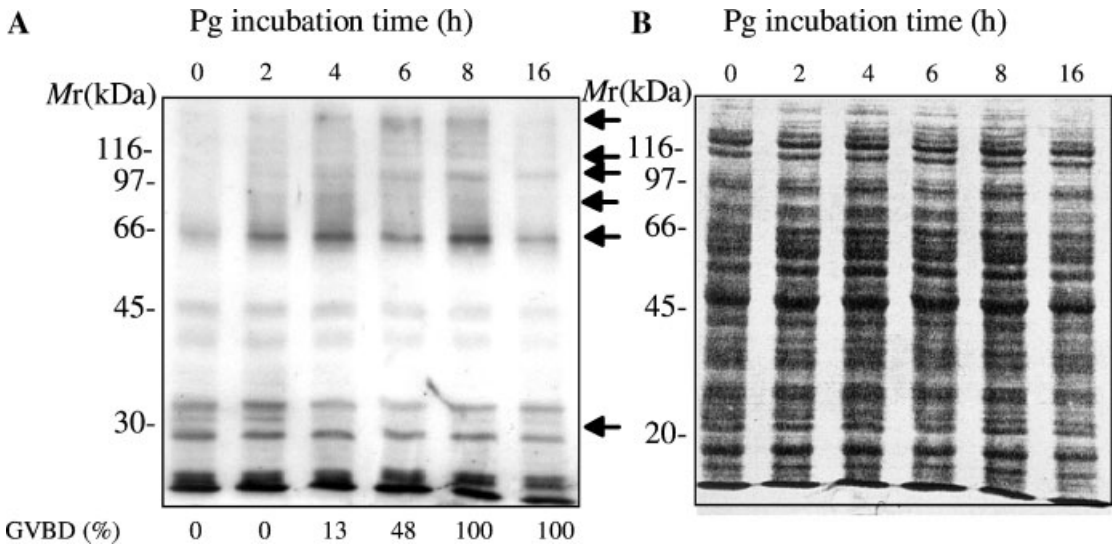


**Fig. 3.** O-GlcNAc glycosylation during the maturation process. The O-GlcNAc content of oocytes at different times of the maturation was analyzed as indicated in Figure 1. The WGA staining is shown in Figure 3A, and ponceau red staining in Figure 3B. Arrows indicate the main profile changes observed by WGA staining. The evolution of O-GlcNAc glycosylation was not similar for all proteins. The 97 kDa protein showed a maximum in O-GlcNAc glycosylation 6–8 h after the progesterone addition. Then, the latter decreased but remained higher than in immature oocytes. The 66 kDa protein was already glycosylated in immature oocytes and this glycosylation increased as early as 2 h after the progesterone application; it sharply decreased at the end of the maturation. The 30 kDa protein showed an O-glycosylated profile in immature oocytes, and this glycosylation decreased after the progesterone treatment.

### Synthesized β-catenin Is O-GlcNAc Modified During *Xenopus* Oocyte Maturation

In order to check whether the increase in the O-GlcNAc content observed during maturation was related to an increase in the synthesis of the corresponding proteins, oocytes were induced to mature in the presence of cycloheximide (CHX, 50 μg/ml) a protein synthesis inhibitor. Since progesterone-induced maturation is dependent upon protein synthesis, maturation was triggered by micro-injection of GST-Cdc25A in this set of experiments. Cdc25 is a dual specificity phosphatase that directly dephosphorylates and activates $p34^{Cdc2}$, the catalytic subunit of the M-phase promoting factor. Results are

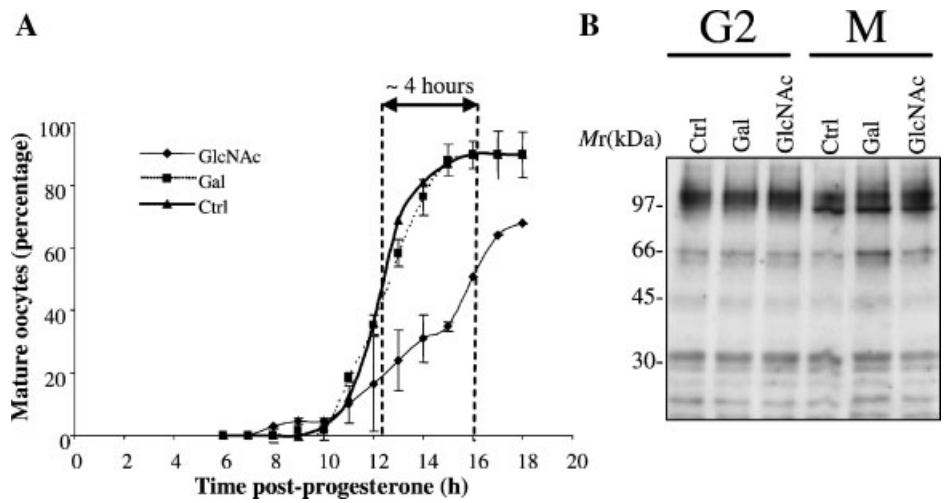


**Fig. 4.** Effect of GlcNAc microinjection on oocyte maturation. **A**: The progesterone treatment was performed after the control microinjection of either water (-▲-) or free galactose (-■-) or after the microinjection of free GlcNAc (-◆-). Maturation was assessed using white spot examination. The results are from three different experiments. **B**: WGA staining of oocytes extracts. The injection of either Gal or GlcNAc did not modify the O-GlcNAc profile in immature oocytes ($G_2$). This did not inhibit the occurrence of the changes of O-GlcNAc glycosylation usually observed in mature oocytes (M).

described in Figure 6A. In the absence of CHX, the injection of Cdc25A induced the changes in O-GlcNAc usually observed following progesterone stimulation (see Fig. 1Aa). The same changes were still observed in the presence of CHX for most of the proteins, except for the protein with an apparent molecular weight of 97 kDa, which was just slightly glycosylated. So, based on the observations that the O-GlcNAc glycosylation of the 97 kDa protein was related in part to its accumulation during maturation (i.e., its synthesis) and according to its apparent molecular weight, we assumed that the protein was β-catenin. This hypothesis was reinforced by the description of the O-GlcNAc modification of β-catenin [Zhu et al., 2001]. In fully grown immature oocytes, the glycogen synthase kinase-3β (GSK-3β) is active, and it must be inactivated to allow maturation [Fisher et al., 1999]. This kinase is responsible for β-catenin phosphorylation, which is the signal for its degradation using the proteasome pathway. The inactivation of GSK-3β allows β-catenin to accumulate in mature oocytes [Fisher et al., 1999]. O-GlcNAc proteins were enriched on agarose beads-coupled WGA, run on SDS–PAGE, transferred and stained with the anti-β-catenin antibody. As shown with the RL2 antibody (Fig. 6B), after WGA-enrichment, β-catenin was better detected in mature oocytes (M-phase) compared to immature oocytes ($G_2$-phase). LiCl, also known as a GSK-3β inhibitor, was used to accumulate β-catenin [Hedgepeth et al., 1997; Davies et al., 2000; Davies et al., 2001]. When oocytes were treated with LiCl, we found greater amounts of glycosylated β-catenin both in mature and immature oocytes. To confirm that the identity of the 97 kDa-protein was actually β-catenin, we performed immunoprecipitations with the anti-O-GlcNAc RL2 antibody on immature and mature extracts. The immunoprecipitated proteins were separated on a 10% SDS–PAGE, electrotransfered on nitrocellulose and stained with an anti-β-catenin antibody (Fig. 6C). These immunoprecipitations clearly showed the accumulation of β-catenin during the maturation process and

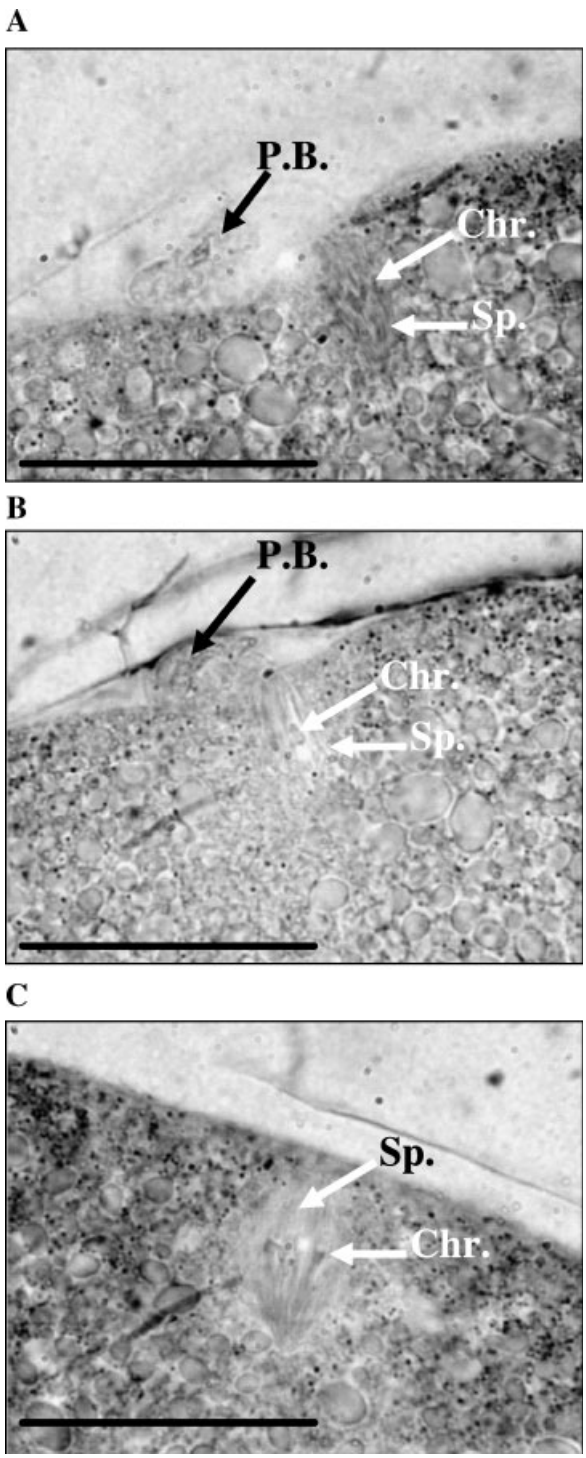


**Fig. 5.** Effects of free GlcNAc injection on the cytological events of the maturation. In this experiment, oocytes were fixed 4 h after control oocytes reached the $GVBD_{50}$ stage. **A**: Galactose-injected oocytes showing the usual aspect of mature oocytes with a polar body and a metaphase spindle with condensed chromosomes (arrows). **B**: Free GlcNAc injected oocytes showing a metaphase II status as in A. This was observed in 50% oocytes. **C**: Free GlcNAc injected oocytes with an unachieved maturation. Here is shown an example with a metaphase I spindle with condensed chromosomes but no polar body. Chr., condensed chromosomes; P.B., polar body; Sp., spindle. bar represents 50 μm.

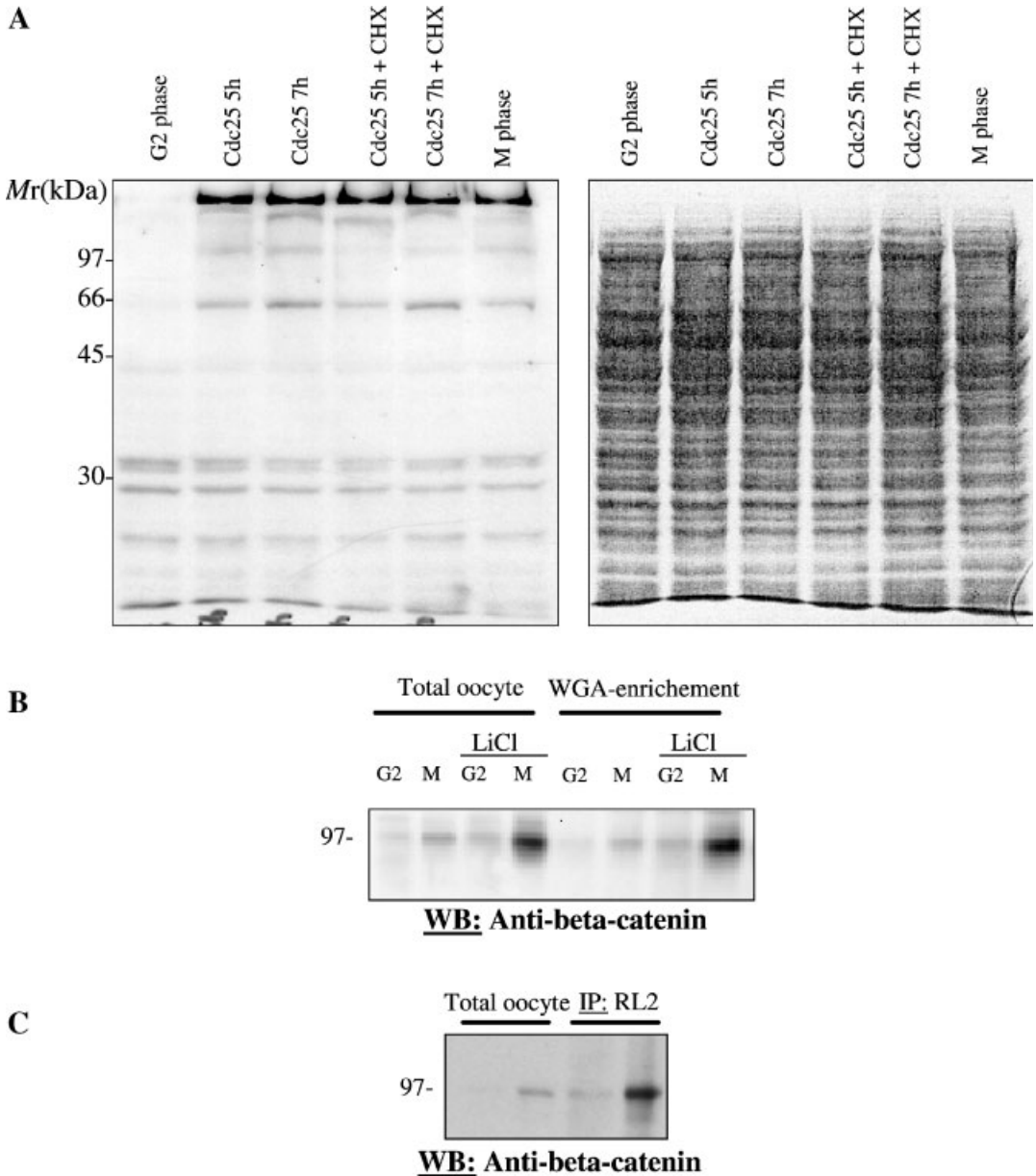


**Fig. 6.** Inhibition of protein synthesis and identification of the 97 kDa protein as β-catenin. **A**: Maturation was performed in the presence of the protein synthesis inhibitor, cycloheximide (CHX) and was triggered using microinjection of GST-Cdc25A. Oocytes were analyzed 5 and 7 h after the injection (**A**). A: **Left panel** represents the WGA staining as described in Figure 1A whereas the **right panel** represents the ponceau red staining. Cdc25 injection induced an increase in the O-GlcNAc glycosylation as was previously observed with progesterone. Except for the 97 kDa protein, the glycosylation was independent from the protein synthesis as CHX had no effect on it. **B**: Anti-β-catenin staining. β-catenin was strongly detected in the WGA-enriched fraction, proving that β-catenin was still present after WGA precipitation. The GSK-3β inhibitor, LiCl, induced an increase in the amount of β-catenin which was observed not only in total homogenates but also following WGA enrichment ($G_2$, immature oocytes; M, mature oocytes). **C**: Immunoprecipitation was performed with the anti-O-GlcNAc antibody (RL2) on the two extracts ($G_2$ and M), the bound proteins were separated on a 10% SDS–PAGE, electroblotted onto nitrocellulose and then stained with the anti–β-catenin.

concomitantly its O-GlcNAc glycosylation. This identification of β-catenin prompted us to make a further experiment. It seemed particularly interesting to investigate into the inhibition of the β-catenin phosphorylation on the O-GlcNAc glycosylation of the protein. These points unambiguously demonstrate that the 97 kDa glycosylated protein is β-catenin.

## DISCUSSION

The aim of this study was to investigate into the O-GlcNAc level of cellular proteins during *Xenopus* oocyte maturation. *Xenopus* oocytes were widely used to study the regulation of the M-phase entry that is triggered by well-characterized and well-conserved pathways of phosphorylations and dephosphorylations [Nebreda and Ferby, 2000]. Several reports have suggested that phosphorylation could be antagonized on the same site, or on an adjacent site, through O-GlcNAc glycosylation [Torres and Hart, 1984; Comer and Hart, 2000; Comer and Hart, 2001]. Hence, resumption of meiosis in oocytes, according to the phosphorylation/dephosphorylation cascade, might be accompanied by a modulation of the O-GlcNAc content of individual proteins, which could play a part in the cell cycle progression.

Unexpectedly, we observed that progesterone-induced oocyte maturation is correlated with an increase of O-GlcNAc glycosylation. Indeed, when Western blots were analyzed after WGA staining, several proteins exhibited an increase in their O-GlcNAc glycosylation in M-phase (mature) oocytes versus $G_2$-phase (immature) ones. The major changes were mainly shown by two proteins of 66 and of 97 kDa (the O-GlcNAc glycosylation for these two proteins was confirmed with the RL2 antibody). These observations were confirmed by gas phase chromatography, which showed a global increase in the O-GlcNAc content in mature oocytes ($\times 4.51 \pm 0.32$). This increase may be partly due to an increase in the O-GlcNAc transferase (OGT) activity: measurements performed on the OGT activity have shown a slight increase in this activity during the maturation process (data not shown). Similar changes in the O-GlcNAc content were observed when the M-phase entry was triggered by the microinjection of Cdc25 that is a direct activator of $p34^{Cdc2}$. The importance of the O-GlcNAc glycosylation-mediated phenomenon for oocyte maturation was strongly suggested by the noticeable delay observed in the maturation process following the microinjection of free GlcNAc (4 h delay).

The conclusion drawn from these results seems to disagree with a competition between phosphorylation and O-GlcNAc for the same sites. Indeed, since maturation is mainly associated with a burst in phosphorylation due to the activation of many kinases (Mos, MEK1, $p42^{Erk2}$, $p90^{rsk}$, $p34^{Cdc2}$, Cdc25C, Plx1, Plkk1; for review, see [Nebreda and Ferby, 2000]), we could have expected to observe a decrease in the O-GlcNAc content. However, such a conclusion should be qualified. First, because the analyses that were performed during time-course experiments showed that the changes in O-GlcNAc were rather complex. For instance, a protein of 30 kDa showed a transient decrease in its O-GlcNAc glycosylation. Secondly, even if a burst of phosphorylation accompanies $G_2$/M transition in *Xenopus* oocytes, kinases such as PKA or GSK-3β have to be inhibited for cell cycle progression [Maller and Krebs, 1977; Fisher et al., 1999]. Interestingly, we characterized the protein of 97 kDa as β-catenin. In immature oocytes, when β-catenin is phosphorylated by GSK-3β, this protein is degraded by an ubiquitin ligase-proteasome system [Aberle et al., 1997; Orford et al., 1997]. Following the progesterone stimulation, GSK-3β is inhibited, β-catenin is synthesized but no longer proteolysed and it consequently accumulates [Fisher et al., 1999]. Later, β-catenin accumulates on the future dorsal side of the *Xenopus* embryo due to the two-cell stage [Larabell et al., 1997]. We found high amounts of glycosylated β-catenin in mature oocytes as well as following treatments with the GSK3 inhibitor, LiCl. This result confirmed the O-GlcNAc glycosylation of β-catenin recently reported in MCF-7, MDA-MB-468, and MDCK cells as well as in 3T3-L1 adipocytes [Zhu et al., 2001; Vosseler et al., 2002]. In the oocyte, the increase in the O-GlcNAc content resulted from the accumulation and stabilization of the protein as was demonstrated by experiments using cycloheximide. This apparent correlation between the glycosylation of β-catenin and its stabilization is in line with the O-glycosylation of the Sp1 transcription factor since it was demonstrated that reduced O-GlcNAc of Sp1 increased the proteasome susceptibility [Han and Kudlow, 1997]. So, at least in the case of β-catenin, the increase in O-GlcNAc might be correlated to a decrease in phosphorylation.

We assumed that O-GlcNAc could promote interactions between several components required for the $G_2$/M transition and subsequent cytological events during oocyte meiosis. These interactions could be disturbed by the presence of high amounts of free GlcNAc in the oocyte and a similar approach was used by Fang and Miller [2001]. Such a role for the modulation of the

protein–protein interaction by O-GlcNAc has already been suggested. For example proteins that link the cytoskeleton to cellular membranes are O-GlcNAc modified but the exact function of O-GlcNAc in these interactions remains obscure [Hagman et al., 1992; Cole and Hart, 1999]. The best-known example is the interaction between Sp1, the TATA-binding-protein-associated factor (TAF110) and holo-Sp1. It was suggested that the removal of O-GlcNAc from an interaction domain was a signal for the protein association [Roos et al., 1997]. In this example O-GlcNAc may thereby prevent untimely and ectopic interactions. Conversely, our findings suggest that O-GlcNAc promotes protein–protein interactions that are involved in the cell cycle kinetic but not in spindle morphogenesis, nor chromosomes condensation.

As a conclusion, this study provides new evidence of the dynamic feature of the O-GlcNAc glycosylation during a cellular process and particularly during the cell cycle. Further experiments are required to investigate into the role of the O-glycosylation-phosphorylation balance in the cell cycle control. The role of O-GlcNAc might be important not only in the resumption of meiosis but also in the events that follow maturation: fertilization and early development. So, the role of the O-glycosylation of β-catenin is interesting since it is an important factor in the Wnt signaling pathway [Akiyama, 2000].

## ACKNOWLEDGMENTS

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## REFERENCES

Aberle H, Bauer A, Stappert J, Kispert A, Kemler R. 1997. Beta-catenin is a target for the ubiquitin-proteasome pathway. EMBO J 16:3797–3804.

Akiyama T. 2000. Wnt/β-catenin signaling. Cytokine Growth Factor Rev 11:273–282.

Bodart JF, Béchard D, Bertout M, Gannon J, Rousseau A, Vilain JP, Flament S. 1999. Activation of *Xenopus* eggs by the kinase inhibitor6-DMAP suggests a differential regulation of cyclin B2 and $p39^{Mos}$ proteolysis. Exp Cell Res 253:413–421.

Cole RN, Hart GW. 1999. Glycosylation sites flank phosphorylation sites on synapsin I: O-linked *N*-Acetylglucosamine residues are localized within domains mediating synapsin I interactions. J Neurochem 73: 418–428.

Comer FI, Hart GW. 2000. O-glycosylation of nuclear and cytosolic proteins. Dynamic interplay between O-GlcNAc and O-phosphate. J Biol Chem 275:29179–29182.

Comer FI, Hart GW. 2001. Reciprocity between O-GlcNAc and O-phosphate on the carboxyl terminal domain of RNA polymerase II. Biochemistry 40:7845–7852.

Comer FI, Vosseler K, Wells L, Accavitti MA, Hart GW. 2001. Characterization of a mouse monoclonal antibody specific for *O*-linked *N*-acetylglucosamine. Anal Biochem 293:169–177.

Davies SP, Reddy H, Caivano M, Cohen P. 2000. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 351:95–105.

Davies G, Jiang WG, Mason MD. 2001. The interaction between beta-catenin, GSK3beta and APC after motogen induced cell-cell dissociation, and their involvement in signal transduction pathways in prostate cancer. Int J Oncol 18:843–847.

Fang B, Miller MW. 2001. Use of galactosyltransferase to assess the biological function of O-linked *N*-acetyl-D-glucosamine: A potential role for O-GlcNAc during cell division. Exp Cell Res 263:243–253.

Ferrell JE, Jr. 1999. *Xenopus* oocyte maturation: New lessons from a good egg. BioEssays 21:833–842.

Fisher DL, Morin N, Doree M. 1999. A novel role for glycogen synthase kinase-3 in *Xenopus* development: Maintenance of oocyte cell cycle arrest by a beta-catenin-independent mechanism. Development 126:567–576.

Flament S, Browaeys E, Rodeau JL, Bertout M, Vilain JP. 1996. *Xenopus* oocyte maturation: Cytoplasm alkalization is involved in germinal vesicle migration. Int J Dev Biol 40:471–476.

Gotoh Y, Nishida E. 1995. The MAP kinase cascade: Its role in *Xenopus* oocytes, eggs, and embryos. Prog Cell Cycle Res 1:287–297.

Griffith LS, Schmitz B. 1999. O-linked *N*-acetylglucosamine levels is upregulated in Alzheimer brains. Eur J Biochem 262:824–831.

Gross SD, Schwab MS, Taieb FE, Lewellyn AL, Qian YW, Maller JL. 2000. The critical role of the MAP kinase pathway in meiosis II in *Xenopus* oocytes is mediated by p90 (Rsk). Curr Biol 10:430–438.

Hagman J, Grob M, Burger MM. 1992. The cytoskeletal protein talin is O-glycosylated. J Biol Chem 267:14424–14428.

Haltiwanger RS, Philipsberg GA. 1997. Mitotic arrest with nocodazole induces selective changes in the level of O-linked *N*-acetylglucosamine and accumulation of incompletely processed N-Glycans on proteins from HT-29 cells. J Biol Chem 272:8752–8758.

Haltiwanger RS, Holt GD, Hart GW. 1990. Enzymatic addition of O-GlcNAc to nuclear and cytoplasmic proteins, identification of a uridine diphospho-*N*-acetylglucosamine: Peptide β-*N*-acetylglucosaminyltransferase. J Biol Chem 265:2563–2568.

Haltiwanger RS, Busby S, Grove K, Li S, Mason D, Medina L, Moloney D, Philipsberg G, Scartozzi R. 1997. O-glycosylation of nuclear and cytoplasmic proteins:

Regulation analogous to phosphorylation? Biochem Biophys Res Commun 231:237–242.

Haltiwanger RS, Grove K, Philipsberg GA. 1998. Modulation of O-linked *N*-acetylglucosamine levels on nuclear and cytoplasmic proteins in vivo using the peptide O-GlcNAc-b-*N*-acetylglucosaminidase inhibitor *O*-(2-acetamido-2-deoxy-D glucosaminopyranossylidene) amino-*N*-phenylcarbamate. J Biol Chem 273:3611–3617.

Han I, Kudlow JE. 1997. Reduced O-glycosylation of Sp1 is associated with increased proteasome susceptibility. Mol Cell Biol 17:2550–2558.

Hausen P, Riebesell M. 1991. The early development of *Xenopus laevis*. An atlas of histology. Berlin: Springer-Verlag.

Heasman J, Crawford A, Goldstone K, Garner-Hamrick P, Gumbiner B, McCrea P, Kintner C, Yoshida-Noro C, Wilie C. 1994. Overexpression of cadherins and underexpression of β-catenin inhibit dorsal mesoderm induction in early *Xenopus* embryos. Cell 79:791–803.

Hedgepeth CH, Conrad LJ, Zhang J, Huang HC, Lee VMY, Klein PS. 1997. Activation of the Wnt signaling pathway: A molecular mechanism for lithium action. Dev Biol 185: 82–91.

Kearse KP, Hart GW. 1991. Lymphocyte activation induces rapid changes in nuclear and cytoplasmic glycoproteins. Proc Natl Acad Sci USA 88:1701–1717.

Larabell CA, Torres M, Rowning BA, Yost C, Miller JR, Wu M, Kimelman D, Moon RT. 1997. Establishment of the dorso-ventral axis in *Xenopus* embryos is presaged by early asymmetries in β-catenin that are modulated by the Wnt signaling pathway. J Cell Biol 136:1123–1136.

Lefebvre T, Alonso C, Mahboub S, Dupire MJ, Zanetta JP, Caillet-Boudin ML, Michalski JC. 1999. Effect of okadaic acid on O-linked *N*-acetylglucosamine levels in a neuroblastoma cell line. Biochim Biophys Acta 1472: 71–81.

Lefebvre T, Cieniewski C, Lemoine J, Guerardel Y, Leroy Y, Zanetta JP, Michalski JC. 2001. Identification of *N*-acetyl-D-glucosamine-specific lectins from rat liver cytosolic and nuclear compartments as Heat-shock proteins Biochem J 360:179–188.

Maller JL, Krebs EG. 1977. Progesterone-stimulated meiotic cell division in *Xenopus* oocytes. Induction by regulation subunit and inhibition by catalytic subunit 3′, 5′-monophosphate dependent protein kinase. J Biol Chem 252:1712–1718.

Masui Y. 2001. From oocytes maturation to the in vitro cell cycle: The history of discoveries of maturation-promoting factor (MPF) and cytostatic factor (CSF). Differentiation 69:1–17.

Miller MW, Caracciolo MR, Berlin WR, Hanover JA. 1999. Phosphorylation and glycosylation of nucleoporins. Arch Biochem Biophys 367:51–60.

Nebreda AR, Ferby I. 2000. Regulation of the meiotic cell cycle in oocytes. Curr Opin Cell Biol 12:666–675.

Orford K, Crockett C, Jensen JP, Weissman A, Byers SW. 1997. Serine phosphorylation-regulated ubiquitination and degradation of beta-catenin. J Biol Chem 272:24735–24738.

Roos MD, Su K, Baker JR, Kudlow JE. 1997. O-glycosylation of an Sp1-derived peptide blocks the removal of O-GlcNAc. Mol Cell Biol 17:6472–6480.

Snow CM, Senior A, Gerace L. 1987. Monoclonal antibodies identify a group of nuclear pore complex glycoproteins. J Cell Biol 104:1143–1156.

Torres CR, Hart GW. 1984. Topography and distribution of terminal *N*-acetylglucosamine residues on the surface of intact lymphocytes, evidence of O-linked GlcNAc. J Biol Chem 259:3308–3317.

Vosseler K, Wells L, Lane MD, Hart GW. 2002. Elevated nucleocytoplasmic glycosylation by O-GlcNAc results in insulin resistance associated with defects in Akt activation in 3T3-L1 adipocytes. Proc Natl Acad Sci USA 99: 5313–5318.

Wells L, Vosseler K, Hart GW. 2001. Glycosylation of nucleocytoplasmic proteins: Signal transduction and O-GlcNAc. Science 291:2376–2378.

Zhu W, Leber B, Andrews DW. 2001. Cytoplasmic O-glycosylation prevents cell surface transport of E-cadherin during apoptosis. EMBO J 20:5999–6007.

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# The tumor suppressor HIC1 (hypermethylated in cancer 1) is *O*-GlcNAc glycosylated

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*HIC1* (hypermethylated in cancer 1) is a transcriptional repressor containing five Krüppel-like $C_2H_2$ zinc fingers and an N-terminal dimerization and autonomous repression domain called BTB/POZ. Here, we demonstrate that full-length HIC1 proteins are modified both *in vivo* and *in vitro* with O-linked *N*-acetylglucosamine (*O*-GlcNAc). This is a highly dynamic glycosylation found within the cytosolic and the nuclear compartments of eukaryotes. Analysis of [$^3$H]Gal-labeled tryptic peptides indicates that HIC1 has three major sites for *O*-GlcNAc glycosylation. Using C-terminal deletion mutants, we have shown that *O*-GlcNAc modification of HIC1 proteins occurred preferentially in the DNA-binding domain. Nonglycosylated and glycosylated forms of full-length HIC1 proteins separated by wheat germ agglutinin affinity purification, displayed the same specific DNA-binding activity in electrophoretic mobility shift assays proving that the *O*-GlcNAc modification is not directly implicated in the specific DNA recognition of HIC1. Intriguingly, N-terminal truncated forms corresponding to BTB-POZ-deleted proteins exhibited a strikingly differential activity, as the glycosylated truncated forms are unable to bind DNA whereas the unglycosylated ones do. Electrophoretic mobility shift assays performed with separated pools of glycosylated and unglycosylated forms of a construct exhibiting only the DNA-binding domain and the C-terminal tail of HIC1 (residues 399–714) and supershift experiments with wheat germ agglutinin or RL-2, an antibody raised against *O*-GlcNAc residues, fully corroborated these results. Interestingly, these truncated proteins are *O*-GlcNAc modified in their C-terminal tail (residues 670–711) and not in the DNA-binding domain, as for the full-length proteins. Thus, the *O*-GlcNAc modification of HIC1 does not affect its specific DNA-binding activity and is highly sensitive to conformational effects, notably its dimerization through the BTB/POZ domain.

*Keywords*: HIC1; BTB/POZ; *O*-GlcNAc; transcriptional repression; DNA binding.

O-Linked *N*-acetylglucosamine (*O*-GlcNAc) is the most abundant glycosylation found within the cytosolic and the nuclear compartments of eukaryotes. It consists of the attachment of a single residue of *N*-acetylglucosamine on serine and threonine of the peptidic backbone. Hundreds of proteins are modified by this type of glycosylation [1], including structural proteins such as keratins [2] and highly numerous neuronal structural proteins such as neurofilaments [3], synapsin [4] or Tau5; proteins playing a role in transcription such as RNA polymerase II [6]; transcription factors such as Elf1 [7], c-Myc [8], Pax6 [9] or the cAMP response element binding protein (CREB) [10]; corepressors such as mSin3A [11] and even histone deacetylases such as HDAC1 [11]. *O*-GlcNAc is particularly interesting given that this glycosylation is abundant, reversible and highly dynamic; it could compete with phosphorylation on the same or on neighboring amino acids [6,8]. The enzymes of the cycling *O*-GlcNAc, i.e. the *O*-GlcNAc transferase (OGT) and β-*N*-acetylglucosaminidase (*O*-GlcNAcase) are nucleoplasmic enzymes that are particularly enriched in the brain [12–14].

*O*-GlcNAc could have different functional consequences regarding transcription factor activity [1,15]. First, a relationship between *O*-GlcNAc glycosylation and the sensitivity to proteasomal degradation has been described. Sp1 is hyperglycosylated when cells are treated with glucosamine, whereas under glucose starvation hypoglycosylation occurred [16]. Correlating with this hypoglycosylated state, Sp1 is rapidly degraded by the proteasome and this degradation can be prevented by glucose or glucosamine treatment [16]. Another example is the murine β-estrogen receptor (mER-β) where the glycosylation occurs on Ser16, a known phosphorylation site located in the sequence PSST(14–17) that is related to a PEST sequence, which seems to be responsible of the rapid degradation of certain

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*Abbreviations*: BTB/POZ, broad complex-tramtrack-bric a brac/Poxviruses and zinc fingers; CREB, cAMP response element binding protein; GFAT, glutamine:fructose-6-phosphate amidotransferase; HIC1, hypermethylated in cancer 1; HiRE, HIC1 responsive element; mER-β, murine beta-estrogen receptor; *O*-GlcNAc, O-linked *N*-acetylglucosamine; OGT, *O*-GlcNAc transferase; WGA, wheat germ agglutinin.

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proteins. The alternate *O*-GlcNAc/O-phosphorylation of Ser16 appears to be involved in both degradation and transactivation functions of mER-β [17]. Second, *O*-GlcNAc could play a critical function in the regulation of protein–protein interactions. The glutamine-rich transactivation domain of Sp1 (B-c) contains a single *O*-GlcNAc residue whose modification inhibits hydrophobic interactions between Sp1 and two partners, the TATA binding protein-associated factor ($TAF_{II}110$) and holo-Sp1 [18]. Similarly, CREB is *O*-GlcNAc glycosylated at two sites within its Q2 domain and *O*-GlcNAc disrupts the interaction between $TAF_{II}130$ and CREB, thereby inhibiting its transcriptional activity [10]. In addition, a direct link between *O*-GlcNAc and transcriptional repression has been recently deciphered. Indeed, OGT interacts with the corepressor mSin3A and this complex is targeted to promoters where OGT inactivates transcription factors and RNA polymerase II by *O*-GlcNAc modification [11]. This HDAC-independent mechanism acts in concert with histone deacetylation to repress gene transcription. Finally, another function of *O*-GlcNAc in the regulation of transcriptional activity could implicate interactions of transcription factors with DNA. The tumor suppressor p53 contains a C-terminal basic region that inhibits its DNA-binding activity. It has been shown that *O*-GlcNAc glycosylation of this C-terminal region can abrogate this repression [19]. A correlation has also been found between glycosylation of Sp1 and its ability to bind DNA. Its DNA-binding activity can be enhanced by palmitate, via the activation of the hexosamine pathway by increasing the expression of glutamine:fructose-6-phosphate amidotransferase (GFAT) that results in elevated UDP-GlcNAc (the donor of *O*-GlcNAc). Conversely, this DNA-binding activity is abrogated when Sp1 is deglycosylated by enzymatic treatment [20].

The 'hypermethylated in cancer 1' gene (*HIC1*) is a candidate tumor suppressor gene located on chromosome 17p13.3, a region frequently hypermethylated or deleted in many types of solid tumors [21–23]. In addition, *HIC1* expression can be upregulated by p53 [21,24]. Knockout experiments have recently demonstrated that *HIC1* is a 'bona fide' tumor suppressor gene. Homozygous disruption of *HIC1* impairs development and results in embryonic and perinatal lethality [25] whereas heterozygous $HIC1^{+/-}$mice develop malignant tumors, after 1 year [26].

*HIC1* encodes a major 714 amino acid protein, which can be subdivided in three main functional regions: (a) the N-terminal BTB/POZ domain of about 120 amino acids is a dimerization domain known to play a direct or indirect (through conformational effects) role in protein–protein interactions and is an autonomous transcriptional repression domain [27,28]; (b) the C-terminal end contains five Krüppel-like $C_2H_2$ zinc fingers which bind a recently defined specific-DNA sequence [29] and a tail that displays no obvious functional domain but has been phylogenetically conserved [30]; and (c) a central region which is poorly conserved between the HIC1 proteins from different species. However, it contains a conserved GLDLSKK motif reminiscent of the consensus sequence, PxDLSxK, and allowing the recruitment of the corepressor, CtBP (C-terminal binding protein) [28].

In this paper, we demonstrate that the full-length HIC1 protein is *O*-GlcNAc glycosylated in many cellular systems. Although this modification particularly affects residues located in the zinc fingers domain, this *O*-GlcNAc glycosylation did not significantly affect the binding of the full-length protein to its cognate specific DNA sequence. These results suggest that the *O*-GlcNAc residues did not interfere directly or indirectly with the DNA-binding activity, but their involvement in protein stability or in protein–protein interaction had to be investigated. By contrast, BTB/POZ-truncated proteins generated either during the synthesis in rabbit reticulocyte lysates or derived from an *in vitro* constructed mutant, displayed a strikingly differential activity, as the glycosylated truncated forms are *O*-GlcNAc-modified in their extreme C-terminal tail (residues 670–711) and yet are unable to bind DNA. This intriguing finding raises two major functional consequences. First, the difference in the DNA-binding activities of the full-length and the truncated HIC1 forms underscores the crucial implication of *O*-GlcNAc-modified C-terminal tail in DNA interaction with the truncated HIC1 forms, demonstrating the implication of the glycosylation in the binding. Second, as the glycosylation does not occur in the same region for the full-length proteins or for the truncated ones, it emphasizes the sensibility of the *O*-GlcNAc glycosylation to conformational effects and undoubtedly to the dimerization of HIC1 through its BTB/POZ domain in the localization of the glycosylation.

## Materials and methods

### Cell culture and transfections

Cos7 cells and CHO cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum at 37 °C in a 5% (v/v) $CO_2$-enriched atmosphere. Cos7 were transfected in 2.5 mL of Opti-MEM® (Gibco/BRL, Grand Island, NY, USA) by the polyethyleneimine (Euromedex, Mundolsheim, France) method (10 μL), in 100 mm diameter dishes with 2.5 μg of DNA, as previously described [27]. Cells were transfected for 6 h and then incubated for 48 h in 10 mL of fresh complete medium.

### Glucosamine treatment

Glucosamine (Sigma Chemical Co., St Louis, MO, USA) was used at a final concentration of 20 mM as previously described [31]. Concentrated solutions (800 mM) were prepared in physiological water. The control experiments were performed by adding equal volumes of physiological water in the culture medium.

### *In vitro* transfer of tritiated galactose on GlcNAc residues using galactosyltransferase

Flag-HIC1 proteins expressed in Cos7 cells were enriched using anti-Flag Igs covalently coupled to agarose beads. After elution with 150 $\mu g \cdot mL^{-1}$ of the Flag peptide, the bound proteins were labeled with 50 mU of preauto-galactosylated bovine galactosyltransferase (Sigma) and 5 μCi of UDP-[6-$^3$H]galactose (Amersham; Little Chalfont, Buckinghamshire, UK) at 37 °C for 2 h in Buffer L

(56.25 mM HEPES, 11.25 mM $MnCl_2$, 250 mM galactose, 12.5 mM adenosine mono-phosphate, pH 6.0) [9]. Samples were run on an 8% (w/v) SDS/PAGE, and the gel was incubated in Amplify® (Amersham) and then fluorographed.

### Determination of the *O*-GlcNAc site numbers on HIC1

The procedure was essentially as previously described [32]. Briefly, Flag-HIC1 proteins were purified and labeled with tritiated galactose as detailed above. After protein denaturation (6 M guanidine chlorhydrate, 50 mM Tris/HCl, 2 mM dithiothreitol, pH 8.0) for 20 min at 100 °C, tryptic digestion was performed with sequencing grade modified trypsin (Promega, Madison, WI, USA) overnight at 37 °C in 50 mM Tris/HCl, 1 mM $CaCl_2$, pH 7.6, until the concentration in guanidine chlorhydrate was below 1 M. The resultant peptides were separated on a C18 column by reverse phase HPLC (Dionex corporation, Sunnyvale, CA, USA). Detection was performed at 225 nm and fractions were counted after collecting in polyethylene vials by liquid scintillation detection.

### Rabbit reticulocyte lysate expression

Various HIC1 proteins were produced in rabbit reticulocyte lysate complemented with [$^{35}$S]methionine (Amersham) according to the manufacturer's recommendations (Promega; Madison, WI, USA).

### Immunoprecipitation

Before immunoprecipitation, rabbit reticulocyte lysate products were diluted in radioimmunoprecitation assay buffer [RIPA: 20 mM Tris, 150 mM NaCl, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, pH 8.0, one tablet of Complete (Roche) protease inhibitors per 50 mL] to a final volume of 500 μL. For cultured cells, Cos7 or CHO cells were lysed on ice with 1 mL of RIPA buffer directly in the dishes. The lysates were centrifuged at 20 000 ***g*** for 30 min at 4 °C, and the supernatants were recovered.

Immunoprecipitations were performed overnight at 4 °C with the anti-Flag (M2) (Sigma) or the anti-(*O*-GlcNAc) (RL-2) (MA1-072; Affinity BioReagents, Golden, CO, USA) monoclonal antibodies (dilution 1 : 1000, w/v) and with the anti-HIC1 polyclonal serum (325 pAb), raised against a C-terminal peptide of HIC1 (dilution 1 : 500, w/v) [28]. Twenty microliters of protein G or protein A Sepharose beads (Amersham) were added for 1 h at 4 °C. The beads were washed four times successively with RIPA, NaCl-enriched RIPA (500 mM final concentration of NaCl), RIPA/TNE (20 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 8.0) (v/v) and TNE alone.

### β-Hexosaminidase treatment

After enrichment of HIC1 proteins produced in Cos7 cells on an M2 affinity column, the proteins were incubated in 100 mM acetate, pH 5.2, with *Escherichia coli* recombinant beta-hexosaminidase (Calbiochem, San Diego, CA, USA) for 2 h at 37 °C.

### SDS/PAGE and electroblotting

Proteins were separated by SDS/PAGE. For radiolabeled proteins, the gel was immersed in 10 mL of Amplify® for 30 min, dried under vacuum and exposed to a film. In the other cases, proteins were electroblotted onto nitrocellulose sheet (Amersham) for 1 h at 100 V under cooling to perform Western blot analyses. The nitrocellulose sheets were saturated for 45 min at room temperature in Tris-buffered saline (TBS)-Tween [15 mM Tris, 140 mM NaCl, 0.05% (w/v) Tween] containing 5% (w/v) nonfat milk. The first antibody was incubated overnight at 4 °C at a final dilution of 1 : 1000 (w/v) for the mAb anti-(*O*-GlcNAc) (RL-2) and 1 : 5000 (w/v) for the mAb anti-Flag (M2) or for the HIC1 (pAb 325; [28]) in TBS/Tween containing milk or bovine serum albumin. After washing in TBS/Tween, horseradish peroxidase-labeled secondary antibody raised against either mouse or rabbit antibodies (Amersham) was incubated at room temperature for 1 h at a dilution of 1 : 10 000 (w/v) in TBS/Tween containing milk. After washing in TBS/Tween, the detection was carried out using the Western lightning chemiluminescence reagents plus kit (Perkin Elmer; Aurora, OH, USA). For the use of WGA-peroxidase (Sigma), the procedure was essentially as described above, except that the nitrocellulose sheet was blocked with 3% (w/v) bovine serum albumin and incubated with WGA-peroxidase at a dilution of 1 : 10 000 (w/v) for 1 h at room temperature. The specificity of WGA-peroxidase binding was controlled by incubation in presence of 0.2 M of free GlcNAc (ICN; Boston, MA, USA).

### Electrophoretic mobility shift assays (EMSA)

Two microliters of each rabbit reticulocyte lysate product were incubated with the HIC1-specific radiolabeled probes HIC1 responsive element (HiRE) or 5×HiRE (containing five concatemerized response elements [29]) in a final volume of 20 μL of binding buffer [20 mM Tris, 80 mM NaCl, 0.1% (v/v) Triton X-100, 2 mM dithiothreitol, 10 μM $ZnCl_2$, 5% (v/v) glycerol, 5 $\mu g \cdot mL^{-1}$ poly(dI/dC)] for 30 min on ice. The reaction mixture was then subjected to electrophoresis in a 4% or in an 8% nondenaturing polyacrylamide gel at 4 °C. After drying, the gel was exposed to a film for autoradiography. For supershift assays, the reaction mixtures were incubated with the specific antibodies for 20 min before the addition of the labeled probe.

### Purification of the HIC1 glycosylated forms by affinity chromatography on WGA-beads

The full-length HIC1 protein and the 399–714 construct were produced in rabbit reticulocyte lysates. The lysates were diluted in phosphate-buffered saline (NaCl/$P_i$: 20 mM phosphate, 150 mM NaCl, pH 7.5) before loading on a column containing WGA-labeled agarose beads (Sigma) at 4 °C. After collecting the unbound fractions (unglycosylated proteins), the column was washed with NaCl/$P_i$, and finally bound proteins (glycosylated proteins) were eluted with NaCl/$P_i$ containing free GlcNAc (0.2, 0.5 and 1 M, respectively).

## Results

### HIC1 is *O*-GlcNAc glycosylated *in vitro* and *in vivo*

To clearly establish that HIC1 is glycosylated with *O*-GlcNAc, rabbit reticulocyte lysates that are known to catalyze the transfer of *O*-GlcNAc residues [33] were programmed with a pcDNA$_3$Flag-HIC1 vector expressing the full-length HIC1 protein tagged with an N-terminal Flag epitope (Flag-HIC1 1–714) and passed through a WGA-agarose affinity column as association with this lectin has been widely used to detect *O*-GlcNAc modification of various proteins [1]. Total rabbit reticulocyte lysates (input, In), the bound (B) and the unbound (NB) fractions (Fig. 1A) were analyzed by SDS/PAGE. As shown in Fig. 1A (lane 2), a significant portion of HIC1 proteins is retained on WGA.

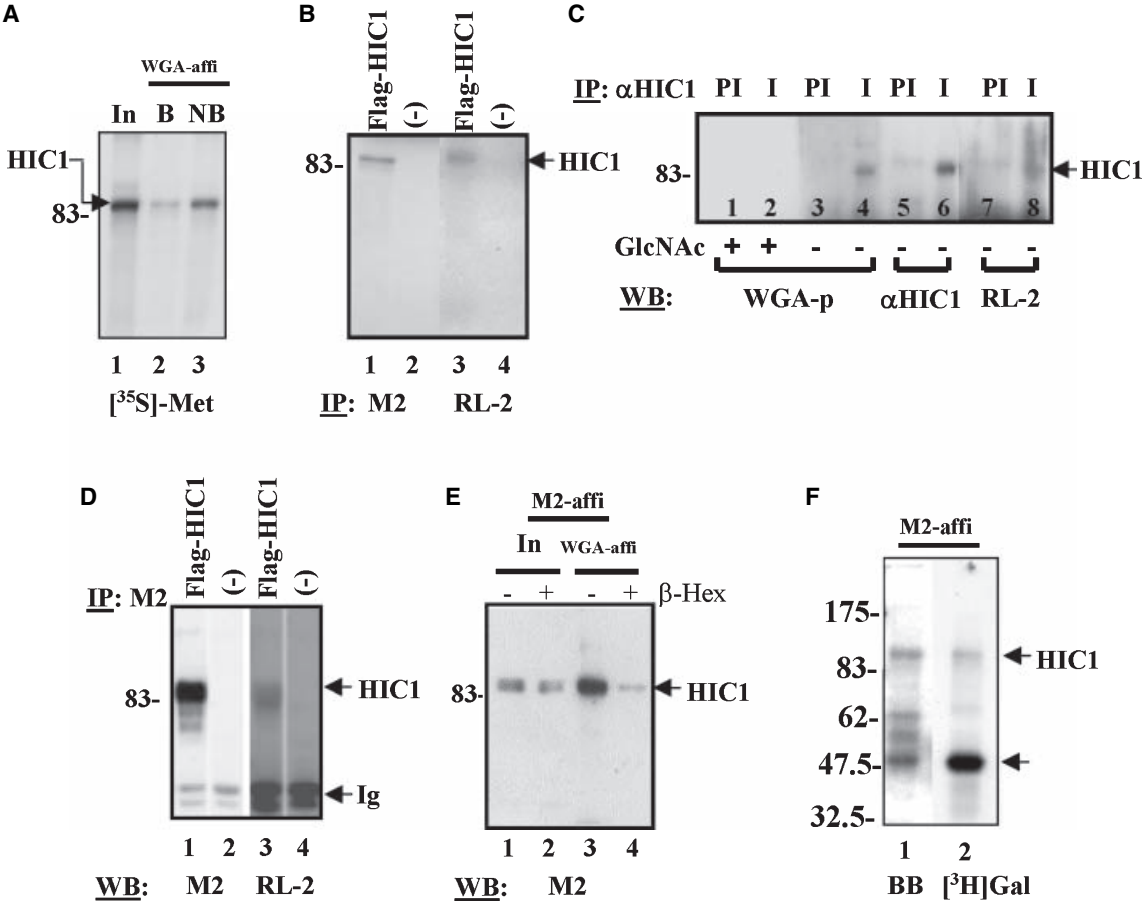



**Fig. 1. HIC1 is an *O*-GlcNAc-glycosylated transcriptional repressor.** (A) Full-length HIC1 proteins tagged with an N-terminal Flag epitope were produced in rabbit reticulocyte lysates programmed with the pcDNA$_3$Flag-HIC1 1–714 vector supplemented with [$^{35}$S]methionine (input, In) and incubated with a WGA affinity matrix (WGA-affi). After centrifugation, the unbound (NB) fraction was recovered. After washing with NaCl/P$_i$, the beads were incubated with 0.5 M free GlcNAc to recover the bound (B) fraction. The proteins were separated on an 8% SDS/PAGE. The gel was dried under vacuum and exposed to a film. (B) Immunoprecipitations were performed on the same reticulocyte lysates using anti-Flag (M2) (lanes 1 and 2) or anti-(*O*-GlcNAc) (RL-2) (lanes 3 and 4). (C) A stably transfected CHO cell line containing an integrated and inducible HIC1 expression vector, EcRCHO-pINDFlag-HIC1 clone 6 [28] was induced with ponasterone. Total extracts were incubated with immune (I) rabbit sera directed against HIC1 (325 pAb) or with preimmune sera from the same rabbit (PI) [28]. The immunoprecipitated proteins were run on an 8% SDS/PAGE and analyzed by Western blotting with peroxidase-labeled WGA in presence of free GlcNAc to compete for the HIC1/WGA interaction (lanes 1 and 2) or without free GlcNAc (lanes 3 and 4), with the anti-HIC1 Igs (lanes 5 and 6) or with anti-(*O*-GlcNAc) (RL-2) (lanes 7 and 8). (D) Total extracts from Cos7 cells transiently transfected for 48 h with the empty pcDNA$_3$Flag (–) or the pcDNA$_3$Flag-HIC1 1–714 vector were submitted to immunoprecipitation using the mAb anti-Flag (M2). The immunoprecipitated proteins were separated on an 8% SDS/PAGE and analyzed by Western blotting with anti-Flag (M2) (lanes 1 and 2) or anti-(*O*-GlcNAc) (RL-2) (lanes 3 and 4). (E) Flag-HIC1 1–714 proteins were expressed in Cos7 cells, purified on M2 affinity columns (M2-affi). Equal amounts were subjected or not to digestion by recombinant β-hexosaminidase and enriched on WGA-agarose beads (lanes 3 and 4). Controls (In) are shown on lanes 1 and 2. (F) Flag-HIC1 1–714 proteins expressed in Cos7 cells were purified using anti-Flag Igs covalently coupled to agarose. The bound proteins were specifically eluted with the Flag peptide. *In vitro* labeling of the GlcNAc residues was then performed with bovine galactosyltransferase. The labeled proteins were separated on an 8% SDS/PAGE, stained with Coomassie Brilliant Blue (BB, lane 1) and fluorographed after immersion of the gel in Amplify® (lane 2). The arrowhead indicates a cleavage product which is highly labeled.

To confirm these results, the same lysates were immunoprecipitated with the anti-Flag Ig (M2) or with the anti-(*O*-GlcNAc) (RL-2) mAbs. A band of similar size was detected by both antibodies only in the Flag-HIC1 lysates (Fig. 1B, lanes 1 and 3). These experiments demonstrate that HIC1 proteins are glycosylated *in vitro* with *O*-linked *N*-acetylglucosamine. The glycosylation of HIC1 was also tested in a previously described stable CHO cell line with inducible expression of a chromatinized endogenous HIC1 gene [28]. After induction with ponasterone, total cell extracts were immunoprecipitated with the HIC1 polyclonal antibody (pAb325) directed against a C-terminal peptide of human HIC1 or with preimmune serum from the same rabbit as control [28]. Western blot analyses were performed with WGA-peroxidase (in either the presence or absence of free GlcNAc, used as a competitor of *O*-GlcNAc–HIC1/WGA interaction), with the anti-HIC1 or with the anti *O*-GlcNAc antibodies (Fig. 1C). The induced endogenous HIC1 proteins were clearly detected only in the HIC1 immunoprecipitates by the anti-HIC1 Ig (Fig. 1C, lane 6) and by the WGA-peroxidase only in absence of the GlcNAc competitor (Fig. 1C, compare lanes 2 and 4). Again a faint band of similar size was also detected by the RL-2 antibody (Fig. 1C, lane 8).

Similar results were obtained *in vivo* in Cos7 cells transiently transfected with the empty or the Flag-HIC1 vectors. As expected, a promiscuous expression of HIC1 is detected in the transiently transfected Cos7 cells by the anti-Flag mAbs (Fig. 1D, lane 1). A weaker but significant band of roughly similar size is detected by the RL-2 antibodies, corresponding to the *O*-GlcNAc modified HIC1 proteins (Fig. 1D, lane 3). Using transient transfection in Cos7 cells, we also showed that HIC1 could be enriched on WGA-beads (Fig. 1E, lane 3), and that this binding was dramatically decreased when samples were previously treated with beta-hexosaminidase, reinforcing the fact that HIC1 is *O*-GlcNAc modified (Fig. 1E, lane 4).

Bovine galactosyltransferase is a specific and sensitive probe frequently used in the detection of *O*-GlcNAc residues on cytosolic and nuclear proteins [9,34,35]. Full-length Flag HIC1 proteins were purified from extracts of transfected Cos7 cells using an anti-(Flag M2) affinity column. The bound proteins recovered by a specific elution with the Flag peptide were labeled *in vitro* by bovine galactosyltransferase in the presence of UDP-[6-$^3$H]galactose and run on an 8% SDS/PAGE. We can see an upper band corresponding to full-size HIC1 (Fig. 1F, lanes 1 and 2), which provides another clear piece of evidence for the *O*-GlcNAc glycosylation of HIC1. Notably, several truncated HIC1 forms are also generated during this purification scheme which includes a 2 h incubation at 37 °C (Fig. 1F, lane 1) and one of these bands with an apparent molecular mass of 48 kDa is heavily labeled (Fig. 1F, lane 2).

Taken together these results demonstrate that HIC1 is an *O*-GlcNAc-modified transcriptional repressor both *in vitro* and *in vivo*.

The number of sites that were modified with *O*-GlcNAc on HIC1 was estimated using the approach described by Gao *et al.* [32]. Full-length Flag HIC1 proteins were purified from extracts of transfected Cos7 cells using an anti-Flag (M2) affinity column. The silver staining of the affinity chromatography preparation of HIC1 demonstrates that it was devoid of any other contaminating proteins (Fig. 2A). It should be noted that this silver stained gel was performed on freshly purified HIC1 proteins and before the labeling step. After digestion with trypsin, the resulting peptides were separated on reverse phase HPLC and analyzed. The HPLC profiles clearly show that HIC1 contained three major *O*-GlcNAc sites shown by arrows (Fig. 2B,C).

### HIC1 is upglycosylated when cells are cultured in glucosamine-containing medium

The *O*-GlcNAc glycosylation occurs via the hexosamine pathway and could be enhanced by direct addition of free glucosamine ($GlcNH_2$) in the cell culture medium [31,35]. To address this issue, Cos7 cells were transfected with the empty pcDNA$_3$Flag vector or with the pcDNA$_3$Flag-HIC1 vector in Dulbecco's modified Eagle's medium containing 20 mM glucosamine or physiological water (mock control). Two days after transfection, cell extracts were immunoprecipitated with anti-Flag (M2) and analyzed by Western blot with the M2 or RL-2 monoclonal antibodies. In high glucosamine medium conditions, the total amount of transiently expressed HIC1 protein is slightly less abundant (Fig. 3, lanes 3 and 4). However, we observed a clear increase in the HIC1 glycosylated forms detected by the RL-2 antibody in presence of glucosamine (Fig. 3, compare lanes 7 and 8). These results further demonstrate that HIC1 can be *O*-GlcNAc modified *in vivo* and that the glycosylation status could be enhanced by culturing in glucosamine-enriched medium.

### HIC1 *O*-GlcNAc glycosylation preferentially occurs within the DNA-binding domain

Using deletion mutants of HIC1, affinity chromatography analyses on WGA-agarose beads have shown that the *O*-GlcNAc glycosylation of HIC1 was more pronounced in the C-terminal region (data not shown), i.e. the zinc fingers domain and the C-terminal end. To confirm these results, the full-length HIC1 protein and two C-truncated HIC1 mutants (1–714, 1–616 and 1–400; Fig. 4A) were produced in reticulocyte lysates and then immunoprecipitated with the anti-(*O*-GlcNAc)-specific monoclonal antibody, RL-2. Notably, these constructs all contain the N-terminal BTB/POZ domain which is a dimerization domain instrumental for the functional properties of these proteins. As shown in Fig. 4B (lanes 1–4), all three constructs are produced at similar levels. However, only the 1–714 and 1–616 are efficiently and equally immunoprecipitated with the RL-2 antibody (Fig. 4B, lanes 5 and 7). Notably, the 1–400 HIC1 mutant is only very poorly recognized by the RL-2 antibody (Fig. 4B, lane 8). Taken together, these results thus suggest that most of the *O*-GlcNAc glycosylation occurs in the DNA-binding domain containing the five Krüppel-like $C_2H_2$ zinc fingers (amino acids 401–616).

### *O*-GlcNAc glycosylation of full-length HIC1 proteins does not affect their DNA binding activity

As the *O*-GlcNAc glycosylation occurs in the DNA-binding domain, the DNA binding activity of both glycosylated and

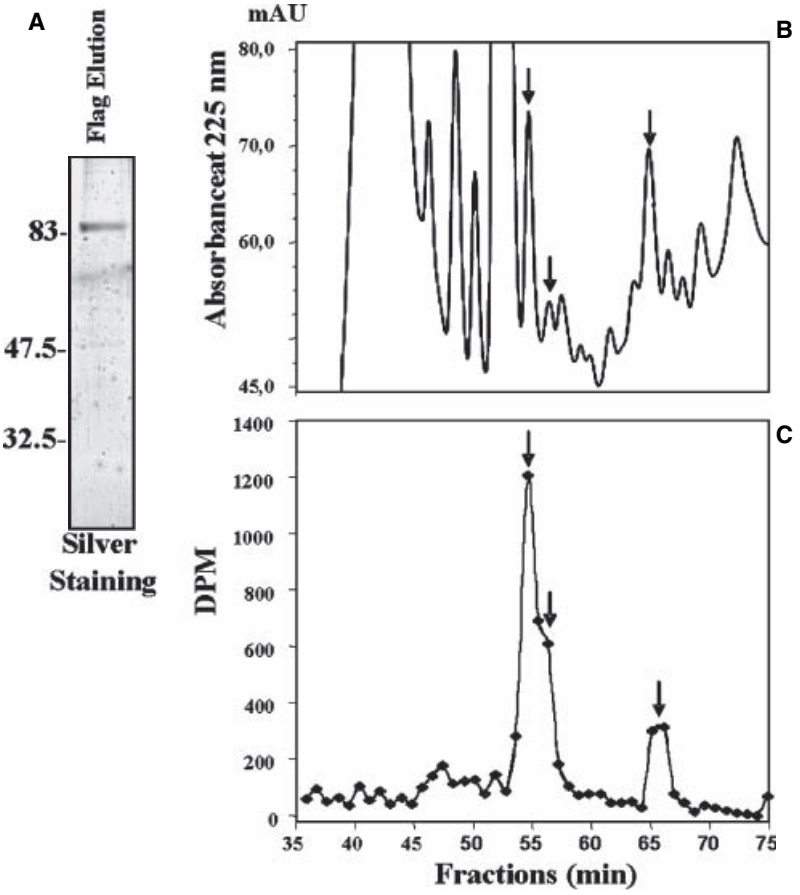



**Fig. 2. HIC1 is modified with at least three major *O*-GlcNAc residues.** (A) Flag-HIC1 1–714 proteins expressed in Cos7 cells were enriched on M2-affinity beads. After extensive washing, the Flag-HIC1 proteins were specifically eluted with an excess of Flag peptide. The purity of the preparation was checked by silver staining an 8% SDS/PAGE. *O*-GlcNAc residues were extended by *in vitro* galactosylation with bovine galactosyltranferase and [$^3$H]galactose. A digestion with trypsin was performed and the resultant peptides were separated using reverse-phase HPLC on a C18 column. (B) This represents the detection of the total peptides at 225 nm, and (C) the detection of the radiolabeled-peptides by radioactivity counting. Three major glycosylation peaks are shown by arrows.

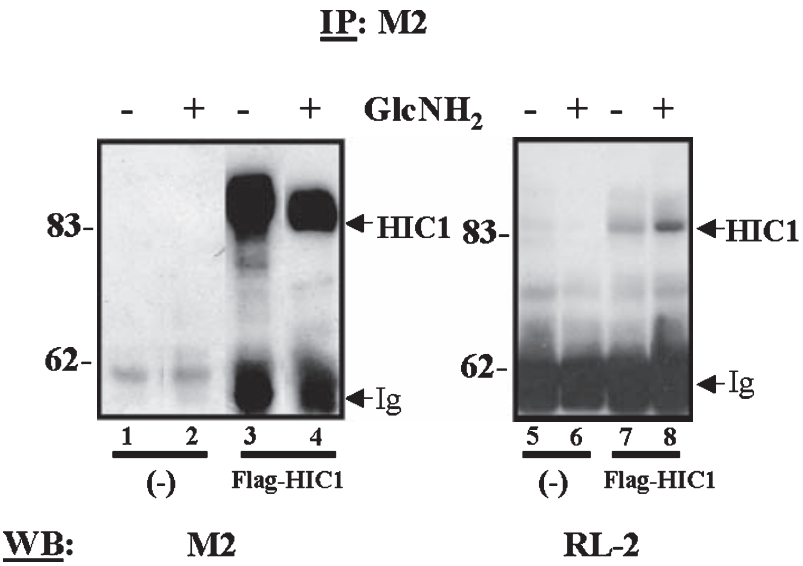



**Fig. 3. Cos7 cells cultured in enriched-glucosamine medium upglycosylate HIC1.** Cos7 cells were transiently transfected with an empty pcDNA$_3$Flag vector (–) or with the pcDNA$_3$Flag-HIC1 1–714 vector. Twenty-four hours after transfection, glucosamine was added at a final concentration of 20 mM (+ $GlcNH_2$; lanes 2, 4, 6 and 8) and equal volumes of physiological water were added to the dishes as mock control (– $GlcNH_2$; lanes 1, 3, 5 and 7). Cells were then lysed and immunoprecipitations were performed using anti-Flag (M2). The immunoprecipitated proteins were run on an 8% SDS/PAGE, electroblotted on nitrocellulose sheets and Western blotted with anti-Flag (lanes 1–4) or with anti-(*O*-GlcNAc) (RL-2) (lanes 5–8) mAbs. Ig, immunoglobulins.

nonglycosylated forms was thus investigated, after purification by WGA-affinity chromatography. Full-length (1–714) Flag-HIC1 programmed reticulocyte lysates were applied on a WGA-agarose bead column and the nonretained fraction was considered as the unglycosylated proteins. After washing with NaCl/$P_i$, increasing concentrations of free GlcNAc-containing NaCl/$P_i$ were applied to the column to elute the retained proteins, i.e. the glycosylated forms. An aliquot of each fraction (including the washes) was separated on an 8% SDS/PAGE and autoradiographed to detect HIC1 (Fig. 5A). Equal amounts of nonglycosylated and glycosylated HIC1 proteins, as demonstrated by

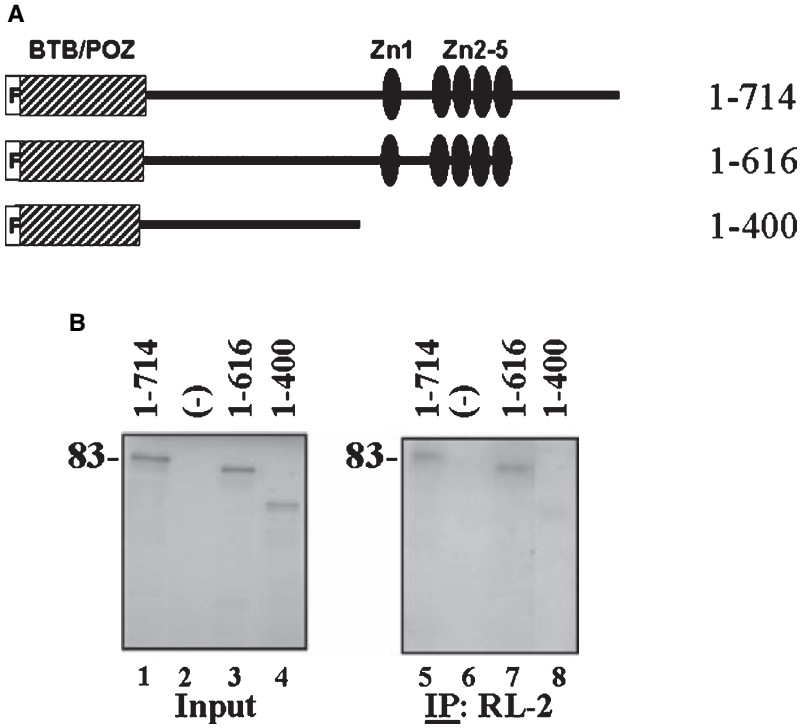


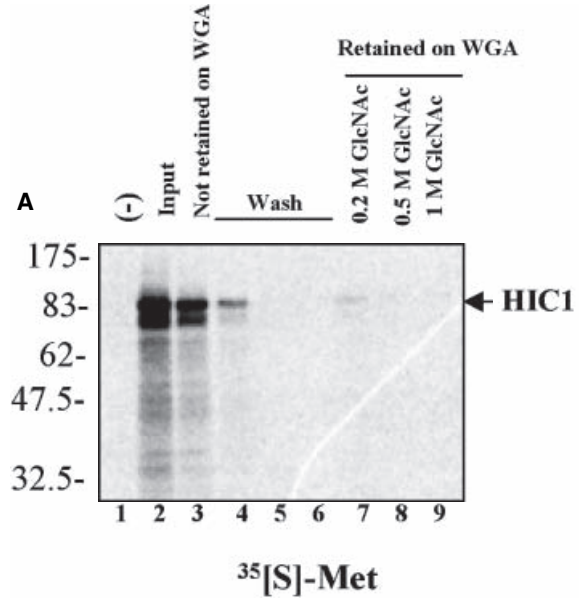

**Fig. 4. *O*-GlcNAc modification of full-length HIC1 proteins is predominantly localized in the DNA-binding domain.** (A) Diagram of the HIC1 deletion mutants used in the study. The top lane shows the full-length HIC1 protein. Zinc fingers (Zn 1 and Zn 2–5) are shown as black ovals, the BTB-POZ domain is shown as a hatched box and the Flag epitope tagged at the N-terminus of the proteins is represented as a white box. (B) Full-length HIC1 proteins and the various deletion mutants produced in reticulocyte lysates were immunoprecipitated with the anti-(*O*-GlcNAc) Ig (RL-2) and separated on a 12.5% SDS/PAGE (lanes 5–8). 2 μL of each lysate (input) were also run for control (lanes 1–4). The gels were dried under vacuum and exposed to a film. (–), empty pcDNA$_3$Flag vector.

SDS/PAGE analyses (Fig. 5B, left), were tested for their capacity to bind a HIC1 specific DNA sequence by EMSA. Full-length HIC1 proteins, as several BTB/POZ proteins, bind poorly *in vitro* a probe containing a single binding site but bind cooperatively a probe containing multimerized sites, thus yielding slow mobility complexes [29,37,38]. Therefore, we used a probe called 5×HiRE, which contains five copies of the recently defined HIC1 binding sequence [29]. As shown in Fig. 5B (lane 2), we observed a specific band of very weak mobility (at the top of the gel) corresponding to the binding of full-length HIC1 proteins to their specific DNA-target. No obvious differences in the DNA-binding activity could be detected between the glycosylated and the nonglycosylated forms of HIC1 (Fig. 5B, lanes 3 and 4), indicating that the *O*-GlcNAc glycosylation did not play a major role in the DNA-binding activity of full-length HIC1 proteins. These complexes are not observed with a mutated 5×HiRE probe (Fig. 5B, lane 8) [29], demonstrating that they do not correspond to nonspecific stacking of proteins to this probe. In addition, it is worth pointing out that the presence of very low mobility complexes, some even retained at the top of the gel, has been already observed with other BTB/POZ proteins, e.g. PLZF [38]. However, we also observed specific complexes of higher mobility that strikingly showed a differential binding activity with the specific sequence, as in that case, the glycosylated forms did not bind the probe (Fig. 5B, lanes 3 and 4). These high mobility complexes could correspond to a minor population of truncated forms of HIC1 able to bind this probe with a high affinity and generated during the synthesis of the proteins in reticulocute lysates (Fig. 5A). Fully consistent with this prediction, the anti-Flag M2 did not super-shift these complexes (Fig. 5B, lane 6), demonstrating that they do not contain full-length proteins with the N-terminal Flag and most likely correspond to truncated proteins (Fig. 1F), also observed *in vivo* [29]. Such *in vitro* constructed mutants, as, for example, the isolated zinc fingers domain, display a very high binding activity in EMSA as compared with full-length proteins [29].

Thus, the *O*-GlcNAc glycosylation of HIC1, even though it occurs preferentially in the zinc finger domain involved in specific DNA-binding, does not significantly affect this functional property in the context of the full-length protein.

### *O*-GlcNAc glycosylation within the DNA-binding domain requires the presence of the BTB/POZ domain

As a model with which to study the *O*-GlcNAc glycosylation of truncated forms of full-length HIC1 proteins (Fig. 6), several deletion mutants were constructed in the region encompassing the five zinc fingers and the C-terminal end of HIC1 (amino acids 399–714) and were tagged at the N-terminal with a Flag epitope (Fig. 6A). All these constructs were produced at a similar level in rabbit reticulocyte lysates (data not shown). After immunoprecipitation with the M2 mAb, the resulting immunoprecipitates were analyzed by 12.5% SDS/PAGE followed by Western blotting with either the anti-Flag (M2) or the RL-2 monoclonal antibodies (Fig. 6B). The 399–714 construct is *O*-GlcNAc modified (Fig. 6B, lane 1), but in striking contrast with the results obtained with proteins containing the BTB/POZ domain (Fig. 4), the 399–669 deletant, although it includes the five zinc fingers, is absolutely not glycosylated (Fig. 6B, lane 4). Thus, in the context of the full-length HIC1 protein, the *O*-GlcNAc glycosylation occurs mostly in the DNA-binding domain (residues 401–616) (Fig. 4), whereas in BTB/POZ-truncated proteins this modification is rather located in the C-terminal end (Fig. 6) (see Discussion). *In silico* analyses with the YINOYANG program (http://www.cbs.dtu.dk/services/YinOYang/) identified the SPT sequence (amino



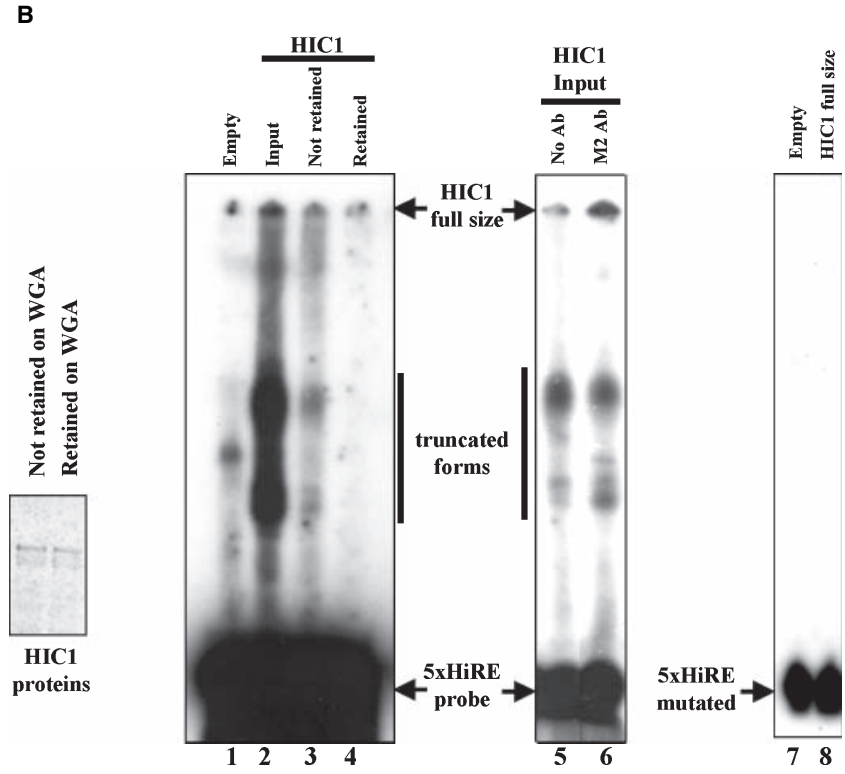




**Fig. 5. The full-length HIC1 proteins bind DNA both in their glycosylated and in their unglycosylated forms.** (A) The full-length HIC1 proteins were produced in reticulocyte lysates and unglycosylated and glycosylated HIC1 forms were separated by WGA-affinity chromatography. The non-retained fraction was collected and after extensive washing of the column with NaCl/$P_i$, the bound fraction was eluted with free GlcNAc. An aliquot of each fraction was run on an 8% SDS/PAGE, and the gel was dried under vacuum and exposed to a film (lanes 1–9). (–), reticulocyte lysate programmed with the empty pcDNA$_3$Flag vector. (B) Equal amounts, as shown by SDS/PAGE analysis (left panel), of unglycosylated (lane 3) and glycosylated (lane 4) HIC1 were tested for their ability to bind a specific DNA probe containing five HIC1 responsive elements (5×HiRE) in EMSA experiments (4% reticulated gel in TBE buffer). A positive control was performed with 2 μL of the input (lane 2) and a negative control with the empty pcDNA$_3$Flag vector (lane 1). A supershift experiment was performed with the input (no antibody, lane 5) and with the anti-Flag (M2) mAb (lane 6). (–), empty vector. As a control, no retarded bands were observed with the 5×HiRE mutated probe (lanes 7 and 8).

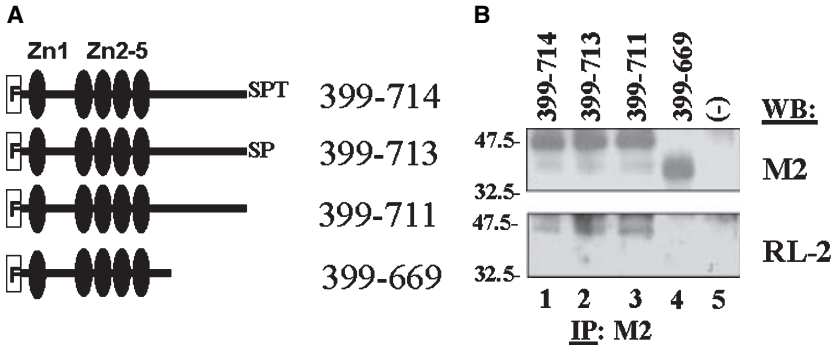



**Fig. 6. The N-terminal HIC1 truncated forms are glycosylated but in their C-terminal tail.** (A) HIC1 deletion mutants used in the study. Symbols and numbering are as in Fig. 4. (B) The various deletion mutants produced in reticulocyte lysates were immunoprecipitated with anti-Flag (M2), separated on a 12.5% SDS/PAGE and Western blotted with the anti-Flag (M2) (lanes 1–5, top panel) or with the anti-(*O*-GlcNAc) (RL-2) mAbs (lanes 1–5, bottom panel). (–), empty pcDNA$_3$Flag vector.

acids 712–714) as potentially good substrates for OGT. However, the 399–714 construct and two deletion mutants (construct 399–713 and construct 399–711) were equally detected by the RL-2 antibodies (Fig. 6B, lanes 1–3) suggesting that residues 712–714 were not *O*-GlcNAc modified. As the 399–669 deletion mutant is not recognized by RL-2, all these results demonstrate that the *O*-GlcNAc modified residue(s) is(are) preferentially localized in the region 670–711. Interestingly enough, this region contains several potential target residues and in particular the sequence SLYP(670–673), which is perfectly conserved between the human, avian and zebrafish HIC1 proteins [28,30]. Thus, truncated HIC1 proteins devoid of the BTB/POZ domain are efficiently *O*-GlcNAc modified, but in their C-terminal tail.

### Truncated HIC1 proteins that are *O*-GlcNAc modified in their C-terminal tail are unable to bind their specific DNA target

During the purification of the full-length HIC1 proteins on WGA affinity columns, N-terminal truncated and glycosylated forms unable to bind the specific DNA-binding sequence are generated (Fig. 5B). To test the role of this *O*-GlcNAc modification on the DNA-binding activity of these 'artificial' HIC1 proteins, we produced the 399–714 construct in reticulocyte lysates. Then, equal amounts of the glycosylated and the unglycosylated 399–714 HIC1 proteins, separated using WGA-agarose beads as described above, were tested by EMSA with the HiRE specific probe. The unglycosylated proteins bind DNA (Fig. 7A, lane 3) whereas the glycosylated forms retained on WGA do not (Fig. 7A, lane 4), exactly as observed with the truncated forms generated during the WGA-affinity purification of the full-length proteins (Fig. 5B). To fully validate these results, a rabbit reticulocyte programmed with this 399–714 construction was incubated with the specific $^{32}$P-labeled HiRE probe. With this mixture of glycosylated and unglycosylated HIC1 proteins, a specific retarded complex is observed (Fig. 7B, compare lanes 1 and 7). However, when increasing amounts of WGA, the lectin that specifically binds GlcNAc residues, are added, no supershift can be detected (Fig. 7B, lanes 2–4); nor can they be detected with the anti-(*O*-GlcNAc) (RL-2) monoclonal antibody (Fig. 7B, lane 13), although this antibody has been successfully used in such experiments in the case of Elf1 [7]. As a positive control, we show that the anti-Flag (M2) monoclonal antibody is able to supershift the complex (Fig. 7B, lane 12).

These results indicate that the *O*-GlcNAc forms of the 399–714 construct cannot bind DNA.

## Discussion

*O*-GlcNAc is a nuclear and cytosolic-specific glycosylation found in eukaryotes that has been widely described in terms of glycosylation on numerous proteins, and particularly on transcription factors, however, its role remains elusive.

In this work, we looked at the glycosylation of HIC1, a recently described transcriptional repressor, with regard to the growing list of transcription factors that are modified with *O*-GlcNAc, and whose activity seems to be modulated by this post-translational modification. First, we demonstrated that full-length HIC1 proteins, produced in reticulocyte lysates, bind to WGA, a lectin extracted from wheat germ (*Triticum vulgaris*) that specifically recognizes terminal GlcNAc residues (Fig. 1A). To confirm that the glycosylation beard by HIC1 was actually *O*-GlcNAc and not more complex glycans with terminal GlcNAc residues (even if these complex glycans are not preferentially found in the nucleus), we used the *O*-GlcNAc-specific monoclonal antibody RL-2 (Fig. 1B), which has been originally raised against an *O*-GlcNAc peptide of the nucleoporin p62 but is now recognized as able to bind *O*-linked *N*-acetylglucosamine residues on many proteins. HIC1 is glycosylated when produced in reticulocyte lysates *in vitro* and also in a stably transfected CHO clone, as well as *in vivo* in transiently transfected Cos7 cells (Fig. 1C–E). Finally, the glycosylation status of HIC1 could be increased when Cos7 cells were cultured in presence of glucosamine that bypasses GFAT, the key enzyme in the hexosamine pathway (Fig. 3). Collectively, these experiments unambiguously demonstrate the *O*-GlcNAc glycosylation of HIC1.

To localize the region(s) that is(are) glycosylated in the full-length HIC1 proteins, several mutants were analyzed. Because the BTB/POZ domain is a dimerization domain absolutely required for the correct folding of the protein, we

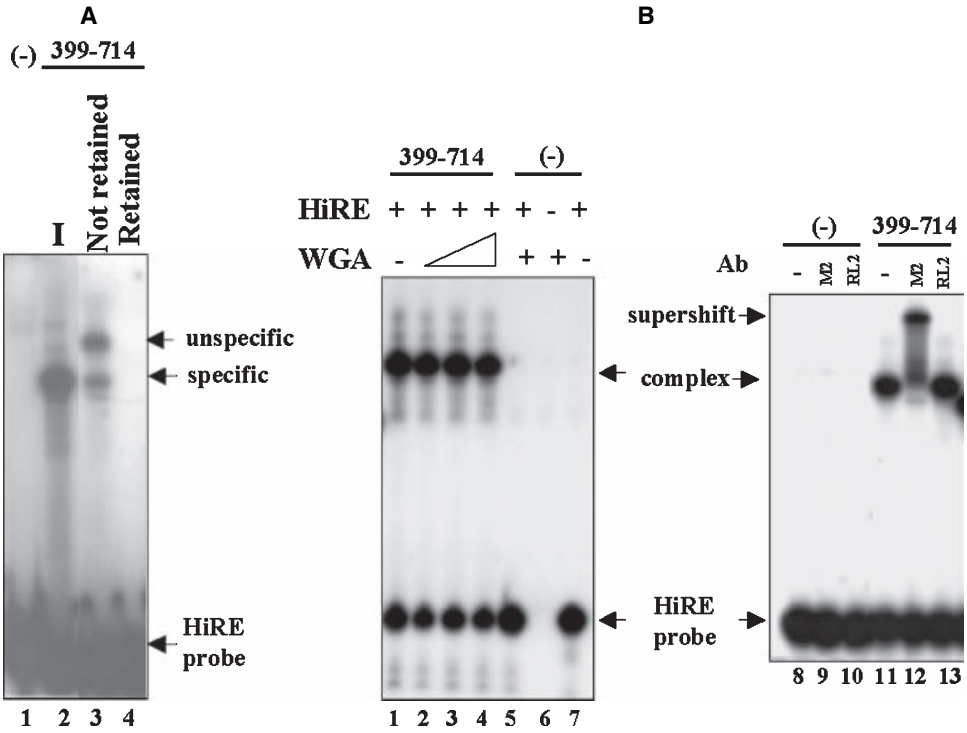



**Fig. 7. The glycosylated truncated forms of HIC1 are unable to bind their specific DNA sequence.** (A) The 399–714 mutant encompassing the DNA-binding domain and the C-terminal tail of HIC1 was produced in reticulocyte lysate and the unglycosylated and the glycosylated forms were fractionated on WGA-agarose beads. Equal quantities of the unbound (lane 3) and of the bound (lane 4) fractions were tested in EMSA (8% reticulated gel in TBE buffer) with the specific radiolabeled oligonucleotide probe (HiRE). A positive control was performed with 2 μL of the input (lane 2) and a negative control with the empty pcDNA$_3$Flag vector (–, lane 1). Note that a nonspecific band is observed in the unbound fraction. (B) Total rabbit reticulocyte lysates programmed with the pcDNA$_3$Flag 399–714 HIC1 vector (lanes 1–4 and 11–13) or the empty pcDNA$_3$ Flag vector (–) (lanes 5–7 and 8–10) were incubated with HiRE probe. The complexes formed were run on an 8% acrylamide gel in a TBE buffer and increasing amounts of WGA (lanes 2–6) or anti-Flag (M2) (lanes 9 and 12) or anti-(*O*-GlcNAc) (RL-2) (lanes 10 and 13) were added. The gels were dried under vacuum and exposed to film. A super-shift is observed only with anti-Flag (M2) (lane 12).

first decided to focus our work on various C-terminal deletion mutants. In that context, we demonstrated by immunoprecipitation experiments with the monoclonal antibody RL-2, anti-(*O*-GlcNAc), that the DNA-binding domain (residues 401–616) is the major region glycosylated with single *O*-GlcNAc (Fig. 4).

The identification of a higher density of *O*-GlcNAc in the DNA-binding domain suggested that the glycosylation could modulate interactions between HIC1 and its target DNA sequence. Indeed, it appears that the *O*-GlcNAc glycosylation and the phosphorylation of Elf1, a member of the ETS transcription factor family, allow it to migrate to the nucleus and then to bind the TCR ζ chain promoter [7]. EMSAs performed with nuclear proteins from Jurkat T-cells demonstrated that the forms that bind the Elf1 binding site of the TCR ζ chain promoter could be glycosylated, as the observed complex could be supershifted by an antibody directed against Elf1 and by the RL-2 monoclonal antibody. A more complex situation has been described for YY1, a zinc finger transcription factor essential for development of mammalian embryos that is also modified by *O*-GlcNAc [38]. Indeed, the glycosylated YY1 forms did not bind the retinoblastoma protein Rb, as the YY1-Rb complex is significantly more abundant in glucose-deprived cultures [38]. In addition, the glycosylated forms of YY1 are free to bind DNA. These results suggest that O-glycosylation could regulate the transcriptional activity of YY1 by disrupting the Rb-YY1 complex, thus favoring the binding of free YY1 to its consensus DNA sequence. Finally, the *O*-GlcNAc modification of the pancreatic/duodenal homeobox transcription factor PDX-1 increases its DNA-binding affinity and directly correlates with an increase in insulin secretion in pancreatic β cells [32].

In the case of HIC1, EMSA experiments performed on purified pools of glycosylated and nonglycosylated full-length proteins did not unravel salient differences in their DNA-binding properties, demonstrating that the glycosylation is neither directly nor indirectly involved in the DNA-binding activity. In these experiments, complexes of high mobility due to the presence of N-terminal HIC1 truncated forms were also observed (Fig. 5). Notably, these truncated proteins, when glycosylated, cannot bind the specific DNA probe. To confirm these results obtained with a naturally occurring HIC1 proteolysis, we constructed a mutant (399–714) corresponding to the C-terminal half of the protein. This truncated protein is *O*-GlcNAc modified but, in contrast with the full-length protein, this modification occurs in the extreme C-terminal tail (residues 670–711) and not in the DNA-binding domain (Fig. 6). These results provide another convincing example highlighting the

pivotal role played by the BTB/POZ domain, particularly its dimerization properties, in generating the correct conformation and folding of the protein required for its interaction with partners, as already shown for HIC1 and CtBP [28]. Another hypothesis could be that the BTB/POZ per se is required for the interaction between HIC1 and OGT that itself possesses tetratricopeptide repeats (TPR) for interacting with partners. Indeed, the strict requirement for an appropriate conformation of the full-length HIC1 protein mediated mainly by the BTB/POZ dimerization domain has been demonstrated by its interaction with the corepressor CtBP, even though this interaction takes place in a central region located between the BTB/POZ and the zinc fingers domains [28]. Similarly, in the truncated proteins, the true target residues for glycosylation in the DNA-binding domain (residues 401–616) could be not accessible to OGT which could therefore modify non target residues exposed in the C-terminal tail (residues 670–711).

Purified pools of glycosylated 399–714 HIC1 proteins cannot bind the specific DNA-binding sequence (Fig. 7A). In addition, whereas the complex formed between the non fractionated 399–714 proteins and the labeled oligonucleotide can be supershifted by the anti-Flag M2, no supershift could be detected with WGA or with the anti-(*O*-GlcNAc) RL-2 monoclonal antibody (Fig. 7B). Thus, the glycosylated 399–714 truncated proteins cannot bind DNA. As in many cases, the site of *O*-GlcNAc modification is also a phosphorylation site (e.g. c-myc [8]), a plausible hypothesis could be that a residue in the C-terminal tail must be phosphorylated to allow efficient DNA-binding, at least in the context of the truncated proteins.

Several studies have pointed to strong evidence for the importance of *O*-GlcNAc in protein–protein interactions, as discussed above for YY1. For Sp1, it modulates hydrophobic interactions with the TATA binding-protein-associated factor, $TAF_{II}110$ or holo-Sp1 [18]. This protein–protein interaction is inhibited by *O*-GlcNAc, thus reducing the RNA-polymerase II-dependent transcription [18]. In addition, the overexpression of OGT reduces the activity of Sp1, whereas a Sp1 mutant with reduced *O*-GlcNAc exhibits an increased transcriptional activity [39]. Likewise, the *O*-GlcNAc modification of the transcription factor STAT5 on Thr92 is essential for the STAT5-mediated gene transcription, as only the glycosylated form of STAT5 can bind the CBP coactivator [41]. Thus, the *O*-GlcNAc modification of HIC1 which occurs in the zinc fingers without affecting the sequence specific DNA-binding properties could modulate the recruitment of some partners via this domain. Krüppel $C_2H_2$ zinc fingers are not only involved in sequence-specific DNA-binding, but can also mediate protein–protein interactions, as shown for the BCL6 BTB/POZ transcriptional repressor whose zinc fingers can interact with c-Jun and class II HDACs [42]. This latter hypothesis appears highly attractive in the light of the connection recently established between OGT and repressive complexes [11]. In terms of protein stability, the glycosylation of the full-length HIC1 protein could also contribute to its stabilization as shown for Sp1 [16] or the beta-estrogen receptor [17]. Examination of the HIC1 sequence with the PEST FIND program (http://www.at.embnet.org/embnet/tools/bio/PESTfind/) clearly reveals two potential PEST sequences. One of this sequence with a high score is located just upstream of the DNA-binding domain that appears to be *O*-GlcNAc modified. Thus, *O*-GlcNAc could protect the protein against the proteasomal degradation by preventing ubiquitinylation. Indeed, it is clearly known that phosphorylation usually activates PEST sequences for degradation and that a reciprocal balance relationship between phosphorylation and *O*-GlcNAc can regulate the stability of a protein, as shown for m-ER-β [17].

In conclusion, *O*-GlcNAc could play a critical role in transcriptional regulation, even though it is hard to draw a general scheme for the function of this glycosylation as it can play either a negative or a positive role in the function of a transcription factor. Many transcription factors are modified by *O*-GlcNAc, and even if HIC1 completes this long list, to our knowledge it is one of the first transcriptional repressors and only the second tumor suppressor in addition to p53 [19] that has been described to be *O*-GlcNAc. The major point of our work was to describe the *O*-GlcNAc modification of HIC1, which is highly sensible to the dimerization status of the protein.

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## References

1. Wells, L., Whelan, S.A. & Hart, G.W. (2003) *O*-GlcNAc: a regulatory post-translational modification. *Biochem. Biophys. Res. Commun.* **302**, 435–441.
2. Omary, M.B., Ku, N.O., Liao, J. & Price, D. (1998) Keratin modifications and solubility properties in epithelial cells and in vitro. *Subcell. Biochem.* **31**, 105–140.
3. Dong, D.L., Xu, Z.S., Hart, G.W. & Cleveland, D.W. (1996) Cytoplasmic *O*-GlcNAc modification of the head domain and the KSP repeat motif of the neurofilament protein neurofilament-H. *J. Biol. Chem.* **271**, 20845–20852.
4. Cole, R.N. & Hart, G.W. (1999) Glycosylation sites flank phosphorylation sites on synapsin I: *O*-linked N-acetylglucosamine residues are localized within domains mediating synapsin I interactions. *J. Neurochem.* **73**, 418–428.
5. Lefebvre, T., Ferreira, S., Dupont-Wallois, L., Bussiere, T., Dupire, M.-J., Delacourte, A., Michalski, J.-C. & Caillet-Boudin, M.-L. (2003) Evidence of a balance between phosphorylation and *O*-GlcNAc glycosylation of Tau proteins: a role in nuclear localization. *Biochim. Biophys. Acta* **1619**, 167–176.
6. Comer, F.I. & Hart, G.W. (2001) Reciprocity between *O*-GlcNAc and *O*-phosphate on the carboxyl terminal domain of RNA polymerase II. *Biochemistry* **40**, 7845–7852.
7. Juang, Y.T., Solomou, E.E., Rellahan, B. & Tsokos, G.C. (2002) Phosphorylation and *O*-linked glycosylation of Elf-1 leads to its translocation to the nucleus and binding to the promoter of the TCR zeta-chain. *J. Immunol.* **168**, 2865–2871.
8. Kamemura, K., Hayes, B.K., Comer, F.I. & Hart, G.W. (2002) Dynamic interplay between *O*-glycosylation and *O*-phosphorylation of nucleocytoplasmic proteins: alternative glycosylation/phosphorylation of THR-58, a known mutational hot spot of c-Myc in lymphomas, is regulated by mitogens. *J. Biol. Chem.* **277**, 19229–19235.
9. Lefebvre, T., Planque, N., Leleu, D., Bailly, M., Caillet-Boudin, M.-L., Saule, S. & Michalski, J.-C. (2002) *O*-Glycosylation of the

nuclear forms of Pax-6 products in quail neuroretina cells. *J. Cell. Biochem.* **85**, 208–218.
10. Lamarre-Vincent, N. & Hsieh-Wilson, L.C. (2003) Dynamic glycosylation of the transcription factor CREB: a potential role in gene regulation. *J. Am. Chem. Soc.* **125**, 6612–6613.
11. Yang, X., Zhang, F. & Kudlow, J.E. (2002) Recruitment of *O*-GlcNAc transferase to promoters by corepressor mSin3A: coupling protein *O*-GlcNAcylation to transcriptional repression. *Cell* **110**, 69–80.
12. Kreppel, L.K., Blomberg, M.A. & Hart, G.W. (1997) Dynamic glycosylation of nuclear and cytosolic proteins: cloning and characterization of a unique *O*-GlcNAc transferase with multiple tetratricopeptide repeats. *J. Biol. Chem.* **272**, 9308–9315.
13. Gao, Y., Wells, L., Comer, F.I., Parker, G.J. & Hart, G.W. (2001) Dynamic *O*-glycosylation of nuclear and cytosolic proteins: cloning and characterization of a neutral, cytosolic beta-*N*-acetylglucosaminidase from human brain. *J. Biol. Chem.* **276**, 9838–9845.
14. Iyer, S.P. & Hart, G.W. (2003) Dynamic nuclear and cytoplasmic glycosylation: enzymes of *O*-GlcNAc cycling. *Biochemistry* **42**, 2493–2499.
15. O'Donnell, N. (2002) Intracellular glycosylation and development. *Biochim. Biophys. Acta* **1573**, 336–345.
16. Han, I. & Kudlow, J.E. (1997) Reduced *O*-glycosylation of Sp1 is associated with increased proteasome susceptibility. *Mol. Cell. Biol.* **17**, 2550–2558.
17. Cheng, X. & Hart, G.W. (2001) Alternate *O*-glycosylation/*O*-phosphorylation of serine 16 in the murine estrogen receptor-Beta. *J. Biol. Chem.* **276**, 10570–10575.
18. Roos, M.D., Su, K., Baker, J.R. & Kudlow, J.E. (1997) *O*-Glycosylation of an Sp1-derived peptide blocks known Sp1 protein interactions. *Mol. Cell Biol.* **17**, 6472–6480.
19. Shaw, P., Freeman, J., Bovey, R. & Iggo, R. (1996) Regulation of specific DNA binding by p53: evidence for a role for *O*-glycosylation and charged residues at the carboxy-terminus. *Oncogene* **12**, 921–930.
20. Weigert, C., Klopfer, K., Kausch, C., Brodbeck, K., Stumvoll, M., Haring, H.U. & Schleicher, E.D. (2003) Palmitate-induced activation of the hexosamine pathway in human myotubes: increased expression of glutamine: fructose-6-phosphate aminotransferase. *Diabetes* **52**, 650–656.
21. Wales, M.M., Biel, M.A., el Deiry, W., Nelkin, B.D., Issa, J.P., Cavenee, W.K., Kuerbitz, S.J. & Baylin, S.B. (1995) p53 activates expression of HIC1, a new candidate tumour suppressor gene on 17p13.3. *Nat. Med.* **1**, 570–577.
22. Baylin, S.B. & Herman, J.G. (2001) Promoter hypermethylation: can this change alone ever designate true tumor suppressor gene function? *J. Natl Cancer Inst.* **93**, 664–665.
23. Herman, J.G. & Baylin, S.B. (2003) Gene silencing in cancer in association with promoter hypermethylation. *N. Engl. J. Med.* **349**, 2042–2054.
24. Guérardel, C., Deltour, S., Pinte, S., Monte, D., Begue, A., Godwin, A.K. & Leprince, D. (2001) Identification in the human candidate tumor suppressor gene HIC1 of a new major alternative TATA-less promoter positively regulated by p53. *J. Biol. Chem.* **276**, 3078–3089.
25. Carter, M.G., Johns, M.A., Zeng, X., Zhou, L., Zink, M.C., Mankowski, J.L., Donovan, D.M. & Baylin, S.B. (2000) Mice deficient in the candidate tumor suppressor gene Hic1 exhibit developmental defects of structures affected in the Miller–Dieker syndrome. *Hum. Mol. Genet.* **9**, 413–419.
26. Chen, W.Y., Zeng, X., Carter, M.G., Morrell, C.N., Chiu Yen, R.W., Esteller, M., Watkins, D.N., Herman, J.G., Mankowski, J.L. & Baylin, S.B. (2003) Heterozygous disruption of Hic1 predisposes mice to a gender-dependent spectrum of malignant tumors. *Nat. Genet.* **33**, 197–202.
27. Deltour, S., Guerardel, C. & Leprince, D. (1999) Recruitment of SMRT/N-CoR-mSin3A-HDAC-repressing complexes is not a general mechanism for BTB/POZ transcriptional repressors: the case of HIC-1 and γFBP-B. *Proc. Natl Acad. Sci. USA* **96**, 14831–14836.
28. Deltour, S., Pinte, S., Guerardel, C., Wasylyk, B. & Leprince, D. (2002) The human candidate tumor suppressor gene HIC1 recruits CtBP through a degenerate GLDLSKK motif. *Mol. Cell. Biol.* **22**, 4890–4901.
29. Pinte, S., Stankovic-Valentin, N., Deltour, S., Rood, B.R. & Guerardel and Leprince, D. (2004) The tumor suppressor gene HIC1 (hypermethylated in cancer 1) is a sequence-specific transcriptional rerpressor: definition of its consensus binding sequence and analysis of its DNA-binding and repressive properties. *J. Biol. Chem.* in press.
30. Bertrand, S., Pinte, S., Stankovic-Valentin, N., Deltour-Balerdi, S., Guérardel, C., Begue, A., Laudet, V. & Leprince, D. (2004) Identification and developmental expression of the zebrafish orthologue of the tumor suppressor gene *HIC1*. *Bioch. Biophys. Acta* **1678**, 57–66.
31. Han, I., Oh, E.S. & Kudlow, J.E. (2000) Responsiveness of the state of *O*-linked N-acetylglucosamine modification of nuclear pore protein p62 to the extracellular glucose concentration. *Biochem. J.* **350**, 109–114.
32. Gao, Y., Miyazaki, J. & Hart, G.W. (2003) The transcription factor PDX-1 is post-translationally modified by *O*-linked N-acetylglucosamine and this modification is correlated with its DNA binding activity and insulin secretion in min6 beta-cells. *Arch. Biochem. Biophys.* **415**, 155–163.
33. Starr, C.M. & Hanover, J.A. (1990) Glycosylation of nuclear pore protein p62: reticulocyte lysate catalyzes *O*-linked N-acetylglucosamine addition *in vitro*. *J. Biol. Chem.* **265**, 6868–6873.
34. Torres, C.R. & Hart, G.W. (1984) Topography and polypeptide distribution of terminal N-acetylglucosamine residues on the surfaces of intact lymphocytes: evidence for O-linked GlcNAc. *J. Biol. Chem.* **259**, 3308–3317.
35. Hanover, J.A., Cohen, C.K., Willingham, M.C. & Park, M.K. (1987) *O*-Linked N-acetylglucosamine is attached to proteins of the nuclear pore: evidence for cytoplasmic and nucleoplasmic glycoproteins. *J. Biol. Chem.* **262**, 9887–9894.
36. Wells, L., Vosseller, K. & Hart, G.W. (2003) A role for N-acetylglucosamine as a nutrient sensor and mediator of insulin resistance. *Cell. Mol. Life Sci.* **60**, 222–228.
37. Li, J.-Y., English, M.A., Ball, H.J., Yeyati, P.L., Waxman, S. & Licht, J.L. (1997) Sequence-specific DNA binding and transcriptional regulation by the promyelocytic leukemia zinc finger protein. *J. Biol. Chem.* **272**, 22447–22455.
38. Ivins, S., Pemberton, K., Guidez, F., Howell, L., Krumlauf, R. & Zelent, A. (2003) Regulation of Hoxb2 by APL-associated PLZF protein. *Oncogene* **22**, 3685–3697.
39. Hiromura, M., Choi, C.H., Sabourin, N.A., Jones, H., Bachvarov, D. & Usheva, A. (2003) YY1 is regulated by *O*-linked N-acetylglucosaminylation (*O*-GlcNAcylation). *J. Biol. Chem.* **278**, 14046–14052.
40. Yang, X., Su, K., Roos, M.D., Chang, Q., Paterson, A.J. & Kudlow, J.E. (2001) *O*-Linkage of N-acetylglucosamine to Sp1 activation domain inhibits its transcriptional capability. *Proc. Natl Acad. Sci. USA* **98**, 6611–6616.
41. Gewinner, C., Hart, G., Zachara, N., Cole, R., Beisenherz-Huss, C. & Groner, B. (2004) The coactivator of transcription CREB binding protein interacts preferentially with the glycosylated form of Stat5. *J. Biol. Chem.* **279**, 3563–3572.
42. Lemercier, C., Brocard, M.P., Puvion-Dutilleul, F., Kao, H.Y., Albagli, O. & Khochbin, S. (2002) Class II histone deacetylases are directly recruited by BCL6 transcriptional repressor. *J. Biol. Chem.* **277**, 22045–22052.