Université des Sciences et Technologies de Lille I
Unit for Structural and Functional Glycobiology

Katholieke Universiteit Leuven
Group Biomedical Sciences
Faculty of Medicine
Center for Human Genetics

Characterization of novel CDG-I defects

New insights in dolichol cycle regulation and ER quality control

Wendy Vleugels

Doctoral Thesis in Biology and Health
Lille, 2009-2010

Doctoral Thesis in Biomedical Sciences
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Cover HPLC profile of the LLO detected in a control, a RFT1-deficient patient and a patient with a defect in ALG11.
The dolichol cycle, inspired from Helenius, A. and Aebi, M. 2004.

Met dank aan An Lommelen.

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Wendy

Table of contents

List of abbreviations

ApoC-III	apolipoprotein C-III
CDG	Congenital Disorders of Glycosylation
CDG-I	CDG type I
CDG-Ix	unsolved CDG-I
CDG-II	CDG type II
CHO	chinese hamster ovary
CNX	calnexin
CRT	calreticulin
CZE	capillary zone electrophoresis
DHPLC	denaturing HPLC
Dol-P	dolichol phosphate
Dol-PP	dolichol pyrophosphate
DPM	Dol-P-Man synthase
ENGase	endo-β-N-acetylglucosaminidase
ER	endoplasmic reticulum
ERAD	ER-associated degradation
ESI-MS	electrospray ionization mass spectrometry
Gal	galactose
GalNAc	N-acetylgalactosamine
GDP	guanosine diphosphate
Glc	glucose
GlcNAc	N-acetylglucosamine
GMPP	GDP-Man pyrophosphorylase
GTP	guanosine triphosphate
HPLC	high-performance liquid chromatography
IEF	iso-electric focusing
LLO	lipid-linked oligosaccharides
Man	mannose
MRI	magnetic resonance imaging

OST	oligosaccharyltransferase
PCR	polymerase chain reaction
PNGase	peptide: N-glycanase
POS	phosphorylated oligosaccharides
Sia	sialic acid
SNP	single nucleotide polymorphism
SRP	signal recognition particle
UDP	uridine diphosphate
UGGT	UDP-glucose: glycoprotein glucosyltransferase

General introduction

What is glycosylation? A highly diverse post-translational modification.

The number of proteins encoded by the human genome is estimated at 25000 (Stein, L.D. 2004), a surprisingly low number regarding the complexity of our species. How does a relatively small number of proteins generate an enormous biological diversity essential for the development, growth and functioning of an intact organism? Newly synthesized proteins in the cell are modified either during or after protein synthesis to extend their range of biological functions. During the past decades, many co- and/or post-translational modifications were identified. They include phosphorylation, sulfation, methylation, ubiquitylation, N-acetylation and glycosylation .

Glycosylation, defined by the covalent attachment of glycans onto proteins (or lipids), is one of the most common co- or post-translational modifications since 80 to 90% of all proteins are estimated to be glycosylated (Gupta, R. and Brunak, S. 2002). In addition, glycosylation is found in every cell type of an organism and is distributed throughout the entire phylogenetic spectrum, ranging from archea and eubacteria to higher eukaryotes. In spite of this strong conservation, glycans are the most structurally diverse biopolymers formed in nature. To fulfill a glycosylation reaction, the following components are needed: a monosaccharide donor, a protein or lipid acceptor, an enzyme and a specific environment to carry out the reaction. The huge diversity of glycosylation is created by combining different monosaccharide donors with multiple acceptors in a variety of ways. In protein glycosylation, thirteen monosaccharides can be linked to eight amino acids, generating at least thirty-one different glycosylation types (Lis, H. and Sharon, N. 1993). The attached monosaccharides can subsequently be elongated with additional monosaccharides differing in sequence, chain length and type of linkage, generating an even broader spectrum of glycoprotein linkages. Since most glycosyltransferases catalyze only one specific sugar-sugar or sugar-protein linkage, a multiplicity of glycosyltransferases is needed to cover this diversity of glycosylation. Species- and cell-specific glycosylation is also greatly variable. Vertebrates synthesize a highly complex repertoire of glycan structures that are structurally distinct from those of lower eukaryotes or bacteria. Cell-specific glycosylation is created by regulating the expression of glycan synthesizing enzymes according to the type and physiological state of the cell.

In mammals, the different glycosylation types can be divided into two major forms: cytosolic glycosylation and compartmentalized glycosylation occurring in specific cellular organelles

(Figure 1). In 1983, Hart and co-workers identified the first type of glycosylation occurring outside the secretory pathway (Hart, G.W., *et al.* 1988): on many cytosolic and nuclear proteins, binding of N-acetylglucosamine (GlcNAc) in β to serine or threonine residues of the protein occurs. In contrast to most other forms of glycosylation, this GlcNAc residue is not elongated by additional monosaccharides. O-GlcNAc glycosylation is involved in signal transduction and modulates protein function analogous to protein phosphorylation (Zachara, N.E. and Hart, G.W. 2004).

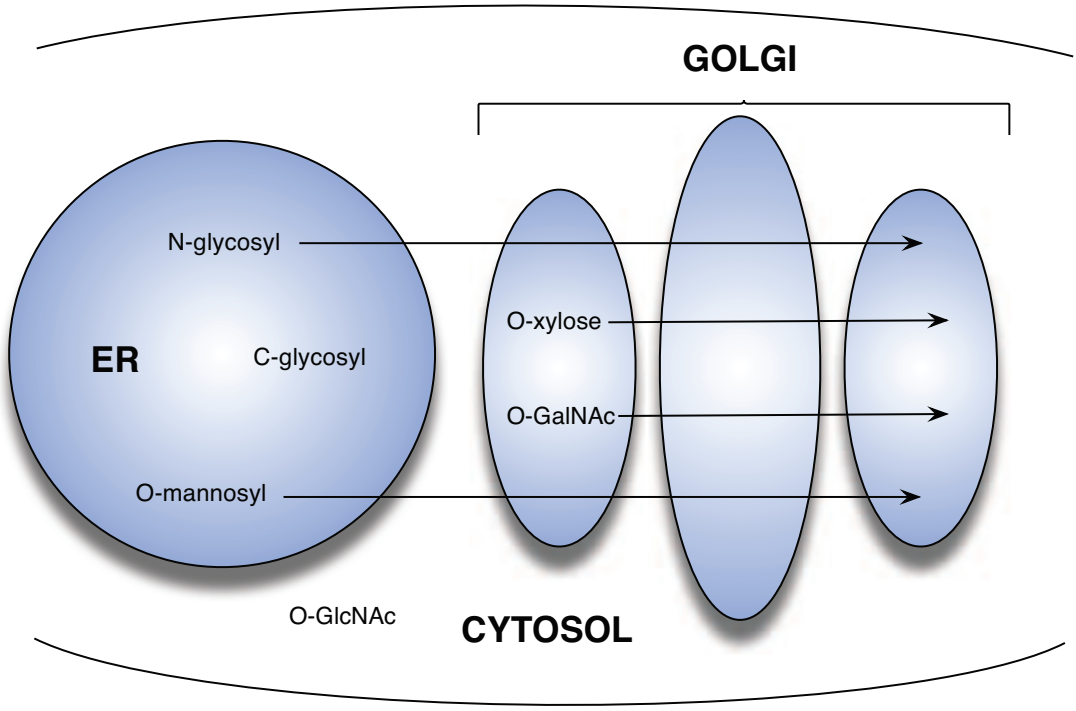

Figure 1: Sub-cellular localization of the different protein glycosylation types. N-glycosylation, C-glycosylation and O-mannosylation is initiated in the ER and proceeds in the Golgi compartment, while O-xylosylation and O-GalNAc glycosylation only takes place in the Golgi compartment.

All other types of glycosylation occur in two different organelles of the cell: the endoplasmic reticulum (ER) and/or the Golgi compartment (Figure 1). Among these types, three major forms can be distinguished: 1) N-glycosylation or the addition of glycans to the amide group of asparagine residues, 2) O-glycosylation in which the glycan is linked to the hydroxyl group of serine or threonine residues and 3) C-glycosylation or the binding of a glycan to the C2 atom of tryptophan via an unusual C-C bond. During N-glycosylation, an oligosaccharide precursor consisting of three glucoses (Glc), nine mannoses (Man) and two GlcNAc residues is linked in β to an asparagine residue of the protein. This reaction occurs in the ER and the resulting glycoprotein is further modified in the Golgi compartment (Figure 1) (Yan, A. and

Lennarz, W.J. 2005). N-glycosylation is the most widespread form of glycosylation and will later be discussed in detail.

O-glycosylation is complex and divided in different subtypes according to the first monosaccharide linked to serine or threonine. One of the most frequent subtypes of O-glycosylation is the binding of N-acetylgalactosamine (GalNAc) in α to serine or threonine. In contrast to N-glycosylation, O-GalNAc is synthesized, and further elongated, in the Golgi compartment without a prior biosynthetic step in the ER (Figure 1). Since O-GalNAc glycosylation is mainly found in mucins, it is also called the mucin type of glycosylation (Spiro, R.G. 1973). In glycosaminoglycans like heparan sulphate and chondroitin sulphate, xylose is linked in β to serine or threonine residues. After xylose addition in the Golgi compartment, two galactoses (Gal) and one glucuronic acid (GlcA) are added and subsequently extended with the repeating disaccharides GlcA-GalNAc in the case of chondroitin sulphate or GlcA-GlcNAc in the case of heparan sulphate (Kjellen, L. and Lindahl, U. 1991). Another well-studied subtype of O-glycosylation is O-mannosylation in which Man is linked in α to serine or threonine. As shown in Figure 1, this reaction takes place in the ER and O-mannosylation is further elongated in the Golgi compartment by the sequential addition of GlcNAc, Gal and sialic acid (Sia) (Lommel, M. and Strahl, S. 2009).
Man can also be linked to a tryptophan residue of the protein via an unusual C-C bond . This type of glycosylation, called C-mannosylation, exclusively takes place in the ER lumen (Figure 1) (Doucey, M.A., *et al.* 1998).

Roles of glycosylation: from protein folding to endocytosis

The huge repertoire of glycan structures and glycosylation types results in a broad spectrum of biological roles for glycosylation. For a long time, only a decorative role was assigned to glycosylation but numerous studies during the last thirty years demonstrated the essential role of glycosylation in protein function. Glycosylation modulates protein-protein interactions and thereby influences several cellular functions ranging from protein folding and intracellular trafficking to gene transcription, cell adhesion, protein clearance, receptor activation and endocytosis (Figure 2) (Varki, A. 1993).

Protein folding

Glycosylation and particularly N-glycosylation plays a role in protein folding and oligomerization. Many examples illustrated that the removal of glycans from the folded protein had no effect on protein activity but altered protein stability and folding kinetics. These observations pointed to a chaperone-like activity of glycans. Deletion of one or more N-glycosylation sites of the simian virus 5 hemagglutinin sialidase by site-directed

mutagenesis resulted for example in an impaired folding and subsequent retention in the ER. The severity of the impairment depended on the number of glycosylation sites deleted as well as on their location within the protein (Ng, D.T., *et al.* 1990). Glycosylation also modulates protein oligomerization by mediating inter-subunit interactions. The human hormone chorionic gonadotropin (hCG) is a heterodimer consisting of non-covalently associated α and β subunits. The hCG heterodimer association is prevented or stimulated depending on the type of glycan present on the α subunit (Jentoft, N. 1990). Introduction of an extra N-glycan, normally not present in the protein, can also affect protein oligomerization. A mutated form of fibrinogen bearing an extra N-glycosylation site is converted into fibrin but its aggregation, essential for blood clotting, is impaired (Maekawa, H., *et al.* 1991).

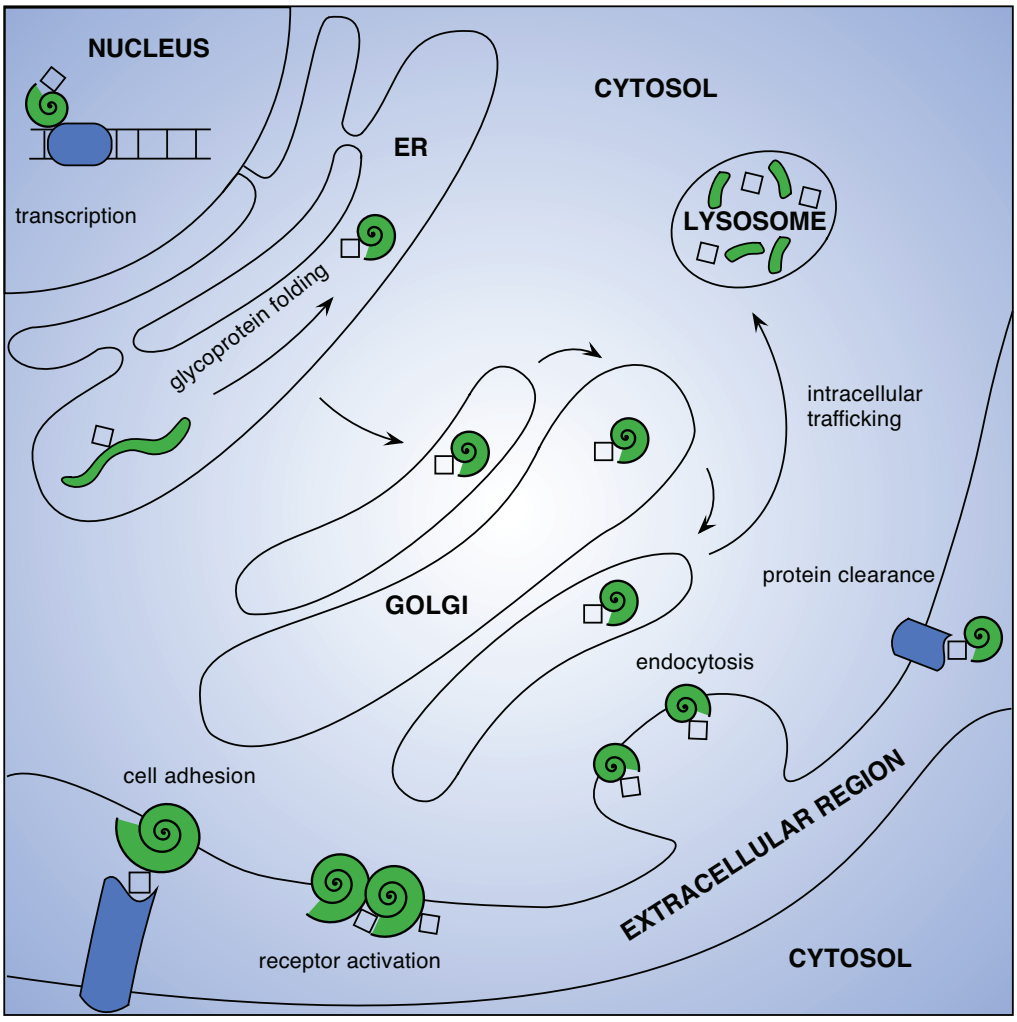

Figure 2: The huge repertoire of glycan structures and glycosylation types results in a broad spectrum of biological roles for glycosylation. The main cellular functions requiring glycosylation are: gene transcription in the nucleus, glycoprotein folding in the ER, intracellular trafficking of glycoproteins destined for the lysosomes, protein clearance from the extracellular region, endocytosis of transmembrane proteins expressed at the cell membrane, receptor activation and cell adhesion (figure inspired from Ohtsubo, K. and Marth, J.D. 2006).

Intracellular trafficking

Another important function of glycosylation concerns the targeting of proteins to the appropriate cellular location. For example, Man-6-P residues on newly synthesized N-glycoproteins signal their targeting to the lysosomes. In lysosomal membranes, a Man-6-P specific receptor facilitates the translocation of the newly synthesized proteins into the lysosomes (von Figura, K. 1991). A deficiency in N-acetylglucosamine-1-phosphotransferase, called I cell disease, results in an impaired trafficking of proteins to the lysosomes (Kornfeld, S. 1986). Although still poorly understood, O-GlcNAc is also believed to be involved in intracellular trafficking, more specifically in nuclear transport. For example, a decrease in the O-GlcNAc modification of Tau, a brain microtubule-associated protein, correlated with a decrease in its nuclear transport (Lefebvre, T., *et al.* 2003). In addition, the O-GlcNAc glycosylated forms of Pax6, a transcription factor involved in eye morphogenesis, are exclusively located in the nucleus, while unglycosylated Pax6 is located in the cytosol. O-GlcNAc could thus be involved in maintaining glycosylated Pax6 forms in the nucleus (Lefebvre, T., *et al.* 2002).

Gene transcription

O-GlcNAc plays also a crucial role in the regulation of transcription. To date, numerous transcription factors have been shown to be modified by O-GlcNAc. The best studied example is Sp-1 for which O-GlcNAc modification modulates its transactivation capability by blocking protein-protein interactions (Yang, X., *et al.* 2001). In addition, increased O-GlcNAc modification of the transcription factors p53 and NF-κβ is associated with increased transcriptional activity (Vosseller, K., *et al.* 2002). Transcriptional regulation by O-GlcNAc is especially involved in glucose sensing and glucotoxicity (Issad, T. and Kuo, M. 2008).

Cell adhesion

Glycosylation is also important for cell adhesion. Cell adhesion is achieved through sugar binding proteins or lectins that are highly specific for a certain glycan structure (Goldstein, I.J. 2002). Lectin-glycan interactions are for example essential for the proper functioning of our immune system. Leukocyte trafficking to sites of inflammation is mediated by the endogenous lectin E-selectin on endothelial cells, which recognizes the O-glycan structure sialyl-Lewis^x expressed on leukocytes (Vestweber, D. and Blanks, J.E. 1999). Lectin-glycan interactions are involved in cell adhesion during fertilization. The interaction of O-glycans on the zona pellucida of the egg with lectins on the sperm is essential for species-specific fertilization in mammals (Miller, D.J., *et al.* 1992).

Protein clearance

Protein clearance or the removal of proteins from circulation is another process that relies on glycosylation. Newly synthesized serum glycoproteins like erythropoietin bear sialylated glycans. During vascular circulation, enzymes on the blood vessel wall sequentially remove these sialic acids from the glycoprotein resulting in the formation of asialoglycoproteins. These asialoglycoproteins are recognized and cleared from circulation by specific receptors in the liver (Ashwell, G. and Harford, J. 1982). Another protein clearance system relies on the sulfatation of glycoproteins. Luteinizing hormone levels are for example regulated by receptors in the liver that are specific for sulfated glycans (Drickamer, K. 1991).

Receptor activation and signal transduction

Glycans can also modulate receptor-ligand interactions and signal transduction. The affinity of the luteinizing hormone receptor for its ligand is for example decreased by the removal of its glycosylation sites by site-directed mutagenesis (Zhang, R., *et al.* 1991). Removal of all N-glycosylation sites of the β subunit of the insulin receptor however did not affect ligand affinity but prevented the stimulation of glucose transport and glycogen synthesis (Leconte, I., *et al.* 1992).

Endocytosis

Endocytosis is a process by which cells internalize extracellular material by invagination of the plasma membrane. Glycosylation is involved in modulating the endocytosis of cell-surface glycoproteins thereby influencing their expression and turnover. Expression of the Golgi glycosyltransferase GlcNAcT-V for example decreases the endocytosis of EGF and TGF-beta receptors, altering receptor activation and signaling among epithelial carcinoma cells (Partridge, E.A., *et al.* 2004).

N-glycosylation in general

N-glycosylation or the linkage of glycans to asparagine residues of the protein is the most widespread form of glycosylation responsible for the modification of secreted and transmembrane proteins. During evolution, N-glycosylation evolved from a process implicated in pathogenesis and host invasion in archea and bacteria to a complex and multi-functional protein modification in eukaryotic cells (Weerapana, E. and Imperiali, B. 2006). In bacteria and archea, N-glycans are added onto proteins at the cell surface, while this process occurs in specialized cellular organelles in eukaryotes (Weerapana, E. and Imperiali, B. 2006). In eukaryotes, N-glycans are added to nascent proteins in the ER by means of an oligosaccharide precursor consisting of 3 Glc, 9 Man and 2 GlcNAc residues. After protein

transfer, the oligosaccharide precursor is trimmed in the ER and Golgi compartment to build it up again with different monosaccharides (Herscovics, A. 1999). Why did cells evolve this complex and apparently wasteful biosynthetic pathway? Because the glycan structures produced by monosaccharide trimming and/or addition are not biosynthetic intermediates but have specific functions on their own (Helenius, A. and Aebi, M. 2004).

In the ER, partial trimming of the oligosaccharide precursor plays a role in glycoprotein folding and quality control. Since glycan trimming differs according to the folding state of the protein, glycans serve as tags to control the fate of the glycoprotein (Figure 3). Depending on the folding state, glycoproteins are secreted, retained in the ER or translocated to the cytosol for degradation (Helenius, A. and Aebi, M. 2001). In the Golgi compartment, a diversity of glycan structures is generated by monosaccharide trimming and/or addition allowing to fine-tune glycoprotein activity and function, thereby generating species- and cell-type specific characteristics (Figure 3) (Herscovics, A. 1999). In mammalian systems, glycosyltransfeases in the Golgi compartment catalyze the addition of different monosaccharides such as Gal, GlcNAc and Sia, while in yeast only polymannose is added and no elongation occurs in insect cell (Lehle, L. 1992, Marchal, I., *et al.* 2001).

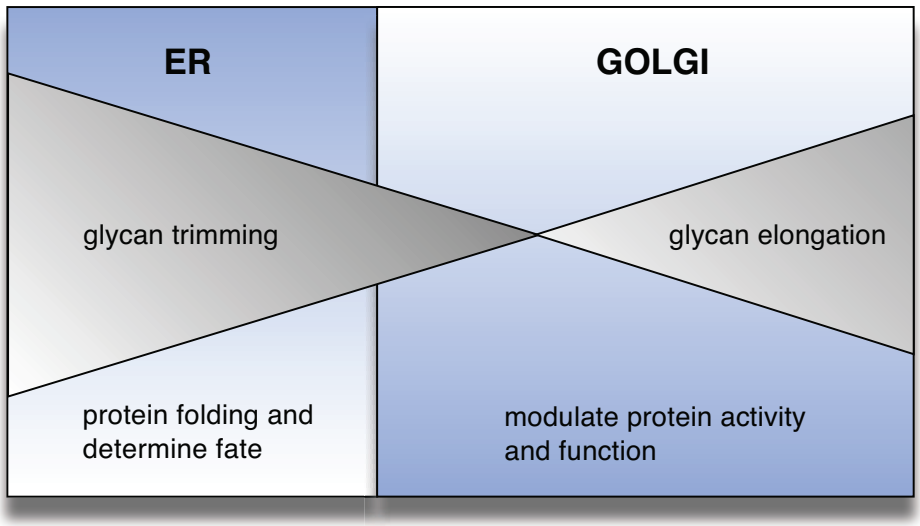

Figure 3: In eukaryotes, N-glycans are added to nascent proteins in the ER by means of an oligosaccharide precursor, which is subsequently trimmed in the ER and Golgi compartment to build it up again with different monosaccharides. The glycan structures produced by monosaccharide trimming and/or addition are not biosynthetic intermediates but have specific functions in protein folding and determining glycoproteins' fate in the ER as well as in modulating protein activity and protein function in the Golgi compartment.

N-glycoprotein biosynthesis: a multi-step process

The biosynthesis of an eukaryotic N-glycoprotein can be divided into four phases. In a first phase, the protein is synthesized in the cytosol and co-translationally translocated to the lumen of the ER. In parallel, an oligosaccharide precursor is assembled on the ER membrane

and in the next phase transferred onto the protein in a co-translational manner. In the fourth and last phase, the proper folding of the glycoprotein is evaluated by a quality control system.

Protein synthesis and translocation to the ER

The synthesis of all proteins starts on free ribosomes in the cytosol (Figure 4). However, secretory proteins contain a specific hydrophobic sequence at their N-terminus, called the signal sequence, involved in directing proteins to the ER. As soon as the signal sequence emerges from the ribosome, it is recognized by the signal recognition particle (SRP) which stops translation and directs the ribosomes to the translocon complex via binding to the SRP receptor (Meacock, S.L., *et al.* 2000). The translocon complex is an aqueous protein conducting channel in the ER membrane, allowing the co-translational translocation of newly synthesized proteins to the lumen of the ER. As translation proceeds, the newly synthesized protein is inserted into the translocon pore and subsequently enters the lumen of the ER (Figure 4). In eukaryotes, the translocon complex is a hetero-oligomeric complex consisting of three transmembrane proteins: Sec61α, Sec61β and Sec61γ in mammals or Sec61p, Sbh1p and Sss1p in yeast (Rapoport, T.A., *et al.* 1996). In yeast, a second complex with high similarity to the trimeric Sec61p complex has been identified. This complex is composed of the subunits Ssh1p, Sbh2p and Sss1p and was shown to specifically translocate proteins with a strong hydrophobic signal sequence (Finke, K., *et al.* 1996). In humans, two isomers of the Sec61α subunit are annotated in sequence databases: Sec61A1 and Sec61A2. During translocation, the permeability barrier of the ER membrane must be retained. To this end, the ribosomes and the translocon complex form an ion-tight association that seals off the cytosolic side of the translocon pore. As shown in figure 4, the luminal side of the translocon pore is sealed by the Hsp70 chaperone BiP, which is released as soon as nascent protein chain length reaches about seventy amino acid residues. When translation is terminated, the ribosomes dissociate from the translocon complex and BiP again seals the luminal side of the pore (Hamman, B.D., *et al.* 1998).

Oligosaccaride precursor assembly: the dolichol cycle

The biosynthesis of the N-glycan is initiated by a highly regulated and step-wise process known as the dolichol cycle in which an oligosaccharide precursor $Glc_3Man_9GlcNAc_2$ is assembled on a dolichol-pyrophosphate (Dol-PP) carrier in the ER membrane (Burda, P. and Aebi, M. 1999). The dolichol cycle has most extensively been studied in yeast and is strongly conserved among higher order eukaryotic systems. This has been an advantage for the identification of novel congenital disorders of glycosylation (see below).

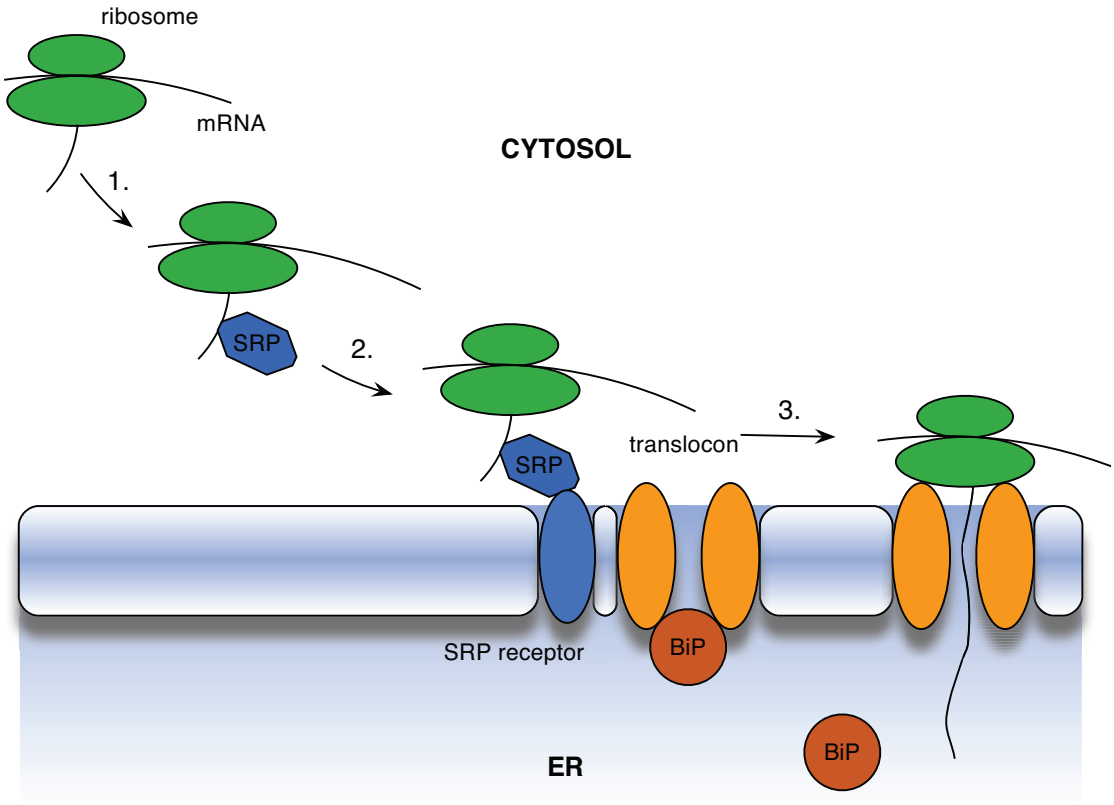

Figure 4: Synthesis of secretory proteins in the cytosol and its translocation to the ER. 1. Binding of SRP to the signal sequence. 2. Targeting the ribosomes to the translocon complex by interacting with the SRP receptor. 3. Insertion of the protein in the translocon pore and BiP dissociation.

a. Synthesis of the building blocks

The assembly of Dol-PP-$GlcNAc_2Man_9Glc_3$ requires five building blocks: dolichol-P (Dol-P) and four different sugar donors. Dolichol is a neutral lipid consisting of α-saturated (S) polyisoprenyl-phosphates containing 18-21 isoprene units in mammals. Dolichol biosynthesis is initiated by the condensation of three isopentylpyrophosphates resulting in the formation of farnesylpyrophosphate (Rilling, H.C. and Chayer, L. 1985). As shown in figure 5, farnesylpyrophosphate is successively elongated by cis-prenyltransferase catalyzing the head-to-tail condensation of farnesylpyrophosphate with several isopentylpyrophosphate units (Crick, D.C., *et al.* 1991). When its final length is achieved, the resulting polyprenylpyrophosphate is dephosphorylated by a yet unknown protein and the α-isoprene unit is subsequently reduced by an NADPH dependent polyprenyl-reductase, thereby forming dolichol (Figure 5) (Sagami, H., *et al.* 1993). Before dolichol can serve as a substrate for oligosaccharide precursor assembly, it is phosphorylated by the action of a dolichol-specific kinase, SEC59, resulting in the formation of Dol-P (Kranz, C., *et al.* 2007). The availability of Dol-P at the cytosolic face of the ER membrane is a rate limiting factor in the dolichol cycle and N-glycosylation (Rush, J.S., *et al.* 2008).

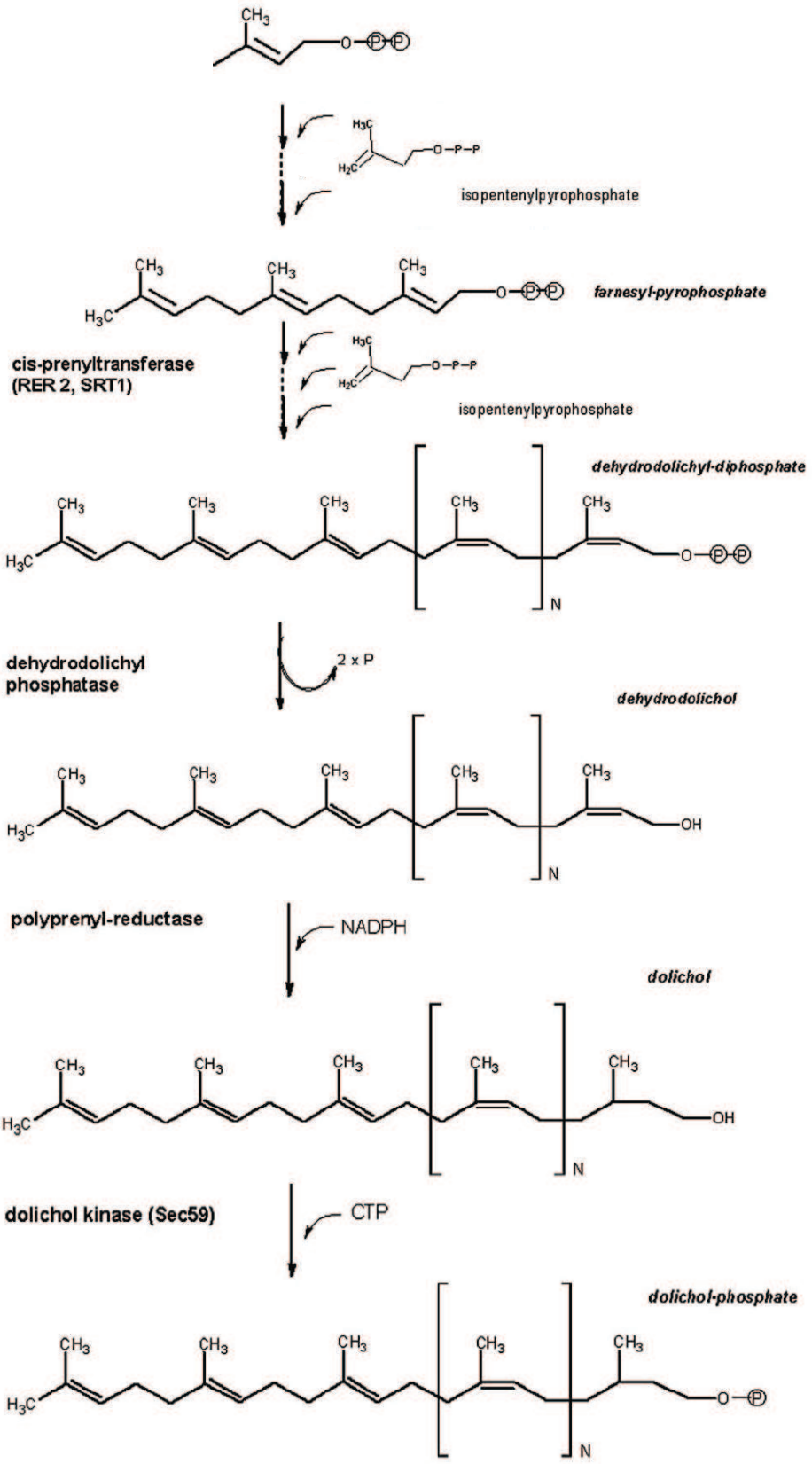

Figure 5: The biosynthesis of dolichol is initiated by the formation of farnesylpyrophosphate, which is successively elongated with several isopentylpyrophosphate units in order to form dehydrodolichol-diphosphate. Dehydrodolichol-diphosphate is subsequently converted into dolichol by dephosphorylation and reduction of the α-isoprene unit. In a final step, dolichol is phosphorylated by the dolichol kinase Sec59 (Denecke, J. and Kranz, C. 2009).

The sugar donors required for oligosaccharide precursor assembly are uridine-diphospho-GlcNAc (UDP-GlcNAc), guanosine-diphospho-Man (GDP-Man), Dol-P-Man and Dol-P-Glc. The nucleotide-activated sugar donors UDP-GlcNAc and GDP-Man are synthesized from monosaccharides that are taken up by the cell. Subsequently, they are phosphorylated by GlcNAc kinase and hexokinase respectively, yielding monosaccharide-6-P. The biosynthesis of UDP-GlcNAc is initiated by the conversion of GlcNAc-6-P to GlcNAc-1-P by N-acetylglucosamine phosphomannomutase (Hofmann, M., *et al.* 1994). GlcNAc-1-P is then converted into UDP-GlcNAc by a UDP-GlcNAc pyrophosphorylase that uses UTP as a donor. GDP-Man can either be synthesized from Man-6-P or fructose-6-P that is converted into Man-6-P by phosphomannoisomerase (Smith, D.J., *et al.* 1992). Phosphomannomutase subsequently catalyzes the conversion of Man-6-P into Man-1-P (Kepes, F. and Schekman, R. 1988). GDP-Man pyrophosphorylase or GMPP catalyzes the final step in GDP-Man synthesis in which Man-1-P is transferred to GTP. In mammals, GMPP is composed of two subunits (GMPPA and GMPPB) but their exact functions in the complex have not yet been identified (Ning, B. and Elbein, A.D. 2000). The dolichol-linked monosaccharides are generated from Dol-P and the corresponding nucleotide-activated sugar at the cytosolic face of the ER membrane. Once synthesized, they are flipped to the ER lumen by an unknown mechanism. Dol-P-Man is synthesized by the Dol-P-Man synthase, which transfers Man from GDP-Man to Dol-P. In lower eukaryotes like yeast, Dol-P-Man synthase consists of a single component, the Dpm1 protein, which possesses a transmembrane region for anchoring the protein to the ER membrane (Orlean, P., *et al.* 1988). The human homologue DPM1 lacks this transmembrane region, suggesting the existence of additional proteins to anchor the enzyme to the ER membrane. Hence, the human Dol-P-Man synthase consists of three proteins: the catalytic and soluble protein DPM1 in addition to two transmembrane proteins DPM2 and DPM3 (Maeda, Y., *et al.* 2000). Dol-P-Glc is on the other hand synthesized by the transmembrane protein ALG5 Dol-P-Glc synthase from UDP-Glc and Dol-P (Heesen, S., *et al.* 1994).

b. Oligosaccharide precursor assembly at the cytosolic face of the ER membrane

The assembly of the oligosaccharide precursor on Dol-P starts at the cytosolic face of the ER membrane with the transfer of GlcNAc-P from UDP-GlcNAc to Dol-P, a reaction catalyzed by the ALG7 GlcNAc-P transferase (Figure 6) (Burda, P. and Aebi, M. 1999). The resulting Dol-PP-GlcNAc then acts as a substrate for the hetero-oligomeric glycosyltransferase ALG13/ALG14, which catalyzes the addition of the second β(1,4)GlcNAc residue (Bickel, T., *et al.* 2005). ALG13 is the cytosolic and catalytic subunit, while the membrane protein ALG14 recruits ALG13 to the ER (Gao, X.D., *et al.* 2005). As shown in figure 6, the first mannosylation step is carried out by the ALG1 mannosyltransferase using GDP-Man as a

nucleotide donor (Burda, P. and Aebi, M. 1999). For a long time it was unclear which proteins catalyze the further elongation of Dol-PP-GlcNAc$_2$Man to Dol-PP-GlcNAc$_2$Man$_5$. Recently, only two enzymes (ALG2 and ALG11) were shown to orchestrate the four remaining mannosylation reactions at the cytosolic face of the ER membrane, also using GDP-Man as a nucleotide donor (O'Reilly, M.K., *et al.* 2006). ALG2 is responsible for both the α(1,3) and α(1,6) mannosylation, forming the first branched intermediate in oligosaccharide precursor assembly, and ALG11 catalyzes the elongation of Dol-PP-GlcNAc$_2$Man$_3$ to Dol-PP-GlcNAc$_2$Man$_5$ by the sequential addition of two α(1,2) mannose residues (Figure 6).

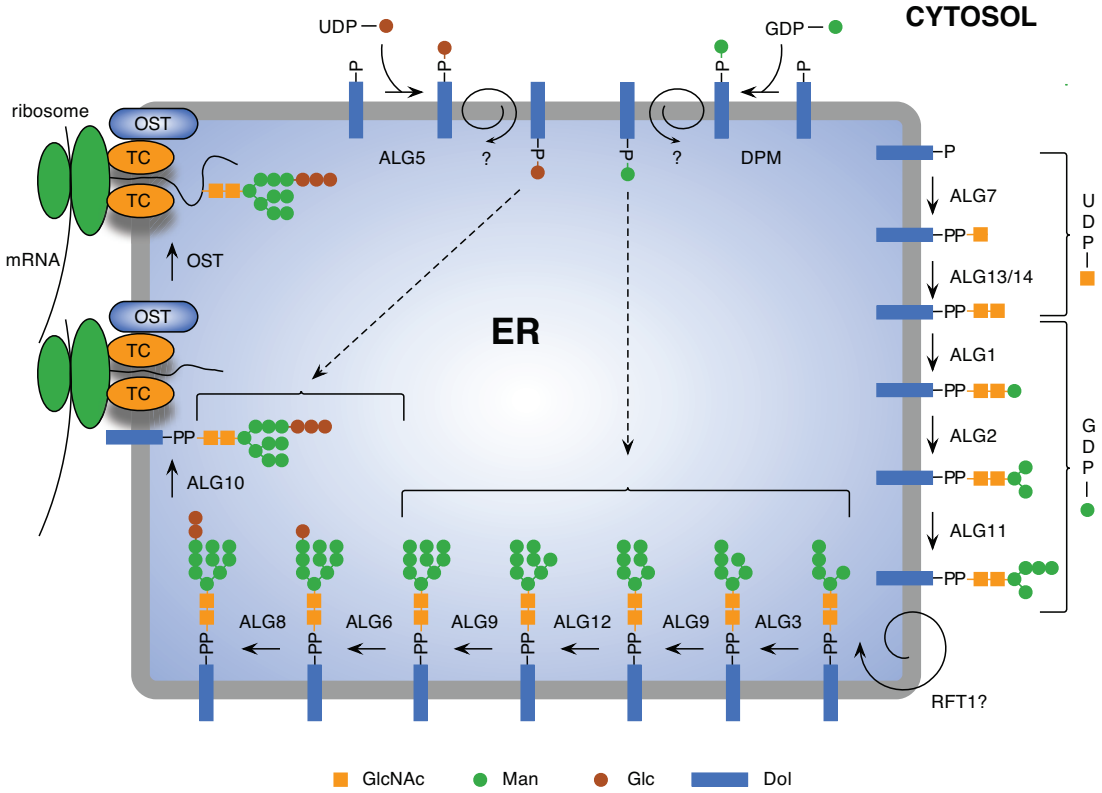

Figure 6: The biosynthesis of an N-glycan is initiated by a highly regulated and step-wise process known as the dolichol cycle in which an oligosaccharide precursor consisting of three Glc, nine Man and two GlcNAc residues (Glc$_3$Man$_9$GlcNAc$_2$) is assembled on a dolichol-pyrophosphate (Dol-PP) carrier in the ER membrane. The first five steps of the dolichol cycle take place at the cytosolic face of the ER membrane, while further elongation occurs in the ER lumen. Once the complete oligosaccharide precursor is synthesized, it is transferred onto newly synthesized proteins by the OST complex. TC: translocon complex (figure inspired from Helenius, A. and Aebi, M. 2004).

c. Translocation of Dol-PP-GlcNAc$_2$Man$_5$ to the ER lumen

Elongation of Dol-PP-GlcNAc$_2$Man$_5$ occurs in the lumen of the ER, thus requiring the translocation of the assembly intermediate from the cytosolic to the luminal face of the ER membrane. This transmembrane movement is of fundamental importance for the dolichol cycle and is extremely slow when occurring unassisted (Hanover, J.A. and Lennarz, W.J.

1979). Hence, this key step is facilitated by a flippase translocating Dol-PP-$GlcNAc_2Man_5$ in a bidirectional and ATP independent manner. In addition, this flippase is not strictly specific for Dol-PP-$GlcNAc_2Man_5$ but also translocates, although inefficiently, smaller assembly intermediates like Dol-PP-$GlcNAc_2Man_3$ (Helenius, J., *et al.* 2002). In yeast, Helenius and co-workers identified the Rft1 protein as the putative flippase, but this hypothesis is currently under debate. A recent study performed by Sanyal and co-workers suggested that Rft1 is not the flippase itself but plays only an accessory role in Dol-PP-$GlcNAc_2Man_5$ translocation (Sanyal, S., *et al.* 2008). In addition, Rush and co-workers found that suppression of Rft1 expression did not impair Dol-PP-$GlcNAc_2Man_5$ translocation in yeast microsomes (Rush, J.S., *et al.* 2009). Further studies are thus needed to define the exact physiological role of Rft1 and to identify the flippase.

d. Elongation to the complete oligosaccharide precursor in the ER lumen

In the ER lumen, four mannosyltransferases and three glucosyltransferases catalyze the elongation of Dol-PP-$GlcNAc_2Man_5$ to the complete oligosaccharide precursor (Figure 6) (Burda, P. and Aebi, M. 1999). These luminally oriented glycosyltransferases differ from those acting on the cytosolic face of the ER membrane by the nature of their oligosaccharide substrate: they use dolichol-linked monosaccharides (Dol-P-Man and Dol-P-Glc) instead of nucleotide-activated sugar donors (Burda, P. and Aebi, M. 1999). The Dol-P-Man dependent elongation of Dol-PP-$GlcNAc_2Man_5$ is initiated by the ALG3 mannosyltransferase, catalyzing the addition of α(1,3)mannose and releasing Dol-P. The resulting Dol-PP-$GlcNAc_2Man_6$ acts as a substrate for the bi-functional α(1,2)mannosyltransferase ALG9. Just like ALG2 and ALG11, ALG9 is involved in two steps during oligosaccharide precursor assembly: the addition of the seventh and ninth mannose residue (Frank, C.G. and Aebi, M. 2005). The last branch of the oligosaccharide precursor is formed by the ALG12 mannosyltransferase, adding the eighth mannose in α(1,6) linkage and releasing Dol-P (Burda, P. and Aebi, M. 1999). Sequential addition of three glucoses from Dol-P-Glc governs the final steps in oligosaccharide precursor assembly. The addition of the first two α(1,3) glucoses is carried out by the glucosyltransferases ALG6 and ALG8, releasing two molecules of Dol-P. ALG10 finally adds the terminal α(1,2)glucose, forming the complete oligosaccharide precursor (Burda, P. and Aebi, M. 1999). Once the fully assembled oligosaccharide precursor is formed in the lumen of the ER, it is transferred onto protein by the oligosaccharyltransferase (OST) complex, thereby releasing Dol-pyrophosphate (Dol-PP).

e. Dolichol cycle regulation

Since the dolichol cycle is of crucial importance in N-glycosylation, it is highly regulated in a complex and multi-faceted way. Regulation occurs at different levels ranging from Dol-P to the sugar donors and the enzymes catalyzing the reactions. The synthesis of one oligosaccharide precursor requires eight molecules of Dol: one as Dol-P, four as Dol-P-Man residues and three as Dol-P-Glc residues. A substantial amount of Dol-P is recycled to supply enough Dol-P sufficient for N-glycosylation (Schenk, B., *et al.* 2001). Dol-PP, released in the ER lumen by the OST complex, is dephosphorylated by the DolPP1 phosphatase resulting in the formation of Dol-P and anorganic P. A recent study performed by Rush and co-workers suggested that this Dol-P, as well as the luminally oriented Dol-Ps released by mannosyl- and glucosyltransferases, are retranslocated to the cytosolic face of the ER membrane, without cleavage of the phosphate, in order to reinitiate another round of the dolichol cycle (Rush, J.S., *et al.* 2008). Numerous studies showed that the level of Dol-P available at the cytosolic face of the ER membrane is a rate controlling factor for N-glycosylation, determining the flux through the dolichol cycle (Rush, J.S., *et al.* 2008). The availability of Dol-P influences protein glycosylation by regulating the synthesis of the assembly intermediate Dol-PP-GlcNAc and the synthesis of the sugar donors Dol-P-Man and Dol-P-Glc (Rosenwald, A.G., *et al.* 1990). The amount of Dol-P is on the other hand regulated by changes in the activity of Dol-P synthesizing enzymes. Increases in cis-prenyltransferase activity during mammalian development for example correlate with an increased level of Dol-P (Crick, D.C. and Waechter, C.J. 1994), while changes in the balance between dolichol phosphorylation and dephoshorylation are reported to control the level of Dol-P (Bhat, N.R., *et al.* 1991). The sugar donors Dol-P-Man and Dol-P-Glc regulate the dolichol cycle by respectively activating and inhibiting the ALG7 GlcNAc transferase activity in an allosteric manner (Kean, E.L. 1985, Kean, E.L., *et al.* 1994). Moreover, Dol-P-Man synthesis is enhanced by the assembly intermediate Dol-PP-GlcNAc and both Dol-PP-GlcNAc and Dol-PP-$GlcNAc_2$ inhibit their own *de novo* synthesis by feedback inhibition (Kean, E.L., *et al.* 1999).

Transfer onto protein: the OST complex

Once the complete oligosaccharide precursor is assembled on Dol-PP, $Glc_3Man_9GlcNAc_2$ is transferred "en bloc" to an asparagine residue of newly synthesized proteins by the OST complex. Sequencing of N-glycopeptides derived from eukaryotic glycoproteins revealed that the glycosylated asparagine is located within the specific protein sequence Asn-X-Ser/Thr, where X can be any amino acid except proline (Marshall, R.D. 1972).

The OST complex has most extensively been studied in yeast and is an integral membrane protein complex closely associated to the translocon pore. Although largely conserved among

higher eukaryotes, small differences in the composition of the OST complex were reported between yeast and mammals (Kelleher, D.J. and Gilmore, R. 2006). The Ost5p subunit has for example not been detected in mammalian OST and the mammalian genome encodes two isoforms of the STT3 subunit. As shown in figure 7, the mammals OST complex consists of seven proteins: ribophorin 1, DAD1, N33 or IAP, OST4, STT3A or STT3B, OST48 and ribophorin 2 (Kelleher, D.J. and Gilmore, R. 2006). The STT3A/B subunit defines the catalytic site of the OST complex , while OST48 and ribophorin 1 are thought to play a role in donor and acceptor substrate recognition (Wilson, C.M., *et al.* 2008, Yan, Q., *et al.* 1999). A recent study in yeast showed in addition that Ost3p, the homologue of N33 and IAP, determines OST substrate specificity at the level of individual glycosylation sites (Schulz, B.L. and Aebi, M. 2009). It is however unclear why the other three OST subunits are required for the transfer of the oligosaccharide precursor onto protein. Nevertheless, the maximal rate of N-glycosylation requires the presence of all subunits. The OST subunits encoded by essential genes in yeast (ribophorin 1, DAD1, STT3A/STT3B, OST48 and ribophorin 2*)* are proposed to participate directly in N-glycosylation, facilitating critical reactions, while the subunits encoded by non-essential genes in yeast (N33/IAP and OST4*)* may perform important accessory functions.

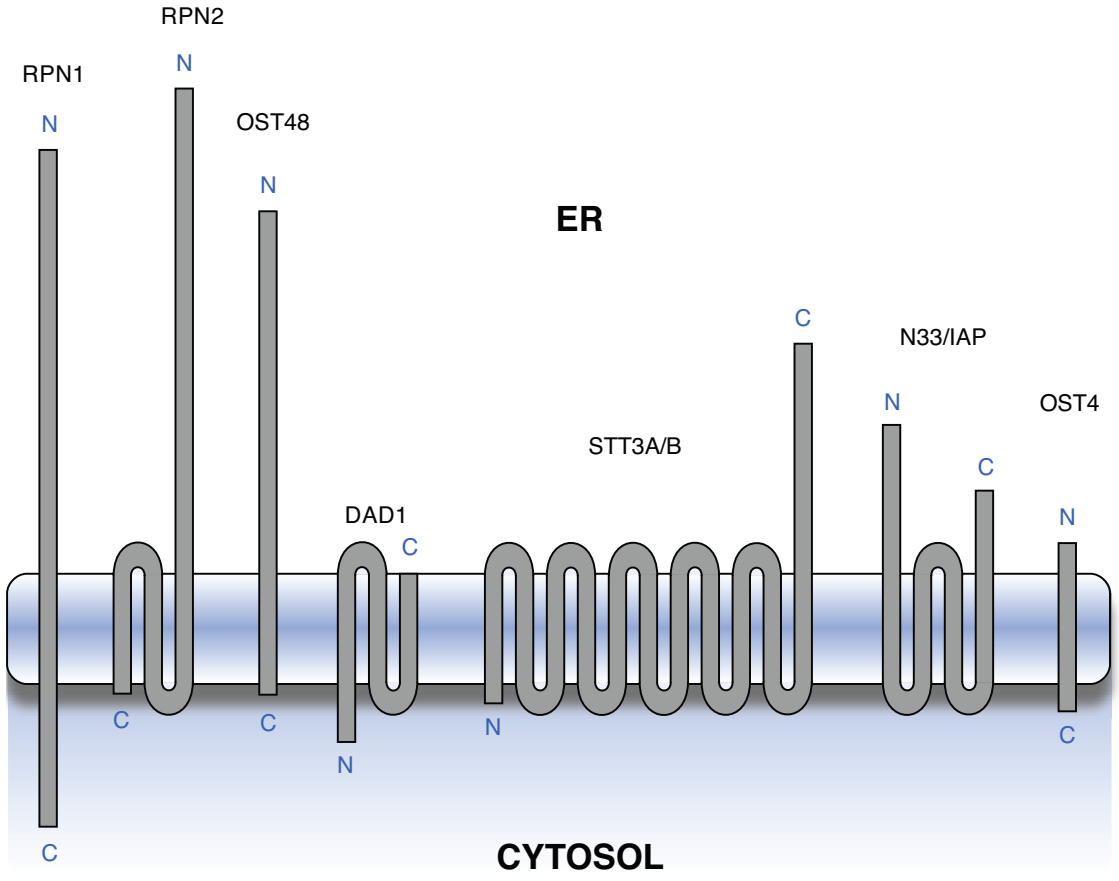

Figure 7: The mammalian OST complex is composed of seven transmembrane proteins: RPN1 (ribophorin 1), RPN2 (ribophorin 2), OST48, DAD1, STT3A or STT3B, N33 or IAP and OST4.

The seven OST subunits are assembled in a dimeric complex and are organized in three different subcomplexes (Kelleher, D.J. and Gilmore, R. 2006). Protein cross-linking studies revealed direct interactions between ribophorin 2, OST48 and DAD1, pointing to the formation of a first subcomplex. In addition, several lines of evidence indicate that N33/IAP and OST4 interact directly with STT3A/B in a second subcomplex. The third and final subcomplex constitutes of ribophorin 1 and Ost5, an additional subunit in yeast for which no homologues protein has been identified in mammals

Mammalian (and other eukaryotic) organisms do not have only one OST, but instead express several OST isoforms (Kelleher, D.J. and Gilmore, R. 2006). The homologues STT3A and STT3B are assembled into two alternative OST complexes, differing in donor-substrate selection and specific activity. OST isoforms that incorporate the STT3B subunit are for example more active and less specific for the fully assembled oligosaccharide precursor then the OST isoforms that incorporate STT3A. In addition, northern blot experiments revealed tissue-specific differences in the relative expression of the STT3 isoforms: STT3B is more uniformly expressed, while the expression of STT3A greatly varied among the tested tissues. Besides the STT3 subunit, two homologs of the yeast Ost3p subunit (N33 and IAP) are expressed in mammals and incorporated into different OST isoforms. Similarity in mRNA expression profiles suggested that IAP is preferentially associated with the STT3A OST isoform. N33 and IAP are suggested to influence OST substrate specificity at the level of individual glycosylation sites (Schulz, B.L. and Aebi, M. 2009). By expressing different OST isoforms, organisms may respond to alterations in glycoprotein flux through the secretory pathway and this may contribute to tissue-specific differences in the glycan repertoire.

I*n vitro* assays using donor substrate mixtures have shown that the fully assembled oligosaccharide precursor is transferred 5-20 times faster than assembly intermediates (Kelleher, D.J. and Gilmore, R. 2006). How does the OST complex assures this preferential transfer of the fully assembled oligosaccharide precursor? The OST complex has a regulatory binding site that influences donor substrate binding to the catalytic site. The regulatory binding site has an equal affinity for the fully assembled precursor and for the assembly intermediates. Hence, regulatory site binding reflects the pool of donor oligosaccharides. When the regulatory binding site is occupied by a fully assembled oligosaccharide precursor, the affinity of the catalytic site for the fully assembled precursor is only enhanced by 1.5 fold (Figure 8). However, when an assembly intermediate binds the regulatory binding site, the catalytic binding site will show a 7-10 fold increase in affinity for the fully assembled oligosaccharide precursor, assuring the transfer of the less abundant fully assembled precursor.

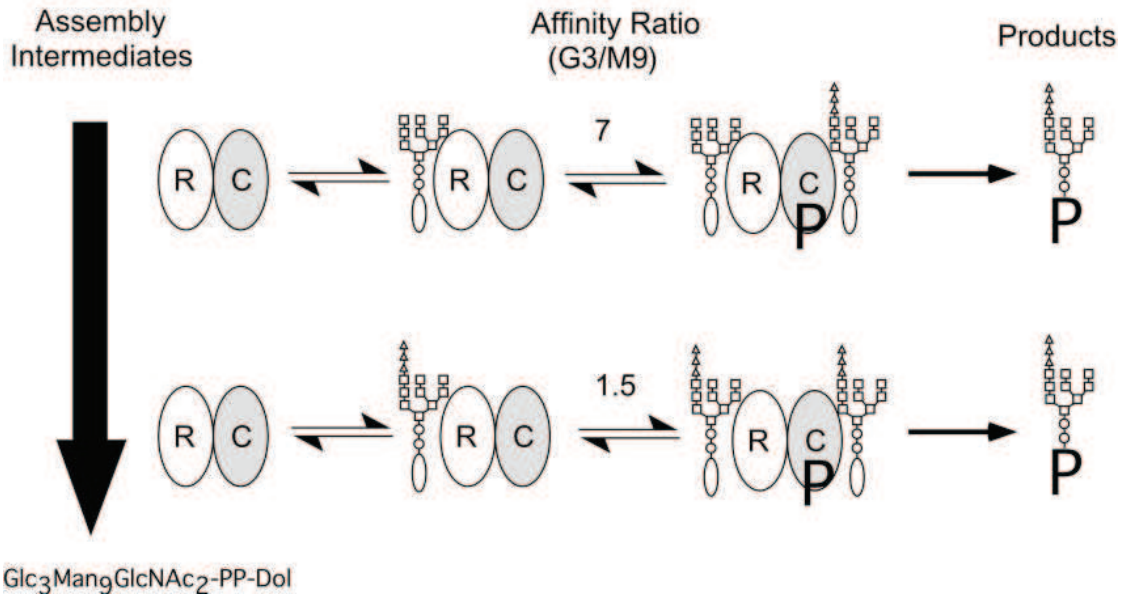

Figure 8: In the donor substrate activation model proposed by Kelleher, D.J. and Gilmore, R. (2006), the OST complex bears two binding sites: a regulatory (R) and a catalytic (C) binding site. When an assembly intermediate like Dol-PP-$GlcNAc_2Man_9$ binds to the regulatory binding site, this binding site increases the affinity of the catalytic binding site for the fully assembled oligosaccharide precursor. In this way, the preference of the OST complex for the fully assembled oligosaccharide precursor is assured, even if its abundance is low.

Quality control: determine the fate of the glycoprotein

In the final step of eukaryotic glycoprotein synthesis, the protein is folded in the ER and its folding state is evaluated by a quality control system. Already during protein translation and ER translocation, newly synthesized proteins start to fold in the ER. The ER is a cellular compartment ideal for protein folding because of its unique oxidizing environment that supports disulfide-bond formation, essential for proper protein folding (Tu, B.P. and Weissman, J.S. 2004). In addition, the ER harbors a multiplicity of chaperones and folding enzymes that prevent aggregation of unstructured polypeptides in aberrant homo- and hetero-oligomeric protein complexes, hence allowing efficient protein folding (Kleizen, B. and Braakman, I. 2004). At least two main chaperone systems are involved in ER quality control: the BiP chaperone complex and the lectin chaperone complex. Most proteins exclusively rely on one system, whereas others may need both for proper folding. BiP is one of the most abundant ER chaperones and mainly facilitates the folding of non-glycosylated proteins. The lectin chaperone system is on the other hand specific for glycoprotein folding by interacting with the protein-bound glycans (Kleizen, B. and Braakman, I. 2004). This latter system will be discussed in detail.

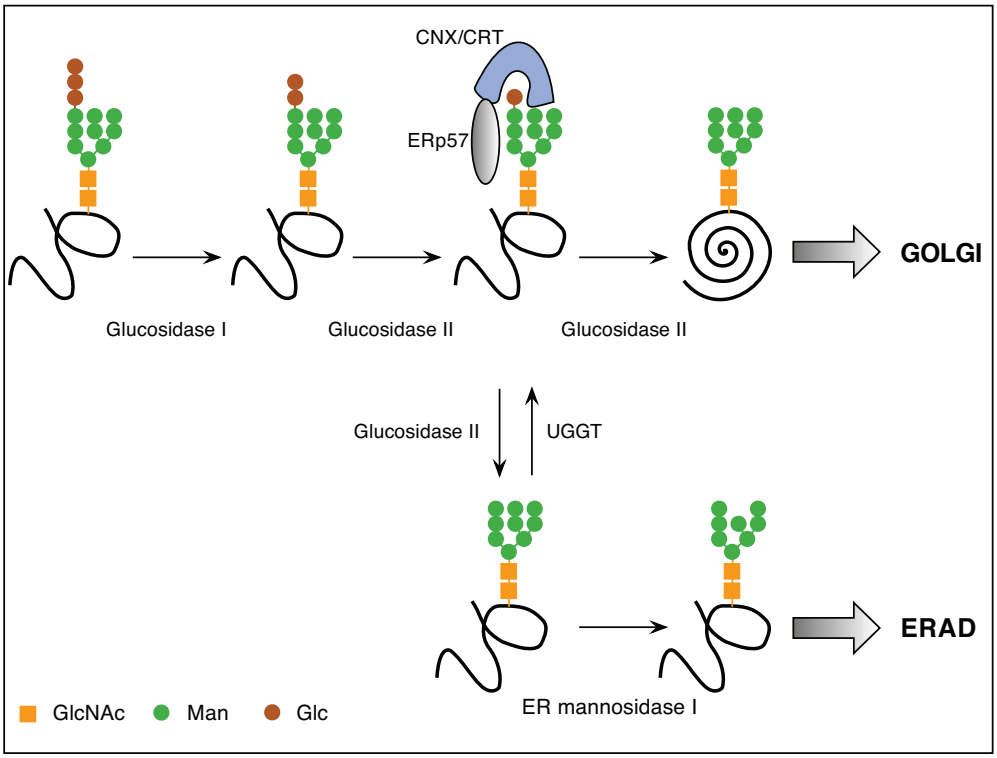

Figure 9: The ER quality control system in higher eukaryotes. In a first step, monoglucosylated glycans are formed, allowing the protein to interact with molecular chaperones like CNX and CRT that assist in protein folding. Glucosidase II subsequently releases the glycoprotein from the chaperone system, enabling its trafficking to the Golgi compartment. However, if folding is not complete, the glycan on misfolded glycoproteins becomes again monoglucosylated by the action of UGGT, allowing another round of association with CNX/CRT. If the protein is unable to fold correctly, even after several rounds of glucosylation/deglucosylation, ER mannosidase I removes a Man residue, signaling the glycoprotein for degradation by the ERAD pathway.

To enable the interaction between ER resident chaperones and the folding glycoprotein, $Glc_3Man_9GlcNAc_2$ is trimmed in the ER (Ellgaard, L. and Helenius, A. 2001). As shown in figure 9, processing of $Glc_3Man_9GlcNAc_2$ is initiated immediately after protein transfer by the removal of the reducing α(1,2)glucose residue, a reaction catalyzed by glucosidase I. The second deglucosylation step governs the removal of α(1,3) glucose by glucosidase II (Figure 9). This reaction is slower and requires the transactivation of glucosidase II by another N-glycan on the same polypeptide chain (Deprez, P., *et al.* 2005). In higher eukaryotes, the resulting $Glc_1Man_9GlcNAc_2$ is able to interact with either the membrane-bound chaperone calnexin (CNX) or the soluble chaperone calreticulin (CRT) to prevent protein aggregation (Figure 9) . In yeast, only one CNX homologue, called Cne1p, has been described (Xu, X., *et al.* 2004). CNX and CRT are also associated with ERp57, a protein disulfide isomerase catalyzing the formation of inter- and intramolecular disulfide bonds (Ellgaard, L. and

Helenius, A. 2001). The glycoprotein is again released from the chaperone system by the action of glucosidase II which removes the last α(1,3) glucose residue (Figure 9). Glucosidase II thus plays a double role in glycoprotein folding: preparing the glycoprotein for entry into the chaperone system and allowing its release.

For certain glycoproteins, one round of association with CNX/CRT is however not sufficient for protein folding. Hence, the ER contains a folding sensor, UDP-glucose:glycoprotein glucosyltransferase (UGGT), which scans the glycoproteins released from the chaperone system for nonnative conformations (Ellgaard, L. and Helenius, A. 2001). As shown in figure 9, if the glycoprotein is not completely folded, it is retained in the ER and UGGT will again transfer an α(1,3)glucose residue from UDP-glucose to $Man_9GlcNAc_2$, allowing an additional interaction with CNX and CRT. The majority of newly synthesized glycoproteins is not affected by deletion of UGGT, suggesting that most proteins acquire a native conformation after a single round of association with CNX/CRT (Molinari, M., *et al.* 2005). In addition, this reglucosylation activity is absent in *Saccharomyces cerevisae*.

The quality control system detailed above entirely relies on the formation of $Glc_1Man_9GlcNAc_2$. Still, gradual removal of mannose residues was reported to retain CNX/CRT binding affinity *in vitro*: mannose trimming till $Glc_1Man_5GlNAc_2$ only reduced binding capacity to 65%. On the other hand, a deficiency in glucose removal resulting in the formation of $Glc_2Man_9GlcNAc_2$ did prevent the interaction with CNX/CRT (Labriola, C., *et al.* 1999, Spiro, R.G., *et al.* 1996, Ware, F.E., *et al.* 1995). Hence, monoglucosylated glycan formation is the only demand for CNX/CRT binding. Another *in vitro* study on the activity of UGGT revealed that mannose trimming completely abolished reglucosylation. The relative reglucosylation rates of $Man_9GlcNAc_2$, $Man_8GlcNAc_2$ and $Man_7GlcNAc_2$ were reported 1.0, 0.5 and 0.15 respectively (Sousa, M.C., *et al.* 1992). These studies thus suggest that mannose trimming enables chaperone binding but prevents the glycoprotein from entering the reglucosylation/deglucosylation cycle. When glycans with trimmed mannose branches bind CNX/CRT, they need to be released after protein folding. Glucosidase II, responsible for the release of glycoproteins from the chaperone system, however, shows a substantially reduced activity towards substrates with trimmed mannose branches (Grinna, L.S. and Robbins, P.W. 1980). Glucose removal can, however, also be achieved by endomannosidase, a processing enzyme that releases Glcα(1,3)Man from the glycoprotein resulting in the formation $Man_8GlcNAc_2$. Interestingly, the endomannosidase acts most favorably on oligosaccharides with trimmed mannose branches down to $Glc_1Man_5GlcNAc_2$ (Lubas, W.A. and Spiro, R.G. 1988). The endomannosidase is predominantly a Golgi enzyme but was also found in the intermediate compartment between the ER and Golgi (Zuber, C., *et al.* 2000).

Since cycling between the ER and Golgi was reported to take place in the quality control of certain glycoproteins, it is suggested that proteins bearing monoglucosylated glycans still associated to the chaperone would be dissociated in the post ER compartment by the endomannosidase. Nevertheless, endomannosidase is only found among members of the phylum *Chordata* (Dairaku, K. and Spiro, R.G. 1997).

If glycoprotein folding is successful, the glycoprotein will leave the ER and be transported to the Golgi compartment (Figure 9). In the Golgi compartment, the N-glycans are further modified by the ordered removal and/or addition of monosaccharides. If the glycoprotein fails to fold, even after several rounds of glucosylation/deglucosylation, the unfolded glycoprotein is retained in the ER and removed from the secretory pathway by the ER-associated degradation (ERAD) pathway (Figure 9). The ERAD pathway prevents the accumulation of unfolded proteins in the ER which would interfere with efficient protein folding and secretion. In mammalian cells, the actual fraction of newly synthesized proteins that fail to adopt a native conformation range from 30% to a much lower amount (Schubert, U.*, et al.* 2000, Vabulas, R.M. and Hartl, F.U. 2005).

Degradation of misfolded glycoproteins: the ERAD pathway

The ERAD pathway can be divided into three major steps: the recognition of misfolded glycoproteins, its retranslocation to the cytosol and its subsequent degradation by the ubiquitinin-proteasome system (Figure 10). Misfolded proteins are recognized by molecular chaperones like BiP that recognize hydrophobic regions normally buried inside the native protein (Fewell, S.W.*, et al.* 2001). When the misfolded proteins bear glycans, they can also be recognized by a specific lectin system in the lumen of the ER. This latter system will be discussed in detail.

How does the ER distinguishe between folded and unfolded glycoproteins? The distinction is based on the length of time a glycoprotein stays in the ER. The ER contains an α(1,2) mannosidase, ER mannosidase I, which removes the terminal mannose of the B-branch of $Man_9GlcNAc_2$, generating the ER degradation signal $Man_8GlcNAc_2$ isomer B (Jakob, C.A.*, et al.* 1998) (Figure 9 and Figure 10). The activity of mannosidase I is relatively low, hence it only acts after a lag period ranging from 10 minutes in yeast to more than an hour in some mammalian cells (Cabral, C.M.*, et al.* 2001, Jakob, C.A.*, et al.* 1998). Mannosidase I thus serves as a timer to protect newly synthesized glycoproteins from degradation. In contrast to yeast, the mammalian ER also harbors two additional mannosidases: ER mannosidase II and the Man_9 mannosidase (Bause, E.*, et al.* 1992, Weng, S. and Spiro, R.G. 1993). ER mannosidase II preferentially releases the terminal mannose of the C-branch thus generating

$Man_8GlcNAc_2$ isomer C, while the Man_9 mannosidase can trim $Man_9GlcNAc_2$ to $Man_5GlcNAc_2$. The exact function of these additional mannosidases in the mammalian ER however remains elusive.

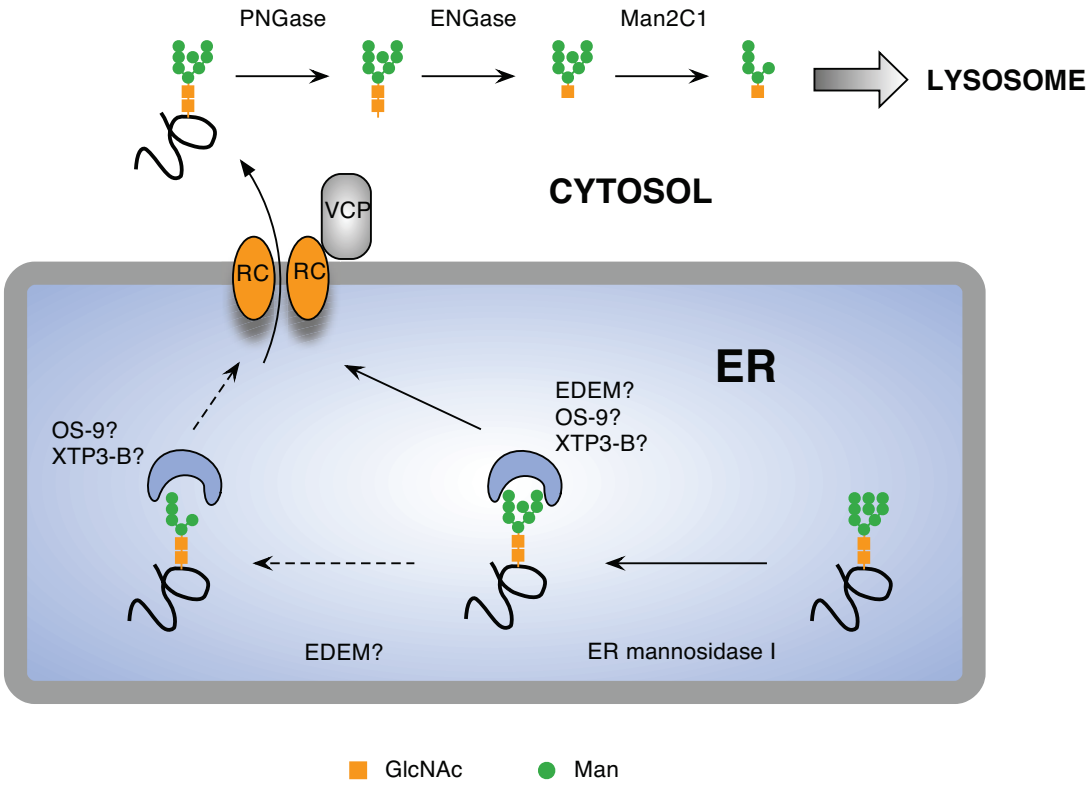

Figure 10: The ERAD pathway as predicted by the current knowledge. Continuously misfolded glycoproteins are signaled for degradation by demannosylation of the glycan structure, a reaction catalyzed by ER mannosidase I. Recent studies indicate that the EDEM proteins might be involved in further demannosylation. The demannosylated glycoprotein is subsequently recognized by a lectin (potentially EDEM, OS-9 or XTP3-B), facilitating their retranslocation to the cytosol. In the cytosol, the glycan is removed from the protein and further degraded into $Man_5GlcNAc_1$, which is transferred to the lysosomes. RC: retranslocation channel.

Mannose trimming subsequently initiates a series of events, still incompletely characterized, leading to the retranslocation of misfolded glycoproteins to the cytosol. The ER resident mannosidase homologues termed EDEM1-3 in mammals and Mnl1p/Htm1p in yeast accelerate the disposal of misfolded glycoproteins to the cytosol (Hirao, K., *et al.* 2006, Hosokawa, N., *et al.* 2001). Hence, they were thought to serve as lectins that recognize the degradation signal $Man_8GlcNAc_2$ isomer B and direct misfolded glycoproteins for retranslocation to the cytosol (Figure 10). Although the EDEM proteins are mannosidase homologues, they were initially reported to lack mannosidase activity *in vitro* and *in vivo* (Hosokawa, N., *et al.* 2001). Recent studies however suggested that the EDEM proteins could be active mannosidases: EDEM1 and EDEM3 over-expression enhances mannose removal from misfolded glycoproteins (Hirao, K., *et al.* 2006, Olivari, S., *et al.* 2006) and

several misfolded glycoprotein were reported to bear trimmed mannose branches (Frenkel, Z., *et al.* 2003). In addition, a recent study performed by Clerc and co-workers suggested that the yeast EDEM homologue Htm1p is an α(1,2) exomannosidase that generates a novel degradation signal $Man_7GlcNAc_2$ isomer C from the initially described $Man_8GlcNAc_2$ isomer B (Clerc, S., *et al.* 2009). Instead of the EDEM proteins, the yeast Yos9p or its mammalian homologues OS-9 and XTP3-B are now proposed to be the lectins that direct proteins for retranslocation (Figure 10) (Hosokawa, N., *et al.* 2009).

Despite extensive studies, the molecular mechanisms of retranslocating terminally misfolded glycoproteins into the cytosol also remain incompletely characterized. In yeast and mammalian cells, retranslocation of secretory and membrane proteins has long been associated with the Sec61 translocon complex that is also involved in the co-translational translocation of newly synthesized proteins in the ER (Pilon, M., *et al.* 1997). However, final proof for the constitution of the retranslocation channel is currently lacking and the involvement of the Sec61 complex was challenged by the identification of other protein conducting channels in the ER membrane. The mammalian protein Derlin-1 and its yeast homologue Der1p form a protein conducting channel in the ER membrane and were reported to be involved in the retranslocation of misfolded proteins (Knop, M., *et al.* 1996, Lilley, B.N. and Ploegh, H.L. 2004). In addition, Derlin-1 is known to interact with the EDEM proteins (Oda, Y., *et al.* 2003). Nevertheless, a study in yeast revealed that several proteins are degraded independently of Der1p and Sec61p, but require Doa10 (Kreft, S.G., *et al.* 2006). Doa10 is an ER-localized E3 ubiquitin ligase possessing several transmembrane domains that may contribute to an additional retranslocation channel in the ER. Derlin-1 and Doa10 are thus alternative retranslocation channels that may act alone or in concert with the Sec61 complex. Protein translocation requires a lot of energy and is therefore facilitated by an AAA-ATPase known as Cdc48p/p97 in yeast and VCP in mammals (Figure 10). The AAA-ATPase binds the polypeptide backbone and subsequently drives protein retranslocation by ATP hydrolysis (Ye, Y., *et al.* 2001).

After retranslocation, the misfolded glycoprotein is degraded in the cytosol (Suzuki, T. and Funakoshi, Y. 2006). As shown in figure 10, glycoprotein degradation is initiated by a deglycosylation reaction catalyzed by the cytosolic peptide:N-glycanase (PNGase). PNGase was first characterized in yeast and was shown to cleave the amide bond in the side chain of glycosylated asparagine residues, thereby releasing the glycan from the protein. The resulting protein is degraded by the 26S proteasome complex , while the free oligosaccharide $Man_8GlcNAc_2$ is sequentially degraded by a series of cytosolic enzymes (Figure 10). Free oligosaccharide degradation starts with the removal of the reducing GlcNAc residue by the endo-β-N-acetylglucosaminidase or ENGase forming $Man_8GlcNAc_1$. ENGase activity has

been described in multiple animal cells but the identification of a yeast homologue is currently lacking. In animal cells, $Man_8GlcNAc_1$ is subsequently demannosylated by the cytosolic, neutral mannosidase Man2C1 forming a linear $Man_5GlcNAc_1$ structure. Man2C1 only acts on the products of the ENGase enzyme, more specifically on oligosaccharides bearing only one GlcNAc residue (Oku, H. and Hase, S. 1991). As shown in figure 10, $Man_5GlcNAc_1$ is subsequently translocated into the lysosomes for further degradation into monosaccharides. Despite several kinetic studies, the molecular nature of the transporter responsible for this translocation reaction remains elusive. In yeast, the Man2C1 homologue Ams1p is located in the vacuole and is required for the degradation of free oligosaccharides formed by the yeast PNGase (Chantret, I., *et al.* 2003). Structural studies on the products of Ams1p activity in yeast and the fate of these products are currently lacking.

Although the ERAD pathway has been most extensively studied, there is increasing evidence for the existence of an alternative pathway to degrade misfolded glycoproteins in the lumen of the ER (Schmitz, A. and Herzog, V. 2004). This alternative ERAD pathway, called the non-proteasomal pathway, consists of proteolytic degradation of misfolded glycoproteins in the lumen of the ER, independent of the proteasome. It is however unclear how this non-proteasomal pathway distinguishes between native and misfolded glycoproteins in the ER lumen. Cabral and co-workers suggesed that the ER mannosidase II, which generates $Man_8GlcNAc_2$ isomer C, could be involved (Cabral, C.M., *et al.* 2000).

Deficiencies in glycosylation: Congenital Disorders of Glycosylation (CDG)

The former chapters demonstrated that glycosylation is a highly diverse and complex process involved in numerous cellular functions. It is therefore not surprising that a deficiency in glycosylation is detrimental and leads to multi-system disorders with a broad spectrum of clinical phenotypes. These disorders have biochemically and clinically been gathered into Congenital Disorders of Glycosylation or CDG. CDG was initially described by Prof. Jaeken in 1980. He reported on the identification of identical twin sisters with psychomotor retardation and multiple serum glycoprotein abnormalities (Jaeken, J., *et al.* 1980). Since this first report, CDG have rapidly expanded to a group of almost forty genetic diseases characterized by a huge clinical diversity. CDG now encompasses defects in the synthesis of N-glycoproteins, O-glycoproteins, or both, and recently defects in the synthesis of glycolipids were also identified (Jaeken, J. and Matthijs, G. 2007).

CDG diagnosis: IEF and ApoCIII

Because the first CDG patients showed multiple serum glycoprotein abnormalities, the molecular nature of these abnormalities was further investigated. The serum transferrin of

these patients turned out to undergo a cathodal shift upon iso-electric focusing (IEF) (Jaeken, J., *et al.* 1984). IEF is a technique in which proteins are separated according to their charge. Serum transferrin, one of the prominent serum glycoproteins, has two N-glycosylation sites each occupied by a bi-antennary glycan structure with terminal sialic acids (Figure 11). Tetrasialotransferrin (S4) is thus the main serum transferrin form. A deficiency in N-glycan synthesis results in a deficient incorporation of sialic acids leading to a cathodal shift in the IEF pattern of serum transferrin. This observation provided insight into the biochemical basis of CDG and IEF of serum transferrin became the prominent tool for CDG screening. In addition to the inexpensive, low-resolution method of IEF, more powerful techniques like electrospray ionization mass spectrometry (ESI-MS) and capillary zone electrophoresis (CZE) of serum transferrin are currently also used for CDG diagnosis. Moreover, analysis of other N-glycoproteins such as α_1-antitrypsin, thyroxine-binding globulin and hexosaminidase is performed to evaluate a general N-glycosylation defect (Grünewald, S., *et al.* 2002 for an overview).

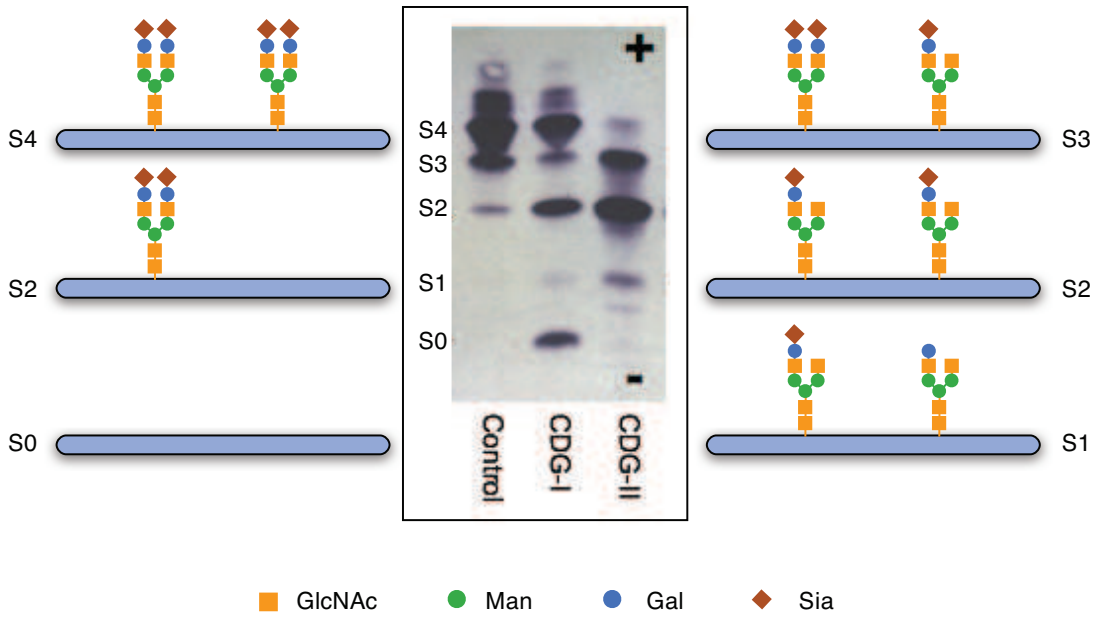

Figure 11: The IEF of serum transferrin revealing a CDG-I and CDG-II pattern. The glycan structures corresponding to the different S bands are also shown.

On the basis of the IEF/CZE/ESI-MS pattern of serum transferrin, N-glycosylation disorders are divided into two types: CDG type I (CDG-I) and CDG type II (CDG-II) defects (Grünewald, S., *et al.* 2002). As shown in Figure 11, a CDG-I pattern is characterized by a decrease of tetrasialotransferrin (S4) associated with an increase of disialo- (S2) and asialotransferrin (S0) forms. This pattern indicates a hypoglycosylation of serum transferrin

or the absence of entire glycans. Hence, CDG-I defects are caused by the synthesis of insufficient and/or incomplete oligosaccharide precursors that are poorly transferred onto proteins. A CDG-II pattern is a combination of a CDG-I pattern with an increase of trisialo- (S3) and monosialotransferrin (S1) forms, pointing to the presence of abnormal glycan structures (Figure 11). CDG-II defects thus comprise all defects in the processing of the protein-bound glycans during N-glycoprotein biosynthesis. Although many more genes are involved in glycan processing in comparison to oligosaccharide precursor assembly, most known CDG patients are of type I, with about 800 patients known worldwide.

Due to the identification of O-glycosylation disorders, an alternative method for the diagnosis of a specific subset of O-glycosylation defects was developed. This method consists of the IEF of apolipoprotein C-III (ApoC-III) (Wopereis, S., *et al.* 2003). ApoC-III is a plasma protein bearing a mucin-type O-glycan with either one or two sialic acids at the end. Deficiencies in O-glycan biosynthesis affect sialic acid incorporation, resulting in a cathodal shift of ApoC-III on IEF. IEF of ApoC-III is commonly used for the screening of mucin type O-glycosylation defects. So far, a rapid screening method for the identification of defects in glycolipid synthesis is lacking.

Clinical phenotype of glycosylation disorders: a wide range of features

The first patients diagnosed with CDG were deficient in phosphomannomutase 2 (PMM2) and presented similar clinical features including inverted nipples, subcutaneous fat pads, psychomotor retardation, seizures, cerebellar hypoplasia, failure to thrive, strabismus and hypotonia (Jaeken, J., *et al.* 1987, Kristiansson, B., *et al.* 1989, Matthijs, G., *et al.* 2000). This phenotype was considered the "classical" presentation of CDG. Today we however know that the clinical spectrum of CDG is very broad: literately all organs can be affected (Grunewald, S. 2007). N-glycosylation disorders especially affect the central nervous system in addition to the gastrointestinal, hepatic, visual and immune systems, demonstrating that these systems heavily depend on normal N-glycosylation (Grunewald, S. 2007). The clinical phenotype of O-glycosylation disorders is usually distinct from those that are seen in N-glycosylation disorders. The O-mannosylation disorders are characterized by muscular dystrophy, an uncommon finding in N-glycosylation disorders. O-xylose based defects usually cause bone, cartilage and other extracellular matrix abnormalities. The recently identified glycolipid disorders are characterized by epilepsy or thrombosis, depending on the type of defect (Freeze, H.H. 2007).

Due to this enormous variation in clinical features, it is sometimes difficult to recognize CDG patients. Since the ubiquitous nature of glycosylation results in a general dysfunction, CDG often lead to childhood mortality because of multiple organ failure and/or severe infections.

In the first 5 years of life, mortality is estimated at 20% but then decreases (Matthijs, G., *et al.* 2000). Many adults with CDG probably remain undiagnosed because these disorders were unknown at the time a diagnosis was sought.

CDG-I disorders: diagnostic tools and (sub)types so far identified

Since 1980, almost forty glycosylation disorders have been identified. We will focus on the N-glycosylation defects, more specifically on the CDG-I disorders. The yeast species *Saccharomyces cerevisiae* has contributed a lot to the identification of the various CDG-I subtypes (Aebi, M. and Hennet, T. 2001). The dolichol cycle is strongly conserved from yeast to human and almost all genes involved in oligosaccharide precursor assembly have been identified in yeast. Moreover, yeast mutants with dolichol cycle defects were created in the eighties and were characterized by the accumulation of specific assembly intermediates or lipid-linked oligosaccharides (LLO). By comparing the LLO structure accumulating in yeast and patients' cells, the candidate genes for CDG-I could be pinpointed (Aebi, M. and Hennet, T. 2001). Once serum transferrin analysis reveals a CDG-I disorder, LLO analysis is thus performed to identify the assembly intermediate accumulating in the patients' cells (Grünewald, S., *et al.* 2002). During LLO analysis, patients' cells are labelled with radioactive mannose to allow the structural analysis of the LLO. Since LLO analysis is expensive and labour intensive, and because a deficiency in PMM2 is by far the most frequent form of CDG, an enzymatic activity measurement of PMM2 in fibroblasts or leukocytes is generally performed prior to LLO analysis. In the same assay, phosphomannoismerase (PMI) is measured.

On the basis of the methodology explained above, several CDG-I (sub)types have been identified. They were labeled with lower case letters according to the chronological order of identification (Figure 12 and Table 1). All known N-glycosylation disorders are autosomal recessive and the most frequent N-glycosylation disorder is CDG-Ia with about 700 patients known worldwide (Grunewald, S. 2007).

Nomenclature: reconsideration required

So far, N-glycosylation defects were classified on the basis of the IEF/CZE/ESI-MS of serum transferrin and were alphabetically labeled in order of identification. The field of CDG has however significantly expanded and the O-glycosylation and glycolipid disorders do not fit in the current classification system. Hence, a new nomenclature was suggested (Jaeken, J., *et al.* 2008). Jaeken and co-authors propose to use CDG for all protein and lipid glycosylation disorders and to specify the disorders by using the gene name. CDG-Ia is, for example, converted into phosphomannomutase deficiency or PMM2-CDG (Table 1).

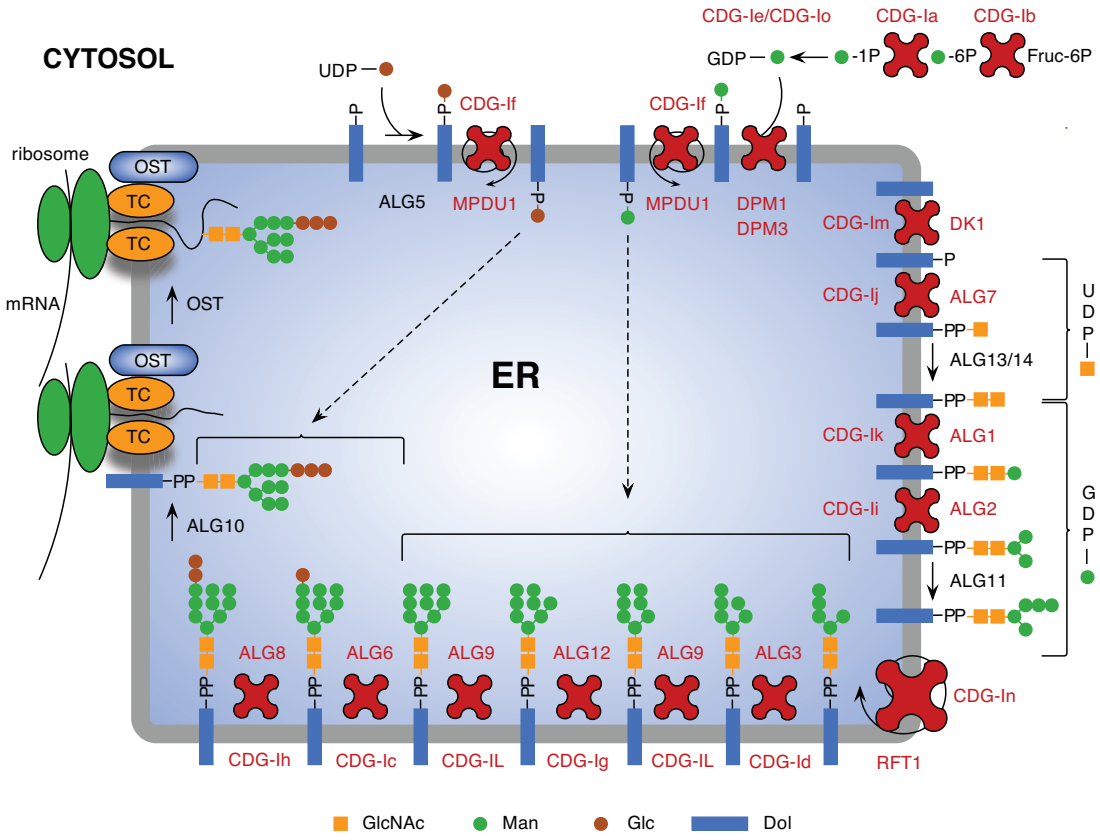

Figure 12: Localization of the CDG-I defects that have so far been discovered in the dolichol cycle.

Disorder		Protein	Gene
CDG-Ia	**PMM2-CDG**	Phosphomannumutase 2	PMM2
CDG-Ib	**PMI-CDG**	Phosphomannoisomerase	PMI
CDG-Ic	**ALG6-CDG**	Dol-P-Glc:Man9GlcNAc2-PP-Dol glucosyltransferase	ALG6
CDG-Id	**ALG3-CDG**	Dol-P-Man:Man5GlcNAc2-PP-Dol manosyltransferase	ALG3
CDG-Ie	**DPM1-CDG**	Dol-P-Man synthase 1	DPM1
CDG-If	**MPDU1-CDG**	Dol-P-Man utlization 1/Lec53	MPDU1
CDG-Ig	**ALG12-CDG**	Dol-P-Man:Man7GlcNAc2-PP-Dol manosyltransferase	ALG12
CDG-Ih	**ALG8-CDG**	Dol-P-Glc:Glc1Man9GlcNAc2-PP-Dol glucosyltransferase	ALG8
CDG-Ii	**ALG2-CDG**	GDP-Man:Man1GlcNAc2-PP-Dol mannosyltransferase	ALG2
CDG-Ij	**DPAGT1-CDG**	UDP-GlcNAc: Dol-P-GlcNAc-P transferase	DPAGT1
CDG-Ik	**ALG1-CDG**	GDP-Man:GlcNAc2-PP-Dol mannosyltransferase	ALG1
CDG-IL	**ALG9-CDG**	Dol-P-Man:Man6/8GlcNAc2-PP-Dol manosyltransferase	ALG9
CDG-Im	**DK1-CDG**	Dolichol kinase	DK1
CDG-In	**RFT1-CDG**	putative flippase for the translocation of Man5GlcNAc2-PP-Dol	RFT1
CDG-Io	**DPM3-CDG**	Dol-P-Man synthase 3	DPM3

Table 1: The CDG-I defects identified so far and their new nomenclature.

Therapy for CDG-I (sub)types

CDG-Ib is the only disorder for which a therapy has been found: patients suffering from a phosphomannoisomerase deficiency show significant improvement on treatment with oral mannose (Grunewald, S. 2007). Mannose treatment not only bypasses the enzymatic defect but also reverses several clinical symptoms such as protein-losing enteropathy, hypoglycemia and coagulopathy. Since mannose supplementation is probably a life-long therapy, side effects such as osmotic diarrhea have to be carefully monitored. The treatment of the other CDG-I disorders is generally limited to managing the disease complications.

Objectives

Three years ago, at the start of the project, nine different CDG-I defects had been identified, all affecting the assembly of the oligosaccharide precursor. The number of unsolved CDG-I (CDG-Ix) cases was however significantly increasing. Hence, this study aimed to characterize the molecular defect(s) in these CDG-Ix patients.

Among the CDG-Ix patients in our laboratory in Leuven, twenty-seven patients showed both a normal level and intact structure of the oligosaccharide precursor on lipid-linked oligosaccharides (LLO) analysis. They were therefore considered good candidates for a deficiency in the transfer of the fully assembled oligosaccharide precursor onto proteins. Two main hypotheses were investigated. We hypothesized that these patients could be deficient in the oligosaccharyltransferase (OST) complex since studies in yeast revealed that point mutations in the OST subunits cause a hypoglycosylation of proteins, which is characteristic for CDG-I patients. Our second hypothesis was a deficiency in the ERAD pathway. It is likely that defects in the translocation or degradation of misfolded glycoproteins can lead to an accumulation of unfolded glycoproteins in the ER, possibly disturbing the transfer of the oligosaccharide precursor onto proteins.

For another subgroup of CDG-Ix patients (twenty-three patients in total), LLO analysis had not been performed. To pinpoint the molecular defect in these patients, structural analysis of the LLO was needed. On the basis of the LLO analysis, candidate genes could be selected and subsequently screened. When the molecular defect would be found, the new cases could in addition be used as models for fundamental studies on N-glycosylation.

To increase the strength of the project, this study has been elaborated in a close collaboration between the '*Laboratory for Molecular Diagnosis*' from the University of Leuven (Belgium), specialized in genetics, and the '*Unité de Glycobiologie Structurale et Fonctionnelle (UGSF)*' from the University of Lille (France), specialized in glycan analysis.

Results

Chapter A

ANALYZING CANDIDATE GENES IN CDG-IX PATIENTS HAVING A NORMAL LLO PROFILE

Chapter B

SCREENING OF CANDIDATE GENES IN CDG-IX PATIENTS ACCUMULATING DOL-PP-$GLCNAC_2MAN_3$ ON LLO

Chapter C

IDENTIFICATION OF THE MOLECULAR DEFECT IN CDG-IX PATIENTS ACCUMULATING DOL-PP-$GLCNAC_2MAN_5$ ON LLO

Chapter D

QUALITY CONTROL OF GLYCOPROTEINS BEARING TRUNCATED GLYCANS IN AN ALG9-DEFECTIVE (CDG-IL) PATIENT

Chapter A

ANALYZING CANDIDATE GENES IN CDG-IX PATIENTS HAVING A NORMAL LLO PROFILE

A1

SCREENING FOR OST DEFICIENCIES IN CDG-IX PATIENTS

A2

ABSENCE OF ERAD DEFECTS IN CDG-IX PATIENTS

A1

SCREENING FOR OST DEFICIENCIES IN CDG-IX PATIENTS

Abstract

Congenital Disorders of Glycosylation (CDG) are a group of inherited disorders caused by deficiencies in glycosylation. Since 1980, fourteen CDG type I (CDG-I) defects have been identified in the ER, all affecting the assembly of the oligosaccharide precursor. However, the number of unsolved CDG-I (CDG-Ix) patients displaying protein hypoglycosylation in combination with an apparently normal assembly of the oligosaccharide precursor is currently expanding.

We hypothesized that the hypoglycosylation observed in some of these patients could be caused by a deficiency in the transfer of the oligosaccharide precursor onto protein, a reaction catalyzed by the oligosaccharyltransferase (OST) complex. For this purpose, the different subunits of the OST complex were screened in twenty-seven CDG-Ix patients for whom structural analysis of the lipid-linked oligosaccharides revealed a normal level and intact structure of the oligosaccharide precursor. Among these twenty-seven patients, one was identified with a homozygous missense mutation (c.1121G>A; p.G374D) in the ribophorin 2 (RPN2) subunit of the OST complex. The pathogenic nature of this mutation remains unproven due to the complexity of tackling a possible OST defect.

Adapted from

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Screening for OST deficiencies in unsolved CDG-I patients

Biochem Biophys Res Commun, in press

Introduction

Congenital Disorders of Glycosylation (CDG) define a group of inherited disorders caused by deficiencies in the glycosylation of proteins and lipids. Since 1980, several defects in protein N-glycosylation have been identified of which fourteen are located in the endoplasmic reticulum (ER) leading to protein hypoglycosylation (CDG type I defects) (Eklund, E.A. and Freeze, H.H. 2006, Jaeken, J. and Matthijs, G. 2007). These fourteen disorders are autosomal recessive and the clinical phenotype is characterized by neurological features associated with a multi-organ involvement (Grunewald, S. 2007).

Protein N-glycosylation is defined by the attachment of a glycan onto an asparagine residue of nascent proteins. This conserved process is initiated by the dolichol cycle in which an oligosaccharide precursor ($Glc_3Man_9GlcNAc_2$) is assembled on a dolichol-pyrophosphate (Dol-PP) carrier in the ER membrane (Burda, P. and Aebi, M. 1999). Once synthesized, $Glc_3Man_9GlcNAc_2$ is transferred en bloc to the specific protein sequence Asn-X-Ser/Thr (where X can be any amino acid except proline) of nascent polypeptide chains (Gavel, Y. and von Heijne, G. 1990). In eukaryotic cells, this critical step is catalyzed by the oligosaccharyltransferase (OST) complex, an integral membrane protein complex closely associated to the translocon pore (Chavan, M. and Lennarz, W. 2006, Menetret, J.F.*, et al.* 2005). The mammalian OST complex is composed of seven transmembrane proteins: ribophorin 1 (RPN1), DAD1, N33 or IAP, OST48, STT3A or STT3B, OST4 and ribophorin 2 (RPN2). The STT3A/B subunit defines the catalytic site of the OST complex (Yan, Q. and Lennarz, W.J. 2002), while OST48 and ribophorin 1 are thought to play a role in donor and acceptor substrate recognition (Beatson, S. and Ponting, C.P. 2004, Pathak, R.*, et al.* 1995, Yan, Q.*, et al.* 1999). The OST complex has most extensively been studies in yeast and the introduction of point mutations in different yeast OST subunits resulted in a severe hypoglycosylation of N-glycoproteins (Chi, J.H.*, et al.* 1996, Karaoglu, D.*, et al.* 1995, Kelleher, D.J. and Gilmore, R. 2006, Reiss, G.*, et al.* 1997, Silberstein, S.*, et al.* 1995, te Heesen, S.*, et al.* 1993).

The fourteen known CDG type I (CDG-I) defects all affect oligosaccharide precursor assembly. However, the number of unsolved CDG-I (CDG-Ix) patients displaying protein hypoglycosylation in combination with a normal assembly of the oligosaccharide precursor is is growing steadily. Since studies in yeast showed that a deficiency in different OST subunits leads to protein hypoglycosylation (Chi, J.H.*, et al.* 1996, Karaoglu, D.*, et al.* 1995, Kelleher, D.J. and Gilmore, R. 2006, Reiss, G.*, et al.* 1997, Silberstein, S.*, et al.* 1995, te Heesen, S.*, et al.* 1993), we hypothesized that an OST defect could be causal for CDG in some of these patients.

Materials and methods

Mutation analysis

Mutation analysis was performed on DNA extracted from blood. Based on the genomic sequence, primers were designed to amplify the different exons of the OST subunits *RPN2* (GenBank accession nos. **NM_002951.3**, **NM_001135771.1**), *OST48* (GenBank accession no. **NM_005216.4**), *DAD1* (GenBank accession no. **NM_001344.2**), *STT3A* (GenBank accession no. **NM_152713.3**), *STT3B* (GenBank accession no. **NM_178862.1**), *N33* (GenBank accession nos. **NM_006765.2**, **NM_178234.1**), *IAP* (GenBank accession nos. **NM_006765.2**, **NM_178234.1**) and *RPN1* (GenBank accession no. **NM_002950.3**), including at least 50 bp of the flanking intronic regions. Primer sequences are available on request. The exons were amplified using standard PCR conditions, subsequently sequenced with Big Dye Terminator Ready reaction cycle sequencing kit V3.1 (Applied Biosystems, California, USA) and analyzed on an ABI3100 Avant and ABI3130xL. Screening of *OST4* was not performed since the human *OST4* sequence was not annotated in the available sequence databases.

Cell culture

Primary skin fibroblasts from healthy controls and the RPN2-deficient patient were cultured at 37°C under 5% CO_2 in DMEM/F12 (Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum (Clone III, HyClones, Utah, USA).

Metabolic labelling of cells

Fibroblasts (8 x 10^6 per labelling) were grown overnight in a 175 cm^2 tissue culture flask. After 24 h, cells were pre-incubated in 0.5 mM glucose for 45 min and then pulse labelled for 1h with 150 µCi of 2-[^{3}H]-mannose (16 Ci/mmol, Amersham Biosciences, UK). After metabolic labelling, the cells were scraped with 1.1 ml MeOH/H_2O (8:3) followed by the addition of 1.2 ml $CHCl_3$. Sequential extraction of oligosaccharide material was performed as previously described (Cacan, R. and Verbert, A. 1997).

LLO analysis

LLO fractions obtained at the end of the sequential extraction were subjected to mild acid treatment (0.1 M HCl in THF) for 2 h at 50°C in order to release the oligosaccharides from the lipid. The oligosaccharides were subsequently desalted on Bio-Gel P2 columns and eluted with 5% acetic acid. The oligosaccharides were separated by HPLC on an amino derivated Asahipak NH_2P-50 column (250 mm x 4,6 mm; Asahi, Kawasaki-ku, Japan) applying a

gradient of acetonitrile/H_2O ranging from 70:30 to 50:50 over 90 min at a flow rate of 1 ml/min. Oligosaccharides were identified on the basis of their retention times compared to standard glycans (Foulquier, F., *et al.* 2002). Elution of the labelled oligosaccharides was monitored by continuous β-counting with a flo-one β detector (Packard, Les Ullis, France).

RNA extraction and cDNA synthesis

RNA extraction from control and patient's fibroblasts was performed with the RNeasy kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. cDNA was subsequently synthesized from 5 µg RNA with oligo-dT priming (Amersham Biosciences) and Superscript III RNase (Invitrogen) according to the manufacturer's protocol. The synthesized cDNA was diluted 1/16 and 5 ul of this solution was used for the real-time PCR reaction.

Real time PCR

RT-PCR was performed with SYBR green for detection and quantification of PCR products. Primers were designed with Primer Express v 2.0 (Applied Biosystems) amplifying the boundaries of two exons to prevent DNA amplification (primer sequences are available upon request). The GADPH gene was used as an internal standard. Reactions were carried out in a 96-well microtiter plate in a final volume of 25 µl containing 12.5 µl qPCR MasterMix Plus for SYBR Green I Low ROX (Eurogentec, Seraing, Belgium), mixed with 2.5 µl of both forward and reverse primer (5 pmol/µl), 2.5 µl of sterile water and 5 µl of diluted control or patient cDNA. Thermo-cycling included 40 cycles of 60 °C for 1 min followed by 15 s at 95 °C on a 7500 real-time PCR system (Applied Biosystems). A melting curve was implemented during the reactions to check for the possibility of primer dimer formation. The $2^{-\Delta\Delta Ct}$ method was used to determine relative transcript abundance (Livak, K.J. and Schmittgen, T.D. 2001).

Western blotting

Control and patient's fibroblasts were washed twice with ice-cold PBS and then lysed in cell lysis buffer (Tris-buffered saline supplemented with 1%Triton X-100) for 60 minutes at 4 °C. The lysed cells were subsequently passed through a 26G needle and insoluble material was removed by centrifugation at 13,000 x *g* for 15 min at 4 °C. Proteins were quantified using the BCA Kit (Pierce, Rockford, USA). 20 µg of total protein extract was mixed with 4x reducing sample loading buffer (Invitrogen, California, USA), subsequently incubated at 90 °C for 5 min and separated on 4–12% precast gels (Invitrogen). Proteins were transferred to nitrocellulose membranes and further processed for western blotting using 1:500 rabbit anti-RPN2 antibody and 1:5000 HRP-conjugated goat anti rabbit antibody. Detection was performed using the ECL PLUS detection kit (Amersham Biosciences, UK) according to the

manufacturer's instructions. The RPN2 expression was normalized against the expression of actin.

Metabolic double labelling

Fibroblasts (0.6 x 10^6 per labelling) were grown overnight in a 6-well plate. After 24 h, cells were either pre-incubated in 5 mM glucose for 45 min and subsequently pulse labelled for 1h with 25 µCi of [^{35}S]-methionine (14,28 µCi/µl, Amersham Biosciences) or pre-incubated in 0.5mM glucose for 45 min and subsequently pulse labelled for 1h with 50 µCi of 2-[^{3}H]-mannose (16 Ci/mmol, Amersham Biosciences). After the labelling, cells were washed twice with ice cold PBS and glycoproteins were extracted using a mixture of chloroform/methanol/water (v/v/v; 3/2/1). The extracted glycoproteins were subsequently incubated in 5% SDS for 1h and then counted using a β counter.

Retroviral transduction

The retroviral expression vector was constructed by subcloning the human wildtype RPN2 cDNA, amplified by PCR from human control cDNA and flanking XhoI and EcoRI restriction sites, into the pMSCVpuro vector (Clontech, California, USA).
HEK293T cells were subsequently transfected with 7 µg of pMSCV-RPN2, 7 µg pVPackAMPHO (Stratagene, California, USA) and 7 µg pVPack-GP (Stratagene) using the Fugene 6 kit (Roche Diagnostics, Indianapolis, USA) according to the manufacturer's instructions. Twenty-four hours post transfection, the medium was replaced with fresh DMEM containing 10% FCS and another 24 hours later, the supernatant was collected.
The collected supernatant was used to transduce healthy control and patient's fibroblasts. Subsequently, the infected cells were selected with 5 mg/ml puromycin (Sigma, Missouri, USA) for 10 days.

Results

In our cohort of CDG-Ix patients, twenty-seven patients presenting protein hypoglycosylation despite a normal assembly of the oligosaccharide precursor were identified. In comparison to healthy controls, all investigated patients showed a hypoglycosylation of serum transferrin as demonstrated by the increase in asialotransferrin forms on isoelectric focussing (Figure 1A). In addition, HPLC analysis of the lipid-linked oligosaccharides (LLO) revealed a normal level and intact structure of the oligosaccharide precursor: both in healthy control and patients' cells, the major peak in the HPLC profile represented the fully assembled oligosaccharide precursor Dol-PP-$GlcNAc_2Man_9Glc_3$ (Figure 1B). Since OST defects in yeast are associated with protein hypoglycosylation, the different OST subunits were considered potential candidates for CDG. For this purpose, mutation

analysis of the OST subunits was performed in the twenty-seven selected CDG-Ix patients. Genomic sequencing of *RPN2, OST48, DAD1, STT3A, STT3B, N33, IAP* and *RPN1* identified several nucleotide variants or SNPs. A summary of the identified base pair changes is shown in Table 1.

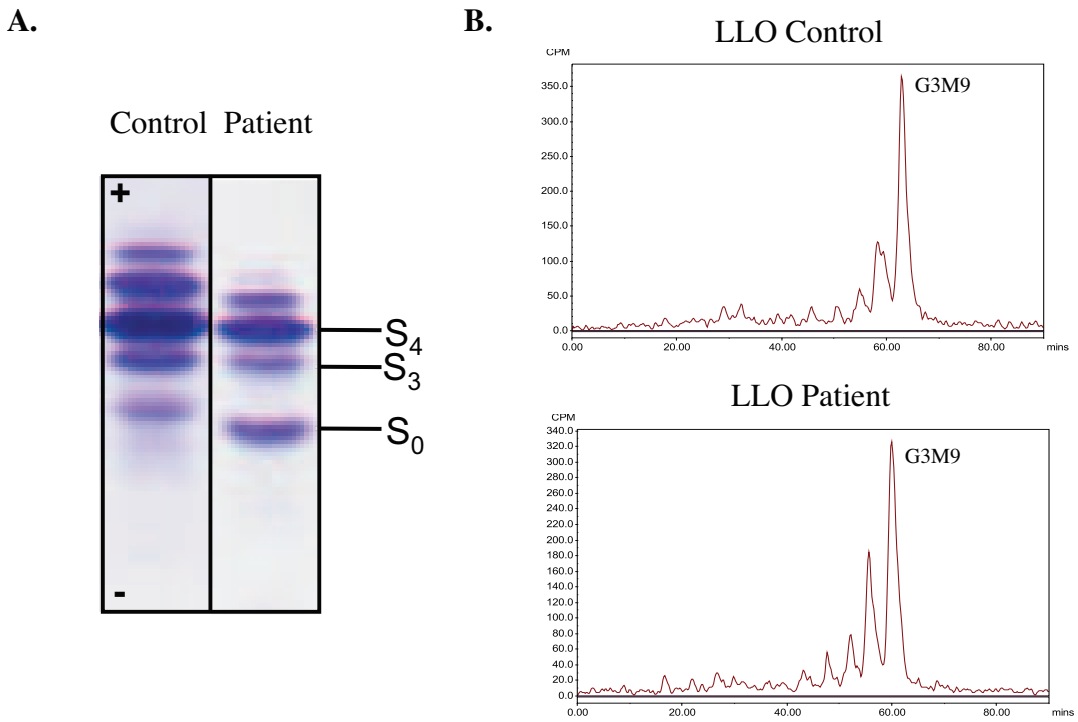


Figure 1: General characteristics of the twenty-seven selected CDG-Ix patients. **(A)** Iso-electric focusing of serum transferrin in a control and a representative CDG-Ix patient reveals protein hypoglycosylation in the patients by the increase in asialotransferrin forms. **(B)** Normal oligosaccharide precursor assembly detected by HPLC analysis of the lipid-linked oligosaccharides in control and patient after metabolic labelling. Symbols: S0: asialotransferrin; S3: triasialotransferrin; S4: tetrasialotransferrin; G3M9: Dol-PP-$GlcNAc_2Man_9Glc_3$.

However, one patient was found to carry a potentially deleterious mutation in the *RPN2* subunit of the OST complex (Table 1). As shown in Figure 2, this patient was homozygous for the missense mutation c.1121G>A in exon 10 of the *RPN2* gene, causing the conversion of glycine into aspartic acid at position 374 on the protein level. The substituted glycine residue was strictly conserved among different species ranging from *Homo sapiens* over *Drosophila melanogaster* to *Saccharomyces cerevisiae* (Figure 3A).

Gene	Base pair change	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
RPN2 ex 10	c.1121G>A; p.G374D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+/+
RPN2 ex 16	c.1825C>T; p.L608L	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/+	+/+	+/+	+/+	+/+	+/-	+/-	+/+	-	+/+	-	-	+/-	+/+	+/-	+/-	+/+	-	-
OST48 ex 3	c.366C>T; p.G122G	-	-	-	-	-	-	+/-	-	+/-	+/+	+/-	-	+/-	-	-	-	+/+	-	-	+/+	+/-	+/-	+/-	+/+	-	+/-	+/+
OST48 ex 8	c.875C>G; p.A292G	-	-	-	-	-	+/-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OST48 ex 9	c.861C>G; p.G287G	-	-	-	-	-	+/-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OST48 ex 10	IVS9-5C>T	-	-	-	-	+/-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OST48 ex 9	c.1050C>T; p.G350G	-	-	-	-	-	-	-	-	+/-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N33 ex 3	IVS3-6C>T	+/+	-	+/-	-	+/+	+/-	+/+	-	-	-	+/-	+/-	+/-	-	+/-	+/-	+/-	+/-	-	-	+/-	-	-	-	+/-	-	-
RPN1 ex 3	c.423G>A; p.P140P	-	+/-	+/+	-	-	+/+	+/-	+/+	+/+	+/-	+/-	+/+	+/+	+/+	+/+	+/-	+/-	+/-	+/-	+/-	+/+	-	-	-	-	-	+/-
RPN1 ex 7	c.1206C>T; p.D402D	-	+/-	-	-	+/+	-	-	+/-	+/-	-	-	-	-	+/+	-	-	-	-	+/-	-	-	-	-	+/-	-	+/+	-
STT3A ex 2	IVS2+7G>T	-	-	+/-	+/-	-	-	-	-	-	-	-	-	-	-	-	+/-	+/-	-	-	+/-	-	-	-	-	-	-	-
STT3A ex 3	c.52T>G; p.L18L	-	-	+/-	+/-	-	-	-	-	-	-	-	-	-	-	-	+/-	+/-	-	-	+/-	-	-	-	-	-	-	-
STT3A ex 9	c.672C>G; p.L224L	-	-	+/-	-	-	-	-	-	-	-	-	-	-	-	-	+/-	-	-	-	+/-	-	-	-	-	+/-	-	-
STT3A ex 11	c.996G>A; p.S332S	-	+/-	-	+/+	-	-	+/-	-	+/-	-	-	-	+/-	-	+/-	+/+	+/-	-	-	+/-	+/-	+/+	+/-	+/-	-	-	-
STT3A ex 14	c.1464T>C; p.I488I	-	-	-	-	-	-	-	-	+/-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
STT3A ex 19	IVS19-5T>C	-	-	-	+/-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
STT3B ex 13	IVS13+5G>A	-	+/-	+/-	-	-	+/-	-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	-	-	-	+/-	-	-	-	-	-	+/-	+/-
IAP ex 2	c.216G>A; p.P72P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+/+	-

Table 1: Summary of the identified SNPs in the twenty-seven selected CDG-Ix patients. All identified nucleotide variants are non-pathogenic, except for the c.1121 G>A mutation detected in the *RPN2* gene of one patient. Symbols: +/+: homozygous; +/- heterozygous; -: absent.

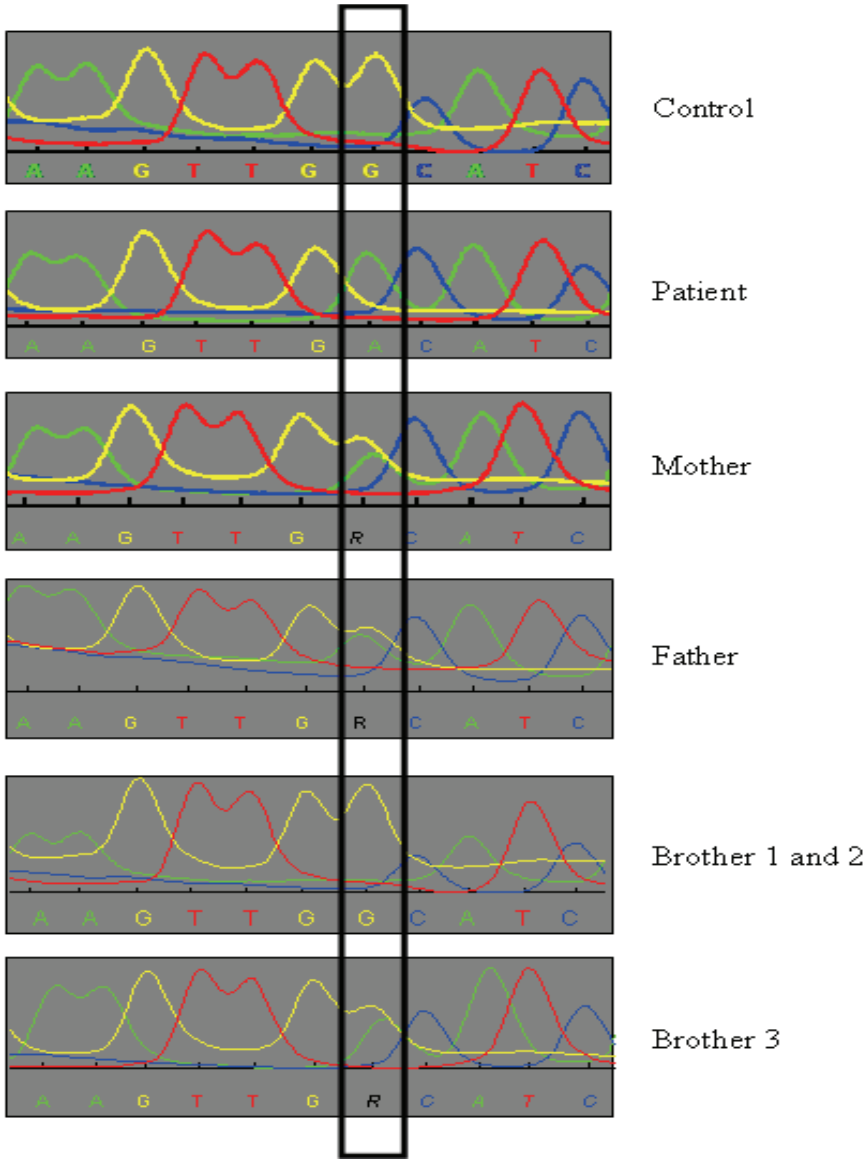
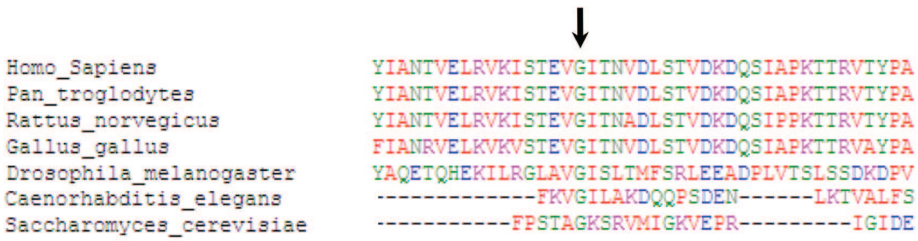


Figure 2: Sequence alignment of the *RPN2* gene in control and patient as well as the parents and healthy siblings. The patient is homozygous for the missense mutation c.1121G>A (p.G374D), while the parents are carriers for the missense mutation and no homozygosity was detected in the three brothers.

Assmann and coworkers previously described this patient as the 4th son of healthy consanguineous parents presenting with a mild clinical phenotype including mental retardation, strabismus, hypotonia and mild ataxia (Assmann, B., *et al.* 2001). Sequencing of the parents and three healthy brothers confirmed the segregation of the mutation within the family: both parents were carriers for the mutation and no homozygosity was detected in the healthy brothers (Figure 2). Moreover, this base pair change was not detected in eighty control alleles (data not shown). However, it was impossible to obtain an ethnically matched control group, so we cannot exclude that this base pair change is a (rare) polymorphism in this particular population.

A.



B.

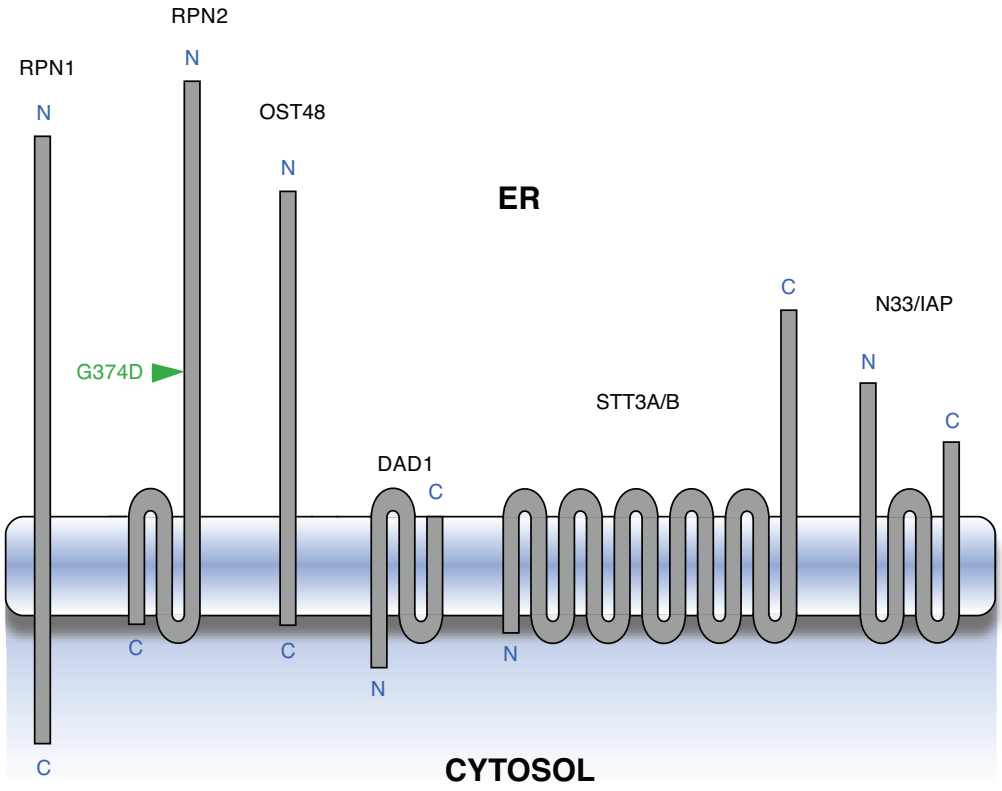


Figure 3 (A) Alignment of the RPN2 protein sequence in different species ranging from *Homo Sapiens* to *Saccharomyces cerevisiae*. The glycine residue, indicated by the arrow, is perfectly conserved. **(B)** Topology of the different OST subunits in mammals. The missense mutation p.G374D is shown in green, while the N- and C-termini are marked in blue.

The missense mutation is located in the luminal tail of the RPN2 protein (Figure 3B), which could influence the interaction with other OST subunits or luminal glycoproteins (Fu, J., *et al.* 1997). We analyzed the influence of this missense mutation on RPN2 stability at mRNA and protein level. Real-time PCR on mRNA derived from healthy control and patient's cells did not reveal differences in *RPN2* expression. In comparison to the average expression in three healthy controls, *RPN2* expression in the patient's cells was only decreased by 10.5% (+/-2.3%; n=3) (Figure 4A). This result excluded mRNA decay. In addition, western blot analysis of the RPN2 protein in healthy control (n=3) and patient's fibroblasts did not reveal instability of the mutated RPN2 protein. RPN2 expression in the patient, normalized against actin, was only decreased to 91% (+/-3.0%; n=3) (Figure 4B). Still, the patient's cells did

express a hypoglycosylation phenotype. Metabolic double labelling using (2-^{3}H)-Man and (^{35}S)-Met was performed to measure *in vivo* the transfer of the oligosaccharide precursor onto proteins. (2-^{3}H)-Man incorporation monitored glycoprotein synthesis, while (^{35}S)-Met labelling reflected protein synthesis. Metabolic double labelling in healthy control and patient's fibroblasts revealed a 2.64-fold decrease in the level of (2-^{3}H)-Man incorporation in the patient, normalized against (^{35}S)-Met incorporation (Figure 4C). When the average normalized (2-^{3}H)-Man incorporation of three controls was set at 100%, the normalized (2-^{3}H)-Man incorporation in the patient was only 38% (+/- 5.2%; n=3).

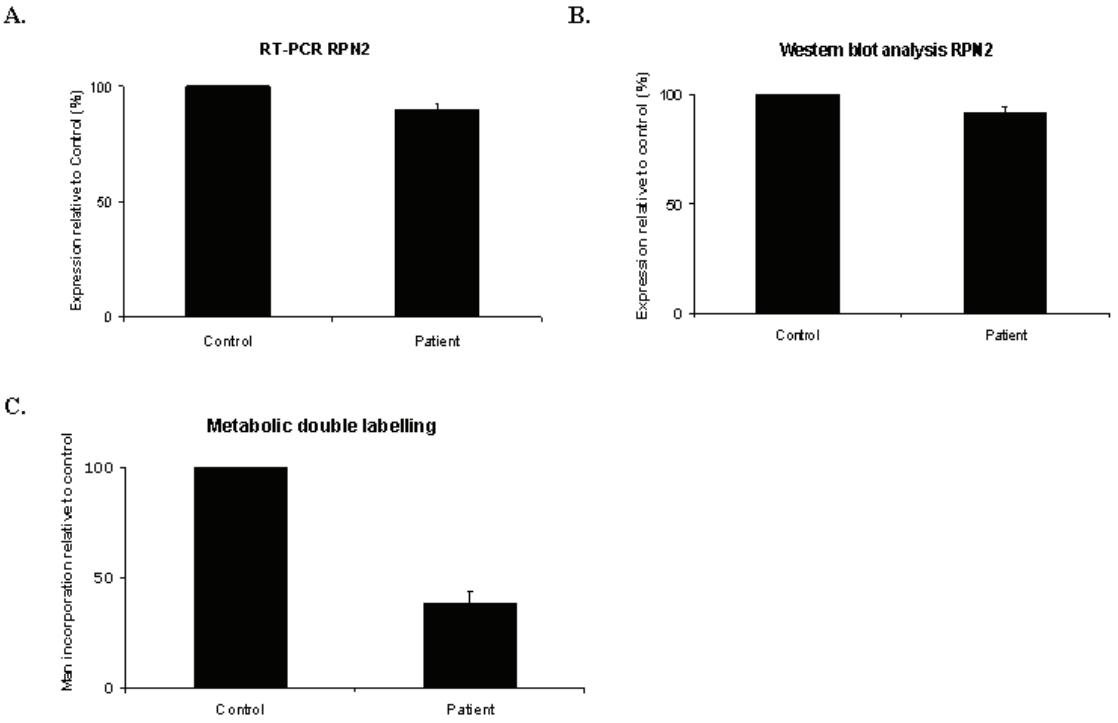


Figure 4: (A) Real-time PCR analysis of *RPN2* mRNA in control and patient, revealing a 10,50% (+/-2.26%; n=3) reduced expression in the patient. The average *RPN2* expression in three controls was set at 100%. **(B)** Western blot analysis of the RPN2 and loading control β-actin in controls (n=2) and patient. **(C)** Metabolic double labelling of healthy control and patient's fibroblasts using (2-^{3}H)-Man and (^{35}S)-Met. The (2-^{3}H)-Man incorporation was normalized against (^{35}S)-Met incorporation and the average incorporation in three controls was set at 100%.

Finally, we aimed to rescue the observed hypoglycosylation in the patient's cells. For this purpose, healthy control and patient's cells were transduced with retroviruses overexpressing wildtype RPN2. However, overexpression of wildtype RPN2 resulted in cellular apoptosis, both in control and patient's cells. Hence, it was practically impossible to measure changes in cellular hypoglycosylation.

Discussion

With the number of CDG-Ix patients displaying protein hypoglycosylation in combination with a normal assembly of the oligosaccharide precursor increasing, we decided to study these patients systematically at the molecular level. Twenty-seven patients were selected on the basis of the normal LLO profile and were screened for mutations in the different subunits of the OST complex. Only one patient with a potential OST defect was found. This patient was homozygous for a missense mutation (c.1121G>A; p.G374D) in *RPN2*. Although essential in yeast (te Heesen, S., *et al.* 1993), this human RPN2 defect does not cause embryonic lethality but results in a rather mild phenotype. The RPN2-deficient patient presented a mild clinical phenotype characterized by mental retardation, strabismus, hypotonia and mild ataxia (Assmann, B., *et al.* 2001). This could be explained by the relatively late addition of RPN2 to the OST complex during evolution: RPN2 is for example not encoded by the genomes of lower eukaryotes such as *Cryptosporidium*, *Trichomonas*, *Giardia and Kinetoplastidia*. In addition, nematode and fungi genomes encode a truncated form of the RPN2 protein, lacking an N-terminal luminal domain (Kelleher, D.J. and Gilmore, R. 2006).
Since RPN2 overexpression in both control and patient's fibroblasts caused cellular apoptosis, we could not perform a rescue experiment to prove the pathogenicity of the identified missense mutation. In addition, RPN2 knockdown is lethal in yeast and *C. elegans* and no other RPN2-deficient cell lines are currently available (Fraser, A.G., *et al.* 2000, te Heesen, S., *et al.* 1993). However, the evidence for the pathogenic character of this missense mutation stems from the inheritance pattern and the phylogenetic conservation. The missense mutation segregated in the family because both parents were carriers and none of the three healthy brothers were homozygous for the missense mutation. In addition, the glycine residue was perfectly conserved among lower and higher eukaryotes.

Interestingly, Molinari and co-workers recently identified the first OST deficiency in patients with autosomal recessive nonsyndromic mental retardation: two patients showed a deficiency in the N33 subunit, while two other patients were deficient in IAP (Molinari, F., *et al.* 2008). Remarkably, none of these patients showed a general glycosylation defect. Hence, these patients potentially suffer from a hypoglycosylation of brain-specific N-glycoproteins. In addition, the function of most OST subunits remains elusive. We thus hypothesize that the STT3A/B subunit, the catalytic subunit, is the only subunit essential for general protein N-glycosylation in humans. The absence of STT3A/B defects in CDG patients could however be explained by redundancy. The two STT3 homologs could compensate for each other since they only show differences in kinetic properties: the OST complex incorporating STT3B is more active but less specific for the fully assembled oligosaccharide precursor then the OST

complex that incorporates STT3A (Kelleher, D.J., *et al.* 2003). The other OST subunits may provide accessory roles essential for the glycosylation of a specific subset of N-glycoproteins. We thus hypothesize that OST defects should be looked for in patients with a specific clinical phenotype restricted to one or few organs.

In summary, molecular screening of twenty-seven CDG-Ix identified revealed one patient with a homozygous missense mutation in RPN2, but the pathological nature of this mutation remains elusive. We thus conclude that the different OST subunits were not good candidates for CDG in the twenty-seven selected CDG-x patients. Current advances in full genome sequencing could help us to identify the genetic defect in the remaining CDG-Ix patients.

Acknowledgements

We thank Dr. B. Assmann for providing material of the patient and family and we are grateful to Prof. AV. Nikonov for the generous gift of the RPN2 antibody. We also thank Ms. E. Reynders for her technical assistance. This work was supported by the Mizutani Foundation for Glycoscience; the European Commission [Sixth Framework Programme, contract LSHM-CT.2005-512131 to EUROGLYCANET; http://www.euroglycanet.org]; Marie Curie [Marie Curie European Reintegration Grant to F.F.]; the "Ministère de la Recherche et de l'Enseignement supérieur" ["Allocation de Recherche" to W.V.] and the Research Foundation (FWO) Flanders [Grant G.0553.08 to G.M.].

A2

ABSENCE OF ERAD DEFECTS IN CDG-IX PATIENTS

Abstract

N-glycosylation or the covalent attachment of glycans onto asparagine residues of the protein is an essential post-translational modification found in almost all living organisms. A deficiency in N-glycosylation results in a group of inherited human disorders called Congenital Disorders of Glycosylation (CDG). All the CDG type I (CDG-I) disorders so far identified mainly comprise defects in the assembly of the oligosaccharide precursor in the ER. A large number of unsolved CDG-I (CDG-Ix) patients however show a normal assembly and normal level of the oligosaccharide precursor. The hypoglycosylation observed in these patients could therefore be explained by a deficiency in the transfer of the oligosaccharide precursor onto proteins. Since the accumulation of misfolded glycoproteins in the lumen of the ER could interfere with the transfer of the oligosaccharide precursor onto proteins, we screened these CDG-Ix patients for a deficiency in the ERAD pathway, a pathway responsible for the degradation of misfolded glycoproteins. A deficiency in ERAD could however not be identified.

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Unpublished data

Introduction

Proteins in all living organisms are generally modified in a co- or post-translational way to increase their range of biological functions. One of the most common co- or post-translational modification is protein N-glycosylation or the covalent attachment of glycans onto asparagine residues of the protein (Spiro, R.G. 2002).

The biosynthesis of N-glycoproteins starts in the endoplasmic reticulum (ER) with the assembly of an oligosaccharide precursor consisting of three glucoses, nine mannoses and two N-acetylglucosamine residues ($Glc_3Man_9GlcNAc_2$) on a dolichol pyrophosphate carrier (Burda, P. and Aebi, M. 1999). Once assembled, $Glc_3Man_9GlcNAc_2$ is transferred onto the specific protein sequence Asn-X-Ser/Thr by the oligosaccharyltransferase complex thereby forming an N-glycoprotein (Kelleher, D.J. and Gilmore, R. 2006). The N-glycoprotein subsequently acquires its native conformation through the assistance of molecular chaperones like calnexin (CNX) and calreticulin (CRT) that assist in protein folding (Ellgaard, L. and Helenius, A. 2001). If protein folding is successful, the glycoprotein is transported to the Golgi compartment, where the N-glycans are further modified by the ordered removal and/or addition of monosaccharides. If the protein is however unable to fold in a proper way, the unfolded glycoprotein is retranslocated to the cytosol and degraded by the ER-associated degradation of misfolded glycoproteins (ERAD) pathway (Spiro, R.G. 2004).

Protein degradation by the ERAD pathway is signalled by demannosylation of the protein-bound glycan. Mannosidase I removes the terminal mannose of the B-arm of $Man_9GlcNAc_2$, thereby forming $Man_8GlcNAc_2$ isomer B which has so far been estimated to be the signal for degradation. Since mannosidase I activity is low, demannosylation occurs only on proteins residing in the ER for a long time, thus acting as a timer (Spiro, R.G. 2004). Recent studies however suggest that further demannosylation by the EDEM proteins or other mannosidases in the ER lumen is essential for the disposal of unfolded proteins to the cytosol (Hirao, K., *et al.* 2006, Olivari, S., *et al.* 2006). It is thus currently unclear whether $Man_8GlcNAc_2$ isomer B or the further demannosylated structure is the real signal for degradation. The mammalian lectins OS-9 and XTP3-B are thought to recognize the degradation signal and subsequently direct the proteins for retranslocation (Christianson, J.C., *et al.* 2008, Hosokawa, N., *et al.* 2009). The molecular mechanism of protein retranslocation is also unclear but this process has long been associated with the Sec61 complex (Pilon, M., *et al.* 1997). Besides retranslocation, the Sec61 complex is responsible for the translocation of newly synthesized proteins to the ER lumen and is composed of three transmembrane proteins in mammals: Sec61α1 or Sec61α2, Sec61β and Sec61γ (Johnson, A.E. and van Waes, M.A. 1999). After retranslocation, the misfolded glycoproteins are immediately degraded in the cytosol. Degradation of the protein backbone only occurs after the action of the cytosolic peptide:N-

glycanase (PNGase) which removes the glycan moiety from the protein. The resulting protein is degraded by the proteasome, while the free oligosaccharide is sequentially dismantled. The first GlcNAc residue is removed by the endo-β-N-acetylglucosaminidase or ENGase forming $Man_8GlcNAc_1$. $Man_8GlcNAc_1$ is subsequently demannosylated by the cytosolic, neutral mannosidase Man2C1, forming a linear $Man_5GlcNAc_1$ structure which is translocated to the lysosomes for further degradation into monosaccharides (Spiro, R.G. 2004).
N-glycosylation is thus a complex process in which numerous enzymes and other proteins are involved. A deficiency in N-glycosylation leads to a group of inherited human disorders called Congenital Disorders of Glycosylation (CDG). CDG defects can be classified into two subgroups on the basis of serum sialotransferrin analysis, a method for diagnosing CDG patients. One subgroup, formerly known as CDG type I (CDG-I) disorders, comprise defects in the assembly and/or transfer onto protein of the oligosaccharide precursor in the ER and are thus characterized by protein hypoglycosylation. The subgroup formerly known as type II disorders (CDG-II) arise from defects in the processing of the protein-bound glycans, mainly in the Golgi compartment (Jaeken, J. and Matthijs, G. 2007).

During the last years, the number of unsolved CDG-I (CDG-Ix) patients has been growing steadily. One subgroup of CDG-Ix patients showed a normal level and intact structure of the oligosaccharide precursor. Hence, the hypoglycosylation observed in these patients could be explained by a deficiency in the transfer of the oligosaccharide precursor onto proteins, a reaction catalyzed by the oligosaccharyltransferase (OST) complex. However, screening of the different OST subunits identified only one patient with a mutation in the OST complex (see chapter A1). The genetic defect in the twenty-six other patients thus remained to be determined. We hypothesized that is was likely that defects in the retranslocation or disposal of misfolded glycoproteins lead to an accumulation of unfolded glycoproteins in the ER, possibly disturbing the transfer of the oligosaccharide precursor onto proteins and leading to protein hypoglycosylation (Moore, S.E. 1999). To this end, the twenty-six OST negative CDG-Ix patients were screened for deficiencies in ERAD.

Materials and Methods

Mutation analysis

Mutation analysis was performed on DNA either extracted from blood or fibroblasts by a combination of DHPLC and genomic sequencing. Based on the genomic sequence, primers were designed to amplify the different exons of the Sec61 subunits (Sec61α1, Sec61α2, Sec61β and Sec61γ), including at least 50 bp of the flanking intronic regions. The exons were amplified using standard touch down PCR conditions and the PCR products of different

patients with equal concentrations were pooled two by two. Pooling of patients is necessary to identify homozygous mutations which are often observed in rare genetic disorders like CDG. Prior to DHPLC analysis, the mixed PCR fragments were melted (2' at 95°C) and slowly cooled-down to 45°C to allow heteroduplex formation. DHPLC analysis was performed on the Transgenomic Wave system (Transgenomic, Cheshire, UK) by loading 10 µl of the slow-cooled PCR products on the DNASep column (Transgenomic). Heteroduplex separation was achieved by applying a linear acetonitrile gradient in 0.1mM trietylamine acetate (TEAA) at a constant flow rate of 0.9 ml/min. A linear acetonitrile gradient was formed by mixing buffer A (0.1 mM TEAA) and buffer B (0.1 mM TEAA, 25% acetonitrile). The optimal oven temperature for heteroduplex separation was deduced from the melting profiles of the DNA sequence using the Wavemaker 4.0 Software (Transgenomic). Precise conditions are available on request. Amplicons showing abnormal DHPLC profiles were sequenced to identify the mutation. Sequencing was performed with the Big Dye Terminator Ready reaction cycle sequencing kit V3.1 (Applied Biosystems, California, USA) and analyzed on an ABI3100 Avant.

Cell culture

See 'Materials and Methods' in section A1, page 40.

Metabolic labelling of cells

See 'Materials and Methods' in section A1, page 40.

Analysis of oligosaccharide material

The free oligosaccharide fractions obtained after sequential extraction of the oligosaccharide material were desalted on Bio-Gel P2 and eluted with 5% (v/v) acetic acid. Glycoprotein fractions were first digested overnight at room temperature with trypsin (1 mg/ml; Sigma) in 0.1 M ammonium bicarbonate buffer, pH 7.9. The resulting glycopeptides were then treated with 0.5 U PNGase F (Roche) in 50 mM phosphate buffer pH 7.2 for 4 h to release the oligosaccharides from the protein. The released oligosaccharides were finally purified and desalted on the Bio-Gel P2 column. The eluted oligosaccharides (originating either from the free oligosaccharides or the glycoproteins) were subsequently separated by HPLC on an amino derivated Asahipak NH_2P-50 column (250 mm x 4,6 mm; Asahi, Kawasaki-ku, Japan) applying a gradient of acetonitrile/H_2O ranging from 70:30 to 50:50 over 90 min at a flow rate of 1 ml/min. Oligosaccharides were identified on the basis of their retention times compared to standard glycans (Foulquier, F., *et al.* 2002). Elution of the labelled oligosaccharides was monitored by continuous β-counting with a flo-one β detector (Packard, Les Ullis, France).

Results

The accumulation of unfolded glycoproteins in the lumen of the ER could have different causes. One hypothesis was a deficiency in the retranslocation of unfolded glycoproteins to the cytosol. An accumulation of unfolded proteins in the ER lumen could however also be explained by a deficiency in the disposal of unfolded glycoproteins to the cytosol, thus by a deficiency upstream the retranslocation step. Our hypotheses include a deficiency in ER mannosidase I, the EDEM proteins, OS-9 or XTP3-B. Since the steps occurring prior to retranslocation are incompletely characterized, we opted for a general screening method being the analysis of the free oligosaccharides. Since free oligosaccharides are the end products of the degradation of misfolded glycoproteins, they give a clear representation of the ERAD pathway. By checking the amount and structure of the free oligosaccharides, we should be able to pinpoint defects occurring upstream the retranslocation step.

To identify a defect in the retranslocation of misfolded glycoproteins, the OST-negative CDG-Ix patients were screened, at the genetic level, for mutations in one of the subunits of the translocon complex. The genes encoding for the different translocon subunits (*Sec61A1*, *Sec61A2*, *Sec61B* and *Sec61G*) were analyzed on the basis of DHPLC and genomic sequencing. One nucleotide variant or SNP was identified during this genetic screen. This SNP was however not pathogenic since it did not encode an amino acid change (Table 1).

Since free oligosaccharides give a clear representation of the ERAD pathway, their structural analysis could reveal deficiencies upstream the retranslocation step. For this purpose, the fibroblasts of all OST-negative patients were metabolically labeled with (2-^{3}H)-Man and the labelled glycoproteins and free oligosaccharides were subsequently analyzed by HPLC. In control fibroblasts, mainly $Man_9GlcNAc_{1/2}$ and $Man_8GlcNAc_{1/2}$ structures were found on the free oligosaccharides while $Glc_1Man_9GlcNAc_2$, $Man_9GlcNAc_2$ and $Man_8GlcNAc_2$ were detected on the glycoproteins (Figure 1). Structural analysis of the protein-bound glycans was performed as a control to exclude aberrant free oligosaccharide formation originating from abnormal protein-bound glycan structures. In the patients' cells, neither aberrant glycoprotein structures nor abnormal free oligosaccharides could be detected (Figure 1). In addition, the amount of free oligosaccharides formed in all patients was normal. These results thus point to the absence of ERAD defects in the twenty-six CDG-Ix patients.

Gene	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	Base pair change
Sec61A1 exon 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Sec61A1 exon 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Sec61A1 exon 4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Sec61A1 exon 5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Sec61A1 exon 6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Sec61A1 exon 7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Sec61A1 exon 8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Sec61A1 exon 9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Sec61A1 exon 10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Sec61A1 exon 11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Sec61A1 exon 12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Sec61A2 exon 4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Sec61A2 exon 5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Sec61A2 exon 6	+/-	+/+	+/-	+/-	+/-	+/+	+/-	+/+	-	-	+/-	+/-	+/-	+/-	-	-	-	-	-	-	+/-	-	+/-	+/+	+/+	+/+	c.435C>T; p.A145A
Sec61A2 exon 7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Sec61A2 exon 8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Sec61A2 exon 9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Sec61A2 exon 10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Sec61A2 exon 11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Sec61A2 exon 12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Sec61B exon 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Sec61B exon 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Sec61B exon 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Sec61B exon 4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Sec61G exon 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Sec61G exon 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Sec61G exon 4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

Table 1: Genetic screening of the translocon subunits revealed one nucleotide variant or SNP.

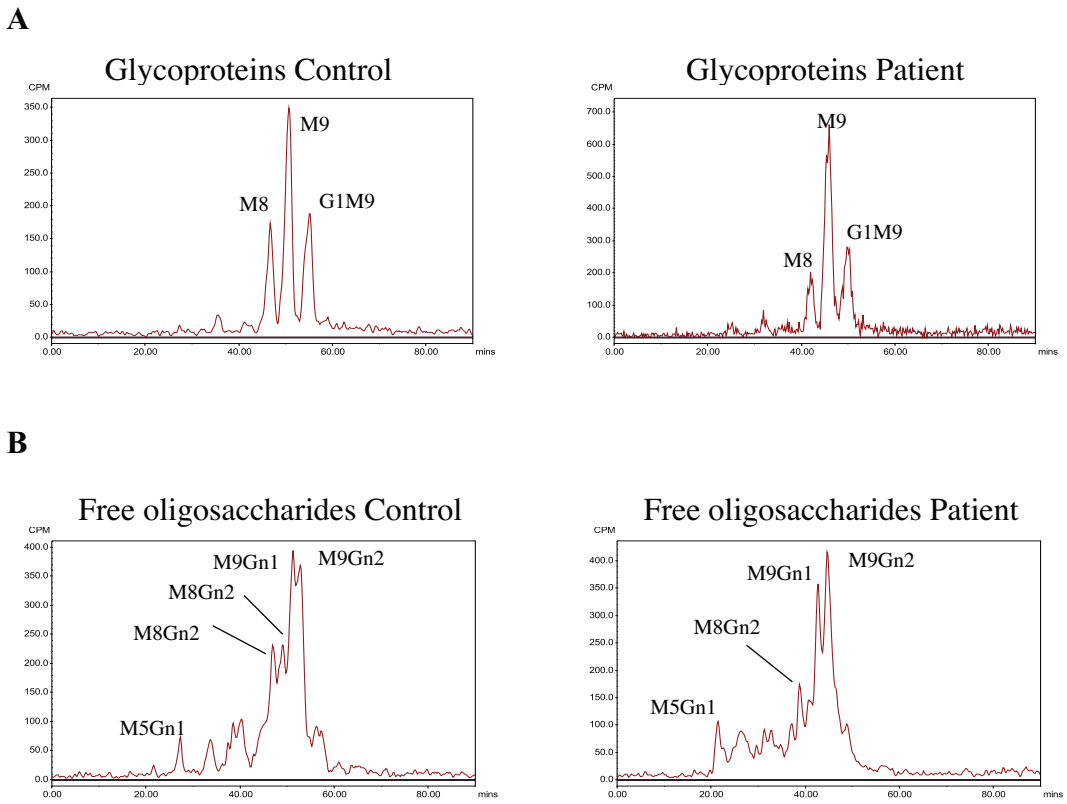

Figure 1: Structural analysis by HPLC of the protein-bound glycans (A) and free oligosaccharides (B) found in the fibroblasts of control and CDG-Ix patients. Symbols: M8-9: $Man_{8-9}GlcNAc_2$; G1M9: $Glc_1Man_9GlcNAc_2$; M5-9Gn1: $Man_{5-9}GlcNAc_1$; M5-9Gn2:$Man_{5-9}GlNAc_2$.

Discussion

In our laboratory in Leuven, an increasing number of CDG-Ix patients were identified displaying protein hypoglycosylation despite a normal level and intact structure of the oligosaccharide precursor. We hypothesized that the primary defect in these patients was caused by a deficiency in the transfer of the oligosaccharide precursor onto proteins. Genetic screening of the OST subunits in twenty-seven CDG-Ix patients however revealed only one patient with a potential OST defect (see chapter 1). The molecular defect in the remaining CDG-Ix patients thus stayed unidentified. All CDG-I defects so far identified are deficient in biosynthetic enzymes of the N-glycosylation process (Eklund, E.A. and Freeze, H.H. 2006, Jaeken, J. and Matthijs, G. 2007). The glycosylation defect in the OST-negative CDG-Ix patients could however be caused by a deficiency in another pathway indirectly affecting the transfer of the oligosaccharide precursor onto proteins. Recent discoveries in CDG-II revealed that a deficiency in a pathway important for the proper localization of glycosyltransferases, thus indirectly involved in glycosylation, also causes a glycosylation disorder (Zeevaert, R., *et al.* 2008). Since an accumulation of unfolded proteins in the lumen

of the ER could interfere with the transfer of the oligosaccharide precursor onto proteins (Moore, S.E. 1999), we screened the twenty-six OST-negative CDG-Ix patients for a deficiency in the ERAD pathway. Genetic screening of the different subunits of the Sec61channel did not reveal a new disorder nor did analysis of the free oligosaccharides, the degradation products of the misfolded glycoproteins, identify an ERAD defect. The molecular defect in these OST-negative CDG-Ix patients thus remains to be determined. Fundamental studies on the glycosylation process could identify other proteins or pathways directly or indirectly affecting the transfer of the oligosaccharide precursor onto proteins.

Chapter B

SCREENING OF CANDIDATE GENES IN CDG-IX PATIENTS ACCUMULATING DOL-PP-$GLCNAC_2MAN_3$ ON LLO

B1

IDENTIFICATION OF AN UNUSUAL LLO PROFILE IN FIVE CDG-IX PATIENTS

B2

A DEFICIENCY IN ALG11 IN A CDG-IX PATIENT

B1

IDENTIFICATION OF AN UNUSUAL LLO PROFILE IN FIVE CDG-IX PATIENTS

Abstract

Lipid-linked oligosaccharide (LLO) analysis is a frequently used tool for the identification of the molecular defect in patients with a Congenital Disorder of Glycosylation type I (CDG-I). CDG is a group of inherited human disorders caused by a deficiency in glycosylation and CDG-I disorders comprise all defects in the assembly of the oligosaccharide precursor during N-glycosylation. LLO analysis in a group of unsolved CDG-I patients identified five patients with an unusual profile characterized by the accumulation of Dol-PP-$GlcNAc_2Man_3$ in combination with Dol-PP-$GlcNAc_2Man_5$. In these patients, three candidate genes for CDG-I were screened at the genetic level. Sequencing of *ALG11* and *GMPPA/B* did however not reveal a mutation nor did homozygosity mapping contributed to the identification of the genetic defect. The cause of CDG-I in these patients thus remains to be determined.

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Unpublished data

Introduction

Protein N-glycosylation is an essential post-translational modification required for the proper functioning of glycoproteins (Spiro, R.G. 2002). In eukaryotes, the attachment of glycans to asparagine residues of the protein is initiated by the assembly of an oligosaccharide precursor, $Glc_3Man_9GlcNAc_2$, which is later co-translationally transferred onto proteins. The oligosaccharide precursor $Glc_3Man_9GlcNAc_2$ is assembled on a lipid carrier (dolichol pyrophosphate) in the ER membrane via a highly regulated and well-ordered pathway known as the dolichol cycle (Burda, P. and Aebi, M. 1999). The dolichol cycle starts at the cytosolic face of the ER membrane with the elongation of Dol-P to Dol-PP-$GlcNAc_2Man_5$ using nucleotide activated sugar donors synthesized in the cytosol. The molecular mechanisms by which Dol-PP-$GlcNAc_2Man$ is elongated to Dol-PP-$GlcNAc_2Man_5$ remained unclear for a long time. Recent studies however identified two enzymes that catalyze the addition of these four mannose residues (Kampf, M., *et al.* 2009, O'Reilly, M.K., *et al.* 2006). ALG2 is responsible for the elongation of Dol-PP-$GlcNAc_2Man$ to Dol-PP-$GlcNAc_2Man_3$, while ALG11 catalyzes the addition of the remaining mannose residues thereby forming Dol-PP-$GlcNAc_2Man_5$ (Kampf, M., *et al.* 2009, O'Reilly, M.K., *et al.* 2006). Both enzymes transfer mannose to the growing oligosaccharide precursor from the nucleotide activated sugar donor GDP-Man. GDP-Man is synthesized in the cytosol from GTP and mannose-1-P by the GDP-mannose pyrophosphorylase (GMPP) (Hashimoto, H., *et al.* 1997). In contrast to the yeast and bacterial GMPP, the mammalian homologue is composed of two subunits (GMPPA and GMPPB). The GMPPB subunit is thought to be the catalytic subunit while GMPPA may play a regulatory role (Ning, B. and Elbein, A.D. 2000). The exact function of both subunits however remains to be determined. Once the intermediate Dol-PP-$GlcNAc_2Man_5$ is synthesized, it is flipped to the lumen of the ER where assembly proceeds: four additional mannoses and three glucoses are added using dolichol-linked sugar donors to form the complete oligosaccharide precursor on dolichol pyrophosphate (Burda, P. and Aebi, M. 1999).

In our laboratory in Leuven, we collected material from many unsolved CDG-I (CDG-Ix) cases. For twenty-three of them, lipid-linked oligosaccharides (LLO) analysis had not yet been performed. During LLO analysis, the patients' fibroblasts are labelled with radioactive mannose which is subsequently incorporated into the oligosaccharide precursor and its assembly intermediates, generally called LLO. Structural analysis of LLO could reveal an abnormal accumulation of assembly intermediates, facilitating the identification of the molecular defect in the patients. LLO analysis in twenty-three CDG-Ix patients identified five patients with an unusual LLO profile. To identify the cause of CDG-I in these patients, several candidate genes were screened.

Materials and Methods

Mutation analysis

Mutation analysis was performed on DNA either extracted from blood or fibroblasts. Based on the genomic sequence, primers were designed to amplify the different exons of the *ALG11* gene and the GMPP isoforms (*GMPPA* and *GMPPB*), including at least 50 bp of the flanking intronic regions. Primer sequences are available on request. The exons of *ALG11* were amplified using the PCR buffer (minus $MgCl_2$) and the TaqPCRx polymerase of the TaqPCRx recombinant polymerase kit (Invitrogen, California, USA) according to the manufacturers' instructions. Exon 2, the end of exon 3 and exon 4 were amplified using standard PCR conditions, while exon 1 and the beginning of exon 3 were amplified with a standard touch down PCR program. Exon 1 was in addition amplified in presence of 2.5mM $MgCl_2$, while 1.75mM $MgCl_2$ was sufficient for the amplification of the other exons. *GMPPA* and *GMPPB* were on the other hand amplified using standard touch down PCR conditions. Sequencing was performed with the Big Dye Terminator Ready reaction cycle sequencing kit V3.1 (Applied Biosystems, California, USA). The sequences were subsequently analyzed on an ABI3100 Avant.

Cell culture

See 'Materials and Methods' in section A1, page 40.

Metabolic labelling of cells

See 'Materials and Methods' in section A1, page 40.

LLO analysis

See 'Materials and Methods' in section A1, page 40 and 41.

Homozygosity mapping

Homozygosity mapping by means of a single nucleotide polymorphism (SNP) array (Affymetrix Genechip) was performed by Dr. M. Amyere and Prof. M. Vikkula of the de Duve Institute (Université Catholique de Louvain, Brussels, Belgium). For this purpose, DNA from both affected siblings and their parents were used for SNP analysis.

Results

LLO analysis in twenty-three CDG-Ix patients identified five patients who accumulated the assembly intermediate Dol-PP-$GlcNAc_2Man_3$ in combination with Dol-PP-$GlcNAc_2Man_5$

(Figure 1). This profile has never been observed in CDG-I patients, but an accumulation of Dol-PP-GlcNAc$_2$Man$_3$ in yeast was known to be caused by a deficiency in the *Alg11p* gene (Cipollo, J.F., *et al.* 2001). In this mutant yeast strain, other assembly intermediates also accumulated due to the translocation of Dol-PP-GlcNAc$_2$Man$_3$ to the ER lumen and its further elongation by the ER localized mannosyltransferases. The LLO profile seen in these five CDG-Ix patients could thus fit with a deficiency in the human homologue ALG11. Sequencing of the *ALG11* gene did however not reveal a mutation.

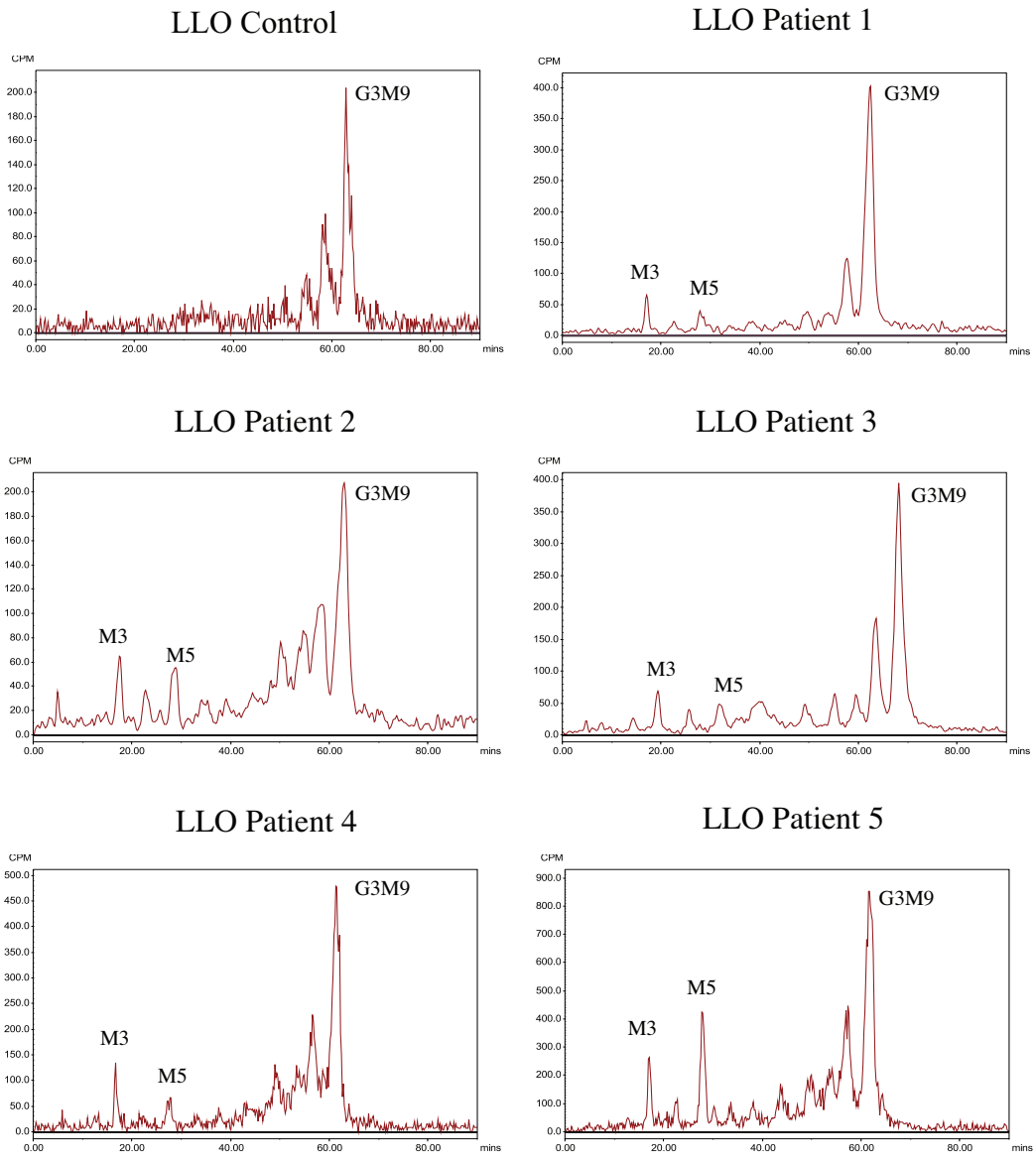

Figure 1: Comparison of the LLO profile in a control and five CDG-Ix patients revealed an accumulation of Dol-PP-GlcNAc$_2$Man$_3$ and Dol-PP-GlcNAc$_2$Man$_5$ in the CDG-Ix patients.

Another hypothesis for the observed LLO profile is a defect in the synthesis of GDP-mannose (GDP-Man). Patients with a mild defect in phosphomannomutase 2 (PMM2-CDG), an enzyme involved in GDP-Man synthesis, also show an accumulation of Dol-PP-GlcNAc$_2$Man$_3$

in combination with Dol-PP-$GlcNAc_2Man_5$ (own observations). Although *PMM2* sequences were normal in these patients, other enzymes involved in the synthesis of GDP-Man could cause CDG in these patients. For this reason, both subunits of the GDP-Man synthase (GMPPA and GMPPB) were screened at the genetic level. Nevertheless, no mutations were found.

Since we insisted on identifying the gene deficient in these CDG-Ix patients, we had chosen another, more powerful approach. Patient 2 and 3 belong to the same family and could therefore be used for homozygosity mapping, which was performed in collaboration with Dr. M. Amyere and Prof. M. Vikkula from the UCL in Brussels. Thanks to this approach, a list of of 167 candidate genes could be identified, but the majority of them had unknown functions and none of the other candidate genes were known to be involved in the dolichol cycle. Hence, we were still unable to find the causal gene in these CDG-Ix patients

To determine whether these patients could bear the same deficiency, we compared their clinical phenotype. With the help of Prof. Jaeken, we concluded that these patients were all very similar, with exception of patient 5. As shown in table 1, patients 1 to 4 showed a major neurological phenotype characterized by psychomotor retardation, hypotonia and epilepsy. Patient 1 and 4 showed in addition feeding difficulties, while ophthalmological problems (absence of visual contact in patient 2 and 3 and strabismus and nystagmus in patient 1) were reported in patient 1,2 and 3. Brain MRI was abnormal in patient 1 and 2 with cerebellar hypoplasia, cortico-subcortal atrophy and delayed myelination reported in patient 1 and a slight increase of lateral ventricles and slight frontal and temporal atrophy observed in patient 2. Kyphoscoliosis was also reported in patient 1. The clinical phenotype of patient 5 is on the other hand remarkably different and consists of only liver problems and no neurological features (Table 1).

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Psychomotor retardation	+	+++	++	+	-
Hypotonia	+	+	+	+	-
Epilepsy	+	++	++	-	-
Feeding problems	+	-	-	+	-
Ophtalmological problems	+	++	++	ND	-
Liver problems	-	transient	transient	++	++
Kyphoscoliosis	+	-	-	-	-
Atrophy of the brain	+	+	-	ND	-

Table 1: Comparison of the clinical phenotype in five patients accumulating Dol-PP-$GlcNAc_2Man_3$ and Dol-PP-$GlcNAc_2Man_5$ on LLO. ND: not determined.

Discussion

LLO analysis in CDG-Ix patients identified five patients with an unusual accumulation of Dol-PP-$GlcNAc_2Man_3$ and Dol-PP-$GlcNAc_2Man_5$. It should however be noted that the accumulation of Dol-PP-$GlcNAc_2Man_3$ and Dol-PP-$GlcNAc_2Man_5$ is rather small, pointing to a mild defect or a defect in a non-essential gene of the glycosylation pathway. Due to the marked similarity in LLO profile, these patients could bear the same genetic defect. In patient 5, the peak corresponding to Dol-PP-$GlcNAc_2Man_3$ was however smaller in comparison to Dol-PP-$GlcNAc_2Man_5$, which is in contrast to the LLO profile detected in the other four patients. Moreover, this patient showed a very specific clinical phenotype characterized by liver problems and the absence of neurological features, which is totally different from the neurological presentation found in the other four patients. These results thus suggest that patient 5 bears a genetic defect which is different from the deficiency in the other four patients.

On the basis of the LLO profile, we identified three candidate genes (*ALG11* and *GMPPA/B*) for CDG-I in the five CDG-Ix patients. In yeast, a deficiency in Alg11p was known to result in an accumulation of Dol-PP-$GlcNAc_2Man_3$ in combination with other assembly intermediates arisen from the translocation of Dol-PP-$GlcNAc_2Man_3$ to the ER lumen and its subsequent elongation. On the other hand, a deficient GDP-Man synthesis in mild PMM2-CDG patients results in a combined accumulation of Dol-PP-$GlcNAc_2Man_3$ and Dol-PP-$GlcNAc_2Man_5$ on LLO. A defect in other proteins involved in GDP-Man synthesis like GMPPA or GMPPB could thus also cause CDG-I. All three candidate genes were therefore screened at the genetic level but no mutations could be found. Since two out of these five CDG-Ix patients belong to the same family, homozygosity mapping could contribute to the identification of the molecular defect in these patients. This powerful tool was however unsuccessful in identifying a restricted set of candidate genes. Both patients belong to a small family with two affected siblings and no healthy child. The presence of a healthy sibling thus appears to be crucial for the efficiency of homozygosity mapping.

We hypothesize that the defect in these CDG-Ix patients should be sought in proteins involved in the early steps of the dolichol cycle, occurring at the cytosolic face of the ER membrane. Besides a deficiency in proteins involved in nucleotide-activated sugar synthesis, a deficient cytosolic homeostasis could also result in the observed LLO profile.

After the screening of *ALG11* in these five CDG-Ix patients, we identified an ALG11 defect in another CDG-I patient showing an accumulation of Dol-PP$GlcNAc_2Man_3$ and Dol-PP-$GlcNAc_2Man_4$ on LLO (see chapter B1.2). This LLO profile thus clearly differs from the profile observed in the five CDG-Ix patients discussed within this chapter.

B2

A DEFICIENCY IN ALG11 IN A CDG-IX PATIENT

Abstract

Protein glycosylation is an important post-translational modification and deficient protein glycosylation is at the basis of a group of genetic disorders called CDG (Congenital Disorders of Glycosylation). Most CDG are caused by a deficiency in the assembly of the oligosaccharide precursor (Dol-PP-$GlcNAc_2Man_9Glc_3$) and are classified as CDG type I (CDG-I) disorders. In the present study, we described the genetic defect in a CDG-I patient presenting with severe psychomotor retardation, feeding problems, epilepsy, sensorineural deafness and hypotonia. This patient was deficient in the mannosyltransferase ALG11, an enzyme that catalyzes the elongation of Dol-PP-$GlcNAc_2Man_3$ to Dol-PP-$GlcNAc_2Man_5$ during oligosaccharide precursor assembly. Lipid-linked oligosaccharides (LLO) analysis showed an accumulation of the assembly intermediates Dol-PP-$GlcNAc_2Man_3$ and Dol-PP-$GlcNAc_2Man_4$ and sequencing of *ALG11* revealed compound heterozygosity for the missense mutation c. 479G>T (p.G160V) and the splice site mutation IVS1-2A>T. A rescue experiment is needed to prove that both mutations are the primary cause of CDG in this patient.

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Unpublished data

Introduction

N-glycosylation or the synthesis of N-glycans and their attachment to asparagine residues of the protein is an important post translational modification involved in numerous cellular functions like protein folding, protein clearance, receptor activation, intracellular targeting, ... (Lis, H. and Sharon, N. 1993). The biosynthesis of an N-glycoprotein is initiated by the assembly of an oligosaccharide precursor (Dol-PP-$GlcNAc_2Man_9Glc_3$), which is subsequently transferred onto proteins by the oligosaccharyltransferase complex. The oligosaccharide precursor is assembled on a lipid carrier, dolichol-P (Dol-P), during a multi-step and highly ordered process called the dolichol cycle (Burda, P. and Aebi, M. 1999). The dolichol cycle starts with the elongation of Dol-P to Dol-PP-$GlcNAc_2Man_5$ at the cytosolic face of the ER membrane. Once Dol-PP-$GlcNAc_2Man_5$ is synthesized, it is flipped to the lumen of the ER where further elongation into the complete oligosaccharide precursor takes place (Burda, P. and Aebi, M. 1999). One of the enzymes involved in the assembly process at the cytosolic face of the ER membrane is ALG11, which catalyzes the elongation of Dol-PP-$GlcNAc_2Man_3$ to Dol-PP-$GlcNAc_2Man_5$ (O'Reilly, M.K., *et al.* 2006).

Congenital disorders of glycosylation or CDG comprise a group of genetic disorders deficient in the glycosylation of protein or lipids and one subgroup of CDG defects, classified as CDG type I (CDG-I), are deficient in oligosaccharide precursor assembly and/or in its transfer onto proteins (Eklund, E.A. and Freeze, H.H. 2006, Jaeken, J. and Matthijs, G. 2007). So far, thirteen CDG-I disorders have been identified, but a deficiency in ALG11 is still lacking. In the present study, we identified an unsolved CDG-I (CDG-Ix) patient that accumulated Dol-PP-$GlcNAc_2Man_3$ and Dol-PP-$GlcNAc_2Man_4$ on LLO, pointing to a deficiency in ALG11.

Materials and Methods

Mutation analysis

Mutation analysis was performed on DNA either extracted from blood or fibroblasts. Based on the genomic sequence, primers were designed to amplify the different exons of *ALG11*, including at least 50 bp of the flanking intronic regions. Primer sequences are available on request. The exons of *ALG11* were amplified using the PCR buffer (minus $MgCl_2$) and the TaqPCRx polymerase of the TaqPCRx recombinant polymerase kit (Invitrogen, California, USA) according to the manufacturers' instructions. Exon 2, 3 part 2 and 4 were amplified using standard PCR conditions, while exon 1 and 3 part 1 were amplified with a standard touch down PCR program. Exon 1 was in addition amplified in presence of 2,5mM $MgCl_2$, while 1,75mM $MgCl_2$ was sufficient for the amplification of the other exons. Sequencing was

performed with the Big Dye Terminator Ready reaction cycle sequencing kit V3.1 (Applied Biosystems, California, USA). The sequences were subsequently analyzed on an ABI3130XL.

Cell culture

See 'Materials and Methods' in section A1, page 40.

Metabolic labelling of cells

See 'Materials and Methods' in section A1, page 40.

LLO analysis

See 'Materials and Methods' in section A1, page 40 and 41.

Results

LLO analysis in control fibroblasts revealed the fully assembled oligosaccharide precursor Dol-PP-$GlcNAc_2Man_9Glc_3$, while the cells of one CDG-Ix patient accumulated the assembly intermediates Dol-PP-$GlcNAc_2Man_3$ and Dol-PP-$GlcNAc_2Man_4$ (Figure 1). Subsequent analysis of the glycoproteins in control and patient revealed in addition a small accumulation of $Man_5GlcNAc_2$ and $Man_6GlcNAc_2$ in the patient's fibroblasts (Figure 1). Since ALG11 was recently shown to catalyze the elongation of Dol-PP-$GlcNAc_2Man_3$ to Dol-PP-$GlcNAc_2Man_5$, we hypothesized that this patient was deficient in ALG11.

Sequencing of *ALG11* indeed revealed that this patient was compound heterozygous for the missense mutation c.479G>T (p.G160V) and the splice site mutation IVS1-2A>T (Figure 2). The mother is a carrier of the missense mutation, while the splice site mutation was of paternal origin. To investigate the potential pathogenic character of the missense mutation, *ALG11* was screened at the genomic level in seventy controls having the same ethnic background as the identified patient. The missense mutation could not be found in any of the controls. Moreover, the glycine residue at position 160 on the protein level is perfectly conserved among higher eukaryotes and was changed into an alanine residue in lower eukaryotes (Figure 3). A glycine-into-valine change was however never observed. These results thus point to the pathogenic character of the missense mutation. A rescue experiment is however needed to confirm that both the missense mutation and the splice site mutation are pathogenic.

The ALG11-deficient patient was the first child of healthy, unrelated Belgian parents. At birth, he showed an important axial hypotonia and developed a poorly responsive epilepsy. A clinical examination at the age of 5 months showed in addition severe psychomotor

retardation characterized by the absence of eye contact, sensorineural deafness and persistent feeding problems. Brain MRI revealed a neuronal migration disturbance.

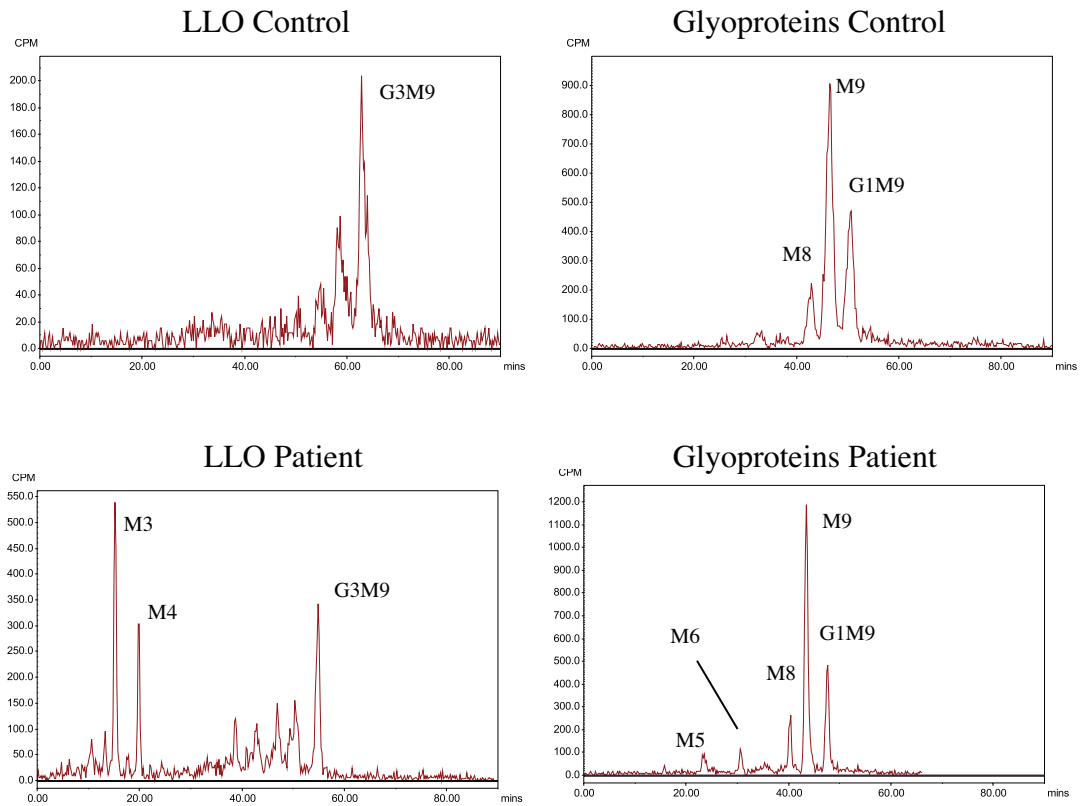

Figure 1: Structural analysis of the LLO and glycoproteins in control and patient's fibroblasts revealed an accumulation of Dol-PP-GlcNAc$_2$Man$_3$ and Dol-PP-GlcNAc$_2$Man$_4$ in the patient, while only a small accumulation of Man$_5$GlcNAc$_2$ and Man$_6$GlcNAc$_2$ was detected on the patient's glycoproteins. Symbols: G3M9: Dol-PP-GlcNAc$_2$Man$_9$Glc$_3$; M3-4: Dol-PP-GlcNAc$_2$Man$_{3-4}$; G1M9: Glc$_1$Man$_9$GlcNAc$_2$; M5-9: Man$_{5-9}$GlcNAc$_2$.

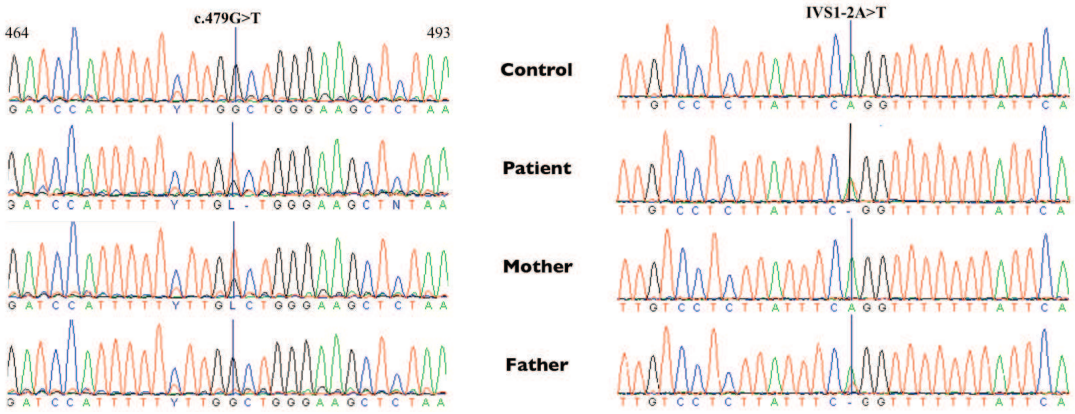

Figure 2: Sequence analysis of *ALG11* in control, patient, mother and father. The patient showed compound heterozygosity for the missense mutation c.479G>T, which was from maternal origin, and the splice site mutation IVS1-2A>T that segregated from the father.

Figure 3: Protein sequence alignment of ALG11 among eukaryotes shows that the glycine residue at protein position 160 is conserved among higher eukaryotes and converted into alanine in some lower eukaryotes.

Discussion

In the present study, we identified the first human ALG11 disorder in a CDG-I patient accumulating the assembly intermediates Dol-PP-$GlcNAc_2Man_3$ and Dol-PP-$GlcNAc_2Man_4$ during LLO analysis. Sequencing of *ALG11* showed that this patient was compound heterozygous for the missense mutation c.479G>T (p.G160V) and the splice site mutation IVS1-2A>T. Although a rescue experiment has not yet been performed, several results already point to the pathogenic character of the missense mutation. The missense mutation has not been observed in 70 controls and a glycine-to-valine change was not detected upon alignment of the ALG11 homologues found in higher and lower eukaryotes. We thus postulate to assign this disorder ALG11-CDG according to the new nomenclature (Jaeken, J., *et al.* 2008).

It should be noted that the LLO profile observed in the ALG11-deficient patient slightly differs from the profile seen in the yeast Alg11p mutant. In yeast, the accumulating Dol-PP-$GlcNAc_2Man_3$ is translocated to the ER lumen and elongated into Dol-PP-$GlcNAc_2Man_7$. Structural analysis of the LLO in the Alg11p mutant thus revealed an accumulation of Dol-PP-$GlcNAc_2Man_3$ (30%), Dol-PP-$GlcNAc_2Man_6$ (13%) and Dol-PP-$GlcNAc_2Man_7$ (46%) (Cipollo, J.F., *et al.* 2001, Helenius, J., *et al.* 2002). In the ALG11-deficient patient, efficient translocation and further elongation of the accumulating assembly intermediates could however not be detected. Moreover, structural analysis of the glycoproteins in the ALG11-CDG patient revealed only a small accumulation of $Man_5GlcNAc_2$ and $Man_6GlcNAc_2$, while the majority of protein-linked glycans in the Alg11p mutant beared a $Man_6GlcNAc_2$ structure formed by demannosylation of $Man_7GlcNAc_2$ (Cipollo, J.F., *et al.* 2001, Helenius, J., *et al.* 2002). We thus hypothesize that, in contrast to yeast, the accumulating Dol-PP-$GlcNAc_2Man_3$ and Dol-PP-$GlcNAc_2Man_4$ are inefficiently transferred across the ER membrane in human cells. Since the potential flippase RFT1, which facilitates the translocation of Dol-PP-$GlcNAc_2Man_5$ during oligosaccharide precursor assembly, was

postulated to catalyze the translocation of Dol-PP-$GlcNAc_2Man_3$ in yeast (Helenius, J., *et al.* 2002), we hypothesize that human RFT1 is highly specific for Dol-PP-$GlcNAc_2Man_5$.

The clinical phenotype of ALG11-CDG is characterized by severe psychomotor retardation, feeding problems, epilepsy, sensorineural deafness and hypotonia. Sensorineural deafness has only rarely been observed in CDG patients, with the exception of RFT1-CDG that results in an accumulation of Dol-PP-$GlcNAc_2Man_5$ at the cytosolic face of the ER membrane (Vleugels, W., *et al.* 2009, Jaeken, J., *et al.* 2009). The observation of sensorineural deafness in RFT1- and ALG11-deficient patients thus suggests an otoneurotoxic effect of LLO (or their derivatives) accumulating at the cytosolic face of the ER membrane. However, since we know only one ALG11-deficient patient, an occasional association of sensorineural deafness with ALG11-CDG remains possible.

Chapter C

IDENTIFICATION OF THE MOLECULAR DEFECT IN CDG-IX PATIENTS ACCUMULATING DOL-PP-$GLCNAC_2MAN_5$ ON LLO

C1

RFT1 DEFICIENCY IN THREE NOVEL CDG PATIENTS

C2

RFT1-CDG: DEAFNESS AS A NOVEL FEATURE OF CDG

C3

IDENTIFICATION OF PHOSPHORYLATED OLIGOSACCHARIDES IN THE CELLS OF CDG-I PATIENTS

C4

A DEFICIENCY IN DPM2 CAUSES A NEW SUBTYPE OF CDG

C1

RFT1 DEFICIENCY IN THREE NOVEL CDG PATIENTS

ABSTRACT

The medical significance of N-glycosylation is underlined by a group of inherited human disorders called Congenital Disorders of Glycosylation (CDG). One key step in the biosynthesis of the Dol-PP-$GlcNAc_2Man_9Glc_3$ precursor, essential for N-glycosylation, is the translocation of Dol-PP-$GlcNAc_2Man_5$ across the endoplasmic reticulum membrane. This step is facilitated by the RFT1 protein. Recently, the first RFT1-deficient CDG (RFT1-CDG) patient was identified and presented a severe N-glycosylation disorder.

In the present study, we describe three novel CDG patients with an RFT1 deficiency. The first patient was homozygous for the earlier reported *RFT1* missense mutation (c.199C>T; p.R67C), while the two other patients were homozygous for the missense mutation c. 454A>G (p.K152E) and c.892G>A (p.E298K), respectively. The pathogenic character of the novel mutations was illustrated by the accumulation of Dol-PP-$GlcNAc_2Man_5$ and by reduced recombinant DNase 1 secretion. Both the glycosylation pattern and recombinant DNase 1 secretion could be normalized by expression of normal *RFT1* cDNA in the patients' fibroblasts. The clinical phenotype of these patients comprised typical CDG symptoms in addition to sensorineural deafness, rarely reported in CDG patients. The identification of additional RFT1-deficient patients allowed to delineate the main clinical picture of RFT1-CDG and confirmed the crucial role of RFT1 in Dol-PP-$GlcNAc_2Man_5$ translocation.

Adapted from

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RFT1 deficiency in three novel CDG patients

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Introduction

N-glycosylation is a post-translational modification of proteins found in eukaryotic and prokaryotic organisms (Weerapana, E. and Imperiali, B. 2006). In eukaryotes, protein N-linked glycans are involved in many essential biological processes including the immune response, intracellular targeting, cell-cell recognition, protein folding and protein stability (Varki, A. 1993).

The eukaryotic N-glycosylation pathway starts with the assembly of the $Glc_3Man_9GlcNAc_2$ oligosaccharide precursor on a dolichol-PP (Dol-PP) carrier in a well-ordered process known as the dolichol cycle (Burda, P. and Aebi, M. 1999). This dolichol cycle starts with the elongation of Dol-P to Dol-PP-$GlcNAc_2Man_5$ on the cytosolic face of the endoplasmic reticulum (ER) membrane, while the elongation of Dol-PP-$GlcNAc_2Man_5$ to the complete Dol-PP-$GlcNAc_2Man_9Glc_3$ occurs in the ER lumen. For this reason, the Dol-PP-$GlcNAc_2Man_5$ intermediate has to be translocated across the ER membrane. In yeast, the ER membrane protein Rft1 was shown to facilitate the translocation of Dol-PP-$GlcNAc_2Man_5$ to the ER lumen in a bidirectional and ATP-independent manner (Helenius, J., *et al.* 2002). Once the fully assembled oligosaccharide precursor is synthesized, $Glc_3Man_9GlcNAc_2$ is transferred onto selected asparagine residues of polypeptide chains by the oligosaccharyltransferase (OST) complex (Burda, P. and Aebi, M. 1999).

Congenital Disorders of Glycosylation (CDG) are a group of inherited human disorders characterized by deficient protein glycosylation. Up to now, 14 different CDG types deficient in protein N-glycosylation site occupancy have been identified. The analysis of the serum sialotransferrin pattern is the most widely used method to screen for N-glycosylation disorders, which can be classified into two subgroups: defects of oligosaccharide precursor assembly and transfer to proteins (formerly known as CDG-I) and defects of N-glycoprotein processing (formerly known as CDG-II) (Eklund, E.A. and Freeze, H.H. 2006, Freeze, H.H. 2007, Jaeken, J. and Matthijs, G. 2007).

Recently, the first RFT1-deficient patient (OMIM 612015) was identified (Haeuptle, M.A., *et al.* 2008). Haeuptle and co-workers described a young girl presenting with marked psychomotor retardation, hypotonia, seizures, hepatomegaly and coagulopathy. This patient was homozygous for the missense mutation c.199C>T (p.R67C) in the *RFT1* gene and accumulated Dol-PP-$GlcNAc_2Man_5$ as shown by lipid-linked oligosaccharides (LLO) analysis. However, no $Man_5GlcNAc_2$ was transferred onto glycoproteins, which pointed to a deficient translocation of the accumulated LLO across the ER membrane.

In the present study, we describe three additional RFT1-deficient patients including two novel pathogenic point mutations. The identification of these additional patients allowed us to

refine the clinical phenotype characteristic for RFT1 deficiency, designated as RFT1-CDG according to the suggested novel nomenclature (Jaeken, J., *et al.* 2008).

Materials and methods

Cell culture

See 'Materials and Methods' in section A1, page 40.

Mutation analysis

Total RNA was isolated from 2 x 10^7 fibroblasts using the TRIzol LS reagent (Invitrogen) according to the manufacturer's instructions. The human *RFT1* cDNA was prepared and the protein-coding region was amplified by PCR as described before (Haeuptle, M.A., *et al.* 2008). The PCR products were sequenced (Synergene Biotech) after removal of the unincorporated nucleotides with QIAquick columns (QIAGEN). Carrier analysis in the parents and healthy siblings was performed on DNA extracted from blood. Primers were designed to amplify exons 3, 4 and 9 of the *RFT1* gene, based on the genomic sequence (NM_052859.2). Primer sequences are available on request. These exons were amplified using standard PCR conditions, subsequently sequenced with the Big Dye Terminator Ready reaction cycle sequencing kit V3.1 (Applera) and analyzed on an ABI3100 Avant (Applera). The numbering of the nucleotide changes is based on the cDNA sequence (NM_052859.2) with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence.

Metabolic radiolabelling

See 'Materials and Methods' in section A1, page 40.

Analysis of oligosaccharide material

Glycoprotein fractions obtained at the end of the sequential extraction were digested overnight at room temperature with trypsin (1 mg/ml; Sigma) in 0.1 M ammonium bicarbonate buffer, pH 7.9. The resulting glycopeptides were treated with 0.5 U PNGase F (Roche) in 50 mM phosphate buffer, pH 7.2 for 4 h to release the oligosaccharides from the peptides. The oligosaccharides were desalted on Bio-Gel P2 columns and eluted with 5% acetic acid. LLO fractions obtained after sequential extraction were subjected to mild acid treatment (0.1 M HCl in THF) for 2 h at 50 °C. Purification of the released oligosaccharides was performed as described above.

The oligosaccharides were separated by HPLC on an amino derivated Asahipak NH_2P-50 column (250 mm x 4.6 mm; Asahi) applying a gradient of acetonitrile/H_2O ranging from

70:30 to 50:50 over 90 min at a flow rate of 1 ml/min. Oligosaccharides were identified on the basis of their retention times compared to standard glycans (Foulquier, F., *et al.* 2002). Elution of the radiolabelled oligosaccharides was monitored by continuous β-counting with a flo-one β detector (Packard).

Complementation of the LLO profile

Lentiviruses containing *RFT1* and *EGFP* cDNAs were prepared as previously described (Haeuptle, M.A., *et al.* 2008). Briefly, 3 x 10^6 HEK293T cells were transfected with 20 µg pLenti6-*hRFT1* or pLenti6-*EGFP* and 36 µg of the packing plasmid mix (Invitrogen) by calcium-phosphate precipitation. Two days after transfection, the supernatants were collected and used to transduce the patient fibroblasts. After selection with 5 µg/ml blasticidin (Invitrogen), the cells were metabolically labelled with 2-[^{3}H]-mannose and the extracted LLO were analysed by HPLC (Zufferey, R., *et al.* 1995).

Complementation of DNase 1 secretion

Bovine *DNase I* cDNA was subcloned into the pSVK3 vector as previously reported (Nishikawa, A., *et al.* 1997). Patients' fibroblasts were transduced with an adenovirus expressing bovine *DNase I* (Eklund, E.A., *et al.* 2005, Fujita, N., *et al.* 2008). Two days post transduction, the cells were washed twice with PBS and incubated at 37 °C with 1 ml DMEM without methionine/cysteine, containing 10% dialysed fetal bovine serum, 10 mM NH_4Cl and 0.2 mCi [^{35}S]-Met/Cys labelling mixture. After a labelling time of 4 h, the culture medium was harvested and DNase 1 was immunoprecipitated as previously described (Nishikawa, A. and Mizuno, S. 2001). The immunoprecipitated samples were subjected to SDS-PAGE using a 13% acrylamide gel. After electrophoresis, the gels were rinsed with a mixture of 7% acetic acid and 10% methanol for 15 min and soaked in Amplify solution (Amersham Pharmacia Biotech) for 15 min. The gels were dried, autoradiography was carried out and the intensity of the bands corresponding to DNase I was quantified by scanning densitometery (using Quantity One software PDI). Band intensity was normalized against protein levels.

Results

Biochemical and molecular diagnosis

Three patients with a type 1 pattern of serum sialotransferrins were further investigated. Subsequently, phosphomannomutase (*CDG-Ia*) and phosphomannose isomerase (*CDG-Ib*)

deficiencies were excluded on the basis of enzymatic activity measurements in the patients' fibroblasts (data not shown).

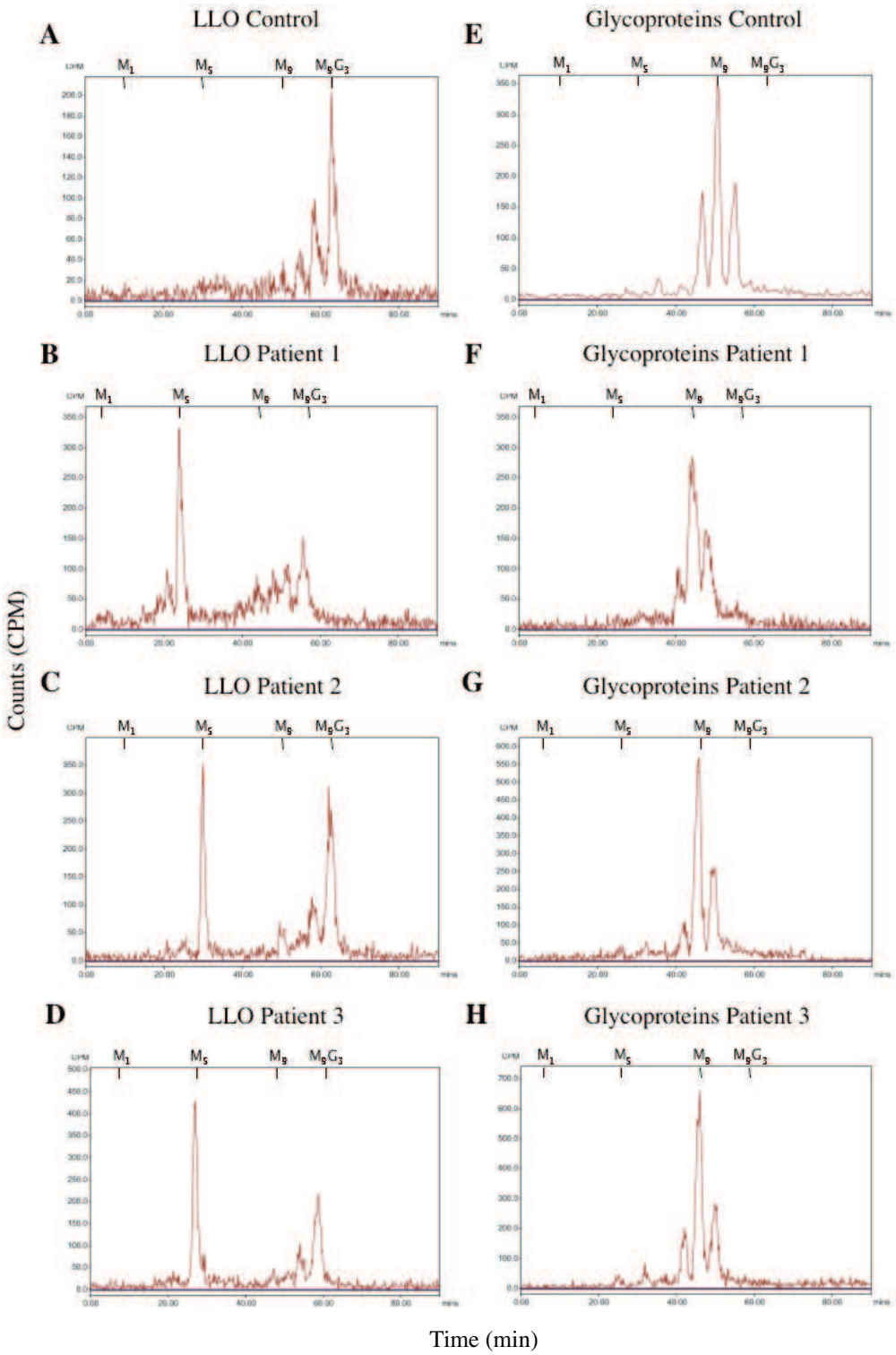

Figure 1: (A-D) HPLC analysis of the LLO in control and patients' fibroblasts revealing the accumulation of Dol-PP-$GlcNAc_2Man_5$ in the three patients. **(E-H)** Protein N-linked oligosaccharides of control and patients were separated by HPLC, demonstrating that no aberrant glycan structures were detected in the patients. The retention times of the standard oligosaccharides Dol-PP-$GlcNAc_2Man_9Glc_3$ (G_3M_9) and Dol-PP-$GlcNAc_2Man_{1-9}$ (M_{1-9}) are marked above the HPLC profiles.

In order to identify a defect in the assembly of the oligosaccharide precursor within the dolichol cycle, structural analysis of the LLO was performed by HPLC after metabolic labelling. In healthy control fibroblasts, the LLO profile is characterized by the fully assembled oligosaccharide precursor Dol-PP-$GlcNAc_2Man_9Glc_3$. In contrast, an accumulation of Dol-PP-$GlcNAc_2Man_5$ in combination with only a minor amount of complete LLO was detected in all three patients (Figure 1, panels A, B, C and D). Additionally, protein-linked glycan structures were analyzed. In both, healthy control and patients, $Man_8GlcNAc_2$, $Man_9GlcNAc_2$ and $Glc_1Man_9GlcNAc_2$ structures were obtained (Figure 1, panels E, F, G and H). Notably, no $Man_5GlcNAc_2$ structure could be detected on the patients' glycoproteins.

The accumulation of Dol-PP-$GlcNAc_2Man_5$ on LLO combined with normal protein-linked glycans is typical for a defect in the translocation of Dol-PP-$GlcNAc_2Man_5$ to the ER lumen and was recently reported in a RFT1-deficient patient (OMIM 612015) (Haeuptle, M.A., *et al.* 2008). To this end, mutation analysis of the *RFT1* cDNA was performed in the present patients and all three carried *RFT1* mutations. The first patient was homozygous for the earlier reported missense mutation (c.199C>T; p.R67C) (Haeuptle, M.A., *et al.* 2008), while the parents were heterozygous carriers of this mutation (Figure 2). Two novel point mutations were identified in two other patients. Sequencing of the second patient's *RFT1* cDNA revealed an A to G transition at nucleotide position 454, leading to the conversion of a lysine into a glutamic acid at position 152 on the protein level (Figure 2). Both parents as well as a healthy sibling were heterozygous for this mutation. In model eukaryotic organisms, the lysine at position 152 is mostly conserved and a conversion of a lysine into a glutamic acid was not observed (data not shown). The third patient was homozygous for a G to A transition at nucleotide position 892, causing a glutamic acid to lysine change at position 298 (Figure 2). The mutated glutamic residue is strictly conserved among eukaryotes (data not shown). No material of the parents and siblings was available for carrier analysis.
In addition, all converted amino acids were found in hydrophilic domains of the RFT1 protein predicted to be oriented to the ER lumen (TMpred (Hofmann, K. and Stoffel, W. 1992); TMHMM (Krogh, A., *et al.* 2001)) (Figure 3). As previously reported, the p.R67C mutation is located in the first luminal loop of the RFT1 protein (Haeuptle, M.A., *et al.* 2008). The p.K152E mutation was found in the second luminally oriented 25 amino acid long hydrophilic stretch and the p.E298K mutation was positioned in the largest luminal loop, bearing a potential N-glycosylation site at position p.N227 and ranging over 130 amino acids (Figure 3).

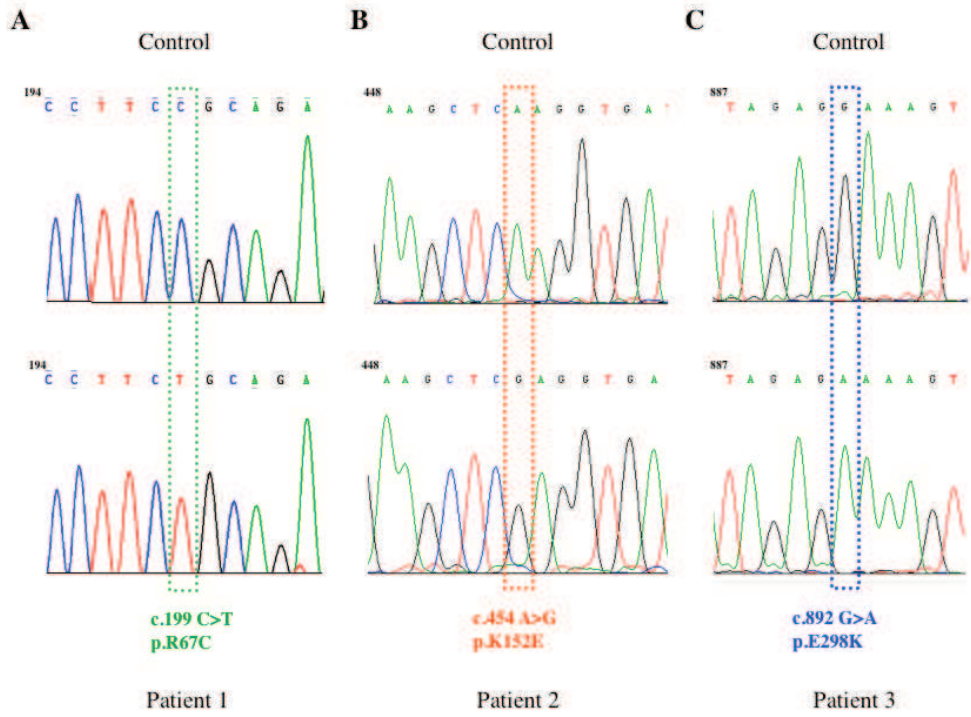

Figure 2: Sequence alignment of the *RFT1* cDNA fragments in control and patients. **(A)** Patient 1 is homozygous for the earlier reported missense mutation c.199C>T (p.R67C). **(B)** In patient 2, the homozygous A to G transition at nucleotide position c.454 was detected, leading to a conversion of a lysine into a glutamic acid at position p.152. **(C)** Analysis of the *RFT1* cDNA in patient 3 revealed a homozygous G to A transition at nucleotide position c.892, causing a glutamic acid to lysine change at protein position p.298.

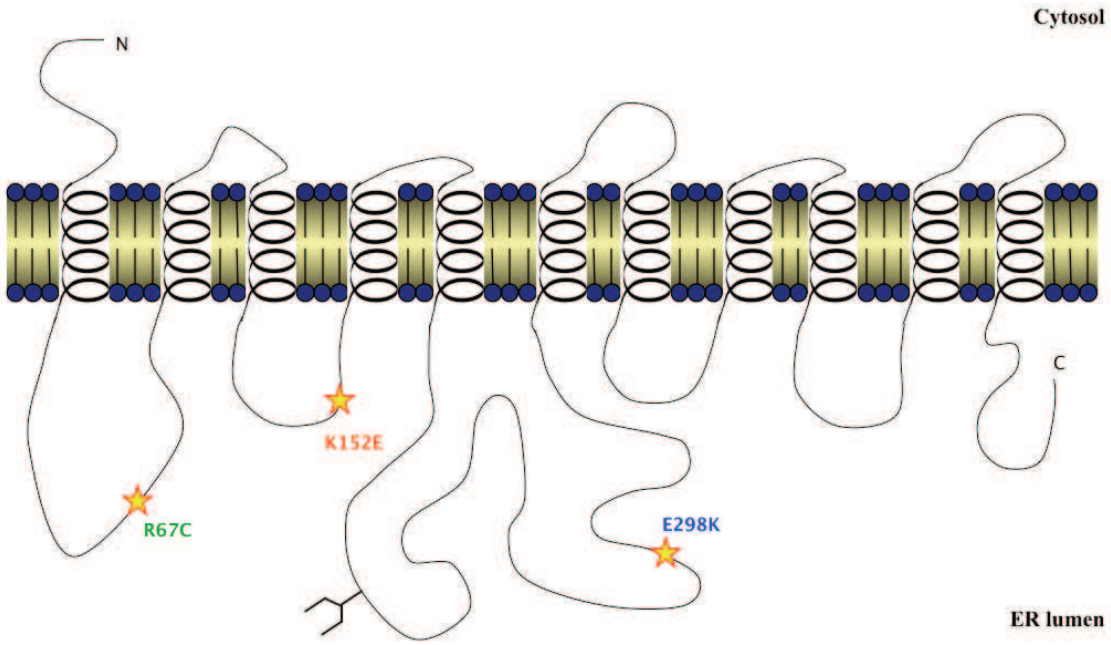

Figure 3: Schematic representation of the identified missense mutations in the predicted topology of the RFT1 protein. All three mutations were found in the predicted luminal loops of the protein. The orientation of the model was supported by the favoured position of the 11 transmembrane domains and the localization of a potential N-glycosylation site (p.N227) (TMpred (Hofmann, K. and Stoffel, W. 1992); TMHMM (Krogh, A., *et al.* 2001)).

Complementation of the LLO profile

To demonstrate the pathogenicity of the two new missense mutations, wild-type *RFT1* cDNA was transduced into patients' fibroblasts using a lentiviral construct to complement the Dol-PP-$GlcNAc_2Man_5$ accumulation. Healthy control and patients' fibroblasts were thus infected with recombinant lentiviruses expressing either the wild-type *RFT1* cDNA or *EGFP* as a negative control. Compared to *EGFP* expression in the patients' fibroblasts, analysis after *RFT1* expression revealed a normalization of the LLO profile characterized by decreased levels of Dol-PP-$GlcNAc_2Man_5$ and increased levels of the complete Dol-PP-$GlcNAc_2Man_9Glc_3$ (Figure 4). *EGFP* expression in the patients' fibroblasts did not affect the LLO profile, as expected.

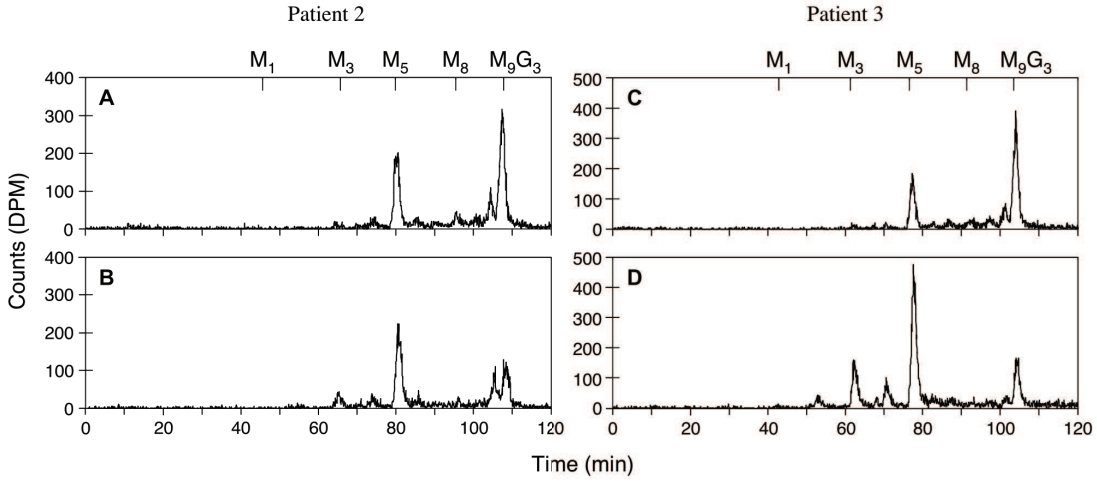

Figure 4: Complementation of the Dol-PP-$GlcNAc_2Man_5$ accumulation. Fibroblasts of patient 2 **(A)** and patient 3 **(C)** expressing wild-type *RFT1* cDNA, leading to a decreased accumulation of Dol-PP-$GlcNAc_2Man_5$. Lentiviral mediated expression of *EGFP* in the fibroblasts of patient 2 **(B)** and patient 3 **(D)** showed no effect. The retention times of the standard oligosaccharides Dol-PP-$GlcNAc_2Man_9Glc_3$ (G_3M_9) and Dol-PP-$GlcNAc_2Man_{1\text{-}9}$ ($M_{1\text{-}9}$) are marked above the HPLC profiles.

Complementation of DNase 1 secretion

In another alternative method to prove the pathogenicity of the two new mutations, secretion of recombinant bovine DNase 1 was investigated in all three RFT1-deficient patients. A modified version of bovine DNase 1 has a single potential N-glycosylation site (p.N106) and is secreted when expressed in human fibroblasts (Nishikawa, A. and Mizuno, S. 2001). However, upon expression in the fibroblasts of CDG patients, DNase 1 secretion was strongly reduced (Eklund, E.A., *et al.* 2005, Fujita, N., *et al.* 2008). As shown in Figure 5 (lanes 3, 5 and 7), DNase 1 secretion was significantly reduced in all three RFT1-deficient patients. Transduction of the patients' fibroblasts with lentiviruses coding for wild-type *RFT1*

restored the levels of DNase 1 secretion (Figure 5, lanes 4, 6 and 8), thus demonstrating the pathogenicity of the new mutations.

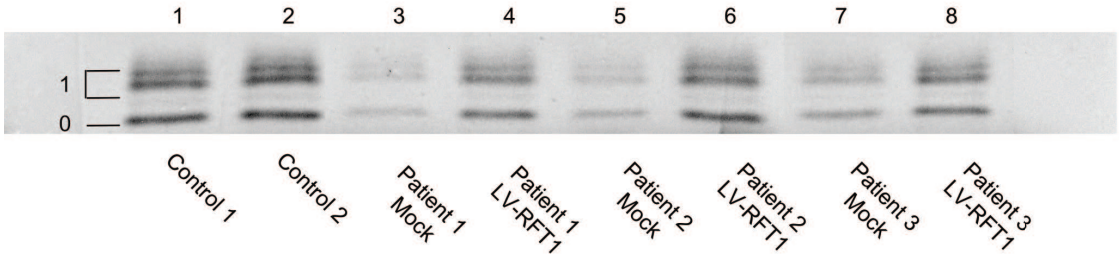

Figure 5: Rescue of DNase 1 secretion in the patients' fibroblasts. Secretion of DNase 1 is reduced in the fibroblasts of the three RFT1-deficient patients (lanes 3, 5 and 7). Mock stands for no lentiviral treatment. Expression of wild-type *RFT1* cDNA (lanes 4, 6 and 8) restored the secretion of DNase 1 to a level equal to controls (lanes 1 and 2). Symbols: o = non-glycosylated DNase 1; 1 = singly glycosylated DNase 1.

Clinical phenotype

To address the main clinical characteristics of RFT1 deficiency, the clinical phenotypes of the three different patients were compared. Patient 1 was the first child of healthy, unrelated North Americans of Scottish-English origin and presented with respiratory insufficiency, severe generalized epilepsy with intractable seizures, infantile spasms, microcephaly, failure to thrive, hypotonia, sensorineural deafness and decreased visual acuity. In addition, this patient showed severe mental retardation, with virtually no development, and had normal liver function. Dysmorphic features included micrognathia, short neck, small nose, drooping eyelids, valgus feet and adducted thumbs. This patient also had severe feeding problems requiring gastrostomy and died at the age of eight months. Brain MRI at early age did not reveal cerebral nor cerebellar atrophy. However, at autopsy, weight and gyral/sulcal pattern consistent with a degree of cerebral atrophy was reported (Table 1).

Patient 2 was the second son of healthy, consanguineous Italian parents. During the first year of life, he presented with severe developmental delay, microcephaly, nystagmus, sensorineural deafness, relapsing aspiration pneumonia, a generalized hypotonia and inverted nipples. Additionally, this patient was frequently hospitalized because of drug-resistant epilepsy and feeding problems requiring gastrostomy. At the age of 4 years, computer tomography scan of the brain revealed a stroke-like episode affecting the left frontal lobe, and the following year he started to suffer from recurrent deep venous thrombosis of the left leg. Clinical examination at the age of five years showed a severe mentally retarded child with microcephaly, spastic tetraparesis and kyphoscoliosis. Serial brain MRI examinations showed

progressive cortical and subcortical atrophy with no cerebellar involvement. Liver function has always been normal (Table 1).

		Patient 1	Patient 2	Patient 3	Patient KS
Gender		female	male	male	female
Origin		Scottish	Italian	Algerian	English
Vital status		died 8 m	alive 5.5 y	alive 2.2y	died 4.3 y
Consanguinity		-	+	+	-
Feeding problems		+	+	+	+
Failure to thrive		+	+	+	+
Severe mental retardation		+	+	+	+
Microcephaly		+	+	-	na
Hypotonia		+	+	+	+
Epilepsy		+	+	+	+
Decreased visual acuity		+	+	+	+
Sensorineural deafness		+	+	+	+
Myoclonic jerks		+	+	+	+
Respiratory insufficiency		+	-	+	na
Pulmonary infections		-	+	+	+
Coagulopathy		na	+	na	+
Dysmophic features:	micrognathia	+	-	+	na
	short neck	+	-	+	na
	valgus feet	+	+	+	na
	adducted thumbs	+	na	+	-
	kyphoscoliosis	na	+	na	+
	inverted nipples	-	+	+	+
Brain MRI:	cerebral atrophy	-*	+	-	+
	cerebellar atrophy	-	-	-	+

Table 1: Clinical phenotype of the three new RFT1-deficient patients compared to the phenotype of the originally reported case (KS) (Clayton and Grunewald, 2009; Haeuptle et al., 2008; Imtiaz et al., 2000). Symbols: na: not available; *: cerebral atrophy reported at autopsy.

The third patient was the ninth child of healthy, consanguineous parents from Algerian origin. This family has two children presenting with the autosomal recessive disorder *Hemophagocytic Lymphohistiocytosis* (OMIM 267700). One child died at the age of three months, while the other is still alive due to bone marrow transplantation at the age of four months. In addition, one son died at the age of four days and presented with similar characteristics as patient 3. Patient 3 presented with respiratory insufficiency, hypotonia, body spasm, failure to thrive, epilepsy, bilateral glaucoma and sensorineural deafness. Dysmophic features included slightly inverted nipples, infiltrated ears, short neck, retrognathism, glossoptosis, adducted thumbs and valgus feet. Brain MRI did not show cerebral or cerebellar atrophy. This patient also had feeding problems and chronic infections of the respiratory tract. In addition, he showed severe mental retardation with the absence of visual contact. Liver function was normal (Table 1).

Discussion

Based on the analysis of LLO and protein-linked glycan profiles in fibroblasts, three potential RFT1-deficient patients were identified. All three patients showed an accumulation of the LLO Dol-PP-$GlcNAc_2Man_5$, while no $Man_5GlcNAc_2$ was detected on glycoproteins. Mutation analysis of the *RFT1* gene revealed that one patient was homozygous for the earlier reported missense mutation (c.199C>T, p.R67C). This is the second report of the p.R67C mutation in a patient of British origin (Imtiaz, F., *et al.* 2000) and there are no indications that both patients are related. This could thus point to a founder effect of the p.R67C mutation. The other patients were homozygous for the new missense mutations c.454A>G (p.K152E) and c.892G>A (p.E298K). The pathogenic character of these novel mutations was demonstrated by the complementation of the abnormal LLO profile and reduced DNase 1 secretion upon expression of wild-type *RFT1* in the patients' fibroblasts.

All three *RFT1* mutations identified so far are located in one of the hydrophilic loops predicted to be within the ER lumen. It can be assumed that these regions are of major importance for the translocation of Dol-PP-$GlcNAc_2Man_5$ in the ER lumen or for the maintenance of Dol-PP-$GlcNAc_2Man_5$ on the luminal side. Further structural analysis will be required to confirm the predicted orientation of the RFT1 protein. Determination of the occupancy of the putative N-glycosylation site at position p.N227 (Figure 2) would certainly contribute to establish the topology of the RFT1 protein.

In yeast, the Rft1 protein was genetically identified as a protein mediating the translocation of LLO across the ER membrane (Helenius, J., *et al.* 2002). However, recent evidence suggests that the RFT1 protein would not be the flippase enzyme itself, but would play a critical accessory role in translocating Dol-PP-$GlcNAc_2Man_5$ to the ER lumen (Frank, C.G., *et al.* 2008, Sanyal, S., *et al.* 2008). Anyhow, the identification of three additional RFT1-deficient patients clearly underscores the major importance of RFT1 in this translocation event.

Finally, the identification of three additional patients allowed us to refine the clinical phenotype characteristic for RFT1 deficiency (OMIM 612015). All four known RFT1-deficient patients showed very similar characteristics including severe mental retardation, hypotonia, epilepsy, myoclonic jerks, decreased visual acuity, sensorineural deafness and feeding problems (Table 1). In comparison to other CDG defects, RFT1 deficiency is thus mainly a neurological disorder. Strikingly, sensorineural deafness was found in all four RFT1-deficient patients (Imtiaz, F., *et al.* 2000) and might represent a characteristic clinical feature of RFT1-CDG, since deafness has been reported in only a few other CDG patients (Hutchesson, A.C., *et al.* 1995, Imtiaz, F., *et al.* 2000, Kranz, C., *et al.* 2007).

Acknowledgements

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C2

RFT1-CDG: DEAFNESS AS A NOVEL FEATURE OF CDG

Abstract

We report two novel patients with a CDG due to defective RFT1. Both patients show a severe, mainly neurological, syndrome as seen in the four other known patients. Strikingly, like these other patients, they also show sensorineural deafness which has not been reported before as a regular feature of any CDG. RFT1-CDG is thus the first CDG in which sensorineural deafness is part of the phenotype.

Adapted from

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RFT1-CDG: deafness as a novel feature of congenital disorders of glycosylation (CDG)

J Inherit Metab Dis, in press

Introduction

Congenital disorders of glycosylation (CDG) are a rapidly growing family of genetic diseases caused by defects in the synthesis of the glycans of proteins and lipids, and in their attachment to these compounds (Freeze, H.H. 2006, Grunewald, S. 2007, Jaeken, J. and Matthijs, G. 2007). At present, some forty CDG have been identified (Jaeken, J., *et al.* 2008). They are mostly multi-system diseases and the number of symptoms and syndromes reported in CDG increases steadily. Although occasional patients with CDG and sensorineural deafness have been reported, deafness is one of the few symptoms that have not been observed as a consistent feature in any CDG. Recently, a defect was identified in RFT1 (Haeuptle, M.A., *et al.* 2008). It is a defect in the assembly part of the N-glycosylation pathway. RFT1 is involved in the transfer of Dol-PP-$GlcNAc_2Man_5$ from the cytoplasmic to the luminal side of the endoplasmic reticulum membrane, although it is not certain that it is the flippase itself (Frank, C.G., *et al.* 2008, Rush, J.S., *et al.* 2009). Recently, three other patients have been reported (Vleugels, W., *et al.* 2009). We report two novel patients and conclude that all six known patients with this defect show a severe neurological syndrome including sensorineural deafness. In this paper, we use the novel CDG nomenclature namely the gene symbol followed by –CDG, in this case RFT1-CDG (Jaeken, J., *et al.* 2008).

Patients

Patient 1

This is a boy of Moroccan descent, born from unrelated parents after a 36 weeks pregnancy with cesarean section because of breech presentation. He has two healthy sisters. At birth weight was 1865 g, length 43 cm and head circumference 31.5 cm. Apgar scores were 5 and 8. He showed retropulsion of the head, a stridorous inspiration, an important generalized (particularly axial) hypotonia, hypertonia of the fingers flexors, hypokinesia, absent Moro reflex, normal glabella reflex and brisk knee tendon reflexes. He had a rather small chin and a mildly limited extension of the elbows but no other dysmorphy. His eye movements were erratic with a tendency to internal strabism. The further evolution was characterized by episodic apnoeas (necessitating several reanimations), feeding difficulties (gastroesophageal reflux, difficult co-ordination between respiration and swallowing, relative constipation). The patient was partly fed via nasogastric tube. Epileptic seizures were easily controlled with valproic acid.

At the age of 8 months weight was 3.87 kg (3^{rd} centile is 7 kg), length 59.7 cm (3^{rd} centile is 67 cm) and head circumference 39.5 cm (3^{rd} centile is 43 cm). Psychomotor development

was less than 4 weeks (he did not show eye contact) and there was no response to auditory stimuli. He had a tympanic abdomen; palpation of liver and spleen was normal.

Biochemical investigation showed normal blood count and normal levels of blood lactate, serum albumin, cholesterol, transaminases, creatine kinase, lactate dehydrogenase, arylsulphatase A, thyroid stimulating hormone, total T4, thyroxine binding globulin, insulin, growth hormone, IGF-1, and plasma amino acids. Blood clotting factor XI (14%; normal range for age: 25-93%), protein C (11%; normal range for age: 37-81%) and antithrombin (27%; normal range for age: 52-128%) were decreased. Capillary zone electrophoresis showed a type 1 pattern of serum sialotransferrins.
Technical investigations: ultrasonography of liver, spleen and kidneys, echocardiography, ophthalmology, electromyography (EMG)(initially interpreted as myopathic), single-fiber EMG, sensory and motor nerve conduction velocities, and electroencephalography were normal. However, the brainstem evoked response audiometry (BERA) showed evidence for a severe bilateral hearing loss (no peak V at 80 dB) with normal tympanometry. Radiology of the skeleton showed no abnormalities. Brain MRI at the age of 3 weeks showed a cavum septum pellicidum, but no other abnormalities, including normal myelination for age.

Patient 2

This girl was born from Italian parents after a 36 weeks pregnancy with polyhydramnios. Apgar scores were only 1 and 5. Birth weight was 2490 g, length 44 cm and head circumference 33 cm. There was no respiratory distress syndrome. Nevertheless the patient had to be ventilated from birth on and tracheostomy was performed at 4 months. There was facial dysmorphy (anteverted nostrils, depressed and broad nasal bridge, small mouth) as well as inverted nipples. Epilepsy started in the first week of life and was therapy-resistant. The newborn was extremely hypotonic and showed no spontaneous movements. She was never able to suck and swallow, and was fed by gastrostomy. Knee tendon reflexes were not elicitable. A femoral and iliac vein thrombosis was diagnosed at 4 months. At 11 months weight was 8,87 kg (25th centile), length 66 cm (3rd centile is 67.6) and head circumference 39 cm (3rd centile is 43.1).
Biochemical investigations showed a normal metabolic screening except for a type 1 pattern of serum sialotransferrins on capillary zone electrophoresis. Blood coagulation factor XI was 11% (normal range for age: 46-110%), antithrombin 17% (normal range: 52-128%) and protein C 10% (normal range: 31-83%).
Technical investigations: ultrasonography of liver, spleen and kidneys, echocardiography, electromyography, and motor and sensory nerve conduction velocities were normal.

Radiology of the skeleton was normal. Ophtalmological investigation showed pale optic nerves.

The electroencephalogram was epileptic without specific features.

Brain stem evoked response audiometry showed severe bilateral hearing loss. Brain MRI: symmetrical lesions of the basal ganglia, cortical atrophy, diffuse supratentorial white matter hyperintensity, unmyelinated capsula interna and normal cerebellum.

Materials and Methods

Lipid-linked oligosaccharide analysis (LLO) was performed in fibroblasts as reported (Grubenmann, C.E., *et al.* 2004).

Mutation analysis of RFT1 was performed by direct sequencing of the RFT1 cDNA. In the parents, carrier analysis was performed by sequencing of the corresponding exon at the genomic level.

Results

In controls, LLO analysis showed the formation of Dol-PP-$GlcNAc_2Man_9Glc_3$, the fully assembled oligosaccharide precursor for N-glycosylation. However, LLO analysis revealed in both patients an accumulation of Dol-PP-$GlcNAc_2Man_5$, associated with a decreased formation of Dol-PP-$GlcNAc_2Man_9Glc_3$ (Figure 1). Structural analysis of the patients' glycoproteins showed in addition an absence of $Man_5GlcNAc_2$, pointing to an accumulation of Dol-PP-$GlcNAc_2Man_5$ at the cytosolic face of the ER membrane (Figure 1).

Mutation analysis of *RFT1* showed in patient 1 homozygosity for the previously reported missense mutation c.454A>G (p.K152E) (se chapter B2.1) and heterozygosity for this mutation in his parents, and patient 2 is compound heterozygous for two novel missense mutations affecting the same nucleotide: c.887T>A (p.I296K) and c.887T>G (p.I296R) (Figure 2). Carrier analysis revealed heterozygosity for the c.887T>A mutation in the father and for the c.887T>G mutation in the mother. The isoleucine at position p.296 is perfectly conserved from humans over *Drosophila melanogaster* to *Saccharomyces cerevisiae,* with the exception of *Rattus norvegicus* and *Mus musculus* (data not shown). In these two latter species, a change into valine was observed. However, no isoleucine-into-lysine or isoleucine-into-arginine variation was detected, pointing to the pathogenic character of both missense mutations. The two novel missense mutations are just like the other known RFT1 mutations (see chapter B2.1) predicted to be located in the luminal loops of the RFT1 protein.

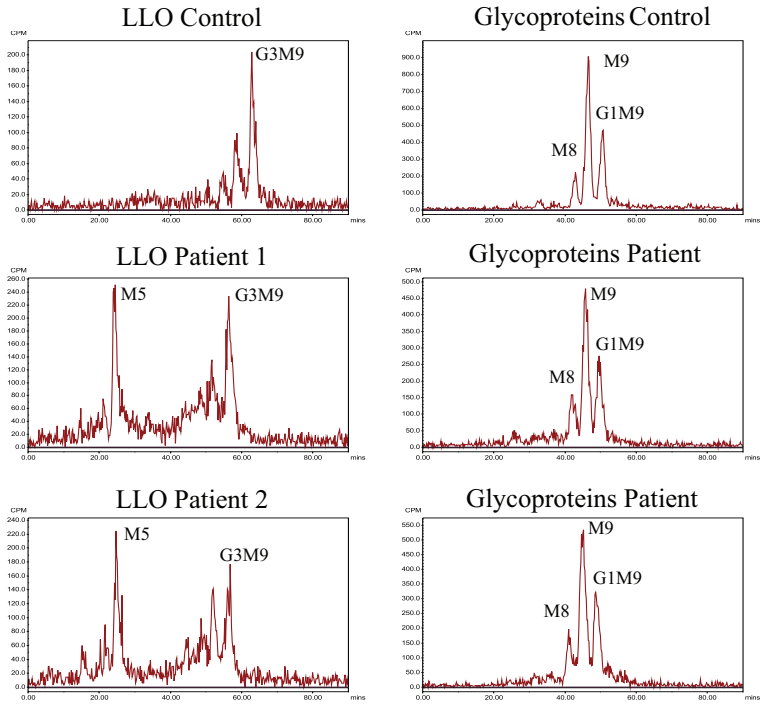

Figure 1: LLO analysis in the cells of a control and two CDG-I patients revealed the formation of Dol-PP-$GlcNAc_2Man_9Glc_3$ in the control and an accumulation of Dol-PP-$GlcNAc_2Man_5$ in both patients. The accumulating Dol-PP-$GlcNAc_2Man_5$ was however not transferred onto proteins since $Man_5GlcNAc_2$ was not detected on the patient's glycoproteins. Symbols: G3M9: Dol-PP-$GlcNAc_2Man_9Glc_3$; M5: Dol-PP-$GlcNAc_2Man_5$; G1M9: $Glc_1Man_9GlcNAc_2$; M5-9: $Man_{5\text{-}9}GlcNAc_2$.

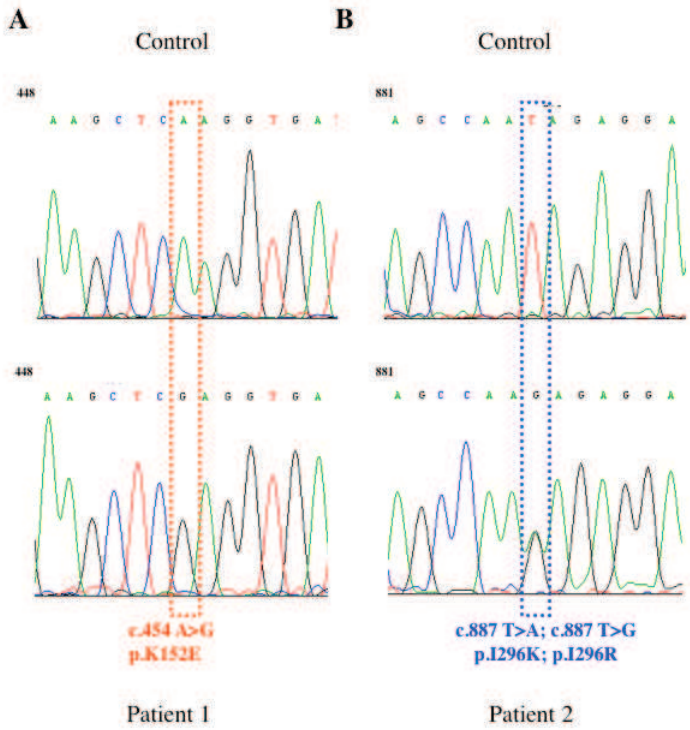

Figure 2: Mutation analysis of RFT1 showed that patient 1 is homozygous for the earlier reported missense mutation (c.454A>G; p.K152E) **(A)** while patient 2 is compound heterozygous for two missense mutations affecting the same nucleotide (c.887T>A; p.I296K and c.887T>G; p.I296R) **(B)**.

Discussion

The present patients show a very similar, essentially neurological, clinical syndrome as the four other known patients with an RFT1 defect (Clayton, P.T. and Grunewald, S. 2009, Haeuptle, M.A., *et al.* 2008, Imtiaz, F., *et al.* 2000, Vleugels, W., *et al.* 2009). Common features are feeding problems, failure to thrive, severe developmental delay, poor to absent visual contact, epilepsy, hypotonia and sensorineural deafness. Less constant features are microcephaly, brisk tendon reflexes, variable dysmorphy, respiratory problems, gastrointestinal problems and venous thromboses. Except for sensorineural deafness, all these symptoms belong to the phenotype of a number of other CDG (Freeze, H.H. 2006, Grunewald, S. 2007, Jaeken, J. and Matthijs, G. 2007). Sensorineural deafness has not been reported as a regular feature in any CDG type, although some 40 different types have been described. In fact, it has been reported only rarely in CDG: in 1 patient with ALG12-CDG (*CDG-Ig*) (Kranz, C., *et al.* 2007), in 1 of 10 patients with ATP6V0A2-CDG (cutis laxa type II) (Morava, E., *et al.* 2008), in a few patients with severe PMM2-CDG (*CDG-Ia*), the most frequent CDG (Imtiaz, F., *et al.* 2000, Kjaergaard, S., *et al.* 2001) and in one unsolved case (Hutchesson, A.C., *et al.* 1995). Deafness was not consistent between siblings affected with PMM2-CDG. There is little doubt that this is (mostly) a chance association since deafness is a relatively common disorder; about 1 in 800 children are born with serious permanent hearing impairment (Steel, K.P. and Kros, C.J. 2001).

The fact that none of the other CDG is systematically associated with hearing loss, may suggest that glycosylation is not particularly important for hearing, and/or that RFT1 may have a specific otoneurological function. Alternatively, the cytoplasmic accumulation of Dol-PP-$GlcNAc_2Man_5$ or of a derivative of this compound may have an otoneurotoxic effect.

In conclusion, these data indicate that hearing loss belongs to the phenotype of RFT1-CDG which, in turn, is the first CDG firmly associated with deafness in humans. Mice with mutated LARGE, coding for a putative glycosyltransferase, show besides a neuromuscular phenotype also sensorineural deafness (Grewal, P.K., *et al.* 2001). However, the only reported human patient with mutated LARGE did not have sensorineural deafness (Longman, C., *et al.* 2003). With this novel symptom added to the already impressive list of signs and symptoms, CDG comes closely to: “It’s all in it” (Jaeken, J. 2003). CDG should be included in the work-up of congenital, particularly syndromic, hearing loss. In unexplained, isolated deafness, on the other hand, the possibility should be considered of an (as yet to be identified) defect in otospecific glycosylation.

C3

IDENTIFICATION OF PHOSPHORYLATED OLIGOSACCHARIDES IN THE CELLS OF CDG-I PATIENTS

Abstract

Protein N-glycosylation is initiated by the dolichol cycle in which the oligosaccharide precursor $Glc_3Man_9GlcNAc_2$-PP-dolichol is assembled in the endoplasmic reticulum (ER). One critical step in the dolichol cycle concerns the availability of Dol-P at the cytosolic face of the ER membrane. In RFT1-deficient patient's cells, the lipid-linked oligosaccharide (LLO) intermediate $Man_5GlcNAc_2$-PP-Dol accumulates at the cytosolic face of the ER membrane. Since Dol-P is a rate-limiting intermediate during protein N-glycosylation, continuous accumulation of $Man_5GlcNAc_2$-PP-Dol would block the dolichol cycle. Hence, we investigated the molecular mechanisms by which accumulating $Man_5GlcNAc_2$-PP-Dol could be catabolized in RFT1-deficient patient's cells. On the basis of metabolic labelling experiments and in comparison to human control cells, we identified phosphorylated oligosaccharides (POS) and demonstrated that they originated from the accumulating LLO intermediates. In addition, POS were also detected in other CDG patients' cells accumulating specific LLO intermediates at different cellular locations. Moreover, the enzymatic activity that hydrolyses oligosaccharide-PP-Dol into POS was identified in human microsomal membranes and required Mn^{2+} for optimal activity. In CDG patients' cells, we thus identified and characterized POS that could result from the catabolism of accumulating LLO intermediates.

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Identification of phosphorylated oligosaccharides in cells of patients with a Congenital Disorders of Glycosylation (CDG-I)

Unpublished data

Introduction

The endoplasmic reticulum (ER) is a sub-cellular compartment essential for the synthesis and modification of proteins and lipids. One of the most common protein modification in eukaryotic cells is the glycosylation on asparagine residues, also called N-glycosylation (Helenius, A. and Aebi, M. 2004).

Protein N-glycosylation can be divided into two main phases and starts with the assembly of an oligosaccharide precursor $Glc_3Man_9GlcNAc_2$ on dolichol pyrophosphate (Dol-PP). This biosynthetic process, called the dolichol cycle, is initiated at the cytosolic face of the ER membrane with the elongation of Dol-P to Dol-PP-$GlcNAc_2$ using UDP-GlcNAc and the additional transfer of five mannose residues from GDP-Man (Burda, P. and Aebi, M. 1999). Once formed, the lipid-linked oligosaccharide (LLO) intermediate Dol-PP-$GlcNAc_2Man_5$ is translocated to the luminal face of the ER membrane. Aebi and co-workers showed that this reaction was catalyzed by the RFT1 protein (Helenius, J., *et al.* 2002), but recent studies however suggested that RFT1 is not the flippase itself but plays an important accessory role in the translocation reaction (Rush, J.S., *et al.* 2009, Sanyal, S., *et al.* 2008). Following translocation, four additional mannose and three glucose residues are added from Dol-P-Man and Dol-P-Glc to build the complete oligosaccharide precursor Dol-PP-$GlcNAc_2Man_9Glc_3$ (Burda, P. and Aebi, M. 1999). In a second phase, the fully assembled $Glc_3Man_9GlcNAc_2$ is cotranslationally transferred onto newly synthesized proteins. This reaction, catalyzed by the oligosaccharyltransferase (OST) complex, results in the formation of an N-glycoprotein and the release of Dol-PP (Kelleher, D.J. and Gilmore, R. 2006).

One critical step in the dolichol cycle is the availability of Dol-P at the cytosolic face of the ER membrane: the synthesis of one N-glycan requires eight Dol-P molecules. Besides de novo synthesis, a substantial amount of the Dol-P pool relies on the recycling of luminally oriented Dol-P and Dol-PP. Dol-PP, released at the luminal face of the ER membrane by the OST complex, is converted into Dol-P by the Dol-PP phosphatase named CWH8 in yeast and DolPP1 in mammalian cells (Fernandez, F., *et al.* 2001, Rush, J.S., *et al.* 2002). This Dol-P as well as the Dol-Ps released in the ER lumen by mannosyl- and glucosyltransferases are translocated back to the cytosolic face of the ER membrane in order to reinitiate another round of the dolichol cycle (Rush, J.S., *et al.* 2008).

Congenital Disorders of Glycosylation (CDG) represent a group of inherited human disorders characterized by deficient protein glycosylation. One subgroup of CDG patients (known as CDG-I) is deficient in oligosaccharide precursor assembly, leading to the accumulation of LLO intermediates and causing protein hypoglycosylation (Freeze, H.H. 2006, Jaeken, J. and Matthijs, G. 2007). Since Dol-P is a rate limiting intermediate during protein N-glycosylation, continuous accumulation of LLO intermediates would block the

dolichol cycle causing apoptosis and cell death in the patient (Rosenwald, A.G., *et al.* 1990). In CDG-I patients' cells, blocking of the dolichol cycle is thus prevented by a mechanism assuring the catabolism and recycling of accumulating LLO.

How are the accumulating LLO intermediates catabolized in RFT1-deficient patient's cells? In these cells, we identified phosphorylated oligosaccharides (POS) bearing a $Man_5GlcNAc_2$ structure and originating from the accumulating LLO intermediates. In addition, POS formation was not restricted to RFT1 deficiency. We also identified POS in other CDG-I patients' cells accumulating different LLO intermediates. Moreover, a pyrophosphatase activity was identified in human microsomal membranes.

Materials and Methods

Patients and cells

Primary skin fibroblasts from healthy controls and CDG-I patients were cultured at 37 °C under 5% CO_2 in DMEM/F12 (Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum (Clone III, HyClones, Utah, USA).

The first RFT1-deficient (RFT1-CDG) patient was homozygous for the p.R67C mutation, while the second was homozygous for the missense mutation p.E298K. The third RFT1-deficient patient was homozygous for the conversion of lysine into glutamic acid at position 152 on the protein level (p.K152E). The PMM2-CDG (*CDG-Ia*) patient was compound heterozygous for the p.R141H and p.F119L mutations in *PMM2*. The ALG6-CDG (*CDG-Ic*) patient was compound heterozygous for the missense mutations p.A333V and p.R18Q in *ALG6*. The DPM1-CDG (*CDG-Ie*) patient was compound heterozygous for the missense mutation p.R92G and the deletion p.G111LfsX45 in *DPM1*. The MPDU1-CDG (CDG-If) patient was homozygous for the missense mutation p.G73E in *MPDU1*. The ALG12-CDG (*CDG-Ig*) patient was compound heterozygous for the mutations p.T67M and p.V284X in *ALG12*. The ALG2-CDG (*CDG-I i*) patient was compound heterozygous for the mutations p.G393T and p.G347VfsX27 in *ALG2*. The ALG9-CDG (*CDG-IL*) patient was homozygous for the missense mutation p.Y286C in *ALG9*.

Research on patients' cells was prospectively reviewed and approved by the Ethics Committee of the University of Leuven.

Metabolic labelling

See 'Materials and Methods' in section A1, page 40.

Structural analysis of neutral oligosaccharides

Free oligosaccharide fractions, obtained at the end of the sequential extraction of oligosaccharide material, were desalted on Bio-Gel P2 columns and eluted with 5% acetic acid. The oligosaccharides were separated by HPLC on a polymer based amino column (Asahipak NH_2P-50 250 mm x 4.6 mm; Asahi, Kawasaki-ku, Japan) applying a gradient of acetonitrile/H_2O ranging from 70:30 to 50:50 over 90 min at a flow rate of 1 ml/min. Oligosaccharides were identified on the basis of their retention times compared to standard glycans (Foulquier, F., *et al.* 2002). Elution of the radiolabelled oligosaccharides was monitored by continuous β-counting with a flo-one β detector (Packard, Les Ullis, France).

Ion exchange chromatography

After desalting on Bio-Gel P2 columns, free oligosaccharides were dissolved in 10 mM Tris/HCl pH 7.4 and passed through a 3 ml column of QAE-Sephadex equilibrated in 10 mM Tris/HCl pH 7.4. POS were eluted stepwise with increasing concentrations of NaCl, ranging from 20 to 100 mM, in 10 mM Tris/HCl. [^{14}C]Glc-1-P, used for co-loading, was purchased from Amersham Biosciences (UK). When comparing the percentages of charged oligosaccharides in the free oligosaccharide fraction of different CDG patients, these percentages were normalized according to the number of mannose residues in the oligosaccharide structure.

Analysis of phosphorylated oligosaccharides

Alkaline phosphatase digestion of POS (eluted from the QAE-Sephadex column) was performed overnight at 37°C in water using 20 U of alkaline phosphatase from Calf intestine (Roche Diagnostics, Indianapolis, USA). After alkaline phosphatase digestion, structural analysis of POS was performed by HPLC as described above.

Thin layer chromatography

After desalting on Bio-Gel P2 columns, the free oligosaccharide fractions were collected, evaporated and dissolved in PBS pH 7,4. Subsequently, thin layer chromatography (TLC) (Merck, Nottingham, UK) was performed in n-butanol/ethanol/acetic acid/pyridine/water (10/100/3/10/30; v/v/v/v/v) for two times 2 hours and the identification of the oligosaccharide species was carried out by scraping equal pieces of the TLC plate for direct scintillation counting. Inorganic pyrophosphatase (Sigma, St. Louis, MO, US) treatment was performed overnight at 37°C in presence of 5U inorganic pyrophosphatase and 4mM of $ZnCl_2$. Alkaline phosphatase digestion was performed as described above. To test the specificity of the inorganic pyrophosphatase used, AMP (Sigma) and ADP (Sigma) were

digested as described above and subsequently applied on TLC according to the above described method. In contrast to the free oligosaccharides, AMP and ADP were detected using 0,1% orcinol in 20% H_2SO_4 and subsequent heating at 100-110°C for 10 minutes.

Pyrophosphatase activity measurement

B3F7 cells (*CHO* cells deficient in DPM1) and *RFT1* deficient patient cells were radiolabelled and the accumulating LLO intermediate $Man_5GlcNaAc_2$-PP-Dol was extracted as described above. Approx. 100000 dpm of labelled $Man_5GlcNaAc_2$-PP-Dol was dissolved in 70 µl of 1 % Triton X-114 in 20 mM Tris/HCI pH 7.4; 154 mM NaCl; 1mM $MnCl_2$ and mixed with 30 µl of human microsomes or cytosol (20mg protein/ml) (BD Biosciences, Erembodegem, Belgium). To inhibit dephosphorylation by phosphatases, 5.5 µl of the phosphatase inhibitor cocktail set II (Calbiochem, San Diego, California, USA) was added to the mixture. This mixture was subsequently incubated overnight at 35 °C and oligosaccharides were extracted according to the phase-separation technique previously described (Belard, M., *et al.* 1988).

Results

In most CDG-I patients' cells, the accumulating LLO intermediates have a luminal orientation and their transfer onto proteins has been repeatedly reported (Chantret, I., *et al.* 2003, Chantret, I., *et al.* 2002, Frank, C.G., *et al.* 2004, Grubenmann, C.E., *et al.* 2002, Korner, C., *et al.* 1999, Kranz, C., *et al.* 2001). However, in RFT1-deficient cells, the LLO intermediate Dol-PP-$GlcNAc_2Man_5$ accumulates at the cytosolic face of the ER membrane and can therefore not be transferred onto newly synthesized proteins (Haeuptle, M.A., *et al.* 2008, Helenius, J., *et al.* 2002). The molecular mechanisms by which accumulating LLO intermediates are catabolized in RFT1-deficient patient's cells are unknown. Nevertheless, three plausible mechanisms can be envisaged: 1) the conversion of accumulating LLO intermediates into free, neutral oligosaccharides, 2) the formation of free, monophosphorylated oligosaccharides and 3) the formation of free, pyrophosphorylated oligosaccharides (Figure 1). To discriminate between these possibilities, the level and structure of neutral oligosaccharides were first investigated.

Neutral oligosaccharide analysis

In comparison to control cells, metabolic labelling of RFT1-deficient cells showed a slightly increased level of free oligosaccharides. In control cells, 7% (+/-0.5; n=3) of total radioactivity incorporated into the free oligosaccharide fraction, while 9.8% (+/- 0.8; n=5) of radioactivity was found in the free oligosaccharides of RFT1-deficient cells (data not shown). To investigate whether the conversion of accumulating LLO intermediates into free, neutral

oligosaccharides could account for this difference, structural analysis of the neutral oligosaccharides was carried out. In both control and RFT1-deficient cells, $Man_9GlcNAc_{1/2}$, $Man_8GlcNAc_{1/2}$ and $Man_5GlcNAc_1$ structures were observed (Figure 2). Surprisingly, neither $Man_5GlcNAc_2$ nor a substantial increase in $Man_5GlcNAc_1$ were detected in the RFT1-deficient cells. This result strongly suggested that neutral oligosaccharide formation could not account for the catabolism of accumulating Dol-PP-$GlcNAc_2Man_5$ in RFT1-CDG cells.

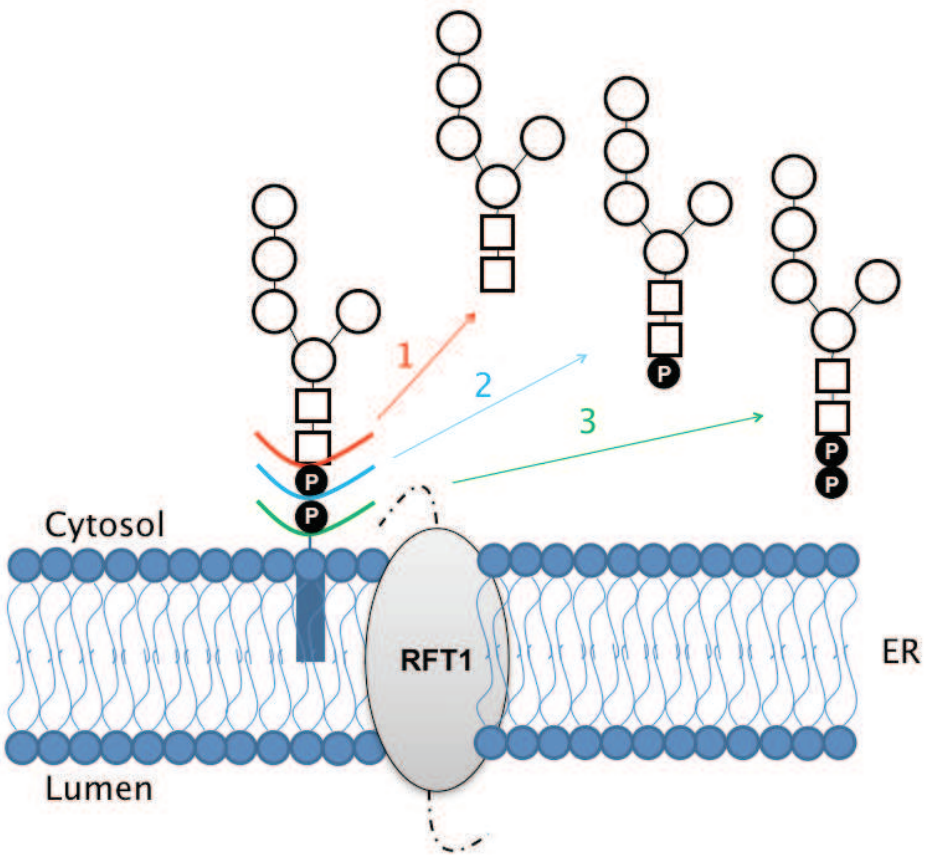

Figure 1: Three potential mechanisms for the catabolism of accumulating LLO in RFT1-deficient cells: 1) formation of free, neutral oligosaccharides, 2) formation of free, monophosphorylated oligosaccharides and 3) formation of free, pyrophosphorylated oligosaccharides.

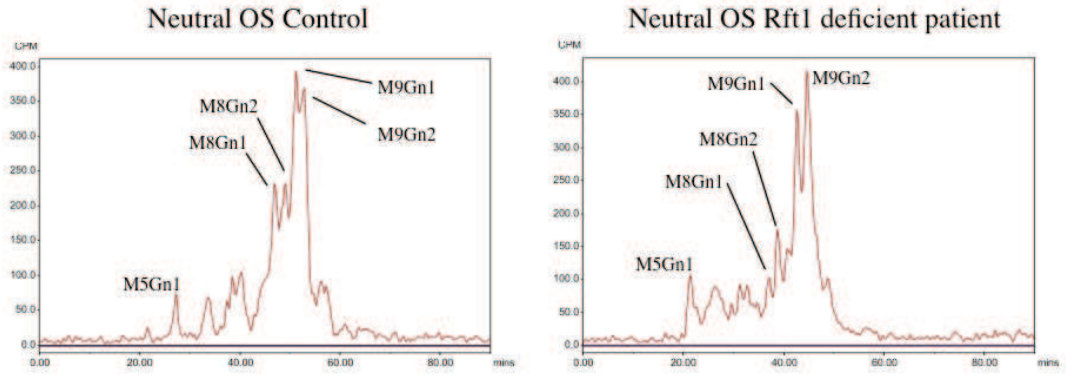

Figure 2: Structural analysis of the neutral oligosaccharides by HPLC after metabolic labelling in control and RFT1-deficient cells. Symbols: M5-9Gn1: $Man_{5\text{-}9}GlcNAc_1$ and M8-9Gn2: $Man_{8\text{-}9}GlcNAc_2$.

Phosphorylated oligosaccharide analysis

Another hypothesis for the fate of accumulating LLO intermediates was the formation of free, phosphorylated oligosaccharide structures (Figure 1, hypothesis 2 and 3). To confirm these hypotheses, ion exchange chromatography of free oligosaccharides formed in control and RFT1-deficient cells was performed. In control cells, only 1.4% (+/- 0.2, n=4) of the free oligosaccharides consisted of charged oligosaccharides, while 23.3% (+/- 2.0; n=3) of the free oligosaccharides were charged in the RFT1 deficient cells (Figure 3A).

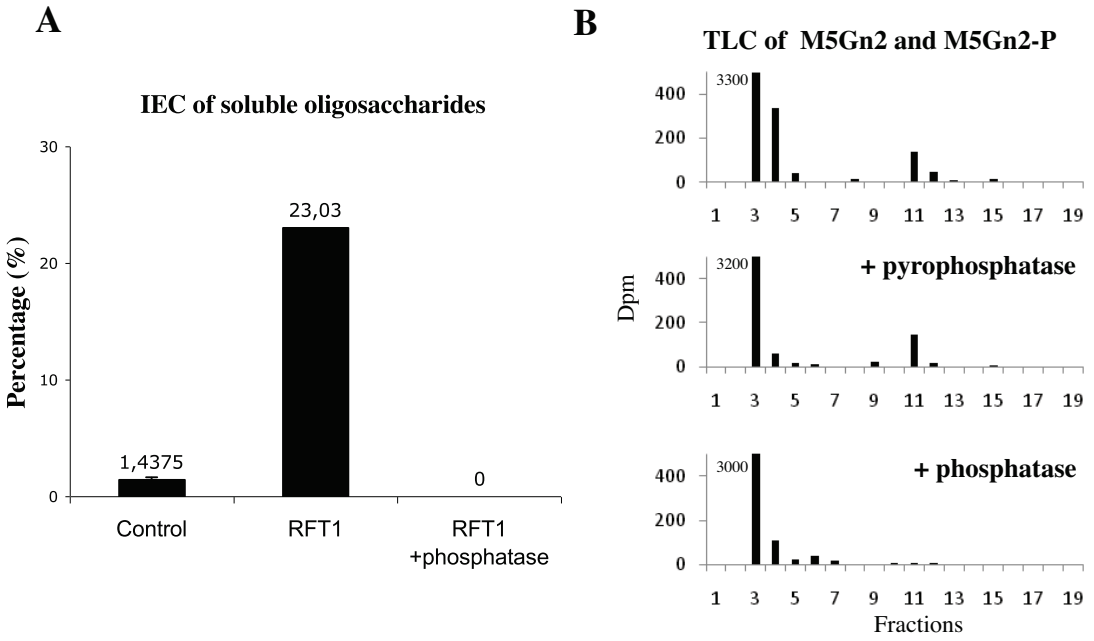

Figure 3: (A) Ion exchange chromatography of free oligosaccharides in control and RFT1-deficient cells after metabolic labelling. The percentage of charged oligosaccharides in comparison to the total amount of free oligosaccharides is shown. Alkaline phosphatase treatment of the charged oligosaccharides resulted in the release of the charge. **(B)** Thin layer chromatography of neutral and phosphorylated oligosaccharides without treatment (upper panel), after inorganic pyrophosphatase treatment (middle panel) and after alkaline phosphatase treatment (lower panel). Symbols: M5Gn2: $Man_5GlcNAc_2$ and M5Gn2-P or PP: $Man_5GlcNAc_2$-P or $Man_5GlcNAc_2$-PP.

To confirm the charged nature of these free oligosaccharides, an *in vitro* alkaline phosphatase treatment was performed and the products were subsequently reanalyzed by ion exchange chromatography. As shown in Figure 3A, no charge was observed after alkaline phosphatase treatment, indicating that the oligosaccharide species were indeed phosphorylated. However, no distinction could be made between monophosphorylated and pyrophosphorylated species. Hence, co-loading of the phosphorylated oligosaccharides and [^{14}C]Glc-1-P on ion exchange chromatography was performed, revealing a co-elution of both species (data not shown). Moreover, a mixture of neutral and phosphorylated oligosaccharides was subjected to a treatment with an inorganic pyrophosphatase, unable to hydrolyze monophosphorylated

species. The specificity of the inorganic pyrophosphatase was tested using AMP and ADP (data not shown). Thin layer chromatography of the free oligosaccharide mixture without treatment revealed two peaks, corresponding to the neutral and phosphorylated oligosaccharides. In presence of inorganic pyrophosphatase, neither changes in the level of phosphorylated oligosaccharides nor the appearance of another peak was observed (Figure 3B). However, alkaline phosphatase treatment clearly resulted in the disappearance of the phosphorylated oligosaccharides. These results therefore demonstrated that the phosphorylated oligosaccharides bear only one phosphate, confirming our second hypothesis (Figure 1).

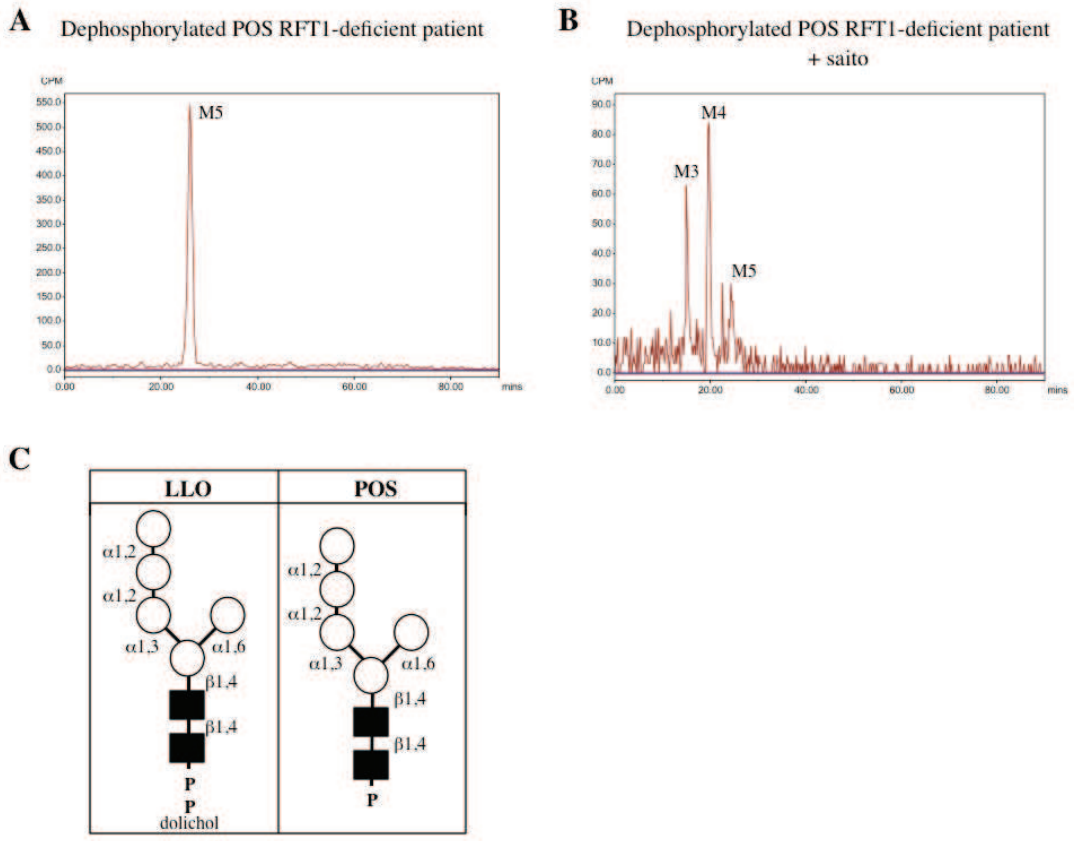

Figure 4: (**A**) HPLC analysis of the charged oligosaccharides after metabolic labelling and alkaline phosphatase treatment in RFT1-deficient cells. **(B)** Profile A after α1,2-mannosidase treatment. **(C)** Structure of the LLO intermediate accumulating in RFT1-deficient cells compared to the structure of POS formed in this patient. Symbols: M5: $Man_5GlcNAc_2$.

To further characterize the glycan structure of these monophosphorylated oligosaccharides (POS), HPLC analysis was performed after dephosphorylation and revealed solely a $Man_5GlcNAc_2$ structure (Figure 4A). Two different $Man_5GlcNAc_2$ structures exist: the linear $Man_5GlcNAc_2$, formed during oligosaccharide precursor assembly and the branched $Man_5GlcNAc_2$, formed in the Golgi compartment by demannosylation of $Man_9GlcNAc_2$ linked to proteins. To discriminate between both possibilities, treatment of these POS with the

α1,2-mannosidase saito was performed. An increase in $Man_3GlcNAc_2$ and $Man_4GlcNAc_2$, concomitant with a decrease in $Man_5GlcNAc_2$, was observed (Figure 4B). This result demonstrated that the POS formed in the RFT1-deficient cells corresponded to the linear $Man_5GlcNAc_2$-PP-Dol structure found on LLO (Figure 4C).

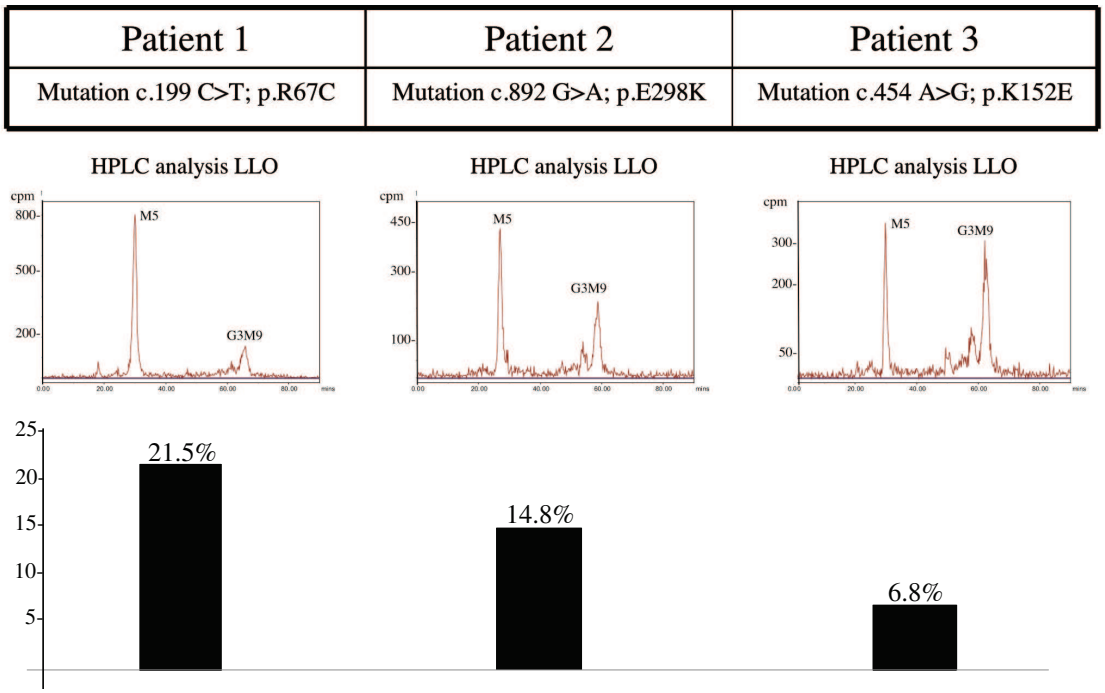

Figure 5: Comparison of POS formation in three RFT1-deficient patients' cells harbouring different RFT1 mutations and accumulating different levels of $Man_5GlcNAc_2$-PP-Dol on LLO. The percentage of POS in comparison to the total amount of free oligosaccharides is shown. Patient one is homozygous for the p.R67C mutation in RFT1, while patient two is homozygous for the missense mutation p.E298K. Patient three is homozygous for the conversion of lysine into glutamic acid at position 152 on the protein level (p.K152E). Symbols: M5: $Man_5GlcNAc_2$-PP-Dol and G3M9: $Glc_3Man_9GlcNAc_2$-PP-Dol.

Since the glycan structure of LLO and POS were identical, we hypothesized that the POS formed in the RFT1-deficient cells originated from the accumulating LLO intermediate. To demonstrate this, we compared the POS levels in three different RFT1-deficient patients' cells harboring different RFT1 mutations and accumulating different levels of $Man_5GlcNac_2$-PP-Dol on LLO. A strong correlation between $Man_5GlcNAc_2$-PP-Dol accumulation and the level of POS formation was observed (Figure 5). The highest amount of POS was formed in the patient's cells accumulating the highest level of $Man_5GlcNAc_2$-PP-Dol, while the patient with the smallest accumulation of $Man_5GlcNAc_2$-PP-Dol formed only a low amount of POS (Figure 5). In addition, a pulse chase experiment confirmed that the POS originated from the LLO, while the neutral oligosaccharides are the degradation products of the misfolded glycoproteins (data not shown).

To further address the fate of the POS, a Folch extraction on the medium of RFT1-deficient patient's cells was performed after metabolic labelling. However, no detectable amount of free oligosaccharides, neither neutral nor phosphorylated, could be found (data not shown).

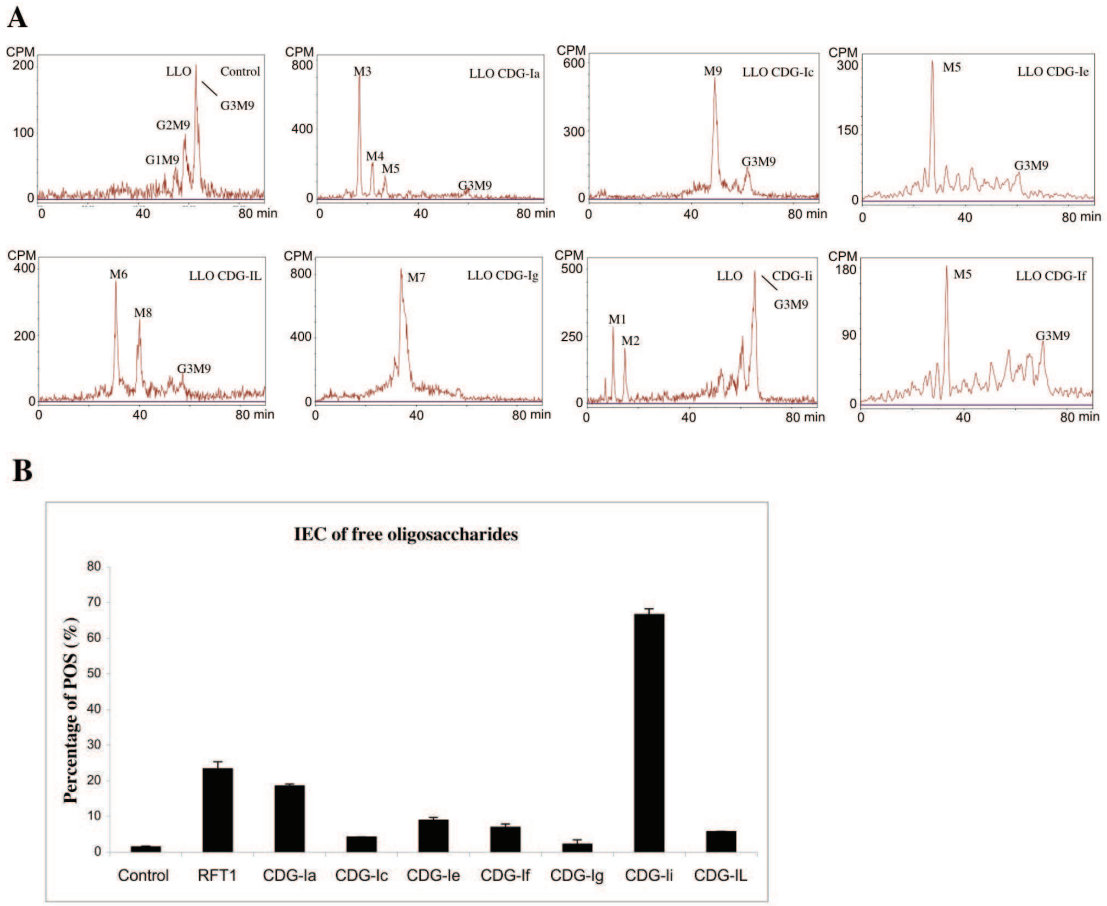

Figure 6: (A) HPLC analysis of the LLO formed in eight different CDG-I patients' cells. **(B)** Ion exchange chromatography of the free oligosaccharides in these patients' cells revealed a various amount of POS. The values were normalized according to the number of mannose residues in the oligosaccharide structure. Symbols: G1-3M9 on LLO: $Glc_{1-3}Man_9GlcNAc_2$-PP-Dol; M1-9 on LLO: $Man_{1-9}GlcNAc_2$-PP-Dol; G1M5-9 on glycoproteins: $Glc_1Man_{5-9}GlcNAc_2$ and M4-9 on glycoproteins: $Man_{4-9}GlcNAc_2$.

POS formation in other CDG-I patients

To determine whether POS formation was specific for RFT1 deficiency, POS levels were analyzed in other CDG-I patients' cells accumulating different LLO intermediates. For eight different CDG-I patients' cells, LLO structures were analyzed after metabolic labelling. As previously reported and as shown in Figure 6A, HPLC analysis of LLO revealed mainly the fully assembled oligosaccharide precursor $Glc_3Man_9GlcNAc_2$-PP-Dol in control cells. CDG-Ia (PMM2-CDG) patient's cells accumulated $Man_3GlcNAc_2$-PP-Dol on LLO, while CDG-Ic (ALG6-CDG) cells accumulated the LLO intermediate $Man_9GlcNAc_2$-PP-Dol. CDG-Ie (DPM1-CDG) and CDG-If (MPDU1-CDG) patients' cells both accumulated $Man_5GlcNAc_2$-

PP-Dol in the ER lumen and CDG-Ig (ALG12-CDG) patient's cells accumulated $Man_7GlcNAc_2$-PP-Dol on LLO. Finally, CDG-Ii (ALG2-CDG) patient's cells accumulated the small LLO intermediates $Man_1GlcNAc_2$-PP-Dol and $Man_2GlcNAc_2$-PP-Dol, while CDG-IL (ALG9-CDG) cells accumulated $Man_6GlcNAc_2$-PP-Dol and $Man_8GlcNAc_2$-PP-Dol.

In all tested CDG-I patients' cells, POS were detected but their amounts varied according to the deficiency (Figure 6B). Moreover, structural analysis of the POS formed in four selected patients' cells revealed a $Man_3GlcNAc_2$-P structure in CDG-Ia patient's cells, $Man_1GlcNAc_2$-P and $Man_2GlcNAc_2$-P structures in CDG-Ii patient's cells, a $Man_6GlcNAc_2$-P structure in CDG-IL patient's cells and CDG-Ig patients formed POS bearing a $Man_7GlcNAc_2$-P structure (Figure 7). This result again pointed to the conversion of accumulating LLO into POS.

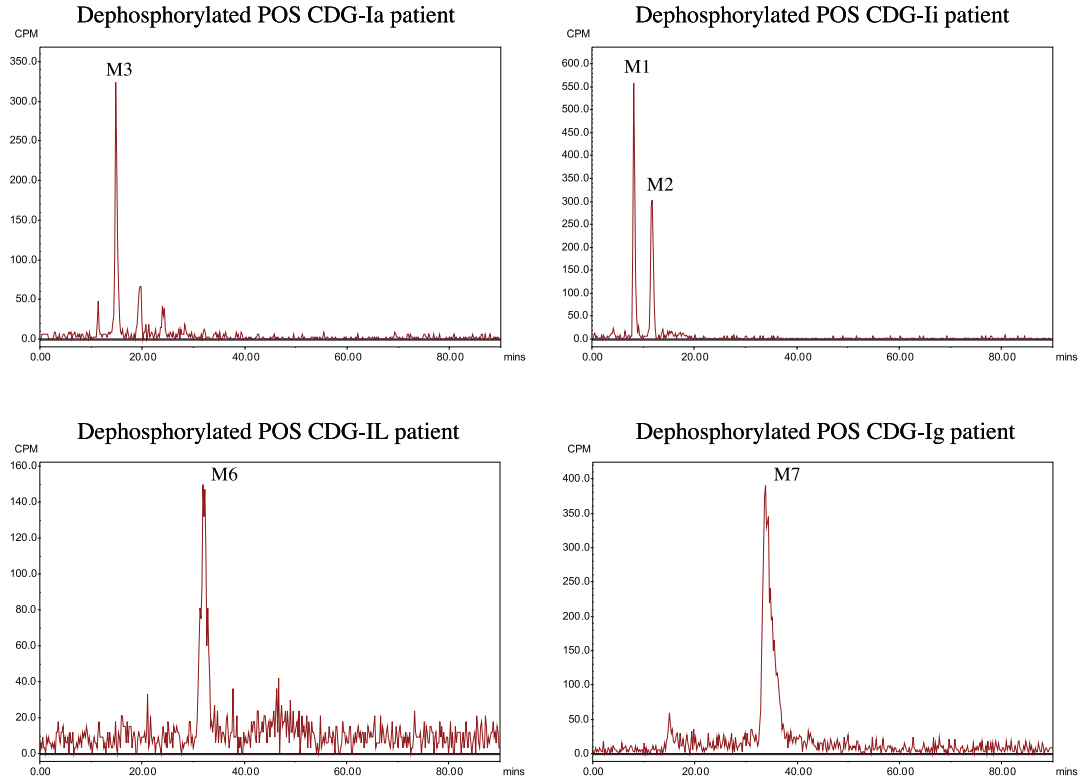

Figure 7: HPLC analysis of the dephosphorylated POS formed in CDG-Ia, CDG-Ii, CDG-IL and CDG-Ig patients' cells. Symbols: M1-7: $Man_{1-7}GlcNAc_2$.

Enzymatic activity measurement

To further characterize the enzymatic activity responsible for POS formation in human cells, an *in vitro* assay was performed using human cytosol and microsomes as described in the materials and methods. For this purpose, labelled $Man_5GlcNAc_2$-PP-Dol, prepared from the *CHO* cell line *B3F7*, were incubated in presence of either human cytosol or human microsomes. Free oligosaccharides were then extracted according to the phase-separation

technique described by Belard and co-workers in 1988. In presence of human microsomes, 74.45% (+/- 7.0; n=2) of the labelled LLO was converted into free oligosaccharides after overnight incubation at 35°C (Figure 8A). In contrast, incubation in presence of cytosol resulted in the formation of only 1.93% (+/- 3.35; n=3) of free oligosaccharides. In absence of microsomes and cytosol, representing the negative control, only 1.96% (+/- 0.23; n=2) of free oligosaccharides were formed. Moreover, heat inactivation (15 minutes at 95°C) of the microsomes prior to incubation or incubation at 4°C significantly reduced the free oligosaccharide formation (Figure 8A). The hydrolase required Mn^{2+} ions for its optimal activity, since incubation in absence of 1mM Mn^{2+} resulted in the formation of only 21.3% (+/- 0.15; n=2) of free oligosaccharides (Figure 8A). Moreover, other bivalent cations like Ca^{2+} and Mg^{2+} resulted in the formation of respectively 28.4% and 18% of free oligosaccharides.

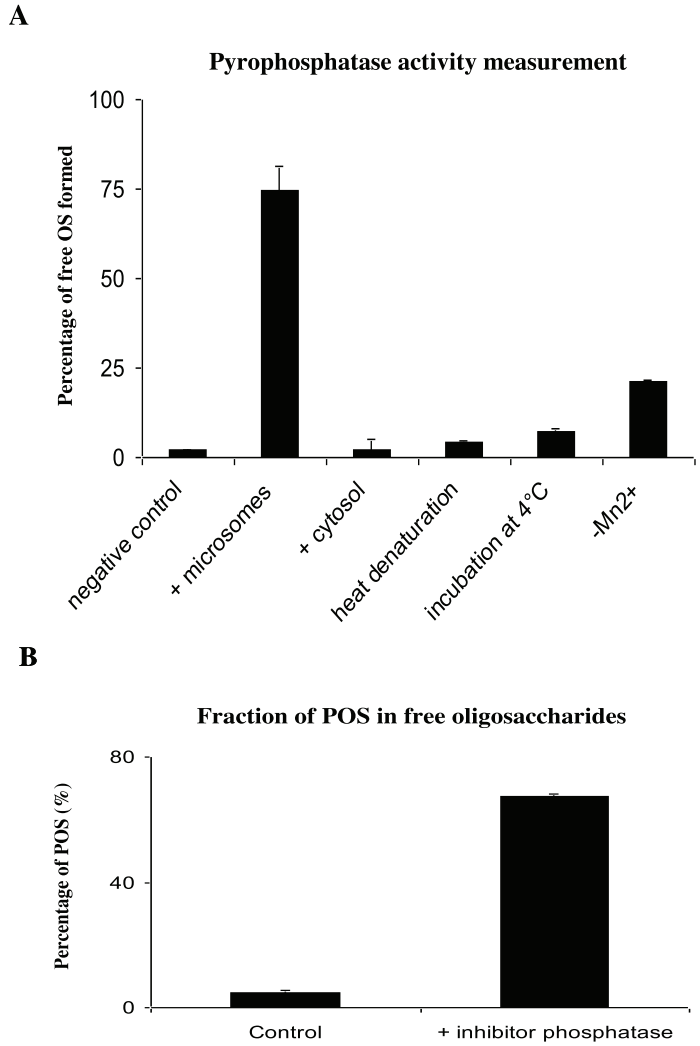

Figure 8: (A) *In vitro* assay measuring the pyrophosphatase activity in the microsomes and cytosol derived from HeLa cells. Incubation in absence of microsomes and cytosol was used as a negative control. **(B)** Only 4.2% of the free oligosaccharides formed during this *in vitro* assay accounted for POS. Incubation in presence of a phosphatase inhibitor cocktail however increased the fraction of POS to 66.8%.

To test whether the released oligosaccharide material corresponded to POS, the free oligosaccharides were submitted to ion exchange chromatography. Surprisingly, the majority of the free oligosaccharides formed consisted of neutral oligosaccharides. Since the observed neutral oligosaccharides could arise from the dephosphorylation of POS species, incubation was performed in presence of a phosphatase inhibitor cocktail. As shown in Figure 8B, this treatment considerably increased the fraction of POS from 4.83% (+/-0.56; n=3) to 67.4% (+/-0.85; n=2), pointing to a quick dephosphorylation of POS. Subsequent alkaline phosphatase treatment abolished the charged nature of the POS formed and further structural analysis by HPLC showed a $Man_5GlcNAc_2$ structure, which is in accordance to the $Man_5GlcNAc_2$-PP-Dol structure initially used.

This assay clearly demonstrated the presence of an enzymatic activity capable of hydrolyzing LLO into phosphorylated oligosaccharides in human microsomal membranes.

Discussion

Since 1970, it was known that the amount of Dol-P presented at the cytosolic face of the ER membrane was a critical factor in N-glycosylation (Carson, D.D., *et al.* 1981, Harford, J.B. and Waechter, C.J. 1980, Harford, J.B., *et al.* 1977, Hubbard, S.C. and Robbins, P.W. 1980, Lucas, J.J. and Levin, E. 1977, Rosenwald, A.G., *et al.* 1990, Rush, J.S., *et al.* 2008, Spiro, M.J. and Spiro, R.G. 1986). To prevent Dol-P depletion in CDG-I patients' cells, they must have a mechanism to assure the recycling of Dol-P. Since RFT1-deficient patient's cells cannot transfer the accumulating Dol-PP-$GlcNAc_2Man_5$ onto proteins, they presented a good model to study the molecular mechanism by which accumulating LLO could be catabolized.

Our first hypothesis was the conversion of accumulating LLO into free, neutral oligosaccharides. Since three decades, protein N-glycosylation has been associated with the production of neutral oligosaccharides and the majority of these oligosaccharides is formed by the degradation of terminally misfolded N-glycoproteins in the cytosol (Anumula, K. and Spiro, R. 1983, Cacan, R., *et al.* 1980, Hanover, J.A., *et al.* 1982, Suzuki, T. and Funakoshi, Y. 2006). However, one group also reported on the formation of neutral oligosaccharides by transfer of the fully assembled oligosaccharide precursor onto water (Spiro, M.J. and Spiro, R.G. 1991). We therefore analyzed the level and structure of free, neutral oligosaccharides in control and RFT1-deficient patient's cells, but no major differences were found. Formation of free, neutral oligosaccharides could therefore not account for the catabolism of accumulating LLO.

On the other hand, several groups reported on the identification of free, charged oligosaccharides in mammalian cell lines, mainly *in vitro* (Cacan, R., *et al.* 1987, Cacan, R., *et al.* 1980, Cacan, R., *et al.* 1992, Hoflack, B., *et al.* 1981, Hsu, A.F., *et al.* 1974, Oliver, G.J.,

et al. 1975). These free, phosphorylated oligosaccharides were never observed in human cells and, although still controversial, it was suggested that they originated from the LLO by the action of a pyrophosphatase activity located in the ER membrane (Belard, M., *et al.* 1988, Cacan, R., *et al.* 1992). Nevertheless, the physiological significance of POS formation remained to be determined. In our study, POS were for the first time identified, *in vivo*, in human cells, more specifically in the RFT1-deficient patient's cells. In addition, we showed that the structure of these POS reflected the structure of accumulating LLO. A study of three RFT1 deficient patients' cells accumulating different levels of Dol-PP-$GlcNAc_2Man_5$ showed that these POS indeed originated from the accumulating LLO intermediate.

In Figure 9A, we propose a model for POS formation in RFT1 deficient patients' cells. In these cells, the pyrophosphate linkage of Dol-PP-$GlcNAc_2Man_5$ accumulating at the cytosolic face of the ER membrane is cleaved by a potential pyrophosphatase activity. This reaction results in the release of Dol-P and free $Man_5GlcNAc_2$-P. Dol-P could be recycled for another round of the dolichol cycle, but what is the fate of the POS? Since POS (or their dephosphorylated form) were not detected in the medium of RFT1-deficient patient's cells, they could be further degraded in the lysosomes, just like the free, neutral oligosaccharides formed by the degradation of misfolded glycoproteins (Moore, S. and Spiro, R. 1994). Since the transporter specific for the translocation of these free, neutral oligosaccharides cannot translocate phosphorylated oligosaccharides (Saint-Pol, A., *et al.* 1999), POS should be dephosphorylated prior to translocation.

Interestingly, Rft1 depletion in yeast resulted in a 30-fold increase in the amount of LLO in comparison to control cells (Rush, J.S., *et al.* 2009). However, we never observed such a huge accumulation of LLO in human RFT1-deficient patient's cells. During evolution, human cells could have developed a mechanism, the formation of POS, to catabolize accumulating LLO in order to prevent dolichol cycle blocking.

POS formation was however not restricted to RFT1 deficiency. We also detected POS in other CDG-I patients' cells accumulating different LLO intermediates. As shown in Figure 9B, two plausible mechanisms can be envisaged for the formation of POS derived from LLO accumulating in the ER lumen. The first mechanism concerns the formation of POS by a pyrophosphatase activity located in the ER lumen and the second one involves the translocation of accumulating LLO intermediates from the ER lumen to the cytosol where POS could subsequently be formed and Dol-P recycled (Figure 9B). Since unassisted translocation of LLO across the membrane bilayer is energetically unfavorable and extremely slow (Hanover, J.A. and Lennarz, W.J. 1979, McCloskey, M.A. and Troy, F.A. 1980), we propose that this action is catalyzed by a flippase, possibly the RFT1 flippase. In addition to Dol-PP-$GlcNAc_2Man_5$ translocation, the RFT1 flippase is also

found to facilitate the flip-flop of Dol-PP-$GlcNAc_2Man_3$, although at reduced rate (Helenius, J., *et al.* 2002). However, another flippase specific for the translocation of accumulating LLO may also exist. Because flippase activity remains difficult to measure, extensive studies on the identification of other flippases are lacking.

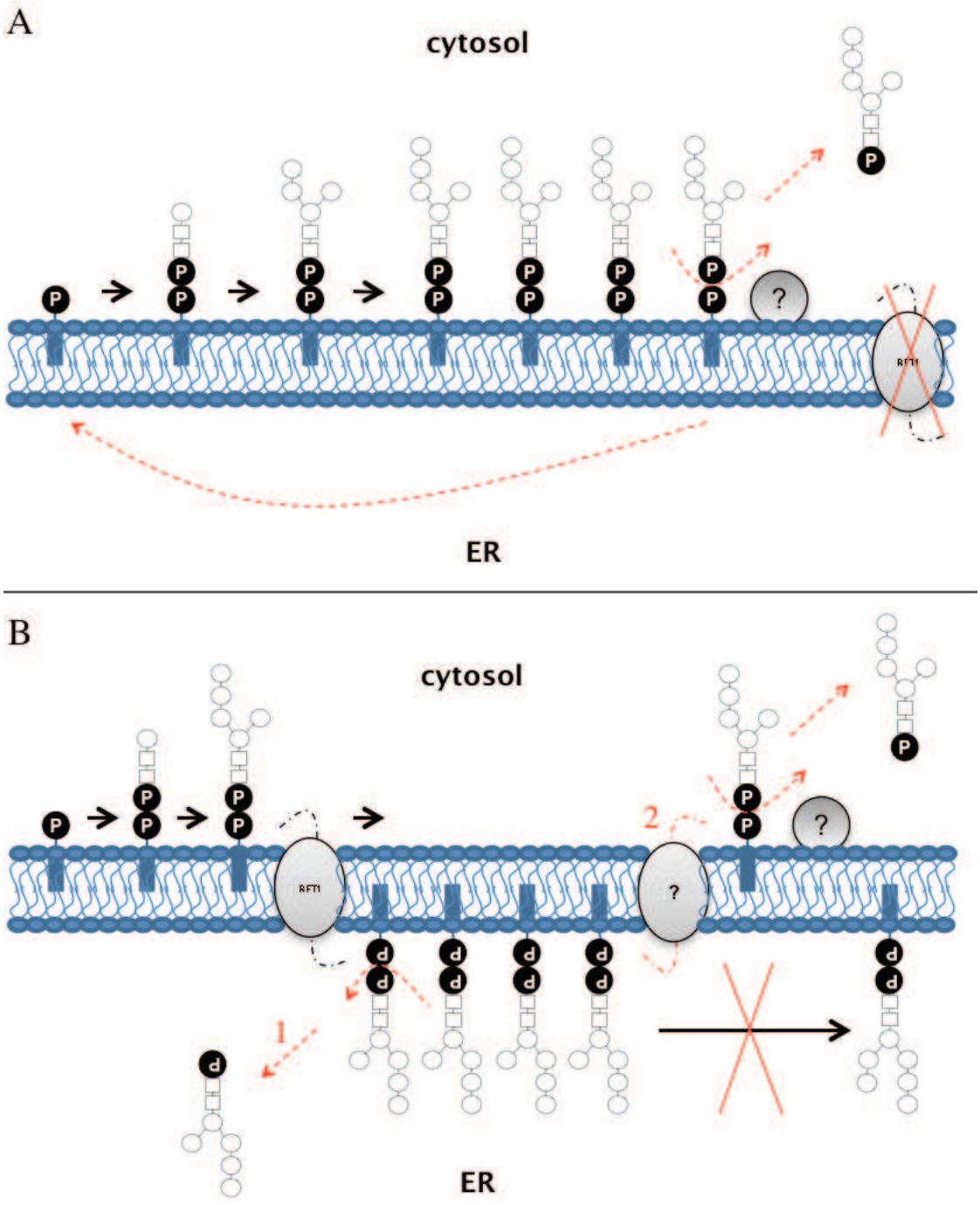

Figure 9: Model representing the conversion of accumulating LLO, located either at the cytosolic **(A)** or luminal face **(B)** of the ER membrane, into POS by a potential ER membrane localized pyrophosphatase activity.

In the model shown in Figure 9, we propose that a potential pyrophosphatase activity catalyzes the cleavage of the pyrophosphate bound. A pyrophosphatase activity that uses LLO

intermediates as a substrate has formerly been detected in the microsomes of yeast (Belard, M., *et al.* 1988). In the present study, we detected a similar pyrophosphatase activity in human microsomes, presumably pointing to a location in the ER membrane, and showed in addition that it depended on Mn^{2+} for its optimal catalytic activity. Since the POS structures identified in different CDG-I patients' cells ranged from $Man_1GlcNAc_2$ to $Man_7GlcNAc_2$, we can assume that the pyrophosphophosphatase activity is not specific for a certain oligosaccharide structure. The human DolPP1 pyrophosphatase, located in the ER membrane and involved in the recycling of Dol-PP released in the ER lumen, could account for the detected pyrophosphatase activity. However, DolPP1 has a luminally-oriented active site (Fernandez, F., *et al.* 2001). Another pyrophosphatase that could account for POS formation is the polyprenylpyrophosphate phosphatase. This pyrophosphatase is involved in the *de novo* synthesis of Dol-P. Unfortunately, only its enzymatic activity has been demonstrated (Adair, W.L., Jr. and Cafmeyer, N. 1983, Kato, S., *et al.* 1980, Scher, M.G. and Waechter, C.J. 1984, Wedgwood, J.F. and Strominger, J.L. 1980). So far, neither a protein accounting for this activity nor its topological orientation has been identified.

In the present study, we identified for the first time POS in CDG-I patients' cells and demonstrated that they originated from the accumulating LLO. All together, this study highly suggests that POS formation would result from a molecular mechanism by which accumulating LLO are catabolized. This mechanism would assure the recycling of Dol-P in order to prevent dolichol cycle blocking. Much additional work is however required to identify the pyrophosphatase and to gain further insight in the mechanisms of dolichol cycle regulation.

Acknowledgements

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C4

A DEFICIENCY IN DPM2 CAUSES A NEW SUBTYPE OF CDG

Abstract

N-glycosylation of proteins is an essential post-translational modification involved in many cellular processes. It is therefore not surprising that a deficiency in N-glycosylation results in a group of severe genetic disorders called Congenital Disorders of Glycosylation (CDG). One subgroup of CDG patients, called CDG type I (CDG-I) patients, have a defect in the early steps of the N-glycosylation process, more specifically in the assembly of the oligosaccharide precursor. In the present study, we identified the molecular defect in an unsolved CDG-I patient accumulating Dol-PP-$GlcNAc_2Man_5$ on lipid-linked oligosaccharides. Genetic screening of the Dol-P-Man synthase subunit *DPM2* revealed that this patient was compound heterozygous for the missense mutation c.68A>G (p.Y23C) and the splice site mutation IVS2-1G>C. The clinical phenotype of the DPM2-deficient patient is characterized by severe developmental delay, hypotonia, postnatal microcephaly and epilepsy. This patient thus shows many similarities with DPM1-CDG but clearly differs from the phenotype of the recently identified DPM3-deficient patient.

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Unpublished data

Introduction

Congenital Disorders of Glycosylation or CDG are a rapidly growing group of genetic diseases due to a deficient biosynthesis of the glycans linked to glycoproteins and/or glycolipids (Eklund, E.A. and Freeze, H.H. 2006, Jaeken, J. and Matthijs, G. 2007). One subgroup of CDG patients is deficient in the initial steps of N-glycoprotein biosynthesis and are called CDG type I (CDG-I) patients (Jaeken, J. and Matthijs, G. 2007). N-glycoprotein biosynthesis is initiated by the assembly of an oligosaccharide precursor ($Glc_3Man_9GlcNAc_2$) on dolichol pyrophosphate (Dol-PP) during a well-defined pathway known as the dolichol cycle (Burda, P. and Aebi, M. 1999). The dolichol cycle starts at the cytosolic face of the ER membrane with the elongation of Dol-P to Dol-PP-$GlcNAc_2Man_5$ using UDP-GlcNAc and GDP-Man, two nucleotide-activated sugar donors. Since the assembly process continues in the lumen of the ER, Dol-PP-$GlcNAc_2Man_5$ needs to be translocated from the cytosolic to the luminal face of the ER membrane. This translocation reaction is facilitated by the RFT1 protein, although it remains unclear whether RFT1 is the flippase itself or plays an important accessory role (Frank, C.G., *et al.* 2008, Helenius, J., *et al.* 2002, Rush, J.S., *et al.* 2009). In the lumen of the ER, three mannosyltransferases (ALG3, ALG9 and ALG12) and three glucosyltransferases (ALG6, ALG8 and ALG10) subsequently catalyze the conversion of Dol-PP-$GlcNAc_2Man_5$ to the complete oligosaccharide precursor. These luminally oriented mannosyl- and glycosyltransferases cannot appeal to the nucleotide-activated sugar donors localized in the cytosol but instead rely on dolichol-linked monosaccharides (Dol-P-Man and Dol-P-Glc). These dolichol-linked monosaccharides are generated from Dol-P and the corresponding nucleotide-activated sugar at the cytosolic face of the ER membrane and are subsequently flipped to the ER lumen by a yet unknown mechanism requiring the MPDU1 protein (Burda, P. and Aebi, M. 1999). Dol-P-Man is synthesized by the Dol-P-Man synthase (DPM). In lower eukaryotes like yeast, DPM consist of only one subunit, the transmembrane protein Dpm1 (Orlean, P., *et al.* 1988). The human homologue DPM1 lacks the transmembrane region and therefore relies on two other transmembrane proteins (DPM2 and DPM3) for its anchoring to the ER membrane (Maeda, Y., *et al.* 2000). DPM1 directly interacts with DPM3 which is on its turn bound to DPM2. In the absence of DPM3, DPM1 is degraded by the proteasome and DPM2 is involved in stabilizing the DPM3 protein (Maeda, Y., *et al.* 2000, Maeda, Y., *et al.* 1998). While both essential for Dol-P-Man synthesis, the exact function of DPM2 and DPM3 remains unknown. Once Dol-PP-$GlcNAc_2Man_9Glc_3$ is completely assembled on the ER membrane, it is transferred onto proteins in a co-translational manner by the oligosaccharyltransferase complex.

Materials and Methods

Mutation analysis

Mutation analysis was performed on DNA either extracted from blood or fibroblasts. Based on the genomic sequence, primers were designed to amplify the different exons of *SAC1* and *DPM2,* including at least 50 bp of the flanking intronic regions. Primer sequences are available on request. The exons were amplified using standard touch down PCR conditions. Sequencing was performed with the Big Dye Terminator Ready reaction cycle sequencing kit V3.1 (Applied Biosystems, California, USA) and sequences were subsequently analyzed on an ABI3130XL.

Cell culture

See 'Materials and Methods' in section A1, page 40.

Metabolic labelling of cells

See 'Materials and Methods' in section A1, page 40.

LLO analysis

See 'Materials and Methods' in section A1, page 40 and 41.

Results

To unravel the molecular defect(s) in a group of unsolved CDG-I (CDG-Ix) patients, we performed structural analysis of the lipid-linked oligosaccharides (LLO) in the patients' fibroblasts. In contrast to the fully assembled oligosaccharide precursor observed in control fibroblasts, fourteen patients accumulated the assembly intermediate Dol-PP-$GlcNAc_2Man_5$ (Figure 1). In nine of them, structural analysis of the protein-bound glycans also revealed an accumulation of $Man_5GlcNAc_2$, pointing to the transfer of Dol-PP-$GlcNAc_2Man_5$ onto proteins and thus excluding a deficiency in the RFT1 protein (see chapter B2.1 and B2.2). An accumulation of (Dol-PP)-$GlcNAc_2Man_5$ on LLO and glycoproteins has already been reported in CDG-I patients with a deficiency in *ALG3* (ALG3-CDG or CDG-Id), *DPM1* (DPM1-CDG or CDG-Ie) and *MPDU1* (MPDU1-CDG or CDG-If) (Imbach, T., *et al.* 2000, Kim, S., *et al.* 2000, Korner, C., *et al.* 1999, Kranz, C., *et al.* 2001). However, sequence analysis of these three genes was normal in all patients. Since a deficiency in DPM1 and very recently also in DPM3 has been associated with a glycosylation disorder (Imbach, T., *et al.* 2000, Kim, S., *et al.* 2000, Lefeber, D.J., *et al.* 2009), the third subunit of the DPM complex, DPM2, was also a good candidate for CDG-I. Moreover, the lipid phosphatase

SAC1 is known to interact with DPM1 and SAC1 deficient cells accumulate the assembly intermediate Dol-PP-GlcNAc$_2$Man$_5$ (Faulhammer, F., *et al.* 2005). Hence, we hypothesized that SAC1 could also be deficient in these nine CDG-Ix patients.

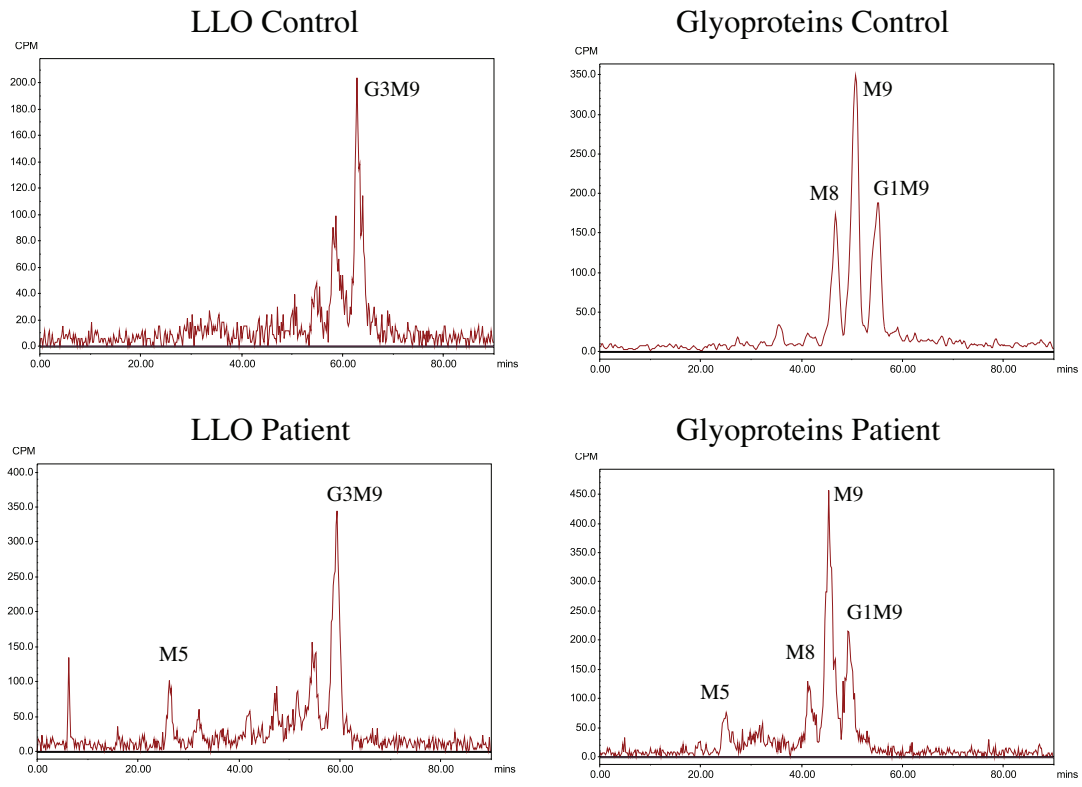

Figure 1: Structural analysis of the LLO and glycoproteins in the fibroblasts of a control and the DPM2-deficient patient. These profiles are similar to the ones observed in the eight other CDG-Ix patients. Symbols: G3M9 on LLO: Dol-PP-GlcNAc$_2$Man$_9$Glc$_3$; M5 on LLO: Dol-PP-GlcNAc$_2$Man$_5$; G1M9 on glycoproteins: Man$_9$GlcNAc$_2$; M5-9 on glycoproteins: Man$_{5\text{-}9}$GlcNAc$_2$.

While the genomic sequencing of *SAC1* did not reveal any mutation, a defect in the DPM2 subunit of the DPM complex was observed in one patient. The corresponding LLO and glycoproteins profiles are shown in figure 1. This patient was compound heterozygous for the missense mutation c.68A>G (p.Y23C) in exon 1 of *DPM2* and the intronic mutation IVS1-1G>C, disrupting the acceptor splice site of exon 2 (Figure 2). The missense mutation was of maternal origin, while the father was heterozygous for the splice site mutation and no mutation was found in the healthy sibling (Figure 2). The tyrosine at position 23 on the protein level is strongly conserved among higher eukaryotes ranging from *Homo sapiens* to *Danio rerio* and *Xenopus tropicalis*, pointing to the pathogenic character of the identified missense mutation (Figure 3). Certainty is however only provided by performing a rescue experiment.

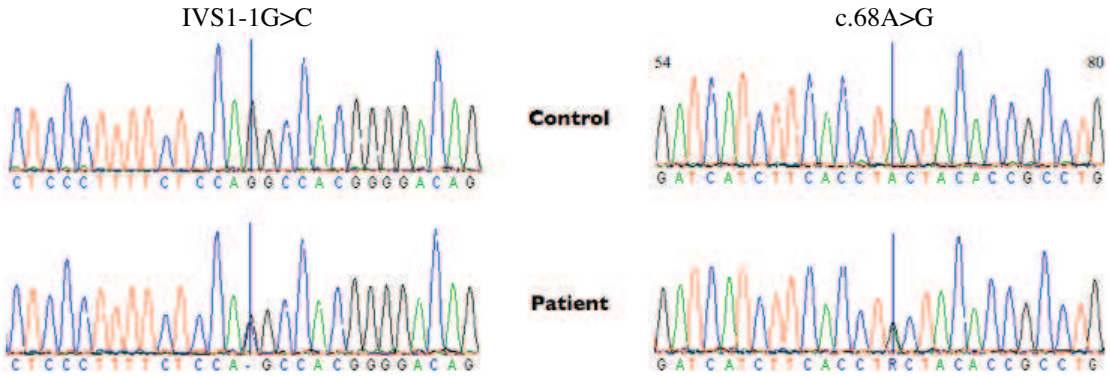

Figure 2: Sequence analysis of *DPM2* revealed one patient who is compound heterozygous for the missense mutation c.68A<G (p.Y23C) and the splice site mutation IVS1-1G>C.

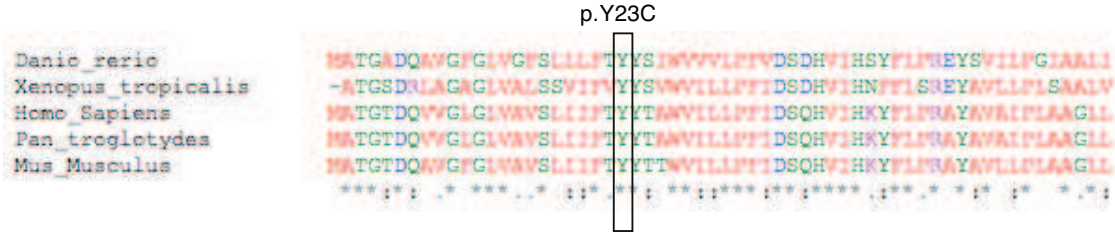

Figure 3: Protein sequence alignment of DPM2 among higher eukaryotes reveals a strong conservation of the tyrosine residue at position 23 at the protein level.

The DPM2-deficient patient is the second child of healthy unrelated Sicilian parents. She presented with severe psychomotor retardation, marked hypotonia, postnatal microcephaly, epilepsy and dysmorphy. Brain MRI showed cerebral atrophy without cerebellar atrophy.

Discussion

In the present study, we identified the first patient with a deficiency in the DPM2 subunit of the DPM complex. This patient was compound heterozygous for the missense mutation c. 68A>G (p.Y23C) and the splice site mutation IVS1-1G>C (Figure 2). Despite the perfect conservation of the tyrosine residue at position p.23 among higher eukaryotes, a rescue experiment is needed to prove the pathogenic character of both mutations. At the cellular level, the phenotype of the DPM2-deficient patient is characterized by the accumulation of Dol-PP-$GlcNAc_2Man_5$ on LLO and the transfer of this truncated intermediate onto proteins (Figure 1). The clinical phenotype of this patient on the other hand comprises severe psychomotor retardation, pronounced hypotonia, postnatal microcephaly, epilepsy and dismorphy. We propose to call this new type DPM2-CDG, according to the new nomenclature suggested by Jaeken and co-autors (Jaeken, J., *et al.* 2008).

The DPM complex catalyzes the synthesis of Dol-P-Man at the cytosolic face of the ER membrane and is composed of three subunits in mammals: DPM1, DPM2 and DPM3 (Maeda, Y., *et al.* 2000). A deficiency in DPM1 (DPM1-CDG or CDG-Ie) has long been associated with CDG and very recently also a defect in DPM3 was shown to cause a glycosylation disorder (Imbach, T., *et al.* 2000, Kim, S., *et al.* 2000, Lefeber, D.J., *et al.* 2009). The clinical phenotype of the DPM2-deficient patient resembles the phenotype observed in DPM1-CDG (CDG-Ie) but is quite different from the one observed in the recently identified DPM3-deficient case. DPM1-CDG is strongly similar to DPM2-CDG and mainly characterized by severe developmental delay, hypotonia, microcephaly and cerebral atrophy (Imbach, T., *et al.* 2000, Kim, S., *et al.* 2000). In contrast, a deficiency in DPM3 results in muscular dystrophy and a normal psychomotor development (Lefeber, D.J., *et al.* 2009). Even-though DPM2 is indirectly bound to DPM1 via the DPM3 subunit of the DPM complex, a deficiency in DPM2 mostly resembled DPM1-CDG.

Besides the clinical phenotype, also the LLO profile showed significant differences among the DPM-deficient patients. A deficiency in DPM1 and DPM2 both leads to an accumulation of Dol-PP-$GlcNAc_2Man_5$ on LLO, while the LLO profile observed in the DPM3-CDG case was completely normal (Imbach, T., *et al.* 2000, Kim, S., *et al.* 2000, Lefeber, D.J., *et al.* 2009). In contrast to DPM1-CDG and DPM2-CDG, a deficiency in DPM3 thus only mildly affects N-glycosylation resulting in mild serum transferrin hypoglycosylation and a normal LLO profile (Lefeber, D.J., *et al.* 2009). Dol-P-Man is however not only a donor for N-glycosylation, but is also used for O-glycosylation such as O-mannosylation of alpha dystroglycan, a protein involved in muscle contraction. The DPM3-deficient patient shows a strongly reduced O-mannosylation of dystroglycan (Lefeber, D.J., *et al.* 2009), suggesting that DPM3 is specifically involved in O-glycosylation. The discovery of this new deficiency in Dol-P-Man synthase highlights the different roles of the DPM subunits in protein glycosylation.

Chapter D

QUALITY CONTROL OF GLYCOPROTEINS BEARING TRUNCATED GLYCANS IN AN ALG9-DEFECTIVE (CDG-IL) PATIENT

Abstract

We describe an *ALG9* defective (Congenital Disorders of Glycosylation type IL) patient who is homozygous for the p.Y286C (c.860A>G) mutation. This patient presented with psychomotor retardation, axial hypotonia, epilepsy, failure to thrive, inverted nipples, hepatomegaly and pericardial effusion. Due to the *ALG9* deficiency, the cells of this patient accumulated the lipid-linked oligosaccharides Dol-PP-$GlcNAc_2Man_6$ and Dol-PP-$GlcNAc_2Man_8$.

It is known that the oligosaccharide structure has a profound effect on protein glycosylation. Therefore we investigated the influence of these truncated oligosaccharide structures on the protein transfer efficiency, the quality control of newly synthesized glycoproteins and the degradation of the truncated glycoproteins formed in this patient. We demonstrated that lipid-linked $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ are transferred onto proteins with the same efficiency. In addition, glycoproteins bearing these $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ structures efficiently entered in the glucosylation/deglucosylation cycle of the quality control system to assist in protein folding. We also showed that in comparison to control cells, patient's cells degraded misfolded glycoproteins at an increasing rate. In comparison to $Man_6GlcNAc_2$, the $Man_8GlcNAc_2$ isomer C on the patient's glycoproteins was found to promote the degradation of misfolded glycoproteins.

Adapted from

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Quality control of glycoproteins bearing truncated glycans in an ALG9 defective (CDG-IL) patient

Glycobiology, 2009, 19(8):910-17

Introduction

N-glycosylation of proteins is a widespread posttranslational modification, crucial for the development, growth, function and survival of organisms (Varki, A. 1993). The N-linked glycosylation pathway starts with the assembly of the $Glc_3Man_9GlcNAc_2$ oligosaccharide precursor on a dolichol-P (Dol-P) carrier in the Endoplasmic Reticulum (ER) membrane. Once synthesized, this structure is transferred onto nascent polypeptide chains by the oligosaccharyltransferase (OST) complex. Subsequently, the $Glc_3Man_9GlcNAc_2$ oligosaccharide is deglucosylated by the combined action of glucosidase I and II, leaving a $Man_9GlcNAc_2$ oligosaccharide on the proteins (Parodi, A.J. 2000).

The fate of newly synthesized glycoproteins is determined during the quality control system in the ER lumen. This quality control system ensures 1) that only correctly folded glycoproteins leave the ER, 2) that misfolded glycoproteins are assisted in their folding and 3) that irreversibly misfolded proteins are retro-translocated to the cytosol for degradation. One of the key steps in the quality control of newly synthesized glycoproteins is mediated by the action of UDP-Glc:glycoprotein glucosyltransferase (UGGT) and glucosidase II. UGGT, considered as a sensor for protein folding, recognizes hydrophobic regions on incorrectly folded glycoproteins and reglucosylates the protein-linked $Man_9GlcNAc_2$ oligosaccharide. This reglucosylation event allows the resulting $Glc_1Man_9GlcNAc_2$ to interact with molecular chaperones like calnexin and calreticulin, which assist in protein folding. Finally, glucosidase II removes again the glucose residue to allow the release of the folded glycoproteins. Correctly folded glycoproteins then leave the ER and are transported to the appropriate destination, while partially folded glycoproteins enter once more in the glucosylation/ deglucosylation cycle until folding is complete (Parodi, A.J. 2000).

If glycoproteins fail to fold correctly after several cycles of reglucosylation and deglucosylation, the $Man_9GlcNAc_2$ oligosaccharide is converted into the $Man_8GlcNAc_2$ isomer B by the action of ER mannosidase I. The formation of this $Man_8GlcNAc_2$ isomer B signals the unfolded glycoproteins for degradation via the ER-associated degradation (ERAD) pathway (Cabral, C.M., *et al.* 2001, Jakob, C.A., *et al.* 1998). This ERAD pathway constitutes of the retro-translocation of misfolded glycoproteins from the ER to the cytosol, followed by the release of the oligosaccharides by a peptide N-glycanase activity (Cacan, R. and Verbert, A. 2000, Suzuki, T. and Funakoshi, Y. 2006). The resulting free oligosaccharides bear two GlcNAc residues at their reducing ends (called OSGn2). Further degradation of these free oligosaccharides in the cytosol occurs via the trimming by a chitobiase, resulting in the formation of oligosaccharides having one GlcNAc residue at their reducing ends (called OSGn1). Moreover, these OSGn1 species are converted into a specific

$Man_5GlcNAc_1$ structure by a cytosolic mannosidase (Cacan, R., *et al.* 1996, Kmiecik, D., *et al.* 1995).

The oligosaccharide structures play an important role during the glycosylation pathway. On one hand, the integrity of the lipid-linked oligosaccharides (LLO) influences the protein transfer rate and therefore the appearance of hypoglycosylated protein forms: non-glucosylated oligosaccharide structures are 20-25x fold less efficiently transferred onto proteins in comparison to their fully glucosylated forms (Karaoglu, D., *et al.* 2001, Trimble, R.B., *et al.* 1980, Turco, S.J., *et al.* 1977). On the other hand, the structure of the protein-bound oligosaccharide has a profound effect on the protein quality control and the recycling of misfolded glycoproteins in the glucosylation/deglucosylation cycle. It has been shown that UGGT and glucosidase II act poorly on substrates with trimmed mannose branches (Grinna, L.S. and Robbins, P.W. 1980, Sousa, M.C., *et al.* 1992), while extensive mannose trimming retained the chaperone binding activity (Spiro, R.G., *et al.* 1996).

The importance of glycosylation is dramatically illustrated by a group of inherited human disorders called Congenital Disorders of Glycosylation (CDG). Up to now, thirty-one different CDG types have been identified, of which fourteen are caused by deficiencies in oligosaccharide precursor formation. These defects in oligosaccharide precursor formation were formerly classified as CDG type I (CDG-I)(Eklund, E.A. and Freeze, H.H. 2006, Freeze, H.H. 2007, Jaeken, J. and Matthijs, G. 2007).

In 2004, Frank and coworkers described a new type of CDG, called CDG-IL, in an infant with neuro-developmental impairment, seizures and hepatomegaly (Frank, C.G., *et al.* 2004). CDG-IL was caused by a mutation (c.1567G>A; p.E523K) in the *ALG9* gene, which codes for the α1,2 mannosyltransferase catalyzing the addition of the seventh and ninth mannose residue during oligosaccharide precursor formation. One year later, Weinstein and collaborators described a second CDG-IL patient who was homozygous for the p.Y286C (c. 860A>G) mutation, differing from the originally described mutation (Weinstein, M., *et al.* 2005). Due to recent changes in nomenclature, CDG-IL is now called *ALG9* deficiency (CDG-IL) (Jaeken, J., *et al.* 2008).

In this study, we described an *ALG9* defective (CDG-IL) patient who is homozygous for the p.Y286C (c.860A>G) mutation. Due to the accumulation of both Dol-PP-$GlcNAc_2Man_6$ and Dol-PP-$GlcNAc_2Man_8$ on LLO, the patient's fibroblasts were used to study *in vivo* the protein transfer efficiency of these assembly intermediates, the quality control of newly synthesized glycoproteins and the eventual degradation of the truncated glycoproteins formed in this patient. We demonstrated that lipid-linked $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ are transferred onto proteins with the same efficiency. In addition, glycoproteins bearing these

$Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ structures efficiently entered in the glucosylation/ deglucosylation cycle of the quality control system to assist in protein folding. We also showed that in comparison to control cells, patient's cells degraded misfolded glycoproteins at an increasing rate. The $Man_8GlcNAc_2$ isomer C on the patient's glycoproteins was found to promote the degradation of misfolded glycoproteins.

Materials and methods

Cell culture

See 'Materials and Methods' in section A1, page 40.

Metabolic radiolabelling of cells

For this purpose, 8 x 10^6 fibroblasts were grown overnight in a 175 cm^2 tissue culture flask. The next day, cells were pre-incubated in 0.5 mM glucose for 45 min and then pulse-radiolabelled with 150 µCi of 2-[^{3}H] mannose (16 Ci/mmol, Amersham Biosciences) at the same glucose concentration for 1 h. Where appropriate, glycosidase inhibitors kifunensin (20 µM, Calbiochem) and castanospermine (50 µg/ml,Sigma) were added to the cells at the beginning of the pre-incubation step and lasted throughout the experiment. After metabolic labelling, cells were scraped with 1.1 ml MeOH/H_2O (8:3; v/v) followed by the addition of 1.2 ml $CHCl_3$. Sequential extraction of oligosaccharide materials was performed as previously described (Cacan, R. and Verbert, A. 1997).

Analysis of oligosaccharide material

Free oligosaccharide fractions obtained after sequential extraction of the oligosaccharide material were desalted on Bio-Gel P2 and eluted with 5% (v/v) acetic acid. Glycoprotein fractions obtained at the end of the sequential extraction were digested overnight at room temperature with trypsin (1 mg/ml; Sigma) in 0.1 M ammonium bicarbonate buffer, pH 7.9. The resulting glycopeptides were then treated with 0.5 U PNGase F (Roche) in 50 mM phosphate buffer, pH 7.2 for 4 h to release the oligosaccharides from the protein. Purification of the released oligosaccharides was performed like described above. Lipid-linked oligosaccharide fractions obtained after the sequential extraction were subjected to a mild acid treatment (0.1 M HCl in THF) at 50 °C for 2 h. Purification of the released oligosaccharides was performed like described above.

The oligosaccharides were separated by HPLC on a polymer based amino column (Asahipak NH_2P-50 column; 250 mm x 4.6 mm; Asahi) applying a gradient of acetonitrile/H_2O ranging from 70:30 (v/v) to 50:50 (v/v) at a flow rate of 1 ml/min over 90 min. Oligosaccharides were identified on the basis of their retention times compared to well-defined standards.

Elution of the radiolabelled oligosaccharides was monitored by continuous flow detection of radioactivity with a flo-one β detector (Packard).

Calculation peak counts

HPLC chromatograms were analyzed using the ProFSA software (Perkin Elmer). The counts in each peak were calculated on the basis of the peak area and normalized against the total number of counts in the injected sample. As protein linked $Glc_1Man_6GlcNAc_2$ and $Glc_1Man_8GlcNAc_2$ are formed by reglucosylation of $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$, the peak counts for the glucosylated forms were taken into account for calculating the $Man_6GlcNAc_2/Man_8GlcNAc_2$ ratios on the glycoproteins and free oligosaccharides. In addition, the difference in mannose residues between $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ were taken into account.

Mutation analysis

Primers were designed to amplify the 15 exons of *ALG9*, including at least 50 bp of the flanking intronic regions, based on the genomic sequence of *ALG9* (NM_024740). Primer sequences are available on request. The exons were amplified using standard PCR conditions, subsequently sequenced with Big Dye Terminator Ready reaction cycle sequencing kit V3.1 (Applera) and analyzed on an ABI3100 Avant (Applera).

Results

Clinical phenotype

The patient is the daughter of Caucasian parents that are distantly related. She was admitted at three months with failure to thrive, vomiting and watery stools. Further investigations at 6.5 months revealed mild dysmorphy, mild central hypotonia and psychomotor retardation. She was on nasogastric feeding with good catch-up growth and had less frequent episodes of inconsolable crying. In addition, she suffered from several seizures, echocardiography showed a small resolving pericardial effusion and CT scan of the brain was normal. Metabolic investigations were normal.

Biochemical and molecular diagnosis

Iso-electric focusing of serum transferrin revealed a type 1 sialotransferrin pattern in the patient, confirming the clinical suggestion of CDG-I (Figure 1A). Moreover, PMM2 deficiency (CDG-Ia), the most frequent type of CDG-I, was excluded on the basis of normal phosphomannomutase activity in the patient's fibroblasts.

In order to detect a possible defect during oligosaccharide precursor formation in the ER,

LLO analysis was performed. Compared to control fibroblasts, an accumulation of the assembly intermediates Dol-PP-$GlcNAc_2Man_6$ and Dol-PP-$GlcNAc_2Man_8$ was detected in the patient's fibroblasts (Figure 1, panels B and C), suggesting a deficiency in the *ALG9* α(1,2)-mannosyltransferase. Sequencing of the patient's DNA revealed indeed a homozygous missense mutation c.860A>G (p.Y286C) in the *ALG9* gene and both parents were shown to be carriers for this mutation (Figure 1D).

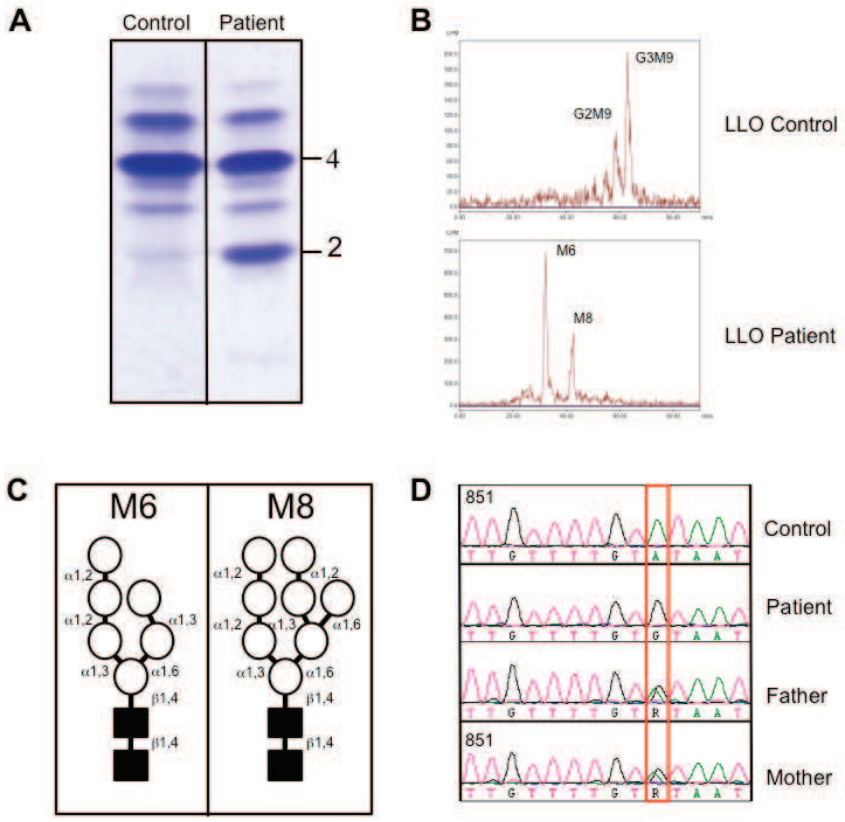

Figure 1: (**A**) Isoelectric focusing of serum transferrin in control and patient. The number of negative charges is indicated on the right. (**B**) HPLC analysis of the lipid-linked oligosaccharides in control and patient's fibroblasts. (**C**) Structure of the lipid-linked oligosaccharides accumulating in the patient's fibroblasts. (**D**) Sequence alignment of the *ALG9* cDNA fragment in control, patient and the patient's parents. The patient is homozygous for an A to G transition at position c.860, while both parents are heterozygous for this base pair change. Symbols: 4, tetrasialotransferrin forms; 2, disialotransferrin forms; G2-3M9, Dol-PP-$GlcNAc_2Man_9Glc_{2-3}$; M6-8, Dol-PP-$GlcNAc_2Man_{6-8}$; squares represent GlcNAc residues and circles represent Man residues.

Transfer of $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ onto proteins

Due to the accumulation of both lipid-linked $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$, the fibroblasts of this patient provided a good model to analyze and compare *in vivo* the transfer rate of these intermediates onto proteins. Hence, the structure of the oligosaccharides transferred onto proteins was analyzed after metabolic labelling of the patient's fibroblasts. $Man_8GlcNAc_2$, $Man_9GlcNAc_2$ and $Glc_1Man_9GlcNAc_2$ structures were found on the

glycoproteins of control fibroblasts, whereas mainly $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ structures were detected on the patient's glycoproteins (Figure 2, panels A and B). Moreover, kifunensin treatment did not influence the patient's HPLC profile (Figure 2C) We thus concluded that the species accumulating on LLO were transferred onto proteins, although they were not optimal substrates for the OST complex.

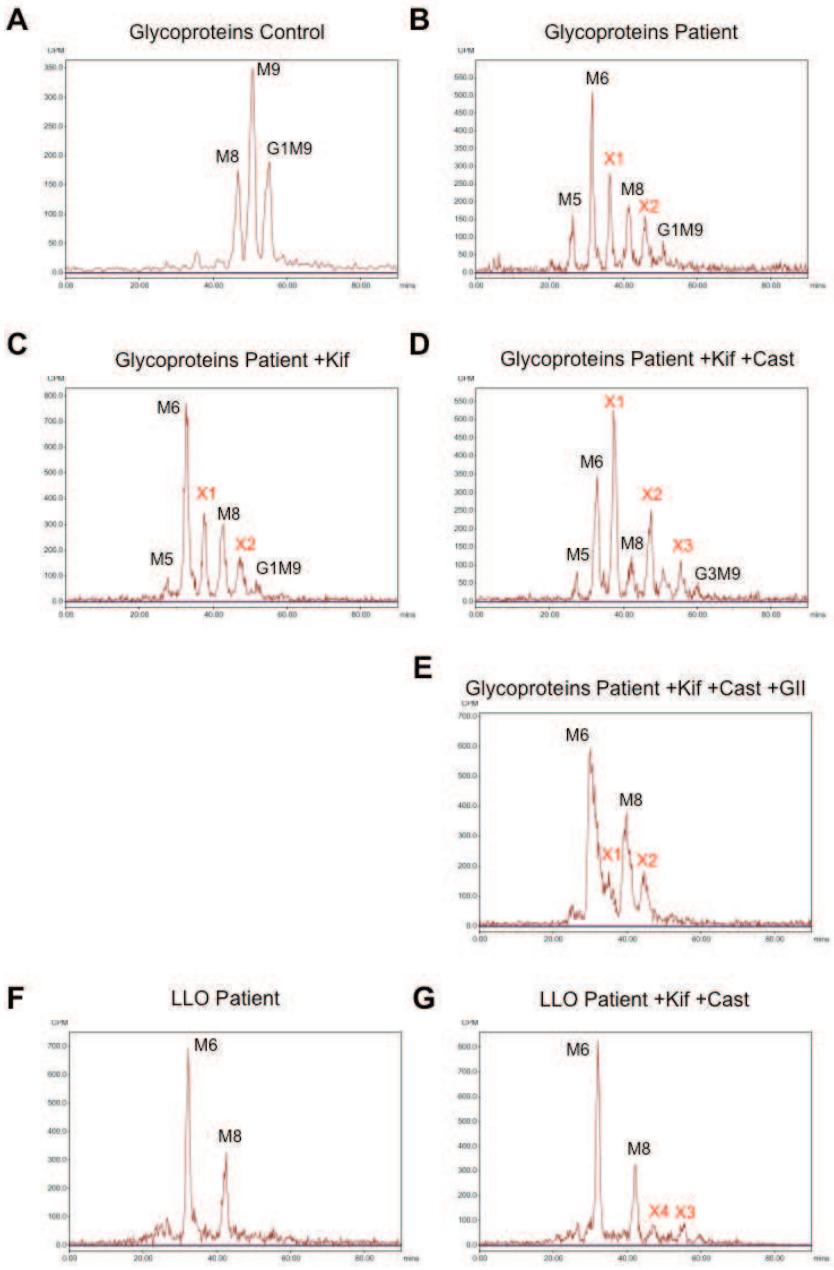

Figure 2: (**A-E**) HPLC analysis of the oligosaccharides linked to proteins. (**A**) Control fibroblasts. (**B**) Patient's fibroblasts. (**C**) Patient's fibroblasts in presence of the ER mannosidase I inhibitor kifunensin (**D**) Patient's fibroblasts in presence of kifunensin and castanospermine, the latter being an inhibitor of glucosidase I and II. (**E**) Profile D after treatment with glucosidase II. (**F**) HPLC analysis of the lipid-linked oligosaccharides in the patient's fibroblasts. (**G**) HPLC analysis of the lipid-linked oligosaccharides in the patient's fibroblasts in presence of kifunensin and castanospermine. Symbols: M5-9, $Man_{5-9}GlcNAc_2$; G1-3M9, $Glc_{1-3}Man_9GlcNAc_2$ and X1-X4, peaks of unknown identity shown in red.

Intriguingly, both species were transferred with the same efficiency. By comparing the ratios of $Man_6GlcNAc_2$/$Man_8GlcNAc_2$ on LLO and glycoproteins, a value of 2.98 (+/- 0.27; n=3) was obtained for the LLO, while a value of 2.99 (+/- 0.14; n=3) was found on the glycoproteins (for the calculation of the ratios, see Materials and Methods). The addition of kifunensin during these metabolic labellings was essential to avoid a mixture of the transferred $Man_8GlcNAc_2$ structure with the $Man_8GlcNAc_2$ formed by demannosylation of the $Man_9GlcNAc_2$ structure. The obtained ratios suggested an equal protein transfer rate of $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ species. Changing the duration of metabolic labelling from 1 hour to 30 minutes, 2 hours or 4 hours did not influence the obtained ratios (data not shown).

Reglucosylation of $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ on proteins

Besides the $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ oligosaccharide structures observed in Figure 2B, two other peaks (X_1 and X_2) were detected on the patient's glycoproteins. Peak X_1 and X_2 migrated as $Glc_1Man_6GlcNAc_2$ and $Glc_1Man_8GlcNAc_2$ respectively. To confirm their identity, a metabolic labelling in presence of kifunensin and castanospermine, the latter being an inhibitor of glucosidase I and II, was carried out. In presence of kifunensin and castanospermine, mainly $Glc_3Man_9GlcNAc_2$ was found on the glycoproteins of control fibroblasts (data not shown), whereas only a small amount of $Glc_3Man_9GlcNAc_2$ was detected in the patient. However, the levels of X_1 and X_2 increased significantly in the glycoproteins fraction of the patient (Figure 2D). Additionally, treatment of the glycoproteins with glucosidase II resulted in a significant decrease in the levels of X_1 and X_2 in combination with an increase of $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ (Figure 2E). From these results we concluded that X_1 and X_2 referred to $Glc_1Man_6GlcNAc_2$ and $Glc_1Man_8GlcNAc_2$ respectively.
Because $Glc_1Man_6GlcNAc_2$ and $Glc_1Man_8GlcNAc_2$ can be formed by deglucosylation of $Glc_3Man_6GlcNAc_2$ and $Glc_3Man_8GlcNAc_2$ respectively, we checked the presence of these latter species on the LLO and/or glycoproteins in the patient's fibroblasts. In presence of kifunensin and castanospermine, triglucosylated oligosaccharides (X_3: potentially $Glc_3Man_8GlcNAc_2$ and X_4: potentially $Glc_3Man_6GlcNAc_2$) did appear on the LLO and the glycoproteins, although at very low level (Figure 2, panels D, F and G). This result excluded that $Glc_1Man_6GlcNAc_2$ and $Glc_1Man_8GlcNAc_2$ oligosaccharides were formed by deglucosylation of triglucosylated species. Moreover, we did not detect monoglucosylated $Man_6GlcNAc_2$ or $Man_8GlcNAc_2$ species on the LLO after metabolic labelling in presence of kifunensin and castanospermine (Figure 2, panels F and G).
All together, these results demonstrated that $Glc_1Man_6GlcNAc_2$ and $Glc_1Man_8GlcNAc_2$ structures were formed by reglucosylation of the $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ structures transferred onto proteins. When comparing the efficiency of reglucosylation, only minor

differences in the reglucosylation of $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ were detected. Comparison of the ratios $Glc_1Man_6GlcNAc_2/Man_6GlcNAc_2$ and $Glc_1Man_8GlcNAc_2/Man_8GlcNAc_2$ in presence of castanospermine revealed a value of 1.25 (+/- 0.12; n=3) and 1.78 (+/- 0.08; n=3) respectively (for the calculation of the ratios, see Materials and Methods). These data suggested that the $Man_6GlcNAc_2$ structure was only 30% less glucosylated than the $Man_8GlcNAc_2$ structure.

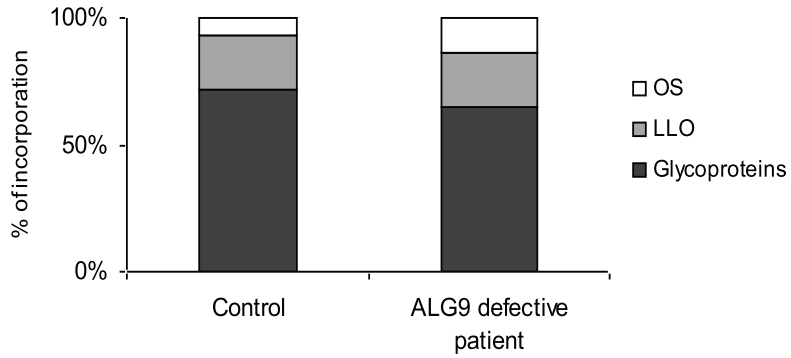

Figure 3: Distribution of the incorporated radioactivity between the various fractions (LLO, glycoproteins and free oligosaccharides) after metabolic labelling of control and patient's fibroblasts.

Preferential degradation of $Man_8GlcNAc_2$

To study the fate of the truncated glycoproteins in the patient, we analyzed the free oligosaccharides, which are the degradation products of the misfolded glycoproteins (Cacan, R. and Verbert, A. 2000, Suzuki, T. and Funakoshi, Y. 2006). First, we compared the total amount of free oligosaccharides formed in control and patient's fibroblasts. After metabolic labelling, patient's cells incorporated 14,20 % (+/- 0.58; n=3) of total radioactivity in the free oligosaccharides, while only 6.98 % (+/- 0.54; n=3) of total radioactivity was found in control free oligosaccharides (Figure 3). In addition, this increased formation of free oligosaccharides in the patient resulted in a proportional decrease in the glycoproteins fraction, while the LLO remained unchanged (Figure 3). Subsequently, the structure of the free oligosaccharides was analyzed by HPLC. The free oligosaccharide fraction of control fibroblasts contained mainly $Man_8GlcNAc_{1/2}$ and $Man_9GlcNAc_{1/2}$ structures, which was in accordance to the glycan structures found on the glycoproteins (Figure 4A). In contrast, $Man_6GlcNAc_{1/2}$ and $Man_8GlcNAc_{1/2}$ structures were found in the free oligosaccharides of the patient's fibroblasts, reflecting the glycan structures on the patient's glycoproteins (Figure 4B). However, $Man_8GlcNAc_{1/2}$ species were better represented in the free oligosaccharide fraction. The ratio $Man_6GlcNAc_{1/2}/Man_8GlcNAc_{1/2}$ reached indeed a value of 2.02 (+/- 0.05: n=3) in the free oligosaccharide fraction, which is in contrast to the ratio of 2.99

(+/-0.14; n=3) value obtained for the glycoproteins (for the calculation of the ratios, see Materials and Methods).

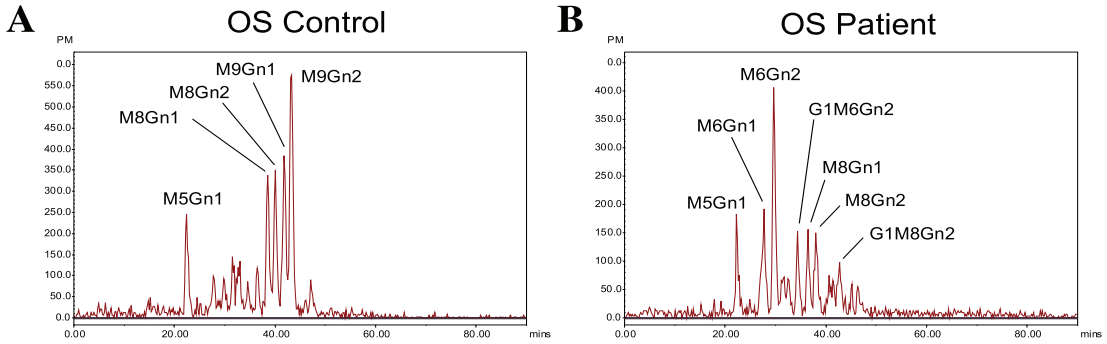

Figure 4: HPLC analysis of the free oligosaccharides in control (**A**) and patient (**B**). Symbols: M5-9Gn1, $Man_{5-9}GlcNAc_1$; M5-9Gn2, $Man_{5-9}GlcNAc_2$; G1M6-9Gn2, $Glc_1Man_{6-9}GlcNAc_2$ and G1M8Gn1, $Glc_1Man_8GlcNAc_1$.

Discussion

In this paper, we described an *ALG9* defective (CDG-IL) patient who is homozygous for the p.Y286C (c.860A>G) mutation, like the patient described by Weinstein M *et al.* (2005). The new *ALG9* defective (CDG-IL) patient has a Canadian nationality, like the first patient, which could point to a founder effect of the p.Y286C mutation. Both patients showed very similar clinical pictures including psychomotor retardation, axial hypotonia, epilepsy, failure to thrive, inverted nipples, hepatomegaly, and pericardial effusion. The patient reported by Weinstein M *et al.* (2005) showed in addition intermittent esotropia and splenomegaly, while the present patient had more, albeit mild dysmorphic features. The *ALG9* defective (CDG-IL) patient reported by Frank CG *et al.* (2004) had psychomotor retardation, axial hypotonia, epilepsy and hepatomegaly in common with the other two patients. Other features were not mentioned in this very short report, which does not exclude their presence. Altogether, the reported *ALG9* defective (CDG-IL) patients did not show a specific clinical picture.

Due to the accumulation of both Dol-PP-$GlcNAc_2Man_6$ and Dol-PP-$GlcNAc_2Man_8$ on LLO, the patient's cells were used to analyze and compare *in vivo* the transfer rate of these accumulating intermediates onto proteins. Analysis of the patient's glycoproteins revealed the transfer of the accumulating $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ oligosaccharides onto proteins (Figure 2B). Assembly intermediates have a low affinity for the OST complex. However, transfer of accumulating assembly intermediates onto proteins has been repeatedly reported in different CDG patients (Chantret, I., *et al.* 2003, Chantret, I., *et al.* 2002, Frank, C.G., *et*

al. 2004, Grubenmann, C.E., *et al.* 2002, Korner, C., *et al.* 1999, Kranz, C., *et al.* 2001). A comparison of the $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ transfer rate has however never been done. Analysis of the glycoproteins in the *ALG9* defective patient revealed an equal transfer rate for both accumulating oligosaccharides. This proved that the OST had no preference for the most entire assembled intermediate, which is the $Man_8GlcNAc_2$ oligosaccharide. The donor-substrate activation model for the OST complex only favours the selective utilization of the fully assembled oligosaccharide precursor. In this model, the OST has a regulatory binding site that favours the transfer of the fully assembled oligosaccharide precursor in function of the donor population. For this reason, the transfer of the fully assembled oligosaccharide precursor is promoted, even when this structure is not abundant(Karaoglu, D., *et al.* 2001).

To address whether or not the $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ oligosaccharides were transferred onto proteins in their triglucosylated forms, a metabolic labelling in presence of kifunensin and castanospermine was performed. Only minor triglucosylated $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ structures were detected on the LLO and glycoproteins (Figure 2). These triglucosylated forms could therefore not account for the transfer of $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ onto proteins. Furthermore, the absence of triglucosylated $Man_7GlcNAc_2$ was reported in *ALG12* defective (CDG-Ig) fibroblasts labelled in presence of castanospermine (Chantret, I., *et al.* 2002, Grubenmann, C.E., *et al.* 2002). From these results we could postulate that the accumulating assembly intermediates $Man_6GlcNAc_2$, $Man_7GlcNAc_2$ and $Man_8GlcNAc_2$ were not good substrates for the α1,2 glucosyltransferase I and that the presence of nine mannose residues was essential for its catalytic activity (Figure 5A). Glucosyltransferase I catalyzes the addition of the first glucose residue during oligosaccharide precursor formation.

Analysis of the patient's glycoproteins after metabolic labelling also revealed the presence of $Glc_1Man_6GlcNAc_2$ and $Glc_1Man_8GlcNAc_2$ (Figure 2B). Due to the absence of monoglucosylated and triglucosylated species on LLO, we hypothesized that the monoglucosylated structures ($Glc_1Man_6GlcNAc_2$ and $Glc_1Man_8GlcNAc_2$) observed onto proteins were formed by the reglucosylation of $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ structures. While UGGT reglucosylates oligosaccharide structures transferred onto protein, these results showed that the truncated glycoproteins bearing $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ were incorrectly folded and entered into the glucosylation/deglucosylation cycle to improve folding. In addition, we demonstrated that UGGT efficiently reglucosylated small oligosaccharide structures like $Man_6GlcNAc_2$: $Man_6GlcNAc_2$ was only 30% less glucosylated in comparison to $Man_8GlcNAc_2$. This is in contrast to the report of Sousa MC *et al.* (1992)

where they reported a strong diminution in UGGT activity when smaller oligosaccharides were used. In this study, the relative rates for reglucosylation of $Man_9GlcNAc_2$, $Man_8GlcNAc_2$ and $Man_7GlcNAc_2$ were 1.0, 0.5 and 0.15 respectively. We could thus hypothesize that, in contrast to glucosyltransferase I, mannose trimming does not impair the catalytic activity of UGGT. Moreover, studies in CHO demonstrated that $Man_4GlcNAc_2$ could not be reglucosylated (Duvet, S., *et al.* 2000). Only a fully assembled α1,3 branch is thus essential for reglucosylation by UGGT (Figure 5A).

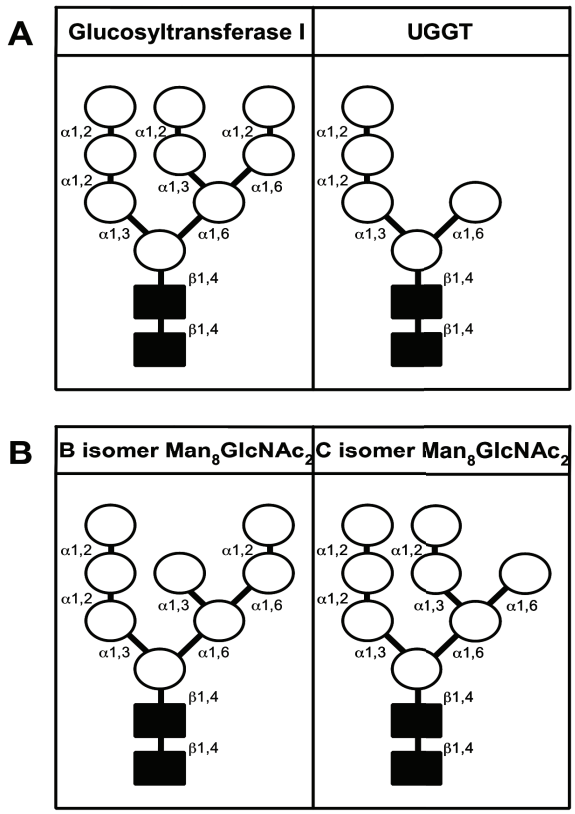

Figure 5: (**A**) Model of the minimal structure necessary for the catalytic activity of the glucosyltransferase 1 and UDP-Glc:glycoprotein glucosyltransferase (UGGT). (**B**) Structure of the B and C isomer of $Man_8GlcNAc_2$. Symbols: squares represent GlcNAc residues and circles represent Man residues.

In parallel, analysis of the patient's glycoproteins revealed that the reglucosylated $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ structures were also efficiently deglucosylated. In absence of castanospermine, only a small fraction of $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ structures were glucosylated. On the other hand, addition of castanospermine caused a marked increase in the fraction of reglucosylated oligosacchararides. These results tended to prove that $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ structures were also efficiently deglucosylated, which is in contrast to the report of Grinna LS and Robbins PW (1980). In this study, a marked decrease in deglucosylation efficiency was obtained for substrates with trimmed mannose residues. Thanks to the efficient reglucosylation and deglucosylation of truncated glycoproteins, the

cell ensures that every truncated glycoprotein can enter into the quality control system and can be recycled in the glucosylation/deglucosylation pathway. In addition, the specific activity of glucosyltransferase I ensures that only correctly assembled LLO are glucosylated during oligosaccacharide precursor assembly.

We also investigated the fate of $Man_6GlcNAc_2$ and $Man_8GlcNAc_2$ bearing glycoproteins by analyzing the free oligosaccharides, the degradation products of misfolded glycoproteins. Analysis of the total amount of free oligosaccharides formed in control and patient's fibroblasts revealed an increased formation of free oligosaccharides in the patient. These results showed that the degradation of misfolded glycoproteins was increased in this patient, reflecting a higher amount of misfolded glycoproteins. In addition, metabolic labelling of the patient's fibroblasts revealed that the oligosaccharide structure influences the degradation rate of misfolded glycoproteins. Glycoproteins bearing $Man_8GlcNAc_2$ oligosaccharides were shown to be 34% more efficiently degraded than glycoproteins bearing a $Man_6GlcNAc_2$ oligosaccharide. In a yeast study, comparing carboxypeptidase Y (CPY) bearing $Man_6GlcNAc_2$, $Man_7GlcNAc_2$ $Man_8GlcNAc_2$ and $Man_9GlcNAc_2$ oligosaccharides, the $Man_8GlcNAc_2$ oligosaccharide also promoted degradation of the misfolded glycoproteins (Jakob, C.A., *et al.* 1998). However, the $Man_8GlcNAc_2$ structure detected in our patient is not identical to the one reported by Jakob CA *et al.*: in yeast, the B-isomer of $Man_8GlcNAc_2$, generally known as the degradation signal, was investigated, while we analyzed the C isomer of $Man_8GlcNAc_2$ (Figure 5B). Based on these results, we could postulate that the C isomer of $Man_8GlcNAc_2$ also promoted degradation via the ERAD pathway or that the C-isomer is the degradation signal of another degradation pathway, like for example the non-proteasomal degradation of misfolded glycoproteins. These data were reinforced by the recent publication of Clerc S *et al.* (2009). They reported that N-glycans bearing a terminally α1,6 linked mannosyl residue, like $Man_7GlcNAc_2$ and $Man_8GlcNAc_2$ isomer C, were a determinant directing misfolded glycoproteins to degradation.

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Concluding discussion

Introduction

Over the last thirty years, CDG rapidly expanded to a group of around forty genetic diseases (Jaeken, J. and Matthijs, G. 2007). Compared to the large number of glycosylation steps, the number of identified diseases is however limited. Recent advances in CDG type II (CDG-II) research revealed that pathways indirectly involved in glycosylation could also lead to glycosylation disorders (Kornak, U., *et al.* 2008, Zeevaert, R., *et al.* 2008), indicating that many more CDG-I diseases thus remain to be discovered. This work aimed to identify novel CDG-I defects by screening different candidate genes in a group of unsolved CDG-I (CDG-Ix) patients. As shown in figure 1, the potential candidate genes for CDG-I are widespread and directly or indirectly involved in N-glycosylation.

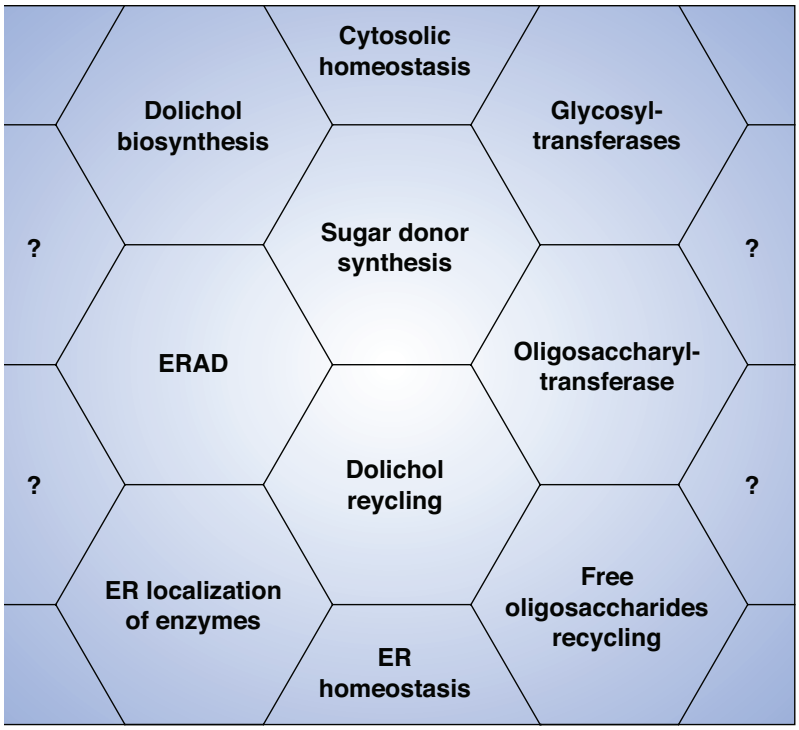

Figure 1: Schematic representation of the candidate genes directly or indirectly involved in N-glycosylation.

Defects in oligosaccharide precursor assembly

The CDG-I defects so far identified are all caused by a decreased synthesis of the fully assembled oligosaccharide precursor (Dol-PP-$GlcNAc_2Man_9Glc_3$), which results in protein

hypoglycosylation. The molecular defect could be situated at different levels: 1) a deficiency in the synthesis of dolichol-P (Dol-P) (DK1-CDG or CDG-Im) (Kranz, C., *et al.* 2007), 2) a deficient synthesis of nucleotide-activated or dolichol-linked sugar donors, found in PMM2-CDG (CDG-Ia), PMI-CDG (CDG-Ib) and DPM1/3-CDG (CDG-Ie/o) (Kim, S., *et al.* 2000, Lefeber, D.J., *et al.* 2009, Matthijs, G., *et al.* 2000, Schollen, E., *et al.* 2000), 3) a defect in the translocation of glycosylation intermediates to the ER lumen, which is observed in MPDU1-CDG (CDG-If) and RFT1-CDG (CDG-Im) (Haeuptle, M.A., *et al.* 2008, Schenk, B., *et al.* 2001) and finally 4) a deficiency in the glycosyltransferases that catalyze the elongation of the oligosaccharide precursor. This latter group is deficient in the majority of known CDG-I defects ranging from a deficiency in the first step of Dol-P elongation (ALG7-CDG or CDG-Ij) to a deficient addition of the second glucose residue in ALG8-CDG or CDG-Ih (see introduction, figure 12, page 32) (Eklund, E.A. and Freeze, H.H. 2006, Jaeken, J. and Matthijs, G. 2007). Despite the large number of defects so far identified, several steps in oligosaccharide precursor assembly have not yet been associated with CDG-I.

Dol-P synthesis: four remaining candidate genes

A total of five enzymes are for example involved in the biosynthesis of Dol-P, however, only a deficiency in DK1 which catalyzes the final step in Dol-P biosynthesis has been associated with CDG-I (Denecke, J. and Kranz, C. 2009, Kranz, C., *et al.* 2007). Proteins like farnesylpyrophosphate synthase, cis-prenyl transferase, dehydrodolichol phosphatase and polyprenyl reductase thus remain good candidates for CDG-I. A CHO mutant deficient in polyprenyl reductase showed for example a decreased synthesis of LLO and subsequent hypoglycosylation of glycoproteins (Stoll, J. and Krag, S.S. 1988).

Synthesis of sugar donors: three genes remain unassociated with CDG-I

On the other hand, several proteins involved in sugar donor synthesis like GMPPA/B, ALG5 and DPM2 were so far not deficient in one of the CDG-I patients. In the present study, we identified the first patient with a deficiency in the DPM2 subunit of the DPM complex (chapter B2.4). The DPM complex synthesizes Dol-P-Man from Dol-P and GDP-Man and consists of three proteins (DPM1, DPM2 and DPM3) in humans (Maeda, Y., *et al.* 2000). The DPM2-deficient patient showed an accumulation of Dol-PP-$GlcNAc_2Man_5$ during LLO analysis of the patient's fibroblasts and presented with severe psychomotor retardation, hypotonia, microcephaly and coagulopathy. This patient resembled the DPM1-CDG or CDG-Ie patients, both on clinical and biochemical level, and significantly differed from the recently identified DPM3-CDG case for whom LLO analysis was normal and whose clinical phenotype consisted of normal psychomotor development and severe muscular dystrophy

(Imbach, T., *et al.* 2000, Kim, S., *et al.* 2000, Lefeber, D.J., *et al.* 2009). We suggest to continue the screening of GMPPA/B and ALG5 in CDG-Ix patients to enable the identification of a novel CDG-I defect.

Translocation of lipid intermediates across the ER membrane: all unidentified flippases and accessory proteins are candidates for CDG-I

A third class of proteins essential for oligosaccharide precursor assembly is involved in the translocation of glycosylation intermediates such as Dol-P-Man, Dol-P-Glc and Dol-PP-$GlcNAc_2Man_5$ across the ER membrane. Translocation of lipids across the ER membrane is very slow when occurring unassisted (Hanover, J.A. and Lennarz, W.J. 1979, McCloskey, M.A. and Troy, F.A. 1980). Hence, this reaction is thought to be facilitated by proteins called flippases. Since flippase activities are difficult to measure, extensive studies on the identification of flippases involved in protein N-glycosylation remained unsuccessful. However, two proteins, MPDU1 and RFT1, were reported to take part in the translocation reaction (Helenius, J., *et al.* 2002). Nevertheless, it remains unclear whether they are the flippases themselves or play accessory roles in lipid translocation (Rush, J.S., *et al.* 2009, Sanyal, S., *et al.* 2008). The identification of five additional patients with an RFT1 deficiency (chapter B2.1 and B2.2) points to the major importance of RFT1 in the translocation of Dol-PP-$GlcNAc_2Man_5$ across the ER membrane. Clinical comparison of all the identified patients allowed in addition to delineate the main clinical phenotype of RFT1-CDG or CDG-Im: severe mental retardation, hypotonia, epilepsy, myoclonic jerks, decreased visual acuity, feeding problems and sensorineural deafness. This latter characteristic has only rarely been associated with CDG and might be the hallmark for RFT1 deficiency, together with the absence of Dol-PP-$GlcNAc_2Man_5$ transfer onto proteins on the cellular level. Besides RFT1 and MPDU1, all flippases or accessory proteins that remain unidentified are candidates for CDG-I in the CDG-Ix patients.

Glycosyltransferases: three remaining candidate genes

Although the majority of CDG-I defects comprise defects in the glycosyltransferases that catalyze the elongation of Dol-P to Dol-PP-$GlcNAc_2Man_9Glc_3$ (Jaeken, J. and Matthijs, G. 2007), still a few enzymes have not yet been associated with CDG-I. These enzymes comprise two proteins involved in the early steps of the dolichol cycle (the ALG13/14 complex and ALG11) and one enzyme catalyzing the final step in oligosaccharide precursor assembly, the ALG10 glucosyltransferase. In our study, we very recently identified the first patient with a deficiency in the mannosyltransferase ALG11 (chapter B1.2). The LLO profile of the patient's fibroblasts was characterized by an accumulation of the assembly intermediate Dol-

PP-$GlcNAc_2Man_3$ in addition to Dol-PP-$GlcNAc_2Man_4$ and the clinical phenotype of the ALG11-deficient patient consisted of severe psychomotor retardation, feeding problems, epilepsy, sensorineural deafness and hypotonia. The observation of sensorineural deafness in ALG11-CDG and RFT1-CDG could point to an otoneurotoxic effect of LLO accumulation at the cytosolic face of the ER membrane. Besides the ALG11-deficient patient, we also identified a new patient with a deficiency in the ALG9 mannosyltransferase, causing ALG9-CDG or CDG-IL and resulting in the accumulation of Dol-PP-$GlcNAc_2Man_6$ and Dol-PP-$GlcNAc_2Man_9$ on LLO (chapter B3). This patient was the third ALG9-CDG patient so far identified and was homozygous for the earlier reported missense mutation p.Y286C (Frank, C.G., *et al.* 2004, Weinstein, M., *et al.* 2005). The identical nationality of both ALG9-CDG patients homozygous for the missense mutation p.Y286C points to a founder effect of the mutation. So, eventually, only ALG13/14 and ALG10 remain unlinked to disease.

Fundamental studies on new CDG-I patients: insight in ER quality control and dolichol cycle regulation

The identification of novel CDG-I patients in our laboratory allowed to perform fundamental studies on the CDG-I patients' cells to gain insight in ER quality control and dolichol cycle regulation. Since the ALG9-deficient patient's cells accumulate the assembly intermediates Dol-PP-$GlcNAc_2Man_6$ and Dol-PP-$GlcNAc_2Man_9$, they presented an interesting model to study *in vivo* the protein transfer efficiency of the assembly intermediates, the quality control of newly synthesized glycoproteins and the eventual degradation of the truncated glycoproteins formed in this patient. We demonstrated that both assembly intermediates were transferred onto proteins at the same efficiency and showed that the truncated glycoproteins formed in this patient efficiently enter the reglucosylation/deglucosylation cycle of the quality control system in the ER (chapter B3). This latter observation contrasted with the report of Sousa and co-workers demonstrating *in vitro* that truncated glycans are inefficiently reglucosylated during the quality control of newly synthesized proteins (Sousa, M.C., *et al.* 1992). We also demonstrated that, in comparison to control cells, patient's cells showed an increased degradation of misfolded glycoproteins. This observation could be explained by the formation of the $Man_8GlcNAc_2$ isomer C on the patient's glycoproteins. We detected that the $Man_8GlcNAc_2$ isomer C was promoting degradation of the truncated glycoproteins formed in patient's cells. These results are in accordance to the recently published study of Clerc and co-workers where they postulated that removal of the C-arm terminal mannose creates the signal for degradation of misfolded glycoproteins (Clerc, S., *et al.* 2009).

A fundamental study in the RFT1-deficient patients' cells contributed on the other hand to the identification of novel free oligosaccharide species with a potential role in dolichol cycle regulation (chapter B2.3). We demonstrated that Dol-PP-$GlcNAc_2Man_5$, accumulating at the cytosolic face of the ER membrane in RFT1-deficient patients' cells, was catabolized into Dol-P and Man_5 $GlcNAc_2$-P. These phosphorylated oligosaccharides (POS) were formed in the cytosol in order to prevent dolichol cycle blocking. Additional studies demonstrated that this catabolic pathway is also present in other CDG-I patients' cells accumulating LLO either at the cytosolic or the luminal face of the ER membrane. In the seventies and eighties, several *in vitro* studies reported on the formation of POS during glycosylation, but neither their presence in human cells nor their physiological role could be assigned (Cacan, R.*, et al.* 1987, Cacan, R.*, et al.* 1980, Hoflack, B.*, et al.* 1981, Hsu, A.F.*, et al.* 1974, Oliver, G.J.*, et al.* 1975). The identification of POS in CDG-I patients' cells sheds a new light on dolichol cycle regulation and the catabolism of accumulating LLO intermediates in CDG-I patients' cells.

Defects in the transfer of the oligosaccharide precursor onto proteins

Oligosaccharyltransferase defect(s): only one potential disorder found

Surprisingly, no glycosylation disorder has so far been found in the key enzyme of N-glycosylation: the oligosaccharyltransferase (OST) complex. The OST complex catalyzes the transfer of the fully assembled oligosaccharide precursor onto specific asparagine residues of the protein, thereby forming an N-glycoprotein (Kelleher, D.J. and Gilmore, R. 2006). Since a deficiency in OST would result in protein hypoglycosylation despite a normal assembly of Dol-PP-$GlcNAc_2Man_9Glc_3$, we screened for OST defects in a group of twenty-seven CDG-Ix patients having a normal LLO profile (chapter A1). To our surprise, we only found one patient with a potential OST defect. This patient was homozygous for a missense mutation in ribophorin 2 and presented a mild clinical phenotype. We concluded that the OST subunits were no good candidates for CDG in these CDG-Ix patients and hypothesized that OST defects should be looked for in patients with a specific clinical phenotype restricted to one or few organs. A deficiency in the IAP and N33 subunits of the OST complex was indeed recently identified in patients with non-syndromic mental retardation (Molinari, F.*, et al.* 2008). These patients potentially have a deficiency in the glycosylation of brain-specific N-glycoproteins. This restricted clinical phenotype is in contrast to the general dysfunction observed in CDG-I patients, which could explain the absence of multiple OST defects in our group of CDG-Ix patients.

Deficiency in ERAD: absence of ERAD defects in CDG-Ix patients

The transfer of the oligosaccharide precursor onto proteins could also be affected when unfolded proteins accumulate in the lumen of the ER, thereby interfering with the normal substrate for the oligosaccharyltransferase complex (Moore, S.E. 1999). Hence, we screened the ERAD pathway, which is responsible for the clearance of misfolded glycoproteins from the ER, in the group of CDG-Ix patients showing a normal assembly of the oligosaccharide precursor on LLO analysis (chapter A2). Neither Sec61 sequencing nor free oligosaccharides analysis revealed an ERAD defect in those patients, suggesting that the deficiency is located in other proteins or pathways interfering with the transfer of the oligosaccharide precursor onto proteins.

Where to look for in the future?

Recent discoveries in CDG-II demonstrated that a deficiency in pathways indirectly involved in the biosynthesis of N-glycoproteins could also result in a glycosylation deficiency. A deficiency in the Golgi pH was for example reported to affect Golgi glycosyltransferases resulting in aberrant protein glycosylation (Kornak, U., *et al.* 2008). On the other hand, the subunits of the COG complex, essential for the correct cellular localization of glycosyltransferases, were also indispensable for proper protein glycosylation (Zeevaert, R., *et al.* 2008). So far, CDG-I screening has always focused on the proteins involved in the biosynthesis of N-glycoproteins. Hence, the time has come to broaden our field and explore pathways that could indirectly result in a glycosylation disorder. Several hypotheses can be brought forward. Proteins involved in the recycling of Dol-P are for example good candidates for CDG-I. Dol-P is a rate limiting factor in protein glycosylation and, besides *de novo* synthesis, a major fraction of Dol-P is recycled (Rush, J.S., *et al.* 2008, Schenk, B., *et al.* 2001). A deficiency in the recycling of Dol-P could thus result in Dol-P depletion and subsequent protein hypoglycosylation. If Dol-P recycling is important, one can easily imagine that also the proper recycling of monosaccharides could be pivotal. Metabolic labelling experiments on fibroblasts of patients having Salla disease, a deficiency in the export of sialic acids out of the lysosomes in order to be re-used by the cell, indeed revealed a decreased incorporation of sialic acids into glycoproteins (Chigorno, V., *et al.* 1996).

Another hypothesis is a deficiency in ER homeostasis. Since oligosaccharide precursor assembly and the transfer of the oligosaccharide precursor onto proteins takes place in the ER, ER homeostasis is in important factor for proper N-glycosylation. ER homeostasis could for example be disrupted by abnormal pH and/or calcium levels. Calcium release is for example reported to result in a loss of ER continuity (Terasaki, M., *et al.* 1996), which could

interfere with proper N-glycosylation, and glycosyltransferases require divalent cations like Ca^{2+} for optimal enzymatic activity (Wiggins, C.A. and Munro, S. 1998). Since the initial steps in the biosynthesis of N-glycoproteins take place at the cytosolic face of the ER membrane, deficiencies in cytosolic homeostasis could also lead to CDG-I.

Finally, we hypothesize that a defect in the sub-cellular localization of enzymes involved in glycoprotein biosynthesis could cause a glycosylation disorder of type I. The ER is a cellular organelle involved in numerous functions: protein translation, protein folding, glycosylation of proteins and degradation of misfolded glycoproteins as well as calcium storage, glycolipid synthesis, ... It is likely that these different functions occur in specialized compartments of the ER. Kamhi-Nesher and co-workers indeed proposed that the ER quality control takes place in the ER-derived quality control compartment (ERQC) (Kamhi-Nesher, S., *et al.* 2001). Mislocalization of enzymes among the sub-compartments might cause a glycosylation disorder.

Tools used to study CDG-I in the future

When serum transferrin analysis revealed a CDG-I profile and enzymatic activity measurements excluded PMM2-CDG (CDG-Ia) and PMI-CDG (CDG-Ib), LLO analysis was subsequently used to pinpoint the molecular defect in the CDG-Ix patient. On the basis of LLO analysis, the assembly intermediate(s) accumulating in the CDG-I patient's cells due to a deficiency in the biosynthetic enzymes of the dolichol cycle could be identified. By comparing the observed LLO profile with the profile detected in yeast mutants, candidate genes could be selected. This strategy enabled the identification of most CDG-I defects known so far. A substantial amount of CDG-Ix patients however show a normal assembly of the oligosaccharide precursor and several CDG-Ix patients accumulate assembly intermediates on LLO but do not have a defect in the biosynthetic enzymes of the dolichol cycle. To identify the molecular defect in the remaining CDG-Ix patients, not fitting in any known CDG-I, a different approach should be applied. Since the candidate genes of CDG-I are widespread and involved in numerous biochemical pathways, powerful tools like homozygosity mapping and full exome sequencing could be appropriate. Moreover, these techniques allow the identifications of defect(s) in genes of unknown function and in genes at first sight not involved in N-glycosylation. The last years of CDG research were ruled by biochemists, but geneticists are now back into business.

Summary

Not only the field of CDG-I but CDG in general is expected to become even more broader, with the identification of additional defects in N- and O-glycosylation and the further

exploration of defects in glycolipid biosynthesis. In addition, deficiencies in pathways indirectly involved in glycosylation are believed to constitute a major part of CDG defects. We have probably only seen the tip of the iceberg.

Summary

Most proteins expressed in an organism are modified in a co- or post-translational manner to extend their range of biological functions. One of these post-translational modifications is glycosylation, which consists of the covalent attachment of glycans onto proteins. Protein glycosylation is strongly conserved throughout the entire phylogenetic spectrum and is involved in numerous cellular functions such as protein folding, intracellular trafficking, cell adhesion, receptor activation, endocytosis, ... Hence, it is not surprising that a deficiency in glycosylation results in disorders, called Congenital Disorders of Glycosylation (CDG), with a broad spectrum of clinical features affecting nearly every organ system. In 1980, Prof. Jaeken identified the first CDG patients displaying a deficiency in N-glycosylation. The identification of this first N-glycosylation disorder triggered the discovery of many more defects in this complex biosynthetic pathway. So far, more than twenty genetic defects in protein N-glycosylation have been identified and the field of CDG has now expanded to disorders in O-glycosylaton and lipid glycosylation. Most CDG types are deficient in the early steps of N-glycosylation, more specifically in the assembly of the oligosaccharide precursor Dol-PP-$GlcNAc_2Man_9Glc_3$. Defects in oligosaccharide precursor assembly are called CDG type I (CDG-I) disorders and are characterized by protein hypoglycosylation. Despite the extensive exploration of CDG-I defects, the number of unsolved CDG-I (CDG-Ix) cases has been significantly increasing. Hence, the aim of this study was the identification of the molecular defect(s) in a cohort of fifty CDG-Ix patients.

Among our cohort of CDG-Ix patients, twenty-seven showed both a **normal level** and **intact structure of the oligosaccharide precursor** during lipid-linked oligosaccharides (LLO) analysis. How could we then explain the hypoglycosylation observed in these CDG-Ix patients? We postulated that these patients were deficient in the oligosaccharyltransferase (OST) complex which catalyzes the transfer of the oligosaccharide precursor onto proteins. Surprisingly, genetic screening of the OST subunits in these twenty-seven patients identified only one patient with a potential **deficiency in** the **ribophorin 2** subunit. Hence, we hypothesized that the remaining CDG-Ix patients accumulated unfolded proteins in the lumen of the endoplasmic reticulum (ER) which also interferes with the transfer of glycans onto proteins. An accumulation of unfolded proteins could be caused by a deficiency in the retranslocation of misfolded glycoproteins to the cytosol or by a deficiency in the disposal of misfolded glycoproteins for degradation. Neither a genetic screening of the Sec61 complex, involved in protein retranslocation, nor structural analysis of the free oligosaccharides, the

degradation products of misfolded glycoproteins, revealed the genetic defect in these CDG-Ix patients.

For another twenty-three CDG-Ix patients, a deficiency in oligosaccharide precursor assembly had not yet been excluded on the basis of LLO analysis. Structural analysis of the LLO in this group of CDG-Ix patients identified five patients with an unusual LLO profile **accumulating both Dol-PP-$GlcNAc_2Man_3$ and Dol-PP-$GlcNAc_2Man_5$**. Genetic screening of the candidate genes did not reveal a mutation, but the similarity in LLO profile and clinical phenotype allowed us to suppose that four out of these five patients share the same genetic defect. LLO analysis in another CDG-Ix patient also revealed an **accumulation** of **Dol-PP-$GlcNAc_2Man_3$**, now associated with **Dol-PP-$GlcNAc_2Man_4$.** This patient was shown to be the first CDG-I patient with a **deficiency in** the mannosyltransferase **ALG11**.

In the cells of fourteen patients, an accumulation of **Dol-PP-$GlcNAc_2Man_5$** was detected. Five of them displayed a **deficiency in RFT1**, a protein facilitating the transfer of Dol-PP-$GlcNAc_2Man_5$ from the cytosolic to the luminal face of the ER membrane. So far, only one RFT1-deficient patient had been described. The identification of these five additional RFT1-CDG patients allowed us to delineate the clinical characteristics of RFT1 deficiency. Sensorineural deafness, only rarely observed in CDG patients, was found to be the hallmark for RFT1-CDG. An additional fundamental study on the RFT1-deficient patients' cells identified novel free oligosaccharide species originating from the accumulation of Dol-PP-$GlcNAc_2Man_5$. Dol-PP-$GlcNAc_2Man_5$ accumulating in RFT1-CDG cells was converted into Dol-P and P-$GlcNAc_2Man_5$. This catabolic pathway was also observed in other CDG-I patients' cells accumulating different LLO structures either at the cytosolic or luminal face of the ER membrane. We postulated that the formation of phosphorylated oligosaccharides in CDG-I patients' cells was involved in the recycling of Dol-P, a rate-limiting substrate in N-glycosylation.

The accumulation of Dol-PP-$GlcNAc_2Man_5$ on LLO was also observed in another CDG-Ix patient shown to be **deficient in DPM2**. DPM2 is a subunit of the DPM complex which synthesizes Dol-P-Man from GDP-Man and Dol-P. In contrast to the other subunits of the DPM complex (DPM1 and DPM3), DPM2 was not yet associated with CDG-I. DPM2-CDG strikingly resembled a deficiency in DPM1 (CDG-Ie), both at the clinical and biochemical level, while it completely differed from a deficiency in DPM3.

LLO analysis in our group of CDG-Ix patients also revealed a patient who accumulated **Dol-PP-$GlcNAc_2Man_6$ and Dol-PP-$GlcNAc_2Man_8$** on LLO. This patient was deficient in ALG9 and was the third known **ALG9-CDG** (CDG-IL) patient. Due to the accumulation of two assembly intermediates onto LLO, the ALG9-deficient patient's cells presented a good model for a fundamental study on ER quality control. The most striking feature identified during this

study was the increased degradation of misfolded glycoproteins bearing $Man_8GlcNAc_2$ isomer C. These results strongly suggest that the $Man_8GlcNAc_2$ isomer C could be a novel degradation signal for misfolded glycoproteins.

From the fifty CDG-Ix patients investigated, we localized the biochemical defect in twenty-two patients and identified the genetic defect in nine of them. Moreover, fundamental studies on CDG-I patients' cells have shed a new light on ER quality control and dolichol cycle regulation. The forty-one remaining CDG-Ix patients potentially bear defects in still unknown proteins involved in N-glycosylation and/or defects in other pathways indirectly involved in N-glycosylation. The use of powerful genetic tools like homozygosity mapping and full exome and genome sequencing could contribute to the identification of the genetic defect in these CDG-Ix patients.

Samenvatting

De meeste proteïnen die tot expressie komen in een organisme ondergaan een reeks veranderingen op een co- of post-translationele manier om hun waaier aan biologische functies uit te breiden. Eén van deze co- of post-translationele modificaties is de aanhechting van suikerketens, ook wel glycosylatie genoemd. De glycosylatie van proteïnen is sterk geconserveerd doorheen het gehele fylogenetisch spectrum en is essentieel voor een aantal cellulaire functies zoals het opvouwen van proteïnen, intracellulair transport, cel adhesie, de activering van receptoren, endocytose, ... Het is dan ook logisch dat een defect in de glycosylatie van proteïnen leidt tot aandoeningen, 'Congenital Disorders of Glycosylation' (CDG) genaamd, met een breed spectrum aan klinische kenmerken waarbij zo goed als elk orgaan kan aangetast worden. In 1980 identificeerde Prof. Jaeken de eerste CDG patiënten met een aangeboren defect in de N-glycosylatie van proteïnen. De ontdekking van dit eerste N-glycosylatie defect leidde tot de identificatie van een brede waaier aan aandoeningen in dit complex proces. Tot op heden werden meer dan twintig N-glycosylatie defecten geïdentificeerd en breidde het CDG veld zich ook uit tot aandoeningen in de O-glycosylatie van proteïnen en in de glycosylatie van lipiden. De meeste CDG types vertonen een defect in de eerste stappen van het N-glycosylatie proces, namelijk in de opbouw van het precursor oligosaccharide Dol-PP-$GlcNAc_2Man_9Glc_3$. Defecten in de opbouw van deze precursor worden CDG type I (CDG-I) aandoeningen genoemd en deze worden gekenmerkt door een hypoglycosylatie van proteïnen. Ondanks de identificatie van de vele CDG-I defecten is er een duidelijke stijging in het aantal onopgeloste CDG-I (CDG-Ix) gevallen. Met deze studie trachtten we dan ook het genetisch defect in een groep van vijftig CDG-Ix patiënten te identificeren.

Bij zevenentwintig van deze vijftig CDG-Ix patiënten vertoonde de analyse van de oligosacchariden gebonden aan lipiden ('lipid-linked oligosaccharides' of LLO) geen enkele afwijking, noch op kwalitatief als kwantitatief vlak. Hoe kunnen we dan de hypoglycosylatie van proteïnen bij deze reeks patiënten verklaren? We suggereerden dat deze patiënten mogelijk een defect vertoonden in het oligosaccharyltransferase (OST), een proteïne complex dat de transfer van het precursor oligosaccharide naar de proteïnen katalyseert. Een genetische screening van de verschillende OST subeenheden bij deze zevenentwintig patiënten leidde slechts tot de identificatie van één patiënt met een mogelijk **defect in** de **ribophorin 2** subeenheid. De transfer van het precursor oligosaccharide op proteïnen kan

echter ook aangetast worden door een opstapeling van fout opgevouwen glycoproteïnen in het lumen van het endoplasmatisch reticulum (ER). Een opstapeling van fout opgevouwen glycoproteïnen kan veroorzaakt worden door een defect in de retranslocatie van deze glycoproteïnen naar het cytosol of door een defect in de afbraak van fout opgevouwen glycoproteïnen. Noch een genetische screening van het Sec61 complex, dat betrokken is bij de retranslocatie van fout opgevouwen glycoproteïnen, noch een analyse van de vrije oligosacchariden, de afbraak producten van fout opgevouwen glycoproteïnen, onthulde het moleculair defect bij deze patiënten.

Voor drieëntwintig andere CDG-Ix patiënten werd een defect in de opbouw van het precursor oligosaccharide nog niet uitgesloten op basis van LLO analyse. Een structurele analyse van de LLO bij deze patiënten leidde tot de identificatie van vijf patiënten met een ongewoon LLO profiel waarbij de intermediairen **Dol-PP-$GlcNAc_2Man_3$ en Dol-PP-$GlcNAc_2Man_5$** zich opstapelden. Een genetische screening van de verschillende kandidaat genen identificeerde geen enkele mutatie, maar de sterke gelijkenis in LLO profiel en klinisch fenotype suggereerde dat vier van deze vijf patiënten eenzelfde genetische aandoening hebben. LLO analyse in een andere CDG-Ix patiënt toonde ook een opstapeling van **Dol-PP-$GlcNAc_2Man_3$** aan, maar nu in combinatie met **Dol-PP-$GlcNAc_2Man_4$**. Deze patiënt is de eerste CDG-I patiënt met een defect in het mannosyltransferase **ALG11.**

In de cellen van veertien andere CDG-Ix patiënten werd tijdens de LLO analyse een opstapeling van **Dol-PP-$GlcNAc_2Man_5$** gevonden. Vijf van deze patiënten vertoonden een een **defect in RFT1**, een proteïne dat betrokken is bij de translocatie van Dol-PP-$GlcNAc_2Man_5$ van de cytosolische naar de luminale kant van het ER membraan. Voordien was slechts één patiënt met een deficiëntie in RFT1 beschreven. De identificatie van deze vijf bijkomende RFT1-CDG patiënten liet ons toe de klinische kenmerken van RFT1-CDG te bepalen. Sensorineurale doofheid, wat slechts zeer zelden voorkomt bij CDG patiënten, bleek karakteristiek te zijn voor een defect in RFT1. Een fundamentele studie op de cellen van RFT1-CDG patiënten leidde bovendien tot de identificatie van een nieuw type vrije oligosacchariden die afkomstig zijn van Dol-PP-$GlcNAc_2Man_5$: in de RFT1-CDG patiënten werd Dol-PP-$GlcNAc_2Man_5$ omgevormd tot Dol-P en P-$GlcNAc_2Man_5$. Dit katabool proces werd eveneens aangetroffen bij andere CDG-I patiënten die intermediairen opstapelen zowel langs de cytosolische als de luminale kant van het ER membraan. De vorming van gefosforyleerde oligosacchariden in de cellen van CDG-I patiënten is hoogstwaarschijnlijk betrokken bij de recyclage van Dol-P, een snelheidsbepalend substraat voor N-glycosylatie.

Een opstapeling van Dol-PP-$GlcNAc_2Man_5$ werd ook aangetroffen bij een andere CDG-Ix patiënt, maar deze patiënt bleek een **DPM2 deficiëntie** te vertonen. DPM2 is een subeenheid van het DPM complex dat de synthese van Dol-P-Man vanuit GDP-Man en Dol-P katalyseert.

In tegenstelling tot de andere subeenheden van het DPM complex (DPM1 en DPM3) werd een DPM2 defect tot dusver nog niet geassocieerd met CDG. DPM2-CDG vertoont sterke gelijkenissen met DPM1-CDG (CDG-Ie), zowel op klinisch als biochemisch vlak, en verschilt sterk van een deficiëntie in DPM3.
LLO analyse bij de groep CDG-Ix patiënten onthulde eveneens een patiënt die de intermediairen **Dol-PP-$GlcNAc_2Man_6$ en Dol-PP-$GlcNAc_2Man_6$** opstapelt. Deze patiënt vertoonde een deficiëntie in ALG9 en is de derde beschreven **ALG9-CDG** (CDG-IL) patiënt. Door de opstapeling van de twee verschillende intermediairen vertoonde de cellen van deze ALG9 deficiënte patiënt een interessant model om de kwaliteitscontrole in het ER verder te bestuderen. Het meest opmerkelijke resultaat van deze studie is de verhoogde afbraak van fout opgevouwen glycoproteïnen met $Man_8GlcNAc_2$ isomeer C, wat suggereert dat het $Man_8GlcNAc_2$ isomeer C een nieuw afbraak signaal is voor fout opgevouwen glycoproteïnen.

Van de vijftig bestudeerde CDG-Ix patiënten werd het biochemisch defect gelokaliseerd in tweeëntwintig patiënten terwijl voor negen onder hen tevens het genetisch defect werd geïdentificeerd. Bovendien hebben fundamentele studies op de cellen van een aantal CDG-I patiënten een nieuw licht geworpen op de kwaliteitscontrole in het ER en de regulatie van de dolichol cyclus. De eenenveertig overblijvende CDG-Ix patiënten vertonen mogelijk een defect in proteïnen met een nog ongekende functie in de N-glycosylatie of hebben een probleem in andere pathways die (in)direct de N-glycosylatie beïnvloeden. Het gebruik van krachtige genetische methoden zoals 'homozygosity mapping' en 'full exome/genome sequencing' zouden in de toekomst kunnen bijdragen tot de identificatie van het genetisch defect bij deze patiënten.

Résumé

La plupart des protéines synthétisées dans un organisme sont modifiées de façon co- ou post-traductionnelle en vue d'élargir leur gamme de fonctions biologiques. Une de ces modifications, appelée glycosylation, concerne la liaison de sucres aux protéines. La glycosylation des protéines est un processus extrêmement conservé tout au long du spectre phylogénétique et impliquée dans plusieurs fonctions cellulaires comme le repliement des protéines, le transport intracellulaire, l'adhésion cellulaire, l'activation des récepteurs et l'endocytose. Il apparaît donc évident qu'une déficience en glycosylation conduit à des anomalies, nommée 'Congenital Disorders of Glycosylation' ou CDG, avec un large spectre de caractéristiques cliniques affectant la majorité des organes. En 1980, Prof. Jaeken identifia les premiers patients CDG atteints d'une anomalie congénitale de N-glycosylation. La découverte de cette première déficience déclencha l'identification d'un grand nombre de défauts génétiques au sein de cette voie de biosynthèse. Jusqu'à présent, vingt anomalies de N-glycosylation ont été identifiées et le domaine des CDG s'élargit maintenant aux maladies de O-glycosylation des protéines et de glycosylation des lipides. La plupart des patients CDG sont déficients dans les premières étapes de la N-glycosylation, et plus particulièrement dans l'assemblage du précurseur oligosaccharidique Dol-PP-$GlcNAc_2Man_9Glc_3$. Les déficiences en assemblage du précurseur sont nommées les CDG de type I (CDG-I) et sont caractérisées par une hypoglycosylation des protéines. Malgré les efforts entrepris, le nombre de cas de CDG-I non résolus (CDG-Ix) augmente de manière significative. Par conséquent, l'objectif de cette étude a été d'identifier le(s) déficit(s) moléculaire(s) dans une cohorte de cinquante patients CDG-Ix.

Parmi les patients CDG-Ix, l'analyse des oligosaccharides liés aux lipides (en anglais lipid-linked oligosaccharides ou LLO) se révéla normale chez vingt-sept patients tant au niveau quantitatif que qualitatif. Comment alors expliquer chez ces patients une hypoglycosylation des protéines ? Nous avons émis l'hypothèse que ces patients étaient déficients dans l'oligosaccharyltransferase (OST), un complexe catalysant le transfert du précurseur oligosaccharidique sur les protéines. L'analyse génétique des sous-unités de l'OST chez ces patients conduisit à l'identification d'un patient atteint d'une **déficience** potentielle **en ribophorin 2**, une sous-unité du complexe OST. Surpris que cette stratégie ne permette l'identification que d'un seul patient, nous avons émis l'hypothèse qu'une accumulation de protéines malconformées au sein de la lumière du RE pourrait interférer avec le transfert du

précurseur glycanique. Une accumulation de protéines mal conformées dans le RE peut être causée soit par une déficience en rétrotranslocation des glycoprotéines mal conformées, soit par une déficience dans les mécanismes de dégradation de ces glycoprotéines mal conformées Ni l'analyse génétique du complexe Sec61, impliqué dans la retrotranslocation de protéines mal conformées, ni l'analyse des oligosaccharides libres, les produits de dégradation des glycoprotéines mal conformées, n'ont révélé des déficiences.

Pour vingt-trois autres patients atteints de CDG-Ix, l'analyse structurale des LLO n'a pas encore été réalisée. L'analyse des LLO au sein de ces patients CDG-Ix identifia cinq patients avec un profil inhabituel **accumulant les intermédiaires Dol-PP-$GlcNAc_2Man_3$ en combinaison avec Dol-PP-$GlcNAc_2Man_5$**. En raison de la similitude entre le profil LLO et le phénotype clinique, quatre de ces cinq patients partageraient potentiellement la même anomalie génétique. Cette anomalie reste malheureusement inconnue car le séquençage des gènes candidats n'a révélé aucune mutation. L'accumulation de **Dol-PP-$GlcNAc_2Man_3$** a aussi été détecté dans un autre patient CDG-Ix, maintenant **en combinaison avec Dol-PP-$GlcNAc_2Man_4$**. Ce patient fut le premier avec une **déficience en ALG11**, une mannosyltransfease impliquée dans les premiers étapes du cycle dolichol.

Dans les cellules de quatorze autres patients CDG-Ix, une **accumulation de Dol-PP-$GlcNAc_2Man_5$** a été trouvée. Cinq d'entre eux étaient **déficients en RFT1**, une protéine impliquée dans le transfert de Dol-PP-$GlcNAc_2Man_5$ du côté cytosolique vers le côté luminal de la membrane du RE. Jusqu'à présent, un seul patient déficient en RFT1 était identifié. L'identification de ces cinq patients supplémentaires nous ont permis d'affiner les caractéristiques cliniques de patients RFT1-CDG. La surdité de perception, rarement observée chez les patients CDG, fut caractéristique pour une déficience en RFT1. Une étude fondamentale sur les cellules de patients RFT1-CDG nous a permis aussi d'identifier une nouvelle espèce d'oligosaccharides libres provenant du Dol-PP-$GlcNAc_2Man_5$. S'accumulant dans les cellules de patients déficients en RFT1, le Dol-PP-$GlcNAc_2Man_5$ serait converti en Dol-P et P-$GlcNAc_2Man_5$. Cette nouvelle voie catabolique fut également observée dans d'autres patients CDG-I Cette formation d'oligosaccharides phosphorylés dans les cellules de patients atteints de CDG-I permettrait le recyclage de Dol-P, un substrat limité dans le processus de N-glycosylation.

L'accumulation de Dol-PP-$GlcNAc_2Man_5$ au niveau des LLO fut également observée chez un autre patient CDG-Ix où l'analyse génétique révéla une **déficience en DPM2.** La protéine DPM2 est une sous-unité du complexe DPM qui synthétise Dol-P-Man à partir de GDP-Man et Dol-P. En contraste aux autres sous-unités du complexe DPM (DPM1 et DPM3), DPM2 n'a jusqu'à présent pas été associé à aucun CDG-I connu. D'un point de vue clinique et

biochimique, DPM2-CDG ressemble parfaitement à une déficience en DPM1 (CDG-Ie) mais est complètement différent de DPM3-CDG qui.

L'analyse des LLO révéla un autre patient accumulant à la fois **du Dol-PP-$GlcNAc_2Man_6$ et Dol-PP-$GlcNAc_2Man_8$**. Ce patient est le troisième patient identifié avec une **déficience en ALG9** (CDG-IL). En raison de l'accumulation des deux intermédiaires d'assemblage, les cellules du patient déficient en ALG9 présentaient un bon modèle pour étudier le contrôle qualité du RE. La caractéristique la plus frappante identifiée au cours de cette étude fut l'augmentation de la dégradation des glycoprotéines mal conformées portant le $Man_8GlcNAc_2$ isomère C. En effet, notre étude suggère fortement que le $Man_8GlcNAc_{2\&}$ isomère C pourrait être un nouveau signal de dégradation pour les protéines mal conformées.

En conclusion, a partir des cinquante patients CDG-Ix étudiés, nous avons donc localisé l'anomalie biochimique dans vingt-deux patients et identifié le déficit génétique dans neuf d'entre eux. En outre, les études fondamentales sur les cellules de patients atteints de CDG-I ont permis d'apporter un nouveau regard sur le contrôle qualité dans le RE et la régulation du cycle dolichol. Le reste des quarante-et-un patients CDG-Ix portent potentiellement des déficiences en protéines inconnues impliquées dans la N-glycosylation et des déficits dans d'autres voies qui participent (in)directement à la N-glycosylation. L'utilisation d'outils génétiques puissants comme la cartographie par homozygotie et le séquençage complet du génome pourrait contribuer à l'identification du déficit génétique dans ces patients.

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PUBLICATIONS

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INTER)NATIONAL CONGRESSES

Congenital Disorders of Glycosylation: the past and the future. **Presentation.** Symposium 'Biomedical glycobiology and glyco-biotechnology', Gent, April 23, 2009.

Identification of novel charged oligosaccharide structures: contribution of two new RFT1-deficient patients. **Presentation.** 19th Joint Meeting, Wageningen (the Netherlands), December 1 and 2, 2008.

Quality control of glycoproteins bearing truncated glycans in a new CDG-IL patient. **Presentation.** Euroglycanet Meeting, Worms (Germany), October 2 and 3, 2008.

Two new patients with a deficieny in the RFT1 protein. **Presentation.** Euroglycanet Meeting, Worms (Germany), October 2 and 3, 2008.

Screening of unsolved CDG-I patients identified a potential mutation in the ribophorin 2 subunit of the OST complex. **Poster.** 18th Joint Meeting, Lille (France), November 5 and 6, 2007.

Normal phenotype of Pmm1 deficient mice points towards functional redundancy and no direct role in CDG syndromes. **Presentation.** Euroglycanet Meeting, Athens (Greece), March 28 and 29, 2005.