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ETUDE DES CELLULES DE NEUROBLASTOMES HUMAINS, LES SKNSH-SY 5Y ET KELLY, COMME MODELES POTENTIELS DE DEGENERESCENCE NEUROFIBRILLAIRE: 1- MISE EN EVIDENCE DE LA SYNTHESE DE L'APOLIPOPROTEINE E ET CARACTERISATION DES TRANSCRITS DE TAU; 2- ETUDE DE LA PHOSPHORYLATION DES PROTEINES TAU ENDOGENES OU TRANSFECTEES.

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ANNEXES

PUBLICATIONS PUBLIEES

Annexe 1: Dupont-Wallois L., Sautière P.-E., Cocquerelle C., Bailleul B., Delacourte A. and Caillet-Boudin M.-L. (1995) : Shift from fetal-type to Alzheimer-type phosphorylated tau proteins in SKNSH-SY 5Y cells treated with okadaic acid. FEBS Letters 357: 197-201.

Annexe 2: Dupont-Wallois L., Soulié C., Sergeant N., Wavrant-de Vrieze F., Chartier-Harlin M.-C., Delacourte A.and Caillet M-L.: Apo E synthesis in human neuroblastoma cells. Neurobiology of Disease: sous presse.

ANNEXE 1

Neurobiology of Disease 00, 1–10 (1997)
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ApoE Synthesis in Human Neuroblastoma Cells

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Apolipoprotein E (apoE) is associated with the two hallmarks of Alzheimer's disease: Aβ deposits and neurofibrillary tangles. ApoE synthesis was detected in astrocytes by *In situ* hybridization but was not detected in neurons. Nevertheless, different studies on apoE immunoreactivity reported the presence of apoE in neurons of Alzheimer, control, and necrosis pontisubicular brains. In this study, we addressed the question of potential synthesis of apoE in neurons and its possible involvement in or in response to pathological conditions. To this purpose, we have studied human neuronal cell lines (SY 5Y and Kelly cells) originating from neuroblastoma. Using monoclonal and polyclonal antibodies, a 32-kDa band was detected in SY 5Y and Kelly cells, before and after NGF differentiation. Two-dimensional gel electrophoresis analysis showed a typical profile of apoE spots resolved to the exact isoelectric points. By reverse transcription-polymerase chain reaction experiments, we demonstrated the presence of apoE mRNA in these cell lines. SY 5Y cells synthesized the apoE3 variant, whereas Kelly cells expressed both apoE3 and apoE4 isoforms, corroborating the two-dimensional gel results. These results suggested that apoE synthesis could occur in human neuronal cell lines under certain conditions. © 1997 Academic Press

INTRODUCTION

Apolipoprotein E (apoE) is the most abundant apolipoprotein in the human brain, which is the second major site of its synthesis (Elshourbagy *et al.*, 1985). ApoE exists as three major isoforms which differ from one another by the presence of a Cys or an Arg at the 112 and 158 AA positions (Weisgraber *et al.*, 1981). These isoforms are named E2 (Cys 112, Cys 158), E3 (Cys 112, Arg 158), and E4 (Arg 112, Arg 158).

In recent years, many reports have provided evidence of a potential role for apoE in Alzheimer's disease (AD). An increase of apoE mRNA was observed in astrocytes of AD brains (Diedrich *et al.*, 1991). Numerous genetic studies clearly demonstrated a modulation of the apoE effect according to the expressed alleles: the ϵ4 allele acts as a risk factor (Strittmatter *et al.*, 1993; Saunders *et al.*, 1993; Corder *et al.*, 1993; Mayeux *et al.*, 1993; Poirier *et al.*, 1993), whereas the ϵ2 allele may have a protective effect (Chartier-Harlin *et al.*, 1994; Corder *et al.*, 1994). Immunohistochemical studies showed that apoE is present in the two neuropathological abnormalities characteristic of this disease: extracellular amyloid deposits and intracellular neurofibrillary tangles (Namba *et al.*, 1991; Strittmatter *et al.*, 1993). Furthermore, allele ϵ4 may have an effect on the duration of the disease and on the amyloid deposits (Frisoni *et al.*, 1995; Schmechel *et al.*, 1993).

In vitro studies have shown a possible direct interaction of apoE with the major component of both neuropathological markers of AD: Aβ peptide, the main component of amyloid deposits (Strittmatter *et al.*, 1993; Wisniewski *et al.*, 1994); and Tau proteins, the main constituent of the paired helical filaments, which are themselves assembled in neurofibrillary tangles (Strittmatter *et al.*, 1994; Huang *et al.*, 1995; Richey *et al.*, 1995; Fleming *et al.*, 1996; Ledesma *et al.*, 1996). These interactions are dependent on the apoE isoform and indicate the presence of apoE in extracellular space and within neurons. Thus, it is important to determine whether neurons are able to synthesize apoE. To date, apoE synthesis has been detected only in glial cells, in

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particular in astrocytes, by *in situ* hybridization, but it has never been observed in neurons (Diedrich *et al.*, 1991; Poirier *et al.*, 1991). Nevertheless, in addition to detection of apoE in astrocytes, the presence of apoE in human neurons is now supported by numerous immunohistochemical studies. Since the first descriptions by Namba *et al.* (1991) and Strittmatter *et al.* (1993), apoE has been detected in neurons (bearing NTFs or not) from Alzheimer subjects (Han *et al.*, 1994a,b; Benzing & Mufson, 1995; Schmechel *et al.*, 1993), in cortical neurons from normal individuals (Metzger *et al.*, 1996), and in neurons of neonates with pontosubicular neuron necrosis (Arai *et al.*, 1996). Pyramidal neurons seem to be preferentially apoE-immunoreactive and the neuron staining intensity in different cortical layers appears laminar (Schmechel *et al.*, 1996; Metzger *et al.*, 1996). In rodent brains, immunocytochemistry for apoE revealed immunoreactivity in several cellular types, in particular in astrocytes, but not in neurons (Schmechel *et al.*, 1996; Xu *et al.*, 1996). When human apoE is expressed in transgenic mice on an apoE knockout background, the apoE-immunopositive neurons are located in specific cortical layers, as was similarly described for primates, whereas no neurons are immunostained for apoE in wild mice (Xu *et al.*, 1996). ApoE present in the neurons seems to be preferentially located in the cell cytoplasm, as demonstrated by electron microscopy studies (Han *et al.*, 1994a; Xu *et al.*, 1996). The presence of cytoplasmic apoE can be explained either by neuronal synthesis of a cytoplasmic form or by direct insertion into the cytoplasm. The latter happens with some bacterial toxins, in particular with *Pseudomonas* exotoxin A, which binds to the same LRP receptor as apoE-enriched lipoproteins (but without intermediate proteoglycan binding). After endocytosis, it escapes lysosomal degradation by translocating across intracellular membranes into the cytoplasm (review in Krieger & Herz, 1994).

Thus, to understand how apoE could interact with Tau or other cytoskeleton proteins and to determine which mechanisms are implicated, it is important to verify that apoE synthesis can take place in the neurons themselves. To investigate the ability of neurons to synthesize apoE, a cell culture system has been created to study neuronal cells of human origin in the absence of glial cells. In this article, we describe the intracellular presence of apoE in SY 5Y and Kelly cells. Reverse transcription-polymerase chain reaction (RT-PCR) experiments directly proved that these two human neuroblastoma cell lines have the ability to synthesize apoE.

MATERIALS AND METHODS

Cell Cultures

SY 5Y and Kelly cells were respectively maintained in Dulbecco's modified Eagle's medium and RPMI 1640 (Gibco BRL) supplemented with 10% fetal calf serum (Boehringer Mannheim). Differentiation of SY 5Y cells was performed with NGF treatment for 4 or 8 days, in the absence of serum, as described elsewhere (Dupont-Wallois *et al.*, 1995).

Protein Extraction and Western Blot Analysis

The cells were harvested at 4°C and collected by centrifugation. Laemmli sample buffer (5% SDS, 0.25% dithiothreitol) was added to the cell pellet. Human or fetal calf serum (±20 μg) was directly diluted in the Laemmli buffer. Human serum came from a control subject. Brain tissue was homogenized in Laemmli buffer (1% wt/vol). The samples were denatured by heating at 100°C for 10 min as described in Dupont-Wallois *et al.* (1995).

After electrophoresis and transfer onto nitrocellulose, the Western blotting method was used to determine the presence of different proteins. The presence of Tau proteins was examined using M19G serum (Sautière *et al.*, 1994; Dupont-Wallois *et al.*, 1995). The polyclonal antiserum 345 recognizes neurofilament subunits NF-L, NF-M, and NF-H, whereas SMI31 monoclonal recognizes a phosphorylated epitope in the carboxy-terminal domain of NF-M and NF-H (Sternberger & Sternberger, 1983). Monoclonal GF5 antibody was specific for the glial fibrillary acidic protein (GFAP) (David *et al.*, 1994). Rabbit polyclonal antibody to NSE (neuron-specific enolase) was purchased from Affiniti (TEBU, France). The presence of apoE was investigated using two distinct antibodies: a rabbit polyclonal antiserum raised against the whole apoE and the EO1 monoclonal antibody for which the epitope is located on the amino-terminal part of apoE (Leroy *et al.*, 1988). Both apoE antibodies were a generous gift from Dr. J. C. Fruchart, and EO1 in particular was used in numerous works (Buée *et al.*, 1996; Lefranc *et al.*, 1996; Gracia *et al.*, 1994). The specificity of apoE binding was checked in simultaneous incubations of EO1 antibodies (1/5000) and apoE4 recombinant protein (0.5 μg/3 ml) purchased from Panvera (Medgene Science S.A.).

Two-Dimensional Electrophoresis

After washing with PBS buffer, the cells were collected by centrifugation, resuspended in Laemmli

buffer
sample, and heat-treated at 100°C for 5 min. Two-dimensional gel electrophoresis was performed as described in Sergeant *et al.* (1997). Briefly, for the first dimension, samples were adjusted to a final concentration of 8 *M* urea and 2% Triton X-100 and were laid onto an isoelectric focusing gel containing 4% (wt/vol) acrylamide and 2.5% (wt/vol) bisacrylamide, 9.5 mol/L urea, 2% (vol/vol) Triton X-100, 4% (vol/vol) pH 3–10 Pharmalytes, and 1% (vol/vol) pH 4–6.5 Pharmalytes (Pharmacia). The second dimension was performed on a 10–20% gradient SDS–PAGE.

Culture Medium Delipidation

Two milliliters of culture medium was centrifuged at 10,000*g* for 15 min at 4°C. The pellet was resuspended in ether and frozen at −20°C for 2 h. After a second centrifugation, the pellet was resuspended in 70 µl of Laemmli buffer and heat-denaturated. The sample was analyzed by dot-blot experiments.

Analysis of Different Transcript Expression

Total RNA was extracted from cells with the RNAzol B method (Cinna/Biotecx) according to the manufacturer's instructions. The yield and the quality of RNA preparations were determined by spectrophotometry. Analysis of the transcripts was performed using RT-PCR.

The primers used in this study were chosen for analysis of the cellular expression of apoE and GFAP. The primers used for the detection of GFAP cDNA were described in Reeves *et al.* (1989), and those for apoE cDNA were described in Pérez-Tur *et al.* (1995). A 1.5- to 2-µg amount of each RNA sample was reverse-transcribed with the Mu-MLV reverse transcriptase (Gibco BRL) using the antisense-specific primer. As the primers used for apoE cDNA detection are located within the same exon of the gene (exon 4, described in Pérez-Tur *et al.* (1995)), total cellular RNA was first treated with DNase I to ensure the absence of genomic DNA contamination (Eurogentec) and then used for RT-PCR experiments. To check the efficiency of the DNase treatment, positive control PCR experiments were performed directly using the treated total RNA samples for PCR amplification by omitting the reverse transcription step. The synthesized cDNA was then subjected to 30 cycles of amplification using the ready sense primer for each amplification. In each RT-PCR assay, a negative control was performed by replacing total RNA with water. The PCR products were analyzed on 2% (wt/vol) agarose gels and visualized by ethidium bromide.

ApoE Genotype

Exon 4 of the *APOE* gene was amplified by PCR from genomic DNA as described in Pérez-Tur *et al.* (1995). The PCR products were specifically digested with restriction enzyme *Cfo*I and subjected to electrophoresis on a 10% nondenaturing polyacrylamide gel. The cell genotype was identified according to the digested product sizes.

RESULTS

Schwab et al 1973)

Neuronal Marker Expression

SY 5Y and Kelly cells are described as neuronal-type cells of tumoral origin (Biedler *et al.*, 1973). In preliminary experiments, we checked that both cells always expressed neuronal markers, such as neurofilaments and NSE, and not glial proteins, such as GFAP.

On Western blots, the three species of neurofilaments (NF-H, 200 kDa; NF-M, 160 kDa; and NF-L, 70 kDa) were revealed in SY 5Y and Kelly cellular extracts. SMI31 monoclonal antibody detected two bands of 200 and 160 kDa corresponding to NF-H and NF-M, respectively, whereas the polyclonal 345 antibody detected three species: NF-H, NF-M, and NF-L (Fig. 1A). As expected, neurofilament detection increased after cellular differentiation, as shown in Fig. 1A, using the monoclonal SMI31. F1

The NSE was also immunodetected by Western blotting before and after cellular differentiation (data not shown).

No trace of GFAP was detected either by Western blotting (Fig. 1B) or by RT-PCR in cells (Fig. 1C). The GFAP detection from brain homogenates was used as positive control for Western blotting and RT-PCRs.

Intracellular Presence of ApoE

Western blotting was used to investigate the presence of apoE in the cellular extracts. Well-characterized monoclonal and polyclonal antibodies detected bands of equal apparent molecular weights in SY 5Y and Kelly cell extracts (Fig. 2A). The same band was also detected in SY 5Y cells differentiated by 4 (Fig. 2A) or 8 days (not shown) of NGF treatment. However, this band showed a lower molecular weight (32 kDa) than apoE from brain tissue homogenates (35 kDa) or human serum (34 kDa). Anti-apoE antibodies were also tested on fetal calf serum and no immunoreactive band could be observed (Fig. 2A). F2

The specificity of the recognition was proved by the

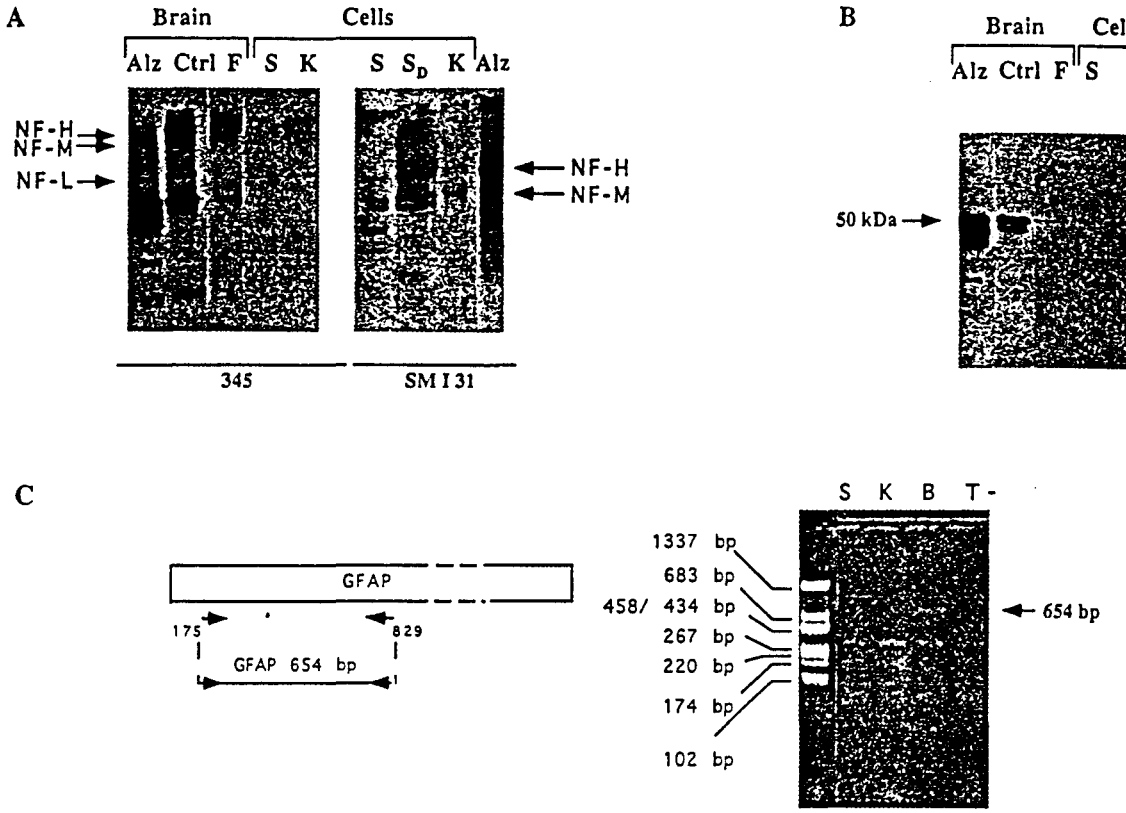

FIG. 1. Neuronal and glial markers analysis. (A) Neuronal markers: presence of the three neurofilament proteins in SY 5Y (S), NGF-differentiated SY 5Y (S_D), and Kelly (K) cell extracts as detected by Western blotting with polyclonal antiserum 345, which recognizes the NF-L, NF-M, and NF-H subunits, or with monoclonal SMI31, which reacts with extensively phosphorylated NF-H and, to a lesser extent, with NF-M. In cells, the three subunits of NF were seen with the polyclonal antibodies 345, whereas NF-H and NF-M are detected with SMI31. Note an increase in SMI31 detection after cellular differentiation (S_D). Homogenates from Alzheimer (Alz), control (Ctrl), and fetal (F) brains were used as positive control. (B) Glial marker: Western blotting with monoclonal GF5 antibody. GFAP was detected only in Alzheimer (Alz), control (Ctrl), and Fetal (F) brain homogenates, not in SY 5Y (S) or Kelly (K) cell extracts. (C) RT-PCR experiments for GFAP detection. No amplification products were obtained with cellular RNAs, whereas a band of the correct size was observed with brain RNA preparations.

absence of the cellular 32-kDa band in the simultaneous presence of EO1 monoclonal antibodies and apoE4 recombinant purchased from Panvera (Medgene Science S.A.) as described under Materials and Methods (Fig. 2B). However, the extinction of the apoE signal was observed only with a narrow ratio of apoE4/antibodies.

The last argument in favor of a cellular apoE presence came from two-dimensional electrophoresis (Fig. 2D). We compared apoE profiles using the polyclonal serum on human brain homogenates for which the genotype was $\epsilon3/\epsilon3$ or $\epsilon3/\epsilon4$ and on Kelly cells. For the three samples, apoE was detected in a gel region corresponding to *pI* 5.25–5.45 and of molecular weight around 34–38 kDa, but the pattern differed in the function of the apoE genotype, the degree of sialylation, and the degree of glycosylation as described in Zannis *et al.* (1981, 1986), Zanni *et al.* (1989), and Visviskis *et al.* (1986). We observed a clear resemblance of cellular and $\epsilon3/\epsilon4$ brain apoE patterns: we could distinguish the apoE3 isoform (spot 2) from the apoE4 isoform (spot 1), and these proteins from their sialylated products (spots 3, 4, and 5). Note that $\epsilon3/\epsilon3$ brain apoE was different because of the absence of the apoE4 variant.

In addition to the 32-kDa band, some bands of higher molecular weight were faintly detected in the different cellular extracts (not shown). The bands of higher molecular weight were also detected in brain homogenates, in particular with EO1 antibodies. Attempts to correlate these bands with the apoE genotype of the brain samples or with the disease were unsuccessful (data not shown).

The presence of secreted apoE in the culture me-

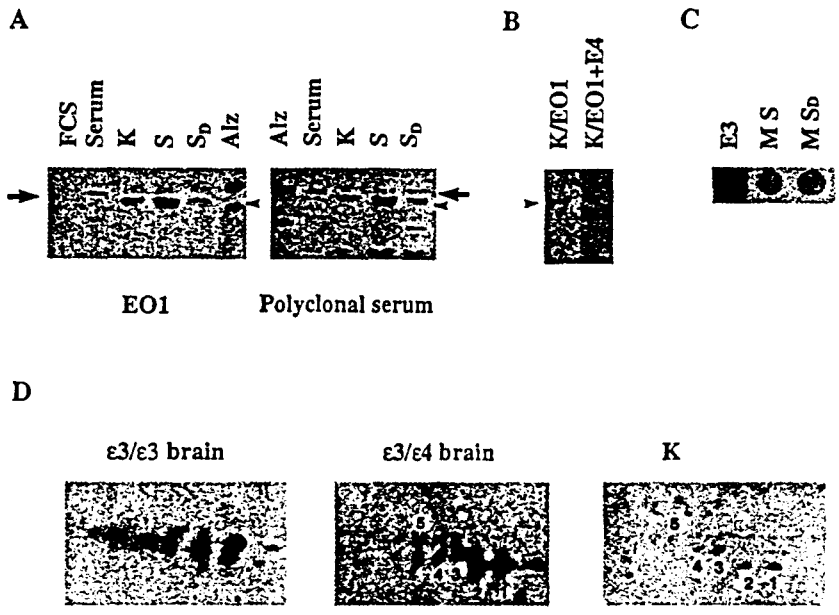

FIG. 2. Analysis of apoE expression in neuroblastoma cells. (A) Analysis of apoE expression in neuroblastoma cells (SY 5Y, S; NGF-differentiated SY 5Y, S_D; Kelly, K) by Western blotting with both monoclonal antibody EO-1 and polyclonal serum. Alzheimer brain (Alz), human serum (serum), and fetal calf serum (FCS) were also analyzed with both antibodies. The arrowheads show the immunoreactive bands corresponding to cell apoE, and the arrows indicate the apoE of human serum or Alzheimer brain homogenate. (B) Specificity of the EO1 antibody was checked by comparison of Western blots revealed using the antibody alone (K/EO1) or previously incubated with apoE4 recombinant protein (K/EO1 + E4). Note the disappearance of the 32-kDa band when antibody was saturated with apoE4. (C) Dot-blot detection of apoE secreted into the cellular culture medium. E3 corresponded to an apoE3 recombinant sample (80 ng), and MS and MS_D to SY 5Y- and NGF-differentiated SY 5Y-delipidated mediums, respectively. (D) Two-dimensional gel electrophoretic patterns of apoE from brain genotyped ε3/ε3, brain genotyped ε4/ε4, and Kelly cells. Only the area of the gel in the vicinity of apoE is presented. Spot 1 corresponds to the apoE4 isoform, Spot 2 corresponds to the apoE3 isoform, and spots 4, 5, and 6 correspond to the sialylated products.

dium was investigated by dot-blot experiments. Only dot-blot experiments performed after sample delipidation suggested a possible but faint apoE secretion in the medium (Fig. 2C).

Cellular Synthesis of ApoE

To demonstrate that human neuroblastoma cells are able to synthesize apoE, the apoE transcripts were investigated by specific amplification of exon 4 by RT-PCR (Fig. 3B). A band of 244 bp, similar to the expected size, was amplified with each total cellular mRNA. The absence of the amplified product in the control experiments and the restriction analysis of the PCR products (not shown) indicated that the RT-PCR-amplified products resulted from the apoE transcripts.

Identification of the Isoforms Expressed by Each Line

We further investigated which apoE variants were expressed in neuroblastoma cell lines. First, the APOE genotype was determined for each cell line as described under Material and Methods. After genomic APOE amplification, the analysis of the *Cfo*I-digested products showed that the genotype of SY 5Y was ε3/ε3 (characterized by the 91- and 48-bp bands) whereas that of Kelly was ε3/ε4 (characterized by the 91-, 72-, and 48-bp bands) (Fig. 3C). The nature of the apoE transcripts of Kelly and SY 5Y had to be investigated by the RT-PCR product analysis after digestion by the *Cfo*I enzyme. The result of the experiment on Kelly cDNA is shown in Fig. 3D. The presence of 91-, 72-, and 48-bp fragments agreed with the expression of both apoE3 and apoE4 mRNA in Kelly neuroblastoma cells. A similar analysis of SY 5Y mRNAs (RT-PCR and *Cfo*I digestion) confirmed the expression of only the apoE3 variant by the SY 5Y cells (not shown).

DISCUSSION

This report is the first demonstration of apoE synthesis by neuronal-type cells. The two SY 5Y and Kelly cell

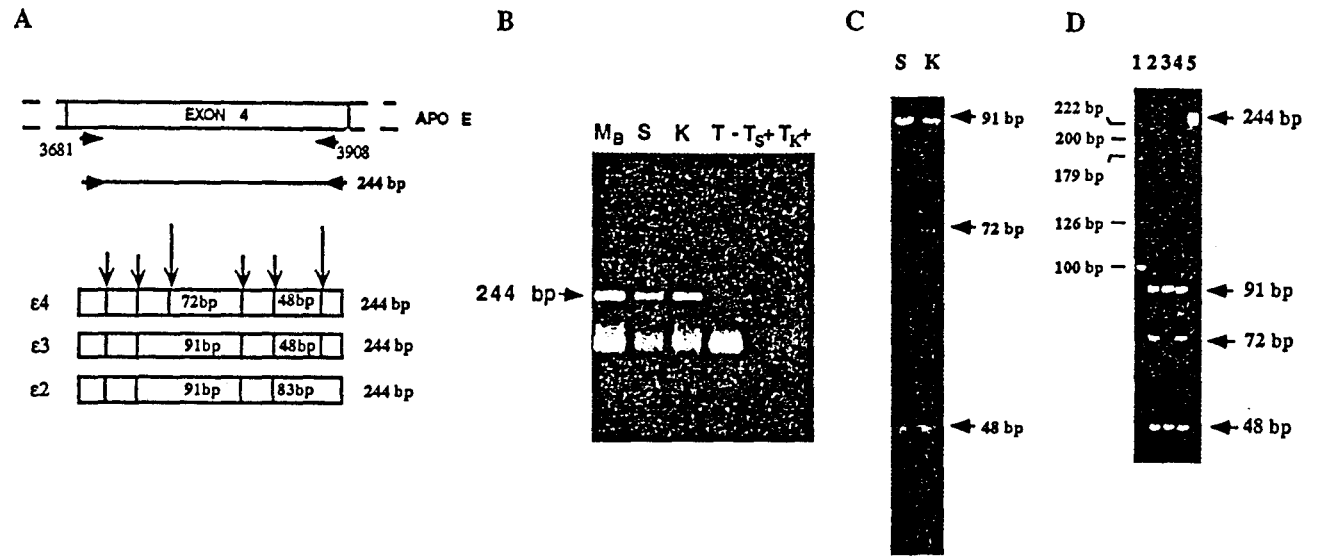

FIG. 3. Analysis of apoE transcripts in both SY 5Y and Kelly cells. (A) Schematic draft representing the amplified polymorphic area (exon 4) of apoE by RT-PCR. (Bottom) Representation of the different *Cfo*I restriction sites located on exon 4 of the three *APOE* alleles. Small and large arrows show the common and polymorphic *Cfo*I restriction sites, respectively. The specific product sizes are annotated on the schematic draft. (B) ApoE synthesis investigation by RT-PCR in SY 5Y (S) and Kelly (K) cells. The cellular RT-PCR products are loaded on a 2% (w/v) agarose gel and their migration is compared to that of ApoE RT-PCR product amplified from mRNAs of adult brain (M_B). T- corresponds to the negative control performed by replacing cellular RNA by water; T_S+ and T_K+ correspond to direct amplification on SY 5Y and Kelly RNAs, respectively, by omitting the reverse transcription step as described under Materials and Methods. (C) SY 5Y (S) and Kelly (K) cell genotype analysis. The cell genotype was identified according to the *Cfo*I-digested product sizes analyzed on a 10% nondenaturing polyacrylamide gel as described under Materials and Methods. (D) Identification of the Kelly apoE transcripts. Kelly RT-PCR products (244 bp) were digested by restriction enzyme *Cfo*I and compared to the ε3/ε4 and ε3/ε3 size markers after migration through a 10% nondenaturing polyacrylamide gel. ε3/ε4 and ε3/ε3 markers corresponded to the *Cfo*I-digested ApoE cDNA from human brains genotyped ε3/ε4 and ε3/ε3, respectively. Lane 1, mix of 100-bp ladder and pGEM DNA marker (Promega); lane 2, ε3/ε4 marker size; lane 3, ε3/ε3 marker size; lane 4, amplified products from Kelly total RNA digested by *Cfo*I; lane 5, RT-PCR product before digestion.

lines used for our study were derived from a human neuroblastoma. SY 5Y cells have been widely described in the literature. These cells are an adrenergic cell line established from human neuroblastoma cells, SKNSH (Biedler *et al.*, 1973), and many of their neuronal features have been reported (West *et al.*, 1977; Biedler *et al.*, 1978; Ammer & Schulz, 1994). They can be differentiated by NGF treatment. Kelly cells are less well known but were described for the first time by Schwab *et al.* (1983). In this study, to confirm the neuronal feature of SY 5Y and Kelly cells, we have chosen to check for the presence of neuronal markers such as neurofilaments and NSE and the lack of glial markers such as GFAP. The three neurofilament subunits (NF-L, NF-M, and NF-H) were effectively detected by polyclonal and monoclonal antibodies in the cells. NF immunodetection was amplified after SY 5Y NGF treatment with monoclonal antibody SMI 31, which binds to phosphorylated neurofilaments (Sternberger & Sternberger, 1983). The initial appearance of NF proteins is known to occur early during neuronal development *in vivo* (Cochard & Paulin, 1984; Carden *et al.*, 1987). Therefore, the presence of NF and NSE and the lack of GFAP confirmed the neuronal type of both cell lines.

The presence of intracellular apoE in Kelly and SY 5Y cell lines, differentiated or not, was first investigated using Western blotting. The specificity of the detected band was checked (1) by the use of two distinct antibodies; (2) by inhibition of cellular apoE detection in simultaneous incubations of antibodies and recombinant apoE; and (3) by two-dimensional gel electrophoresis. Two-dimensional electrophoresis experiments allowed us to assume that (1) the 32-kDa band revealed by the anti-apoE antibodies used in this study actually corresponded to apo E since the immunodetected spots have isoelectric points which exactly correspond to those given by the Swiss-2DPage database (Sanchez *et al.*, 1995), and (2) the apo E sialylation occurred in neuroblastoma cells. However, the apparent molecular weight of apoE in SDS–polyacrylamide gel in our cell cultures was slightly different from that

of human serum. Several factors are known to influence electrophoretic migration, and thus several explanations are possible: a difference in the degree of oxidation, the substitution of one of the amino acids, or a difference in posttranslational modifications, excluding sialylation, as most of the apoE in the serum (Zannis & Breslow, 1981) or in the cells (Fig. 2D) does not seem to be sialylated. Thus, this difference in the molecular weight probably indicates a subtle difference in the molecular structure.

The cellular apoE probably corresponded to the cell-synthesized apoE and not to an uptake from the culture medium. First, in our experiments, apoE was never detected in the fetal calf serum. This result agrees with the fact that this serum is known to be poor in lipoproteins. Second, apoE is present after 4 or 8 days of cell culture in the absence of serum, during the differentiation experiments. In a model of apoE uptake, the extracellular apoE internalized was completely degraded after 24 h of cell culture in the absence of exogenous apoE (Jensen *et al.*, 1994). Third, the phenotype of Kelly cells, determined by two-dimensional gel analysis, corresponded to the transcripts, determined by RT-PCR experiments.

In addition, the capacity of the human neuroblastoma cells to synthesize apoE was demonstrated by the detection of apoE mRNA in the cells, using RT-PCR. Together, our data strongly support *in vivo* apoE synthesis by neuronal cells in culture, but, of course, this does not mean that all brain neurons are able to synthesize apoE *in vivo* or at all stages of their life. Indeed, apoE expression by neuronal cells in the nervous tissues is probably regulated in a different way than in an *in vitro* cellular model. This could explain why apoE synthesis was never detected in neurons although neuronal apoE was detected by different authors in human brain slices using immunohistochemical methods (Namba *et al.*, 1991; Strittmatter *et al.*, 1993; Han *et al.*, 1994a,b; Benzing & Mufson, 1995; Schmechel *et al.*, 1993; Metzger *et al.*, 1996; Arai *et al.*, 1996). Nevertheless, the possibility that some pathologies might restore or exacerbate the ability of the neuron to synthesize apoE proteins cannot be excluded. An effect of pathology on apoE expression has already been reported in AD and in a sciatic nerve injury model (Diedrich *et al.*, 1991; Muller *et al.*, 1985).

In addition to the demonstration of apoE synthesis by neuronal-type cells, an interesting outcome of this study came from the difference in apoE genotypes of these cells: SY 5Y cells expressed apoE3 protein, whereas Kelly cells synthesized both apoE3 and apoE4.

The analysis of a third human neuroblastoma cell line, LA-N-2 cells, characterized previously as a cholinergic neuronal cell line (Seeger *et al.*, 1977; Singh *et al.*, 1990), confirmed that the neuronal synthesis of apoE was not restricted to one neuroblastoma cell type but could be representative of a more general phenomenon (not shown). These last cells were genotyped $\epsilon3/\epsilon3$ (not shown), like the SY 5Y cells.

In conclusion, this article is the first report of apoE synthesis by neuronal-type cells. This result may be important in understanding AD mechanisms. Neuronal apoE expression may differ during development, aging, or pathology. In the present biological system, we show that a physiological level of apoE is compatible with normal development of the cells. Such cell lines (genotyped $\epsilon3/\epsilon3$ for SY 5Y cells or $\epsilon3/\epsilon4$ for Kelly cells) are likely to be of great interest in studies of the role of apoE in a cellular model of neurodegeneration.

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Study of ApoE ... electrophoresis: Title of the article

Electrophorèse bidimensionelle: Title of the book

ANNEXE 2

VARIATION OF APOLIPOPROTEIN E SYNTHESIS DURING NEURONAL DIFFERENTIATION OF HUMAN SKNSH-SY 5Y NEUROBLASTOMA CELLS

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ABSTRACT

As apolipoprotein E (apoE) is the major apolipoprotein expressed in the central nervous system and acts an important role 1) in maintaining the integrity of the aging central nervous system; 2) in repair, growth and maintenance of myelin and axonal membranes during development and after injury; 3) in neurite outgrowth and 4) in neuronal toxicity 5) in pathological processes such as Alzheimer's disease, we have investigated the ability of the neuronal-type cells such as SKNSH-SY 5Y to synthesize apoE. SKNSH-SY 5Y (SY 5Y) cells originated from a human neuroblastoma tumor. The synthesis of molecules characteristic of neuronal cells, the ability to differentiate and the activation of high-voltage channels inductible by several neurotransmitters make these cells an apropriate model of neuronal-type cells.

In this paper, we clearly showed the intracellular presence of apoE in these human neuroblastoma cells and this presence was due to a cellular synhesis. Quantification of apoE synthesis during the neuronal differentiation showed that cellular differentiation induced a variation of cellular apoE synthesis as determined by in situ hybridization experiments. This result suggested that, in vivo, neuronal differentiation could shut off the neuronal apoE expression but this synthesis could be enhanced during stress or pathology. Then, in vivo apoE synthesis could be regulated during development or pathology process.

INTRODUCTION

The SKNSH-SY 5Y, derived from the human neuroblastoma cell line SKNSH (Biedler et al., 1978), have a phenotype ressembling immature sympathetic neuroblasts. When these cells are induced to differentiate with phorbol ester 12-O-teradecanoyl-phorbol-13-acetate (TPA) (Pahlman et al., 1981, 1983), nerve growth factor (NGF) or retinoic acid (Biedler et al., 1978), insulin or insulin like growth factor (Recio-Pinto and Ishii, 1984), they exhibit morphological, neurochemical and electrophysiological properties which are characteristic of neuronal noradrenergic lineage (Pahlman et al., 1990). Neuron specific enolase (Oldestad et al., 1981) and neurofilaments (Wang et al., 1992), two characteristic proteins of neuronal cells, are synthesized by these cells. SY 5Y cells express also receptors for various neuromodulators or neurotransmitters, like μ and γ opioid receptors (Kazmi and Mishra, 1986; Breivogel et al., 1997), muscarinic receptors (Serra et al., 1988; Lambert et al., 1989; Kukkonen et al., 1992; Steel and Buckley, 1993), glutamatergic (Naarala et al., 1993) and nicotinic receptors (Gould et al., 1992). High voltage-gated ion channel are activated by various treatments, such as opioid (Reuveny and Narahashi, 1993; Keren et al., 1997), oxonol (Kukkonen et al., 1996) or inhibitor of Ca2+/calmodulin dependent protein kinase which interact with muscarinic receptors (Puhl et al., 1997). These observations led us to think that SKNSH-SY 5Y might constitute a well characterized model of certain aspects of neuronal cells.

For a few years, apolipoprotein E (apoE) has been thought to play an important role in the central nervous system. It exists as three major structural isoforms: E2, E3, E4 (Weisgraber et al., 1981). According to the expressed isoforms, apo E might play an important role 1) in maintaining the integrity of the ageing central nervous system (Roses, 1995; Masliah et al., 1995); 2) in repair, growth and maintenance of myelin and axonal membranes during development and after injury system (Müller et al., 1985; Poirier et al., 1991; Weisgraber, 1994); 3) in neurite outgrowth in presence of β-VLDL (Nathan et al., 1994) 4) in neurotoxicity in absence of β-VLDL (Tolar et al., 1997); 5) in pathological processes such as Alzheimer's disease (AD) (Strittmatter et al., 1993; Roses, 1995) or Creutzfeld-Jakob disease (Amouyel et al., 1994). The apo E effects were dependent of the

isoform expressed. For example, genetic studies have shown that allele ε4 acts as an important risk factor of the AD whereas allele ε2 would be protector (Saunders et al., 1993; Chartier-Harlin et al., 1994; Corder et al., 1993, 1994). By immunochemical studies of Alzheimer's brain, apoE was located in the two histological markers of the disease: the extracellular amyloid deposits and neurofibrillary tangles (Namba et al., 1991; Han et al., 1994a,b). Thus, extraneuronal and intraneuronal apoE could interact, in a possibly isoform-specific fashion, with the two hallmarks of AD. The interaction of apoE with the different actor proteins of AD led the hypothesis that apoE would be the same cellular compartments as these proteins.

Since the different roles of apoE in cerebral nervous sytem elucidated up to now are always associated to the development and to the stability ot to the degeneration of neurons, it seemed interesting to know if this protein was synthesized in neuronal-type cells such as SY 5Y cells. To answer the question, immunofluorescence, RT-PCR and in situ hybridization were realized. Because one of the hypotheses of the apoE role is an interaction between apoE and cytoskeleton proteins (Roses, 1995), apoE synthesis and cellular localization were compared with those of Tau proteins. Tau are cytoskeletal proteins involved in the neuritic stabilization and extension. Indeed, Tau proteins are mainly, but not only, found in neuron cells (Gu et al., 1996). They are associated to the microtubules and play a major role in the regulation of microtubule assembly, axonal stabilization, formation of bundles and neuritic extension (Drubin et al., 1988; Caceres and Kosik, 1990; Shea et al., 1992; Kanai et al., 1992; Lee and Rook, 1992).

This study shows the ability of some neuronal-type cells to synthesize apoE. These results could be relevant to a regulation of apoE expression during development or pathology of the neuron, in vivo.

MATERIALS AND METHODS

Cell cultures

SKNSH-SY 5Y cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) supplemented with 10% fœtal calf serum. Differentiation was performed by adding 10 ng/ml NGF 2,5S (Sigma, St Louis, MO) in the serum-free medium as described in Sautière et al. (1994). Total cellular extracts were prepared by heating at 100°C the cell pellet (3-4 x 10^6 cells) resuspended in Laemmli's buffer (0,1 ml) as described previously (Sautière et al., 1994).

SDS-Page and Western blotting

Electrophoresis, transfer, Ponceau red staining and Western blotting were performed as described previously (Sautière et al., 1994).

A monoclonal antibody directed against apoE (E01) was used as primary antibody (Leroy et al., 1988). The secondary anti-mouse antibody was purchased from Diagnostic Pasteur. Immunobinding was revealed using the ECL (Enhanced Chemiluminescence) detection kit from Amersham. Similar protocol was applied with apoE specific polyclonal serum. Both apoE antibodies were a generous gift from Dr. J.C. Fruchart's laboratory (Lille, France).

Specificity of the reaction was proven by using E01 antibodies (1/5000) preliminary incubated with apoE recombinant (0,5 µg/ 3 ml) purchassed from Panvera corporation, Madison, USA, (Biogene Science, Paris, France).

Two-dimensional electrophoresis

After washing with PBS buffer, the cells were collected by centrifugation and resuspended in Laemmli sample and heat-treated at 100°C, 5 min. Two-dimensional electrophoresis gels were performed as described in Sergeant et al., (1997). Briefly, for the first dimension, samples were adjusted to a final concentration of 8M urea and 2% Triton X-100 and were laid onto an isoelectric focusing gel containing 4% (wt/vol) acrylamide and 2,5% (wt/vol) bis-acrylamide, 9,5 M/L urea, 2% (vol/vol) Triton X-100, 4 % (vol/vol) pH 3-

10 Pharmalytes™ and 1% pH 4-6,5 (vol/vol) Pharmalytes ™ (Pharmacia). The second dimension was performed onto a 10-20% gradient SDS-PAGE.

Commercial apoE were processed in the same way.

Immunofluorescence

Cell cultures were fixed at room temperature with a 4% paraformaldehyde solution in phosphate buffer (PB) for 10 minutes. Before incubation with the primary antibodies, cells were first treated by glycine 0,1M, then, saturated by donkey serum (5% in PB) (Interchim, France) during 30 minutes to decrease the background. The primary antibodies used were: M14 monoclonal antibody which detects neurofilaments (NF) (Riederer's gift) (Riederer et al., 1993), M19G polyclonal serum (1/1000), directed against the N-terminal end of Tau (Sautière et al., 1994; Dupont-Wallois et al.,1995), and the E01 monoclonal antibodies specific of apoE and previously described in SDS-Page and Western blotting section. The antibodies were diluted in 0.25% Triton X-100 phosphate solution. The incubation lasted 90 minutes at room temperature and was followed by 3x PB washes and by incubation for 90 minutes with FITC- or TRITC-conjugated donkey anti-mouse IgG or anti-rabbit IgG (1/300) (Interchim, France). After 3x PB washes, samples were mounted in Vectashield buffer (Vector Laboratories, Burlingame, USA) and examined under an axiophot (Zeiss) epifluorescence microscope.

After saturation by donkey serum, double staining was obtained by cell incubation with a mix of mouse monoclonal E01 antibodies and rabbit M19G polyclonal serum. Then, the cells were washed 3 x by PB buffer and incubated with FITC-conjugated donkey anti-mouse IgG and TRITC-conjugated donkey anti-rabbit IgG.

The specificity of the immunoreactivity was controlled either by omitting the primary antibody or by using a normal mouse serum as primary antibody (Fig. 2A, 2B). For apoE, control experiment was also performed by using E01 antibody (1×10^{-12} M) preadsorbed with apoE3 protein (10×10^{-12} M) (Panvera corporation, Biogene Science, Paris, France) (not shown).

Reverse transcriptase-polymerase chain reaction and *apoE* genotypage.

Total cellular RNA was extracted by the RNAzol method (Cinna/ Biotecx) according to the manufacturer's instructions. To eliminate possible contamination by cellular DNA, RNA samples were treated by DNase I (Eurogentec) before reverse transcription. The primers used for apoE mRNA detection were the same as those used by Pérez-Tur et al. (1995) and allowed the amplification of a DNA fragment of 244 bp containing the *apoE* polymorphism Control experiments were performed either by omitting RNA in RT-PCR experiments or in presence of RNA but by passing over the cDNA synthesis step. PCR was carried out in a Perkin Elmer thermal cycler using 30 cycles consisting of denaturation at 94°C for 1 minute, followed by annealing at 65°C for 1 minute and DNA extension at 72°C for 2 minutes. The cDNA amplified was analyzed by migration through agarose gel. The amplified products was checked by *Cfo I* restriction enzyme digestion.

Control of *apoE* genotype was performed using genomic DNA, obtained from SKNSH-SY 5Y cells by the method of Miller et al. (1988). After digestion by *Cfo I* enzyme and migration through a 10% non denaturing polyacrylamide gel, the fragment pattern was compared to those of previously genotyped brain DNA (Pérez-Tur et al., 1995).

In situ hybridization

1- Probes

Three 40-mer antisense oligonucleotide probes (^{5}'CCAGGAATGTGACCA GCAACGCAGCCCACAGAACCTTCAT^{3}', ^{5}'TTCAACTCCTTCATGGTCTCGTCCATCA GCGCCCTCAGTT^{3}', ^{5}'CATGTCTTCCACCAGGGGCTCGAACCAGCTCTTGAGGCGG^{3}) complementary to sequences encoding apoE mRNA and one complementary to Tau mRNA (^{5}'TGGTTTGTAGACTATTTGCACCTTCCCGCCTCCCGGCTG^{3}') were employed for hybridization experiments. Sense oligonucleotide corresponding to the last one was used for control experiments. The probes (50 ng) were labelled at the 3' end with [^{35}S]dATP (Amersham) using terminal transferase (Amersham) following manufacturer's protocol. After 90 minutes incubation at 37°C, the reaction was stopped by the addition of 1µl of 200mM EDTA, pH 8, and 1µl yeast tRNA (5 µg/µl). The labelled nucleotides were separated from

non-incorporated nucleotides chromatographically using a quick spin column sephadex G 25 fine (Boehringer Mannheim, France). The probes were labelled to a specific activity of approximatively $2x10^5$ cpm/µl and stored at -20°C.

2- Cell fixation

Cells grown on glass slides were fixed with 4% paraformaldehyde in PB for 5 minutes, rinsed in PB, and stored at -80°C. Slides were defrozen 15 minutes at room temperature. Each area of cultured cells was incubated in glycine 0.1 M, Tris 0.2 M, pH7.4 for 10 minutes and then 15 minutes with proteinase K at 0.05 µg/ml in Tris/EDTA buffer. The cells were fixed again with 4% paraformaldehyde for 15 minutes.

3- Hybridization

The slides were first incubated for 55 minutes in prehybridation buffer constituted of 4x standard saline citrate buffer (1x SSC= 0.15 M NaCl and 0.015 M sodium citrate, pH7). Then, hybridization experiments were performed with each of the oligonucleotide probes (10^6 cpm per 30 µl of the hybridization buffer) and one experiment with a mixture of the three apoE probes ($0.5.10^6$ cpm of each probe per 30 µl of the hybridization mixture). The hybridization buffer was constituted by 50% deionized formamide, 1x Denhardt's solution (2% each of polyvinyl pyrolidone, bovine serum albumin and Ficoll), 4xSSC, 1x Sarkosyl, 0.1M phosphate buffer and 10 mM dithiothreitol (DTT). The slides were placed in a humid chamber for 16 hours at 42°C. Following hybridization, slides were rinsed in 1xSCC with 10 mM DTT, then in 1xSSC for twice 55 minutes at 20°C and 45°C, and allowed to dry. Slides were dipped in LM1 (Amersham) and exposed for 4 weeks. The slides were developed in D19 (Kodak), for 4 minutes and fixed in 30% sodium thiosulfate. Finally, the slides were stained with Azur blue 2/1000, mounted and coverslipped.

4- Controls

Specificity of the probes was established by use of either a sense probe or an excess of unlabelled probes (20 times more) over the labelled probes, which resulted in the abolition of the specific signal.

5- Quantification

Quantification of apoE and Tau mRNAs was performed under epifluorescence by counting silver grains in at least 80 cells by slide using the computer-based image analysis Biocom system (Biocom, Paris, France). As after 8 days of cell culture, some cells began to suffer and could be distinguish from the other ones by the nucleus size. Quantification was only performed on the healthy cells (i.e with a normal size of the nucleus). The results are presented as a silver grain density (grain number per μm^2) which is representative of the mRNA level. The standart deviation and the probability p factor was determined by Student's t-test.

RESULTS

Biochemical evidence for apoE presence in SKNSH-SY 5Y cells

Using monoclonal E01 antibodies specific of apoE protein, the western blotting analysis of the total cellular extract revealed a band of about 32-34 kDa (Fig. 1A). The correct identity of the band was confirmed by detecting the same band using a specific polyclonal serum directed against the apoE (Fig. 1A), by inhibition of the detection of this band when antibodies were saturated by recombinant apoE3 proteins (purchased from Panvera) (Fig. 1B), by 2D-electrophoresis experiments which showed that the immunospot detected with the polyclonal serum specific to apoE is located in the gel region with a pI (5,25-5,45) and M_r (34 kDa) corresponding to apoE protein according to the SWISS-bank database (Sanchez *et al.*, 1995) (Fig. 1B). Furthermore, the immunospot was located in the same region that the main spot of recombinant apoE3 (Fig. 1C).

To check if apoE detection was dependent or not on the differentiation state, SY 5Y cells were differentiated by NGF treatment for different days (0, 4, 8 days) and total cellular extracts were analyzed by western blotting. ApoE was detected in each extract (Fig. 1C).

ApoE detection by immunofluorescence

Immunodetection was performed on both undifferentiated and NGF-differentiated cells. No immunoreactivity was detected using a normal serum of mouse (Fig. 2A, B). Using E01 antibodies, apoE immunolabelling was observed in the cytoplasm (Fig. 2C). In addition, neuritic processes, grown during NGF treatment, were clearly immunostained (Fig. 2D). The immunofluorescent signal was abolished when using preadsorbed serum with apoE3 protein in a strict ratio of antigen/antibodies (not shown).

ApoE cellular localization was compared with those of Tau proteins and neurofilaments. Anti-Tau antibodies immunolabelled mainly cytoplasm in both undifferentiated and differentiated cells (Fig. 2E, F). Neuritic processes of differentiated cells were also Tau-immunoreactive but the labelling was most often observed only in the growth cone (Fig. 3D). However, in some cases a labelling was also observed in the proximal part of

the processes (Fig. 2F). Using monoclonal specific antibodies, M14, cellular neurofilaments are observed in the cytoplasm of undifferentiated cells but limited at the basis of neurite elongation site (Fig. 2G). A labelling of the neuritic extensions together a large cytoplasmic signal was obtained after NGF treatment (Fig. 2H).

Experiments of double staining for apoE and Tau proteins confirmed the presence of the both proteins in the cytoplasm (Fig. 3A, C). In neuritic processes, apoE was present all along whereas Tau protein was mostly restricted to the growth cone (Fig. 3B, D).

Variation of apoE mRNA synthesis during the NGF-differentiation of the SKNSH-SY 5Y

Cellular apoE mRNA synthesis was first examined by RT-PCR experiments. Cellular RNA was purified, then analyzed by RT-PCR. To avoid a false positive reaction because of DNA contamination, RNA samples were previously treated by DNAse. Control experiment using RNA for direct PCR assay confirmed the absence of DNA in our preparation. By RT-PCR, a band of 244 bp, similar to the expected size of apoE band, was obtained by amplification of the cDNA corresponding to the DNAse-treated cellular RNA (Fig. 4B). Analysis by *CfoI* restriction enzyme confirmed the amplification of the correct product corresponding to the expression of only the ε3 allele (not shown). This product corresponded to the the restriction pattern ε3-ε3 genotype of the apoE fragment amplified from cellular DNA and digested by *Cfo* I effectively corresponded to the *apoE* (Fig. 4C).

To confirm the presence of apoE mRNA in the SKNSH-SY 5Y cells, and to test whether all cells and not only a subpopulation could synthesize apoE protein, in situ hybridization experiments were performed. A better detection was observed when using the three apoE oligonucleotide mixture when compared to each oligonucleotide used separately. ApoE mRNA was visualized in all SKNSH-SY 5Y cells and silver grains counterstained cell perikarya (Fig. 5A). Positive and negative controls were performed using anti-sense and sense Tau oligonucleotides probes: numerous grains were detected with Tau antisens probe (Fig.

5B) whereas only an aspecific background was observed with Tau sens oligonucleotide (Fig. 5C).

Quantification of apoE and Tau were then performed during the differentiation process by counting the silver grains as described in materials and methods (Table 1, Fig. 6). A significant decrease of the apoE mRNAs occurred during the differentiation time: the apoE mRNA density mean was of 37.7 ± 11.2 for undifferentiated cells but of 17.61 ± 4.8 for NGF-4 day-differentiated cells, p=0.001 and 26.4 ≠ 8.58 for NGF-8 day-differentiated cells, p=0.001. Then, apoE synthesis first decreased during the 4 days of differentiation but increased again after 8 days. Conversely, Tau mRNA density first increased after 4 days of differentiation and then decreased: Tau mRNA density mean was of 20.143 ± 4.593, 43.578 ± 12.51 (p=0.001) and 25.2 ± 7.14 (p=0.001) respectively for undifferentiated, NGF-4day-differentiated cells, NGF-8day-differentiated cells.

DISCUSSION

Using a multidisciplinary approach, we demonstrate here that human neuroblastoma SKNSH-SY 5Y cells in cultures are able to synthesize apoE and this synthesis seems to be regulated during the differentiation process.

Synthesis of ApoE in neuronal-type cells

The neuronal characteristics of the SKNSH-SY 5Y cells are largely admitted in the literature. Our studies confirm the neuronal feature of these cells by detecting neurofilaments and Tau proteins (Fig. 2G,H). We have also checked that these cells always express Neuronal Specific Enolase (NSE) and not Glial fibrillary acidic protein (GFAP), specific of glial cells (not shown).

The cellular synthesis of apoE has been investigated by two different techniques: RT-PCR and in situ hybridization. The in situ hybridization showed that all cells synthesize apoE. The specificity of the probes was controlled as described in material and methods. The cellular synthesis of apoE occurred in differentiated or undifferentiated cells but with a different degree of expression: a significant decrease of the apoE mRNA was observed after 4 days of differentiation and then an increase after 8 days of differentiation. Similar results were observed in distinct experiments. This could mean that cellular apoE synthesis is first downregulated during the differentiation before being stimulated again after four NGF treatment days. This stimulation appeared during the long time of NGF cell treatment, suggesting apoE synthesis could stimulate the cellular survival. Unlike apoE mRNA, Tau mRNA increased during the first days of NGF treatment and then decreased. This last result agrees with Przyborski and Cambraydeakin' s data (1995) obtained in another cellular model: during the differentiation of cerebellar granule cell neurons, Tau mRNA level first increases and then decreases and more especially fœtal Tau mRNA. In SY 5Y, the main isoform of synthesized Tau proteins corresponds to the fœtal form (Dupont-Wallois et al., 1995).

Are the SY 5Y cells relevant to an in vivo apoE expression by the neurons?

Cerebral apoE synthesis was mainly described in glial cells, but immunohistochemical studies showed apoE presence in neurons of Alzheimer brain (Han et al., 1994a,b), control brain (Metzger et al., 1996), patients with pontosubicular necrosis (Arai et al., 1996) and in rat pyramidal neurons after a transient ischaemia injury (Horsburgh and Nicoll, 1996). Since hybridization in situ was not performed in these studies, we don't know whether it is cellular uptake or neuronal synthesis. If apoE mRNA synthesis takes place in the neurons, several hypotheses might explain why it is not detected in neurons of brain slices. First, it cannot be totally excluded that apoE synthesis in SY 5Y cells resulted from a dysregulation of the cell lineage metabolism during successive cell culture passages even if these cells had retained neuronal features as discussed above. In this case, cellular synthesis of apoE would be specific to these neuroblastoma cells. But other explanations are possible. The first explanation could be that too faint a labelling in neurons might not be discerned from background, especially when a strong labelling is detected in glial cells. If a degradation of mRNA occurred in brain neurons during the post-mortem delay, the low apoE mRNA level would decrease and explain the apparent absence of apoE mRNA. In our experiments, the detection sensitivity is improved by the use of three oligonucleotides, complementary to three distinct regions along the apoE mRNA. A second possibility is that, in SKNSH-SY 5Y cells, apoE synthesis is not regulated in a same way as in brain tissue, allowing an expression which could normally be shut off in physiological conditions. The changes of apoE cellular expression might indicate that in vivo differentiation lead to level modification of apoE expression. In this way, a complete or nearly complete extinction of apoE synthesis could occur in healthy neurons. Thus, it cannot be excluded that in some pathologies, this synthesis would be restored even to a limited extent. This might explain neuronal apoE immunolabelling described in Alzheimer brain neurons (Han et al., 1994a,b), in neonates with pontosubicular neuron necrosis (Arai et al., 1996), and in degenerating pyramidal neurons in the CA1 region after a transient ischaemic injury in rat (Horsburgh and Nicoll, 1996). A third explanation is that only a subclass of neurons is able to synthesize apoE: in this context, it can be mentioned that apoE protein has recently been detected in many small pyramidal neurons

in cortical layer III and in few larger pyramidal cells with long projection in layer V of the human brain frontal cortex in normal subjects (Metzger et al., 1996). Surprisingly, the human apoE expressed in transgenic mice after the inhibition of the endogenous apoE protein (knock-out mice) was also immunodetected in pyramidal neurons in layers III and V whereas no neuronal apoE was immunodetected in wild mice (Xu et al., 1996). Therefore, these last two observations might be in favour of apoE presence in some well defined population of neurons.

Cellular localization of apoE proteins is consistent with its eventual implication in the neuritic growth and interactions with cytoskeletal components

By immunofluorescence, we show that all SKNSH-SY 5Y human neuroblastoma cells contained apoE in the cytoplasm. In NGF-differentiated cells, neuritic processes were also labelled. This cellular localization of SY 5Y apoE is in agreement with other observations made in brain in which apoE was confined to the cytoplasm and in proximal cellular processes (Han et al., 1994b; Metzger et al., 1996). The presence of apoE in neuritic process is compatible with its role in neuritic growth or regeneration (Ignatius et al., 1986; Nathan et al., 1994, 1995; Bellosta et al., 1995; Narita et al., 1997). Our results differ from those of William's et al. (1997) who did not detect apoE in a human neuronal cell line derived from human embryonic carcinoma: Ntera2/D1 cells. This could be explained by the fact that these cells corresponded to another type of neuronal cells and this would be in favour of a neuron-type dependent apoE expression as dicussed above. In the other hand, we had also to notice that the experimental conditions were different for the two studies: in particular, cell Ntera2/D1 fixation and permeabilization were quite drastic using methanol fixation during 20 minutes and Triton 4% permeabilization whereas SY 5Y cells were fixed by a 4% paraformaldehyde solution in phosphate buffer (PB) for 15 minutes and Triton was used to 0,1%. Indeed, we had observed that cytoplasmic detection of apoE SY 5Y cells was nearly abolished by the 4% paraformaldehyde/(0,1%) picric acid mix and the morphology of SY 5Y cells were damaged by a methanol fixation.

ApoE localization was compared with that of Tau proteins which are also involved in the neuritic growth (Caceres and Kosik, 1990; Kanai et al., 1992). Tau immunofluorescence was detected in the cytoplasm of both differentiated and not differentiated SY 5Y cells. After cell differentiation, Tau was also located in neuritic processes but, its detection was not along neuritic processes. This result agrees with recent reports which described Tau in the distal part of the axon (Black et al., 1996; Kempf et al., 1996) and more especially in growth cone (as seen in fig. 3D) or in swellings on neuritic processes. The cellular localization for Tau and apoE proteins in our model are consistent with a role of these two proteins in the neuritic growth.

Conclusion

The present study clearly demonstrate an apoE synthesis in human neuroblastoma SY 5Y cells. The neuronal feature of these cells are largely admitted in the literature. Our results allow to hypothetize that, in vivo, a neuronal apoE expression could be regulated during the differentiation and perhaps nearly shut off in a healthy mature neuron. In this case, apoE synthesis could be stimulated in reponse to a degenerative process such as Alzheimer's disease and could then have a predominant role for neuron survival. The fact that apoE could also be synthesized by neurons would be important in the understanding of apoE role in neuronal growth, differentiation and survival.

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FIGURE LEGENDS:

Fig. 1: Detection and cellular localization by western blotting.

(A) Detection of cellular apoE. Cell extracts were analyzed by western blotting using monoclonal E01 antibody or polyclonal serum directed against apoE. A band of about 32 kDa was detected.

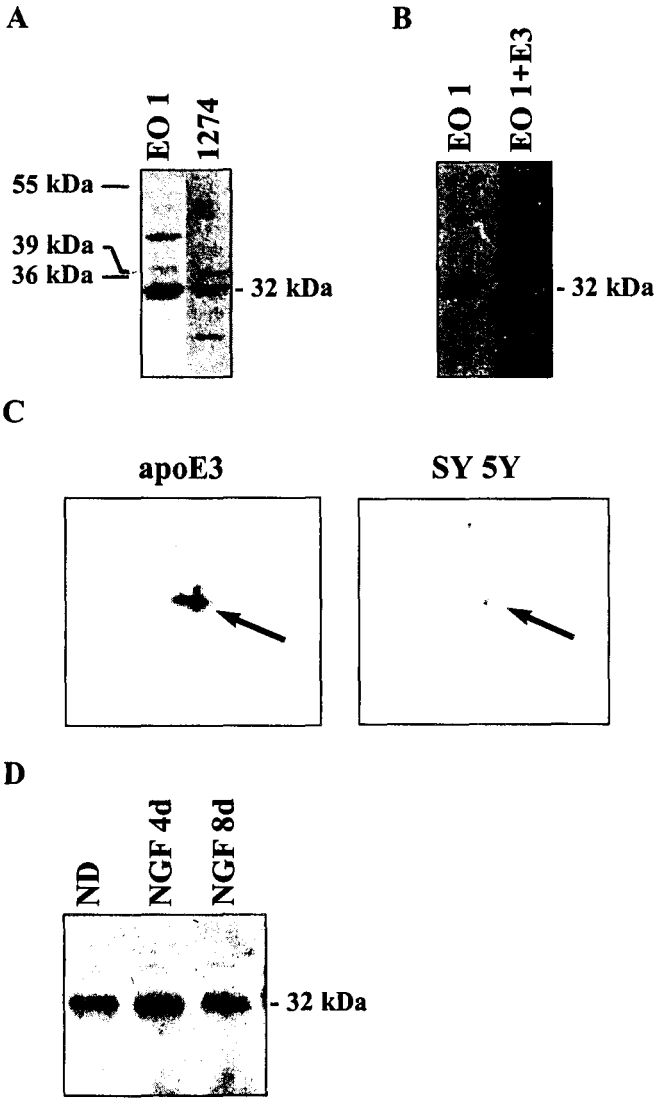
(B) Saturation of the E01 antibody with apoE3 recombinant protein. The 32 kDa band was detected before (E01) and not after antibody saturation (E01+E3).

(C) Two-dimensional electrophoresis of commercial recombinant apoE3 and SY 5Y cellular extract. The SY 5Y spot was detected by polyclonal serum exactly to the same region that this of the main spot of recombinant apoE3. The minor acidic isoforms seen with apoE3 recombinant sample could be due to glycosylation and deamination differences according to the furnisher's data sheet.

(D) Analysis of differentiated-cell extracts by polyclonal serum directed against apoE. Cells were undifferentiated (ND) or differentiated by NGF treatment during 4 days (NGF 4d) or 8 days (NGF 8d). ApoE was detected in each extracts.

Fig. 2: Comparison of apoE (C, D), Tau (E, F) and neurofilaments (G, H) immunoreactivities of SKNSH-SY 5Y neuroblastoma cells undifferentiated (A, C, E, G) or differentiated (B, D, F, H) by a 2 day-NGF treatment. Cells stained with mouse pre-immunserum showed no immunoreactivity (A, B). The E01 monoclonal antibody mainly detected the cytoplasm (C, D) and neuritic processes of differentiated cells (D). Tau antibodies (M19G) (E, F) and neurofilament antibodies (M14) (G, H) strongly detected cytoplasmic elements and neuritic processes when differentiation was performed. Magnification: 400X.

Fig.3: Double label Tau-apoE immunofluorescence. Experiments were performed in un-differentiated (A, C) and differentiated cells (B, D). Tau antibodies were detected by TRITC-conjugated donkey anti-rabbit IgG (C, D) whereas ApoE antibodies (E01) were recognized by FITC-conjugated anti-mouse IgG (A, B).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1274
55 kDa
39 kDa
36 kDa
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B
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EO 1+E3
32 kDa
C
apoE3
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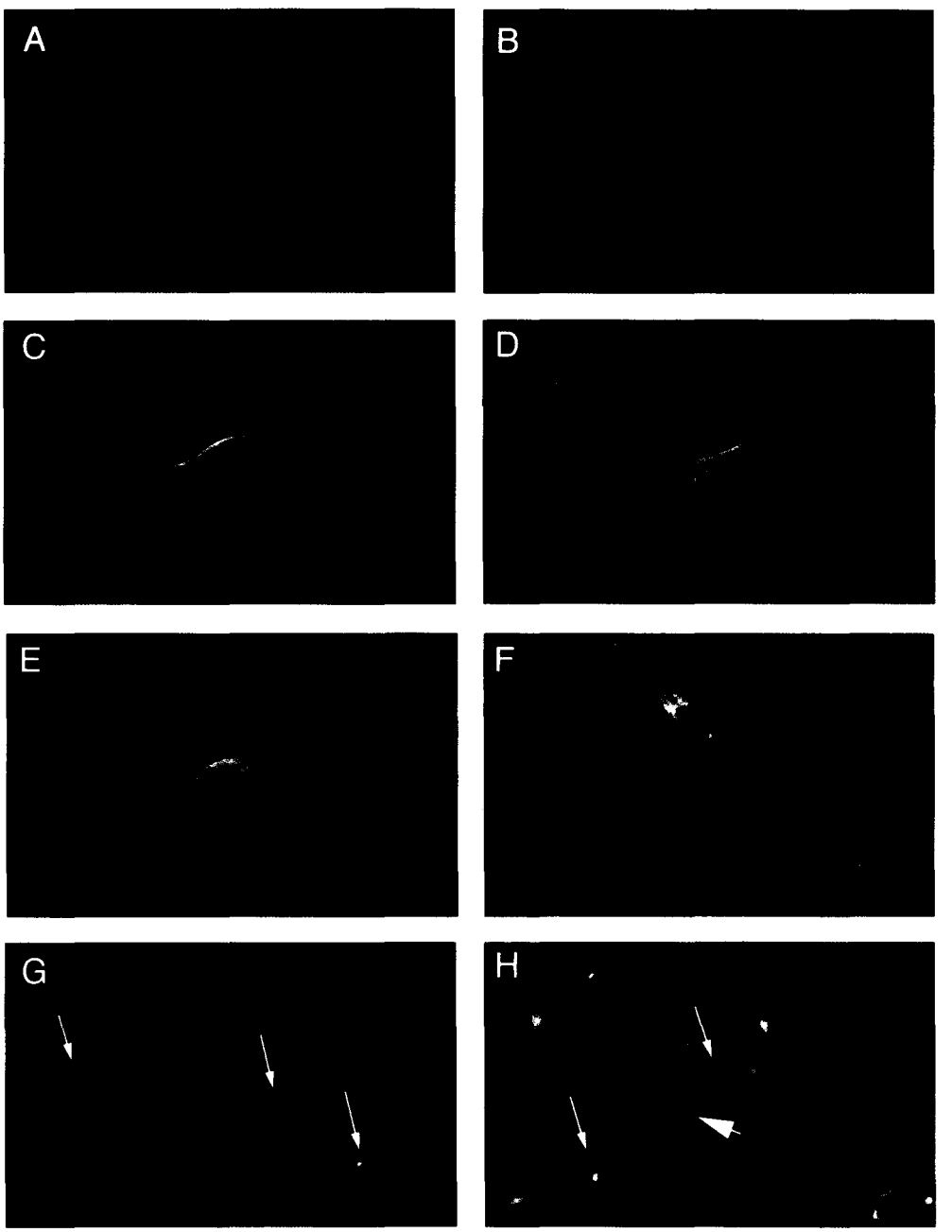
D
ND
NGF 4d
NGF 8d
32 kDa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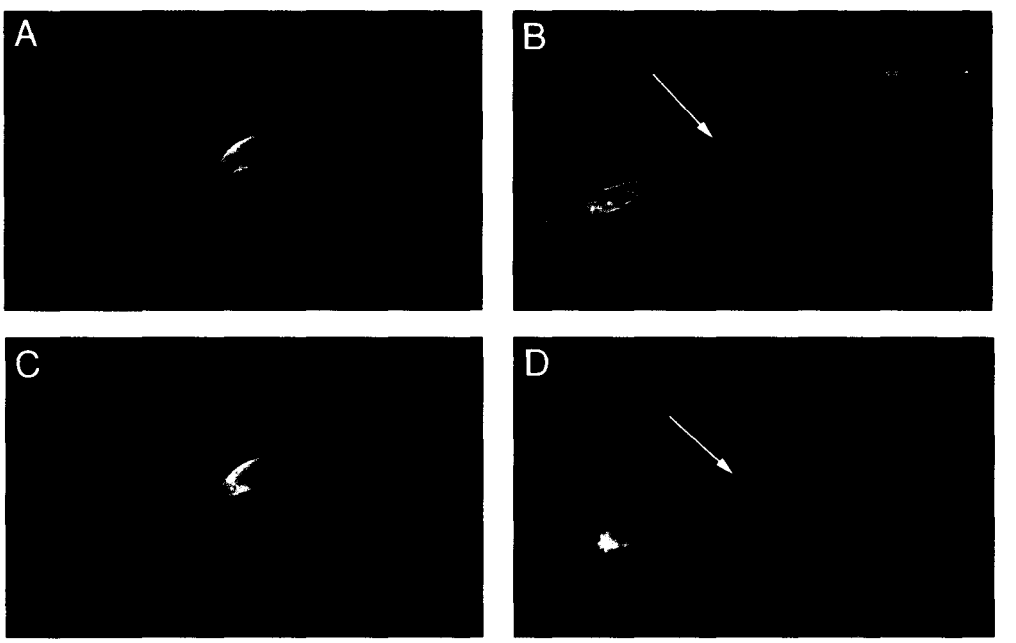
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Fig. 4: Detection of apoE mRNAs by RT-PCR.

(A) Schematic representations of the region studied: mapping of the oligonucleotides used and indications of native and digested product sizes.

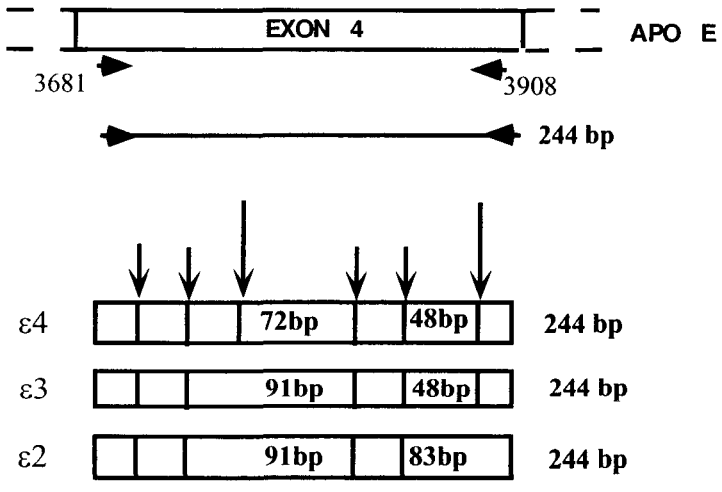
(B) Detection of apoE mRNA in SY 5Y cells by RT-PCR: lane 1: Control of apoE size: apoE PCR amplification of brain DNA; lane 2: RT-PCR using SY 5Y cell mRNA; lane 3: RT-PCR control in absence of mRNA, lane 4: direct PCR assay using mRNA. The 4th lane allowed to assume that the apoE band detected in lane 2 was effectively due to apoE mRNA and not to a DNA contamination.

(C) ApoE genotyping: Analysis of *Cfo I* restriction patterns of DNA from cells and brains. A brain sample, genotyped ε3/ε4 was used as migration control of the different characteristic bands. Because the 48 and 96 bp but not the 72 bp band were detected, the restriction pattern of the cell cDNA corresponded to the genotype ε3/ε3.

Fig. 5: Detection of apoE mRNA (A) and Tau mRNAs (B) by in situ hybridization experiments. Silver grains were located on nucleus stained with Azur blue. Hybridization with Tau oligonucleotide sens (C) constituted a negative control and demonstrated only a few silver grains corresponding to the experimental background.

Fig. 6: Quantification of apoE and Tau mRNA. The cells were undifferentiated (ND) or differentiated by NGF treatment for 4 days (NGF 4d) or for 8 days (NGF 8d). White histograms corresponded to apoE mRNA, black ones to Tau mRNA. Data are mean ± SEM values (bars). * corresponded to $p = 0.001$. For each sample, p value was calculated versus corresponding undifferentiated cell sample.

A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APO E
3681
3908
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ε4
72bp
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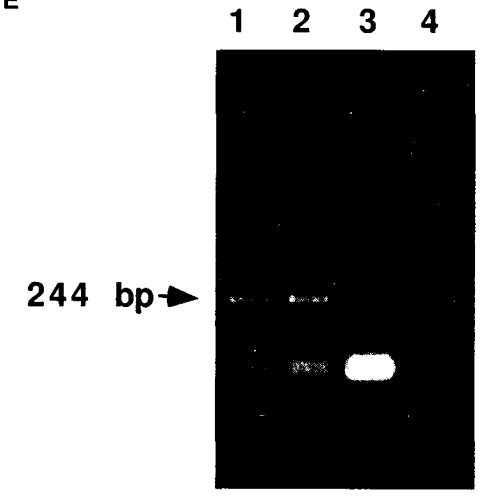
ε2
91bp
83bp
244 bp

B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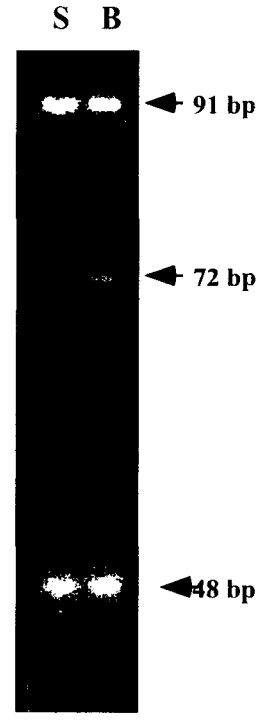
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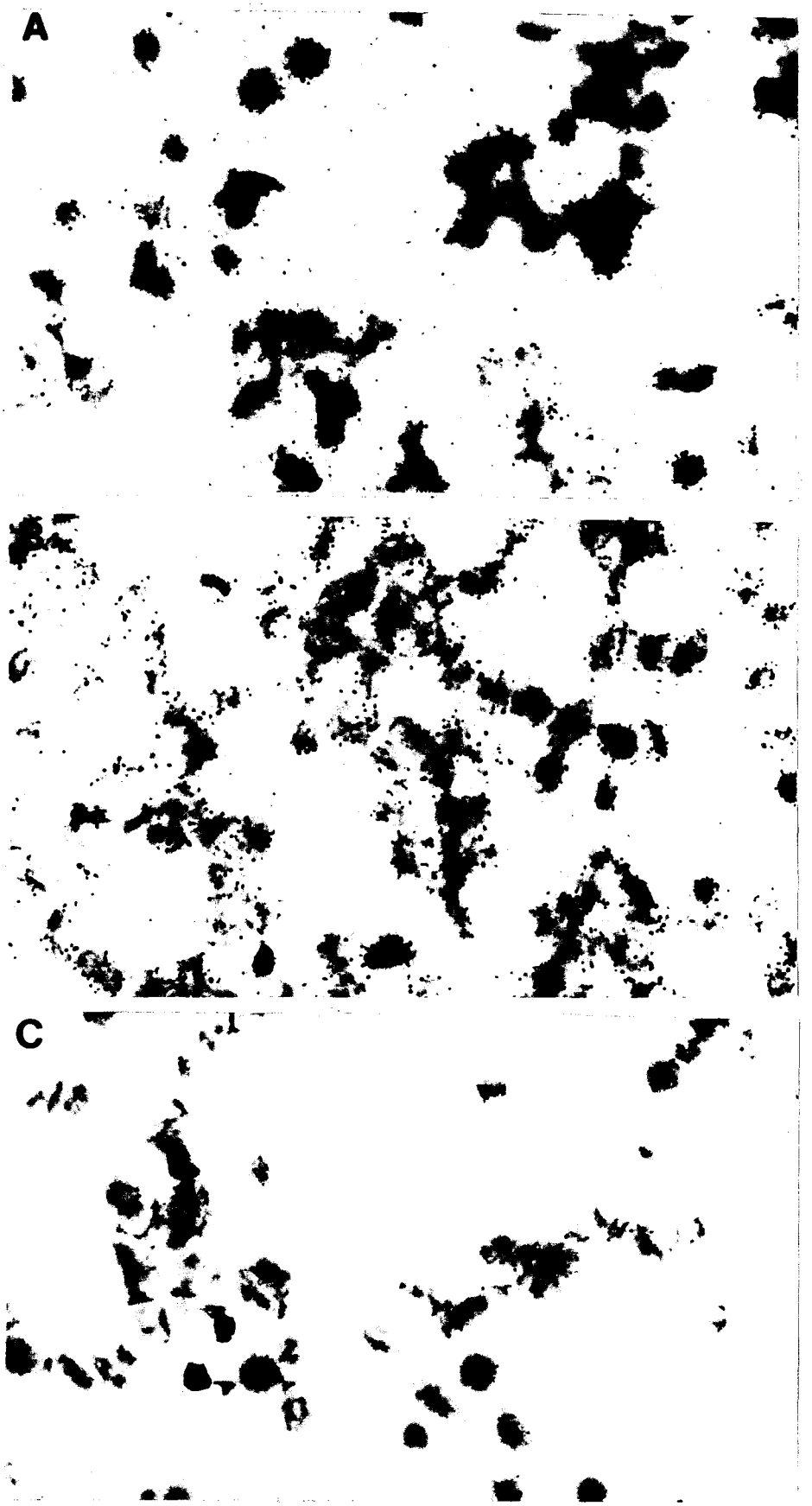
A
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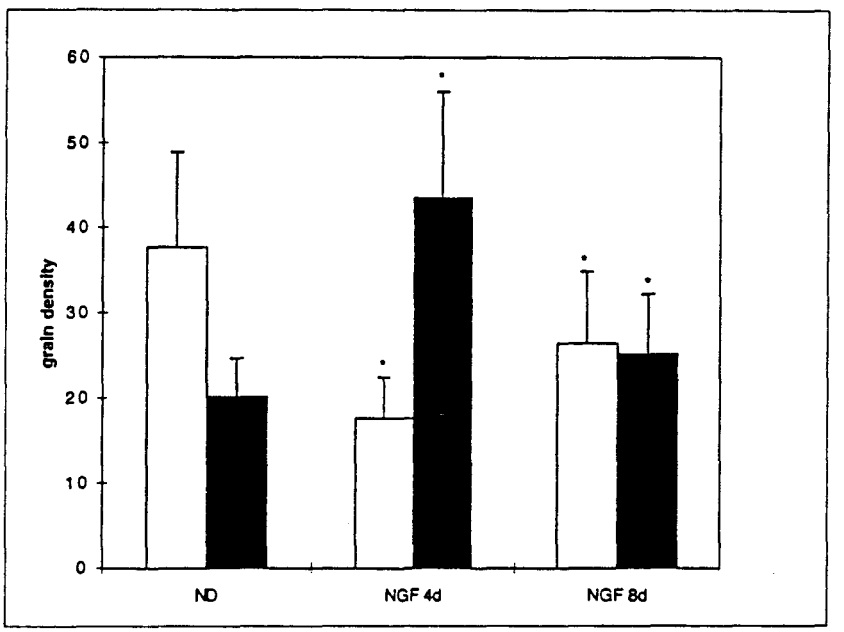

Supplemental Fig 5

mRNA	Cellular differentiation	Grain density ± SEM
ApoE	ND	37.7 ± 11.2
	NGF 4d	17,6 ± 4,8
	NGF 8d	26,4 ± 8,5
Tau	ND	20,1 ± 4,5
	NGF 4d	43,5 ± 12,5
	NGF 8d	25, 2 ± 7,14

Table 1: Quantification of apoE and Tau mRNAs, on hybridization slides. Cells were not differentiated (ND) or NGF-differentiated during 4 days (NGF 4d) or 8 days (NGF 8d). Standard deviation was represented.

PUBLICATIONS SOUMISES

Annexe 3: Soulié C., Dupont- Wallois L., Mitchell V., Wavrant-de-Vriez, Chartier-HarlinM.-C., Lépagnol J., Delacourte A., Beauvillain J.C. and Caillet-Boudin M.-L.: Apolipoprotéine E Synthesis in human neuroblastoma cells, the SKNSH-SY 5Y line: soumise à la revue: Journal of Cell Science.

Annexe 4: Dupont-Wallois L., Sergeant N., Goedert M., Delacourte A. and Caillet-Boudin M.-L.: Expression, phosphorylation and hyperphosphorylation of tau proteins in humanneuroblastoma cells: study of endogenous tau and transfected longest tau isoform: soumise à la revue: Journal of Neurochemistry.

ANNEXE 3

Shift from fetal-type to Alzheimer-type phosphorylated Tau proteins in SKNSH-SY 5Y cells treated with okadaic acid

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Abstract **Tau proteins are abnormally phosphorylated in Alzheimer's disease. Pathological Tau proteins named PHF-Tau 55, PHF-Tau 64, and PHF-Tau 69, are the main constituents of the paired helical filaments (PHF). When treating SKNSH-SY 5Y cells with okadaic acid (OA), Tau 55 protein was clearly induced whereas Tau 64 protein was only faintly induced. Here, we show that the absence of Tau 69 could be explained by the fact that adult isoforms containing N-terminal inserts are not detected. Phosphorylation is similar for untreated cellular Tau proteins and fetal Tau proteins, while OA cell treatment transformed fetal-type into Alzheimer-type phosphorylated proteins.**

Key words: Tau protein; Okadaic acid; Phosphorylation; Alzheimer's disease; SKNSH-SY cell

1. Introduction

Tau proteins are microtubule-associated proteins of 50,000–64,000 Da, mainly found in axons of neurons. In human, six isoforms arise by alternative splicing of a primary transcript originating from a single gene [1]. Exons 2, 3 and 10 (nomenclature according to Andreadis's paper [2]) are under developmental regulation and they are only expressed in some adult isoforms. The fetal isoform (FF) (which expresses no exon among the alternatively expressed exons) is found in both fetal and adult brains. The other five isoforms found in adult brain correspond to the fetal isoform modified by insertions corresponding to the expression of 1, 2 or 3 alternative exons, i.e. the exons 2, 3, and 10 [1]. In this paper, these isoforms are named FF-10; FF-2; FF-2,10; FF-2,3 and FF-2,3,10 according to the expressed alternative exon (as described in Fig. 1).

In Alzheimer's disease, abnormal phosphorylation of Tau proteins leads to their aggregation in paired helical filaments (PHF) [3,4]. All six isoforms are abnormally phosphorylated and then migrate in SDS-polyacrylamide gels as a triplet named PHF-Tau 55, PHF-Tau 64 and PHF-Tau 69. Both Goedert et al. [5] and Brion et al. [6] have identified the lower PHF-Tau band (here named PHF-Tau 55) as the abnormally phosphorylated Tau molecules containing neither exon 2 nor 3 (i.e. FF and FF-10). The upper band (here named PHF-Tau 69) was identified as the phosphorylated products of Tau proteins containing exon 2 and exon 3 (i.e. FF-2,3 and FF-2,3,10) [5]. The middle band (here named PHF-Tau 64) would correspond to the abnormally phosphorylated isoforms containing exon 2 (i.e. FF-2 and FF-2,10). The distribution of the different isoforms in the triplet, as proposed by Goedert et al. [5], is shown in Fig. 1.

The mechanisms leading to abnormal phosphorylation are unknown. Some purified members of the proline-directed protein kinase family (MAP kinase, cyclin-dependent kinase, GSK3 kinase) were successfully tested for their capacity to phosphorylate in vitro Tau proteins and to generate Alzheimer-type epitopes [7–12]. The molecular weight of in vitro phosphorylated Tau protein and PHF-Tau triplet was only compared in the two following systems. First, in human brain slices treated by okadaic acid (OA), an inhibitor of protein phosphatases-1 and -2A [13], PHF-Tau triplet was induced: Tau 64 band was mainly detected at low OA concentrations whereas Tau 55 and Tau 69 only appeared at higher OA concentrations [14]. Second, in NGF-differentiated SKNSH-SY 5Y cells treated by OA, only Tau 55 and Tau 64 were detected, Tau 55 being the major band and Tau 64 the minor one [15,16]. The differences between the two systems could be due to a different ratio between the Tau isoforms present. According to Goedert et al.'s results [1] (Fig. 1), in OA treated cells, the absence of Tau 69 might be due to the lack of isoforms containing exons 2 and 3 (i.e. FF-2,3, FF-2,3,10 isoforms). In a similar way, the low quantity of Tau 64 might reflect a low quantity of Tau isoform(s) with exon 2 (FF-2 and FF-2,10). To verify this assumption, we characterized the Tau isoforms present in our cell model by biochemical and molecular biology methods. Here, we show that cellular Tau proteins are similar to fetal Tau proteins and that adult isoforms containing N-terminal inserts are not detected in SKNSH-SY 5Y. Moreover, the treatment of the neuroblastoma cells by OA allowed a shift from fetal-type to Alzheimer-type phosphorylated proteins.

2. Materials and methods

2.1. Cell cultures, cell- and Alzheimer brain extracts

SKNSH-SY 5Y cells were maintened in Dulbecco's modified Eagle's medium (DMEM) (Boehringer, Mannheim) supplemented with 10% fetal calf serum. Differentiation, OA treatment, and cell extraction were performed as described in [16]. Briefly, NGF-differentiated cells were treated with 0.25 μM OA for 6 h over 4 days. Cells and brain tissues were homogenized in Laemmli's buffer with 0.25% dithiothreitol, and heat treated before loading onto polyacrylamide gels.

2.2. PAGE and Western blotting

Electrophoresis, transfer, Ponceau red staining and Western blotting were performed as described in [16]. The antibodies used were: the absorbed anti-PHF serum (abs PHF) (specific for PHF-Tau [4]), the polyclonal amino-terminal Tau serum (N-term) (specific for both Alzheimer and normal Tau proteins [16]), Tau 1 antibodies (specific for normal Tau [17,18]), AT8 (from Innogenetics, specific for Alzheimer-type Tau proteins [19]) and AD2 (monoclonal antibody specific for an abnormal site of phosphorylation in Alzheimer Tau proteins (V. Buée

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et al., manuscript in preparation)). Tau 1 and AT8 are located in the same region (amino acids 198–202) but AD2 is located in the C-terminal part of the Tau molecule. After incubation with the anti-rabbit or anti-mouse antibodies conjugated with peroxydase (Diagnostic Pasteur), visualization was performed using the ECL (Enhanced chemiluminescence) detection kit from Amersham.

2.3. Alkaline phosphatase treament

OA cell extracts were dialysed against a buffer containing 50 mM Tris, pH 8.3, 50 mM NaCl, 1 mM $MgCl_2$ and 0.2 mM DTT, overnight at 4°C, and were dephosphorylated using calf intestine alkaline phosphatase (Boehringer, Mannheim) at 100 U/ml as described by Flament and Delacourte [20].

2.4. mRNA isolation and reverse transcriptase-polymerase chain reaction

Total cellular RNA was extracted by the RNAzol B method (Cinna/Biotecx) according to the manufacturer's instructions. For first-strand cDNA synthesis, 10 μl reaction mixture contained the provided enzyme buffer, 1.5 μg of total RNA, 20 pM of reverse primer, 100 U of reverse transcriptase Mu-MLV, 2.5 mM deoxynucleotide triphosphates and 1 μg serum albumin. The reaction mixture was first incubated at 80°C for 5 min, then at 37°C for 90 min. For the 100 μl PCR mixture, 1 U of *Taq*I polymerase, 20 pM of forward primer and the provided *Taq*I buffer were added to the 10 μl of reverse transcription reaction mixture. Forward and reverse primers were located, respectively, in exon 1 and in exon 4, i.e. on each side of the alternatively expressed exons 2 and 3 and corresponded, respectively, to bases 5′ TACGGGTTGGGGGACAGGAAAGAT 3′ and to bases 5′GGGGTGTCTCCAATGCCTGCTTCT 3′. PCR was carried out in a Perkin Elmer thermal cycler using cycles consisting of denaturation at 94°C for 1 min, followed by annealing at 65°C for 1 min and DNA extension at 72°C for 2 min for 30 cycles. The obtained DNA was characterized by gel agarose or acrylamide electrophoresis, digestion with endonuclease restriction and cDNA sequencing.

2.5. cDNA sequencing

The ampligen was first cloned into pBluescript II SK (Stratagene), then sequenced with the Sequenase Kit (USB) following the manufacturer's protocol.

3. Results and discussion

The electrophoretic pattern of SKNSH-SY 5Y cell Tau proteins was compared with that of fetal, adult and Alzheimer

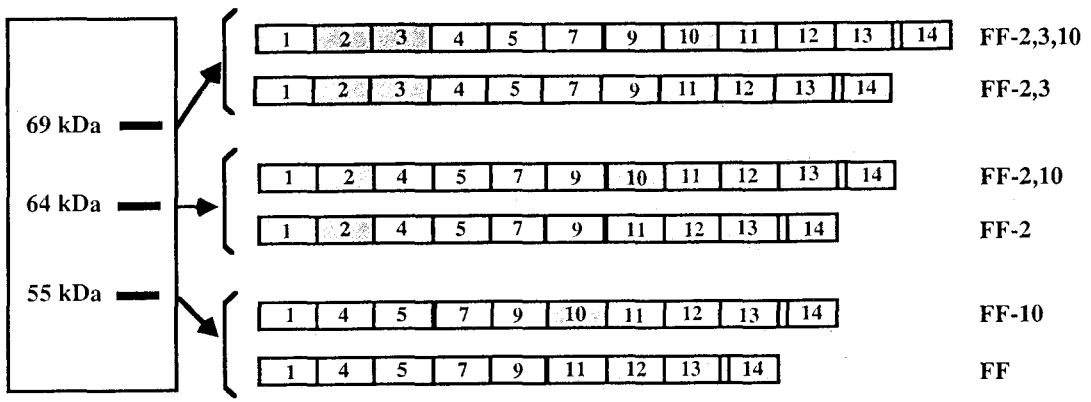

Fig. 1. Correspondance between pathological Tau triplet and different Tau isoforms according to Goedert et al. [1,6]. The exon numbers are indicated according to Andreadis et al.'s nomenclature [2].

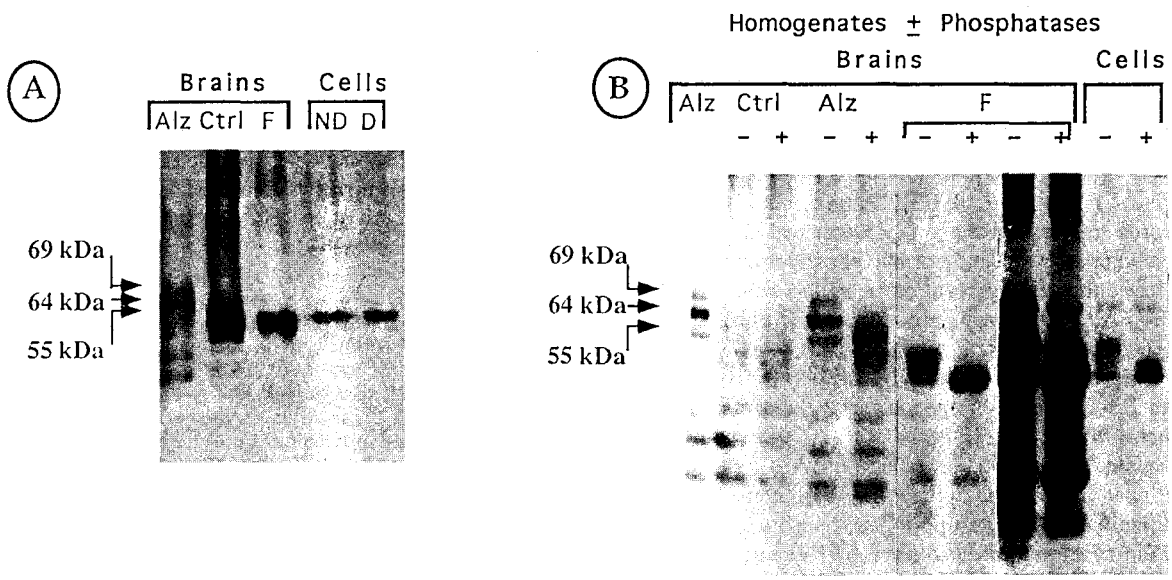

Fig. 2. Western blot analysis of homogenates from brains and SKNSH-SY 5Y cell cultures. (A) Electrophoretic pattern of Tau proteins from Alzheimer (Alz), control (Ctrl) and fetal (F) brain homogenates and from 4 days NGF-differentiated (D) or not differentiated (ND) cell homogenates. SKNSH-SY 5Y Tau proteins migrated as fetal Tau proteins. (B) Migration of Tau proteins from brain (Alz, Ctrl, F) and differentiated cell (Cell) homogenates before (−) or after phosphatase treatment (+). Two fetal Tau proteins were revealed after two different exposure times. Tau proteins in A and B were revealed by N-term serum.

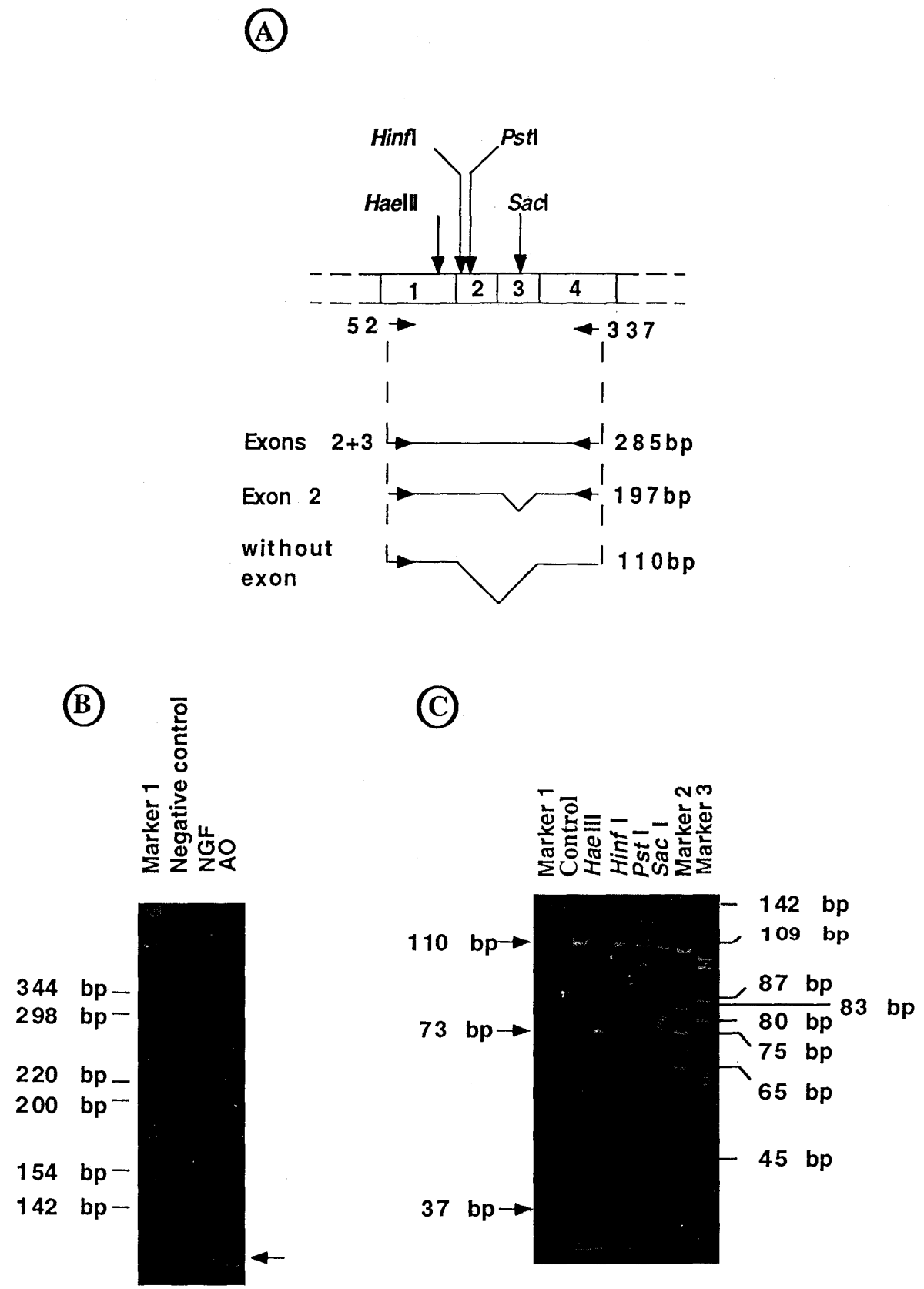

Fig. 3. Analysis of the exons 2 and 3 presence in SKNSH-SY 5Y Tau mRNA. (A) Drawing of the studied region indicating the restriction enzyme sites and the sizes of PCR products corresponding to the different possibilities of splicing. (B) RT-PCR product of Ctrl and OA treated-cells. Marker 1, DNA ladder from BRL. (C) Enzyme restriction analysis of the 110 bp band. Plasmid pblcat5 cut by *Hae*III or *Hin*fI enzymes was used as the size marker (respectively marker 2 and marker 3).

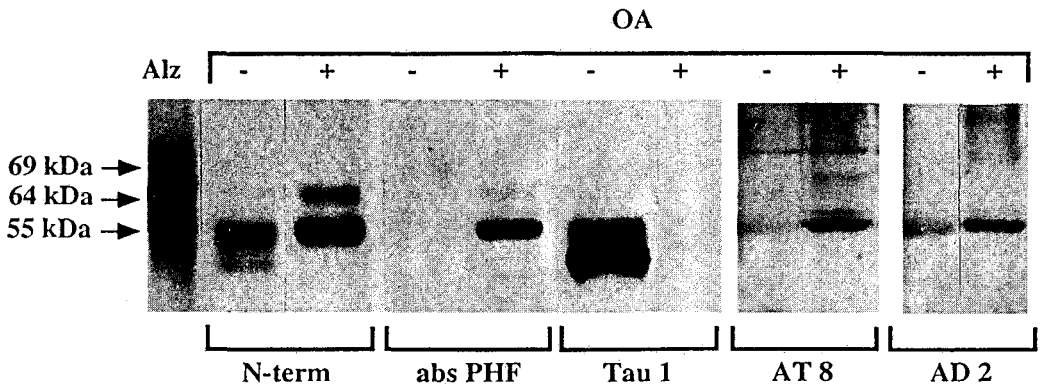

Fig. 4. Alzheimer specific antibodies' immunoreactivity on non-treated (−) and OA-treated (+) cells. NGF-differentiated cells were treated with 0.25 μM OA for 6 h over 4 days. Tau 64 was only detected with the N-term and abs PHF polylonal antibodies whereas Tau 55, the major band, was also detected by AT8 and AD2 monoclonal antibodies .

brains. As seen in Fig. 2A, fetal and cell Tau proteins migrated in a similar way. By comparison of Tau patterns of fetal and cellular samples after alkaline phosphatase, we confirm a similar migration for both dephosphorylated fetal and cellular Tau proteins (Fig. 2B). Therefore, the result of alkaline phosphatase treament demonstrates a similar phosphorylation degree and the presence of the same isoforms in cellular and fetal Tau proteins. Cellular Tau protein did not then contain the N-terminal inserts.

To ascertain the absence of isoforms containing exons 2 and 3 in SKNSH-SY 5Y cells, we examined the 5′ extremity of cellular mRNA by RT-PCR. Using primers on each side of exon 2 and 3, only one amplified product of about 110 bp was obtained (Fig. 3B) and corresponded to the size expected in the absence of exons 2 and 3 (Fig. 3A). The restriction enzyme digestion profile effectively corresponded to this isoform (Fig. 3C). This result was confirmed by cloning and sequencing this band of 110 bp. The sequence was identical to the one published by Goedert et al. [21] and confirmed the absence of exons 2 and 3 in this DNA and then in the cellular mRNA (not shown). Nevertheless, isoforms containing exons 2 and 3 could be newly synthesized during OA treatment. To check this point, RT-PCR analysis was performed on mRNA prepared from OA treated cells. The absence of exons 2 and 3 was confirmed since no new band was detected (Fig. 1B).

In SKNSH-SY 5Y cells, only the isoform without exons 2 and 3 was detected unless both Tau 55 and Tau 64 (corresponding to isoforms without exon 2 and to isoforms with exon 2, according to Goedert et al.'s results [5]) were induced when treating cells with OA ([16] and Fig. 3). Tau 55 was the major band, Tau 64 the minor one. Thus, in OA treated cells, the detection of Tau 64 did not seem to be due to the phosphorylation of the exon 2-containing isoform as reported for PHF-Tau 64 by Goedert et al. [5]. Then, Tau 64 detection might then be due to a hyperphosphorylation of the 55 kDa band. This would support the idea of the existence of different states of phosphorylation of the same isoform. Such a hypothesis is compatible with (i) the observation reported by Ksiezak-Reding et al. [22] that phosphatase treatment of PHF-Tau proteins induced a relative increase in the immunoreactivity of Tau 64 polypeptide and a decrease in the immunoreactivity of the Tau 69 band; and (ii) a multiple step phosphorylation of Tau proteins by in vitro kinase assays [7,10].

Some common phosphorylation sites were described between fetal and Alzheimer Tau molecules [23–27]. Fetal Tau phosphorylation is highly heterogeneous and only a proportion of fetal Tau molecules is phosphorylated in the majority of the Alzheimer common sites. Since in SKNSH-SY 5Y cells, fetal-type Tau proteins were mainly detected (this paper), it was important to know if the Tau phosphorylation induced by OA treatment [16] was more related to a fetal stage or to a pathological phosphorylation. In untreated SKNSH-SY 5Y cells, Tau proteins are already phosphorylated, as shown by the action of phosphatase on Tau migration (Fig. 2B). Tau protein phosphorylation degree seemed similar to fetal phosphorylation since we observed a similar migration of both proteins before and after phosphatase treatment (Fig. 2B). Indeed, detection by Tau 1 was increased by phosphatase treatment, (not shown) then some of the Tau 1 sites must already be phosphorylated before OA treament. This was confirmed by AT8 binding to control cellular Tau proteins (Fig. 4). In the same way, a weak AD2 immunoreactivity is seen on control Tau proteins (Fig. 4). Thus, like fetal Tau proteins, cellular Tau proteins are partially phosphorylated in some Alzheimer specific sites. Yet OA treatment induced a hyperphosphorylation and the OA-modified Tau protein electrophoretic migration was very similar to Tau 55 and Tau 64 migration of the Alzheimer triplet as described in [16]. After treatment, all the Tau 1 sites were phosphorylated since Tau proteins were no longer detected by Tau 1 antibodies. We also observed a clear increase of Tau 55 phosphorylation in Alzheimer-specific AT8 and AD2 sites after OA treatment on Tau 55 (Fig. 4). We did not succeed in obtaining the same strong immunodetection of Tau 55 with the AT8 and AD2 monoclonal antibodies as with the N-term and abs PHF polyclonal sera (perhaps because of the antibodies' concentration). This might explain why the minor Tau 64 band was only detected by using polyclonal sera.

In the OA treated cells, the apparent molecular weight change, the loss of Tau 1 immunoreactivity and the increased detection of Alzheimer epitopes on Tau molecules are in favour of a shift from fetal-type into Alzheimer-type phosphorylated Tau proteins. These results further reinforce the value of our

SKNSH-SY 5Y model for the in vitro study of Alzheimer-type Tau phosphorylation mechanisms.

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ANNEXE 4

EXPRESSION, PHOSPHORYLATION AND HYPERPHOSPHORYLATION OF TAU PROTEINS IN HUMAN NEUROBLASTOMA CELLS: STUDY OF ENDOGENOUS TAU AND TRANSFECTED LONGEST TAU ISOFORM

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Abbreviations:2-D gel electrophoresis, 2D- gel electrophoresis; PHF, paired helical filament; PHF-Tau, abnormally phosphorylated tau proteins aggregated into PHF; SDS, sodium dodecyl sulfate; MW: apparent molecular weight, RT-PCR: Reverse Transcriptase inverse-PCR; a.a., amino acid; IEF, isoelectrofocalisation.

ABSTRACT

Microtubule-associated Tau proteins in human brain consist of six isoforms derived from alternative spicing of a unique primary transcript. Phosphorylation is the major post-translational modification of Tau isoforms. This phosphorylation is developmentaly and spatialy regulated and is dysregulated in numerous neurodegenerative disorders. In order to investigate Tau phosphorylation events, we have transfected the longest human Tau isoform cDNA (Tau441) in human neuroblastoma cells which mainly synthesize the shortest one in a constitutive manner. Degree of phosphorylation of both endogenous Tau and transfected Tau proteins expressed in these cells was compared by mono- and 2D-electrophoresis using different phosphorylation dependent anti-tau antibodies. Here, we show that, like normal Tau proteins, both endogenous and transfected cellular Tau proteins were phosphorylate. No preferential phosphorylation on the studied epitopes takes place on one isoform as compared to the other. Cellular treatment by okadaic acid induced an hyperphosphorylation state of both endogenous and transfected Tau proteins. These proteins co-migrated with Tau55 and Tau74 bands of Tau-PHF, two among the hyperphosphorylated proteins characteristic of Alzheimer's disease, and shared with those proteins common phosphorylated epitopes.

Key Words: Alzheimer's disease, cellular model, Tau isoforms, phosphorylation, okadaic acid, transfection.

Running title: Phosphorylation of Tau proteins in transfected cells

INTRODUCTION

Tau proteins belong to the microtubule-associated proteins (MAP) family and are mainly expressed in neuronal cells. They are resolved on a sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) as several bands. This Tau electrophoretic pattern complexity arises from alternative splicing of a primary transcript and from different phosphorylation events (For review., see Delacourte and Buée, 1997).

Alternative splicing of Tau mRNAs is developmentally regulated (Goedert et al., 1989a) and differs according to its tissue origin (Couchie et al., 1992; Gu et al., 1996). In a human adult brain, six Tau isoforms, ranging from 352 to 441 amino acids (a.a.) in length are expressed. They differ from each other by the expression or not of three alternatively spliced exons: exons 2, 3 and 10 (Goedert *et al.*, 1989a,b). Only the shortest isoform, i.e. without any insert, is found in the fœtal human brain. Addition of exon 2 or exons 2 and 3 into the mRNAs leads to the insertion of 29 or 58 a.a., respectively, in the amino-terminal region of the protein. The carboxy-terminal part of Tau isoforms without exon 10 contains three tandem repeats of 31 or 32 a.a.. Presence of exon 10 in Tau mRNA leads to insertion of a fourth repeat between the repeat 1 and 2. These repeats represent the tubulin binding domain of Tau proteins. In the peripheral nervous system (PNS), Tau proteins of higher molecular weight resulting from exons 4A and 6 insertions in mRNA may also be expressed (Couchie et al., 1992; Goedert et al., 1992).

Phosphorylation is the major post-translational modification of Tau proteins and plays an important role in Tau biological functions in decreasing its affinity for microtubules (Lindwall and Cole *et al.*, 1984; Drewes *et al.*, 1995). Tau phosphorylation is submitted to a developmental regulation and is dysregulated in many neurodegenerative disorders. In fœtal brain, Tau fœtal molecules were found phosphorylated at a higher extent than the adult ones from biopsic cerebral tissue (Matsuo *et al.*, 1994; Mawal-Dewan et al., 1994). Moreover, adult Tau is submitted to an extensive dephosphorylation during post-mortem delay (Matsuo et al., 1994). In Alzheimer's disease (AD), all six Tau proteins are more extensively phosphorylated on the sites common to fœtal and adult biopsic Tau proteins and are referred as PHF-Tau, (Matsuo *et al.*, 1994; Sergeant et al.

1995; Moroshima-Kawashima *et al.*, 1995). Moreover, additional Serine and Threonine residues are phosphorylated only on these PHF-Tau proteins (Hasegawa et al., 1996; Moroshima-Kawashima *et al.*, 1995; Caillet-Boudin and Delacourte, 1996; Hoffmann et al., 1997). This hyperphosphorylation of Tau proteins probably preceeds their aggregation into insoluble paired helical filaments (PHF), feature of degenerating neurons in Alzheimer brain (Brion et al., 1985; Delacourte and Defossez, 1986). These PHF-Tau are not sensitive to the post-mortem phosphatase activity and are resolved as a major triplet of polypeptides referred as tau 55, tau 64 and tau 69 and an additional Tau component, namely tau 74 using mono and bi-dimensional SDS gel electrophoresis (Delacourte *et al.*, 1990; Sergeant et al., 1997a).

To understand the Tau phosphorylation mechanism and its role on Tau proteins functions, Tau proteins were overexpressed in a number of non-neuronal cell lines. In such cell systems, several electrophoretic Tau species issued from an unique Tau cDNA were synthesized, generated by different phosphorylation events (Sygowski *et al.*, 1993; Medina *et al.*, 1995). This phosphorylation greatly increases during the cell cycle (Preuss et al., 1995), after co-tranfection with GSK3 kinases (Latimer et al., 1995; Lovestone et al., 1994; Wagner et al., 1996) or cell treatment with okadaic acid (Medina *et al.*, 1996). But, Tau proteins are mainly found in neurons (Gu *et al.*, 1996). Furthermore, the kinases/phosphatases activities seem to be more efficient on Tau epitopes in *in situ* neurons or neuronal-type cell culture (Brion *et al.*, 1993; Trojanowski *et al.*, 1994; Baum *et al.*, 1995). Then, it seemed to be important to examine the phosphorylation events on Tau proteins in two human neuroblastoma cell lines, whose neuronal feature is largely described in the literature: the SKNSH-SY 5Y and Kelly cells (Pahlman *et al.*, 1990; Schwab *et al.*, 1983). After characterization of the Tau isoforms expressed in each cell line, we have chosen to transfect cells with the longest human Tau isoform, Tau441. In this system, we could analyze and compare the phosphorylation extent of the two distinct Tau isoforms: the transfected one, Tau441, and the endogenous Tau ones, the fœtal Tau isoform. Hyperphosphorylation of Tau proteins, induced by okadaic acid treatment, resulted in antigenic and electrophoretic changes of the cellular Tau proteins.

MATERIALS AND METHODS

- Characterization of Tau isoforms expressed in cell lines:

Total RNA was extracted from cells by the RNAzol B method (Cinna/ Biotecx) according to the manufacturer's instructions. Analysis of the different Tau transcripts was performed by Reverse Transcription-Polymerase Chain Reaction (RT-PCR). 1.5 to 2 µg of each RNA sample was reverse transcribed with the Mu-MLV reverse transcriptase (Gibco BRL) using the antisense specific primer. The synthesized cDNA were then subjected to 30 cycles of amplification using the ready sense primer for each amplification. The different primers used are summarized in Table 1: the Tau52/Tau337, Tau52/Tau220, Tau747/Tau1326, Tau308/Tau415 and Tau388/Tau541 primer pairs were used to look for the presence of exons 2 and 3, 10, 4A and 6, respectively. Next, the PCR products were analyzed on 2% (w/v) agarose gels and visualized by ethidium bromide. In each RT-PCR assay, negative control was performed replacing total RNA by water.

- Construction of the expression vector:

Tau441 cDNA (htau40) was cloned into the Nde I/ EcoR I sites of the pRK 172 plasmid (Goedert *et al.*,1990). For direct subcloning of Tau441 cDNA into the eukaryotic expression pcDNA3 vector (Invitrogen), a BamH I site was introduced above its initiator site, eliminating the initial Nde I site. Sequencing performed on the obtained pcDNA3-Tau441 showed identical sequences between the subcloned cDNA and that published by Goedert et al. (1989). Therefore, CsCl-purified plasmid was used for the transfection experiments.

- Cell Culture and Transfection

Human neuroblastoma KELLY and SY 5Y cells were maintained in RPMI 1640 (Gibco BRL) and DMEM medium, respectively, supplemented with 10% fœtal calf serum (Boehringer Mannheim).

Transfection experiments were performed by adding 5 µg of pcDNA3-Tau40 and 39,4 µg of Tfx^{TM}-50 Reagent (Promega) on 80% confluent Kelly or SY 5Y cells grown in 60 mm dishes in serum-free medium. Additional medium supplemented with serum was added one hour after this step.

Treatment of cells with okadaic acid (OA) was performed as described in Dupont-Wallois *et al.* (1995) for 6 hours. This phosphatase inhibitor was added to cells at 48h post-transfection.

- Protein extraction and Western blot analysis.

The cell pellet was collected and denatured as described in Dupont-Wallois *et al.* (1995). Samples were loaded onto a SDS-polyacrylamide mini-gel electrophoresis (PAGE) containing 7.5% (wt/v) acrylamide and 0.104%(wt/v) bis-acrylamide. After electrophoresis and transfer onto nitrocellulose, Tau proteins were analyzed by western blotting using different antibodies.

Detection of both transfected and endogeneous Tau molecules, independently of their phosphorylation state, was performed with two rabbit polyclonal antibodies: M19G (Sautière *et al* ., 1994) raised against the first 19 a.a. of Tau proteins and the 134 antiserum raised against the last 14 residues (Goedert et al., 1992). Presence of Tau inserts was investigated with three polyclonal antiserum: the 304 (Goedert et al., 1992), Tau-E3 and Tau-E10 (Sergeant et al., 1997b) raised against the additional inserts translated from the exon 2, exon 3 and exon 10, respectively.

Phosphorylation state of both transfected and endogeneous Tau proteins was investigated with several phospho-dependent anti-Tau antibodies. Tau-1, a monoclonal antibody recognizes the 192-204 a.a. stretch in an unphosphorylated state (numbering of the a.a. position according to the longest human Tau isoform) (Szendrei *et al.*, 1993). It is specific of normal Tau protein and is undetected on PHF-Tau. AT8, AT270, AT180 (Goedert *et al.*, 1994), AD2 (Buée-Scherrer *et al.*, 1996) and 12E8 (Seubert *et al.*, 1995) monoclonal antibodies bind to phosphorylated sites Ser 202-Thr 205, Thr 231, Thr 181, Ser 396-Ser 404, Ser 262-Ser 356, respectively (Figure 4A). These sites are highly

phosphorylated on PHF-Tau and more weakly on normal biopsic Tau proteins. Lastly, a polyclonal antiserum, namely Ser422P, raised against the phosphorylated Ser 422 was obtained by immunizing rabbits with synthetic peptides (Neosystem, France). This serum was similar to the AP422 serum and is more specific of PHF-Tau (Moroshima-Kawashima *et al.*, 1995; Caillet-Boudin and Delacourte, 1996). The corresponding anti-mouse or anti-rabbit secondary antibodies were purchased from Diagnostic Pasteur (Sigma). Visualization of immunolabelling was performed using the ECL (Enhanced Chemiluminescence) detection kit (Amersham).

- 2D- electrophoresis:

The cells were harvested at 4°C and collected by centrifugation. Cell pellet was essentially treated in a similar protocol as described in Dupont-Wallois et al. (1997) except that the cell samples were treated by DNAse I before denaturation, in order to optimize the resolution of cell Tau variants. Cell samples were then resolved on isoelectric focusing gels in the first dimension and on a 10-20% gradient SDS-PAGE in the second dimension. After transfert onto nitrocellulose membrane, Tau phosphorylation was analyzed in the following way: AD2 monoclonal antibody and M19G polyclonal serum western blotting were successively performed on the same nitrocellulose replica, after stripping of the first primary antibodies by an overnight incubation in Guanidine Chlorhydrate 3M, Dithioerythritol 50mM. In the same way, Tau-1, 12E8, AP422 monoclonal antibodies and 134 polyclonal serum incubation were successively performed on a second membrane.

RESULTS

1- Identification of Tau isoforms expressed in the SY 5Y and Kelly cells:

Cellular Tau mRNA were purified and analyzed for the presence of the alternatively spliced exons on 5' and 3' sides by independent RT-PCR experiments.

5' part of the mRNA coding region which encompass exons 2 and 3 was first studied using Tau52/Tau337 primer pair. A band of 112 bp was mainly amplified indicating that isoforms without exons 2 and 3 were present in both SY 5Y and Kelly cells (Fig. 1A). Restriction enzyme analysis confirmed the identity of this 112 bp band (Fig. 1B). Furthermore, in some experiments, a very faint additional band of 199 bp was amplified suggesting that Kelly and SY 5Y cells could, in addition, express isoforms with exon 2. Then, a second amplification of the RT-PCR products using more internal primers (Tau52/ Tau220 primer pair) was needed to confirm the presence of exon 2. A band of 169 bp, identical to the expected size of Tau mRNA with exon 2, was effectively amplified from Kelly and SY 5Y RT-PCR products (Fig. 1A). The restriction digestion pattern of this band corresponded to this isoform (Fig. 1C). In conclusion, both cell lines mainly synthesized mRNA without exons 2 and 3 and few mRNA with exon 2.

3' part of the mRNA coding region which encompass exon 10 was studied using Tau747/Tau1326 primer pair. Two fragments of 580 bp and 487 bp were amplified by RT-PCR from both SY 5Y and KELLY RNA. These bands corresponded to the expected size for the isoforms with and without exon 10 (Fig. 2A). The specificity of the RT-PCR products was checked by enzymatic digestion as shown in Fig. 2B. The 580 bp band, corresponding to the exon 10 presence, was always more weakly amplified than the 487 bp band corresponding to the exon 10 absence.

The eventual presence of exons 4A and 6, exons specific of PNS, on cellular Tau mRNA was looked for by RT-PCR with Tau308/Tau415 and Tau388/Tau 541 primer pairs, respectively. No amplified product containing exon 4A or exon 6 was detected (data not shown).

2- Transfection of both human neuroblastoma cell lines by the longest human Tau isoform cDNA, Tau441 :

Kelly and SY 5Y cells were transiently transfected with Tau441 cDNA, containing exons 2, 3 and 10 as described in material and methods. Transfected and endogenous Tau proteins were analyzed by immunoblotting and compared with Tau proteins found in AD brain homogenate.

The endogenous Tau proteins present in both untransfected SY 5Y and Kelly cells migrated as a large band with a MW of 52-53 kDa, as detected by M19G serum. Other antibodies specific of the amino and carboxy terminal inserts (304, Tau-E3 and Tau-E10) failed to detect any bands of higher molecular weight (Fig. 3B).

After transfection with Tau441 cDNA, one diffuse band with a MW of 67-70 kDa was revealed by M19G antiserum in both transfected -Kelly and -SY 5Y cell extracts. This band was also recognized by the three anti Tau antibodies raised against the amino and carboxy terminal inserts. Accordingly, this additional but diffuse band actually corresponded to the transfected Tau441 protein (Fig. 3B).

Addition of either phosphatase inhibitor (okadaic acid, sodium orthovanadate) or protease inhibitors (Aprotinin, Leupeptin, PMSF) to the buffers during the cell collect did not alter the electrophoretic patterns of both Tau isoforms (data not shown). This eliminated the hypothesis of a dephosphorylation or proteolysis event during the protein extraction.

Altough similar cell extracts quantities (20 µg of total homogenate) were run through 7.5 % SDS-PAGE, the anti Tau M19G and 134 sera always labeled more strongly transfected Tau441 proteins from transfected Kelly cell extracts than those from SY5Y. Furthermore, efficiency of transfection for an experiment to another one was more fluctuant for the SY 5Y cells than for the Kelly cells. Therefore, the thorough analysis of the Tau phosphorylation state was conducted only on transfected Kelly cells.

3- Phosphorylation and hyperphosphorylation of both Tau441 and endogeneous Tau proteins after okadaic acid treatment:

3-1- Mono-dimensional analysis:

Before OA-treatment, both Tau 441 and endogenous Tau proteins were detected by AD2, Tau-1, 12E8 monoclonal antibodies and also very faintly by AT8, AT270 and AT180 antibodies. When we compared the two more reactive phospho-dependent antibodies, AD2 and Tau-1, we could note a light shift between Tau-1 and AD2 reactive bands. AD2 was reactive with the upper part of the corresponding M19G bands whereas Tau-1 detected the lower part. Ser422P serum, specific of an Alzheimer epitope, failed to detect some bands of these Tau441 and endogenous Tau bands (Fig. 4).

After cell treatment by OA, a decrease in the mobility of both Tau isoforms was observed. The constitutive and transfected Tau proteins comigrated with PHF-tau55 and -tau74 proteins, respectively (Fig. 4B). Tau441 proteins migrated as a broad band whereas the endogenous Tau proteins were resolved in a single and thinner band. This mobility decrease is concomittant with a change in the phosphorylation level. Indeed, AD2, AT8, AT180, AT270 and 12E8 phospho-dependent anti Tau antibodies strongly detected these cellular OA-modified Tau441 and endogenous proteins (Fig.4). Furthermore, Tau-1 immunoreactivity, specific of normal Tau proteins, disappeared whereas Ser422P epitope, specific of PHF-Tau proteins, was detected on both OA-modified cellular Tau bands (Fig. 4B).

3-2- 2D-electrophoresis analysis:

3-2-1- Analysis of endogeneous and transfected Tau proteins before OA-treatment:

Isoelectric variants of both transfected and endogenous Tau proteins were analyzed after immunoblotting with different anti-tau antibodies.

The analysis of 2D-electrophoresis blotting with M19G and 134 polyclonal antisera allowed us to detect numerous spots corresponding to the unproteolyzed Tau proteins. Each spot was characterized by its isoelectric point (pI) and its Mr. Endogenous Tau proteins corresponded to the spots with a MW ranging from 52 to 58 kDa whereas transfected Tau441 variants had a MW ranging from 62 to 74 kDa. As compared to the endogenous Tau variants, the Tau 441 variants were more numerous and could be subdivided into two groups: Tau A group with a MW ranging from 69 to 74 kDa and Tau B group with a MW from 62 to 68 kDa (Fig. 5). The different spots were located in a gel region with a pH ranging **from 5.33 to 7.4 for Tau441 proteins and 6,1 to 7.4 for** the endogenous Tau ones (Fig. 5).

The degree of phosphorylation of these different Tau variants was analyzed with AD2, 12E8 and Tau-1 antibodies. AD2 clearly detected the endogenous and Tau441 variants with the upper molecular weight. Thus, for Tau441 proteins, all variants belonging to the Tau A group were detected with AD2 antibody whereas those of the Tau B group were not (Fig. 6). The 12E8 monoclonal antibody, raised against the phosphorylated Ser262/356, recognized all spots of the endogenous and transfected Tau441 proteins (Fig. 6). Tau-1 antibody, raised against an unphosphorylated epitope, detected Tau441 spots in both Tau A and Tau B groups. But, Tau-1 immunoreactivity only labelled the most basic variants of both groups (Fig. 7).

3-2-2- Analysis of Tau proteins after hyperphosphorylation by OA treatment:

After OA treatment, cell Tau proteins were resolved into three main bands of 56, 71 and 75 kDa in the pH gradient ranging from 4,0 to 8,0 as revealed with the polyclonal antisera (Fig. 5).

All the hyperphosphorylated endogeneous Tau spots, detected by both M19G and 134 antisera, migrated with the same apparent molecular weight of 56 kDa. They were separated in more acidic pH region (from 5.3 to 6.8) when compared to the unmodified Tau proteins (6,1 to 7.4) (Fig. 5). In the same way, all the OA-modified Tau441 spots were resolved between more acidic pI values ranging from 4.65 to 7.1 than the unmodified ones. Moreover, all these Tau441 spots could always be separated into two groups, Tau A and Tau B groups, with a maximum MW of 75 and 71 kDa respectively.

The phosphorylated degree of the Tau proteins were also investigated using several phosphorylated-dependent antibodies. As before the cellular OA-treatment, 12E8 monoclonal antibody detected all the Tau441 and endogeneous Tau spots resolved in our pH gradient (compare Fig. 5 and Fig 6.). On the other hand, AD2 recognized all the endogeneous Tau variants whereas only the Tau441 variants of the Tau A group were detected (Fig. 6) suggesting they were the only ones which were phosphorylated on the Ser396/404 residues. When immunoblotted with the AP422 monoclonal antibody, specifically raised against a PHF-Tau epitope, all endogeneous Tau variants and all the Tau A variants were detected whereas only the most acidic variants of the Tau B were recognized by this antibody.

Discussion

The present study is the first report of transfection of a Tau isoform in human neuroblastoma cell lines namely SY 5Y and Kelly cells.

1- Characterization of Tau isoforms expressed in human neuroblastoma cell lines: SY 5Y and Kelly cells:

Both cell lines used in the present study, SY 5Y and Kelly cells, were described as human neuronal-type cells of tumoral origin (Biedler *et al.*, 1973; Schwab *et al.*, 1983). By RT-PCR and western blotting experiments, we showed in this study that SY 5Y and Kelly cells synthesize Tau proteins and mainly the fœtal-type Tau isoform (i.e. without the alternatively spliced exons 2, 3 and 10: $2^-3^-10^-$). This result agrees with previous studies about SY 5Y cells (Smith et al., 1995; Dupont-Wallois et al., 1995). Neverthless, additionnal RT-PCR products were amplified. Some products containing exon 10 were detected but always in weak ratio when compared to the amplified products without exon 10. mRNA with exon 2 may also be expressed in both cell lines but in very few copies since its detection needed two successive amplifications using two sets of primers. These results suggest that, in addition to the fœtal mRNA ($2^-3^-10^-$), these cells synhesized some mRNA Tau specific of adult isoforms ($2^-3^-10^+$), and at a very low level $2^+3^-10^-$ and/or $2^+3^-10^+$ mRNA. The proteins corresponding to these minor populations of mRNA were not detected by Western blotting. This could be explained either by an insufficient sensitivity of this technique or by a translation regulation of these adult isoforms.

2- Transfection experiments:

We have chosen to transfect these cells by Tau441 cDNA (i.e. $2^+3^+10^+$) because it gave us the possibility to distinguish easily the endogenous from the transfected Tau proteins and to compare the phosphorylation level of two distinct Tau isoforms containing the three or none insert. Here, we successfully show that neuroblastoma cells are able to

support the lipotransfection procedure and to express Tau isoform absent of the native cells. The transfection of a unique Tau cDNA in these cells induced the apparition of a large diffuse band. A better efficiency and reproductibility of Tau441 expression was observed with Kelly cells as compared to SY 5Y cells. Therefore, we have thoroughly examined phosphorylation state of both endogenous Tau proteins and transfected Tau441 in transiently transfected Kelly cells by mono- and 2D- electrophoresis.

3- Analysis of the phosphorylation state of both transfected and endogenous Tau proteins in Kelly cells:

The phosphorylation of both endogenous and transfected Tau isoforms expressed in transfected Kelly cells has been analyzed by the use of different specific antibodies. The different bands corresponded to different states of Tau phosphorylation. Both Tau441 and endogenous Tau proteins were phosphorylated on residues Thr181, S202/Thr205, Thr231, Ser262/356 and Ser396/Ser404 recognized by AT270, AT8, AT180, 12E8 and AD2 antibodies, respectively. All these sites are faintly phosphorylated in normal adult brain but highly phosphorylated in PHF-Tau (Matsuo, 1994; Seubert et al., 1995; Buée-Scherrer et al., 1996). Some of Tau species were unphosphorylated in the 199-204 stretch as shown by the Tau-1 immunodetection. These species constituted the lower MW part of the large M19G reactive band whereas AD2 only labeled the upper part. This probably means that the diffuse bands were due to an heterogeneously phosphorylated Tau population as was already described in previous studies using non-neuronal type cells, such as 3T3, L-cells, COS cells (Kanaï et al., 1989; Medina et al., 1995; Lovestone et al., 1994; Sygowski et al., 1993). This result was confirmed by the numerous spots observed in 2D-electrophoresis blotting as it will be discussed below. No band seemed to contain phosphorylated Ser422, a specific site of Alzheimer PHF-Tau proteins. Altogether, these results showed that in neuronal-type cells, such as Kelly cells, phosphorylation of Tau proteins constituvely occurs on several Ser and Thr residues located on each side and within the tubulin binding domain. This phosphorylation might be similar to that found on adult Tau proteins from biopsy samples or fœtal Tau.

2D-electrophoresis experiments allowed us to resolve both transfected and endogenous Tau proteins in numerous spots. Because we have chosen the shortest and the longest Tau isoforms, there was no overlap of spots corresponding to one or to other isoform and thus each spot really corresponded to a tau molecule in a well-definite state of phosphorylation. Indeed, for each isoform, we observed various spots with the same pI but with a different MW whereas for a given MW corresponded various spots with different pI. The pI of one spot was representative of the number of phosphates by Tau molecule but not of the phosphorylated site whereas the MW increases reflected conformational modifications induced by phosphorylation of some specific sites.

As detected by polyclonal sera raised against amino- and carboxy-terminal part of Tau proteins, Tau 441variants were resolved between more acidic extreme pI values (pI 5.5 to pI 7.4) than endogenous Tau proteins (p I6.4 to pI 7.4). This apparent acidification of Tau441 proteins as compared to endogenous Tau proteins might result either from addition of the acidic amino-terminal inserts or (and) a higher extent of phosphorylation. Indeed, the addition of these inserts lowers the calculated pI of Tau441 (pI 8.12) with regard to the calculated pI of endogenous one (pI 9.76). Nevertheless, as demonstrated by mono- and 2D- electrophoresis, a higher heterogeneity in the MW is observed for Tau441 variants than in endogenous ones. This could be due to a greater conformational effect of phosphorylation or to (an) additional phosphorylation(s) on Tau441 as compared to Tau endogenous. Additional phosphorylation sites might be located within the additional inserts: Ser46, located in the first amino-terminal insert or Ser285 and Ser305 residues located in the C-terminal insert, corresponding to the fourth repeat, are good candidates (Gustke et al., 1992; Paudel, 1997). Furthermore, phosphorylation on site(s) common to both Tau isoforms may induce a greater conformational change in Tau441 proteins due to the insert presence.

In our 2D-electrophoresis experimental conditions, Tau441 variants were distributed into two groups, namely Tau A and Tau B groups according to their MW and their pI. If the phosphorylation of one site was strictly dependent upon the phosphorylation of another site, the MW and pI of spots would progressively shift to

higher MW and more acidic pI giving a spot pattern with the appearance of stair steps. This was not really observed. Thus, the phosphorylation did not seem to process in well-ordered steps, resulting in a heterogenous population of proteins with a difference in the degree and sites of phosphorylation. Then, several hypothesis can be raised about the cellular regulation on the phosphorylation process. The first hypothesis suggest that, after each step of phosphorylation, kinases could phosphorylate one site among 2, 3 or n sites on which phosphorylation would lead or not to a MW change. In this case, Tau 441 variants would shift from group B to group A whatever their pI. In the second hypothesis, group A and B would result from two independent phosphorylation processes. A crucial step would switch on the variants either to group A or to group B excluding a latter passage from group B to group A.

Using phosphorylation-dependent antibodies, some conclusions can be drawn about the order of site phosphorylation and about the effect of phosphorylation on protein conformation change.

The analysis of Tau phosphorylation with Tau-1 antibodies confirmed the heterogeneity in the phosphorylation cascade. Indeed, Tau-1 partially immunolabelled Tau441 variants of both Tau A and Tau B groups. Some of the phosphorylation events which occurred before the phosphorylation of Tau-1 site resulted in MW change since the Tau-1 immunodetected spots belonged to both Tau A and Tau B groups. But for a given MW, only the most basic spots are Tau-1 immunodetected. This means that all Tau-1 epitopes (unphosphorylated Ser199/202 residues) were phosphorylated after a restricted number of phosphorylation events. This event would induce or not a MW shift.

Labeling with AD2 was concomittant to the electrophoretic shift from Tau B variants to Tau A variants suggesting that these Ser396 and/or Ser 404 residues are directly implicated in this electrophoretic shift and that other phosphorylation events are needed before that the phosphorylation on these residues could take place. Nevertheless, we cannot conclude on the separated role of Ser396 and/or Ser 404 in this shift which were independently implicated in the mobility shift of Tau proteins as reported in previous *in vitro* studies (Mandelkow et al., 1992; Gustke et al., 1992; Biernat et al., 1993). AD2

epitope phosphorylation could be correspond to the crucial strep in the second hypothesis about the phosphorylation process.

Because, 12E8 antibodies detects all the Tau441 spots whatever their pI and MW, Ser262 phosphorylation can occur quickly and do not modify the electrophoretic migration of the protein. This result agrees with the data reported in Gutske et al. (1992). Thus, phosphorylation on this site which plays an important role in the tubulin-Tau binding (Biernat et al., 1993; Drewes et al., 1995) did not lead to a perceptible conformational change of the molecule.

4- Analysis of the hyperphosphorylation induced by OA cell treatment:

Okadaic acid cell treatment induced a change of Tau phosphorylation level from normal-like to Alzheimer-like phosphorylated state. This was demonstrated by an increase of the MW, antigenic modifications and acidification of both OA-modified endogenous and OA-modified transfected Tau as compared to the corresponding Tau molecules of untreated cells. The increase of MW induced a co-migration of OA-modified Tau 441 and endogenous Tau proteins with Tau74 and Tau55 bands of PHF-Tau. This means that Tau55 corresponded to the hyperphosphorylation of fœtal isoform whereas Tau74 corresponded to Tau441 isoform. These comigrations confirmed, in a cellular model, the correlation between Tau isoforms and PHF-Tau bands proposed in Mulot et al. (1994) after an *in vitro* phosphorylation assay of Tau recombinant proteins. The antigenic variations consisted of 1) an increase of phosphorylation on some sites: Thr181, S202/Thr205, Thr231, Ser262 and Ser396/Ser404; 2) the disappearance of Tau-1 epitope and 3) the appearance of an Alzheimer specific epitope (phosphorylated Ser422). These three antigenic characteristics are specific to PHF-Tau as compared with biopsic Tau.

Nevertheless, after cellular OA treatment, Tau441 and endogenous Tau proteins were still resolved into numerous distinct spots, after 2D-electrophoresis. It was surprising that the acidification and MW increase due to OA treatment did not lead to a less heterogenous spot population. The heterogeneity consisted only in the pI for

endogenous Tau proteins but still in pI and MW for Tau441. The analysis of the Tau441 variants with the different phosphorylation-dependent antibodies showed that they resulted from different phosphorylation events. AD2 antibody only labeled the Tau441 variants of Tau A group. These result confirmed that phosphorylation on the Ser396/404 residues lead to a large shift on the mobility shift. In the other hand, AP422 antibodies labeled, in addition to Tau A group, the Tau B variants which had the highest apparent MW. This suggest that the Ser422 phosphorylation can occure before these of Ser396/404. At last, 12E8 antibody, raised against the phosphorylated Ser262, immunodetected all the OA-modified Tau441 variants. This result supported that this phosphorylation is not implicated in the molecular shift and can occur in the first phosphorylation steps.

This higher heterogenity for Tau441 variants as compared to the endogenous Tau may result from an incomplete hyperphosphorylation of the Tau441 variants. Three hypotheses could explain this phenomenom. The first one was about the kinase activities. Indeed, some kinases could be activated by OA treatment (such as MAP kinase) whereas other ones could be inactivated (such as GSK3 kinase°. Then, in tis model, we can consider either that the kinases activated during OA treatment would be saturated due to their high expression level or that phosphorylation on some sites specific to Tau441 could need the action of kinases inactivated by this treatment. The second hypothesis consisted to consider that phosphatase 2B (PP 2B) (calcineurin) was efficient to dephosphorylate Tau proteins on AD2 epitope (Ser396/404). Indeed, PP 2B is very faintly inhibited by the OA treatment (Cohen *et al.*, 1990) and is able to dephosphorylate Ser396 on Tau proteins from primary cortical neurons (Saito et al., 1995). At last, *in vitro* studies showed that phosphorylation on some sites of Tau proteins may prevent other kinases activities limiting then phosphorylation of Tau molecule (Singh *et al.*, 1995). In this case, the heterogeneity of Tau molecules might result from different sequential cascade of kinases activities.

In order to compare hyperphosphorylation extent of both isoforms in transfected cells, we have brought into alignement the more basic spots detected by AD2 antibodies

of each isofom (Fig. 8). This was possible because, after OA treatment, all the spots were located in the well-resolutive part of the gel. Three or four supplementar acidic spots were immunodetected for Tau441 variants as compared to the endogenous ones. This was not due to a too faint phosphorylation of the endogenous Tau isoform because of the transfected protein presence since endogenous Tau isoforms of both transfected or untransfected cells were phospharylated in a similar way (Fig. 8). Thus, this result suggest that about 3 additional phosphorylation events occured on the longest isoform as compared to the shortest one when cells were treated with OA.

5- Conclusion

In conclusion, the present study is the first demonstration of the expression of an adult isoform in neuroblastoma cell lines after transfection experiments. The endogenous protein as well as the transfected Tau441 isoform are constitutively phosphorylated in a similar extent to that Tau from normal biopsic or fœtal brain sample and Alzheimer-type phosphorylation was triggered by OA treatment. Comparison of the electrophoretic patterns of both Tau isoforms showed a role of the additional inserts in the increase of Tau441 isovariants heterogeneity resulting from phosphorylation processing. Moreover, additional phosphorylation events, more detectable after cellular OA-treatment, take place on the longest human Tau isoform with regard to the shortest one. Thus, these cells contain kinases and phosphatases able to phosphorylate Tau proteins in a normal or pathological level. But the phosphorylation step order was heterogenous, resulting in an heteogenous Tau population.

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When compared the more acidic pI of OA-modified cellular Tau proteins (Tau441: 4,94 and endogenous Tau: 5,27) to this of Tau-PHF tau74 and tau55 reported in Sergeant et al. (1997) (6,15 and 5,81 respectively), we observed that OA treatment induced a Tau phosphorylation level bigger than this of Tau-PHF although the MW in monodimensional gel was identical. This probably means that the phosphorylation events associated with a conformational change were similar in OA-treated cells and in Tau-PHF but additional site phosphorylation probably occured in OA-cells as compared to Tau-PHF without modified the conformation proteins.

The hyperphosphorylation induced by OA cellular treatment is greater than that observed during the Alzheimer's disease. Indeed, when compared the more acidic pI of OA-modified cellular Tau proteins (Tau441: 4,94 and endogenous Tau: 5,27) to this of Tau-PHF tau74 and tau55 reported in Sergeant et al. (1997) (6,15 and 5,81 respectively), we observed that OA treatment induced a Tau phosphorylation level bigger than this of Tau-PHF although the MW in monodimensional gel was identical. This probably means that the phosphorylation events associated with a conformational change were similar in OA-treated cells and in Tau-PHF but additional site phosphorylation probably occured in OA-cells as compared to Tau-PHF without modified the conformation proteins. The second observation is that the acidic pI of Tau-PHF is similar to that of the oA-untreated cells. Then the normal phosphorylation

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LEGENDS OF FIGURES

FIG.1. Analysis of the Tau isoforms expressed in neuroblastoma cells on their 5' side. (A) Schematic draft of the transcripts 5' extremity: the RT-PCR products corresponding to the different possibilities of splicing are represented on the left for the Tau52/Tau337 primer pair and on the right for the Tau52/Tau220 primer pair. The sizes of the different products are indicated. For each used primer pair Tau52/Tau337 (52/337) and Tau52/Tau220 (52/220), 12µl of each RT-PCR-amplified product are loaded onto a 2% (w/v) agarose gel and their sizes were determined thanks to two size markers (La: 100 bp ladder, M: pGEM markers (Promega)). A band of 112 bp, corresponding to the 2-3- form, was mainly detected from the SY 5Y (S) and KELLY (K) Tau mRNA after the first amplification with the Tau52/Tau337 primer pair . A sample of this amplified product was then used for a second amplification with Tau52/Tau220 primer pair. A band of 169 bp, corresponding to forms including exon 2, was then obseved. The absence of these bands in the corresponding negative control (T) proved the specificity of the reactions. (B) Analysis by restriction enzyme of the 112 bp band. Schematic draft shows the position of the exon specific-restriction enzyme sites wich are located with arrows on the longest Tau isoform and on the predicted isoform. As shown beside, only *Hae III* located on exon 1 was efficient to cut this band corresponding to the isoform without exons 2 and 3. (C) Enzymatic analysis of the 169 bp band: localization of *Hae III*, *Pst I* and *Sac I* restriction enzyme sites on the longest Tau isoform and on the predicted isoform. *Hae III* and *Pst I* cut the 169 bp band giving the digestion fragments of expected size and demonstrating that this band corresponded to the isoform with exon 2.

FIG. 2. Analysis of the Tau isoforms expressed in neuroblastoma cells on their 3' side. (A) Schematic draft of the transcript 3' extremity which represents the alternatively spliced forms, their corresponding size by RT-PCR with Tau747/Tau1326 primers. On the right, the RT-PCR products were loaded onto a 2% agarose gel and migrated as two bands of 487 bp and 580 bp. These both bands were amplified from SY 5Y (S) and KELLY (K)

total RNA. (B) Restriction analysis: two *Hae III* restriction enzyme sites are present on both Tau forms and are indicated with arrows on the draft . The *Hae III* digestion of these PCR products lead to three fragments of 305 bp, 279 bp and 166 bp, as shown on the 2% (w/v) agarose gel and confirmed that both cell lines express isoforms with and without exon 10.

FIG. 3: Western blot analyses of Tau proteins isolated from untransfected and transiently transfected Kelly and SY 5Y cells. Equal quantities of transfected and untransfected Kelly and SY 5Y cell homogenates (Kelly tr, Kelly, SY 5Y tr, SY 5Y respectively) were loaded onto a 7,5% SDS-PAGE. Cellular Tau proteins were immunodetected with M19G, E10, 304 and E3 antibodies raised against the amino-terminal part, exon 2-, exon 3- and exon 10- translated sequences, respectively. Endogenous Tau proteins were only immunodetected by M19G serum. As control of cellular Tau migration, Alzheimer brain homogenates (Alz brain) was analyzed with M19G serum.

FIG. 4: Comparison to phosphorylation of both cellular transfected and endogenous Tau proteins, before and after cell treatment by the OA phosphatase inhibitor. (A) Schematic draft of Tau proteins. The different antibodies used and the nature of their epitope (phosphorylated (P) or unphosphorylated(P)) were located on Tau protein. Both M19G and 134 antisera, raised respectively against the first 19 a.a. and the last 14 a.a. of Tau proteins, were also annoted and detected Tau independently of its phosphorylated state. The grey circles or square represent the inserts due to the alternetive spilcing and the squares to the tubulin binding domain repeats (B) Electrophoretic pattern of both transfected and endogenous Tau proteins, issued from OA-treated Kelly cells (+) or untreated Kelly cells (-). Similar amount of transfected cell extracts were resolved onto a 7,5% SDS-PAGE. Phosphorylation state of tau proteins was investigated using AD2, Tau-1, Ser422P, AT8, AT180, AT270 and 12E8. Note the increase of immunodetection with AD2, AT8, AT180, AT270 and 12E8 antibodies. Both cellular Tau proteins were no

more reactive for Tau-1 antibodies after OA treatment whereas conversely immunodetection by Ser422P was effective only after OA treatment. As control of cellular Tau migration, Alzheimer brain homogenates (Alz) was analyzed with M19G serum.

FIG. 5. Comparative 2-D profiles of Tau proteins isolated from transiently Kelly cells before or after treatment by okadaic acid. For analysis of Tau molecules with M19G and 134 antibodies, similar quantities of OA-treated (OA+) or untreated (OA-) transiently transfected Kelly cells homogenates were loaded onto a similar NEPHGE gradient gels for the first dimension, and onto a 10-20% gradient SDS-PAGE, for the second dimension. For each gel, only the area in the vicinity of Tau proteins was represented. Endogenous Tau proteins migrated as a main band whereas the Tau441 variants could be reparted into two groups of different molecular weight, Tau A and Tau B groups. A shift to more acidic extreme values of both endogenous and Tau441 proteins was observed after the cellular OA-treatment as visualized by dotted line. Note that the 3 more acidic spots of Tau B group detected by M19G were not revealed by 134 antibodies and then they did not corresponded to intact Tau proteins.

FIG. 6. Analysis of 2D-electrophoresis western blotting with AD2 and 12E8 monoclonal antibodies. The identification of the spots detected by these phosphorylation-dependent antibodies by comparison with Fig. 5. Before OA treatment (OA-), AD2 labeled the Tau A group of Tau441 variants and the endogenous Tau variants of highest Mr. After OA treatment (OA+), AD2 was immunoreactive with all spots detected with M19G for the endogenous Tau proteins whereas only the Tau A group of Tau 441 was detected. Tau B group spots were never revealed by AD2. 12E8 antibodies reacted with all the endogenous and Tau441 variants detected by 134 polyclonal serum (compare with Fig. 5), before and after OA treatment.

Fig. 7. 2D-electrophoresis analysis of cellular Tau proteins with Tau-1 and AP422 monoclonal antibodies. As for fig. 6, the identification of the spots detected by these

phosphorylation-dependent antibodies by comparison with Fig. 5. With Tau-1 antibodies, only the most basic variants of Tau A and Tau B groups were immuno-labeled whereas any endogeneous Tau variants was never detected in this experiment probably because of a too faint reactivity. Note that Tau-1 antibody failed also to detect Tau A variants with the highest apparent molecular weight. The Ser422P antibody detected all endogenous Tau variants whereas only some Tau441 variants (Tau A variants and upper Tau B variants) were immunodetected.

Fig. 8. Comparison of 2D-profiles of OA-modified Tau. Tau 441 group A variants and endogeneous Tau proteins of transfected cells, immuno-detected by AD2 antibody, were brought into alignement from their extreme basic variants. Three to four additional spots with more acidic values were immunodetected for Tau 441 as compared to the endogeneous ones. The profile of endogenous Tau proteins is not modified by cell transfection as is demonstrated by comparison of OA-modified endogenous Tau from both transfected and not transfected cells.

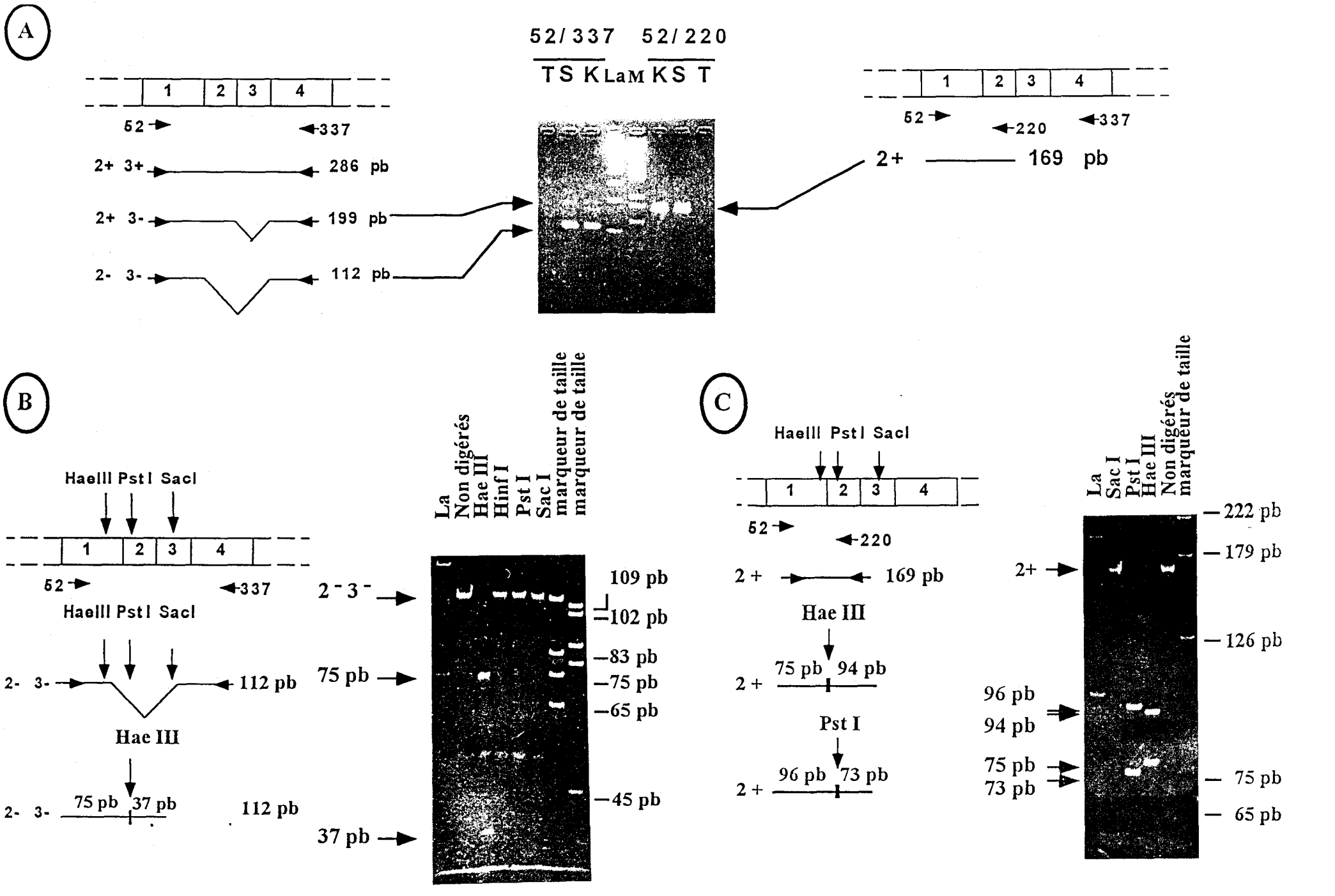
TABLE 1

Primers used in PCR. Tau oligonucleotide primers are given according to the numbering of the longest human tau cDNA sequence.

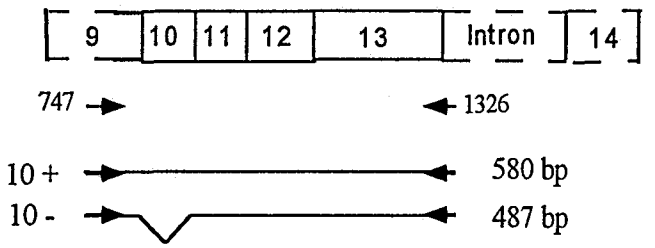
Name	Sequence	Orientation	Localization	Exons researched
Tau 52	5'TACGGGTTGGGGGACAGGAAAGA T 3'	Sense	Exon 1	Exons 2, 3
Tau 337	5'GGGGTGTCTCCAATGCCTGCTTCT 3'	Antisense	Exon 4	Exons 2, 3
Tau 220	5'CTTCCGCTGTTGGAGTGCTCTT 3'	Antisense	Exon 2	Exon 2
Tau 747	5'CATGCCAGACCTGAAGAATGTCAA G 3'	Sense	Exon 9	Exon 10
Tau 1328	5'TCACAAACCCTGCTTGGCCA 3'	Antisense	Exon 13	Exon 10
Tau 308	5'CTGAAGAAGCAGGCATTGGAGAC ACCCC 3'	Sense	Exon 4	Exon 4A
Tau 415	5'TCATCGCTTCCAGTCCCGTCTTT 3'	Antisense	Exon 5	Exon 4A
Tau 388	5'AAAAGCAAAGACGGGACTGG 3'	Sense	Exon 5	Exon 6
Tau 541	5'TCTTTGGAGCGGGCGGGGTTTTTG 3'	Antisense	Exon 7	Exon 6

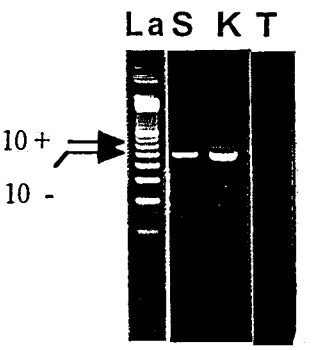
TABLE 2: Experimental and calculated pI of Tau proteins detected by M19G.

	Calculated	Experimental	
		Without OA	With OA
	pI	pI	pI
Tau 441	8.12	5.33-7.4	4.65-7.1
Endogenous Tau	9.76	6.1-7.4	5.3-6.8
Difference between more acidic pI of Tau441 and endogenous Tau proteins	1.64	0,77	0.65

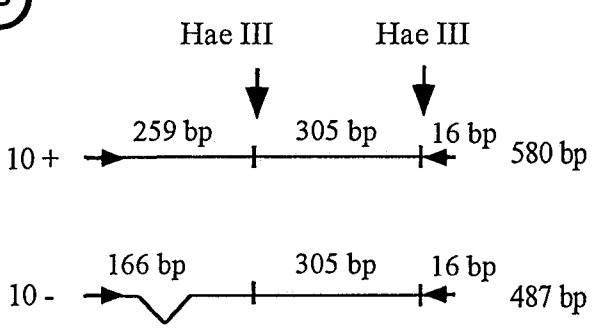

A
52/337 52/220
TS KLaM KS T
1 2 3 4
52
337
2+ 3+
286 pb
2+ 3-
199 pb
2- 3-
112 pb
220
2+
169 pb
B
HaeIII PstI SacI
2- 3-
109 pb
102 pb
83 pb
75 pb
65 pb
45 pb
37 pb
Hae III
75 pb 37 pb
La
Non digérés
Hae III
Hinf I
Pst I
Sac I
marqueur de taille
C
Pst I
96 pb 73 pb
222 pb
179 pb
126 pb
96 pb
94 pb
75 pb
73 pb
65 pb

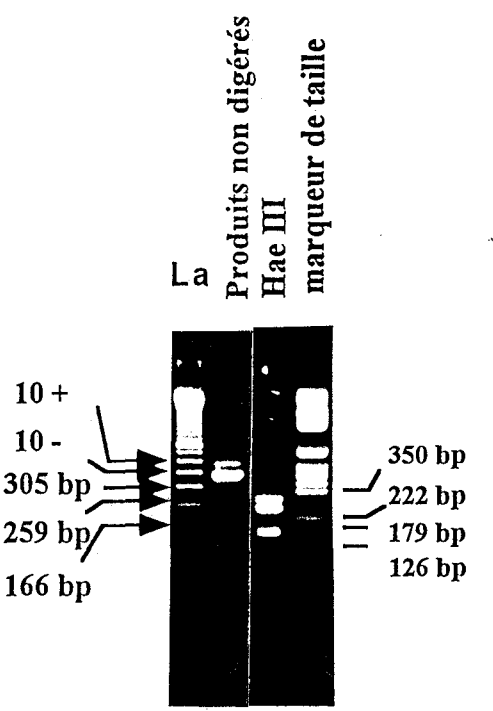
A


9
10
11
12
13
Intron
14
747
1326
10 +
580 bp
10 -
487 bp


La S K T
10 +
10 -

B


Hae III
Hae III
259 bp
305 bp
16 bp
10 +
580 bp
166 bp
305 bp
16 bp
10 -
487 bp


La
Produits non digérés
Hae III
marqueur de taille
10 +
10 -
305 bp
259 bp
166 bp
350 bp
222 bp
179 bp
126 bp

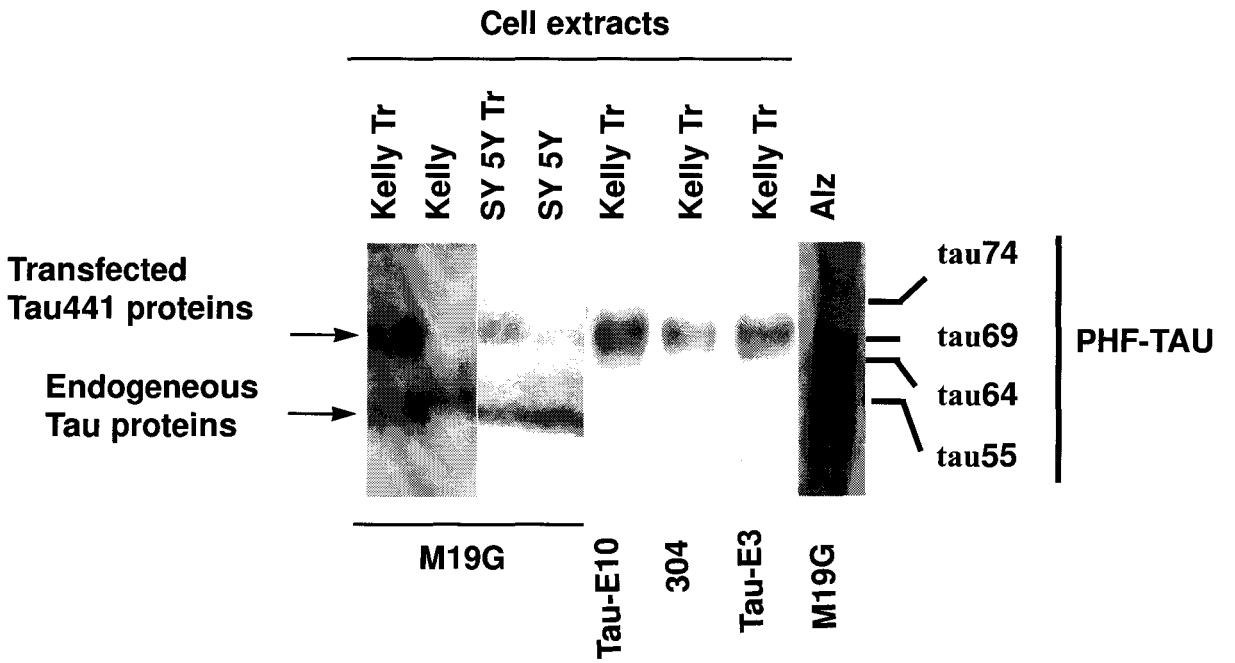

Cell extracts
Kelly Tr
Kelly
SY 5Y Tr
SY 5Y
Kelly Tr
Kelly Tr
Kelly Tr
Alz
Transfected
Tau441 proteins
Endogeneous
Tau proteins
tau74
tau69
tau64
tau55
PHF-TAU
M19G
Tau-E10
304
Tau-E3
M19G

FIGURE 3

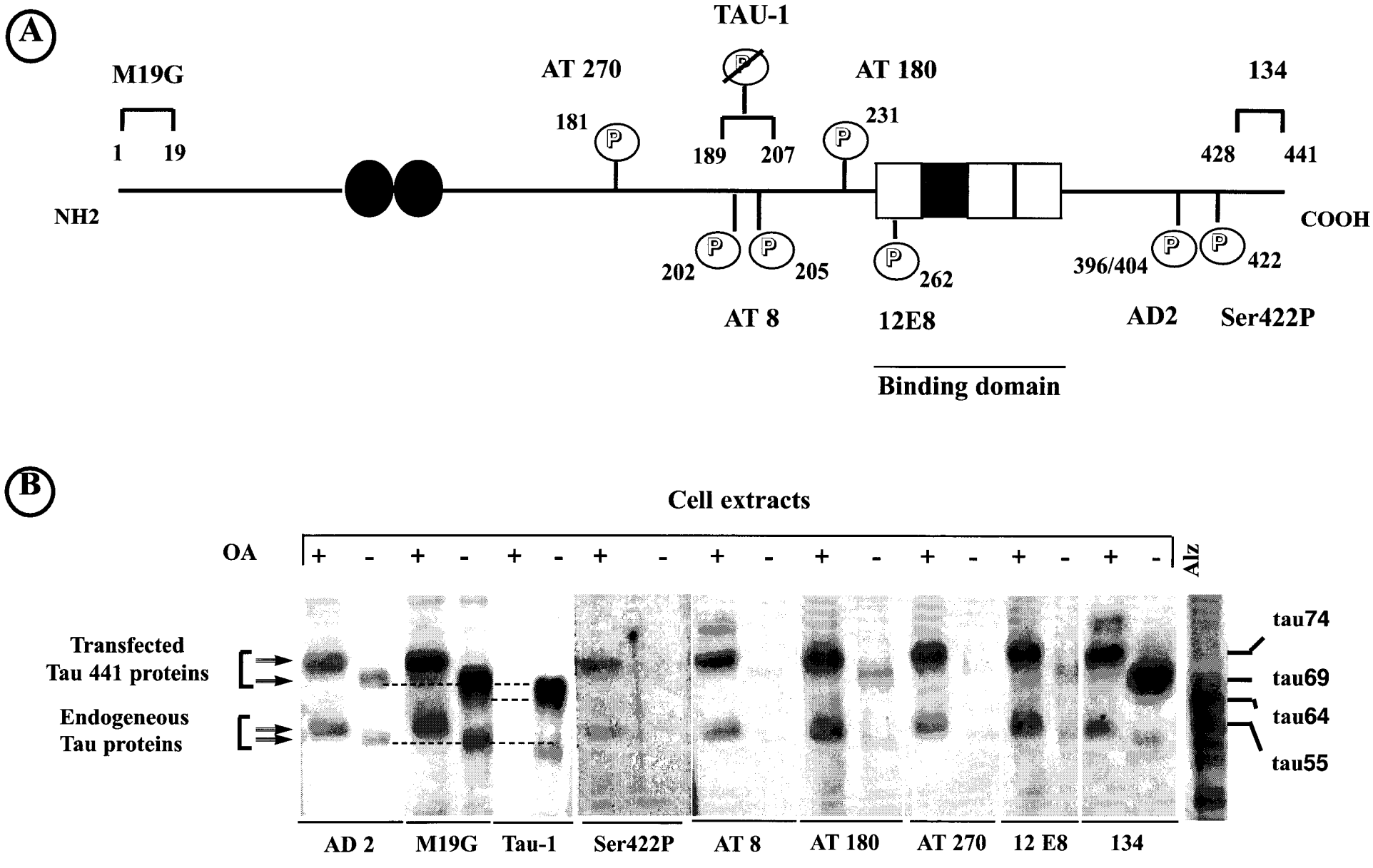

A
TAU-1
M19G
AT 270
AT 180
134
181
231
1
19
189
207
428
441
NH2
COOH
202
205
262
396/404
422
AT 8
12E8
AD2
Ser422P
Binding domain
B
Cell extracts
OA
+ - + - + - + - + - + - + - + - + -
Alz
Transfected
Tau 441 proteins
Endogeneous
Tau proteins
tau74
tau69
tau64
tau55
AD 2
M19G
Tau-1
Ser422P
AT 8
AT 180
AT 270
12 E8
134

FIGURE 4

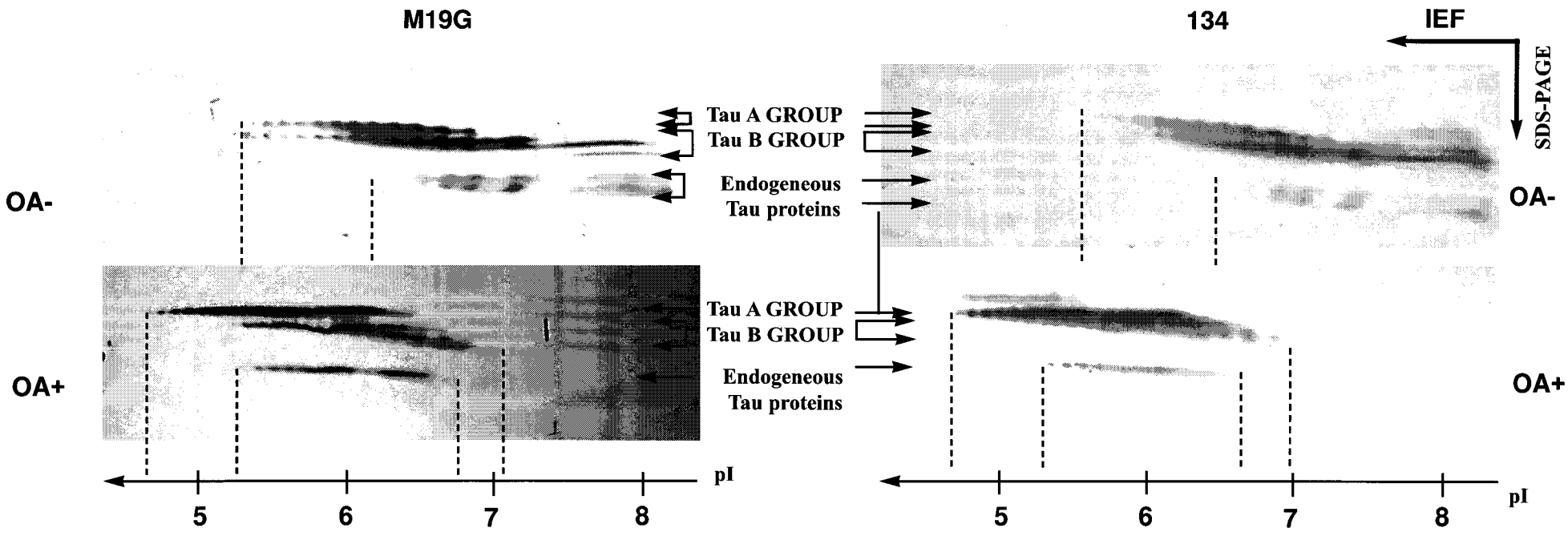

M19G
134
IEF
SDS-PAGE
OA-
OA+
Tau A GROUP
Tau B GROUP
Endogeneous
Tau proteins
Tau A GROUP
Tau B GROUP
Endogeneous
Tau proteins
OA-
OA+
pI
5
6
7
8
pI
5
6
7
8

FIGURE 5

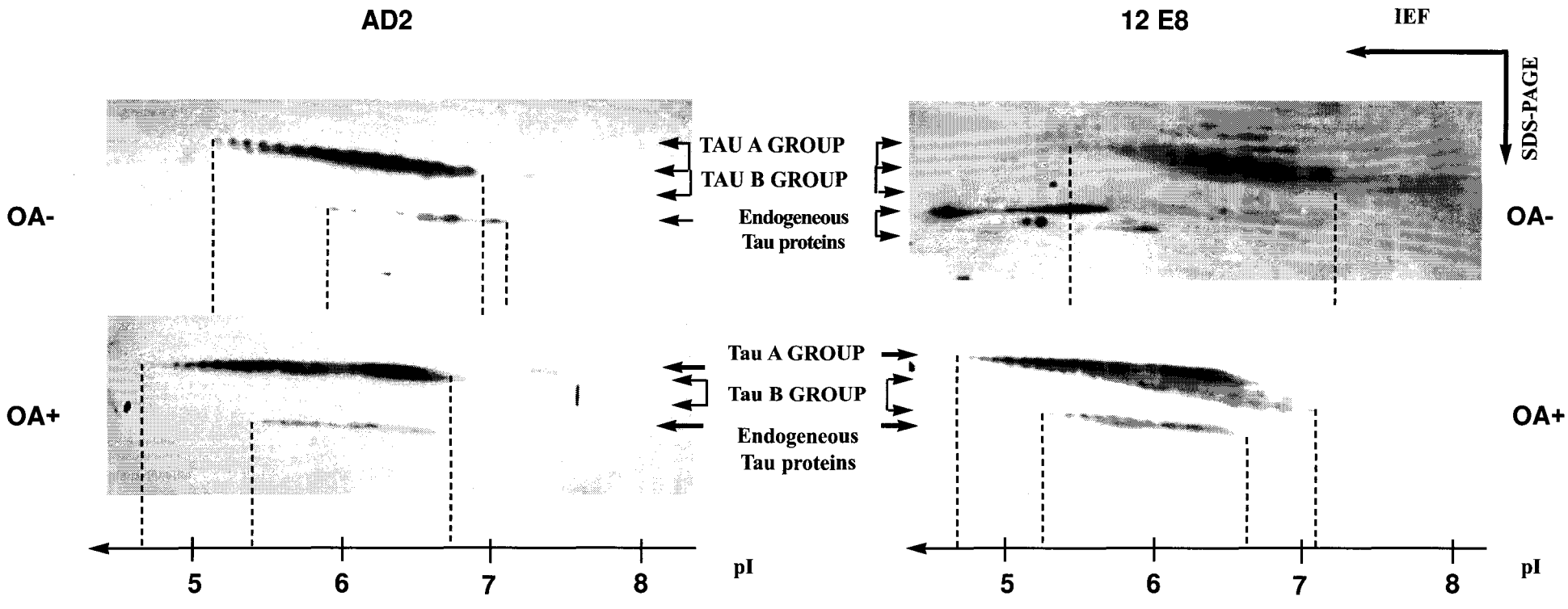

AD2
12 E8
IEF
SDS-PAGE
OA-
OA+
TAU A GROUP
TAU B GROUP
Endogeneous
Tau proteins
Tau A GROUP
Tau B GROUP
Endogeneous
Tau proteins
OA-
OA+
5
6
7
8
pI
5
6
7
8
pI

FIGURE 6

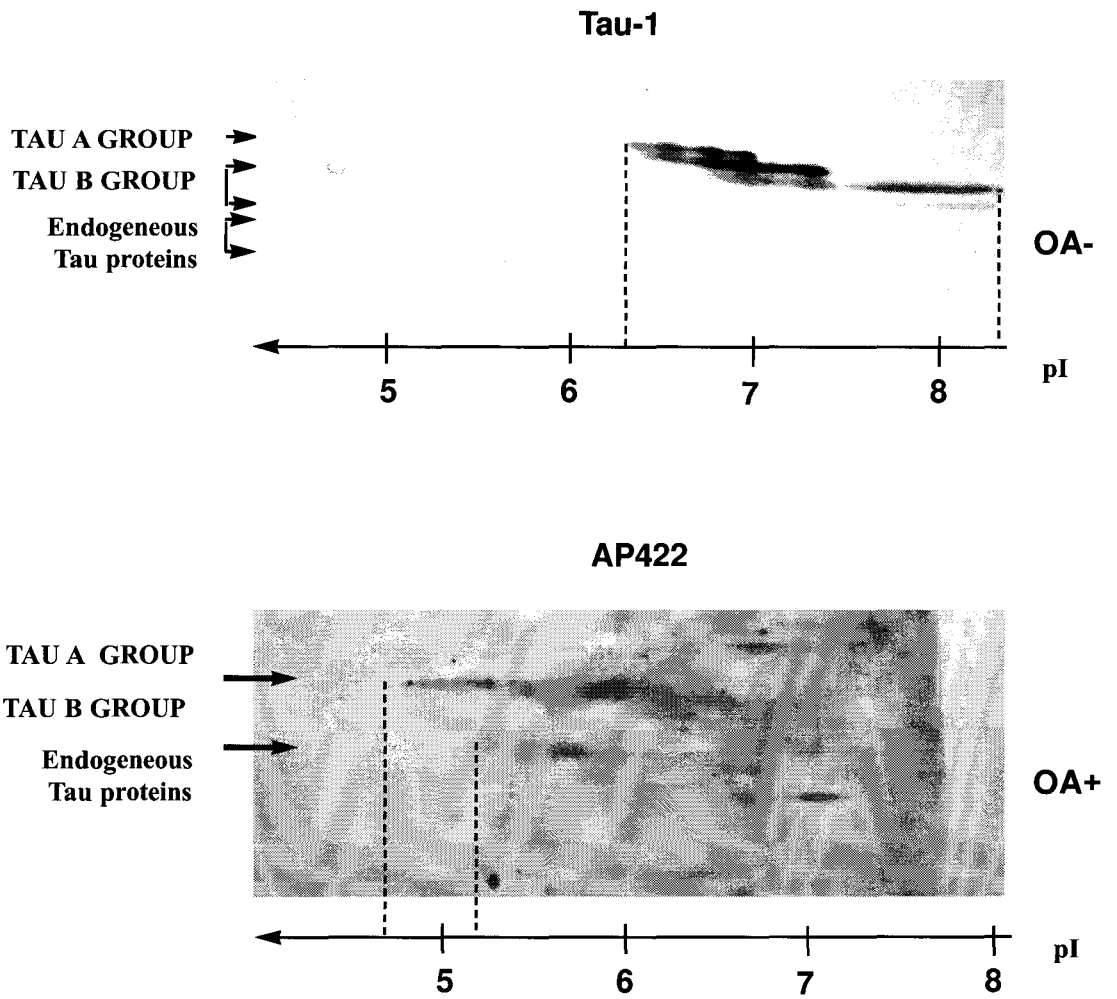

Tau-1
TAU A GROUP
TAU B GROUP
Endogeneous
Tau proteins
OA-
5
6
7
8
pI
AP422
TAU A GROUP
TAU B GROUP
Endogeneous
Tau proteins
OA+
5
6
7
8
pI

FIGURE 7

OA TREATED CELLS

AD2

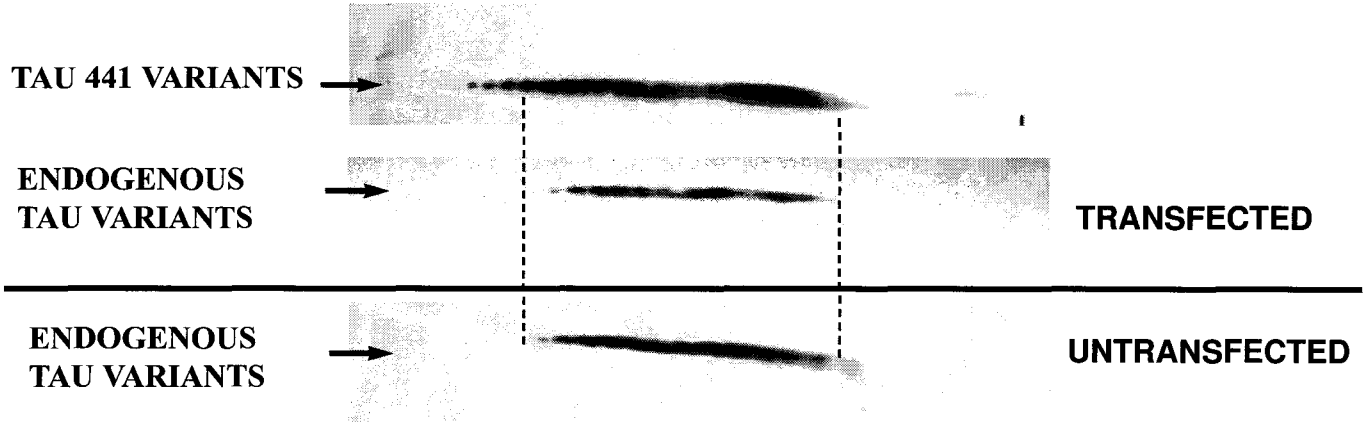


FIGURE 8