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Biochimica et Biophysica Acta 1619 (2003) 167–176

BIOCHIMICA ET BIOPHYSICA ACTA
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Evidence of a balance between phosphorylation and *O*-GlcNAc glycosylation of Tau proteins—a role in nuclear localization

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Received 21 June 2002; received in revised form 30 September 2002; accepted 16 October 2002

Abstract

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

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

1. Introduction

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

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

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doi:10.1016/S0304-4165(02)00477-4

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

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

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

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

2. Materials and methods

2.1. Materials

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

2.2. Expression vector construction

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

2.3. Cell culture and transfection

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

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

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

2.4. Cell fractionation

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

2.5. Polyacrylamide gel electrophoresis

2.5.1. 1-D electrophoresis

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

2.5.2. 2-D electrophoresis

For 2-D electrophoresis, samples were diluted in 20% (v/v) IsoA (0.34 M sodium dodecyl sulfate (SDS), 0.15 M DTT) and 80% (v/v) IsoE (0.108 M DTT, 0.108 M CHAPS, 15 M urea, 83.3 ml/L pI 3–10 range ampholytes). The samples were run on a 4% gel (containing 9.5 M urea, 1% (v/v) 5–7 range ampholytes, 4% (v/v) 3–10 range ampholytes, 5% (v/v) detergent solution—0.3 g CHAPS, 100 μl Nonidet P40 to 900 μl ddH_2O) at 200 V for 1 h, then at 500 V for 1 h and finally at 800 V for 16 h. The lower and upper buffers were 20 mM phosphoric acid and 20 mM NaOH, respectively. After isoelectrofocusing, gels were extracted from their capillary and placed in transfer solution (0.5 M Tris/HCl, 10% SDS, 0.05% bromophenol blue, pH 8.8) for 10 min: proteins were then resolved on 10% SDS/PAGE, as described by Laemmli [33].

2.6. Western blotting

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

2.6.1. O-GlcNAc residue detection

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

2.6.2. Tau protein detection

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

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

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

2.6.3. Neurofilaments and Lamin B detection

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

2.6.4. Densitometric analysis

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

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

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

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

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

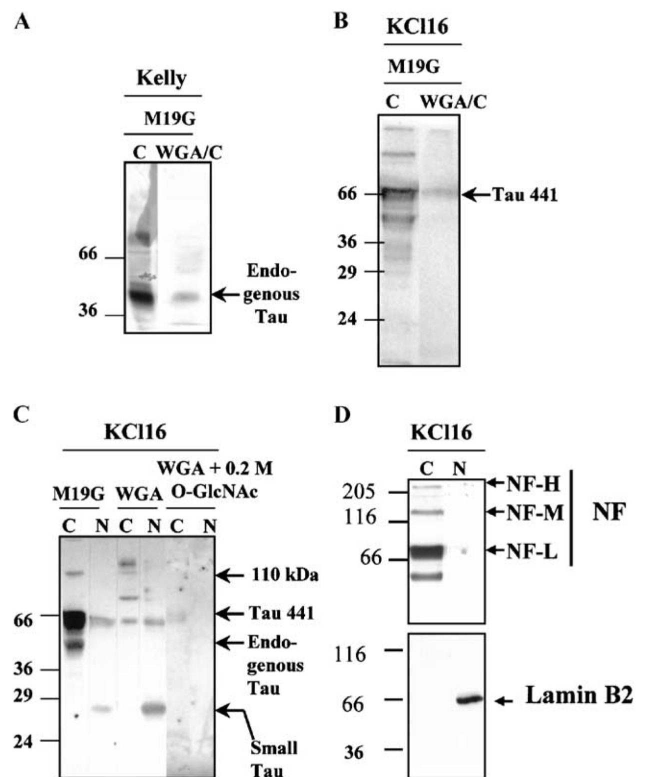

Fig. 1. WGA binding to cytosolic and nuclear Tau proteins. (A) Analysis of WGA-precipitated proteins from cytosolic Kelly extracts. The cytosolic fraction of human neuroblastoma Kelly cells was incubated with WGA–agarose beads. The proteins bound to WGA were analysed by Western blotting. A 52 kDa band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

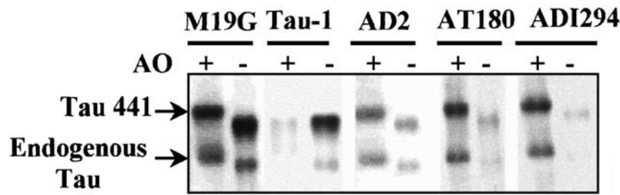

Fig. 2. Effect of okadaic acid on Tau protein phosphorylation. K Cl16 Cells were treated (+) or not (−) with OA for 4 h. Tau phosphorylation was tested by using several phospho-dependent antibodies: Tau-1, which recognizes an unphosphorylated epitope; AD2, specific for phosphorylated Ser-396/Ser-404;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

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

3. Results

3.1. WGA staining of cytosolic and nuclear Tau proteins

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

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

3.2. Effect of okadaic acid on Tau protein glycosylation

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

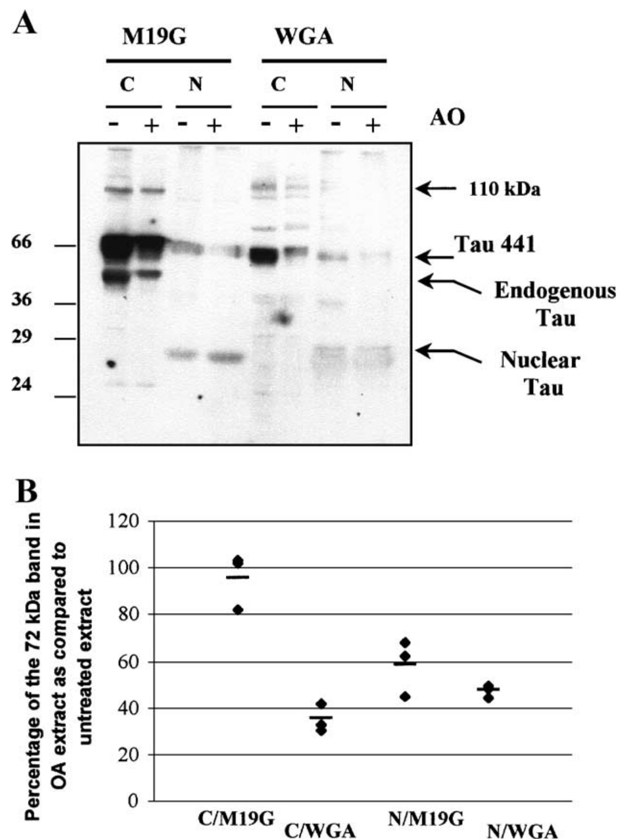

Fig. 3. Effect of okadaic acid on *O*-GlcNAc glycosylation of Tau proteins. (A) K Cl16 Cells were treated (+) or not (−) with OA for 4 h and then fractionated into cytosolic (C) and nuclear (N) fractions. M19G serum and peroxidase–WGA were used for detection of Tau and *O*-GlcNAc bearing proteins, respectively.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

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

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

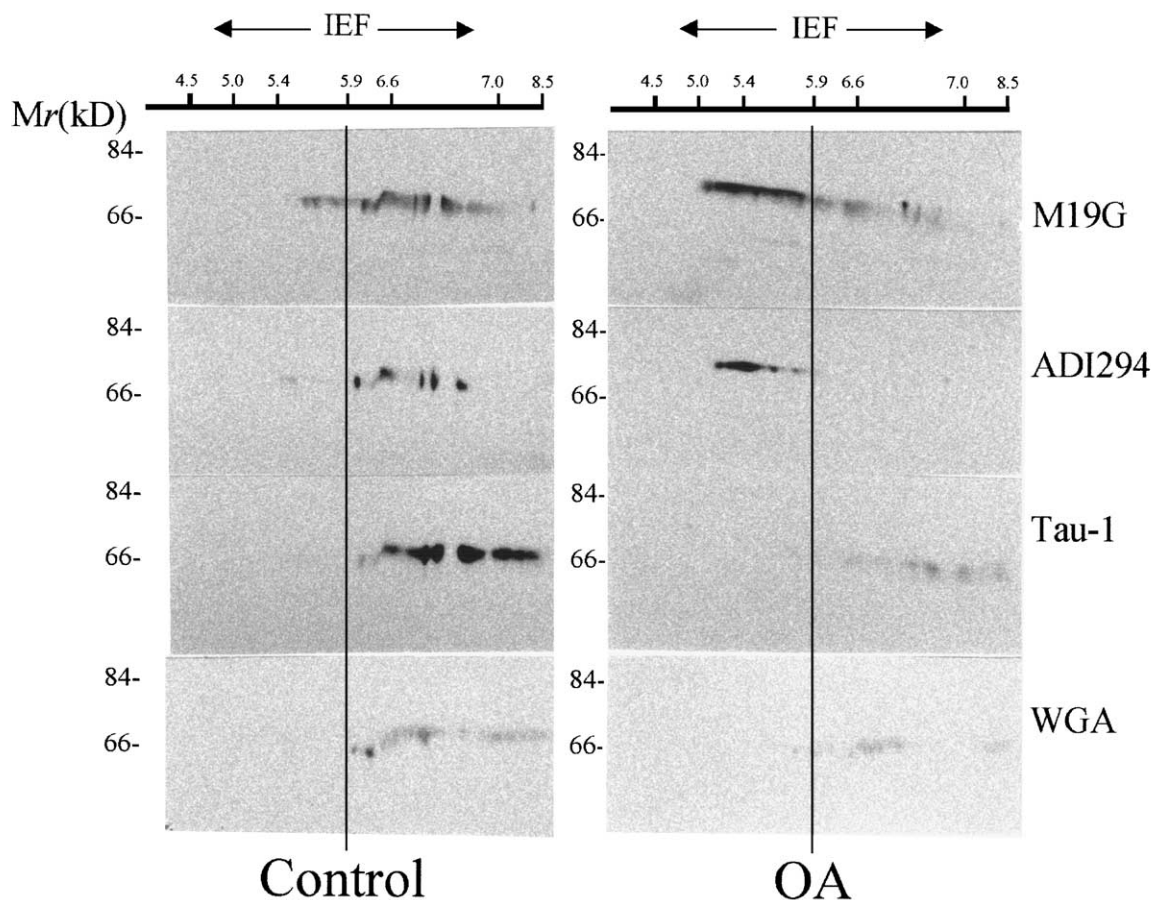

Fig. 4. Two-dimensional electrophoretic analysis of cytosolic Tau 441 variants. To investigate the balance between phosphorylation and *O*-GlcNAc glycosylation, cytosolic extracts were analysed using two-dimensional electrophoresis, as described in Materials and methods. Tau proteins from control and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

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

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

3.3.1. Western blot analysis

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

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

3.3.2. Analysis using the galactose transfer method

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

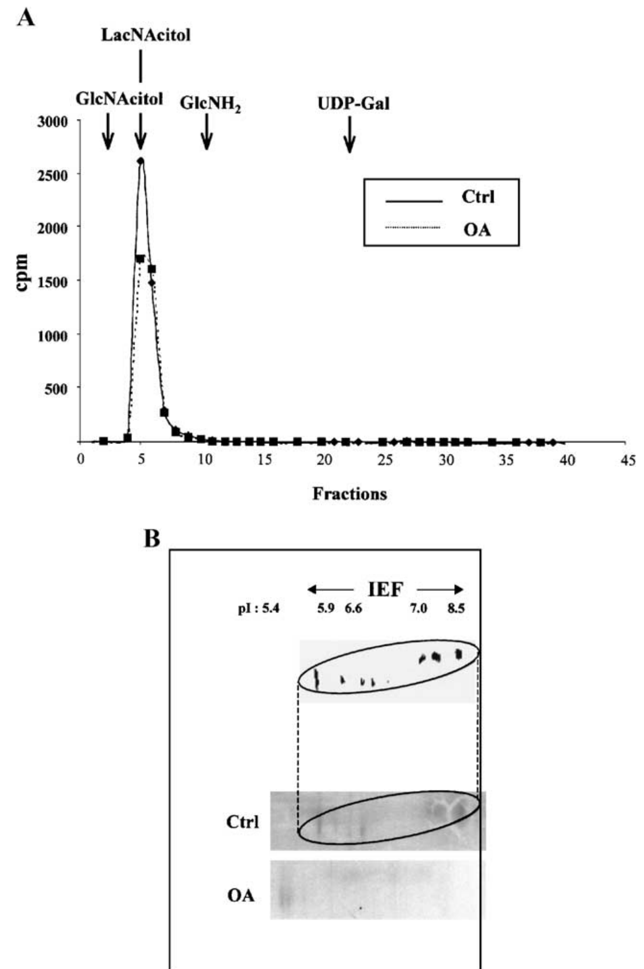

Fig. 5. Radiolabelled galactose transfer onto Tau 441. (A) Radioactive [^{3}H]-galactose labelling. Radioactive [^{3}H]-galactose was transferred by galactosyltransferase reaction onto the *O*-GlcNAc bearing cytosolic proteins of both control and OA-treated cells. Unincorporated UDP-6 [^{3}H]-galactose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

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

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

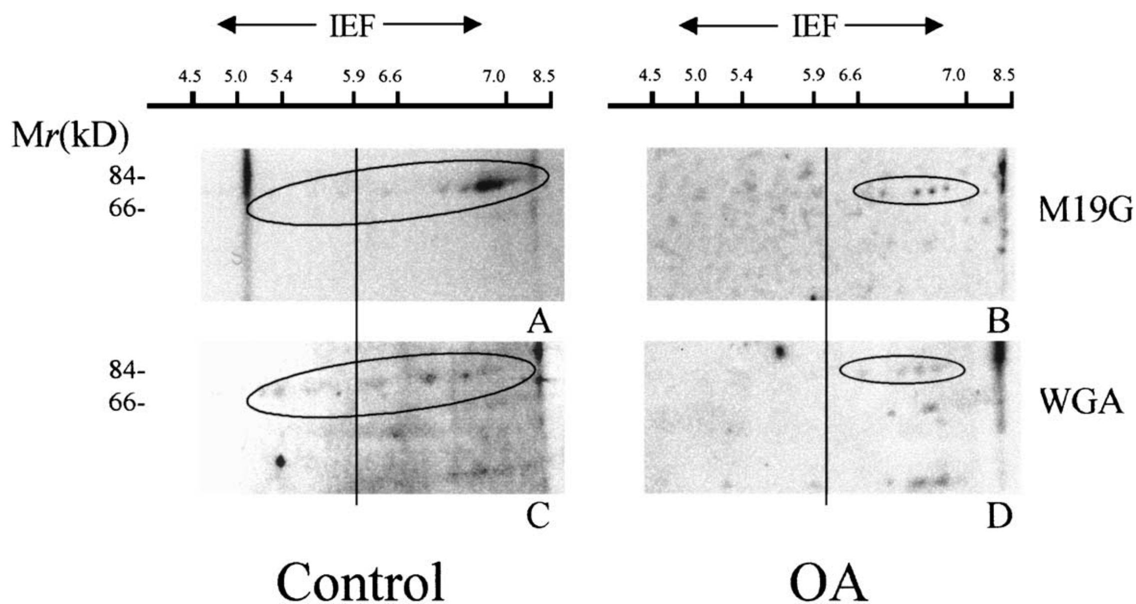

Fig. 6. 2D analysis of nuclear Tau proteins. Nuclear Tau isoforms were analysed by two-dimensional analysis. Nuclear Tau 441 isoforms were detected with both M19G and WGA.

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

4. Discussion

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

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

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

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

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

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

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

Acknowledgements

This work was supported in part by the CNRS (Unité Mixte de Recherches CNRS no. 8576, director J.C. Michalski, the Université de Lille I, the INSERM (U422, director J.C. Beauvillain), and the Lille Génopole. We are very grateful to Dr. Michel Goedert for his generous gift of pRK 172 plasmid (hTau40). ADI294 antibody was obtained in collaboration with Immunotech (E. Rouvier and F. Jean in particular). We also thank Dr. David Fraser (SARL Biotech Communication) for helpful criticism of this manuscript. Stéphanie Ferreira is a recipient of a fellowship from the French Research Ministry.

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