

Tau isoform expression and phosphorylation state during differentiation of cultured neuronal cells

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Abstract The axonal microtubule-associated protein, tau, is thought to play an important role in axonal growth and in the establishment of neuronal polarity. In adult human brain there are six alternatively spliced tau isoforms, which have different microtubule binding affinities *in vitro*. The tubulin–tau interaction is further modified by phosphorylation of tau and, compared to adult brain tau, both foetal brain tau and paired helical filament (PHF) tau, characteristic of Alzheimer's disease, are hyperphosphorylated. *In vivo* both the expression of tau isoforms and their phosphorylation states are developmentally regulated. In order to establish the correlation between the expression of tau isoforms and their pattern of phosphorylation, we have characterised these two features in several *in vitro* models of neuronal differentiation, including the human neuroblastoma cell lines, SK-N-SH, SH-SY5Y and IMR32 cells, rat PC12 cells and primary rat cortical neurones. Sensitive RT-PCR analysis revealed a different complement of tau isoforms in the different cell lines and neuritogenesis was associated mainly with an increase in the overall tau protein level with no apparent phosphorylation changes. A switch in tau isoform expression occurred only at the terminal stages of neuronal development, when it may be important in reinforcing the previously established axonal cytoarchitecture.

Key words: Tau; Neuronal differentiation; Cytoskeleton; Phosphorylation; Alzheimer's disease

1. Introduction

The neuronal microtubule-associated protein, tau, appears to play a major role in the polymerisation and stabilisation of microtubules during axonal elongation. Indeed, inhibition of tau expression in cultured cerebellar neurones results in a loss of axon formation [1,2] whereas over-expression of tau in non-neuronal insect cells leads to production of neurite-like processes [3]. Because of the different requirement for microtubule dynamics and stability during axonal elongation [4,5], the binding of tau to microtubules has to be modulated during development; this is achieved at both the transcriptional and post-translational levels.

Tau is encoded by a single gene but, in the adult human brain, is expressed as six alternatively spliced isoforms. The isoforms differ in having either three or four imperfect homologous repeat sequences in the C-terminal half of the molecule

and each of these two variants have either none, one or two inserts towards the N-terminus [6,7]. Isoforms with four C-terminal repeats have a higher affinity for microtubules than isoforms with three such repeats; the N-terminal inserts do not appear to contribute to microtubule binding [8]. Tau is a phosphoprotein and dephosphorylated tau has a higher microtubule assembly promoting activity than native tau [9]. In addition, tau phosphorylated *in vitro* by certain kinases has a reduced affinity for microtubules [10–12].

Only the smallest isoform, i.e. with no N-terminal inserts and three C-terminal repeats, is expressed in the foetal brain and during early postnatal development (approximately postnatal day 10 in the rat) [6,13]. There is a switch in RNA splicing to produce adult tau isoforms that is concomitant with a marked reduction in tau phosphorylation. Decrease in the level of tau phosphorylation during development essentially occurs by loss of phosphorylation at (Ser/Thr)-Pro sites [13–16] leaving only a proportion of tau in a similar phosphorylation state in the adult brain [17,18].

Tau is the principal component of the paired helical filaments (PHF), characteristic of the neurodegenerative pathology of Alzheimer's disease [19–21]. Tau associated with PHF (PHF-tau), is phosphorylated at the same (Ser/Thr)-Pro sites as foetal brain tau [13–16]. Despite this similarity in phosphorylation state, PHF are not formed in foetal brain. This is possibly because only the single shortest isoform is expressed in foetal brain or because PHF-tau is phosphorylated at additional sites compared to foetal tau [22]. Nevertheless, this has led to the proposal that a pathological neurotrophic response in Alzheimer's disease may re-activate the kinases/phosphatases responsible for tau phosphorylation in the juvenile brain, thus contributing to PHF formation via the inappropriate hyperphosphorylation of the full complement of adult brain tau isoforms.

The expression of different tau isoforms and the regulation of their phosphorylation in developing rat brain has been well documented [6,7,13–16]. However, since neurones from different areas of the brain do not extend their axons in a coordinated manner, a precise correlation between isoform expression and pattern of phosphorylation of tau has not been established. In the present study we characterised the expression and phosphorylation of tau during neurite outgrowth in several cultured neuronal cells. Our results suggest that, whereas early neurite outgrowth is associated with an overall increase in tau protein expression, the change in the splicing pattern of tau isoforms is a later event. Moreover, there were no detectable changes in the pattern of tau phosphorylation with respect to differentiation.

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2. Materials and Methods

2.1. Cell culture

SH-SY5Y, their parent cell line, SK-N-SH [23] and IMR32 human neuroblastoma cells were routinely grown in DMEM supplemented with 10% (v/v) foetal bovine serum (FBS). In order to induce differentiation, SH-SY5Y cells were plated at 4×10^5 cells/cm² and incubated with 10 μ M retinoic acid (RA) in DMEM supplemented with 1% (v/v) FBS. Cells were maintained in retinoic acid for up to three weeks at which stage a stable neuronal population had been established and retinoic acid was omitted from the medium. IMR32 cells were induced to differentiate by a single addition of 10 μ M bromodeoxyuridine (BrDU). PC12 cells were routinely grown on collagen in RPMI1640 supplemented with 10% (v/v) FBS and 5% (v/v) horse serum (HS). For differentiation, cells were replated at 2×10^4 cells/cm² and grown in RPMI supplemented with 1% (v/v) HS and 0.5% (v/v) FBS in the presence of 50 ng/ml β -NGF. Fresh differentiation medium was added every 3–4 days and control cells were cultured similarly in the absence of NGF. Primary dissociated rat brain cortical neuronal cultures were routinely established from embryonic day 17–18 (E17–E18) fetuses and maintained in DMEM/F12 (4:1) supplemented with 2% (v/v) FBS and essential supplements [24]. Postnatal cultures were established from 3-day-old (P3) pups by using a similar protocol.

2.2 Analysis of tau isoform expression

Total RNA was extracted from cells by the guanidium isothiocyanate/acid phenol method [25] and 2 μ g of each sample was reverse transcribed using an oligo(dT) primer and AMV reverse transcriptase (Promega, Southampton, England). The synthesised cDNAs were then subjected to 30 cycles of amplification by PCR using two pairs of primers to detect specifically either the N-terminal inserts or the C-terminal repeats in the tau cDNA, as previously described [13]. Oligonucleotide primer pairs were chosen according to the published rat [6] and human [7] tau cDNA sequences. PCR products were analyzed on 2% (w/v) Nu-sieve agarose gels and visualised with ethidium bromide.

2.3 Tau protein extraction and Western blot analysis

Cells were harvested in PBS and an aliquot was retained for subsequent protein determination by the Bradford method (Bio-Rad, Hemel Hempstead, England). Laemmli sample buffer was added to a second aliquot of the cells which was then boiled. The remaining cells were used to prepare a tau-enriched heat-stable fraction, as previously described [13]. Briefly, cells were lysed in Mes/NaCl buffer (100 mM Mes, 0.5 mM MgCl₂, 1 M NaCl, 2 mM DTT, 1 mM PMSF, 50 mM NaF, 0.1 mM NaVO₃, pH 6.5) and immediately boiled for 5 min before cooling on ice. Following centrifugation at $20,000 \times g_{av}$ for 20 min, the supernatant, containing tau, was diluted with Laemmli sample buffer and boiled. Equivalent loadings of total cell protein fractions or heat-stable preparations were run on 10% (w/v) SDS-PAGE gels and electrophoretically transferred to nitrocellulose. Nitrocellulose membranes were then blocked in 10% (w/v) semi-fat dried milk, 0.2% (w/v) Tween-20 in TBS and probed with various tau antibodies. Immunoreactivity was revealed using an alkaline phosphatase conjugated secondary antibody and BCIP/NBT substrate (Bio-rad). For dephosphorylation, pre-blocked nitrocellulose membranes were incubated for 16 h at 37°C with 1 U/ml of *E. coli* alkaline phosphatase (type III-N, Sigma, Poole, England) in 50 mM Tris/HCl, pH 8.3, containing 50 mM NaCl, 1 mM MgCl₂, 1 mM ZnCl₂ and 1 mM PMSF [26]. Control membranes were treated identically except for the addition of 0.2 M Na/K-phosphate, pH 8.3, to inhibit the phosphatase activity. Dephosphorylation of heat-soluble tau preparations prior to gel migration was carried out under similar conditions after spin dialysis against the alkaline phosphatase buffer using a Centricon-10 microconcentrator (Amicon, Stonehouse, England).

2.4 Antibodies

Antibodies 8073 [27] and TP70 [24] are rabbit polyclonal antisera raised against normal human brain tau or a carboxy-terminal 14 amino acid human tau peptide, respectively. The monoclonal antibody Tau 1 recognises a potentially phosphorylated epitope at Ser-199/Ser-202 of tau when it is not phosphorylated. 8D8, SMI31 and SMI310 are all neurofilament monoclonal antibodies that recognise phosphorylated epitopes in both neurofilaments and tau [28,29].

3. Results

In order to determine which tau isoforms were expressed in the different cultured cells examined and to investigate changes in their pattern of expression in relation to neuronal differentiation, mRNA was qualitatively analyzed by a sensitive reverse transcription-PCR (RT-PCR) technique. For this purpose, reverse transcribed mRNA was amplified by using pairs of primers flanking the N-terminal insert or the C-terminal repeat domains of tau. Typical results are shown in Fig. 1. Undifferentiated human neuroblastoma cells, SH-SY5Y (lanes 1) and their parent cell line, SK-N-SH (lanes 2) expressed principally the shortest tau isoform (i.e. tau isoforms with no N-terminal inserts and three C-terminal repeats). Forms with four C-terminal repeats are also expressed, but, given the limitation of PCR as a quantitative technique, at a much lower level than three repeat isoforms. After 3 to 4 weeks in culture in the presence of retinoic acid, these cells establish a non-proliferative neuronal population with extensive neurites, expressing the adult neurofilament triplet proteins [30]. No significant changes in the pattern of tau expression following differentiation of SH-SY5Y cells for up to 25 days were observed. Undifferentiated IMR32 cells also mainly express the shortest tau isoform, however, in cells terminally differentiated following a 28 day treatment with BrDU, tau isoforms with N-terminal inserts, particularly two inserts, began to appear (Fig. 1A, lanes 3). Even considering the difficulty in quantifying PCR data, there was also a marked increase in isoforms with four C-terminal repeats

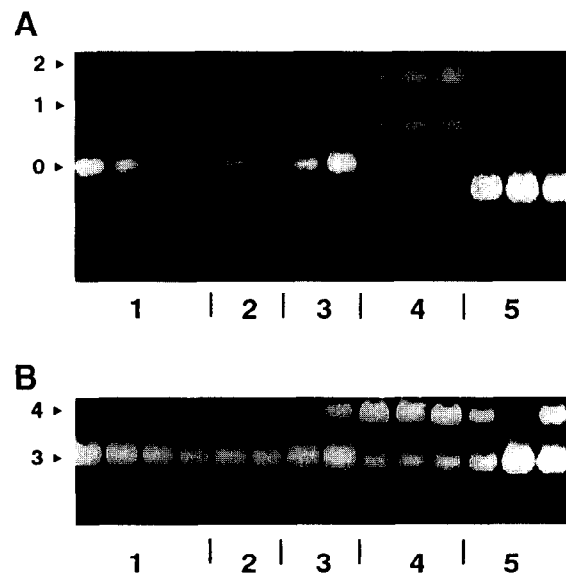


Fig. 1. RT-PCR analysis of tau isoform expression in cultured neuronal cells. (A) Reverse transcribed DNA amplified with a pair of primers detecting the N-terminal insert domains of tau. Arrowheads on the left-hand side indicate the positions of human tau isoforms with no, one or two N-terminal inserts. Arrowheads on the right-hand side indicate the positions of similar isoforms of rat tau, that are slightly smaller than the corresponding human isoforms. (B) Reverse transcribed DNA amplified with a pair of primers detecting the C-terminal repeats of tau. Arrowheads to the left of the figure indicate the positions of tau isoforms with three or four C-terminal inserts. Lanes 1: SH-SY5Y cells exposed to RA for 0, 9, 17 and 25 days; lanes 2: SK-N-SH cells, control and 28 days with RA; lanes 3: IMR32 cells, control and 28 days with BrDU; lanes 4: PC12 cells, 0, 10 and 17 days with NGF; lanes 5: postnatally (P3) established primary rat cortical cultures after 10 days in vitro; rat brain extracts from P3 and P10 animals.

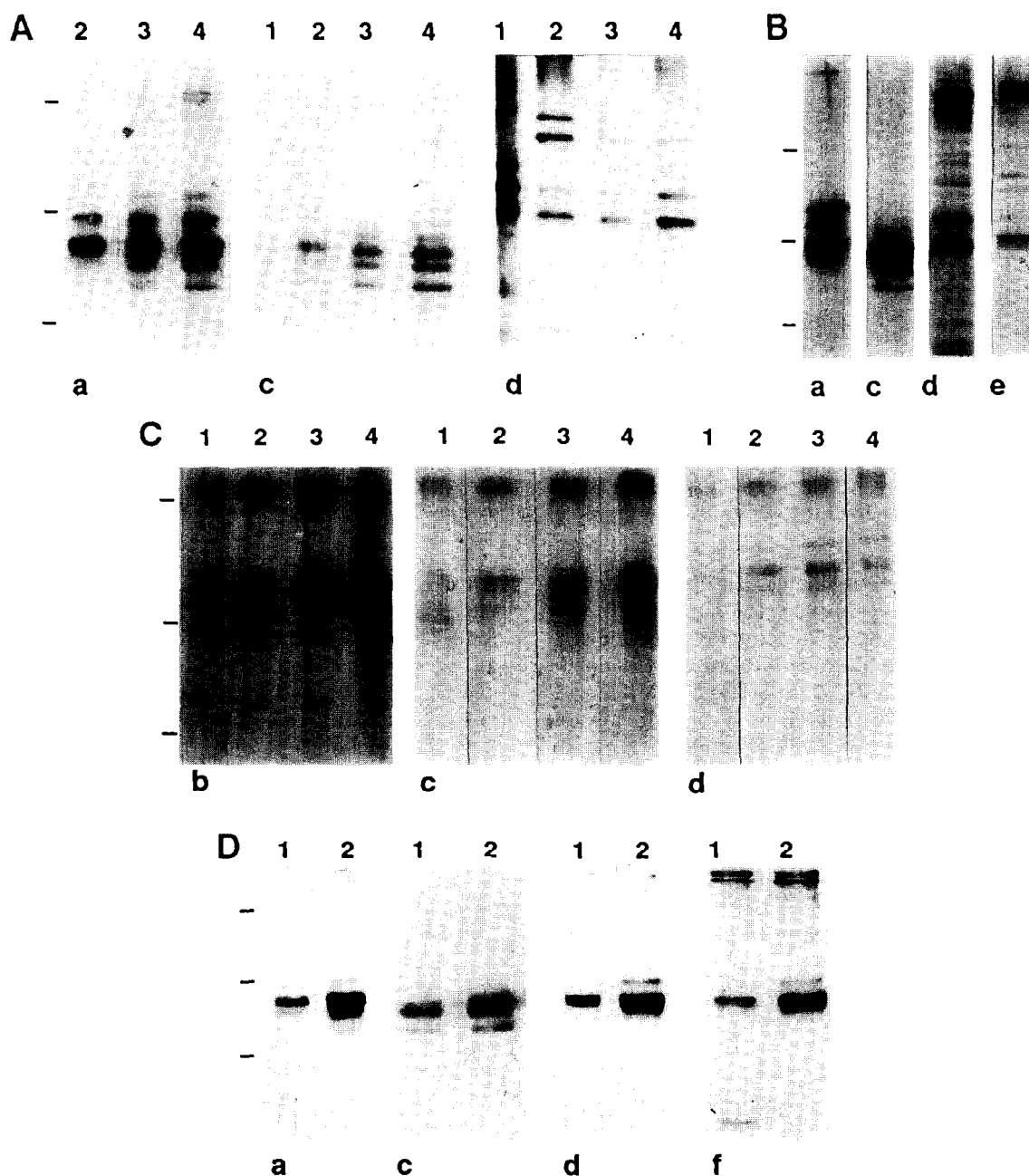


Fig. 2. Western blots showing tau protein expression and phosphorylation in cultured neuronal cells. (A) SH-SY5Y cells; lanes 1: PHF-tau; lanes 2: undifferentiated cells; lanes 3 and 4: cells differentiated for 14 and 21 days, respectively. (B) IMR 32 cells differentiated for 28 days with BrDU. (C) PC12 cells; lanes 1: undifferentiated cells; lanes 2, 3 and 4: cells differentiated for 7, 11 and 17 days, respectively. (D) P3 rat primary neurones after 10 (lanes 1) and 18 (lanes 2) days in vitro. Western blots were stained with the following antibodies: 8073 (a), TP70 (b), Tau 1 (c), 8D8 (d), SMI310 (e) and SMI31 (f). The bands at about 140 kDa detected with 8D8, SMI310 and SMI31 are neurofilament subunits. In addition, 8D8 labels two unidentified slower-migrating species in undifferentiated SH-SY5Y cells (A, d, lane 2). Bars to the left of the panels indicate the positions of the molecular weight markers: phosphorylase b, 97.4 kDa; catalase, 58.1 kDa and alcohol dehydrogenase, 39.8 kDa.

(Fig. 1B, lanes 3). Undifferentiated rat pheochromocytoma 1/C12 cells expressed a mixture of longer tau isoforms (i.e. one and two N-terminal inserts with both three and four C-terminal repeats) but little of the shortest isoform (Fig. 1, lanes 4). Differentiation of PC12 cells using low density cultures and low serum resulted in the formation of an extensive neuritic network which could be maintained for at least one month in culture. However, as in SH-SY5Y and SK-N-SH cells, there was no change in tau isoform expression in relation to differentiation.

Rat cortical neurones in primary cultures established from P3 animals and maintained for 10 days in vitro contained isoforms with three and four C-terminal repeats; forms with one or two N-terminal inserts just began to appear (Fig. 1A and B, lanes 5). This pattern is similar to that of P10 rat brain, whereas tau in P3 rat brain is still principally composed of the shortest isoform. However, an adult pattern of tau isoforms was not observed in cultures established from E18 foetal brain tissue and maintained for up to one month in vitro (data not shown).

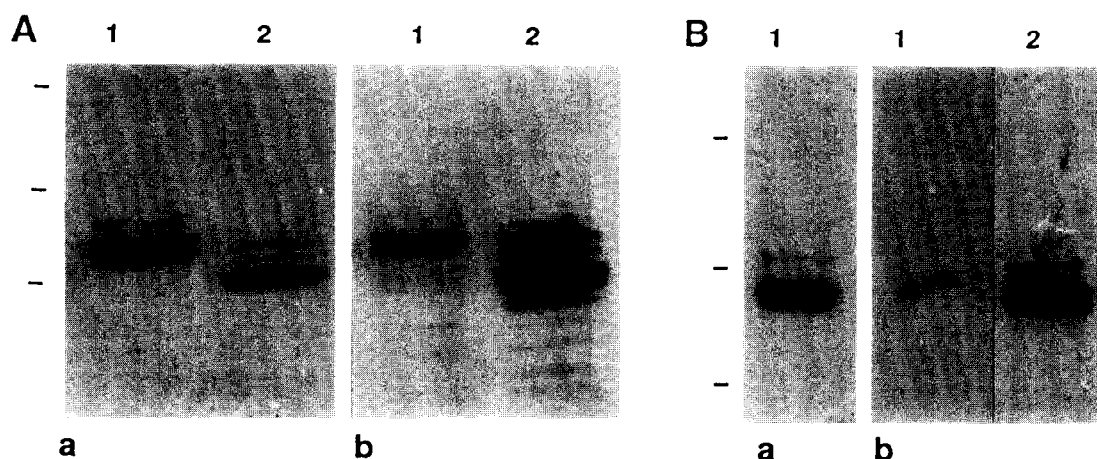


Fig. 3. Tau is partially hyperphosphorylated in SH-SY5Y cells. Heat-stable tau preparations from undifferentiated SH-SY5Y cells were dephosphorylated with alkaline phosphatase either prior to SDS-PAGE (A) or on nitrocellulose subsequent to Western blotting (B). Lanes 1: control; lanes 2: dephosphorylated preparation. The blots were stained with 8073 (a) or Tau 1 (b). Note the increased migration rate of dephosphorylated tau in (A) as well as increased Tau 1 immunoreactivity following dephosphorylation. Molecular weight markers are as in Fig. 2.

When examined by Western blotting, tau from the various cell lines appears as multiple bands. The number of bands detected in tau is determined by its isoform composition and its state of phosphorylation, that affects electrophoretic mobility [9]. This is illustrated in Fig. 2 for SH-SY5Y, IMR32 and PC12 cells as well as for P3 rat brain primary cortical neurones. The principal tau species in SH-SY5Y cells (Fig. 2A), as well as in IMR 32 cells (Fig. 2B) or in rat cortical neurones (Fig. 2D), migrated as a broad band or doublet at around 50 kDa. Consistent with the RT-PCR analyses, PC12 tau migrated as several bands in the 55–65 kDa range, together with high molecular weight tau at around 110 kDa (Fig. 2C).

In SH-SY5Y and PC12 cells differentiated for up to 3 weeks, as well as in primary cortical neurones, a major feature of neuronal differentiation was an overall increase in the level of tau protein with little change in the band pattern of tau species. This is best illustrated by the labelling with the polyclonal antibodies, 8073 and TP70, which recognise all brain tau isoforms largely independently of their phosphorylation state (Fig. 2A, C and D, a and b). For each cell type, the same total amount of protein was loaded in each lane so that increased immunoreactivity represents an increase in the proportion of tau in the samples.

Western blots were also probed with a panel of antibodies binding to tau in a phosphorylation-dependent manner (Fig. 2A–D, c–f). These included the monoclonal antibody, Tau 1 (Fig. 2A–D, c), recognizing Ser-199/Ser-202 of the longest human tau isoform when unphosphorylated and the neurofilament monoclonal antibodies, 8D8, SMI310 and SMI31, that recognise phosphorylated neurofilaments as well as tau phosphorylated at specific sites (Fig. 2A–D, d–f). For instance 8D8 recognises Ser-396 of human tau in a phosphorylated state and the epitope for SMI31 and SMI310 is centred around Ser-396 and Ser-404. These sites are in regions conserved between rat and human tau. The fraction of tau labelled by the neurofilament antibodies was always the slowest-migrating component of the total cellular tau. Tau in all the cultured cells stained with either Tau 1 or the neurofilament antibodies, indicating that a proportion of tau was phosphorylated. However, the pattern of immunoreactive species was different for each

cell type. For example, in SH-SY5Y cells, the slow migrating tau species stained by 8D8 (Fig. 2A, d) had a weak reactivity for 8073 which strongly labelled faster migrating tau (Fig. 2A, a), indicating that most of the tau in SH-SY5Y cells was not phosphorylated at Ser-396. On the other hand, most of the tau in rat cortical neurones appears phosphorylated at Ser-396 (Fig. 2D, a and d).

Although an absolute quantification was not performed, there was no overt change in the proportion of hyperphosphorylated tau occurring during differentiation of the cell type examined (Fig. 2A, C and D, d and f). The 110 kDa high molecular weight tau present in PC12 cells was also partially phosphorylated (Fig. 2C, c and d). The monoclonal antibodies 8D8, SMI310 and SMI31 also detected neurofilament polypeptides at about 140 kDa, this is particularly clear for IMR 32 cells (Fig. 2B, d and e) and for rat cortical neurones (Fig. 2D, d and f).

Faster-migrating and slower-migrating tau species were also detectable in the neuroblastoma cells and primary neurones, albeit in minor quantities, suggesting the presence of dephosphorylated and longer tau isoforms, respectively. In order to investigate further the basis for this heterogeneity, tau extracted from undifferentiated SH-SY5Y cells, which expressed almost exclusively the shortest tau isoform, was dephosphorylated *in vitro* and analyzed by Western blotting. Treatment of tau from SH-SY5Y cells with alkaline phosphatase resulted in a dramatic electrophoretic shift and in an altered immunoreactivity, as illustrated in Fig. 3A. Following dephosphorylation, the principal tau species migrated at about 42 kDa, as opposed to 50 kDa in untreated samples, and labelled intensely with the Tau 1 antibody. Partial phosphorylation of tau at the Tau 1 epitope in SH-SY5Y cells was confirmed by the increase in Tau 1 immunoreactivity after treatment of nitrocellulose-bound tau with alkaline phosphatase (Fig. 3B).

4. Discussion

In the developing brain there is a switch in tau expression from the single shortest isoform to multiple adult isoforms,

which occurs at about postnatal day 10 in the rat [6,13]. This switch is coincident with reduced tau phosphorylation and has been related to a reduction in neuronal plasticity and reinforcement of mature neuronal cytoarchitecture.

Our results in several different *in vitro* models of neuronal differentiation, indicate that an overall increase in tau protein levels is coincident with neurite outgrowth. This could either be due to an increase in tau expression or to a reduced sensitivity of tau to proteolysis after its transport into neurites. Another microtubule-associated protein, MAP2, has been shown to be more stable in dendrites than in axons and neuronal cell bodies [31]. A switch in tau isoform expression would be a much later event; it is even possible that, in some neuronal systems, including primary cultures established from foetal brain, that the expression of mature isoforms may never occur [32].

The presence of multiple tau isoforms in PC12 cells contrasts with the single shortest isoform present in other cell lines. However, the presence of mature tau isoforms in undifferentiated PC12 cells does not inhibit neurite extension. It thus appears that the absolute level rather than the complement of tau isoforms is the key factor in early neurite outgrowth. This is consistent with tau transfection experiments, where over-expression of tau induces processes in the non neuronal insect Sf9 cells [3] and accentuates neurite outgrowth in PC12 cells [33]. Likewise, the inhibition of tau expression in cerebellar neurones inhibits differentiation of exploratory neurites into axons [1].

It thus appears that altered tau isoform expression is not necessary for extensive neuritic development although equally, the presence of multiple tau isoforms does not impede early neuronal differentiation in PC12 cells.

Our observations on tau phosphorylation in the human neuroblastoma cell line, SH-SY5Y confirmed those reported by Tanaka et al. [34], who also found phosphorylation of tau at Ser-199/Ser-202, Ser-396 and Ser-404 in undifferentiated SH-SY5Y cells and during their early differentiation. In addition we showed that, after long term differentiation, when a mature neuronal phenotype is obtained, there is no overt change in the pattern of tau phosphorylation. However, the immunofluorescence reactivity of SH-SY5Y tau for PHF-1, a monoclonal antibody to tau phosphorylated at Ser-396, increases during differentiation to a neuronal phenotype and redistributes from the soma to elaborating neurites [35]. This, together with our results indicates that phosphorylated tau may undergo conformational changes or changes in its interaction with other proteins during differentiation that would, in turn, result in the unmasking of the PHF-1 epitope. We found no evidence for a significant change in the phosphorylation pattern of tau in association with neuronal differentiation. Thus, a minor fraction of the total cellular tau was phosphorylated at Ser-199/Ser-202 and/or at Ser-396/Ser-404 in all of the cell lines investigated, independently of their differentiation state. As the developmental change in tau phosphorylation *in vivo* coincides with the expression of mature tau isoforms [13], it is perhaps not surprising that no phosphorylation changes *in vitro* were observed. However, alterations in tau phosphorylation at these serine residues may not be an important regulatory control for microtubule properties during neuronal differentiation. Regulation of tau phosphorylation at other residues, particularly Ser-262 which influences microtubule binding *in vitro*, may be more significant [11,12]. It is also possible that differentially phosphorylated forms of tau are associated with different sub-

populations of microtubules or localized in different subcellular compartments. It is interesting that tau phosphorylation is not obviously affected by the arrest in cell division associated with the initiation of neuronal differentiation in neuroblastoma and PC12 lines. Proline-directed kinases, including cell cycle-dependent kinases, phosphorylate tau *in vitro* [10,26,36,37] and there is a recent report of increased immunoreactivity for the antibody PHF-1 (recognizing phosphorylated Ser-396) in mitotic SH-SY5Y cells [38] therefore classical cell cycle-dependent kinases may have a role in tau phosphorylation.

With regard to unravelling the processes that underlie tau hyperphosphorylation in Alzheimer's disease, it is noteworthy that all of these neuronal culture models possess a fraction of PHF-like tau which is phosphorylated at Ser-202 and/or Ser-396/Ser-404. Furthermore, in the presence of phosphatase inhibitors, the majority of tau accumulates in a hyperphosphorylated state [39,40]. Therefore it appears that all of these cell lines possess some of the kinases necessary to generate PHF-tau epitopes. The effect of okadaic acid on tau phosphorylation, as has been reported previously, is only apparent when cells are irreversibly poisoned.

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