

Tau protein is phosphorylated by cyclic AMP-dependent protein kinase and calcium/calmodulin-dependent protein kinase II within its microtubule-binding domains at Ser-262 and Ser-356

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Phosphorylation of tau protein at Ser-262 has been shown to diminish its ability to bind to taxol-stabilized microtubules. The paired helical filaments (PHFs) found in Alzheimer's disease brain are composed of PHF-tau, which is hyperphosphorylated at multiple sites including Ser-262. However, protein kinase(s) able to phosphorylate this site are still under investigation. In this study, the ability of cyclic AMP-dependent protein kinase (cAMP-PK) and calcium/calmodulin-dependent protein kinase II (CaMKII) to phosphorylate tau at Ser-262, as well as Ser-356, is demonstrated by use of a monoclonal antibody (12E8) which has been shown to recognize tau when these sites are phosphorylated. Cleavage of cAMP-PK-phosphorylated tau at

cysteine residues by 2-nitro-5-thiocyanobenzoic acid, which cuts the protein into essentially two fragments and separates Ser-262 from Ser-356, revealed that cAMP-PK phosphorylates both Ser-262 and Ser-356. In addition, phosphorylation with cAMP-PK or CaMKII of recombinant tau in which Ser-262, Ser-356 or both had been mutated to alanines, clearly demonstrated that cAMP-PK and CaMKII were able to phosphorylate both sites. Mitogen-activated protein kinase or protein kinase C did not phosphorylate tau at Ser-262 and/or Ser-356. Finally, evidence is presented that phosphorylation of both these sites occurs in cultured nerve cells under certain conditions, indicating their potential physiological relevance.

INTRODUCTION

Tau proteins are extended, microtubule-associated proteins with little or no secondary structure [1]. This family of structural proteins is formed by alternative mRNA splicing of a single gene, and in human brain there are six known tau isoforms [2]. All these isoforms contain three or four imperfect tandem repeats in the carboxyl half of the molecule, and either no, one or two inserts in the N-terminal portion [2]. The expression of tau isoforms is developmentally regulated [2,3]. In adult human brain, all six isoforms ranging from 352 to 441 amino acids are present, while only the shortest form is found in fetal brain [2]. Although there is now evidence that tau is glycosylated [4] and in certain situations glycated [5] or ubiquitinated [6], the most prevalent post-translational modification is phosphorylation (for reviews, see [7,8]).

Tau is a substrate *in vitro* for a number of protein kinases, including casein kinase II [9], cyclin-dependent protein kinase 5 (cdk5) [10], glycogen synthase kinase-3 (GSK3) [11], mitogen-activated protein kinase (MAP kinase) [12,13], calcium/calmodulin-dependent protein kinase II (CaMKII) [14,15], p34^{cdc2} kinase [16] and cyclic AMP-dependent protein kinase (cAMP-PK) [17,18]. Site-specific phosphorylation has been shown to modulate the function and metabolism of tau *in vitro*. Recombinant tau phosphorylated by MAP kinase has been shown to have a 10-fold reduced ability to bind to microtubules [19], although another study reported little change in the ability of tau to bind to taxol-stabilized microtubules following phosphorylation by MAP kinase [20]. Phosphorylation of tau by CaMKII significantly inhibits intermolecular tau-tau inter-

actions [21] and phosphorylation by p34^{cdc2} kinase decreases tau-induced microtubule assembly [22]. Phosphorylation of tau by cAMP-PK significantly decreases the binding of tau to tubulin [15] and inhibits the ability of tau to promote microtubule assembly and nucleation [23]. Additionally, phosphorylation of tau significantly inhibits its proteolysis by the calcium-dependent protease calpain [17]. However, the specific kinases which phosphorylate tau *in situ* have not yet been identified.

Paired helical filaments (PHFs), which are abnormal fibrous structures found in brains of patients with Alzheimer's disease, are composed primarily of all six brain tau forms in a hyperphosphorylated state (PHF-tau) [24–26]. PHFs exist both in a soluble form, and as the principal structural component of neurofibrillary tangles, a predominant neuropathological lesion found in the brains of patients with Alzheimer's disease [27]. Recent studies have identified numerous phosphorylation sites in PHF-tau and fetal tau (for a review, see [7]). PHF-tau has been shown to be phosphorylated at as many as 19 different sites, and fetal tau at at least 11 sites [28,29]. However, only a single site in the microtubule-binding region of tau, Ser-262 (amino acids are numbered according to the largest human brain tau isoform [2]), has been shown to be phosphorylated in a fraction of PHF-tau [29,30]. Notably, this site was not found to be phosphorylated in fetal or adult rat tau using mass spectrometry [28]. This is intriguing, since it has been reported that phosphorylation of Ser-262 strongly inhibits the binding of tau to microtubules [20]. It is also significant that fetal tau, which is phosphorylated at many of the same Ser/Thr-Pro sites as PHF-tau [28], is able to bind to microtubules and to promote microtubule assembly [31]. PHF-tau is unable either to bind to microtubules or to promote

Abbreviations used: PHFs, paired helical filaments; cAMP-PK, cyclic AMP-dependent protein kinase; CaMKII, calcium/calmodulin-dependent protein kinase II; NTCB, 2-nitro-5-thiocyanobenzoic acid; GSK3, glycogen synthase kinase 3; MAP kinase, mitogen-activated protein kinase; PKI, protein kinase inhibitor.

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microtubule assembly, unless it is first dephosphorylated [31,32]. These results suggest that the hyperphosphorylation of non-Ser/Thr-Pro sites, especially those within the microtubule-binding domains, may play a role in rendering PHF-tau dysfunctional. Indeed, it can be hypothesized that phosphorylation of a few key regulatory sites in tau may prevent it from appropriately binding and stabilizing microtubules, and thus may play a role in its abnormal accumulation and extensive hyperphosphorylation in Alzheimer's disease brain [33].

Although an enriched fraction containing a protein kinase activity capable of phosphorylating Ser-262 has been described, the enzyme in question was not initially identified [20]. Subsequent work [34] suggests a portion of this activity may be due to cAMP-PK. It has also been demonstrated previously that cAMP-PK is able to phosphorylate tau at Ser-325 and Ser-356 within the microtubule-binding domains. However, Ser-262 was not found to be phosphorylated by cAMP-PK in this study [18].

By using a monoclonal antibody (12E8) under conditions in which it recognizes tau only when Ser-262 and/or Ser-356 are phosphorylated [35], we demonstrate that cAMP-PK and CaMKII phosphorylate both sites *in vitro*. Evidence is also presented that these sites are continually undergoing phosphorylation/dephosphorylation *in situ*.

MATERIALS AND METHODS

Proteins

Tau was purified directly from bovine brain (referred to as 'total tau') and from twice-cycled microtubules prepared from rat brain as previously described [36] with the following exceptions: 10 mM sodium pyrophosphate was added to all buffers when purifying tau directly from bovine brain and a Sephacryl-S200 HR column (Pharmacia) was used to fractionate the samples. Heat-stable, tau-enriched extracts were prepared from the prefrontal cortex of an autopsied control human brain (65-year-old) as previously described [37]. Protein concentrations were determined by the method of Lowry et al. [38] after acid precipitation of the protein. The catalytic subunit of cAMP-PK was purified to homogeneity from beef heart using the method of Reimann and Beham [39], CaMKII was isolated from rat brain by the method of Goldenring et al. [40] and protein kinase C was purchased from Calbiochem. Recombinant p42 MAP kinase that had been activated by incubation with MAP kinase kinase and MgATP [41] was provided by P. Cohen.

A full-length human brain tau cDNA clone (htau 24) which encodes for the isoform containing four microtubule-binding repeats and no N-terminal inserts (T4) [2], was used for site-directed mutagenesis, as described [35,42]. Site-directed mutagenesis was used to change the codons for Ser-262 and/or Ser-356 to alanine. Recombinant tau proteins were purified as described [43].

Phosphorylation

Tau protein was phosphorylated for 2 h at 37 °C with either cAMP-PK or CaMKII exactly as described previously [15]. In some experiments tau was incubated with cAMP-PK in the presence of 1 μ M protein kinase inhibitor (PKI), a selective cAMP-PK inhibitor [44]. Phosphorylation of tau by protein kinase C (1 unit of kinase/mg of tau) was carried out for 4 h at 37 °C as described by Correas et al. [45]. Phosphorylation of recombinant tau by 1 unit/ml p42 MAP kinase for up to 24 h at 30 °C was performed as described [13].

Cell culture

Human mixed brain cell cultures were grown as previously described [46]. Cultures were treated with 100 nM okadaic acid for 24 h, rinsed once with phosphate-buffered saline and harvested in 10 mM Tris/HCl, pH 8.0, 140 mM NaCl, 0.8 mM EDTA, 0.1 mM PMSF (3 ml per T-75 flask). The extract was spun at 300 g for 5 min and the pellet stored at -80 °C until required for use.

2-Nitro-5-thiocyanobenzoic acid (NTCB) cleavage

Human mixed brain cells from two T-75 flasks were resuspended in 2 ml of 20 mM Mes, pH 7.0, containing 1 mM MgSO₄, 1 mM EGTA, 1 M NaCl and 4 mM dithiothreitol, sonicated and incubated in a boiling-water bath for 10 min. Homogenates were cooled, spun for 10 min at 16000 g, and the supernatants collected. Perchloric acid was added to the supernatants to a final concentration of 2.5% while stirring on ice. The mixture was spun at 16000 g, the supernatants collected and dialysed against 20 mM Mes, pH 7.0, 1 mM MgSO₄, 1 mM EGTA until the pH was 6.5-7.0. The samples were then dialysed for 1 h against water, frozen and lyophilized.

Lyophilized protein samples from the cells or 10 μ g of purified bovine tau were resuspended in 25 μ l of 40 mM EDTA, 20 mM dithiothreitol and incubated in a boiling-water bath for 2 min prior to the addition of 225 μ l of 7.04 M guanidine hydrochloride, 110 mM Tris/HCl, pH 8.0, 11 mM NTCB. Samples were incubated for 12 h at 37 °C, 50 μ l of 60 mM NTCB in 6.4 M guanidine hydrochloride, 100 mM Tris/HCl, pH 8.0, was added to each tube and the incubation continued for another 48 h. The protein was precipitated by the addition of deoxycholate to a final concentration of 125 μ g/ml and trichloroacetic acid to a concentration of 6% followed by centrifugation. The precipitated protein was resuspended in 200 μ l of 100 mM Tris, pH 8.0, and extracted with 4 vol. of methanol:chloroform:water (4:1:3, by vol.). The samples were spun for 2 min at 16000 g, the top layer was removed, leaving the interface, and 500 μ l of ice-cold methanol was added to each tube. After incubation on ice for 1 h, the samples were spun at 16000 g for 8 min, the supernatant removed and discarded, and the pellets air-dried prior to resuspension in sample buffer and electrophoresis [47].

Immunoblot analysis

Aliquots were run on SDS/8%-polyacrylamide gels (unless otherwise indicated), transferred to nitrocellulose [48] and immunoblots were probed with one of the following monoclonal antibodies to tau: 12E8 which recognizes tau phosphorylated at Ser-262 and/or Ser-356 [35], and either T14, T46 [32,49], Tau 2 [50] or 8C11 [51] which are phosphate-independent antibodies to tau. After incubation with the primary antibody the blots were probed with horseradish peroxidase-conjugated goat anti-(mouse IgG) and the immunoreactive bands visualized either by colour development with 3,3'-diaminobenzidine in the presence of hydrogen peroxide, or by enhanced chemiluminescence (ECL Western Blot Kit, Amersham).

³²P phosphorylation of T4 and mutants

T4 and each mutant (2.5 μ M) was incubated with the catalytic unit of cAMP-PK (0.85 μ M) in phosphorylation buffer containing 25 mM Tris/HCl, pH 7.2, 10 mM MgCl₂, 0.5 mM dithiothreitol and 50 μ M [γ -³²P]ATP (0.1 μ Ci/nmol) at 37 °C for 3 h in a final volume of 25 μ l. The reaction was terminated by the addition of an equal volume of 2 \times SDS Stop, and the samples

incubated in a boiling-water bath for 5 min and run on SDS/8% polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue R250, destained, dried and exposed to X-ray film. To determine differences in the extent of phosphorylation between T4 and the mutants, the autoradiographs and the Coomassie-stained gels were analysed using a Bio-Rad model GS-670 imaging densitometer. The data were expressed as ratios of the relative absorbances of the two measurements to normalize the autoradiographic values based on the amount of protein in each lane. Mol ^{32}P /mol T4 ratios incorporated by cAMP-PK were determined as described previously [17].

Kinetic determinations

Samples (100 μM) of the peptide KSKIGSTENLKHQPGGG, or the phosphoform KSKIGS(P)TENLKHQPGGG, representing amino acids 257 to 273 in tau [35], were incubated with the catalytic subunit of cAMP-PK (30 $\mu\text{g}/\text{ml}$) at 37 °C in phosphorylation buffer in a total volume of 30 μl . Aliquots (5 μl) were removed at 2, 5, 15, 30 and 60 min, spotted on to P81 paper (0.5 cm \times 1 cm) and immediately placed into 0.5% phosphoric acid. The papers were washed extensively with 0.5% phosphoric acid, followed by two washes in 95% ethanol. The papers were air-dried prior to determination of ^{32}P incorporation into the peptides by liquid scintillation counting. Autophosphorylation was allowed for by subtracting the counts of control samples, incubated in the absence of peptide, from sample values.

RESULTS

Monoclonal antibody 12E8 was raised against the phosphorylated peptide, KSKIGS(P)TENLKHQPG, which corresponds to amino acids 257–271 of tau located within the first microtubule-binding repeat [35]. Figure 1 shows representative immunoblots of total bovine tau, twice-cycled microtubule rat tau, or of a tau-enriched fraction from human brain which were incubated in the absence or presence of cAMP-PK and probed with either 12E8 (Figure 1A), or the phosphate-independent tau antibody 8C11 (Figure 1B). 12E8 immunoreactivity of rat tau isolated from twice-cycled microtubules, and of the tau-enriched fraction from human brain, was weak or absent. In contrast, total bovine tau showed significant 12E8 immunoreactivity (Figure 1). This was probably because the bovine brains were processed rapidly in the presence of 10 mM sodium pyrophosphate to inhibit phosphatase activity. Indeed, bovine tau isolated from twice-cycled microtubules showed only weak 12E8 immunoreactivity, similar to that observed for rat tau isolated from twice-cycled microtubules (results not shown). These findings indicate that tau is dephosphorylated at the 12E8 epitope(s) during the microtubule cycling procedures. However, in all cases, incubation with cAMP-PK greatly increased 12E8 immunoreactivity. Given that previous findings have demonstrated that cAMP-PK phosphorylates Ser-356, among other sites [18], this suggested that 12E8 recognized tau due to Ser-356 phosphorylation [35]. To determine whether cAMP-PK also phosphorylates Ser-262, bovine tau was phosphorylated with cAMP-PK and then cleaved at cysteine residues with NTCB. Since NTCB cleaves selectively at cysteine residues, and there is only one cysteine residue located within the second (which is alternatively spliced to form three or four repeat tau [2]) and third microtubule-binding domains of tau, cleavage with this reagent separates tau into amino and carboxyl fragments, with Ser-262 being located in the N-terminal portion and Ser-356 in the C-terminal fragment. Representative results from these experiments are shown in Figure 2. The N-terminal domain was

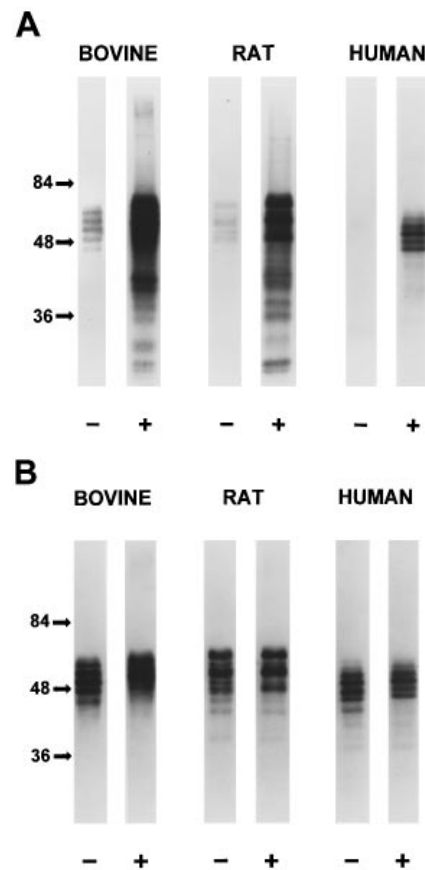


Figure 1 Immunoblots demonstrating the increase in 12E8 immunoreactivity and decreased electrophoretic mobility of tau after phosphorylation by cAMP-PK

Total bovine tau, twice-cycled microtubule rat tau, or a tau-enriched fraction from human brain were incubated in the absence (–) or presence (+) of cAMP-PK prior to electrophoresis and immunoblotting. (A) Samples (0.5 μg) of total bovine or twice-cycled microtubule rat tau or of the tau-enriched fractions from human brain (1 μg) were run in each lane and the blots probed with 12E8 (1:2500). (B) Samples (0.1 μg) of bovine or rat tau or of the tau-enriched fraction from human brain (0.5 μg) were run in each lane and the blots probed with the phosphate-independent antibody, 8C11 (1:10000). The positions at which molecular-mass standards (kDa) migrated are indicated on the left.

identified using Tau 2 which recognizes an epitope at amino acids 106–119 [50], and the C-terminal domain was identified with T46 which binds to an epitope in the extreme carboxyl region of the molecule [32,49]. Immunoreactivity with 12E8 was present in both the amino and carboxyl fragments, indicating that cAMP-PK was able to phosphorylate Ser-262, as well as Ser-356 (Figure 2). Uncleaved tau is also evident (Figure 2, τ), since NTCB treatment always produces incomplete fragmentation with approx. 60–80% of the initial substrate being cleaved [47,52]. Some 12E8 immunoreactivity is also evident in unphosphorylated tau, but is only associated with the N-terminal fragments (Figure 2). Although the reactivity is significantly less than that observed after phosphorylation with cAMP-PK, this finding indicates that Ser-262 is phosphorylated in a fraction of the tau isolated from bovine brain. Further evidence for the phosphorylation of tau at Ser-262 by cAMP-PK was obtained using a peptide containing amino acids 257–273 of tau. Under conditions where the concentration of cAMP-PK was limiting, the peptide was phosphorylated at a rate of 0.252 nmol/min per mg of enzyme

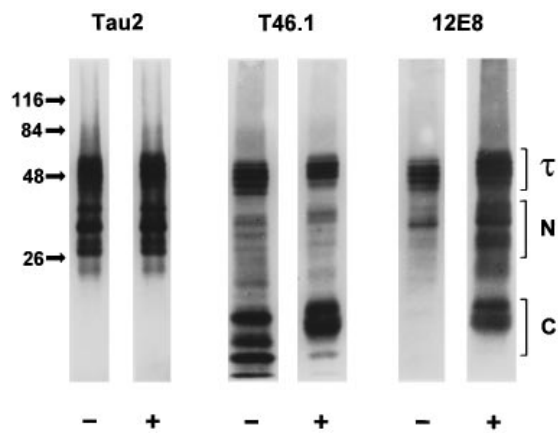


Figure 2 Immunoblots of total bovine tau cleaved at cysteine residues with NTCB after incubation in the absence (–) or presence (+) of cAMP-PK

Blots were probed with Tau 2 (1:1000) which recognizes the N-terminal fragments (N) containing Ser-262, T46 (1:250) which recognizes the C-terminal fragments (C) containing Ser-356, or 12E8 (1:500). τ indicates the uncleaved tau. The positions at which molecular-mass standards (kDa) migrated are indicated on the left.

($n = 3$). This rate of phosphorylation compares favourably with the rate at which cAMP-PK phosphorylates histone (0.239 nmol/min per mg of protein), the substrate that is classically used to determine the activity of different preparations of the enzyme [39]. There was no detectable phosphate incorporation into the phosphoform of the peptide by cAMP-PK.

To demonstrate unequivocally that the cAMP-PK-induced increase in 12E8 immunoreactivity was due to phosphorylation of Ser-262 and Ser-356, and not other sites, mutants of the human tau isoform were used. In these studies T4 and T4 mutants 262A, 356A and 262A/356A, in which alanines were substituted for the indicated serines, were phosphorylated by cAMP-PK in the absence or presence of the selective cAMP-PK peptide inhibitor, PKI. Representative immunoblots from these

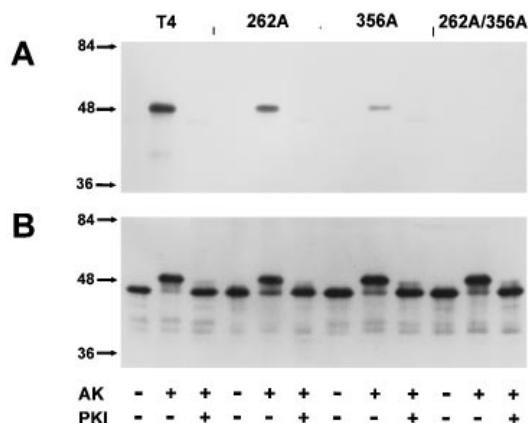


Figure 3 Immunoblots of the human recombinant tau isoform, T4 and mutant isoforms containing an alanine residue at 262 (262A), 356 (356A) or in both positions (262A/356A) incubated in the absence (–) or presence (+) of cAMP-PK (AK) and/or the selective cAMP-PK inhibitor (PKI)

A sample (0.05 μ g) of protein was run in each lane and the blots probed with either 12E8 (1:3000) (A) or 8C11 (1:50000) (B). The positions at which molecular-mass standards (kDa) migrated are indicated on the left.

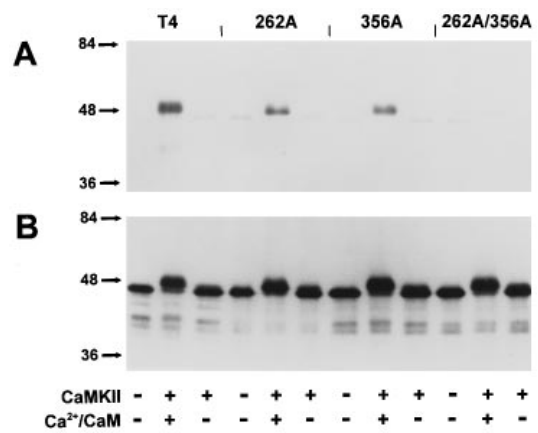


Figure 4 Immunoblots of the human recombinant tau isoform, T4 and mutant isoforms containing an alanine residue at 262 (262A), 356 (356A) or in both positions (262A/356A) incubated in the absence (–) or presence (+) of CaMKII and/or calcium and calmodulin (Ca²⁺/CaM)

A sample (0.05 μ g) of protein was run in each lane and the blots probed with either 12E8 (1:3000) (A) or 8C11 (1:50000) (B). The positions at which molecular-mass standards (kDa) migrated are indicated on the left.

experiments are shown in Figure 3. Phosphorylation of T4, 262A and 356A by cAMP-PK resulted in increased 12E8 immunoreactivity, which in all cases was inhibitable by the addition of PKI. Phosphorylation of 262A/356A with cAMP-PK did not result in increased reactivity with 12E8. Phosphorylation of T4 and the mutants with cAMP-PK resulted in a decrease in the electrophoretic mobility of tau. Phosphorylation of wild-type or mutant forms of T4 with protein kinase C or MAP kinase did not alter 12E8 reactivity. However, phosphorylation of T4 and the mutants with MAP kinase resulted in reactivity with phosphorylation-dependent antibodies such as AT8 [42], AT180 and AT270 [43] (results not shown). Phosphorylation with CaMKII yielded results similar to those obtained with cAMP-PK (Figure 4). Phosphorylation of T4 or the single mutants with CaMKII resulted in an increase in 12E8 immunoreactivity. No increase in 12E8 immunoreactivity was evident when 262A/356A was phosphorylated with CaMKII. However, as with cAMP-PK, phosphorylation of T4 and the mutants with CaMKII resulted in a decrease in electrophoretic mobility (Figure 4). In addition, incubation with cAMP-PK resulted in a time-dependent increase in 12E8 immunoreactivity of T4, indicating that there is a correlation between antibody binding and the extent of phosphorylation (results not shown). Stoichiometric determinations revealed that cAMP-PK phosphorylated T4 to a level of 2 ± 0.3 mol of ³²P/mol of tau ($n = 3$) [53]. Quantitative determination of the relative differences in the phosphorylation of T4, and the T4 mutants, demonstrated that $8 \pm 1\%$ ($n = 4$) of the total phosphate was incorporated into Ser-262 by cAMP-PK, while $17 \pm 2\%$ ($n = 4$) was incorporated into Ser-356.

To determine the potential physiological relevance of the phosphorylation of Ser-262 and Ser-356, tau was extracted from control and okadaic acid-treated human mixed brain cells and cleaved at cysteine residues with NTCB. The C-terminal fragments which contain Ser-356, were identified by T46 immunoreactivity, and the N-terminal fragments which contain Ser-262, by reactivity with T14 [49]. Phosphorylation at Ser-262 was clearly evident in control cells (Figure 5). Okadaic acid treatment resulted in a significant increase in the extent of endogenous

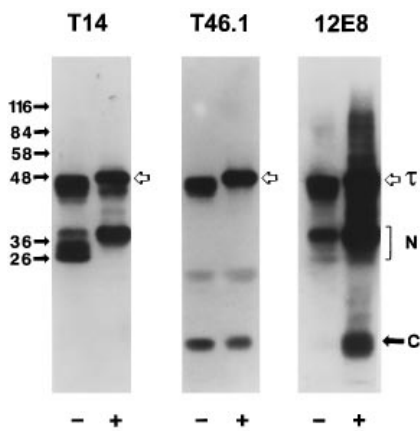


Figure 5 Immunoblots of tau isolated from control (–) or okadaic acid-treated (+) human mixed brain cell cultures cleaved at cysteine residues with NTCB

Samples were run on SDS/12%-polyacrylamide gels and blots were probed with T14 (1:1000) which recognizes the N-terminal fragments (N) containing Ser-262, T46 (1:250) which recognizes the C-terminal fragments (C) containing Ser-356, or 12E8 (1:500). The open arrow indicates uncleaved tau. The positions at which molecular-mass standards (kDa) migrated are indicated on the left.

phosphorylation at both Ser-262 and Ser-356, as evidenced by increased 12E8 reactivity of both the amino and carboxyl fragments. Interestingly, NTCB cleavage of tau from control cells produced three T14-reactive amino fragments; however, only the slowest-migrating fragment, which was the least abundant, was recognized by 12E8. Treatment with okadaic acid shifted all the N-terminal fragments into a single band that migrated identically to the slowest-migrating N-terminal fragment generated from control tau (Figure 5).

DISCUSSION

The present results demonstrate that both Ser-262 and Ser-356 in tau are phosphorylated by cAMP-PK and CaMKII *in vitro*. In addition, the phosphorylation state of both sites was enhanced in cultured brain cells following treatment with okadaic acid. This suggests that Ser-262 and Ser-356 are continually undergoing phosphorylation/dephosphorylation *in situ*. Phosphorylation of these sites therefore may be of physiological relevance and may play a role in modulating the function of tau.

In a previous study, the human recombinant tau isoform with three microtubule-binding domains and no N-terminal inserts, was shown to be phosphorylated by cAMP-PK on serines 214, 324, 356, 409 and 416 using liquid secondary-ion mass spectrometry and solid-phase N-terminal sequencing [18]. Ser-324 and Ser-356 are located within the second and third microtubule-binding domains respectively. However, only 8% of the total phosphate was incorporated into these two sites, and Ser-262 was not found to be phosphorylated [18]. In the present study, recombinant human tau was found to be phosphorylated by cAMP-PK at Ser-262, as well as Ser-356. This is not surprising given the extensive similarities between the sequences surrounding Ser-262 and Ser-356 [2]. The reason why phosphorylation of tau at Ser-262 by cAMP-PK was not observed in the previous study is unknown. Relative stoichiometric determinations in the present study demonstrated that 17% of the total phosphate incorporated into T4 by cAMP-PK was on Ser-356, while 8% was on Ser-262. Given that approx. 50% of the phosphate

incorporated into tau by cAMP-PK is on Ser-214, and there are six sites in tau that are phosphorylated by the kinase [18], these values are reasonable. In addition, the peptide encompassing amino acids 257–273 was readily phosphorylated by cAMP-PK, confirming that Ser-262 in tau is phosphorylated to a significant extent by this protein kinase.

Biernat et al. [20] prepared an enriched fraction containing protein kinase activity which was capable of phosphorylating Ser-262, which was subsequently suggested to contain the catalytic subunit of cAMP-PK as well as a novel kinase (p110^{mark}) [34]. Yang et al. [54] recently reported that in the presence of heparin, GSK3 α phosphorylated Ser-262 as well as several other Ser/Thr-Pro and non-Ser/Thr-Pro sites. However, protein kinase C and MAP kinase failed to phosphorylate Ser-262 or Ser-356. By contrast, CaMKII did phosphorylate both Ser-262 and Ser-356 as evidenced by an increase in 12E8 immunoreactivity. Although only Ser-416 has been identified previously as a CaMKII site in tau [55], it is not surprising that CaMKII also phosphorylates Ser-262 and Ser-356, given the similarity of the consensus sequences for cAMP-PK and CaMKII [56]. Indeed, Ser-416 is also phosphorylated to a significant extent by cAMP-PK [18].

In vitro phosphorylation of tau by cAMP-PK significantly decreases the binding of tau to tubulin [15] and reduces the promotion of microtubule nucleation and growth [23]. These functional changes in tau induced by cAMP-PK phosphorylation correlate well with phosphorylation of sites within the microtubule-binding domain. However, Brandt et al. [23] also presented evidence that phosphorylation of Ser-214 was primarily responsible for decreasing the ability of tau to nucleate microtubules. These findings support the hypothesis that both the flanking and microtubule-binding domains of tau are required for proper microtubule binding and promotion of microtubule assembly [57].

The phosphorylation of Ser-262 in a fraction of PHF-tau has been demonstrated by means of mass spectrometry and solid-phase sequencing [30]. However, this site was not found to be phosphorylated in fetal or adult rat tau using these analytical techniques [28]. In contrast to this finding, immunoblot analysis with 12E8 revealed that Ser-262 was phosphorylated in fetal rat and human tau, and to a lesser extent in adult biopsy-derived tau [35]. In addition, Ser-262 was also found to be phosphorylated in bovine tau which was prepared from rapidly processed brain in the presence of phosphatase inhibitors. Tau prepared from twice-cycled microtubules showed virtually no 12E8 reactivity (Figure 1). These data suggest that tau is dephosphorylated at these sites during the microtubule cycling procedure, and/or that tau which is phosphorylated at Ser-262 and/or Ser-356 partitions preferentially in the microtubule-binding incompetent pool of tau.

Tau extracted from untreated human mixed brain cell cultures revealed that Ser-262 was endogenously phosphorylated (Figure 5). The lack of detectable 12E8 immunoreactivity at the Ser-356 epitope in tau from untreated cultures could be due to the rapidity with which phosphates are turned over at the site, the level of phosphorylation at Ser-356, and/or the fact that 12E8 is apparently more reactive towards the Ser-262(P) epitope than the Ser-356(P) epitope [35]. Treatment of cells with 100 nM okadaic acid resulted in a significant increase in the endogenous phosphorylation state of both Ser-262 and Ser-356, indicating that these sites are probably dephosphorylated by protein phosphatases 1 or 2A [58]; however, it does not preclude the possibility that they are also dephosphorylated by phosphatase 2B. It is also interesting to note that in control cells only the slowest-migrating T14-reactive N-terminal fragment was 12E8 reactive, and that this was the least abundant of the T14

fragments. The most likely explanation for the slowest-migrating tau fragment being 12E8 reactive is that phosphorylation of Ser-262 results in a further decrease in electrophoretic mobility, and that phosphorylation of other sites probably precedes Ser-262 phosphorylation. Treatment of the cells with okadaic acid resulted in a single slow-migrating T14 fragment that was intensely immunoreactive with 12E8. This suggests that only a fraction of tau is phosphorylated at Ser-262 at any given time under control conditions.

In conclusion, we have demonstrated that cAMP-PK and CaMKII phosphorylate Ser-262 and Ser-356 in tau. Both protein kinases are abundant in nerve cells [59,60] and could therefore phosphorylate tau *in situ* at these sites. Since many of the sites phosphorylated in PHF-tau, including Ser-262, are phosphorylated in a significant fraction of fetal tau, and in a small fraction of biopsy-derived adult human tau [28,29,35,37], it is likely that the hyperphosphorylation of tau observed in PHFs is due to an imbalance in specific kinase/phosphatase activities, inappropriate compartmentation of tau or a combination of both [33]. Whether phosphorylation at Ser-262 and Ser-356 is achieved by cAMP-PK, CaMKII, or the recently described p110^{mark} [34], or by some combination of these or other undescribed kinases, will be an important question in Alzheimer's disease research.

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