FEBS Letters 349 (1994) 359–364

FEBS 14316

PHF-tau from Alzheimer's brain comprises four species on SDS-PAGE which can be mimicked by in vitro phosphorylation of human brain tau by glycogen synthase kinase-3β

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Received 9 June 1994; revised version received 24 June 1994

Abstract

Extensive in vitro phosphorylation of a purified preparation of control human brain tau consistently produces four rather than, as previously believed, three tau species on SDS-PAGE. The species thus generated are shifted on SDS-PAGE to positions that match those of PHF-tau isolated from Alzheimer's disease brain. A mixture of recombinant human tau isoforms phosphorylated by GSK-3β gave similar results to those obtained with control human brain tau. In vitro phosphorylation of the individual recombinant isoforms by GSK-3β showed that the four bands of PHF-tau are likely to consist of isoforms 3R,0 alone; 4R,0 with 3R,29; 4R,29 with 3R,58 and 4R,58 alone.

Key words: Microtubule-associated protein tau; Paired helical filament; Glycogen synthase kinase-3β; Protein phosphorylation; Alzheimer's disease

1. Introduction

Neurofibrillary tangles and senile plaques are the two histopathological hallmarks of Alzheimer's disease brain. Neurofibrillary tangles consist of paired helical filaments (PHF) a major constituent of which is the microtubule-associated protein, tau (PHF-tau) [1–5]. PHF-tau is composed of several tau isoforms that are in a hyperphosphorylated state compared to tau from control human brain [6–10]. PHF-tau usually separates into three major species on SDS-PAGE with a fourth and less prominent, but often observable, band possessing a lower mobility than the three main bands.

Tau promotes tubulin assembly and stabilizes microtubules in vitro (see [11] for review). Phosphorylation regulates the ability of tau to bind to microtubules and promote tubulin assembly [12–15]. Some of the abnormal phosphorylation sites on PHF-tau have been identified [16–19] and these are mainly at serine-proline and threonine-proline motifs. For this reason, several members of the proline-directed protein kinase family, including glycogen synthase kinase-3α (GSK-3α) and β (GSK-3β), have been investigated for their ability to phosphorylate tau in vitro [20–27].

The highest level of expression of both GSK-3 isoforms is found in brain [28]. GSK-3α and GSK-3β are present in neurones of both control and Alzheimer's disease brain [20] and have been reported to co-purify with PHF [21]. We have previously shown that a single isoform of recombinant human brain tau is phosphorylated in vitro by both GSK-3α and GSK-3β in a manner that generates the characteristic shift in mobility of tau on SDS-PAGE and also induces phosphorylated epitopes on tau which are associated with PHF [20]. In this report, we show that PHF-like tau comprising four electrophoretically distinct species can be generated, after 24 hours incubation with GSK-3β, from both human brain tau isolated from normal brain and from a mixture of recombinant human tau isoforms. Phosphorylation of the individual recombinant isoforms of tau enabled us to assign probable identities to the four PHF-tau species that are different from previous assignments [7,9,10]. The phosphorylation state of GSK-3β-phosphorylated tau protein was assessed using a panel of phosphate-dependent and -independent monoclonal and polyclonal antibodies and was demonstrated to have an antigenic profile similar to that of PHF-tau.

2. Materials and Methods

2.1. Purification of tau from control human brain

Tau was prepared from control human brain (obtained from the MRC Alzheimer's Disease Brain Bank, Institute of Psychiatry, London) by homogenizing 1 g grey matter from cerebral cortex in 2 ml of 100 mM MES, pH 6.5, containing 1 M NaCl, 0.5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 10 mM sodium pyrophosphate and 20 mM sodium fluoride. The homogenate containing α-mercaptoethanol was

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Abbreviations: SDS-PAGE, sodium dodecyl polyacrylamide gel electrophoresis; DTT, dithiothreitol; PHF, paired helical filament; GSK-3, glycogen synthase kinase-3; EGTA, ethylene glycol-bis-(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; ATP, adenosine 5'-triphosphate.

SSDI 0014-5793(94)00702-0

heated to 100°C for 10 min and centrifuged at 20,000 × g_{av} for 35 min at 4°C. The heat-stable fraction was precipitated by addition of saturated ammonium sulphate solution to 35% saturation and centrifugation at 20,000 × g_{av} for 35 min at 4°C. The precipitate was dissolved in 100 μl of 50 mM MES, pH 6.5 containing 1 mM DTT and loaded onto a Mono S column (Pharmacia) equilibrated in the same buffer. Tau was eluted with a linear gradient (0.05 M to 0.5 M) of NaCl and finally purified on a reversed-phase C18 column (Vydac) with a gradient of acetonitrile containing 0.1% (v/v) trifluoroacetic acid. Fractions containing tau were detected by Western blotting with the monoclonal antibody Tau.1.

2.2. Preparation of recombinant human tau

cDNA from the six human tau isoforms in the plasmid pRK 172 were expressed in *E. coli* (BL21 DE3) [29] and are referred to as 3R,0; 4R,0; 3R,29; 4R,29; 3R,58 and 4R,58 (see also Fig. 4) following the nomenclature in [29]. Tau protein was purified by a modification of the method of Scott et al. [30]. The bacterial cell suspension (500 ml) was pelleted at 3,320 × g_{av} for 20 min at 4°C, resuspended in 50 mM MES, pH 6.5 and centrifuged as above. The pelleted cells were weighed and resuspended (10 ml/g pellet) in 50 ml 50 mM MES, pH 6.5, containing 1 mM PMSF, 1 mM DTT, 1 mM EGTA, 10 mM sodium pyrophosphate and 20 mM sodium fluoride. The suspension was sonicated for 5 min at room temperature and centrifuged at 29,900 × g_{av} for 20 min at 4°C. NaCl was added to the supernatant to produce a final concentration of 0.5 M, the mixture was heated to 100°C for 10 min and centrifuged at 182,000 × g_{av} for 2 h at 4°C. The heat-stable supernatant was precipitated with ammonium sulphate (35% saturation) and centrifuged at 16,400 × g_{av} for 20 min at 4°C. The pellet was resuspended in 3 ml 50 mM MES, pH 6.5, containing 1 mM DTT and 50 mM NaCl and dialysed overnight at 4°C against 500 ml of the same buffer. The dialysate was loaded onto a Mono S column and eluted with a linear gradient (0.05–0.5 M) of NaCl.

2.3. Purification of PHF-tau

PHF-tau was prepared from Alzheimer's disease brain essentially according to the method of Brion et al. [31].

2.4 Activity of GSK-3β

The enzyme GSK-3β was assayed by incubating 5 μl of diluted enzyme with 50 μM ATP (containing 1 μCi = 0.037 MBq [γ-^{32}P]ATP), 5 mg/ml bovine serum albumin, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 10 μg/ml heparin and 8 μM GS-1 peptide in 25 mM Tris-HCl, pH 7.4, at 30°C in a volume of 20 μl. After 30 min, the reaction was terminated by adding 20 μl of 20% (w/v) trichloroacetic acid containing 5 mM ATP, standing on ice for 5–10 min and centrifuging on a microfuge. The supernatant (25 μl) was pipetted onto pieces of phosphocellulose paper (P81 Watman), washed 5 times in 0.5% (v/v) phosphoric acid, and immersed in water for Cerenkov counting. The GSK-3β sample used in experiments in which tau was phosphorylated had an activity of 36 pmol of phosphate transferred per minute per μl of stock enzyme.

The GS-1 peptide (described in [32]) was synthesised as the phosphopeptide with its casein kinase II site fully phosphorylated.

2.5 Phosphorylation of tau by GSK-3β

Control human brain tau and individual recombinant human tau isoforms (8 μg) were incubated with 4 μl of GSK-3β (prepared as described in [32]) in 20 mM HEPES buffer, pH 7.5, containing 5 mM $MgCl_2$, 10 μM leupeptin, 1 μM pepstatin, 1 mM PMSF, 0.5 mM DTT, 5 μM okadaic acid, 1 μM sodium orthovanadate and 3 mM ATP, for 24 h at 30°C. Appropriate controls containing heat-inactivated enzyme were incubated in parallel. The reaction was stopped by heating at 100°C for 5 min. Samples were analysed by SDS-PAGE (10% gels) [33] and Western blotting [8]. Blots were probed with the monoclonal antibodies SMI 31, SMI 310 and SMI 34 (Affiniti Research Products Limited), which recognise phosphorylated epitopes and SMI33 which recognises an unphosphorylated epitope on tau [17,34]. Monoclonal antibodies RT97, BF10, 8D8 and 1215 [35,36] recognise phosphorylated epitopes on tau, the exact location of which is known only for 8D8 as serine 396 (numbering corresponds to the largest isoform of human brain tau) [20]. 5E2 is a phosphate-independent monoclonal antibody to tau [37]. The monoclonal antibody, AT8 [38], recognises phosphorylated serine 202 on tau [39] whereas Tau.1 [40] recognises serine 199 and/or 202 only when they are non-phosphorylated [41,42]. The polyclonal antibody to tau has been previously described [43].

3. Results

Following phosphorylation by GSK-3β, recombinant human tau and control human brain tau (Fig. 1, lanes 1–4) shifted to align with PHF-tau isolated from Alzheimer's disease brain (Fig. 1, lane 5). Both types of phosphorylated tau protein as well as PHF-tau appeared as four bands when immunolabelled with the polyclonal antibody to tau (Fig. 1, lanes 3–5).

GSK-3β-phosphorylated human brain tau was strongly immunolabelled by 5E2 and the phosphate-dependent antibodies 1215, 8D8, RT97, BF10, AT8, SMI 31, SMI 310 and SMI 34 (Fig. 2A). The reactivity of tau with AT8, 8D8, SMI 31 and SMI 310 indicates the presence of phosphorylated serines 202, 396 and 404 in tau. SMI 34 which is both phosphorylation and conformation-dependent reacted strongly with phosphorylated human brain tau suggesting that GSK-3β had generated both the necessary phosphorylated residue(s) and the required conformational change. SMI 33 had a decreased immunoreactivity for the GSK-3β-phosphorylated protein indicating that serine 235 was phosphorylated to some extent but not completely. Tau.1 was only immunoreactive with the unphosphorylated protein indicating that possibly both serines 199 and 202 are phosphorylated by GSK-3β.

The pattern of immunoreactivity generated by phosphorylation with GSK-3β of both human brain tau and recombinant human tau is very similar. Human brain tau, incubated with heat inactivated GSK-3β, showed some immunoreactivities with antibodies 1215 and 8D8, and a strong reactivity with RT97 and BF10 (Fig. 2A), indicating that the respective epitopes are at least partially phosphorylated in normal brain tau, whereas

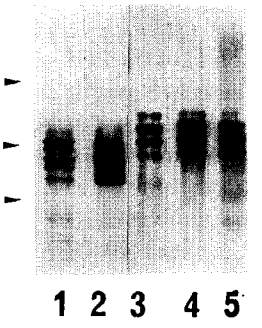

Fig. 1. Western blot labelled with polyclonal antibody to tau. Lane 1- all six recombinant human tau isoforms; lane 2- purified control human brain tau; lane 3- the same sample as in lane 1, phosphorylated by GSK-3β; lane 4- the same sample as in lane 2, phosphorylated by GSK-3β; lane 5- PHF-tau. Molecular weight markers are indicated by arrowheads and correspond to phosphorylase b (97.4 kDa), catalase (58.1 kDa) and alcohol dehydrogenase (39.8 kDa).

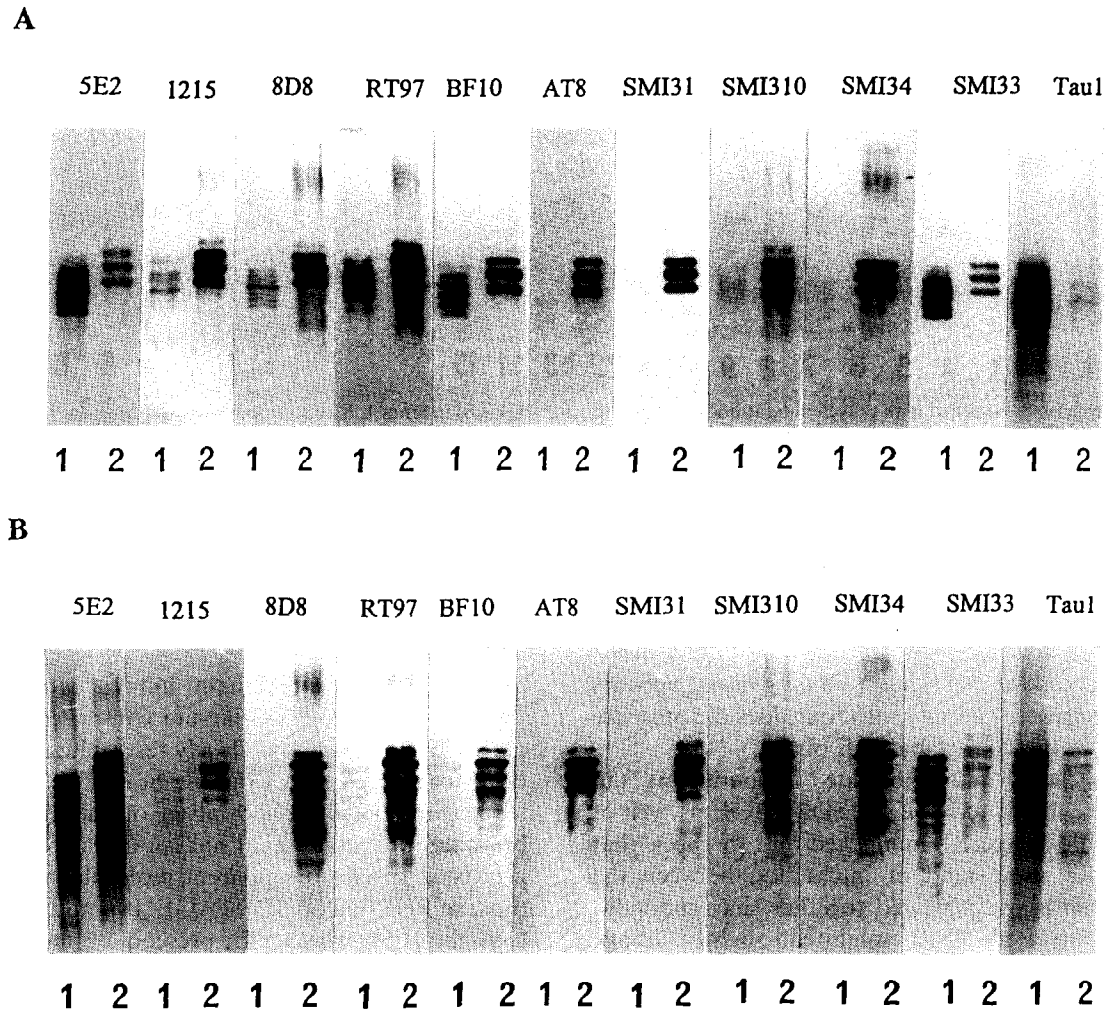

Fig. 2. Immunoblots of control human brain tau (panel A) and recombinant human brain tau (panel B) incubated with heat-inactivated GSK-3β (lanes 1) or with active GSK-3β (lanes 2). Blots were labelled by the antibodies indicated.

unphosphorylated recombinant human tau had a very weak reactivity only with 1215 (Fig. 2B). 5E2 labelled recombinant and human brain tau in the same way (Fig. 2A,B). Thus, the pre-existing phosphorylated sites in human brain tau do not appear to prime for phosphorylation by GSK-3β, as has been found necessary for some substrates, at the epitopes detected by this panel of monoclonal antibodies [44–46].

Phosphorylated human brain tau showed either 3 or 4 bands on the blots, depending on the antibody. The fourth (upper) band is detected by the polyclonal antibody to tau (Fig. 1), 1215, 8D8, RT97 and SMI 310 (Fig. 2A), whereas all the antibodies recognise the fourth band generated from the recombinant protein (Fig. 2B). The stronger intensity of the slowest migrating band of phosphorylated recombinant human tau (Fig. 2B) compared with phosphorylated human brain tau (Fig. 2A) probably results from the individual isoforms being mixed in similar quantities, whereas human brain tau contains relatively less of both 3R,58 and 4R,58 isoforms and hence are detected less well by certain antibodies after phosphorylation. Several antibodies also detected numerous bands migrating faster than the smallest tau isoform and these are the result of some limited proteolysis of brain tau and recombinant tau.

Individual recombinant human tau isoforms were incubated with GSK-3β or with heat-inactivated enzyme as control (Fig. 3A). Following phosphorylation, the smallest tau isoform, 3R,0 (Fig. 3A, lane 3) migrated to the same position or very close to that of the lower band of PHF-tau (Fig. 3A, lanes 1, 8 and 15). Isoforms 4R,0 and 3R,29 (Fig. 3A, lanes 5 and 7, respectively) aligned close to (from bottom to top on SDS-PAGE and Fig. 4) the second band of PHF-tau, with 4R,0 migrating slightly above 3R,29. Isoforms 4R,29 and 3R,58 (Fig. 3A, lanes 10 and 12, respectively) aligned with the third band of PHF-tau. Finally, phosphorylated isoform 4R,58 (Fig. 3A, lane 14) aligned with the fourth and slowest migrating band of PHF-tau. Note that not all tau isoforms have an equal mobility shift on SDS-PAGE; the

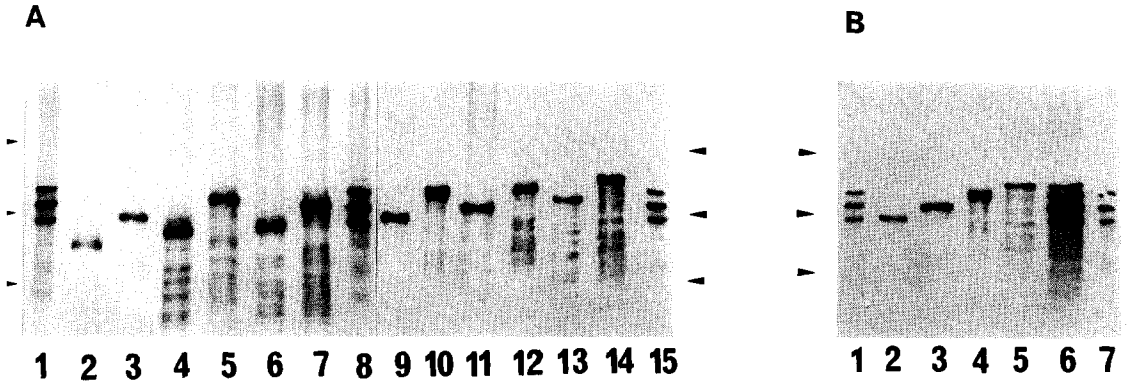

Fig. 3. (A) Immunoblots labelled with polyclonal antibody to tau, showing: PHF-tau in lanes 1, 8 and 15; unphosphorylated single recombinant tau isoforms 3R,0; 4R,0; 3R,29; 4R,29; 3R,58 and 4R,58 in lanes 2, 4, 6, 9, 11 and 13, respectively; and the corresponding isoforms phosphorylated by GSK-3β in lanes 3, 5, 7, 10, 12 and 14, respectively. Lanes 1–8 and 9–15 were electrophoresed separately, molecular weight markers are indicated for both gels and are as in Fig. 1. (B) Immunoblot labelled with polyclonal antibody to tau showing the reconstitution of bands matching the electrophoretic mobility of PHF-tau (lanes 1 and 7) from combinations of phosphorylated tau isoforms. 3R,0 alone (lane 2); 4R,0 with 3R,29 (lane 3); 4R,29 with 3R,58 (lane 4); 4R,58 alone (lane 5) and the mixture of all six isoforms (lane 6). Note that the slowest migrating PHF-tau species is not always visible and may depend on both the antibody used and the amount loaded onto the gel.

presence of an additional tubulin-binding repeat appears to produce a greater mobility shift than does the presence of an insert in the N-terminal half of tau.

The individually phosphorylated recombinant tau isoforms were mixed to see if the SDS-PAGE pattern of PHF-tau could be exactly mimicked and to check that there was not some kind of interaction between the isoforms when phosphorylated together since individually phosphorylated 4R,0 migrated slightly above phosphorylated 3R,29. Isoform 3R,0 was run on its own (Fig. 3B, lane 2) and aligned with the fastest PHF-tau band (Fig. 3B, lane 6). When mixed, isoforms 4R,0 and 3R,29 ran as a single band on SDS-PAGE aligning with the second fastest band of PHF-tau, and the mixture of isoforms 4R,29 plus 3R,58 aligned as a single band with the third fastest-migrating species of PHF-tau (Fig. 3B, lanes 3 and 4). Isoform 4R,58 (Fig. 3B, lane 5) aligned with the slowest-migrating species of PHF. Thus, with regard to electrophoretic mobility, the PHF-tau pattern can be reproduced by GSK-3β-phosphorylated human brain tau and recombinant human tau and the relationship between the individual tau isoforms and the PHF-tau species is illustrated schematically in Fig. 4.

4. Discussion

The isoform composition of PHF-tau has only been partially resolved because PHF-tau sometimes separates as three and sometimes as four bands on SDS-PAGE and attempts to assign isoforms to each species have only been made for the three principal PHF-tau bands. The fourth, slowest migrating, species of PHF-tau is always present in much smaller amounts compared to the other three main bands. Here we have described a method to generate, from recombinant human tau and control human brain tau, PHF-like-tau comprised of four species on SDS-PAGE and closely resembling PHF-tau isolated from Alzheimer's disease brain. We found no difference in the phosphorylation of the control human brain tau or recombinant human tau as both substrates produced species of apparent molecular mass equal to those of PHF-tau on SDS-PAGE. It is not possible to say from this study whether the presence of phosphate on tau isolated from human brain altered the kinetics of phosphorylation by GSK-3β with this substrate compared to the recombinant protein. Individual recombinant tau isoforms phosphorylated by GSK-3β had characteristic migrations on SDS-PAGE which matched, either singly or in pairs, with one or other of the four bands of PHF-tau.

We have previously shown that cAMP-dependent protein kinase (PKA) also induces a marked reduction in the electrophoretic mobility of recombinant and human brain tau [47]. However, when we incubated human brain tau or a mixture of all six recombinant human tau isoforms with PKA under the conditions described here, we did not generate phosphorylated tau with a pattern resembling that of PHF-tau (data not shown). Hence, the migration of PHF-tau on SDS-PAGE is dependent upon a specific pattern of phosphorylation which can be reproduced in vitro by GSK-3β but not by PKA. However, we cannot exclude the possibility that other kinases such as cdk5 [48,49], 35 kDa/41 kDa protein kinase [50], MAP kinase [51] or other, as yet unidentified, protein kinases might also be able to phosphorylate tau in a manner which generates both the precise shift in electrophoretic mobility and the immunoreactivity with antibodies that recognise PHF-tau.

The PHF-like-tau generated by in vitro phosphoryla-

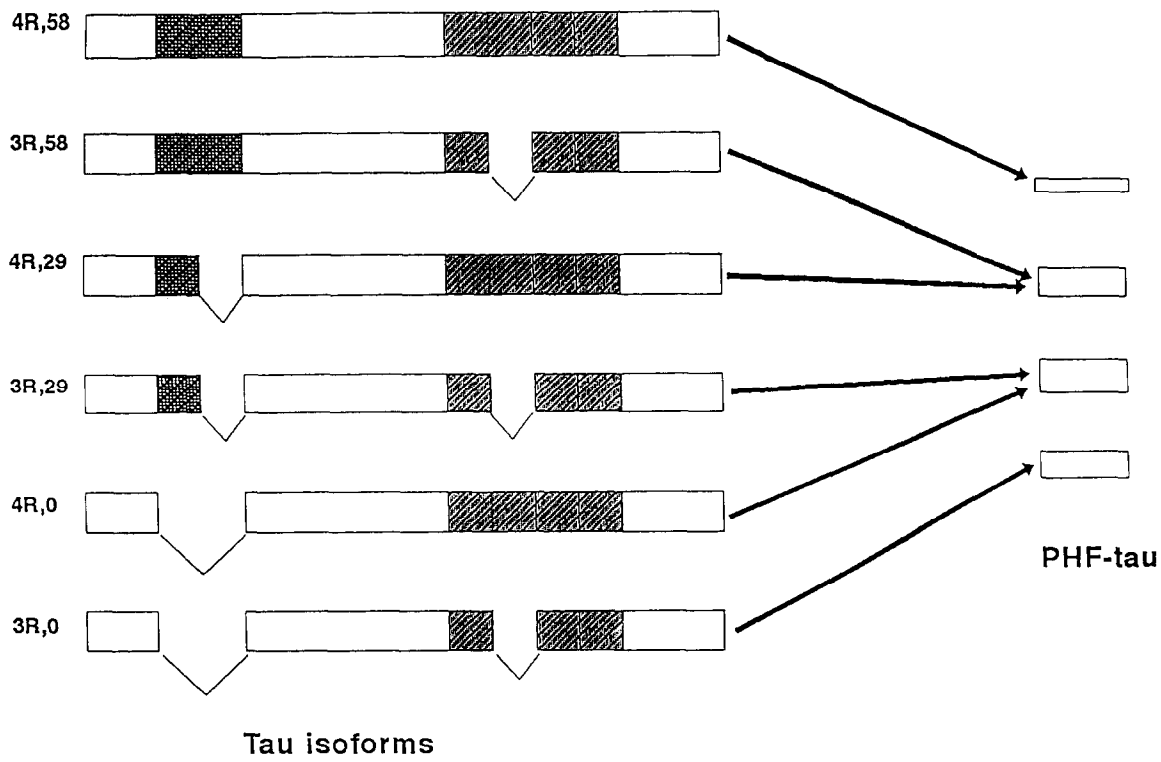

Fig. 4. Schematic representation of the six human tau isoforms and PHF-tau. GSK-3β-phosphorylated tau isoforms align with each of the four PHF-tau species as indicated by the arrows.

tion suggests that the four species of PHF-tau are composed of isoforms 3R,0 alone; 4R,0 with 3R,29; 4R,29 with 3R,58; and isoform 4R,58 alone (Fig. 4). The tau isoforms isolated from control human brain are not all present in equal amounts. Isoforms 4R,0, 3R,29 and 4R,29 are more abundant than 3R,0, which is itself more abundant than 3R,58 and 4R,58. Dephosphorylation of PHF-tau using alkaline phosphatase shows that all six tau isoforms are present in PHF-tau in the relative quantities described above [29]. Our model suggests that the second band of PHF-tau is composed of both isoforms 4R,0 and 3R,29 which could explain why this band is always the most abundant on SDS-PAGE. The fourth and slowest migrating PHF-tau band corresponds to isoform 4R,58 which is one of the two least abundant isoforms. The first and third PHF-tau bands usually have similar intensities on SDS-PAGE and this observation again fits with the composition of the model described here. It was previously suggested that PHF-tau was composed of (3R,0 plus 4R,0), (3R,29 plus 4R,29) and (3R,58 plus 4R,58) for the fastest migrating, the middle and the slowest migrating bands of PHF-tau, respectively [10]. This conclusion was based on labelling with antibodies specific for certain tau isoforms and the composition suggested here would give the same immunoreactive profile with the antibodies used in that study.

We found that RT97 always labelled the four bands of both phosphorylated control brain and recombinant tau. This result contrasts with RT97 labelling of PHF-tau in which only the band of lowest apparent molecular mass is unlabelled by RT97 [31]. The discrepancy in labelling by RT97 indicates that although the phosphorylated tau generated in vitro by GSK-3β is very similar to PHF-tau, it may not be identical. Under the conditions used of extensive phosphorylation of tau in vitro by GSK-3β, it is possible that additional RT97-reactive sites are generated. However, although the precise phosphorylation pattern of in vitro-generated PHF-like tau may not be identical to PHF-tau isolated from Alzheimer brain, the remarkable similarity in electrophoresis profile of these forms of tau implies that sites essential for producing the necessary changes in electrophoretic mobility are generated by GSK-3β.

We propose therefore that PHF-tau comprises four bands on SDS-PAGE and that the isoform composition of the individual bands is different from that previously suggested. Our results also imply that the conformational changes necessary for the reduction in electrophoretic mobility of PHF-tau can be generated by GSK-3β alone and does not require priming by another protein kinase.

Acknowledgements: This research was funded by the European Union, Glaxo Research and Development Limited, The Wellcome Trust and the Canadian MRC. We thank Dr. M. Goedert for providing the human tau cDNA and Innogenetics for the AT8 antibody. The hybridoma clones 5E2 and Tau.1 were the generous gifts of Drs. K.S. Kosik

and L.I. Binder, respectively. We are grateful to Dr. N. Cairns (MRC Alzheimer's Disease Brain Bank, Institute of Psychiatry, London, UK) for kindly providing us with the brain tissue used in these studies and Dr. H. Reynolds for performing the enzyme activity assay.

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