Tau in Alzheimer neurofibrillary tangles

N- and *C*-terminal regions are differentially associated with paired helical filaments and the location of a putative abnormal phosphorylation site

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To investigate the extent to which whole tau proteins, structurally abnormal tau and fragments of tau are incorporated into neurofibrillary tangles in Alzheimer's disease, an immunocytochemical mapping study using a panel of antibodies to several synthetic human tau peptides has been performed. Neurofibrillary tangles were immunolabelled *in situ*, and paired helical filaments (PHF), the principal structural component of tangles, were immunolabelled after isolation and Pronase treatment. *N*-Terminal and *C*-terminal domains of tau were found to be present in tangles *in situ*. SDS-treated PHF were found to contain most of the *C*-terminal half of tau and were also labelled by antibodies to ubiquitin. Only some of these PHF were labelled by antisera to tau sequences towards the *N*-terminus, and this enabled the identification of a region of tau in which proteolytic cleavage may occur. The ultrastructural appearance of the immunolabelling suggested that both the *N*- and *C*-terminal domains of tau extend outwards from the axis of PHF. After Pronase treatment, PHF were strongly labelled only by an antiserum to PHF and by the antiserum to the most *C*-terminal tau synthetic peptide. The latter antiserum also strongly labelled extracellular tangles *in situ*, whereas these extracellular tangles were poorly labelled by the antisera to the other synthetic peptides. One anti-(tau peptide) serum labelled a population of neurofibrillary tangles *in situ* only after alkaline phosphatase pretreatment of tissue sections. Our results show that, although peptides along the length of the tau molecule are associated with neurofibrillary tangles *in situ*, only the *C*-terminal one-third of the molecule is tightly associated with PHF, since this region of tau is resistant to SDS treatment of PHF. We also report the existence in PHF *in situ* of a masked tau epitope which is partially unmasked by dephosphorylation. These results are indicative of post-translational changes in tangle-associated tau in degenerating neurons in Alzheimer's disease.

INTRODUCTION

Alzheimer's disease is the most common cause of dementia in people in developed countries. The severity of dementia is correlated with the brain content of two characteristic neuropathological lesions, neurofibrillary tangles and senile plaques. Neurofibrillary tangles are made of abnormal filaments (the so-called paired helical filaments, PHF), the composition of which has been partially deciphered. Initial immunocytochemical studies demonstrated that the microtubule-associated protein (MAP) tau is a major antigenic constituent of PHF (Brion *et al.*, 1985a; Delacourte & Defossez, 1986; Kosik *et al.*, 1986). The presence of tau in PHF was confirmed by the findings that amino acid sequences of peptides derived from enriched PHF fractions are similar to amino acid sub-sequences of the tau protein (Wisichik *et al.*, 1988; Kondo *et al.*, 1988), and that some PHF-derived polypeptides cross-react immunocytochemically with tau proteins (Grundke-Iqbal *et al.*, 1986b).

Tau proteins consist of a family of developmentally regulated proteins which *in vitro* promote the polymerization of, and co-assemble with, microtubules, and which *in vivo* are known to stabilize the microtubule network (Cleveland *et al.*, 1977; Drubin & Kirschner, 1986). The different isoforms of tau (at least four) differ not only by *N*-terminal sequence variability but also by the presence of three or four identical tandem repeats in the *C*-

terminal half of the molecule (Lee *et al.*, 1988; Goedert *et al.*, 1989b; Himmler *et al.*, 1989; Kosik *et al.*, 1989; Himmler, 1989). These isoforms are generated by alternative splicing (Himmler, 1989) of a primary transcript originating from a single gene (Neve *et al.*, 1986). Additional tau heterogeneity is thought to be due to differential phosphorylation of these isoforms (Baudier & Cole, 1987).

The extent to which all isoforms of tau are integrated into PHF and whether this includes only part of tau or the whole polypeptide has still to be resolved. Some studies suggest that the whole tau protein is present in PHF (Kosik *et al.*, 1988; Goedert *et al.*, 1989a; Iqbal *et al.*, 1989), whereas other studies have indicated that tau fragments isolated from PHF are restricted to the *C*-terminal domain of tau (Kondo *et al.*, 1988; Wisichik *et al.*, 1988). We have performed an immunocytochemical mapping study of neurofibrillary tangles *in situ* and of PHF after isolation and after proteinase treatment using polyclonal antisera raised to six synthetic tau peptides, to whole tau proteins, to isolated PHF fractions and to ubiquitin. The results indicate that the whole tau protein is associated with tangles *in situ*, but that the *N*-terminal domain of tau is lost in SDS-extracted PHF. Proteinase treatment of isolated PHF released ubiquitin epitopes, but the *C*-terminal domain of tau was not removed. We also found evidence of a previously undetected site that may be abnormally phosphorylated in tau and which is associated with PHF.

Abbreviations used: PHF, paired helical filaments; TBS, Tris-buffered saline; MAP, microtubule-associated protein.

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EXPERIMENTAL

Preparation and characterization of antisera

Six peptides corresponding to fragments of a human tau sequence (Goedert *et al.*, 1988) were synthesized with the addition of a C-terminal cysteine (see Fig. 1). A search for amino acid identity between these peptides and other known sequences was performed using the PIR databank; no complete sequence identity was encountered for any of the peptides other than with tau proteins, although TP60 did show some sequence similarity with the related cytoskeletal element MAP 2.

The peptides were conjugated to keyhole-limpet haemocyanin with glutaraldehyde and injected into rabbits (approx. 500 µg of peptide/injection) in order to produce anti-TP10, -TP20, -TP30, -TP40, -TP50 and -TP60 sera. To affinity-purify these sera, peptides TP10–TP60 were coupled to activated thiol-Sepharose (Pharmacia) via their C-terminal cysteine residue. Antisera were diluted in 0.1 M-Tris/HCl/0.5 M-NaCl (pH 7.5) filtered (0.2 µm pore size) and applied to the prepared affinity columns. Bound antibody was eluted with 50 mM-diethylamine/0.5 M-NaCl (pH 11.5) into 0.5 M-NaH₂PO₄ (pH 4.0); high-affinity antibody was eluted with 6 M-guanidine/HCl, and fractions were tested for absorbance at 280 nm and immunoreactivity against the original peptides by e.l.i.s.a. Active fractions were pooled, dialysed against Tris-buffered saline (TBS) and, after addition of BSA to a final concentration of 1% (w/v), the purified antibodies were lyophilized. For Western blotting with these antibodies, total proteins from human brain as a 20% (w/v) homogenate in SDS sample buffer were used, and human tau was prepared from *post mortem* brain tissue. To purify tau, the heat stability of this protein was used to provide a thermostable extract enriched in tau, followed by a modification of the method of Lindwall & Cole (1984), which exploits the solubility of tau in 2.5% (v/v) HClO₄. Briefly, tissue was homogenized (1 mg/ml) in 0.1 M-Mes/0.5 mM-MgCl₂/1 mM-EGTA/1 M-NaCl (pH 6.5) and centrifuged at 100 000 *g*_{av.} at 4 °C for 90 min. The supernatant was made up to 0.5% (v/v) 2-mercaptoethanol, heated at 100 °C for 5 min and centrifuged at 20 000 *g*_{av.} at 4 °C for 30 min. This second supernatant was brought to 45% saturation in (NH₄)₂SO₄ and centrifuged at 20 000 *g*_{av.} as above, and the resulting pellet was resuspended (0.1 ml/g of starting material) in Mes buffer without NaCl. After precipitation with 2.5% (v/v) HClO₄ and a further centrifugation at 20 000 *g*_{av.}, the final supernatant was dialysed against TBS. The total brain proteins and the tau preparation were run on SDS/PAGE [10% (w/v) gels] and blotted on to nitrocellulose, and the nitrocellulose filters were blocked with 3% (w/v) dried milk in TBS containing 0.2% (w/v) Tween 20. Strips of nitrocellulose were incubated with dilutions of whole sera raised to TP10–TP60 and with the lyophilized affinity-purified antibodies reconstituted directly in blocking buffer; the final IgG concentration could therefore not be accurately ascertained. Immunoreactive bands were visualized using biotinylated donkey anti-(rabbit IgG) and streptavidin-alkaline phosphatase, with bromochloroindolyl phosphate/Nitroblue Tetrazolium as chromogen.

Two antisera were raised to bovine tau proteins. Bovine microtubules were prepared by two cycles of polymerization-depolymerization and thermostable MAPs were obtained by heating at 95 °C (Fellous *et al.*, 1977). HClO₄ was added to a concentration of 2.5% (v/v) (Lindwall & Cole, 1984) and, after centrifugation, the supernatant containing purified tau was dialysed against phosphate-buffered saline (0.14 M-NaCl/2.7 mM-KCl/1.5 mM-KH₂PO₄/8.1 mM-Na₂HPO₄) and used as immunogen (50 µg/injection). One antiserum (B19) was tested for its immunoreactivity against the tau synthetic peptides by e.l.i.s.a., and against total human brain proteins and purified tau

by Western blotting. Both sera were also tested against bovine thermostable MAPs by Western blotting.

The antiserum to isolated PHF (Brion *et al.*, 1985b) was also tested for its reaction with the tau synthetic peptides by e.l.i.s.a. The antiserum to rat tau proteins (Brion *et al.*, 1985a) reacted with bovine thermostable MAPs on Western blots. An antiserum raised to ubiquitin has been described previously (Brion *et al.*, 1989).

Preparation of neurofibrillary tangles

Neurofibrillary tangles were prepared from frozen human brain samples taken at autopsy from patients with Alzheimer's disease, using previously described methods (Miller *et al.*, 1986). Some tangle preparations were extracted with SDS at room temperature, while others were suspended in 20 mM-Tris/HCl (pH 7.0)/1 mM-CaCl₂/3 mM-MgCl₂ and digested with micrococcal nuclease (200 units/ml, Boehringer) for 1 h at 37 °C followed by digestion with Pronase E (100 µg/ml, Sigma) for 1 h at 37 °C as described by Wischik *et al.* (1988). The digest was then adjusted to 100 mM-NaCl/3 mM-EGTA/3 mM-EDTA/50 mM-Tris/HCl, pH 8.0, and cholic acid was added to a final concentration of 10 mg/ml.

Immunohistochemistry

Hippocampal and neocortical tissue blocks from patients with Alzheimer's disease were fixed in 10% (v/v) formalin and embedded in paraffin wax. The immunolabelling was performed on paraffin sections using the peroxidase-antiperoxidase method with diaminobenzidine as chromogen. Whole antisera to tau synthetic peptides were used at 1:100–1:500 dilutions, and sera to tau proteins were used at a 1:2000 dilution. Some sections were counterstained with Congo Red and viewed under crossed Nicoll filters. Other sections labelled with anti-TP30 were counterstained with Luxol Fast Blue to demonstrate myelinated axonal tracts.

Immunolabelling was also performed after formic acid treatment of paraffin sections (Kitamoto *et al.*, 1987) or combined trypsin and alkaline phosphatase treatment (Sternberger *et al.*, 1985). After incubation with trypsin [400 µg/ml in 0.05 M-Tris/HCl (pH 7.6)/0.3 M-NaCl/0.02 M-CaCl₂] for 10 min at 37 °C, sections were incubated overnight at 37 °C with alkaline phosphatase (30 units/ml; Sigma, from calf intestine) diluted in 0.1 M-Tris/HCl (pH 8.0)/0.01 M-phenylmethanesulphonyl fluoride. Some sections were also labelled after trypsin treatment alone.

Thick vibratome sections (30 µm) were cut from unembedded tissue blocks fixed in 4% (w/v) paraformaldehyde and labelled with the same antibodies.

The electron microscopy of isolated and immunolabelled PHF with and without Pronase treatment was carried out by the Immunogold staining method (Brion *et al.*, 1985a,b). PHF were adsorbed on carbon/formvar grids and incubated overnight with the primary antibody followed by goat anti-rabbit antibody coupled to 15 nm gold particles (Janssens, Beerse, Belgium).

Adsorption of antisera on to tau was performed by incubating the diluted antisera overnight with nitrocellulose-bound thermostable MAPs. Antisera to synthetic peptides were absorbed on peptides coupled to CH-Sepharose (Pharmacia).

RESULTS

Antisera

The sequences of the six synthetic peptides corresponding to portions of human tau are shown in Fig. 1. All of the antisera raised to synthetic tau peptides reacted with their corresponding peptides by e.l.i.s.a. and with the different tau isoforms on

(a)

Synthetic peptide	Amino acid residues in tau sequence	Antiserum	
TP10	EVMDHAGTY	9-18	Anti-TP10
TP20	HQDQEGD ^{TP20} DTDA	32-41	Anti-TP20
TP30	AGHVTQAR	61-68	Anti-TP30
TP40	TPSLPTPPTR	154-163	Anti-TP40
TP50	QTAPVMPDL	186-195	Anti-TP50
TP60	TDHGAEIVY	297-305	Anti-TP60

(b)

MAEPRQEFV	TP10 MEDHAGTYG	GDRKDQGGYT	TP20 MHQDQEGD ^{TP20} TD	AGLKAEEAGI	50
GDTPSLEDEA	TP30 AGHVTQARMV	SKSKDGTGSD	DKKAKGADGK	TKIATPRGAA	100
PPGQKGQANA	TRIPAKTPPA	PKTPPSSGEP	PKSGDRSGYS	SPGSPGTGPGS	150
RSRT ^{TP40} PSLPTP	PTREPKKVAV	VRTPPKSPSS	AKSRLQTAPV ^{TP50}	PMPDLKNVKS	200
KIGSTENLKH	QPGGGKQVIV	YKPV ^{TP60} DL ^{TP60} SKVT	SKCGSLGNIH	HKPGGGQVEV	250
KSEKLDPKDR	VQSKIGSLDN	ITHVPGGNGK	KIETHKLTFR	ENAKAKTDHG ^{TP60}	300
AEIVYKSPVV	SGDTPSPRHS	NVSSTGSIDM	VDSPQLATLA	DEVASLAKQ	350
GL					352

Fig. 1. Sequences of the synthetic tau peptides used to raise polyclonal antisera (a) and the human tau sequence (b)

The amino acid sequence numbering in (a) corresponds to that in the human tau sequence shown in (b) (Goedert *et al.*, 1988).

Western blots of human tau protein (Fig. 2, lanes A–F). The antisera labelled predominantly tau bands on Western blots of total human normal brain proteins (Fig. 2, lanes H–M), although there was some variation in the relative intensities of the tau bands labelled by the different anti-peptide sera. Others have noted that certain anti-tau antibodies label polypeptides of higher molecular mass than tau, although such species still remain to be characterized (Viereck *et al.*, 1988). Lower-molecular-mass bands may be degradation products of tau, particularly since the protein substrate is *post mortem* brain. The anti-TP30 was the antiserum that had the lowest titre of those used, and labelled only weakly some tau isoforms in total brain protein homogenates. Affinity-purified antibodies from these

antisera to the six synthetic tau peptides also reacted with the different tau isoforms, including antibodies from the low-titre anti-TP30 serum (Fig. 2, lanes O–T). The sequences we selected for the synthesized peptides seem to be present in all tau isoforms. The antisera to rat and bovine tau proteins and the antiserum to isolated PHF labelled the different tau isoforms on Western blots of bovine thermostable MAPs (results not shown). The labelling of tau isoforms on Western blots of human tau and total human brain proteins by one such anti-tau serum (B19) is shown in Fig. 2 (lanes G, N and U). The latter serum reacted with the TP40 and TP50 peptides (but not with the other peptides) by e.l.i.s.a. The anti-PHF serum did not react with any of the tau peptides by e.l.i.s.a.

Immunolabelling in light microscopy

The antisera raised to rat and bovine whole tau proteins strongly labelled most neurofibrillary tangles and the neurites of many senile plaques in paraffin sections, as previously reported (Brion *et al.*, 1985a). Alkaline phosphatase treatment of tissue sections did not significantly alter the labelling, but formic acid treatment enhanced the labelling, especially of extracellular ‘ghost’ tangles.

With the exception of anti-TP30, all antisera to the synthetic tau peptides labelled many tangles on paraffin sections. Plaque neurites (but not the amyloid core) were also labelled by these antisera, but neuritic labelling in plaques tended to be stronger with anti-TP10, anti-TP20 and anti-TP40 than with the antisera to the other tau peptides (Figs. 3, and 4, and Table 1). Extracellular ghost tangles were strongly labelled only by anti-TP60 (Fig. 4c). Treatment of sections with formic acid tended to increase the labelling with the antisera to the synthetic tau peptides; alkaline phosphatase treatment only slightly enhanced the labelling. The anti-TP30 serum strongly labelled white matter tracts and neurites in grey matter on paraffin and vibratome sections, but did not label tangles or senile plaque neurites. However, a subpopulation of tangles and plaque neurites was labelled with anti-TP30 after combined trypsin and alkaline phosphatase treatment (Figs. 4a and 4b). Trypsin treatment alone before labelling with anti-TP30 did not induce this labelling. Counterstaining of sections with Luxol Fast Blue (a dye for myelin sheaths) after anti-TP30 labelling showed a super-

Fig. 2. Western blots of human tau proteins (lanes A–F and O–U) and total human brain proteins (lanes G–N)

Note that lanes G–J and K–N are from two different gels. The proteins were separated by SDS/PAGE (10% gels) and blotted on to nitrocellulose. Blots were incubated with dilutions of the six whole antisera to synthetic tau peptides TP10 (1:100 dilution, lanes A and H), TP20 (1:500, lanes B and K), TP30 (1:100, lanes C and L), TP40 (1:1000, lanes D and M), TP50 (1:100, lanes E and I) and TP60 (1:100, lanes F and J), affinity-purified antibodies to the same synthetic tau peptides (approx. 100–500 µg of IgG/ml) (O, TP10; P, TP20; Q, TP30; R, TP40; S, TP50; T, TP60), and an antiserum to bovine tau protein (1:1000) (lanes G, N and U). Bands below tau proteins are probably degradation products.

Fig. 3. Immunolabelling on paraffin (a–d) and vibratome sections (e) of Alzheimer brain tissue with antisera to TP10 (a and b), TP40 (c and d) and TP50 (e)

Both neurofibrillary tangles (a,c and e) and senile plaque neurites (b and d) are labelled. The labelling of plaque neurites is strongest with anti-TP10. Numerous small neuropil threads are clearly labelled on vibratome sections (e). Scale bar = 10 µm.

Fig. 4. Immunolabelling on paraffin sections of Alzheimer brain tissue

(a) Anti-TP30 labelling. The arrow points to an unlabelled tangle. The arrowhead shows a labelled myelinated axon. (b) Anti-TP30 labelling after alkaline phosphatase treatment. The arrow points to a labelled tangle. (c) Anti-TP60 labelling of extracellular tangles (entorhinal star cell cluster). Scale bar = 10 µm (a, b) or 25 µm (c).

position of the dye staining and the immunoreactivity of white matter tracts.

On vibratome sections, tangles and numerous small neurites in the neuropil were labelled strongly by anti-TP40, anti-TP50 (Fig. 3e) and anti-TP60, but only weakly by anti-TP10 and anti-TP20. Senile plaque neurites, however, were stained by all of these anti-(tau peptide) sera.

White matter tracts in paraffin and vibratome sections were not labelled by the anti-(tau peptide) (with the exception of anti-

Table 1. Labelling with antisera to tau peptides

Tangle and plaque neurite labelling was on paraffin sections. Key: –, none; +/-, rare; +, some; ++, many.

Antibody	Tangle labelling	Plaque neurite labelling	Labelling of isolated PHF	
			SDS-treated	Pronase-treated
Anti-tau	++	++	++	+
Anti-TP10	++	++	+	–
Anti-TP20	++	++	+	–
Anti-TP30	+/-*	+/-*	+	–
Anti-TP40	++	++	++	–
Anti-TP50	++	+	++	+/-
Anti-TP60	++	+	++	++
Anti-PHF	++	++	++	++
Anti-ubiquitin	++	++	++	–

* Observed only after alkaline phosphatase treatment.

Fig. 5. Immunolabelling of isolated tangles in electron microscopy with antisera to TP20 (a and b), TP40 (c and d), TP50 (e and f) and TP60 (g and h)

PHF in (a), (c), (e) and (g) were extracted with SDS and PHF in (b), (d), (f) and (h) were digested with Pronase. SDS-extracted PHF were labelled by the antisera to synthetic tau peptides from the C-terminal domain of tau (TP40, TP50 and TP60), but after Pronase treatment only anti-TP60 still labelled PHF. Scale bar = 100 nm.

Fig. 6. Immunolabelling of isolated tangles (SDS-extracted) in electron microscopy with anti-TP10 (a, b) and anti-TP50 (c) sera

(a) Example of a PHF that is labelled with anti-TP10 (arrow) is shown adjacent to a more commonly unlabelled PHF (arrowhead). Scale bar = 100 nm. (b) Higher magnification of the labelled PHF shown in (a). Note the distance between the gold granule and the axis of the PHF. (c) High magnification of anti-TP50 labelling. Note that the gold granules are nearer to the PHF axis than in (b). Scale bar = 50 nm.

TP30 serum, see above) or the anti-(bovine tau) sera. This was to be expected, since it is well established that tau immunoreactivity other than for PHF-associated tau is labile in *post mortem* human brain and is dependent on the tissue processing (Trojanowski *et al.*, 1989).

Pre-absorption with MAPs adsorbed on to nitrocellulose decreased the labelling of tangles and plaque neurites by the anti-(tau peptide) sera, and completely abolished the labelling with the antisera to whole tau proteins. Absorption of anti-(tau peptide) sera with their corresponding synthetic peptides completely abolished the labelling of tangles and plaque neurites.

Immunolabelling in electron microscopy

On ultrathin sections of Alzheimer's disease cortical brain tissue, anti-TP30 was observed by electron microscopy to label myelin sheaths (results not shown).

Isolated PHF were labelled by the antisera to whole tau proteins, ubiquitin and PHF, as previously reported (Mori *et al.*, 1987; Perry *et al.*, 1987; Brion *et al.*, 1989) (see Table 1). After Pronase treatment, the labelling with anti-ubiquitin was abolished and with anti-tau it was diminished (some variation arose with different preparations of PHF). In addition, Pronase-treated PHF often appeared to be narrower than untreated PHF and were more difficult to demonstrate by negative staining. Pronase treatment also led to a substantial decrease in the number of PHF (approx. 75 %).

Isolated PHF were occasionally labelled with anti-TP10, anti-TP20 and anti-TP30, but were strongly labelled by anti-TP40, anti-TP50 and anti-TP60 (Figs. 5 and 6). Pronase treatment of isolated PHF abolished the labelling of PHF by anti-TP40, significantly decreased the labelling with anti-TP50, but did not

Table 2. Mean perpendicular distance between each gold granule and the axis of SDS-extracted PHF

Results are means $\pm$ S.D. for numbers of measurements given in parentheses. Unpaired *t* tests (two tails) showed highly statistically significant differences between anti-TP10 and anti-TP40 ($P < 0.0001$), between anti-TP10 and anti-TP50 ($P < 0.0001$), between anti-TP40 and anti-TP60 ($P < 0.0005$), and between anti-TP50 and anti-TP60 ($P < 0.0025$). Other comparisons were not found to be statistically significant.

Antiserum	Mean distance (nm)
Anti-TP10	36.5 $\pm$ 4.2 (60)
Anti-TP40	16.7 $\pm$ 1.8 (41)
Anti-TP50	16.5 $\pm$ 2.1 (30)
Anti-TP60	29.3 $\pm$ 4.5 (38)

affect the labelling by anti-TP60 (Figs. 5*d*, 5*f* and 5*h*; Table 1). The mean perpendicular distance between each gold particle and the axis of SDS-extracted PHF was measured for anti-TP10, -TP40, -TP50 and -TP60 (Table 2). Anti-TP10 and anti-TP60 gave results indicating that the corresponding epitopes were equidistant and farthest from the PHF axis, with the epitopes for TP40 and TP50 at an intermediate distance.

DISCUSSION

The abnormal filaments constituting the principal component of neurofibrillary tangles in Alzheimer's disease undoubtedly contain protein derived from the MAP tau (Brion *et al.*, 1985*a*; Delacourte & Defossez, 1986; Kosik *et al.*, 1986; Wischik *et al.*, 1988; Kondo *et al.*, 1988). We have used a panel of polyclonal antisera to epitopes spanning the tau molecule to assess the extent to which the complete tau polypeptide is incorporated into the PHF structure and to probe for structural abnormalities in PHF-associated tau. Identification of structural modifications in tau may lead to a better understanding of the transition from a normal neuronal cytoskeleton to the formation of PHF.

Tau exists as a set of polypeptide isoforms generated by alternative splicing of a primary transcript, and post-translational phosphorylation increases further the heterogeneity of tau. All known tau isoforms contain either three or four tandem repeats of a 31–32 identical amino acid sequence in the C-terminal half of the molecule (Lee *et al.*, 1988; Goedert *et al.*, 1989*b*; Himmler *et al.*, 1989; Kosik *et al.*, 1989). These repeats are the tubulin-binding domains, with only the 3-repeat forms being present in immature brain but both 3- and 4-repeat forms constituting adult brain tau (Kosik *et al.*, 1989; Goedert *et al.*, 1989*a*; Mori *et al.*, 1989). Goedert *et al.* (1989*b*) have shown that PHF contain both 3- and 4-repeat forms of tau as well as all other known human isoform variants generated as primary transcripts. Thus there is no obvious abnormality of tau gene expression and transcript processing that might account for abnormal tau structures and hence underlie PHF formation; rather, post-translational modification of tau may be defective in neurons that develop PHF.

Kosik *et al.* (1988) and Goedert *et al.* (1989*b*) have shown that the majority of tau is apparently incorporated into PHF *in situ*. We have extended these findings, since our studies investigated epitopes encompassing much of the tau molecule (from residue 8 to 305, the complete sequence of the isoform used for the basis of this study being of 352 amino acids) by labelling of paraffin sections, vibratome sections and isolated PHF. The latter were

also immunolabelled after Pronase treatment. Our findings are indicative of certain post-translational changes in the tau that is associated with PHF.

In contrast with the strong labelling of tangles on paraffin sections by all anti-peptide antibodies except anti-TP30, neurofibrillary tangles in vibratome sections were only strongly labelled by antibodies to TP40, TP50 and TP60, although neurites in senile plaques were still stained by anti-TP10 and anti-TP20. The tissue used for vibratome sections was less extensively fixed and processed than for paraffin sections, and we interpret the findings as indicating that the N-terminal domain of tau is less tightly associated with PHF in tangles than is the case for the C-terminal half of the molecule and so is lost during processing due to the light fixation conditions. Ghost tangles were the most strongly labelled by anti-TP60; since these ghost tangles are cell remnants after neuronal death, they probably represent the most insoluble part of tangles and would have already lost N-terminal domains of tau *in vivo* or during tissue processing.

This differential pattern of labelling was also found with isolated SDS-extracted PHF, since this material was labelled by anti-TP40, anti-TP50 and anti-TP60, but only a few PHF were decorated by anti-TP10, anti-TP20 and anti-TP30. The tau immunoreactivity of isolated PHF to a tau monoclonal antibody (Tau-1) has also been reported to be removed partially after SDS extraction and completely in the presence of SDS and sulphhydryl reducing agents, whereas ubiquitin immunoreactivity was retained (Shaw & Chau, 1988). The epitope for Tau-1 lies between amino acids 130 and 149 (Kosik *et al.*, 1988), i.e. N-terminal to the TP40 sequence. These observations suggest that tau may be proteolytically cleaved in PHF in tangles, although perhaps not in plaque neurites, and it is of interest to determine if cleavage occurs *in vivo* or if it is only a *post mortem* artefact, since if it is *in vivo* this suggests that metabolism of PHF-associated tau is different in plaque neurites compared with perikaryal tangles. This cleavage would occur between the TP30 and TP40 sequences, which would explain why the domain of tau N-terminal to TP40 is absent from many isolated SDS-extracted PHF. The identification of the precise cleavage point might be resolved by further epitope mapping and amino acid sequence analysis followed by identification and investigation of the distribution and levels of the proteolytic enzyme responsible. Kondo *et al.* (1988) observed that SDS-treated PHF were rarely labelled by antibodies to a tau peptide (tau C1) whose sequence is located 7 residues N-terminal to TP40, and so a putative cleavage site could lie between the tau C1 and TP40 sequences or at either of their near-neighbour ends.

Treatment of isolated PHF with Pronase resulted in strong labelling by only anti-TP60, which is located in the C-terminal region of tau. This is consistent with the findings of Wischik *et al.* (1988), who identified peptide sequences corresponding to the C-terminal half of tau that were isolated from the Pronase-resistant PHF 'core'. Our anti-PHF serum also labelled Pronase-treated PHF, but we were unable to demonstrate activity in this antiserum to any of our synthetic peptides; presumably the anti-tau activity of this antiserum is directed towards other epitopes in the C-terminal half of tau. Pronase treatment also abolished labelling with anti-ubiquitin sera, suggesting that the ubiquitin in PHF is in a peripheral location rather than being in the core.

In some preparations of SDS-extracted PHF, labelling of a fraction of PHF was observed with anti-TP10, anti-TP20 and anti-TP30. The labelling with anti-TP10 and anti-TP60 was interesting, since the gold particles were localized at a statistically significantly greater distance from the PHF axis than were the gold particles from labelling with anti-TP40 and anti-TP50. The tau molecules would therefore seem to be in at least a partially extended conformation, which in native tau is related to it being in a phosphorylated state (Hagestedt *et al.*, 1989); in PHF the

central region of tau would appear to be located closest to the PHF core. Since the *N*-terminal half of tau seems to be labile in PHF, whereas the *C*-terminal half is resistant even to Pronase treatment, this suggests that the lability is not simply determined by the extent to which tau polypeptide domains are extended from the PHF axis. The labelling of a fraction of isolated PHF with anti-TP10, anti-TP20 and anti-TP30 probably does not simply reflect the presence of weakly adhering material, since the density of labelling with these antibodies was high for a subpopulation of isolated PHF and weak or absent for others. Therefore the simplest interpretation is that the labelled PHF most probably contain uncleaved tau, and these PHF may originate from plaque neurites rather than from tangles.

The results with anti-TP30 are interesting, since they identify a site in tau that may be abnormally phosphorylated or modified in PHF-associated tau. Anti-TP30 clearly recognizes all of the tau isoforms labelled by all of the other antibodies, as shown by Western blotting, demonstrating that the TP30 epitope is not spliced out of any particular isoforms. Kosik *et al.* (1988) reported that a monoclonal antibody to tau (antibody 60) that is directed at an epitope in a region of tau spanning the TP30 epitope labels tangles, and yet anti-TP30 labelled few tangles *in situ*. However, alkaline phosphatase treatment of sections induced labelling of an increased number, although still a minority, of tangles, implying that the TP30 epitope may be phosphorylated in PHF; the failure to induce labelling of all tangles by alkaline phosphatase implies that the phosphorylated residue is not readily accessible to the enzyme. The labelling of a fraction of SDS-extracted PHF with anti-TP30 also indicates that the corresponding epitopes are more accessible on these isolated PHF than in tissue sections. This observation may be due to the TP30 epitope being partially modified in sections or partially blocked by the fixation procedure. Although there seems to be a cross-reactive epitope present in myelin to which anti-TP30 binds, it is unlikely that labelling of tangles *in situ* and in isolated PHF is the result of a myelin protein being a constituent of PHF. There are no reports of myelin antibodies labelling tangles, and the Western blot results with anti-TP30 clearly show it to recognize all tau polypeptides. We conclude that the results of labelling of PHF with anti-TP30 are due to the tau component of PHF and not to a myelin-derived molecule being in PHF.

The TP30 sequence contains a single threonine residue but also lies just *N*-terminal to putative phosphorylation sites for protein kinase C and calcium/calmodulin protein kinase (Goedert *et al.*, 1988). A monoclonal antibody, Tau-1, has also been shown to behave similarly to anti-TP30 in that it recognizes tau on Western blots but only labels tangles following alkaline phosphatase treatment (Grundke-Iqbal *et al.*, 1986a). The epitope for Tau-1 lies between amino acids 130 and 149 in the tau sequence (Kosik *et al.*, 1988), whereas TP30 encompasses residues 61–68, and the adjacent kinase consensus sequences are serine residues at positions 71 and 73. Thus our findings with anti-TP30 suggest that a second site in tau may be subject to an excessive level of phosphorylation in the tau associated with PHF, implying an additional disturbance in protein phosphorylation in tangle-bearing neurons.

In summary, our results have identified two regions of tau that deserve further investigation in relation to PHF structure and biochemistry. One region identified by the TP30 epitope may be subject to abnormal phosphorylation, and the other, *N*-terminal to the TP40 epitope, may undergo proteolytic cleavage.

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