Tau in Alzheimer's disease and Down's syndrome is insoluble and abnormally phosphorylated

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Some investigators have described the presence in Alzheimer's disease brain extracts of several abnormal forms of the microtubule-associated protein tau, based on their unusual mobility in SDS/PAGE. It has been proposed that these abnormal forms of tau may be the result of aberrant tau phosphorylation. In this study we show that tau in extracts of Alzheimer's disease brain can be separated into two fractions based upon its solubility (100 000 g × 1 h supernatant) in non-denaturing conditions (100 mM-Mes, pH 6.5, 0.5 mM-MgCl₂, 1 mM-EGTA and 1 M-NaCl). The tau isoforms with decreased mobility in SDS/PAGE are predominantly in an insoluble fraction, whereas the soluble tau is indistinguishable by its mobility in SDS/PAGE from tau in soluble extracts of control brain. Insoluble tau displaying abnormal mobility on SDS/PAGE was only found in Alzheimer and adult Down's syndrome brains and was absent from the brains of age-matched controls and from foetal and infant Down's syndrome brains. There was a good correlation between the presence of insoluble tau in brain extracts and the abundance of neurofibrillary tangles and senile neuritic plaques. The monoclonal antibody Tau.1 stained insoluble tau on Western blots only after treatment of the nitrocellulose transfers with alkaline phosphatase, implying that this insoluble tau is in a particular state of phosphorylation. We conclude that, in Alzheimer's disease, a fraction of tau has a modified phosphorylation state and a decreased solubility; these modifications may precede formation of the neurofibrillary tangles characteristic of Alzheimer's disease and Down's syndrome in adults.

INTRODUCTION

Two of the characteristic histopathological features of Alzheimer's disease are intraneuronal neurofibrillary tangles and neuritic plaques, the latter consisting of an extracellular amyloid core surrounded by dystrophic neurites. Neurofibrillary tangles are composed of paired helical filaments (PHF) which consist of two strands, each about 10 nm wide, wound around each other with a periodicity of approx. 70 nm (Wischnik *et al.*, 1985). A fraction, if not all, of PHF are insoluble, and it is this insolubility which has hampered the elucidation of their chemical composition. However, it has been possible by the application of immunocytochemistry (Brion *et al.*, 1985; Grundke Iqbal *et al.*, 1986; Wood *et al.*, 1986; Kosik *et al.*, 1986, 1988; Delacourte & Defossez, 1986) and by protein chemical studies (Wischnik *et al.*, 1988; Ihara & Kondo, 1989; Mori *et al.*, 1989) to establish that the neuron-specific cytoskeletal microtubule-associated protein (MAP), tau, is an integral component of the neurofibrillary tangles of Alzheimer's disease. Tau appears on Western blots of brain homogenate as a set of 4–7 bands of molecular mass 55–62 kDa (Cleveland *et al.*, 1977); the precise number and pattern of bands varies according to the stage of development and to the animal species. Tau is thought to function as a promoter and stabilizer of tubulin polymerization (Weingarten *et al.*, 1975).

It is not yet known whether the properties of PHF-associated tau are different from those of the tau forms which co-purify with microtubules from human and bovine brain. It is sometimes stated that tau in PHF is abnormally phosphorylated (Ihara *et al.*, 1986; Grundke Iqbal *et al.*, 1986; Baudier & Cole, 1987; Parent *et al.*, 1988), supported by the fact that labelling of the

majority of neurofibrillary tangles by the monoclonal antibody Tau.1 under immunoelectron microscopy occurs only if they are first treated with alkaline phosphatase (Wood *et al.*, 1986; Merz *et al.*, 1987; Bancher *et al.*, 1989). Although Tau.1 labels axons, it has also been shown to label dendrites and neuronal cell bodies after alkaline phosphatase treatment of the histological substrate (Papazosomenos & Binder, 1987). The implication of this is that tau is not restricted solely to the axonal domain, but that it is also present in the somato-dendritic compartment of neurons in a different phosphorylation state to that of the tau found in axons. We have identified another site, N-terminal to the Tau.1 epitope, that is phosphorylated in tangle-associated tau (Brion *et al.*, 1991). Like the labelling with Tau.1, tangles are decorated by an antibody to this site only if they are first treated with alkaline phosphatase; it remains to be established whether this site is phosphorylated only in tau that is associated with Alzheimer pathology.

Biochemical studies of extracts from Alzheimer's disease brain have shown that a proportion of tau migrates on SDS/PAGE more slowly than tau from control brain (Grundke Iqbal *et al.*, 1986; Flament *et al.*, 1989a). Treatment of Alzheimer's disease brain homogenates with alkaline phosphatase before analysis on SDS/PAGE results in a shift of the slower-migrating species to positions more characteristic of normal tau. These findings have been interpreted as showing that, in Alzheimer's disease, tau is abnormally phosphorylated. A recent report describes the isolation of a fraction of PHF from Alzheimer's disease brain which are soluble in SDS/PAGE sample buffer, and which contain predominantly slower-migrating tau isoforms (Greenberg & Davies, 1990), reinforcing the association between slower-migrating tau and PHF pathology.

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

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We now report that the slower-migrating tau species in Alzheimer's disease and Down's syndrome brain extracts are, unlike normal tau, insoluble in aqueous buffers; we refer to this slower-migrating tau as insoluble tau. A semi-quantitative correlation was demonstrated between the presence of insoluble tau and the quantity of PHF and neuritic plaques in these brains. The insoluble tau, but not the soluble tau, was only labelled on Western blots by the monoclonal antibody Tau.1 after pretreatment of the blots with alkaline phosphatase. This demonstrated that the insoluble tau is selectively composed of tau molecules that are in a particular state of phosphorylation.

EXPERIMENTAL

Human brain material

Post-mortem human brain samples were obtained from the Medical Research Council Alzheimer's Disease Brain Bank. The 14 Alzheimer's disease cases had been clinically assessed using the criteria of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (McKhann *et al.*, 1984). Histopathological classification of tissue sections stained by silver impregnation methods, which identified dystrophic neurites, neurofibrillary tangles and neuritic plaques, were made known only after the biochemical analyses of the tissues were completed. The Alzheimer's disease cases ranged in age from 76 to 91 years (81 ± 2 years, mean $\pm$ S.E.M.) and were compared with nine age-matched controls of 64–91 years old (78 ± 3 years). Of the 10 confirmed (trisomic chromosome 21) Down's syndrome cases, three samples were from foetal (21 and 23 weeks) and infant (6.5 months) brains, and seven were from adults of 46–69 years old (55 ± 3 years). The post-mortem delays were 11–57 h (35 ± 4 h) for the Alzheimer's disease cases, 24–96 h (45 ± 7 h) for the age-matched controls, 6–9 h (7 ± 1 h) for the foetal and infant Down's syndrome and 24–72 h (43 ± 8 h) for the adult Down's syndrome cases. Brain samples were stored at -70°C until required.

Antibody preparation

The rabbit polyclonal anti-(bovine tau) serum, B19, was raised to purified tau proteins from a HClO_4 -treated thermostable preparation of bovine MAPs, as described previously (Brion *et al.*, 1991). This antibody recognizes human, bovine and rat tau on Western blots of thermostable MAP preparations. It also stains neurofibrillary tangles and dystrophic neurites in the periphery (but not the amyloid core) of neuritic plaques in paraffin sections of Alzheimer's disease brain; in addition, the B19 antiserum labels isolated PHF. This antiserum was used at a dilution of 1:5000 on Western blots of human brain extracts.

Tau.1, a monoclonal antibody raised to bovine tau, was a gift from Dr. L. I. Binder (University of Alabama, Birmingham, AL, U.S.A.) and has been described previously (Binder *et al.*, 1985). Tau.1 stains normal human tau on Western blots, but the majority of neurofibrillary tangles in tissue sections require dephosphorylation with alkaline phosphatase before they are labelled by this antibody (Wood *et al.*, 1986). Tau.1 was used at a dilution of 1:1000 on Western blots of soluble and insoluble human brain fractions.

Preparation of human brain extracts

Brain tissue (predominantly grey matter) was homogenized (1 g/5 ml) in cold (0°C) Mes buffer (100 mM-Mes, pH 6.5, 0.5 mM- MgCl_2 and 1 mM-EGTA) containing 1 M-NaCl with an all-glass tissue grinder. The homogenate was centrifuged at 100000 g (Beckman Type 40 rotor) for 60 min at 4°C , the supernatant (Mes/NaCl supernatant) was retained, and the

pellet was re-homogenized at ambient temperature in the original volume of Mes buffer containing 5% (w/v) SDS but without NaCl. The SDS-treated material was centrifuged at 100000 g (Beckman Type 40 rotor) for 90 min at 20°C , and the supernatant was retained (SDS-solubilized Mes/NaCl pellet).

SDS/PAGE and Western blotting of extracted proteins

Brain extracts (Mes/NaCl supernatants and SDS-solubilized Mes/NaCl pellets) were heated at 100°C for 5 min with 0.25 vol. of sample buffer [156 mM-Tris/HCl, pH 6.8, 5% (w/v) SDS, 12.5% (v/v) 2-mercaptoethanol, 25% (v/v) glycerol and 0.005% (w/v) Bromophenol Blue] and analysed on SDS/PAGE (10% gels) using a discontinuous buffer method (Laemmli, 1970). Gels were either stained with Coomassie Brilliant Blue R or the separated proteins were blotted on to nitrocellulose membranes (Schleicher and Schuell, $0.45 \mu\text{m}$, grade BA 85) at 15 V for 1 h using a combination of buffers: 40 mM-aminohexanoic acid/25 mM-Tris, pH 9.4 (cathode), 300 mM-Tris, pH 10.4 (anode 1) and 25 mM-Tris, pH 10.4 (anode 2), each containing 20% (v/v) methanol. Non-specific binding was blocked by incubating the nitrocellulose in Tris-buffered saline (TBS: 25 mM-Tris/HCl, pH 8.0, 140 mM-NaCl and 5 mM-KCl) containing 0.2% (v/v) Tween 20 and 3% (w/v) dried skimmed milk, for a minimum of either 1 h at 37°C or 2 h at 20°C . The nitrocellulose was incubated for 16 h at 4°C with primary antiserum diluted in blocking buffer. Blots were developed with either donkey anti-rabbit or sheep anti-mouse IgG (biotinylated) (Amersham: 1:200 dilution in blocking buffer), followed by streptavidin-alkaline phosphatase (Amersham: 1:1500 dilution in blocking buffer). Bromochloroindolyl phosphate ($50 \mu\text{g/ml}$), Nitroblue Tetrazolium ($500 \mu\text{g/ml}$) and 4 mM- MgCl_2 in 100 mM-diethanolamine, pH 9.5, was used as the alkaline phosphatase chromogenic substrate.

Alkaline phosphatase treatment of blotted proteins

For the solid phase dephosphorylation of blotted proteins separated by SDS/PAGE, nitrocellulose membranes were blocked as above and incubated for 16 h at 37°C with 1 unit of *Escherichia coli* alkaline phosphatase/ml (Sigma, Type III-N) in 50 mM-Tris/HCl, pH 8.3, containing 50 mM-NaCl, 1 mM- MgCl_2 , 1 mM- ZnCl_2 , 1 mM-phenylmethanesulphonyl fluoride (PMSF) and $10 \mu\text{g}$ of leupeptin/ml. In order to ensure in controls that any changes in tau immunoreactivity were due to alkaline phosphatase and not to proteinase activity, alkaline phosphatase was inhibited by the inclusion of 200 mM-sodium/potassium phosphate, pH 8.3. The blots were washed for 3×1 h in TBS containing 0.2% (v/v) Tween 20. The nitrocellulose was blocked again and then incubated for 16 h at 4°C with monoclonal antibody Tau.1 diluted 1:1000 in blocking buffer and developed as described above.

Alkaline phosphatase treatment of solubilized extracts

This was a modification of the protocol used by Flament *et al.* (1989a) for the alkaline phosphatase treatment of total brain homogenate in SDS/PAGE buffer. SDS-solubilized Mes/NaCl pellets from cases of Alzheimer's disease and adult Down's syndrome were dialysed for 16 h at 20°C against 500 vol. of 100 mM-Mes, pH 6.5, containing 0.75 M-NaCl. After heating at 100°C for 5 min and centrifuging at 10000 g (Eppendorf 5410) for 30 min at 20°C , the heat-stable supernatants were dialysed for 16 h at 4°C against 500 vol. of 50 mM-Tris/HCl, pH 8.3, containing 50 mM-NaCl, 1 mM- MgCl_2 and 1 mM- ZnCl_2 . The diffusates were incubated with 15 units of *E. coli* alkaline phosphatase/ml at 37°C for 4 h in the presence of 1 mM-PMSF and $10 \mu\text{g}$ of leupeptin/ml; for controls, 200 mM-sodium/potassium phosphate, pH 8.3, was included. After incubation the

samples were heated to 100 °C for 5 min, SDS/PAGE sample buffer was added, followed by a further 5 min incubation period at 100 °C, and the extracts were subjected to SDS/PAGE and Western blotting with the anti-tau serum B19, as described above.

RESULTS

The cases and brain areas examined in this study of tau protein are described in Table 1.

The SDS/PAGE separation of the total proteins in the Mes/NaCl supernatant and in the SDS-solubilized Mes/NaCl pellet from a representative Alzheimer's disease brain and from a representative age-matched control brain are shown in Fig. 1. Variation in the relative intensities of some Coomassie Blue-stained protein bands was observed between brain specimens, but the differences were not consistent and it was not possible to distinguish between Alzheimer's disease, Down's syndrome and control brain samples from such gels. The major band, at

Fig. 1. Coomassie Brilliant Blue R-stained SDS/10% (w/v)-polyacrylamide gels of extracted proteins

C, age-matched control brain; A, Alzheimer's disease brain. Samples used were Mes/NaCl supernatant (lanes 1 and 3) and SDS-solubilized Mes/NaCl pellet (lanes 2 and 4). Molecular mass markers were phosphorylase *b* (97.4 kDa), catalase (58.1 kDa), alcohol dehydrogenase (39.8 kDa) and carbonic anhydrase (29.0 kDa). There were no overt qualitative or quantitative differences between the comparable extracts from the two brains.

Table 1. Cases and brain areas examined for the presence of tau protein

Brain samples from cases of Alzheimer's disease (A1–A14), age-matched controls (C1–C9) and cases of Down's syndrome (D1–D10) are shown, indicating post-mortem delay (PMD) and region of brain examined (TC, temporal cortex; FC, frontal cortex). The presence of neurofibrillary tangles (NFT) and neuritic plaques (NP) is shown as: ++ abundant; + some; +/- few; - none. Insoluble tau was detected by Western blotting of the SDS-solubilized Mes/NaCl pellets with a polyclonal antiserum to bovine tau. Down's syndrome cases indicated by *, ** and *** are from a 23-week foetus, a 21-week foetus and a 6.5-month infant respectively. N/A, data not available.

Case no.	Age (years)	Sex	PMD (Hours)	Brain area tested	Lesions		Insoluble tau
					NFT	NP	
A1	76	F	31	TC	++	++	+
A2	81	M	57	TC	+/-	+/-	-
A3	90	M	48	TC	++	+	+
A4	77	F	46	TC	+/-	+/-	-
A5	87	F	29	TC	-	+	-
A6	88	F	45	FC	+/-	+	-
A7	81	F	52	FC	++	++	+
A8	78	F	35	FC	+	+	+
A9	80	M	44	FC	++	++	+
A10	91	F	13	FC	+/-	+/-	-
A11	73	M	11	FC	++	+	+
A12	71	F	33	TC	+/-	++	+
A13	79	F	16	TC	++	++	+
A14	78	F	27	FC	++	++	+
C1	79	M	32	TC	-	-	-
C2	84	M	24	TC	-	-	-
C3	73	M	48	TC	-	-	-
C4	91	M	47	TC	-	-	-
C5	75	M	44	FC	-	-	-
C6	68	F	42	FC	-	-	-
C7	83	F	48	FC	-	-	-
C8	64	M	24	FC	-	-	-
C9	82	M	96	FC	-	+/-	-
D1	*	M	6	FC	N/A	N/A	-
D2	**	M	6	FC	N/A	N/A	-
D3	***	M	9	FC	N/A	N/A	-
D4	47	F	24	FC	++	++	+
D5	46	M	72	FC	++	++	+
D6	57	M	31	FC	++	++	+
D7	56	M	72	FC	++	++	+
D8	69	M	30	FC	++	++	+
D9	54	F	24	FC	++	++	+
D10	53	F	48	FC	++	++	-

Fig. 2. Western blot of tau immunoreactivity in fractions from Alzheimer's disease, control and Down's syndrome brains

Samples were Mes/NaCl supernatants (lanes 1, 3 and 5) and SDS-solubilized Mes/NaCl pellets (lanes 2, 4 and 6) from brains of a case of Alzheimer's disease (A), on age-matched control (C) and a case of adult Down's syndrome (D). Tau immunoreactivity was detected with the polyclonal anti-tau serum B19 (diluted 1:5000). Molecular mass markers are as in Fig. 1. The two slower-migrating tau species in the Mes/NaCl pellets are indicated by arrowheads.

approx. 50 kDa, present in the insoluble fraction of both Alzheimer's disease and control brains has been identified as tubulin by Western blotting with monoclonal antibodies to α - and β -tubulin (results not shown).

Western blots with the polyclonal antiserum to tau, B19, on representative Mes/NaCl supernatants from an Alzheimer's disease brain, an age-matched control brain and an adult Down's syndrome brain gave similar patterns of tau immunoreactivity (Fig. 2, lanes 1, 3 and 5 respectively). A characteristic set of between four and six tau protein bands of approx. 48–62 kDa and three strong bands of approx. 34–43 kDa, which are presumed to be tau degradation products, were consistently observed in these soluble extracts, irrespective of the source of brain tissue. Hence it was not possible to discriminate between Alzheimer's disease, Down's syndrome and age-matched control brains on the basis of the Mes/NaCl-extracted tau. Quantitative differences

Fig. 3. Monoclonal antibody Tau.1 immunoreactivity on untreated and dephosphorylated nitrocellulose transfers of human tau in Alzheimer's disease, control and Down's syndrome brain extracts

Samples were Mes/NaCl supernatants (lanes 1, 3, 5 and 7) and SDS-solubilized Mes/NaCl pellets (lanes 2, 4, 6 and 8) from brains of a case of Alzheimer's disease (lanes 3 and 4), an age-matched control (lanes 1, 2, 5 and 6) and a case of adult Down's syndrome (lanes 7 and 8). Nitrocellulose transfers were labelled with monoclonal antibody Tau.1 (diluted 1:1000). Pre-treatment of the blot was carried out with alkaline phosphatase (b); 200 mM-sodium/potassium phosphate was included in the controls (a). Insoluble tau bands with decreased mobility are only seen in the Alzheimer's disease and Down's syndrome brain SDS-solubilized Mes/NaCl pellets after dephosphorylation (arrowheads).

between individual cases were often observed, with a tendency for lower amounts of soluble tau in those cases which exhibited insoluble tau immunoreactivity. However, these differences were not sufficiently consistent to be diagnostic of the source of the brain extract. The tau-immunoreactive patterns of Mes/NaCl supernatants obtained from the foetal and infant Down's syndrome brains were different both from each other and from those of the other brain extracts examined in this study (results not shown), but were not investigated further.

Western blots of SDS-solubilized Mes/NaCl pellets from each category of brain examined showed a substantial overall increase in tau immunoreactivity in Alzheimer's disease and in adult Down's syndrome (Fig. 2, lanes 2 and 6 respectively) compared with age-matched controls (Fig. 2, lane 4) and with the foetal and infant Down's syndrome brain extracts (results not shown). This abnormal Mes/NaCl-insoluble tau immunoreactivity was characterized by the presence of at least two clearly defined bands with a decreased mobility on SDS/PAGE (apparent molecular masses of 69 and 63 kDa) compared with soluble tau; occasionally a third band of 58 kDa was also seen. There was also a smear of tau labelling throughout the electrophoretic tracks on Western blots of those Alzheimer's disease and adult Down's syndrome brain extracts which contained distinct insoluble tau bands. The Mes/NaCl-soluble fractions from the same brains did not contain these slower-migrating tau-immunoreactive bands, demonstrating that these insoluble tau isoforms were completely separated from soluble tau by the centrifugation conditions employed. The SDS-solubilized Mes/NaCl pellets from the age-matched control group contained minimal tau immunoreactivity on Western blots and the slower-migrating insoluble tau bands were never seen in these controls, nor was there the characteristic smearing of tau-immunoreactive material throughout the electrophoretic track.

The abnormality associated with the insoluble tau is not due solely to self-aggregation via thiol links between the cysteine groups in the C-terminal portion of tau. The Mes/NaCl pellet from an Alzheimer's disease brain was extracted in Mes buffer containing 5% (v/v) 2-mercaptoethanol, and a Western blot of this extract showed that the insoluble tau was not solubilized by this procedure (results not shown).

Nine of the 14 Alzheimer's disease brain SDS-solubilized Mes/NaCl pellet extracts were positive for an increased tau immunoreactivity, as were six of the seven adult Down's syn-

drome brains (Table 1). The presence of insoluble tau was found to be correlated with the neuronal load of neurofibrillary tangles and neuritic plaques in the Alzheimer's disease and Down's syndrome cases. The results from Alzheimer's disease and Down's syndrome brains contrast with those from the age-matched control group, in which none of the nine cases contained insoluble tau immunoreactivity.

Post-mortem delays of less than 20 h are not an absolute requirement in order to visualize normal and insoluble tau on Western blots. Several cases with a post-mortem delay of greater than 40 h contained insoluble tau and, of the total of 15 positive results, with a range of 11–72 h, only two cases had a post-mortem delay of less than 20 h.

The insoluble tau in the SDS-solubilized Mes/NaCl pellets from Alzheimer's disease and Down's syndrome brains was labelled by the monoclonal antibody Tau.1 only after dephosphorylation of Western blots with alkaline phosphatase before incubation with antibody (Fig. 3b, lanes 4 and 8 respectively). The alkaline phosphatase treatment of the blots also led to an increase in the smeared Tau.1 immunoreactivity in the lanes containing the insoluble tau species, indicating that the smeared tau-immunoreactive material is also phosphorylated similarly to the distinct insoluble tau species. Alkaline phosphatase treatment of the Western blot of an SDS-solubilized Mes/NaCl pellet from a control brain did not result in any additional labelling of bands by Tau.1 or in an increase in smeared immunoreactive material (Fig. 3b, lanes 2 and 6), confirming that control brain does not contain insoluble tau. Alkaline phosphatase treatment of Western blots of Mes/NaCl-soluble tau extracted from age-matched control, Alzheimer's disease and Down's syndrome brains did not increase labelling by Tau.1 (Fig. 3b, lanes 1, 3 and 7 respectively) compared with the untreated immunoblots (Fig. 3a, lanes 1, 3 and 7 respectively). Thus the only tau species more intensely labelled by Tau.1 after alkaline phosphatase treatment were the abnormal Mes/NaCl-insoluble tau polypeptides and the smeared material in the electrophoretic tracks from brain samples with abundant Alzheimer pathology. The Tau.1 antibody did not reveal any differences between control and either Alzheimer's disease or Down's syndrome brain extracts without prior alkaline phosphatase treatment, implying that all of the insoluble tau is in a state of phosphorylation that prevents recognition by the Tau.1 antibody.

Fig. 4. Effects of alkaline phosphatase treatment on electrophoretic mobility of insoluble tau in Alzheimer's disease and Down's syndrome

Samples were SDS-solubilized Mes/NaCl pellets from Alzheimer's disease (lanes 1–3) and adult Down's syndrome brains (lanes 4–6): untreated extracts, lanes 1 and 4; extracts incubated with alkaline phosphatase before SDS/PAGE and immunoblotting, lanes 2 and 5; extracts treated with alkaline phosphatase in the presence of 200 mM-sodium/potassium phosphate, lanes 3 and 6. The blots were labelled with the anti-(bovine tau) serum B19 (diluted 1:5000). The slower-migrating tau bands are indicated by arrowheads and the main tau bands produced after alkaline phosphatase treatment are indicated by asterisks.

Dephosphorylation of the SDS-solubilized Mes/NaCl pellets from Alzheimer's disease and Down's syndrome brain before Western blotting revealed differences between the insoluble tau in the two disease states (Fig. 4). After alkaline phosphatase treatment of the SDS-solubilized Mes/NaCl pellet, the insoluble tau in the Down's syndrome brain extract showed an increased mobility on SDS/PAGE, moving into the normal molecular mass range for soluble tau; this change was inhibited by the inclusion of 200 mM-phosphate. However, the equivalent extract from the Alzheimer's disease brain produced a less distinct result after dephosphorylation, as the two insoluble tau bands became more diffuse and formed a new region of tau immunoreactivity at the interface of the positions for normal and insoluble tau; this latter result may be due to incomplete dephosphorylation.

DISCUSSION

In this study we were able to separate tau from Alzheimer's disease and adult Down's syndrome brain extracts into two fractions according to its solubility in aqueous buffer at high ionic strength. The presence of 1 M-NaCl in the buffer does not affect the amount of insoluble tau, but decreases non-specific ionic association of soluble proteins with particulate material. Analysis of the fractions by Western blotting showed that in control brain samples the tau is mainly soluble, whereas Alzheimer's disease and adult Down's syndrome brain extracts contain significant amounts of insoluble tau, the quantity of which is correlated with the degree of Alzheimer pathology.

Tubulin precipitation was observed as a broad 50 kDa band in the insoluble fractions of all brain extracts examined. There was no difference in the proportion of tubulin in the insoluble fraction of extracts from Alzheimer's disease, adult Down's syndrome or age-matched controls. Hence the marked increase in tau in the insoluble fraction of extracts from Alzheimer's disease and adult Down's syndrome does not appear to be related to tubulin precipitation.

The insoluble tau was found to migrate more slowly on SDS/PAGE than soluble tau. The results with the monoclonal

antibody Tau.1, which only labels Alzheimer neurofibrillary tangles in tissue sections after the sections have been treated with alkaline phosphatase, showed that all of the slower-migrating insoluble tau is in a state of phosphorylation that prevents recognition by Tau.1. This suggests that the insoluble tau is in a physical conformation at least partially resembling that in PHF. Antibodies to tau have repeatedly been shown to label on Western blots material smeared throughout the electrophoretic track of PHF fractions (Kosik *et al.*, 1986; Grundke Iqbal *et al.*, 1986; Parent *et al.*, 1988; Flament & Delacourte, 1989). Our observations with the Tau.1 antibody indicate that this smeared material is tau that is in a phosphorylation state similar to that of the distinct insoluble tau species and is probably a range of aggregation states of the abnormal tau. While this paper was in preparation, Greenberg & Davies (1990) published a new protocol for isolating a fraction enriched in PHF that can be solubilized in SDS/PAGE sample buffer and found that the tau in this fraction is predominantly of the slower-migrating type.

Flament and co-workers have described slower-migrating forms of tau in total SDS-solubilized protein extracts from Alzheimer's disease and adult Down's syndrome brains (Flament *et al.*, 1989a, 1990) and showed that these tau forms can be converted to faster-migrating species by treatment with alkaline phosphatase (Flament *et al.*, 1989b; Flament & Delacourte, 1989). We have shown that alkaline phosphatase treatment of the insoluble tau after it is solubilized by SDS converts it to faster-migrating species. It is known that increasing amounts of tau phosphorylation lead to its mobility in SDS/PAGE becoming lowered (Baudier & Cole, 1987), and so our findings and those of Flament *et al.* (1990) imply that a fraction of tau in Alzheimer's disease and adult Down's syndrome brains is excessively or abnormally phosphorylated. It is not yet known if this apparent abnormal phosphorylation is the reason why the monoclonal antibody Tau.1 does not label the slower-migrating tau or neurofibrillary tangles, since it has been suggested that Tau.1 recognizes only tau present in axons and that the somato-dendritic compartment of neurons contains tau unreactive with Tau.1 (Papazozomenos & Binder, 1987). This nevertheless would imply that the fraction of tau that is unreactive with the Tau.1 antibody is selectively sequestered into the insoluble PHF-containing fraction of brain affected by Alzheimer pathology.

The protein A68, which was first identified by the monoclonal antibody Alz-50 (Wolozin *et al.*, 1986), is a polypeptide of 68 kDa on SDS/PAGE and is apparently of high molecular mass in extracts (obtained under non-denaturing conditions) from Alzheimer's disease brain; this 68 kDa form of the antigen is absent from control brain. Alz-50 stains tangle-bearing neurons in Alzheimer brain but does not stain control brain (Wolozin *et al.*, 1986), although on Western blots of Alzheimer's disease and control brain extracts Alz-50 labels tau bands (Nukina, 1989). Other workers have recently found that A68 is only recognized by Tau.1 after alkaline phosphatase treatment (Ksiezak Reding *et al.*, 1990), suggesting that A68 may be identical with the insoluble form of tau that we have now found to be specifically associated with neurofibrillary pathology in diseased brain.

Our results, and the recent findings from several other laboratories, demonstrate that a fraction of tau becomes insoluble and is abnormally phosphorylated as pathology develops in Alzheimer's disease and Down's syndrome. There seems to be an association between tau insolubility and its phosphorylation state. Increased amounts of phosphorylation are known to decrease the ability of tau to promote tubulin assembly into microtubules *in vitro* (Iqbal *et al.*, 1986), and this may be a factor in the disappearance of microtubules from regions afflicted with PHF (Flament Durand & Couck, 1979; Gray *et al.*, 1987). It is

now important to identify the amount and sites of phosphorylation in insoluble tau and compare these with those in soluble tau, to establish if tau insolubility is the direct result of abnormal phosphorylation and to determine if either or both of these altered properties confer upon tau the ability to form PHF *in vitro*.

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