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Expression of separate isoforms of human tau protein: correlation with the tau pattern in brain and effects on tubulin polymerization

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We have expressed six previously cloned isoforms of human microtubule-associated tau protein in *Escherichia coli* and purified them to homogeneity in a biologically active form. They range from 352 to 441 amino acids in length and differ from each other by the presence of three or four tandem repeats in the carboxy-terminal half and by the presence or absence of 29 or 58 amino acid inserts in the amino-terminus. When mixed together they gave a set of six bands on SDS-PAGE gels with apparent molecular weights of 48-67 kd and with a characteristic pattern of spacings. Four of these bands aligned with the major tau bands found in adult human cerebral cortex following perchloric acid extraction and alkaline phosphatase treatment. They consisted of isoforms with three repeats and no insertions, four repeats and no amino-terminal insertions and three- and four-repeat containing isoforms with the 29 amino acid insertion. In fetal human brain extracts treated with alkaline phosphatase one of the two major tau bands aligned with the three-repeat containing isoform with no insertions, whereas the molecular nature of the second major tau band remains to be established. The recombinant tau isoforms were biologically active at micromolar concentrations, as assessed by their ability to promote microtubule assembly. The rates of assembly were 2.5-3.0 times faster for isoforms containing four repeats when compared with three-repeat containing isoforms, with no significant contribution by the amino-terminal insertions.

Key words: alternative mRNA splicing/Alzheimer's disease/microtubule assembly/microtubule-associated proteins/tau protein

Introduction

Microtubule-associated protein tau promotes microtubule assembly and stability in the nervous system (Weingarten *et al.*, 1975; Cleveland *et al.*, 1977a,b; Horio and Hotani, 1986; Caceres and Kosik, 1990). Multiple isoforms are generated from a single gene through alternative RNA splicing, leading to the stage and cell type specific expression of different forms (Lee *et al.*, 1988; Goedert *et al.*, 1988, 1989a,b; Himmler, 1989; Himmler *et al.*, 1989; Kanai *et al.*, 1989; Kosik *et al.*, 1989; Mori *et al.*, 1989). The most striking feature of the primary structure of tau as predicted from molecular cloning is a stretch of 31 or 32 amino acids that is repeated three or four times in the carboxy-terminal half of the molecule. Experiments using tau fragments synthesized *in vitro* and synthetic peptides have shown that the repeats constitute microtubule binding units (Aizawa *et al.*, 1989; Ennulat *et al.*, 1989; Himmler *et al.*, 1989; Lee *et al.*, 1989). Additional isoforms exist which contain 29 or 58 amino acid insertions in the amino-terminal region in conjunction with three or four repeats, giving rise in humans to a total of six different isoforms identified from full-length cDNA clones (Goedert *et al.*, 1989b). The shortest human form is 352 amino acids in length and contains three repeats, whereas the largest form is 441 amino acids in length and contains four repeats and the 58 amino acid insertion. By RNase protection assay on human brain, mRNAs encoding three-repeat containing isoforms are found both in fetal and adult brain, whereas mRNAs encoding four-repeat containing isoforms and isoforms with amino-terminal insertions are found in adult but not in fetal brain (Goedert *et al.*, 1989b).

In normal cells tau protein is associated with axonal microtubules (Binder *et al.*, 1985), whereas in Alzheimer's disease it is immobilized in the somatodendritic compartment of certain nerve cells, where it forms part of the paired helical filament, the major constituent of the neurofibrillary tangle (Brion *et al.*, 1985; Delacourte and Défossez, 1986; Grundke-Iqbal *et al.*, 1986; Ihara *et al.*, 1986; Kosik *et al.*, 1986; Wood *et al.*, 1986). Protein sequencing and molecular cloning have shown that the repeat region of tau lies in the protease resistant core of the paired helical filament (Goedert *et al.*, 1988; Kondo *et al.*, 1988; Wischik *et al.*, 1988a,b).

Experiments with tau protein have mostly been performed with mixtures of isoforms extracted from brain microtubule preparations or with single isoform species electroeluted from polyacrylamide gels. In all cases these isoforms existed in ill defined states of phosphorylation and their primary amino acid sequences were not known. The cloning and sequencing of cDNAs encoding different tau protein isoforms has allowed us to express, isolate and purify six discrete species, from a bacterial expression system, in milligram quantities.

We demonstrate here that these recombinant isoforms are functional in promoting tubulin assembly and that there are marked differences in their relative potencies depending on the presence of three or four tandem repeats. Amino-terminal insertions make no contribution to tubulin assembly. We also show that the larger than predicted molecular size of tau protein on denaturing gels constitutes an intrinsic property of the protein and does not result from multiple post-translational modifications. Following alkaline phosphatase treatment the four major species of tau protein from adult human brain extracts were found to align with recombinant isoforms.

Results

To express six human tau isoforms site-directed mutagenesis was used to introduce a *Nde*I site in the context of the initiator

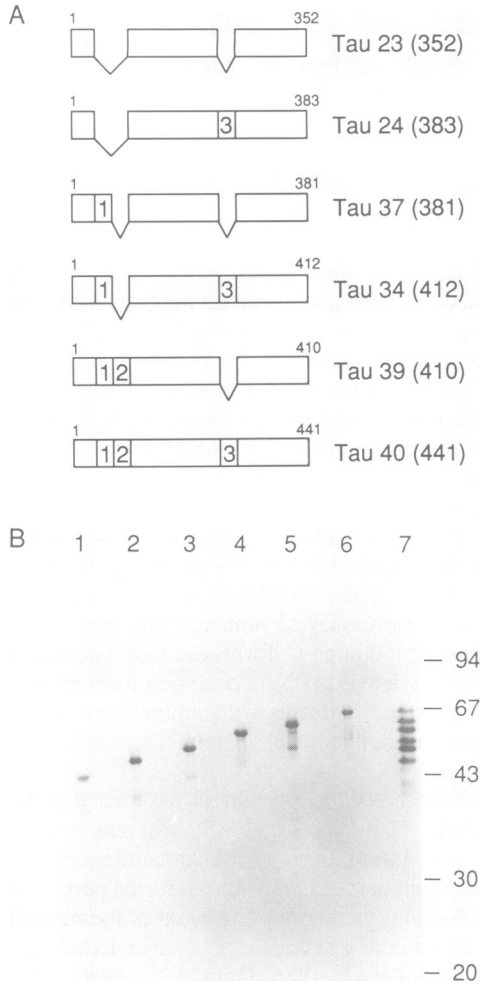

Fig. 1. Expression of six human tau isoforms in total cell protein from induced *E.coli* cultures. (**A**) Schematic representation of the six human tau isoforms arranged according to increasing apparent molecular weights. They differ from each other by the presence or absence of inserts 1–3 (Goedert *et al.*, 1989b). (**B**) Lane 1, 352 amino acid form; lane 2, 383 amino acid form; lane 3, 381 amino acid form; lane 4, 412 amino acid form; lane 5, 410 amino acid form; lane 6, 441 amino acid form; lane 7, all six isoforms mixed together. Following electrophoresis on a 10% SDS–PAGE gel, the tau isoforms were visualized by Western blotting.

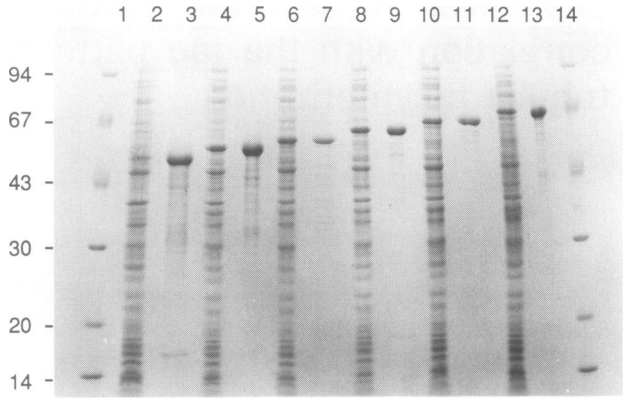

Fig. 2. SDS–PAGE of the purification of the expressed tau isoforms. Lanes 1 and 14, standard markers. Lanes 2, 4, 6, 8, 10 and 12, total cell protein from induced *E.coli* cultures expressing the 352, 383, 381, 412, 410 and 441 amino acid isoforms. Lanes 3, 5, 7, 9, 11 and 13, purified 352, 383, 381, 412, 410 and 441 amino acid isoforms. The proteins were run on a 10–20% gradient gel and stained using Coomassie blue.

Table I. Comparison between true and apparent molecular weights of recombinant tau isoforms

Clone name	Inserts 1 2 3	Length (amino acids)	True mol. wt	Apparent mol. wt
htau23	– – –	352	36 760	48 000
htau24	– – +	383	40 007	52 000
htau37	+ – –	381	39 720	54 000
htau34	+ – +	412	42 967	59 000
htau39	+ + –	410	42 603	62 000
htau40	+ + +	441	45 850	67 000

The six isoforms have been arranged according to increasing apparent molecular weights expressed in daltons, as judged from SDS–PAGE gels.

codon of full-length cDNA clones. The resulting *Nde*I–*Eco*RI fragments were subcloned downstream of the T7 RNA polymerase promoter in the expression vector pRK172. Expression was then induced with isopropyl-β-D-thiogalactoside (IPTG) and the tau protein detected by SDS–PAGE and identified by Western blotting (Figures 1 and 2). The expressed protein represented 5–10% of the total cellular protein and the estimated level of expression was of the order of 30–60 μg/ml cell culture. Tau isoforms were extracted and fractionated to 95% purity using column chromatography (Figure 2). The amino-terminal sequence of the different tau isoforms was determined and read as AEPRQEFEVMEDHA, indicating that the initiating methionine residue had been cleaved away. On SDS–PAGE gels the six tau isoforms ran with apparent molecular weights of 48 kd (three-repeat containing form), 52 kd (four-repeat containing form), 54 kd (three-repeat containing form with short amino-terminal insertion), 59 kd (four-repeat containing form with short amino-terminal insertion), 62 kd (three-repeat containing form with long amino-terminal insertion) and 67 kd (four-repeat containing form with long amino-terminal insertion). When mixed together a total of six distinct protein bands with a characteristic spacing pattern was obtained (Figure 1). The molecular weights of the different isoforms predicted from their sequences range from 37–46 kd (Table I), indicating that all tau isoforms run abnormally on SDS–PAGE gels. Of the three inserts, the two amino-terminal ones, each 29 amino acids long, produced a greater retardation than the extra repeat, 31 amino acids long (Table I). The pattern thus consists of a triplet of bands, corresponding to the three-repeat containing isoforms and an equivalent but displaced triplet of bands corresponding to the four-repeat containing isoforms.

The pattern of recombinant tau bands was compared with that of tau extracted from fetal human brain, as well as from normal adult cerebral cortex and from cerebral cortex of patients who had died with Alzheimer's disease. The extraction procedure made use of the known solubility of tau in perchloric acid (Lindwall and Cole, 1984b). Tau protein was prepared by homogenizing the tissues in perchloric acid and detected by Western blotting. In fetal brain tau ran as two closely spaced bands with apparent molecular weights of 54 and 56 kd (Figure 3). The tau patterns in cerebral cortex from a control patient and from

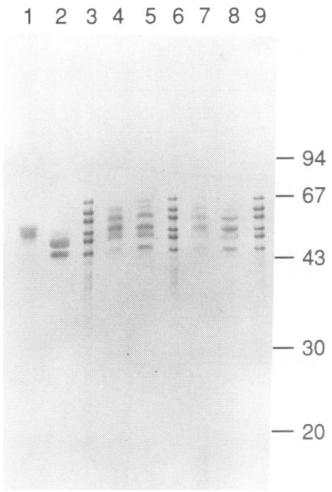

Fig. 3. Comparison of recombinant human tau isoforms with native human tau protein. Lanes 3, 6 and 9, mixture of recombinant isoforms; lanes 1 and 2, tau extracted from fetal brain before and after alkaline phosphatase treatment; lanes 4 and 5, tau extracted from adult control cerebral cortex before and after alkaline phosphatase treatment; lanes 7 and 8, tau extracted from Alzheimer's disease cerebral cortex before and after alkaline phosphatase treatment. Following electrophoresis on a 10% SDS−PAGE gel the tau isoforms were visualized by Western blotting.

a patient who had died with Alzheimer's disease were similar to each other and consisted of four major and two minor bands running with apparent molecular weights of 48−67 kd. Alkaline phosphatase treatment of fetal tau produced a downwards shift of the two bands into bands with apparent molecular weights of 48 kd and 50 kd, with the latter being stronger and broader than the former. In cerebral cortex from control patients and from Alzheimer's disease patients alkaline phosphatase treatment resulted in a stronger 48 kd molecular weight band (Figure 3). The intensities of the four bands of lowest apparent molecular weight were similar to one another but those of the two bands of highest apparent molecular weight were always weaker. No differences were observed between cerebral cortex from a control patient and from a patient who had died with Alzheimer's disease (Figure 3). Similar results were obtained using cerebral cortex from a further two control patients and a further two Alzheimer's disease patients. Control experiments had established that the alkaline phosphatase did not carry any protease activity.

The patterns of native tau following alkaline phosphatase treatment were compared with those given by the mixture of recombinant tau isoforms. The tau bands extracted from fetal and adult human brain were contained within the apparent molecular weight range (48−67 kd) of the recombinant isoforms. Of the two fetal tau bands the 48 kd band aligned with the recombinant three-repeat containing form, whereas the 50 kd band did not align with any of the recombinant tau isoforms (Figure 3). The four strong tau bands obtained from normal adult and Alzheimer's disease cerebral cortex aligned with four of the lowest recombinant tau isoform bands (Figure 3). These results suggest that the four major tau bands present in adult human brain following alkaline phosphatase treatment correspond to (with increasing apparent molecular weights): tau protein isoforms containing three repeats and no inserts, four repeats and no amino-terminal inserts, three repeats and the short amino-terminal insert and four repeats and the short amino-terminal insert.

Since tau protein was originally defined by its ability to promote tubulin assembly we compared three of the six recombinant isoforms for their ability to promote the polymerization of microtubules, as followed kinetically by turbidimetry (Figure 4). The isoforms tested promoted microtubule assembly at micromolar concentrations similar to those of native tau, indicating that the recombinant tau isoforms were fully biologically active. The four-repeat containing isoforms were found to promote microtubule polymerization at a rate 2.5−3.0 times faster than the isoform with three repeats (Figure 4). No difference was observed between four-repeat containing isoforms with or without the long amino-terminal insert (Figure 4).

Discussion

We have expressed six human tau protein isoforms in *E. coli* and purified them to homogeneity in a biologically active form. Two hours following induction with IPTG the expressed proteins represented 5−10% of the total cellular protein, with an estimated 30−60 μg tau protein/ml bacterial culture. Amino-terminal sequencing indicated that all six isoforms lacked the initiating methionine residue; this is in agreement with the known post-translational processing of *E. coli* proteins, in which the terminal methionine before an alanine is removed (Hirel *et al.*, 1989), but this is unlikely to affect the biological functions of the recombinant isoforms. When run on SDS−PAGE gels the tau isoforms gave a set of six characteristically spaced bands, ranging from 48 to 67 kd in apparent molecular weights. The pattern consisted of a triplet of bands corresponding to isoforms with three repeats and an equivalent but displaced triplet of bands corresponding to isoforms with four repeats. Ever since the cloning of the first tau isoforms (Goedert *et al.*, 1988; Lee *et al.*, 1988) it has been known that tau is anomalously retarded on denaturing gels, with the predicted molecular weights of the different isoforms ranging from 37 to 46 kd. This behaviour has been explained by the extended configuration of tau (Lee *et al.*, 1988; Lichtenberg *et al.*, 1988). The present results confirm and extend these findings by showing that the presence of amino-terminal inserts produces larger apparent molecular weight shifts than the presence of the extra repeat (~7 kd versus 5 kd). This may be related to the unusual amino acid composition of the inserts, which are rich in proline and charged amino acids.

The running pattern on SDS−PAGE gels of the recombinant isoforms was compared with that of tau protein extracted from fetal human brain and from normal adult cerebral cortex, as well as from cerebral cortex of patients who had died with Alzheimer's disease. Native tau was treated with alkaline phosphatase, since phosphorylation is a known post-translational modification of tau that can lead to a change in its mobility (Lindwall and Cole, 1984b). In fetal brain tau protein ran as two closely spaced bands with apparent molecular weights of 54 and 56 kd. It was shifted into two bands of 48 kd and 50 kd by alkaline phosphatase, with the former aligning with the recombinant tau isoform with three repeats. These findings are consistent with studies showing that fetal tau from various mammalian species runs as one broad band (Francon *et al.*, 1982; Couchie and Nunez, 1985) and indicate that perchloric acid soluble human fetal tau can be resolved into two bands and that it is probably

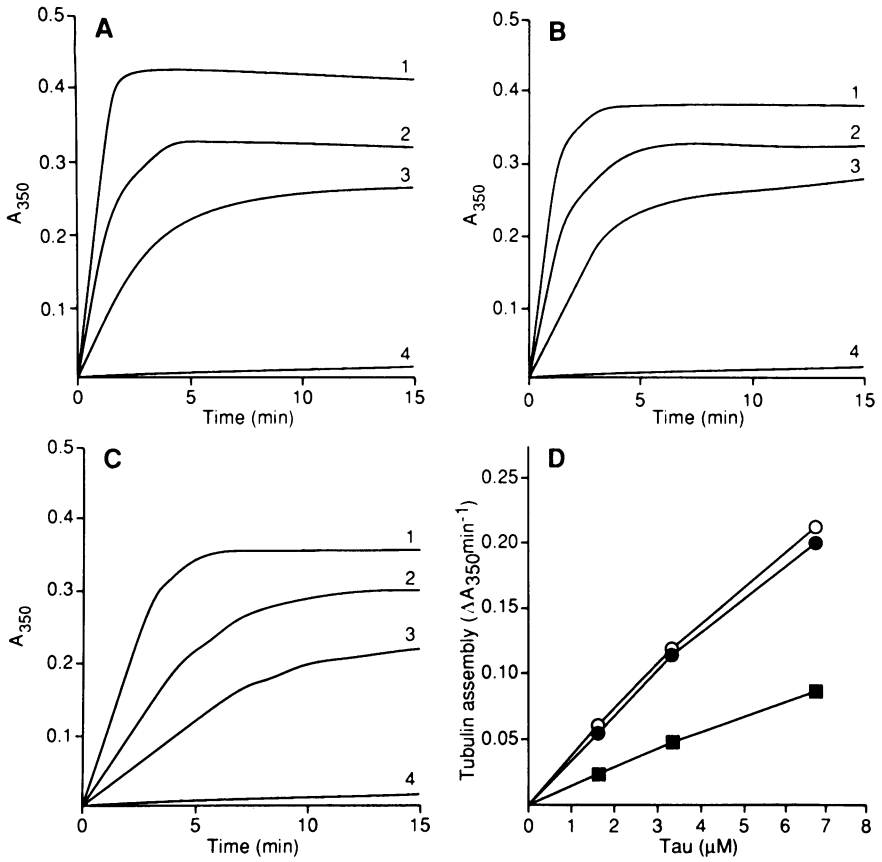

Fig. 4. Polymerization of tubulin induced by recombinant tau isoforms and monitored by turbidimetry. (**A**) Tau isoform with four repeats and no amino-terminal inserts (383 amino acid form). (**B**) Tau isoform with four repeats and long amino-terminal insert (441 amino acid form). (**C**) Tau isoform with three repeats and no inserts (352 amino acid form). The numbered curves correspond to the following tau concentrations: 1, 6.8 μM; 2, 3.4 μM; 3, 1.7 μM; 4, 0 μM. (**D**) The optical density after 1 min as plotted against the tau isoform concentration. ○, 383 amino acid form; ●, 441 amino acid form; ■, 352 amino acid form. A typical experiment is shown; similar results were obtained in four separate experiments.

totally phosphorylated. RNase protection assays have shown that transcripts encoding three-repeat containing tau isoforms are found in fetal brain (Goedert *et al.*, 1989a); this is consistent with the appearance of the 48 kd band following alkaline phosphatase treatment. At present, it is unclear whether the 50 kd band represents a three-repeat containing isoform of tau that carries a post-translational modification resistant to alkaline phosphatase or if it constitutes an isoform that has so far gone undetected by molecular cloning. In adult cerebral cortex, following alkaline phosphatase treatment, four major tau bands ranging from 48 to 59 kd were observed, in agreement with a previous study on human tau (Ksiezak-Reding *et al.*, 1988). The four tau bands aligned with four of the recombinant isoforms, namely the three- and four-repeat containing isoforms without amino-terminal insertions and the three- and four-repeat containing isoforms with the short amino-terminal insertion. These findings are in good agreement with results from RNase protection assays showing mRNAs encoding isoforms with three and four repeats and the short amino-terminal insertion to be much more abundant than mRNAs encoding isoforms with the long amino-terminal insertion (Goedert *et al.*, 1989b); bands aligning with the latter were also observed in some human samples, albeit at low levels. The present results thus indicate that the major isoforms of human brain tau protein can be accounted for by the expressed cDNA clones and that the regulation of tau synthesis occurs through the control of alternative mRNA splicing. A study that compared the patterns of *in vitro* translated mRNAs transcribed from clones encoding various bovine tau isoforms with those of tau extracted from bovine brain concluded that the major bovine isoforms contain three and four repeats in conjunction with short or long amino-terminal insertions (Himmler, 1989). It therefore would appear that there exists a species difference between cow and man with respect to the nature of the major tau isoforms.

The pattern of tau bands in cerebral cortex from patients with Alzheimer's disease was indistinguishable from that observed in cerebral cortex from control patients either before or after alkaline phosphatase treatment. It has been reported that there are abnormally phosphorylated forms of tau that lead to a shift in mobility in brain tissue from patients with Alzheimer's disease (Flament and Delacourte, 1989). Using our extraction procedure we have not been able to observe any difference in the pattern of tau bands when comparing control cerebral cortex with Alzheimer's disease cerebral cortex before or after alkaline phosphatase treatment. This difference can be accounted for by the differential solubility of abnormally phosphorylated tau under different extraction conditions.

The development of the vertebrate nervous system is characterized by a change in expression of several microtubule-associated proteins from early to late forms, with alternative mRNA splicing representing the major mechanism underlying these changes (Matus, 1990). Tau constitutes no exception in that the three-repeat containing

isoform with no insertions is the major fetal form, whereas isoforms with four repeats and amino-terminal insertions are adult-specific (Goedert *et al.*, 1989a,b). To assess a possible functional significance of this developmentally regulated expression we have compared the abilities of recombinant tau isoforms with three and four repeats to promote microtubule assembly. The four-repeat containing isoforms promoted microtubule assembly at a rate 2.5- to 3.0-fold faster than the three-repeat containing isoform, whereas the amino-terminal insertions did not appear to contribute towards assembly. This suggests that adult tau isoforms are more potent in promoting microtubule assembly than the isoform expressed in fetal brain. This difference is probably even greater *in vivo*, as fetal tau appears to be more heavily phosphorylated than adult tau; phosphorylation of tau has been shown to reduce its ability to promote microtubule assembly (Lindwall and Cole, 1984a).

Tau isoforms with three repeats are also found in the adult nervous system; *in situ* hybridization studies on human hippocampus have shown that mRNAs encoding three-repeat containing isoforms are found in granule and pyramidal cells, whereas mRNAs encoding four-repeat containing isoforms are only found in pyramidal cells (Goedert *et al.*, 1989a). This implies that in the adult nervous system there are likely to exist different degrees of microtubule polymerization in different cell types and that such differences may also be found at the level of microtubule assembly in individual nerve cells.

Materials and methods

Expression constructs

Full-length cDNA clones encoding six human tau protein isoforms (Goedert *et al.*, 1989b) were subcloned into the *Eco*RI site of M13mp18. Site-directed mutagenesis was used to introduce a *Nde*I site in the context of the initiator codon. Following cleavage with *Nde*I and *Eco*RI the resulting cDNA fragments were subcloned downstream of the T7 RNA polymerase promoter into *Nde*I/*Eco*RI cut expression plasmid pRK172 (McLeod *et al.*, 1987) and the recombinant plasmids were transformed into *E.coli* BL21 (DE3) cells (Studier *et al.*, 1990).

Expression and purification of tau protein isoforms

The *E.coli* cells were grown to an optical density of 0.6–1.0 at 600 nm. Expression was induced by adding IPTG to a final concentration of 0.4 mM. After shaking for 2 h at 37°C the cells were collected by centrifugation. Expression was checked by resuspending the pellet from 1 ml culture in 100 μl sample buffer (Laemmli, 1970), followed by a 20 s sonication using a MSE Soniprep 150 at full power. One μl of the supernatant was run on a 10% SDS–PAGE gel; the gel was blotted and the tau bands were visualized using the anti-human tau monoclonal antibody 7/51 (Bondareff *et al.*, 1990).

The large-scale purification of tau isoforms was peformed either using a combination of ammonium sulphate precipitation and ion exchange chromatography (C.Scott and D.P.Blowes, in preparation), or using the following protocol. Frozen *E.coli* pellets were suspended in 30 ml 50 mM PIPES, 1 mM dithiothreitol (DTT) pH 6.8, (10 ml/g pellet) and sonicated 2 × 5 min on ice using a Kontes Micro Ultrasonic Cell Disrupter. The homogenate was centrifuged at 15 000 *g* for 20 min and the resultant supernatant filtered through a 0.45 μm Acrodisc. The filtrate was loaded onto a phosphocellulose packed column (bed volume 3 ml) equilibrated in extraction buffer. After exhaustive washing with the same buffer protein was eluted batchwise with 3 ml aliquots of extraction buffer containing 0.5 M NaCl. Fractions 2 and 3, which contained the recombinant tau isoforms, were pooled and dialysed overnight against 50 mM MES/1 mM DTT, pH 6.25. After centrifugation the dialysate was loaded onto a fast flow carboxymethyl-Sepharose HR 5/5 column (Pharmacia). The column was washed with 50 mM MES, 1 mM DTT, 50 mM NaCl, pH 6.25, and the protein was eluted using a 100–300 mM NaCl in 50 mM MES, 1 mM DTT, pH 6.25, gradient. Column fractions were screened by gel electrophoresis, the peak tau fractions were pooled, dialysed against 25 mM MES/1 mM DTT, pH 6.25, and freeze-dried. The freeze-dried material was reconstituted in water at a nominal concentration of 5 mg/ml and centrifuged at 10 000 *g* for 10 min. The supernatant was then aliquoted and stored at −20°C until use. The protein concentrations were measured by amino acid composition and the amino-terminal sequences of the various isoforms checked as described (Matsudaira, 1987). Typically 10–12 mg purified recombinant tau protein was recovered from 1 l bacterial culture.

Extraction of tau protein from human brain

The frontal cortex was used from three control patients who had died with no neurological or psychiatric disorders (ages 63, 72 and 79 years) and from three patients who had died with a histologically confirmed diagnosis of Alzheimer's disease (ages 65, 70 and 74 years). Whole brain was from a 15 week old human fetus. All human tissues were frozen <7 h after death. Frozen brain tissue (200 mg) was thawed in 0.5 ml 2.5% perchloric acid (Lindwall and Cole, 1984b), dounce homogenized, allowed to stand on ice for 20 min and centrifuged for 10 min at 10 000 *g*. The supernatant was dialysed overnight at 4°C against 5% formic acid and recentrifuged. The resulting supernatant was concentrated for Western blot analysis using a speed vacuum apparatus.

Alkaline phosphatase treatment was performed by incubating the tau fractions overnight at 37°C in 50 mM Tris, 5 mM $MgCl_2$, pH 8.3, containing calf intestinal alkaline phosphatase (Boehringer) at a concentration of 400 U/ml. Recombinant tau isoforms were run in parallel under identical conditions to test for endogenous proteolytic activity.

Microtubule assembly assay

Tubulin was purified by phosphocellulose ion exchange chromatography of microtubule protein prepared from pig brain using a modification of the temperature cycle method (Mandelkow *et al.*, 1985). Polymerization of phosphocellulose column-purified tubulin was carried out in 500 μl reassembly buffer (100 mM PIPES, 1 mM $MgSO_4$, 2 mM EGTA, 1 mM DTT, pH 6.9) containing 1 mM GTP, 2 mg/ml tubulin and various concentrations of recombinant tau. The time-course of tubulin polymerization was followed over 15 min at 37°C by the change in turbidity at 350 nm using a Philips PU 8740 UV/VIS scanning spectrophotometer.

Other methods

SDS–PAGE (Laemmli, 1970) was carried out using 10% or 10–20% gradient minigels (Matsudaira and Burgess, 1978). For Western blotting the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore) at 150 mA for 1 h. Residual protein binding sites were blocked with 1% gelatin in phosphate-buffered saline (PBS). The blots were then incubated for 5 h at room temperature with the anti-human tau monoclonal antibody 7/51 (Bondareff *et al.*, 1990). Bound antibody was detected by the peroxidase technique.

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