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Mutation-dependent aggregation of tau protein and its selective depletion from the soluble fraction in brain of P301L FTDP-17 patients

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Mutations in the gene for the microtubule-associated protein tau are associated with frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17). In this study we compared the presence of the P301L mutated tau protein from brain material of patients with that of the normal 4-repeat, using polyclonal antibodies specific for the P301L point mutation and its normal counterpart. We determined the relative ratio of mutated versus normal tau protein in the sarkosyl-soluble and -insoluble protein fractions from several brain regions. Although mutated and normal tau proteins are both present in the sarkosyl-insoluble deposits, quantitative analysis showed that the mutated protein is the major component. In the sarkosyl-soluble fraction of frontal and temporal cortex the overall ratio of 3-repeat versus 4-repeat tau isoforms is unchanged but there is a dramatic depletion of mutant tau protein. Furthermore, we observed an increase in tau-immunoreactive cleavage products with the P301L antibody, suggesting that the mutant protein is partly resistant to degradation and this is confirmed by pulse–chase experiments. This is the first direct evidence using patient material that shows a selective aggregation of mutant tau protein resulting in sarkosyl-insoluble deposits and the specific depletion of mutated tau protein in the soluble fraction.

INTRODUCTION

The term frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), covers a number of neurodegenerative syndromes with diverse but overlapping clinical and neuropathological features. Early behavioral changes, later accompanied by cognitive and motor disturbances, and often by atrophy in the frontal and temporal lobes on neuroimaging, are characteristic for this condition (1,2). Pathological hallmarks of most FTDP-17 brains are insoluble filamentous aggregates of hyperphosphorylated protein tau, in neurons or in both neurons and glial cells, similar to those found in other neurodegenerative disorders such as Alzheimer's disease (AD), Pick' s disease, progressive supranuclear palsy, corticobasal degeneration and amyothrophic lateral sclerosis (ALS), Parkinsonism-dementia complex of Guam (3).

Genetic analysis of FTDP-17 has revealed that the disorder is caused by mutations in the gene for the microtubule-associated protein tau (4–6). The tau protein is encoded by a single gene but, due to differential RNA splicing, is expressed in the adult human brain in six isoforms. These isoforms differ by the presence of three or four imperfect repeats in the C-terminus, which are part of the microtubule-binding region and by the presence or absence of two N-terminal inserts of 29 and 58 amino acids (7).

The tau protein regulates the dynamic stability of the neuronal cytoskeleton and plays an important role in neuronal differentiation and axonal development (8) through its ability to promote microtubule assembly. Moreover, tau protein is involved in positioning of cell organelles, axonal transport and the maintenance of neuron polarity (9–12).

The mutations identified in the *tau* gene are localized both in the coding and non-coding sequences of the gene (13). The intronic mutations are all located close to the 5′ splice-donor site of the intron following exon 10. In addition, several mutations within exon 10 have been shown to cause a shift in the normal splicing ratio of transcripts containing exon 10, mostly resulting in an increased ratio of 4-repeat (4R) with respect to 3-repeat (3R) tau (4,6,14–16).

The missense mutations and a small deletion are localized in the C-terminal part of the protein containing the microtubule-binding domains and are likely to alter tau's interactions with tubulin. The mutations in exon 10 only affect the 4R tau isoforms, but other mutations affect both 3R and 4R tau isoforms.

The P301L mutation in FTDP-17 kindreds has been most extensively described (4,17–21). Perinuclear deposits of hyperphosphorylated tau are seen in neurons, glial cells and neurites of frontal and temporal cortex, hippocampal formation and substantia nigra in brains of P301L patients. The deposits consist of slender twisted filaments 15 nm wide with variable

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periodicity and a few straight filaments (22). Tau extracted from these filaments appears on immunoblot as two major bands of 64 and 68 kDa and a minor band of 72 kDa, that after alkaline phosphatase treatment can be resolved in two 4R (4R, 4R + 28 amino acids) and one 3R tau isoforms (3R + 28 amino acids).

The proline residue at codon 301 is part of a highly evolutionary conserved PGGG motif that is found in all microtubule-binding repeats and is located in the microtubule–binding repeat specific for 4R tau. Several studies (15,16,23,24) have been performed to investigate the possible effect of the tau mutations on microtubule polymerization and binding and the results indeed showed impairment in these parameters.

Tau filaments, similar to those found in brains of patients, can be generated *in vitro* as well. 4R tau aggregates more readily than 3R tau and, compared with this, tau with missense mutations aggregates even faster. Results from three independent studies demonstrate that the P301L mutation has a high potential for fibril formation (25–27). Until now, however, it has not been shown that the P301L mutated tau protein has these properties *in vivo*.

For the present study, we developed specific rabbit polyclonal antibodies raised against the P301L point mutation and its normal counterpart and showed that although both normal 4R and mutated P301L tau are present in the insoluble deposits of the cerebral cortex, the main component of these deposits is the mutated tau protein. Conversely, in the soluble fraction we observed a strong reduction in the level of mutated P301L protein, especially in the frontal cortex. These findings suggest that the low level of mutated protein in the soluble fraction is due to a selective depletion, resulting from the selective aggregation of mutant protein in the sarkosyl-insoluble deposits. In addition, we observed an increase of tau-immunoreactive cleavage products for the mutant protein compared with the normal protein, strongly suggesting that an inappropriate digestion of the mutated protein plays an important role in the pathogenesis of FTDP-17 as well.

RESULTS

Characterization of the antibodies tau-P301 and tau-P301L

To determine the specificity of tau-P301 and tau-P301L antibodies, transient and stable transfection experiments were performed using COS and PC12 cell lines. Immunocytochemical (Fig. 1) and immunoblot (Fig. 2A and B) analyses showed that the tau-P301 and tau-P301L antibodies specifically recognize the normal 4R tau or the P301L mutant protein, respectively.

In order to compare the strength of the tau-P301 and tau-P301L antibodies, both normal and mutant P301L tau (longest isoform), were synthesized by *in vitro* transcription and translation in the presence of [^{35}S]methionine. Identical amounts of both tau proteins were loaded on SDS–polyacrylamide gel, blotted (Fig. 2C) and incubated with tau-P301 and tau-P301L antibodies (using the same dilutions), respectively (Fig. 2D). Quantitative analysis showed that the ratio tau-P301L:tau-P301 was 0.86; thus, no significant difference was found in the binding of the two antibodies to their respective epitopes, making them suitable for quantitative western blots studies.

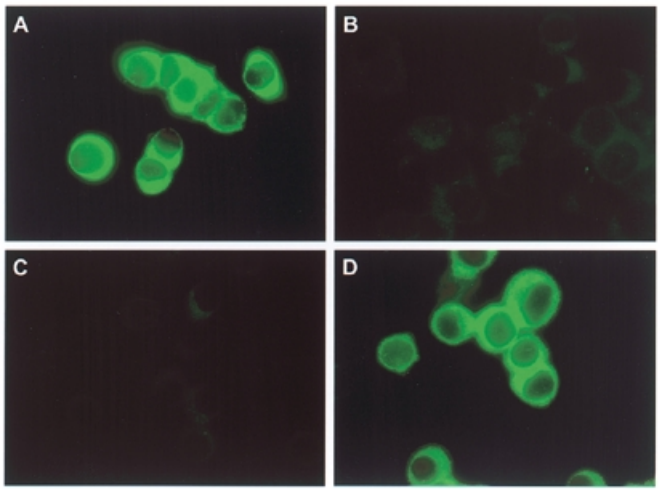


Figure 1. PC12 stably transfected cells with normal 4R (**A** and **C**) and mutant P301L tau (**B** and **D**) were stained with tau-P301 antibody (A and B) and tau-P301L antibody (C and D).

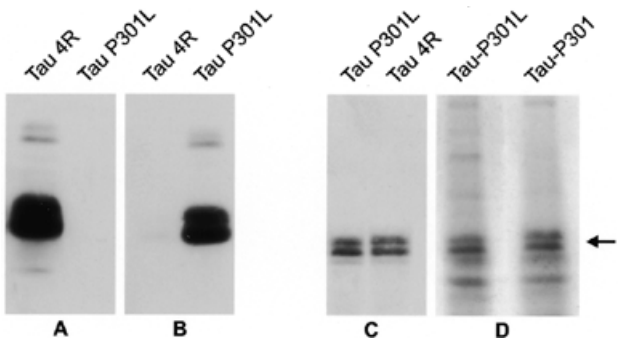


Figure 2. Immunoblot of COS cells transfected with normal 4R and mutated P301L tau and incubated with tau-P301 (**A**) and tau-P301L (**B**) antibodies. (**C**) Autoradiograph of SDS–PAGE gel showing P301L mutated and normal 4R tau proteins synthesized by *in vitro* transcription and translation in the presence of [^{35}S]methionine. The two bands seen in each lane might be the result of an additional translation initiation due to the presence of a second methionine 10 amino acids downstream from the first. (**D**) Immunoblot of *in vitro* synthesized P301L mutated and normal 4R tau proteins, detected with tau-P301L and tauP301 antibodies, respectively. The bands indicated by an arrow were quantified to compare the strength of both antibodies.

Histochemical analysis of mutated and normal tau proteins

In patients carrying the P301L mutation, the tau-P301 and the tau-P301L antibodies gave a similar staining patterns in brain slides from the cerebral cortex and the hippocampus. They recognized especially the aggregates in the superficial layers 2–3 and deep layer 6 of the temporal cortex (Fig. 3A) and to a lesser extent aggregates in the frontal and entorhinal cortex. These aggregates were mainly located in the perinuclear region, cell body and sometimes extending to the apical dendrites of neurons. In one patient occasionally a diffuse staining with both antibodies was observed in the axons. Tau-

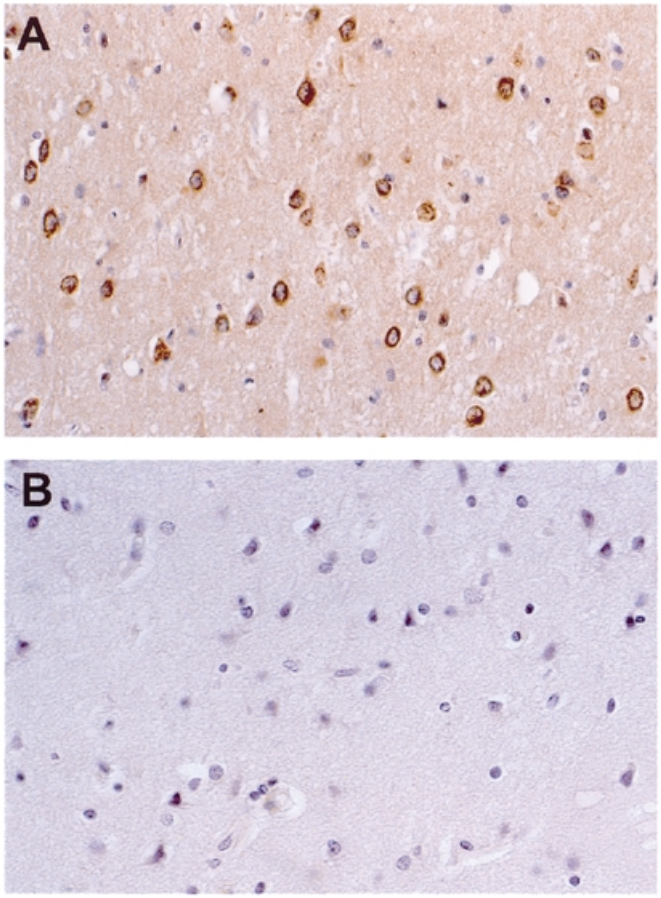


Figure 3. Sections of the temporal cortex of P301L patient 3 (**A**) and an AD patient (**B**) stained with tau-P301L antibody. Magnification, ×40.

P301L antibody did not show any signal in AD brain (Fig. 3B), non-demented control and R406W cases.

Biochemical analysis of mutated and normal tau proteins

Sarkosyl-insoluble tau, isolated from frontal cortex, temporal cortex and cerebellum of three patients carrying the P301L mutation, one AD patient and one disinhibition dementia Parkinsonism amyotrophy complex (DDPAC) patient, was run on an SDS–PAGE gel and immunoblotted with the antibodies H-7, tau-P301 and tau-P301L.

With H-7 and tau-P301 antibodies, three major bands (60, 64 and 68 kDa) were seen in the AD patient and two bands (64 and 68 kDa) in the DDPAC patient. These brains did not show any signal with the tau-P301L antibody, as expected (Fig. 4A).

In the material extracted from frontal cortex of the P301L patients, all three antibodies recognized two major bands of 64 and 68 kDa and a minor band of 72 kDa, with the 68 kDa band being the strongest (Fig. 4A). After alkaline phosphatase treatment of the material from P301L patients and staining with H-7 antibody, the insoluble tau was resolved into four bands corresponding to the 4R, 3R + 29 amino acids, 4R + 29 amino acids and 4R + 58 amino acids isoforms (Fig. 4B). The bands corresponding to the 4R and to the 4R + 29 amino acids appeared to be the strongest.

Quantitative analysis was used to determine the relative amount of mutated tau-P301L protein in the insoluble deposits for all the three patients with the P301L mutation (Fig. 4C). The P301L mutated tau protein was found to be on average four times more abundant than the normal 4R tau.

These results show that in the sarkosyl-insoluble deposits of the frontal cortex both normal 4R and mutated P301L tau protein are present, but that 4R protein consists mainly of mutated P301L protein.

Similar experiments performed on the sarkosyl-insoluble tau isolated from the temporal cortex showed that also in tau deposits of the temporal lobe the mutated tau is the major 4R tau component. The sarkosyl-insoluble fraction from the relatively unaffected cerebellum of P301L patients did not show the presence of hyperphosphorylated tau protein, as expected (data not shown).

Sarkosyl-soluble tau from the frontal, temporal cortex and cerebellum of the three P301L patients, stained with the H-7 antibody, showed a pattern similar to that of the control case (Fig. 5): six bands aligned with the six isoforms from the recombinant tau. Staining with tau-P301 and tau-P301L antibodies (Fig. 6A–C) resolved this sarkosyl-soluble dephosphorylated tau into two major bands corresponding to 4R and 4R + 29 amino acids isoforms. A quantitative analysis was performed to compare the 3R:4R ratio in the soluble fractions among the P301L cases and the age-matched control. No significant differences were observed in all cases, the ratio was ~1 in the frontal cortex. A single patient showed a decrease of 20% for 4R tau, similar to what has been described previously (16). The cerebellum of the FTDP-17 cases and the control contained more 4R tau than 3R, again in agreement with Hong *et al.* (16).

The same experiment was repeated to determine the ratio between mutated P301L and normal 4R tau (Fig. 6D) and a striking decrease of mutated P301L protein was observed in the soluble fraction from the frontal cortex where the amount of the normal 4R tau was on average 5-fold higher than the mutated tau. In the temporal cortex the difference was less strong, showing a 2- to 5-fold decrease of mutated P301L tau compared with the normal 4R. In the non-affected area of the cerebellum a 2-fold decrease was observed for the P301L protein with respect to the normal 4R tau.

Below 45 kDa, two bands were detected with both the tau-P301 and tau-P301L antibodies but their intensity was significantly stronger with the tau-P301L antibody. Since we carefully matched the total amount of protein loaded on the gel for each sample and we have showed that both antibodies have similar strength, it is unlikely that the differences in the intensity of the two bands seen with the two antibodies are caused by proteolysis in the experimental procedure, suggesting therefore an alteration of the proteolytic processing of the mutated P301L protein compared with the normal protein.

Therefore, we performed pulse–chase experiments and determined the half-life of normal 4R tau and its mutant P301L counterpart in transiently transfected COS cells. Newly synthesized tau proteins were harvested at different time points (0, 6, 10 and 22 h) and immunoprecipitated with H-7 monoclonal antibody. After 22 h only little amounts of normal 4R (4.9 ± 2.4%) and P301L (6.6 ± 3%) tau proteins were present. However, after 10 h of chase a significant delay in the degradation of P301L tau (40 ± 13%) compared with the normal 4R (67 ± 1.75%) was observed (Fig. 7).

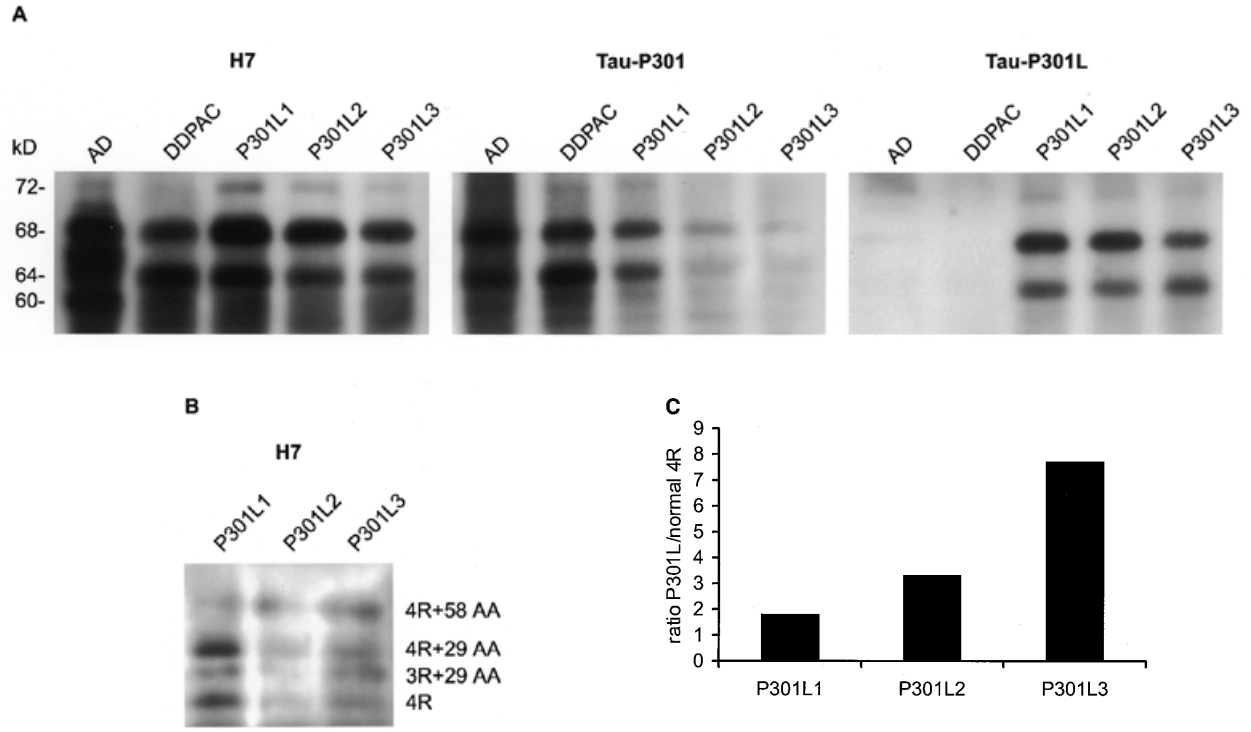


Figure 4. (**A**) Immunoblot of sarkosyl-insoluble tau from the frontal cortex of one AD, one DDPAC and three FTDP-17 patients carrying the P301L mutation (P301L1, P301L2 and P301L3), using the phosphorylation independent antibody H-7, the tau-P301 and the tau-P301L antibodies. (**B**) Immunoblot of sarkosyl-insoluble tau from the frontal cortex of the three P301L patients after alkaline phosphatase treatment, stained with the phosphorylation-independent antibody H-7. (**C**) Ratio between the relative amounts of mutated P301L protein and normal 4R tau in the sarkosyl-insoluble deposits from the frontal lobe.

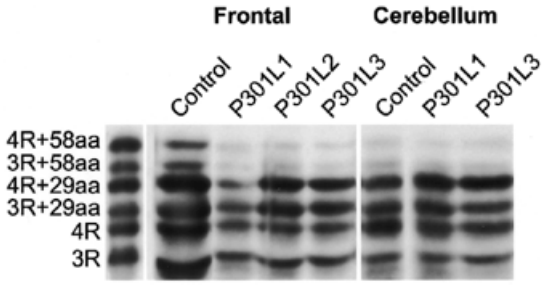


Figure 5. Quantitative immunoblot of sarkosyl-soluble tau from frontal cortex of one control and three P301L patients and cerebellum from two P301L patients and one control. Sarkosyl-soluble tau was dephosphorylated and stained with the H-7 antibody. Six bands were obtained that aligned with the six isoforms of the recombinant tau.

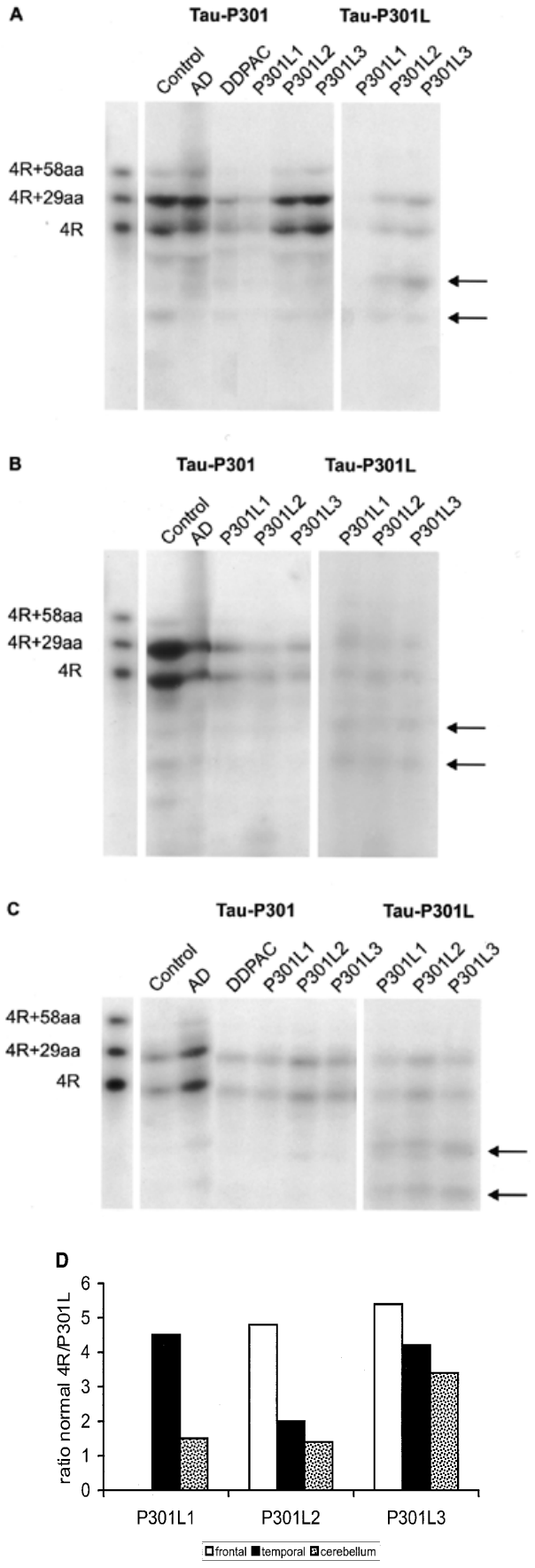
DISCUSSION

The mutations found in the gene for the microtubule-associated protein tau in FTDP-17 patients have provided us with new opportunities to study the role of tau protein in neurodegenerative disorders characterized by tau pathology. Several *in vitro* studies have demonstrated that most missense mutations result in impaired binding and microtubule polymerization capacities compared with the normal 4R protein (15–17,23,24), suggesting a possible role for mutated tau protein in the neurodegeneration process through a progressive disruption of microtubule function and stability. However, in a recent study (24) cells transiently transfected with wild-type or mutated tau constructs could not be distinguished in terms of tau-decorated microtubule networks and bundles, suggesting that mutations do not have a large and immediate effect on the interactions between tau and microtubules.

More recently, *in vitro* studies demonstrated that most missense mutations also increase tau's potential for the formation of fibrils, both at the nucleation and the elongation phases and this 'gain-of-function' of mutant tau might explain the observed aggregation of tau protein in brain.

For a better understanding of the role of the mutated protein in the pathogenesis of the disease we studied the localization and levels of the P301L and normal 4R tau proteins in brain material from patients with P301L mutation using rabbit polyclonal antibodies specifically directed against the P301L mutation and its normal 4R counterpart.

Our immunohistochemistry experiments clearly show the presence of both mutated and normal 4R tau protein in the perinuclear deposits in the affected areas of frontal and temporal cortex and hippocampal formations. No clear differences were observed in the cellular localization of the mutated P301L protein versus the normal 4R tau. The diffuse staining seen occasionally in the axons with both antibodies, suggests that both normal and mutated protein are present in the axons. This finding suggests that the P301L mutation does not result in the absence of mutated protein from the axon, which can be



explained by the observation that tau mRNA is selectively translated in the axons of neurons. The fact that the axonal staining is seen only in one patient might reflect the limits of using these antibodies on paraffin-embedded material.

In our biochemical experiments, we show that the aggregates, in patients with the P301L mutation, mainly consist of mutant 4R tau and that only small amounts of normal 4R and 3R proteins are present. This preferential incorporation of mutated protein is, however, not reflected in the overall ratio of 3R:4R tau protein in the soluble fraction of frontal and temporal lobe and cerebellum. The 3R:4R tau ratio remained unchanged in two of the P301L patients with respect to the age-matched control and one patient showed only a 20% decrease of 4R tau. FTDP-17 is an autosomal dominant disorder and, therefore, the P301L protein in patients is produced by a single allele. Assuming that the normal ratio of 3R:4R tau is ~1, a 2-fold change in ratio of 3R:4R protein would be expected if all P301L proteins were effectively incorporated in aggregates or selectively degraded. The fact that this change in 3R:4R ratio is not observed suggests that upregulation of the expression level of the normal 4R tau isoform might compensate for the depletion of mutant 4R tau. However, to determine whether our finding is a general finding for the P301L mutation more patient material should be investigated.

Using the tau-P301L and tau-P301 antibodies, we demonstrate that in the soluble fraction the 4R tau mainly consists of normal protein and the P301L protein is almost not present. The most notable depletion of the mutant P301L protein with respect to the normal 4R protein was observed in the frontal lobe (~5-fold) and temporal lobe (on average 3-fold), the most affected areas of the brain. In cerebellum, where no tau aggregates were observed, the depletion of mutant protein was the smallest (2-fold).

The observation that P301L protein is selectively trapped in the insoluble deposits of the frontal and temporal cortices and the finding that tau protein with the P301L mutation has a high potential to form fibrils *in vitro*, provides an attractive explanation for the formation of the characteristic tau aggregates in brains of patients. However, we then would expect that during the period of 40–60 years before the death of the patient the selective trapping of the mutated protein from the soluble tau fraction into insoluble aggregates would lead to extensive deposit formation in all neurons. Instead, insoluble tau aggregates are visible only in a small subset of neurons and in specific areas of the brain. So other factors must be involved as well.

Our finding that the immunoblots of the soluble tau material show two bands of <45 kDa might point towards an alternative explanation. These two bands are strongly stained with the

Figure 6. Quantitative immunoblot of sarkosyl-soluble tau from the frontal cortex (**A**), the temporal cortex (**B**) and the cerebellum (**C**) of one control case and three P301L patients, using the tau-P301 and tau-P301L antibodies after alkaline phosphatase treatment. The three isoforms containing 4R + 0, + 29 and + 58 amino acids, respectively, aligned with the corresponding isoforms of the recombinant tau. The two intermediate cleavage products, <45 kDa, are indicated by arrows. (**D**). Ratio between normal 4R and mutated P301L protein in the soluble fraction from frontal cortex, temporal cortex and cerebellum. The amount of mutated P301L tau in the soluble fraction of frontal cortex in P301L1 patient was below detection level.

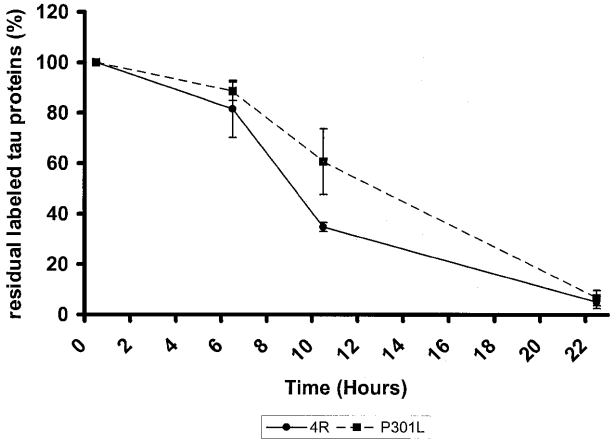


Figure 7. Pulse–chase experiments on COS cells transfected with normal 4R and P301L tau. Cells labeled with [^{35}S]methionine were incubated in unlabeled chase medium for intervals up to 22 h and harvested at time points indicated. NT, non-transfected COS cells used as negative control. The amount of residual ^{35}S-labeled 4R and P301L tau proteins in COS cells are expressed as percentage of the label measured immediately after pulse labeling (0 h).

antibody specific for the point mutation and only weakly with the tau-P301 that recognizes the normal 4R. They are present in the frontal and temporal cortex as well as in the cerebellum, an area that does not show any deposits. These bands may be intermediate fragments generated during the normal proteolysis of the mutated protein. Tau is sensitive to calpain and caspase proteolysis in neurons (28), resulting in the generation of multiple fragments, the major fragment being 42 kDa in size. The relative increase in mutant tau positive cleavage products might suggest that the mutant protein is more resistant to further calpain degradation with respect to the normal protein and this resistance might cause accumulation of intermediate proteolysis products. Yen *et al.* (29), using an *in vitro* assay, indeed reported that the P301L protein is more resistant to calpain degradation than the normal 4R tau. Our pulse–chase experiment showed that there is a delay in the degradation of mutated P301L protein in COS cells. Although our data are based on overexpression studies, the finding that there is a delay in the degradation of the mutated P301L tau protein compared with the normal 4R under the same experimental conditions suggests a potential mechanism by which the mutated protein accumulates in the cytoplasm at higher concentrations.

It is tempting to speculate that due to the presence of mutated protein and the accumulation of intermediate cleavage products, cells are more vulnerable to stress agents associated with aging, which in time might trigger alterations and modifications in the proteolytic degradation of the mutated tau versus the normal protein. Over time the combined effects of reduced microtubule assemblage and accelerated filament formation characteristic of the P301L mutation might lead to an increase of unbound cytosolic mutated P301L in the form of aggregates.

The question arises of whether for FTDP-17, as for many other neurodegenerative disorders such as AD, prion diseases, ALS and the polyglutamine repeat disorders, the aggregates are the pathogenic agents or simply markers of the cell's demise. For polyglutamine disorders evidence is emerging that inhibiting the aggregation of the mutated protein will not prevent disease (30) but instead accelerates neurodegeneration. Moreover, in a recent paper, Passani *et al.* (31) observed that modification of mutant huntingtin in target neurons promotes an abnormal interaction with one or all of huntingtin's WW domain partners, with toxic consequences, demonstrating that aberrant protein interactions play an important role in the pathogenesis of Huntington's disease.

In this study we presented evidence that mutant tau protein not only is deposited in insoluble aggregates but must for the major part be partially digested by the cell. Detailed research into the processes that result from the presence of mutated tau, including factors that are involved in tau proteolysis and the formation of aggregates, will help us to understand the critical events that lead to neurodegeneration.

MATERIALS AND METHODS

Patient materials

Paraffin-embedded sections of frontal, temporal and parietal cortices, hippocampus and substantia nigra from three FTDP-17 patients with P301L mutation, (64, 66 and 73 years) (18,22,32), two AD, two non-demented controls and one FTDP-17 patient with an R406W mutation (18) were used for immunohistochemistry. Fresh frozen tissue from frontal and temporal cortices and cerebellum of three P301L patients (60, 63 and 73 years), one AD case, one DDPAC (E10 + 14 intronic mutation in tau) (4) and 1 non-demented control were used for biochemical studies. The ages of the non-demented patients, the AD, DDPAC and R406W FTD patients were matched with the age of the P301L patients.

Antibodies

The following anti-tau antibodies were used: mouse monoclonal H-7 and AT8, rabbit polyclonal tau-P301 and tau-P301L. H-7 is a phosphorylation-independent antibody which epitope maps at amino acids 157–163 (33) (Innogenetics, Gent, Belgium). AT8 recognizes human tau isoforms when phosphorylated at Ser202 and Thr205 (34) (Innogenetics). Two rabbit polyclonal antibodies were generated in our laboratory using synthetic peptides encoding amino acids 291–305, numbered according to the longest tau isoform: CGSKDNIKHVPGGGS, for tau-P301 and CGSKDNIKHVLGGGS for tau-P301L.

Immunohistochemistry

For immunohistochemistry experiments paraffin sections were incubated with the polyclonal tau-P301 and tau-P301L antibodies (1:2000 dilution). After endogenous peroxidase inhibition (35), the sections were subjected to antigen retrieval treatment using a microwave oven. (35). Primary antibodies were incubated at 4°C overnight and visualized with an indirect immunoperoxidase technique utilizing the Histostain–Plus (Zymed, San Francisco, CA).

As a test for labeling specificity, primary antibodies were absorbed with their respective peptides as described by Hoogeveen *et al.* (36). Background labeling was negligible. Staining with the AT8 antibody was carried out as described (22).

Plasmids and constructs

Site-directed mutagenesis on a cDNA clone expressing the longest tau isoform was used to change P301 to a leucine in the longest tau isoform, with the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutagenesis was performed according to the manufacturer's instructions. DNA was isolated from the wild-type clone and its mutant, verified by sequencing using the BigDye Terminator Cycle sequencing kit (PE Biosystems, Foster City, CA) on an ABI 377 automated sequencer (PE Biosystems) and the tau cDNA was sub-cloned into CMV, pCDNA3 (Invitrogen, Carlsbad, CA) and pTRE vectors (Clontech, Palo Alto, CA).

In vitro transcription and translation

The longest tau isoform and its mutant P301L cloned in pCDNA3, were synthesized by *in vitro* transcription and translation in presence of [^{35}S]methionine (Amersham Pharmacia Biotech, Little Chalfont, UK) using the TNT Coupled Reticulocyte Lysate system (Promega, Madison, WI) for 90 min at 30°C. After adding 2× sample buffer, the samples were run on 10% polyacrylamide gel, dried and exposed to film.

Cell culture and transfection

COS cells were cultured in Dulbecco's minimal Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) (Clontech), 100 IU/ml penicillin and 100 µg/ml streptomycin (all from Gibco BRL, Bethesda, MD) and were kept at 37°C in 5% CO_2. PC12 cells were grown in DMEM supplemented with10% (v/v) horse serum (Sigma, St Louis, MO) and 5% (v/v) FCS (Clontech), 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco BRL), and kept in 10% CO_2. COS cells were transiently transfected with pCDNA3 constructs mentioned above, using Lipofectamine reagent (Gibco BRL) according to the manufacturer's recommendations. PC12 cells were stably transfected with the wild-type and P301L tau cloned in pTRE vectors using the Tet-On Gene expression system (Clontech) (37).

Immunofluorescence

Cells were rinsed twice in cold phosphate-buffered saline (PBS), fixed and permeabilized, essentially as described by Preuss *et al.* (38). The fixed cells were then incubated with tau-P301 and tau-P301L antibodies (1:100 dilution) for 1 h at room temperature and after washing in PBS with 0.5% bovine serum albumin and 0.15% glycine, were incubated with secondary fluorescein-conjugated goat anti-rabbit antibody (1:100 dilution; Sigma). Cells were mounted in Vectashield mounting media (Vector, Burlingame, CA) and examined with a Leica DMXRA fluorescence microscope equipped with digital camera.

Gel electrophoresis and immunoblotting

Homogenates from transfected cells were prepared by lysing subconfluent cells on ice in lysis buffer (50 mM Tris–Cl pH 7.4, 1% Nonidet P-40, 1 mM $MgCl_2$, 5 mM EGTA, 5 mM dithiothreitol, 120 mM NaCl, 100 µM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin). Extracts were centrifuged immediately at 13 000 *g* for 10 min and the supernatants were used directly for SDS–PAGE. No cross-reactivity was observed for tau-P301 antibody. A faint band was visible in the tau 4R lane after incubation with tau-P301L antibody. However, quantitative analysis showed that the band intensity was <1% compared with the band in the tau P301L lane.

Sarkosyl-insoluble tau from brain was extracted and dephosphorylated as previously described (39). Samples were run on 10% SDS–PAGE and electrophoretically transferred to a nitrocellulose membrane (Scheicher & Schuell, Dassell, Germany). Residual protein-binding sites were blocked by incubation in low fat milk for 1 h at room temperature. After 30 min of washing at room temperature in PBS containing 0.1% Tween 20, the first antibody (1:1000 and 1:2000 dilution for the polyclonal and monoclonal H-7 antibodies, respectively) was incubated for 1 h at room temperature or overnight at 4°C in PBS + 0.1% Tween 20. Following 30 min of washing in PBS + 0.1% Tween 20 at room temperature, the nitrocellulose membrane was incubated 1 h at room temperatuare with the appropriate secondary antibody conjugated with horseradish peroxidase and the reaction products were visualized by using the ECL kit (Amersham Pharmacia Biotech).

To determine the isoform composition of soluble tau present both in the affected and unaffected areas, the sarkosyl-soluble tau was concentrated ~10× using Centricon devices (Millipore, Bedford, MA) and treated with protease inhibitor (Complete; Boehringer, Mannheim, Germany). Protein concentration was determined by using BCA protein assay (Pierce, Rockford, IL). After dephosphorylation, equal amounts of protein for each patient were resolved by 10% SDS–PAGE and the antibodies tau P301, tau-P301L and H-7 were used as described above.

For quantitative immunoblot analysis Protein A labeled with ^{125}I (Amersham Pharmacia Biotech) was used. The radiolabeled tau bands were analyzed and quantified using Image master 1D elite software (Amersham Pharmacia Biotech).

[^{35}S]methionine pulse–chase experiments

COS cells transiently transfected with wild-type and P301L tau, were rinsed in PBS and incubated for 3 h in 5 ml of Met–Leu-free DMEM containing 200 µCi of [^{35}S]methionine-*trans*-labeled methionine (ICN, Irvine, CA). Cells were then rinsed with 5 ml of PBS and incubated for chase intervals of 0, 6, 10 and 22 h in 10 ml of non-radioactive DMEM supplemented with 10% FCS. Cells lysates were precipitated with H-7 monoclonal antibody overnight at 4°C. The antibody–antigen complex was extracted from the lysate by incubating with Protein G–Sepharose (Amersham Pharmacia Biotech) for 1 h at 4°C, followed by centrifugation for 1 min at 10 000 *g*. Protein G–Sepharose pellets were suspended in lysis buffer and rinsed three times. After adding sample buffer the sample was then electrophoresed in 10% polyacrylamide gel and vacuum-dried. Radiolabeled tau bands were visualized and quantified by Image Master 1D elite software (Amersham Pharmacia Biotech).

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