Accelerated filament formation from tau protein with specific FTDP-17 missense mutations

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Abstract Tau is the major component of the neurofibrillar tangles that are a pathological hallmark of Alzheimers' disease. The identification of missense and splicing mutations in *tau* associated with the inherited frontotemporal dementia and Parkinsonism linked to chromosome 17 demonstrated that tau dysfunction can cause neurodegeneration. However, the mechanism by which tau dysfunction leads to neurodegeneration remains uncertain. Here, we present evidence that frontotemporal dementia and Parkinsonism linked to chromosome 17 missense mutations, P301L, V337M and R406W, cause an accelerated aggregation of tau into filaments. These results suggest one mechanism by which these mutations can cause neurodegeneration and frontotemporal dementia and Parkinsonism linked to chromosome 17.

Key words: Frontotemporal dementia and Parkinsonism linked to chromosome 17; Neurodegeneration; Tau polymerization; Tau mutation; Tauopathy

1. Introduction

Frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17) is a neurological disorder characterized by early personality changes as well as deterioration of memory and language [1–6]. In this disease, hyperphosphorylated tau inclusions are observed in neurons and variably in glia in affected cortical and subcortical regions [3,7–9].

The tau isoform composition and filament morphology in these inclusions varies in different forms of FTDP-17. The explanation for this variability was recently determined with the identification of missense and 5′ splice-site mutations of exon 10 in the *tau* gene [10–12]. There is a clear correlation between the type of tau mutation and the biochemical and ultrastructural findings in FTDP-17. Mutations that affect exon 10, either missense (P301L and N279K) or 5′ splice-site mutations (+3, +13, +14 and +16), are associated with inclusions that contain predominantly tau isoforms with four microtubule-binding repeats [8,13] and filaments with a longer periodicity (twisted ribbons) [7,8] than the paired helical filaments (PHFs) observed in Alzheimer's disease (AD) [14,15]. In addition, these mutations are associated with the presence of glial tau pathology [7,8]. In contrast, missense mutations outside exon 10 (G272V, V337M and R406W) are associated with inclusions largely restricted to neurons [3,5] that contain all six tau isoforms [13,16,17] as in AD [18] and the filaments morphologically resemble PHF [5,16,17].

Tau is capable of forming filaments in vitro, especially when incubated with polyanions such as heparin [19–21], RNA [22] or arachidonic acid [23]. The morphology of in vitro polymers depends on the tau isoform used, 3-repeat tau generates PHF-like filaments while 4-repeat isoforms generate straight filaments [19]. The microtubule-binding domain is essential for the formation of tau filaments in vitro [19,20,24]. Since the missense mutations in FTDP-17 are within or adjacent to the microtubule-binding domain, these might be expected to influence the ability of tau to form filaments. We therefore compared the polymerization potential of recombinant wild-type and mutant tau in the presence and absence of polymerization-inducing agents.

2. Materials and methods

2.1. Generation of constructs and mutagenesis

A *Nde*I-*Eco*RI human tau cDNA fragment (exons 1–5, 7, 9–13) in pUC19 was mutagenized with the Gene Editor site-directed mutagenesis kit (Promega) and the selection primer (5′-CCGCGAGACCCACCCTTGGAGGCTCCAGATTTATC-3′). The sequences of mutagenesis oligos were: P301L 5′-AACACGTCCTGGGAGGCG-3′, R406W 5′-GGGACACGTCTCCATGGCATCTCAGCAAT-3′, V337M 5′-CAGGAG GTGGCCAGATGGAAGTAAAATCTG-3′. Mutagenesis was performed according to the manufacturer's instructions. DNA was isolated from mutant and wild-type clones and the tau cDNA sub-cloned in frame into the expression vector pRK172. Tau cDNA constructs were sequenced using the BigDye Terminator Cycle Sequencing Kit (Perkin Elmer). Sequencing was performed on an ABI377 automated sequencer with Sequence Navigator software (Perkin Elmer).

2.2. Expression and purification of recombinant tau

Recombinant wild-type and mutant tau (longest isoform, 441 residues) was expressed in *Escherichia coli* as described previously [25]. Protein was extracted from the bacterial pellet with 20 mM 4-morpholine ethane sulfonic acid (MES), 0.8 M NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), pH 6.8. The extract was dialyzed against the same buffer without salt. Ammonium sulfate was added to 50% saturation and the precipitated proteins were collected by centrifugation at $40\,000 \times g$. The pellet was resuspended in 50 mM MES, 5 mM dithiothreitol (DTT), pH 6.25 and dialyzed overnight against the same buffer. The sample was chromatographed on CM Sepharose, using a salt gradient of 0–0.5 M NaCl [26]. Fractions containing tau were pooled, dialyzed against water and lyophilized. The purity of the tau was determined by SDS-PAGE and Western blotting.

2.3. Polymerization of tau

Wild-type and mutant tau (0.1 mg/ml) was incubated with or without heparin (0.01 mg/ml) in 30 mM MOPS, 2 mM PMSF, pH 7.4, at 37°C. For arachidonic acid-induced polymerization, tau was incubated at a concentration of 0.2 mg/ml with or without 80 μM arachidonic acid in 50 mM borate buffer, 10 mM DTT, 80 mM NaCl, 1 mM

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$MgCl_2$, 2 mM PMSF, 1 mM EDTA, 2 mM EGTA, pH 8.0, at 37°C. Aliquots were removed at indicated time intervals and placed on 200-mesh copper grids coated with formvar and carbon for 2 min [23]. The grids were drained and stained with 2% uranyl acetate for 2 min. Finally, the grid was rinsed with water and dried. The filaments on the grids were monitored using a Philips EM208S electron microscope. After examining the whole grid at 4400× magnification, at least five representative fields for each grid were recorded using the Digital Micrograph software (Gatan). The total filament length was determined for each field using SigmaScan Pro (Jandel Scientific) to give an indication of the relative filament mass. Tau filaments measuring at least 100 nm were included in the data [23]. The average filament length per field was calculated. The average value from duplicate experiments was plotted.

3. Results

3.1. Heparin-induced polymerization of wild-type and mutant recombinant tau

The longest tau isoform (441 residues) and mutants, P301L, V337M and R406W, were polymerized at 0.1 mg/ml in the presence of heparin (0.01 mg/ml). All tau species formed filaments but the kinetics was different in each case (Fig. 1). After incubation for 1 day, the P301L mutant generated a ~7-fold higher mass of filaments than either wild-type tau or the other mutants (Fig. 2). V337M gave no significant differences in polymerization compared to wild-type tau after 1 day, but displayed a 2-fold increase after 2 days incubation. The R406W mutant generated a smaller mass of filaments than wild-type tau after 2–3 days of incubation, but thereafter displayed a dramatic acceleration in filament formation (Fig. 1). The most rapid phase of net growth of filament mass was on days 0–1 for P301L, days 1–2 for V337M, days 2–3 for wild-type and days 3–4 for R406W. The total mass of filaments formed after 4 days was in the order R406W > P301L > wild-type = V337M (2.5:1.8:1.1:1, respectively) (Fig. 1). Polymerization of P301L and R406W tau remained active in samples with 4 days of incubation. However, the growth of filaments in V337M samples reached a plateau on day two and in wild-type sample on day three. The differences in the kinetics of tau polymerization were reproducible in duplicate experiments.

Polymers generated by wild-type and mutant tau with heparin were similar in ultrastructure (data not shown). They appeared as straight filaments with a diameter of ~20 nm [19]. The filament length increased as the incubation time was extended. When placed on grids, the filaments usually distributed evenly. Only in samples containing numerous filaments, as in mutants P301L and R406W after 4 days incubation, were filament clusters observed (Fig. 2). The filaments in these clusters, however, did not form bundles.

3.2. Arachidonic acid-induced polymerization of wild-type and mutant recombinant tau

Wild-type and mutant tau (0.2 mg/ml) was also polymerized in the presence of arachidonic acid (80 μM). Under these conditions, P301L tau again formed filaments most rapidly (Fig. 3). After a 2 day incubation, the mass of filaments generated had the following order: P301L > V337M > wild-type > R406W. These results are similar to those obtained with heparin after this incubation period (2 day). After a 4 day incubation, a qualitative assessment indicated that the R406W mutant had formed more polymers than wild-type tau but less than the P301L or V337M mutants (data not shown). This result is consistent with the time course observed with the R406W mutant incubated with heparin, where the mutant exhibited a dramatic but delayed acceleration in polymerization compared to wild-type tau. The morphology of filaments generated with arachidonic acid and heparin was almost indistinguishable. However, arachidonic acid-induced tau polymers often formed bundles (Fig. 3) which prevented a detailed quantitative assessment.

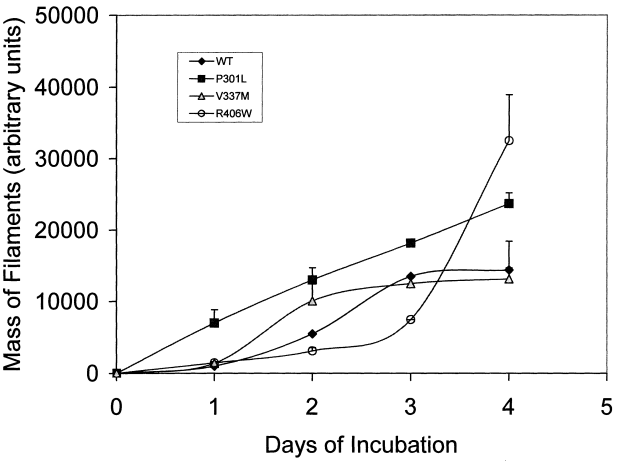

Fig. 1. Kinetics of polymerization of wild-type and mutant tau. Tau (0.1 mg/ml) was incubated with heparin (0.01 mg/ml) at pH 7.4 and 37°C. Aliquots removed at the indicated time intervals were stained with 2% uranyl acetate on copper grids. The digital electron micrographs of 5–10 representative low magnification fields of each grid were recorded. The filament length was measured and the average length per field was calculated. The average of the values from duplicate experiments was plotted. The T bar represents the difference between the individual value and the corresponding average.

3.3. Polymerization of wild-type and mutant tau in the absence of inducing agents

In the absence of polymerization-inducing agents, no filaments were detected with wild-type or mutant tau after 4 days incubation. However, with a prolonged incubation (9 days), an appreciable number of short filaments were observed with the P301L mutant (Fig. 4) but not with wild-type or the other tau mutants. In addition, P301L tau uniquely formed a large number of spherical structures interpreted as protofilaments (Fig. 4). These results are consistent with the observation that the initial rate of polymerization is fastest with P301L in the presence of either heparin or arachidonic acid.

4. Discussion

Our results demonstrate that the FTDP-17 missense mutations tested have a pronounced effect on tau self-interaction. Each mutation has a unique effect on the kinetics of in vitro tau polymerization. The P301L mutant displayed the highest initial rate of polymerization under all conditions tested. Most patients with P301L mutations develop disease at a relatively early age (40–50 years), compared with other FTDP-17 cases with missense mutations, and display a massive fronto-temporal cell loss [6,10]. Interestingly, soluble tau isolated from the brains of patients with this mutation has been reported to have unusually low levels of 4-repeat isoforms presumably because the mutant P301L tau is readily incorporated into insoluble tau filaments [13]. This observation is entirely consistent with the results presented here.

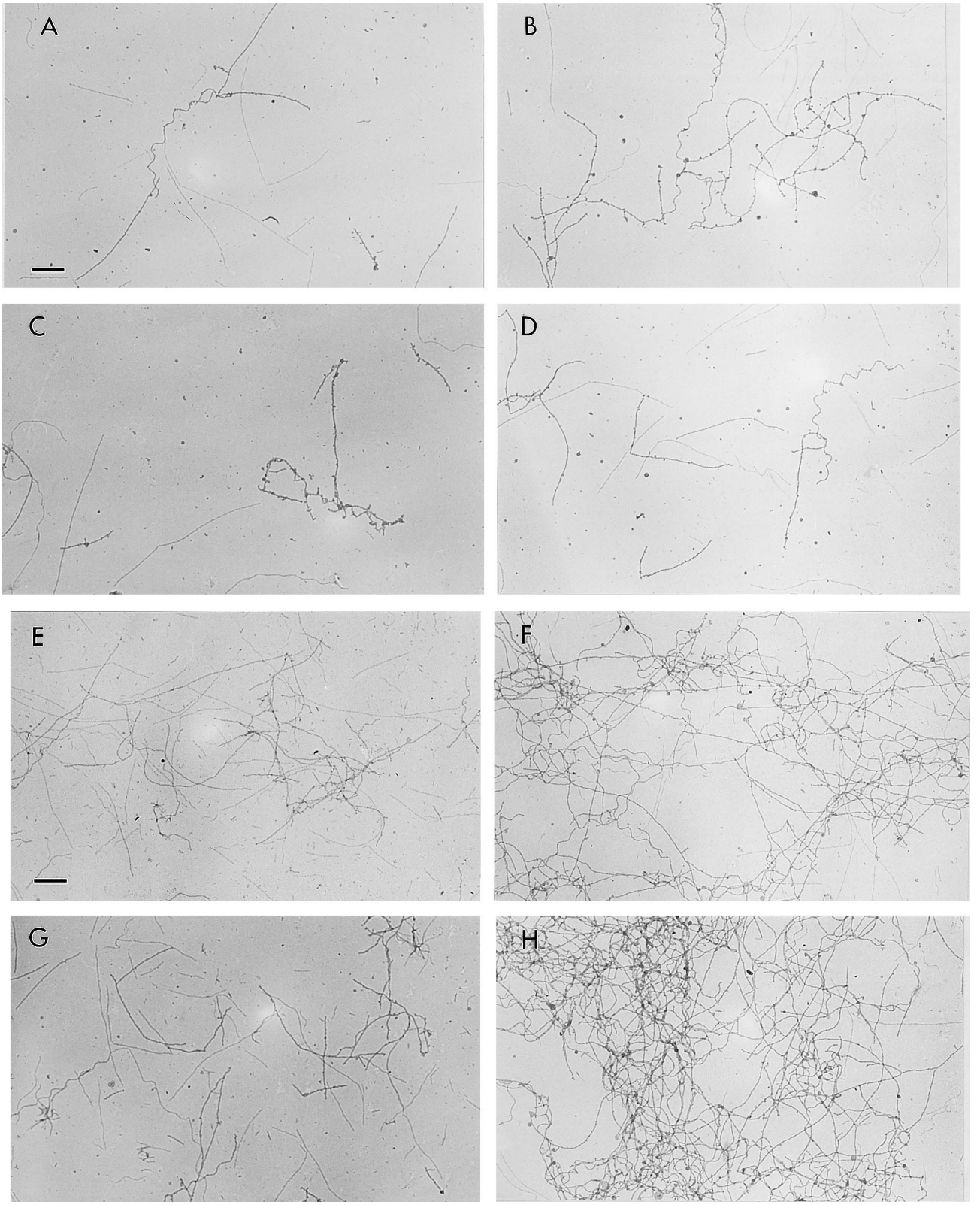

Fig. 2. Tau filaments formed in the presence of heparin. Tau proteins were polymerized as described for Fig. 1. The representative fields from wild-type (A, E) and corresponding mutants P301L (B, F), V337M (C, G) and R406W (D, H) after 1 day (A–D) and 4 days incubation (E–H) were shown. Scale bar = 0.75 μm.

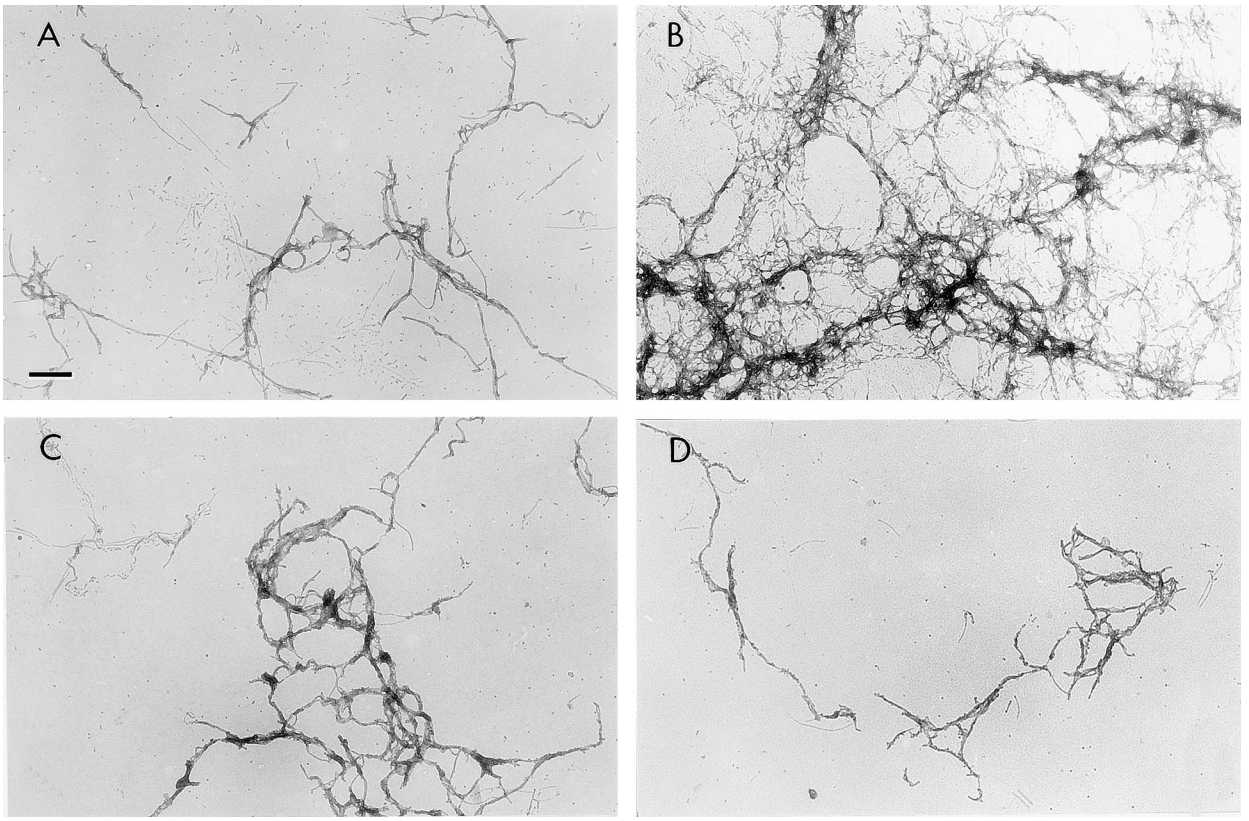

Fig. 3. Representative fields of tau filaments formed in the presence of arachidonic acid. Wild-type tau (A) and its mutants P301L (B), V337M (C) and R406W (D) were incubated at a concentration of 0.2 mg/ml with arachidonic acid (80 μM) at pH 8.0 and 37°C for 2 days. Note that the filaments were bundled. Scale bar = 0.75 μm.

The greater potential of P301L tau for self-interaction may be due to the location of this mutation in a conserved PGGG motif in the microtubule-binding domain. This motif is present in all the four microtubule-binding repeats. Previous studies have shown that in vitro tau filament formation is dependent on the microtubule-binding domain [19,20,24]. Thus, the conformational change generated by replacement of leucine for proline in one of these domains may destabilize tau-tubulin interactions and favor tau-tau self-interaction. Accordingly, the P301L mutation has also been reported to cause the most dramatic reduction (of the mutations tested) in tau-induced in vitro tubulin polymerization [27].

The V337M mutation is located in the variable region of the fourth microtubule-binding repeat. The V337M mutant also exhibited a higher rate of tau filament polymerization than wild-type, but only in the initial stages of polymerization. It remains unclear why polymerization of V337M mutant tau reaches a plateau sooner than that of other recombinant tau proteins. However, it seems likely that the V337M mutation accelerates the nucleation of filaments but has little effect on the subsequent phases of tau polymerization.

Initial polymerization of the R406W mutant was slower than other types of tau but after a delay, the rate increased rapidly in the presence of either heparin or arachidonic acid. Indeed, the mass of filaments formed in the presence of heparin was greater than with other recombinant tau proteins after 4 days incubation. The R406W mutation thus seems to be inhibitory to nucleation. The reason for this could be due to the loss of a positively charged residue from the C-terminal region of tau, the region that contains one of the heparin binding sites of tau [19]. This may affect the interaction between tau and the anionic polymerization inducer [19–22] which may in turn result in a reduced rate of nucleation.

The slow initial rate of R406W tau filament formation is interesting in the light of the clinical phenotype in the FTD004 family that carries this mutation [5]. Patients from this family develop disease with a relatively late age of onset (55 years) and the disease duration is longer than observed in most other FTDP-17 families [5]. Thus, the slower initial kinetics of R406W tau filament formation may be reflected in the clinical phenotype of this family. It must be acknowledged, however, that correlations drawn between these in vitro assays and the clinical features in different FTDP-17 families must be treated with caution since numerous genetic and environmental factors presumably influence the pathogenesis of this disease.

Analysis of the biophysical properties of wild-type, P301L, V337M, R406W and G272V recombinant tau using HPLC elution and circular dichroic spectra has revealed that each mutation causes a similar alteration in tau confirmation (Peter Davies, MH, unpublished results). Each missense mutation results in an increase in the apparent α-helical content of the tau molecule. The result of this conformational change seems to shift the structure of tau away from that which favors microtubule-binding/polymerization to a structure that favors self-interaction.

The results presented here on tau polymerization and previous observations that tau missense mutations reduce micro-

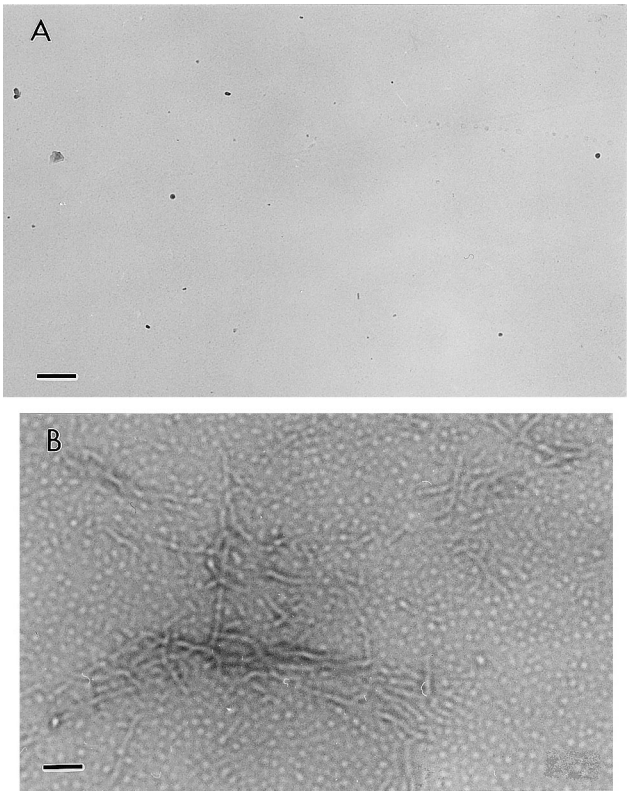

Fig. 4. Polymerization of the P301L mutant without polymerization inducing agents. Wild-type tau (A) and mutant P301L (B) were incubated at a concentration of 0.2 mg/ml in 50 mM borate buffer, 10 mM DTT, pH 8.0 at 37°C for 9 days. Note the short filaments and spherical structures formed with P301L (B). Scale bar = 0.38 μm (for A) and 0.18 μm (for B).

tubule-binding and polymerization [13,27] are consistent with a dual effect of the FTDP-17 mutations. However, further studies will be needed to investigate which of these two mechanisms, or both, are critical to the pathogenesis of FTDP-17.

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