Frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), an autosomal, dominantly inherited neurodegenerative disorder caused by tau gene mutations, is neuropathologically characterized by intraneuronal filamentous inclusions of hyperphosphorylated tau protein. Biochemical and immunocytochemical analyses have shown that only mutant tau is deposited in patients harboring P301L missense mutation, whereas both wild-type and mutant tau are deposited in patients harboring R406W mutation (Miyasaka, T., Morishima-Kawashima, M., Ravid, R., Kamphorst, W., Nagashima, K., and Ihara, Y. (2001) J. Neuropathol. Exp. Neurol. 60, 872–884 and Miyasaka, T., Morishima-Kawashima, M., Ravid, R., Heutink, P., van Swieten, J. C., Nagashima, K., and Ihara, Y. (2001) Am. J. Pathol. 158, 373–379). Here we have tested the nucleation ability of monomeric tau and the seeding ability of fibrilligenic nuclei obtained from bacterially expressed human tau. P301L mutant tau showed a higher nucleation ability than wild-type tau, whereas R406W mutant tau shows similar ability to wild-type tau. Surprisingly, fibrilligenic nuclei composed of P301L mutant tau enhanced the assembly of P301L mutant tau into filaments but did not promote filament formation from wild-type tau. In contrast, nuclei composed of R406W mutant tau supported filament formation from both wild-type tau and R406W mutant tau, as did nuclei composed of wild-type tau. Proteolytic analyses indicated that the substructure of nuclei composed of P301L mutant tau was different from that of nuclei composed of wild-type or R406W mutant tau. Thus, the interaction between fibrilligenic nuclei and monomeric protein appears to play an important role in the mechanism of tau filament assembly.

Neurofibrillary tangles, i.e. bundles of paired helical filaments and straight filaments, are one of the major neuropathological hallmarks of Alzheimer disease. They are composed of the microtubule-associated protein tau in a hyperphosphorylated state (1–7). Because the temporal and spatial accumulation of tau correlates well with nerve cell loss and severity of dementia, formation of neurofibrillary tangle is considered to be involved in the pathways leading to neuronal death (8, 9).

Intracellular filamentous inclusions composed of tau are also characteristic of a number of other neurodegenerative diseases, including Pick disease, progressive supranuclear palsy, corticobasal degeneration, and frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17)1 (10). FTDP-17 is an autosomal, dominantly inherited neurodegenerative disorder caused by tau gene mutations. Currently, more than 30 exonic and intronic pathogenic mutations in the tau gene have been identified in more than 100 families with FTDP-17. Most of the exonic mutations reduce the physiological activity of tau to interact with microtubules and to promote their assembly (11) and also increase the propensity of tau to aggregate into abnormal filaments (12–15). The other mutations, including intronic ones, affect the alternative splicing of exon 10 on tau mRNA, which contains a second repeat of the microtubule binding domain, resulting in disturbance of the normal ratio of tau isoforms (16, 17). However, it remains unclear that how these tau mutations lead to assembly of tau protein and why different tau mutations cause different tau pathologies.

Among the tau gene mutations, the proline-to-leucine mutation at codon 301 (numbered according to the longest isoform) (P301L) is thought to be the most frequent mutation in FTDP-17 (18–20). Using site-specific polyclonal antibodies against wild-type tau and P301L mutant tau, it has been shown that P301L mutant tau is selectively deposited in the brains of patients with P301L mutation, although there was no difference in expression level between wild-type and P301L mutant tau (21, 22). In contrast, in the case of the arginine-to-tryptophan mutation at codon 406 (R406W), which has been described in an American (23), a Dutch (20), and a Japanese family (24), almost equal amounts of wild-type and R406W mutant tau were detected in the Sarkosyl-insoluble fraction of the brains (25). Elucidation of the underlying cause of these phenomena is important to understand not only the molecular mechanism of

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9 The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1.

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3 The abbreviations used are: FTDP-17, frontotemporal dementia with parkinsonism linked to chromosome PIPES, piperazine-1,4-bis(2-ethanesulfonic acid); DTT, dithiothreitol; GSK3β, glycogen synthase kinase 3β; Cdk5, cyclin-dependent kinase 5; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MS, mass spectrometry; CBB, Coomassie Brilliant Blue; WT, wild type.
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tau assembly induced by tau mutations in FTDP-17 but also the isoform-specific tau deposition in sporadic tauopathies.

As in the cases of many other amyloid fibrils, tau fibrillation has been modeled as a nucleation-dependent process that is characterized by the requirement for a nucleus (26). Once the nucleus forms, subsequent addition of monomeric tau to nascent filament ends follows in a reaction termed elongation. Bacterially expressed recombinant tau is able to assemble into filaments in the presence of polyanions, including glycosaminoglycans such as heparin (27), peptides such as polyglutamate (28), nucleic acids such as RNA (29), or free fatty acids such as arachidonic acid (30) and detergents (31). These molecules accelerate the nucleation reaction and shorten the lag time, i.e. the time until monomeric protein begins to aggregate spontaneously in a detectable form. The addition of external nuclei obtained by sonication of heparin-induced tau filaments also promoted tau aggregation into filaments (26). It is possible to circumvent the self-nucleation step by adding external nuclei, since they can act as fibrillogenic seeds for tau assembly.

In the current study we examined the basis of the selective accumulation of P301L mutant tau and the non-selective accumulation of wild-type and R406W mutant tau in FTDP-17 patient brains by using an in vitro aggregation system of recombinant tau in the presence of heparin and fibrillogenic nuclei to analyze the nucleation and elongation steps of tau filament formation. Our results indicate that the interaction between fibrillogenic nuclei and monomeric protein plays an important role of the mechanisms of tau protein aggregation.

EXPERIMENTAL PROCEDURES

Antibodies—Site-specific polyclonal antibodies against wild-type and P301L mutant tau (AP301 and AL301) were gifts from Dr. Ihara (21).Other site-specific polyclonal antibodies recognizing wild-type and R406W mutant tau (AR406 and AW406) were also gifts from Dr. Ihara (25). An anti-tau antibody HT7 was obtained from Innogenetics (Ghent, Belgium). Phosphorylation-dependent anti-tau antibodies Thr(P)-181, Ser(P)-199, Thr(P)-205, Thr(P)-212, and Ser(P)-396 were purchased from BIOSOURCE International (Camarillo, CA).

Cloning and Construction of Human Wild-type and Mutant Tau—Wild-type human tau cDNA containing four repeats without amino-terminal inserts was amplified from a human brain cDNA library (Clontech) and cloned into pENTR1A vector (Invitrogen) using BamHI and NotI. The resultant recombinant plasmid was digested with Ncol and Xhol and cloned into Escherichia coli expression vector pET-15b that had been digested with the same enzymes. Site-directed mutagenesis was used to change Pro-301 to leucine and Arg-406 to tryptophan in the four-repeat 383-amino acid isoform of human tau. The structures of all constructs were verified by DNA sequencing.

Expression and Purification of Wild-type and Mutant Tau Proteins—The 4-repeat 383-amino acid isoform of human tau was expressed from cDNA in E. coli BL21 Star (DE3) as described (11). Bacterial pellets were resuspended in extraction buffer (50 mM PIPES, 1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, pH 6.8) followed by a 1-min sonication on ice twice using an Astrason ultrasonic processor XL2020 (Misonix Inc.) at output level 2. The homogenates were centrifuged at 15,000 rpm for 15 min (Beckman XL-90), and the supernatants was loaded onto a phosphocellulose column (bed volume 2 ml) equilibrated in extraction buffer. The column was washed in extraction buffer followed by extraction buffer containing 0.1 M NaCl. Protein was eluted batch-wise with 6 ml of extraction buffer containing 0.5 M NaCl. This was followed by the addition of ammonium sulfate to 50% saturation and then centrifugation for 30 min, at 50,000 rpm (Beckman XL-90). The pellet was resuspended in extraction buffer containing 0.5 M NaCl and 1% 2-mercaptoethanol, and the suspension was boiled for 5 min. The supernatant of a 15-min centrifugation at 15000 rpm (TOMY MRX-152) was dialyzed overnight against 30 mM Tris-HCl, pH 7.5. Tau protein concentration was determined by bichinonic acid protein assay, measuring the absorbance at 540 nm. In all experiments, wild-type and mutant tau proteins were expressed and purified in parallel.

Tau Filament Assembly Induced by Heparin—Purified recombinant tau (10 μM) and heparin (100 μg/ml) were incubated at 37 °C for 48 h in 30 mM Tris-HCl, pH 7.5, containing 20 mM DTT. Aliquots of assembly mixture (100 μl) were washed in 10 volumes of 30 mM Tris-HCl, pH 7.5, and centrifuged at 150,000 × g for 20 min. The pellet was resuspended in 30 mM Tris-HCl, pH 7.5, and sonicated for 3 s (output level 1.5) with an Astrason ultrasonic processor XL2020 (Misonix). Concentrations of the seeds were based on the amount of monomeric protein used.

Preparations of Fibrillogenic Seeds—Solutions of wild-type or mutant tau protein at 25 μM were incubated at 37 °C for 72–96 h in 30 mM Tris-HCl, pH 7.5, containing 20 mM DTT. Aliquots of assembly mixture (100 μl) were washed in 10 volumes of 30 mM Tris-HCl, pH 7.5, and centrifuged at 150,000 × g for 20 min. The pellet was resuspended in 30 mM Tris-HCl, pH 7.5, and sonicated for 3 s (output level 1.5) with an Astrason ultrasonic processor XL2020 (Misonix). Concentrations of the seeds were based on the amount of monomeric protein used.

Electronic Microscopy—Electron microscopy was used for qualitative assessment of filament formation. Aliquots (2 μl) of assembly mixture were diluted to 10 μl with 30 mM Tris-HCl, pH 7.5. Aliquots (2 μl) of these dilutions were placed on colloid-coated 300-mesh copper grids and stained with 2%
sodium phosphotungstate. Micrographs were recorded at a nominal magnification of ×50,000 on a JEM-1230 transmission electron microscopy (JEOL).

**Tau Phosphorylation by GSK3β and Cdk5**—Wild-type and mutant tau (27.5 μm) were incubated with 4 units/ml GSK3β (New England Biolabs) and 4 units/ml activated Cdk5 (Upstate Biotechnology, Inc.) in 30 mM Tris–HCl, pH 7.5, containing 0.1 mM EGTA, 10 mM MgCl₂, 0.8 mM phenylmethylsulfonyl fluoride, 2 mM ATP, and complete protease inhibitor mixture (Roche Applied Science) at 37 °C for 4 h as described (33). Reactions were stopped by boiling for 10 min. After a 15-min centrifugation at 15,000 rpm (TOMY MRX-152), the supernatants were analyzed by immunoblotting and used for filament formation.

**Proteolytic Digestion of Monomeric Tau and Fibrilligenic Nuclei**—Monomeric tau or seeds (25 μm) were digested with 50 μg/ml Pronase (Novagen) at room temperature for various times (0, 10, 30, and 60 min). The proteolysis reactions were stopped by the addition of SDS sample buffer containing 5% 2-mercaptoethanol followed by boiling for 10 min.

**Protein Chemical Analyses of Pronase-resistant Core of Fibrilligenic Nuclei Composed of Wild-type or Mutant Tau**—Aliquots of Pronase-treated fibrilligenic nuclei (100 μl) were washed in 5 volumes of 30 mM Tris–HCl, pH 7.5, and centrifuged at 150,000 × g for 20 min. The pellet was resuspended in 30 mM Tris–HCl, pH 7.5, by sonication for 3 s (output level 1.5) with an Astrason ultrasonic processor XL2020 (Misonix) and solubilized in 6 M guanidine hydrochloride. The Pronase-resistant peptides were purified on an Aquapore RP-300 column (2 × 30 mm, PerkinElmer Life Sciences) by reverse–phase high performance liquid chromatography (HPLC) (Model 1100, Agilent) (34) and analyzed by a 492 Protein Sequencer (Applied Biosystems) (35) and matrix-assisted laser desorption ionization-time of flight/mass spectrometry (MALDI-TOF MS) using a Voyager-DE Pro mass spectrometer (PerSeptive Biosystems) (36).

**RESULTS**

**Nucleation of Wild-type, P301L, and R406W Tau**—To compare the nucleation properties of wild-type, P301L, and R406W tau proteins, we examined the lag time of fibrillogenesis in the absence or presence of heparin. We assessed tau assembly by measuring the Sarkosyl-insoluble tau as described (32). No Sarkosyl-insoluble tau was observed with wild-type, P301L, or R406W mutant tau in the absence of heparin (data not shown). Incubation of tau with heparin resulted in an increase in Sarkosyl-insoluble tau in all three species, with the highest level being that in P301L mutant tau (Fig. 1A). Although wild-type and R406W mutant tau did not aggregate up to 24 h, P301L mutant tau started to assemble from 4 h (Fig. 1B), in good agreement with previous reports (12, 13). The 48-h incubation of wild-type, P301L, or R406W mutant tau in the presence of heparin gave predominantly straight filaments. There was no detectable difference in their morphologies (see Fig. 3, A–C). These data demonstrate that P301L mutant tau can assemble into nuclei more rapidly than wild-type or R406W mutant tau.

**Elongation by Exogenous Nuclei Composed of Wild-type Tau or P301L Mutant Tau**—Elongation of tau filaments can proceed via assembly of soluble tau either by de novo formation or exogenous addition of nuclei. Therefore, exogenous nuclei composed of wild-type or P301L mutant tau were prepared as seeds by sonicating preformed heparin-induced tau filaments and incubated with wild-type or P301L mutant tau. No increase of Sarkosyl-insoluble tau was detected in the absence of seeds (Fig. 2, A and D). Seeds composed of wild-type tau promoted assembly of both wild-type and P301L mutant tau (Fig. 2, B and E). In contrast, seeds composed of P301L mutant tau led to a drastic fibrillation of P301L mutant tau, although they cross-seeded wild-type tau aggregation only slightly (Fig. 2, C and F). The filament formation was assessed morphologically by electron microscopy. Filaments formed by exogenous nuclei showed straight morphology which was similar to that formed in the presence of heparin (Fig. 3, D, E, G, and H). There was no difference in the morphologies of filaments between any combinations of seeds and monomeric tau. These data indicate that wild-type nuclei have the ability to seed both wild-type and P301L mutant tau; meanwhile, P301L mutant tau seeds can promote the assembly only of tau-containing P301L mutations.

**Elongation Reaction by Exogenous Nuclei Composed of Wild-type Tau or R406W Mutant Tau**—We next tested the cross-seeding effects on wild-type and R406W mutant tau. In the absence of seeds, no increase of Sarkosyl-insoluble tau was detected in the incubation of wild-type or R406W tau up to 48 h...
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Co-incubation of Wild-type and Mutant Tau in the Presence of Fibrillogenic Nuclei—FTDP-17 is an autosomal dominant disorder, i.e. patients carrying both wild-type and mutant gene develop the disease. In such patients both wild-type and mutant proteins are expressed equally in the brain. To investigate whether nucleation-dependent filament formation as described above can also be observed in an environment more closely resembling the physiological condition, we co-incubated a mixture of wild-type and P301L mutant tau with or without fibrillogenic nuclei. The total amount of assembled tau was assessed by detecting Sarkosyl-insoluble tau with Coomassie Brilliant Blue (CBB) staining, and the amount of wild-type or mutant tau contained in the insoluble fraction was determined by using two sets of antibodies, a specific antibody to the Pro-301 site of wild-type tau (AP301) and a Leu-301 site-specific antibody to P301L mutant tau (AL301) or a specific antibody to the Arg-406 site of wild-type tau (AR406) and a Trp-406 site-specific antibody to R406W mutant tau (AW406).

When P301L mutant tau seeds were added to the mixture of wild-type tau and P301L mutant tau, a large amount of Sarkosyl-insoluble tau was detected with CBB staining, whereas only a small increase of the Sarkosyl-insoluble tau was observed when wild-type tau seeds were added (Fig. 4, A and B). To analyze which tau assembled into filaments with the seeds, the insoluble tau was probed with site-specific antibodies, AP301 and AL301. The Sarkosyl-insoluble tau assembled with P301L mutant tau seeds was labeled strongly with AL301 but only faintly with AP301. Quantitative analysis revealed that more than 80% of the assembled tau with P301L mutant tau seeds was the P301L mutant (Fig. 4, A and D). Insoluble tau assembled with wild-type tau seeds was undetectable with these site-specific antibodies (Fig. 4, A and C).

Similar experiments were performed using wild-type and R406W mutant tau. Wild-type tau seeds produced almost the same amount of total Sarkosyl-insoluble tau as was produced with R406W tau seeds (Fig. 4, E and F). The amount of wild-type tau in the Sarkosyl-insoluble fraction formed after seeding by wild-type seeds was similar to that of R406W mutant tau.
R406W tau seeds also led to aggregation of both wild-type and R406W mutant tau, and there was little difference in amount between the two (Fig. 4, E and H).

Pronase Digestion of Seeds Composed of Wild-type or Mutant Tau—We hypothesized that the above results can be explained in terms of differences in the conformation of seeds. Treatment of Alzheimer paired helical filaments (PHFs) with Pronase digests the fuzzy coat and reveals the core fragment of PHFs. Therefore, we thought it might be possible to reveal the differences in the structure of the seeds by comparing the core patterns. To address this issue, we digested seeds composed of wild-type, P301L, or R406W tau with Pronase and compared the digestion patterns by SDS-PAGE.

Monomeric tau was digested within 10 min with Pronase, and no tau band was detected. In contrast, 5–9-kDa core bands remained when the seeds were digested with Pronase for up to 60 min (Fig. 5A). The digestion of the seeds composed of wild-type tau afforded two major bands of 7 and 9 kDa, and a similar band pattern was observed in the digestion of the seeds composed of R406W mutant tau (Fig. 5A). One major 7-kDa band and one minor 9-kDa band were detected in the digestion of P301L tau seeds, although the mobilities of these bands were slightly larger than those of the bands derived from wild-type tau seeds (Fig. 5A).

To further analyze the core of the seeds, the Pronase-resistant fragments were purified by reverse-phase HPLC and subjected to protein sequencer. The Pronase-resistant fragments of wild-type tau seeds gave four sequences, i.e., ENLKHQPGGG, LKHQPGGGKV, HQPGGGKVQI, and IGSTENLKHQ (in order of their relative abundance), which corresponded to residues 264–273, 266–275, 268–277, 262–271, and 260–269 (numbered according to the longest isoform). The Pronase-resistant R406W mutant tau seeds

FIGURE 3. Qualitative assessment of tau filament formation in the presence of heparin or seeds by electron microscopy. Wild-type (A, D, G, and I), P301L (B, E, and H), or R406W (C, F, and J) mutant tau was incubated with 100 μg/ml heparin (A–C), 0.5 μM Seed-WT (D–E), 0.5 μM Seed-P301L (G and H), or 0.5 μM Seed-R406W (I and J). Negatively stained tau filaments after 48 h of incubation were assessed by transmission electron microscopy.

FIGURE 4. Tau assembly in a mixture of wild-type and mutant tau in the presence of various seeds. A–D, wild-type and P301L mutant tau were co-incubated in the presence of Seed-WT or Seed-P301L. Total insoluble tau was assessed by CBB staining (A and B). AP301 and AL301 were used to detect wild-type and P301L mutant tau, respectively, in the insoluble fraction (A, C, and D). E–H, wild-type and R406W mutant tau were co-incubated in the presence of Seed-WT or Seed-R406W. Total insoluble tau was assessed by CBB staining (E and F). AR406 and AW406 were used to detect wild-type and R406W mutant tau, respectively, in the insoluble fraction (E, G, and H). Intensities of the bands of insoluble tau were measured and assessed, and the results are expressed as the means ± S.E. (*, p < 0.05).
showed similar results to those of the wild-type tau seeds, whereas the Pronase-treated P301L mutant tau seeds gave three sequences, LKHQPGGGKV, HQPGGGKVQI, and ENLKHQPGGG, which corresponded to residues 266–275, 268–277, and 264–273 in order of their relative abundance.

The MALDI-TOF MS analyses showed that the Pronase-resistant cores of tau seeds were composed of heterogeneous multiple fragments (Fig. 5B). In the analyses of the Pronase-resistant WT tau and R406W mutant tau seeds, mass signals ranging from 7392.8 to 8985.1 were detected. Although in MS analysis of the Pronase-resistant P301L mutant tau seeds, mass signals ranging from 6213.8 to 7979.7 were detected (Fig. 5B, supplemental Table S1).

From MALDI-TOF MS and amino-terminal sequence data, it was determined that the fragments of wild-type and R406W mutant tau seeds were composed of Ile-260—Val-337, Glu-264—Glu-338, Glu-264—Val-339, Leu-266—Glu-338, Leu-266—Val-339, His-268—Glu-338, and His-268—Val-339 (Fig. 5C). These protein chemical data strongly suggest that the core structure of P301L mutant tau seeds is distinct from that of wild-type tau seeds, whereas R406W mutant tau seeds have a similar core structure to wild-type tau seeds.

Pronase Digestion of Seeds Composed of Wild-type or Mutant Tau Phosphorylated by GSK3β/H9252 and Cdk5—To investigate tau seeds composed of phosphorylated tau, wild-type and mutant tau were phosphorylated by GSK3β/H9252 and Cdk5 concurrently.

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![Figure 5](image.png)
Phosphorylated tau epitopes were observed by immunoblotting with phosphorylation-dependent anti-tau antibodies to phosphorylated Thr-181, Ser-199, Thr-205, Ser-212, or Ser-396 (Fig. 6). The phosphorylation states of P301L and R406W mutant tau were similar to that of wild-type tau (Fig. 6).

Phosphorylated wild-type, P301L, or R406W mutant tau seeds were prepared using the tau phosphorylated by GSK3β and Cdk5 as described above followed by Pronase digestion. The digestion of the seeds composed of phosphorylated wild-type tau showed an ~7-kDa single band, and a similar band was observed in the Pronase digestion of phosphorylated R406W tau seeds (Fig. 7A). On the other hand, the digestion of phosphorylated P301L mutant tau seeds gave an ~6-kDa single band (Fig. 7A).

The digestion of phosphorylated wild-type or mutant tau seeds were purified by reverse-phase HPLC and analyzed by MALDI-TOF MS and amino-terminal sequence data. Although SDS-PAGE of the Pronase digestions of phosphorylated tau seeds gave a single band in each lane, MALDI-TOF MS analyses revealed that the Pronase-resistant core of phosphorylated wild-type, P301L, or R406W mutant tau seeds contained a few kinds of fragments (Fig. 7B). Although the patterns of mass signals (ranging from 7638.9 to 8167.0) were observed in the Pronase digestion of phosphorylated R406W tau seeds (Fig. 7A). On the other hand, the digestion of phosphorylated P301L mutant tau seeds gave an ~6-kDa single band (Fig. 7A).

From MALDI-TOF MS and amino-terminal sequence data, it was determined that the core structures of phosphorylated wild-type and R406W mutant tau seeds were composed of Ser-262—Glu-338, Ser-262—Val-339, Glu-264—Glu-338, Glu-264—Val-339, Leu-266—Glu-338, and Leu-266—Val-339, whereas the core structure of phosphorylated P301L mutant tau seeds were composed of Glu-264—Glu-338, Leu-266—Ser-324, Leu-266—Glu-338, Leu-266—Val-339, His-268—Glu-338, and His-268—Val-339 (Fig. 7C). These data demonstrated that even in the phosphorylated state the core of P301L mutant tau seeds have a different structure from that of wild-type tau seeds, whereas the core structure of R406W mutant tau seeds was similar to that of wild-type tau seeds.

**DISCUSSION**

The discovery of tau gene mutations in FTDP-17 has confirmed the idea that tau protein dysfunction causes neurodegeneration (23, 37, 38). This is relevant not only to FTDP-17 but also to other diseases with a neurofibrillary pathology, such as Alzheimer disease, Pick disease, progressive supranuclear palsy, and corticobasal degeneration. Hence, there is great interest in the molecular mechanisms through which tau mutations lead to frontotemporal dementia.

In the case of FTDP-17 patients carrying P301L mutation, only mutant tau was detected in the Sarkosyl-insoluble fraction of their brains, although both wild-type and P301L mutant tau were expressed at the mRNA level (21, 22). On the other hand, almost equal amounts of wild-type and mutant tau were present in the Sarkosyl-insoluble fraction of the brain affected by R406W mutation (25). Hence, we hypothesized that the nucleation and elongation rates of tau into filaments may be different between the mutants, and the assembly rates of tau may be dependent on the combination of monomeric tau and fibrillogenic nuclei.

In this study we first demonstrated that P301L mutant tau nucleates faster than wild-type tau. On the other hand, the nucleation speed of R406W mutant tau was similar to that of wild-type tau. These data are consistent with previous findings (13, 39). We next assessed the elongation reaction of wild-type and mutant tau. Interestingly, P301L mutant tau seeds initiated elongation of only P301L mutant tau, but not wild-type tau, whereas seeds composed of wild-type tau were able to promote assembly of both wild-type and P301L mutant tau equally efficiently. In contrast, R406W mutant tau seeds initiated elongation of wild-type tau as effectively as that of R406W mutant tau.

Based on these observations, we propose a model that can at least partly explain the accumulation of tau in FTDP-17 patients. In the brain affected by P301L mutation, mutant tau is assumed to form a large number of fibrillogenic nuclei as compared with wild-type tau because of its higher propensity for nucleation. The subsequent elongation step is induced mainly by nuclei composed of P301L mutant tau. Thus, the elongation of P301L mutant tau proceeds on the P301L mutant tau seeds, whereas wild-type tau cannot assemble efficiently on the P301L mutant tau seeds. In contrast, in the case of R406W mutation, both wild-type and R406W tau form fibrillogenic nuclei at similar rates in the brain, and the elongation of both wild-type and R406W mutant tau can occur on either wild-type tau seeds or...
mutant tau seeds with similar frequency. To test this model, we co-incubated wild-type and mutant tau in the presence of fibrillogenic nuclei, because wild-type and mutant tau are assumed to co-exist in the brains of patients.

As expected, seeds composed of P301L mutant tau induced aggregation of P301L mutant tau more effectively than wild-type tau upon co-incubation. When wild-type tau was co-incubated with R406W mutant tau, both wild-type and R406W mutant tau appeared in Sarkosyl-insoluble aggregates in the presence of seeds composed of wild-type tau, and similar results were obtained with seeds composed of R406W mutant tau. These results were consistent with the findings when wild-type or R406W mutant tau were incubated alone in the presence of nuclei composed of wild-type tau. This result is contrary to the results obtained when wild-type or P301L mutant tau was incubated alone with wild-type nuclei. The reasons for this discrepancy are unclear, although it is possible that the co-existence of wild-type and P301L mutant tau affects the kinetics of tau assembly into filaments. Monomeric wild-type tau and P301L mutant tau might interact or compete with one another for nuclei. Taken together, our findings in the co-incubation experiments suggest that nuclei composed of P301L mutant tau can promote assembly of P301L mutant tau into filaments much more efficiently than assembly of wild-type tau.

Proteolytic digestion is a widely used technique to examine the substructures of proteins and protein assemblies. Pronase or trypsin digestion of paired helical filaments from the brains of Alzheimer disease patients resulted in the isolation of paired helical filaments. The results of the digestion were analyzed by SDS-PAGE (CBB staining) (A). The Pronase-resistant fragments of 60 min of digestion were purified and analyzed by MALDI-TOF MS and protein sequencing. The major signals of MALDI-TOF MS from Pronase-resistant cores composed of phosphorylated wild-type, P301L, or R406W mutant tau seeds were numbered from 1 to 7 individually (B). The Pronase-resistant fragments identified by MALDI-TOF MS and amino-terminal sequence analyzes were shown (C). Arrows represent the sites of the P30L and R406W mutations.
helical filaments core structure, which largely overlaps with the repeat domain of tau (40–43). To examine the effect of tau mutation in the conformation of fibrillogenic nuclei, we used SDS-PAGE, MALDI-TOF MS, and a protein sequencer to compare the patterns of the Pronase-resistant core. The results revealed that the conformation of P301L mutant tau seeds was different from that of wild-type tau, whereas R406W mutant tau seeds were suggested to have a similar substructure to that of wild-type tau seeds.

Indeed, it is reasonable that the P301L mutation affects the conformation of nuclei, because proline frequently induces a bending point in peptide structure and the P301L mutation is located in the second repeat domain of tau protein, which comprises the Pronase-resistant core. On the other hand, it seems reasonable that R406W mutation does not influence the conformation of nuclei, because the R406W mutation is located outside the Pronase-resistant core.

In the brains of patients tau protein is accumulated in a highly phosphorylated state (1–7). To elucidate the effect of P301L mutation on the core structure of tau seeds in a pathological condition, we used wild-type and mutant tau seeds that were composed of recombinant tau phosphorylated by two kinases, GSK3β and Cdk5, which are assumed to have an important role in a pathological tau phosphorylation (44, 45). Incubation of wild-type or mutant tau with GSK3β and Cdk5 generated epitopes of several phosphorylation sites shown in pathological tau (and similar levels of phosphorylation were detected in wild-type, P301L, and R406W mutant tau). SDS-PAGE and subsequent protein chemical analyses of Pronase-resistant cores of the seeds indicated that, even in a pathologically phosphorylated state, P301L mutation affects the conformation of fibrillogenic nuclei, whereas R406W mutation does not. The Pronase-resistant cores of the seeds formed with phosphorylated tau exhibited similar characteristics to those of seeds formed with non-phosphorylated tau.

Collectively, these data suggest that a single amino acid substitution can lead to a conformational change of fibrillogenic nuclei. Furthermore, changes of the substructure can dramatically influence the elongation reaction. The importance of the substructure of nuclei is well established for other amyloidogenic proteins, especially in prion diseases. Prion protein, which deposits in the brains of patients with various kinds of neurodegenerative diseases, is natively a large α-helical, monomeric protein termed PrP^C,. PrP^C becomes fibrillogenic when it is converted into PrP^Sc, an isofrom containing a higher level of β-sheet (46). Our study is the first to describe the substructure of tau filaments containing a mutation that affects the assembly of tau protein.

Although the importance of the conformation of fibrillogenic nuclei is clear, we also observed that elongation reactions in the protein aggregation process differ even when the same nuclei are used. A large difference in elongation rate between wild-type and P301L monomeric tau was observed when the two were seeded with nuclei carrying the P301L mutation. This suggests that seeding effects are determined not only by the substructure of fibrillogenic nuclei but also by the conformation of monomeric tau. The strength of the interaction between fibrillogenic nuclei and monomeric tau is presumably an important factor in the elongation reaction. It is possible that the interaction between P301L mutant nuclei and P301L mutant monomeric tau is stronger than the interaction between P301L mutant nuclei and wild-type monomeric tau, resulting in greater elongation of P301L mutant tau than wild-type tau in the presence of P301L mutant tau seeds.

In conclusion, we emphasize the importance of the strength of the interaction between fibrillogenic nuclei and monomeric protein that can potentially aggregate into filaments. This concept is consistent with the idea that tau aggregation plays a critical role in neurodegeneration.

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