Familial FTDP-17 Missense Mutations Inhibit Microtubule Assembly-promoting Activity of Tau by Increasing Phosphorylation at Ser^{202} in Vitro^{*}§

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In Alzheimer disease (AD), frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) and other tauopathies, tau accumulates and forms paired helical filaments (PHFs) in the brain. Tau isolated from PHFs is phosphorylated at a number of sites, migrates as ~60-, 64-, and 68-kDa bands on SDS-gel, and does not promote microtubule assembly. Upon dephosphorylation, the PHF-tau migrates as ~50–60-kDa bands on SDS-gels in a manner similar to tau that is isolated from normal brain and promotes microtubule assembly. The site(s) that inhibits microtubule assembly-promoting activity when phosphorylated in the diseased brain is not known. In this study, when tau was phosphorylated by Cdk5 in vitro, its mobility shifted from ~60-kDa bands to ~64- and 68-kDa bands in a time-dependent manner. This mobility shift correlated with phosphorylation at Ser^{202}, and Ser^{202} phosphorylation inhibited the microtubule assembly-promoting activity of FTDP-17 mutants more than of WT. Upon dephosphorylation, the PHF-tau regain its ability to bind to and promote microtubule assembly (6, 7). Tau hyperphosphorylation is suggested to cause microtubule instability and PHF formation, leading to NFT pathology in the brain (1–3).

PHF-tau is phosphorylated on at least 21 proline-directed and non-proline-directed sites (8, 9). The individual contribution of these sites in converting tau to PHFs is not entirely clear. However, some sites are only partially phosphorylated in PHFs (8), whereas phosphorylation on specific sites inhibits the microtubule assembly-promoting activity of tau (6, 10). These observations suggest that phosphorylation on a few sites may be responsible and sufficient for causing tau dysfunction in AD.

The major fibrous component of NFTs are paired helical filaments (PHFs) (for reviews see Refs. 1–3). Initially, PHFs were found to be composed of a protein component referred to as “A68” (4). Biochemical analysis revealed that A68 is identical to the microtubule-associated protein, tau (4, 5). Some characteristic features of tau isolated from PHFs (PHF-tau) are that it is abnormally hyperphosphorylated (phosphorylated on more sites than the normal brain tau), does not bind to microtubules, and does not promote microtubule assembly in vitro. Upon dephosphorylation, PHF-tau regains its ability to bind to and promote microtubule assembly (6, 7).

Neurofibrillary tangles (NFTs) and senile plaques are the two characteristic neuropathological lesions found in the brains of patients suffering from Alzheimer disease (AD).

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† The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S3.

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4 The abbreviations used are: NFT, neurofibrillary tangle; AD, Alzheimer disease; Cdk5, cyclin-dependent protein kinase 5; AU, absorption unit; FTDP-17, frontotemporal dementia and Parkinsonism linked to chromosome 17; PHF, paired helical filament; PKA, cAMP-dependent protein kinase; Pipes, 1,4-piperazinediethanesulfonic acid; WT, wild type.
mobility shift. Identification of such sites will significantly enhance our knowledge of how NFT pathology develops in the brain.

PHFs are also the major component of NFTs found in the brains of patients suffering from a group of neurodegenerative disorders collectively called tauopathies (2, 11). These disorders include frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17), corticobasal degeneration, progressive supranuclear palsy, and Pick disease. Each PHF-tau isolated from autopsied brains of patients suffering from various tauopathies is hyperphosphorylated, displays ~60-, 64-, and 68-kDa bands on SDS-gel, and is incapable of binding to microtubules. Upon dephosphorylation, the above-referenced PHF-tau migrates as a normal tau on SDS-gel, binds to microtubules, and promotes microtubule assembly (2, 11). These observations suggest that the mechanisms of NFT pathology in various tauopathies may be similar and the phosphorylation-dependent mobility shift of tau on SDS-gel may be an indicator of the disease. The tau gene is mutated in familial FTDP-17, and these mutations accelerate NFT pathology in the brain (15–18). Understanding how FTDP-17 mutations promote tau phosphorylation can provide a better understanding of how NFT pathology develops in AD and various tauopathies. However, when expressed in CHO cells, G272V, R406W, and P301L tau mutations reduce tau phosphorylation (19, 20). In COS cells, although G272V, P301L, and V337M mutations do not show any significant effect, the R406W mutation caused a reduction in tau phosphorylation (21, 22). When expressed in SH-SY5Y cells subsequently differentiated into neurons, the R406W, P301L, and V337M mutations reduce tau phosphorylation (23). In contrast, in hippocampal neurons, R406W increases tau phosphorylation (24). When phosphorylated by recombinant GSK3β in vitro, the P301L and V337M mutations do not have any effect, and the R406W mutation inhibits phosphorylation (25). However, when incubated with rat brain extract, all of the G272V, P301L, V337M, and R406W mutations stimulate tau phosphorylation (26). The mechanism by which FTDP-17 mutations promote tau phosphorylation leading to development of NFT pathology has remained unclear.

Cyclin-dependent protein kinase 5 (Cdk5) is one of the major kinases that phosphorylates tau in the brain (27, 28). In this study, to determine how FTDP-17 missense mutations affect tau phosphorylation, we phosphorylated four FTDP-17 tau mutants (G272V, P301L, V337M, and R406W) by Cdk5. We have found that phosphorylation of tau by Cdk5 causes a tau mobility shift to ~64- and 68-kDa bands. Although the mobility shift to a ~64-kDa band is achieved by phosphorylation at Ser396/404 or Ser202, the mobility shift to a 68-kDa band occurs only in response to phosphorylation at Ser202. We show that in vitro, FTDP-17 missense mutations, by promoting phosphorylation at Ser202, enhance the mobility shift to ~64- and 68-kDa bands and inhibit the microtubule assembly-promoting activity of tau. Our data suggest that Ser202 phosphorylation is the major event leading to NFT pathology in AD and related tauopathies.

**MATERIALS AND METHODS**

*cdDNA Cloning—*The longest isoform of human tau and FTDP-17 tau mutants G272V, P301L, V337M, and R406W, each in the pQE32 vector, were gifts from Dr. Peter Davies (Albert Einstein College of Medicine, Bronx, NY). Cloning of tau mutants S202A, T231A, and S396A in pcDNA3.1 vector is described previously (29). Tau mutants S262A and S214A in pcDNA3.1 vector were gifts from Dr. Nicole Leclerc (University of Montreal). Each DNA fragment from the WT or mutant tau was amplified by PCR using pfu DNA polymerase (Stratagene), with a forward primer (5’-AAAAACGCCCATAATGGCTGAGCCCGGC-3’) that contained an Ndel site and a reverse primer (5’-AAA AAA GGA TCC TCACAA ACC CTG CTT GG-3’) that contained a BamHI site, and subcloned into bacterial expression vector pET9a (Promega). Various double mutants, each containing the indicated FTDP-17 and S202A mutations, were cloned by PCR using their respective FTDP-17 mutant in pET9a vector as the template and the QuikChange II site-specific mutagenesis kit (Stratagene) following the manufacturer’s instruction manual. Primers used for PCR were 5’-CAG CGG CTA CAG CGC CCC AGG CAC TCC CGG CAG CGG CCG C-3’ and 5’-GCC GCT GCG GAG AGT GCC TGG GGC GCCGG GCT GCT GTA GCC GCT G-3’. All cDNA clones and mutations were confirmed by DNA sequencing.

*Proteins and Enzymes—*Tau(WT) and various tau mutants were purified from lysates of *Escherichia coli* overexpressing their respective tau species essentially as described previously (28). Briefly, tau expression was induced by adding isopropyl-1-thio-β-D-galactopyranoside (0.2 mM) to the overnight bacterial culture. The culture containing isopropyl-1-thio-β-D-galactopyranoside was allowed to grow for 3 h at 37 °C with shaking and then was centrifuged. The pellet was suspended in Pipes buffer (100 mM Pipes [pH 6.8], 1 mM EDTA, 1 mM MgSO4) containing 5 mg/ml benzamidine, 1 μg/ml leupeptine, 1 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, and 20 μg/ml lysozyme. The bacterial suspension was lysed by sonication and then clarified by centrifugation (15,000 rpm, 15 min at 4 °C). The supernatant was placed in a boiling water bath for 20 min and subsequently centrifuged. The heat-stable proteins in the supernatant were loaded onto an SP-Sepharose Fast Flow column (1 ml; Amersham Biosciences) equilibrated previously in Pipes buffer. The flow-through containing tau was loaded onto an SP-Sepharose Fast Flow column (~1 ml) equilibrated in Pipes buffer. The column was washed with ~20 column volumes of the Pipes buffer and then eluted with Pipes buffer containing 0.2 M NaCl. Fractions containing tau were pooled, concentrated with Aquacade III (Calbiochem) by dialysis, dialyzed against Heps buffer (25 mM Heps [pH 7.2], 0.1 mM EDTA, 0.5 mM dithiothreitol, and 100 mM NaCl), and stored at ~80 °C until use. Microtubules were purified from fresh bovine brain extract by three cycles of temperature-induced microtubule polymerization and depolymerization as described previously (28, 30). Tubulin was purified from microtubules through phosphocellulose chromatography (28, 30). Monoclonal tau 5 antibody against total tau and tau phosphorylation-dependent monoclonal antibodies AT8, PHF-1, MC6, and TG3
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have been described previously (29, 31). Polyclonal antibodies pS202 and pT212 against tau phosphorylated at Ser202 and Thr212, respectively, were purchased from BIOSOURCE. Cdk5 was purified from the extract of fresh bovine brain (28). The active catalytic subunit of PKA was purchased from Sigma-Aldrich. Purification of protein phosphatase 1 (PP1) from E. coli extract overexpressing human PP1α has been described previously (32, 33).

Protein Concentrations—Tau(WT) concentration is based on its absorption at 280 nm as described previously (28). The concentrations of various FTDP-17 tau mutants were determined by Bio-Rad protein assay using tau(WT) as the standard. Concentrations of phosphorylated tau and tau mutants were also determined by Bio-Rad protein assay using tau(WT) as the standard. The concentration of Cdk5 is based on its activity (28). PKA concentration was determined by its dry weight. The concentrations of all other proteins were determined by Bio-Rad proteins assay using bovine serum albumin as the standard.

Tau Phosphorylation—Tau(WT) and various tau mutants were phosphorylated by Cdk5 under identical conditions. Each phosphorylation mixture contained 25 mM Hepes (pH 7.2), 0.1 mM EDTA, 0.2 mM dithiothreitol, 0.1 mM NaCl, 10 mM MgCl₂, 0.5 mM \([\gamma^{32}P]\) ATP, 1.0 mg/ml tau, and 400 units/ml Cdk5. The reaction was initiated by adding an aliquot of Cdk5 to a vial containing the rest of the phosphorylation mixture at 30 °C. At the indicated time points, aliquots were withdrawn and analyzed for phosphorylation by filter paper assay (34) or subjected to SDS-PAGE followed by Western blot analysis. Gel and blot bands were scanned, and the band intensity values were used to determine the relative amounts of various proteins. Phosphorylation of tau and tau mutants by PKA was also performed as described above, except Cdk5 was replaced by PKA at a concentration of 10 μg/ml each.

Microtubule Assembly Assay—The microtubule assembly was monitored by measuring the increase in absorbance at 350 nm as described previously (35). The vial containing all of the components of the assay except tau was incubated at 37 °C for 1 min in a water bath. To the incubated vial, the indicated prewarmed tau species was added. After gentle mixing, the content of the vial was transferred immediately to a quartz cuvette placed in a spectrophotometer at 37 °C. The increase in the absorbance at 350 nm of the transferred sample was recorded at 1-min intervals for 32 min. The final concentrations of various components in the assay were 0.75 mg/ml tubulin, 100 mM Pipes (pH 6.8), 1 mM EGTA, 1 mM dithiothreitol, 2 mM MgSO₄, 1 mM GTP, 10 mM taxol, and 0.2 mg/ml tau (indicated species). The lag phase of polymerization is defined as the time at which the absorbance at 350 nm reaches a minimum, followed by a steady increase in absorbance (35). The rate of polymerization is the slope of the absorbance at 350 nm as a function of time (35). The concentration of tubulin was kept constant so that no detectable turbidity was observed in the absence of tau.

Microtubules in the presence of tau(WT) assembled with a lag time of 2 min at a rate of 0.0363 AU/min. When P301L was used, the lag phase was extended to 6 min and the polymerization rate was reduced to 0.0036 AU/min (Fig. 1 and supplemental Table S1). These data determined that in the presence of P301L, microtubule nucleation and polymerization occurred 3 and 1.75 times, respectively, slower than in the presence of tau(WT). This, in turn, indicates that the microtubule nucleation-promoting activity of P301L is 33.3% of that of the WT. Likewise, microtubule polymerization-promoting activity of P301L is 57% of that of the WT. In the presence of P301L, these data indicate that the microtubule formation-promoting activity of P301L is 57.1% of that of the WT. The

![FIGURE 1. Microtubule assembly in the presence of phosphorylated and nonphosphorylated tau(WT) or FTDP-17 tau mutants.](image-url)
Phosphorylation of FTDP-17 Tau Mutants

![Phosphorylation of FTDP-17 Tau Mutants](image)

**FIGURE 2. SDS-PAGE of phosphorylated tau(WT) and FTDP-17 tau mutants.** Indicated tau species (5 µg each) phosphorylated by Cdk5 under identical conditions for 60 min (lanes 2–5) or control samples incubated with all the components of the phosphorylation mixture except Cdk5 (lanes 6–9) were subjected to 10% SDS-PAGE. The resulting gel was stained with Coomassie Brilliant Blue. M (lane 1) represents standard molecular weight marker.
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To determine the basis of the mobility shift, we wanted to know first whether FTDP-17 mutations stimulate tau phosphorylation. We phosphorylated tau(WT) and various FTDP-17 tau mutants by Cdk5 under identical conditions for different time periods and determined the number of phosphate incorporated into each tau species. Tau(WT) incorporated \(1.1 \text{ mol of phosphate/mol of protein in 15 min. This value increased to } \sim 2.4 \text{ in 30 min and became } \sim 4.1 \text{ in 60 min. On SDS-gel, tau(WT) phosphorylated for 15 min migrated as a } \sim 60-\text{kDa band but became } \sim 60-\text{ and 64-kDa bands after 60 min of phosphorylation (data not shown). G272V incorporated } \sim 1.5, 3.4, \text{ and } 5.2 \text{ mol of phosphate/mol of protein in 15, 30, and 60 min, respectively. In 15, 30, and 60 min, P301L contained } \sim 1.3, 2.5, \text{ and } 4 \text{ mol of phosphate/mol of protein, respectively, whereas V337M incorporated } \sim 1.2, 3.2, \text{ and } 3.9 \text{ mol of phosphate/mol of protein, respectively. R406W, on the other hand, incorporated more phosphate, whereas P301L and V337M incorporated amounts similar to the WT. R406W, on the other hand, incorporated less phosphate than the WT. On SDS-gel, however, all FTDP-17 mutants phosphorylated for 60 min migrated as } \sim 60-\text{, 64-, and 68-kDa bands (data not shown, but see Fig. 2).}

These data indicated that the increased mobility shift of phosphorylated FTDP-17 mutants was not due to the higher extent of total phosphorylation of mutants compared with the WT.

Effect of FTDP-17 Mutations on Site-specific Phosphorylation of Tau—To evaluate whether FTDP-17 mutations, by promoting tau phosphorylation at any specific site(s), increase the tau mobility shift, we phosphorylated tau(WT) and various FTDP-17 tau mutants by Cdk5 under identical conditions. Controls were S214A and S262A. Phosphorylated products were analyzed by Western blot using antibodies that recognize phosphorylated tau on proline-directed sites Ser396, Ser404, Ser235, Thr231, Ser202/205, and Thr212, which are potential targets of Cdk5 (27).

As shown in Fig. 3, compared with the WT, G272V and P301L are more phosphorylated and R406W is less phosphorylated at Ser\textsuperscript{396/404}. At Ser\textsuperscript{235}, G272V and V337M are more phosphorylated and R406W is less phosphorylated than the WT. At Thr\textsuperscript{231}, WT and all mutants are phosphorylated to similar extents. However, at Ser\textsuperscript{202}, G272V, P301L, V337M, and R406W are 1.8-, 2.2-, 2.1-, and 2.5-fold more phosphorylated than the WT. At Thr\textsuperscript{212}, on the other hand, neither WT nor any of the FTDP-17 mutants was phosphorylated. Thus, at Ser\textsuperscript{235} and Ser\textsuperscript{396/404}, some mutations promoted and some inhibited phosphorylation. At Ser\textsuperscript{202}, neither the S214A nor the S262A control showed any effect, whereas all FTDP-17 mutations pro-
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Phosphorylation of tau and FTDP-17 tau mutants by Cdk5 on Ser\textsuperscript{202}. The indicated tau species were phosphorylated by Cdk5. At the indicated time points, aliquots were withdrawn, and 1 \( \mu \)g of each sample was subjected to Western blot analysis using AT8 antibody that recognizes tau phosphorylated at Ser\textsuperscript{202}. Based on the intensities of various bands at the 60-min time point, the relative amount of a ~68-kDa band of each sample was determined. A, Western blots. B, relative amount. To calculate the relative amount, the intensity value of the ~68-kDa band of each sample in each blot was normalized against the sum of the band intensity values of ~60-, 64-, and 68-kDa bands of that sample in that blot. The values are the average of three determinations.

Promoted phosphorylation. To substantiate these data, we analyzed all of the above phosphorylated samples by using a monoclonal AT8 antibody specific for Ser\textsuperscript{202}-phosphorylated tau. Like pS202, AT8 antibody indicated that all FTDP-17 mutants promote tau phosphorylation at Ser\textsuperscript{202} (data not shown).

When Western blotted using tau 5 antibody for total tau, phosphorylated tau(WT) showed two major bands of sizes ~60 and 64 kDa and a faint ~68-kDa band, whereas V337M, R406W, P301L, and G272V displayed prominent ~60-, 64-, and 68-kDa bands (Fig. 3A). PHF-1 antibody, specific for Ser\textsuperscript{396/404}-phosphorylated tau, stained ~60- and 64-kDa bands and failed to recognize the ~68-kDa band of all tau species. Similarly, MC6 and TG3 antibodies, which recognize tau phosphorylated at Ser\textsuperscript{235} and Thr\textsuperscript{231}, respectively, stained only a ~60-kDa band for WT and all FTDP-17 mutants. However, pS202 antibody, specific for tau phosphorylated at Ser\textsuperscript{202}, recognized all three, the ~60-, 64-, and 68-kDa bands. These data indicate that the ~60-kDa band of all tau species is phosphorylated at Ser\textsuperscript{202} (Fig. 3A, lane 4). Thus, unlike tau(WT), S202A failed to display the ~68-kDa mobility shift in response to Cdk5 phosphorylation. These data indicate that tau does not display mobility shift to the ~68-kDa band upon phosphorylation by Cdk5 if phosphorylation at Ser\textsuperscript{202} is blocked.

Tau is phosphorylated at multiple sites (8). Studies have shown that tau phosphorylation on some sites affect subsequent phosphorylation on other sites (29). To determine whether blocking Ser\textsuperscript{202} phosphorylation may inhibit phosphorylation at other sites, which may prevent mobility shift, phosphorylated tau(WT) and S202A were Western blotted using antibodies that recognize phosphorylated tau (Fig. 6). Tau(WT) was phosphorylated at Ser\textsuperscript{202}, Ser\textsuperscript{396/404}, Ser\textsuperscript{235}, and Thr\textsuperscript{231} (Fig. 6, lane 3). S202A, on the other hand, was phosphorylated on all of the above sites except Ser\textsuperscript{202} (Fig. 6, lane 4). Thus, blocking Ser\textsuperscript{202} phosphorylation did not prevent phosphorylation at any of the major sites known to be phosphorylated by Cdk5.

Cdk5 phosphorylates tau on a number of sites (27). Among these sites, Ser\textsuperscript{396} and Thr\textsuperscript{231} were reported to cause tau conformational change (6, 29, 40). To evaluate whether mobility shift caused by Cdk5 phosphorylation is specific for Ser\textsuperscript{202}, we phosphorylated tau(WT), S202A, S396A, and T231A by Cdk5 and analyzed the products by Western blot analysis (Fig. 7). Phosphorylated tau(WT) migrated as ~60-, 64-, and 68-kDa bands (Fig. 7, lanes 12–15). Phosphorylated S202A was not phosphorylated at Ser\textsuperscript{202}, and it displayed ~60- and 64-kDa but not ~68-kDa bands (Fig. 7, lanes 7–10). S396A was phosphorylated tau(WT), ~40% remained as a ~60-kDa band and the rest shifted to ~64 (~50%)- and 68 (~10%)-kDa bands (Fig. 4B). G272V and P301L, on the other hand, displayed a ~68-kDa band with a relative amount (48 and 49%), respectively, of the total. The relative amount of ~68-kDa bands was 45 and 44% of the total in V337M and R406W, respectively. When compared with the WT, G272V, P301L, V337M, and R406W displayed, respectively ~4.8-, 4.9-, 4.5-, and ~4.4-fold more Ser\textsuperscript{202}-phosphorylated ~68-kDa band upon phosphorylation by Cdk5 (Fig. 4B). Thus, all FTDP-17 mutations promoted phosphorylation at Ser\textsuperscript{202} as well as mobility shift to a ~68-kDa band.

Effect of Ser\textsuperscript{202} Phosphorylation on Tau Structure—Tau(WT) displayed a mobility shift that correlated with phosphorylation at Ser\textsuperscript{202}, and FTDP-17 mutations accelerated this process (Figs. 3 and 4). To determine the significance of this phenomenon, we wanted to know the role of Ser\textsuperscript{202} phosphorylation on the tau mobility shift on SDS-gel/Western blot. We phosphorylated tau(WT) and the site-specific tau mutant S202A under identical conditions and analyzed the products by Western blot analysis. Tau(WT) became increasingly phosphorylated with time (Fig. 5A, lanes 2–5). At 15 min, tau(WT) displayed mobility shift to ~64 kDa, which became prominent at 60 min (Fig. 5B, lanes 2–4). At 120 min, the ~60-kDa band of tau(WT) faded significantly with the appearance of a ~68-kDa band (Fig. 5B, lane 5). S202A showed mobility shift, and a ~64-kDa band became visible at 15 min. With increasing time, the relative amount of ~64-kDa band increased progressively, and that of the ~60-kDa band decreased. However, even with phosphorylation at 120 min the ~68-kDa band was not formed significantly (Fig. 5B, lane 11). Thus, unlike tau(WT), S202A failed to display the ~68-kDa mobility shift in response to Cdk5 phosphorylation. These data indicate that tau does not display mobility shift to the ~68-kDa band upon phosphorylation by Cdk5 if phosphorylation at Ser\textsuperscript{202} is blocked.
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FIGURE 5. Effect of Ser\textsuperscript{202} phosphorylation on SDS-gel mobility of tau. Tau(WT) and tau(S202A) phosphorylated by Cdk5 using \([\gamma\textsuperscript{32}P]ATP\) for the indicated time points were analyzed by Western blot (IB). The blot was subsequently autoradiographed to monitor radioactivity in each band.

FIGURE 6. Site-specific phosphorylation of tau(WT) and tau(S202A) by Cdk5. Tau(WT) and tau(S202A), phosphorylated for 120 min, were analyzed by Western blot (IB) using the indicated antibodies.

we phosphorylated tau WT and S202A by Cdk5 or PKA for 120 min. Products were analyzed by Western blot using tau 5 antibody (data not included). Tau(WT) phosphorylated by Cdk5 for 120 min showed ~60-, 64-, and 68-kDa bands. S202A phosphorylated by Cdk5 under identical conditions showed only ~60- and 64-kDa bands. Both tau(WT) and S202A phosphorylated by PKA, on the other hand, showed two bands of sizes ~60 and 64 kDa (data not shown). Thus, Cdk5 phosphorylates tau at Ser\textsuperscript{202} and causes mobility shift to a ~68-kDa band, and PKA, which does not phosphorylate Ser\textsuperscript{202}, does not cause this shift. Based on these results, we concluded that phosphorylation at Ser\textsuperscript{202} is a major determinant for the tau mobility shift to the ~68-kDa band upon Cdk5 phosphorylation.

Effect of Ser\textsuperscript{202} Phosphorylation on Microtubule Assembly-promoting Activity of Tau—To determine why FTDP-17 mutations promote phosphorylation at Ser\textsuperscript{202}, we examined the effect of Ser\textsuperscript{202} phosphorylation on the microtubule assembly-promoting activity of tau. We phosphorylated WT and S202A by Cdk5. As controls, we phosphorylated T231A, S396G, and S214A. Thr\textsuperscript{231} and Ser\textsuperscript{396} are phosphorylated \textit{in vivo} (8) and \textit{in vitro} by Cdk5 (27). Ser\textsuperscript{214} is not phosphorylated by Cdk5, and hence it was used to monitor the effect of the Ser to Ala mutation on tau activity. Microtubule assembly-promoting activities of non-phosphorylated and phosphorylated tau were monitored.

As shown in Fig. 8 and supplemental Table S2, nonphosphorylated S202A, T231A, S396G, S214A, and WT promoted microtubule assembly with similar nucleation time and polymerization rate, leading to the formation of similar amounts of microtubules. Likewise, the microtubule assembly-promoting activities of phosphorylated WT and phosphorylated S214A are similar. These data demonstrate that mutation of any nonspecific Ser to Ala does not significantly affect the microtubule assembly-promoting activity of tau.

The microtubule assembly-promoting activity of all phosphorylated WT, S214A, T231A, and S396G was significantly less than their respective nonphosphorylated counterparts (Fig. 8A). This observation indicated that phosphorylation inhibits the microtubule assembly-promoting activities of all the above tau species. Moreover, although the microtubule assembly-promoting activity of phosphorylated T231A is similar to that of phosphorylated WT, those of phosphorylated S396G and S202A are higher (Fig. 8A). Thus, the microtubule assembly-promoting activity of phosphorylated tau is not affected significantly by blocking Thr\textsuperscript{231} phosphorylation. Blocking phosphorylation at Ser\textsuperscript{396} or Ser\textsuperscript{202}, however, increases the microtubule assembly-promoting activity of phosphorylated tau. These
data, in turn, indicate that phosphorylation at Ser\textsuperscript{396} or Ser\textsuperscript{202} decreases the microtubule assembly-promoting activity of tau.

Microtubules, in the presence of phosphorylated WT, nucleated at 6 min (supplemental Table S2). In the presence of phosphorylated S202A, nucleation time was reduced to 5 min, an increase of 16.5% nucleation promoting activity. This means that Ser\textsuperscript{202} phosphorylation inhibits 16.6% of tau microtubule nucleation-promoting activity (Fig. 8B). In the presence of phosphorylated WT, microtubules polymerized with a rate of 0.016 AU/min. In the presence of phosphorylated S202A, the microtubule polymerization rate was increased 1.5-fold to 0.024 AU/min. These data indicate that Ser\textsuperscript{202} phosphorylation inhibits 50% of tau microtubule polymerization-promoting activity (Fig. 8B). Finally, the amount of microtubules formed in the presence of phosphorylated S202A was 1.4 times more than that formed in the presence of phosphorylated WT, indicating that Ser\textsuperscript{202} phosphorylation inhibits 40% of tau microtubule formation-promoting activity. Furthermore, the microtubule nucleation polymerization and formation-promoting activities of phosphorylated S202A are higher than that of phosphorylated Ser\textsuperscript{396} (supplemental Table S2 and Fig. 8B). These data show that the microtubule assembly-promoting activity of tau is inhibited more by phosphorylation at Ser\textsuperscript{202} than by phosphorylation at Ser\textsuperscript{396}. Taken together, these data indicate that phosphorylation at Ser\textsuperscript{202} has the major inhibitory impact on the microtubule assembly-promoting activity of tau in vitro.

Effect of Ser\textsuperscript{202} Phosphorylation on SDS-gel Mobility Shift of FTDP-17 Tau Mutants—To determine whether missense FTDP-17 mutations promote mobility shift by promoting phosphorylation at Ser\textsuperscript{202}, we mutated Ser\textsuperscript{202} of each of the FTDP-17 single mutants, WT, and S202A by Cdk5. All phosphorylated proteins were then analyzed by Western blot. All nonphosphorylated single and double mutants migrated as single ~60-kDa band on SDS-gel (Fig. 9A). Phosphorylated WT migrated as ~60- and 64-kDa bands and a relatively weak ~68-kDa band (Fig. 9B, lane 3), whereas phosphorylated S202A...
of all FTDP-17 tau mutants. These data indicate that FTDP-17 mutations lose their abilities to promote mobility shift to a ~68-kDa band if phosphorylation at Ser²⁰² is blocked. This in turn indicates that FTDP-17 mutations promote the ~68-kDa band mobility shift by enhancing phosphorylation at Ser²⁰².

**Effect of Ser²⁰² Phosphorylation on Microtubule Assembly-promoting Activity of FTDP-17 Tau Mutants**—Finally, we monitored the microtubule assembly-promoting activities of phosphorylated double mutants and their respective FTDP-17 single mutants. Nonphosphorylated WT and WT(S202A) promoted microtubule assembly with similar nucleation time and polymerization rate and caused the formation of similar amounts of microtubules. Likewise, nonphosphorylated double mutants G272V/S202A, P301L/S202A, V337M/S202A, and R406W/S202A supported microtubule assembly in a manner similar to their respective FTDP-17 single mutants (data not included). Thus, mutation of Ser²⁰² to Ala did not affect the microtubule assembly-promoting activity of WT or any of the FTDP-17 mutants.

Phosphorylated WT(S202A) displayed microtubule nucleation, polymerization, and formation-promoting activities higher than that of phosphorylated WT (Fig. 10A and supplemental Table S3). These data are consistent with the observation made in Fig. 8, demonstrating that blocking Ser²⁰² phosphorylation enhances the microtubule assembly-promoting activity of phosphorylated tau. As shown in Fig. 10A and supplemental Table S3, microtubule nucleation-promoting activity of phosphorylated G272V/S202A is more than that of phosphorylated G272V. Likewise, microtubule polymerization and formation-promoting activities of phosphorylated G272V/S202A are higher than that of phosphorylated G272V. Moreover, microtubule nucleation, polymerization, and formation-promoting activities of phosphorylated P301L/S202A, V337M/S202A, and R406W/S202A are higher than those of their respective phosphorylated FTDP-17 single mutants. Thus, as with phosphorylated WT, blocking Ser²⁰² phosphorylation increased the microtubule assembly-promoting activity of all phosphorylated FTDP-17 mutants. This indicates that
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Ser\textsuperscript{202} phosphorylation inhibits the microtubule assembly-promoting activity of tau(WT) and all its FTDP-17 mutants. Microtubule nucleation-promoting activity of phosphorylated WT is 6 min, whereas that of phosphorylated WT(S202A) is 5 min (supplemental Table S3). This indicates that blocking Ser\textsuperscript{202} phosphorylation increases the nucleation activity of phosphorylated WT by 16.6%. This, in turn, indicates that 16.6% of the microtubule nucleation-promoting activity of tau(WT) is inhibited by Ser\textsuperscript{202} phosphorylation. As shown in Fig. 10B, Ser\textsuperscript{202} phosphorylation inhibits the microtubule nucleation-promoting activity of G272V, P301L, V337M, and R406W by 22.2, 18.2, 27.3, and 25.0%, respectively. Ser\textsuperscript{202} phosphorylation inhibits the microtubule polymerization-promoting activity of WT by 37.5% and that of G272V, P301L, V337M, and R406W by 45.0, 63.6, 44.2, and 70.0%, respectively. Similarly, Ser\textsuperscript{202} phosphorylation inhibits the microtubule formation-promoting activity of WT by 40.6%, which is increased in G272V, P301L, V337M, and R406W mutants to 53.8, 71.4, 65.2, and 88.9%, respectively. Thus, compared with the WT, Ser\textsuperscript{202} phosphorylation inhibits the microtubule nucleation-promoting activity of G272V, P301L, V337M, and R406W 1.64-, 1.60-, 1.50-fold more, respectively, and microtubule formation-promoting activity 1.34-, 1.10-, 1.05-fold more, respectively, and microtubule formation-promoting activity 1.32-, 1.75-, 1.60-, and 2.20-fold more, respectively, and microtubule formation-promoting activity 1.32-, 1.75-, 1.6-, and 2.20-fold more, respectively (Fig. 10C). These data indicate that phosphorylation at Ser\textsuperscript{202} has a more profound inhibitory effect on the microtubule assembly-promoting activity of FTDP-17 mutants than on that of the WT.

DISCUSSION

The presence of ~64- and 68-kDa tau bands is a characteristic feature of the AD brain, and studies suggest that the appearance of these species correlates with the disease progression (12, 13). Because these tau species are formed due to abnormal tau phosphorylation, tau sites that are responsible for causing their formation are suggested to be involved in the development of AD pathology in brain (12, 13).

Cdk5 is one of the kinases suggested to phosphorylate tau in the AD brain (27, 39). In vitro, Cdk5 phosphorylates tau on several sites that are phosphorylated in PHFs including Ser\textsuperscript{202}, Thr\textsuperscript{231}, and Ser\textsuperscript{396} (27). In this study, nonphosphorylated tau migrated as a ~60-kDa band on SDS-gel. Upon phosphorylation by Cdk5, tau migrated as ~60, ~64-, and ~68-kDa bands (Fig. 4). These data indicate that phosphorylation by Cdk5 cause mobility shift of tau to ~64- and 68-kDa bands. Although phosphorylation at Ser\textsuperscript{396}/404 also promoted tau mobility shift to a ~64-kDa band, only Ser\textsuperscript{202}-phosphorylated tau displayed both ~64- and 68-kDa bands, and phosphorylation at Ser\textsuperscript{202} correlated with their formation (Figs. 3 and 4). Furthermore, blocking Thr\textsuperscript{231} or Ser\textsuperscript{396} did not affect tau mobility shift to ~64- or 68-kDa bands. Blocking Ser\textsuperscript{202} phosphorylation blocked mobility shift to a ~68-kDa band (Fig. 7). This indicates that tau mobility shift to ~64-kDa band can occur without Ser\textsuperscript{202} phosphorylation. However, mobility shift to a ~68-kDa band in response to Cdk5 phosphorylation requires Ser\textsuperscript{202} phosphorylation.

As shown in Fig. 3, the ~68-kDa band formed by Cdk5 phosphorylation is phosphorylated on Ser\textsuperscript{202} but not at Ser\textsuperscript{396}/404, Thr\textsuperscript{231}, Ser\textsuperscript{396}, or Thr\textsuperscript{212}. This indicates that the ~68-kDa band is formed as a result of phosphorylation at Ser\textsuperscript{202} but not at Ser\textsuperscript{396}/404, Thr\textsuperscript{231}, Ser\textsuperscript{396}, or Thr\textsuperscript{212}. Similarly, the ~64-kDa band is phosphorylated at Ser\textsuperscript{202} and Ser\textsuperscript{396}/404 but not at Thr\textsuperscript{231}, Ser\textsuperscript{396}, and Thr\textsuperscript{212}, indicating that the ~64-kDa band is formed due to phosphorylation at Ser\textsuperscript{202} and Ser\textsuperscript{396}/404 and not at Thr\textsuperscript{231}, Ser\textsuperscript{396}, or Thr\textsuperscript{212}. Furthermore, the Ser\textsuperscript{202}-phosphorylated tau first migrates as a ~60-kDa band (Fig. 4A, lane 2). With the increase in phosphorylation time, ~64-kDa band followed by ~68-kDa band appear. These data suggest that Ser\textsuperscript{202}-phosphorylated tau migrates as a ~60-kDa band and that phosphorylation on this site alone does not cause a tau band shift. The mobility shift of tau may, therefore, occur by a sequential mechanism. First-step phosphorylation at Ser\textsuperscript{202} may allow Cdk5 to perform second-step phosphorylation on new sites that are not accessible in non-Ser\textsuperscript{202}-phosphorylated tau. First-plus second-step-phosphorylated tau may then migrate as a ~64-kDa band. The ~64-kDa phosphorylated tau may then undergo third-step phosphorylation on additional sites and become hyperphosphorylated. The hyperphosphorylated tau may then migrate as a ~68-kDa band. Note that in this mechanism, the ~68-kDa band will be expected to contain phosphate on all the sites, including Ser\textsuperscript{202}, that are involved in the mobility shift from ~60 to ~64-kDa and then to 68-kDa bands. As shown in Fig. 3, the ~68-kDa band is phosphorylated at Ser\textsuperscript{202} but not at Ser\textsuperscript{396}/404, Ser\textsuperscript{396}, Thr\textsuperscript{231}, or Thr\textsuperscript{212}. This observation suggests that phosphorylation on the new, additional sites that occurs after phosphorylation at Ser\textsuperscript{202} and results in a ~60–68-kDa band shift, has to occur at sites other than Ser\textsuperscript{396}/404, Ser\textsuperscript{396}, Thr\textsuperscript{231}, and Thr\textsuperscript{212}. In fact, in addition to the sites mentioned above, PHF-tau is phosphorylated on a number of proline-directed sites, including Ser\textsuperscript{199}, Thr\textsuperscript{181}, Thr\textsuperscript{217}, and Ser\textsuperscript{222}, which are potential targets of Cdk5 (8).

Our data have demonstrated that Thr\textsuperscript{231} phosphorylation did not have a significant effect on microtubule nucleation or formation-promoting activities and only slightly affected the microtubule polymerization-promoting activity of tau (Fig. 8A). Likewise, Ser\textsuperscript{396} phosphorylation inhibited microtubule polymerization and formation-promoting activities but did not affect microtubule nucleation-promoting activity (Fig. 8A and supplemental Table S2). Ser\textsuperscript{202} phosphorylation, on the other hand, not only inhibited microtubule nucleation-promoting activity, but it inhibited microtubule polymerization and formation-promoting activities 2- and 1.5-fold more than Ser\textsuperscript{396} phosphorylation (Fig. 8B). Our results indicate that Ser\textsuperscript{202} phosphorylation significantly inhibits the microtubule assembly-promoting activity of tau in vitro. It should be noted that under our experimental conditions, Cdk5 may have phosphorylated tau at Ser\textsuperscript{202} to a higher extent than at Ser\textsuperscript{396} and Thr\textsuperscript{231}. The observed difference in the ability of each of the above sites to inhibit tau microtubule assembly-promoting activity may be due, in part, to the differences in extent of phosphorylation.

The pathological significance of Ser\textsuperscript{202} phosphorylation in the brain is not very clear. Studies suggest that mild memory impairment is the earliest clinical feature of AD and is associated with subtle cytoskeletal alterations in pre-tangle neurons. This alteration can be detected by immunohistochemical anal-
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ysis of the brain using AT8 monoclonal antibody (43, 44). The AT8 epitope first appears in the pre-AD brain areas that do not show any brain degeneration and are devoid of NFTs. But slowly as the disease progresses and the brain begins to degenerate, the intensity of AT8 reactivity increases (43–45). Among several tau phosphorylation-sensitive antibodies tested, AD brains stain strongest with AT8 (46). When purified PHF-tau is Western blotted, ~60-, 64-, and 68-kDa tau bands cross-react with AT8 antibody (47). AT8 immunoreactivity in the brain is regarded as the abnormal cytoskeletal change that occurs during the AD development (43, 44). In vitro AT8 specifically recognizes tau phosphorylated at Ser181 and/or Ser202 (48). However, mass spectrometric studies have determined that PHF-tau is phosphorylated at Ser202 but not at Ser205 (8, 9). Thus, AT8 immunoreactivity in the brain represents tau phosphorylated at Ser202. Moreover, among several sites tested, only phosphorylation at Ser202 correlates with mobility shift of tau to ~64- and 68-kDa bands upon Cdk5 phosphorylation in vitro (Figs. 3 and 4). Also, all pathogenic FTDP-17 tau missense mutations that accelerate NFT pathology in the brain promote phosphorylation at Ser202 (Figs. 3 and 4). Taken together, these observations suggest that Ser202 phosphorylation is the major pathological event in the brain leading to brain degeneration in AD.

Exonic and intronic mutations have been discovered in the familial type of FTDP-17. Although intronic mutations interfere with pre-mRNA splicing leading to an increase in four-repeat tau in the brain, the mechanism by which exonic mutations promote NFT pathology is an area of current research in neurobiology. Studies using antibodies directed against tau phosphorylated at various sites have determined that V337M FTDP-17 brains stain most intensely with AT8 (9), less intensely with PHF-1 (Ser396/404), AT100 (Thr212/Ser214), AT180 (Thr231/Ser235), AT270 (Ser181), and 12E8 (Ser262), indicating that V337M in the brain is most strongly phosphorylated at Ser202 and less strongly at Ser396/404, Thr212/Ser214, Thr231/Ser235, Ser181, and Ser262 (46). Likewise, P301L in the brain is most extensively phosphorylated at Ser202, Ser396/404, and Thr212/Ser214 and less extensively at Thr231/Ser235 and Ser262 (49, 50). G272V in the brain is most extensively phosphorylated at Ser202, less extensively at Ser396/404 and Thr212/Ser214, but not at all at Ser262 (17). Finally, R406W in the brain is most strongly phosphorylated at Ser202, Ser396/404, and Thr212/Ser214 (50). Thus, among all of the different sites examined, all FTDP-17 tau mutants in the brain are most extensively phosphorylated at Ser202 recognized by AT8 antibody. In addition, PHF-tau isolated from all FTDP-17 mutant brains migrates as ~60-, 64-, and 68-kDa bands on SDS-gels (11, 17, 46, 49, 50), and in vitro Ser202 phosphorylation promotes tau mobility shift to ~64- and 68-kDa bands (Fig. 4). These studies indicate that all missense FTDP-17 mutations promote tau phosphorylation at Ser202 in the brain.

In this study, we examined four FTDP-17 tau mutations, G272V, P301L, V337M, and R406W, and found that these mutations, by promoting phosphorylation at some sites and inhibiting at some sites, do not significantly affect the total amount of phosphate incorporated into the tau molecule. Although phosphorylation at Thr231 is not influenced by any mutation, phosphorylation at Ser396/404 is promoted by G272V and P301L but inhibited by R406W (Fig. 3). Ser235 phosphorylation is promoted by V337M, G272V, and P301L but is inhibited by R406W. Phosphorylation at Ser202, on the other hand, is promoted by all of the FTDP-17 missense mutations, and by enhancing phosphorylation at Ser202 each mutation promoted a tau mobility shift to ~64- and 68-kDa bands (Figs. 3, 4, and 9). Furthermore, compared with the phosphorylated WT, each phosphorylated FTDP-17 mutant displayed reduced microtubule assembly-promoting activity (Fig. 10). When Ser202 phosphorylation was blocked, each phosphorylated mutant recovered more relative amounts of microtubule assembly-promoting activity than the phosphorylated WT (Fig. 10a and supplemental Table S3). Moreover, compared with the WT, G272V is 175% more phosphorylated at Ser202 (Fig. 3) and causes 1.4 times less microtubule formation than the phosphorylated WT (supplemental Table S3). Likewise, P301L, V337M, and R406W are phosphorylated 225, 219, and 259% more than the WT at Ser202. The amount of microtubules formed in the presence of phosphorylated P301L, V337M, and R406W is 1.7-, 1.5-, and 2.2-fold less than formed in the presence of phosphorylated WT. Thus there is a correlation between the amount of Ser202 phosphorylation and the amount of loss in the microtubule formation-promoting activity of various FTDP-17 tau mutants. Our data indicate that FTDP-17 missense mutations inhibit tau microtubule assembly-promoting activity by promoting tau phosphorylation at Ser202, suggesting that FTDP-17 mutations may accelerate NFT pathology by increasing Ser202 phosphorylation in the brain.

Previous studies have shown that ~60-, 64-, and 68-kDa tau bands of AD brain are also immunoreactive to PHF-1 antibody specific for Ser396/404-phosphorylated tau (51). This observation suggests that Ser396/404 phosphorylation may also promote the formation of ~60-, 64-, and 68-kDa tau bands in the brain. However, phosphorylation by Cdk5 at Ser396/404 does not cause tau mobility shift to the ~68-kDa band (Fig. 3). Our data indicate that Cdk5 phosphorylation alone is not sufficient for the Ser396/404-phosphorylated ~68-kDa mobility shift. It is possible that for the formation of the Ser396/404-phosphorylated ~68-kDa tau band, in addition to Cdk5, phosphorylation by another or other brain kinases may be required. Alternatively, the Ser396/404-phosphorylated ~68-kDa band in the brain may result from phosphorylation by kinases other than Cdk5.

In this study we used the longest human tau isoform, which migrates as a ~60-kDa band on SDS-gels. We showed that upon Cdk5 phosphorylation, this isoform migrates as ~60-, 64-, and 68-kDa bands on an SDS-gel. We also demonstrated that various FTDP-17 mutations enhance the mobility shift of this isoform from ~60- to 64-kDa and ~68-kDa bands upon Cdk5 phosphorylation. Because PHF-tau isolated from AD brain migrates as ~60-, 64-, and 68-kDa bands on an SDS-gel (4, 5, 11), we argued that Cdk5 phosphorylation converts tau to a PHF-like state. However, in adult human brain there are six tau isoforms, and all are present in PHFs (52). Migration of PHF-tau as ~60-, 64-, and 68-kDa bands may also be due in part to the difference in the sizes of various tau isoforms present in PHFs. More studies will be required to determine how Cdk5 phosphorylation affects the SDS-gel mobility of tau isoforms not analyzed in this study.
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