Phosphorylation of FTDP-17 Mutant tau by Cyclin-dependent Kinase 5 Complexed with p35, p25, or p39*

Fumika Sakaue‡, Taro Saito‡, Yutaka Sato‡, Akiko Asada‡, Koichi Ishiguro‡, Masato Hasegawa‡, and Shin-ichi Hisanaga‡

From the ‡Department of Biological Sciences, Graduate School of Science, Tokyo Metropolitan University, Hachioji, Tokyo 192-0397, ‡Department of Molecular Neurobiology, Tokyo Institute of Psychiatry, Setagaya, Tokyo 156-8585, and §Mitsubishi Kagaku Institute of Life Sciences, Machida, Tokyo 194-8511, Japan

One of the major pathological hallmarks of Alzheimer disease is neurofibrillary tangles. Neurofibrillary tangles are bundles of paired helical filaments composed of hyperphosphorylated tau. Cyclin-dependent kinase 5 (Cdk5) is one of the tau protein kinases that increase paired helical filament epitopes in tau by phosphorylation. Recently, various mutations of tau have been identified in frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17). Here, we investigated the phosphorylation of FTDP-17 mutant tau proteins, K257T, P301L, P301S, and R406W, by Cdk5 complexed with p35, p25, or p39 in vitro and in cultured cells. The extent of phosphorylation by all Cdk5 species was slightly lower in mutant tau than in wild-type tau. Major phosphorylation sites, including Ser202, Ser235, and Ser404, were the same among the wild-type, K257T, P301L, and P301S tau proteins phosphorylated by any Cdk5. On the other hand, R406W tau was less phosphorylated at Ser404 than were the other variants. This was not due to the simple replacement of amino acid Arg406 with Trp close to the phosphorylation site, because Ser404 in a R406W peptide was equally phosphorylated in a wild-type peptide. The decreased phosphorylation of mutant tau by Cdk5s was canceled when tau protein bound to microtubules was phosphorylated. These results indicate that FTDP-17 mutations do not affect the phosphorylatability of tau by Cdk5 complexed with p35, p25, or p39 and may explain part of the discrepancy reported previously between in vivo and in vitro phosphorylation of FTDP-17 tau mutants.

 Neurofibrillary tangles are one of the major pathological hallmarks of Alzheimer disease (AD).1 Neurofibrillary tangles are bundles of paired helical filament composed of the microtubule (MT)-associated protein tau in a hyperphosphorylated state (1, 2). Intracellular inclusions made of tau are also found in several other neurodegenerative diseases, including Pick disease, progressive supranuclear palsy, corticobasal degeneration, and frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17), collectively called tauopathies (3, 4). Exonic and intronic mutations in the tau gene have recently been identified in familial FTDP-17 (5–7), indicating that dysfunction of the tau protein can cause the above mentioned neurodegenerative diseases. Many of the exonic mutations reduce the ability of tau to promote MT assembly (8–10), but why these mutations cause the formation of tau inclusions is unknown.

The tau protein inclusions is hyperphosphorylated. Around 25 phosphorylation sites have been identified in paired helical filament tau from AD brains (11–13). Characteristic phosphorylation sites are Ser or Thr residues followed by Pro that are phosphorylated by the proline-directed protein kinase activities of extracellular signal-regulated kinase, glycogen synthase kinase 3β (GSK3β), and cyclin-dependent kinase 5 (Cdk5). Phosphorylation reduces the ability of tau to bind to and polymerize MTs, resulting in an increase in the soluble form of tau dissociated from MTs. However, it is unclear how phosphorylated soluble tau assembles into filamentous aggregates of neurofibrillary tangles and then induces neurodegeneration. Elucidating the relationship between tau phosphorylation and aggregate formation is critical to our understanding of the pathogenesis. Phosphorylation of FTDP-17 mutant tau has been studied mainly with GSK3β or in non-neuronal cultured cells (14–17). Mutant tau proteins were not phosphorylated more than wild-type (WT) tau either in transfected cultured cells or in vitro. Among them, R406W mutant tau showed significantly reduced phosphorylation in those experiments. However, both mutant tau and wild-type tau deposited in FTDP-17 brains are also hyperphosphorylated (18, 19). It is important to resolve the discrepancy between the reduced phosphorylation of mutant tau in vitro and in cultured neurons and the high phosphorylation of mutant tau in pathological brains.

Cdk5 is a proline-directed Ser/Thr kinase activated by a p35 or p39 Cdk5 activator (20–22). Cdk5 activity is primarily detected in differentiated neurons because p35 and p39 show limited expression in neurons. As described above, Cdk5 is one of the tau protein kinases that phosphorylate tau in living neurons and is also able to generate several paired helical filament epitopes of tau in AD (23, 24). However, the phosphorylation of FTDP-17 mutant tau by Cdk5 has not yet been examined. This question should be addressed because the involvement of Cdk5 in the pathogenic phosphorylation of tau has recently become more evident (25, 26). In particular, it is important to investigate the phosphorylation of tau by Cdk5 bound to p25 or p39. p25, the N-terminal-truncated form of p35...
Phosphorylation of FTDP-17 tau by Cdk5

generated by cleavage with calpain, a calcium-activated cysteine protease, accumulates in AD brains (27) and neurons undergoing cell death (28–30). It is also thought to induce the hyperphosphorylation of tau. Although the generation of p25 in human brains is still controversial because of possible obfuscation by postmortem delay (31–33), p25 is also generated in the brains of mouse models of amyotrophic lateral sclerosis and Niemann-Pick disease type C (34, 35). Furthermore, transgenic mice overexpressing p25 or p25 with P301L FTDP-17 mutant tau have recently been reported to display enhanced phosphorylation and the formation of tau tangles (25, 26). Although p25-Cdk5 has a higher kinase activity for tau than p35-Cdk5 in vitro (36), its phosphorylation of FTDP-17 mutant tau proteins has not been investigated. On the other hand, it was recently shown that Cdk5 bound to p39 phosphorylates tau in vivo in p35−/− mice (37). This suggests that p39-Cdk5 is the major Cdk5 that phosphorylates tau in the brain and may also contribute to the pathological phosphorylation observed in neurodegenerative diseases. However, the phosphorylation of tau by p39-Cdk5 has not been investigated in detail.

In this study, we examined the phosphorylation of K257T, P301L, P301S, and R406W mutant tau by Cdk5 complexed with p35, p25, or p39. There was no difference in tau phosphorylation among the Cdk5s used. P301L, P301S, and R406W mutant proteins were less phosphorylated than was WT tau, indicating that the association with MTs cancels the effects of the FTDP-17 mutation in reducing phosphorylation. These results may explain part of the previously described discrepancy between in vitro and in vivo phosphorylation.

EXPERIMENTAL PROCEDURES

Chemicals and Antibodies—Roscovitine, an inhibitor of Cdk5, was purchased from Calbiochem. Synthetic peptides were obtained from Qiao- gen (Herucles, CA). The anti-tau antibody, tau-C, has been described previously (38). All of the other reagents were of analytical grade.

Expression and Purification of Recombinant Human tau—The pRK172 bacterial expression vector containing recombinant human wild-type and FTDP-17 mutant tau cDNAs (encoding a tau protein with one N-terminal insert and four repeat domains [1N4R], WT, K257T, P301L, P301S, or R406W) was used to transform competent E. coli BL21-DE3 cells (8). tau protein expression was induced with 0.4 mM isopropyl-1-thio-β-D-galactoside for 2 h at 37 °C. Bacterial cells collected by centrifugation at 6,000 × g for 15 min at 4 °C were lysed in MOPS buffer (10 mM MOPS (pH 6.8), 1 mM MgCl2, and 0.1 mM [γ-32P]ATP to a prewarmed mixture of tau and MTs in the presence of 20 μM Taxol. The reaction was stopped by boiling in the presence of 0.5 M NaCl. Boiled samples were centrifuged at 100,000 × g for 30 min, and the heat-stable supernatants were collected. 10% SDS-PAGE on 10% polyacrylamide gel, the extent of tau phosphorylation was quantified by Cerenkov counting using a liquid scintillation counter (Beckman Coulter). Cell Culture and Metabolic Phosphorylation of tau—Cerebral cortical neurons were prepared from embryonic day-17 rat brains and labeled 11 days after plating with [32P]orthophosphate, as described previously (39). COS-7 cells cultured in Dulbecco’s modiﬁed Eagle’s medium containing 10% fetal bovine serum were transfected with cDNAs for tau, Cdk5, and each Cdk5 activator using PolyFect transfection reagent (Qiagen) according to the manufacturer’s protocol. Cells were labeled by incubation for 4 h with [32P]orthophosphate 24 h after transfection.

Immunoprecipitation of tau—32P-Labeled cell lysate was boiled in the presence of 0.5 M NaCl. The boiled samples were centrifuged at 100,000 × g for 30 min, and the heat-stable supernatant was collected. Polyclonal tau-C antibody was added to the supernatant and incubated at 4 °C for 1 h. After the addition of protein A-Sepharose CL-4B (Amersham Biosciences), the mixture was incubated further at 4 °C for 1 h. The immunocomplexes were washed five times with radioimmuno precipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.1% deoxycholate, 0.1% SDS, 20 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 10 mM β-glycerophosphate, 5 mM NaF), and the tau proteins bound to protein A beads were eluted by boiling in Laemmli sample buffer.

Two-dimensional Phosphopeptide Map Analysis—The tau proteins phosphorylated by Cdk5 were digested with trypsin (Sigma) in 50 mM NH4HCO3 (pH 8.4) at 30 °C. The digested peptides were subjected to two-dimensional phosphopeptide map analysis using a thin-layer-cellulose plate (Merck, Darmstadt, Germany) as described previously (41).

RESULTS

WT and Mutant tau Proteins and Cdk5—Fig. 1A shows CBB staining of tau proteins (WT, K257T, P301L, P301S, and R406W) on 10% SDS-PAGE. Each tau protein was purified as a single band. The CBB staining of Cdk5 and its activators, p35, p25, and p39, purified from Sf9 cells is shown in Fig. 1B. Cdk5 and each activator were isolated as major bands in these fractions. The specific activities of these Cdk5 fractions for WT tau protein were determined to be 10.6, 15.5, and 0.38 nmol/min/mg protein with p35-Cdk5, p25-Cdk5, and p39-Cdk5, respectively. These values for Sf9 p35-Cdk5 and p25-Cdk5 are similar to the value of 22.00 nmol/min/mg protein for tau phosphorylated with p25-Cdk5 purified from bovine brains (24), which is the only available datum to our knowledge. p39-Cdk5 showed a lower kinase activity because the formation of this complex is more labile.2 Cdk5 Is the Major Kinase Phosphorylating tau in Cultured Rat Cortical Neurons—tau is phosphorylated by many protein kinases in vivo and in vitro (2, 13). To determine the extent to which Cdk5 contributes to tau phosphorylation in neurons, we

2 M. Yamada, T. Saito, Y. Sato, A. Sekigawa, Y. Hamazumi, A. Asada, and S. Hisanaga, manuscript in preparation.
used two-dimensional phosphopeptide mapping to compare the phosphorylation of tau labeled metabolically in cultured rat brain cortical neurons (Fig. 2B) with that of tau phosphorylated by p35-Cdk5 in vitro (Fig. 2A). The major spots, spots 1–5 but not spot 6, that were detected in cultured neurons comigrated with in vitro phosphorylation spots produced by p35-Cdk5 (Fig. 2C). Our in vitro phosphopeptide map pattern was almost identical to the map patterns of previous reports for Cdk5 (41, 42) but distinct from those reported for the proline-directed protein kinase activities of extracellular signal-regulated kinase and GSK3β (42). These results indicate that Cdk5 is a major tau kinase in rat brain cortical neurons.

On the basis of previous reports (41, 42) and immunoblots with site-specific phosphorylation-dependent antibodies (data not shown), we identified the phosphorylation site in each spot: Ser202 and Thr205 in spot 1, Ser202 in spot 2, Ser404 in spot 3, and Ser235 in spot 4. There were two unidentified, discrete spots in the two-dimensional phosphopeptide maps, spot 5 in in vitro phosphorylated tau (Fig. 2A) and spot 6 in metabolically labeled tau (Fig. 2B). We used site-directed mutagenesis to determine their phosphorylation sites. Because spot 6 was also weakly detected in the two-dimensional phosphopeptide map of in vitro phosphorylated tau (longer exposure around spot 6) (Fig. 2A, inset), we examined the phosphorylation sites reported for Cdk5. The two-dimensional phosphopeptide maps of Ala mutants at Thr181 (T181A) and Ser404 (S404A) are shown in Fig. 2, D and E, respectively. Spot 5 disappeared in the two-dimensional phosphopeptide map of S404A tau (Fig. 2E), and spot 6 was lost in the map of T181A (Fig. 2D, inset). These results indicate that spot 6 is the peptide containing phosphorylated Thr181 and spot 5 is the peptide containing phosphorylated Ser404, which might be cleaved differently from the peptide of spot 3.

**Mutant tau Is Less Phosphorylated by Cdk5 than Is WT tau in Vitro**—We phosphorylated WT and FTDP-17 mutant tau (0.1 mg/ml) by p35-Cdk5, p25-Cdk5, or p39-Cdk5 at 35 °C and measured the initial rate of phosphorylation after a 5-min incubation and the plateau level of phosphorylation after a 2-h incubation. The initial rates of phosphorylation are compared in Fig. 3. As was reported previously (36), p25-Cdk5 showed stronger kinase activity for tau than did p35-Cdk5. p39-Cdk5 displayed tau phosphorylation activity similar to that of p35-Cdk5 when the kinase activity was normalized to histone H1 phosphorylation, although the specific activity was low. Here, we focused on comparing the phosphorylation between WT and mutant tau. The phosphorylation of mutant tau is expressed as a ratio relative to that of WT tau (Fig. 3, mean ± S.E., n = 3). Although K257T showed a phosphorylation level similar to that of WT tau, other mutant tau proteins were less phosphorylated by all of the complexes of Cdk5 and its activators. The extent of phosphorylation of P301L, P301S, and R406W was also reduced after incubation for 2 h, at which time phosphorylation had almost reached plateau levels (data not shown).

**Cdk5 Phosphorylation Sites in Mutant tau**—The sites at which tau is phosphorylated by Cdk5 were examined by two-dimensional phosphopeptide mapping. Tau was phosphorylated by each Cdk5 complex for 5 min at 35 °C and subjected to two-dimensional phosphopeptide mapping. We applied the same amount of radioactivity to the two-dimensional phosphopeptide maps to compare the relative phosphorylation intensity of each spot. Four major phosphorylation spots were detected, and the phosphorylation patterns were identical for WT, K257T, P301L, and P301S, indicating that K257T, P301L, and P301S mutations do not change the Cdk5 phosphorylation sites (Fig. 4). Furthermore, no difference was observed among tau proteins phosphorylated by p35-Cdk5, p25-Cdk5, or p39-Cdk5. One additional spot was observed in each tau variant phosphorylated by p39-Cdk5 (Fig. 4, white arrow in bottom panels), but this spot was not the result of p39-Cdk5-dependent phosphorylation because the phosphorylation of this spot varied depending on the preparation of p39-Cdk5 and was not reduced in the presence of the Cdk5 inhibitor, roscovitine, in the reaction mixture. The spot may have been phosphorylated by another kinase contaminating the p39-Cdk5 fraction. p39-Cdk5 is an unstable complex compared with p35-Cdk5 and p25-Cdk5. To purify the active p39-Cdk5 complex, we needed to use a purification procedure milder than that used for p35-Cdk5 or p25-Cdk5. Except for this spot, other spots were the same as those phosphorylated by p35-Cdk5 or p25-Cdk5. Because mutant tau proteins were less phosphorylated than WT tau even though the phosphorylation sites were the same, each site might be equally less phosphorylated.
Phosphorylation of R406W tau by Cdk5s—
Two phosphopeptide spots containing Ser404, spots 3 and 5, were missing in R406W tau phosphorylated by p35-Cdk5, p25-Cdk5, or p39-Cdk5 (Fig. 4, R406W). The R406W mutation changes the trypptic peptide pattern by eliminating the cleavage site just C-terminal of the mutation site, which could result in a different migration of the peptide on two-dimensional phosphopeptide mapping. To determine whether the mutant peptide moved in the other dimension of electrophoresis, we changed the spotting origin, but we did not detect a new spot. It is also possible that the longer peptide that was generated by the elimination of the cleavage site might remain in the acrylamide gel even after trypsin digestion. However, there was no difference between R406W tau and other tau proteins in the radioactivity remaining in the acrylamide gel after digestion. Diffuse and weak spots observed above the origin (Fig. 4, arrowheads in R406W) were suspected to be peptides containing phosphorylated Ser404. Even if this were the case, however, the signal intensity was considerably weaker than the sum of spots 3 and 5 of the other tau variants. Therefore, we concluded that Ser404 is not or is much less phosphorylated in R406W tau than in other tau variants.

Mutant tau Proteins Bound to MTs Are Phosphorylated at the Same Levels as WT tau by All Cdk5s—We examined whether the binding of mutant tau proteins to MTs affected the phosphorylation of mutant tau proteins. When WT tau or mutant tau (0.1 mg/ml) was incubated with MTs (1 mg of tubulin/ml, i.e. 10 μM) in the presence of 20 μM Taxol, all of the tau species bound to MTs (Fig. 6A, Unphospho). Firstly, we examined whether the binding to MTs would increase the phosphorylation of R406W tau by Cdk5s.

Phosphorylation of R406W tau by Cdk5s—Two phosphopeptide spots containing Ser404, spots 3 and 5, were missing in R406W tau phosphorylated by p35-Cdk5, p25-Cdk5, or p39-Cdk5 (Fig. 4, R406W). The R406W mutation changes the trypptic peptide pattern by eliminating the cleavage site just C-terminal of the mutation site, which could result in a different migration of the peptide on two-dimensional phosphopeptide mapping. To determine whether the mutant peptide moved in the other direction in the first dimension of electrophoresis, we changed the spotting origin, but we did not detect a new spot. It is also possible that the longer peptide that was generated by the elimination of the cleavage site might remain in the acrylamide gel even after trypsin digestion. However, there was no difference between R406W tau and other tau proteins in the radioactivity remaining in the acrylamide gel after digestion. Diffuse and weak spots observed above the origin (Fig. 4, arrowheads in R406W) were suspected to be peptides containing phosphorylated Ser404. Even if this were the case, however, the signal intensity was considerably weaker than the sum of spots 3 and 5 of the other tau variants. Therefore, we concluded that Ser404 is not or is much less phosphorylated in R406W tau than in other tau variants.

The Ser404 phosphorylation site is located two amino acids closer to the N terminus than is the R406W mutation site. The R406W mutation could decrease the Cdk5-dependent phosphorylation of Ser404 by changing the primary sequence. To test this possibility, we prepared two peptides with corresponding amino acid residues 396–409 containing the WT and R406W sequences (Fig. 5A) and phosphorylated them with p35-Cdk5. The R406W peptide was phosphorylated as much as the WT tau peptide (Fig. 5, B and C). We confirmed that Ser404 was the only Cdk5-dependent phosphorylation site in these peptides using a peptide with Ala at the Ser404 site. These results indicate that the mutation of Arg406 to Trp diminished the phosphorylation of Ser404 by Cdk5 only in the full-length tau protein but not in the short peptide.

Phosphorylation of FTDP-17 tau by Cdk5

**Fig. 3.** The extent of phosphorylation of mutant tau proteins by p35-Cdk5, p25-Cdk5, or p39-Cdk5. WT or mutant tau (0.1 mg/ml) was phosphorylated by p35-Cdk5 (left), p25-Cdk5 (middle), or p39-Cdk5 (right) for 5 min at 35 °C. The extent of phosphorylation was measured with a scintillation counter using the Cerenkov method and is expressed as a ratio relative to WT tau phosphorylation (mean ± S.E., n = 3). a.u., arbitrary units.

**Fig. 4.** Two-dimensional phosphopeptide maps of mutant tau proteins phosphorylated by p35-Cdk5, p25-Cdk5, or p39-Cdk5. The tau proteins WT, K257T, P301L, P301S, and R406W were phosphorylated by p35-Cdk5 (top row), p25-Cdk5 (middle row), or p39-Cdk5 (bottom row) using [γ-32P]ATP, and the phosphorylation sites were analyzed by two-dimensional phosphopeptide mapping. The phosphorylation patterns were almost identical for WT, K257T, P301L, and P301S. Two phosphopeptide spots, spots 3 and 5, missing in the R406W mutant, are indicated by black arrows. Arrowheads indicate spots detected only in R406W. The spot indicated by the white arrows in tau phosphorylated by p39-Cdk5 (bottom row) resulted from the phosphorylation by a contaminating kinase.
We confirmed that Cdk5s was similar to that by WT tau (Fig. 6). The extent of phosphorylation of mutant tau proteins by all of the Cdk5s was given the value of 1 (Fig. 6A), mean 15, 30, 60 (min). Phosphorylation was expressed relative to that of WT tau, which was detected with tau phosphorylated by p39-Cdk5 using [γ-32P]ATP. Phosphorylation did not dissociate tau proteins from MTs polymerized with Taxol under these conditions (Fig. 5). MTs were phosphorylated by p35-Cdk5, p25-Cdk5, and p39-Cdk5, mutant tau was compared (Fig. 6). The tau proteins bound to MTs increased the extent but not the sites of phosphorylation of WT tau. Thus, we detected no differences in the phosphorylation of FTDP-17 tau mutants by the different Cdk5 species used. However, we found that the phosphorylation of mutant tau by Cdk5 was enhanced to the same level as was that of WT tau by binding to MTs. Phosphorylation at Ser404 was reduced in full-length R406W tau protein but not in an R406W peptide, suggesting that the R406W mutation induces a local conformational change that reduces its phosphorylation by Cdk5s at Ser404. Binding to MTs increased the extent but not the sites of phosphorylation of P301L, P301S, and R406W mutant tau proteins more than it increased the phosphorylation of WT tau. These results suggest that the conformational change in tau induced by FTDP-17 mutations is canceled by an association with MTs, resulting in greater phosphorylation. The present results may explain part of the discrepancy described previously in the phosphorylation of FTDP-17 mutant tau in vivo and in vitro (14–19).

Phosphorylation-specific anti-tau antibodies are now available for most phosphorylation sites. Therefore, the phosphorylation status of tau has been examined mainly by immunoblotting with phosphorylation-dependent antibodies. It is a simple and sensitive method with which to detect changes in phosphorylation levels at each site. However, the method does not give the relative extent of phosphorylation among different phosphorylation sites. In this study, we used two-dimensional phosphopeptide mapping to analyze the phosphorylation states in tau. This method determines the relative extent of phosphorylation at most phosphorylation sites. Previous detailed analyses with this method have made it possible to identify the major Cdk5 phosphorylation sites (41, 42). Using site-directed mutagenesis, we first identified the two unidentified spots as Ser201 for spot 5 and Thr181 for spot 6 in Fig. 6B. Neither spot 3 nor spot 5 was detected again in R406W tau coexpressed in COS-7 cells with Cdk5 and each activator (Fig. 7C, right, an example with p25-Cdk5).

**Phosphorylation of Mutant tau Proteins by Cdk5s in COS-7 Cells**—To compare the extent and sites of phosphorylation under cellular conditions, we cotransfected mutant tau cDNA into COS-7 cells with Cdk5 cDNA in combination with cDNAs for each activator (p35, p25, and p39). Cells were metabolically labeled for 4 h with [32P]orthophosphate 24 h after transfection. The phosphorylation of tau was examined by autoradiography of the heat-stable supernatant after SDS-PAGE (Fig. 7). There was no phosphorylated band around the molecular weight of tau in mock-transfected cells (Fig. 7A, normal lane). The extent of tau phosphorylation was normalized to the amount of tau protein detected by immunoblotting with phosphorylation-independent anti-tau antibody (Fig. 7A, WB). All mutant tau proteins (K257T, P302L, P301S, and R406W) were phosphorylated to a similar extent as WT tau by all Cdk5s (Fig. 7B). The phosphorylation sites were also examined by two-dimensional phosphopeptide mapping (Fig. 7C). The phosphorylation sites of the different tau proteins were almost identical to those phosphorylated in vitro, as shown above. WT tau transfected with Cdk5-p25 is shown in Fig. 7C as an example. Neither spot 3 nor spot 5 was detected again in R406W tau coexpressed in COS-7 cells with Cdk5 and each activator (Fig. 7C, right, an example with p25-Cdk5).

**DISCUSSION**

In this study, we examined the phosphorylation of FTDP-17 mutant tau (K257T, P301L, P301S, and R406W) by Cdk5 complexed with either p35, p25, or p39. All Cdk5 complexes phosphorylated K257T, P301L, and P301S tau at the same sites as in WT tau. Thus, we detected no differences in the phosphorylation of FTDP-17 tau mutants by the different Cdk5 species used. However, we found that the phosphorylation of mutant tau by Cdk5 was enhanced to the same level as was that of WT tau by binding to MTs. Phosphorylation at Ser404 was reduced in full-length R406W tau protein but not in an R406W peptide, suggesting that the R406W mutation induces a local conformational change that reduces its phosphorylation by Cdk5s at Ser404. Binding to MTs increased the extent but not the sites of phosphorylation of P301L, P301S, and R406W mutant tau proteins more than it increased the phosphorylation of WT tau. These results suggest that the conformational change in tau induced by FTDP-17 mutations is canceled by an association with MTs, resulting in greater phosphorylation. The present results may explain part of the discrepancy described previously in the phosphorylation of FTDP-17 mutant tau in vitro and in vivo (14–19).

Phosphorylation-specific anti-tau antibodies are now available for most phosphorylation sites. Therefore, the phosphorylation status of tau has been examined mainly by immunoblotting with phosphorylation-dependent antibodies. It is a simple and sensitive method with which to detect changes in phosphorylation levels at each site. However, the method does not give the relative extent of phosphorylation among different phosphorylation sites. In this study, we used two-dimensional phosphopeptide mapping to analyze the phosphorylation states in tau. This method determines the relative extent of phosphorylation at most phosphorylation sites. Previous detailed analyses with this method have made it possible to identify the major Cdk5 phosphorylation sites (41, 42). Using site-directed mutagenesis, we first identified the two unidentified spots as Ser201 for spot 5 and Thr181 for spot 6 in Fig. 2. These sites have already been identified as Cdk5 phosphorylation sites (23, 38, 44), and our results confirm that Ser202, Ser202, and Ser404 are major phosphorylation sites for Cdk5 in vitro and in neurons (23, 38, 41, 42).
When we commenced the study, our major interest was the phosphorylation of FTDP-17 mutant tau by p25-Cdk5 and p39-Cdk5. p25-Cdk5 phosphorylates tau more than does p35-Cdk5 in cultured neurons (27) and in vitro (36), and p25 transgenic mice and mice doubly transgenic for P301L and p25 display AD-like neurofibrillary pathology in the brain (25, 26). However, how p25-Cdk5 phosphorylates FTDP-17 mutants has not been examined. On the other hand, p39-Cdk5 phosphorylates tau in the brains of p35-deficient mice (37), but the extent of this phosphorylation and the sites in tau that are phosphorylated by p39-Cdk5 have not been evaluated. Using Cdk5s expressed and purified from Sf9 cells, we showed that all Cdk5s (p35-Cdk5, p25-Cdk5, and p39-Cdk5) have the same substrate preference for phosphorylation sites on tau in both WT and

**Fig. 6.** The extent of phosphorylation by Cdk5s of mutant tau bound to MTs. WT tau or various mutant tau proteins (0.1 mg/ml) were incubated with MTs (1 mg of tubulin/ml) polymerized with 20 μM Taxol at 35 °C for 30 min. A, CBB staining of tau and tubulin (Tu) separated into the MT pellet (ppt) and supernatant (sup) by ultracentrifugation at 100,000 × g for 30 min before (Unphospho) or after phosphorylation (Phospho). B, the ratio of phosphorylation in MT-bound tau and free tau. Tau was phosphorylated in the presence or absence of MTs by p35-Cdk5 for 5 min at 35 °C with 0.1 mM [γ-32P]ATP. The extent of phosphorylation was measured with a scintillation counter using the Cerenkov method. C, the ratio of the phosphorylation of mutant tau to that of WT tau. Tau bound to MTs was phosphorylated by p35-Cdk5 (left), p25-Cdk5 (middle), or p39-Cdk5 (right) and measured as described in B. Phosphorylation is expressed as a ratio relative to the phosphorylation of WT tau (mean ± S.E.) (n = 3 or 4). D, two-dimensional phosphopeptide maps of WT tau and mutant tau proteins. Tau bound to MTs was phosphorylated by p35-Cdk5 (top row), p25-Cdk5 (middle row), or p39-Cdk5 (bottom row). Major phosphorylation spots are numbered in the top left panel.
mutant proteins. Our results support the proposition that p39-Cdk5 is responsible for the compensatory phosphorylation of tau proteins in p35/H11002/H11002 mouse brains or suggest that p39-Cdk5 is one of the intrinsic tau protein kinases in neurons. These results also indicate that the activator does not change the phosphorylation sites in tau and that FTDP-17 mutations in the MT-binding repeat domain do not affect the sites of phosphorylation by Cdk5s in vitro. If p25-Cdk5 contributes to the hyperphosphorylation of tau in pathological brains, mislocalization or stabilization of p25-Cdk5 in neurons, which has been suggested (27), would be a major cause of the increase in the extent of phosphorylated tau. This would lead to further phosphorylation by other protein kinases (2, 13).

Several in vitro phosphorylation experiments have been reported involving FTDP-17 mutant tau proteins using GSK3β as the protein kinase (14, 45). In general, mutant tau proteins were less phosphorylated in vitro. Our results with P301L and P301S, which have the mutation in the second MT-binding repeat domain, are consistent with these in vitro experiments even though a different protein kinase was used. Based on the decreased phosphorylation of mutant tau, it has been proposed that mutations cause conformational changes (45, 46). Although the precise conformational change is not yet known, our present results suggest that conformational change decreases the phosphorylatability of tau equally at those sites.

In addition to the mutation at Pro301 in the repeat domain, which reduces the total phosphorylation of tau in vitro, the R406W mutation may induce another local conformational change affecting Ser404 phosphorylation by Cdk5. That the phosphorylation of Ser404 is reduced only in R406W tau protein and not in the R406W peptide suggests a decreased accessibility of Cdk5 to Ser404 in the full-length R406W mutant tau protein. A similar approach was used to study phosphorylation by GSK3β (14). In contrast to our results, however, phosphorylation by GSK3β was reduced in a short peptide containing the R406W mutation as it was in the full-length R406W protein. This difference may be caused by a difference in major phosphorylation sites, Ser404 for Cdk5 and Ser396 for GSK3β. The peptide used in that study spanned amino acid residues 392–408 and was therefore able to form an aberrant conformation like that in R406W protein.

We demonstrate here for the first time that the binding of tau to MTs increases the level of phosphorylation of P301L, P301S, and R406W tau to a level similar to that observed in WT tau. On the other hand, R406W is less phosphorylated at Thr231 and Ser396 when bound to MTs in transfected Chinese hamster ovary cells (17). This apparently contradicts our present results with COS-7 cells. However, Matsumura et al. (17) detected the decrease in phosphorylation at Thr231 and Ser396 by immuno blotting with phosphorylation-dependent anti-tau antibodies; they did not demonstrate the phosphorylation at Ser404 and did not measure total phosphorylation by 32P incorporation. Therefore, our present results do not necessarily contradict theirs. We think it is important to study in detail the absolute extent of phosphorylation, as well as any changes in it at each site.

Krishnamurthy and Johnson (47) reported that R406W tau is more strongly phosphorylated than WT tau when stably expressed in immotile neuronal cells. This suggests that there

---

**FIG. 7.** Phosphorylation of mutant tau by Cdk5 kinases in COS-7 cells. WT or mutant tau was coexpressed in COS-7 cells with Cdk5 and its activator p35, p25, or p39. COS-7 cells were incubated for 4 h with [32P]orthophosphate 24 h after transfection. A, the heat-stable cell lysate was subjected to autoradiography (ARG, upper panel) and immunoblotting (WB, Western blotting) with phosphorylation-independent anti-tau antibody (lower panel) after SDS-PAGE on 10% polyacrylamide. B, the extent of tau phosphorylation was estimated with a scintillation counter and normalized to the amount of tau protein. Quantification is expressed as a ratio relative to the phosphorylation of WT tau (mean ± S.E.) (n = 3). a.u., arbitrary units. C, two-dimensional phosphopeptide maps of WT and R406W tau phosphorylated by p25-Cdk5 in COS-7 cells.
are some unknown factors in neuronal cells that enhance the phosphorylation of mutant tau. Alonso et al. (46) recently reported that mutant tau proteins are phosphorylated more strongly than WT in vitro when brain extract is used as the source of protein kinases. Although their experimental conditions involved the repeated addition of phosphatase inhibitors and ATP to induce hyperphosphorylation, it is also possible that tubulin or some other proteins in the brain extract masked the mutational effects on tau by binding during incubation. In fact, tau is suggested to bind to monomeric or oligomeric tubulin dimers and many other proteins in brain extracts (17, 48). It would be interesting to identify such proteins in brain extracts and to extend our present results to other mutants or other tau protein kinases.

The following mechanism is proposed for the MT-dependent enhancement of mutant tau phosphorylation. FTDP-17 mutations are located within or flanking the MT-binding domain, such as at Pro301 and Arg406. These mutations would induce a conformational change in tau that would reduce phosphorylation in the regions flanking the MT-binding repeats. Although these mutations reduce the ability of tau to bind to MTs (8–10), mutation sites can be masked by MTs when tau binds to them. The mutation-induced conformational change would then be canceled, restoring the normal conformation, which is phosphorylated as much as is WT tau. We do not know how mutant tau is further phosphorylated abnormally, but our results may explain part of the discrepancy reported previously between the in vivo and in vitro phosphorylation of FTDP-17 mutant tau proteins.

REFERENCES
1. Goedert, M. (2001) Curr. Opin. Genet. Dev. 11, 343–351
2. Stoothoff, W. H., and Johnson, G. V. (2005) Biochem. Biophys. Acta 1739, 280–287
3. Lee, V. M., Goedert, M., and Trojanowski, J. Q. (2001) Annu. Rev. Neurosci. 24, 1121–1159
4. Iqbal, K., Alonso, A. del C., Chen, S., Chohan, M. O., El-Akkad, E., Gong, C. X., Khatoo, S., Li, B., Liu, F., Rahman, A., Tanimmukai, H., and Grundke-Iqbal, I. (2005) Biochem. Biophys. Acta 1739, 198–210
5. Hutton, M., Lendon, C. L., Rizzu, P., Betts, J. C., Wilhelmsen, K. C., Schellenberg, G. D., Trojanowski, J. Q., and Lee, B. H. (1998) J. Neurochem. 74, 2583–2589
6. Maca, T. G., Dayanandan, R., Van Slegtenhorst, M., Whone, A., Hutton, M., Lovestone, S., and Anderton, B. H. (2001) Neuroscience 108, 701–712
7. DeTure, M., Ko, L.-W., Eason, C., and Yen, S.-H. (2002) Am. J. Pathol. 161, 1711–1722
8. Matsumura, N., Yamazaki, H., and Iwahara, Y. (1999) Am. J. Pathol. 154, 1849–1856
9. Reynolds, L. A., Grabowski, T. J., Schmid, M. L., Morris, J. C., Goate, A., Solodkin, A., Van Hoesen, G. W., Schelper, R. L., Talbot, C. J., Wragg, M. A., and Trojanowski, J. Q. (1997) Ann. Neurol. 42, 564–572
10. Miyasaka, T., Morishima-Kawashima, M., Ravid, R., Heutink, P., van Swieten, J. C., Nagashima, K., and Iwahara, Y. (2001) Am. J. Pathol. 158, 373–379
11. Tang, D., and Wang, J. H. (1996) Prog. Cell Cycle Res. 2, 205–216
12. Dhavan, R., and Tsai, L. H. (2001) Nat. Rev. Mol. Cell. Biol. 2, 749–759
13. Hanganu, S., and Saito, T. (2003) Neurosignals 12, 221–229
14. Paudel, H. K., Lew, J. A., and Wang, J. H. (1993) J. Biol. Chem. 268, 25512–25518
15. Ishiguro, K., Takamatsu, M., Tomizawa, K., Omori, A., Takahashi, M., Arioka, M., Uchida, T., and Iwahori, K. (1992) J. Biol. Chem. 267, 10987–10991
16. Noble, W., Olm, V., Takata, K., Casey, E., Mary, O., Meyerson, J., Gaynor, K., LaFrancois, J., Wang, L., Kondo, T., Davies, P., Burns, M., Veeranna, R., Dickson, D., Matsuka, Y., Ahlijanian, M., Lau, L. F., and Duff, K. (2003) Neuron 38, 555–565
17. Cruz, J. C., Tseng, H. C., Goldman, J. A., Shih, H., and Tsai, L. H. (2003) Neuron 40, 471–478
18. Patrick, G. N., Zukerberg, L., Nikolic, M., de la Monte, S., Dikkes, P., and Tsai, L. H. (1999) Nature 402, 615–622
19. Lee, M. S., Kwon, Y. T., Li, M., Peng, J., Friedlander, R. M., and Tsai, L. H. (2000) Nature 405, 360–364
20. Kusakawa, K., Saito, T., Onuki, R., Ishiguro, K., Kishimoto, T., and Hinsagana, S. (2000) J. Biol. Chem. 275, 17166–17172
21. Nath, R., Davis, M., Probert, A. W., Kopina, N. C., Ren, X., Schiele, G. P., and Wang, K. K. (2000) Biochem. Biophys. Res. Commun. 274, 16–21
22. Taniguchi, S., Fujita, Y., Hayashi, S., Kakita, A., Takahashi, H., Murayama, S., Saio, T. C., Hinsagana, S., Iwashuho, T., and Hasegawa, M. (2001) FEBS Lett. 498, 46–50
23. Yoo, B. C., and Lubec, G. (2001) Nature 411, 763–764
24. Tseng, H. C., Zhou, Y., Shen, Y., and Tsai, L. H. (2002) FEBS Lett. 523, 58–62
25. Nguyen, M. D., Lariviere, R. C., and Julien, J. P. (2001) Annu. Rev. Neurosci. 24, 1189–1197
26. Ishiguro, K., Takamatsu, M., Tomizawa, K., Omori, A., Takahashi, M., Arioka, M., Uchida, T., and Iwahori, K. (1992) FEBS Lett. 282, 81–84
27. Saito, T., Onuki, R., Fujita, Y., Kusakawa, G., Ishiguro, K., Bibb, J. A., Kishimoto, T., and Hinsagana, S. (2003) J. Neurosci. 23, 1189–1197
28. Schagger, H., and von Jagow, G. (1987) Anal. Biochem. 166, 468–477
29. Wada, Y., Ishiguro, K., Itoh, T. J., Uchida, T., Hatan, H., Saito, T., Kishimoto, T., and Hinsagana, S. (1998) J. Biol. Chem. 273, 179–188
30. Fahrenholz, S., Zheng-Fischhofer, Q., Arends, M., van der Bilt, M., Mandelkow, E. M., and Mandelkow, E. (1992) FEBS Lett. 290, 205–216
31. Connell, J. W., Gibb, G. M., Betts, J. C., Blackstock, W. P., Gallo, J., Lovestone, S., Hutton, M., and Anderton, B. H. (2001) FEBS Lett. 493, 40–44
32. Alonso, A. del C., Mederlyova, A., Novak, M., Grundke-Iqbal, I., and Iqbal, K. (2004) J. Biol. Chem. 279, 34873–34881
33. Johnson, G. V. W., and Hartigan, J. A. (1998) Alzheimer’s Dis. Rev. 3, 125–141

Downloaded from http://www.jbc.org/ on July 26, 2018
Phosphorylation of FTDP-17 Mutant tau by Cyclin-dependent Kinase 5 Complexed with p35, p25, or p39
Fumika Sakaue, Taro Saito, Yutaka Sato, Akiko Asada, Koichi Ishiguro, Masato Hasegawa and Shin-ichi Hisanaga

J. Biol. Chem. 2005, 280:31522-31529.
doi: 10.1074/jbc.M504792200 originally published online July 1, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M504792200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 48 references, 13 of which can be accessed free at http://www.jbc.org/content/280/36/31522.full.html#ref-list-1