Promotion of Hyperphosphorylation by Frontotemporal Dementia Tau Mutations*

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Mutations in the tau gene are known to cosegregate with the disease in frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17). However, the molecular mechanism by which these mutations might lead to the disease is not understood. Here, we show that four of the FTDP-17 tau mutations, R406W, V337M, G272V, and P301L, result in tau proteins that are more favorable substrates for phosphorylation by brain protein kinases than the wild-type, largest four-repeat protein τ4L and τ4S more than τ3L. In general, at all the sites studied, mutant tau proteins were phosphorylated faster and to a higher extent than τ4L and τ4S > τ3L. The most dramatic difference found was in the rate and level of phosphorylation of τ4L compared at positions Ser-396, Ser-400, Thr-403, and Ser-404. Phosphorylation of this mutant tau was 12 times faster and 400% greater at Ser-396 and less than 30% at Ser-400, Thr-403, and Ser-404 than phosphorylation of τ4L. The mutated tau proteins polymerized into filaments when 4–6 mol of phosphate per mol of tau were incorporated, whereas wild-type tau required ~10 mol of phosphate per mol of protein to self-assemble. Mutated and wild-type tau proteins were able to sequester normal tau upon incorporation of ~4 mol of phosphate per mol of protein, which was achieved at as early as 30 min of phosphorylation in the case of mutant tau proteins. These findings taken together suggest that the mutations in tau might cause neurodegeneration by making the protein a more favorable substrate for hyperphosphorylation.

Pathological tau aggregation in the form of filaments is increasingly recognized as a common denominator underlying a variety of dementias called tauopathies, which include Alzheimer disease (AD),1 frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), amyotrophic lateral sclerosis, cortical basal degeneration, dementia pugilistica, Pick disease, progressive supranuclear palsy, and tangle-only dementia. Despite their diverse topographic and phenotypic manifestations, these tauopathies are linked to the progressive accumulation of filamentous, hyperphosphorylated tau inclusions. The discovery of the co-segregation of certain mutations in the tau gene with the disease in inherited cases of FTDP-17 has confirmed that tau abnormalities that are seen in tauopathies as neurofibrillary degeneration are disease-causative (1–3).

Tau promotes the assembly of and stabilizes microtubules. It is a family of six proteins derived from a single gene by alternative mRNA splicing (4, 5). The human brain tau isoforms range from 352 to 441 amino acids. They differ in whether they contain 3 (τ3L, τ3S, or τ3R) or 4 (τ4L, τ4S, or τ4R) tubulin binding domains/repeats (R) of 31 or 32 amino acids each near the C terminus and 2 (τ3L, τ4L), 1 (τ3S, τ4S), or no (τ3R, τ4R) inserts of 29 amino acids each in the N-terminal portion of the molecule. In AD, all six isoforms have been reported to be present in a hyperphosphorylated state in paired helical filaments (PHFs), which form neurofibrillary tangles (6–8). Microtubules are required for axoplasmic transport, and in the tangle-bearing neurons of patients with AD, the microtubule system is destroyed and replaced by PHFs.

In inherited FTDP-17 certain mutations in tau gene cosegregate with the disease. These mutations are missense, intronic, and deletions of single amino acids. The missense mutations result in the substitution of one amino acid, and it has been reported that these mutations compromise tau ability to promote microtubule assembly (9, 10) and, in the presence of polyanions, promote its ability to polymerize (11–13). The intronic 5’ to exon 10 mutations result in overexpression of 4 R tau (1, 3). The effect of the deletion mutations in tau gene in patients is as yet unknown. In vitro ΔK280 and ΔN296 result in a change of 7R/7R ratio and a decrease in binding of tau to microtubules (14–16). The exact molecular mechanism of neurodegeneration in the affected patients is not yet understood. Like patients with AD, FTDP-17 patients show accumulations of hyperphosphorylated tau in the form of neurofibrillary tangles. All the mutations discovered in tau are dominant, suggesting that the effect of these mutations results in a gain of toxic function (17). In AD, hyperphosphorylation of tau appears to precede the appearance of tangles (18, 19), and abnormally hyperphosphorylated tau (AD P-tau) sequesters normal tau, microtubule-associated protein 1 and 2 and depolymerizes microtubules in vitro (20–22).

In the present report, we show that the gain of toxic function by FTDP-17 mutations rests on the increased susceptibility of mutant tau to be hyperphosphorylated as follows. (i) The FTDP-17 mutations R406W, V337M, G272V, and P301L make

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tau more favorable for hyperphosphorylation, (ii) rIL is a more favorable substrate for kinases than rIL, (iii) the mutated tau proteins polymerize into filaments at lower levels of phosphate incorporation (4–6 mol of phosphate/mol of tau) than wild-type tau (−10 mol of phosphate per mol of protein), and (iv) upon incorporation of −4 mol of phosphate per mol of protein, the hyperphosphorylated tau is able to sequester normal tau. These findings taken together suggest that mutations in tau might cause neurodegeneration through rendering a molecule that is a favorable substrate for kinases which polymerizes at lower levels of phosphorylation and that becomes “toxic” to the microtubule system by sequestering normal tau.

MATERIALS AND METHODS

Antibodies—The following phosphorylation-dependent tau antibodies were employed: Tau-1, 1,500,000 (Ser-195, Ser-198, Ser-199, or Ser-202 (7, 23, 24)); PHF-1 (Ser(P)-396/404 (25), 1,250; AT100 (Thr(P)-212–Ser(P)-214; Inigenetics) 1 μg/ml; AT270 (Ser(P)-181; Inigenetics), 1 μg/ml; antibodies from BIOSOURCE (all 0.5 μg/ml), Thr(P)-181, Ser(P)-199, Ser(P)-202, Thr(P)-205, Ser(P)-212, Ser(P)-214, Thr(P)-217, Thr(P)-219, Ser(P)-235, Ser(P)-262, Ser(P)-396, Ser(P)-404, R165 (Ser(P)-422 (26)), 1,300. Phospho-independent polyclonal antibodies were 92e (27) 134d (28), and 111e (29), each 1:5,000.

Plasmid Construction and DNA Mutagenesis—All wild-type and mutant tau genes were cloned in pET17b vector (Novagen) through NdeI-EcoRI restriction sites. The pET17b vector in combination with suitable Escherichia coli strains (DH5alpha for cloning and BL21DE3 for protein expression) represents an inducible T7-promoter expression system (30).

The pET17b plasmids carrying cDNA of the human genes for rIL and rIL isoforms were produced by PCR amplification of tau cDNAs from pHK172/rIL or pRK172/rIL vectors kindly supplied by Dr. Michel Goedert. Specific primers, including translation initiation start (ATG) and stop (TGA) codons and NdeI and EcoRI cloning restriction sites, were used.

Four pET17b plasmids bearing genes for rIL with FTDP17 point mutation G272V, P301L, V337M, or R406W (Fig. 1A) were produced.

Mapping of Phosphorylation Sites with Phospho-dependent Antibodies—The sites phosphorylated in tau were mapped by using the phospho-dependent antibodies listed above by Western blots. A 5-μl sample of the phosphorylation reaction mixture was taken at 1, 5, 10, 15, 20, and 22 h of incubation and subjected to SDS-PAGE (50 ng of tau per lane). The rate of phosphorylation was compared at Thr-181, Ser-199, Ser-202, Thr-205, Thr-212, Ser-214, Thr-217, Thr-219, Ser-235, Ser-262, Ser-396, Ser-404, and Ser-422 using specific phospho-dependent antibodies. Once we identified the specific sites that were differentially phosphorylated, we quantitated the specific differences.

The phosphorylated proteins were detected in different amounts (0.5, 1, 5, 10 ng per dot) on nitrocellulose membranes, blocked with bovine serum albumin, and developed with the phospho-dependent antibodies listed above. We employed a mixture of rabbit polyclonal antibodies, 92e, 134d, and 111e, against total tau to assay the levels of total tau. The latter was counted, and the mol of phosphate per mol of protein was estimated by subtracting the corresponding value of the enzyme alone control.

RESULTS

Expression and Isolation of rIL, rIL, and Mutant Tau rIL, rIL, and rIL with FTDP17 point mutation G272V, P301L, V337M, or R406W were produced (Fig. 1A). Each selected construct was verified by DNA sequencing with one primer in the middle of the tau gene and two primers complementary to vector sequences flanking the insert (BigDye Terminator Ready Reaction kit, ABI Prism377 Sequencer, PerkinElmer Life Sciences).

Association of Normal Tau to the Hyperphosphorylated Tau—The binding of the in vitro hyperphosphorylated tau to normal tau was studied by a dot overlay assay (20, 34). For the dot overlay assay, in vitro hyperphosphorylated tau proteins (10–100 ng) were spotted on nitrocellulose and blocked with 5% fat-free dry milk in 100 mM Mes buffer, pH 6.7, for 1 h. After blocking, the membrane was overlayed with normal tau (3–6 μg/ml) for 3 h. The bound tau was removed by washing the membrane, and the bound tau was fixed with 0.5% formaldehyde. After fixation, the bound tau was detected with Tau-1 antibody, which recognizes tau unphosphorylated at Ser-195/198/199/202. Samples in which overlay with normal tau was substituted with bovine serum albumin were used to deduct any background binding of Tau-1 to the hyperphosphorylated tau.

Immunoblots, Protein, andTau Assays—Protein concentration was estimated by the method of Bensadoun and Weinstein (35). Sample preparation and immunoblots were carried out as described previously (6). The levels of recombinant tau isoforms and AD P-tau were determined by the radioimmuno-slot blot method of Khatoon et al. (36) using a mixture of three phospho-independent tau antibodies, 92e, 134d, and 111e.

RESULTS

Expression and Isolation of rIL, rIL, and Mutant Tau rIL, rIL, and rIL with FTDP17 point mutation G272V, P301L, V337M, or R406W were produced (Fig. 1A). Each selected construct was verified by DNA sequencing with one primer in the middle of the tau gene and two primers complementary to vector sequences flanking the insert (data not shown). The tau proteins (Fig. 1A) were expressed and purified from E. coli. We previously showed that although tau is heat- and acid-stable, these treatments abolished its ability to self-polymerize into short PHF-like filaments (32). For this reason, we purified recombinant proteins without any steps using heat or acid treatment. The isolation procedure yielded ~5 mg of >95% pure protein per 500 ml of bacterial culture (Fig. 1B).

The FTDP17 point mutations in recombinant tau40 protein did not cause any drastic property change compared with rIL. The bacterial expression yield and behavior in the fast protein
liquid chromatography purification were identical to those of normal tau. The point mutations did not increase toxicity for E. coli or different apparent thermostabilities of purified protein. All the FTDP-17 mutant tau proteins migrated to the same apparent molecular weight in SDS-PAGE, suggesting no apparent conformational change in the mutant versus the wild-type tau proteins, at least in the denaturing conditions (Fig. 1B).

**Comparison of Mutated r4L,R406W, r4L,V337M, r4L,G272V, and r4L,P301L with Wild-type r4L and r3L as Substrates for Hyperphosphorylation**—We postulated that FTDP-17-mutated tau proteins are more favorable substrates than the wild-type tau for abnormal hyperphosphorylation. To test this hypothesis, we in vitro hyperphosphorylated recombinant wild-type and mutated tau proteins with normal rat brain extract as a source of kinases. Phosphorylation of r4L with the brain extract resulted in ~12 mol of phosphate per mol of the protein during ~20 h, whereas the extent of phosphorylation was much higher for all the mutated tau proteins examined, i.e., ~13–18 mol of phosphate per mol of r4L,R406W, r4L,V337M, r4L,P301L, and r4L,G272V (Fig. 2A). Not only the stoichiometry but also the kinetics of phosphorylation were more favorable for phosphorylation of the mutated tau proteins. The kinetics of phosphorylation was more favorable for r4L than for r3L, although the net levels of phosphorylation were only ~10% less for r3L. Phosphorylation is known to retard the mobility of tau in SDS-PAGE (7). We analyzed the electrophoretic patterns of wild-type and mutant tau proteins as a function of the phosphorylation time by Western blots using antibodies against total tau (Fig. 2B). As the phosphorylation reaction progressed, there was an upward mobility shift of tau that corresponded to the higher levels of phosphorylation. For example, at 3 h of phosphorylation two bands were seen, and the proportion of the upper band was ~50% for wild-type r4L and 27% for r3L. These mobility shift data suggested that the higher phosphorylation stoichiometry of mutant tau proteins compared with wild-type r4L and r3L.

The higher phosphorylation stoichiometry of mutant tau proteins compared with wild-type tau indicated an increase in phosphorylation of more than a single site. To identify the phosphorylation sites responsible for hyperphosphorylation, we studied tau phosphorylation at various sites. For each phospho-epitope, the immunoreactivity was determined at each time point of the phosphorylation reaction, and that amount was normalized to the total amount of tau present in the sample (specific immunoreactivity). The specific immunoreac-
activity was obtained for each antibody and each time point and plotted as a function of time. From these plots, three different parameters could be determined; they are the rate of phosphorylation at each particular site from the slope of the curve at the starting time (initial velocity), the highest amount of phosphorylation at that site (from the plateau of the same curve), and the time needed to reach 50% of the total phosphorylation of the particular site in $\tau$H9270.4L. Because there are no standards for each antibody, only the relative level of phosphorylation, but not the absolute values, can be calculated. The values obtained for $\tau$L at each site were taken as 100%, and all the others were expressed relative to those of $\tau$L. $\tau$L, $\tau$L, $\tau$L$^{G272V}$, $\tau$L$^{P301L}$, $\tau$L$^{V337M}$, and $\tau$L$^{R406W}$ were phosphorylated with rat brain extract as a source of kinases. The phosphorylation at each site was determined using phospho-dependent antibodies to specific sites. The rates of phosphorylation (A) were calculated from the slope of initial reaction of specific phosphorylation at each site as a function of time. The total phosphorylation level (B) was calculated from the saturation levels of phosphorylation as a function of time. Both values are expressed relative to $\tau$L (100% at each site). The positions of the amino acids phosphorylated are indicated on the x axis. The bar represents S.D.; *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$. C, time needed for each site of the tau indicated in each panel to achieve 50% of the maximal phosphorylation of that site in $\tau$L (open bars). At each position, the second bar (filled bars) represents the time for $\tau$L to achieve 50% of its maximal phosphorylation level. The bar represents S.D.; *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$.  

Fig. 3. A, rate of phosphorylation (A) and total phosphorylation (B) at positions Thr-181, Ser-199, Ser-202, Thr-205, Thr-212, Thr-217, Ser-262, Thr-231, Ser-235, Ser-386, Ser-404, and Ser-422 of $\tau$L and mutant tau proteins ($\tau$L$^{G272V}$, $\tau$L$^{P301L}$, $\tau$L$^{V337M}$, and $\tau$L$^{R406W}$) compared with $\tau$L. $\tau$L, $\tau$L, $\tau$L$^{G272V}$, $\tau$L$^{P301L}$, $\tau$L$^{V337M}$, and $\tau$L$^{R406W}$ were phosphorylated with rat brain extract as a source of kinases. The phosphorylation at each particular site from the slope of the curve at the starting time (initial velocity), the highest amount of phosphorylation at that site (from the plateau of the same curve), and the time needed to reach 50% of the total phosphorylation of the particular site in $\tau$L. Because there are no standards for each antibody, only the relative level of phosphorylation, but not the absolute values, can be calculated. The values obtained for $\tau$L at each site were taken as 100%, and all the others were expressed relative to those of $\tau$L. The calculated rates of phosphorylation, average results of three to six independent experiments, are shown in Fig. 3A. In general $\tau$L was a more favorable substrate for kinases than $\tau$L, since the rate of phosphorylation at almost all the sites investigated was lower (less than 100%) for $\tau$L (Fig. 3A). The total level of phosphorylation was slightly lower for $\tau$L than for $\tau$L (from 70–90%, Fig. 3B) at most of the sites studied. All the mutant tau proteins investigated were found to be more favorable substrates...
for phosphorylation than r4L in both the rate (Fig 3A) and the total level of phosphorylation (Figs. 2 and 3B). In general, r4L was a more favorable substrate than r3L, and the mutant tau proteins were more favorable than r4L for phosphorylation.

To compare the rates of phosphorylation of tau at each site, we determined the time it took for each mutant and for r3L (Fig. 3C) to achieve 50% of the phosphorylation level of r4L at a particular site. These results suggested that mutations in tau induced conformational changes, making it a more favorable substrate for overall phosphorylation. The most remarkable change was the one induced by the R406W mutation. This mutation reduced the time for half-maximal phosphorylation at Ser-396 from ~8 h in r4L to ~2 h in r4L<sub>R406W</sub> (see panel r4L<sub>R406W</sub>). The dramatic increase in the phosphorylation of r4L<sub>R406W</sub> at Ser-396 (Fig. 3C) resulted in a marked decrease in phosphorylation at positions 400, 403 (data not shown), and 404 (Fig. 3C).

**Polymerization of Mutated Taus upon Hyperphosphorylation**—We previously showed that polymerization of tau can be induced by hyperphosphorylation when at least 10 mol of phosphate per mol of this protein have been incorporated (32). Because we found (see above in “Results”) that the mutated tau proteins were more favorable substrates for phosphorylation, we proceeded to test the hypothesis that the mutated proteins on phosphorylation self-assemble into filaments more readily than does wild-type tau. For this purpose, r3L, r4L, r4L<sub>R406W</sub>, r4L<sub>G272V</sub>, and r4L<sub>P301L</sub> were phosphorylated as described above, and 10-µl aliquots were taken at different incubation times (0, 1/2, 1, 2, 4, 6, and 22 h) to grow for negative stain electron microscopy. No filaments were detected at the 0-min incubation time (data not shown). As early as 1/2 h after phosphorylation, polymerization of r4L<sub>G397M</sub> into filaments that had portions very well defined of about 4-nm-width was detected (data not shown). At 1 h of phosphorylation, r4L<sub>G397M</sub> (Fig. 4Ai) appeared to polymerize more than r4L<sub>R406W</sub> (Fig. 4Aii) and r4L<sub>G272V</sub>, but filaments were observed in all these tau proteins. At 4 h of phosphorylation, filaments were detectable in r4L<sub>P301L</sub> and lateral association of filaments was detectable for r4L<sub>R406W</sub> (Fig. 4Aiii). At 6 h of phosphorylation, association of filaments into bundles was easily visible (Fig. 4, Aiv). No filaments were detected in r3L or r4L in up to 6 h of incubation or in any of the mutant tau proteins incubated in the absence of ATP (figure not shown). As the phosphorylation progressed, the filaments detected were more abundant, and 10–15-nm filaments were also found (Fig. 4Aiii). Generally, the 10–15-nm filaments were associated into bundles. PHP-like structures could be detected in some of the bundles (Fig. 4, Aiv, inset). At 22 h of phosphorylation, filaments could be seen in both mutated and wild-type tau proteins (data not shown). These findings revealed that mutant tau proteins required fewer moles of phosphate per mole of protein to self-assemble into filaments, probably because the conformation of mutated tau proteins made them more prone to aggregate.

To quantitate aggregation of tau, aliquots of the phosphorylation mixture were taken at different times and centrifuged for 1.1 times more tau than r3L. As phosphorylation progressed, the levels of binding ranged from 2.1 (r4L<sub>V337M</sub>) to 1.4 (r4L<sub>P301L</sub>) times more than that by r4L, and the latter bound ~1.1 times more tau than r3L. As phosphorylation progressed, mutant tau proteins showed a marked decrease in their ability to bind normal tau, probably due to their self-assembly into filaments.

**DISCUSSION**

Dementia in AD and in other tauopathies such as FTDP-17 appears to require neurofibrillar degeneration. Like AD,
shown the association of neurotoxicity to hyperphosphorylation of tau filaments. However, to date, there is no evidence linking third postulated mechanism of toxicity is increased formation through some other mechanism, because 4R tau proteins bind mutations of tau have been shown to be autosomal dominant, formation in the presence of polyanions 4R/3R tau proteins (1, 3, 38), and 3) an increase in tau filament with the majority of the mutations resulting in increased in- coerse normal tau, and the ability to sequester normal tau was mutatd tau proteins > 4L > 3L. Mutated tau proteins acquired the conformation needed to polymerize into filaments at lower levels of incorporated phosphate. Therefore, these results taken together suggest that FTDP-17 mutations of tau render tau more ready to acquire toxic-like behavior induced by abnormal hyperphosphorylation.

We observed that FTDP-17 mutant tau proteins phosphorylated faster and to a higher extent than did 4L in vitro using brain extract as a source of kinases. These results suggested that the FTDP-17 mutations induce a conformational change in the tau proteins that make them more favorable for phosphorylation and that the presence of the extra microtubule binding domain, i.e. the second repeat, similarly leads to hyperphosphorylation. Previously, Jicha et al. (47) showed by circular dichroism that there is a change in the conformation of tau due to the mutations in 4L 4LG272V, 4L 4LP301L, 4L 4L337M, and 4L 4LR406W. The alteration in tau structure was so significant that it also altered its elution profile in a C8 reverse-phase column chromatography, probably leading to increased exposure of hydrophobic residues in extended areas of the molecule. The present study suggests that the mutation-induced conformational changes probably make tau more prone to hyperphosphorylation and to assemble at lower levels of phosphate incorporation (4-6 mol of phosphate/mol of tau) than wild-type tau (~10 mol of phosphate per mol of protein). Phosphorylation-induced polymerization of filamentous protein in the pathogenesis of neurodegenerative diseases is not exclusive to tau and to AD. Recently, it has been shown that α-synuclein is phosphorylated in synucleinopathy lesions and that its phosphorylation promotes fibril formation in vitro (48).

Previous studies of the effect of phosphorylation on mutant tau proteins are quite variable. In cellular systems, it has been reported that mutant tau proteins are phosphorylated equally or less than wild-type tau (49-51). The discrepancy between the findings of these previous studies and our results might be due to the absence of certain kinases in the cell lines used, to the different isoforms of tau transfected, and/or to the fact that tau can be microtubule-bound in the cell system. In the present study, one of the most remarkable differences we found with 4LR406W transgenic mice, the protein was found to be phosphorylated at Ser-396, although another study found it to be phosphorylated at several specific sites. At around 4 mol of phosphate per mol of tau, this protein was able to sequester normal tau, and the ability to sequester normal tau was mutatd tau proteins > 4LR406W. The alteration in tau structure was so significant that it also altered its elution profile in a C8 reverse-phase column chromatography, probably leading to increased exposure of hydrophobic residues in extended areas of the molecule. The present study suggests that the mutation-induced conformational changes probably make tau more prone to hyperphosphorylation and to assemble at lower levels of phosphate incorporation (4-6 mol of phosphate/mol of tau) than wild-type tau (~10 mol of phosphate per mol of protein). Phosphorylation-induced polymerization of filamentous protein in the pathogenesis of neurodegenerative diseases is not exclusive to tau and to AD. Recently, it has been shown that α-synuclein is phosphorylated in synucleinopathy lesions and that its phosphorylation promotes fibril formation in vitro (48).
found to be highly phosphorylated at Ser-396 but was not aggregated into filaments. \( \tau^4_{\text{V337M}} \), \( \tau^4_{441} \), and \( \tau^4_{\text{P301L}} \) were also expressed in mice, and increased tau phosphorylation and tau inclusions were found in these animals (54–58).

For tau to assemble into filaments or to acquire the ability to bind normal tau, it is possible that phosphorylation of a particular site in a homologous or heterogeneous manner within a portion of the molecule must occur. If it were a particular site, it should have been consistently phosphorylated early in all three mutant tau proteins studied. If the distribution of charges is important, some sites nearby in the molecule could balance the rate of phosphorylation of a particular site with others. Although it is very difficult to distinguish between both alternatives, it appears that the need of a region of tau to be phosphorylated is more compatible with the results presented in the present study. For example, in the case of \( \tau^4L_{\text{R406W}} \), the phosphorylation of Ser-404 was impaired, but that of Ser-396 was markedly increased. These findings are compatible with the need for a balance of charges in the C-terminal region of tau more than the need of Ser-404 to be phosphorylated. Except for this effect on \( \tau^4L_{\text{R406W}} \), the effect on the other mutant tau proteins seemed to be general for all sites. It appears that these mutations affect conformation-facilitating phosphorylation in general. In the case of \( \tau^4L \), at least 10 mol of phosphate are required for its polymerization (32), and according to phosphorylation kinetics, 10 mol of phosphate per mol of protein are added during about 10 h of phosphorylation (see Fig. 2A). \( \tau^4L \) acquires the maximal ability of binding normal tau at about 3–4 h of phosphorylation (see Fig. 5B), i.e. after the incorporation of about 4 mol of phosphate per mol of protein. These results suggest that at least two different conformational states of tau are induced by phosphorylation, one in which the hyperphosphorylated tau is able to bind normal tau and one in which it is able to self-assemble into filaments. These results combined with the data of phosphorylation kinetics (see Fig. 3C) suggest that the conformation of tau needed to sequester normal tau might involve phosphorylation of \( \tau^4L \) at positions 199, 202, 205, 212, 235, 262, and 404, and for self-assembly, further phosphorylation at positions 231, 396, and 422. Phosphorylation at Thr-181 or Thr-217, sites with a highly basic segment that interfere with polymerization. The conformation of the mutant tau proteins (mutation positions indicated in red) is more favorable for the action of kinases than \( \tau^4L \). Upon hyperphosphorylation (phosphorylation positions indicated in red), both wild-type and mutated tau proteins adopt the conformation needed to polymerize into filaments.
conformational states of tau in the case of the mutants appears to follow a similar pattern to that of r4L, except that in the case of r4Ld120N, Ser-404 is barely phosphorylated, and the rate of Ser-396 phosphorylation is greatly increased. For the mutant tau proteins, it appears that phosphorylation at sites such as Thr-181 and Thr-217 is achieved on the polymer. This further phosphorylation might stabilize the polymers and induce lateral interactions.

The ability of P-tau to bind normal tau in all the tau proteins studied had a maximum at −3.5–4 mol of phosphate per mol of protein and decreased upon higher mol of phosphate incorporation (Fig. 5). It is possible that once tau starts forming the polymer, the protein loses its ability to bind normal tau. These results are in agreement with our observations that AD P-tau binds normal tau more as a monomer than when it is aggregated into filaments.2

We propose the scenario depicted in Fig. 6. Tau has been previously shown to undergo intermolecular association through the microtubule binding domains, and the flanking regions appear to be inhibitory of tau self-assembly (32, 51, 59). If we consider tau to have little secondary structure and that segments of tau are separated by prolines that induce a bend in the amino acid chain, the theoretical isoelectric points of these segments are strongly basic at the N-terminal side of microtubule binding domains. We propose that these very basic segments (pI > 9) in the proline-rich region of tau mask the intermolecular attraction of the microtubule binding domains. Considering that the mutant tau proteins start polymerizing after 1 or 2 h of incubation (4–6 mol of phosphate per mol of protein), the sites that are phosphorylated at a level of about 50% of the total phosphorylation of r4L in less than 2 h are Thr-212, Ser-235, and Ser-262 in the N-terminal side. The pI value for the segment containing Thr-212 is 12, Ser-235 is 9.18, and Ser-262 is 9.56. Phosphorylation of these sites decreases the theoretical pI and, consequently, the negative effect of the N-terminal region on tau self-assembly. The C-terminal region is basic up to Pro-397, and the rest of the segments are acidic. It is possible that the acidic segment “masks” the interacting part of tau and that the phosphorylation at Ser-396 and/or Ser-404 opens up this segment, allowing the intermolecular interaction through the microtubule binding domains. In FTDP-17 mutant tau, the conformation is more prone to polymerize than the wild-type protein. In short, the FTDP-17 tau mutations induce a conformational change in tau molecule that makes it easier to polymerize and a more favorable substrate for kinases that polymerize at lower levels of phosphorylation. This implies that mutated tau proteins are more sensitive to changes in phosphorylation than wild-type tau and r4L more than r3L. Any imbalance that increases phosphorylation levels might trigger the polymerization of tau.

Clinically, FTDP-17 phenotypes appear to be quite variable, not only between mutations, but also within a single mutation and even within individual families either with intronic (60) or missense mutations (61). Despite the variability, some similarities are observed, for example parkinsonism is present more frequently in families with intronic mutations (for review, see Ref. 62). This evidence suggests that there is a clinical overlap among the various tauopathies and that modifications of tau by other genetic and/or epigenetic factors, such as different expression levels of tau proteins, stress-activated kinases, and different levels of phosphatase activity, might also be involved in the disease.

In conclusion, the present study suggests that mutations alter tau conformation, making it more susceptible to hyperphosphorylation, and that the conformation is further altered by phosphorylation, making this phosphorylated tau able to sequester normal tau and then assemble into filaments at lower phosphorylation levels than wild-type tau. The autosomal dominant inheritance could be explained by the generation of the toxic form of tau that sequesters normal tau. Re-establishment of tight regulation of phosphorylation levels could arrest or prevent tau-induced neurofibrillary degeneration.

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