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Tau 6D and 6P Isoforms Inhibit Polymerization of Full-Length Tau *in Vitro*[†]

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ABSTRACT: Alzheimer's disease and other tauopathies are characterized by the intracellular accumulation of insoluble filaments of the microtubule-associated protein tau. The six canonical tau isoforms in the adult brain consist of an N-terminal "projection" domain followed by a proline-rich region, a microtubule-binding repeat region, and a C-terminal tail. However, alternative splicing in exon 6 produces an additional set of tau isoforms, termed 6D and 6P, which contain only the N-terminus and part of the proline-rich region. We have previously shown that constructs representing N-terminal fragments of tau, which resemble the naturally occurring 6P and 6D isoforms, inhibit polymerization of the full-length protein in an *in vitro* filament formation assay and traced the inhibitory activity to amino acids 18−42. Here we report that 6P and 6D tau isoforms inhibit polymerization of full-length tau (hTau40) in a similar manner, likely by stabilizing full-length tau in a soluble conformation. The absence of exons 2 and 3 decreased the effectiveness of the 6D isoforms but not the 6P variants or the N-terminal tau fragments from our previous study, indicating that the 18−42 region is not the sole determinant of inhibitory ability. Finally, this paper demonstrates that inhibition is blocked by pseudophosphorylation of tyrosines 18 and 29, providing a potential link between tyrosine phosphorylation and disease progression. Taken together, these results indicate that the 6P/6D isoforms are potential endogenous inhibitors of tau filament formation and suggest a mechanism by which this ability may be disrupted in disease.

The microtubule-associated protein tau forms intracellular, filamentous aggregates in Alzheimer's disease (AD)[1] and other tauopathies. The formation of tau pathology is thought to be intimately linked to neurodegeneration, in part because the appearance of tau pathology in AD follows a spatial and temporal progression through the anatomical regions that underlie the clinical symptoms. Filamentous tau deposition is first found in areas associated with learning and memory and later spreads through much of the cerebral cortex (reviewed in ref *1*). Yet even in severe AD some areas, including the cerebellum and primary sensory-motor areas, remain relatively free of tau pathology (*2*−*4*). Identifying the differences between susceptible and protected neuronal populations, including endogenous triggers and inhibitors of tau aggregation, may be crucial to understanding selective cellular vulnerability in AD.

Although tau was historically considered to be an unstructured protein in solution, recent evidence indicates that soluble tau occupies a number of folded states and that these conformations influence tau's propensity to form filaments. The amino terminus of tau has emerged as an important regulator of tau folding. The association of the extreme amino terminus (5−15) with the microtubule binding repeat region (MTBR), recognized by the conformational antibody Alz50, is thought to underlie the transition from soluble to filamentous tau (reviewed in ref *5*). The amino terminus is also an important negative regulator of tau aggregation. When present in molar excess, constructs containing only the N-terminus inhibit polymerization of the full-length protein in an *in vitro* filament formation assay. This inhibitory effect requires amino acids 18−42 in the N-terminal fragments and residues 392−421 in the C-terminus of full-length tau (*5*). The N-terminal fragments may act by stabilizing a "paperclip" conformation of tau (*5*), in which the C-terminus associates with the MTBR region and the N-terminus comes into close proximity to the C-terminus. This conformation is observed when tau is a soluble monomer and may discourage polymerization (*6*−*8*).

The majority of tau studies to date focus on six canonical isoforms in the adult CNS produced by alternative splicing in the amino terminus (exons 2 and 3) and the MTBR region (exon 10) of the protein (reviewed in ref *9*). This picture grew more complicated with the discovery of additional tau isoforms that contain all or part of the sequence encoded by exon 6, which is absent from the canonical isoforms. Inclusion of the entire exon (6+) extends the proline-rich region of tau and results in isoforms that are otherwise identical to their canonical counterparts. The 6+ isoforms may play a regulatory role in neurite elongation (*10*). In addition to the 3′ splice site at the end of exon 6, there are two alternate 3′ splice sites within exon 6 itself, termed "6P" and "6D" according to location (Figure 1). Use of either of these splice sites results in a frame shift that introduces a unique amino acid sequence, PCCVPRATFLS (6P) or FWSKGDETQGG (6D),

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[1]Abbreviations: AA, arachidonic acid; AD, Alzheimer's disease; CNS, central nervous system; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FTDP-17, frontotemporal dementia and Parkinsonism linked to chromosome 17; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; hTau40, the 441 amino acid isoform of human tau; i_s, intensity of scattered light; MTBR, microtubule binding repeat; PHF, paired helical filament.

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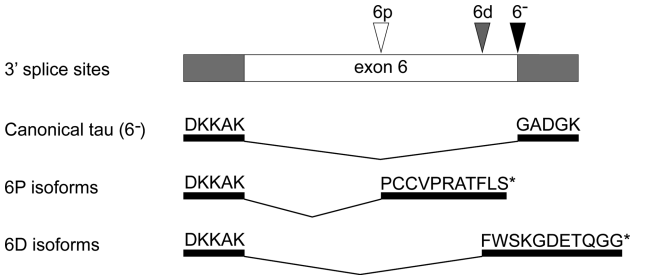

FIGURE 1: Schematic illustrating the relative positions of the 3′ splice sites in exon 6, with the amino acid sequences resulting from use of each site indicated below. Flanking areas of exons 5 and 7 are also shown. Use of either splice site internal to exon 6 (6p or 6d) results in a frame shift in the message that introduces a unique 11 amino acid sequence followed by a stop codon (denoted by an asterisk).

followed by a stop codon. As a result, tau 6P and 6D isoforms are truncated, lacking part of the proline-rich region, the MTBR region, and C-terminus (*11*, *12*). Of these, the MTBR region is absolutely essential to tau-microtubule binding (*13*−*15*) and tau aggregation (*16*, *17*).

The expression of 6P/6D tau isoforms is spatially and developmentally regulated. At the mRNA level, 6P levels are similar in fetal and adult brain, while 6D is more prominent in fetal brain (*18*). In adult brain, 6P and 6D mRNA levels are highest in cerebellum and spinal cord but are detectable in all CNS tissues examined, including the cerebral cortex and the hippocampus (*11*). Using an antibody to the C-terminus of 6D, Luo et al. compared the expression of 6D and full-length isoforms in various human tissues. 6D levels were comparable to full-length isoforms in cerebellum, and levels were reduced but detectable in the cerebral cortex and hippocampus. It is notable that 6D protein expression is particularly high in the cerebellum, which is not affected by tau lesions in AD, and lowest in tangle-prone areas (hippocampus, cerebral cortex). Even within AD hippocampus, anti-6D labeling does not colocalize with an antibody that recognizes neurofibrillary tangles (Tau-5 (*19*)).

In light of the polymerization suppressive properties of N-terminal tau fragments and the expression pattern in human brain, we asked whether 6P/6D isoforms might act as endogenous inhibitors of tau aggregation. To begin to address this question, we assessed the effects of 6P and 6D isoforms on the polymerization of full-length tau in an *in vitro* filament formation assay (*20*, *21*). We report that 6P and 6D isoforms inhibit polymerization of full-length tau and that the efficacy of each isoform depends on whether it is of the 6D or 6P variety and on the presence or absence of N-terminal exons 2 and 3. We also demonstrate that inhibition is abolished by mutations in the crucial 18−42 region that mimic phosphorylation. Collectively, our results indicate that the 6P/6D isoforms have the potential to act as endogenous regulators of tau filament assembly and suggest a basis for the disruption of this regulatory ability by disease-related phosphorylation events.

EXPERIMENTAL PROCEDURES

Recombinant proteins. The six canonical tau isoforms contain zero, one, or two alternatively spliced N-terminal inserts (designated 0N, 1N, and 2N, respectively) and either three or four MTBRs (3R or 4R). The full-length tau used in this study (hTau40) is the longest canonical isoform in the human central nervous system, containing 441 amino acids, including both alternatively spliced N-terminal exons (e2 and e3) and four

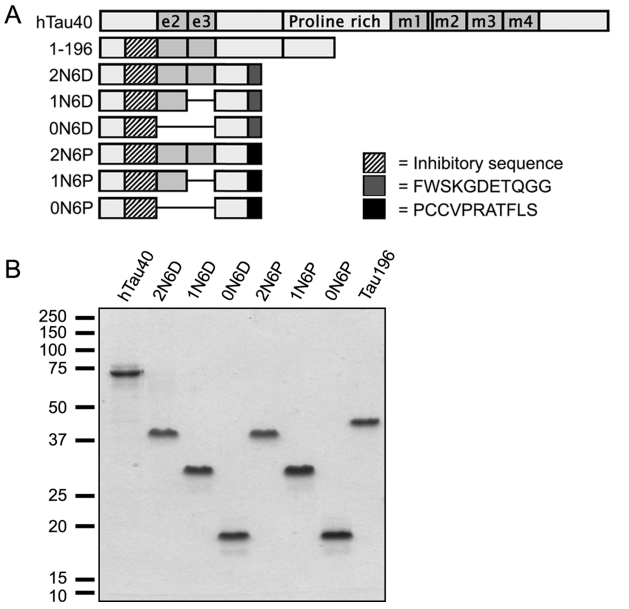

FIGURE 2: Schematic of the tau constructs used in this study. (A) A tau construct containing a stop codon at Y197 (1−196) has been described elsewhere (*5*). Constructs containing zero, one, or two alternately spliced N-terminal exons (e2 and e3) were created on the background of the 6P and 6D isoforms. The key indicates the specific N-terminal sequence required to inhibit polymerization of full-length tau (residues 18−42), as well as sequences unique to 6P and 6D isoforms. (B) Purified proteins used in this study separated by SDS−PAGE electrophoresis and stained with Coomassie Brilliant Blue R.

microtubule binding repeats (m1−m4; Figure 2A). The various 6D and 6P isoforms were generated by restriction digestion and ligation of cDNA constructs described previously (*19*); hTau40 (2N4R) (*22*), hTau23 (0N3R), and hTau37 (1N3R) (*23*). $6D^{Y}18/29^{F}$ and $6D^{Y}18/29^{E}$ were created by site-directed mutagenesis (Stratagene) on the 2N6D background. The construct 1−196 has been described previously (*5*). All constructs were verified by sequencing prior to protein purification. Proteins were expressed in *Escherichia coli* and purified by means of an N-terminal polyhistidine tag (*16*, *22*). Protein concentrations were determined by the Lowry assay (*24*).

Polymerization. Arachidonic acid (AA) was obtained from Cayman Chemical (Ann Arbor, MI) and stored at −20 °C. Working solutions were prepared in 100% ethanol immediately prior to use. Tau polymerization was induced by arachidonic acid as previously described (*21*). Briefly, tau protein (4 μM) was incubated at room temperature in reaction buffer (10 mM HEPES, pH 7.6, 100 mM NaCl, 0.1 mM EGTA, 5 mM DTT) in the presence of 75 μM AA. The final volume of ethanol in these reactions was 3.8%, and this volume was added to control reactions in the absence of AA. Unless otherwise noted, 6P/6D constructs and 1−196 were added at a concentration of 8 μM to the polymerization reaction mixture prior to the addition of arachidonic acid. Polymerization was monitored at room temperature by the intensity of right angle laser light scattering (i_s (*21*)). End point (t = 300 min) laser light scatter data from at least three independent experiments were analyzed. Statistical significance was determined by comparing polymerization in the presence and absence of N-terminal constructs by a Student's two-tailed t test. Time course data were fit with curves using GraphPad Prism 3.0 software. Error bars in all figures represent plus and minus one standard error of the mean.

Electron Microscopy. Polymerization reactions were allowed to proceed at least 5 h, fixed with 2% glutaraldehyde (Electron Microscopy Sciences, EMS, Hatfield, PA), spotted onto 300 mesh Formvar/carbon coated copper grids (EMS), and negatively stained with 2% uranyl acetate (EMS) as previously described (*20*). Grids were examined using a JEOL JEM-1220 electron microscope at 60 kV and 12000× magnification and photographed using a MegaScan 794/20 digital camera and DigitalMicrograph software version 3.9.3 (Gatan). Optimas 6.0 imaging software (Media Cybernetics) was used to automatically identify and measure filaments (defined as objects > 20 nm in length). At least five separate fields from each grid were randomly chosen under low illumination to prevent bias. In experiments where different constructs were analyzed for effects on hTau40, data from each experiment were normalized to control reactions containing hTau40 alone and expressed as percent of the hTau40 value. Results from at least three independent experiments were analyzed by Student's two-tailed *t* tests to determine if polymerization was significantly different from controls (GraphPad Prism 3.0 software).

Filament Sedimentation. Polymerization reaction mixtures were incubated for 5 h in the presence of arachidonic acid. Following assembly, a pretreatment sample was removed, and the remainder of the reaction mixture was centrifuged at 100000*g* for 20 min at 25 °C over a 40% glycerol cushion. Samples of the starting material and supernatants were diluted in 2× Laemmli buffer and boiled. Pellets were resuspended in an amount of polymerization buffer equal to the starting volume, prior to addition of 2× Laemmli buffer. Proteins were separated by SDS−PAGE, transferred to nitrocellulose membranes, and probed with the amino-terminal antibody Tau-12 (4 ng/mL) (*25*) to visualize all tau constructs and the carboxy-terminal antibody Tau-7 (40 ng/mL) (*5*), which recognizes full-length hTau40 but not the 6D/6P isoforms. HRP-conjugated goat anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA) and ECL (GE Healthcare, Amersham, U.K.) were used to detect primary antibody binding.

RESULTS

6D and 6P Isoforms Share Common Features with N-Terminal Fragments That Inhibit the Polymerization of Full-Length Tau. To examine the effects of the 6D and 6P isoforms on the polymerization of full-length tau (hTau40), several protein constructs were created and purified (Figure 2). The 6P and 6D constructs, like the previously described N-terminal tau construct 1−196, lack the MTBR region of tau known to be necessary for polymerization (*16*, *17*). Alternative splicing of exons 2 and 3 produces canonical tau isoforms containing zero, one, or two N-terminal inserts (designated 0N, 1N, and 2N, respectively). Hence, constructs containing zero, one, or two alternately spliced N-terminal exons were created on the background of the 6P and 6D isoforms. An additional tau protein construct, 1−196, that was previously shown to inhibit hTau40 polymerization (*5*) was included as a positive control. All constructs contain the specific amino acid region (18−42) previously identified as crucial for the inhibition of hTau40 polymerization (*5*).

2N6D and 2N6P Inhibit the Polymerization of Full-Length hTau40. To determine whether 6P and 6D isoforms influence tau polymerization, hTau40 (4 μM) was incubated in the presence or absence of a twice molar excess of 2N6D, 2N6P, or 1−196. Polymerization was induced by the addition of arachidonic acid, and right angle laser light scattering (LLS) was used to monitor filament formation (Figure 3A). After 5 h of polymerization, a similar degree of inhibition was apparent in the presence of 2N6P (28.63 ± 6.27%, $P < 0.05$) and 1−196 (33.40 ± 6.59%, $P < 0.05$). 2N6D also significantly inhibited hTau40 polymerization, albeit to a lesser extent (20.20 ± 2.85%, $P < 0.05$).

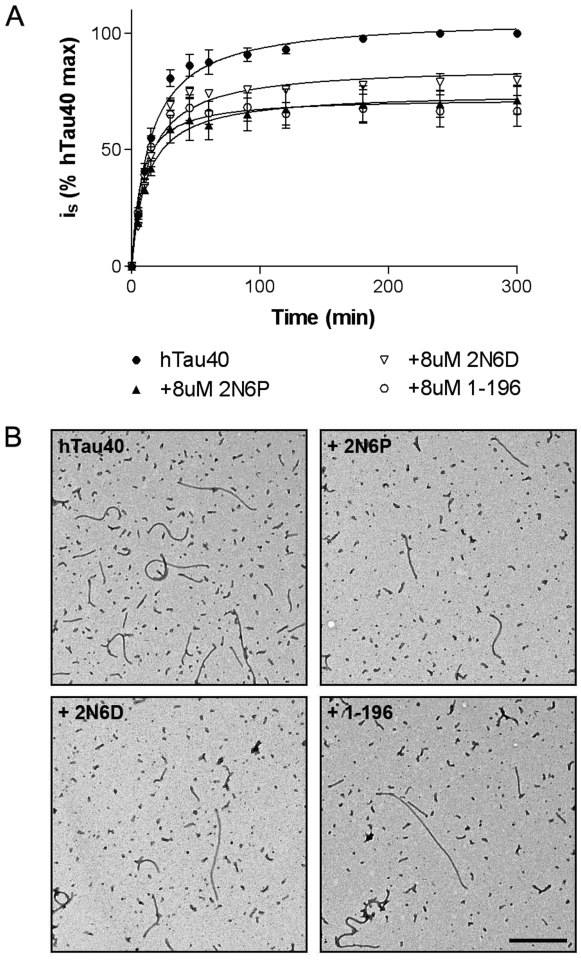

FIGURE 3: 6D and 6P isoforms inhibit the polymerization of hTau40. (A) Laser light scattering (LLS) was used to monitor the polymerization of hTau40 in the absence (●) or presence of a twice molar excess of 2N6P (▲) and 2N6D (▽). An N-terminal construct known to inhibit polymerization, 1-196 (○), was included as an experimental control. (B) Representative electron micrographs of hTau40 filaments formed alone or in the presence of 8 μM 2N6P, 2N6D, or 1−196. Scale bar represents 500 nm.

To verify the results of the LLS experiments and to further characterize the effects of 2N6P and 2N6D on hTau40 polymerization, we performed quantitative electron microscopy (EM) on filaments formed under each experimental condition (Figure 3B). Previous work from our laboratory demonstrated that incubation with N-terminal tau fragments reduced the overall mass of hTau40 filaments formed, with the primary effect being a reduction in the number of filaments per field (*5*). Quantitative analysis revealed similar effects in the present study (Figure 4). Polymerization of hTau40 (4 μM) in the presence of 2N6P (8 μM) caused a significant reduction in the number of filaments per field (36.26 ± 2.36%; $P < 0.001$) and in the overall mass of polymerized material per field (42.80 ± 4.64%; $P < 0.001$). Incubation with 2N6D also reduced filament number (25.20 ± 4.62%; $P < 0.001$) and overall polymer mass (30.12 ± 3.23%; $P < 0.001$). In agreement with our previous report (*5*),

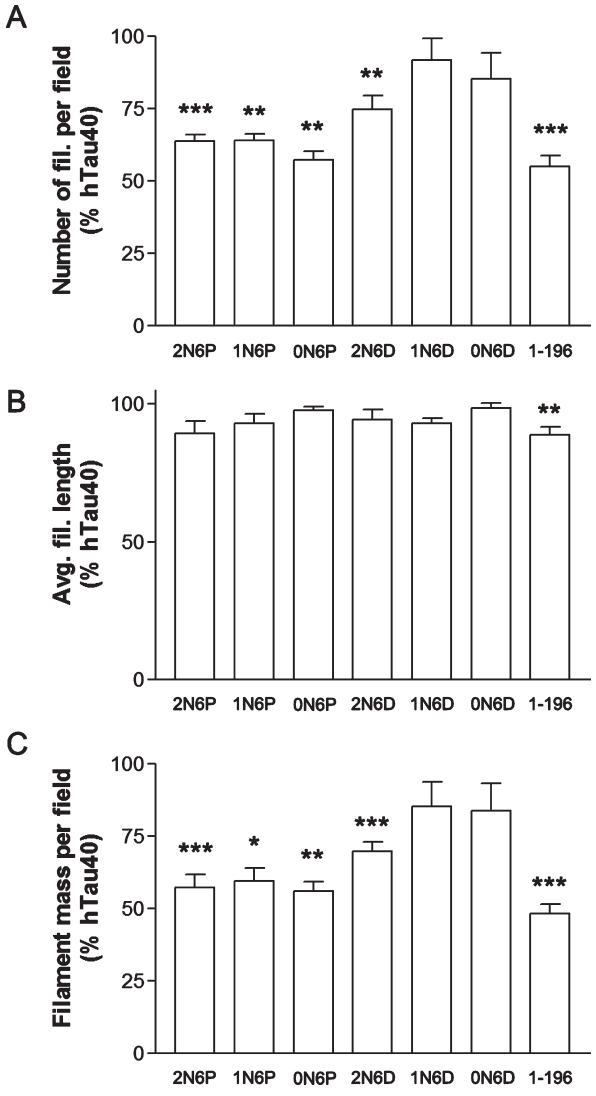

FIGURE 4: The presence of alternatively spliced N-terminal exons differentially impacts the effects of 6D and 6P constructs on hTau40 polymerization. Incubation with 2N6P reduced the number (63.74 ± 2.36% of control) and mass (57.20 ± 4.64% of control) of hTau40 filaments per field. Filament number and mass were also reduced with 0N6P and 1N6P (see Results section). Although 2N6D significantly reduced filament number (74.80 ± 4.62% of control) and mass (69.88 ± 3.23% of control), 6D constructs containing zero or one N-terminal exon (0N6D and 1N6D) failed to significantly inhibit hTau40 polymerization. In agreement with our previous report (*5*), incubation with 1−196 reduced both the number and overall mass of filaments (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

incubation with 1−196 reduced both the number and overall mass of filaments (45.07 ± 3.86% and 51.63 ± 3.27%, respectively; $P < 0.001$). These results are consistent with the reduction in polymer mass as observed by LLS. Taken together, the results of the LLS and EM experiments indicate that, like the N-terminal fragments previously examined, 2N6P and 2N6D inhibit polymerization of full-length tau.

The Presence of Alternatively Spliced N-Terminal Exons Differentially Impacts the Effects of 6D and 6P Constructs on hTau40 Polymerization. Alternative splicing of exons 2 and 3 on the 6D/6P background potentially gives rise to three isoforms apiece (see Figure 2A). To determine whether alternatively spliced 6P and 6D differentially affect hTau40 polymerization, we assayed inhibition by each isoform using quantitative EM (Figure 4). Filament number was significantly

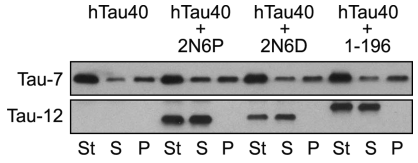

FIGURE 5: 6D/6P isoforms remain in the soluble fraction of the polymerization reaction. Polymerized samples were subjected to ultracentrifugation over a 40% glycerol cushion and separated by gel electrophoresis. From left to right: hTau40 polymerization; hTau40 polymerization with twice molar 2N6P, 2N6D, or 1−196. Short N-terminal constructs were probed with the amino-terminal antibody Tau-12, while full-length tau was probed with the carboxy-terminal antibody Tau-7. Like 1−196, 2N6P and 2N6D do not pellet with the mass of polymerized hTau40 but instead remain in the supernatant. Key: St, prespin starting material; S, supernatant; P, pellet.

reduced by 0N6P and 1N6P in a manner similar to 2N6P (42.72 ± 2.94% and 36.08 ± 2.45%, respectively; $P < 0.01$), as was filament mass (43.95 ± 3.22% and 40.38 ± 4.30%, respectively; $P < 0.01$, $P < 0.05$). This is in agreement with previous results demonstrating that exons 2 and 3 had no effect on the inhibitory abilities of N-terminal fragments (*5*). In contrast, while 2N6D significantly reduced filament number and mass, 6D constructs containing zero or one N-terminal exon (0N6D and 1N6D) failed to significantly inhibit hTau40 polymerization. These experiments indicate that although alternative splicing has no effect on the ability of 6P isoforms to inhibit hTau40 polymerization, the absence of exons 2 and/or 3 reduces the effectiveness of 6D isoforms.

6D and 6P Isoforms Remain in the Soluble Fraction. In a previous study, we demonstrated that N-terminal tau fragments do not associate with hTau40 filaments in a cosedimentation assay (*5*). This result suggested that the fragments act in the soluble fraction of the polymerization mixture to produce their effects. To determine whether 6D and 6P isoforms inhibit polymerization through a similar mechanism, we assembled hTau40 (4 μM) in the presence or absence of 2N6P, 2N6D, or 1−196 (8 μM) and then separated the mixtures into soluble and filamentous fractions through ultracentrifugation. The resulting supernatants and pellets were processed by SDS−PAGE gel electrophoresis, transferred to nitrocellulose membranes, and probed with antibodies against tau (Figure 5). All three constructs, 2N6P, 2N6D, and 1-196, remained in the supernatant fraction and were absent from the filamentous pellet, even at longer exposures (data not shown). These results indicate that 2N6P and 2N6D act in the soluble fraction to inhibit hTau40 polymerization, as did the N-terminal tau fragments previously studied.

Pseudophosphorylation in a Crucial N-Terminal Sequence Modulates the Effects of the 6D/6P Isoforms. We have previously demonstrated that the inhibitory effect of N-terminal tau fragments requires amino acids 18−42 in the amino terminus (*5*). Since 6D and 6P isoforms have similar effects on full-length tau polymerization (i.e., reduce filament number, remain in the soluble fraction), it is likely that amino acids 18−42 are crucial for their effect as well. This sequence contains tyrosine residues at positions 18 and 29, which are potential targets for kinase activity in AD (reviewed in ref *26*). Therefore, we asked whether phosphorylation at these tyrosine residues affects the ability of 6D and 6P isoforms to inhibit polymerization of the full-length protein.

In order to overcome technical problems associated with *in vitro* phosphorylation reactions (i.e., inefficient phosphorylation,

Table 1: Tau Constructs Used in This Study

name	description	purpose
hTau40	full-length tau; 2 N-terminal inserts and 4 MTBRs	
1−196	hTau40 truncated at amino acid 196	positive control for inhibition of hTau40 polymerization
2N6D, 1N6D, 0N6D	6D isoforms containing 0, 1, or 2 N-terminal inserts	isoform inhibition studies
2N6P, 1N6P, 0N6P	6D tau containing 0, 1, or 2 N-terminal inserts	isoform inhibition studies
$6D^{Y}18/29^{E}$	2N6D with two Tyr → Glu mutations	mimics phosphorylation at Tyr18 and 29
$6D^{Y}18/29^{F}$	2N6D with two Tyr → Phe mutations	control construct that does not mimic phosphorylation

phosphorylation at unintended secondary sites), we used pseudophosphorylation, a well-established strategy that employs site-directed mutagenesis to replace a phosphorylatable residue with glutamic acid (*27*). We modeled the effects of tyrosine phosphorylation on the inhibitory ability of 6D/6P isoforms by generating a construct based on 2N6D in which both tyrosine residues were mutated to glutamic acid ($^{Y}18/29^{E}$). As a control, we generated a construct in which both residues were mutated to phenylalanine ($^{Y}18/29^{F}$) (see Table 1 and Figure 6A).

We polymerized hTau40 (4 μM) in the presence or absence of these constructs (8 μM) and measured the effects on filament number, average length, and mass by quantitative EM. Pseudophosphorylation at both tyrosine residues ($^{Y}18/29^{E}$) blocked the ability of 2N6D to inhibit hTau40 (Figure 6B−D). In contrast, the effects of the control construct containing mutations to phenylalanine ($^{Y}18/29^{F}$) were not significantly different than wild-type 2N6D. These results indicate that tyrosine phosphorylation may block the inhibitory effects of the N-terminal isoforms and that residues 18−24 identified in our earlier work using N-terminal hTau40 fragments (*5*) are also important for the inhibitory effects of the 6D and 6P isoforms.

DISCUSSION

6D and 6P Isoforms Inhibit in Vitro Polymerization of Full-Length Tau. Alternative splicing of tau exon 6 produces isoforms that contain the amino terminus of the canonical protein but lack the proline-rich region, MTBR region, and the C-terminal tail. The 6D/6P isoforms bear a striking resemblance to N-terminal tau fragments previously shown to inhibit *in vitro* polymerization of full-length hTau40 (*5*). Based in part on this physical resemblance, we assayed 6D and 6P isoforms for effects on hTau40 filament formation. The results presented here demonstrate that 6D/6P isoforms inhibit *in vitro* polymerization of full-length tau. Like the N-terminal fragments previously studied (*5*), the 6D/6P isoforms reduce the number of filaments formed and the overall filament mass and remain in the soluble fraction of the polymerization mixture, consistent with a model in which these isoforms stabilize a conformation of full-length tau that discourages polymerization (Figure 7).

Surprisingly, although all 6P/6D isoforms contain the region necessary for inhibition (amino acids 18−42), we observe differences in their effectiveness. In general, 6P isoforms are more potent inhibitors than 6D isoforms. Additionally, the absence of exons 2 and 3 decreased the effectiveness of the 6D isoforms but not the 6P variants or the N-terminal tau fragments previously tested (*5*). A simple interpretation is that the C-terminal 6D sequence encourages a protein folding event that limits accessibility of the 18−42 sequence and that this effect is counteracted by the presence of exons 2 and 3. Future insights into the structure of these short isoforms, as well as their interaction with full-length tau, should distinguish between this and other possible explanations.

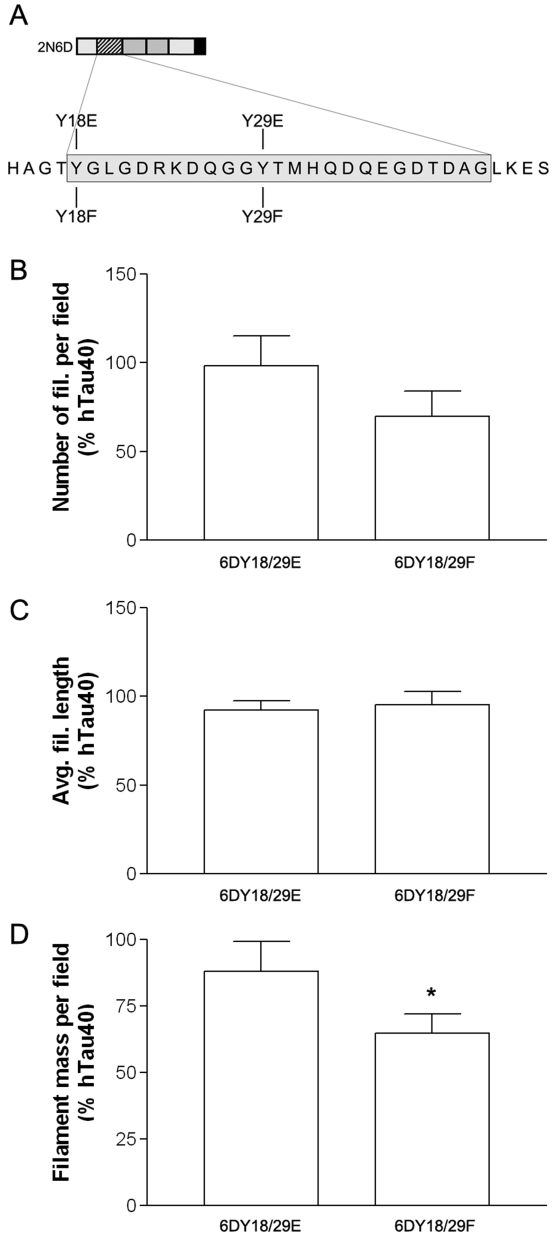

FIGURE 6: Mutations that mimic phosphorylation modifications impair the ability of 2N6D to influence hTau40 polymerization. (A) Schematic illustrating the position of modifications created in the N-terminal inhibitory region of 2N6D. (B) We polymerized hTau40 (4 μM) in the presence or absence of 8 μM wild-type 2N6D (data not shown), $^{Y}18/29^{E}$, or $^{Y}18/29^{F}$ and measured the effects on filament number, average length, and mass by quantitative EM. Pseudophosphorylation at both tyrosine residues ($^{Y}18/29^{E}$) blocked the ability of 2N6D to inhibit hTau40 polymerization. In contrast, $^{Y}18/29^{F}$ decreased filament mass and number similar to wild-type 2N6D (*, $P < 0.05$).

Our results demonstrate that 6P/6D isoforms are potential endogenous inhibitors of filament formation, but the question of

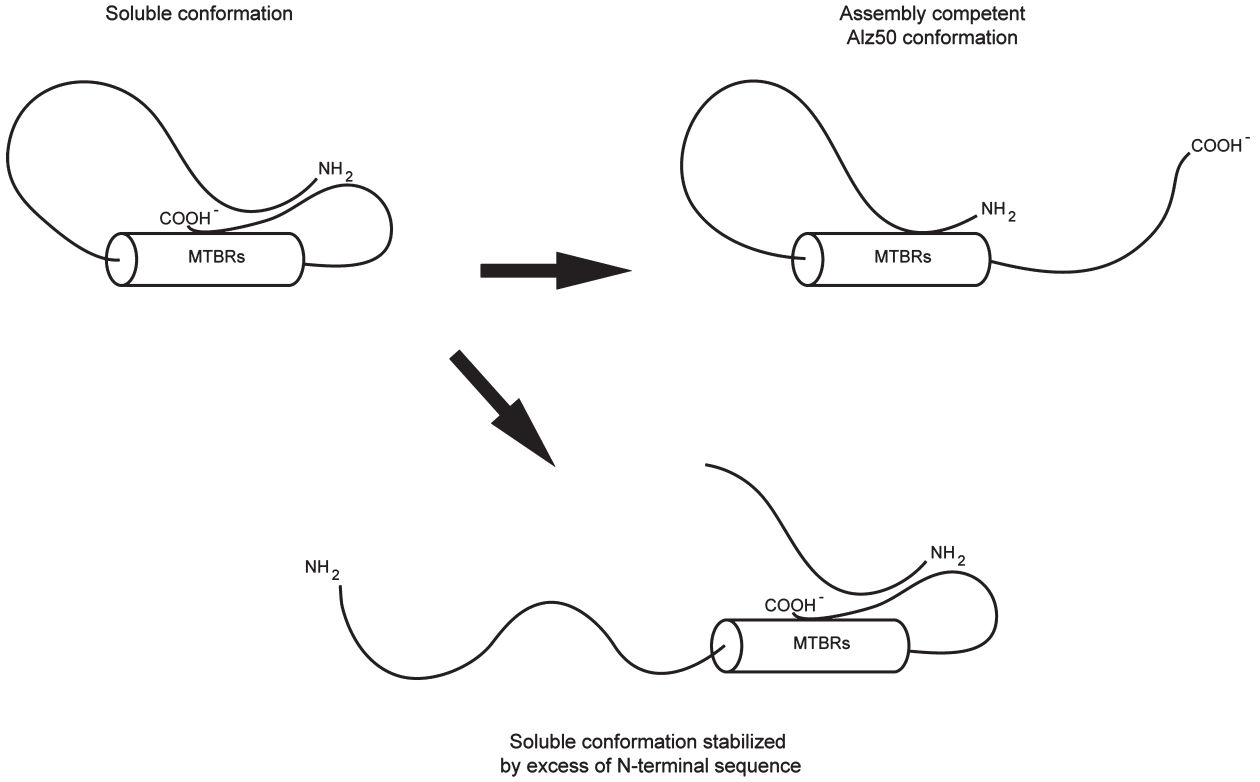

FIGURE 7: Schematic illustrating a potential mechanism by which 6D/6P isoforms or N-terminal tau fragments might promote the solubility of full-length hTau40. In this model, the N-terminus stabilizes an interaction between the C-terminus and the microtubule binding repeat region.

whether they do so in the brain is still unanswered. A twice molar excess of the 6D/6P isoforms was necessary to inhibit *in vitro* polymerization, but with the exception of the cerebellum, 6D/6P levels are low relative to canonical tau isoforms in adult brain (*11*, *18*, *19*, *28*). In this *in vitro* assay system, however, the 6D/6P isoforms (8 μM) must compete with the arachidonic acid (75 μM) that drives polymerization, presumably by inducing hTau40 to adopt an Alz50-like conformation. Lower levels of the 6D/6P isoforms may be required in the context of a cellular system. It is also possible that enrichment of 6D/6P isoforms in certain parts of the cell results in increased local levels relative to canonical tau. Supporting this idea, anti-6D immunoreactivity in cultured cells and in brain is punctate and enriched in the proximal section and distal tips of neuronal processes (*19*). A candidate site for 6D/6P enrichment is the plasma membrane, which interacts with the amino terminus of tau (*29*). Because these truncated isoforms lack the MTBR region, they may even be more likely to interact with the plasma membrane than full-length isoforms. Future studies in cellular or animal models are necessary to determine whether 6D/6P isoforms are endogenous inhibitors of tau aggregation.

Future studies of 6P/6D expression levels and patterns in human brain may reveal more about the potential of these isoforms as endogenous regulators of tau polymerization. Although we propose that expression of 6D/6P may protect the cerebellum in AD, this region develops fibrillar pathology in other tauopathies (*30*, *31*). This indicates either that 6D/6P is not protective against tau aggregation or that this ability is compromised in these diseases. It is not known at present whether 6D/6P expression or phosphorylation (discussed below) is disrupted in these tauopathies, although brain-specific changes in the expression of these isoforms were detected in myotonic dystrophy type 1, a disease marked by tau aggregation (*28*). Comparative neuropathological studies of 6D/6P expression in various tauopathies may inform this issue.

Posttranslational Modifications of 6D/6P Isoforms. In the present study, we demonstrate that pseudophosphorylation of tyrosines 18 and 29 blocks the inhibitory ability of the 6D/6P isoforms. Pseudophosphorylation may disrupt the interaction of 18−42 with full-length tau directly or through conformational effects that reduce the availability of this region. Phosphorylation at tyrosine 18 is elevated in AD (*32*, *33*) and may be targeted by Fyn or other members of the src family of tyrosine kinases (reviewed in ref *26*). Affinity of src tyrosine kinases for the 6D/6P isoforms might be reduced though, because they are truncated prior to an SH3-binding motif (^{233}PKSP^{236}) that binds src family tyrosine kinases (*34*, *35*). In addition to phosphorylation, other factors with the potential to affect the inhibitory ability of the 6D/6P isoforms include nitration at tyrosines 18 and 29 (*36*, *37*), the mutations $^{R}5^{L}$ (*38*) and $^{R}5^{H}$ (*39*), which cause frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17), and removal of the amino terminus, which occurs early in AD progression and may be mediated by caspase 6 (*40*) or puromycin-sensitive aminopeptidase (*41*). If the 6D/6P isoforms represent endogenous inhibitors of filament formation, these changes in the amino terminus could contribute to disease progression by removing a barrier to tau aggregation.

Additional Cell Biological Functions for 6P/6D Isoforms. In isolated axoplasm, 6P/6D isoforms selectively inhibit kinesin-based axonal transport by activating a signaling cascade that results in phosphorylation of kinesin light chains (*42*). Other studies also indicate a role for the N-terminus of tau, and therefore the 6P/6D isoforms, in intracellular signaling. The N-terminus of tau may be part of the cellular response to β-amyloid (Aβ) (*43*−*46*). Expression of N-terminal fragments of tau in cell culture can be neuroprotective (*47*) or toxic,

depending on their length, and can induce cell death through a mechanism involving *N*-methyl-D-aspartate (NMDA) receptors and calpain (*47*, *48*). Finally, the unique C-terminal 6D and 6P sequences may confer isoform-specific cellular roles to these proteins. Further work is necessary to reveal the role of these truncated tau isoforms in human brain.

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