N-Terminal Fragments of Tau Inhibit Full-Length Tau Polymerization in Vitro†

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ABSTRACT: The polymerization of the microtubule-associated protein, tau, into insoluble filaments is a common thread in Alzheimer's disease and in a variety of frontotemporal dementias. The conformational change required for tau to transition from an extended monomeric state to a filamentous state with a high β-sheet content involves the extreme N-terminus coming into contact with distal portions of the molecule; however, these exact interactions are incompletely understood. Here we report that a construct representing amino acids 1−196 (Tau196), which itself does not polymerize, inhibits polymerization of full-length tau (hTau40) in vitro. In addition, we trace the inhibitory effect of Tau196 to amino acids 18−42 of the construct. We also provide evidence that the N-terminal tau fragments require a specific C-terminal region of tau (residues 392−421) to exert their inhibitory effect. The fragments are most effective at inhibiting polymerization when present during the initial 5 min; they remain in the soluble fraction of the polymerization reaction, and they increase the amount of soluble hTau40. The fragments also reduce the number and average length of filaments that are formed. Taken together, these results suggest that the N-terminal tau fragments inhibit hTau40 polymerization by interacting with a specific C-terminal sequence, thereby stabilizing a soluble conformation of tau.

Tau polymerization is a characteristic pathological feature of Alzheimer's disease (AD)[1] (*1*−*4*), several frontotemporal dementias (FTDs) (*5*), and various hereditary tauopathies (*5*−*7*). In AD, the appearance of filamentous tau pathology follows a spatial and temporal progression through the brain regions that underlie cognitive systems affected in AD (*8*−*10*). Therefore, it is widely recognized that an understanding of the biochemical mechanisms underlying tau polymerization may lead to a deeper understanding of disease progression.

In solution, tau monomers have little discernible secondary structure (*11*), although isolated regions of the tau protein, specifically, the third microtubule binding repeat (*12*) and the C-terminus (*13*, *14*), exhibit secondary structure that can be detected by NMR and/or circular dichroism. When not bound to microtubules, monomeric tau is thought to either exist in a largely extended state (*15*), exhibit high mobility in a globally folded state (*16*), or transition rapidly between several conformations (*17*, *18*). In contrast, tau filaments are highly ordered structures, with β-sheets predominating in the MTBR region (*19*−*22*). The transition of tau from a monomeric to a filamentous state therefore must involve extensive conformational rearrangement.

Several lines of evidence suggest that the transition of tau from a soluble monomer to insoluble filaments involves a folding event in which the N-terminus comes into the proximity of the MTBR region (*16*, *23*). Insights into the structures of filamentous tau have come from immunological studies with the conformation-dependent monoclonal antibodies Alz-50 (*23*) and Tau-66 (*24*). Both antibodies recognize tau conformations in which an N-terminal portion of tau is folded over the microtubule binding repeat (MTBR) region. The Alz-50 antibody recognizes a discontinuous epitope involving the extreme amino terminus and the third MTBR (residues 5−15 and 312−322) (*23*, *25*) and labels early tau lesions in AD brain (*26*, *27*). In contrast, the epitope recognized by Tau-66 involves a different N-terminal region and a similar sequence in the third MTBR (residues 155−244 and 305−314) (*24*), and Tau-66 decorates tau pathology at a later stage of maturation (*10*, *27*).

Although immunological evidence suggests that the amino terminus is an integral part of filamentous tau conformations, little is known about how this region regulates the polymerization of tau. We have previously proposed that the N- and C-termini exert antagonistic effects on polymerization by competing for the same region in the MTBRs (*4*, *13*, *28*, *29*). The C-terminus appears to be in the proximity of the MTBRs in the absence of polymerization inducers (*16*), and removing the C-terminus increases the rate and extent of polymerization in vitro (*13*, *30*). In contrast, folding of the extreme N-terminus into proximity with the MTBR region

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[1] Abbreviations: AA, arachidonic acid; AD, Alzheimer's disease; CMC, critical micelle concentration; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FRET, fluorescence resonance energy transfer; FTD, fronto-temporal dementia; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; hTau40, full-length human tau; i_s, intensity of scattered light; MBTR, microtubule binding repeat; PHF, paired helical filament.

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may be an early step in polymerization (*31*), and removal of amino acids 2−18 of tau inhibits, but does not abolish, polymerization in vitro (*28*). Because we have postulated that polymerization involves a folding event that brings the N-terminus and the MTBR region together (*28*, *29*), we hypothesized that a protein fragment containing only the N-terminus and not the MTBRs would inhibit polymerization of the full-length protein. However, we have previously shown that application of an N-terminal peptide consisting of amino acids 1−15 of tau did not affect polymerization in vitro (*28*), suggesting that a different or larger polypeptide fragment may be necessary for observation of such an effect. Therefore, we chose to begin our investigations with a construct representing nearly the entire N-terminal half of the protein.

Here we report that an N-terminal tau fragment truncated after residue 196 (Tau196, which lacks the MTBRs and is therefore assembly-incompetent) inhibits polymerization of full-length human tau (hTau40) in a dose-dependent manner. We show that the inhibitory effect requires amino acids 18−42 in the N-terminal fragment and residues 392−421 of the full-length protein. Furthermore, we provide evidence that the primary effect of the fragments is to inhibit polymerization at an early stage of the reaction, most likely by interacting with and stabilizing a monomeric or soluble oligomeric tau species.

EXPERIMENTAL PROCEDURES

Materials. 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. Synthetic peptides were supplied by Cell Essentials (Boston, MA). Peptide 18−42 represents amino acids 18−42 of hTau40 (YGLGDRKDQGGYTMHQDQEGDTDAG) and was supplied at >90% purity. A peptide with the same amino acid composition in a randomized order (KDQLDGGQQGGDTMHEGRAYDDGTY) was also synthesized.

Recombinant Proteins. The full-length tau used in this study (hTau40) is the longest isoform in the human central nervous system and contains 441 amino acids, including both alternatively spliced N-terminal exons (e2 and e3) and four microtubule binding repeats (m1−m4; Figure 1). The generation and purification of this construct and several others used in this study have been described elsewhere: hTau40, Δ2−18, Δ9−155, Δ155−244, Δ321−441, and Δ430−441 (*23*), Δ18−42, Δ18−30, Δ24−36, and Δ30−42 (*32*), hTau23 and hTau24 (*33*), Δ291−349, Δ376−441, and Δ392−441 (*30*), and Δ422−441 (*34*). The Tau196 construct was generated as previously described (*35*). The Tau196 internal deletion mutant library was generated by restriction digestion and ligation of Tau196 with the various N-terminal deletions listed above, with the exception of Tau196 Δ104−147 ("Tau196B"), which was created using the Tau196 template and phosphorylated primers flanking the desired deletion. All proteins were expressed in *Escherichia coli* and purified by means of an N-terminal polyhistidine tag (*23*, *30*).

Epitope Mapping of Tau-7. Internal deletion mutants of hTau40 were diluted to 2 ng/μL in Tris-buffered saline (pH 7.6) and attached in quadruplicate to Costar 96-well plates

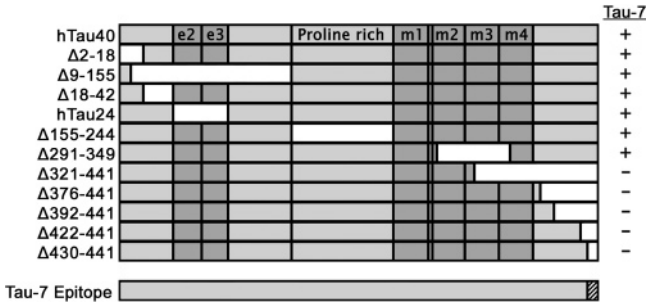

FIGURE 1: Monoclonal antibody Tau-7 recognizes the extreme C-terminus of tau. hTau40 is the longest isoform of tau in the human central nervous system (441 amino acids). Labeled boxes represent (from left to right) alternatively spliced N-terminal exons (e2 and e3), the proline-rich region, and the microtubule-binding repeats (m1−m4). The ability of Tau-7 to react with full-length hTau40 and tau harboring deletions (white, numbers at the left) was assayed by an ELISA. Data are expressed as plus (OD > 3.5) or minus (OD at background levels). The epitope was defined as the smallest deletion which completely abolished Tau-7 binding (hatched, Δ430−441).

overnight at 4 °C. ELISAs were performed as previously described (*24*) using the mouse monoclonal C-terminal tau antibody Tau-7 (40 ng/mL). Results of these ELISAs (Figure 1) confirm that removal of amino acids 430−441 of tau abolishes Tau-7 binding.

Polymerization Reactions. Tau polymerization was induced by arachidonic acid as previously described (*36*). Briefly, tau protein (4 μM) was incubated at room temperature in reaction buffer [final buffer conditions, 10 mM HEPES (pH 7.6), 100 mM NaCl, 0.1 mM EGTA, and 5 mM DTT] in the presence of 75 μM arachidonic acid. The final volume of ethanol in these reactions was 3.8%, and this volume was added to control reaction mixtures in the absence of AA. Unless otherwise noted, N-terminal constructs were added at a concentration of 8 μM to the polymerization reaction mixture, prior to the addition of 4 μM full-length tau and arachidonic acid. Reaction progress was monitored by the intensity of right angle laser light scattering (i_s). End-point ($t = 300$ min) laser light scatter data from at least three independent experiments were analyzed for statistical significance by one-way ANOVA and protected *t*-tests, and time course data were fit with curves using GraphPad Prism 3.0. Error bars in all figures and tables represent one standard error of the mean.

Arachidonic Acid Critical Micelle Concentration (CMC). Arachidonic acid was diluted in polymerization buffer at a range of concentrations and incubated for 10 min at room temperature in the presence or absence of 8 μM protein. The intensity of right-angle laser light scattering was plotted as a function of arachidonic acid concentration, and linear regression was performed to determine the *x*-intercept (*37*, *38*). The data presented here are the combined results of five separate experiments, analyzed using a one-tailed *t*-test (GraphPad Prism 3.0).

N-Terminal Fragment Centrifugation. Reaction mixtures polymerized for 5 h were centrifuged at 355000*g* for 15 min at 25 °C over a 40% glycerol cushion. Supernatant and pellet were boiled in Laemmli buffer, and proteins were separated by SDS−PAGE before being transferred to nitrocellulose membranes. N-Terminal fragments (Tau196B) were probed with amino-terminal antibody Tau-12 (4 ng/mL) (*10*, *32*), while full-length tau was probed with Tau-7 (see Figure 1).

HRP-conjugated goat anti-mouse secondary antibody (Vector) and ECL (Amersham) were used for detection.

Electron Microscopy. Polymerization reactions were allowed to proceed for at least 4 h, and the mixtures were fixed with 2% glutaraldehyde (Electron Microscopy Sciences, EMS), spotted onto 300 mesh Formvar/carbon-coated copper grids (EMS), and negatively stained with 2% uranyl acetate (EMS) as previously described (*31*). 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 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 fields from each grid were chosen for quantitation under low illumination to prevent bias. Results from three separate experiments were analyzed by two-tailed *t*-tests (GraphPad Prism 3.0).

Tau Critical Concentration Assay. Reaction mixtures polymerized for 5 h were centrifuged at 355000*g* for 15 min at 25 °C. To determine the effectiveness of these sedimentation conditions in the removal of polymerized material from the supernatant, the supernatant fractions were analyzed by electron microscopy as described above. The amount of tau in supernatant fractions was quantified by diluting samples in Laemmli sample buffer and spotting onto nitrocellulose membranes, alongside a series of hTau40 standards of known concentrations. Full-length tau was visualized with Tau-7 (which does not react with the N-terminal fragment) and HRP-conjugated goat anti-mouse secondary antibody. The amount of protein per spot was quantified using Adobe Photoshop: a box of fixed size was centered on each spot, and the average pixel intensity was determined using the histogram function. The tau standard curve and GraphPad Prism 3.0 were used to determine tau concentrations in the soluble fractions of the polymerization reaction mixtures.

RESULTS

N-Terminal Tau Fragments Inhibit Full-Length Tau Polymerization in Vitro. To determine whether an amino-terminal fragment of tau would inhibit polymerization of full-length tau (hTau40), a protein construct corresponding to the N-terminal half of hTau40 was generated by changing the tyrosine at position 197 to a stop codon. The resulting construct ("Tau196", Figure 2A) lacks the MTBR region, including the sequence required for arachidonic acid-induced filament assembly (residues 314−320) (*30*). As predicted, Tau196 itself failed to polymerize in the presence of arachidonic acid (data not shown). When Tau196 was added at a 2- or 4-fold molar ratio, this N-terminal fragment of tau inhibited the polymerization of full-length tau protein as measured by right-angle laser light scattering (Figure 2B).

Inhibition of Polymerization Depends on Amino Acids 18−42 of Tau196. To identify the specific sequence in Tau196 responsible for interfering with hTau40 polymerization, we generated a library of internal deletions on the Tau196 background (Figure 2A) and screened these proteins for their ability to inhibit hTau40 polymerization. Tau196 mutants harboring deletions of residues 2−18, 41−103 (exons 2 and 3), or 104−147 reduced the extent of hTau40 polymerization by 57−63% ($p < 0.01$) at a 2-fold molar ratio. However,

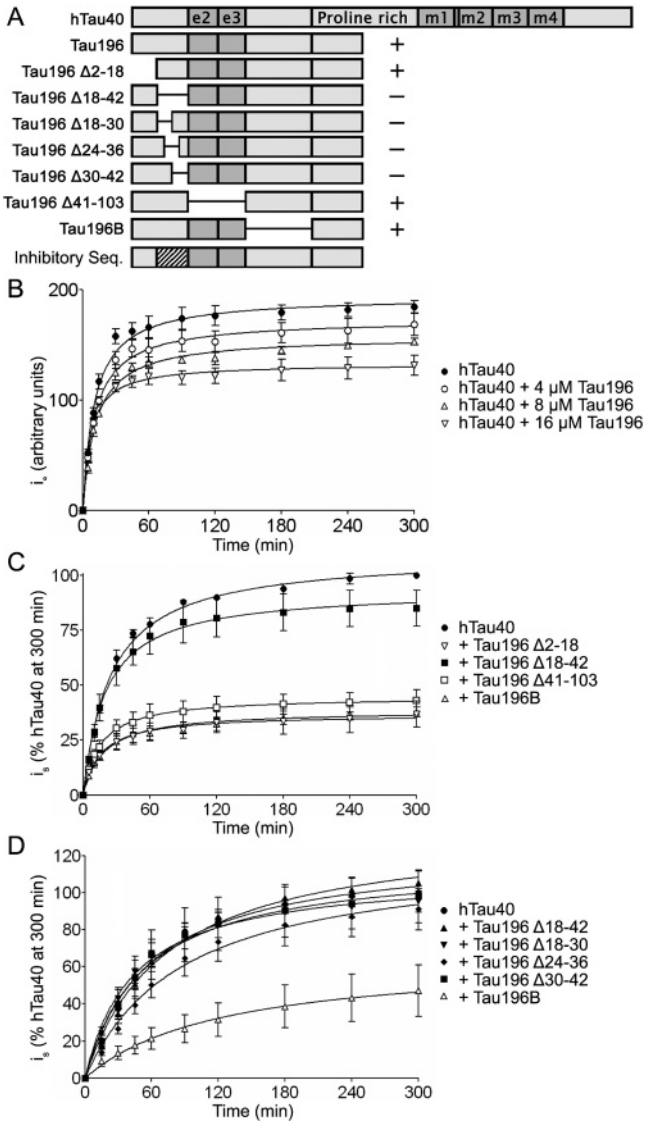

FIGURE 2: N-Terminal fragments of tau specifically inhibit arachidonic acid-induced tau polymerization as measured by laser light scattering. (A) Schematic of the N-terminal tau constructs used in this study. A tau construct containing a stop codon at Y197 (Tau196) and several internal deletion mutations on the Tau196 background were created and purified, including Tau196 Δ104−147 (Tau196B). Constructs that inhibited (+) or failed to inhibit (−) polymerization (shown below) are indicated, as is the specific N-terminal sequence required for inhibition (hatched, residues 18−42). (B) Tau196 was added at a 1-, 2-, or 4-fold molar ratio to a polymerization reaction mixture of 4 μM full-length tau (hTau40). Polymerization was significantly inhibited in the presence of 8 ($p < 0.05$) and 16 μM Tau196 ($p < 0.01$). (C) Internal deletion mutations in Tau196 (8 μM) were utilized to map the sequence specificity of the inhibitory effect. Only the Tau196 Δ18−42 fragment failed to significantly inhibit hTau40 polymerization ($p < 0.01$ vs other deletion constructs, not significant vs hTau40 control). (D) Smaller deletions in the region of residues 18−42 of the tau fragment also eliminate the inhibitory effect of the fragment ($p < 0.01$ vs Tau196B, not significant vs hTau40 control), suggesting that this entire sequence is necessary to inhibit hTau40 polymerization.

removal of residues 18−42 resulted in loss of the inhibition (Figure 2C; $p < 0.01$ vs other deletions, not significant vs hTau40 control). Constructs containing smaller deletions in the region of residues 18−42 (18−30, 24−36, or 30−42) also failed to significantly decrease the extent of hTau40 polymerization (Figure 2D, not significant vs hTau40

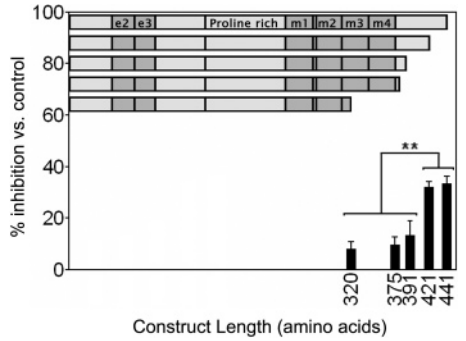

FIGURE 3: Inhibition requires the C-terminal region of residues 392−421 of hTau40. A schematic of full-length tau and the C-terminal deletion constructs used is shown. Tau196B inhibits full-length hTau40 and Δ422−441 tau polymerization ($p < 0.01$) but not the polymerization of Δ321−441, Δ376−441, or Δ392−441 tau ($p < 0.01$ vs hTau40 and Δ422−441, not significant vs control).

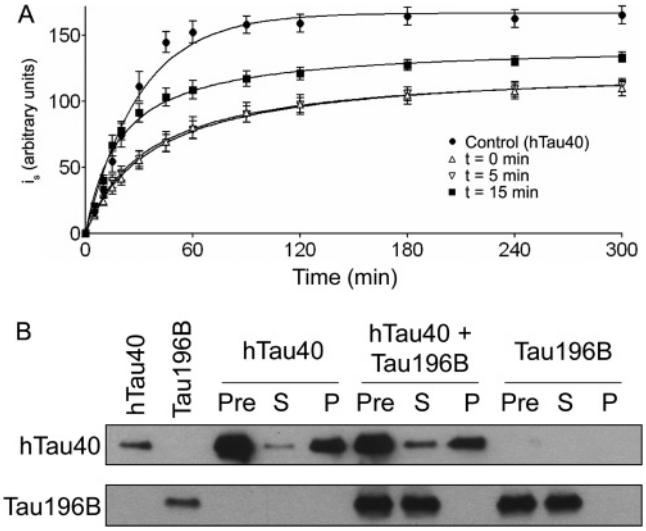

FIGURE 4: N-Terminal fragments act at an early step in polymerization and remain in the soluble fraction. (A) Tau196B was added just prior to ($t = 0$ min) or after induction of polymerization ($t =$ 5 or 15 min). Delaying addition of the fragment by 5 min did not reduce its efficacy; however, 15 min after induction, the fragment was significantly less effective ($p < 0.05$). (B) Polymerized samples were subjected to ultracentrifugation over a 40% glycerol cushion and separated by gel electrophoresis. From left to right are shown hTau40 and Tau196B standards, hTau40 polymerization, hTau40 polymerization with a 2-fold molar excess of Tau196B, and Tau196B alone. Pre denotes a prespin polymerization reaction, S the supernatant, and P the pellet. N-Terminal fragments (Tau196B) were probed with the amino-terminal antibody Tau-12, while full-length tau was probed with the carboxy-terminal antibody Tau-7. Tau196B does not pellet with the mass of polymerized hTau40 but instead remains in the supernatant.

control), indicating that this entire sequence may be required for inhibition. The inhibition caused by Tau196 Δ104−147 was more robust than for Tau196, and for this reason, it was used as a positive control for polymerization inhibition in this experiment ($p < 0.01$) and for the remainder of the study and Tau196 Δ104−147 was renamed to Tau196B.

Since these data suggested a crucial role for residues 18−42, we next asked whether a peptide corresponding to these residues would also inhibit hTau40 polymerization. A second peptide containing the same amino acids in a randomized order was used as a control. At a 10-fold molar ratio (40 μM), neither peptide inhibited hTau40 polymerization as measured by laser light scattering and electron microscopy (data not shown). This result is consistent with our previous report that another N-terminal peptide (residues 1−15) does not affect hTau40 polymerization (*28*). The failure of these peptides to inhibit polymerization may indicate that a larger sequence is necessary to stabilize a direct interaction with hTau40 or to recapitulate the native conformation of the N-terminus of tau.

N-Terminal Fragments Require Residues 392−421 of hTau40 To Inhibit Polymerization. In our working model of tau polymerization, the N-terminus and C-terminus interact with the MTBR region of the full-length protein, with the N-terminus promoting polymerization and the C-terminus promoting solubility (*4*, *13*, *28*, *29*). We therefore hypothesized that the N-terminal fragments likewise interact with the MTBRs of hTau40, blocking the intramolecular interaction of these two regions that facilitates polymerization. If this is the case, removal of the MTBR binding site on hTau40 should ameliorate the inhibitory effects of the N-terminal fragment.

To identify this site, we employed a series of C-terminally truncated tau constructs (Δ321−441, Δ376−441, Δ392−441, and Δ422−441), the last two of which are also found in AD brains (*34*, *39*). All of these truncated tau proteins are known to polymerize effectively in vitro (*30*, *34*). These C-terminally truncated tau proteins were polymerized in the absence or presence of a 2-fold molar ratio of Tau196B, and the extent of polymerization was measured by laser light scattering. Tau196B inhibited polymerization of full-length tau and tau truncated at aspartic acid 421 (Figure 3; $p <$ 0.01). However, the N-terminal fragment failed to inhibit polymerization of tau proteins truncated prior to residue 392 ($p < 0.01$), suggesting that residues 392−421 are required for N-terminal inhibition. The simplest explanation for this result is that the N-terminus interacts directly with residues 392−421, although more complicated scenarios are possible. Because we have previously shown that residues 321−375 (and not 376−441) are required for C-terminal inhibition of polymerization (*13*), this result suggests that different regions of tau are required for polymerization regulation by the N- and C-termini.

N-Terminal Fragments Are Most Effective at Early Time Points. Once the specific sequences involved in the inhibition were identified, we turned our attention to the mechanism of tau polymerization and its inhibition. We began by examining the efficacy of the N-terminal fragments at various time points in tau polymerization. Specifically, Tau196B (8 μM) was added to an hTau40 polymerization reaction just prior to the induction of polymerization, or 5 min, 15 min, or 24 h after induction. Tau196B was equally effective when added prior to polymerization induction or 5 min after induction (Figure 4A); however, when the fragment was added 15 min after induction, it was 44% less effective in inhibiting polymerization ($p < 0.05$). Addition of Tau196B to fully polymerized hTau40 (24 h after induction) did not result in depolymerization of hTau40 filaments 6 h later, as measured by laser light scattering and quantitative electron microscopy (data not shown). Overall, the results of this experiment indicate that the N-terminal tau fragments are most effective at an early step in polymerization, suggesting that they hinder the generation or elongation of nascent polymers rather than destabilize existing filaments.

Table 1: The Critical Micelle Concentration of Arachidonic Acid Is Depressed in the Presence of either Tau196 or Tau196 Δ18−42

condition	AA CMC (μM)
no protein	80.03 ± 5.79
Tau196	55.54 ± 8.80^a
Tau196 Δ18−42	38.88 ± 12.50^a

[a] $p < 0.05$ vs no protein.

N-Terminal Fragments Remain in the Soluble Fraction. Next, we asked whether the fragments associate with the mass of polymerized material or with a soluble component of the reaction mixture. To address this question, hTau40 polymerization reaction mixtures were subjected to ultracentrifugation, and the prespin reaction mixtures and postspin supernatants and pellets were analyzed by Western blotting (Figure 4B). While the majority of the hTau40 in the polymerization reaction sediments under these conditions, we found no evidence of Tau196B in the pellet. As predicted, more hTau40 remains in the supernatant in the presence of the fragment, and the fragment does not sediment in the absence of hTau40. Our results strongly suggest that the fragments are not incorporated into the growing filaments but rather exert their effects on a soluble component of the polymerization reaction mixture.

The Region of Residues 18−42 Does Not Affect the Arachidonic Acid Critical Micelle Concentration (CMC). Since the fragments remain in the soluble fraction and act at an early step in polymerization, we suspected they may interact with the polymerization inducer, arachidonic acid (AA). AA forms micelles under physiological buffer conditions, and tau filaments are thought to nucleate at the micelle surface (*38*, *40*−*42*). Tau lowers the CMC of AA and other anionic detergents, and this effect correlates with the ability of these detergents to induce tau polymerization (*38*); however, the regions of tau that mediate the effect on AA CMC remain unidentified. If the N-terminal fragments also lower the AA CMC, they could coat the AA micelles or sequester AA and thereby have a substantial effect on filament nucleation.

To determine whether the inhibitory effect of the fragment involves such an interaction with arachidonic acid, we measured the AA CMC in the absence of protein, in the presence of an inhibitory fragment (Tau196), and in the presence of a fragment that failed to inhibit polymerization (Tau196 Δ18−42). Although both fragments lowered the AA CMC, the effects of Tau196 and Tau196 Δ18−42 did not differ significantly (Table 1). These results suggest that the observed inhibition of polymerization cannot be explained simply by an effect on arachidonic acid.

Tau196B Decreases the Number and Average Length of Filaments. Electron microscopy is a useful tool for verifying results obtained by laser light scattering and further examining the effects of molecules that regulate polymerization (*28*). Filaments formed in the absence or presence of 8 μM Tau196B were visualized by electron microscopy. The filaments formed under these two conditions appeared to be morphologically similar (Figure 5A). However, quantitative analysis (Figure 5B) revealed several differences; the addition of the N-terminal tau fragment greatly reduced the number of filaments per field (to $51.0 \pm 5.6\%$ of control; $p < 0.01$) and had a small but significant effect on average filament length (to $81.8 \pm 1.9\%$ of control; $p < 0.05$). Under both conditions, exponential length distributions were observed (data not shown). Combined, the effects on filament number and length resulted in a substantial decrease in the mass of polymerized material (to $41.8 \pm 5.2\%$ of control; $p < 0.01$), consistent with the light scattering data (Figure 2C,D). The electron microscopy data suggest that the N-terminal fragments primarily act by reducing the number of filaments formed but also slightly decrease the average length of filaments.

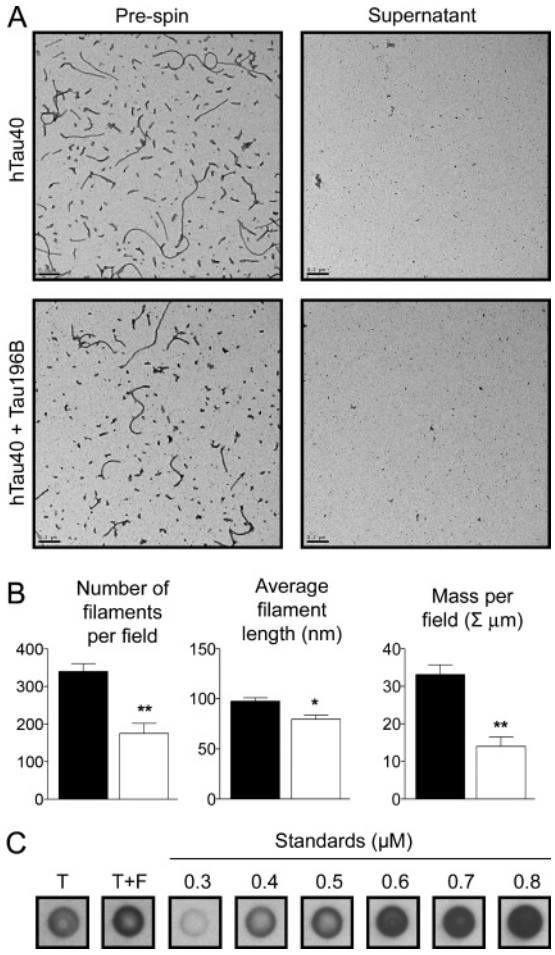

FIGURE 5: N-Terminal tau fragments decrease the mass of polymerized material and increase the hTau40 critical concentration. (A) The left column shows representative electron microscopy fields from polymerization reactions of hTau40 in the absence and presence of Tau196B. In the right column, electron microscopy confirms that ultracentrifugation removes the vast majority of polymerized material from the supernatant fraction. The scale bars are 200 nm. (B) Quantitation of 5 h polymerization reactions reveals that Tau196B caused a significant reduction in the number of filaments per field (to $51.0 \pm 5.6\%$ of control; $p < 0.01$) as well as a smaller decrease in the average length per filament (to $81.8 \pm 1.9\%$ of control; $p < 0.05$). The combined effects on filament number and length resulted in a reduced overall mass of polymerized material per field (to $41.8 \pm 5.2\%$ of control; $p < 0.01$): (black bars) hTau40 alone and (white bars) hTau40 with Tau196B. (C) The hTau40 critical concentration in the absence and presence of Tau196B was quantified by blotting the supernatant fraction with the Tau-7 C-terminal antibody: (T) hTau40 alone and (T+F) hTau40 plus N-terminal fragments (Tau196B). The hTau40 critical concentration (0.45 ± 0.03 μM) is similar to previously published results. Tau196B increased the tau critical concentration to 0.56 ± 0.05 μM ($p < 0.01$).

N-Terminal Fragments Increase the Tau Critical Concentration. Since Tau196B increased the amount of hTau40 in

the supernatant of the polymerization reaction (Figure 4B), we sought to quantify this result to determine the effect of N-terminal tau fragments on the hTau40 critical concentration. Though critical concentration measurements are frequently used as reflections of the growth constant (*43*), there is debate in the literature about how best to estimate the amount of unpolymerized, soluble tau in a polymerization reaction mixture (*29*). We chose as our measure of tau critical concentration the amount of tau remaining in the supernatant fraction following ultracentrifugation. Since the centrifugal force required to sediment all of the polymerized material (oligomeric and filamentous) is not known, electron microscopic analysis was performed to determine the efficacy of the protocol used here at sedimenting polymerized material (Figure 5A). The micrographs demonstrate that the vast majority of polymerized protein is removed from the supernatant under the conditions that were employed (see Experimental Procedures). Furthermore, this protocol is externally validated by noting that the critical concentration of hTau40 measured by this method ($0.45 \pm 0.03\ \mu$M, Figure 5C) is comparable to several previously published values of the hTau40 critical concentration (0.50 ± 0.09 and $0.47 \pm 0.14\ \mu$M) as estimated by laser light scattering (*31*, *44*).

To determine the effect of Tau196B on hTau40 critical concentration, the amount of full-length tau in the supernatants was quantified with the monoclonal antibody Tau-7 (Figure 5C), which recognizes only the C-terminus of hTau40 (and therefore not the N-terminal fragment). The presence of a 2-fold molar excess of Tau196B significantly increased the hTau40 critical concentration to $0.56 \pm 0.05\ \mu$M ($p < 0.01$). Since the tau critical concentration is inversely proportional to the growth constant (*43*), this signifies a 19.6% decrease in the growth constant for hTau40 filament elongation. These data support the quantitative electron microscopy findings which show that the fragments exert a small but significant effect on filament length, in addition to a primary effect on the number of filaments.

DISCUSSION

Although the path from tau monomer to filament is incompletely understood, a picture of the regions of the tau protein governing this transition is beginning to emerge. A salient feature of this model is competition between the extreme termini of the protein for proximity to the MTBR region. The MTBR region of tau (specifically residues 314−320) (*30*) is required for arachidonic acid-induced polymerization, and folding of distal parts of tau onto the MTBRs is an important regulator of tau solubility and polymerization (*13*, *28*, *29*). Interaction of the extreme C-terminus with the MTBRs promotes solubility (*13*). Conversely, polymerization is favored when the N-terminus is in the proximity of the MTBRs, as recognized by the Alz-50 antibody (*23*, *31*). The goal of this study was to further characterize the role of the N-terminus by assessing the effects of a truncated construct representing the N-terminal half of tau (Tau196) in an in vitro polymerization assay. Our results demonstrate that a specific N-terminal tau region inhibits polymerization of the full-length protein, suggesting a novel regulatory role for the amino terminus of tau.

We initially hypothesized that an excess of Tau196, which contains the N-terminal portion of the Alz-50 epitope (residues 1−15) but lacks the corresponding MTBR portion, would inhibit polymerization of hTau40 by binding to nascent filaments and preventing the addition of full-length tau. However, our data show that both N- and C-terminal sequences that are required for the fragments to inhibit hTau40 polymerization (18−42 and 392−421; Figures 2 and 3) differ considerably from the sequences required for Alz-50 binding (1−15 and 312−322) (*23*). Furthermore, the filament "capping" mechanism is unlikely given that we were unable to detect any N-terminal fragments associated with tau filaments in the pellet of a polymerization reaction following ultracentrifugation. Moreover, a capping mechanism would be expected to produce a pronounced effect on elongation, which seems not to be the case. Overall, these results suggest that if filament capping occurs, it is not the primary mechanism by which the N-terminal fragments inhibit polymerization.

Instead, the data presented here are consistent with a mechanism in which the N-terminal fragments act on a soluble component of the polymerization reaction mixture. The fragments increase the amount of soluble tau in the polymerization reaction mixture and are most effective when present at the induction of polymerization. Furthermore, the fragments are not incorporated into or associated strongly with filaments; instead, they remain in the soluble fraction. Since the inhibitory effect of the fragments cannot be explained by an effect on the arachidonic acid critical micelle concentration and instead requires specific sequences of both hTau40 and the N-terminal fragments, our results are most consistent with a mechanism in which the fragments interact with full-length soluble tau. Collectively, these results suggest that the N-terminal fragments stabilize full-length tau in a soluble conformation, removing hTau40 molecules from the pool of polymerizable protein.

One known conformation of soluble tau involves folding of the C-terminus of tau onto the MTBR region (*13*). Stabilizing this interaction could conceivably increase hTau40 solubility in the face of polymerization inducers. Recently, fluorescence resonance energy transfer (FRET) analysis of tau in solution has shown that residue 432 of the C-terminus and residue 310 of the third MTBR are in the proximity of each other, confirming that the C-terminus of tau folds over the MTBRs in the absence of polymerization inducers (*16*). However, the N-terminus has also been implicated in this C-terminal−MTBR folding; while the N-terminus and MTBRs have low FRET efficiency, residues 17 and 18 of the N-terminus are in the proximity of residue 432 of the C-terminus (*16*). Thus, in this "paperclip folded" conformation of soluble tau, the N-terminus is near the C-terminus as the C-terminus associates with the MTBRs.

The N-terminal fragment data presented here are consistent with the N-terminal involvement in the folding of the C-terminus over the MTBR region (*16*). The association of residues 17, 18, and 432 of the N- and C-termini in the paperclip folding of soluble tau (*16*) suggests that residues 18−42 and 392−421, which have yet to be investigated by FRET, may also be in the proximity of each other in this conformation. We therefore propose a scenario in which the N-terminal fragments bind to the C-terminus of hairpin-folded soluble tau and stabilize the C-terminal−MTBR interaction (Figure 6). This explanation is consistent with our observations that the fragments increase the amount of

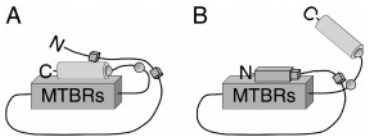

FIGURE 6: Diagrammatic representations of the proposed conformations of tau in soluble and polymerized states. (A) Association of the MTBR region with the extreme C-terminus (cylinder) promotes solubility (*13*), while the N-terminal region between residues 18 and 42 (both cubes) may stabilize this conformation by associating with the C-terminal region between residues 392 and 421 (both spheres). This arrangement of the MTBRs and both termini of tau has also been demonstrated independently by structural analyses of soluble tau (*16*). (B) When the C-terminus vacates its position along the MTBRs, the extreme N-terminus may be allowed access to the MTBR core of polymerization. This conformation, recognized by the Alz-50 antibody (*23*), is associated with early tau polymerization both in vitro (*31*) and in vivo (*26*, *27*).

soluble tau while the fragments themselves remain in the soluble fraction (Figures 4 and 5). Furthermore, this interpretation, along with evidence from other structural studies (*16*), places the extreme N-terminus of soluble tau in a precarious location, near the MTBR "core" of polymerization. It is easy to imagine how the N-terminus of tau could be induced to replace the carboxy terminus as the binding partner of the MTBR core to form the Alz-50 conformation (*23*). However, the influence of the N-terminus on the polymerization of tau is complex, and further experimentation is needed to fully understand its involvement in the conformational shifts that take place during tau polymerization.

Although we have shown that residues 18−42 are necessary for polymerization inhibition, a peptide corresponding to this sequence alone was not sufficient to recapitulate the inhibitory effect of the N-terminal fragments. This contrasts with a C-terminal tau peptide (residues 422−441) which we have shown inhibits polymerization of the full-length protein (*13*). In the latter case, the C-terminal "tail" peptide is thought to inhibit polymerization by forming an amphipathic α-helix (*14*). Unlike the tail peptide, the N-terminus has little predicted secondary structure, and the N-terminal peptide may be unable to adopt a stable conformation outside the context of the larger N-terminal fragment. Alternatively, a longer sequence of the N-terminal fragment may be required to promote a stable interaction with the full-length molecule and thereby inhibit polymerization.

Our results suggest a novel role for the N-terminus in the regulation of tau solubility. These findings are particularly interesting in light of the numerous disease-related modifications of tau that affect the regions at either end of the molecule in vivo. The sequences of amino acids 18−42 and 392−421 include residues which may be regulated by phosphorylation (*45*, *46*) or nitration (*35*). Our previous work has also shown that the N-terminus of tau in neurofibrillary tangles may be subject to regulation by enzymatic cleavage in early AD (*32*), and proteolysis after glutamic acid 391 (*47*−*49*) is known to occur at an advanced stage in AD tangle evolution (*10*, *27*). It will be interesting to determine how these modifications influence the ability of the fragments to inhibit tau polymerization.

The inhibitory N-terminal constructs described here also bear an intriguing resemblance to a recently discovered group of alternatively spliced tau isoforms which lack the sequences encoding the MTBR region and C-terminal portion of the protein (*50*, *51*). At least one of these alternatively spliced constructs (the 6d tau isoform, which contains residues 1−143 of canonical tau followed by 11 additional unique amino acids) is expressed in human brain tissue, and the level of expression is particularly high in areas that are not affected by tau lesions in AD (cerebellum and spinal cord) and lowest in tangle-prone areas (hippocampus and cerebral cortex) (*52*). Even within affected areas, the 6d isoform does not colocalize with cells bearing tangles (*52*). In light of our results, it is tempting to speculate that expression of this alternatively spliced N-terminal fragment of tau could be responsible for preventing tau polymerization in these unaffected cells and brain regions in vivo.

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