Site-specific Phosphorylation and Caspase Cleavage Differentially Impact Tau-Microtubule Interactions and Tau Aggregation*

Received for publication, October 28, 2005, and in revised form, March 29, 2006 Published, JBC Papers in Press, May 10, 2006, DOI 10.1074/jbc.M511697200

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The microtubule-associated protein tau is hyperphosphorylated and forms neurofibrillar tangles in Alzheimer disease. Additionally caspase-cleaved tau is present in Alzheimer disease brains co-localized with fibrillar tau pathologies. To further understand the role of site-specific phosphorylation and caspase cleavage of tau in regulating its function, constructs of full-length tau (T4) or tau truncated at Asp231/T4C3 to mimic caspase-3 cleavage with and without site-directed mutations that mimic phosphorylation at Thr231/Ser235, Ser396/Ser404, or at all four sites (Thr231/Ser235/Ser396/Ser404) were made and expressed in cells. Pseudophosphorylation of T4, but not T4C3, at either Thr231/Ser235 or Ser396/Ser404 increased its phosphorylation at Ser262 and Ser199. Pseudophosphorylation at Thr231/Ser235 impaired the microtubule binding of both T4 and T4C3. In contrast, pseudophosphorylation at Ser396/Ser404 only affected microtubule binding of T4C3 but did make T4 less soluble and more aggregated, which is consistent with the previous finding (Abrah, A., Ghoshal, N., Gamblin, T. C., Cryns, V., Berry, R. W., Kuret, J., and Binder, L. I. (2000) J. Cell Sci. 113, 3737–3745) that pseudophosphorylation at Ser396/Ser404 enhances tau polymerization in vitro. In situ T4C3 was more prevalent in the cytoskeletal and microtubule-associated fractions compared with T4, whereas purified recombinant T4 bound microtubules with higher affinity than did T4C3 in an in vitro assay. These data indicate the importance of cellular factors in regulating tau-microtubule interactions and that, in the cells, phosphorylation of T4 might impair its microtubule binding ability more than caspase cleavage. Treatment of cells with nocodazole revealed that pseudophosphorylation of T4 at both Thr231/Ser235 and Ser396/Ser404 diminished the ability of tau to protect against microtubule depolymerization, whereas with T4C3 only pseudophosphorylation at Ser396/Ser404 attenuated the ability of tau to stabilize the microtubules. These results show that site-specific phosphorylation and caspase cleavage of tau differentially affect the ability of tau to bind and stabilize microtubules and facilitate tau self-association.

One of the major neuropathological hallmarks of Alzheimer disease (AD) is the intracellular neurofibrillary tangle (NFT), which is mainly composed of hyperphosphorylated microtubule-associated protein tau (1, 2). A pathological role for tau in AD is supported by strong evidence including clinicopathological studies showing that the amount of abnormal tau protein and the abundance of NFTs correlate positively with the severity of cognitive impairment in AD (3) and the finding that tau is essential to Aβ-induced toxicity in cultured hippocampal neurons from human tau transgenic mice (4). Furthermore a recent study demonstrated that attenuation of tau overexpression in a neurodegenerative mouse model prevented further neuronal loss and memory impairment (5). These and other studies support the hypothesis that pathogenic alterations in tau contribute to the etiology of AD. Nonetheless the mechanisms leading to tau dysfunction and neurodegeneration have not been clearly elucidated.

Tau is predominantly expressed in neurons where its primary function is to promote tubulin polymerization and microtubule stability (6). This physiological function of tau is regulated by tau phosphorylation. In AD tau is hyperphosphorylated at a number of sites (2); this impairs its ability to bind and stabilize microtubules. It has been proposed that in AD brain this loss of tau function may result in the breakdown of axonal transport of vesicles and organelles (loss of function) and lead to neurodegeneration (7, 8). In addition, in vitro studies showed that hyperphosphorylated tau can self-assemble into paired helical filaments (PHFs), which are similar in structure to those found in AD brain (gain of function) (9, 10). Although these and other studies clearly indicate that extensive hyperphosphorylation of tau occurs in pathological states, it is becoming increasingly evident that the phosphorylation of specific sites on tau play essential roles in regulating its function. For example, phosphorylation of tau at Thr231 by glycogen synthase kinase 3β (GSK3β) plays a critical role in regulating the ability of tau to bind and stabilize microtubules (11). Phosphorylation of tau at Ser262 by microtubule affinity-regulating kinase not only decreases the microtubule binding ability of tau but also attenuates its assembly into PHFs (12, 13). Although in situ studies using protein kinases to phosphorylate tau have provided important information about how site-specific phosphorylation of tau affects tau function, there are drawbacks to

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* This work was supported by a grant from the Alzheimer’s Disease Association and National Institutes of Health Grant N5051279. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: AD, Alzheimer disease; GSK3β, glycogen synthase kinase 3β; NFT, neurofibrillary tangle; PHF, paired helical filament; Mes, 4-morpholineethanesulfonic acid; HEP, human embryonic kidney; PIPES, 1,4-piperazinediethanesulfonic acid; GST, glutathione S-transferase.
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this approach. For example, tau is not the only substrate that the kinase phosphorylates, and therefore the observed changes in tau function may not be due to direct effect of phosphorylation. Furthermore protein kinases that phosphorylate tau invariably phosphorylate several up to many different sites, and phosphorylation events are often less than stoichiometric, which can complicate the interpretation of those data. Therefore, complementary approaches are needed to determine how the phosphorylation of specific sites affects tau function. One such approach is mutating serine or threonine residues to glutamic acid or aspartic acid (pseudophosphorylation) to mimic the effects of the addition of a phosphate group (14, 15). Previously it has been demonstrated that pseudophosphorylation of both Ser396/Ser404 and Ser422 enhances tau aggregation in vitro (16–18). Therefore it is critically important to understand how the phosphorylation of specific sites on tau modulates its physiological and pathological functions, and the use of pseudophosphorylation of specific residues is an important tool in this process.

In addition to abnormal phosphorylation, aberrant cleavage is another pathological posttranslational modification of tau. In vitro, tau is a substrate for caspase-3 (19–22). Tau truncated at Asp421, the caspase-3 cleavage site in tau, aggregates more rapidly and to a greater degree than full-length tau, and at the same time, the truncated tau is capable of accelerating filament formation of full-length tau (19, 20). In vivo, antibodies that specifically recognize tau truncated at Asp421 stain AD brains, and this staining frequently colocalizes with active caspase-3 and fibrillar tau pathologies (19, 20). Furthermore there is evidence in cultured cell models suggesting that caspase-3-cleaved tau may be neurotoxic (21, 22). In cultured cortical neurons Aβ treatment results in caspase activation and tau cleavage (19). Given these findings it has been suggested that the cleavage of tau by these proteases may be a key link between Aβ deposits and tau pathology in AD. Therefore, it is important to investigate how tau cleavage regulates its physiological function as a microtubule stabilizer as well as its pathological role in tau aggregation. In a previous study, it was demonstrated that tau truncated at Asp421, but not full-length tau, partitioned into Sarkosyl-insoluble fraction and formed thioflavin S-positive aggregates when co-expressed with GSK3β and GSK3β, but not T4 or T4C3 alone, caused tau to form Sarkosyl-insoluble aggregates. Most interestingly, the 2EC mutation resulted in the partitioning of T4, but not T4C3, in the Sarkosyl-insoluble fraction, whereas the 2EM and 4E mutations had no effects on the partitioning pattern of either T4 or T4C3. Our data suggest that phosphorylation and caspase cleavage differentially affect tau function. In addition these findings support the hypothesis that tau self-association as an initiating step in aggregation may occur on microtubules, and thus microtubules may serve as a nucleation center for seeding tau aggregation (19, 24).

MATERIALS AND METHODS

Plasmid Constructs—pcDNA3.1(−)-T4 containing human tau with four microtubule-binding repeats but without exons 2 and 3 and pcDNA3.1(+)-T4C3 with the last 20 amino acids deleted and thus mimicking caspase-cleaved tau have been described previously (23, 25). Mutant constructs T4-2EM (T231E/S235E), T4-2EC (S396E/S404E), T4-4E (T231E/S235E/S396E/S404E), T4C3-2EM (T231E/S235E), T4C3-2EC (S396E/S404E), and T4C3-4E (T231E/S235E/S396E/S404E) were generated by mutating the indicated threonine or serine to glutamic acid using the QuikChange site-directed mutagenesis kit according to the manufacturer’s instructions (Stratagene, La Jolla, CA). All the mutations were confirmed by DNA sequencing analysis.

Cell Culture and Transient Transfection—Human embryonic kidney (HEK) cells were grown in Dulbecco’s modified Eagle’s medium/F-12 medium (Cellgro, Herndon, VA) supplemented with 5% bovine growth serum (HyClone, Logan, UT), 2 mM L-glutamine, 10 units/ml penicillin, and 100 units/ml streptomycin (Cellgro). Cells at 50–70% confluency were transiently transfected with the different constructs using Effectene reagent according to manufacturer’s protocol (Qiagen, Valencia, CA). After transfection, the cells were collected and processed as described below.

Immunoblotting—Cells were collected in 2× SDS stop buffer (0.25 M Tris-Cl, pH 6.8, 10% glycerol, 2% SDS, 5 mM EDTA, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin, aprotonin, and pepstatin). Cell lysates were sonicated briefly and centrifuged. Protein concentrations in the supernatants were determined using the bicinchoninic acid (BCA) assay (Pierce). Equal amounts of proteins were diluted with 2× protein loading buffer (2× SDS stop buffer, 25 mM dithiothreitol, and 0.01% bromphenol blue), incubated in a boiling water bath for 5 min, separated on 8% SDS-polyacrylamide gels, transferred to nitrocellulose membrane, and probed with the indicated antibodies. After incubation with the appropriate horseradish peroxidase-conjugated secondary antibody, the blots were developed using enhanced chemiluminescence (Amersham Biosciences).
The following antibodies were used in this study. Tau5 (from Dr. L. Binder) and 5A6 are phospho-independent tau antibodies (26, 27); PHF-1 (from Dr. P. Davies) recognizes tau phosphorylated at Ser^396/Ser^404 (28); AT-180 (Pierce) recognizes tau phosphorylated at Thr^31 (29, 30); 12E8 (from Dr. P. Seubert) recognizes tau phosphorylated at Ser^199. Anti-GSK3β was from BD Biosciences Transduction Laboratories; anti-actin was from Chemicon; anti-α-tubulin (B-5-1-2) was purchased from Sigma.

Cytoskeleton Association Assay—The assay was carried out as described previously with some modifications (32). 30 h after transfection, cells were rinsed with phosphate-buffered saline; scraped off the plate; collected by centrifugation; resuspended in prewarmed extraction buffer (0.1 m PIPES/KOH, pH 6.75, 2 m glycerol, 1 mM MgSO_4, 2 mM EGTA, 0.1 mM EDTA, 0.1% Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each of aprotinin, leupeptin, and pepstatin, and 0.5 μM okadaic acid; and then incubated for 8 min at 37 °C followed by centrifugation at 15,000 × g for 15 min at 25 °C. The supernatant fractions (soluble) were collected, and the pellet fractions (cytoskeleton-associated) were resuspended in 2× SDS stop buffer and sonicated briefly. Equal amounts of pellet and supernatant fractions were separated by SDS-PAGE, transferred, and blotted with the Tau5/5A6 antibodies.

In Situ Microtubule Binding Assay—The assay was carried out as described previously with some modifications (33). Briefly 24 h after transfection, cells were rinsed with warm phosphate-buffered saline and suspended in warm microtubule-stabilizing buffer (80 mM PIPES/KOH, pH 6.8, 1 mM GTP, 1 mM MgCl_2, 1 mM EGTA, 0.5% Triton X-100, and 30% glycerol) containing 1 mM phenylmethyisulfonil fluoride, 10 μg/ml each of aprotinin, leupeptin, and pepstatin, and 0.5 μM okadaic acid, and 10 μM Taxol. The samples were then centrifuged at 5,000 × g for 10 min at room temperature, and an aliquot of the supernatant was retained as the postnuclear lysate (T). The remaining postnuclear lysate was further centrifuged at 100,000 × g for 1 h at room temperature. The supernatant (S) was collected, and the pellet (P) was resuspended in microtubule-stabilizing buffer, and sonicated briefly. Equal amounts of each of the fractions were separated by SDS-PAGE, transferred, and blotted with the Tau5/5A6 antibodies.

In Vitro Microtubule Binding Assay—The coding regions for T4, T4C3, and T4C3-E4 were subcloned into pGEX-6P-2 vector with glutathione S-transferase tagge to the N terminus of tau. The recombinant GST-tau fusion proteins were expressed in Escherichia coli strain BL21(DE3) after induction overnight with 0.3 mM isopropyl 1-thio-D-galactopyranoside at 16 °C and purified using an affinity column packed with glutathione-Sepharose 4B. The glutathione S-transferase tags were cleaved by using PreScission protease (Amersham Biosciences) to obtain purified tau proteins. Purified microtubules were prepared from mouse brain and stabilized with Taxol according to a protocol described previously (34). For the in vitro microtubule binding assay, purified tau protein and Taxol-stabilized microtubules were incubated in reaction buffer (80 mM PIPES/KOH, pH 6.8, containing 1 mM EGTA, 1 mM MgCl_2, 1 mM GTP, and 10 μM Taxol) for 10 min at 37 °C. The mixture was subse-quence loaded onto 30% sucrose in reaction buffer and centrifuged for 30 min at 100,000 × g at room temperature (35). The resultant supernatant (microtubule-unbound) and pellet (microtubule-bound) were collected, and the same percentage of the two fractions was separated by SDS-PAGE followed by immunoblotting with Tau5 antibody.

Nocodazole Treatment—40 h after transfection with the tau constructs, cells were treated with 2.5 μg/ml nocodazole (Sigma) for 10 min at 37 °C followed by the cytoskeleton association assay as described above. The pellet and supernatant fractions were collected, and protein concentrations were determined using the BCA assay. Equal amounts of proteins were separated by SDS-PAGE and probed with Tau5 and anti-α-tubulin antibodies.

Sarkosyl Fractionation Assay—The assay was carried out as described previously (23, 36). HEK cells were transfected with tau constructs alone or in combination with GSK3β as indicated. Cells were rinsed with ice-cold phosphate-buffered saline; scraped off the plate; collected by spinning at 2000 × g for 10 min at 0 °C; resuspended in Mes buffer (20 mM Mes at pH 6.8, 80 mM NaCl, 1 mM MgCl_2, 2 mM EGTA, 10 mM Na_2HPO_4, and 20 mM NaF) containing 1 mM phenethylsulfonyl fluoride and 10 μg/ml each of aprotinin, leupeptin, and pepstatin; and then homogenized with 30 strokes using a tissue grinder. The homogenates were centrifuged at 500 × g for 10 min to remove nuclei. Postnuclear lysates were incubated at 4 °C for 20 min to depolymerize the microtubules and then centrifuged at 200,000 × g for 30 min at 4 °C. Supernatants (soluble fraction) were collected, diluted with 2× protein loading buffer, and incubated in a boiling water bath for 5 min. The pellets were resuspended in Mes buffer containing 500 mM NaCl, 10% sucrose, and 1% N-lauroylsarcosine (Sarkosyl); vortexed for 30 min at room temperature; incubated overnight at 4 °C; and then centrifuged at 200,000 × g for 30 min at 4 °C. The supernatants (Sarkosyl-soluble fraction) were collected and diluted with 2× protein loading buffer, and the pellets (Sarkosyl-insoluble fraction) were resuspended in 2× protein loading buffer and incubated in a boiling water bath for 5 min. Samples were separated by SDS-PAGE, transferred, and blotted with the Tau5/5A6 antibodies.

Statistics—Data were analyzed using Student’s t test, and values were considered significantly different when p was <0.05.

RESULTS

Generation and Expression of Tau Constructs—To further understand the role of site-specific phosphorylation and caspase cleavage in regulating tau function, we made constructs of full-length tau (T4) or tau truncated at Asp^421 (T4C3) that mimics caspase-3 cleavage in the absence or presence of site-directed pseudophosphorylation (T231E/S235E) (23, 36). HEK cells were transfected with these tau constructs to understand the role of site-specific phosphorylation and caspase cleavage in regulating tau function, we made constructs of full-length tau (T4) or tau truncated at Asp^421 (T4C3) that mimics caspase-3 cleavage in the absence or presence of site-directed pseudophosphorylation (T231E/S235E) (23, 36). HEK cells were transfected with these tau constructs to...
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examine their expression and phosphorylation patterns (Fig. 1B). The Tau5/5A6 blot shows that these constructs were expressed at approximately equivalent levels. As expected, the T4C3 constructs migrated faster than the T4 constructs. T4C3-2EC and T4C3-4E exhibited decreases in electrophoretic mobility compared with their parent tau forms, and T4-2EC and T4-4E presented as a single slow migrating band that corresponded to the top band of the parent construct. These mobility changes are likely due to conformational changes caused by the pseudophosphorylation of Ser396/Ser404 sites on tau, similar to what occurs when these sites are phosphorylated (23, 25). As expected, PHF-1 and AT-180 did not recognize T4/T4C3-2EC/4E and T4/T4C3-2EM/4E, respectively, due to the indicated mutations. The extent of endogenous phosphorylation of T4 at both the PHF-1 and AT-180 sites was greater than that of T4C3; this is consistent with a previous finding showing that truncation of tau at Asp421 attenuates GSK3β-mediated phosphorylation (23). Interestingly compared with T4, T4-2EC was phosphorylated more efficiently than Thr231 as detected by AT-180, indicating phosphorylation at one site may affect phosphorylation at other sites. To further examine the differential phosphorylation of the tau constructs, the 12E8 (phospho-Ser262) and pS199 antibodies were also used. In general, pseudophosphorylation of T4, but not T4C3, at the indicated sites increased phosphorylation at both Ser262 and Ser199. Together these data indicate that the phosphorylation at key sites may have different effects on full-length and caspase-cleaved tau.

**Site-specific Phosphorylation and Caspase Cleavage Differentially Regulate the Ability of Tau to Associate with Cytoskeleton Network**—Although studies have shown that hyperphosphorylated tau forms the PHFs and NFTs in AD brains and that caspase-cleaved tau is present in AD brains (19), the importance of phosphorylation at key sites and the possible combination effect of phosphorylation and caspase cleavage on tau function are not well understood. To further investigate this question, we first compared the ability of different tau forms described above to associate with the cytoskeleton. PIPES buffer containing 0.1% Triton X-100 was used to disrupt cell membranes while keeping the cytoskeleton network intact followed by centrifugation to separate cellular content into a soluble fraction and an insoluble fraction that contains the cytoskeleton. The insoluble to soluble ratio was calculated as an indicator of association ability with a higher insoluble to soluble ratio indicating a stronger association with the cytoskeleton. A portion of the T4 was distributed into the soluble fraction, whereas unexpectedly T4C3 was found almost exclusively in the insoluble fraction, suggesting more efficient association of T4C3 with the cytoskeletal network than T4 (Fig. 2, A and D). The 2EM, 2EC, and 4E mutations all significantly decreased the partitioning of tau into the insoluble fractions (Fig. 2, B, C, E, and F), but the degree of decrease for the 2EC mutation on T4 was less than that for 2EM or 4E (Fig. 2, E and F).
Site-specific Phosphorylation and Caspase Cleavage Differentially Regulate the Ability of Tau to Bind Microtubules. A defining function of tau is to bind microtubules and promote their stability. To examine the effects of site-specific phosphorylation and caspase cleavage on the ability of tau to bind microtubules, an in situ microtubule binding assay was carried out. Microtubules were stabilized in situ with Taxol, and the cellular content was separated into postnuclear lysate (Input) and supernatant, which was further separated into microtubule-bound (Pellet) and -unbound (Supernatant) fractions and immunoblotted for tau. Compared with T4, T4C3 was more prevalent in the microtubule-bound fraction. The 2EM mutations impaired the microtubule binding ability of both T4 and T4C3, whereas the 2EC mutations only resulted in less microtubule binding of T4C3 with no obvious effect on T4. A synergistic effect of 2EM and 2EC in decreasing microtubule binding was apparent for T4C3-4E but not for T4-4E. B, in the in vitro microtubule binding assay, purified recombinant tau proteins and purified microtubules were incubated and subsequently loaded onto sucrose cushion to separate microtubule-bound (P) and -unbound (S) fractions. More T4C3-4E protein distributed in the microtubule-unbound fraction than that of T4 and T4C3. Compared with T4, T4C3 bound microtubules less efficiently.

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FIGURE 3. Site-specific phosphorylation and caspase cleavage differentially regulate the ability of tau to bind microtubules. A, in the in situ microtubule binding assay, microtubules were stabilized in situ with 10 μM Taxol, and the cellular content was separated into postnuclear lysate (Input) and supernatant, which was further separated into microtubule-bound (Pellet) and -unbound (Supernatant) fractions and immunoblotted for tau. Compared with T4, T4C3 was more prevalent in the microtubule-bound fraction. The 2EM mutations impaired the microtubule binding ability of both T4 and T4C3, whereas the 2EC mutations only resulted in less microtubule binding of T4C3 with no obvious effect on T4. A synergistic effect of 2EM and 2EC in decreasing microtubule binding was apparent for T4C3-4E but not for T4-4E. B, in the in vitro microtubule binding assay, purified recombinant tau proteins and purified microtubules were incubated and subsequently loaded onto sucrose cushion to separate microtubule-bound (P) and -unbound (S) fractions. More T4C3-4E protein distributed in the microtubule-unbound fraction than that of T4 and T4C3. Compared with T4, T4C3 bound microtubules less efficiently.

FIGURE 4. Site-specific phosphorylation differentially regulates the ability of tau to stabilize microtubules. Cells transfected with each tau construct were treated with 2.5 μg/ml nocodazole for 10 min followed by the cytoskeleton association assay. The stability of microtubules was indicated by the level of α-tubulin in the cytoskeleton-associated fractions. In the absence of nocodazole (−noco), the amounts of α-tubulin in the cytoskeleton fractions were similar for all the tau constructs. Treatment with nocodazole (+noco) resulted in the disappearance of α-tubulin from the cytoskeleton-associated fractions of mock-transfected cells, whereas cells transfected with tau constructs were protected against nocodazole as shown by the different levels of α-tubulin in the cytoskeleton-associated fractions (A and B). The ability of T4 to stabilize microtubules was impaired by both the 2EM and 2EC mutations (A), whereas the ability of T4C3 to stabilize microtubules was impaired by the 2EC but not the 2EM mutation (B).
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**Figure 5. T4-2EC forms Sarkosyl-insoluble particulates.** HEK cells overexpressing tau were homogenized, and the postnuclear lysates were further separated into soluble, Sarkosyl-soluble, and Sarkosyl-insoluble fractions. Co-expression of T4C3 and GSK3β, but not T4 or T4C3 alone (B), caused tau to form Sarkosyl-insoluble aggregates. The 2EC mutation resulted in the partitioning of T4 (A), but not T4C3, into the Sarkosyl-insoluble fraction, whereas the 2EM and 4E mutations had no effects on the partitioning pattern of either T4 or T4C3.

**DISCUSSION**

The purpose of this study was to elucidate the effect of site-specific phosphorylation and caspase cleavage of tau on its physiological function in binding and stabilizing microtubules and its pathological function in tau aggregation. Our data demonstrate that in cultured cell model truncation at Asp421 increases the ability of tau to associate with cytoskeleton, especially microtubules. Pseudophosphorylation at Thr231/Ser235 impairs the microtubule binding ability of both truncated and full-length tau, supporting the finding by phosphorylation by GSK3β (23, 25). Although phosphorylation at Ser396/Ser404 only affected truncated tau and not full-length tau with regard to its microtubule binding function, phosphorylation at this site did result in partitioning of full-length tau in the Sarkosyl-insoluble fraction. These findings suggest that tau function can be differentially regulated by site-specific phosphorylation, and different tau forms (intact versus cleaved) may respond differently to site-specific phosphorylation.

**The 2EC Mutation Causes Full-length Tau to Partition in the Sarkosyl-insoluble Fraction**—Tau aggregates into NFTs in AD brain (1, 2). But how site-specific phosphorylation and caspase cleavage regulate tau self-association is still unclear. To investigate this question, we took advantage of the property of Sarkosyl, a detergent used to solubilize most cellular content, to separate cell lysate into postnuclear lysate, soluble, Sarkosyl-soluble, and Sarkosyl-insoluble fractions. In agreement with a previous study, expression of T4C3 and GSK3β together, but not T4 or T4C3 alone, causes tau to form Sarkosyl-insoluble aggregates (23) (Fig. 5B). The 2EM and 4E mutations had no effects on tau-tau interaction for both T4 and T4C3. However, the 2EC mutation resulted in partitioning of T4, but not T4C3, in the Sarkosyl-insoluble fraction (Fig. 5, A and B). This result is consistent with the finding of an in vitro study showing that pseudophosphorylation at Ser396/Ser404 enhances tau polymerization (16).

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Compared with wild-type full-length tau (T4), T4 with pseudophosphorylation at either Thr231/Ser235 or Ser396/Ser404 showed a decreased ability to associate with the cytoskeleton, whereas only Thr231/Ser235 pseudophosphorylation, but not Ser396/Ser404, impaired the binding of T4 to microtubules. T4 pseudophosphorylated at Thr231/Ser235/Ser396/Ser404 exhibited a partitioning pattern similar to that of T4 pseudophosphorylated only at Thr231/Ser235. On the other hand, pseudophosphorylation at either Thr231/Ser235 or Ser396/Ser404 site attenuated both cytoskeleton and microtubule binding of tau truncated at Asp421 (T4C3) and when combined together showed a synergistic effect on T4C3 in impairing its interaction with microtubules (Figs. 2 and 3A). The obvious effect of Ser396/Ser404 phosphorylation on the function of T4C3, but not T4, in regulating the microtubule binding ability of tau is likely due to the conformational change of T4C3 caused by this phosphorylation as indicated by the much slower migration of T4C3-2EC (Fig. 1). Taken together, these findings suggest that for both full-length and caspase-cleaved tau, phosphorylation of Thr231/Ser235 plays an important role in regulating their interaction with microtubules, whereas phosphorylation leading to tau dysfunction and tau pathologies in neurodegenerative diseases are not clearly understood. Several studies provided evidence that phosphorylation of key sites on tau has a strong impact on the normal function of tau and likely contributes to its pathological role. For example, phosphorylation of tau at Thr231 (11) or Ser262 (12) negatively regulates tau-microtubule interactions, and phosphorylation at both sites is required to maximally reduce this interaction (37). Furthermore, phosphorylation at both Ser396/Ser404 and Ser422 enhances tau aggregation, whereas phosphorylation at Ser262 may decrease the ability of tau to polymerize (13, 16–18). Recently it has been found that caspase cleavage of tau at Asp421 occurs in AD brain, and this is likely a critical event linking Aβ and tau pathology because Aβ accumulation is able to induce caspase activity, resulting in tau cleavage, which makes tau more fibrillogenic (19, 20). Taken together these data demonstrate the importance of both tau phosphorylation and cleavage in modulating its function, and therefore it is important to understand how site-specific phosphorylation and cleavage of tau individually and cooperatively affect tau function.

Pseudophosphorylation by mutating Ser/Thr residues to Glu or Asp residues has been used as an approach to mimic phosphorylation and is an important complementary approach to protein kinase-mediated phosphorylation. It has been shown that pseudohyperphosphorylated tau mimics key structural and functional aspects of hyperphosphorylated tau (14, 15, 32). In addition, pseudophosphorylation allows a more accurate evaluation of the effects of site-specific phosphorylation on tau function in cellular model systems. Therefore, in this study we generated tau constructs in the absence or presence of site-specific pseudophosphorylation and then expressed these constructs in mammalian cells. By taking this approach, this study extends and complements previous work in which the impact of phosphorylation on tau function was examined by overexpression of protein kinases that likely phosphorylate substrates other than tau and phosphorylate tau on multiple sites that are not always defined.
of Ser\textsuperscript{396}/Ser\textsuperscript{404} is able to differentially regulate tau-microtubule interactions of full-length and cleaved tau. An interesting observation in the microtubule binding assays with T4 and T4C3 is that, in the \textit{in vitro} assay using cell lysate transfected with tau constructs, T4C3 was more concentrated in the microtubule-bound fraction than T4 (Fig. 3A), whereas in the \textit{in vitro} experiments using purified recombinant tau proteins and microtubules, T4C3 was found to bind microtubules less efficiently than T4 (Fig. 3B). One explanation for this is that although T4 protein purified from bacterial expression system does bind microtubules with higher affinity than T4C3, this affinity is compromised in cultured cell model by the phosphorylation of tau, which is regulated by protein kinases and phosphatases that are absent in the \textit{in vitro} system. This idea is supported by the finding that, compared with T4C3, T4 is a significantly better substrate for GSK3\textbeta{} both \textit{in vitro} and \textit{in situ} (23). This was also evident in this study when T4 and T4C3 were overexpressed in HEK cells (Fig. 1B). Thus, the combination of cell models and \textit{in vitro} systems will be beneficial to investigate functions of different tau forms \textit{per se} as well as the roles of cellular factors in regulating tau-microtubule interactions.

Pseudophosphorylation at Thr\textsuperscript{231}/Ser\textsuperscript{235} or Ser\textsuperscript{396}/Ser\textsuperscript{404} on T4 and Ser\textsuperscript{396}/Ser\textsuperscript{404} or Thr\textsuperscript{231}/Ser\textsuperscript{235}/Ser\textsuperscript{396}/Ser\textsuperscript{404} on T4C3 impaired the ability of tau to stabilize microtubules, but T4 with all four sites pseudophosphorylated and T4C3 with pseudophosphorylation at Thr\textsuperscript{231}/Ser\textsuperscript{235} each stabilized microtubules to a similar extent compared with their wild-type tau forms (Fig. 4). Taken together with the effect of pseudophosphorylation at different sites on the binding of tau to microtubules, it suggests that strong binding to microtubules might help tau to stabilize microtubules, but impairment of tau-microtubule interactions does not necessarily decrease the stability of microtubules. Because it was demonstrated previously that a conformation optimized by the flanking regions of the repeat domain on tau is required for strong microtubule binding and nucleation (38), it is not hard to imagine that phosphorylation on key residues in the flanking regions would change the conformation of tau in different manners. This could have a different impact on microtubule binding and stabilization, thus adding to the complication of the way that tau function is regulated. The relationship between microtubule binding and microtubule stabilization clearly needs to be investigated further.

It has been suggested previously that \textit{in vitro}, pseudophosphorylation of tau at Ser\textsuperscript{396}/Ser\textsuperscript{404} potentiates the rate of filament formation, whereas phosphorylation of Ser\textsuperscript{202} by microtubule affinity-regulating kinase inhibits tau aggregation into PHFs (13, 16). But how phosphorylation at specific sites on tau affects its self-association in a cell model has not been reported. In this study we found that overexpression of T4 pseudophosphorylated at Ser\textsuperscript{396}/Ser\textsuperscript{404}, but not Thr\textsuperscript{231}/Ser\textsuperscript{235}, resulted in formation of Sarkosyl-insoluble aggregates. Thus, pseudophosphorylation of Thr\textsuperscript{231}/Ser\textsuperscript{235} detached T4 from microtubules but had no effect on promoting tau aggregation. In contrast, pseudophosphorylation of Ser\textsuperscript{396}/Ser\textsuperscript{404} did not alter microtubule binding ability of T4 but enhanced its aggregate formation. These findings support the idea that phosphorylation reducing the affinity of tau for microtubules does not necessarily prime tau for PHF assembly, and a mechanism other than impaired microtubule binding might be accounting for tau filament formation (13).

Based on \textit{in vitro} studies showing that microtubule-binding repeats of tau are essential in tau filament formation and that the C terminus of tau inhibits its polymerization (16, 39), a model for tau polymer assembly was proposed previously (16, 40). According to this model, phosphorylation of Ser\textsuperscript{396}/Ser\textsuperscript{404} on tau may change its conformation and thus release the inhibitory effect of the C terminus and increase the polymerization of tau based on those repeats (16, 40). Here given the finding that phosphorylation of Thr\textsuperscript{231}/Ser\textsuperscript{235} and Ser\textsuperscript{396}/Ser\textsuperscript{404} on T4 affected its microtubule binding and aggregation in opposite ways, we extend the previous model by proposing that the initiation of tau filament formation is dependent on the association of tau with microtubules, and microtubules might serve as a nucleation center for seeding tau aggregation. Several layers of evidence support this intriguing idea. First, in our study, T4 with all four sites pseudophosphorylated did not form aggregates, likely due to its decreased binding to microtubules caused by Thr\textsuperscript{231}/Ser\textsuperscript{235} phosphorylation, thus masking the effect of Ser\textsuperscript{396}/Ser\textsuperscript{404}. Additionally none of the pseudophosphorylated constructs of T4C3 were capable of aggregating when overexpressed alone, possibly because their interactions with microtubules were impaired. The formation of tau aggregates when T4C3 was co-expressed with GSK3\textbeta{} can be explained by the indirect effect of GSK3\textbeta{} on numerous substrates besides tau that may play a role in facilitating tau aggregation directly or indirectly. Indeed T4C3 is a relatively poor substrate of GSK3\textbeta{}, suggesting that mechanisms other than direct phosphorylation of the tau construct contributed to its aggregation. Although co-expression of T4C3 with GSK3\textbeta{} resulted in aggregation, co-expression of T4 with GSK3\textbeta{} did not result in tau aggregation (23). Again this can be attributed to the attenuated association of T4 with microtubules after phosphorylation by GSK3\textbeta{}, whereas the less efficient phosphorylation of T4C3 by GSK3\textbeta{} resulted in tau staying associated with microtubules (23). Second, a previous study using atomic force microscopy suggested that tau may form oligomers upon binding to microtubules, indicating that disruption of the dynamic formation of normal tau complexes around microtubules might contribute to altered microtubule-dependent axonal transport and the formation of pathological tau aggregates (41). Third, it has been observed that C-terminal truncation of tau promotes filament assembly \textit{in vitro}, and detergent-insoluble truncated tau partially co-localizes with microtubules in primary neuron cultures (19), also suggesting that the aggregation of truncated tau into pathological filaments may be initiated on microtubules, which is consistent with what was suggested previously for full-length tau (24). Taken together, these studies strongly implicate an important role for microtubules and the ability of tau to bind to microtubules in initiating tau filament formation. It would be interesting and critically important to determine the stage of pathological tau self-association and find out how this process is regulated by tau phosphorylation, caspase cleavage, and other factors.

Because hyperphosphorylated tau is a major component of NFTs in AD, it is generally proposed that hyperphosphoryla-
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tion of tau causes its “drop-off” from microtubules, and the increased tau concentration in free pool promotes its assembly. However, our study and studies from other groups suggest that phosphorylation at specific sites on tau may play a key role in the early stage of tau pathology.

Acknowledgments—We thank Dr. L. Binder, Dr. P. Davies, and Dr. P. Seubert for the generous gifts of tau antibodies.

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