

Molecular Interactions among Protein Phosphatase 2A, Tau, and Microtubules

IMPLICATIONS FOR THE REGULATION OF TAU PHOSPHORYLATION AND THE DEVELOPMENT OF TAUOPATHIES*

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Hyperphosphorylated forms of the neuronal microtubule (MT)-associated protein tau are major components of Alzheimer's disease paired helical filaments. Previously, we reported that AB α C, the dominant brain isoform of protein phosphatase 2A (PP2A), is localized on MTs, binds directly to tau, and is a major tau phosphatase in cells. We now describe direct interactions among tau, PP2A, and MTs at the submolecular level. Using tau deletion mutants, we found that AB α C binds a domain on tau that is indistinguishable from its MT-binding domain. AB α C binds directly to MTs through a site that encompasses its catalytic subunit and is distinct from its binding site for tau, and AB α C and tau bind to different domains on MTs. Specific PP2A isoforms bind to MTs with distinct affinities *in vitro*, and these interactions differentially inhibit the ability of PP2A to dephosphorylate various substrates, including tau and tubulin. Finally, tubulin assembly decreases PP2A activity *in vitro*, suggesting that PP2A activity can be modulated by MT dynamics *in vivo*. Taken together, these findings indicate how structural interactions among AB α C, tau, and MTs might control the phosphorylation state of tau. Disruption of these normal interactions could contribute significantly to development of tauopathies such as Alzheimer's disease.

flanking both sides of the repeats to support higher affinity MT binding (4–6). All tau isoforms in human brain contain 21 serine/threonine phosphorylation sites (7), some of which modulate MT binding of tau (8–11). Only a few sites on tau are phosphorylated at any moment in normal adults (12, 13). In Alzheimer's disease brain, however, tau is more heavily phosphorylated (12, 13), due in part to decreased tau phosphatase activity (13, 14). Hyperphosphorylated tau is the principal component of Alzheimer's disease paired helical filaments and neurofibrillar lesions present in several other neurodegenerative disorders (15) and has very low affinity for MTs (16, 17). Although non-phosphorylated tau can assemble into paired helical filament-like filaments *in vitro* (18–21), it is reasonable to hypothesize that changes in tau phosphorylation are decisive events in paired helical filament biogenesis *in vivo*.

To study how tau phosphorylation is regulated, we have been focusing on protein phosphatase 2A (PP2A), a heterotrimeric enzyme that comprises one catalytic C subunit, one non-catalytic A subunit, and one of several structurally distinct, regulatory B subunits (22). We previously reported that PP2A is likely to be a major tau phosphatase *in vivo* (23). Initially, we found that a pool of AB α C, the major PP2A isoform in brain (22), is associated with MTs in brain and cultured cells (17). Subsequently, we determined that tau binds with high affinity to AB α C and AB β C; less tightly to AB' C; and poorly, if at all, to AC or individual PP2A subunits (23). Finally, we found that the relative affinities of PP2A isoforms for tau correlated with their tau phosphatase activities, and suppression of PP2A activity in cells stimulated Alzheimer's disease-like phosphorylation of tau and prevented tau from binding MTs (23).

Here, we describe the use of tau deletion mutants, specific PP2A enzymes, and intact and proteolyzed MTs to define binding sites on tau for PP2A, on PP2A for tau and MTs, and on MTs for PP2A. When considered collectively, the results indicate how structural interactions among PP2A, tau, and MTs can control the phosphorylation of tau. The results suggest, moreover, that disruption of the normal interactions could contribute significantly to the development of tauopathies such as Alzheimer's disease.

EXPERIMENTAL PROCEDURES

Binding of PP2A to Tau—Purified bovine brain or bovine cardiac (24, 25) or human recombinant (22) AB α C (300 nM) in storage buffer (25 mM Tris, 1 mM dithiothreitol, 1 mM EDTA, and 50% glycerol, pH 7.5) was incubated for 15 min on ice in a final volume of 5 μ l with a 600 nM concentration of either purified bovine brain tau (26) or any of several previously described human recombinant tau (rTau) fragments (27, 28). We also used one new recombinant tau fragment, rTau9 (see Fig. 1),

The axonal microtubule (MT)¹-associated protein (MAP) tau (1, 2) is encoded by one alternatively spliced gene that directs the synthesis of six tau isoforms in human brain (3). The C-terminal half of brain tau encompasses three or four contiguous MT-binding repeats that act synergistically with regions

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¹ The abbreviations used are: MT, microtubule; MAP, microtubule-associated protein; PP2A, protein phosphatase 2A; PP1, protein phosphatase 1; rTau, recombinant tau; PIPES, 1,4-piperazinediethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

which was made following the same method used to generate previously produced fragments (27). In some assays, PP2A was incubated on ice for 15 min with 1–5 μM okadaic acid before adding tau. For competition experiments with the synthetic tau peptide ²²⁴KKVAVVRTPPKSP²³⁶ (numbered according to the longest isoform of human brain tau (3)), 300 nM AB α C was first incubated for 15 min with 1 or 10 μM peptide and then for an additional 15 min in the presence of peptide plus 600 nM tau. After all incubations were completed, samples were applied directly onto pre-cast, nondenaturing 8–25% polyacrylamide gels (Amersham Pharmacia Biotech); subjected to native gel electrophoresis using the Amersham Pharmacia Biotech PhastSystem; and transferred to nitrocellulose for immunoblotting with antibodies to the C or B α subunits of PP2A (23). Immunoreactive proteins were detected using enhanced chemiluminescence reagents (ECL, Amersham Pharmacia Biotech). Blots were densitometrically scanned and quantitatively analyzed using a PhosphorImager and ImageQuant software (Molecular Dynamics, Inc.).

Assembly and Limited Proteolysis of Tubulin—Purified bovine brain tubulin (29) at 5 μM (equal to 0.5 mg/ml) was polymerized into MTs by incubation for 10 min at 37 °C in PEM buffer (0.1 M PIPES, pH 6.9, 2 mM EGTA, and 5 mM MgCl₂) containing 1 mM GTP and 20 μM Taxol (provided by Nancita Lomax, NCI, National Institutes of Health). When used in PP2A enzymatic assays, MTs were first washed free of GTP by centrifugation at 100,000 $\times g_{\text{max}}$ for 20 min at 30 °C in a Beckman TLA 100.3 rotor, followed by resuspension in PEM buffer lacking GTP, but containing 20 μM Taxol. This step was essential because high levels of free GTP interfered with PP2A enzymatic assays. To remove C-terminal domains of polymerized tubulin, Taxol-stabilized MTs were incubated overnight at 30 °C with ~1% (w/w) subtilisin (Roche Molecular Biochemicals) (30). Proteolysis was then terminated by addition of phenylmethylsulfonyl fluoride to 5 mM. Subtilisin-digested MTs were sedimented for 45 min at 100,000 $\times g_{\text{max}}$ in a Beckman TLA 100.3 rotor, resuspended in PEM buffer containing 2 mM phenylmethylsulfonyl fluoride and 20 μM Taxol, and used immediately for co-sedimentation assays. The extent of MT proteolysis was verified by SDS-polyacrylamide gel electrophoresis (PAGE) as described previously (30–32).

MT Co-sedimentation Assays—PP2A and tau were incubated either individually or together for 15–20 min at room temperature with PEM buffer alone or with PEM buffer containing F-actin (33) or intact or subtilisin-digested MTs. The samples were then centrifuged at ~50,000 $\times g_{\text{max}}$ for 20 min at 25 °C in a Beckman TLA 100.3 rotor. Next, the supernatants were collected, and each pellet was resuspended to the starting volume (20–50 μl). The samples were then resolved by SDS-PAGE using 12% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with antibodies to the C subunit of PP2A or with the Tau-1 (1) or Tau-5 (28) antibodies.

Dephosphorylation of Tau by PP2A—Radiolabeled soluble tau (~5 mol of phosphate/mol of protein) was produced by incubating bovine brain tau with protein kinase A (Sigma) in the presence of 20 μM [γ -³²P]ATP, 10 mM MgCl₂, 10 mM dithiothreitol, and 10 μM cAMP and then purifying phosphorylated tau as described previously (23). Radiolabeled, MT-bound tau was obtained by incubating radiolabeled soluble tau with Taxol-assembled MTs, centrifuging MTs for 10 min in a Beckman TLA 100.3 rotor at ~50,000 $\times g_{\text{max}}$, and resuspending MTs in GTP-free PEM buffer. For the experiment shown in the upper panel of Fig. 6, ~1000 cpm each of radiolabeled soluble and MT-bound tau were incubated for 5–45 min at 30 °C with 14 nM AB α C, and dephosphorylation of tau was halted at various time points by addition of 3 \times sample buffer for SDS-PAGE. For the experiment shown in the lower panel of Fig. 6, 200 nM radiolabeled soluble tau was mixed with 40 nM AB α C and 0–10 μM tubulin that had been polymerized in the presence of Taxol. The samples were incubated at 30 °C for 15 min, after which tau dephosphorylation was terminated by addition of 3 \times sample buffer for SDS-PAGE. The samples were resolved by SDS-PAGE using 12% polyacrylamide gels, and ³²P incorporation into tau was measured on dried gels using a PhosphorImager.

Dephosphorylation of Tubulin by PP2A—Purified AB α C, AC, or C subunits (25 nM) in storage buffer were incubated for 15 min with either polymerized or dimeric tubulin (20 μM) in a final volume of 50 μl of phosphatase assay buffer (20 mM MOPS, 0.02% β -mercaptoethanol, and 0.25 mg/ml bovine serum albumin, pH 7.0). The reactions were performed in 96-well U-bottom microtiter plates. Green reagent (BIOMOL Research Labs, Inc.) was used in a quantitative colorimetric assay for free phosphate (P_i) released after 30-min incubations at room temperature. P_i levels were determined by measuring A_{620 nm} according to the manufacturer's instructions. Control wells containing only tubulin, MTs, or buffer alone, but no phosphatases, were used to determine the background values of P_i, and the PP2A activities reported here were

background-corrected.

Effects of MTs on PP2A Activity—AB α C, AC, or C subunits were incubated for 15 min with or without 5 μM polymerized or dimeric tubulin. The samples were then incubated for 5 min at 30 °C with a 100 μM concentration of either of two substrates: radiolabeled phosphorylated myosin light chain (24) or the synthetic phosphopeptide RRREEE(pT)EEE (Biosynthesis Inc.). Dephosphorylation of myosin light chain was assayed by measuring the release of ³²P_i as described previously (17). Dephosphorylation of the phosphopeptide was determined by measuring the release of P_i using the colorimetric assay described above for tubulin. Dephosphorylation of polymerized or dimeric tubulin by PP2A enzymes represented, at most, ~4% of the total phosphatase activity measured for phosphorylated myosin light chain or the phosphopeptide.

RESULTS

AB α C and MTs Bind to the Same Region within Tau—To localize the binding site on tau for AB α C, a gel mobility shift assay (23) coupled with immunoblotting (see "Experimental Procedures") was used to monitor binding of AB α C to 13 different rTau proteins, all but one of which (rTau9) have been previously described (27, 28). These recombinant proteins are derived from adult (rTau1–rTau6) or fetal (rTau7–rTau13) isoforms of human brain tau. The largest recombinant tau that was used, rTau1, contains four MT-binding repeats (four-repeat tau) and two 29-mer N-terminal inserts. As shown in Fig. 1, each of the other rTau proteins contained one or more unique deletions. Their N- and C-terminal amino acids and the boundaries of their deletions are numbered relative to the amino acid sequence of the largest isoform of brain tau (3), which is equivalent to rTau1.

Fig. 1 summarizes the results of the binding assays in which AB α C and the pertinent rTau proteins were used at 300 and 600 nM, respectively. Densitometry of the resulting immunoblots was used to estimate the percentage of PP2A that was bound to each rTau protein. Maximal binding of AB α C ($\geq 95\%$) was observed for every rTau protein that contains all four MT-binding repeats plus extensive sequence contiguous with the N terminus of the repeats. Included in this group are rTau1 and rTau2, which do not have any C-terminal deletions, and rTau6 and rTau8, which are missing part or all of the C-terminal 45 amino acid residues of native tau. A modest decrease in binding (to ~80%) was observed for rTau7, which is equivalent to the fetal isoform of brain tau (3), contains only three MT-binding repeats (three-repeat tau), and lacks the two N-terminal inserts. A similar level of AB α C binding (~75%) was observed for rTau5, the C terminus of which is in the middle of the third MT-binding repeat, but contains no other deletions relative to rTau1. By comparison, rTau4, which contains a large internal deletion and includes just part of the last MT-binding repeat, was able to bind only ~45% of AB α C. The minimal protein that retained the ability to bind AB α C (~38%) was rTau12, which lacks all four MT-binding repeats, but, near its C terminus, contains a proline-rich sequence that has MT-binding activity independent of the repeats (4, 5). In stark contrast, rTau13, which lacks residues 221–242 of rTau12, but is otherwise identical, failed to bind any AB α C.

Deletion of the N-terminal 29-mer inserts (rTau8) or residues 84–161 (rTau2), which include part of the second N-terminal repeat, did not impair binding of four-repeat tau to AB α C. Other deletions located N-terminal to the MT-binding repeats yielded demonstrable, albeit modest effects. A slight reduction in binding (to ~90%) was observed for rTau3, which contains all four MT-binding repeats, but lacks most of the proline-rich MT-binding domain located immediately N-terminal to those repeats. Likewise, binding to AB α C correlated roughly with protein length for the three-repeat proteins (rTau9, rTau10, and rTau11) that have extensive N-terminal deletions.

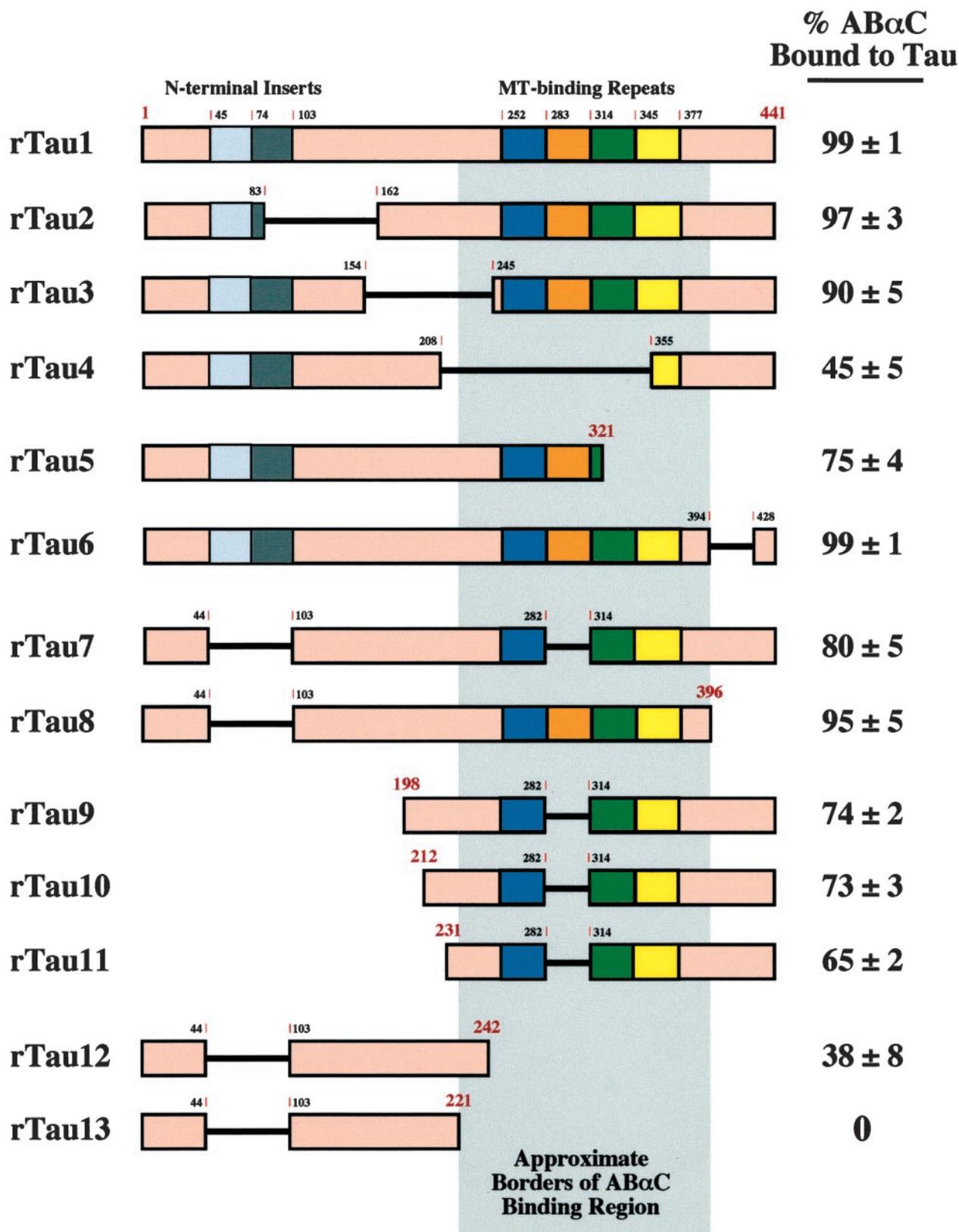


FIG. 1. AB α C binds to the MT-binding domain on tau. Purified bovine brain AB α C (300 nM) was incubated on ice for 15–30 min in the presence of various recombinant proteins (600 nM) derived from adult (rTau1–rTau6) or fetal (rTau7–rTau13) isoforms of human brain tau. For each combination of AB α C and a rTau species, nondenaturing gel electrophoresis was used to separate complexes containing both proteins from free AB α C and rTau. The resulting gels were immunoblotted with a monoclonal antibody specific for the C subunit of PP2A, and densitometry of the immunoblots was used to estimate the percentage of total AB α C that was bound to each rTau. Values shown are the means \pm S.D. of at least three separate experiments. For each rTau species, the N- and C-terminal amino acids and the boundaries of deletions (indicated by *black lines*) are numbered relative to the 441-amino acid sequence of the largest human adult tau isoform (3), which is equivalent to rTau1.

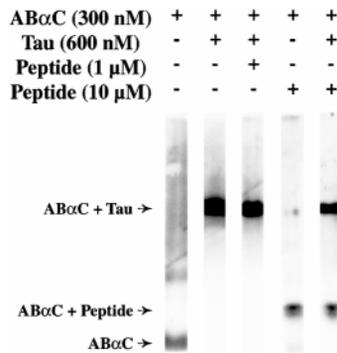


FIG. 2. A synthetic tau peptide competes with native tau for AB α C binding. Bovine brain AB α C (300 nM) was preincubated for 15 min with a 1 or 10 μ M concentration of the synthetic peptide ²²⁴KKVAV-VRTPPKSP²³⁶, corresponding to the 224–236-amino acid sequence of the longest human adult tau isoform, and then further incubated for 15 min with 600 nM native bovine brain tau or buffer alone. The samples were analyzed by nondenaturing gel electrophoresis, followed by immunoblotting with a polyclonal antibody to the β subunit of PP2A. Note the presence of AB α C-peptide complexes and a decreased amount of AB α C-tau complex in the presence of 10 μ M peptide.

Taken together, these data demonstrate that the overall PP2A-binding region on tau encompasses the MT-binding repeats and a short sequence N-terminal to the repeats. It is thus indistinguishable, within the limits of experimental resolution, from the MT-binding region on tau (4–6). Interestingly, lower affinity binding of AB α C can be achieved by proteins that contain only the extreme N-terminal (rTau12) or C-terminal (rTau4) part of the overall binding region on tau for AB α C. In addition, the binding affinity of PP2A for tau increases with the number of MT-binding repeats present in tau. It is also important to note that inclusion of 1 μ M okadaic acid in the binding reactions completely suppressed PP2A activity, but had no effect on the extent of PP2A interaction with tau (data not shown).

To seek further evidence that sequences on tau immediately N-terminal to the MT-binding repeats actually bind AB α C, the synthetic peptide ²²⁴KKVAVVRTPPKSP²³⁶, which corresponds to a portion of this region, was tested for its ability to compete with native tau for binding to AB α C. The sequence of this portion of tau is invariant among all isoforms of human, rat, mouse, and bovine tau. Bovine brain AB α C (300 nM) was preincubated first with 1 or 10 μ M peptide, after which bovine brain tau (600 nM) or an equivalent volume of buffer was added. After an additional 15-min incubation, binding of AB α C to tau was monitored by native gel electrophoresis and immunoblotting with an anti- β antibody (23). As shown in Fig. 2, 10 μ M (but not 1 μ M) peptide partially inhibited binding of PP2A to tau. In addition, 10 μ M peptide induced a shift in the electrophoretic mobility of PP2A on native gels, consistent with the formation of an AB α C-peptide complex.

Direct, Isoform-specific Binding of PP2A to MTs—Our previous finding that a pool of AB α C copurifies with MTs *in vitro* and is associated with MTs in cells (17) raised the question of how AB α C, and possibly other PP2A isoforms as well, might bind to MTs. The results presented in Figs. 1 and 2 strongly imply that tau cannot serve as a MT-anchoring protein for PP2A because they show that AB α C and MTs bind to the same region on tau. To determine whether PP2A might bind directly to MTs, a series of MT co-sedimentation assays were performed with various purified PP2A enzymes. As shown in Fig. 3 (*upper panel*), when bovine brain or human recombinant AB α C heterotrimers were mixed with MTs and centrifuged, ~50% of PP2A was recovered in the MT pellets under the experimental conditions utilized. The possibility that AB α C may have non-specifically co-sedimented with MTs under these conditions

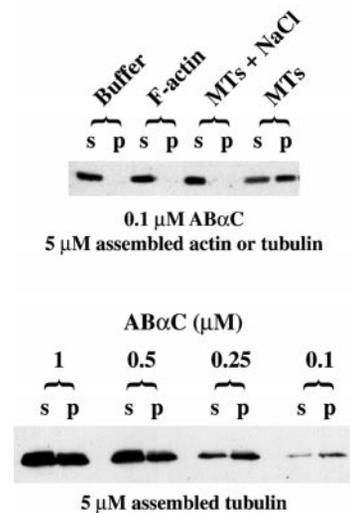


FIG. 3. AB α C co-sediments with MTs. *Upper panel*, purified recombinant AB α C (100 nM) was incubated with buffer alone, 5 μ M F-actin, or 5 μ M Taxol-polymerized tubulin (MTs) in the absence or presence of 0.5 M NaCl. The samples were then centrifuged, and fractions corresponding to the supernatants (s) and pellets (p) were analyzed by SDS-PAGE and immunoblotting with a monoclonal antibody to the C subunit of PP2A. All pellets were resuspended to original sample volumes, and equal aliquots of supernatants and pellets were loaded on the gel. *Lower panel*, MT co-sedimentation assays with increasing concentrations of purified AB α C were performed and analyzed as described in the *upper panel*.

was assessed in parallel control experiments, in which MTs were omitted or F-actin was substituted for MTs. AB α C proteins did not sediment in either of these cases, emphasizing the specificity of the direct interaction between AB α C and MTs. In addition, 0.5 M NaCl was found to prevent co-sedimentation of PP2A with MTs, indicating that ionic interactions are important for association of the enzyme with MTs. As was observed for the binding of AB α C to tau, inclusion of 5 μ M okadaic acid in the assays completely suppressed PP2A activity, but had no effect on the extent of interaction of AB α C with MTs (data not shown).

In the next series of experiments, increasing concentrations of AB α C were incubated with MTs and then centrifuged to generate MT-bound (pellet) and unbound (supernatant) fractions. Fig. 3 (*lower panel*) shows that AB α C co-pelleted with MTs in a concentration-dependent manner. The highest concentration of AB α C that we were able to use for these experiments (1 μ M) was not sufficient for saturation binding to ~5 μ M assembled tubulin (see Fig. 4). Nevertheless, ~40% of 1 μ M AB α C bound to ~5 μ M assembled tubulin, indicating that MTs must be able to accommodate >1 AB α C heterotrimer/12.5 tubulin dimers. Furthermore, because ~80% of AB α C bound to MTs when the total concentration of AB α C was 0.1 μ M (Fig. 3, *lower panel*), the binding of AB α C to MTs must be tight. Efforts to use Scatchard analysis to determine saturation binding and a dissociation constant more accurately were unsuccessful for AB α C and other forms of PP2A (Figs. 3 (*lower panel*) and 4) because the Scatchard plots could not be described accurately by simple linear equations (data not shown).

To assess whether AB α C is the only form of PP2A that can bind MTs, we compared the behavior of distinct PP2A enzymes in the MT co-sedimentation assay. As shown in Fig. 4, all enzymatically active proteins tested, including the AB β C and AB' C holoenzymes, the AC dimer, and the catalytic C subunit, were able to bind MTs to some extent. However, distinct PP2A isoforms appeared to have distinct affinities for MTs because at fixed molar concentrations of PP2A and polymerized tubulin, the ratio of MT-bound to soluble enzyme varied considerably

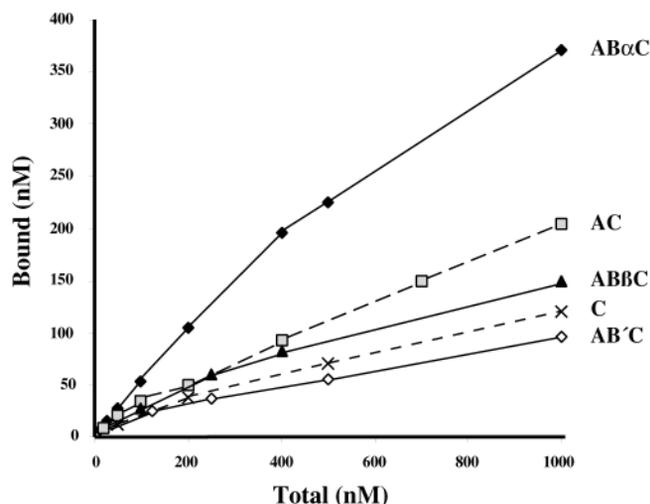


FIG. 4. Differential binding of distinct PP2A enzymes to MTs. Co-sedimentation assays were performed with 10 μ M Taxol-polymerized tubulin and increasing concentrations (10–1000 nM) of purified AB α C, AB β C, AB' C, AC, or C subunits and analyzed by SDS-PAGE and immunoblotting with a monoclonal antibody to the C subunit of PP2A. The relative amounts of MT-bound and unbound enzymes were calculated by quantitative densitometric analysis of the immunoblots. The results shown are representative of a typical experiment.

among the phosphatases tested. Based on the results presented in Fig. 4, the ability of PP2A to bind to MTs can be ranked as follows: AB α C > AC > AB β C > C > AB' C. When actin filaments were substituted for microtubules, virtually none of the PP2A enzymes pelleted, demonstrating that their binding to microtubules was specific (data not shown).

The fact that the monomeric C subunit co-sedimented with MTs indicates that it contains a binding site for MTs. However, our findings suggest that the presence of A and B subunits modulates interactions of the catalytic C subunit with MTs and that each type of B subunit does so in its own unique way. As reported previously for binding of various forms of PP2A to tau (23), the AB α C heterotrimer bound more tightly to MTs than any of the other forms of PP2A that were assayed. In addition, we found that neither AB α C nor AC forms detectable complexes with unpolymerized tubulin during nondenaturing gel electrophoresis (data not shown), implying that PP2A can efficiently interact with tubulin only when the tubulin has polymerized.

Tau and PP2A Bind to Different Sites on MTs—Binding to MTs of several MAPs such as tau and MAP2 can be partially inhibited by prior exposure of either unassembled (32) or polymerized (30, 31) tubulin to the protease subtilisin, which removes a small C-terminal fragment from both α - and β -tubulin. To compare the binding sites on MTs for PP2A and tau, co-sedimentation experiments were therefore performed using untreated or subtilisin-treated MTs, bovine brain AB α C, and bovine brain tau. The resulting supernatants and pellets were analyzed for the presence of tau, intact or cleaved tubulin, and AB α C by SDS-PAGE and immunoblotting. Representative results are shown in Fig. 5, and equivalent results were obtained when AC was substituted for AB α C (data not shown). As expected from previous studies (30–32), the electrophoretic mobilities of nearly all of the α - and β -tubulin increased after exposure of MTs to subtilisin (data not shown), but subtilisin did not alter the proportion of total tubulin that sedimented (\sim 90%) (data not shown). Binding of tau to MTs was significantly weakened, but not completely abolished, by pretreatment of MTs with subtilisin, as reported previously (31). In contrast to tau, AB α C co-sedimented with subtilisin-treated MTs as efficiently as with untreated MTs. We also found that

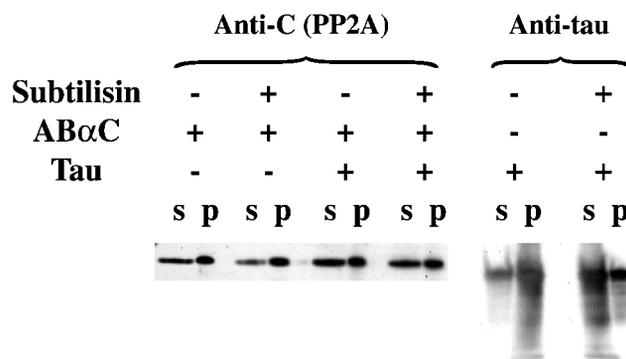


FIG. 5. AB α C and tau bind to different sites on the MT wall. Aliquots of Taxol-stabilized MTs (20 μ M tubulin) were incubated overnight with or without subtilisin, which removes C-terminal sequences from both α - and β -tubulin (30, 32). Co-sedimentation assays were then performed in the presence of bovine brain tau or recombinant AB α C (\sim 128 nM) that had been preincubated with or without bovine brain tau (\sim 500 nM). MT-bound and unbound AB α C and tau were detected by SDS-PAGE and immunoblotting using a monoclonal antibody to the C subunit of PP2A or the monoclonal tau-5 antibody. Note that subtilisin treatment of MTs led to decreased binding of tau, but did not affect binding of PP2A. s, supernatant; p, pellet.

preincubation of AB α C with tau did not prevent the phosphatase from co-sedimenting with either intact or subtilisin-digested MTs. Taken together, these results lead us to conclude that AB α C can bind to a site on MTs that overlaps minimally, if at all, with the binding site on MTs for tau.

MTs Inhibit Dephosphorylation of Tau by AB α C—Because the binding domain on tau for PP2A (Figs. 1 and 2) cannot be distinguished from the MT-binding domain on tau (4–6) and PP2A must bind to that site in order to dephosphorylate tau (23), we postulated that MTs would inhibit the tau phosphatase activity of PP2A by competing with PP2A for binding to tau. To test this possibility, bovine brain tau was phosphorylated by protein kinase A in the presence of [γ - 32 P]ATP (34). Radiolabeled soluble tau was then incubated with MTs, after which the mixture was centrifuged to pellet MTs. The pellet was then resuspended in a buffer that contained 20 μ M Taxol, but lacked free GTP, which interferes with the enzymatic activity of PP2A. MT-associated tau was incubated with AB α C for varying periods of time. In parallel, equal counts (\sim 1000 cpm) of soluble tau were similarly treated with AB α C in the absence of MTs. Finally, dried SDS-polyacrylamide gels were analyzed quantitatively using a PhosphorImager to measure the extent of tau dephosphorylation in each sample. As shown in Fig. 6 (upper panel), AB α C was able to remove \sim 70% of the radiolabeled, covalently bound phosphate from soluble tau within 15 min, whereas only \sim 50% of the phosphate had been removed from MT-associated tau after 45 min. Thus, MTs inhibited the rate of tau dephosphorylation by AB α C. Control experiments demonstrated that the tau phosphatase activity of PP2A was not affected by 20 μ M Taxol (data not shown).

The inhibitory effect of MTs on the tau phosphatase activity of PP2A was also analyzed by incubating 200 nM protein kinase A-phosphorylated bovine brain tau with 40 nM AB α C and a concentration series of MTs for 15 min. Fig. 6 (lower panel) shows that tau dephosphorylation by PP2A was inhibited by MTs in a concentration-dependent manner. In the absence of MTs, <15% of the original 32 P levels remained covalently bound to tau. In contrast, \sim 20% of 32 P remained when 1 μ M assembled tubulin was present, and \sim 45% remained at assembled tubulin concentrations of 2 μ M or higher.

Although the MT-mediated inhibition of tau dephosphorylation by AB α C likely resulted, at least in part, from competition between MTs and AB α C for binding to tau, we hypothe-

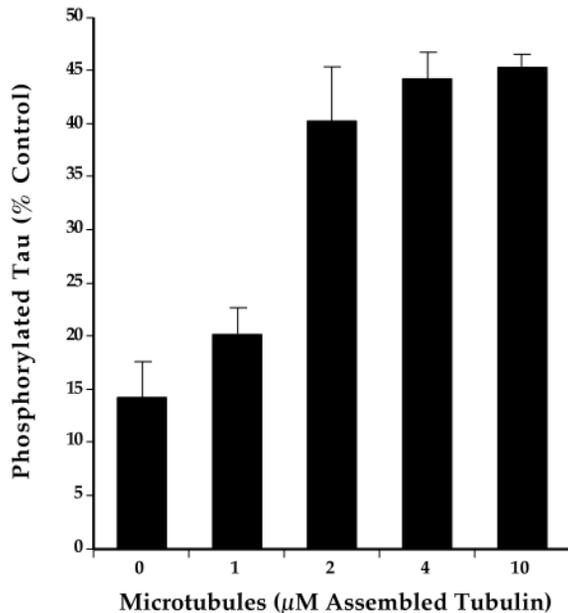
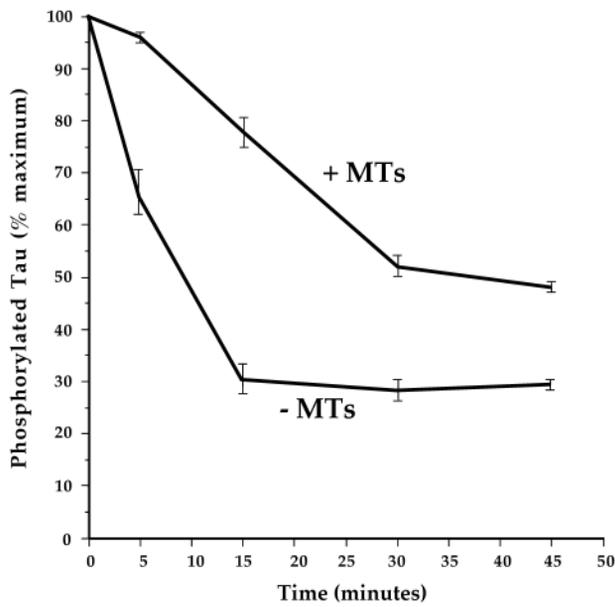


FIG. 6. MTs inhibit dephosphorylation of tau by AB α C. *Upper panel*, bovine brain tau was phosphorylated by protein kinase A in the presence of [γ - 32 P]ATP. \sim 1000 cpm (400 nM) of MT-bound (+MTs) and soluble (-MTs) tau were incubated for the indicated times at 30 °C with \sim 14 nM purified bovine brain AB α C. Samples were then resolved by SDS-PAGE, and 32 P levels in tau were measured on dried gels using a PhosphorImager. *Error bars* indicate the S.D. values for data from two independent experiments. *Lower panel*, radiolabeled, protein kinase A-phosphorylated tau (200 nM) was incubated with or without Taxol-stabilized MTs at the indicated concentrations of polymerized tubulin, after which bovine brain AB α C was added to 40 nM. The dephosphorylation reactions were performed for 5 min at 30 °C as described above. The data shown are the means \pm S.D. of results from three separate experiments and are expressed as the percentage of phosphate on tau that was not exposed to AB α C (control).

sized that the direct interaction of PP2A with MTs may also affect its catalytic activity in general. To test this hypothesis, purified AB α C, AC, and C subunits were incubated with buffer alone or with buffer containing 5 μ M unassembled or Taxol-polymerized tubulin. The phosphatase activity of each sample was then measured using either of two characterized PP2A

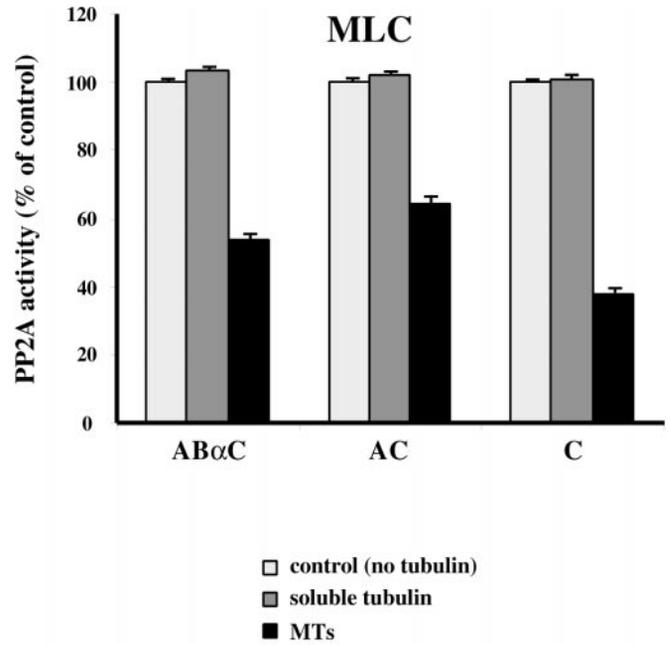


FIG. 7. MTs inhibit the catalytic activity of PP2A. Purified AB α C, AC, and C subunits (25 nM each) were incubated for 15 min in buffer alone or in buffer containing 5 μ M soluble or Taxol-polymerized tubulin. Then, a 100 μ M concentration of either of two phosphorylated substrates, myosin light chain (MLC) or the synthetic RRREEE(pT)EEE peptide, was added, and the samples were incubated for 5 min at 30 °C to allow substrate dephosphorylation. The data shown are the means \pm S.E. of triplicate determinations from two separate experiments and are expressed as the percentage of PP2A activity measured with each substrate in the absence of tubulin (control).

substrates that do not bind MTs: phosphorylated myosin light chain or the synthetic phosphopeptide RRREEE(pT)EEE (35). As shown in Fig. 7, incubation of PP2A enzymes with unpolymerized tubulin did not inhibit phosphatase activity for the substrates. In contrast, preincubation of PP2A enzymes with Taxol-stabilized MTs significantly inhibited their ability to dephosphorylate both substrates. Interestingly, the extent of inhibition varied between \sim 20 and 60%, depending on both which form of PP2A was tested and the chosen substrate. The phosphatase activities of AB α C, AC, and C for myosin light chain and the synthetic peptide were insensitive to Taxol in these assays (data not shown).

PP2A Preferentially Dephosphorylates Unassembled Versus Polymerized Tubulin—Neuronal MTs exist as a mixture of different populations of α - and β -tubulin isoforms (36). It has

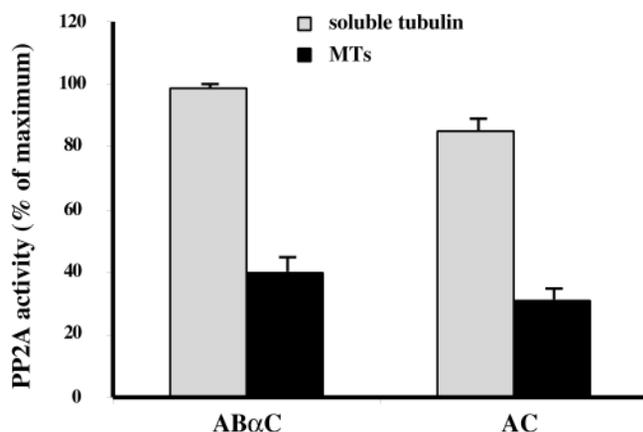


FIG. 8. **PP2A dephosphorylates unassembled tubulin more rapidly than assembled tubulin.** Purified AB α C and AC enzymes (25 nM) were incubated for 15 min at 30 °C with 20 μ M soluble or Taxol-polymerized bovine brain tubulin, which includes naturally phosphorylated β III-tubulin. PP2A activity was then measured as described under "Experimental Procedures." Values are expressed as the percent of maximal PP2A activity and are the means \pm S.E. of duplicate determinations from two separate experiments.

been reported previously that the neuron-specific β III-tubulin isoform, which represents \sim 25% of neuronal β -tubulin, is phosphorylated in cultured cells and *in vivo* (37–39). Remarkably, phosphorylated β III-tubulin can be dephosphorylated by PP2A, but not by any of several other protein phosphatases that have been tested to date (37). Based on these results, we measured dephosphorylation of unassembled or Taxol-polymerized brain tubulin by bovine brain AB α C and AC. As shown in Fig. 8, both forms of tubulin were dephosphorylated by AB α C and AC. During the 30 min in which the reactions were allowed to proceed, however, polymerized tubulin was dephosphorylated to just 40% the level of unassembled tubulin.

DISCUSSION

Previously, we reported that a pool of AB α C is localized on intracellular MTs and binds to tau *in vitro* (23). A question that naturally arose from those observations was, "Does tau anchor PP2A to neuronal MTs?" There is precedent for MAPs acting as bridges between MTs and enzymes that control protein phosphorylation. For example, tau and MAP2 anchor PP1 (40) and protein kinase A (41) to MTs, respectively. As far as PP2A is concerned, however, the results presented here demonstrate that tau cannot be responsible for linking the enzyme to MTs. Instead, the data presented in Figs. 1 and 2 imply that PP2A and MTs bind to tau in a mutually exclusive manner. The interaction site on tau for PP2A corresponds approximately to amino acid residues 221–396 of adult human tau and thus encompasses the MT-binding repeats and nearby flanking regions that form the overall MT-binding domain on tau (4–6). Interestingly, AB α C (Fig. 1), like MTs (5, 6), was able to bind tau variants containing truncated MT-binding regions as small as only one repeat or the proline-rich domain located immediately N-terminal to the repeats. Since the C subunit of PP2A does not bind to tau alone (23), the MT-binding domain on tau can be viewed as a site that anchors the PP2A holoenzyme and enables its catalytic subunit to dephosphorylate residues located predominantly elsewhere on tau. This might explain why the tau phosphatase activities for various forms of PP2A are correlated with their affinities for tau (23) and why okadaic acid, which binds tightly to the catalytic site on PP2A and abolishes its enzymatic activity (42), does not interfere with binding of PP2A to tau.

One mechanism that apparently can account for the MT-binding activity of PP2A is direct association via the C subunit.

This conclusion is supported by the finding that three distinct PP2A holoenzymes, the AC complex, and free C subunits all bound to MTs, albeit with varying affinities (Fig. 4). These *in vitro* data should not be assumed to mean that all PP2A enzymes efficiently interact with MTs *in vivo*, however. For example, in contrast to AB α C, the AB' C holoenzyme showed very low affinity for MTs in our *in vitro* assays (17) and is not known to be associated with MTs *in vivo* (43). Our finding that the affinity for MTs of PP2A holoenzymes varied according to their regulatory B subunits (Fig. 4) is consistent with the model that distinct PP2A isoforms are differentially targeted to specific subcellular compartments through their regulatory subunits (17, 22, 23, 43). It also must be noted that although direct binding of PP2A to MTs has now been shown to occur, other possible binding mechanisms cannot be formally excluded. For example, perhaps PP2A can also be linked to MTs indirectly through a MAP intermediate, as has been described for PP1 and tau (40).

One potential physiological consequence of the binding of PP2A to MTs is reduced phosphatase activity of the enzyme (Figs. 6 and 7). In the case of tau, this phosphatase inhibition could result from at least two factors: immobilization of the catalytic subunit of PP2A on MTs and competition between MTs and PP2A for tau binding. It is likely that inhibition of catalytic activity occurs subsequent to direct binding of the C subunit to MTs. Such an interaction may induce conformational changes in PP2A, which partially or completely conceal the catalytic site, preventing efficient access to substrates. The inhibition of PP2A activity by MTs also provides an explanation for reports that MT depolymerization induces okadaic acid-sensitive dephosphorylation of tau in cultured cells (11, 44). Together, these results underscore the possible importance of MT dynamics in the regulation of the phosphorylation state of PP2A-sensitive substrates, including tau. In addition to the control of PP2A activity by regulatory proteins, post-translational modifications, and biochemical factors (22, 25, 43), selective anchoring of PP2A to MTs may represent a novel way to regulate specific subcellular pools of PP2A.

Whereas MT assembly dynamics may regulate PP2A activity, PP2A, in turn, might modulate MT stability in axons by regulating the MT-binding and -stabilizing activities of tau (23, 45). Moreover, cycles of tubulin phosphorylation and dephosphorylation have been proposed to regulate MT functions during neuronal differentiation and to mediate interactions of MTs with other cellular components (38, 46). Interestingly, AB α C and AC preferentially dephosphorylated depolymerized as compared with polymerized brain tubulin (Fig. 8). This difference may result from the binding of PP2A to MTs, but not to soluble tubulin. β III-Tubulin is the only known form of phosphorylatable brain tubulin and is found primarily in the assembled pool of tubulin *in vivo* (37–39). Because phosphate can turn over rapidly on β III-tubulin, it was hypothesized that higher levels of phosphorylation in assembled MTs likely resulted from slower dephosphorylation of tubulin phosphate in polymers rather than in monomers (38). Since AB α C is the only known phosphatase capable of dephosphorylating β III-tubulin (37, 46), this hypothesis is supported by our *in vitro* data showing the preferential dephosphorylation of unassembled tubulin by AB α C. Thus, AB α C may be an important modulator of MT phosphorylation levels and functions in neurons. Yet, the lack of effect of okadaic acid on the ability of PP2A to co-sediment with MTs and the presence of AB α C on non-neuronal MTs both suggest that the interactions between PP2A and MTs are not simply restricted to dephosphorylation of tubulin. Their functional significance for the regulation of the cytoskeleton remains to be defined.

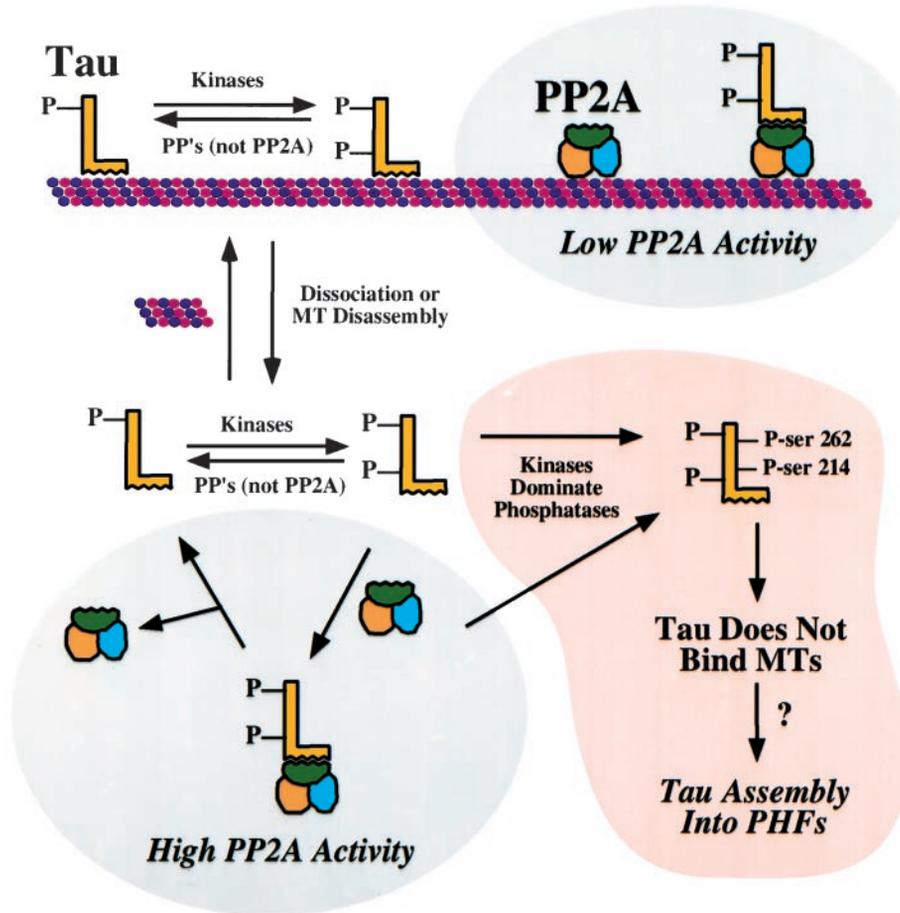


FIG. 9. Model for the role of structural interactions among A β C, tau, and MTs for the regulation of tau phosphorylation. The model emphasizes the following points. 1) Normal and hyperphosphorylated tau are depicted as containing one to two and four covalently bound phosphates, respectively, even though actual *in vivo* levels may be much higher. 2) Both MT-bound and soluble tau are presumed to be accessible to protein kinases. 3) PP2A binds to tau and MTs through distinct sites and therefore might be able to anchor tau to MTs. 4) Since MTs and PP2A compete for binding to the same region on tau and binding of PP2A to MTs inhibits its catalytic activity, PP2A can efficiently dephosphorylate tau only when neither protein is bound to MTs. Depolymerization of MTs or dissociation of PP2A from MTs thus potentiates the phosphatase activity of PP2A for soluble tau. In contrast to PP2A, other tau phosphatases such as PP1 (40) may be able to dephosphorylate both MT-bound and soluble tau. 5) As long as the tau kinases and phosphatases remain in proper balance, the phosphorylation state of tau will remain within limits that favor binding of tau molecules to MTs, as opposed to other tau molecules. 6) If the balance becomes altered in favor of the kinases, however, phosphates may accumulate on tau at specific sites such as serine 214 or serine 262, which, when phosphorylated, dramatically diminish the MT-binding activity of tau (48). The elimination of MTs as favored binding partners for tau may then contribute to an environment that permits tau to self-associate into paired helical filaments. Disruption of the normal structural interactions between PP2A and tau may also lead to tau hyperphosphorylation. *PHFs*, paired helical filaments.

Based on the collective results presented here and in related reports from our laboratories and others, we propose a model in which A β C regulates the phosphorylation state of tau by a complex mechanism involving structural interactions as well as enzyme-substrate interactions among A β C, tau, and MTs (Fig. 9). Because A β C binds to the MT-binding domain of tau and MTs inhibit the tau phosphatase activity of PP2A, the model presumes that A β C can dephosphorylate tau primarily, if not exclusively, when tau is dissociated from MTs. This is in stark contrast to the tau phosphatase activity of PP1 because tau acts as a bridge between PP1 and MTs; PP1 binds to a portion of tau that is distinct from the MT-binding site on tau; and PP1 has the potential to dephosphorylate both soluble and MT-bound tau (40). For simplicity's sake, however, the model does not take into account other factors that are involved in the regulation of PP2A and MTs. Many neurodegenerative disorders besides Alzheimer's disease are characterized by the presence of filaments assembled from hyperphosphorylated tau. The recent discovery of a direct link between tau mutations and neurodegenerative disorders such as FTDP-17 has underscored the importance of functional tau for neuronal integrity and survival (15). In

this context, tau mutations, especially those occurring within the MT-binding domain of tau, could affect its ability to bind to PP2A. Although it has been proposed earlier that a decrease in tau phosphatase activity, especially that contributed by PP2A, could underlie the biogenesis of hyperphosphorylated tau in Alzheimer's disease (23, 44, 47), the data we present here and in a prior report (17) suggest specific molecular mechanisms by which this could occur, namely, any alteration of the MT-binding site on tau, by mutation or post-translational modification, might compromise the ability of PP2A to bind and thereby dephosphorylate tau. It is easy to imagine how such a situation could lead to the accumulation of highly phosphorylated tau, as occurs in Alzheimer's disease. Likewise, deregulation of MT dynamics could indirectly affect endogenous levels of PP2A activity and deregulate PP2A-controlled signaling pathways. The fate of tau thus appears to be intimately linked to the complex interrelationships existing among tau, MTs, and PP2A. Disruption of the normal structural and enzymatic interactions among these factors might be a major underlying cause of the development of tauopathies.

REFERENCES

1. Binder, L. I., Frankfurter, A., and Rebhun, L. I. (1985) *J. Cell Biol.* **101**, 1371–1378
2. Weingarten, M. D., Lockwood, A. H., Hwo, S.-Y., and Kirschner, M. W. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72**, 1858–1862
3. Goedert, M., Spillantini, M. G., Jakes, R., Rutherford, D., and Crowther, R. A. (1989) *Neuron* **3**, 519–526
4. Mandelkow, E.-M., Biernat, J., Drewes, G., Gustke, N., Trinczek, B., and Mandelkow, E. (1995) *Neurobiol. Aging* **16**, 355–363
5. Goode, B. L., Denis, P. E., Panda, D., Radeke, M. J., Miller, H. P., Wilson, L., and Feinstein, S. C. (1997) *Mol. Biol. Cell* **8**, 353–365
6. Gustke, N., Trinczek, B., Biernat, J., Mandelkow, E.-M., and Mandelkow, E. (1994) *Biochemistry* **33**, 9511–9522
7. Goedert, M., Trojanowski, J. Q., and Lee, V. M.-Y. (1997) in *Molecular Mechanisms of Dementia* (Wasco, W., and Tanzi, R. E., eds) pp. 199–218, Humana Press, Inc., Totowa, NJ
8. Illenberger, S., Zheng-Fischöfer, Q., Preuss, U., Stamer, K., Baumann, K., Trinczek, B., Biernat, J., Godemann, R., Mandelkow, E.-M., and Mandelkow, E. (1998) *Mol. Biol. Cell* **9**, 1495–1512
9. Biernat, J., Gustke, N., Drewes, G., Mandelkow, E.-M., and Mandelkow, E. (1993) *Neuron* **11**, 153–163
10. Leger, J., Kempf, M., Lee, G., and Brandt, R. (1997) *J. Biol. Chem.* **272**, 8441–8446
11. Xie, H., Litersky, J. M., Hartigan, J. A., Jope, R. S., and Johnson, G. V. (1998) *Brain Res.* **798**, 173–183
12. Morishima-Kawashima, M., Hasegawa, M., Takio, K., Suzuki, M., Yoshida, H., Titani, K., and Ihara, Y. (1995) *J. Biol. Chem.* **270**, 823–829
13. Matsuo, E. S., Shin, R.-W., Billingsley, M., Van de Voorde, A., O'Connor, M., Trojanowski, J. Q., and Lee, V. M.-Y. (1994) *Neuron* **13**, 989–1002
14. Goedert, M., Jakes, R., Qi, Z., Wang, J. H., and Cohen, P. (1995) *J. Neurochem.* **65**, 2804–2807
15. Spillantini, M. G., and Goedert, M. (1998) *Trends Neurosci.* **21**, 428–433
16. Busciglio, J., Lorenzo, A., Yeh, J., and Yankner, B. A. (1995) *Neuron* **14**, 879–888
17. Sontag, E., Nunbhaki-Craig, V., Bloom, G. S., and Mumby, M. C. (1995) *J. Cell Biol.* **128**, 1131–1144
18. Goedert, M., Jakes, R., Spillantini, M. G., Hasegawa, M., Smith, M. J., and Crowther, R. A. (1996) *Nature* **383**, 550–553
19. Wilson, D. M., and Binder, L. I. (1995) *J. Biol. Chem.* **270**, 24306–24314
20. Wilson, D. M., and Binder, L. I. (1997) *Am. J. Pathol.* **150**, 2181–2195
21. Friedhoff, P., von Bergen, M., Mandelkow, E.-M., Davies, P., and Mandelkow, E. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 15712–15717
22. Kamibayashi, C., Estes, R., Lickteig, R. L., Yang, S.-I., Craft, C., and Mumby, M. C. (1994) *J. Biol. Chem.* **269**, 20139–20148
23. Sontag, E., Nunbhakdi-Craig, V., Lee, G., Bloom, G. S., and Mumby, M. C. (1996) *Neuron* **17**, 1201–1207
24. Mumby, M. C., Russell, K. L., Garrard, L. J., and Green, D. D. (1987) *J. Biol. Chem.* **262**, 6257–6265
25. Kamibayashi, C., Estes, R., Slaughter, C., and Mumby, M. C. (1991) *J. Biol. Chem.* **266**, 13251–13260
26. Kim, H., Binder, L. I., and Rosenbaum, J. (1979) *J. Cell Biol.* **80**, 266–276
27. Brandt, R., and Lee, G. (1993) *J. Biol. Chem.* **268**, 3414–3419
28. Carmel, G., Mager, E. M., Binder, L. I., and Kuret, J. (1996) *J. Biol. Chem.* **271**, 32789–32795
29. Fullerton, A. T., Bau, M. Y., Conrad, P. A., and Bloom, G. S. (1998) *Mol. Biol. Cell* **9**, 2699–2714
30. Paschal, B. M., Obar, R. A., and Vallee, R. B. (1989) *Nature* **342**, 569–572
31. Melki, R., Kerjan, P., Waller, J., Carlier, M.-F., and Pantaloni, D. (1991) *Biochemistry* **30**, 11536–11545
32. Serrano, L., Montejo de Garcini, E., Hernandez, M. A., and Avila, J. (1985) *Eur. J. Biochem.* **153**, 595–600
33. Spudich, J. A., and Watt, S. (1971) *J. Biol. Chem.* **246**, 4866–4871
34. Scott, C. W., Spreen, R. C., Herman, J. L., Chow, F. P., Davison, M. D., Young, J., and Caputo, C. B. (1993) *J. Biol. Chem.* **268**, 1166–1173
35. Pinna, L. A., and Donella-Deana, A. (1994) *Biochim. Biophys. Acta* **1222**, 415–431
36. Ludueña, R. F. (1993) *Mol. Biol. Cell* **4**, 445–457
37. Khan, I. A., and Luduena, R. F. (1996) *Biochemistry* **35**, 3704–3711
38. Gard, D. L., and Kirschner, M. W. (1985) *J. Cell Biol.* **100**, 764–774
39. Diaz-Nido, J., Serrano, L., Hernandez, M. A., and Avila, J. (1990) *J. Neurochem.* **54**, 211–222
40. Liao, H., Li, Y., Brautigam, D. L., and Gundersen, G. G. (1998) *J. Biol. Chem.* **273**, 21901–21908
41. Vallee, R. B., DiBartolomeis, M. J., and Therukauf, W. E. (1981) *J. Cell Biol.* **90**, 568–576
42. Bialojan, C., and Takai, A. (1988) *Biochem. J.* **256**, 283–290
43. McCright, B., Rivers, A. M., Audlin, S., and Virshup, D. M. (1996) *J. Biol. Chem.* **271**, 22081–22089
44. Merrick, S. E., Demoise, D. C., and Lee, V. M.-Y. (1996) *J. Biol. Chem.* **271**, 5589–5594
45. Merrick, S. E., Trojanowski, J. Q., and Lee, V. M.-Y. (1997) *J. Neurosci.* **17**, 5726–5737
46. Yamamoto, H., Saitoh, Y., Fukunaga, K., Nishimura, H., and Miyamoto, E. (1988) *J. Neurochem.* **50**, 1614–1623
47. Gong, C.-X., Shaikh, S., Wang, J.-Z., Grundke-Iqbal, I., and Iqbal, K. (1995) *J. Neurochem.* **65**, 732–738
48. Mandelkow, E.-M., and Mandelkow, E. (1998) *Trends Cell Biol.* **8**, 425–427