Phosphorylation Affects the Ability of Tau Protein to Promote Microtubule Assembly*

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Tau is a family of closely related proteins known for its ability to copolymerize with tubulin, inducing the formation of microtubules. When tau was stripped of phosphate by treatment with alkaline phosphatase it underwent a pronounced change in electrophoretic mobility, probably reflecting a conformational change. The dephosphorylated tau promoted significantly more rapid and more extensive polymerization of microtubules than untreated MAPs from a crude MAP preparation stimulated greater tubulin polymerization than did additionally phosphorylated MAPs.

Materials and Methods

Tau proteins were discovered as nontubulin components of microtubule protein purified by cycles of depolymerization and polymerization (1). Microtubule protein can readily be isolated since it remains in the supernatant upon cold centrifugation but polymerizes and can be sedimented at warm temperatures (2). Starting with microtubule protein, Cleveland et al. (3, 4), further purified tau and characterized it as a family of four closely related proteins with molecular weights of 55,000–62,000.

Evidence has been accumulating that tau is an important component of microtubules in vivo and that in vitro copolymerization with tubulin is not merely an artifact. Nearly all mammalian and avian cells stain for tau by immunofluorescence (5). Tau antibodies and tubulin antibodies, though not cross-reactive, give similar patterns in the fluorescent staining of living cells (6); colcemid, which depolymerizes microtubules but which does not interact directly with tau, eliminates both staining patterns. Intact microtubules isolated by recently developed methods that avoid a depolymerization step also contain tau (7, 8). A question remains whether in addition to being an integral part of microtubules, tau is an active component of a system that regulates microtubule assembly/disassembly or one that determines microtubular interactions with other cellular components.

If tau is involved in microtubule regulation, one possibility is that its activity might in turn be controlled, perhaps by phosphorylation. Phosphorylation has emerged as a major contributor to several intracellular regulatory systems ranging from enzyme regulation in the classic example of glycogen metabolism to musculor control as mediated by myosin kinase. Microtubule protein has been found to undergo phosphorylation; though the major incorporation of phosphate is into a high molecular weight group of MAPs (9) and tau (11–13) and tau (4) have also been reported to undergo phosphorylation. There is evidence that phosphorylation of microtubule-associated proteins affects their interaction with the microtubules since untreated MAPs from a crude MAP preparation stimulated greater tubulin polymerization than did additionally phosphorylated MAPs (14–16).

The phosphorylation of tau has been recognized (4), and an effect on tubulin polymerization has been noted (17). The present paper reports an additional mode of phosphorylation, one that seems to cause a profound conformational change in the protein. This mode of phosphorylation significantly affects the ability of tau to promote the polymerization of tubulin.

Materials and Methods

Protein Purification—Microtubule protein was purified from the cerebral hemispheres of beef brain by a modification of the method of Shelanski et al. (18). Brains from freshly slaughtered cattle were chilled on ice, the meninges and superficial blood vessels were removed, and the brains were minced. Buffer A (20 mM MES, 80 mM NaCl, 2 mM EGTA, 0.1 mM EDTA, 1 mM MgCl2, pH 6.75) containing 0.1 mM GTP was added 1:1 (v/w), and the mixture was homogenized in a Waring blender for two 20-s pulses separated by 20 s. The homogenate was centrifuged at 27,000 × g for 20 min at 4 °C. The resulting crude extract was brought to 1.0 mM with GTP, and the extract was passed through the following cold/warm cycling procedure. After centrifugation for 60 min at 4 °C and 60,000 × g, glycerol was added 1:3 (v/v) to the supernatant, the mix was incubated for 20 min at 37 °C, and centrifuged again for 90 min at 60,000 × g. The pellets were resuspended in 1.0 mM GTP. A second cycle was done as above, but centrifugations were 75 min at 75,000 × g. The final pellets were frozen in liquid nitrogen and stored at −75 °C for later use. All nucleotides were from Sigma except radioulnucleotides as noted which were from Amersham Corp.

The phosphorylation of tau protein was purified from the cycled microtubule protein by the method of Sandoval and Weber (19). Tubulin was purified essentially by the method of Witman et al. (20) except that immediately upon elution from the phosphocellulose column the peak tubulin fractions were passed over a Bio-Gel G-25 column equilibrated with polymerization buffer (0.1 mM MES, 1 mM mercaptoethanol, 0.5 mM MgCl2, 0.1 mM EDTA, 1.0 mM EGTA, pH 6.4). Purified tubulin was frozen in liquid nitrogen and stored at −80 °C; it was clarified by centrifugation prior to use. The method of Lowry et al. (21) was used to estimate purified tau and tubulin concentrations using crystalline bovine serum albumin (Armour) as a standard.

Gel Electrophoresis—Gel electrophoresis was done by the SDS-polyacrylamide gel system of Laemmli (22) on 1-mm thick slab gels containing 12.5% acrylamide in the separation gel. Greater separation was achieved by continuing (at 30 mA) for 1 h after the dye front had

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run off the gel. Gels were stained for a minimum of an hour in aqueous 0.03% (w/v) Coomassie blue R-250, 25% (v/v) isopropanol, 10% (v/v) acetic acid followed by several hours or overnight in 0.003% Coomassie blue, 10% isopropanol, 10% acetic acid and finally fully destained in 10% acetic acid.

Alkaline Phosphatase Treatment of Tau—Purified tau protein was suspended in or dialyzed against Buffer A in which the chelating agents had been omitted. This was mixed 1:1 with 0.1 M Tris buffer, pH 8.2, containing Escherichia coli alkaline phosphatase (Sigma) at a concentration of at least 10 units/ml; 0.02% Na azide was included to inhibit bacterial growth. The mixture was incubated for 18–24 h at 37 °C. Tau was separated from alkaline phosphatase by precipitation with 45% saturated ammonium sulfate.

Phosphorylation of Tau—Tau protein was mixed approximately 1:1 with cycled microtubule protein, in buffer A, and diluted with buffer to about 0.1 mg/ml of total protein. ATP was added to 1 mM, and 0.02% Na azide was included to preclude bacterial growth. One to three days incubation at room temperature were required for complete reaction. To remove the microtubule protein, samples in microfuge tubes were incubated on ice a minimum of 45 min; the tubes were then placed directly in rapidly boiling water for 10 min followed immediately by 30 min of incubation in ice water. The tubes were then spun in the microfuge for a minimum of 15 min, and the supernatant was transferred to clean microfuge tubes and spun again. The supernatant was then transferred again to clean microfuge tubes, and the tau protein was precipitated with 20% trichloroacetic acid followed by two ethanol washes. The dried samples were resuspended in buffer A or directly in SDS-gel buffer for examination by electrophoresis.

Electron Microscopy—Samples were fixed by mixing 10:1 with 8% glutaraldehyde. The samples were drop loaded onto carbon-coated parlodion films on 400-mesh copper grids which had been glow discharged. The excess liquid was removed with filter paper after 3–5 s, and the grids were washed in rapid succession with 2 drops of 1.5% uranyl acetate and drained dry. Specimens were examined on a Phillips EM 301 electron microscope.

**RESULTS**

In light of the potential significance of phosphorylation in microtubule regulation, a series of experiments was designed to determine what effect changes in the phosphorylation state of tau would have upon its ability to interact with tubulin. To compare different phosphorylation states of tau, it seemed appropriate to begin with minimally phosphorylated tau. Therefore, tau was treated with alkaline phosphatase to remove phosphate residues. The tau treated with alkaline phosphatase, EC 3.1.3.1 (Fig. 1C), gave a faster set of four electrophoretic bands than did the untreated tau (Fig. 1A). When tau is in this dephosphorylated state we will call it state I tau.

When incubation was done in the presence of chelating agents, which slow the action of the zinc-requiring alkaline phosphatase, electrophoretic analysis revealed a composite of the two four-band patterns (Fig. 1B). Since one of the phosphorylated components was not well resolved from one of the dephosphorylated components, seven electrophoretic bands were seen rather than eight.

To demonstrate that the dephosphorylation is reversible and, therefore, to rule out proteolysis and other transformations as the cause of the change in electrophoretic mobility, tau was rephosphorylated. Tau that had been dephosphorylated by alkaline phosphatase treatment was incubated with cycle-purified microtubule protein and ATP. After 18 h, phosphorylation was only partially complete as revealed by a seven-band electrophoretic pattern (Fig. 2A). Sixty-nine hours were sufficient for full conversion characterized by the conventional (3) four-band electrophoretic pattern (Fig. 2B). We will call this phosphorylated form state II tau. The proteins represented by each of these patterns were dephosphorylated to give the state I electrophoretic pattern when treated again with alkaline phosphatase (see Fig. 2, C and D). Thus the phosphorylation process is fully reversible.

**Fig. 1 (left). Dephosphorylation of tau by alkaline phosphatase-electrophoretic analysis.** Tau was incubated with alkaline phosphatase. Lane A, untreated tau; lane B, tau incubated with phosphatase and ATP; lane C, tau incubated with phosphatase in the absence of chelating agents. Electrophoretic migration is downward.

**Fig. 2 (right). Reversibility of tau phosphorylation/dephosphorylation.** Tau extensively treated with alkaline phosphatase was incubated with cycled microtubule protein and ATP. Lane A, incubated 18 h; lane B, incubated 69 h. These two samples were subsequently retreated with alkaline phosphatase: lane C, phosphatase treatment of material represented by lane A; lane D, phosphatase treatment of material represented by lane B.

To confirm further that the changes being observed were due to phosphorylation, radio-labeling studies were conducted. Incubation of fully dephosphorylated tau with microtubule protein and \( \gamma^{32}P \)ATP led to a slow conversion of the state I tau to state II tau (Fig. 3A). The autoradiogram of the same gel showed the same set of components regardless of the incubation time (Fig. 3B). This pattern corresponded closely to the electrophoretic pattern of the state II tau. This result suggests that the change in electrophoretic mobility is a consequence of phosphorylation and that the phosphorylation affecting mobility occurs preferentially to other phosphorylations. Additional phosphorylation did appear to occur in this system, however. Fig. 4 shows phosphate incorporation into alkaline phosphatase-treated tau (Fig. 4A) and untreated (presumably already phosphorylated) tau (Fig. 4B). Isotope was incorporated into both preparations. For 69 h of incubation, measurement of radioactivity in gel slices (25) indicated an approximate incorporation of 2.5 mol of ATP per mol of alkaline phosphatase-treated tau and 1.2 mol of ATP per mol of untreated tau.

Since tau was originally defined by its ability to promote tubulin assembly and since this remains its most studied property, alkaline phosphatase-treated tau and untreated tau were compared in their ability to promote the polymerization of tubulin as followed kinetically by turbidimetry. The difference between the two tau samples was unexpectedly dramatic.
Phosphorylation of Tau

FIG. 3. Incorporation of $^{32}$P into tau protein; electrophoretic patterns of protein and radioactivity. Tau treated with alkaline phosphatase was incubated with cycled microtubule protein and [γ-32P]ATP. Aliquots were taken at 0, 1, 7, and 69 h. A, protein stain; B, radioactivity. Autoradiograms were exposed for different lengths of time to allow scanning of equivalently exposed lanes; even unincubated samples incorporated slight amounts of isotope during handling. Electrophoretic migration was left to right.

Dephosphorylated tau was found to promote significantly more rapid and more extensive polymerization than did the untreated tau. To verify that the polymer being formed was actually microtubular, the samples represented by Fig. 5 were fixed in glutaraldehyde and examined by electron microscopy. As shown in Fig. 6, both tau preparations induced the formation of microtubules. There was no obvious difference in the microtubules formed by the two different tau preparations.

FIG. 4. Incorporation of $^{32}$P into both alkaline phosphatase-treated and untreated tau. Samples were incubated as in Fig. 3. The samples were then electrophoresed and autoradiographed. A, tau that had been fully dephosphorylated by alkaline phosphatase; B, untreated tau. The high molecular weight species, probably MAP-2, was introduced with the microtubule protein used as kinase.

DISCUSSION

It is now clear that tau can exist in two different states and that those states are readily interconvertible by a phosphorylation/dephosphorylation process. The two phosphorylation states have a marked difference in electrophoretic mobility, even in SDS-containing buffers. A precedent for such an effect of phosphorylation is found with an H1 histone in which the mobility change was shown to result from a conformational rather than a charge difference (24), suggesting an SDS-resistant folded domain which is eliminated by phosphorylation. It is intriguing that the regulatory subunit of type I CAMP-dependent protein kinase has also been shown to undergo a change in electrophoretic mobility upon phosphorylation (25); this kinase is also a constituent of microtubule protein (10, 26, 27) and has a molecular weight in the same range as the tau family.

It is likely that the shift in mobility reflects incorporation of a single phosphate since no intermediates were detected and since, under similar conditions, tau stripped of its phosphate by alkaline phosphatase incorporated approximately 1 mol of phosphate per mol of protein more than did untreated tau under the same conditions. The fact that the state II tau continues to incorporate phosphate indicates that a second mode of phosphorylation occurs without causing a substantial mobility shift. The kinase that catalyzes the transformation from state I to state II appears to be distinct from kinase(s)
Phosphorylation of Tau

The types of kinases involved in tau phosphorylation are not yet known. A number of kinases from brain have been reported (see e.g. Ref. 29). Preliminary attempts to identify the kinase that acts on tau detected no effects from cAMP and only small inconsistent effects of Ca\(^{2+}\) and/or calmodulin in converting tau from state I to state II. It must be kept in mind, however, that the phosphorylation of tau proceeded very slowly when partially purified microtubule protein was used as the source of enzyme; the reaction required more than a full day to approach completion. Most likely, much of the kinase was lost while purifying microtubule protein. It is also possible, however, that some necessary additional element was missing or that some inhibitor of the reaction was present.

Though tau might simply promote microtubule assembly, the property which led to its discovery, it may have more complex functions. Perhaps it serves primarily to stabilize microtubules once they are formed; perhaps it serves to communicate between or interconnect microtubules and other cytoskeletal elements. While as reported here the phosphorylation state had a significant effect upon the rate and extent of microtubule polymerization in vitro, the effect in vivo has yet to be determined. In one phosphorylation state tau might stabilize microtubules, while in the alternative state it might allow rapid microtubule depolymerization. For instance, Paalanivelu and Luduena (30) proposed that tau promotes longitudinal interaction of tubulin subunits, a proposal also supported by data of Sandoval and Vandekerckhove (31). A change in tau phosphorylation state might allow microtubules to open up into ribbons with coincident rapid depolymerization of microtubules along their entire length or at selected points, thus obviating slow depolymerization from the ends. Support for tau as a stabilizing element arose from an observation made while the procedures for this study were being developed; the longer that tau was incubated with ATP and cycled microtubule protein, the more difficult it became to remove the tubulin from the tau by heat treatment. Perhaps phosphorylated tau protein, especially when multiply phosphorylated, associated more strongly with tubulin or perhaps it protected tubulin from the denaturing effects of heat; tubulin itself incorporated virtually no phosphate under these conditions.

The potential consequences of tau phosphorylation involve multiple components of the cytoskeletal system. It was reported that tau binds calmodulin in the presence of Ca\(^{2+}\) and that addition of calmodulin to a tau/tubulin mix inhibits polymerization (32, 33). Therefore, in addition to phosphorylation, interaction with Ca\(^{2+}\)/calmodulin could control tau in its effects on tubulin. Moreover, the function of tau might extend beyond its tubulin-related effects, e.g. tau interacts with actin (34). Thus the place of tau and its phosphorylation in regulation of the cytoskeleton may be quite complex and requires much further study.

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