In Alzheimer’s disease, hyperphosphorylated tau is an integral part of the neurofibrillary tangles that form within neuronal cell bodies and fails to promote microtubule assembly. Dysregulation of the brain-specific tau protein kinase II is reported to play an important role in the pathogenesis of Alzheimer’s disease (Patrick, G. N., Zukerberg, L., Nikolic, M., De La Monte, S., Dikkes, P., and Tsai, L.-H. (1999) Nature 402, 615–622). We report here that in vitro phosphorylation of human tau by human recombinant tau protein kinase II severely inhibits the ability of tau to promote microtubule assembly as monitored by tubulin polymerization. The ultrastructure of tau-mediated polymerized tubulin was visualized by electron microscopy and compared with phosphorylated tau. Consistent with the observed slower kinetics of tubulin polymerization, phosphorylated tau is compromised in its ability to generate microtubules. Moreover, we show that phosphorylation of microtubule-associated tau results in tau’s dissociation from the microtubules and tubulin depolymerization. Mutational studies with human tau indicate that phosphorylation by tau protein kinase II at serine 396 and serine 404 is primarily responsible for the functional loss of tau-mediated tubulin polymerization. These in vitro results suggest a possible role for tau protein kinase II-mediated tau phosphorylation in initiating the destabilization of microtubules.

Six isoforms of human tau are expressed in adult human brain (1). These arise from alternate splicing of the mRNA transcribed from a single gene located on the long arm of chromosome 17 (1–5). Tau isoforms differ from each other with respect to the presence of three or four tandem repeats of 31 amino acids each in combination with two N-terminal inserts of 29 amino acids. The longest form of tau contains four tandem repeats, and the 58-amino acid N-terminal insert that yields a full-length protein of 441 amino acids (1). The four 31-amino acid repeat domains have been shown to bind microtubules, whereas most of the phosphorylation sites in tau are located outside this microtubule binding repeat region. One of the functions of the tau protein is to promote microtubule assembly in vivo and to stabilize microtubules in the nervous system (6–10).

Neurofibrillary tangles and senile plaques constitute two prominent neuropathological characteristics of Alzheimer’s disease (AD) (11, 12). The main fibrous component of all neurofibrillary lesions is paired helical filament (PHF), which contains predominantly the abnormally phosphorylated tau (13–18). It has been hypothesized that aberrant phosphorylation of tau leads to its aggregation into PHF, resulting in destabilization of microtubules and the death of neurons (1, 19). Notably, hyperphosphorylated tau has been detected in other tau-positive filamentous lesions, in a group of diseases collectively known as the tauopathies. These neurodegenerative diseases include progressive supranuclear palsy, corticobasal degeneration, Down’s syndrome, frontotemporal dementia and Parkinsonism linked to chromosome 17, and Pick’s disease (20). Recent studies show that these various phenotypes might be the result of phosphorylation of specific tau isoforms in different nerve cells in distinct brain regions (21).

Tau-associated with PHF from AD brain is hyperphosphorylated at several serine/threonine sites that are followed by a proline (22). AD tau protein in its hyperphosphorylated state fails to promote microtubule (MT) assembly in vitro (23) and this phosphorylation-dependent tau dysfunction is considered one of the critical events leading to neuronal degeneration (24). It is not known which kinase initiates tau hyperphosphorylation and MT disassembly and which phosphorylated amino acids in tau contribute to tau’s dysfunction. Understanding the molecular basis for tau dysfunction requires identification and availability of highly purified candidate tau protein kinase, careful mapping of phosphorylation sites, mutational studies, and functional characterization of the corresponding phosphorylated tau mutants. There are two proline-directed kinases, tau protein kinase II (TPK II) and glycogen synthase kinase 3β (TPK I), which have been found to be associated with microtubules (25, 26) and are known to phosphorylate tau in a cellular environment (27, 28).

One of the hypotheses is that TPK II initiates tau hyperphosphorylation and MT disassembly. To the best of our knowledge, a highly purified and well-characterized source of human recombinant TPK II has not been reported. Therefore, the definition of TPK II-mediated phosphorylation sites in human tau and their potential role in tau dysfunction remain unknown. Recently, we have been successful in producing milligram quantities of highly purified human recombinant TPK II by in...
vitro reconstitution using separately expressed regulatory (Cdk5) and activator (p20) subdomains of this kinase. This critical step has allowed us to map TPK II phosphorylation sites in human tau by a combination of mass spectrometric techniques.\(^2\) We found that the six TPK II-mediated phosphorylation sites in tau are Thr-181, Thr-205, Thr-212, Thr-217, Ser-396, and Ser-404, based on the numbering used in the longest form (1–441) of human tau (1).

The aims of the present study were to test the TPK II hypothesis for initiation of tau hyperphosphorylation and MT disassembly and to understand the molecular basis for phosphorylation-dependent tau dysfunction and its relevance to tauopathies. We report here that phosphorylation of human tau by TPK II inhibits tau’s ability to promote microtubule assembly and that TPK II-mediated phosphorylation of microtubule-associated tau results in tau’s dissociation from the microtubules and tubulin depolymerization. Based on the knowledge gained from mapping human TPK II–mediated phosphorylation sites in human tau,\(^2\) the molecular basis for this tau dysfunction has been determined using mutational studies on human tau. We show that TPK II–mediated tau phosphorylation at Ser-396 and Ser-404 is primarily responsible for the loss of tau function in MT assembly and dissociation of tau from microtubules. These results are discussed with regard to TPK II as a potential target for inhibiting initiation of tau hyperphosphorylation and the implications for tauopathies endowed with Ser-396 and Ser-404 phosphoepitopes (20, 21).

**EXPERIMENTAL PROCEDURES**

**General Chemicals—** General laboratory chemicals and molecular biology reagents were purchased from Sigma and Life Technologies, Inc., respectively. The oligonucleotides were ordered from Genosys Biotechnologies Inc. The baculovirus gold DNA was obtained from Pharmingen Inc. The expression vectors were purchased from Amersham Pharmacia Biotech and Novagen. Competent BL-21 cells were obtained from Stratagene. The template p35 DNA was kindly provided by Dr. Tsai (Harvard Medical School). The clone for human tau (1–383) was obtained from Dr. Virginia Lee (University of Pennsylvania). Protein concentrations were determined using the BCA assay from Pierce. All the electrophoresis reagents were from Bio-Rad. Recombinant glyceron synthase kinase 3β (TPK I) was from New England BioLabs (5000 units/ml). Tubulin used for the polymerization assays was from Cytoskeleton (catalog no. T238), and the tubulin polymerization assays were done using POLYTRAX Plus microtubule polymerization reagent from Molecular Devices.

**In Vitro Reconstitution of TPK II (cdk5/p20)—** The full-length human cdk5 gene was inserted into the baculovirus vector using standard baculovirus expression vector technology. A sample plaque isolate producing the highest level of cdk5 was plaque-purified three times, and a single virus plaque was used to make stock virus for cdk5 production. For in vitro reconstitution of TPK II, human recombinant native cdk5 was partially purified by Q-Sepharose from baculovirus-infected insect cells. The truncated version of human p35 activator protein, p20 (G137-R307), was cloned and expressed in Escherichia coli as a ubiquitin fusion containing an internal hexa-histidine sequence. For in vitro reconstitution and purification of human recombinant TPK II (cdk5/p20), the clone DE-93, containing the p20 construct, was grown in NS-35 medium (29) until the A\(_{600}\) reached 0.4 and induced for 3 h with 1 mM isopropyl-β-D-thiogalactoside. The crude E. coli extract was adjusted to pH 8.0 using 2 M Tris and centrifuged at 12,000 \(\times\) g for 1 h, and the supernatant was loaded onto a nickel immobilized metal affinity chromatography (IMAC) column equilibrated in 50 mM Tris, pH 8.0. The column was washed overnight with 50 mM Tris, pH 8.0, followed by buffer containing 50 mM imidazole and was then re-equilibrated with 50 mM Tris, pH 8.0. Partially purified cdk5 was diluted 1:2 with 50 mM Tris, pH 8.0, and loaded onto the IMAC. The column was washed with buffer containing 50 mM imidazole, and the complex was eluted with buffer containing 300 mM imidazole. Fractions containing TPK II (cdk5/p20) by SDS-polyacrylamide gel electrophoresis (PAGE) were pooled and dialyzed in 20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM dithiothreitol (DTT). Further purification of the IMAC-isolated TPK II was carried out using immobilized streptavidin (Pierce) followed by affinity chromatography on an ATP-agarose column (Sigma).

**Cloning, Expression, and Purification of Tau and Its Mutants—** Wild type tau (1–383) with an octa-histidine C-terminal tag was cloned and expressed in E. coli by inserting it in-frame behind the T7 tag of the bacteriophage expression vector (26, 27) into pDH Clone-601R fragment. Plasmid DNA from the DE-105.2 clone was isolated and used to transform competent BL-21 (DE-3) cells used for expression with 1 mM isopropyl-β-D-thiogalactoside for 4 h in NS-85 media (29). Cells were collected by centrifugation, and the cell pellets were stored at \(-80^\circ\text{C}\) and used for IMAC purification as described elsewhere (30). The tau protein was eluted with buffer containing 300 mM imidazole, and protein-containing fractions were determined by absorbance at 280 nm. Samples were run on 12% SDS-PAGE, and the tau-containing samples were pooled and dialyzed in 50 mM Tris, pH 8.0, 1 mM DTT. All the tau mutants were obtained using 1–383 tau DNA, mutagenized primers, and the QuikChange site-directed mutagenesis kit from Stratagene. Expression and purification of the mutant proteins and truncated tau (244Q–390A) were carried out as described above for wild type (1–383) tau.

**TPK II Phosphorylation of Tau Associated with Polymerized Tubulin—** Tubulin was polymerized in the presence of 7 mM (1–383 isomorph) tau under the conditions described above. After 30 min, either buffer, TPK II (100 nM final), ATP (1 mM final), or TPK II + ATP was added to the polymerization mixture, and the mixture was incubated at 27°C in a Molecular Devices microplate reader, and the A\(_{440}\) measurement was determined every 1 min for 30 min.

**RESULTS**

Wild type human tau (1–383) was expressed at high levels in E. coli and purified by IMAC using nickel as the immobilized metal ion. The 1–383 isomorph of human tau used in this work lacked the two N-terminal repeats, but contained the single microtubule binding repeats. It had the same number of proline-directed phosphorylation sites as the longest form (1–441) of human tau. Throughout this discussion, the numbering of phosphorylation sites in human recombinant tau is based on the longest form (1–441) of tau (1). The mass spectroscopy analysis of IMAC-purified human recombinant tau (1–383) confirmed that it was devoid of any phosphorylation.

Fig. 1 shows SDS-PAGE of purified recombinant TPK II, a complex of cdk5, and its p20 activator protein. The recombinant enzyme was reconstituted on an IMAC column and eluted with imidazole (lane 1). This partially purified complex was enzymatically active but required further affinity purification on immobilized streptavidin (lane 2) and ATP agarose (lane 3) columns. This highly purified (>90%) and well-characterized TPK II (lane 3) was used for in vitro tau phosphorylation studies in the presence of 1 mM ATP. The TPK II–phosphorylated tau was subjected to mass spectrometry analysis, which showed phosphorylation of tau at six proline-directed sites.
Fig. 1. SDS-PAGE (12%) of recombinant TPK II obtained by in vitro reconstitution from cdK5 and its p20 activator protein. Lane 1, TPK II isolated by in vitro reconstitution on IMAC; lane 2, TPK II eluted from immobilized streptavidin in the presence of biotin; lane 3, purified TPK II obtained after elution from an ATP-agarose column; lane 4, molecular mass markers.

Although the extent of phosphorylation at each site is unknown, these results were consistent with the incorporation of 5–6 mol of phosphate/mol of tau.

It is well known that microtubules (MT) are formed in vitro by noncovalent polymerization of tubulin dimers (23, 32–35). Formation of MT from tubulin solution can be followed by observing an increase in absorbance at 340 nm in the presence of GTP and tau (23, 32, 34, 35). This continuous spectrophotometric tubulin polymerization assay was used for functional characterization of TPK II-phosphorylated human recombinant tau. Fig. 2 shows that, under defined conditions, TPK II-phosphorylated tau failed to promote tubulin polymerization. Fig. 2 (inset, lane 3) shows that TPK II-phosphorylated tau has a significantly higher molecular mass than does unphosphorylated tau (inset, lane 2). There was a requirement for wild type tau, tubulin, and GTP, because no polymerization occurred when tubulin alone was incubated with GTP (data not shown). The polymerization rate (absorbance units per minute) for TPK II-phosphorylated tau was 5% compared with wild type tau, and the level of polymerized tubulin at steady state (A_{340} maximum) was 10% compared with wild type tau. In contrast, tau-dependent tubulin polymerization was observed when tau was phosphorylated with TPK I (data not shown), in agreement with a recent report (36). We also studied the effect of tau concentration on tubulin polymerization under defined assay conditions. The increase in turbidity (A_{340}) was linear with tau concentration for unphosphorylated tau, whereas TPK II-phosphorylated tau inhibited MT assembly at all the tau concentrations tested (data not shown).

As shown in Fig. 2, measurement of turbidity changes allowed us to differentiate unphosphorylated tau from TPK II-phosphorylated tau by its ability to promote tubulin polymerization. However, to directly demonstrate the effect of TPK II-phosphorylated tau on microtubule assembly, we carried out negative-stain electron microscopy at steady-state tubulin polymerization. Fig. 3 shows electron micrographs of the MT assembly reaction at steady state for TPK II-phosphorylated tau and unphosphorylated tau. As shown, large microtubules were seen in assembly promoted by unphosphorylated tau (Fig. 3A), demonstrating that the recombinant human tau is fully functional. In contrast, MT assembly was significantly impaired in terms of length and mass in the presence of TPK II-phosphorylated tau (Fig. 3B). In the tubulin control (data not shown), no microtubule assembly was observed.

The above results led us to investigate the effect of TPK II on MT-associated tau. Fig. 4A shows that addition of TPK II and ATP to preformed MT caused a decrease in A_{340} as a function of time. These results indicate that, as tau gets phosphorylated on MTs, it disrupts MT assembly as monitored by a time-dependent decrease in absorbance. When either TPK II or ATP was added alone either at t = 0 or t = 30 min, no significant change in absorbance from the buffer control was observed. Furthermore, the buffer control sample and TPK II-phosphorylated sample after dissociation from MTs were analyzed by SDS-PAGE followed by Western blotting. Tau was probed with anti-T7 monoclonal antibody known to recognize the N-terminal epitope tag engineered on human tau. As shown in Fig. 4B, the TPK II-treated sample showed a higher molecular mass than did the control, suggesting that detachment of tau from MT is indeed due to phosphorylation of tau by TPK II. In contrast, when a truncated form of tau (244Q-390A), consisting of the four MT binding repeats but none of the TPK II phosphorylation sites, was used for MT assembly, the addition of

Fig. 2. Effect of TPK II phosphorylation on the ability of wild type human tau (1–383 isoform) to promote microtubule assembly. Data represent the average of three experiments ± S.D. Open circles, unphosphorylated tau; filled circles, TPK II-phosphorylated tau. Inset, a 12% SDS-PAGE of the 1–383 isoform of wild type human tau. Lane 1, molecular mass markers; lane 2, unphosphorylated wild type tau; lane 3, TPK II-phosphorylated wild type tau.

Fig. 3. Transmission electron microscopy of steady state microtubules of tubulin plus wild type unphosphorylated (A) and TPK II-phosphorylated wild type tau (B). Samples were applied to Formvar-coated grids, dried, negatively stained with aqueous 1% uranyl acetate, and examined using a JEOL transmission electron microscope. The bar along the right side of each micrograph corresponds to 1 μm.
TPK II had no effect on preformed MTs (Fig. 4A). Taken together, these in vitro results show that TPK II phosphorylation impairs functional activity of tau, which is critical for stabilizing the microtubule transport system. To our knowledge, TPK II is the only known example of a proline-directed kinase that can impair the ability of tau to promote MT assembly and induce MT disassembly in vitro under defined conditions.

In the TPK II phosphorylated tau (Fig. 2), the relative importance of each of the six phosphorylated sites on tubulin polymerization cannot be determined. Therefore, in an attempt to understand the molecular basis for the loss of functional activity of tau upon phosphorylation, we prepared a number of tau mutants based on identification of TPK II phosphorylation sites in tau. Five of these mutants (T181A, T205A, T212A, T217A, and S396A) contain Ala for Thr or Ser, whereas the S404G mutant contained Gly for Ser. Although the S404A tau mutant was cloned, expressed, purified, and characterized, this unphosphorylated tau mutant (S404A) was dysfunctional in the tubulin polymerization assay for unknown reason(s). Therefore, we were unable to use it as a control to study the effect of TPK II phosphorylation on tubulin polymerization and decided to prepare and use S404G for our studies. These tau mutants were phosphorylated with TPK II, and results for T181A, T205A, T212A, and T217A are shown in Fig. 5A. A similar shift in molecular mass was observed when the S396A mutant was phosphorylated with TPK II (Fig. 5B). However, as shown in Fig. 5B, the extent of TPK II phosphorylation was relatively less in single and double mutants containing a mutation at Ser-404. We interpret these results to mean that TPK II phosphorylation of tau at Ser-404 is responsible for the observed higher molecular mass in mutants containing Ser-404. However, other alternative explanations such as the effect of the S404G or S404A mutation on other TPK II sites cannot be ruled out from these studies.

All six unphosphorylated tau mutants were competent to elicit tubulin polymerization (data not shown), and the results were comparable with wild type tau (Fig. 2). Therefore, we studied the effect of TPK II-phosphorylated tau mutants (T181A, T205A, T212A, T217A, S396A, and S404G) on tubulin polymerization. Specifically, we determined the effect of these mutations on nucleation time (lag phase) and extent of polymerization (A$_{340}$ maximum). These studies showed (Table I) that, like wild type tau (Fig. 2), TPK II phosphorylation of the T181A, T212A, and T217A tau mutants inhibited tau’s ability to promote tubulin polymerization as determined by absorbance at steady state. As shown in Table I for these three tau mutants, both the lag time and the extent of polymerization were severely impaired and these results were comparable to the TPK II-phosphorylated tau shown in Fig. 2. These results suggest that phosphorylation at the other TPK II sites (Thr-205, Ser-396, and Ser-404) might play an important role in inhibiting MT assembly. Indeed, the inhibition of tubulin polymerization was less with the TPK II-phosphorylated forms of T205A, S396A, and S404G mutants than with TPK II-phosphorylated WT tau (Table I). Of these three sites, based on mutational studies, phosphorylation at Ser-404 had a more dramatic effect on nucleation time (lag phase). Also, in this case the rate of polymerization after the onset of the nucleation event was significantly higher relative to the other five single tau mutants (data not shown). Taken together, the data suggest that TPK II phosphorylation of tau at Thr-205, Ser-396, and Ser-404 seems to be responsible for impairment of microtubule assembly.

The above studies led us to prepare double (T205A/S404A; S396A/S404A) and triple (S396A/S404A/T205A) tau mutants. Their effects on tubulin polymerization are included in Table I
TPK II Phosphorylated Tau and Its Effect on Microtubules

TABLE I

<table>
<thead>
<tr>
<th>WT tau and mutants</th>
<th>Effect on tubulin polymerization</th>
<th>Lag time (min)</th>
<th>$A_{405} \text{ max}^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-WT</td>
<td></td>
<td>13</td>
<td>0.093 ± 0.003</td>
</tr>
<tr>
<td>P-T181A</td>
<td></td>
<td>12</td>
<td>0.133 ± 0.003</td>
</tr>
<tr>
<td>P-T212A</td>
<td></td>
<td>12</td>
<td>0.116 ± 0.005</td>
</tr>
<tr>
<td>P-T217A</td>
<td></td>
<td>12</td>
<td>0.103 ± 0.001</td>
</tr>
<tr>
<td>P-T205A</td>
<td></td>
<td>10</td>
<td>0.266 ± 0.002</td>
</tr>
<tr>
<td>P-S396A</td>
<td></td>
<td>12</td>
<td>0.244 ± 0.058</td>
</tr>
<tr>
<td>P-S404G</td>
<td></td>
<td>8</td>
<td>0.269 ± 0.020</td>
</tr>
<tr>
<td>WT</td>
<td></td>
<td>3</td>
<td>0.500 ± 0.021</td>
</tr>
<tr>
<td>S396A/S404A</td>
<td></td>
<td>2</td>
<td>0.508 ± 0.038</td>
</tr>
<tr>
<td>P-S396A/S404A</td>
<td></td>
<td>4</td>
<td>0.373 ± 0.054</td>
</tr>
<tr>
<td>P-T205A/S404A</td>
<td></td>
<td>9</td>
<td>0.178 ± 0.010</td>
</tr>
<tr>
<td>P-T205A/S396A/S404A</td>
<td></td>
<td>5</td>
<td>0.351 ± 0.010</td>
</tr>
</tbody>
</table>

* Data represent the average of steady state (30 min) ± S.D. (n = 3).

for comparison with single mutants. Interestingly, the rate and the extent of tubulin polymerization were both increased using the TPK II-phosphorylated S396A/S404A double mutant relative to the TPK II-phosphorylated wild type tau (Fig. 6). As shown in Table I, relative to the phosphorylated forms of S396A/S404A double and S404G single mutants, an additional T205A mutation in tau does not significantly alter the lag time or the extent of tubulin polymerization in the corresponding TPK II-phosphorylated double (T205A/S404A) and triple (T205A/S396A/S404A) tau mutants. These results suggest that to a large extent phosphorylation at Ser-396 and Ser-404 is responsible for tau’s inability to promote tubulin polymerization. Table I shows that the extent of tubulin polymerization was increased with the TPK II-phosphorylated form of the S396A/S404A double mutant, whereas the lag time was decreased relative to either the TPK II-phosphorylated S396A or the phosphorylated S404G tau mutants. Taken together, we conclude that in vitro TPK II phosphorylation of tau at Ser-396 and Ser-404 is primarily responsible for the inability of TPK II-phosphorylated tau to promote microtubule assembly.

DISCUSSION

Bovine brain TPK II (37–39) and activation of cdk5 in the presence of various truncated recombinant forms of the activator protein (p35) from bovine, mice, and human have been reported (40–45). However, there is no report on the purification and characterization of human recombinant TPK II and mapping of the TPK II phosphorylation sites in human tau. Our mapping studies show that human TPK II phosphorylates six sites in human tau. These sites are Thr-181, Thr-205, Thr-212, Thr-217, Ser-396, and Ser-404. In contrast, a partially purified protein kinase from bovine brain was shown to phosphorylate proline-directed Ser-144, Thr-147, Ser-177, and Ser-315 in tau from bovine brain extracts (46). These phosphorylation sites in bovine tau correspond to Ser-202, Thr-205, Ser-235, and Ser-404 in human tau when the sequences of bovine and human tau were aligned (46). This crude kinase fraction from bovine brain was later termed TPK II (25). A similar proline-directed kinase isolated from bovine brain was reported to phosphorylate seven sites in bovine tau. These sites, when aligned with the human tau sequence, corresponded to Ser-195, Ser-202, Thr-205, Thr-231, Ser-235, Ser-396, and Ser-404 (39). Furthermore, it was shown (47) that bovine TPK II phosphorylates human tau at Thr-212 and Thr-231 only in the presence of heparin as an inducer of tau phosphorylation. From these studies (39, 46, 47) it is extremely difficult to directly compare phosphorylation sites and kinases involved, because the outcome of such results depends upon the enzyme source, purity of kinase, source and purity of tau, and presence of inducers like heparin (47). Therefore, it is not surprising to find that these previous studies on bovine and human tau phosphorylation sites (39, 46, 47) were mutually inconsistent. Notably, our mapping studies of human tau phosphorylation sites were performed with highly (>90%) purified (Fig. 1) and well-characterized human TPK II without inducers. Thus, prior to our studies, the definition of human TPK II-mediated phosphorylation sites in human tau and the key phosphorylated amino acids that affect microtubule assembly and disassembly were unknown.

Tau phosphorylation and MT disassembly by the proline-directed TPK II may be involved in the etiology of neurofibrillary tangle pathology in AD. Recent studies suggest that TPK II activity is elevated in the AD brain compared with age-matched controls (48). Moreover, the activator protein p35/p25 of TPK II is localized only in central nervous system neurons (40, 41) and has been shown to be accumulated in a truncated form (p25) in the AD brain (49). The accumulation of p25 has also been correlated to an increase in TPK II (cdk5/p25) activity in the AD brain (49), and an overexpression of the p25 activator subunit of TPK II in transgenic mice has been shown to result in hyperphosphorylated tau and cytoskeletal disruption (50). In this context, intracellular tau is undefined and is apt to display microheterogeneity in terms of phosphorylation. We have observed distinct differences in tubulin polymerization in vitro (Table I), depending upon the phosphorylation status of human tau. Therefore, cell and animal studies (27, 28, 49, 50) do not reveal which sites are phosphorylated by which kinase and which phosphorylated amino acids in tau contribute to cytoskeleton disruption. Biochemical studies (Figs. 2–6), using purified human TPK II (Fig. 1) and human tau, are unique and have focused on understanding the functional and molecular basis of initial events that trigger tau phosphorylation and microtubule breakdown.

Axonal transport mechanism relies on microtubules as tracks that are stabilized by tau (51). Our results show that human TPK II-phosphorylated tau is impaired in its ability to promote MT assembly (Figs. 2 and 3). All the proline-directed TPK II phosphorylation sites in tau identified from our mapping studies lie outside the MT binding repeat domains. This implies that tau’s ability to maintain microtubule tracks seems to depend on the lack of phosphorylation at critical sites outside of the microtubule-binding domain. Our in vitro results indeed show (Fig. 4) that TPK II phosphorylation of tau at critical amino acids detaches tau from microtubules, leading to the breakdown of microtubules.

There are 12 sites in human tau that contain a Ser/Thr-Pro motif (1), and these are canonical sites for proline-directed kinases. These sites are Thr-175, Thr-181, Ser-199, Ser-202, Thr-205, Thr-212, Thr-217, Thr-231, Ser-235, Ser-396, Ser-404, and Ser-422. Human TPK II phosphorylates six of these sites in human tau. Our in vitro tau mutational and functional studies (Figs. 5 and 6, Table I) are the first to demonstrate that of the six sites phosphorylated by TPK II, phosphorylated Ser-396 and Ser-404 are critical in impairing microtubule assembly. To our knowledge, these studies are the first to show that human TPK II, in contrast to TPK I, can initiate tau phosphorylation of MT-associated tau and dissociation of tau from microtubules (Fig. 4).

Studies (52) indicate that TPK I seems to prefer to phosphorylate Ser and Thr residues positioned N-terminal to another...
phosphoserine and the preferred consensus sequence is (S/T)XXXS-P. Based on the specificity of TPK I, a potential role of TPK II in generating TPK I sites could be envisioned. In earlier studies (53–55), it was predicted that prior phosphorylation of tau by another kinase may be required in the generation of phospho-Thr-231 by TPK I. Recently, it has been shown that phosphorylation of tau by TPK II is required to generate an AD-related TG-3 phosphoepitope by TPK I (55). To inhibit MT-promoting activity by TPK I phosphorylated tau, prior phosphorylation of tau by protein kinase A was required (56). These results are consistent with the notion that TPK I is a secondary kinase. Out of the two proline-directed kinases known to associate with microtubules (25, 26) and to phosphorylate tau in cells (27, 28), phosphorylation at two key amino acids (Ser-396 and Ser-404) by TPK II impairs microtubule assembly (Figs. 2–4, 6, Table I).

A number of phosphorylated epitopes in tau have been observed in tau-based diseases (21, 57, 58) known as tauopathies (Down’s syndrome, frontotemporal dementia and Parkinsonism, Pick’s disease, progressive supranuclear palsy, and corticobasal degeneration). The most commonly observed proline-directed phosphoproteins in tau are Ser-202, Thr-205, Thr-212, Ser-214, Thr-231, Ser-235, Ser-396, and Ser-404. Notably, Ser-396 and Ser-404 are a pair of phosphoepitopes found in hyperphosphorylated tau of AD-related TG-3 phosphoepitope by TPK I (55). To inhibit MT-promoting activity by TPK I phosphorylated tau, prior phosphorylation of tau by protein kinase A was required (56). These results are consistent with the notion that TPK I is a secondary kinase. Out of the two proline-directed kinases known to associate with microtubules (25, 26) and to phosphorylate tau in cells (27, 28), phosphorylation at two key amino acids (Ser-396 and Ser-404) by TPK II impairs microtubule assembly (Figs. 2–4, 6, Table I).

Fig. 6. Effect of TPK II phosphorylation of a double tau mutant (S396A/S404A) on microtubule assembly. Samples were phosphorylated with TPK II as shown in Fig. 5 and assayed for their ability to promote microtubule assembly as described under “Experimental Procedures.” The data represent mean ± S.D. (n = 3).

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Acknowledgments—We are thankful to the laboratories of Dr. R. L. Heirinkins and Dr. J. Slifght for their help in amino acid analysis and DNA sequencing, respectively. We also thank George Melchior and Irene Abraham for helpful discussions. Electron microscopy studies