Phosphorylation Blocks the Activity of Tubulin Polymerization-promoting Protein (TPPP)

IDENTIFICATION OF SITES TARGETED BY DIFFERENT KINASES*

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Tubulin polymerization-promoting protein (TPPP), an unfolded brain-specific protein interacts with the tubulin/microtubule system in vitro and in vivo, and is enriched in human pathological brain inclusions. Here we show that TPPP induces tubulin self-assembly into intact frequently bundled microtubules, and that the phosphorylation of specific sites distinctly affects the function of TPPP. In vitro phosphorylation of wild type and the truncated form (Δ3-43TPPP) of human recombinant TPPP was performed by kinases involved in brain-specific processes. A stoichiometry of 2.9 ± 0.3, 2.2 ± 0.3, and 0.9 ± 0.1 mol P/mol protein with ERK2, cyclin-dependent kinase 5 (CdK5), and cAMP-dependent protein kinase (PKA), respectively, was revealed for the full-length protein, and 0.4–0.5 mol P/mol protein was detected with all three kinases when the N-terminal tail was deleted. The phosphorylation sites Thr14, Ser18, Ser160 for Cdk5; Ser18, Ser160 for ERK2, and Ser32 for PKA, respectively, were identified by mass spectrometry. These sites were consistent with the bioinformatic predictions. The three N-terminal sites were also found to be phosphorylated in vivo in TPPP isolated from bovine brain. Affinity binding experiments provided evidence for the direct interaction between TPPP and ERK2. The phosphorylation of TPPP by ERK2 or CdK5, but not by PKA, perturbed the structural alterations induced by the interaction between TPPP and tubulin without affecting the binding affinity (Kd = 2.5–2.7 μM) or the stoichiometry (1 mol TPPP/mol tubulin) of the complex. The phosphorylation by ERK2 or CdK5 resulted in the loss of microtubule-assembling activity of TPPP. The combination of our in vitro and in vivo data suggests that ERK2 can regulate TPPP activity via the phosphorylation of Thr14 and/or Ser18 in its unfolded N-terminal tail.

Previously we isolated a new, flexible, intrinsically unstructured protein from bovine brain, which was denoted tubulin polymerization-promoting protein (TPPP)3/p25 accordingly to its in vitro function, tubulin polymerization-promoting protein, and its molecular mass (1). In the HGNC data base this protein is assigned as TPPP, the first member of TPPP family. We have shown that TPPP can induce formation of double-walled microtubules (MTs) and aberrant tubulin aggregates (2) as well as that it stabilizes MT network via its bundling activity in human cells (3). There are two TPPP homologues, p18 (TPPP2) and p20 (TPPP3) with distinct structural and functional features concerning their folding and tubulin binding properties (4). The physiological functions of the TPPP protein family are unknown; however, TPPP has been suggested to take part in the stabilization of the MT network (3).

Both α-synuclein and GAPDH directly bind to TPPP and co-localize in the Lewy body (5–7). Our immunohistochemistry studies revealed enrichment of TPPP in α-synuclein-positive inflammasomes characteristic for synucleinopathies but not for tauopathies (5). TPPP is similar to α-synuclein and tau (2, 8) as far as all of them belong to the family of the unstructured proteins.

Protein phosphorylation has a significant impact in the etiology of neurodegenerative processes (9). For example, tau and α-synuclein, the major hallmarks of Parkinson and Alzheimer diseases, respectively, are targets of different protein kinases. One of the kinases phosphorylating tau with pathological relevance in Alzheimer disease is cyclin-dependent kinase 5 (CdK5), which is abundant in brain tissue, associates with tau (10), and plays important role in the signaling of mitogen-activated protein kinases (MAP kinases) also called extracellular signal-regulated kinases (ERKs). MAP kinases and CdK5 phos-

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3 The abbreviations used are: TPPP, tubulin polymerization-promoting protein; MT, microtubule; TEM, transmission electron microscopy; ERK2, extracellular signal-regulated protein kinase 2; PKA, cAMP-dependent protein kinase; CdK5, cyclin-dependent kinase 5; MAP, microtubule-associated protein; PSD, post source decay; CID, collision-induced dissociation; DTT, dithiothreitol; MOPS, 4-morpholinepropanesulfonic acid; MES, 4-morpholineethanesulfonic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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phorylate numerous KSPXK consensus motifs in diverse cytoskeletal proteins and cross-talk in the regulation of neuronal functions (11). The cAMP-dependent protein kinase (PKA) interacts with the so-called A kinase-anchoring proteins via its regulatory subunit, that targets the kinase activity to specific subcellular locations (12) including MTs through a direct binding to MAP2 (13, 14).

Currently there is limited information on the phosphorylation of TPPP, although it has been considered as a phosphoprotein (15). It was partially co-purified with tau kinase II (also denoted Cdk5) from bovine brain extract; and two of the Ser/Thr-Pro sites in synthetic peptides, segments of TPPP, were phosphorylated by Cdk5 (15). Martin et al. (16) suggested that rat brain TPPP could be phosphorylated near to stoichiometrically in vitro by Cdk5 and by PKA but practically not by glycogen synthase kinase 3.

The aim of this work was to demonstrate that TPPP was able to promote the formation of intact-like MTs exhibiting a function characteristic of MAPs, and to establish the effect of phosphorylation on the unfolded “structure” of TPPP, on its interaction with tubulin as well as on its MT promoting and bundling activities. For these purposes we performed in vitro phosphorylation studies with human recombinant TPPP and with its truncated form, searched for the in vivo phosphorylation sites and interacting kinase partner(s), as well as studied the functional consequences of the specific phosphorylation events.

**EXPERIMENTAL PROCEDURES**

**Materials**—DTT, iodoacetamide, NH₄HCO₃ and 2,5-dihydroxybenzoic acid were obtained from Sigma, the sequencing and N-terminal glutathione S-transferase-tagged human p25 protein was obtained from Upstate Biochemicals. The two proteins were co-expressed in baculovirus-infected Sf21 insect cells. The catalytic subunit of PKA isolated from bovine heart was purchased from the Institute of Isotopes. Fine chemicals and buffer components were from Sigma.

**DNA Manipulations**—The coding region of human TPPP (5) was inserted into the XhoI and BamHI restriction sites of pET15b vector (Novagen) producing pET15b-TPPP. A PCR fragment for expression of Δ3-43TPPP, the deletion mutant lacking residues 3-43, was generated using pET15b-TPPP as template with the following primers: 5’-GATACTCGAGATGGCTGATCCTCCCTGAGCTCACGGCCTGAGGAG-3’ and 5’-CCGTGAGCCCTTACCTTGGCTGCTAGG-3’. The PCR fragment was inserted into the XhoI and BamHI restriction sites of pET-15b producing pET15b-Δ3-43TPPP. Correct ligations were verified by DNA sequencing.

**Protein Purification**—Recombinant human TPPP and p20 (TPPP3) were expressed in E. coli BL21 (DE3) cells and isolated as described previously (4, 5). Δ3-43TPPP was expressed in E. coli and was purified on HIS-Selected™ Cartridge (Sigma H8286) as the full-length TPPP.

**Protein Determination**—The protein concentration was measured by the Bradford method (18) using the Bio-Rad protein assay kit.

**Affinity Chromatography**—Human recombinant TPPP was immobilized to CNBr-activated Sepharose 4B (Amersham Biosciences) and used for finding interacting proteins from bovine brain extract as described previously (6). The bound proteins were eluted with 10 mM phosphate buffer, pH 7.0, containing 0.5 mM NaCl, and the protein bands obtained by SDS-PAGE (19) were analyzed by mass spectrometry.

**Protein Phosphorylation**—30 μg of recombinant human TPPP or its truncated form (Δ3-43TPPP) or p20 protein (TPPP3) were phosphorylated with PKA, ERK2, and Cdk5 in vitro. A typical reaction mixture contained 50 mM Tris-HCl, pH 7.5 buffer supplemented with 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 mM EGTA, 10 mM NaF, 0.05 mM sodium vanadate, 25 mM MgCl₂, 0.2 mM ATP, and 0.4 μg PKA or 0.2 μg of ERK2 in a final volume of 100 μl. In one set of experiments 30 μg of recombinant human TPPP was phosphorylated with 0.1 μg of ERK2 to reduce the degree of modification. Phosphorylation with Cdk5 was conducted in the presence of 12 mM MOPS, pH 7.0 buffer, 0.2 mM EDTA, 1 mM EGTA, 5 mM NaF, 0.2 mM sodium vanadate, 0.2 mM dithiothreitol, 27 mM MgCl₂, 0.2 mM ATP, and 0.05 μg of kinase. About 10⁻⁶ cpm of [γ-³²P]ATP was added to the mixture for radioactive labeling. The reactions were initiated by the addition of the kinase and were terminated with 50 mM EDTA. 5-μl samples were withdrawn at regular time intervals for quantitative analysis. Phosphate incorporation into the proteins was determined by the method of Witt and Roskoski (20) using Whatmann P-81 filter paper. The radioactivity was counted by Tschereukov radiation. Aliquots of the samples were separated by SDS-PAGE (19). Dried gels were analyzed by autoradiography using RX Fuji medical x-ray films. According to the densitometric scanning of the films with a Bio-Rad Multi-Analyzer apparatus more than 90% of the incorporated radioactivity was found in the monomeric and dimeric forms of the proteins. A prestained protein ladder (Fermentas) was used for the estimation of the molecular masses. Radioactive and cold phosphorylation reactions were run parallel under identical conditions. The degree of phosphorylation in the latter case was checked by the method of back phosphorylation (21). Only the non-radioactive samples were subjected to functional studies and MS.

**In-gel Digestion**—Digestion with side chain-protected porcine trypsin proceeded at 37 °C for 4 h.

**In-solution Digestion**—Protein was dissolved in 6 M guanidine-HCl, disulfide bridges were reduced with dithiothreitol and free sulfhydryls alkylated with iodoacetamide. Then sample was diluted with 25 mM NH₄HCO₃ and digested with side chain-protected porcine trypsin at 37 °C for 4 h.

**Methyl Esterification**—The protocol of Ficarro (22) was applied.
Phosphopeptide Enrichment by Immobilized Metal Ion Affinity Chromatography—In NTA magnetic agarose beads Ni\(^{2+}\) was displaced by Fe\(^{3+}\) as described by Thompson et al. (23) with final washing steps with a mixture of equal volumes of water, methanol, and acetonitrile. Methyl-esterified sample dissolved in water/methanol/acetonitrile was loaded onto the beads and vortexed for 15 min, then beads were washed three times with the loading solvent. Phosphopeptides were eluted with 0.42% H\(_2\)PO\(_4\) in 50% acetonitrile. An aliquot of the eluate was either directly analyzed by MALDI-TOF MS or diluted with 0.1% formic acid and loaded on the trapping column for LC-MS/MS.

Phosphopeptide Enrichment by TiO\(_2\)—A modified protocol of Larsen et al. (24) was applied. The tryptic digest was dried down, re-dissolved in 1% trifluoroacetic acid/50% acetonitrile and mixed with TiO\(_2\) suspended in the same solvent. After a few minutes vortexing the supernatant was discarded, and TiO\(_2\) was washed three to five times with the same solvent. Phosphopeptides were eluted with 1% NH\(_4\)OH.

**MALDI-TOF MS and PSD Analysis**—These analyses were performed on a Bruker Reflex III MALDI-TOF mass spectrometer in 2.5-dihydroxy-benzoic acid matrix. Three point external calibration was used with standard peptides. PSD analysis was performed in 10–12 steps, lowering the reflectron voltage by 25% at each step, and then stitching the data together. While the MS conditions permitted monoisotopic mass measurements, in PSD mode average masses were determined.

**LC-MS/MS**—Samples were analyzed on an Agilent 1100 nanoLC system on-line coupled to an XCT Plus ion trap mass spectrometer in information-dependent acquisition mode. For phosphopeptide analysis, occasionally a preferred mass list was included in data acquisition with m/z values calculated from MALDI-TOF data or derived from previous analyses. HPLC conditions were as follows: column: C18, 75 \(\mu\)m \(\times\) 150 mm; flow rate 300 nl/min; gradient: 5–45% B in 16 min, up to 90% B for 3 min, then equilibrated at 5% B for 15 min; solvent A was 0.1% formic acid in water, solvent B: 0.1% formic acid in acetonitrile; trapping: 10 \(\mu\)l/min flow rate, for 5 min in solvent A.

**Data Base Search**—MS and MS/MS data were searched against the Swissprot 51.3 (250,296 sequences) and the NCBI 20070120 (4462937 sequences) non-redundant protein databases using the Mascot search engine v2.1. Mass tolerance was set according to the type of instrument and acquisition mode. No species restriction was used. Data were also manually inspected.

**Bioinformatic Methods**—For prediction of phosphorylation and docking sites MotifScan (25) was used. Multiple sequence alignment of vertebrate TPPPs was done by ClustalW (26).

**CD Measurements**—CD spectra were acquired with a Jasco J-720 spectropolarimeter (Tokyo, Japan) in the 190–260 nm wavelength range employing 0.1-cm thermostatted cuvettes at 25 °C, using 10 mm phosphate buffer (pH 7.0) as described previously (4). During the titration of 1 \(\mu\)M tubulin the difference ellipticity at 207 nm was determined as a function of the concentration of TPPP. Difference ellipticities were calculated from the ellipticity measured at 207 nm in the mixtures of two proteins and in the samples of the individual proteins.

**Turbidity Measurements**—A total of 7 \(\mu\)M MAP-free tubulin was polymerized by 3 \(\mu\)M TPPP before and after phosphorylation at 37 °C in 50 mm MES buffer, pH 6.6, containing 1 mm dithiothreitol, 1 mm EDTA, 1 mm MgCl\(_2\), and 50 mm KCl. In the absence of TPPP no tubulin assembly occurs. Absorbance was monitored at 350 nm by a Cary 50 spectrophotometer (Varian, Australia).

**Transmission Electron Microscopy (TEM)**—The polymerized tubulin samples were centrifuged at 30,000 \(\times\) g and 30 °C for 20 min and the pellet fraction was used for TEM. The pellets were fixed for 1 h in a mixture of 2% glutaraldehyde, 0.2% freshly prepared tannic acid, and 0.1 M sodium cacodylate (pH 7.4). After washing in cacodylate, they were postfixed in 0.5% OsO\(_4\), and embedded in Durcupan (Fluka, Switzerland). Thin sections were contrasted with uranyl acetate and lead citrate and examined in a Jeol CX 100 electron microscope (1).

**RESULTS**

**In Vitro Phosphorylation of TPPP**—Recombinant human TPPP was phosphorylated by protein kinases (ERK2, Cdk5, and PKA) in the presence of \([\gamma-32P]ATP\) under conditions to reach maximal phosphorylation within 2 h (see “Experimental Procedures”). Fig. 1 shows typical SDS/PAGE protein staining and autoradiographic images. Two characteristic phosphoprotein bands of 26.5 and 63 kDa appeared in the pictures corresponding to monomeric and dimeric species. By the addition of an excess DTT to the samples all of the dimers were converted to monomers (data not shown). The radioactivity incorporated into TPPP was determined as described under “Experimental Procedures”; the stoichiometry of the phosphorylation was found to be 2.9 ± 0.3, 2.2 ± 0.3, and 0.9 ± 0.1 mol P/mol protein for ERK2, Cdk5, and PKA kinases, respectively (Fig. 1).

The phosphorylated protein bands were excised from the gels and digested with trypsin. After phosphopeptide enrichment, samples were analyzed by MALDI-TOF MS, MALDI-TOF PSD, and by LC-MS/MS on an ion trap mass spectrometer. Phosphopeptides were first identified by the 80 Da mass shift of the appropriate peptide fragments (Table 1). The sites of phosphorylation were assigned considering the 80 Da mass shift of the appropriate peptide fragments (Table 1).

In agreement with the minimal consensus of ERK2 and Cdk5, Ser, and Thr residues followed by Pro were targeted by these kinases. From the total of 4 such sites in TPPP, Ser\(^{160}\) and Ser\(^{160}\) were phosphorylated by ERK2, while Thr\(^{14}\), Ser\(^{18}\), and Ser\(^{160}\) were modified by Cdk5. PKA phosphorylated Ser\(^{35}\) within the single PKA consensus sequence KRLS in addition to Thr\(^{92}\) and Ser\(^{159}\) residues, which are in a less favorable sequence environment and may represent minor or unspecified sites.
we conclude that under in vitro conditions Thr^{14}, Ser^{18}, Ser^{32}, and Ser^{160} of human TPPP are the main phosphorylation sites of the MT-associated kinases.

Our structural prediction (8) suggested a quite extended region in the N terminus of TPPP (1–52 amino acids) with highly unfolded structure. As three of the main phosphorylation sites, Thr^{14}, Ser^{18}, and Ser^{32}, are localized in this tail region we expressed a truncated form of the human TPPP that lacks the first 43 amino acid residues (Δ3-43TPPP) and investigated the consequences of this deletion. Fig. 1 demonstrates that the truncation of the protein significantly reduced both the rate and degree of its phosphorylation. A 0.4–0.5 mol of P/mol protein stoichiometry was reached after 2 h of incubation with ERK2, Cdk5, and PKA. As a negative control, the phosphorylation of recombinant human p20 (TPPP3), a homologue of TPPP that lacks the N-terminal tail, and the phosphorylation site corresponding to Ser^{160} in TPPP was also tested under similar conditions; and as expected virtually no ^{32}P incorporation (0.02 mol of P/mol protein) was detected. These results suggest that ERK2 and Cdk5 phosphorylate the residues Thr^{14} and Ser^{18} in the N-terminal tail more vigorously than Ser^{160}; and PKA efficiently modifies Ser^{32} that is also localized in the N terminus. Our data are consistent with the predictions by MotifScan (25) for the probability of the phosphorylation sites by PKA, ERK2, or Cdk5 (see Table 3).

**In Vivo Phosphorylation of TPPP**—To test if the in vitro phosphorylation sites are also relevant under in vivo conditions we isolated TPPP from bovine brain as described previously (2), and identified the phosphorylated sites of TPPP by MS. Phosphopeptides detected by MS without or after TiO2 enrichment are summarized in Table 2. The PSD spectrum of the peptide at MH^+ = 2954.5 produced fragments at m/z 1165.0 (y_{10}), 1635.2 (y_{16}), 1764.0 (y_{18}), and 2077.2 (y_{24}) revealing phosphorylation at Ser^{160}. A series of CID spectra unambiguously identified phosphorylation at Thr^{12}, as listed in Table 2. Phosphorylation site assignments were based on preferential peptide backbone cleavages between ^7LysPro^8 and ^12ThrPro^13. Determination of the phosphorylation sites in the doubly phosphorylated Ac-(2–24) peptide was performed from the quadruply charged precursor at m/z 655.7 (Fig. 2). The fragment ion observed at m/z 665.9 can only be assigned as y_{12}^2+ with one phosphate in this fragment, i.e. phosphorylation at Ser^{16}. The fragment ion observed at m/z 641.6 corresponds to the doubly phosphorylated y_{17}^3+ thus confirming an additional phosphate at position Thr^{12}.

The human and bovine TPPP proteins exhibit >90% homology (1) and carry the same phosphorylation sites, however, because of the deletion of two amino acid residues at the N terminus of the bovine protein the numbering of the phosphorylated residues is shifted (Fig. 3). Therefore, the in vivo phosphorylation sites identified in the bovine protein are in agreement with those phosphorylated in vitro in human recombinant TPPP (Table 3). Ser^{159} in the bovine protein corresponding to Ser^{160} in the human ortholog was not found to be phosphorylated in vivo.

**TABLE 1**

Identification of phosphorylation sites in human recombinant TPPP phosphorylated in vitro by Cdk5, ERK2, and PKA

Phosphorylated residues are indicated as P, if MS/MS data enabled exact phosphorylation site determination. Residues are underlined where fragmentation produced y and/or b ions that contributed to phosphorylation site determination. Methyl esterified Asp, Glu, and C-terminal residues are marked with apostrophe.

| MH^+   | m/z  | Charge | Sequence                  | Position   | Enrichment | Site | Kinase |
|--------|------|--------|---------------------------|------------|------------|------|--------|
| 996.0  | 499.5| 2+     | ALpSPTVLR                 | 157–165    | TiO2       | Ser-160 | Cdk5, ERK2 |
| 1011.6 | 506.3| 2+     | AIPsPTVLR                 | 157–165    | Fe-NTA     | Ser-159 | PKA    |
| 1559.0 | 780.0| 2+     | NTpVLS'DV'EFSK'           | 90–102     | Fe-NTA     | Thr-92  | PKA    |
| 3081.7 | 1027.9| 3+    | RLpSLE'S.E.'GAE'GAAAPpPSK'DSR | 30–57   | Fe-NTA     | Ser-32  | PKA    |
| 1874.5 | 625.5| 3+     | ALpSptVLR                 | 10–26      | TiO2       | Ser-18  | Cdk5, ERK2 |
| 1462.3 | 488.1| 3+     | TPpKpSDPSK'DR            | 14–26      | TiO2       | Ser-18  | Cdk5, ERK2 |
| 1504.3 | 502.1| 3+     | TPpKpSDPSK'DR            | 14–26      | Fe-NTA     | Ser-18  | Cdk5, ERK2 |
| 1218.6 | 609.2| 3+     | TPpKpSDPSK'DR            | 14–24      | Fe-NTA     | Ser-18  | Cdk5, ERK2 |
| 1918.9 | 1915.9| 1+    | ALpSPTVLR                 | 10–26      | Fe-NTA     | Thr-14  | Cdk5    |
MAP Kinase Regulates MAP Functions of TPPP

Interaction of TPPP with ERK2—Next we investigated whether the post-translational modification of TPPP was assisted by a so-called docking mechanism, i.e. by a direct interaction between TPPP and the kinase(s). For this purpose affinity binding experiments were carried out by loading a crude bovine brain extract on a TPPP-Sepharose column. After washing the column, and then eluted by 0.5M NaCl. The two dominant bands in the gel correspond to tubulin and GAPDH as reported earlier (6). Between these two bands the marked band was excised and in-gel digested with trypsin. The digest was analyzed by LC-MS/MS on an ion trap mass spectrometer. In the digest ERK2 (NCBI acc. NP_786987) was identified from the precursor at m/z 655.7 (4+) confirmed the identity of the doubly phosphorylated Ac-ADSRPKPANKTPPKSPGEPAKDKAA peptide. Fragments assigned as y12 and y17 unambiguously identified Thr12 and Ser16 being phosphorylated.

Interaction of TPPP with Tubulin—For the evaluation of the role of phosphorylation on the characteristics of the TPPP-tubulin interaction, the ellipticity differences were determined from the CD spectra measured at varying concentrations of TPPP before and after its phosphorylation. Fig. 5A). The phosphorylation decreased the binding-induced structural changes (the difference of the ellipticity obtained with the mixture compared with the sum of the individual proteins). The phosphorylation of TPPP by ERK2, Cdk5, or PKA did not perturb the CD spectrum (data not shown) indicating that the phosphorylation did not elicit detectable changes in the secondary structure of the protein. We confirmed our previous data (2) that the addition of tubulin to TPPP altered the CD spectrum significantly, and demonstrated that the phosphorylation of TPPP by ERK2 with a stoichiometry of 2.9 P/mol protein modified the spectrum of the TPPP-tubulin complex (Fig. 5A). The phosphorylation decreased the binding-induced structural changes (the difference of the ellipticity obtained with the mixture compared with the sum of the individual proteins).

Effect of Phosphorylation on the Interaction of TPPP with Tubulin—As shown in Fig. 5A, the human recombinant TPPP displays a CD spectrum with a minimum at 205 nm that is characteristic for the unstructured proteins. We found that phosphorylation by ERK2, Cdk5, or PKA did not perturb the CD spectrum (data not shown) indicating that the phosphorylation did not elicit detectable changes in the secondary structure of the protein. The phosphorylation of TPPP by ERK2 with a stoichiometry of 2.9 P/mol protein modified the spectrum of the TPPP-tubulin complex (Fig. 5A). The phosphorylation decreased the binding-induced structural changes (the difference of the ellipticity obtained with the mixture compared with the sum of the individual proteins).

TABLE 2

**In vivo phosphorylated peptides identified by MALDI-TOF MS and/or LC-MS/MS in tryptic digests of TPPP isolated from bovine brain**

Phosphorylated residues are indicated as pX, if MS/MS data enabled exact phosphorylation site determination. Residues are underlined where fragmentation produced y and/or b ions that contributed to phosphorylation site determination. All of the peptides were enriched by TiO2 except for the last modified peptide that was detected by MALDI-TOF MS without enrichment.

| MH*       | Precursor m/z | Charge | Sequence                        | Position | No. of phosphate | Site(s) |
|-----------|---------------|--------|---------------------------------|----------|------------------|---------|
| 2295.2    | 575.0         | 4+     | Ac-ADSRPKPANKTPPKSPGEPAKDK     | Ac-2–22  | 1                | Thr-12  |
| 2410.5    | 603.6         | 4+     | Ac-ADSRPKPANKTPPKSPGEPAKDK     | Ac-2–23  | 1                | Thr-12  |
| 2410.5    | 603.6         | 4+     | Ac-ADSRPKPANKTPPKSPGEPAKDK     | Ac-2–23  | 1                | Thr-12  |
| 2618.5    | 655.7         | 4+     | Ac-ADSRPKPANKTPPKSPGEPAKDK     | Ac-2–24  | 1                | Thr-12, Ser-16 |
| 2680.6    | 671.0         | 4+     | Ac-ADSRPKPANKTPPKSPGEPAKDK     | Ac-2–26  | 2                | Thr-12 |
| 2760.6    | 691.3         | 4+     | Ac-ADSRPKPANKTPPKSPGEPAKDK     | Ac-2–26  | 2                | Thr-12 |
| 2808.7    | 602.7         | 5+     | Ac-ADSRPKPANKTPPKSPGEPAKDK     | Ac-2–27  | 1                | Ser-30  |
| 2954.9    | 2954.9        | 1+     | RLpSLEAREGQEGAAAAAGELSALEEAFRK  | [28–56]  |                  |         |

**FIGURE 2. Identification of phospho-Thr**12** and phospho-Ser**16** of TPPP isolated from bovine brain. CID of the precursor at m/z 655.7 (4+) confirmed the identity of the doubly phosphorylated Ac-ADSRPKPANKTPPKSPGEPAKDKAA (2–24) peptide. Fragments assigned as y12 and y17 unambiguously identified Thr12 and Ser16 being phosphorylated.
MAP Kinase Regulates MAP Functions of TPPP

Assembly of MTs Induced by TPPP and the Effect of Phosphorylation on This Process—Previously we reported that TPPP promotes tubulin polymerization into aberrant, sometimes double-walled, tubules as well as into large aggregates (1). Now we found the conditions at which TPPP was able to induce the formation of intact-like MTs. The addition of 3 μM TPPP to 7 μM MAP-free tubulin in the polymerization buffer at 37 °C induced tubulin polymerization as detected by turbidimetry (Fig. 5D). TEM analysis of the pellet fraction revealed the appearance of large amounts of MTs, the majority of which formed bundles consisting of dozens of MTs (Fig. 6A). The MTs were frequently decorated by rows of tiny projections and dense particles with a periodicity of about 16 nm. The particles appear to connect MTs in the bundle (Fig. 6B). These pictures provide the first experimental evidence that TPPP induces intact-like MTs. Another characteristic morphological feature of these samples, beside the single and bundled MTs, is the presence of thread-like oligomers. The threads are ~15 nm in diameter and are concentrated in densely packed knobs between the bundles of MTs (Fig. 6A).

The effect of phosphorylation on the ability of TPPP to promote tubulin polymerization was also analyzed by time-dependent turbidity measurements. In contrast to the control (no phosphorylation) the ERK2-mediated modification of TPPP (2.9 mol P/mol protein) completely blocked tubulin polymerization (Fig. 5D). The inhibition was so extensive that it prevented a TEM analysis, as it was impossible to collect polymerized species by centrifugation. The Cdk5-mediated phosphorylation diminished the assembly activity of TPPP as well, however, PKA was ineffective (data not shown).

We tested whether a partially phosphorylated TPPP maintained any MT assembling activity. In a specific set of experi-
ments the concentration of ERK2 added to TPPP was reduced which resulted in the incorporation of 1.3 mol P/mol protein. This partially phosphorylated TPPP exhibited low tubulin polymerization activity (Fig. 5D). TEM showed that, in sharp contrast to the control, the sample mainly consists of thread-like oligomers, which form a loosely arranged network instead of densely packed knobs (Fig. 6C). We conclude that the phosphorylation strongly impedes the ability of TPPP to promote the formation and bundling of intact MTs, as well as to mediate the formation of large tubulin/TPPP aggregates.

DISCUSSION

Previous data, based on the co-purification of TPPP with Cdk5 and on the in vitro phosphorylation of three selected peptides by Cdk5, suggested that TPPP could be a phosphoprotein (15). In this work we identified the specific sites targeted by protein kinases involved in brain signaling processes. Ser/Thr-Pro motifs were found to be phosphorylated by both ERK2 (Ser18 and Ser160) and Cdk5 (Thr14, Ser18, and Ser160). It should be noted, that from the stoichiometry of phosphate incorporation (see Fig. 1) as well as from the bioinformatic prediction, three phosphorylation sites can be inferred for ERK2 (see Table 3). Thus, it is likely that Thr14 is also phosphorylated by ERK2, although the modification of this residue was not found by MS. Cdk5 and ERK1/2 share common sites in the tau protein (29); therefore, the cross-talk between these two kinases may occur in the case of TPPP phosphorylation as well. In addition, we found that TPPP was phosphorylated by PKA at Ser32, Thr92, and Ser159. The fact that the truncated Δ3-43TPPP carrying Ser160 as potential phosphorylation site was modified only to a stoichiometry of 0.4 – 0.5 mol P/mole protein, suggests that the major sites of these kinases are localized in the N-terminal region. This idea was further supported by the in vivo phosphorylation data from our and other laboratories. We isolated TPPP from bovine brain, and identified the phosphorylated amino acid residues as Thr12, Ser16, and Ser30. Recent proteomic analysis of in vivo phosphorylated human and mouse synaptic proteins (30–32) identified the same sites although there is a 1 or 2 amino acids shift between the sequences (Fig. 3). These sites of TPPP obtained by prediction as well as identified in vitro and in vivo (see Table 3) are the major targets of the kinases in the brain.

The N-terminal tail of TPPP (1–52 amino acids) was predicted to be completely disordered (8). The amino acid sequence of disordered proteins and protein regions is considered to be much less conservative than that of the structured ones (33, 34). Indeed, in the polypeptide sequences of vertebrate TPPPs, we found 25% identity between mammals and fishes in the N-terminal segment as compared with 75% identity in the C-terminal part (data not shown). However, the putative phosphorylation sites in the more variable N-terminal region are maintained during evolution (Fig. 3) indicating a physiological role for the phosphorylation of these residues.

Interestingly, Ser159/Ser160 of bovine and human TPPP was not found to be phosphorylated in vivo. As demonstrated in Fig. 3B, the neighboring sequence (163–187 amino acids) has high homology with the tubulin binding domain of tau protein. It is possible that in the intracellular milieu these sites are masked by the association of tubulin/MT to TPPP.

All of these observations suggest the modulation of TPPP activity by phosphorylation. It is likely that in this process ERK2 plays a dominant role because of its direct binding to TPPP. In fact, by MotifScan we identified two regions of TPPP that resemble the ERK docking site called D domain (28). Although it is not known whether all ERK-interacting molecules have D domains, the existence of such a region together with our affinity binding experiment indicate that ERK2 is, indeed, a potential interacting partner and regulator of TPPP.
MAP Kinase Regulates MAP Functions of TPPP

In the present report we provided evidence for the inherent ability of TPPP to induce self-assembly of MAP-free tubulin, and for its extensive bundling activity (Fig. 6, A and B), which is a MAP-like function, similar to that of tau or MAP2 proteins in brain. The resistance of TPPP-bundled MTs against antimitotubular agents was recently reported in human cells (3) suggesting the potential physiological role of this protein in the stabilization of the microtubular network.

Our quantitative data showed that the dissociation constants and the stoichiometries for the complexes of tubulin and TPPP as well as of tubulin and phosphorylated TPPP (2–3 mol P/mol protein) did not differ significantly; however, the secondary structural changes induced by the complex formation were found to be distinct (Fig. 5, B and C). Therefore, it is likely that the phosphorylation resulted in some structural changes in the recognition domain(s) of TPPP. As a consequence of the perturbation of the binding interface by phosphorylation, a dramatic alteration in the tubulin polymerization promoting potency of TPPP was detected (Fig. 5D). In fact, we found that the phosphorylation of TPPP by ERK2 (or by Cdk5) but not by PKA, blocks (Fig. 5D) the MT assembling activity of TPPP. A plausible explanation of this finding is that the phosphorylated TPPP is unable to induce appropriate conformational changes required for the assembly of tubulin dimers into protofilaments and tubes. Our TEM analysis revealed that limited phosphorylation of TPPP by ERK2 (1.3 mol P/mol protein incorporated) exerted on one hand, significant inhibitory effect on the MT assembling activity of TPPP; on the other hand, it inhibited the formation of large amorphous protein aggregates (Fig. 6C). Because our recent immunofluorescent data on HeLa cell and immunohistochemistry results on human brain samples from patients suffering in synucleinopathies provided evidence for the co-enrichment of TPPP with tubulin in aggresome, Lewy body and other inclusions (3, 5, 8), one can hypothesize that within these structures the TPPP molecules are dominantly in unphosphorylated form, and suggest possible pathological relevance of the post-translational modification of TPPP.

FIGURE 5. Effect of phosphorylation on the interaction of tubulin and TPPP (A, B, C) and on the TPPP-promoted tubulin polymerization (D). A, CD spectra of TPPP (dash-dot line), the mixtures of tubulin and TPPP (solid line), of tubulin and ERK2-phosphorylated TPPP (dashed line). Final concentrations were 5 μM and 1 μM for TPPP and tubulin, respectively. The stoichiometry of phosphorylation was 2.9 mol P/mol protein. B, titration of 1 μM tubulin with non-phosphorylated (■, solid line) and phosphorylated (○, dashed line) TPPP. The difference ellipticities at 207 nm calculated from the ellipticities measured with the mixture of the two proteins and with the individual proteins were plotted as a function of the concentration of TPPP. The stoichiometry of phosphorylation was 2.9 mol P/mol protein. C, plots of the data of Fig. 5B according to Wu and Hammes (39). Kd values and the stoichiometries of the complexes obtained from the slope and the intercept on the ordinate, respectively, are 2.7 μM and 1.3, and 2.5 μM and 1.0, for the unphosphorylated and phosphorylated TPPP, respectively. D, tubulin polymerization induced by addition of TPPP before and after phosphorylation. Final concentrations were 7 μM tubulin and 3 μM TPPP (solid line) or 3 μM TPPP phosphorylated by ERK2 to the stoichiometry 2.9 mol P/mol protein (dashed line) or 1.3 mol P/mol protein (dotted line).

FIGURE 6. TEM images of the TPPP-induced tubulin assemblies before (A, B) and after phosphorylation (C) with ERK2 (1.3 mol P/mol protein). A and B, bundles of MTs sectioned at different angles and thread-like oligomers aggregated into dense knobs are shown. The MTs are frequently covered by tiny projections and periodically arranged dense particles (B), which form cross-links between MTs (see inset in panel B). C, sample prepared with phosphorylated TPPP contains loosely arranged oligomeric threads. Bars, 200 nm in A and C; 100 nm in B and 50 nm in the inset in B.
Cdk5 may have the same effect as ERK2 since it phosphorylates identical sites in TPPP. Because of the distinct specificity, PKA has a different substrate recognition preference: it predominantly phosphorylates Ser32 in the KRLS motif. The phosphorylation of TPPP at this site occurs in vivo and may have some important function but according to our in vitro results it has no significant effect on the tubulin/MT system. Therefore, we suggest that the functional consequences of phosphorylation of Thr14 and/or Ser18 residues but not Ser32 in the N-terminal region of TPPP play a crucial role in the regulation of the MT assembly and stabilization of the microtubular network. Because the phosphorylation pattern of a protein with multiple phosphorylation sites depends on the coordinated action of protein kinases and phosphatases (35), further investigations are needed to clarify the detailed regulatory mechanisms modulating the functions of TPPP. The significance of the phosphorylations in the phosphatases has been underlined by the example of tau protein that is dephosphorylated by several protein phosphatases (36, 37).

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