Brain-Specific p25 Protein Binds to Tubulin and Microtubules and Induces Aberrant Microtubule Assemblies at Substoichiometric Concentrations†

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ABSTRACT: Previously, we have demonstrated the presence of a protein factor [tubulin polymerization perturbing protein (TPPP)] in brain and neuroblastoma cell but not in muscle extract that uniquely influences the microtubule assembly. Here we describe a procedure for isolation of this protein from the cytosolic fraction of bovine brain and present evidence that this protein is a target of both tubulin and microtubules in vitro. The crucial step of the purification is the cationic exchange chromatography; the bound TPPP is eluted at high salt concentrations, indicating the basic character of the protein. By IDA-nanoLC−MS analysis of the peptides extracted from the gel-digested purified TPPP, we show the presence of a single protein in the purified fraction that corresponds to p25, a brain-specific protein the function of which has not been identified. Circular dichroism data have revealed that, on one hand, the α-helix content of p25 is very low (4%) with respect to the predicted values (30−43%), and its binding to tubulin induces remarkable alteration in the secondary structure of the protein(s). As shown by turbidimetry, pelleting experiments, and electron microscopy, p25 binds to paclitaxel-stabilized microtubules and bundles them. p25 induces formation of unusual (mainly double-walled) microtubules from tubulin in the absence of paclitaxel. The amount of aberrant tubules formed depends on the p25 concentration, and the process occurs at substoichiometric concentrations. Our in vitro data suggest that p25 could act as a unique MAP in vivo.

Microtubules (MTs),[1] polymers of tubulin heterodimers, are ubiquitous cellular structures involved in several essential functions of the cell (*1*) such as the extension and guidance of neurons at the growth cone (*2*), maintaining of the shape of the cells, transport and motility, and the formation of the mitotic spindle required for chromosomal segregation. The stability and spatial arrangement of the tubules are regulated by microtubule-associated proteins (MAPs)*;* however, a great number of other endogenous proteins are known to interact transiently with MTs (*3*, *4*). These dynamic assemblies can influence both the assembly and ultrastructure of MT and the function and activity of the associated protein (*3*, *5*).

Tubulin, however, is able to form not only MTs but also numerous polymorphic assemblies (*6*−*9* and references therein), including macrotubules, rings, sheets, and double-walled MTs, which can be artificially induced by different treatments, but occasionally, they can also be formed under physiological conditions. Substances with strong basic domains such as polybasic drugs, polycation DEAE-dextran, certain antibiotics, alcian blue, protamine, mellitin, spermidine, and cadaverine are well-known inducers of polymorphic MT assemblies (*9*−*12*). Some cationic proteins such as RNAse, histones, and encephalitogenic protein have also been shown to promote the polymerization of tubulin in vitro and to induce the formation of polymorphic assemblies (*7*, *8*, *13*). There are examples in which cationic compounds display MAP-like properties, stimulate assembly of MAP-free tubulin, and stabilize MTs (*10*, *11*, *14*, *15*). However, MAPs, which react with tubulin also by the basic domain, do not induce double-walled MTs even at high concentrations (*16*).

The p25 protein (NCBI accession number 2498194), originally identified as a major protein in a partially purified fraction of tau protein kinases (*17*), has been cloned (*18*), and was not significantly homologous to previously reported proteins. Recently, a novel human gene, mapped onto the p15.3 region of chromosome 5, was found which is significantly homologous (90%) with bovine p25 (*19*). The target(s) and function as well as physiological role of this protein are still unknown.

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[1] Abbreviations: MT, microtubule; MAP, microtubule-associated protein; TPPP, tubulin polymerization perturbing protein; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; IDA-LC−MS, information-dependent acquisition liquid chromatography−mass spectrometry; CID, collision-induced dissociation; CD, circular dichroism.

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Table 1: Purification of p25 from Bovine Brain

step	volume (mL)	protein concentration (g/L)	total protein (mg)	activating effect on paclitaxel-induced polymerization/mg
extraction	800	24	20000	1.1
pH 4.0	800	16	13000	1.5
heat treatment (90 °C for 10 min)	800	6	4800	1.5−1.8
DEAE anion exchange chromatography	100	5	500	2.4−3.6
P11 cation exchange chromatography	350	0.05	17.5	30−100
HPLC cation exchange chromatography	40	0.02−0.04	0.8−1.6	no paclitaxel

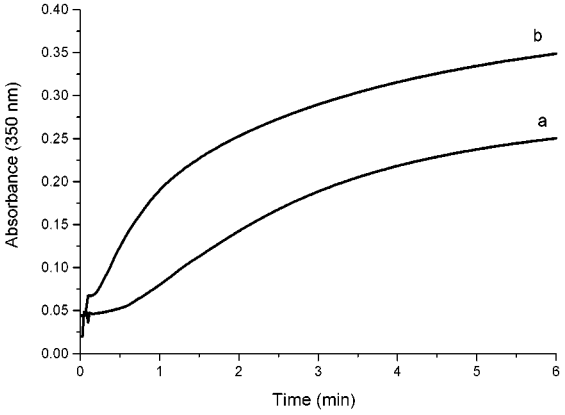

FIGURE 1: Effects of brain extracts on tubulin polymerization as determined via a turbidimetric assay. Paclitaxel-assisted tubulin polymerization in the absence (a) and presence (b) of 1 mg/mL microtubular protein-free brain extract. The experimental conditions are given in Experimental Procedures.

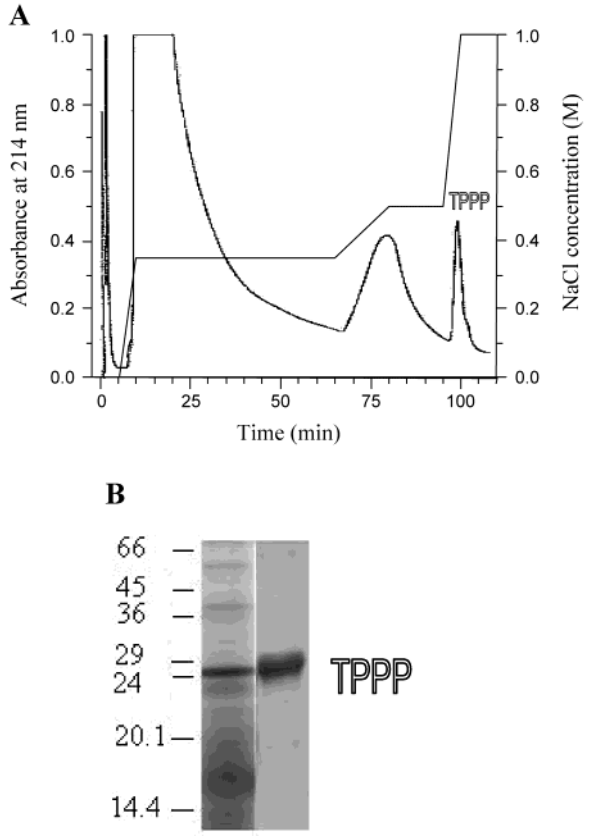

FIGURE 2: Isolation of TPPP from TPPP-enriched bovine brain extract. (A) HPLC of a 15 mg/mL protein solution using the Fractogel EMD SO_3^--650(S) column. The elution was derived with a nonlinear salt gradient. (B) SDS−PAGE picture of the TPPP fraction loaded onto (lane 1) and eluted from (lane 2) the column at a KCl concentration of 1 M. Numbers on the left refer to positions of PAGE protein molecular mass markers (in kilodaltons).

Previously, we have demonstrated that brain extract increases the tubulin polymerization assayed by turbidimetry, and the effect depends on the origin of the cell-free extract. Compared to the brain extract, an ~10-fold smaller amount of neuroblastoma cell extract produced a similar promoting effect, while the muscle extract was inefficient (*20*, *21*). Here we describe purification of a protein factor [tubulin polymerization perturbing protein (TPPP)] from bovine brain extract and present evidence that TPPP is identical with the p25 protein. This brain-specific protein binds to both tubulin and MT. This interaction causes significant secondary structural alterations of p25 and perturbs the physiological tubulin assemblies and microtubular ultrastructures.

EXPERIMENTAL PROCEDURES

GTP, phenylmethanesulfonyl fluoride, and paclitaxel were purchased from Sigma-Aldrich (Dorset, U.K.), and a protease inhibitor cocktail was from ICN (Irvine, CA). All other chemicals were reagent-grade commercial preparations.

The concentrations of tubulin and p25 were determined spectrophotometrically, using a molar absorption coefficient of 1.03×10^5 (*22*) and 0.42×10^5 M^{-1} cm^{-1} at 280 nm, respectively. The concentrations of the purified proteins, tubulin, and p25 are given in micromolar based upon the molecular mass of the polypeptide chains (50 kDa) for tubulin and 25 kDa for p25. The concentration of MT is calculated from the tubulin concentration. The concentrations of other proteins were determined by a Bradford assay (*23*).

Preparation of Tubulin from Bovine Brain. Tubulin was prepared according to the method of Na and Timasheff (*22*). Purified tubulin showed no MAP bands when run on overloaded sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). It was stored in 1 M sucrose, 10 mM sodium phosphate buffer, 0.5 mM $MgCl_2$, and 0.1 mM GTP (pH 7.0) at −80 °C and dialyzed against standard buffer [50 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.8) containing 100 mM KCl, 2 mM dithioerythritol, 1 mM ethylene glycol bis(*β*-aminoethyl ether), and 1 mM $MgCl_2$] before use.

High-Performance Liquid Chromatography (HPLC) Experiments. HPLC was carried out on a Fractogel EMD SO_3^--650(S) column (Merck, Darmstadt, Germany) equilibrated with phosphate buffer [10 mM P_i (pH 5.0) containing 0.5 mM $MgCl_2$] using a Waters (Milford, MA) gradient HPLC system at a flow rate of 2 mL/min, and the effluent was monitored at 214 nm.

Information-Dependent Acquisition Liquid Chromatography−Mass Spectrometry (IDA-LC−MS). IDA-LC−MS experiments were carried out on a Pulsar quadruple orthogonal

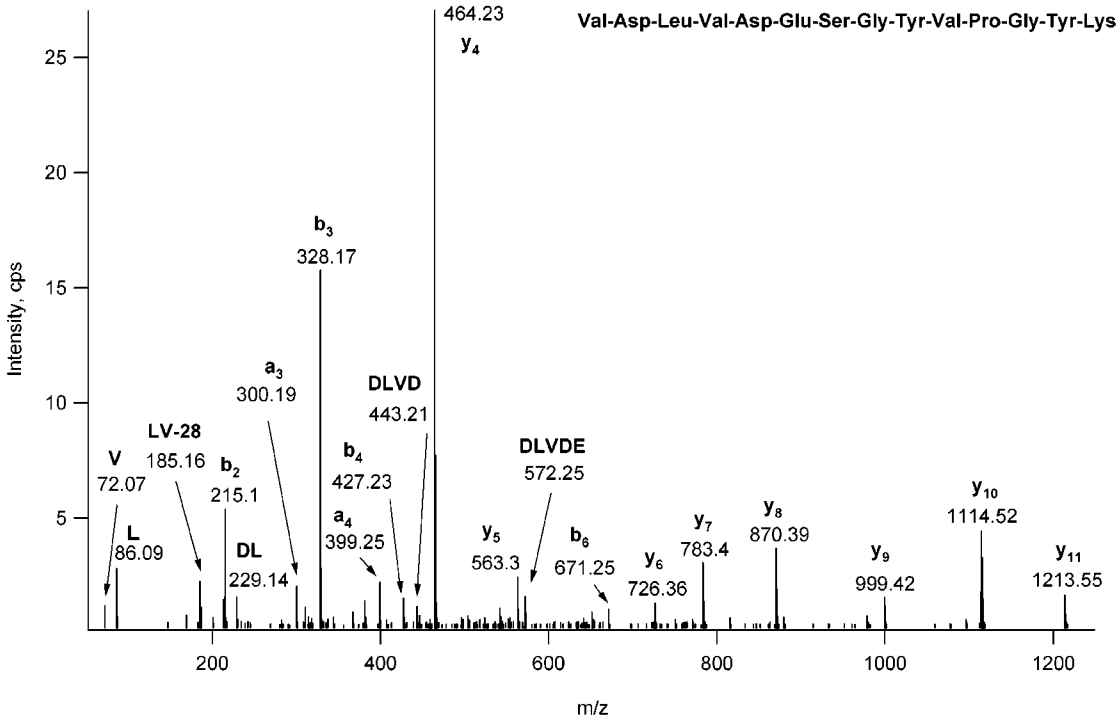

FIGURE 3: Low-energy CID spectrum of one of the tryptic peptides. Precursor ion at *m*/*z* 770.88 (2+), acquired in the IDA-LC−MS experiment. Mascot database search with these MSD/MS data identified its source unambiguously as the bovine 25 kDa brain-specific protein.

acceleration time-of-flight tandem mass spectrometer (MDS Sciex, Toronto, ON). The MS data were first submitted to an automated database search by Mascot (Matrix Science), and then the results were confirmed by careful inspection of the collision-induced dissociation (CID) spectra as described previously (*24*).

The protein was in-gel digested. The resulting peptides were extracted, and the digest was concentrated to ~10 μL. One microliter of the digest was then subjected to IDA-LC−MS analysis. The HPLC was carried out using an Ultimate-nanoHPLC system equipped with a Famous autosampler (both from LC-Packings, San Francisco, CA), using a PepMap 75 μm inside diameter column. Solvent A was 0.1% formic acid in water; solvent B was 0.1% formic acid in acetonitrile. The column was equilibrated at 5% solvent B, and a gradient elution was carried out at a flow rate of ~250 nL/min. The eluent was analyzed with a Pulsar quadruple orthogonal acceleration time-of-flight hybrid tandem mass spectrometer (MDS Sciex). Throughout the LC−MS analysis, 2 s MS acquisitions were followed by 5 s low-energy CID analyses. The precursor ion for these MS/MS experiments was selected by the computer based on the 2 s survey acquisition results. Singly charged ions, trypsin autolysis products, were excluded from the selection process. Doubly charged ions were preferred over multiply charged precursors. The collision energy was adjusted according to the charge state and the selected *m*/*z* value of the precursor ion. The MS/MS data were first submitted to automated database searches by Mascot (over the web to Matrix Science), and then the results were confirmed by careful examination of the spectra. CID spectra not interpreted this way were analyzed manually.

Circular Dichroism (CD) Measurements. CD spectra were acquired with a Jasco J-720 spectropolarimeter (Tokyo, Japan) in the 190−280 nm wavelength range employing 0.1 cm thermostated cuvettes at 25 °C, in 10 mM phosphate buffer (pH 7.0). Protein concentrations were varied between 4 and 10 μM and between 4 and 14 μM for tubulin and p25, respectively. In the control experiment, bovine serum albumin was used instead of p25. Spectra were corrected by subtracting the spectrum of the buffer from that of the sample. Scanning was repeated three times, and the spectra were averaged. Mean molar ellipticity per residue (MRE) in degrees square centimeter per decimole was calculated according to the following equation:

$$\mathrm{MRE} = \Theta/(10ncl)$$

where Θ is the measured ellipticity in millidegrees, n is the number of amino acid residues, c is the concentration in moles, and l is the path length of the cell in centimeters. The α-helical content was calculated from the MRE value at 222 nm using the following equation (*25*):

$$\%\ \alpha\text{-helix} = (|\mathrm{MRE}_{222}| - 2340)/303$$

To look for interaction of proteins, difference spectra were calculated by using the built-in JASCO software; the sum of the spectra of the proteins measured separately was subtracted from the measured spectra of the protein mixture.

Turbidity Measurements. The assembly of 20 μM tubulin was assessed in standard buffer at 37 °C with or without addition of 20 μM paclitaxel in the absence and presence of p25. The optical density was monitored at 350 nm by a Cary 100 spectrophotometer (Varian, Walnut Creek, Australia). At the final state of polymerization, the samples were prepared for electron microscopic analysis.

Pelleting Experiments. Tubulin at 100 μM (5 mg/mL) was preassembled in standard buffer containing 20 μM paclitaxel at 37 °C and centrifuged at 30000*g*, and the pellet fraction was used as the MT fraction. MTs at a final concentration

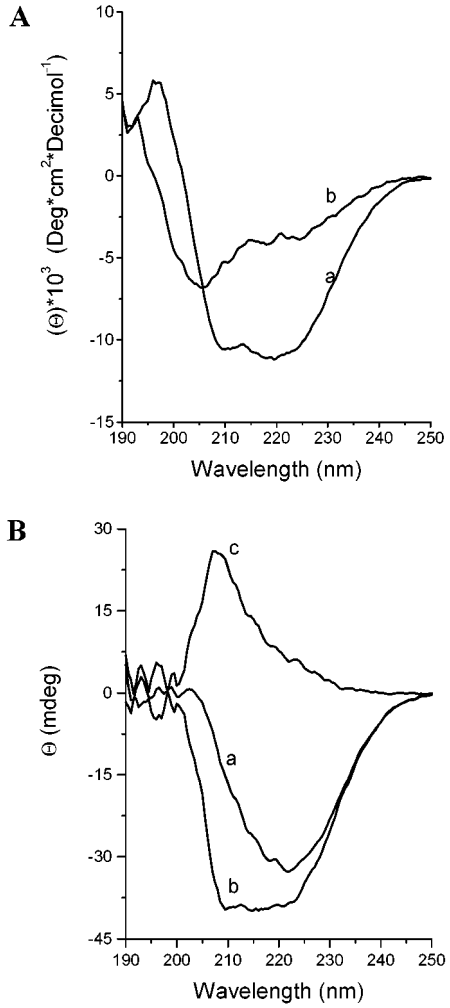

FIGURE 4: Far-UV circular dichroism spectra of tubulin and p25 alone (A) and together (B). (A) Normalized spectra are the average of three spectra measured at 4−10 μM tubulin (a) and 4−14 μM p25 (b). (B) The measured (a) and calculated (b) spectra of 5 μM tubulin and 14 μM p25 after preincubation for 5 min, and their difference spectrum (c).

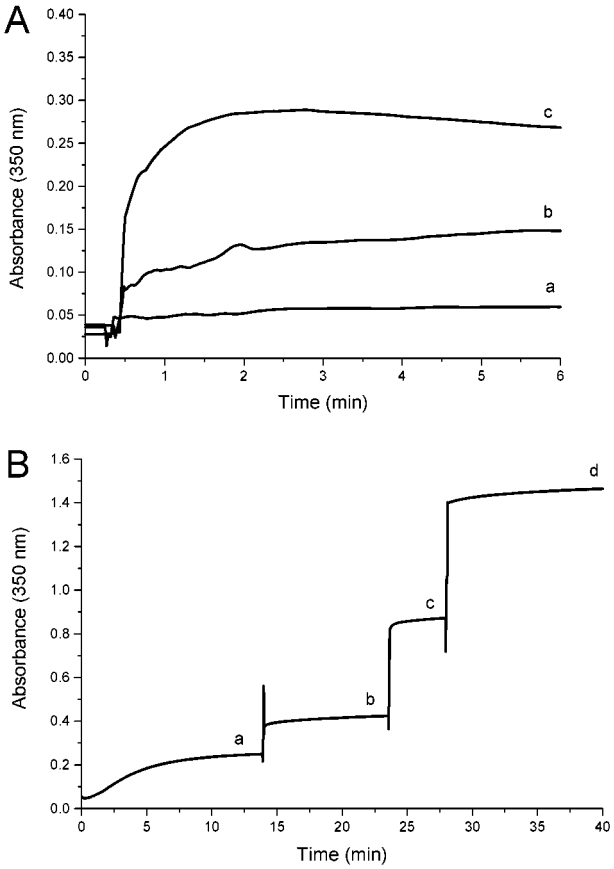

FIGURE 5: Effects of p25 on tubulin oligomerization (A) and the ultrastructure of MTs (B), as determined via a turbidimetric assay. (A) Tubulin (20 μM, 1 mg/mL) oligomerization is induced by 0.4 (a), 0.8 (b), and 1.6 μM p25 (c). (B) Tubulin (20 μM, 1 mg/mL) polymerization in the presence of paclitaxel and p25; 1.6 μM p25 was added subsequently: 0 (a), 1.6 (b), 3.2 (c), and 4.8 μM (d).

of 20 μM were mixed with 4 or 40 μM p25. The samples were preincubated for 20 min at 37 °C and centrifuged at 5000*g* for 20 min at 37 °C as described previously (*26*). The pellet and the supernatant fractions were separated, and the pellets were resuspended in standard buffer. The pellet and the supernatant fractions were analyzed via 12% SDS−PAGE and were identified by staining with Coomassie Brilliant Blue R-250.

Preparation of Samples for Electron Microscopic Studies. Samples were obtained under two different conditions. (a) Tubulin was assembled in the presence of p25 without paclitaxel and centrifuged at 100000*g* and 30 °C for 20 min. (b) MTs assembled in the presence of paclitaxel as described above were treated with p25 and centrifuged at 100000*g* and 30 °C for 20 min.

Electron Microscopy. MT-containing samples were fixed in a mixture of 2% glutaraldehyde, 0.2% tannic acid, and 0.1 M sodium cacodylate (pH 7.4) for 1 h, postfixed in 0.5% OsO_4, and embedded in Durcupan (Fluka, Buchs, Switzerland). Thin sections were contrasted with uranyl acetate and lead citrate and examined in a JEOL CX 100 electron microscope. For negative staining, a drop from the unpelleted samples was applied to Formvar/carbon-coated glow-discharged copper grids for 30 s. The solution was then removed and the grid stained with one drop of freshly filtered 1% aqueous uranyl acetate for 30 s. The excess stain was removed by blotting with filter paper.

RESULTS

Isolation of TPPP from Bovine Brain. Recently, we demonstrated (*20*, *21*) that the paclitaxel-assisted tubulin polymerization visualized by turbidimetry is apparently promoted by the microtubular protein-free cytosolic extract of bovine brain tissue (Figure 1). These observations indicated that a protein factor denoted as tubulin polymerization perturbing protein (TPPP) is present in brain extracts, besides the well-known MAPs, and it affects tubulin assembly.

To identify the TPPP, the protein was isolated from bovine brain according to the procedure summarized in Table 1. The only way to assay the presence of the unknown protein during the purification procedure was to measure its perturbing effect on tubulin polymerization visualized by the standard turbidimetric methods (cf. Experimental Procedures).

Bovine brain (600 g) was homogenized in 0.8 L of acetate buffer [50 mM sodium acetate (pH 5.5) containing 5 mM ethylenediaminetetraacetic acid, and 1 mM phenylmethanesulfonyl fluoride]. The pH of the supernatant fraction was adjusted to 4.0, and after incubation for 1 h at 4 °C, the

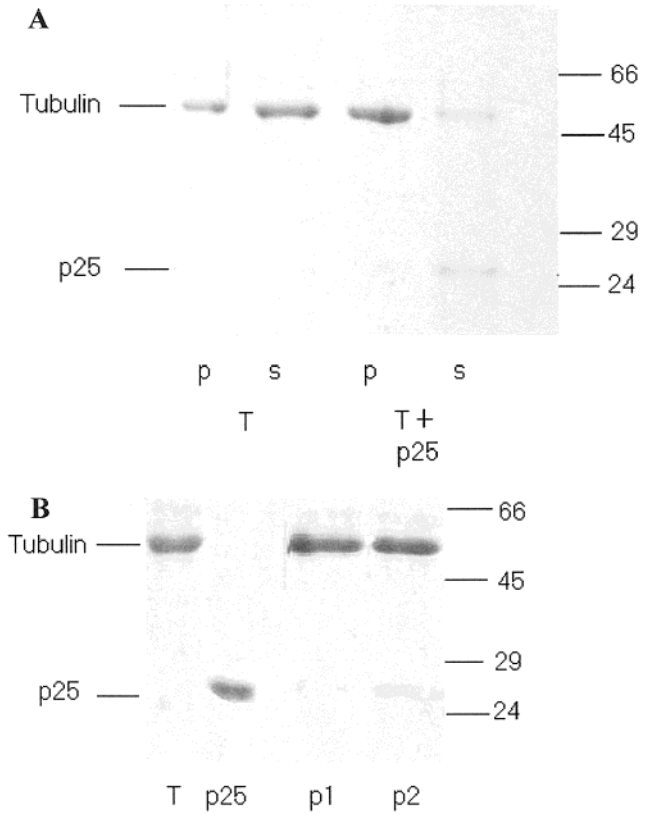

FIGURE 6: SDS−PAGE images of pellets and supernatants of paclitaxel-stabilized MT in the absence and presence of p25. Conditions for MT assembly induced by paclitaxel and for sedimentations at 5000*g* are described in Experimental Procedures. (A) Pellets of 20 μM preassembled MTs were resuspended in the original volumes of the samples, and 5 μL of the pellet and supernatant solutions were applied to a 12% SDS−PAGE gel. Lanes 1 and 3 contained pellets without and with 4 μM p25, respectively, and lanes 2 and 4 contained supernatants without and with 4 μM p25, respectively. (B) Pellets of 20 μM assembled tubulin were resuspended in one-quarter of the original volumes of the samples, and 5 μL of pellets was applied to a 12% SDS−PAGE gel. Lanes 1 and 2 were controls, with tubulin alone and p25 alone, respectively, and lanes 3 and 4 contained pellets of MT samples incubated with 4 and 40 μM p25, respectively.

precipitate was removed by centrifugation (30000*g* and 4 °C for 30 min). The supernatant following neutralization (pH 7.3) was heat-treated at 90 °C for 10 min, and after cooling in ice, the turbid solution was centrifuged (30000*g* and 4 °C for 30 min). The TPPP-enriched supernatant was concentrated with a YM 10 Diaflo membrane (Amicon, Danvers, MA), and then anion exchange (DEAE Sephadex) and cation exchange (P11) chromatographies were carried out in batch systems at pH 7.0 and 6.0, respectively, in the presence of a protease inhibitor cocktail (ICN Products). During anion exchange chromatography, the TPPP was in the unbound fraction; however, on the cation exchanger column, TPPP was retarded on the resin at a salt concentration of up to 0.25 M, and eluted with phosphate buffer [10 mM P_i (pH 6.0) containing 0.5 mM $MgCl_2$] containing 1 M NaCl. The eluted protein fraction was concentrated and extensively dialyzed against the same buffer, and then centrifuged (100000*g* and 4 °C for 20 min). The supernatant protein solution (~20 mg/mL) enriched with 25 kDa protein (cf. Figure 2) was applied to the HPLC system using the Fractogel cation exchanger column, and the proteins were separated by using a salt gradient; TPPP was eluted between 0.5 and 1.0 M NaCl as indicated in Figure 2. The TPPP-containing fractions were dialyzed, concentrated to 1−2 mg/mL, and stored at −80 °C.

Identification of TPPP by LC−MS. The purity of the TPPP fraction was tested via SDS−PAGE. As shown in the inset of Figure 2, the TPPP fraction contains a single band at 25 kDa. The band was cut from the gel and in-gel digested with trypsin (www.prospector.ucsf.edu), and the extracted peptides were subjected to IDA-nanoLC−MS/MS analysis. Only a single protein was identified from the band at 25 kDa: p25, a 25 kDa brain-specific protein (NCBI accession number 2498194). The following peptides were detected in elution order: [131−137], [179−184]], [154−164], [81−88] with acrylamide-modified Cys, [147−155], [117−124], [109−116], [192−205], [89−101], and [29−56]; the CID experiments confirmed a significant portion (~53%) of the sequence. Figure 3 shows the CID of the peptide ^{192}-VDLVDESGYVPGYK^{205}, as an illustration of high-quality data. This peptide was also observed with a methylated Glu197 (measured mass in 10 ppm, site of modification assigned by CID) (data not shown). The significance of this finding has not been determined.

α-*Helix Content of p25 and Its Interaction with Tubulin.* The circular dichroism spectrum of p25 was measured in the far-UV range to obtain information about the secondary structure of the protein. Spectra were measured in a concentration range of 4−14 μM, and the normalized (MRE) spectra are shown in Figure 4A. The spectrum of p25 with a minimum at 205 nm is not typical for globular proteins. The α-helix content is 4% in contrast to the predicted data calculated using the methods described by Garnier (*27*) (43.1%), Maxfield (*28*) (37.6%), Quian (*29*) (30.3%), and PHD (*30*) (29.8%). The normalized spectrum of tubulin is also presented in Figure 4A; the α-helix content calculated from the spectrum corresponds to the literature value (33%) (*31*).

To obtain direct evidence for the binding of p25 to tubulin, the circular dichroism spectrum of the mixture of the two proteins was measured. As shown in Figure 4B, the measured spectrum is significantly different from the calculated one, both of which are the sum of spectra of tubulin and p25 measured separately. A control experiment carried out under similar conditions except with bovine serum albumin instead of p25 showed that the sum of the spectra of tubulin and albumin was the same as the spectrum of the mixture (data not shown). Therefore, the appearance of the positive difference spectrum with tubulin with p25 (curve c) suggests the direct interaction of p25 with tubulin, which results in considerable alteration in the secondary structure of the protein(s) due to their mutual interactions.

Effect of p25 (TPPP) on Tubulin Assemblies: Turbidimetric Measurements. We noticed that the brain extract enhanced the turbidity of the tubulin solution, and the enhancement (the rate of turbidity increase) was more and more effective as the purification proceeded. After the HPLC purification step, the polymerization of tubulin was promoted without paclitaxel. Figure 5A shows that the addition of p25 to 20 μM tubulin causes a concentration-dependent turbidity increase even in the absence of paclitaxel. The shape of the polymerization curve is different from that induced by addition of paclitaxel; there is no nucleation phase, and both

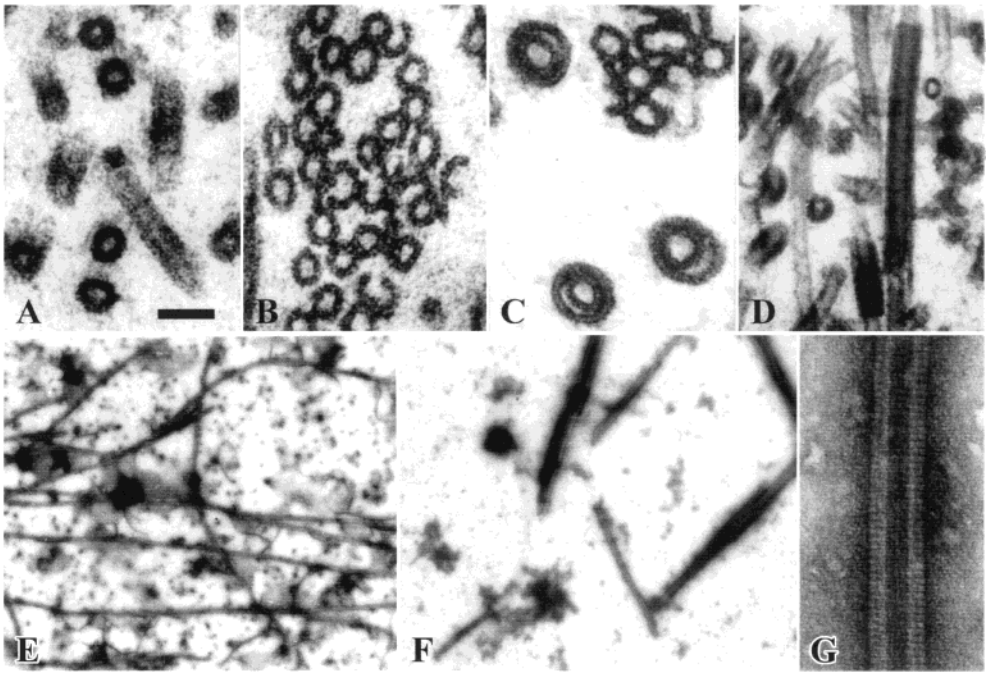

FIGURE 7: Electron microscopic analysis of the effect of p25 on tubulin polymerization. Samples were prepared with (A, B, and E) and without (C, D, F, and G) addition of paclitaxel in the absence (A and E) and presence (B−D, F, and G) of p25. (A−D) Sections from resin-embedded pellets. (E−G) Negatively stained whole mounts. (A and E) Loosely arranged, long straight, and curved MTs formed in the absence of p25. (B) p25 induces the formation of large MT bundles. When added to tubulin, p25 induces the formation of diverse assembly products: straight, short MTs and MT bundles (F), C-shaped ribbons and double-walled MTs (cross section in panel C and longitudinal view in panel D). Rows of tightly packed particles are seen in their outer wall (G). The bar is 50 nm long in panels A−C, 120 nm long in panel D, 700 nm long in panels E and F, and 44 nm long in panel G.

the rate and the final state of turbidity increase show concentration dependence. The effect occurs at a substoichiometric concentration of p25. It is important to note that no spontaneous polymerization of tubulin occurs without p25 under similar conditions. Figure 5B shows that p25 is also able to enhance in a dose-dependent manner the turbidity of samples containing preassembled paclitaxel-stabilized MTs. These findings indicate that p25 can induce, on one hand, tubulin polymerization and, on the other hand, alterations in the ultrastructure of MT assemblies.

Effect of p25 on MT Organization and Its Binding to MTs. To elucidate whether p25 is able to bundle paclitaxel-stabilized MTs forming larger particles, we have carried out sedimentation experiments using a relatively low centrifugation speed (5000*g*). A similar experimental setup had been successfully used for demonstration of cross-linked MTs by phosphofructokinase (*26*) with the following rationale. If the presence of kinase or p25 induces bundle formation, then with a relatively low-speed centrifugation the pelleted amount of the MTs bundled by the proteins will be enhanced. Therefore, low-speed centrifugation may allow discrimination between a single MT and p25-treated (bundled) MTs. As shown in Figure 6A, the MT pelleted almost completely if the sample contained a substoichiometric amount (4 μM) of p25; however, the MT in the control sample was mostly in the supernatant. These data suggest that p25 binding to MTs alters the microtubular ultrastructure by inducing bundle formation.

Similar pelleting experiments rendered it possible to visualize the binding of p25 to MT in the pellet fraction if excess p25 (40 μM) was added to the paclitaxel-stabilized MTs and more protein was applied for SDS−PAGE analysis. Figure 6B illustrates the electrophoretic images of samples: tubulin alone, p25 alone, and pellets of the mixtures containing 20 μM tubulin and 4 or 40 μM p25. Control experiments demonstrated that no p25 pelleted under these conditions (data not shown). Therefore, the appearance of p25, especially in the pellet sample containing more p25, can be taken as direct evidence for the binding of p25 to MTs.

Electron Microscopy of p25-Induced Tubular Assemblies. To visualize the morphological consequences of the interaction between p25 and MTs observed in turbidimetry assays (cf. Figure 5), electron microscopic studies were carried out on sections from resin-embedded pellets and on negatively stained whole-mount preparations. In one set of experiments, paclitaxel-stabilized MTs were prepared, then treated with 4 μM p25, and sedimented at 100000*g*. The control sample did not contain p25. The electron microscopic images of the pelleted MTs are shown in Figure 7. The control sample contains loosely arranged single MTs ~25 nm in diameter (Figure 7A) as described in our previous papers (*32*). On negatively stained samples, they appear as long, smooth-surfaced, straight or slightly curved tubules (Figure 7E). Occasionally, other structures (hooks and C-shaped ribbons) are also seen. The p25-containing sample is crowded with bundles of MTs consisting of dozens of wall-to-wall contacted tubules associated also with C-shaped ribbons probably representing incomplete MT walls (Figure 7B). This bundling can explain the increase in turbidity observed when p25 is added to the paclitaxel-stabilized MTs (see Figure 5). The bundling is probably very fast as seen from the rapid elevation of the turbidity level.

In another set of experiments, p25 was added to tubulin in the absence of paclitaxel, and the pellet fraction was sedimented at 100000*g* and studied in the electron micro-

scope. The population of MTs assembled in the presence of p25 consists of a straight-running single MT or bundled MTs, most of which are shorter (Figure 7F) than those observed in the case of paclitaxel-stabilized MTs (Figure 7E). A remarkable morphologic feature of this sample is the appearance of numerous double-walled MTs (Figure 7C,D). On negatively stained preparations, the outer wall appears as a row of tightly packed small particles covering the surface of the inner wall with a periodicity of ~5 nm (Figure 7G). The length of segments bearing an outer wall on individual MTs depends on the concentration of p25. In samples containing 4 μM p25, most of the MTs are double-walled. At lower concentrations of p25, these segments are shorter and occur at variable intervals (data not shown). These observations suggest that the turbidity increase induced by addition of p25 to tubulin (see Figure 5A) is mainly due to the formation of aberrant, polymorphic tubulin assemblies. The accumulation of many short, double-walled forms may be explained by the stimulating action of this protein upon formation of a second (outer) wall which consumes the pool of tubulin that is available for polymerization and restricts thereby the growth of "conventional" MTs. On the basis of these observations, we conclude that p25 exerted two types of effects in our samples: it induced strong bundling of MTs and promoted the formation of unusual tubulin assemblies.

DISCUSSION

Recently, a novel human gene, the product of which is significantly homologous with the bovine brain-specific protein p25, has been reported (*19*). The p25 protein could be phosphorylated by kinases, tau protein kinase II (*17*) and phosphatidic acid-regulated protein kinase (*33*); however, the function of neither the phosphorylated nor the unphosphorylated form is known. The data in the literature concerning the expression and occurrence of p25 are rather contradictory. Takahashi et al. (*17*, *34*) reported p25 to be a brain-specific protein, the level of expression of which in rat brain was found to increase gradually until 1−2 years of age, and immunohistochemistry revealed distinctly stained regions in the central nervous system. The human p25 gene was suggested, however, to be constantly transcribed in various tissues (*18*). The cause of this discrepancy is unclear.

In the work presented here, we purified p25 from bovine brain until homogeneity and identified it by LC−MS; the CID spectra confirmed more than 50% of the sequence. Circular dichroism experiments revealed largely random coil with α-helix content of <5%. Here we present evidence that p25 physically interacts with tubulin most probably with the tubulin dimer since the conditions used in the CD experiments are unfavorable for tubulin polymerization, and the interaction induces extensive alterations in the secondary structure(s).

An important new finding of this study is that the purified p25 protein perturbs the MT assembly at substoichiometric concentration, and that the effect depends on the assembly state of tubulin: p25 binds to preassembled paclitaxel-stabilized MTs, inducing strong bundling, and promotes the formation of abnormal polymorphic tubulin assemblies when added to tubulin dimers. In this respect, the action of p25 which is a heat-stable cationic protein is similar to that exerted by a variety of other cationic compounds (for review, see ref *6*). The effects of these compounds were detected under various in vitro conditions, in most cases with MAP-containing tubulin in the presence of assembly-promoting factors (with DMSO or paclitaxel and GTP) (*9*). As a rule, the formation of polymorphic tubular assemblies requires concentrations of the basic compounds comparable with that of tubulin. A common morphologic feature of these assemblies is that the inner MT wall is covered by an outer layer of ringlike oligomers or helically arranged protofilaments, and between the two walls, a thin layer of electron-dense material apparently formed by the polycationic compound is deposited. The effect of polycations on MTs may be plausibly explained by their interaction with the acidic C-termini of tubulin dimers, specifically in the cases where their concentrations are comparable with that of tubulin. Charge neutralization may promote the formation of wall-to-wall contacted MTs and assembly of the outer wall. However, the observation that the removal of the C-termini of tubulin subunits by limited subtilisin digestion reduces the potency of polycations in promoting polymerization but cannot fully eliminate it suggests that anionic charges spaced elsewhere in tubulin may participate in the polycation−tubulin interaction (*9*). In addition, MAPs, which also react with the acidic C-termini of tubulin by a basic domain, do not induce and influence the formation of double-walled MTs. These observations strengthen our view that p25 is a unique brain-specific protein concerning its effects on microtubular assemblies. Until now, very few cationic proteins such as RNAse, histones, and enkephalitogenic protein has been identified as polymerization-promoting factors in vitro (*7*, *9*, *13*), and the biological relevance of their action remained elusive. In this context, it is noteworthy that p25 was recognized in our experiments as a tubulin polymerization promoting protein which perturbed the tubulin assembly in reconstituted systems (*20*, *21*). In fact, in an early study, double-walled MTs were occasionally observed in crude brain homogenates (*10*); however, the endogenous factor causing the unphysiological assembly was not identified.

To the best of our knowledge, p25 is the first protein identified in normal brain that can induce the formation of polymorphic tubulin assemblies. The fact that encephalitogenic protein, another basic protein that, however, appears in the brain under pathological conditions (*7*), exerts a similar effect on tubulin polymerization may enforce the studies aimed at understanding the role of the p25 protein in the physiological and pathological processes.

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