Calmodulin Binding and Cdk5 Phosphorylation of p35 Regulate Its Effect on Microtubules*

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In the nervous system, Cdk5 and its neuronal activator p35 are involved in the control of various activities, including neuronal differentiation and migration. Recently, we have reported that p35 is a microtubule-associated protein that regulates microtubule dynamics (Hou, Z., Li, Q., He, L., Lim, H. Y., Fu, X., Cheung, N. S., Qi, D. X., and Qi, R. Z. (2007) J. Biol. Chem. 282, 18666–18670). Here we present two regulatory modes of p35 function as a microtubule-associated protein. First, p35 is Ca\(^{2+}\)-dependent calmodulin (CaM)-binding protein. The CaM- and microtubule-binding domains are localized to overlapping regions at the N terminus of p35. Within the CaM-binding region, Ala substitution for Trp-52 abolishes the CaM-binding activity, corroborating specific CaM-binding of p35. Furthermore, CaM blocks p35 association with microtubules in a Ca\(^{2+}\)-specific manner, suggesting that p35 may be involved in the Ca\(^{2+}\)/CaM-mediated inhibition of microtubule assembly. Second, p35 phosphorylation by Cdk5 interferes with the microtubule-binding and polymerizing activities of p35. Using a mutational approach, we found that only phosphorylation at Thr-138, one of the two residues primarily phosphorylated in vivo, inhibits the polymerizing activity. In PC12 cells, expression of p35 promotes nerve growth factor-induced neurite outgrowth under a Cdk5 inhibitory condition. Such p35 activity is impaired by the phosphomimetic mutation of Thr-138. These data suggest that Thr-138 phosphorylation plays a critical role in the control of the p35 functions in microtubule assembly and neurite outgrowth.

Being a major cytoskeletal element in eukaryotic cells and particularly abundant in nerve cells, microtubules intimately regulate cell mobility and morphology. Microtubule-associated proteins (MAPs)\(^2\) are a family of proteins that bind to, stabilize, and promote assembly of microtubules (1). The microtubule-binding and stabilizing activities of MAPs are regulated by a number of factors in response to internal and external signals. Among them, Ca\(^{2+}\)/calmodulin (CaM) exerts an inhibitory effect on microtubule integrity via binding to MAPs (2–4). In MAPs, the binding domains of Ca\(^{2+}\)/CaM and microtubules are usually adjacent to each other or even overlapping, pointing to competitive binding of Ca\(^{2+}\)/CaM and microtubules to the MAPs (5, 6). Also, MAPs often exist as phosphoproteins in vivo. Phosphorylation of MAPs at different sites may exert different effects on microtubule association. For example, association of tau with microtubules is negatively regulated by glycogen synthase kinase 3\(\beta\)-mediated phosphorylation of tau at primed sites but not by phosphorylation at unprimed sites (7). Therefore, phosphorylation of MAPs may have site-specific effects on their functions.

Cdk5 is one of the protein kinases implicated to participate in the regulation of microtubule dynamics by phosphorylating MAPs. In association with the p35 activator, which is almost exclusively expressed in central nervous system neurons, Cdk5 plays an essential role in proper development of the nervous system (8, 9). During corticogenesis, postmitotic neurons migrate from the neuroepithelium to the cortical plate, where they eventually form the mature cortical layers. Mice deficient of Cdk5 or p35 have abnormally structured cortices because of defects in neuronal migration (10, 11). In addition, blocking of Cdk5 activity in cultured neurons inhibits neurite outgrowth, pointing to an essential role of Cdk5 in neuronal differentiation (12). Several MAPs have been identified to be Cdk5 substrates; the Cdk5-mediated phosphorylation of tau and doublecortin interferes with their microtubule-binding and stabilizing activities, whereas the phosphorylation of MAP1 appears to enhance its microtubule-binding affinity (13–17). Under neurotoxic conditions, p35 is cleaved by the Ca\(^{2+}\)-dependent protease calpain to generate p25, which loses 98 residues at the N terminus (18, 19). The resulting Cdk5-p25 complex shows aberrant control of the kinase activity, causing tau hyperphosphorylation and, thus, microtubule disruption and neuronal cell death (20–22).

After Cdk5 activation, p35 is phosphorylated by Cdk5. This phosphorylation occurs primarily at two residues, Ser-8 and Thr-138, among the four putative targeting sites (23). Thr-138 phosphorylation conversely correlates with brain development, with the highest level detected in fetal brain but undetectable in adult brain; Ser-8 phosphorylation remains unchanged during development (23). Thus, phosphorylation at these two residues is differentially regulated. p35 is a short-lived protein subjected to degradation via the ubiquitin-proteasome pathway (24). Phosphorylation of p35
increases its susceptibility to proteasome-dependent degradation and suppresses the calpain-mediated transformation into p25 (23–25). Moreover, Thr-138 phosphorylation appears to be critical in determining resistance to the calpain-mediated cleavage (23). Therefore, phosphorylation at the two residues contributes differentially to the control of p35 levels.

Recently, p35 has been shown to be a MAP that promotes microtubule assembly and induces bundling of the filaments (26). However, it is unclear how these microtubule-associated functions are regulated. In this study, p35 is identified to interact with CaM in a Ca$^{2+}$-dependent manner, and the CaM binding blocks p35 association with microtubules. In addition, the microtubule-binding and polymerizing activities of p35 are regulated by Cdk5-catalyzed phosphorylation of p35. p35 displays activity independent of Cdk5 activation to promote nerve growth factor (NGF)-induced neurite formation. We characterized the effects of p35 phosphorylation on its microtubule binding and polymerizing activities as well as its Cdk5 activation-independent activity in neurite outgrowth.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**—Construction of Cdk5 and p35 plasmids used in this report was described previously (27). p35(S8A/T138A/S170A/T197A) was a gift from Dr. Li-Huei Tsai (Massachusetts Institute of Technology) (24). Truncations and point mutations were created by PCR methods. The constructs were all verified by DNA sequencing.

**Antibodies**—The following antibodies were used: anti-Cdk5 (J-3, Santa Cruz Biotechnology), anti-β-tubulin (clone TUB2.1, Sigma), anti-CaM (clone 6D4, Sigma), anti-GST (GE Healthcare), anti-CaM (clone 6D4, Sigma), anti-GST (GE Healthcare), anti-V5 (Invitrogen), anti-His$_6$ (H-15, Santa Cruz Biotechnology), and anti-FLAG (Sigma).

**Protein Expression and Purification**—Bacterial expression of GST- and His$_6$-tagged proteins were performed using *Escherichia coli* BL21(DE3). The expressed proteins were purified by binding of His$_6$ to Ni$^{2+}$-nitrilotriacetic acid resin (Qiagen) or GST to GSH-Sepharose (GE Healthcare) (27). The isolated proteins contained full-length p35 and its proteolytic fragments. To determine the contents of full-length p35, the proteins were resolved by SDS-PAGE (10% acrylamide gels), and the resolved patterns were analyzed using the ChemiDoc XRS system and the Quantity One software (Bio-Rad). The p35 preparations contained full-length p35 at 30 and 15%, respectively. To express CaM in bacteria, human CaM gene III was subcloned from a pcDNA3 construct (a gift from Dr. Donald C. Chang, Ref. 28) into the vector pET32a. The construct expresses His$_6$-CaM. After purification using Ni$^{2+}$-nitrilotriacetic acid resin, the tag moiety was removed by cleavage at room temperature using enterokinase (Novagen) in 25 mM trilotriacetic acid resin, the tag moiety was removed by cleavage (23). Therefore, phosphorylation at the two residues contributes differentially to the control of p35 levels.

**Protein Phosphorylation**—Protein phosphorylation was determined using the Bio-Rad (Bradford) protein assay (Bio-Rad) with bovine serum albumin as standard. CaM concentration was also verified by measuring absorbance at 277 nm with 3300 m$^{-1}$ as the extinction coefficient (29).

**CaM-Binding Assays**—In CaM-Sepharose binding assays, p35-derived recombinant proteins (1 μg) were mixed with CaM-conjugated Sepharose (10 μL; GE Healthcare) in 200 μL of binding buffer (25 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, 150 mM NaCl, 1% Triton X-100, and 0.5 mg/ml bovine serum albumin) supplemented with 1 mM CaCl$_2$ or 5 mM EGTA. As a control, blank Sepharose was used instead of CaM-conjugated Sepharose. After incubation for 2 h at 4 °C, the beads were extensively rinsed. The bound proteins were analyzed by immunoblotting. In GST pulldown assays, GST and GST-p35 (2 μg) were allowed to bind to CaM (1 μg) under the above conditions. The GST proteins were then retrieved using GSH beads to analyze bound proteins on anti-CaM immunoblots.

To measure Ca$^{2+}$ concentration required for p35 binding with CaM, we performed equilibrium dialysis. All stock solutions were passed through a Chelex-100 (Bio-Rad) column to remove contaminating divalent cations (30). CaM and p35 were dialyzed separately against binding buffer containing increasing amounts of CaCl$_2$. After the equilibrium was reached, the proteins were subjected to the binding test. To test the effects of Sr$^{2+}$ and Ba$^{2+}$, CaM and p35 were dialyzed against binding buffer containing 0.1 mM EGTA and then subjected to the binding assay in the presence of 1 mM SrCl$_2$ or BaCl$_2$.

**Microtubule Sedimentation**—MAP-free tubulin was purified from porcine brain as detailed by Hou et al. (26). p35 proteins were purified from bacterial expressions or expressed in HEK293T. HEK293T lysates were prepared 24 h post-transfection in cold lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol, 50 mM NaF, 10 mM glycerophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na$_2$VO$_4$, and the protease inhibitor mixture (Roche Applied Science)). The extracts were clarified by centrifugation at 4 °C (14,000 × g; 20 min).

Microtubules, pre-polymerized from MAP-free tubulin (15 μg) in PEM buffer plus 1 mM GTP, 50 μM taxol, and 0.2% Triton X-100, were incubated with recombinant p35 proteins or p35-expressing lysates of HEK293T as indicated in 100 μL of the same buffer at room temperature for 30 min. Microtubules and associated proteins were pelleted at 4 °C (100,000 × g; 15 min) through a cushion of 50% glycerol in the buffer. The supernatants and the pellets were collected separately for immunoblotting.

**Microtubule Assembly**—Microtubule assembly was assayed by monitoring turbidity changes of solutions at 35 °C for 30 min (26, 31). The reaction mixtures contained MAP-free tubulin (10 μM) and p35-derived proteins in 100 μL of PEM buffer plus 1 mM GTP and 10 mM MgCl$_2$. To examine microtubules by fluorescence microscopy, rhodamine-labeled (Cytoskeleton) and unlabeled MAP-free tubulin were used at the ratio of 1:10 in the assembly (31). Polymerized microtubules were observed under an inverted Nikon microscope (Eclipse TE2000).

**Protein Phosphorylation**—Protein phosphorylation was measured in Cdk5-p35 complexes reconstituted from the puri-
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fied recombinant proteins or immunoprecipitated from cells expressing Cdk5 and p35. The Cdk5-p35 complexes were incubated at 30 °C for 1 h in a kinase buffer (20 mM MOPS, pH 7.4, 5 mM MgCl₂, and 1 mM dithiothreitol) containing 100 μM [γ-32P]ATP (~5000 dpm/pmol). The reactions were terminated by boiling for 5 min in the SDS-PAGE sample loading buffer. Before autoradiography, the proteins were resolved by SDS-PAGE.

Immunoblotting—Samples were subjected to electrophoresis on 10% acrylamide gels and then transferred to polyvinylidene difluoride membranes. The following conditions were applied to immunoblotting of CaM (32). Samples were boiled in SDS-PAGE sample loading buffer supplemented with 1 mM EGTA. After electrophoresis of 12% acrylamide gels, proteins were transferred in 25 mM KH₂PO₄, pH 7.0; KP buffer) at 20 V overnight at 4 °C. The blots were incubated at room temperature for 45 min in 0.2% (v/v) glutaraldehyde freshly prepared in KP buffer. After the incubation, the blots were rinsed in KP buffer, blocked in 2% bovine serum albumin/Tris-buffered saline (TBS), and then subjected to incubation with anti-CaM antibody in 2% bovine serum albumin/TBS.

Cell Culture, Transfection, and Immunocytochemistry—HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum under the conditions of 37 °C and 5% CO₂. Transfection medium (Invitrogen) supplemented with 10% fetal bovine serum. Differentiation was induced in low-serum medium (Dulbecco’s modified Eagle’s medium plus 0.5% fetal bovine serum and 0.5% horse serum) containing 50 ng/ml NGF. After differentiation, cells were fixed in phosphate-buffered saline containing 4% paraformaldehyde freshly prepared in 0.2% (v/v) glutaraldehyde. The formaldehyde-fixed cells were then subjected to incubation with anti-CaM (32). Samples were boiled in SDS-PAGE sample loading buffer supplemented with 1 mM EGTA.

†Samples were subjected to electrophoresis on 10% acrylamide gels and then transferred to polyvinylidene difluoride membranes. The following conditions were applied to immunoblotting of CaM (32). Samples were boiled in SDS-PAGE sample loading buffer supplemented with 1 mM EGTA. After electrophoresis of 12% acrylamide gels, proteins were transferred in 25 mM KH₂PO₄, pH 7.0; KP buffer) at 20 V overnight at 4 °C. The blots were incubated at room temperature for 45 min in 0.2% (v/v) glutaraldehyde freshly prepared in KP buffer. After the incubation, the blots were rinsed in KP buffer, blocked in 2% bovine serum albumin/Tris-buffered saline (TBS), and then subjected to incubation with anti-CaM antibody in 2% bovine serum albumin/TBS.

PC12 cells were maintained at 37 °C in 7.5% CO₂ with Dulbecco’s modified Eagle’s medium plus 10% horse serum and 5% fetal bovine serum. Differentiation was induced in low-serum medium (Dulbecco’s modified Eagle’s medium plus 0.5% fetal bovine serum and 0.5% horse serum) containing 50 ng/ml NGF (Sigma). To inhibit Cdk5 activity, GW8510 (4-((7-oxo-6,7-dihydro-8H-[1,3]thiazolo[5,4-e]indol-8-ylidene)methyl)amino)-N-(2-pyridyl)benzenesulfonamide, Sigma) was applied at 2 μM in the culture medium. After differentiation, cells were fixed in phosphate-buffered saline containing 4% paraformaldehyde and 5% sucrose for microscopy analysis. Images were acquired on the Nikon microscope. Neurite length and cell body diameter were determined using MetaMorph (Universal Imaging).

Cells with at least one visible process equal to or greater than two cell bodies were counted as positive for neurite formation. Statistical analysis was performed using analysis of variance followed by Tukey’s post hoc test for comparison among multiple groups and using Student’s unpaired two tails t tests for comparison between two groups. For all comparisons, p < 0.05 was considered statistically significant.

RESULTS

p35 Is a CaM-binding Protein—CaM-binding domains are usually short peptide stretches with defined sequence and structural features (33, 34). Analysis of the p35 protein sequence using a web-based method (33) revealed an unclassified CaM-binding sequence spanning residues 40–50. We tested the predicted binding in a pulldown assay using CaM-conjugated beads and purified recombinant p35. p35 was readily detected to interact with CaM in the presence of Ca²⁺, whereas the binding was abolished when EGTA was added instead of Ca²⁺ (Fig. 1A). In a reciprocal assay, GST-p35 pulled down CaM only in the presence of Ca²⁺, whereas GST did not bind to CaM (Fig. 1B). We proceeded to evaluate the effect of Ca²⁺ concentrations and found that the interaction was detected even at 20 μM but not at 5 μM Ca²⁺ (Fig. 1C). Together, p35 displayed Ca²⁺-dependent association with CaM. It has been known that divalent cations with ionic radii close to Ca²⁺ (e.g. Sr²⁺) can induce conformational change of CaM to effectively stimulate it, as revealed by CaM-dependent

FIGURE 1. p35 binds to CaM. A, p35-His₆ was incubated with CaM-conjugated (CaM) or blank (Blank) Sepharose. In the absence of Ca²⁺, 5 mM EGTA was added. p35 bound to the beads was detected on an anti-His₆ immunoblot. B, CaM was incubated with GST-p35 or GST. After retrieval of the GST proteins, the bound proteins were probed for CaM by immunoblotting. C, p35-His₆ was incubated with CaM-Sepharose at various Ca²⁺ concentrations. EGTA was added in the sample without Ca²⁺. p35 bound to the beads was detected on an anti-His₆ immunoblot. D, the CaM-Sepharose binding assay of p35-His₆ was performed in the presence of 1 mM of the cations as indicated or 5 mM EGTA. The bound proteins were analyzed by anti-His₆ immunoblotting. E, GST proteins of p35 fragments were tested for binding to CaM. After pull down of CaM beads, both bead-bound (Bound) and unbound (Unbound) fractions were subjected to anti-GST immunoblotting. Arrows denote the full-length proteins of the p35-derived constructs. F, alignment of an N-terminal region of p35 sequences. Boxed is the residue mutated for the binding test. GenBank™ accession numbers are CAA56587 (human), NP_034001 (mouse), AAB58715 (Drosophila) and AF231134 (Drosophila). G, mutation of Trp-52 to Ala abolishes the CaM-bind-
phosphodiesterase activation (30, 35). However, cations with different ionic radii (e.g. Ba^{2+}) are ineffective (30, 35). We tested Sr^{2+} and Ba^{2+} in the p35-binding assay. Similar to Ca^{2+}, Sr^{2+} effectively stimulated CaM binding of p35 (Fig. 1D). In contrast, Ba^{2+} exhibited a negligible effect (Fig. 1D). These results indicate that p35 binds specifically to CaM activated by the cations.

To define the CaM-binding region, we assayed CaM binding of several p35 fragments under the Ca^{2+}-containing condition. The p35 fragments were tagged with GST for purification from bacterial expression. The purified proteins contained both the full-length protein constructs and their truncated forms, as shown on the immunoblot (Fig. 1E). GST-p10 bound strongly to CaM, whereas the truncated GST fusion proteins in the preparation did not show binding activities (Fig. 1E). In addition, p25 did not bind to CaM (Fig. 1E). These results indicate the presence of a CaM-binding domain within the p10 region. Next, two fragments, 1–52 and 31–90, both of which contain the predicted CaM-binding sequence, were tested in the assay. CaM was observed to bind with 31–90 but not with 1–52 (Fig. 1E). The CaM-binding sequence appeared to be discrepant from that predicted from the web-based analysis.

Within the identified CaM-binding region, a segment of 30 amino acids is highly conserved in p35 ranging from Drosophila to human (Fig. 1F). A Trp residue, which plays a key role in interaction between CaM and many of its targets (36, 37), is found in the middle of this conserved region and is present in all of the listed species (Fig. 1F). We mutated this Trp residue and found that the mutation W52A eliminated the CaM-binding activity of p35 (Fig. 1G). Collectively, these results demonstrate that CaM binds to a conserved p35 region, within which Trp-52 is critically involved in such interaction.

Microtubule-Binding Regions of p35—Previously, we showed direct association of p35 with microtubules via p10 (26). To delineate the microtubule-binding domains, we constructed several fragments of p10 and tested their microtubule-binding activities. In the sedimentation assay, microtubules were assembled from MAP-free tubulin and stabilized with taxol. Under the assay conditions, most of the tubulin was polymerized into microtubule polymers, as revealed from tubulin distribution between the supernatants and the microtubule pellets. Next, two fragments, 1–52 and 31–90, both of which contain microtubule-binding activities similar to p10 (Fig. 2). When the region of 31–90 is responsible for binding to CaM, the microtubule-binding domains substantially overlap with the CaM-binding domain.

Ca^{2+}-CaM Inhibits p35 Association with Microtubules—We probed whether CaM affects p35 association with microtubules. When the sedimentation assay was performed under the conditions with Ca^{2+} (i.e. 1 mM CaCl_{2}) and without CaM, p35 sedimented with microtubules but did not sediment in from the sedimentation (Fig. 2). After the recombinant proteins derived from p10 were incubated with taxol-stabilized microtubules, the samples were subjected to sedimentation of the microtubules to detect co-sedimented proteins. Two non-overlapping fragments, 1–52 and 53–88, displayed strong microtubule-binding activities similar to p10 (Fig. 2). When the region of 53–98 was split into two constructs, 53–72 but not 72–98 retained such binding activity (Fig. 2). These results indicate that p35 harbors two microtubule-binding domains at the N terminus. Given that the region of 31–90 is responsible for binding to CaM, the microtubule-binding domains substantially overlap with the CaM-binding domain.

**FIGURE 2.** Mapping microtubule binding domains of p35. p35 fragments (1 μg) were incubated with and without taxol-stabilized microtubules (MTs). After microtubule sedimentation, the supernatants (SUP) and the pellets (PEL) were analyzed by anti-GST immunoblotting. Proteins on the transferred membranes were also stained with Ponceau S. The results shown are from a typical experiment conducted three times.

**FIGURE 3.** CaM blocks p35 association with microtubules. Microtubule sedimentation was conducted with p35-His_{6} (0.9 μM). A, CaM was applied at various concentrations. The assay buffer contained 1 mM CaCl_{2}. After centrifugation, the supernatants (SUP) and the microtubule pellets (PEL) were probed for p35-His_{6}, CaM (anti-CaM immunoblotting), and tubulin (protein staining by Ponceau S). The data are representative from one of three independent experiments. WT, wild type.

**A**

| CaM (μM) | SUP | PEL |
|----------|-----|-----|
| 0        | +   | +   |
| 0        | -   | -   |
| 20       | +   | -   |
| 40       | -   | -   |

**B**

|              | p35WT | p35(W52A) |
|--------------|-------|-----------|
| Ca^{2+}      | +     | -         |
| EGTA         | +     | +         |
| MTs          | -     | +         |

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**FIGURE 3.** Mapping microtubule binding domains of p35. p35 fragments (1 μg) were incubated with and without taxol-stabilized microtubules (MTs). After microtubule sedimentation, the supernatants (SUP) and the pellets (PEL) were analyzed by anti-GST immunoblotting. Proteins on the transferred membranes were also stained with Ponceau S. The results shown are from a typical experiment conducted three times.
the absence of microtubules (Fig. 3A), indicative of the microtubule association. When CaM was applied to the sedimentation assay, CaM did not bind to microtubules, and p35 diminished from the microtubule fraction in a manner dependent on the concentration of Ca$^{2+}$/CaM (Fig. 3B). Together, we conclude that the binding of Ca$^{2+}$/CaM to p35 interaction with microtubules.

Effects of p35 Phosphorylation on Its Microtubule-polymerizing and Binding Activities—As a substrate, p35 is phosphorylated by Cdk5 (24, 25, 38). We probed whether p35 phosphorylation affects its microtubule-polymerizing property. In the microtubule assembly assay, Cdk5 itself did not exhibit any activity on microtubule assembly (Fig. 4A). The reconstitution of the Cdk5-p35 complex under the protein phosphorylation condition abolished the microtubule-polymerizing activity of p35 (Fig. 4C). In contrast, the polymerizing activity was not significantly affected under each of the following conditions: 1) p35 was incubated under the phosphorylation conditions without Cdk5, 2) p35 was reconstituted with the kinase-dead mutant Cdk5(N144) under the phosphorylation conditions, and 3) p35 was reconstituted with wild type Cdk5 in the absence of ATP-Mg$^{2+}$ (Fig. 4A). To detect protein phosphorylation, we performed an in vitro kinase reaction using the p35 and Cdk5 proteins. p35 was phosphorylated by Cdk5 but not by Cdk5(N144) or in the absence of the Cdk5 proteins (Fig. 4B). In addition, Cdk5 phosphorylation was not detectable (Fig. 4B). These results revealed phosphorylation of p35 but not Cdk5 in vitro, in agreement with a previous report (38). Furthermore, the phosphorylation abrogates the microtubule-polymerizing activity of p35.

Phosphorylation of p35 by Cdk5 occurs in vivo primarily at Ser-8 and Thr-138, which display different phosphorylation patterns during brain development (23). To analyze the contribution of these phosphorylation events to the inhibition of the microtubule-polymerizing activity, Ser-8 and Thr-138 were individually mutated to Glu to generate phosphomimetic mutants. In the turbidimetric assay, the mutant p35(S8E) displayed activity similar to the wild type, whereas the mutant p35(T138E) completely failed in microtubule polymerization (Fig. 4C). In addition, substitution of Ser-8 or Thr-138 for Ala did not affect the polymerizing activity (Fig. 4C). These assay data were verified by examining the microtubules polymerized with a mixture of rhodamine-labeled and unlabeled tubulin under a fluorescence microscope.
cence microscope (Fig. 4D). Thus, phosphorylation at Thr-138 plays a critical role in the control of the microtubule-polymerizing activity.

We also assessed the potential effects of p35 phosphorylation on microtubule association. To prepare p35 in phosphorylated and unphosphorylated forms, p35 was expressed in HEK293T with Cdk5 wild type and Cdk5(N144), respectively. To examine protein phosphorylation, we immunoprecipitated Cdk5 or its mutant from the transfected cells and subjected the immunoprecipitates to a phosphorylation assay. p35 was readily phosphorylated in the immunoprecipitate of Cdk5 but not in that of Cdk5(N144) (Fig. 5A). In addition, Cdk5 was not phosphorylated in the reaction (Fig. 5A). We also co-expressed Cdk5 with the non-phosphorylatable mutants of p35. The Ala substitutions do not interfere with the Cdk5-binding and activating activity of p35 (24). The mutation of both Ser-8 and Thr-138 to Ala dramatically reduced p35 phosphorylation (Fig. 5A). The mutant p35QUAD, with all four potential sites (i.e. Ser-8, Thr-138, Ser-170, and Thr-197) mutated to Ala, showed little phosphorylation (Fig. 5A). These results revealed that p35 is phosphorylated by Cdk5 at the four proline-directed sites with the preference of Ser-8 and Thr-138.

We subjected the cell extracts to a microtubule sedimentation assay. After the sedimentation, both the microtubule pellets and the supernatants were examined for p35 and Cdk5. When co-transfected with Cdk5 wild type, p35 appeared almost exclusively in the supernatant (Fig. 5B), showing inhibition of the microtubule association by Cdk5 expression. The co-transfection of p35 and kinase-dead Cdk5 resulted in the sedimentation of a large proportion of p35 with microtubules (Fig. 5B). In addition, the mutant p35QUAD was almost completely resistant to the inhibitory effect exerted by Cdk5 (Fig. 5B). These observations revealed that p35 loses its microtubule-binding activity once phosphorylated by Cdk5. Note that Cdk5 and Cdk5(N144) were not present in the microtubule pellet even though p35 co-expressed in the extract sedimented with microtubules (Fig. 5B). This is consistent with the idea that p35 associates with microtubules at the expense of Cdk5 binding (26).

To analyze the phosphorylation effects of Ser-8 and Thr-138, they were mutated to Ala individually or in combination. Ala substitution for one of the two residues has no effect on phosphorylation at the other residue by Cdk5 (23). When overexpressed with Cdk5, a large fraction of the double mutant p35(S8A/T138A) co-sedimented with microtubules (Fig. 5B), further supporting the idea that the phosphorylation at these two residues by Cdk5 is inhibitory to the microtubule association. In the presence of overexpressed Cdk5, p35(S8A/T138A) appeared to have lower microtubule-binding activity than p35QUAD (Fig. 5B), suggesting that the phosphorylation at Ser-170 and Thr-197 may contribute to the impaired association with microtubules. We also performed the assay with single mutants p35(S8A) and p35(T138A). When co-expressed with Cdk5, both of the single mutants were distributed to the microtubule pellets at markedly lower levels than the double mutant (Fig. 5B). Co-expression of Cdk5(N144) dramatically enhanced the microtubule association of the single mutants (Fig. 5B). Therefore, phosphorylation at either Ser-8 or Thr-138 reduces the microtubule-binding affinity of p35.

p35 Has Cdk5 Activation-independent Functions in Neurite Outgrowth—It has been shown in several studies that p35 expression and Cdk5 activation are required for neurite out-
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Given that p35 is a MAP (26) and that p35 phosphorylation by Cdk5 interferes with the function as a MAP, we probed the potential effect of p35 phosphorylation on p35 function in neurite outgrowth using p35 mutants. The mutants were transiently expressed in PC12 cells to examine their effects on NGF-mediated neurite outgrowth under the Cdk5 inhibitory conditions and to compare them with the wild type. Expression of the p35 proteins was validated on the immunoblots (Fig. 6C). The mutation T138E significantly reduced the outgrowth-promoting activity, whereas T138A or S8E did not affect such activity (Fig. 6, A and B). Collectively, our results suggest that Thr-138 phosphorylation impairs the Cdk5 activation-independent function of p35 in neurite outgrowth.

**DISCUSSION**

Cell migration and morphogenesis require dynamic reorganization of the microtubule architecture. This is achieved through sophisticated mechanisms including controlled association and dissociation of MAPs with microtubules. Both Cdk5 and p35 are expressed in the perinuclear soma, neurites, and axonal growth cones of developing neurons, consistent with their functions in neuronal migration, neurite extension, and axonal growth. We have recently found p35 to regulate microtubule assembly and stability via direct association with microtubules (26). We showed in this study that the microtubule-associating and polymerizing activities of p35 are regulated in at least two modes, CaM-binding and Cdk5-catalyzed phosphorylation. In addition, p35 can promote NGF-induced neurite outgrowth in a manner independent of Cdk5 activation. Using site-directed mutagenesis, we found that Thr-138 phosphorylation is inhibitory to the microtubule-assembly function of p35 as well as to the Cdk5 activation-independent neurite outgrowth promoted by p35. These results suggest that microtubule assembly by p35 is involved in neurite outgrowth and that this p35 function is well controlled by phosphorylation.

CaM is a widely distributed protein, serving as a primary Ca\(^{2+}\) sensor in eukaryotic cells. Our results showed that p35 interacts with CaM in a Ca\(^{2+}\)-specific manner and that the binding was mapped to the region 31–90 with the critical CaM-binding sequence.

**FIGURE 6.** Phosphomimetic mutation of Thr-138 impairs the neurite outgrowth-promoting activity of p35. A and B, PC12 cells, transfected with the GFP vector (Vector) or p35 constructs, were induced for 48 h with NGF in the absence or presence of GW8510. Shown are representative images of phase contrast and GFP fluorescence (A). Data shown in the histograms (B) indicate percentages of the transfected cells scored positive for neurite outgrowth. 100 cells were counted in each experiment. Error bars represent ± S.E. from 10 independent experiments. Statistical significance was calculated for GW8510-treated samples; p*, p35WT versus the vector; p**, p35(T138E), p35(T138A), or p35(S8E) versus p35WT. C, PC12 extracts expressing the p35 proteins were probed for p35-GFP (anti-GFP), Cdk5, and actin (anti-β-actin) on the immunoblots. WT, p35 wild type; T138E; p35(T138E); T138A; p35(T138A); S8E; p35(S8E).
binding exerts steric inhibition toward p35 association with microtubules. Indeed, CaM displayed a Ca\(^{2+}\)-dependent and direct inhibitory effect on p35 interaction with microtubules. The effect on p35 is in accordance with the Ca\(^{2+}\)/CaM sensitivity of microtubules, including cold-stable microtubules. Therefore, p35 may play a role in conferring the Ca\(^{2+}\)/CaM-dependent control on microtubule organization.

Protein phosphorylation seems to be a general mechanism in regulating microtubule association of MAPs. We have demonstrated here that the microtubule-associating and polymerizing activities of p35 are controlled by the kinase action of Cdk5 toward p35. Furthermore, phosphorylation at Thr-138 but not at Ser-8 exerts the inhibition of the microtubule-polymerizing activity. Of the two residues phosphorylated primarily in vivo, Thr-138 displays developmentally regulated phosphorylation, which appears to be under tight control by unidentified mechanisms perhaps involving protein phosphatases in adult brain (23). During morphological development of the brain, dynamic reorganization of the microtubule architecture requires dynamic exchange between association and dissociation of MAPs; one way to achieve this is by phosphorylation and dephosphorylation of MAPs. It has been observed that MAPs such as tau and MAP2 from fetal brain are phosphorylated to a greater extent at Ser/Thr-Pro motifs and, thus, are less efficient in promoting microtubule assembly, relevant to the maintenance of a highly dynamic cytoskeletal organization during the period of rapid brain development (44, 45). We propose that the developmentally regulated phosphorylation at Thr-138 may fall into this scheme.

It has been known that p35 phosphorylation by Cdk5 inhibits calpain-mediated p35 cleavage but facilitates proteasome-dependent p35 turnover (24, 25). In addition, the regulated phosphorylation at Thr-138 implicates a triggering role in the control of the calpain and proteasome actions (23). Conceivably, phosphorylation at Thr-138 may not only terminate the microtubule-assembling activity of p35 but also serves in adult brain as a signal of p35 removal. Therefore, p35 phosphorylation at Thr-138 may play a critical role in the control of p35 functions and the protein fate.

Both Cdk5 and p35 are required for neuronal migration and neurite outgrowth. Interestingly, p35 expression overcame at a large extent the neurite-outgrowth inhibitory effect of the Cdk5 inhibition, providing additional support to the idea that p35 has Cdk5-independent functions, such as microtubule-binding and stabilizing activities (26). Furthermore, our data suggest that the microtubule-polymerizing activity of p35 is involved in neurite outgrowth, as the inhibition of such activity by the phosphomimetic mutation of Thr-138 compromised the outgrowth-promoting activity. Therefore, p35 appears to play a multifunctional role in neurite formation. Evidence has accumulated to indicate that Cdk5 plays a role in microtubule stability through phosphorylating several MAPs, including tau and doublecortin, to reduce their affinities for microtubules and, therefore, microtubule-stabilizing activities (8, 9, 17). We suggest here that phosphorylation of p35 by Cdk5 is consistent with Cdk5 actions toward several other cytoskeletal targets. Therefore, Cdk5 exerts regulation of the microtubule network via phosphorylating several cytoskeletal proteins, including p35. In conclusion, p35 may have functions in conferring both Ca\(^{2+}\)/CaM-dependent and phosphorylation-dependent controls to microtubule organization. The findings presented here may contribute to understanding the regulation of p35 functions in microtubule organization.

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