Microtubule Association of the Neuronal p35 Activator of Cdk5*

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Cdk5 and its neuronal activator p35 play an important role in neuronal migration and proper development of the brain cortex. We show that p35 binds directly to α/β-tubulin and microtubules. Microtubule polymers but not the α/β-tubulin heterodimer block p35 interaction with Cdk5 and therefore inhibit Cdk5-p35 activity. p25, a neurotoxin-induced and truncated form of p35, does not have tubulin and microtubule binding activities, and Cdk5-p25 is inert to the inhibitory effect of microtubules. p35 displays strong activity in promoting microtubule assembly and inducing formation of microtubule bundles. Furthermore, microtubules stabilized by p35 are resistant to cold-induced disassembly. In cultured cortical neurons, a significant proportion of p35 localizes to microtubules. When microtubules were isolated from rat brain extracts, p35 co-assembled with microtubules, including cold-stable microtubules. Together, these findings suggest that p35 is a microtubule-associated protein that modulates microtubule dynamics. Also, microtubules play an important role in the control of Cdk5 activation.

As a distinct member of the CDK family, Cdk5 is activated by a neuron-specific protein p35 or the p39 homologue of p35 in the central nervous system (1). Both Cdk5 and p35 are required for neurite outgrowth (2). Studies in animal models have revealed their crucial involvements in neuronal migration during nervous system development as mice deficient of Cdk5 or p35 display abnormal brain cortex (3, 4). To date, a wide range of evidence has been accumulated indicating that Cdk5-p35 is a multifunctional kinase that acts in the regulation of various neuronal activities, including organization of the microtubule cytoskeleton (1). In living cells, the dynamic properties of microtubules are modulated through a sophisticated mechanism involving microtubule-associated proteins (MAPs),

which bind microtubule polymers and promote microtubule polymerization by stabilizing the polymer structure (5). Cdk5 phosphorylates several MAPs including MAP1b, MAP2, tau, and doublecortin, mediating their association with microtubules and their microtubule-stabilizing functions (1, 6, 7).

It is poorly understood how Cdk5 activity is regulated. Although p35 shows little apparent sequence homology to cyclins, it resembles the cyclin A structure with distinct features to bind specifically to Cdk5 (8, 9). The binding of p35 highly stimulates Cdk5 activity (10). Several proteins, including C42, protein kinase CK2, and three importin family members (importin-β, importin-5, and importin-7), show inhibitory effects toward Cdk5 activation via binding to p35 (11–13). Under neurotoxic conditions, p35 is transformed into the N-terminally truncated p25 protein, which causes sustained activation and mislocalization of Cdk5 (14–16). Moreover, p25 deregulation of Cdk5 has been linked to neuronal cell death and pathogenesis of neurodegenerative diseases such as Alzheimer disease (1).

In this report, we have identified direct association of p35 with tubulin and microtubules and have shown the function of p35 as a MAP as well as the regulation of Cdk5 activation by microtubules.

EXPERIMENTAL PROCEDURES

Antibodies—The following antibodies were purchased: anti-α-tubulin from Abcam; anti-β-tubulin (TUB2.1) from Sigma; anti-tau (H-150), anti-p35 (C-19), and anti-Cdk5 (C-8 and J-3) from Santa Cruz Biotechnology; and anti-GST from GE Healthcare.

Recombinant Protein Production—Recombinant proteins of Cdk5, p35, and p35 fragments were expressed in Escherichia coli BL21(DE3) and were purified (11, 17). The expression and purification of the largest human tau isoform hT40 was performed as reported (18). Recombinant proteins used in microtubule sedimentation and polymerization assays were dialyzed in PEM buffer (80 mM K-PIPES, pH 6.9, 1 mM MgCl2) and 1 mM EGTA) supplemented with 1 mM EDTA, 1 mM DTT, and 50 mM NaCl.

Microtubule Isolation from Rat Brain—Brain microtubules were isolated by temperature-dependent assembly/disassembly experiments (19). Rat brain was homogenized on ice in 2-folds (v/w) of homogenization buffer (PEM buffer plus 100 mM NaCl, 0.1% Triton X-100, 1 mM DTT, 10 mM NaF, 0.1 mM Na3VO4, 10 mM β-glycerophosphate, and the protease inhibitor mixture (Roche Applied Science)). The homogenate was cleared by centrifugation at 4 °C first at 20,000 × g for 45 min and then at 100,000 × g for 45 min. Microtubule assembly was initiated in the extract by the addition of 30% (v/v) glycerol and 1 mM GTP and was conducted at 35 °C for 45 min. After microtubules were spun down at 35 °C (100,000 × g for 45 min), the supernatant was removed. The pelleted microtubules were resuspended by homogenization in the ice-cold homogenization buffer and were allowed to disassemble on ice for 45 min. The suspension was then centrifuged at 4 °C (100,000 × g for 45 min) to pellet undisrupted microtubules, which were desig-
Isolation of MAP-free Tubulin—Tubulin was purified from porcine brain by two cycles of microtubule assembly/disassembly followed by phosphocellulose chromatography (19). The temperature-dependent assembly and disassembly were performed as described above except that PEM buffer was used instead of the homogenization buffer, and 1 mm each of ATP and GTP was applied for microtubule polymerization in the first cycle. After two assembly/disassembly cycles, the pre-cleared tubulin sample was applied at less than 3 mg protein/ml and GTP was applied for microtubule polymerization in the first cycle. Above experiments. 

Microtubule Sedimentation—Microtubule cosedimentation was performed as described previously (20). Proteins were centrifuged at 4 °C (100,000 × g; 30 min) to remove aggregates before experiments.

Immunofluorescence Microscopy—Five-day cultures of mouse cortical neurons were immunostained as described previously (13) and were analyzed on a Nikon microscope (Eclipse TE2000).

Extraction of Microtubule Proteins from Cell Cultures—Microtubule proteins and the remaining cytoplasm were differentially extracted from 5-day cultures of cortical neurons as described previously (17). Both extracted fractions were clarified by centrifugation before subjected to Western blotting.

Cdk5 Binding Assay and Immunoprecipitation—GST-Cdk5 (2 μg), prereconstituted with p35-His6 or p25-His6 (2 μg), was incubated with α/β-tubulin or taxol-stabilized microtubules at 25 °C for 1 h in 800 μl of PEM buffer supplemented with 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% Nonidet P-40, and protease inhibitors. GST-Cdk5 was retrieved using GSH beads to detect bound p35/p25 by Western blotting. To perform Cdk5 immunoprecipitation, rat brain extract prepared in the brain extract during the first cycle, temperature-induced assembly and disassembly. When microtubules from adult rat brain through three cycles of temperature-induced assembly and disassembly. When microtubules were assembled in the brain extract during the first cycle, the majority of p35 associated with the polymers, including the cold-stable polymers (Fig. 1F). Most of Cdk5 remained in the supernatant, where Cdk5 was in abundance when compared with p35 and p25 (Fig. 1, F and G). p25 was detected in the cold-labile but not cold-stable fraction of microtubules (Fig.
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In the following cycles, p35 continued to assemble with both cold-labile and cold-stable microtubules; Cdk5 and p25 existed with cold-labile microtubules (Fig. 1F). When probed as a control, tau was found to be present almost exclusively with cold-labile microtubules of each cycle (Fig. 1F). After three cycles of assembly/disassembly, microtubules were essentially free of contaminants. In the isolated cold-labile microtubules, the amount of p35 and p25 combined was more than Cdk5 (Fig. 1G). In the cold-stable microtubules, p25 was undetectable, and p35 was greatly in excess of Cdk5 (p35:Cdk5 was 6:1; Fig. 1, F and G). Thus, p35 associated with the microtubules was free of Cdk5 binding. Taken together, the microtubule co-assembly demonstrates prominent association of p35 with microtubules including cold-stable microtubules. In a previous report, the Cdk5-p25 kinase is shown to associate indirectly with microtubules via binding to tau (22). We reason that the sedimentation of Cdk5 and p25 with cold-labile microtubules was attributed to their association with tau and possibly some other MAPs in the brain extract.

Microtubules Disrupt the Association of p35 with Cdk5—We investigated how Cdk5, p35, and microtubules interact with one another. Cdk5 does not bind directly to microtubules (22). In a binding assay, the preformed complex of Cdk5 and p35 was incubated with taxol-stabilized microtubules or with the α/β-tubulin heterodimer. Interestingly, the microtubules disrupted the interaction between p35 and Cdk5 in a dose-dependent manner, whereas the tubulin dimer did not exhibit any effect (Fig. 2A). In addition, the microtubules did not affect p25 binding to Cdk5 (Fig. 2A). In accordance with these findings, the microtubule polymers but not the tubulin dimer inhibited the activity of Cdk5-p35, and the polymers did not inhibit the activity of Cdk5-p25 (Fig. 2B).

Next, we conducted co-immunoprecipitation of Cdk5 and p35 from a rat brain extract that was pretreated with taxol to induce microtubule assembly or with nocodazole to inhibit the assembly. In the presence of taxol, p35 failed to co-precipitate with Cdk5 (Fig. 2C). In contrast, the co-immunoprecipitation was readily detected under the inhibitory condition of microtubule assembly (Fig. 2C). Therefore, microtubules sequester p35 but not p25 from Cdk5 and thus inhibit p35 activation of Cdk5.

p35 Induces Microtubule Assembly and Bundling—The microtubule and tubulin association prompted us to investigate whether p35 alters microtubule assembly characteristics. In the absence of p35, there was a minimal polymerization of tubulin even after a prolonged incubation because tubulin was below the concentration required for spontaneous polymerization (Fig. 3A). The addition of 3 μg/ml p35 (p35:tubulin at ∼1:400) resulted in significant polymerization of tubulin (Fig. 3A). Both the rate and the extent of polymerization were significantly enhanced in a manner dependent on the input of p35. With 12 μg/ml p35 (p35:tubulin at 1:100), most of the tubulin was polymerized into microtubule polymers. Thus, p35 exhibited a strong activity in inducing microtubule assembly. We also tested the p35 fragments p10 and p25 in the assay. In contrast to p35, neither p10 nor p25 induced microtubule assembly when applied at the same amount as p35 or even at much higher concentrations (data not shown). Given that p10 retains the microtubule and tubulin binding activities (Fig. 1), the microtubule-polymerizing function either involves additional domains from the p25 region or requires it for protein folding. Conceivably, the neurotoxin-induced cleavage of p35 abrogates its microtubule-polymerizing property.
To observe polymerized microtubules, samples from the assembly assay were negatively stained for electron microscopy. In agreement with the turbidimetric assay results, almost no microtubules were found in the assay sample without p35 (Fig. 3B, panel a). Microtubules were readily seen in those polymerized by using taxol or p35 (Fig. 3B, panels b and c). Interestingly, most of the p35-assembled microtubules existed in the form of bundles (Fig. 3B, panel c). As seen in the micrograph, several microtubules were closely attached to each other to form a bundle (Fig. 3B, panel d). In the control, taxol-polymerized microtubules did not form bundles (Fig. 3B, panel b), consistent with the observation in a previous report (23). It appeared that p35 cross-bridges microtubules in addition to the promotion of microtubule assembly.

Given the co-isolation of p35 and cold-stable microtubules from rat brain, we tested whether p35 can stabilize microtubules at low temperature. In the assay, microtubules polymerized in vitro were placed on ice, and solution turbidity was monitored. Most of tau-stabilized microtubules depolymerized within a few minutes (Fig. 3C) as tau does not confer the cold stability (20, 24). The turbidity of the p35-polymerized sample barely changed even after 30 min of incubation (Fig. 3C). Thus, microtubules stabilized by p35 are resistant to cold-induced disassembly.
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DISCUSSION

This report describes for the first time the role of p35 as a MAP and the inactivation of Cdk5-p35 by microtubules. The p10 region of p35, which contains microtubule- and tubulin-binding domains, is rich in basic residues, reminiscent of the microtubule-binding sequences of conventional MAPs. However, scanning of the p10 sequence did not yield any recognizable microtubule-binding motif, implicating that p35 may contain novel microtubule- and tubulin-binding domains. The binding of p35 to microtubules does not confer Cdk5 attachment to microtubules. Instead, microtubules segregate p35 from Cdk5, acting as an inhibitor of Cdk5. Given that a significant proportion of p35 localizes to microtubules, the microtubule cytoskeleton may play an important role in the control of Cdk5 activity. Similar to microtubules, importin-β, importin-5, and importin-7 have been shown to block p35 association with Cdk5 via binding to the N-terminal region of p35 (13). Therefore, this p35 region confers Cdk5 inhibition via interaction with certain protein factors or subcellular structures, which sequester p35 from Cdk5. The truncation of p35 to p25, which loses the N-terminal domain, is a way to relieve the inhibition. Indeed, the production of p25 causes aberrant activation of Cdk5 (16).

p35 has strong microtubule-polymerizing activity. The current model of microtubule polymerization involves the formation of microtubule nuclei (i.e. nucleation) from several tubulin dimers (25). Our preliminary results showed the intermolecular self-association of p35. Therefore, p35 may facilitate microtubule nucleation through its homodimerization/oligomerization, and subsequently, stabilize the microtubules in the form of bundles. In addition, our results suggest that p35 may be one of the microtubule cold stabilizers and that the stabilization is at least partially due to the effect of bundling. In many cell types, there is a family of MAPs called stable tubule-only polypeptides, which render microtubules cold-stable (26). Although studies using transgenic mice have revealed a role of stable tubule-only polypeptides in synaptic plasticity, the precise function of microtubule cold stability is still unclear (27). The function of p35 as a MAP is highly relevant to its role in neuronal migration or morphogenesis. The localization of p35 to microtubules is readily found in axons and dendrites including the growth cone at the leading edge of an extending neurite. As the leading edge extends, microtubules undergo active polymerization to grow into the protrusion. Also, microtubules play a critical role in the movement of the nucleus into the leading process of migrating cells. The results presented here suggest that p35 directly participates in microtubule assembly and stabilization during these processes. It has been shown that the Cdk5-p35 kinase modulates the microtubule architecture through its action toward several molecular targets such as doublecortin and focal adhesion kinase (7, 28). Therefore, p35 may play a multifunctional role in the regulation of microtubule dynamics.

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