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Calpain-dependent Proteolytic Cleavage of the p35 Cyclin-dependent Kinase 5 Activator to p25*

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Cyclin-dependent kinase 5 (CDK5) is a unique CDK, the activity of which can be detected in postmitotic neurons. To date, CDK5 purified from mammalian brains has always been associated with a truncated form of the 35-kDa major brain specific activator (p35, also known as nck5a) of CDK5, known as p25. In this study, we report that p35 can be cleaved to p25 both *in vitro* and *in vivo* by calpain. In a rat brain extract, p35 was cleaved to p25 by incubation with Ca^{2+}. This cleavage was inhibited by a calpain inhibitor peptide derived from calpastatin and was ablated by separating the p35·CDK5 from calpain by centrifugation. The p35 recovered in the pellet after centrifugation could then be cleaved to p25 by purified calpain. Cleavage of p35 was also induced in primary cultured neurons by treatment with a Ca^{2+} ionophore and Ca^{2+} and inhibited by calpain inhibitor I. The cleavage changed the solubility of the CDK5 active complex from the particulate fraction to the soluble fraction but did not affect the histone H1 kinase activity. Increased cleavage was detected in cultured neurons undergoing cell death, suggesting a role of the cleavage in neuronal cell death.

Cyclin-dependent kinases (CDKs)[1] are a group of serine/threonine protein kinases activated by binding to a regulatory subunit cyclin. These kinases are key regulators of the eukaryotic cell cycle progression (1, 2), but only CDK5 appears to function in postmitotic neurons (3). Although the CDK5 protein is expressed at basal levels in most mammalian tissues (4–7), CDK5 activity has been demonstrated only in brains and developing muscle (6, 8), as these are the only tissues that express CDK5 activators (9–11). The major CDK5 activator in the brain is p35 (also termed nck5a) (9, 10, 12). As a deficiency of CDK5 or p35 results in a reversed layer formation of cortical neurons in the cerebrum or a defect in the formation of the cortical laminar structure in the cerebellum (13, 14), p35·CDK5 is thought to play an important role in brain development.

Extensive studies of CDK activation in proliferating cells have shown that the activity of CDKs is tightly regulated by the synthesis/degradation of their respective cyclin partners and the phosphorylation/dephosphorylation of their catalytic subunits (1, 2). However, CDK5 does not need to be phosphorylated to regulate kinase activity. Even though the phosphorylation sites for both activation and inhibition of this protein have been conserved (3–5, 15–17), CDK5 can be activated by binding to p35 or the C-terminal fragment of p35 (a 25-kDa regulatory protein known as p25, formed when p35 is nicked between Phe-98 and Ala-99) (9, 10, 12). Recent studies have shown that p35 level is regulated by proteasome degradation (18, 19), as the level of cyclins in proliferating cells has been shown (1, 2). Cyclins are periodically synthesized and degraded during the cell cycle (1, 2), but the neuronal activities involved in the synthesis and degradation of p35 are unknown.

CDK5 appears to be involved in the migration or positioning of neuronal cells via phosphorylation of cytoskeletal proteins (20–22). Several of these cytoskeletal substrates (microtubule-associated protein tau, the high and middle molecular weight subunits of neurofilaments (NF-H and NF-M), and synapsin I) have been identified (23–30). CDK5 may be targeted to cytoskeletons to enhance their phosphorylation efficiency. This is supported by the following observations: (i) CDK5 was purified from microtubule-enriched fractions of bovine and porcine brain extracts (23, 28); (ii) CDK5 formed a multimolecular complex (~670 kDa) that was eluted at void volume fractions from a gel filtration column (31); (iii) using the yeast two-hybrid system, p35 bound to neurofilaments and α-actinin (20, 32); and (iv) immunofluorescent staining of cultured neurons suggested an association of p35 with actin filaments in growth cone (20). However, how the active complex of p35·CDK5 targets cytoskeletal structures remains to be determined.

Several studies have shown that kinase active CDK5 purified from porcine or bovine brains complexes with p25 but not with p35 (23, 28, 33), even though p35 predominates in the brain (9, 10, 12). CDK5 has been purified from the spinal cord of rats, but the activator associated with it has not been shown (27). Thus, the p35·CDK5 complex has never been purified from mammalian brains, probably because p35 degrades to p25 during purification rather than p25·CDK5 being the active form of CDK5. If p35·CDK5 is active in neurons, it is essential to examine the properties of CDK5 when it is associated with p35. To purify the p35·CDK5 complex, it may be critical to determine why p35 is easily degraded to p25 during purification.

We tried to isolate p35·CDK5 from rat brains that had been homogenized immediately after the rats were decapitated. Af-

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[1] The abbreviations used are: CDK, cyclin-dependent kinase; LDH, lactate dehydrogenase; PAGE, polyacrylamide gel electrophoresis; TUNEL, terminal deoxytransferase-mediated dUTP nick end-labeling; MOPS, 4-morpholinopropanesulfonic acid.

ter fractionation through a hydroxyapatite column composed of calcium phosphate, we found that p35 was completely converted to p25. We thought that and investigated the possibility that p35 might be cleaved to p25 by the Ca^{2+}-activated protease, calpain, and we found that p35 was degraded to p25 by calpain both *in vitro* and *in vivo*. Degradation of p35 to p25 released the kinase active form of CDK5 from the insoluble fraction to the soluble supernatant. Furthermore, we also found that degradation of p35 to p25 occurred in cultured neurons undergoing cell death.

EXPERIMENTAL PROCEDURES

Chemicals and Antibodies—An antiserum against the N terminus of p25, a truncated form of bovine p35, was used as described previously (34). A polyclonal antibody to p35/p25 (C-19), and a monoclonal antibody (DC17) and polyclonal antibody (C-8) to CDK5, were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A monoclonal antibody (9A4H8D3) to the catalytic subunit of μ-calpain, which requires μM levels of Ca^{2+} for activation, was purchased from Funakoshi (Tokyo, Japan). Alkaline phosphatase-conjugated anti-mouse immunoglobulin and peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulin were obtained from DAKO (Glostrup, Denmark). Calpain inhibitor I and cytosine-1-β-D(+)-arabinofuranoside (Ara-C) were obtained from Wako Chemicals (Osaka, Japan). DNase I, a calpain inhibitor peptide, and purified m-calpain were purchased from Sigma. Trypsin was obtained from Life Technologies, Inc., and E64 was obtained from Peptide Institute Inc. (Osaka, Japan). Hydroxyapatite Bio-Gel HT was obtained from Bio-Rad, and histone H1 was obtained from Roche Molecular Biochemicals. Sepharose CL-4B and a Superose 6 column were purchased from Amersham Pharmacia Biotech. The Coomassie protein assay reagent and bovine serum albumin were obtained from Pierce. All other chemicals were reagent grade.

Preparation of the Rat Brain Extract—The brains of 10-week-old Wistar rats (Japan-SLC, Tokyo) were homogenized in 10 volumes of HEPES buffer (20 mM HEPES, pH 7.4, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EGTA) at 4 °C using a glass homogenizer and Teflon pestle. The homogenate was centrifuged at 10,000 × *g* for 15 min, and the resulting supernatant was used as the crude extract.

Cleavage of p35 to p25 was induced in the crude extract by incubating the extract with approximately 0.1–5 mM $CaCl_2$ at 37 °C. The proteolytic reaction was stopped by adding 10 mM EGTA and boiling the extract in SDS sample buffer (34).

Column Chromatographic Fractionation of CDK5—The crude extract was centrifuged at 100,000 × *g* for 60 min, and the pellet was suspended in HEPES buffer supplemented with 0.75 M NaCl. After being cleared by centrifugation at 100,000 × *g* for 60 min, the supernatant was subjected to CDK5 fractionation with a hydroxyapatite or gel filtration column. After the hydroxyapatite column (1.5 × 6 cm) had been washed with 10 mM phosphate buffer, the proteins were eluted from the column with a 10–200 mM linear gradient of Na_3PO_4 (pH 6.8) containing 0.5 M KCl, 5 mM EDTA, 5 mM EGTA, and 1 mM $MgCl_2$. For gel filtration, the high salt extract was concentrated by 50% saturated ammonium sulfate precipitation, suspended in a small volume of HEPES buffer supplemented with 0.5 M NaCl, and dialyzed against the same buffer. The dialyzed fraction was filtered through a Superose-6 column that had been equilibrated with HEPES buffer supplemented with 0.3 M NaCl.

Cell Cultures and Preparation of Cell Extracts—Rat brain cortical neurons were prepared from 17-day-old embryos as described previously (19). Cell death was induced by long term cultivation without medium change from day 6 or 7 or by the addition of 0.5 μM staurosporine (a protein kinase inhibitor) at day 6. Cultured brain neurons were collected and lysed in a solution containing 20 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.1% Nonidet P-40, 20 mM NaF, 20 mM β-glycerophosphate, 1 mM dithiothreitol, 10 mg/ml leupeptin, and 100 μM E-64. The homogenate was centrifuged at 10,000 × *g* for 15 min, and the supernatant was used in the CDK5 kinase activity assay. For SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the neurons were dissolved by sonication in SDS-PAGE sample buffer and boiled for 5 min.

CDK5 Kinase Activity Assay—The brain (350 μg), crude (50 μg), or cultured neuron (50 μg) extracts were prepared in 100 μl of 20 mM MOPS, pH 7.2, containing 0.3 M NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 μg/ml leupeptin, and 1 mM dithiothreitol. Anti-CDK5 antibody C8 (2 μl) was added to the extracts; after 1 h of incubation at 4 °C, 10 μl of a 50% slurry of protein A-Sepharose CL-4B was added to the mixture. After further incubation for 10 h at 4 °C, the beads were removed from the extracts, washed twice with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween-20 and three times with 20 mM MOPS, pH 7.2, 1 mM $MgCl_2$, 1 mM EGTA, and 0.1 mM EDTA. The beads were used for phosphorylation of histone H1 by incubation at 30 °C with 20 mM MOPS, pH 7.2, 1 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP, and 0.4 mg/ml histone H1. The reaction was terminated by the addition of SDS-PAGE sample buffer, followed by 5 min of boiling. The samples were electrophoresed on a 12.5% polyacrylamide gel, and the gel was autoradiographed with RX-U x-ray film or analyzed with a BAS2000 bioimage analyzer (Fuji Film, Tokyo Japan).

Solubility and Molecular Size Determination of p35·CDK5 and p25·CDK5—The crude brain extract was incubated at 37 °C for 30 min in the presence or absence of $CaCl_2$ and centrifuged at 100,000 × *g* for 60 min. The pellet was suspended in HEPES buffer, and p35 and p25 were analyzed by Western blotting using anti-p35 antibody.

The crude extract (incubated in the presence or absence of $CaCl_2$) was applied to a Sepharose CL-4B gel filtration column (1 × 53 cm) that had been equilibrated with HEPES buffer and fractionated into 0.5-ml fractions. The elution positions of p35·CDK5 and p25·CDK5 were estimated by Western blotting using anti-p35 and anti-CDK5 (C-8) antibodies and the CDK5 kinase activity assay. Blue dextran and bovine serum albumin were used as molecular mass markers for the void volume and 68 kDa, respectively.

Detection of Neuronal Cell Death—Cell death was assessed by assaying lactate dehydrogenase (LDH) activity and staining for terminal deoxytransferase-mediated dUTP nick end-labeling (TUNEL). The LDH activity released into the culture medium was measured from day 6 or 7 (the last day, the medium was changed or staurosporine was added) using the LDH cytotoxic test (Wako Chemicals). For TUNEL staining, cultured neurons were fixed in 3.5% formaldehyde for 30 min and permeabilized with 0.5% Triton X-100 in phosphate-buffered saline for 5 min. After the cells were washed three times with phosphate-buffered saline, TUNEL staining was performed using the *In situ* Cell Death Detection Kit, POD (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's protocol. Cells were observed with an AXIOSKOP fluorescence microscope (Zeiss, Jerna, Germany), and the extent of cell death was measured by dividing the number of dead cells by the total number of neuronal cells.

SDS-PAGE, Western Blots, and Protein Concentration Determination—Laemmli's method of SDS-PAGE was performed using 12.5 or 7.5% polyacrylamide gels (35). For Western blot analyses, about 40 μg of proteins were loaded in each lane, and the proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The blots were probed with primary antibodies (anti-p35, anti-CDK5, or anti-μ-calpain), followed by alkaline phosphatase-conjugated anti-mouse IgG or peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies. The reactions were detected using the BCIP/NBT phosphatase substrate system (KPL, Gaithersburg, MD) or the ECL Western blotting system (Amersham Pharmacia Biotech). Protein concentrations were determined with the Coomassie protein assay reagent (Pierce).

RESULTS

Cleavage of p35 to p25 during Column Chromatography—After purification from bovine or porcine brains, CDK5 has been shown to consist of a 32-kDa catalytic subunit and a 23–26-kDa regulatory subunit, known as p25 (23, 28, 33). p25 is the proteolytic C-terminal fragment of p35 (9, 10, 12). There have been no reports of successful purification of CDK5 complexed with p35. We have previously purified CDK5 from porcine brains (28), where several hours elapsed between slaughter and tissue homogenization. This delay could have induced nonspecific activation of proteases, which could explain the p25 detected in the porcine brain extracts when it was prepared (data not shown). In contrast, when we prepared a rat brain homogenate immediately after decapitation in the present study, p35 was a dominant species of the CDK5 activator (Fig. 1, *lane 1*). However, when high salt extracts of these brains were fractionated on hydroxyapatite and gel filtration columns, p25 became a major species of the CDK5 activator. *Lanes 2* and *3* in Fig. 1 show the Western blotting of a peak fraction of the histone H1 kinase activity of CDK5 on hydroxyapatite and Superose 6, respectively, probed with the anti-p35 antibody. Notably, all p35 was converted to p25 after fractionation

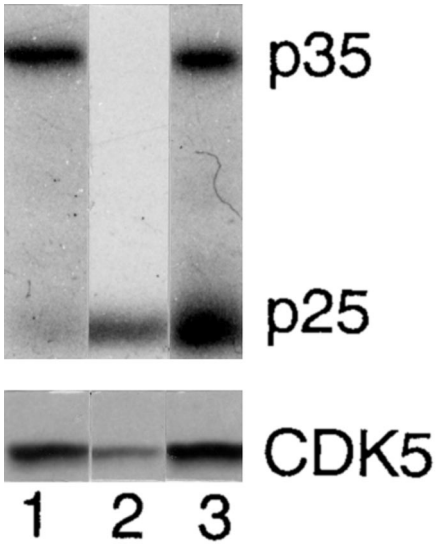

FIG. 1. **Molecular weight of the CDK5 activator in the rat brain extract and the kinase active fractions of CDK5 on the hydroxyapatite and gel filtration columns.** The rat brain extract (*lane 1*) and the peak fraction of CDK5 activity of the hydroxyapatite column (*lane 2*) and the Superose 6 gel filtration column (*lane 3*) were examined by Western blotting using an anti-p35 antibody (*upper panel*) and anti-CDK5 antibody (*lower panel*) after SDS-PAGE. After column chromatography, p35, which was a predominant species in the brain extract, was cleaved to p25.

through the hydroxyapatite column (Fig. 1, *lane 2*).

Proteolytic Cleavage of p35 to p25 by Calpain in Vitro—After observing complete cleavage of p35 during fractionation on a hydroxyapatite column, we suspected that the p35 was cleaved by the Ca^{2+}-activated protease, calpain. When the crude extract was incubated at 37 °C with 0.1 mM $CaCl_2$ (data not shown) or 1 mM $CaCl_2$ (Fig. 2, *lanes 4–6*), p35 was gradually converted to p25. This p25 showed an identical electrophoretic mobility to the p25 detected after column chromatography. The amount of 32-kDa CDK5 protein, however, did not change over the incubation period (Fig. 2, *lower panel*), indicating that the Ca^{2+}-dependent cleavage of p35 to p25 was a selective, but not a general, degradative process. To test the involvement of calpain in this cleavage, a calpain inhibitor peptide (derived from an active domain of calpastatin, an endogenous calpain inhibitor) was added to the brain extract before the Ca^{2+}. As shown in Fig. 2, *lanes 7–9,* the cleavage of p35 was suppressed by the calpastatin peptide. These results suggest that p35 was cleaved to p25 either directly by calpain or indirectly by one or more proteases activated with calpain.

To distinguish between these two possibilities, we first separated the p35·CDK5 complex from calpain. After we had centrifuged the crude extract at 100,000 × *g* for 60 min, the calpain remained in the supernatant (Fig. *A*, *lane 1*) and the p35·CDK5 complex recovered in the pellet could not be cleaved by incubation with $CaCl_2$ (Fig. 3*A*, *lane 2*, and *B, lanes 1–3*). Addition of a purified preactive form of m-calpain, which requires mM levels of Ca^{2+} for activation, could not induce cleavage either (Fig. 3*B, lanes 4–6*), but in the presence of both $CaCl_2$ and m-calpain, the p35 was cleaved to p25 (Fig. 3*B, lanes 7–9*). It is possible that a calpain-activated protease in the pellet cleaved p35 to p25. However, immunoprecipitated p35·CDK5 has also been shown to be cleaved to p25·CDK5 by purified calpain.[2] Considered together, these results indicate that p35 is cleaved directly to p25 by calpain.

The Cleavage of p35 to p25 by Calpain in Cultured Neurons—We also examined whether the cleavage of p35 to p25 in cultured neurons is also catalyzed by calpain. Embryonic rat brain neurons in culture were treated with a Ca^{2+} ionophore (A23187) to activate endogenous calpain. Addition of A23187 to the culture medium, alone and in combination with $CaCl_2$, induced the cleavage of p35 to p25 (Fig. 4*A, lanes 5–12*). This proteolysis was suppressed by 60 μM calpain inhibitor I (Fig. 4*B*), indicating that this cleavage is also mediated by calpain.

[2] R. Onuki, T. Saito, and S. Hisanaga, manuscript in preparation.

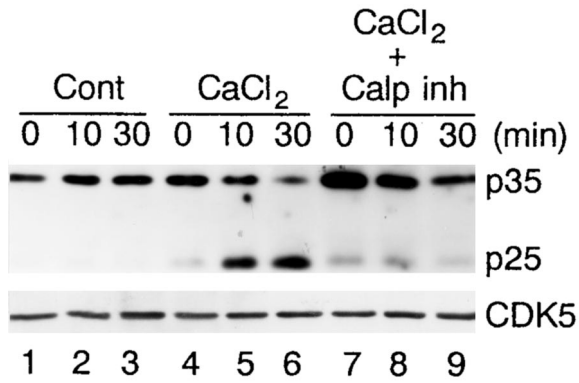

FIG. 2. **Calpain-dependent cleavage of p35 to p25 in the brain extract.** The rat brain extract was incubated in the presence of 1 mM $CaCl_2$ (*lanes 4–6*) or 1 mM $CaCl_2$ and 10 μM calpain inhibitor peptide (*lanes 7–9*) at 37 °C for 0, 10, and 30 min, and Western blotting with anti-p35 antibody (*upper panel*) and anti-CDK5 antibody (*lower panel*) was performed. The controls (*Cont*) (incubation of the brain extract alone) are shown in *lanes 1–3*.

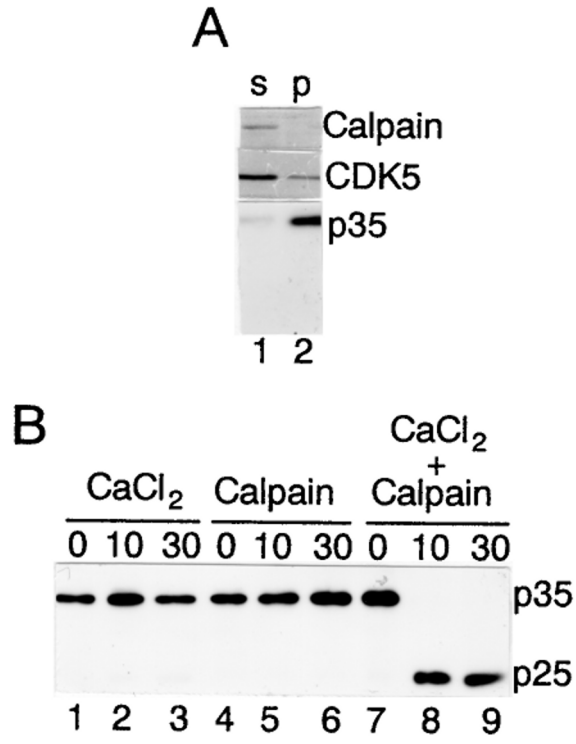

FIG. 3. **Cleavage of p35 to p25 by purified calpain *in vitro*.** *A,* separation of p35·CDK5 from calpain in the brain extract by centrifugation. The rat brain extract was centrifuged at 100,000 × *g* for 60 min, and the supernatant (*lane 1, s*) and the pellet (*lane 2, p*) were examined by Western blotting with anti-calpain (*upper panel*), anti-CDK5 (*middle panel*), and anti-p35 (*lower panel*) antibodies. The p35 and calpain were separated in the pellet and supernatant fractions, respectively. *B,* the pellet was suspended in HEPES buffer and incubated at 37 °C for 0, 10, and 30 min in the presence of 5 mM $CaCl_2$ (*lanes 1–3*), 10 microunits/μl purified calpain (*lanes 4–6*), or 5 mM $CaCl_2$ and 10 microunits/μl m-calpain (*lanes 7–9*). The p35 was degraded to p25 only when both $CaCl_2$ and calpain were present.

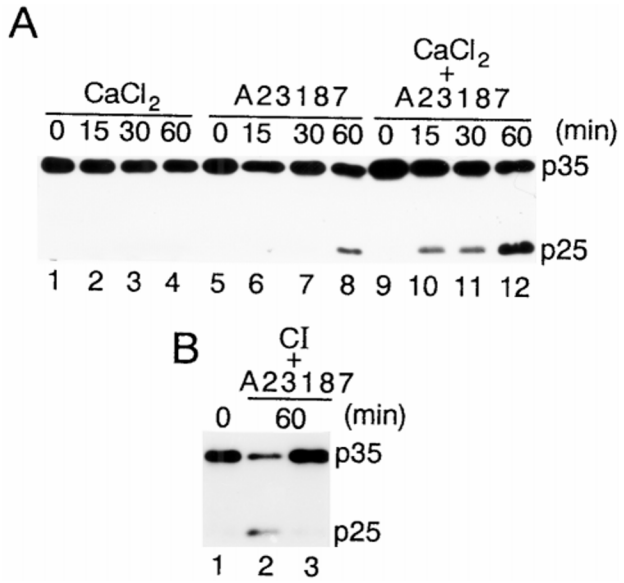

FIG. 4. **Cleavage of p35 to p25 by calpain in cultured neurons.** *A,* embryonic rat brain neurons cultured for 7 days were treated with 2.5 mM $CaCl_2$ (*lanes 1–4*), 5 μM A23187 (*lanes 5–8*), or both 5 μM A23187 and 2.5 mM $CaCl_2$ (*lanes 9–12*) for 0, 15, 30, and 60 min. At the end of the treatment, the neurons were collected, suspended in SDS-PAGE sample buffer, and boiled for 5 min. Each sample was run on a 10% SDS-PAGE gel and blotted with anti-p35 antibody. Cleavage of p35 to p25 was induced by the Ca^{2+} ionophore treatment. *B,* cleavage was suppressed by calpain inhibitor I. The cultured neurons were treated with 5 μM A23187 and 2.5 mM $CaCl_2$ in the absence (*lane 2*) and presence (*lane 3*) of 60 μM calpain inhibitor I (*CI*). Degradation of p35 to p25 was suppressed by calpain inhibitor I.

Overall, these results suggest that p35 is cleaved to p25 by calpain in both the brain extract and the cultured neurons.

The Cleavage of p35 to p25 Changed the Solubility of CDK5—To address what property of CDK5 is changed by the cleavage of p35 to p25, we examined the effect of p35 cleavage on the kinase activity of CDK5. Previous studies have not directly compared the kinase activity of brain p35·CDK5 and p25·CDK5, although kinase activity has been observed in p35·CDK5 complexes prepared from brain extracts by immunoprecipitation (9, 30). We prepared p35·CDK5 and p25·CDK5 complexes from a brain extract by immunoprecipitation before and after Ca^{2+} treatment, respectively, and the kinase activity in the extract was measured using histone H1 or dephosphorylated NF-H as substrate. Each substrate generated nearly equal kinase activity (Fig. 5 and data not shown).

As previously reported (27) and as shown in Fig. 3, almost all p35 and part of the CDK5 was detected in the pellet fraction (also shown in Fig. 6*A*). Most of the CDK5 in the supernatant might correspond to a free monomeric form of CDK5 that has been previously described (30, 34), although the CDK5 in the pellet was shown to be associated with p35 by immunoprecipitation (data not shown). When the 100,000 × *g* pellet was suspended in 1% Triton X-100 or HEPES buffer supplemented with 0.75 M NaCl, the p35·CDK5 complex was solubilized to some extent by Triton X-100 and completely solubilized by the NaCl (Fig. 6*A*). This suggests that p35·CDK5 binds to membranous organelles or to the cytoskeleton, so the effect of cleavage on the association was examined. Incubation of the crude extract with 1 mM $CaCl_2$ induced the cleavage of most of the p35, resulting in p25·CDK5 with a small amount of p35·CDK5. After centrifugation at 100,000 × *g* for 30 min, p25 was detected mainly in the supernatant (Fig. 6*B, lane 1*), whereas p35 could still be recovered in the pellet (Fig. 6*B, lane 4*). After incubation with Ca^{2+}, most of CDK5 was detected in the pellet fraction (Fig. 6*B, lane 2*). This was a nonspecific aggregate of CDK5 induced by incubation. Monomeric soluble CDK5, which remained in the supernatant of the control (Fig. 6*B, lane 3*), precipitated after incubation at 37 °C in the presence of Ca^{2+} (Fig. 6*B, lane 6*).

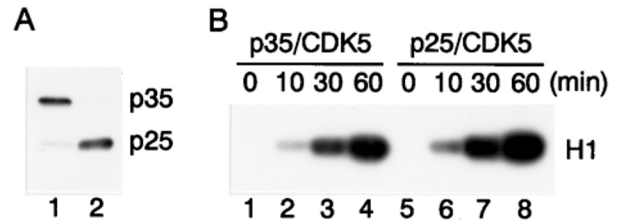

FIG. 5. **The effect of p35 cleavage to p25 on the kinase activity of CDK5.** After p35 cleavage to p25 in the rat brain extract by incubation in the presence of $CaCl_2$ at 37 °C for 40 min, p25·CDK5 was immunoprecipitated using anti-CDK5 antibody (C-8). For comparison, p35·CDK5 was prepared similarly but without the addition of $CaCl_2$. Western blotting with anti-p35 antibody confirmed that all of the p35 was converted to p25 and that similar amounts of p35 and p25 were recovered in the immunoprecipitates (*A*). p35·CDK5 and p25·CDK5 were assayed for histone H1 kinase activity at 37 °C for 0, 30, 60, and 120 min (*B*). They showed similar levels of kinase activity.

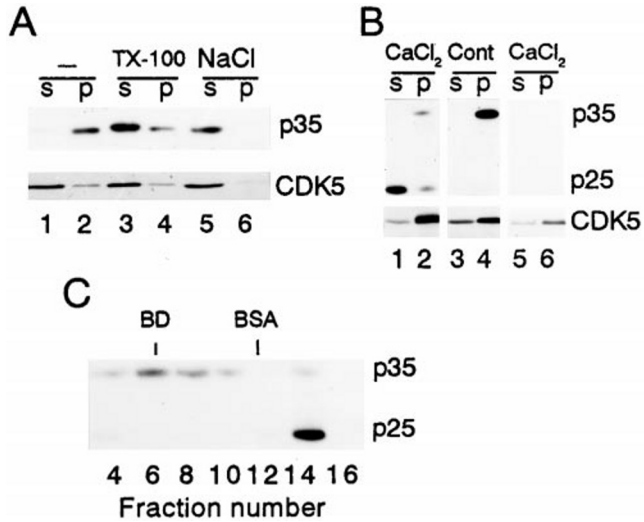

FIG. 6. **The cleavage of p35 to p25 changed the solubility of the p35·CDK5 complex.** *A,* solubilization of p35 by detergent or high salt. The 100,000 × *g* pellet of the brain extract was suspended in HEPES buffer (*lanes 1* and *2*), HEPES buffer containing 1% Triton X-100 (*lanes 3* and *4*), or 0.75 M NaCl (*lanes 5* and *6*) and centrifuged again at 100,000 × *g* for 60 min. The resulting supernatants and pellets were subjected to Western blotting with anti-p35 antibody (*upper panel*) or anti-CDK5 antibody (*lower panel*). Most of the p35 protein was recovered from the supernatant in the presence of 1% Triton X-100, and all of the p35 was recovered from the supernatant in the presence of 0.75 M NaCl. *B,* cleavage of p35 to p25 changed the solubility of the protein. The p35 in the brain extract was cleaved to p25 by incubation at 37 °C for 30 min in the presence of 1 mM $CaCl_2$ (*lanes 1* and *2*) and centrifuged at 100,000 × *g* for 60 min. The supernatants and pellets were analyzed by Western blotting with anti-p35 antibody (*upper panel*) and anti-CDK5 antibody (*lower panel*). Control samples were treated similarly, but without addition of $CaCl_2$ (*lanes 3* and *4*). Most of the p25 remained in the supernatant, whereas the p35 collected in the pellet. The supernatant of the control, which contained a soluble form of CDK5 alone, was incubated in the presence of $CaCl_2$ for 30 min at 37 °C and centrifuged again (*lanes 5* and *6*). Monomeric CDK5 pelleted after incubation. *C,* the elution profile of p35 and p25 after gel filtration. After being incubated in 1 mM $CaCl_2$ at 37 °C for 30 min, the brain extract was gel-filtered on Sepharose CL-4B. Whereas p35 eluted at fraction 6, p25 eluted at approximately fraction 14 and had a slightly smaller molecular size than bovine serum albumin (*BSA*) (68 kDa). The void volume fraction is shown by the blue dextran elute (*BD*).

These results suggest that cleavage of p35 to p25 released p35 from structures present within the pellet, but it is also possible that the calpain cleaved the large cellular structures

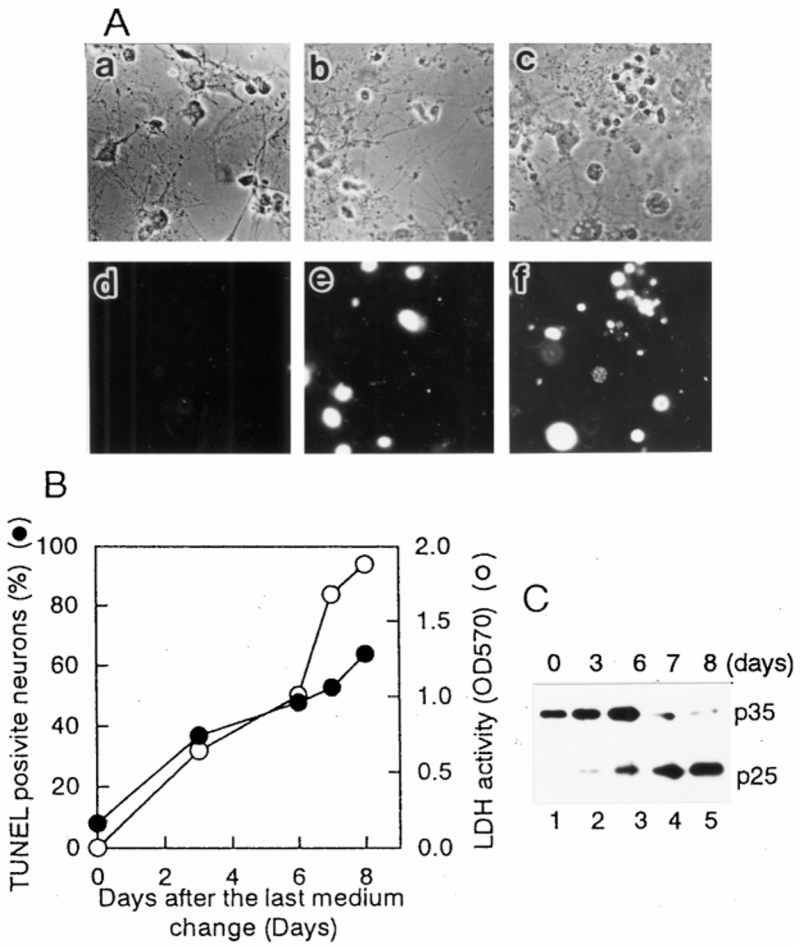

FIG. 7. **Increased cleavage of p35 to p25 in neurons undergoing cell death.** Embryonic brain neurons were maintained for 7 days after plating and further cultured for 8 days without medium change. The neurons were monitored by TUNEL staining and LDH activity assays. *A,* TUNEL staining (*d–f*) and the corresponding phase contrast micrographs (*a–c*): *a* and *d,* just after the last medium change; *b* and *e,* 6 days after the last medium change; *c* and *f,* 8 days after the last medium change. *B,* ratio of TUNEL positive neurons (*closed circles*) and LDH activity detected in the culture medium (*open circles*). *C,* degradation of p35 to p25.

(to which p25·CDK5 was bound) to small pieces. To exclude the latter possibility, we measured the size of p25·CDK5 by gel filtration on Sepharose CL-4B. The control (p35 in the untreated extract) was eluted at the void volume (Fig. 6*C*), indicating again that the p35·CDK5 complex may be associated with large cellular components (30). In contrast, p25 was eluted at molecular mass fractions that were almost the same as or were smaller than than bovine serum albumin (68 kDa) (Fig. 6*C*). Considering that the combined molecular mass of p25 and CDK5 is 57 kDa, this elution profile indicated that the p25·CDK5 complex may be present in a free soluble form.

Occurrence of the Cleavage of p35 to p25 at the Time of Neuronal Cell Death—A small but nearly constant amount of p25 was in the rat brain during development of the embryo through to the adult stages (data not shown). To examine the significance of this low level of p25 expression, we searched for cellular conditions where stimulated degradation could be observed. An increased amount of p25 was observed in culture dishes in which neurons were dying as a result of long term cultivation without a change in medium. Because calpain is reported to be activated during apoptotic cell death (36–39), we suspected a link between the cleavage of p35 to p25 and neuronal cell death. We induced neuronal cell death by long term culture of the neurons without a change in medium or by the addition of staurosporine to the culture medium. Embryonic rat brain cortical neurons, maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum and 5% horse serum for 7 days, were further cultured in the same medium without further exchanges. The LDH activity in the medium increased gradually from 3 days after the last medium change (Fig. 7*B*), as did the number of dead cells, identified by TUNEL staining (Fig. 7, *A* and *B*). Under these apoptotic conditions, increased degradation of p35 to p25 occurred simultaneously with the increase in the number of dead neurons (Fig. 7*C*).

Cleavage of p35 to p25 was also observed in the cultured neurons undergoing cell death induced by staurosporine (Fig. 8). Addition of 0.5 μM staurosporine to the culture medium at day 6 induced DNA fragmentation, as shown by TUNEL staining at 48 h (Fig. 8*A*). Cleavage of p35 to p25 was also detected at 24 h and increased at 48 h after addition of staurosporine (Fig. 8*B*).

DISCUSSION

In brains, the major form of the CDK5 activator is the 35-kDa protein known as p35 (9, 10, 12). However, when CDK5 has been purified from bovine or porcine brains, it has been bound to p25, a 25-kDa activating subunit that is a truncated form of p35 (23, 28, 33). In previous studies using the crude extracts of porcine brains as a starting material for CDK5 purification (28), p35 has already been partially cleaved to p25 (data not shown). This might be due to uncontrolled postmortem degradation in the slaughterhouse prior to homogenization of the tissue in the laboratory. Porcine or bovine brain CDK5 has also been purified from soluble supernatants that predominantly contain the p25·CDK5 complex (23, 28, 33). These observations could partially explain why attempts to purify CDK5 have produced only p25·CDK5. We thought it a possibility to use rats' brains to circumvent postmortem degradation. Although the purification of CDK5 from rat neuronal tissue (the spinal cords) was reported, the activation subunit associated with it was not demonstrated (27).

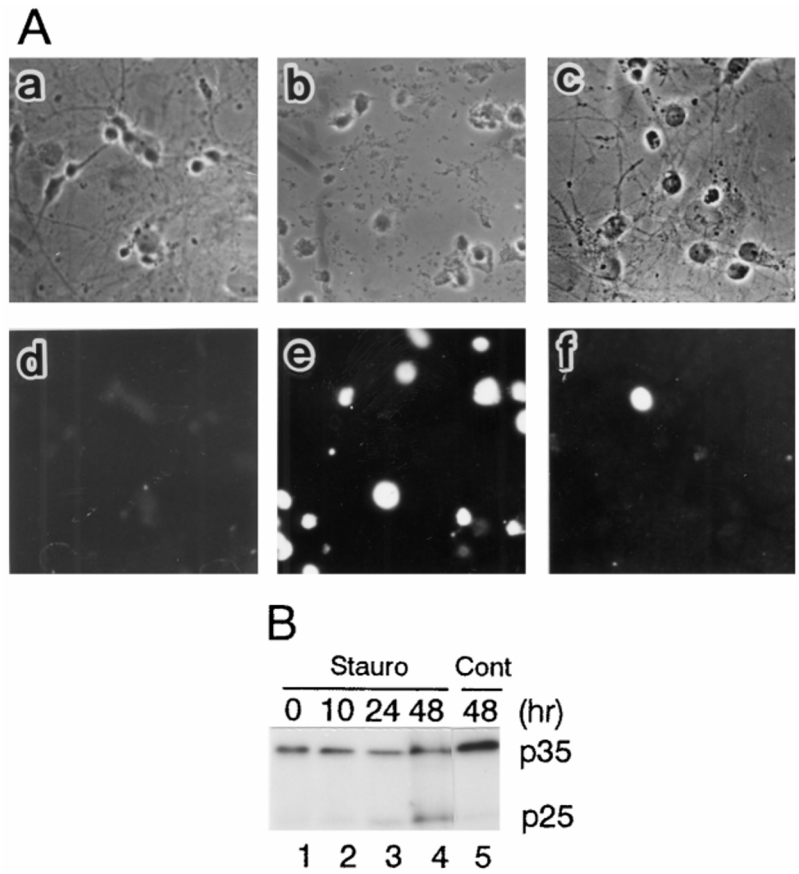

FIG. 8. **Cleavage of p35 to p25 in staurosporine-induced apoptotic neurons.** Staurosporine (0.5 μM) was added to the brain neurons after 6 days in culture. *A,* induction of apoptotic cell death was confirmed by TUNEL staining (*d–f*). The corresponding phase contrast micrographs are shown in *a–c*. *a* and *d,* before addition of staurosporine; *b* and *e,* 48 h after staurosporine addition; *c* and *f,* control neurons 48 h after addition of dimethyl sulfoxide (a solvent of staurosporine). *B,* an increased amount of p25 was evident in neurons undergoing cell death after Western blotting with anti-p35 antibody (*lane 4*).

In the present study, in which rat brain homogenates were prepared immediately after decapitation and probed using an anti-p35 antibody, most of the CDK5 activator detected was p35, with only tiny amount of p25. Using either the rat brain extract or cultured neurons, p35 was shown to be proteolytically cleaved to p25 by calpain, both *in vivo* and *in vitro*. This cleavage did not affect the kinase activity of CDK5 for histone H1 or dephosphorylated NF-H but changed the solubility of the active CDK5 complex. Whereas the p35·CDK5 complex was present in the pellet after ultracentrifugation, the proteolytic form of p25·CDK5 remained in the supernatant as a soluble form. Stimulated cleavage was also observed in the cultured neurons undergoing cell death.

Direct cleavage of p35 to p25 by calpain was indicated by following results. (i) Degradation of p35 to p25 was induced by treatment of the cultured neurons or brain extracts with Ca^{2+}, and in both cases, the Ca^{2+}-induced cleavage was suppressed by calpain inhibitors (Figs. 2 and 4). (ii) Separation of the p35·CDK5 complex from calpain in the brain extract by centrifugation ablated the proteolytic conversion from p35 to p25 (Fig. 3). (iii) p35, prepared by either centrifugation (to remove the calpain) or immunoprecipitation, was proteolysed to p25 by purified calpain *in vitro* (Fig. 3).[2] Although this cleavage was only partial, another cytoplasmic protease called the proteasome has recently been shown to completely degrade p35 (18, 19). In the present study, the cleavage of p35 was stimulated at the time of cell death (Fig. 8), when a series of caspases are specifically activated. Although we did not examine cleavage by caspases in detail, caspase-dependent cleavage of p35 is unlikely because the proteolytic consensus sequence for these caspases is the C terminus of Asp (40). This is distinct from the cleavage site of p35, which is between Phe-98 and Ala-99. Taken together, the results outlined above indicate that p35 is cleaved directly to p25 by calpain.

The cleavage of p35 to p25 was increased in cultured neurons undergoing cell death induced by staurosporine treatment or long term cultivation without a change in medium. The involvement of calpain in apoptotic cell death remains a controversial issue. However, calpain activation does appear to play a role in some types of apoptotic cell death (36–39). The present study suggests neuronal death induced by long term culture without a change in medium is mediated by apoptosis, as evidenced by TUNEL staining of the neuronal nuclei (although we did not perform further studies to confirm the role of apoptosis). Straurosporine-induced neuronal cell death has been shown to be apoptotic (41, 42). Thus, our results support the activation of calpain during apoptotic neuronal cell death. To investigate whether the cleavage of p35 is a cause of cell death or results from cell death, we followed cleavage of p35 over a time course relative to the progression of cell death. However, we could not determine the role p35 plays in cell death because, under both experimental conditions, the cleavage occurred almost simultaneously with the appearance of dead neurons. As there have been various reports about the time of activation of calpain in relation to the activation of caspase (36–39), this is an area that needs to be addressed in the future.

There is a hypothesis that the ectopic activation of cell cycle promoters in postmitotic neurons triggers neuronal cell death (43, 44). If this hypothesis is correct, how can neurons survive when active CDK5, the kinase activity of which is very similar to that of cyclin B/cdc2 kinase, is expressed (28, 45)? Taking the present results into consideration, the following might become possible: CDK5 could be sequestered to a cytoskeletal or membrane fraction that would limit substrate availability or suppress the kinase activity in living neurons (3, 31). This localization could keep neurons alive, even when active CDK5 is expressed. So far, the substrate proteins identified have been cytoskeletal and membranous proteins, including tau, neuro-

filament H and M subunits, synapsin I, and p67^{munc18} (23–30, 46). Upon the onset of apoptosis, however, p35 was cleaved to p25 by activated calpain, resulting in the release of active CDK5 from the particulate fraction to the free soluble form that could be accessible to any proteins the phosphorylation of which is harmful for postmitotic neurons. The involvement of CDK5 in neuronal cell death was suggested in a recent report by Philpott *et al.* (47). This report showed that overexpression of p35 induced cell death in *Xenopus* embryos. A small amount of p25, which was detected in the present study in brain homogenates prepared immediately after decapitation of rats (Fig. 1), might be generated in apoptotic neurons in the brain. If this is correct, it could be possible to estimate the ratio of neurons undergoing apoptotic cell death by measuring the ratio of p25 to p35.

During the revision of this paper, similar results were reported by Patrick *et al.* (48). They demonstrated the active role of p25·CDK5 in promoting cell death by expressing the p25·CDK5 complex in cultured neurons. Their result that cleavage of p35 to p25 changed subcellular distribution from a membrane bound fraction to a cytosolic fraction agrees well with ours. Moreover, they found accumulation of p25 in brains of patients with Alzheimer's disease and hyperphosphorylation of tau by p25·CDK5. Taken together, these results indicate that calpain could be the protease that cleaves p35 to p25 in pathologically degenerative neurons.

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