Suppression of Calpain-dependent Cleavage of the CDK5 Activator p35 to p25 by Site-specific Phosphorylation*

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Cdk5 is a proline-directed Ser/Thr protein kinase predominantly expressed in postmitotic neurons together with its activator, p35. N-terminal truncation of p35 to p25 by calpain results in deregulation of Cdk5 and contributes to neuronal cell death associated with several neurodegenerative diseases. Previously we reported that p35 occurred as a phosphoprotein, phospho-p35 levels changed with neuronal maturation, and that phosphorylation of p35 affected its vulnerability to calpain cleavage. Here, we identify the p35 residues Ser8 and Thr138 as the major sites of phosphorylation by Cdk5. Mutagenesis of these sites to unphosphorylatable Ala increased susceptibility to calpain in cultured cells and neurons while changing them to phosphomimetic glutamate-attenuated cleavage. Furthermore, phosphorylation state-specific antibodies to these sites revealed that Thr138 was dephosphorylated in adult rat, although both Ser8 and Thr138 were phosphorylated in prenatal brains. In cultured neurons, inhibition of protein phosphatases converted phosho-Ser8 p35 to dual phospho-Ser8/Thr138 p35 and conferred resistance to calpain cleavage. These results suggest phosphorylation of Thr138 predominantly defines the susceptibility of p35 to calpain-dependent cleavage and that dephosphorylation of this site is a critical determinant of Cdk5-p25-induced cell death associated with neurodegeneration.

Cyclin-dependent kinase 5 (Cdk5)2 is a unique member of the Cdk family. Its activity in postmitotic neurons is completely dependent upon association with one of two neuronal specific activators, p35 or p39. Cdk5/p35 is involved in a panoply of processes critical to central nervous system function both during development and throughout maturity including neuronal migration during corticogenesis, neurite outgrowth, regulation of the synaptic vesicle cycle, neurotransmitter release, and postsynaptic neurotransmitter receptor regulation and signaling (1–3). The mechanisms by which Cdk5 activity is normally regulated remains to be fully delineated. Furthermore, because aberrant Cdk5 activity has been implicated in the etiology of neurodegenerative diseases (4, 5), identifying the biochemical mechanisms contributing to deregulation of Cdk5 is of substantial biomedical relevance.

Deregulation of Cdk5 results from removal of the first 98 amino acids of p35 by the Ca2+-dependent cysteine protease, calpain, leaving Cdk5 associated with the N-terminal truncated form p25. Cleavage of p35 to p25 changes the subcellular distribution of active Cdk5 from membranes to the cytosolic fraction (6, 7), thereby altering substrate specificity. p25 accumulates in neurons undergoing various types of cell death (6–9). Expression of Cdk5/p25 in cultured cells results in increased phospho-Tau levels in comparison to cells expressing Cdk5/p35 (6). Furthermore, exogenous overexpression of p25 in transgenic mice results in a neurodegenerative phenotype including the formation of paired helical filaments, Tau aggregation, and neuronal loss similar to that observed in Alzheimer disease (10, 11).

Cdk5/p25 has also been implicated in ischemia-induced neuronal loss in the hippocampus via increased phosphorylation of the NR2A subunit of the N-methyl-d-aspartic acid receptor (12). In addition, several recent reports indicate that Cdk5-p25 mediates cell death via translocation to the nucleus (13–15). p25 generation increases nuclear Cdk5 activity in cultured neurons, facilitating phosphorylation and inhibition of the pro-survival transcription factor MEF2 (13, 15, 16). Aberrant Cdk5 activity may also contribute to neuronal cell death via phosphorylation of other survival factors such as the tumor suppressor protein p53 (17) and retinoblastoma protein (14).

Ca2+-dependent activation of the cytoplasmic protease calpain is involved in apoptotic and necrotic cell death (18). Calpain generally recognizes motifs between conformational domains and cleaves substrate proteins in a limited manner, although the physiological function of calpain activity remains unclear. In some cases calpain cleavage is suspected to be a signaling process. Calpain-mediated cleavage of many proteins including neurofilament proteins (19), αI-spectrin (20), NR2 subunits of N-methyl-d-aspartic acid receptors (21), and ezrin (22) is suppressed through phosphorylation. However, how such signaling works and the mechanisms of phosphorylation-dependent inhibition are unknown.

Previously we demonstrated that Cdk5 phosphorylated p35,
that p35 occurred as a phosphoprotein in neurons, and that the phosphorylation state of p35 affected its susceptibility to calpain cleavage (23). Phospho-p35 predominates in fetal rat brain and is resistant to the cleavage by calpain, whereas unphosphorylated p35 present during adulthood is more vulnerable to calpain-dependent cleavage. Here we report that Ser8 and Thr138 of p35 serve as the sites of Cdk5-dependent phosphorylation. Furthermore, phosphorylation at these sites reduces the susceptibility of p35 to calpain cleavage. Moreover, specific dephosphorylation of Thr138 increases the susceptibility of p35 to the cleavage by calpain in adult rat brains, suggesting that the phosphorylation of this site is a particularly critical determinant of Cdk5-dependent neuronal cell death in neurodegenerative diseases.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Antibodies**—cDNAs encoding human Cdk5 and p35 within pCMV vectors were provided by Dr. L.-H. Tsai (Harvard Medical School, Boston, MA) (24). Human m-calpain and the rabbit small subunit of calpain encoded within pSRD vectors using SV40 early promoters were described previously (25). Ionomycin, calpain inhibitor IV, and benzoylloxycarbonyl-leucyl-leucyl-leucinal (MG132) were purchased from Calbiochem. Roscovitine, okadaic acid, and bacterial alkaline phosphatase were obtained from Wako Chemicals (Osaka, Japan). The anti-p35 antibody C19 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Cdk5 antibody DC17 was purchased from Calbiochem. The anti-calpain-cleaved αII-spectrin antibody and the anti-active m-calpain antibody were described previously (26, 27). Horseradish peroxidase-conjugated or alkaline phosphatase-conjugated secondary antibody were from DAKO (Glostrup, Denmark). The enhanced chemiluminescence (ECL) detection system was from Amersham Biosciences (Tokyo, Japan) and the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium phosphatase substrate system was from KPL (Gaithersburg, MD).

**Plasmid Construction of Mutant p35**—The Ala mutants at Ser8, Thr138, Ser170, or Thr197 (S8A, T138A, S170A, or T197A) were generated using p35 as a template by the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. Primers used were 5′-GTGCGTCCCTGGCTCCACGCTACC-3′ and 5′-GGTACCGTGAGGAGCCAGGACGACGAC-3′ for S8A, 5′-TCCGAGGGGCGCCCAACGGGTCTACCAT-3′ and 5′-ATGACAACGTTTGGGCACCCCCGTCGCA-3′ for T138A, 5′-CTGAAGCACCTGGCCATGACCCGTTTGGG-3′ and 5′-GGGTCCTGGTGGGCCAGGTCTCAG-3′-3′ for S170A, and 5′-GGGTTTCTTCGCGGAGCAGGTACGACGACGAC-3′ for T197A. Two-dimensional phosphopeptide map analysis using a thin layer cellulose (TLC) plate (Merck, Darmstadt, Germany) (29).

**Preparation of Antibodies against Phosphorylated Ser8 (Anti-Ser8(P)) and Thr138 (Anti-Thr138(P)) of p35**—Non-phosphorylated and phosphorylated peptides, “LSLPSPSYRKRC” and “LSLPSPSYRKRC” for anti-Ser8(P), and “ITSAGTPKVRIC” and “ITASGpT138(P)”, were chemically synthesized, and the phosphorylated peptides, LSLPSPSYRKRC and ITSAGpTP138KVRIC, were conjugated to keyhole limpet hemocyanin through the cysteine residue attached to the C terminus by AnyGen (Kwang-ju, Korea). Anti-Ser8(P) or anti-Thr138(P) antibody generated by immunizing rabbits was affinity purified by peptide columns, the non-phosphorylated peptide controls, and the phosphorylated peptide positively using SulfoLink (Pierce), according to the manufacturer’s protocol.

**Expression and Metabolic Phosphorylation of p35 in HEK293 Cells**—HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 100 units/ml of penicillin, and 0.1 mg/ml streptomycin. Transfection of Cdk5 and p35 was performed by the Polyfect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. For metabolic phosphorylation of p35, transfected cells were cultured in the presence of 3.7 MBq of [32P]orthophosphate in phosphate-free Dulbecco’s modified Eagle’s medium for 2 h. In some cases, roscovitine (50 μM) was added to inhibit Cdk5 activity 2 h before metabolic labeling. After washing with phosphate-buffered saline, cells were suspended in RIPA buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.15 M NaCl, 1% Nonidet P-40, 0.1% SDS, and 0.1% sodium deoxycholate) containing 10 mM β-glycerophosphate, 5 mM NaF, 0.2 mM Pefabloc SC (Merck, Darmstadt, Germany), 1 μg/ml leupeptin, and 1 mM dithiothreitol, and lysed by freezing and thawing. The extract was obtained as a supernatant of centrifugation at 17,000 × g for 15 min. p35 was isolated from the extract by immunoprecipitation with C19 anti-p35 antibody. 32P incorporation into p35 was detected by a BAS 2000 image analyzer (Fuji Film, Tokyo, Japan) after SDS-PAGE.

**Neuronal Culture, Metabolic Phosphorylation of p35, and Adenoviral Expression of p35**—Cerebral cortical neurons were prepared from rat brains at embryonic day 17 as described previously (28). Neuronal cultures were used 7 days after plating. In some experiments, 0.1 μM okadaic acid was added to the culture medium for the final 2 h of a 4-h labeling period. MG132 (50 μM) was also added 1 h before the addition of okadaic acid to suppress proteosome-dependent degradation of p35. After washing with phosphate-buffered saline, neurons were lysed with RIPA buffer. The supernatant of centrifugation at 17,000 × g was immunoprecipitated with C19 and phosphorylation of p35 was detected as described above. Adenoviral expression vectors encoding p35 or p35S4A were infected into rat primary cortical neurons cultured for 5 days in vitro. Two days after infection, neurons were treated with 5 μM Ca2+/ionophore A23187 for 1 h, and the cleavage of p35 was detected by immunoblotting with anti-p35 antibody (C19).

**Two-dimensional Phosphopeptide Map Analysis**—Metabolically phosphorylated p35 was digested with 50 μg/ml l-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma) in 10 mM (NH4)2CO3 overnight at 37 °C. The digested peptides were subjected to two-dimensional phosphopeptide map analysis using a thin layer cellulose (TLC) plate (Merck, Darmstadt, Germany) (29).

**Expression of Cdk5-p35 in COS-7 Cells, Cleavage of p35 to p25 by Calpain, and Dephosphorylation of p35**—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 0.1 mg/ml streptomycin. Transfection of Cdk5, p35, μ-calpain, and the small subunit of calpain was performed by the Polyfect transfection reagent according to the manufactur-
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RESULTS

p35 Is Phosphorylated at Ser8 and Thr138 by Cdk5—Substantial evidence has been presented to suggest that Cdk5 autophosphorylates its activator p35 in vitro and in cultured cells (24, 30–32). To further investigate this autophosphorylation, we expressed both Cdk5 and p35 in HEK293 cells and cultured them with [32P]orthophosphate for 2 h in the absence or presence of the Cdk5 inhibitor, roscovitine. 32P incorporation was readily detected in p35 immunoprecipitated from the cell extracts by autoradiography (Fig. 1A). The highest levels of radiolabeled p35 were immunoprecipitated from cells cotransfected with Cdk5 (Fig. 1A, lane 4), but not with kinase-dead Cdk5 (Fig. 1A, lane 6). Furthermore, a large portion of the radioactivity associated with p35 could be attenuated by treating the cells with roscovitine (Fig. 1A, lane 5). Interestingly, radiolabeled p35 was detected in cells transfected with p35 alone (Fig. 1A, lane 2). Moreover, treatment of cells transfected with p35 alone with roscovitine reduced radiolabeled p35 to almost undetectable levels (Fig. 1A, lane 3), suggesting that the endogenous Cdk5 was responsible for phosphorylation of p35 in the absence of exogenous Cdk5.

Cdk5 is a proline-directed protein kinase and exhibits the requirement of Pro following the substrate Ser/Thr sites, with a preference for the consensus motif Ser/Thr-Pro-X-His/Lys/Arg. There are only four Ser/Thr-Pro sites, Ser8, Thr138, Ser170, and Thr197, within the primary sequence of p35 (Fig. 1B). Only Thr138 is encompassed within a consensus motif sequence (138TPKR), although Ser8 is also followed by two positively charged amino acid residues (8SPSYRK). Based on these observations, site-directed mutagenesis was used to replace Ser8 or Thr138 with Ala residues and the resulting plasmid was used together with one encoding Cdk5 to cotransfect HEK293 cell cultures. Mutation of Ser8 or Thr138 resulted in a large loss in radiolabel associated with immunoprecipitated p35 (data not shown).

To further characterize the phosphorylation of p35, radiolabeled protein immunoprecipitated from cotransfected cells was next analyzed by two-dimensional phosphopeptide mapping after trypsin digestion (Fig. 1C). Two radioactive spots representing two tryptic phosphopeptides were derived from wild-type p35. The S8A mutation resulted in disappearance of one of these phosphopeptides (spot 1), whereas the T138A mutation caused loss of the other phosphopeptide (spot 2). Taken together, these data indicate that p35 is phosphorylated by Cdk5 in transfected HEK293 cell cultures at two proline-directed sites, Ser8 and Thr138.

Phosphorylation at Ser8 or Thr138 in p35 Suppresses the Calpain-dependent Cleavage of p35 to p25—Previously, we presented data suggesting that Cdk5-dependent autophosphorylation of p35 affected its susceptibility to cleavage by calpain (23). To further investigate the potential role of p35 phospho-
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After 30 min of ionomycin treatment, p25 can be clearly observed, with levels increasing thereafter until 24 h with mild attenuation of p25 levels at 3 h. Based on these results, the 30-min time point with 10 μM ionomycin is shown in Fig. 2A. Further confirmation that calpain-dependent cleavage of p35 is inhibited via autophosphorylation by Cdk5.

To more specifically investigate the regulation of calpain cleavage of p35 by Cdk5-dependent phosphorylation of Ser8 and Thr138, a site-directed mutagenesis approach was again employed in controlling calpain cleavage, p35 and Cdk5 were next exogenously expressed in COS-7 cells. In preliminary analyses, cotransfected COS-7 cells were treated with various concentrations of the Ca2+-ionophores, A23187 or ionomycin, or an inhibitor of intracellular Ca2+ pumps, thapsigargin. For each of these treatments, a small but distinct amount of p25 was found to be produced by immunoblotting the cell lysates (data not shown). However, the largest amount of p35 cleavage was consistently derived using ionomycin. Therefore, 10 μM ionomycin was selected as the method of choice for calpain activation in this paradigm. A time course for p25 generation in COS-7 cells treated with 10 μM ionomycin is shown in Fig. 2A. After 30 min of ionomycin treatment, p25 can be clearly observed, with levels increasing thereafter until 24 h with mild attenuation of p25 levels at 3 h. Based on these results, the 30-min time point with 10 μM ionomycin was chosen for all subsequent experiments as the minimal treatment necessary to induce the cleavage of p35 to p25.

To demonstrate that p25 production in response to ionomycin was indeed calpain-dependent, cells were either left untreated or treated with ionomycin in the absence or presence of 10 μM calpain inhibitor IV (Fig. 2B, lanes 3 and 4). Treatment of cells with the calpain inhibitor resulted in a reduction in p25 levels both in the absence and presence of ionomycin. Furthermore, p35 cleavage was increased substantially by coexpression of μ-calpain (Fig. 2B, lanes 5 and 6). This effect corresponded to increased active μ-calpain levels as assessed in immunoblots using an antibody to this cleaved form of the protease (27). Because the predominant form of endogenous calpain in COS-7 cells is μ-calpain, active μ-calpain was detected only in cultures transfected with the corresponding plasmid encoding μ-calpain (Fig. 2B, second panel). Calpain activity was also assessed by immunoblot analysis of calpain-dependent cleavage of spectrin, a major component of the neuronal cytoskeleton (26). Spectrin cleavage closely mirrored p25 production in response to ionomycin and μ-calpain expression and was similarly blocked by calpain inhibition (Fig. 2B, third panel). These results demonstrate that p25 generation is tightly coupled to calpain activity.

Having established optimal conditions for p25 generation and demonstrated calpain dependence, the effect of phosphorylation on the cleavage of p35 was next assessed (Fig. 3A). Treatment of COS-7 cells expressing p35 and Cdk5 with ionomycin resulted in increased p25 levels, as observed earlier. Pretreatment with roscovitine resulted in increased basal levels of p25, as well as the levels achieved in response to ionomycin (Fig. 3A, compare lane 3 with lane 1 and lane 4 with lane 2). Furthermore, increased basal and ionomycin-induced levels were observed in cells transfected with p35 alone in comparison to those expressing both exogenous Cdk5 and p35. These results further confirm that calpain-dependent cleavage of p35 is inhibited via autophosphorylation by Cdk5.
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Phosphorylation at Ser8 and Thr138 of p35 in Cultured Cortical Neurons and Rat Brains—To further assess the physiological role of Cdk5-dependent phosphorylation of these two sites in regulating the cleavage of p35 to p25, the phosphorylation state of Ser8 and Thr138 was evaluated in cultured cortical neurons and intact brain tissue. Phosphorylation of p35 was first examined in cultured cortical neurons metabolically labeled with [32P]orthophosphate by two-dimensional phosphopeptide map analyses (Fig. 4A). Interestingly, maps of radiolabeled p35 immunoprecipitated from cultured neurons produced a single major spot that corresponded to the phosphopeptide (spot 1 of Fig. 1C) associated with phospho-Ser8. Treatment of the neuronal cultures with the Ser/Thr protein phosphatase 1 and 2A inhibitor okadaic acid (0.1 μM) together with the proteosome inhibitor MG132 (50 μM), to prevent phospho-p35 from being degraded, Refs. 31 and 32) prior to immunoprecipitation of p35 resulted in the appearance of a second spot corresponding to the phosphopeptide (spot 2 of Fig. 1C) associated with phospho-Thr138. The identity of these two phosphopeptides were confirmed by an additional phosphopeptide map, where tryptic digests of p35 immunoprecipitated from cultured neurons treated with okadaic acid and HEK293 cells were mixed and the two spots comigrated to the exact same positions (Fig. 4A, right panel). These results indicate that Ser8 is phosphorylated in cultured cortical neurons and Thr138 becomes phosphorylated in the presence of okadaic acid. These results suggest that whereas both Ser8 and Thr138 are phosphorylated in transfected HEK293 cells, only Ser8 is phosphorylated in cultured cortical neurons under basal conditions.

To further characterize the phosphorylation of Ser8 and Thr138 in intact brain tissue, two rabbit polyclonal phosphorylation state-specific antibodies were generated for these sites. The specificities of these antibodies were defined by immunoblot analyses (Fig. 4B). First lysates of HEK293 cells cotransfected with Cdk5 and p35 were prepared and either left untreated or treated with bacterial alkaline phosphatase prior to immunoblotting. Both phospho-Ser8 and phospho-Thr138 p35 were detected in untreated lysates and the signal for each of these sites was completely removed by phosphatase treatment (Fig. 4B, lane 2). In contrast, addition of the phosphatase had no effect on total p35 levels, although it did result in increased electrophoretic mobility (Fig. 4B, lanes 1 and 2 of the lowest panel), consistent with loss of phosphate moieties and our earlier observations in cortical neurons (23). The specificity of the antibodies was further demonstrated by experiments where addition of the phospho-Ser8 or phospho-Thr138 peptides to the corresponding antibodies in blotting solution resulted in almost complete loss of signal due to preadsorption (Fig. 4B, lanes 3 and 4). Finally, the phosphorylation site specificity was further confirmed by immunoblot analysis of lysates of HEK293 cells transfected with S8A or T138A mutant forms of
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p35. Mutation of each site to Ala resulted in complete loss in the ability of the corresponding antibody to detect p35 (Fig. 4B, lanes 7 and 8). On the other hand, the sensitivity of the phospho-Ser8 and phospho-Thr138 antibodies was unaffected by Ala mutations at the non-targeted site, Thr138 and Ser8, respectively. These results demonstrate that the phospho-Ser8 and phospho-Thr138 antibodies are able to selectively detect p35 and are quite specific for the phosphorylation sites on p35 to which they were raised.

Previous findings have suggested that the phosphorylation state of p35 changes during brain development (23). Therefore, the novel phosphorylation state-specific antibodies were next employed to examine the phosphorylation states of these two sites in fetal and adult brains (Fig. 4C). p35 immunoprecipitated from fetal or adult brain extracts was immunoblotted for phospho-Ser8, phospho-Thr138, and total p35 levels. Fetal p35 was phosphorylated at both Ser8 and Thr138, whereas adult p35 was phosphorylated only at Ser8. To further characterize this observation, the dephosphorylation of Thr138 was examined across postnatal development using p35 immunoprecipitates prepared from rat brains of postnatal days 1 to 20. Dephosphorylation began at postnatal days 5–10 and was almost completed by day 20 (Fig. 4D). These results indicate that p35 is phosphorylated at Ser8 and Thr138 in fetal brains as in HEK293 cells, and phosphorylation at Thr138 decreases with aging.

Okadaic Acid-dependent Suppression of the Cleavage Was Due to the Phosphorylation at Thr138—The observation that inhibition of protein phosphatases resulted in increased phospho-Thr138 levels, as assessed by phosphopeptide map analysis (Fig. 4A) and that phospho-Thr138 levels were selectively reduced in adult tissue (Fig. 4C), together with our previous observations suggesting phosphorylation protects fetal p35 from calpain, prompted further studies of the specific contributions of each of these sites to calpain susceptibility. In these experiments COS-7 cultures cotransfected with Cdk5 and p35 were either left untreated or treated with okadaic acid. The levels of phospho-Ser8 and phospho-Thr138 were then compared by quantitative immunoblot analysis of lysates using the phosphorylation state-specific antibodies. As suspected, phospho-Thr138, but not phospho-Ser8, was increased significantly by treatment with okadaic acid (Fig. 5A). Next, these experimental conditions were employed to investigate the relative contribution of each of the two phosphorylation sites to the vulnerability of p35 to calpain cleavage (Fig. 5B). The effect of okadaic acid on p25 production in response to ionomycin was evaluated in lysates of COS-7 cells cotransfected with Cdk5 and wild-type, S8A, or T138A. Okadaic acid, which selectively increased phospho-Thr138 levels, substantially decreased the ability of ionomycin to induce p25 production in cells transfected with either wild-type or S8A p35. In contrast, okadaic acid had no discernible effect on the ability of ionomycin to induce p35 production in cells expressing T138A p35. These observations strongly suggest that the critical determinant of the susceptibility of p35 to calpain cleavage is Cdk5-dependent phosphorylation of Thr138. These results are in agreement with observations that fetal p35, which is highly phosphorylated at Thr138, is more resistant to the cleavage than adult p35 (23), which exhibits comparatively low phospho-Thr138 levels. In other words, Thr138 is the phosphorylation site most directly involved in the age-dependent cleavage of p35 to p25.

DISCUSSION

Calpain cleavage of p35 is thought to untether Cdk5 from the plasma membrane, resulting in aberrant activity that contributes to neuronal cell death and a variety of neurodegenerative disorders. Previously we have implicated reduction in auto-phosphorylation of p35 in age-dependent susceptibility to p25 generation (23). In the present study, we further characterized phosphorylation of p35 by Cdk5 and investigated the role of this important regulatory mechanism in preventing p35 cleavage by calpain. A combined site-directed mutagenesis/cell culture expression approach was used to identify the phosphorylation sites as Ser8 and Thr138. Inhibition of Cdk5 or mutation of either of these sites to Ala enhanced p25 production in cultured non-neuronal cells and primary neurons in response to ionophore-induced increased intracellular Ca2+ concentrations. Whereas p35 was phosphorylated at Ser8 and Thr138 in fetal brain tissue, only Ser8 was phosphorylated in primary cultured cortical neurons and adult brain. Phosphorylation at Thr138 was induced by the protein phosphatase inhibitor, okadaic acid, suggesting the potential of this site in particular to function as a regulatory signaling mechanism. Furthermore, loss of phosphorylation at Thr138, not Ser8, rendered p35 more susceptible to cleavage by calpain in the presence of okadaic acid. Taken together these observations suggest that phosphorylation of Thr138 of p35 by Cdk5 is a critical regulatory mechanism that prevents p25 formation during brain development.

Whereas p25 generation has been most highly associated
with neuronal cell loss, growing evidence suggests Cdk5 is involved in the mechanisms of synaptic plasticity underlying learning and memory (33–36). Recently, transient overexpression of p25 has been suggested to contribute to enhanced synaptic plasticity and memory before resulting in deleterious effects (37). Given these recent observations, a physiological role for p25 cannot be completely ruled out. On the other hand, calpain has also been thought to be a signaling protease, which generates limited proteolysed fragments that possess altered properties in comparison to their parent molecules (18). Cdk5-p25 is different from Cdk5-p35 with regard to solubility, cellular localization, stability, and kinase activity (6–9, 38). Thus, it is important to understand how proteolytic cleavage of p35 to p25 is regulated.

Calpain has no distinct consensus cleavage sequences, and is thought to target linker regions between conformational domains within proteins (18, 39). The cleavage site between Phe98 and Ala99 of p35 appears to correspond to the linker connecting the N-terminal membrane-binding region and C-terminal Cdk5 activation globular domain (40). In fact, the cleavage of p35 may be suppressed by deletion of about 20 amino acids including the cleavage site but not by mutation at several amino acids prior (i.e. N-terminal) to the cleavage site. Calpain cleavage of a number of other proteins has been shown to be regulated by phosphorylation (19–22). The cleavage of p35 was also suppressed by phosphorylation at Ser8 or Thr138 of p35. Considering that these two phosphorylation sites located on opposite sides of the cleavage site were differently phosphorylated, it is possible that each may suppress p35 cleavage using different or independent mechanisms.

Phosphorylation of Ser8 may serve to repress basal calpain activity toward p35. Ser8 was constitutively phosphorylated when p35 was expressed with Cdk5 in either cultured cells or brains, although the exact stoichiometry of phosphorylation has not been determined. p35 associates with membranes via N-terminal myristoylation (6). Ser8 is close to the Gly2 myristoylation site. Suppression of the cleavage by Ser8 phosphorylation may be related to the interaction of p35 with membranes.

There are several examples of myristoylated proteins whose phosphorylation in the N-terminal region alters the association with membranes (41, 42). Calpain is also translocated to membranes when activated and cleaves proteins on membranes (18), although the manner in which calpain interacts and associates with membranes is unknown. The catalytic site of calpain may be positioned at a particular distance from the surface of membranes. Conceivably, phosphorylation at Ser8 changes the interaction of p35 with membranes so as to render the cleavage site unavailable to the catalytic site of calpain.

Phosphorylation at Thr138 seemed to be the more critical determinant and appeared much more subject to regulation in cultured cells and intact brain tissue. Several possibilities may be considered regarding how p35 phosphorylation prevents calpain cleavage. (i) Phosphorylation at Thr138 may reduce the affinity to calpain by changing the structural conformation around the cleavage site. (ii) The binding of other proteins to the phosphorylated form of Thr138 may mask the region recognized by calpain. Several p35-binding proteins have been reported (43), and it is possible that some of them facilitate phosphorylation of Thr138 during development but not in the adult Cdk5-p35 complex. (iii) It is also possible that phosphorylation of Thr138 simply causes p35 to be degraded so rapidly that this form of the protein is never exposed to active calpain for any length of time. Although this study has focused on the calpain-dependent cleavage, the proteosomal degradation of p35 is also regulated by phosphorylation (31, 32). It has previously been indicated that phosphorylation at Thr138 is required for degradation of p35 (31). In any case, phosphorylation at Thr138 plays an important role as the determinant for the proteolytic fate of p35 and serves as a key point in preventing neurodegeneration.

The question remains of how phosphorylation of Thr138 is differentially regulated in fetal versus adult tissue despite autophosphorylation by Cdk5-p35. Protein phosphorylation is determined by the balance of enzymatic activities between protein kinases and protein phosphatases. Both the activity of Cdk5-p35 and the phosphatase(s) responsible for dephosphorylating p35 may be developmentally regulated. Cdk5-p35 activity peaks during early postnatal days of rat brain development (24, 44), and Cdk5-p35 activity in the fetal brain is greater than that in the adult brain extract.4 These developmental differences in Cdk5-p35 activity appear, at least in part, to depend on the interaction of the protein kinase complex with membranes (45). On the other hand, the increased phosphorylation of p35 observed in cultured neurons and COS-7 cells in the presence of a phosphatase inhibitor suggests that Thr138 is actively dephosphorylated by PP1 or PP2A under basal conditions, raising the modulation of these enzymes as possible mechanisms for regulating the susceptibility of p35 to calpain cleavage. Such putative okadaic acid-sensitive protein phosphatase regulatory mechanisms were also suggested by our previous observations (23). How phosphorylation of p35 is regulated remains an excellent subject for future study.

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