p35, the Neuronal-specific Activator of Cyclin-dependent Kinase 5 (Cdk5) Is Degraded by the Ubiquitin-Proteasome Pathway*

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Cyclin-dependent kinase 5 (Cdk5) was originally isolated by its close homology to the human CDC2 gene, which is a key regulator of cell cycle progression. However, unlike other Cdks, the activity of Cdk5 is required in post-mitotic neurons. The neuronal-specific p35 protein, which shares no homology to cyclins, was identified by virtue of its association and activation of Cdk5. Gene targeting studies in mice have shown that the p35/Cdk5 kinase is required for the proper neuronal migration and development of the mammalian cortex. We have investigated the regulation of the p35/Cdk5 kinase. Here we show that p35, the activator of Cdk5, is a short-lived protein with a half-life (t_{1/2}) of 20 to 30 min. Specific proteasome inhibitors such as lactacystin greatly stabilize p35 in vivo. Ubiquitination of p35 can be readily demonstrated in vitro and in vivo. Inhibition of Cdk5 activity by a specific Cdk inhibitor, roscovitine, or overexpression of a dominant negative mutant of Cdk5 increases the stability of p35 by 2- to 3-fold. Furthermore, phosphorylation mutants of p35 also stabilize p35 2- to 3-fold. Together, these observations demonstrate that the p35/Cdk5 kinase can be subject to rapid turnover in vivo and suggest that phosphorylation of p35 upon Cdk5 kinase activation plays a autoregulatory role in p35 degradation mediated by ubiquitin-mediated protein degradation.

Cyclin-dependent kinase 5 (Cdk5)1 is a small serine/threonine kinase that shares a high degree of homology to the cell cycle regulators Cdc2 and Cdk2 kinases (1). Despite this homology, a role for Cdk5 in cell cycle regulation has not been observed. Rather, expression of Cdk5 is most abundant in the adult nervous system (2, 3), which contains primarily post-mitotic neurons and glial cells. In addition, high levels of Cdk5 kinase activity are only present in brain lysates when compared with other tissues (3, 4).

The D-type cyclins have been shown to bind to Cdk5 in fibroblasts and other tissue culture cell lines (5, 6). However, this association does not result in an active kinase. In an effort to identify the Cdk5 activator in brain, a 35-kilodalton protein species (p35) was found to associate with Cdk5 in cultured primary neurons of embryonic rat cortices whose abundance correlates with the level of Cdk5 kinase activity (7). Purified Cdk5 kinase activity from brain lysates contains two protein entities, Cdk5 and a partial fragment of p35 (4, 8, 9). Finally, recombinant p35 could activate Cdk5 kinase when mixed in vitro, formally establishing the regulatory role of p35 (7, 8). Despite the fact that p35 serves as a regulatory partner for Cdk5, it does not display any primary sequence homology to members of the cyclin family of proteins. However, the predicted tertiary structure of p35 is similar to cyclins (10, 11), suggesting that p35 binds and activates Cdk5 similar to other cyclin-Cdk complexes.

The substrate specificity of the p35/Cdk5 kinase is similar to that of the Cdc2 and Cdk2 kinases, phosphorylating the R/K(D/P)X(K/R) consensus sequence motif (12, 13). The neuronal-specific intermediate filaments neurofilaments and the microtubule-associated proteins τ and mitogen-activated protein 2 have been shown to be substrates of the p35/Cdk5 kinase (15). In addition to these structural proteins, proteins associated with neural transmitter release such as synapsin I and Munc18 have also been shown to be phosphorylated by the p35/Cdk5 kinase (16, 17).

The biological function of the p35/Cdk5 kinase has been addressed in vivo and in vitro. Indeed, when a dominant negative Cdk5 mutant (DNK5) or an antisense p35 construct was introduced into cultured primary cortical neurons, neurite outgrowth was severely inhibited (18). We also created a mouse strain lacking p35 (19). These animals are viable and fertile but display seizures and sporadic lethality that is likely to be the result of terminal seizures. Histological examination reveals that the lamination pattern of the cerebral cortex is disrupted in p35-deficient animals, which results from an inverse packing order of post-mitotic cortical neurons (19, 20). Cdk5-deficient animals die before or around birth with defects in the development of the cortex, cerebellum, and other compartments of the central nervous system (21). The cortex of these Cdk5−/− mice display a lamination defect reminiscent of p35. Together, these results underscore the integral role of the p35/Cdk5 kinase in the migratory behavior of post-mitotic neurons.

Cdk5 activity has recently been shown to be required for muscle development in Xenopus. Homologues of Cdk5 and p35 have also been isolated in Xenopus. One of the p35 homologues in Xenopus, Xp35.1, is highly expressed in developing somites where Cdk5 is also expressed. Overexpression of Xp35.1 or

1 The abbreviations used are: Cdk5, cyclin-dependent kinase 5; LLnL, N-acetyl-l-leucyl-l-leucyl-l-norleucinal; Z-L, V5, carboxybenzyl-leucyl-leucyl-leucyl vinyl sulfone; CMV, cytomegalovirus; GST, glutathione S-transferase.

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DNK5 disrupts muscle organization, and expression of MyoD and MRF4 is suppressed in the presence of DNK5 (23).

In light of the essential function of the p35/Cdk5 kinase during mouse corticogenesis, the regulatory mechanism of this kinase is clearly part of the circuitry that underlies the development of the central nervous system. In addition to cyclin binding, Cdk5s can be regulated by post-translational phosphorylation and dephosphorylation events (24). Cyclin degradation is also used as a unidirectional form of regulation. The fact that the Cdk5 kinase activity can be reconstituted is also used as a unidirectional form of regulation. The availability of p35 appears to be the primary determinant for Cdk5 activation. During neurogenesis, the expression of p35 is stringently regulated at the transcriptional level (25). Spatially, it is restricted to cells of the neuronal lineage. Temporally, p35 mRNA peaks around birth and quickly declines afterward (25, 26). In addition, p35 mRNA is not present in dividing neuroblasts but is expressed when the neuroblasts exit the cell cycle and migrate out of the germinial zone during development of the cerebral cortex (25). In the adult, p35 is only present in certain areas of the forebrain including the hippocampal formation, the pyriform cortex, and layers 2, 3, 4, and 5 of the neocortex, structures that maintain high levels of plasticity (25).

In this study, we show that p35 is regulated at the post-translational level based on the short half-life ($t_{1/2}$) of p35 protein. Our results indicate that the rapid turnover of p35 is mediated at least in part via the ubiquitin-dependent proteasome pathway. Ubiquitin is first activated in an ATP-dependent manner by the ubiquitin-activating enzyme (E1). It is then transferred to a ubiquitin-conjugating enzyme (E2) and then maybe transferred to a ubiquitin ligase (E3) involved in the recognition of substrate and transfer of ubiquitin molecule to the substrate. A multi-ubiquitinated protein can then be recognized by the proteasome, where it is hydrolyzed in a ATP-dependent process. The p35 S8A, T138A, S170A, T197A construct was made by polymerase chain reaction mutagenesis using p35 T138A, S170A, T197A mutant was made by polymerase chain reaction mutagenesis using p35 T138A, S170A, T197A as a template. 5' and 3' p35 primers were used in combination with the mutagenic primers CCGTGTGGGGCCGCCCCGTGCGGAGG and CCTCCGCCGGGCCGCCACCCACACG, respectively. Site-directed mutagenesis of p35 T138A in pBSK for the production of p35 phosphorylation mutants (S8A,T138A; T138A,S170A; and T138A,T197A) was performed as described previously (32). Mutagenic primers used: S8A, GGTAGCTGGGGGCCAGGGACAG; T138A, GCCTTCAGGGGCCAGGGGCGCCCAAACG; and S170A,T197A, CGTTTGGGCGCTCCTGCGGAGG and CCTCCGCCGGGCCGCCACCCACACG, respectively. Site-directed mutagenesis of p35 T138A in pBSK for the production of p35 phosphorylation mutants (S8A,T138A; T138A,S170A; and T138A,T197A) was performed as described previously (32). Mutagenic primers used: S8A, GGTAGCTGGGGGCCAGGGACAG; T138A, GCCTTCAGGGGCCAGGGGCGCCCAAACG; and S170A,T197A, CGTTTGGGCGCTCCTGCGGAGG and CCTCCGCCGGGCCGCCACCCACACG, respectively. All mutations were cloned into CMV (pCDNA3) vectors. The p35 S8A, T138A,S170A,T197A (p35 Quad) construct was made by cloning the Sfi/I/XbaI fragment of the CMV p35 T138A,S170A,T197A mut into the Sfi/I/XbaI site of CMV p35 S8A,T138A. Mutagenesis was confirmed by sequencing.

Cell Cultures, Transfections, and Immunological Procedures—E17-E19 pregnant rats (Long Evans strain) were purchased from Harland Sprague-Dawley. Embryos were surgically removed, and their cortices were dissected out and cultured as described previously by (18). C33A and COS-7 cells were maintained in Dulbecco’s modified Eagle medium supplemented with 10% fetal calf serum. C33A and COS-7 cells were transiently transfected with various plasmid constructs (See Constructs and mutagenesis section above) were performed as described previously (28). For $t_{1/2}$ experiments: primary cortical cultures were grown on 3.5 or 10 cm plates. C33A and COS-7 cells were transfected with various combinations of CMV-p35, CMV-Cdk5, or CMV-Cdk5.
CMV-DNK5. For [35S] pulse-chase experiments, cultures were washed twice with PBS and incubated with methionine free media supplemented with [35S] methionine (110 mcg/ml) for one h. The cultures were then washed twice with PBS and Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Samples were harvested at indicated time points. Cells were lysed in ELB lysis buffer plus inhibitors (250 mM NaCl, 50 mM Tris (pH 7.4), 1 mM EDTA, 0.1% Nonidet P-40, 2 mg/ml of aprotinin, 2 mg/ml of leupeptin, 1 mg/ml of pepstatin, 5 mM NaF, 5 mM NaVO₃, and 100 mcg/ml phenylmethylsulfonyl fluoride). Lysates were precleared with zysorbin (Zymed Laboratories Inc.), immunoprecipitated with 4E3 or 5H8 p35 monoclonal or C8 (Santa Cruz) Cdk5 polyclonal antibodies, and then run on a 12% SDS-polyacrylamide gel, dried, and exposed to film. Cyclohexamide treatment experiments of primary cortical cultures and transfected cells were done as follows. Cultures were treated with cyclohexamide (final concentration of 30 mcg/ml) for the indicated time points. No DNA plus GST-ubiquitin and in vitro transcribed and translated p35 without GST-ubiquitin were used as controls. Cost cells were transfected with CMV-p35 with or without His₆-myc-ubiquitin (pCW7) or with mutant His₆-myc-ubiquitin K48R (pCW8)-expressing plasmids. Lysates were immunoprecipitated with anti-p35 antibodies, the samples were run on a 12% SDS-polyacrylamide gel, and the blots were probed with either p35 or 9E10 antibodies. Lane 1, vector control; lanes 2 and 6, p35; lanes 3 and 7, His₆-myc-ubiquitin; lanes 4, 8, 9, and 11, p35 plus His₆-myc-ubiquitin (asterisks mark 9E10-recognized p35 ubiquitin conjugates); lanes 10 and 12, p35 plus His₆-myc-ubiquitin K48R. Ubiquitin-p35 conjugates are labeled by asterisks, bars, and arrows; the heavy chain is marked by thick black arrowhead.
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In Vitro Translation Assays—In vitro transcribed (T7 promoter from pCDNA3-p35) and translated p35 was performed using Promega TNT/T7 rabbit reticulolysate kit with [35S]methionine for 90 min at 30 °C (50 μl total reaction). After a 90-min incubation, the translation reaction was stopped with cyclohexamide (20 μg/ml final concentration), and samples were split into two. In vitro transcribed and translated lysate of NO DNA sample was used as a control. To each sample the following was added: 10 μl of 10X degradation buffer (100 mM Tris-Cl, 10 mM MgCl2, and 10 mM dithiothreitol), 5 μl of 20X regeneration system (150 mM creatine phosphate (Sigma), 20 mM ATP, and 20 mM MgCl2), 0.5 μl of creatine phosphokinase (Sigma), 15 μl of bacterially purified GST-ubiquitin (0.6 mg/ml) or 15 μl of distilled H2O, and 44.5 μl of E17 rat brain lysate (25 mg/ml). The samples were placed at 37 °C, and 20 μl was taken at 0, 60, and 120 min. 2× sample buffer was added, and the samples were run on a 12% polyacrylamide gel. The gel was transferred to nylon and then probed with anti-p35 antibodies.

Inhibitors of the Proteasome Stabilize p35 in Vivo—To ad-

RESULTS

p35 Is a Short-lived Protein—In light of the observation that association of p35 with Cdk5 is the rate-limiting step for kinase activation, we investigated the regulation of p35 at the protein level. We initially looked at its half-life (t1/2) in dissociated primary rat cortical neurons. E17-E19 rat cortical neurons were cultured for 4 days and then pulse-chased with [35S]methionine (Fig. 1A). Newly synthesized p35 in neurons was immunoprecipitated with a monoclonal antibody to p35 (5H8) at different time points. A rapid drop of p35 protein levels was observed between 20 to 40 min after chase (Fig. 1A). A similar turnover rate of p35 was shown by immunoprecipitation using a Cdk5 antibody that recognizes the p35/Cdk5 complexes (Fig. 1B). When primary cortical neurons were treated with cyclohexamide, the total population of p35 in neurons was also shown to be rapidly turned over with more than half the protein gone after approximately 15 to 20 min. Cdk5 appeared to be a more stable protein whose abundance did not vary throughout the time points analyzed (Data not shown and Fig. 1C). Thus by either method, p35 was found to be a short-lived protein.

Ubiquitination of p35—Proteasome inhibitors have been shown to stabilize proteins destined for degradation by the proteasome (27). To examine the effect of proteasome inhibitors on p35 stability, C33A cells transfected with a p35 expression plasmid were treated with LLnL (250 μM). LLnL stabilized the p35 protein, and slower migrating bands were recognized with p35 antibodies (Fig. 2A). Another proteasome inhibitor Z-Leu-Leu-Leu-H (MG132) (50 μM) produced similar results, as it slowed the p35 turnover rate as well as caused the accumulation of slower migrating species (Fig. 2B). These higher molecular weight species are highly suggestive of ubiquitin modification of p35.

We first determined if p35 could be covalently modified by ubiquitin in vitro. p35 was in vitro transcribed and translated with [35S]methionine in rabbit reticulocyte. Afterward the sample was split into two, and bacteria-purified GST-ubiquitin was either added or not. Aliquots were taken at time 0, 60, and 120 min after the addition of GST-ubiquitin. The in vitro transcribed protein is the only protein that incorporates [35S]methionine as shown in Fig. 3A (no DNA compared with p35 lanes). GST-ubiquitin is approximately 35 kDa, and so the appearance of a labeled band at approximately 70 kDa nicely corresponds to p35 covalently modified by one GST-ubiquitin (Fig. 3A). A labeled band at this weight was not seen in the no DNA plus GST-ubiquitin and in vitro transcribed and translated p35 without GST-ubiquitin control lanes, further confirming that the appearance of the 70-kDa band in the 60-and 120-min time points are indeed GST-ubiquitin-modified p35. Interestingly, p35 is stable when in vitro translated; therefore it is likely that certain components required for degradation are absent in this system, similarly seen for other proteins such as p53, where the in vitro ubiquitination of p53 is dependent on the E6-associated protein (34).

Since polyubiquitinated proteins are quickly degraded by the proteasome, it is sometimes difficult to detect multi-ubiquitinated species in vivo. Ellison and Hochstrasser (35) have shown that addition of NH4-terminal-tagged ubiquitin inhibits degradation, although the epitope-tagged ubiquitin can still be efficiently conjugated to proteins to form multi-ubiquitinated chains. To examine whether the higher molecular weight forms of p35 are indeed p35-ubiquitin conjugates, p35 was co-transfected with NH4-terminal His6-myc-tagged ubiquitin constructs (Fig. 3F). COS7 cells were transiently transfected with p35 alone (Fig. 3B, lanes 2 and 6) or co-transfected with p35 and His6-myc-ubiquitin (Fig. 3B, lanes 4, 8, 9, and 11) or with p35 and His6-myc-ubiquitin containing a K48R mutation (Fig. 3B, lanes 10 and 12), which prevents ubiquitin chain elongation through the Lys-48 residue on ubiquitin (36, 37). Lysates were immunoprecipitated with p35 antibodies and probed with 9E10 antibodies for myc-tagged ubiquitin or with p35-specific antibodies. A p35 ubiquitin ladder can be readily observed by antibodies when p35 was expressed in COS7 cells and conjugated to endogenous ubiquitin (Fig. 3B, lane 6). When p35 was co-transfected with His6-myc-ubiquitin (Fig. 3B, lanes 4 and 8 and 9 and 11), a p35 ubiquitin ladder was also observed. There were also bands immunoprecipitated by p35 antibodies that were recognized by both 9E10 and p35 antibodies. These bands co-migrate precisely (Fig. 3B, asterisks and labeled arrows), indicating that the higher molecular weight species are indeed p35-ubiquitin conjugates. There is no ubiquitin immunoreactivity detected by 9E10 antibodies when COS7 cells are transfected with vector control, p35, or His6-myc-ubiquitin alone (Fig. 3B, lanes 1–3). The K48R mutant His6-myc-ubiquitin prevented polyubiquitination of p35 when co-transfected (Fig. 3B, lane 10). Additional higher molecular weight p35 species with decreased electrophoretic mobility were observed (Fig. 3B, lane 8, labeled arrows) due to the addition of His6-myc moiety to ubiquitin. Only these additional p35-derived species can be recognized by the anti-myc (9E10) antibody (lane 4, asterisks), further confirming that p35 is indeed ubiquitinated in vivo.
were treated with either Me2SO, MG132, lactacystin, Z-L 3VS, and acetylated rat embryonic (E17-E19) cortical neurons 4 days in culture. Neuronal cultures treated with proteasome inhibitors. Dissociation of unidirectional regulation is widely used by the cell to facilitate many events such as cell cycle progression.

We set out to examine whether phosphorylation of p35 might be involved in the regulation of its stability by transfecting COS7 cells with either p35 alone or with wild type Cdk5 or Cdk5 K33T, a dominant negative mutant of Cdk5 (DNK5) that is catalytically inactive but can still bind p35 (18). The turnover rate of p35 transfected alone was about 20 min (Fig. 5A). In contrast, p35 protein was stabilized about 2- to 3-fold when p35 was co-transfected with the catalytically inactive mutant DNK5 (Fig. 5A). As shown in Figs. 1C and 5A, Cdk5 was much more stable than p35, supporting the idea that p35 is rate-limiting for kinase activation.

When we immunoprecipitated p35 from p35 transfected COS7 cells using anti-p35 antibody, p35 was phosphorylated in an in vitro kinase assay (Fig. 5B). This may presumably be due to the endogenous Cdk5 present in COS7 cells. p35 phosphorylation is greatly increased when p35 and Cdk5 were co-transfected; however, p35 was not phosphorylated when p35 was coexpressed with DNK5 (Fig. 5B). Interestingly, p35 was short-lived in the absence of transfected Cdk5 (Fig. 5A). The endogenous Cdk5 in COS7 cells may be sufficient to stimulate degradation.

To discern if inactivation of the p35/Cdk5 kinase in neurons also prolongs p35 stability, primary cortical cultures were treated with a specific Cdk5 inhibitor roscovitine (42), and the turnover rate of p35 was determined. As shown in Fig. 5C, the stability of p35 was increased when the endogenous p35/Cdk5 kinase activity was inhibited. This observation, together with the notion that wild type Cdk5 enhances p35 degradation and a catalytically inactive Cdk5 stabilizes p35, suggests that autophosphorylation of p35 in the kinase complex stimulates its degradation.

Based on the consensus phosphorylation motif of the Cdk5, there are four minimal consensus Cdk phosphorylation sites (SP or TP) in p35 as shown in the schematic (Fig. 6A). To determine whether phosphorylation of these sites facilitates its degradation, serine/threonine to alanine single and combinatorial mutations on p35 were generated, which allowed us to ask whether the inability to phosphorylate these sites in p35 affects its stability. Transient transfection of COS7 cells with all combinations of single, double, triple, and quadruple p35 phosphorylation mutants were performed. Shown are representative cyclohexamide experimental results from co-transfection of

dress the question of whether p35 is degraded by the proteasome in vivo, the turnover rate of p35 was measured in primary neuronal cultures treated with proteasome inhibitors. Dissociated rat embryonic (E17-E19) cortical neurons 4 days in culture were treated with either Me2SO, MG132, lactacystin, Z-L3VS, or E64. Lactacystin is a very specific inhibitor of the proteasome. The aldehyde MG132 and the peptide vinyl sulfone, Z-L3VS (39), are potent inhibitors of the proteasome but are less specific than lactacystin. E64 is a general inhibitor of cysteine proteases found in organelles such as the lysosome (40). The stability of p35 was drastically increased by MG132, lactacystin, and Z-L3VS to various extents in vivo (Fig. 4). Although higher overall levels of p35 were observed when cultures were treated with E64, the turnover rate was not changed when compared with cultures treated with Me2SO alone. Together these results indicate that the proteasome plays a major role in the regulation of p35 stability.

Phosphorylation Stimulates the Degradation of p35—It has been shown previously that p35 can be autophosphorylated within the active kinase complex by Cdk5 (7, 8). Protein phosphorylation is an important post-translational mechanism for regulating protein activity. For instance, autophosphorylation of a cyclin within the cyclin-Cdk complex was shown to result in cyclin destruction and inactivation of the kinase (41). This form of unidirectional regulation is widely used by the cell to facilitate many events such as cell cycle progression.

The aldehyde MG132 and the peptide vinyl sulfone, Z-L3VS (39), are potent inhibitors of the proteasome but are less specific than lactacystin. E64 is a general inhibitor of cysteine proteases found in organelles such as the lysosome (40). The stability of p35 was drastically increased by MG132, lactacystin, and Z-L3VS to various extents in vivo (Fig. 4).

Although higher overall levels of p35 were observed when cultures were treated with E64, the turnover rate was not changed when compared with cultures treated with Me2SO alone. Together these results indicate that the proteasome plays a major role in the regulation of p35 stability.
wild type p35, p35 T138A, or p35 QUAD (which contains alanine substitutions at Ser-8, Thr-138, Ser-170, and Thr-197) without and with wild type Cdk5 (Fig. 6, B and C, respectively). We consistently saw a 2-fold increase in the turnover rate of the p35 T138A single mutant without and with Cdk5 and a 3-fold increase in the stability of the p35 QUAD mutant without and with Cdk5 (Fig. 6, B and C). All of the phosphorylation mutants are competent to bind and activate Cdk5 as determined by in vitro kinase assays of immunoprecipitates from co-transfected COS7 cells (Fig. 6, D). These results support the hypothesis that autophosphorylation of p35 by Cdk5 in the kinase complex stimulates its degradation. The p35 QUAD phosphorylation mutant was not completely stabilized, suggesting that phosphorylation is stimulatory but not obligatory for p35 degradation.

p25, a Deletion Mutant of p35 Is a Stable Protein—p35 consists of an amino-terminal p10 and a carboxyl-terminal p25 region containing the Cdk5 binding site (43) separated by a proline-rich region. When the p10 region was removed from p35, the resulting p25 was a stable protein, even when Cdk5 was overexpressed (Fig. 7), suggesting that the p10 region of p35 was required for degradation. This suggest that the p10 region is important in mediating protein interactions, possibly with components of the ubiquitin machinery that are required for p35 instability.

FIG. 6. Phosphorylation mutants of p35 are more stable. A, schematic of p35 indicating four SP or TP minimal Cdk consensus sites and the Cdk5 binding region. B and C, cyclohexamide experiment. COS7 cells transiently transfected with pCDNA3-p35, pCDNA3-p35 T138A, or pCDNA3-p35 QUAD plus pCDNA3 vector control or pCDNA3-Cdk5 (B and C, respectively) were treated with cyclohexamide (final concentration of 30 μg/ml) for the indicated time. Lysates were run on a 12% polyacrylamide gel, probed with anti-p35 antibodies, and depicted graphically from quantitative measurements of band intensities from gels using NIH Image 1.61 analysis software. open circle, p35 alone; closed circle, p35 T138A; closed triangle, p35 QUAD. D, activity of p35 mutants. Lysates from COS7 cells transfected with p35 or p35 phosphorylation mutants with Cdk5 were immunoprecipitated with p35 antibodies, and H1 kinase reactions were performed.

DISCUSSION

The biological function of the p35/Cdk5 kinase has been implicated in the histogenesis of the central nervous system. The adult mammalian cortex is characterized by a distinct laminar structure generated through a well defined pattern of neuronal migration whereby neurons born later in corticogenesis migrate through and past the older neurons to occupy a more superficial layer of the cortical plate (44, 45). Mice with targeted mutations in either p35 or Cdk5 have a striking disruption of these cortical layers. The earlier born neurons reside in more superficial layers, and the latter born cells reside in more deep layers (19, 21). Furthermore, when primary cortical cultures were transfected with either an antisense construct of p35 or with dominant negative constructs of Cdk5 (Cdk5N144 and Cdk5T33), neurite outgrowth was inhibited. In contrast, when wild type p35 and Cdk5 were ectopically expressed, the neurite length was longer (18). Taken together, these observations indicate that the p35/Cdk5 kinase is clearly involved in dynamic events of neuronal migration and neurite outgrowth.

We have begun to look at the regulation of the p35/Cdk5 kinase. The high homology to cdc2 (1) provides a strong precedence for Cdk5 to be regulated in similar manners as other Cdns. The requirement of an associated protein for kinase activity holds true, but the fact that the activator p35 and the kinase activity is only present in post-mitotic neurons suggests...
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Cysteine proteases in neurons, or the effect of E64 is indirect. Nevertheless degradation through the proteasome pathway appears to be the major route for degradation of p35. We also observed the accumulation of higher molecular weight species that were specifically recognized by p35 antibodies when p35-transfected C33A cells were treated with LlnL or MG132. In His6-myc-ubiquitin/p35 co-transfection experiments, we were able to confirm that higher molecular weight species of p35 were indeed p35-ubiquitin conjugates. Polyubiquitination of p35 was blocked by a K48R mutant version of the His6-myc-ubiquitin construct, which inhibits chain elongation. Taken together, these results suggest that p35 is a target for degradation by the proteasome in a ubiquitin-mediated fashion.

The stability of p35, when transfected in COS7 or C33A cells, was similar to that seen in neurons, which provided an amenable system to study the mechanism of p35 degradation. Co-transfection of p35 with Cdk5 drastically reduced the steady state levels of p35 compared with transfection of p35 alone. Co-transfecting the catalytically inactive Cdk5, Dnk5 (Cdk5 K33T), stabilized p35 by about 2- to 3-fold. Furthermore, inhibition of the endogenous p35/Cdk5 kinase activity in neurons by roscovitine also increased the stability of p35. These observations suggest that kinase activation stimulates p35 degradation. The fact that p35 becomes autophosphorylated by the p35/Cdk5 kinase indicates that phosphorylation of p35 plays a regulatory role in its degradation. When serum or threonine residues in the minimal Cdk phosphorylation consensus sites of p35 were mutated to alanine, the stability of these p35 mutants were increased 2- to 3-fold (p35 T138A and p35 QUAD, respectively). These results were similar to the stabilizing effect of p35 by Dnk5 co-transfection and roscovitine treatment experiments. Taken together this data supports the hypothesis that phosphorylation stimulates p35 degradation. It is possible that phosphorylation may create an entrance point for the degradation machinery to bind and ubiquitinate p35, which awaits to be tested.

Our initial deletion mutagenesis studies suggest that the p10 NH2-terminal region of p35 may be required for degradation, as the p25 fragment of p35 is a much more stable protein even in the presence of overexpressed Cdk5. Purification of the Cdk5 kinase from brain resulted in Cdk5 and the p25 NH2-terminal deletion fragment (8, 9). Additionally, p25 does appear to be physiologically produced.5

Results presented in this study not only demonstrate that the ubiquitin proteasome pathway plays a role in the fast turnover of p35 but also provide a model for the negative feedback regulation of the p35/Cdk5 kinase. Upon activation of Cdk5 by p35 association, the kinase phosphorylates p35 concurrently or soon after it phosphorylates its substrates. Phosphorylation further stimulates p35 degradation by the proteasome in a ubiquitin-mediated fashion, possibly by disassociation of p35 and Cdk5 or by creating an entrance point for ubiquitin-conjugating enzymes, which in turn ubiquitinate and target p35 for degradation. Thus, the Cdk5 kinase is quickly turned off once it is activated. It is likely that the proteasome pathway is broadly involved in the development of the nervous system in light of the regulatory role of this pathway in p35 protein levels and, thus, p35/Cdk5 kinase activity. As the p35/Cdk5 kinase plays a critical role in neurite outgrowth and neuronal migration, it is conceivable that to accommodate these processes, dynamic kinase activation-deactivation is required. The fact that Cdk5 can be activated by p35 association alone is consistent with this notion, provided that there is intricate regulation of p35 synthesis and degradation.

References

1. Y. Ramos and L. H. Tsai, unpublished data.

2. G. Patrick and L. H. Tsai, unpublished data.
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REFERENCES
1. Meyerson, M., Enders, G. H., Wu, C. L., Su, L. K., Gorka, C., Nelson, C., Harlow, E. & Tsai, L. H. (1992) EMBO J. 11, 2909–2917
2. Hellmich, M. R., Pant, H. C., Wada, E. & Battey, J. F. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10867–10871
3. Tsai, L. H., Takahashi, T., Caviness, V. S., Jr. & Harlow, E. (1993) Development 119, 1029–1040
4. Lew, J., Winkfein, R. J., Paudel, H. K. & Wang, J. H. (1992) J. Biol. Chem. 267, 25922–25926
5. Lee, M. H., Nikolic, M., Baptista, C. A., Lai, E., Tsai, L. H. & Massague, J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 3259–3263
6. Xiong, Y., Zhang, H. & Beach, D. (1992) Cell 74, 505–514
7. Tsai, L. H., Delalle, I., Caviness, V. S., Jr., Chae, T. & Harlow, E. (1994) Nature 371, 419–423
8. Lew, J., Huang, Q. Q., Qi, Z., Winkfein, R. J., Abersold, R., Hunt, T. & Wang, J. H. (1994) Nature 371, 423–426
9. Ishiguro, K., Kobayashi, S., Omori, A., Takamatsu, M., Yonekura, S., Anzai, K., Imahori, K. & Uchida, T. (1994) FEBS Lett. 342, 205–208
10. Tang, D., Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D. J., Gonda, D. K. & Varshavsky, A. (1989) Science 243, 1576–1583
11. Beaudette, K. N., Lew, J. & Wang, J. H. (1993) J. Biol. Chem. 268, 20282–20830
12. Songyang, Z., Lu, K. P., Kwon, Y. T., Tsai, L. H., Filhol, O., Cochet, C., Brickey, D. A., Soderling, T. R., Bartleson, C., Graves, D. J., DeMaggio, A. J., Hookstra, M. F., Blenis, J., Hunter, T. & Cantley, L. C. (1996) Mol. Cell. Biol. 16, 4646–4693
13. Songyang, Z., Lu, K. P., Kwon, Y. T., Tsai, L. H., Filhol, O., Cochet, C., Brickey, D. A., Soderling, T. R., Bartleson, C., Graves, D. J., DeMaggio, A. J., Hookstra, M. F., Blenis, J., Hunter, T. & Cantley, L. C. (1996) Mol. Cell. Biol. 16, 4646–4693
14. Omura, S., Matsuoka, K., Fujimoto, T., Kasuga, K., Furuya, T., Fujita, S. & Nakagawa, A. (1991) J. Antibiot. (Tokyo) 44, 117–118
15. Lee, K. Y., Qi, Z., Yu, Y. P. & Wang, J. H. (1997) Int. J. Biochem. Cell Biol. 29, 951–958
16. Matsubara, M., Kusuhara, M., Ishiguro, K., Uchida, T., Titani, K. & Taniguchi, H. (1990) J. Biol. Chem. 271, 21108–21113
17. Shuang, R., Zhang, L., Fletcher, A., Groblewski, G. E., Pevsner, J. & Stuenkel, E. L. (1998) J. Biol. Chem. 273, 4957–4966
18. Nikolic, M., Dudek, H., Kwon, Y. T., Ramos, Y. F. & Tsai, L. H. (1996) Genes Dev. 10, 816–825
19. Chae, T., Kwon, Y. T., Bronson, R., Dikkes, P., Li, E. & Tsai, L. H. (1997) Neuron 18, 29–42
20. Kwon, Y. T. & Tsai, L. H. (1998) J. Comp. Neurol. 395, 510–522
21. Ohshima, T., Ward, J. M., Huh, C. G., Longenecker, G., Veeranna, Pant, H. C., Redy, R. O., Martin, L. J. & Kulkarni, A. B. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11173–11178
22. Omura, S., Fujimoto, T., Ootoguro, K., Matsuizaki, K., Moriguchi, R. & Tanaka, H. & Sasaki, Y. (1991) J. Antimicrob. Chemother. 44, 113–116
23. Philpott, A., Porro, E. B., Kirschner, M. W. & Tsai, L. H. (1997) Genes Dev. 11, 1409–1421
24. Morgan, D. O. (1995) Nature 374, 131–134
25. Delalle, I., Bhude, P. G., Caviness, V. S., Jr. & Tsai, L. H. (1997) J. Neurocytol. 26, 283–296
26. Tomizawa, K., Matsuizaki, M., Matsuizaki, M., Lew, J., Tokuda, H., Tanaka, K. & Hatase, O. (1996) Neuroscience 74, 519–529
27. Coux, O., Tanaka, K. & Goldberg, A. L. (1996) Annu. Rev. Biochem. 65, 801–847
28. Maki, C. G., Huibregtse, J. M. & Howley, P. M. (1996) Cancer Res. 56, 2649–2654
29. Maki, C. G. & Howley, P. M. (1997) Mol. Cell. Biol. 17, 355–363
30. Pagano, M., Tam, S. W., Theodaras, A. M., Beer-Romero, P., Del Sal, G., Chau, V., Yew, P. R., Draetta, G. F. & Rolfe, M. (1995) Science 269, 682–685
31. Sheaff, R. J. & Roberts, J. M. (1996) Chem. Biol. ( Lond.) 3, 869–873
32. Ward, C. L., Omura, S. & Kopito, R. R. (1995) Cell 83, 121–127
33. Kunkel, T. A., Bebenek, K. & McClary, J. (1991) Methods Enzymol. 204, 125–139
34. Huibregtse, J. M., Scheffner, M. & Howley, P. M. (1993) Mol. Cell. Biol. 13, 773–784
35. Ellison, M. J. & Hochstrasser, M. (1991) J. Biol. Chem. 266, 21150–21157
36. Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D. J., Gonda, D. K. & Varshavsky, A. (1989) Science 243, 1576–1583
37. Finley, D., Sado, S., Monia, B. P., Boucher, P., Ecker, D. J., Croteau, S. T. & Chau, V. (1994) Mol. Cell. Biol. 14, 5561–5569
38. Jousselin, G., Standaert, G. S., Van, W. S., Choi, S., Corey, E. J. & Schreiber, S. L. (1995) Science 268, 726–731
39. Bogyo, M., McMaster, J. S., Gazzana, M., Tortorella, D., Goldberg, A. L. & Ploegh, H. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6629–6634
40. Mehdi, S. (1991) Trends Biochem. Sci. 16, 150–153
41. Clurman, B. E., Sheaff, R. J., Thress, K., Groudine, M. & Roberts, J. M. (1996) Genes Dev. 10, 1979–1990
42. De Azevedo, W. F., Leclerc, S., Meijer, L., Havlicek, L., Strnad, M. & Kim, S. H. (1996) J. Biol. Chem. 271, 268, 21108–21113
43. Poon, R. Y., Lew, J. & Hunter, T. (1997) J. Biol. Chem. 272, 5703–5708
44. Luskin, M. B. & Shatz, C. J. (1985) J. Comp. Neurol. 242, 611–631
45. Angevine, B. J. & Sidman, R. L. (1961) Nature 192, 766–768