Two Degradation Pathways of the p35 Cdk5 (Cyclin-dependent Kinase) Activation Subunit, Dependent and Independent of Ubiquitination*

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Cdk5 is a versatile protein kinase that is involved in various neuronal activities, such as the migration of newborn neurons, neurite outgrowth, synaptic regulation, and neurodegenerative diseases. Cdk5 requires the p35 regulatory subunit for activation. Because Cdk5 is more abundantly expressed in neurons compared with p35, the p35 protein levels determine the kinase activity of Cdk5. p35 is a protein with a short half-life that is degraded by proteasomes. Although ubiquitination of p35 has been previously reported, the degradation mechanism of p35 is not yet known. Here, we intended to identify the ubiquitination site(s) in p35. Because p35 is myristoylated at the N-terminal glycine, the possible ubiquitination sites are the lysine residues in p35. We mutated all 23 Lys residues to Arg (p35 23R), but p35 23R was still rapidly degraded by proteasomes at a rate similar to wild-type p35. The degradation of p35 23R in primary neurons and the Cdk5 activation ability of p35 23R suggested the occurrence of ubiquitin-independent degradation of p35 in physiological conditions. We found that p35 has the amino acid sequence similar to the ubiquitin-independent degron in the NNX3.1 homeodomain transcription factor. An Ala mutation at Pro-247 in the degron-like sequence made p35 stable. These results suggest that p35 can be degraded by two degradation pathways: ubiquitin-dependent and ubiquitin-independent. The rapid degradation of p35 by two different methods would be a mechanism to suppress the production of p25, which overactivates Cdk5 to induce neuronal cell death.

Cyclin-dependent kinases (Cdks) are a family of Ser/Thr kinases that are activated by binding a regulatory subunit called cyclin. Most members of Cdk5 are expressed in proliferating cells to promote cell cycle progression (1). In contrast, Cdk5 is activated by p35 or p39 non-cyclin proteins, which are mainly expressed in post-mitotic neurons (2). Cdk5 is a versatile kinase that is involved in many neuronal activities, including neuronal cell layer formation, synaptic transmission, membrane trafficking, and neuron cell death (3). p35 and p39 appear to share

common and/or distinct functions for Cdk5, with p35 being the predominant activator. This is shown by the phenotypes of knock-out (KO) mice; p35 KO mice display abnormal neural layers in the cerebral cortex (4), and p39 KO mice do not show apparent abnormalities, whereas p35 and p39 double KO mice are perinatal lethal with abnormal neural layers, as are the Cdk5 KO mice (5–7). To understand the precise function of Cdk5-p35 in various neuronal activities, it is important to reveal the regulation mechanism of Cdk5 activity.

As well as being cell cycle Cdks, Cdk5 is a stable protein and is expressed more abundantly than p35 in neurons (8, 9). Therefore, Cdk5 activity is determined primarily by the available amount of activator protein p35, and the protein amounts of p35 are regulated by the balance between synthesis and degradation (2). Although the synthesis of p35 is stimulated by NGF or BDNF (10, 11), the degradation of p35 is carried out by proteasomes (12, 13). The degradation is a major determinant of the p35 level, which is reduced by treating neurons with excitatory neurotransmitter glutamate (14). p35 associates with membranes via myristoylation at the N-terminal glycine (15, 16), and this association enhances the degradation of p35 (17). On the other hand, p35 is cleaved by a calcium-dependent protease calpain to produce the C-terminal stable fragment p25 (15, 18, 19). Although the physiological function of Cdk5-p25 has been recently reported (20, 21), its abundance induces neuronal cell death in neurodegenerative diseases (22). Rapid turnover of p35 is suggested to be a mechanism to prevent p25 production (2). Therefore, it is particularly important to determine the degradation mechanism of p35. Interestingly, the addition of the N-terminal hepta-peptide containing the myristoylation site of p35 facilitates p35 lability (17), indicating that the degradation of p35 occurs selectively on membranes. Although p35 has previously been demonstrated to be post-translationally modified by ubiquitination (12), the E3 ligase responsible has not been identified yet in the neuron, and its degradation pathway is not completely understood.

The ubiquitin-proteasome system is a major component of the proteolytic machinery that performs the degradation of proteins in cells (23, 24). Ubiquitin is a small protein that is tagged to substrate proteins to be degraded. The proteasome is a large complex of multicatalytic proteases that degrades proteins to small peptides. The 26S proteasome is a complex of 20S proteasome and 19S particles. The 20S proteasome is the core of the proteasome, and 19S is a regulatory particle (PA700) that recognizes and unfolds ubiquitinated proteins. The unfolded
Ubiquitin-independent Degradation of p35

**TABLE 1**

| Name of primers | Forward sequence | Reverse sequence |
|-----------------|------------------|-----------------|
| K13R            | CCCCAGCTATCGGAGGGCCACACTGTTTG | GAAAACGTGGCGCCTTCCAGATAGCTGAGG |
| K24R            | GGTCTGCTCTCTGGAGGGGCCTGCAGCAC | TGTCTCTCTCTCTGTCCTGTTCCTGACCC |
| K34R/37R        | GAGGCGACGAGGAGGAGGAGGAGGAGGAGG | TGGCTCTCAGTGTGTGTGTGTGTGTGTG |
| K37R/K39R       | GAGGCGACGAGGAGGAGGAGGAGGAGGAGG | TGGCTCTCAGTGTGTGTGTGTGTGTGTG |
| K39R/K42R       | GAGGCGACGAGGAGGAGGAGGAGGAGGAGG | TGGCTCTCAGTGTGTGTGTGTGTGTGTG |
| K53R            | GGTCTGCTCTCTGGAGGGGCCTGCAGCAC | TGTCTCTCTCTCTGTCCTGTTCCTGACCC |
| K61R/K62R/K63R  | GAGGCGACGAGGAGGAGGAGGAGGAGGAGG | TGGCTCTCAGTGTGTGTGTGTGTGTGTG |
| K66R/K67R       | GAGGCGACGAGGAGGAGGAGGAGGAGGAGG | TGGCTCTCAGTGTGTGTGTGTGTGTGTG |
| K68R/K88R       | GAGGCGACGAGGAGGAGGAGGAGGAGGAGG | TGGCTCTCAGTGTGTGTGTGTGTGTGTG |
| K71R/K127R      | GGTCTGCTCTCTGGAGGGGCCTGCAGCAC | TGTCTCTCTCTCTGTCCTGTTCCTGACCC |
| K76R            | GGTCTGCTCTCTGGAGGGGCCTGCAGCAC | TGTCTCTCTCTCTGTCCTGTTCCTGACCC |
| K87R/K88R       | GGTCTGCTCTCTGGAGGGGCCTGCAGCAC | TGTCTCTCTCTCTGTCCTGTTCCTGACCC |
| K90R            | GGTCTGCTCTCTGGAGGGGCCTGCAGCAC | TGTCTCTCTCTCTGTCCTGTTCCTGACCC |
| K254R           | GGTCTGCTCTCTGGAGGGGCCTGCAGCAC | TGTCTCTCTCTCTGTCCTGTTCCTGACCC |
| K271R           | GGTCTGCTCTCTGGAGGGGCCTGCAGCAC | TGTCTCTCTCTCTGTCCTGTTCCTGACCC |
| K290R           | GGTCTGCTCTCTGGAGGGGCCTGCAGCAC | TGTCTCTCTCTCTGTCCTGTTCCTGACCC |
| K298R/K299R     | GGTCTGCTCTCTGGAGGGGCCTGCAGCAC | TGTCTCTCTCTCTGTCCTGTTCCTGACCC |
| MycKtoR         | GGTCTGCTCTCTGGAGGGGCCTGCAGCAC | TGTCTCTCTCTCTGTCCTGTTCCTGACCC |
| R126K/R127K     | GGTCTGCTCTCTGGAGGGGCCTGCAGCAC | TGTCTCTCTCTCTGTCCTGTTCCTGACCC |
| R167K           | GGTCTGCTCTCTGGAGGGGCCTGCAGCAC | TGTCTCTCTCTCTGTCCTGTTCCTGACCC |
| R298K/R299K     | GGTCTGCTCTCTGGAGGGGCCTGCAGCAC | TGTCTCTCTCTCTGTCCTGTTCCTGACCC |
| P247A           | TACCCGCCATCGGACGCGCTCTGGAGGAGG | TCCCTACCAGGGAGGAGGAGGAGGAGGAGG |
| P247R           | TACCCGCCATCGGACGCGCTCTGGAGGAGG | TCCCTACCAGGGAGGAGGAGGAGGAGGAGG |

**Experimental Procedures**

**Antibodies and Chemicals**—Anti-HA (Y11, SC805), anti-p35 (C19), and anti-Cdk5 (DC17) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin (A2066) anti-Myc (9E10) antibodies and cycloheximide (CHX) were purchased from Sigma. Benzyloxycarbonyl-leucyl-leucyl-leucinal (MG132) and epoxomicin were obtained from Calbiochem. Protein A-Sepharose was obtained from GE Healthcare.

**Construction of Mammalian Cell Expression Vectors**—Expression vectors of p35, N7-p25-myc, and Cdk5 were previously described (17). Lys-to-Arg mutants were constructed by polymerase chain reaction (PCR) with Pfu Ultra DNA polymerase (Agilent Technologies, Santa Clara, CA) using the primers listed in Table 1. Mutant p35 P247A and mutant p35 23R P247A were constructed by PCR using pCMV5-p35 and pCMV5-p35 23R, respectively, as a template using primers included in Table 1. pCAG-p35 and pCAG-p35 23R were constructed by inserting p35 and p35 23R, respectively, into the pCAG-MS2 vector (36) at the BamHI/HindIII sites.

**Cell Culture and Transfection**—Neuro2a and HEK293T cells were obtained from the JCRB Cell Bank (Osaka, Japan) and cultured in Dulbecco's modified Eagle's Medium (DMEM, Sigma) containing 10% fetal bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Neuro2a and COS-7 cells were transfected with expression plasmids using Hilly Max transfection regent (DaiNippon Sumitomo, Japan), Lipofectamine 2000 (ThermoFisher, Waltham, MA), or PolyFect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's protocol.

ICR mice were obtained from Sankyo Laboratory Service (Tokyo, Japan). Animal experiments were performed according to the guidelines for Animal Experiments of Tokyo Metropolitan University (approval numbers: 23-13, 24-15, and 25-12). The mice were housed in a temperature-controlled room under a 12-h light/12-h dark cycle with free access to food and water. Primary neurons were prepared from mouse brain cortex at embryonic day 16 (E16) and plated on polyethyleneimine-coated dishes in DMEM and Ham's F-12 (1:1) supplemented with 5% fetal bovine serum, 5% horse serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. 1 million (1 × 10^6) primary cortical neurons were transfected at 3 days in vitro by the calcium phosphate method using the ProFection Mammalian Transfection System (Promega, Madison, WI).

**Immunoprecipitation and Kinase Assay**—Neuro2a cells expressing Cdk5 and N7-p25, N7-p25 11R, p35, or p35 23R were collected and lysed in 20 mM MOPS at pH 6.8, 1 mM...
EGTA, 0.1 mM EDTA, 0.15 M NaCl, 1 mM MgCl₂, 10 μg/ml leupeptin, 0.4 mM Pefabloc, and 0.3% (v/v) Nonidet P-40. After centrifugation, the supernatants were immunoprecipitated using anti-Cdk5 (C8) as previously described (17). The kinase activity of the immunoprecipitates was measured with histone H1 and [γ-32P]ATP as substrates.

For the polyubiquitination assay, Neuro2a cells expressing N7-p25 or its mutants were treated with 20 μM MG132 for 5 h. At the end of the treatment the cells were collected, and the cell extracts were incubated with anti-p35 for p35 or anti-myc for N7-p25, which was followed by coprecipitation with protein A-Sepharose beads (17).

SDS-PAGE, Immunoblotting, and Statistical Analysis—Sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12.5% polyacrylamide gels. The phosphorylation levels of p35 were assessed by Phos-tag SDS-PAGE with 10% polyacrylamide gels containing 50 μM Phos-tag and 100 μM MnCl₂ as previously described (38). Immunoblotting was performed as previously described (17). All of the statistical treatments were performed by one-way analysis of variance with Tukey’s post hoc test.

Results

Effect of Arginine Mutation of the Lysine Residues in the C-terminal p25 Region on the Stability of p35 or N7-p25—p35 has been shown to be degraded by proteasomes through its polyubiquitination (15, 17, 39). To better understand the regulation of p35 degradation, a good goal would be to identify the E3 ligase that catalyzes its ubiquitination. An approach toward identifying the E3 ligase is to determine the ubiquitination sites in p35. Ubiquitin conjugation occurs on the amino group of lysine residue(s) or the N-terminal residue of the protein. The α-NH₂ group of the N-terminal glycine of p35 is blocked by myristoylation (15, 16, 17). Therefore, as candidates for possible ubiquitination sites, we first focused on the lysines at amino acids 126, 127, 140, and 167 because they are relatively close to Thr-138, the phosphorylation of which is involved in the degradation of p35 (37, 40). Also, amino acids 298/299 in the C-terminal region share homology with double Lys residues that act as ubiquitination sites in p35, a tumor suppressor protein with a short half-life (41) (Fig. 1A). We mutated these Lys to Arg and cotransfected the mutant with Cdk5 in Neuro2a cells to observe the degradation in the presence of cycloheximide, a protein synthesis inhibitor, for 1 and 3 h. However, none of the mutations stabilized p35 (Fig. 1B).

Although p25 is a stable C-terminal fragment of p35 (15, 18, 19), the addition of the seven N-terminal amino acids (N7), including the myristoylation motif, confers p25 with susceptibility to proteasomal degradation (17). The results suggest that the ubiquitination site(s) is present in the p25 region because there is no lysine in the N7 sequence. Accordingly, we mutated the Lys residues in N7-p25 in the order described in Fig. 1C. We expected that N7-p25 would become stable when the critical Lys is replaced with Arg. In this experiment we used N7-p25 with a myc tag at the C terminus because we wondered if multiple Lys-to-Arg mutations might affect the reactivity of anti-p35 antibodies. Mutations at the N-terminal Lys residues at 126, 127, 140, and 167 did not change the stability (Fig. 1D, 1R-4R). Arg mutants of N7-p25 became more labile when Lys residues in the C-terminal region were further changed (Fig. 1D, 5R-7R). Additional mutations at Lys at 246, 254, and 271 in the core of the Cdk5 activation domain did not further alter the stability (Fig. 1D, 8R-10R). Although we mutated all of the Lys residues in p25, N7-p25 was still unstable. There was a Lys residue in the myc-tag EQKLISEEDL. The Lys null version of N7-p25 11R was constructed, and its stability was examined. Surprisingly, however, N7-p25 11R was still unstable (Fig. 1D, 11R).
We confirmed that N7-p25 11R was not ubiquitinated. N7-p25 11R as well as 10R was cotransfected with HA-ubiquitin into Neuro2a cells. After immunoprecipitation with anti-HA antibodies and anti-polyubiquitin antibodies, we detected no ubiquitination signals in N7-p25 11R (Fig. 2A). This result suggests that ubiquitination of N7-p25 is not required for degradation. However, we observed the degradation of p35 23R in Neuro2a cells. p35 23R was degraded in the presence of CHX, and this decrease was suppressed with the proteasomal inhibitor MG132 as well as the more specific proteasome inhibitor Epoxomicin (Fig. 3C). These results indicate that p35 23R is degraded by proteasomes without ubiquitination. We confirmed the proper folding of p35 23R by its binding and activation of Cdk5. p35 23R was co-immunoprecipitated with anti-Cdk5, and Cdk5-p35 23R displayed histone H1 phosphorylation activity as much as Cdk5-p35 WT (Fig. 4A).

Degradation of p35 is affected by phosphorylation at Thr-138 (37, 40). Phosphorylation of p35 was assessed by the electrophoretic mobility shift in Phos-tag SDS-PAGE. p35 was separated into three bands; the intense lowest band is unphosphorylated p35, the slightly higher band is Thr-138-phosphorylated p35, and the upper band is p35 phosphorylated at Thr-138 and Ser-91 according to our previous results (38). Co-expression with Cdk5 reduced the mobility of p35 mainly by phosphorylation at Thr-138. However, there are still 13 additional lysine residues in the N-terminal p10 region. To test the possibility that the lysine residues in the N-terminal p10 region provide ubiquitination sites, we constructed a lysine-null mutant of full-length p35 (p35 23R) in which all 23 lysine residues of p35 were changed to arginine (Fig. 3). Here, we used p35 WT and p35 23R without any tag because the Arg mutations in the C-terminal region of p35 did not affect the immunoreactivity to anti-p35 antibodies (C19). Because the N terminus of p35 is blocked as described above, p35 23R has no ubiquitination sites, and in fact, polyubiquitination was not detected in p35 23R (Fig. 3B). We observed the degradation of p35 23R in Neuro2a cells. p35 23R was decreased in the presence of CHX, and this decrease was suppressed with the proteasomal inhibitor MG132 as well as the more specific proteasome inhibitor Epoxomicin (Fig. 3C). These results indicate that p35 23R is degraded by p35 23R with ubiquitination.
Ubiquitin-independent Degradation of p35

Ubiquitin-independent Degradation of p35—We investigated how p35 is recognized by proteasomes without ubiquitination. The number of proteins, which are known to be degraded by proteasomes independent of ubiquitin, have increased recently. Among them, we were interested in a ubiquitin-independent degron that is found in the C-terminal region of NKX3.1, a tumor repressor (42). The ubiquitin-independent degron sequence is composed of 21 amino acids with a PXL motif in the middle (35). p35 has a similar, but not identical, sequence in residues 240–258 (Fig. 6A). To determine if the sequence is involved in the ubiquitin-independent degradation of p35, we mutated Pro-247 to Ala in p35 WT or p35 23R and examined the degradation of the P247A mutants. Pro-247 corresponds to Pro-221 in the degron of NKX3.1, a critical amino acid for the degron activity. p35 P247A was degraded as fast as p35 WT in the presence of exogenous Cdk5; however, the P247A mutation made p35 23R stable (Fig. 6B). This was also found in the absence of exogenous Cdk5. When the activators were expressed alone, the P247A mutation did not affect the half-life of wild-type p35, but the P247A mutation of p35 23R became more stable than p35 23R itself, which had a longer half-life than wild-type p35 (Fig. 6C). These results suggested that the degron-like sequence is involved in degradation of p35 23R whether p35 23R binds to Cdk5 or not.

Discussion

p35 is an unstable protein with a half-life of 30–60 min, and it is degraded by proteasomes after ubiquitination (12, 13, 17). Because the p35 protein amount is a critical determinant of Cdk5 activity, elucidating the p35 degradation mechanism is central for understanding Cdk5 functions. To this end we searched for the polyubiquitination site(s) in p35, but unexpectedly, we found that the degradation of p35 can occur without ubiquitination. We also showed that ubiquitin-independent degradation was mediated by an α-helical degron-like sequence in the C-terminal region of p35. Thus, p35 is subjected to two different degradation mechanisms: ubiquitin-dependent and ubiquitin-independent.
Ubiquitin-independent Degradation of p35

In contrast to cell cycle Cdk5, whose activation is regulated by its phosphorylation/dephosphorylation upon cyclin binding (43), Cdk5 is activated only by binding to its activation subunit p35 (2). On the other hand, similar to cell cycle Cdk5, whose inactivation is induced by the degradation of cyclin, Cdk5 is inactivated by the degradation of p35 by proteasomes. Because Cdk5 is expressed more than p35 in neurons (8, 9), the protein level of p35 is a limiting factor determining the total Cdk5 activity. Cyclins are typical well studied proteins to be targeted by proteasomes via ubiquitination in a cell cycle-dependent manner (44). Therefore, it is natural to expect that p35 is also targeted by proteasomes when it is ubiquitinated. In fact, ubiquitination of p35 has been demonstrated by several previous studies by groups including ours (12, 17, 45). Therefore, it was surprising for us to find that the lysine-less mutant of p35 underwent degradation at a rate similar to wild-type p35 in cultured cell lines and primary neurons.

Polyubiquitin works as a degradation signal for proteins targeted by proteasomes (46, 47). Thus, a question arises as to how proteasomes recognize and degrade p35 without the polyubiquitin tag. Some misfolded or impaired proteins are degraded without ubiquitination by default. There are several examples of proteins that display default degradation, although they are degraded physiologically in a ubiquitin-dependent manner. p53 is a tumor suppressor protein that is degraded by proteasomes via polyubiquitination by E3 ubiquitin ligases, such as Mdm2 (33), but it is also degraded by the 20S proteasome by default if its N-terminal unstructured region is not protected by other proteins or modification. c-Fos proto-oncoprotein is an unstructured protein and degraded independently of ubiquitin by proteasomes when it does not form a transcriptional heterodimer with a partner protein (34). p35 functions exclusively as the activator of Cdk5. Only a few p35 molecules exist as free p35 in vivo because Cdk5 is significantly more abundant than
p35. If p35 fails to bind Cdk5, however, p35 would be recognized as a misfolded protein and degraded without ubiquitination by default. However, p35 23R appeared to maintain the proper conformation to fully bind and activate Cdk5, and it was degraded at a similar rate to that of wild-type p35. In this study we carried out most of the experiments under excess amounts of Cdk5 by co-expression. Thus, we think that it is unlikely that p35 23R is degraded through the default pathway of degradation.

There are at least three types of substrate protein recognition by proteasomes in the ubiquitin-independent degradation systems, which are as follows: by the 19S regulatory particle of the 26S proteasome as an example of ornithine decarboxylase (48, 49); by REGγ, also known as 11S or PA28, complexed with the 20S proteasome that is known for p21 Cdk inhibitor (50); by a core subunit of the 20S proteasome as exemplified by the F protein of hepatitis C virus (51). On the other hand, the amino acid sequence(s) required for degradation has also been investigated with several substrate proteins. In the case of the Rpn4 transcription factor that activates the expression of proteasome genes in yeast, the N-terminal unstructured segment and the following folded domain are essential for ubiquitin-independent degradation (31). A similar requirement of two elements, including an unstructured region and a following α-helical sequence, is shown for thymidylate synthase (30). The two elements of thymidylate synthase function as a degradation signal if they are targeted at the C terminus of a reporter protein and called a ubiquitin-independent degron (52). According to the two-step model of degradation, the α-helical degron region is recognized by the proteasome, and then the disordered region enters into the proteasomal cavity. p35 may be degraded similarly because p35 has an unstructured ~13-amino acids extension at the C terminus downstream of an α-helix-rich domain called the cyclin fold (Fig. 6D).

NKX3.1 is a homeodomain transcription factor that regulates prostate cancer initiation and progression (42). NKX3.1 turnover is regulated by ubiquitination, but it is also proteolyzed by proteasomes independent of ubiquitination. This ubiquitin-independent degradation is mediated by a 21-amino acid sequence in its C-terminal region (35). The proline residue in the sequence is essential for its ubiquitin-independent degron activity. p35 has a homologous (~53% identity) sequence at amino acids 240–258 with Pro-247 in the α-helix-rich domain called the cyclin fold (Fig. 6A). The mutation of Pro-247, which is in the ordered structure of the cyclin fold (53), to Ala slowed the turnover rate of p35 down remarkably. Considering that Pro-247 is positioned in the shallow concave (Fig. 6D), the structure, but not the amino acid sequence, around Pro-247 may provide the proteasome recognition site. Thus, p35 has two elements of an unstructured and a structured region next to each other that conform to the two-step degradation as shown by other proteins displaying ubiquitin-independent degradation.

The physiological role of ubiquitin-independent degradation and its regulation are largely unknown for most proteins (46, 47). In the case of p21, however, it is indicated that the cell cycle-regulated degradation is ubiquitin-dependent (54, 55), and its degradation during resting conditions is ubiquitin-independent (50, 56). Similar differential usage may be in operation for p35. p35 is unstable endogenously in neurons or when...

FIGURE 6. Ubiquitin-independent degron sequence in p35. A, the degron-like amino acid sequence in p35. Amino acids 240–258 of p35 are similar to the ubiquitin-independent degron of NKX3.1. Pro-221 in NKX3.1 is a critical amino acid for degron activity (42). p35 has Pro-247 in a similar position to Pro-221 in the position of Pro-247 in p25. A ribbon structure of p25 is depicted using a Waals software (Altif Laboratories, Inc., Tokyo, Japan) based on the crystal structure of p25 (53). Pro-247 and the...
expressed heterogeneously in cultured cell lines, and p35 P247A showed a similar degradation rate to wild-type p35, suggesting that the degradation of p35 in resting neurons could be ubiquitin-independent. p35 is acutely degraded in neurons when treated with glutamate (14, 57). This stimulated degradation of p35 may be dependent on ubiquitination. In any case, rapid p35 turnover would be crucial for neurons to serve for their long life. Overactivation of Cdk5, which is induced by the p25 C-terminal stable fragment, is toxic for neurons. p25 is produced by the cleavage of p35 with calpain, and Cdk5 activated by p25 acquires a long lasting activity with free accessibility to proteins (15, 18, 19), which Cdk5-p35 cannot access. The longer half-life of p35 may enhance the probability of the overproduction of p25. Two degradation pathways for p35 would be a mechanism to secure the long life of neurons.

Author Contributions—T. T. conceived the study and wrote the paper. S. M. designed, performed, and analyzed the experiments shown in Figs. 1 and 2. A. A. and T. S. provided technical assistance and performed a portion of the experiments shown in Figs. 3 and 4, respectively. T. S. and H. K. provided technical assistance and contributed to the preparation of the figures. S. H. coordinated the study and wrote the paper. All of the authors reviewed the results and approved the final version of the manuscript.

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