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Identification of p10 as a Neurotoxic Product Generated From the Proteolytic Cleavage of the Neuronal Cdk5 Activator

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ABSTRACT

The involvement of cyclin-dependent kinase-5 (Cdk5) and p25, the proteolytic fragment of activator p35, has long been implicated in the development of neuron-fibrillary tangles (NFTs), a hallmark of Alzheimer's disease (AD). Findings in this area over the past decade have been highly controversial and inconclusive. Here we report unprecedented detection of endogenous p10, the smaller proteolytic fragment of the Cdk5 activator p35 in treated primary cortical neurons that underwent significant apoptosis, triggered by proteasome inhibitors MG132 and lactacystin, and protein kinase inhibitor staurosporine (STS). p10 appeared exclusively in the detergent-resistant fraction made up of nuclear matrix, membrane-bound organelles, insoluble membrane proteins, and cytoskeletal components. Intriguingly, transient overexpression of p10 in neural cells induced apoptotic morphologies, suggesting that p10 may play an important role in mediating neuronal cell death in neurodegenerative diseases. We demonstrated for the first time that p10-mediated apoptosis occurred via a caspases-independent pathway. Furthermore, as p10 may contain the myristoylation signal for p35 which is responsible for binding p35 to several intracellular components and the membrane, all in all these novel results present that the accumulation of p10 to the detergent-insoluble fraction may be a crucial pathological event to triggering neuronal cell death. J. Cell. Biochem. 111: 1359–1366, 2010.

KEY WORDS: CYCLIN-DEPENDENT KINASE-5; ALZHEIMER'S DISEASE; NEURODEGENERATION; NEURONAL DEATH; P35; P10

Abbreviations used: AD, Alzheimer's disease; Cdk5, cyclin-dependent kinase-5; DMEM, Dulbecco's modified Eagle's medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NB, neurobasal; NFTs, neuro-fibrillary tangles; PBS, phosphate-buffered saline; PHFs, paired helical filaments; STS, staurosporine; UPS, ubiquitin-proteasome system.

Jenny Chew and Minghui Jessica Chen contributed equally to this study.

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Alzheimer's disease (AD) is typified by an accretion of extracellular deposits of β-amyloid and abundant intracellular neuro-fibrillary tangles (NFTs) in the brain that is associated with a progressive dementia and neuronal loss (Berg et al., 1993). The NFTs are composed of intraneuronal bundles of paired helical filaments (PHFs), which in turn is made up of hyperphosphorylated tau protein. The pathology progress of AD is believed to be initiated by this hyperphosphorylation of the tau protein (Gong and Iqbal, 2008). A key protein implicated in this hyperphosphorylation phenomenon is the tau protein kinase II (Maccioni et al., 2001), which consists of two subunits: the activator subunit, p35 and the catalytic subunit, cyclin-dependent kinase-5 (Cdk5). p35 is expressed almost exclusively in the central nervous system (Ishiguro et al., 1994; Cheng et al., 2002) and is thought to play important roles in brain development (Kusakawa et al., 2000). Proteolytic cleavage of p35 to p25 has been observed to accumulate in brains of AD patients (Patrick et al., 1999; Tsai et al., 2004). Due to its apparent higher stability than its precursor, p25 has been implicated in the prolonged activation and mislocalization of Cdk5 (Town et al., 2002; Camins et al., 2006), leading to hyperphosphorylation of tau and thus the progression of neurodegenerative diseases. The physiological presence of p25 has long been established and thus, the smaller proteolytic fragment, p10, should also theoretically be present in the cell system. However, to current up-to-date, the identification of p10 upon p35 cleavage in cells has not been extensively explored.

Another common feature of AD is the aberrant accumulation of proteins. Studies now suggest that protein aggregation directly impairs the function of the ubiquitin-proteasome system (UPS) (Bence et al., 2001) and that the dysfunction of the UPS is a possible primary mechanism leading to the pathogenesis of various neurodegenerative disorders. Truly, in AD, inhibition of the proteasome has been postulated to be responsible for its pathogenesis (Keller et al., 2000; Halliwell, 2002). In addition, previous studies have shown that the inhibition of proteasome function by lactacystin treatment induces apoptosis of cultured cortical neurons via stimulation of mitochondrial cytochrome *c* release and activation of a caspase-3-like protease activity (Pasquini et al., 2000; Qiu et al., 2000).

Here, we report that neuronal apoptosis induced by the proteasome and protein kinase inhibitors is associated with calpain-mediated cleavage of p35 to a smaller truncated form (p10) that carries the N-terminal myristoylation signal, and accumulation of p10 in the insoluble-membrane fraction. Over-expression studies revealed that the p10 fragment can induce significant cell death in a number of mammalian neural cell lines. Due to the possibility of that p10 may contain the myristoylation signal for p35, which is responsible for binding p35 to several intracellular components and the membrane, these data suggest a novel potential role of p10, in inducing neuronal cell death in pathological conditions such as AD.

EXPERIMENTAL PROCEDURES

MATERIALS

Neurobasal™ (NB) medium was from GIBCO™ (Carlsbad, CA). Cell culture plates were from NUNC (Naperville, IL). Proteasome inhibitors lactacystin and MG-132, staurosporine (STS) and retinoic acid (RA) were from Sigma (St. Louis, MO). Calpeptin and nerve growth factor (NGF) were from Calbiochem (Darmstadt, Germany). The primary antibodies used in Western blot analysis were as follows: anti-p35 (C-19) (cat# sc-820) and anti-c-myc (A14) (cat# sc-789) were obtained from Santa Cruz Biotechnology, Inc. (CA). Anti-caspase-3 (cat# 556425) was obtained from Biosciences. Monoclonal anti-β-tubulin (cat# ATN01) was purchased from Cytoskeleton, Inc. (Denver). All other nutrients, salts and antibiotics used in the culture media or assay buffers were from Sigma.

MURINE PRIMARY CORTICAL NEURONS

Cultures of mouse embryonic days 15–16 cortical neurons were prepared as described previously (Cheung et al., 1998, 2004a). In brief, cortices were microdissected from the sterile brains of mouse fetuses and subjected to trypsin digestion followed by mechanical trituration. The dissociated cells were collected by centrifugation and resuspended in NB medium containing 2.5% B-27 and 0.25% GlutaMAX™-I supplements, 10% fetal calf serum and 1% penicillin–streptomycin. The cells were seeded into poly-D-lysine-coated 6- and 24-well culture plates with 2×10^6 cells/well and 0.4×10^6 cells/well, respectively, and maintained in a humidified 5% CO_2 incubator at 37°C. After 24 h, the culture medium was changed to serum-free NB medium with 2.5% B-27 supplement, 0.25% GlutaMAX™-I supplement and 1% penicillin–streptomycin.

DRUG TREATMENT

Cortical neurons at day 5 in vitro were treated with 1.0 μM lactacystin (Sigma, cat# L6785) and 0.05-2.5 μM MG132 (Sigma, cat# C2211) by diluting with NB medium to the desired concentration. For the inhibitor study, the neurons were co-treated with 10–50 μM calpeptin (Calbiochem®, cat# 03-34-0051). STS (1.0 μM) was used as a positive control. The cells were either harvested 24 h after treatment for protein lysate or subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay.

DNA STAINING

Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). Fixed cells were then incubated with Hoechst 33342 at the final concentration of 2 μg/ml. Stained nuclei were observed and analyzed under a fluorescence microscope (Leica DM IRB).

QUANTITATIVE AND QUALITATIVE ASSESSMENTS OF CELL VIABILITY

MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] reduction was carried out essentially as described (Cheung et al., 2004a). Cultured cells were added with MTT solution (30 μl/well) and incubated at 37°C for 30 min. The culture medium was then removed by aspiration. The formazan formed in each well was dissolved with DMSO and its absorbance at 570 nm was read using the Ultra 384 TECAN (Tecan, Austria) plate reader.

PLASMID CONSTRUCTION

p10, which comprises a.a. 1–98 of p35, was cloned by PCR from a bovine p35 cDNA into the plasmid vectors pEGFP-N3 (Invitrogen)

and pCI-neo (Promega). The resulting plasmids express p10 with GFP or a double myc-tag, respectively, at the carboxy-terminus.

TRANSFECTION AND DIFFERENTIATION OF MURINE NEUROBLASTOMA NEURO-2A CELL LINE

The mouse neuroblastoma Neuro-2a cell lines (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 100 U/ml penicillin, 100 μg/ml streptomycin, 10% fetal bovine serum (FBS) and 5% horse serum. The cells were then seeded at a density of 0.12×10^6 cells/well overnight before treatment and transfection, such that they were about 50% confluent 24 h after seeding. Transfection of the cells was performed using TransFectin Reagent (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocol. After 24 h of incubation with the transfection reagent at 37°C, the medium was changed to complete DMEM medium with 10% FBS. Twenty-four hours later, cells were differentiated by replacing the medium with complete DMEM containing 10 μM retinoic acid and 1 mM *N*-acetylcystine. After 24 h of successful differentiation, cells were used for further experiments.

PREPARATION OF CRUDE CELL LYSATE FOR WESTERN BLOTTING

For whole-cell lysate preparation, neurons were lysed in 5× sample buffer (0.5 M Tris, pH 6.8, 10% SDS, 20% glycerol, 0.05% bromophenol blue, 20% β-mercaptoethanol). When RIPA buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40 (NP40), 0.5% deoxycholate, 0.1% SDS) was used for extraction, cells were lysed in RIPA buffer and the insoluble materials were pelleted by centrifugation at 14,000 rpm for 10 min. Pellets were redissolved in 5× sample buffer. Equal volumes of the whole-cell lysate and the pellet obtained from extraction using RIPA buffer were loaded onto SDS–PAGE gels. An aliquot of the supernatant containing an equal amount of proteins was used for Western blot analysis. After SDS–PAGE, proteins were electrotransferred to PVDF membranes and probed with antibodies. To generate an antibody recognizing the NH_2-terminus of p35, the 6xHis-tagged p10 protein, comprising of a.a. 1–98, was used to immunize rabbits. Affinity purification was performed on antisera using a GST-p10-coupled column to obtain the purified antibody anti-p35N. The resultant affinity purified rabbit polyclonal antibody is confirmed to map against the p10 peptide at the NH_2-terminus of p35 of human origin (identical to corresponding bovine sequence).

TRANSMISSION ELECTRON MICROSCOPY

Neuro2a cells were cultured in 4-well chambered coverglass, non-transfected or transfected with respective plasmid constructs for 24 h and differentiated for 48 h, and fixed with 3% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer for 45 min at room temperature. After osmication in 2% osmium tetroxide, specimens were dehydrated in an ascending series of ethanol and embedded in araldite. Ultrathin sections were cut, mounted on formvar-coated copper grids, and doubly stained with uranyl acetate and lead citrate before viewing in a Philips BioTwin CM120 transmission electron microscope (Phillips Electron Optics, Eindhoven, The Netherlands) (Cheung et al., 2004b).

CASPASE-FAMILY ACTIVITIES MEASUREMENT

Caspase activity of treated cells was measured using caspase-family fluorometric substrate and followed the protocol provided (BioVision). Briefly, control and apoptotic cells lysated using chilled cell lysis buffer were incubated on ice for 10 min. Later the supernatants were collected after centrifuge at 14,000 rpm for 30 min at 4°C. The mixture of reaction buffer containing 10 mM DTT, AFC conjugated substrates, and supernatant was read in a fluorometer (Gemini XS spectrofluorometer, Molecular Devices) equipped with a 400-nm excitation filter and 505-nm emission filter. The protein concentrations were determined by the Lowry method using BSA as the standard. The relative fluorescence unit per microgram of protein (RFU/μg protein) in each sample was then calculated.

STATISTICAL ANALYSES

Results are presented as mean ± SE. All experiments were performed at least three times. Data were analyzed using Tukey test with one-way analysis of variance (ANOVA) to determine significant differences in multiple comparisons. Values of $^*P < 0.05$ were considered as statistically significant.

RESULTS

TREATMENT WITH PROTEASOME INHIBITORS INDUCE APOPTOSIS OF MOUSE CORTICAL NEURONS

Previously, Cheung et al. (2004a) reported that treatment of the cultured neuronal cells with 1 μM lactacystin for 48 h induced apoptosis-associated phenotypic changes including cell shrinkage, DNA condensation and chromatin fragmentations, with a decreasing cell viability that was dose- and time-dependent. To further investigate how proteasome inhibitor induces neuronal cell death, we examined the morphological and biochemical changes of mouse cortical neurons induced by treatment with a proteasome inhibitor, MG132. It was observed that treatment of cultured cortical neuronal cells with 2.5 μM MG132 for 24 h triggered apoptosis-associated phenotypic changes including extensive cell shrinkage, DNA condensation and fragmentation (Fig. 1A). Furthermore, the MTT assay indicated a more than 50% reduction in viability when cells were treated with 0.1 μM MG132 (Fig. 1B). To determine if caspase-3 signaling pathway is involved in the MG132-induced neuronal cell death, we examined the level of the active caspase-3 generated from proteolysis of the procaspase-3. As shown in Figure 2A, Western blotting of the supernatant fraction extracted from RIPA-lysed lactacystin-, MG132-, and STS-treated neurons revealed the presence of a 17 kDa subunit of active caspase-3. Furthermore, expression of p25 corresponded with the activation of caspase-3 (Fig. 2A). Taken together, our results demonstrate that the proteasome inhibitor treatment can activate caspase-3 pathway and can induce apoptosis of cultured neuronal cells.

TREATED CORTICAL NEURONAL CELLS RESULTS IN CALPAIN-MEDIATED CLEAVAGE OF P35

It has been well studied that the neurotoxic molecule β-amyloid induces cleavage of p35 to two fragments, p25 and p10 (Kusakawa

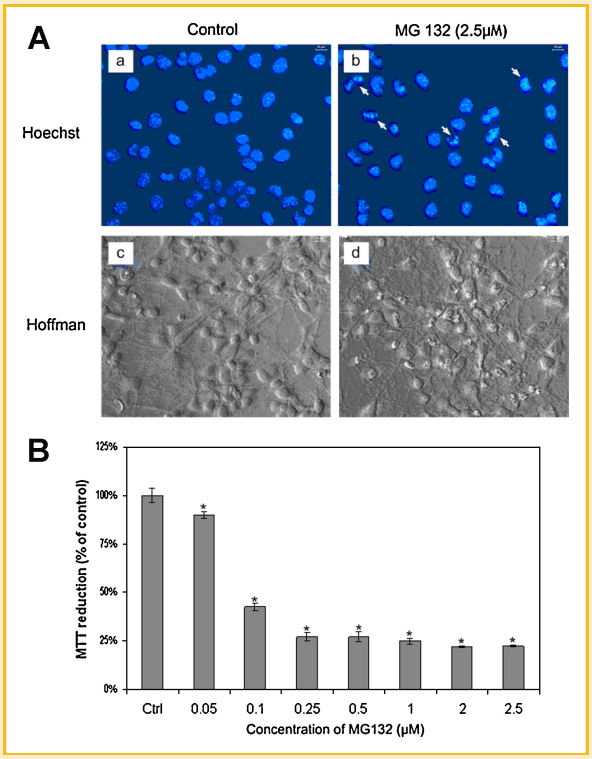

Fig. 1. The effects of MG132 on the morphology and viability of cultured cortical neurons. A: Staining of apoptotic nuclei with Hoechst 33342. Control and treated (2.5 μM MG132 for 24 h) cells were treated and viewed under the fluorescence microscope (Carl Zeiss LSM510). White arrows indicated the apoptotic nuclei in treated cells. B: Cell viability of MG132-treated cultured cortical neurons was determined using the MTT assay (as described in the Experimental Procedures). Values are the means ± SE ($n = 6$). $^{*}P < 0.05$, compared with control.

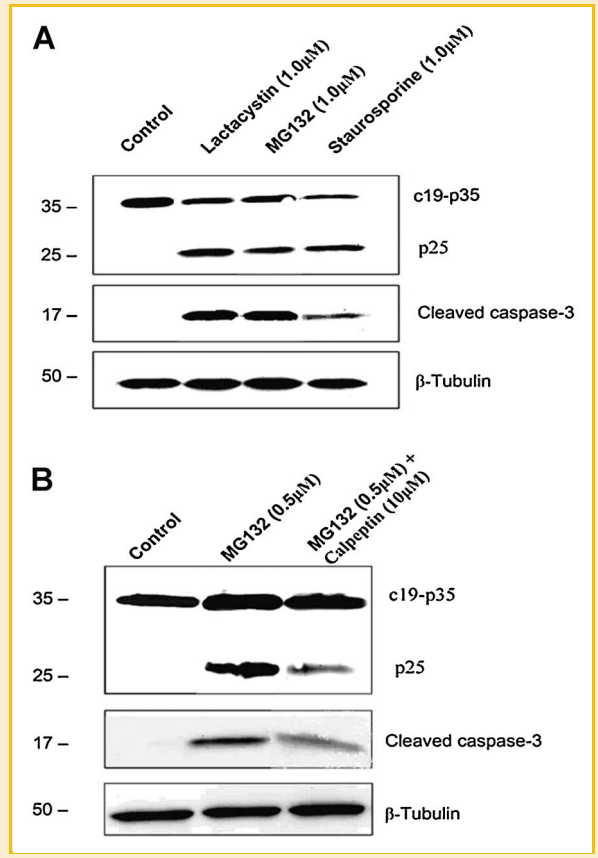

Fig. 2. Western blot analysis of calpain-mediated cleavage of p35 in RIPA-lysed lactacystin- and MG132-treated cultured cortical neurons. A: Detection of the p25 fragment in the supernatant fraction of RIPA-extracted treated samples is accompanied by the activation of caspase-3, as shown by the presence of the cleaved caspase-3. B: Treatment with the calpain inhibitor, calpeptin, significantly inhibits both the amount of p35 cleavage, as well as, the amount of caspase-3 activity in the RIPA-extracted supernatant portion. Ten micrograms of protein sample was loaded.

et al., 2000), which is mediated by a calcium-dependent cysteine proteases called calpain (Kusakawa et al., 2000; Lee et al., 2000; Nath et al., 2000). Therefore, to investigate whether calpain is activated in MG132-treated cells, we examined the amount of cleaved fragment p25 induced by the treatment with and without a calpain inhibitor, calpeptin. Calpeptin is a cell-permeable calpain inhibitor with high affinity for both calpain-I and calpain-II. As shown in Figure 2B, Western blotting of RIPA-lysed supernatant protein fraction extracted from mouse cortical neurons treated concomitantly with 0.5 μM MG132 and 10 μM calpeptin significantly reduced the amount of p25 and cleaved caspase-3.

To further verify if the reduction in p35 cleavage by calpeptin corresponds with a reduction in p10 expression, whole cell lysates extracted by 5× sample buffer containing strong solubilizing cellular detergent SDS to dissolve membranous proteins, were adopted for Western Blot analysis. Increasing the amount of calpeptin treatment to 50 μM completely blocked the cleavage of p35 to p10 in whole cell lysates of both lactacystin- and MG132-treated cells (Fig. 3A). This confirmed earlier findings that calcium-dependent calpain is a key player in inducing the cleavage of p35 to its truncated forms, p25 and p10.

LACTACYSTIN AND MG132 TREATMENTS INDUCE CONVERSION OF P35 TO P10 AND ITS ACCUMULATION IN THE DETERGENT-INSOLUBLE MEMBRANE FRACTION

The Cdk5/p25 pathway has long been implicated in the progression of several neurodegenerative diseases (Dhavan and Tsai, 2001; Camins et al., 2006). However, the exact role played by p25 in enhancing neuronal apoptosis is unknown. As part of our study of the molecular mechanism of lactacystin- and MG132-induced neuronal cell death, we examined the cleavage of p35 to p10 by Western blotting. Whole-cell lysate of neurons in the untreated cells (control; Fig. 3A) revealed the predominant p35 form. Treatment with lactacystin and MG132 revealed formation of the p10 truncated form, which migrated at ~14 kDa by SDS–PAGE (Fig. 3A).

After lysis of the neuronal cells with RIPA buffer, p35 and p10 levels in the soluble and the insoluble fractions were determined by Western blotting. Since RIPA buffer contains detergents (NP40, deoxycholate, and SDS), the soluble portion represents the cytosolic fraction and the detergent-soluble membrane fraction of the cells, while the insoluble portion is designated as the detergent-resistant

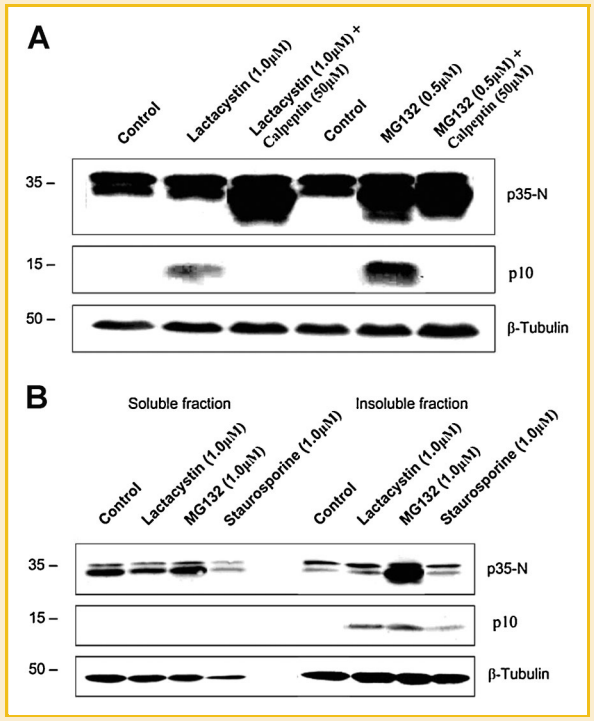

Fig. 3. Detection of endogenous p10 in lactacystin- and MG132-treated cultured cortical neurons, with or without the inhibitor. A: Western blot analysis of whole cell lysates of lactacystin- and MG132-treated cells detects endogenous p10 fragment, which can be blocked completely by the calpain inhibitor, calpeptin. Ten microliters of whole cell lysate protein was loaded. B: Soluble (10 μg) and insoluble (5 μl) fractions of RIPA-extracted cell lysate from cultured cortical neurons treated with lactacystin and MG132 were analyzed by Western blot. Results show the presence of p10 fragment in the insoluble fraction.

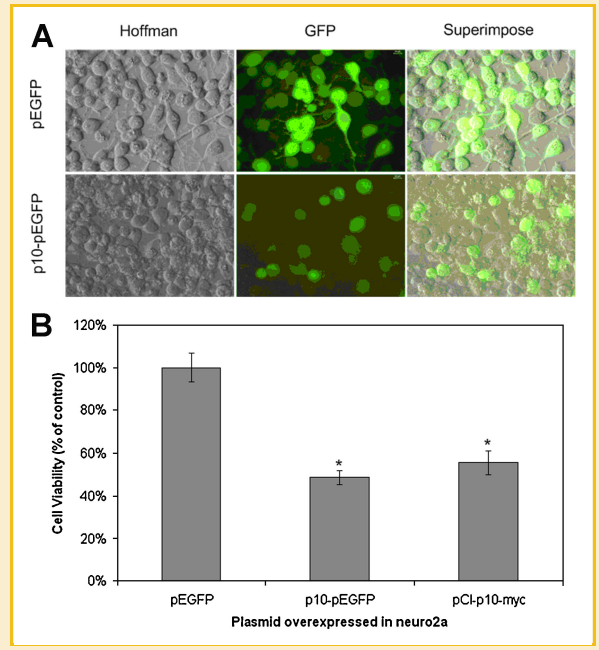

Fig. 4. Transfection efficiency and cell viability of differentiated Neuro2a cells transiently expressing p10. A: Differentiated murine neuroblastoma, Neuro-2a cell line showed neuritic outgrowth when transfected with pEGFP plasmid. However, upon transfection with pEGFP-p10, cells became shrunken and round with no exhibition of neurites. Differentiated neuro2a cells showed a transfection efficiency of about 80% determined using flow cytometry (data not shown). B: Cell viability of differentiated transfected Neuro-2a cells was determined using the MTT assay (as described in the Experimental Procedures). Values are the means ± SE ($n = 4$). $^{*}P < 0.05$, compared with control.

fraction. The 35-kDa species appeared in both soluble and insoluble fractions while the 14-kDa species were present exclusively in the insoluble fraction (Fig. 3B). Thus, we show novel detection of the p10 fragment as well as the existence of p10 in the detergent-resistant fraction, denoting the possibility of p10 interaction with other proteins or complexes to allow precise translocation.

EXPRESSION OF P10 IN NEURO-2A INDUCES APOPTOSIS

These findings suggest a possible accumulation of p10 in proteasome-dysfunctional AD brains. In an attempt to validate the role of p10 in neuronal cell death, an overexpression model employing mammalian neural cell line that demonstrates high transfection efficiency was established. Fluorescence microscopy revealed that transfection efficiency was the highest in Neuro-2a cells (Fig. 4A) and NIE 115 cells (data not shown), with an efficiency of ~80%. Morphological effects of p10 overexpression in Neuro-2a cell line were examined. Intriguingly, Neuro-2a cells transfected with the pEGFP-p10 plasmid construct and subsequently differentiated exhibited similar apoptotic cellular morphology: significant cell shrinkage and round cell morphology as compared with cells transfected with the control vector, pEGFP plasmid (Fig. 4A). The MTT assay confirmed that the viability of the differentiated pEGFP-p10-transfected cells decreased more than 50% relative to the vector control (Fig. 4B). Similar cytotoxic trend on cell survival was observed in cells transfected with cmyc-10 plasmid construct (Fig. 4B). This eliminated the possibility of any contribution to cytotoxicity by the tag moiety of the ectopic p10 proteins.

To further validate if overexpression of p10 induces extensive cell death in neural cell lines, we examined the morphological differences of differentiated non-transfected and transfected cells. Cell imaging in Figure 5A indicated presence of cell shrinkage, DNA fragmentation, and condensation in pEGFP-p10 transfected Neuro-2a cells. Transmission electron microscopy analysis performed on differentiated Neuro-2a cells overexpressing GFP-p10 protein showed membrane blebbing, nuclear condensation, and chromatin fragmentation which were absent in non-transfected and control vector-transfected cells (Fig. 5B). To provide insights into the cell death mechanism induced by p10, we attempted to study in greater detail the activation of various caspases during overexpression of p10 and STS-induced cell death. In Figure 6, we charted the changes in the activities of caspase-1,-2,-3,-4,-5,-6,-8,-9,-10, in p10-overexpressing differentiated Neuro-2a cells and the results showed that none of the caspase members were activated in the p10 overexpressing Neuro-2a cells. Taken together, our results demonstrated that expression of p10 induces apoptosis of neuroblastoma cells via caspase-independent pathways.

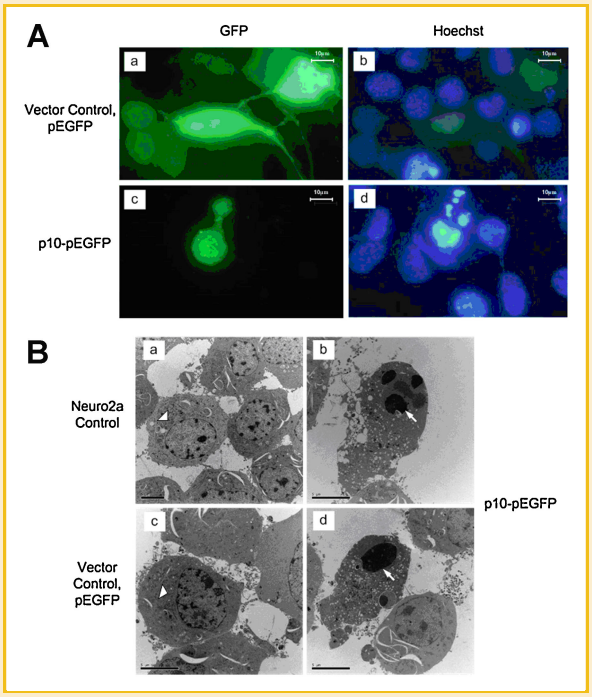

Fig. 5. Morphological changes of differentiated transfected Neuro-2a cell lines. A: Staining of apoptotic nuclei using Hoechst 33342. Cells transfected with the vector control or the p10 plasmid construct and later differentiated were stained and viewed under the fluorescence microscopy (Carl Zeiss LSM510). B: Transmission electron micrographs of differentiated transfected Neuro-2a cells. Differentiated control and vector control-transfected cells showing neuritic outgrowth and normal nuclei, as indicated by arrowheads in (a) and (c). Cells transiently expressing p10 plasmid were round and showed cell shrinkage with fragmented and condensed nuclei in (b) and (d), respectively, indicated by white arrows, all demonstrating apoptotic morphological characteristics.

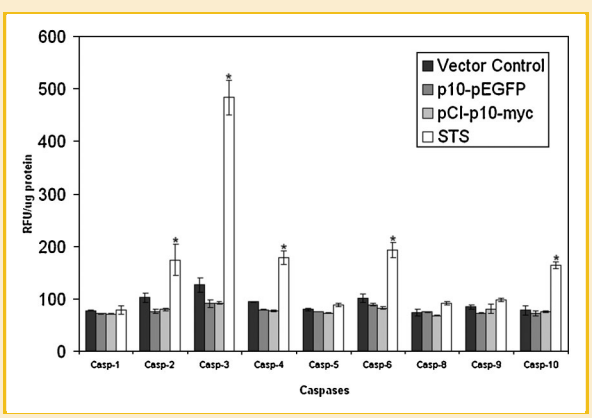

Fig. 6. Determination of activity in various caspases measured by fluorogenic substrates. Fluorescence intensity was expressed as relative fluorescence unit (RFU) per μg protein. All data are the means ± SE of at least two measurements per treatment, from two independent experiments. $^{*}P < 0.05$, compared with control. Casp, caspase.

DISCUSSION

CALPAIN AS A KEY MEDIATOR OF P35 CLEAVAGE TO P25 DURING PROTEASOME INHIBITION IN VITRO

In vivo, cleavage of Cdk5 activator p35 to p25 is known to be induced by the neurotoxic molecule Aβ and mediated by a highly conserved family of calcium-dependent cysteine proteases called calpains (Town et al., 2002). Our initial studies further confirmed the previous finding, through observations of proteasome inhibition by lactacystin and MG132 in vitro leading to an activation of calpain, which has been indirectly demonstrated by the successful inhibition of p35 cleavage upon the calpain inhibitor calpeptin treatment (Fig. 4A). In addition, we also indirectly demonstrated that calpain was involved in the mediation of caspase-3 activation during proteasome inhibition (Fig. 3B). This is in consistent with several studies that suggested calpain could cleave endogenous caspases such as casapase-3, -7, -8, and -9 (McGinnis et al., 1999; Chua et al., 2000).

RELEVANCE OF P25 AND P10 TO NEUROTOXICITY

Truncated activator, p25, has been shown to exhibit greater stability than its precursor and which therefore may result in prolonged Cdk5 activation in addition to the mislocalization of Cdk5 (Town et al., 2002), resulting in continuous hyperphosphorylation of available substrates such as tau (Patrick et al., 1998; Patzke and Tsai, 2002). Many Cdk5 phosphorylation sites on tau have been identified and all of the Ser/Thr-Pro sites, are the most likely to be physiologically relevant (Lund et al., 2001; Hashiguchi et al., 2002; Liu et al., 2002). This would then lead to the formation of PHFs and eventually, promoting neuronal cell death or apoptosis. A possible mechanism for this cell death phenomenon is that phosphorylation of tau by Cdk5 can reduce the binding of tau to microtubules, inhibiting the ability of tau to promote microtubule assembly and decreasing microtubule stability. Since microtubules are important structures maintaining the integrity of the cell, disruption can trigger the cell to undergo programmed cell death or apoptosis. Sobue et al. (2000) further showed that Cdk5 but not its activator interacts directly with tau (Sobue et al., 2000). By far, it has been reported that degradation of p35 by proteasome, and the calpain-mediated cleavage of p35 to p25 are the two discovered proteolytic mechanisms regulating the protein levels of p35 and p25 (Kerokoski et al., 2002a). Consistent with this concept, we hypothesized that the concentration of p10 could also be present in the cellular system and regulated in a similar manner.

However, unlike p25 function, we postulate that p10 does not play a role in the increased tau phosphorylation through Cdk5 during the pathogenesis of AD as earlier studies demonstrated that the Cdk5-binding and activating domain is located within p25 but not in the p10 region (Poon et al., 1997; Tang et al., 1997). Clearly, after calpain-mediated cleavage of p35, Cdk5-p25 but not p10 is involved in tau hyperphosphorylation, which has been substantiated by further findings from several major Cdk5 research groups (Noble et al., 2003; Patrick et al., 1999).

We proposed 3 hypotheses to explain the lack of knowledge with regard to p10. (1) The p10 fragment, being relatively small in size, may exhibit a short lifespan due to rapid proteasomal degradation.

(2) It contains the N-terminal myristylation signal that may target it to detergent-insoluble cellular proteins or structures. Hence upon protein extraction, the p10 protein remains in the insoluble pellet and is left undetected. (3) There is a lack of antibodies that can effectively detect p10 in cell or tissue extracts.

P10, THE N-TERMINAL FRAGMENT OF P35

Our findings have established the novel detection of p10 and its accumulation to the detergent-insoluble fraction of neuronal cells. In addition, transient expression of p10 in neuronal cells induced round cell morphology and DNA condensation, which are classical characteristics of apoptotic cells. Taken together, we have identified p10 as a potential key player in the progression of neurodegenerative diseases by inducing apoptosis of neurons via caspase-independent pathways.

Previously, Paglini et al. (2001) reported that there is possible association of Cdk5-p35 with a component of the Golgi matrix, which is important in regulating actin dynamics. Furthermore, recent study has confirmed Cdk5 activation alone is sufficient to induce robust Golgi fragmentation, a phenomenon observed upon beta-amyloid and glutamate stimulation (Sun et al., 2008). Their results are reminiscent of our findings in that p10, the N-terminal fragment of p35, preferentially accumulates in the detergent-insoluble fraction. This raises an interesting possibility that p10 may associate with subcellular structures or protein complexes in the detergent-insoluble fraction, which may include nuclear matrix (Fu et al., 2006), membrane-bound organelles, insoluble membrane proteins, and cytoskeletal components, thus promoting neuronal apoptosis by interfering with the cellular integrity.

CONCLUSION

The Cdk5/p35 pathway not only plays important roles in brain development, synaptic activity, and neuronal cell death, but has also long been implicated as a major contributor to the accumulation of abnormally hyperphosphorylated tau protein in several neurodegenerative disorders, including AD. Although much research work has been done in this area for the past decade, the involvement of Cdk5 and p35 has not been fully understood and data have been highly conflicting (Takashima et al., 2001; Kerokoski et al., 2002b). Identification of novel neuronal cell death pathways or novel interacting proteins would be important approaches to uncover other possible cell death mechanisms in such neurodegenerative disorders.

Although our findings do not suggest an exact mechanism of how p10 mediates the apoptotic effects of lactacystin and MG132, they suggest for the first time, a novel role for p10 in the progression of neurodegenerative diseases. Further investigations should focus on (i) identifying the activated apoptotic pathway(s), (ii) identifying the protein–protein interactions with the p10 fragment, and (iii) determining the localization of the p10 fragment. These additional studies will be able to provide greater insights to the role p10 in the progressive degeneration of neuronal cells and potential targets for the future therapeutic intervention to curb the onset of various neurodegenerative diseases.

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