E2-25K/Hip-2 regulates caspase-12 in ER stress–mediated Aβ neurotoxicity

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Introduction

Amyloid-β (Aβ) is thought to be the principle cause of the pathogenesis and progression of the neuron and memory loss associated with Alzheimer’s disease (AD). Previously we found that E2-25K/Hip-2, an E2 ubiquitin-conjugating enzyme, mediates Aβ neurotoxicity. Here, we report that E2-25K/Hip-2 modulates caspase-12 activity via the ubiquitin/proteasome system. Levels of endoplasmic reticulum (ER)–resident caspase-12 are strongly up-regulated in the brains of AD model mice, where the enzyme colocalizes with E2-25K/Hip-2. Aβ increases expression of E2-25K/Hip-2, which then stabilizes caspase-12 protein by inhibiting proteasome activity. This increase in E2-25K/Hip-2 also induces proteolytic activation of caspase-12 through its ability to induce calpain-like activity. Knockdown of E2-25K/Hip-2 expression suppresses neuronal cell death triggered by ER stress, and thus caspase-12 is required for the E2-25K/Hip-2–mediated cell death. Finally, we find that E2-25K/Hip-2–deficient cortical neurons are resistant to Aβ toxicity and to the induction of ER stress and caspase-12 expression by Aβ. E2-25K/Hip-2 is thus an essential upstream regulator of the expression and activation of caspase-12 in ER stress–mediated Aβ neurotoxicity.

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Abbreviations used in this paper: AD, Alzheimer’s disease; Aβ, amyloid-β; APP, Aβ precursor protein; β-gal, β-galactosidase; KD, knockdown; MEF, mouse embryonic fibroblast; PS1, presenilin 1; ROS, reactive oxygen species; UPS, ubiquitin/proteasome system.

The online version of this article contains supplemental material.

Supplemental material can be found at:
http://doi.org/10.1083/jcb.200711066
via the sequential actions of ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-ligating (E3) enzymes; and (2) degradation, i.e., recognition of the Lys48 polyubiquitin chain by 26S proteasome and degradation of the target protein with generation of free ubiquitin by ubiquitin-recycling enzymes. Malfunction of the UPS leading to accumulation of aggregation-prone proteins is thought to be involved in some neurodegenerative diseases including AD (Al-Ramahi et al. 2006; Kristiansen et al., 2007; for review see Rubinsztein, 2006). Indeed, it was proposed that E2-25K/Hip-2, an E2 ubiquitin-conjugating enzyme, acts as an essential mediator of Aβ neurotoxicity by promoting the inhibition of proteasome (Song et al., 2003; for review see Song and Jung, 2004). Furthermore, other evidence has shown that the inhibition of proteasome activity promotes ER stress (Nishitoh et al., 2002), suggesting that altered regulation of proteasome activity and ER stress may be associated with Aβ neurotoxicity. However, a critical mediator coordinating ER stress and caspase-12 activity in Aβ neurotoxicity remains unknown. Here we show that E2-25K/Hip-2 regulates the activation of caspase-12 and ER stress responses during Aβ neurotoxicity, and that E2-25K/Hip-2-deficient cortical neurons cultured from E2-25K/Hip-2 knockout mice lack Aβ-induced ER stress responses, including accumulation of caspase-12, and are resistant to Aβ toxicity.

Results
Regulation of caspase-12 expression by E2-25K/Hip-2 during Aβ toxicity
To assess the relationship between E2-25K/Hip-2 and caspase-12, we initially performed an immunohistochemical analysis of the brains of Tg2576 mice (Hsiao et al., 1996). We found that, as with E2-25K/Hip-2 (Song et al., 2003), there was much greater expression of caspase-12 in the hippocampal region of the Tg2576 mouse brain than in their wild-type littermates (Fig. 1 A). Interestingly, the E2-25K/Hip-2 immunoreactivity colocalized with that of caspase-12 around amyloid plaques, but not with that of caspase-2, which is also known to mediate Aβ neurotoxicity (Troy et al., 2000). In addition, Western blotting showed that levels of both caspase-12 and E2-25K/Hip-2 were increased in the brains of 6- and 9-mo-old double transgenic mice expressing Swedish mutant Aβ precursor protein (APPswe) and exon 9-deleted presenilin 1 (PS1dE9; Fig. 1, B and C). These results imply there is concerted regulation of caspase-12 and E2-25K/Hip-2 in the brains of these AD model mice.

We then examined the expression and activation of caspase-12 in rat B103 neuroblastoma cells stably expressing the antisense E2-25K/Hip-2 cDNA (B103/E2-25K-AS cells; Fig. 2 A, left). As compared with control cells, mixed populations of B103/E2-25K-AS cells showed reduced expression of caspase-12 but no changes in the expression of caspase-2 and -8 (Fig. 2 A, right). Treatment with Aβ1-42 induced the accumulation and proteolytic processing of caspase-12 in control cells, but these effects were substantially reduced in B103/E2-25K-AS cells. Conversely, ectopic expression of E2-25K/Hip-2 efficiently induced proteolytic activation of caspase-12 in B103 cells (Fig. 2 B). Collectively, these results suggest that Aβ-induced accumulation and the proteolytic activation of caspase-12 protein may be mediated by E2-25K/Hip-2. RT-PCR analysis showed that the basal levels of caspase-12 mRNA in B103/E2-25K-AS cells were also somewhat lower than in control cells (Fig. 2 C, left) and that caspase-12 mRNA was apparently induced by Aβ in control cells but not in B103/E2-25K-AS #1 cells (Fig. 2 C, right). When reporter assays were performed with pGL3-3.0 (5′ flanking region + 1′ intron + 2′ exon) and pGL3-0.8 (1′ intron + 2′ exon; Oubrahim et al., 2005), the promoter activity of caspase-12 was weak in B103 cells (twofold higher than control) compared with NIH 3T3 cells (sevenfold higher than control).
Caspase-12 is a downstream mediator of E2-25K/Hip-2 in ER stress–induced cell death

Because caspase-12 is known to play an essential role in the apoptosis induced by ER stress, we examined the involvement of E2-25K/Hip-2 in that process. Treating cells with thapsigargin or tunicamycin, which cause ER stress, induced cell death in >80% of control B103 cells but in a much smaller percentage of B103/E2-25K-AS cells (Fig. 2 C, left), suggesting that E2-25K/Hip-2 regulates caspase-12 at both the mRNA and protein levels.

Oubrahim et al., 2005) and the promoter activity of caspase-12 in pGL3-0.8 was slightly increased by E2-25K/Hip-2 (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200711066/DC1). However, a quantitative comparison of our Western and RT-PCR analyses revealed that the reduction in caspase-12 protein was greater than that in caspase-12 mRNA in B103/E2-25K-AS cells (Fig. 2 C, left), suggesting that E2-25K/Hip-2 regulates caspase-12 at both the mRNA and protein levels.

Figure 2. E2-25K/Hip-2 is required for the expression and activation of caspase-12 during Aβ toxicity. (A) Knockdown (KD) of E2-25K/Hip-2 expression reduces Aβ-induced expression and activation of caspase-12 protein. B103 cells were transfected with pcDNA3 (Mock) or antisense [AS] E2-25K/Hip-2 cDNA (pAS-E2-25K/Hip-2) and enriched by incubation with G418 for 10 d (Mixed). The level of E2-25K/Hip-2 expression was examined by Western analysis (left). Cells were incubated with 5 μM Aβ1-42 for 48 h in serum-free culture medium, after which cell extracts were analyzed by Western blotting with anticaspase-2, -8, and -12 and anti-α-tubulin antibodies (right). (B) Forced expression of E2-25K/Hip-2 induces proteolytic activation of caspase-12. B103 cells were transfected with pcDNA3 or pE2-25K/Hip-2 for 48 h and analyzed by Western blotting using the indicated antibodies. (C) E2-25K/Hip-2 regulates the expression of both caspase-12 mRNA and protein. B103 cells stably transfected with pcDNA3 (Mock) or pAS-E2-25K/Hip-2 (#1 and #3) were isolated using single-cell cloning methods. Expression of caspase-12 and E2-25K/Hip-2 was analyzed by Western blotting or RT-PCR in the stable cell lines (left) and in the cells left untreated or exposed to 5 μM Aβ for 36 h (right). (D) E2-25K/Hip-2 KD cells are resistant to cell death induced by ER stress. Mock and stable E2-25K/Hip-2-AS cell lines (#1 and #3) were incubated with 0.1% vehicle (DMSO), 1 μM thapsigargin (Tg), or 2 μg/ml tunicamycin (Tuni.) for 24 h. Cell viability was then determined by trypan blue exclusion (n = 3). Bars depict means ± SD (left). Cell extracts were prepared and analyzed with Western blotting using the indicated antibodies (right). Asterisk indicates the processed form (p20 + p10) of caspase-12.

E2-25K/Hip-2–mediated stabilization of caspase-12 protein via UPS

We next investigated how E2-25K/Hip-2 induces the accumulation of caspase-12 protein. Although we found that both caspase-12 mRNA and protein were regulated by E2-25K/Hip-2, we mainly
focused on the stabilization of the protein because the regulation at the protein level was more pronounced and the higher molecular mass forms of caspase-12 protein were found to accumulate in the brains of 9-mo-old APPswe/PS1dE9 double transgenic mice (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200711066/DC1). E2-25K/Hip-2 is an E2 ubiquitin-conjugating enzyme capable of inhibiting proteasome activity. (Song et al., 2003). We therefore hypothesized that caspase-12 may be regulated by protein degradation via proteasome. Western analysis showed that in contrast to caspase-3 and -8, there was substantial dose-dependent accumulation of caspase-12 protein in B103 cells incubated with MG132 (Fig. 4 C). In addition, an exogenous caspase-12–GFP fusion protein was stabilized in HEK293 cells incubated with MG132 (Fig. 4 C). Also, the level of caspase-12–GFP fusion protein was increased by coexpression with wild-type E2-25K/Hip-2, but not with E2-25K/Hip-2 Ser86→Tyr (S86Y) or Cys92→Ser (C92S) mutant (Fig. 4 C), which lacked the capacity to inhibit proteasome function (Song et al., 2003). These results suggest that the degradation of caspase-12 protein is regulated by UPS and that E2-25K/Hip-2 stabilizes caspase-12 protein by inhibiting proteasome activity.

We used in vitro ubiquitination assays to further characterize E2-25K/Hip-2–dependent regulation of caspase-12 protein. Incubating ubiquitin with purified GST–E2-25K/Hip-2 in the presence of E1, ATP, and an ATP regeneration system led to the appearance of polyubiquitin (Fig. 4 D, left), which confirmed that the purified GST-E2-25K/Hip-2 protein is active and able to generate polyubiquitin. GST and GST-fused E2D (GST-E2D), another E2 ubiquitin-conjugating enzyme, served as negative controls. Incubating B103 cell extract supplemented with ubiquitin, E1, ATP, and an ATP regeneration system with either the purified GST–E2-25K/Hip-2 or MG132 led to the accumulation of caspase-12 protein, but not caspase-3 (Fig. 4 D, middle and right) or IκBα (not depicted). The stability of caspase-12 protein thus appears to reflect the ability of E2-25K/Hip-2 to generate polyubiquitin, which likely interferes with the degradation of caspase-12 through the regulation of UPS. We then determined the subregion of caspase-12 required for MG132-induced accumulation by using full length, prodomain-deleted mutant (ΔPro), prodomain (Pro), or the large subunit (p20) of caspase-12. Compared with Pro, ΔPro and p20 fragments are highly stabilized in the cells exposed to MG132 (10- and 7-fold each; Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200711066/DC1).

**Modulation of ER stress responses and calpainlike activity by E2-25K/Hip-2**

Caspase-12 can be activated by calpain during ER stress (Nakagawa et al., 2000; Nakagawa and Yuan, 2000), which prompted us to test whether E2-25K/Hip-2 is also involved in the regulation of calpain activity. Treatment with Aβ induced the accumulation of the ER stress marker GADD153/CHOP in control B103 cells, but not in B103/E2-25K-AS cells (Fig. 5 A). In addition, a reporter assay using grp78 promoter luciferase showed that the overexpression of E2-25K/Hip-2, but not caspase-12, increased luciferase activity about threefold (Fig. 5 B), indicating that E2-25K/Hip-2 is required for the induction of ER stress markers by Aβ.

Using fluorogenic enzymatic assays, we consistently found that Aβ induced calpainlike activity in control cells (Fig. 5 C), but not in B103/E2-25K-AS cells (#1 and #3). In addition, calpainlike activity was also induced by the ectopic expression of E2-25K/Hip-2, but not by the expression of E2-25K/Hip-2–Δtail mutant (Fig. 5 D), which lacked the ability to inhibit proteasome activity (Song et al., 2003). Furthermore, E2-25K/Hip-2–induced cell death was significantly attenuated by incubating the cells with calpeptin or z-LLL, calpainlike protease inhibitors (Fig. 5 E), or by the overexpression of calpastatin, an endogenous inhibitor of calpain, with the reduction in the numbers of calpain activity–positive cells (Fig. 5 F). E2-25K/Hip-2 thus appears to mediate the induction of calpainlike activity by Aβ, which may lead to activation of caspase-12.

As oxidative stress is another potent regulator of ER stress and a downstream mediator of Aβ, we also investigated the regulation of E2-25K/Hip-2 and caspase-12 expression. We found that treating B103 cells with ascorbic acid, an antioxidant, but not Batpa-AM, a calcium chelator, reduced Aβ-induced expression of E2-25K/Hip-2 and neurotoxicity (Fig. 6 A, top). Also, treatment...
Hip-2 mediates ER stress responses.

of caspase-12 protein were significantly lower in the brains of RT-PCR, and Western analyses (Fig. 7A). We found that the levels of Hip-2 expression were confirmed using genomic DNA-PCR, the genotypes of the gene-trap insertion and depletion of E2-25K/Hip-2 gene (Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200711066/DC1).

To evaluate the role of E2-25K/Hip-2 in the regulation of caspase-12 and ER stress during Aβ-deficient mice from embryonic stem cells in which a gene trap was inserted into the E2-25K/Hip-2 gene (Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200711066/DC1). The genotypes of the gene-trap insertion and deletion of E2-25K/Hip-2 expression were confirmed using genomic DNA-PCR, RT-PCR, and Western analyses (Fig. 7A). We found that the levels of caspase-12 protein were significantly lower in the brains of 7-d-old E2-25K/Hip-2 knockout (−−/−) mice than wild-type (+/+), and that the levels of caspase-12 mRNA were reduced in the brains of 9-mo-old E2-25K/Hip-2–deficient mice (Fig. 7B). Expression of both caspase-12 mRNA and protein thus appears to be regulated by E2-25K/Hip-2 in mice.

Bearing that in mind, we prepared in vitro cultures of primary cortical neurons from E2-25K/Hip-2 wild-type (+/+), heterozygotic (+/−), and homoyzotic (−/−) embryos at embryonic day 15 and examined their sensitivity to Aβ neurotoxicity. We found that E2-25K/Hip-2 (−−/−) neurons were completely resistant to Aβ, whereas E2-25K/Hip-2 (+/−) neurons showed partial resistance (Fig. 7C). In addition, immunocytochemical analysis revealed that caspase-12 and GRP78 proteins, which showed marked accumulation in E2-25K/Hip-2 (+/+) cortical neurons exposed to Aβ, were not induced by Aβ in E2-25K/Hip-2 (−−/−) neurons (Fig. 7D), which confirms that E2-25K/Hip-2 is indeed an essential regulator of caspase-12 and ER stress during Aβ neurotoxicity.

Discussion

The neurotoxicity of Aβ is a fundamental contributor to the pathogenesis and progression of AD (for review see Barnham et al., 2006). To better understand Aβ neurotoxicity, we examined the
mediates the induction of two ER stress markers, GADD153/CHOP and GRP78, as well as the activation of caspase-12 (Fig. 8). In particular, experiments performed with E2-25K/Hip-2 knockout cortical neurons confirmed the essential role of E2-25K/Hip-2 in Aβ-induced accumulation and activation of caspase-12 for neurotoxicity. Interestingly, the accumulation of caspase-12 protein occurred via proteasome inhibition by E2-25K/Hip-2, which also ubiquitinates UBB+1, causing the inhibition of proteasomeal activity in a dose-dependent way (for review see Song and Jung, 2004; van Tijn et al., 2007). Though the accumulation of caspase-12 protein through proteasome inhibition is strongly plausible, it is still possible that E2-25K/Hip-2 may also accumulate caspase-12 protein by generating K63-linked ubiquitin chain (for review see Lim et al., 2006). In contrast, the accumulation of caspase-12 mRNA was not observed in cells.
were prepared from postnatal E2-25K/Hip-2 wild-type (+/+) and homozygotic (−/−) mice analyzed using genomic DNA-PCR and RT-PCR (Tail). Brain extracts were prepared from postnatal E2-25K/Hip-2–deficient mice and analyzed using genomic DNA–PCR and RT–PCR (Brain). (B) Reduction of caspase-12 mRNA in the brains of 9-mo-old E2-25K/Hip-2–deficient mice. Total RNA was analyzed with RT–PCR using synthetic oligonucleotides for E2-25K/Hip-2 and caspase-12, as described in Materials and methods. Anti–E2-25K/Hip-2 and anticaspase-12 antibodies (Brain). (C) Resistance of E2-25K/Hip-2–deficient cortical neurons to Aβ toxicity. Primary cortical neurons from E2-25K/Hip-2 wild-type (+/+) and homozygotic (−/−) embryos at embryonic day 15 were cultured for 3 d and incubated with Aβ for 2 d. Cell viability was examined after staining with Calcein-AM (n = 3; bottom). Bars depict means ± SD. (D) Lack of Aβ-induced increase of caspase-12 and GRP78 in E2-25K/Hip-2–deficient neurons. Primary cortical neurons cultured from E2-25K/Hip-2–deficient mice were exposed to MG132, though the promoter activity of caspase-12 (pGL3-08) was weakly induced by coexpression of E2-25K/Hip-2. Thus, the regulation of caspase-12 mRNA by E2-25K/Hip-2 is separated from its proteasome inhibitory activity.

Although caspase-12 is a proximal caspase and is an important mediator of apoptosis triggered by ER stress, we believe its accumulation may not itself, be sufficient to induce cell death. When we overexpressed caspase-12 in various cell types, including fibroblasts and neuronal cells, it was much less effective in inducing cell death than other proximal caspases, such as caspase-8, -9, and -10 (unpublished data). To be effective, apparently, caspase-12 needs to interact with one or more activators. In our study, E2-25K/Hip-2 was able to induce both calpain-like activity and efficient proteolytic processing of caspase-12. One possible explanation is that the inhibition of proteasome activity by E2-25K/Hip-2 leads to an accumulation of misfolded proteins within cells, which in turn induces ER stress, including the activation of calpain. Consistent with that idea, it has been shown that the inhibition of proteasome activity by aggregation-prone proteins or proteasome inhibitors does indeed induce ER stress (Kourouku et al., 2002; Nishitoh et al., 2002; Nawrocki et al., 2005). Still, the details of the molecular pathway downstream of E2-25K/Hip-2 leading to induction of calpain-like activity remain unclear.

Along with those of an earlier paper showing that calpain directly activates caspase-12 (Nakagawa et al., 2000), our present findings suggest that calpain-like activity is required for proteolytic activation of caspase-12. This means that E2-25K/Hip-2 regulates both the accumulation of caspase-12 and its proteolytic activation during Aβ neurotoxicity. In that sense, calpain inhibitors suppressed Aβ neurotoxicity. Similar accumulation and activation of caspase-12 was observed in neuronal cells exposed to oxidative stress. However, calpain inhibitors still partially suppressed E2-25K/Hip-2–induced cell death, indicating that an additional regulator (e.g., Jun N-terminal kinase) also functions as a downstream mediator of E2-25K/Hip-2 during cell death (Song et al., 2003). We found that treating cultured cells with Aβ led to E2-25K/Hip-2–dependent up-regulation of two ER stress markers, GADD153/CHOP and GRP78, as well as caspase-12, suggesting that E2-25K/Hip-2 is an upstream regulator of ER stress in Aβ neurotoxicity (Fig. 8). If so, the observed accumulation of caspase-12 in the brains of APP transgenic mice might be induced by the increased expression of E2-25K/Hip-2, which would be expected to further increase neuronal susceptibility to cell death or stress. We believe that E2-25K/Hip-2 may induce ER stress via generating unanchored polyubiquitin, which is able to inhibit proteasome activity in vitro like polyubiquitinated UBB14 (Song et al., 2003; van Tijn et al., 2007; for review see Song and Jung, 2004). That said, it is noteworthy that the contributions of E2-25K/Hip-2 to the induction of GADD153/CHOP in cells exposed to various ER stress signals differ. For instance, E2-25K/Hip-2 is required for the induction of GADD153/CHOP by Aβ and ROS, but not by tunicamycin or thapsigargin, implying that E2-25K/Hip-2 might be more specific to ER stress response caused by Aβ than by tunicamycin or thapsigargin. Thus, a possible model is that E2-25K/Hip-2 may be situated upstream of intracellular calcium destabilization or at the accumulation of...
We found that E2-25K/Hip-2 accumulated in B103 cells exposed to ER stress by E2-25K/Hip-2 during Aβ neurotoxicity. The gray box depicts the inhibitory effect of E2-25K/Hip-2 on the proteasome activity via the accumulation of polyubiquitin (Poly Ub) and ubiquitinated UBB+ (Ub-UBB+); Song et al., 2003). Unanchored polyubiquitin is able to interact with proteasome in vitro (Piotrowski et al., 1997), which is similar to the proteasome inhibition by Ubs-UBB+ (van Tijn et al., 2007). Aβ stimulates the expression of E2-25K/Hip-2 via oxidative stresses (ROS) and E2-25K/Hip-2 stimulates up-regulation of caspase-12 mRNA and protein. E2-25K/Hip-2 also mediates the induction of GRP78 and calpain-like activity, which is believed to activate caspase-12 during Aβ neurotoxicity.

This has been proposed that oxidative stress contributes to the progression of pathological processes in neurons, including AD (Manton et al., 2004; for review see Huber et al., 2006). We found that E2-25K/Hip-2 accumulated in B103 cells exposed to ROS and that antioxidants, which suppress Aβ neurotoxicity (Hensley et al., 1994; Bruce et al., 1996), inhibited the accumulation of E2-25K/Hip-2. The regulation of E2-25K/Hip-2 expression may thus be involved in mediating oxidative damage underlying neuronal pathology. Recently, E2-25K/Hip-2 was proposed to be involved in the aggregation of polyglutamine-expanded Huntingtin with proteasome inhibitory activity (de Pril et al., 2007). Thus, it would be interesting to know whether E2-25K/Hip-2 is associated with the pathogenesis of other neurodegenerative ailments, including Huntington’s and Parkinson’s disease, because malfunction of the UPS, its diminished activity, and generation of ROS are common phenomena in neurodegenerative diseases (for review see Song and Jung, 2004; Halliwell, 2006).

Aβ-induced, E2-25K/Hip-2–dependent regulation of caspase-12 and ER stress was confirmed in E2-25K/Hip-2–deficient neurons. Despite the important role of E2-25K/Hip-2 in the regulation of ER stress, E2-25K/Hip-2–deficient (−/−) mice were well bred and healthy until the age of 9 mo. The absence of any detectable defects during the development of these mice suggests that the function of E2-25K/Hip-2 might be compensated for during that period by other E2s, like Mdm2, which interacts with several E2s to ubiquitinate tumor suppressor p53 (Saville et al., 2004). Alternatively, E2-25K/Hip-2 may be mainly associated with neuronal pathogenesis. Although a human homologue of caspase-12 is in debate, our identification of E2-25K/Hip-2 as an upstream regulator of ER stress suggests that E2-25K/Hip-2 could serve as a plausible therapeutic target for the treatment of AD.

Materials and methods

Proteasome and calpain inhibitor

Proteasome inhibitor MG132 (Sigma-Aldrich), calpain inhibitors z-LLY (EMD), and calpeptin (EMD) were purchased.

Plasmid construction and RT-PCR

pE2-25K/Hip-2 and its mutants (Δtail, deletion of tail region; S66Y, Ser→ Tyr; C92S substitution, Cys→Ser) were described previously (Song et al., 2003). pCaspase-12–GFP and its active site mutant (C298S) have also been described previously (Nakagawa et al., 2000). E2-25K/Hip-2 CDNA was subcloned into pcDNA3 in an antisense orientation (pAS-E2-25K/Hip-2). To coexpress with calpastatin, E2-25K/Hip-2 was cloned into the EcoRI and BamHI sites of pDsRed-C2 (Clontech Laboratories, Inc.) using synthetic oligonucleotides mRed-Hip-2 5’-5’GGGATCCCTAACGAGCAATCGGC- GAACTG-3’ and mRed-Hip-2 5’-GGCGGATCCCTAACGAGCAATCGGC- GAACTG-3’. Human calpastatin cDNA cloned in pCMVSPORT6 vector was purchased from Korea Research Institute of Bioscience and Biotechnology.

Total RNA was purified and reverse transcribed as described previously (Song et al., 2003). Levels of E2-25K/Hip-2, caspase-12, β-actin, and glyceraldehyde-3-phosphate dehydrogenase mRNA were analyzed using PCR with gene-specific synthetic oligonucleotides.

Cell culture, DNA transfection, and assessment of cell death

Wild-type MEFs, caspase-11 (−/−) MEFs, and caspase-12 (−/−) MEFs were provided by J. Yuan (Harvard Medical School, Boston, MA). MEFs, B103 cells [rat neuroblastoma, and HEK293 cells (human embryonic kidney cells)] were cultured in DME supplemented with 10% (vol/vol) fetal bovine serum. Cells were transfected using Lipofectamine Reagent (Invitrogen) according to the manufacturer’s protocol, after which their viability was assessed based on the morphology of GFP-positive cells viewed under a fluorescence microscope (DMRBE; Leica), trypan blue exclusion assays, and live/dead cell assays (Invitrogen).

Preparation and treatment with Aβ1-42 peptide

Commercially available Aβ1-42 was purchased from Sigma-Aldrich and dissolved to a concentration of 500 μM in phosphate-buffered saline. The Aβ1-42 stock solution was incubated for 1 wk at 4°C and divided into small aliquots for storage at −70°C. Cells were incubated with Aβ1-42 or other cell death–inducing drugs including tunicamycin (Sigma-Aldrich) and thapsigargin (Sigma-Aldrich) in serum-free DME.

Luciferase and β-galactosidase (β-gal) assays

B103 cells were cotransfected with Grp78/luciferase reporter plasmid, cytomegalovirus β-gal, and effector plasmids. After 32 h, the cells were harvested and the luciferase activities in the cell extracts were determined using a luciferase assay system (Promega). To measure β-gal activity, cell extracts were mixed with equal amounts of β-gal buffer [2X] containing 200 mM sodium phosphate, pH 7.3, 2 mM MgCl2, 100 mM β-mercaptoethanol, and 1.33 mg/ml O-Nitrophenyl-β-D-galactopyranoside, and incubated for 1 h at 37°C. The absorbance at 420 nm was then measured using a microplate reader (Bio-Rad Laboratories).

Enrichment of antisense E2-25K/Hip-2 cDNA–transfected cells

B103 cells were transfected with pAS-E2-25K/Hip-2 or pcDNA3 for 24 h and incubated with 800 μg/ml G418 sulfate (Invitrogen) for 10 d to generate a stable mixed cell population. Single-cell clones (E2-25K-AS #1 and #3) were isolated using standard cell cloning methods and examined for expression of E2-25K/Hip-2 using Western analysis.

Transgenic mice

Tg2576 transgenic mice (13–22 mo of age) were used in our study. Each expressed human APP 695 containing the double K670N/M671L mutation (huAPP695.K670N/M671L) found in a large Swedish family with early onset AD (Hsiao et al., 1996). Double transgenic mice expressing APPswe and PS1ΔE9 exon 9–deleted PS1ΔE9 (The Jackson Laboratory) were purchased and bred to generate transgenic mice and wild-type littersmates.

Antibody generation, Western blotting, and immunocytochemistry

Generation of anti-E2-25K/Hip-2 and anticaspase-8 antibodies was described previously (Kim et al., 1998; Song et al., 2003). Anti-α-tubulin (Sigma-Aldrich), anticaspase-2 (Santa Cruz Biotechnology, Inc.), anticaspase-3 (Biomedia), anticaspase-12 (Santa Cruz Biotechnology, Inc.), and anti-GFP (Santa Cruz Biotechnology, Inc.) antibodies were purchased. Cells were lyzed in sampling buffer [10% glycerol, 2% SDS, 62.5 mM Tris-HCl, and 1% NaN3] and suspended in 2× reducing or nonreducing sample buffer (50 mM Tris, pH 6.8, 10% glycerol, 5% β-mercaptoethanol, 0.1% SDS). Proteins were separated on a 10%–12.5% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (for Western blotting). Membranes were blocked with 5% nonfat milk in TBST (10 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 1 h at room temperature and incubated overnight at 4°C with the indicated primary antibodies. Membranes were washed with TBST and incubated for 1 h with Alexa Flour 488- or 633-labeled donkey anti-rabbit IgG (Invitrogen) or Alexa Flour 546- or 633-labeled donkey anti-goat IgG (Invitrogen) at a 1:2000 dilution. After washing, membranes were incubated with an anti-mouse or an anti-goat secondary antibody and visualized with enhanced chemiluminescence (GE Healthcare). Immunocytochemistry was performed according to a previously described method (Song et al., 2003). Briefly, B103 cells or N2a cells (ATCC) were seeded on coverslips and transfected with indicated plasmids. Two days after transfection, cells were fixed with 4% paraformaldehyde, treated with 0.1% Triton X-100, blocked with 5% normal goat serum, and incubated with the indicated primary antibodies. After washing, cells were incubated with Alexa Flour 488- or 633-labeled secondary antibodies (Invitrogen) and visualized with a fluorescence microscope (DMRBE; Leica).
2% (w-mercaptoethanol, pH 6.8), after which aliquots of lysate containing equal amounts of protein were subjected to SDS-PAGE and Western analysis as described previously (Kim et al., 2002). Primary neurons were analyzed with immunocytochemistry using anti-GRP78 (dilution 1:200; Santa Cruz Biotechnology, Inc.) and anticaspase-12 (dilution 1:50) primary antibodies and Alexa Fluor 488- or 555-conjugated secondary antibodies (1:1,000; Invitrogen) as described previously (Song et al., 2003).

Histology and immunohistochemistry

12 μm-thick frozen brain sections, which were taken from the human Swedish mutant amyloid precursor protein (hAPP695.K670N/M671L) transgenic Tg2576 and their littermate mice at the age of 21 mo, were fixed with 4% PFA and blocked with 3% normal serum and 0.3% Triton X-100 in phosphate-buffered saline, pH 7.4. After immersion with rabbit anti- E2-25K/Hip-2 antibody (dilution 1:200) and rat anticaspase-12 antibody (1:25), the sections were reacted with Alexa Fluor 488- or 555-conjugated secondary antibody (1:1,000). For the double fluorescent immunostaining with rabbit anti-E2-25K/Hip-2 antibody and rabbit anticaspase-2 antibody (1:100), we treated the primary antibodies with Zenon Alexa Fluor rabbit IgG labeling kit (Invitrogen) before the reaction with the tissue sections. All stained samples were mounted using a mounting medium for fluorescence (VECTASHIELD; Vector Laboratories).

Microscopy

Immunohistological studies were performed under a fluorescence microscope (Eclipse 80i with Plan Apo VC 100×/1.40 Oil WD 0.13, lens; Nikon) using the manufacturer’s acquisition system (DS-Fi1/DSU2 digital camera and NISElements F 2.20 program). Fluorescence (W/1S1F3) with two objective lenses ([C]PCanFl, 40×/0.60 Fl2 and 20×/0.45 Fl1; Olympus) and confocal microscopes (UltraVIEW ERS FRET-EH with an oil immersion objective lens [DIC; 100×/1.40]; PerkinElmer) were used for analysis of the immunostained samples. Images were captured using each manufacturer’s acquisition system (DP20 digital camera and DP ver.3.1.1.208 maker program [Olympus]; UltraVIEW ICI [PerkinElmer]). All stained samples were examined at room temperature and Photoshop (Adobe) was used to make combinatory figures (color contrast, medium).

In vitro degradation assay

Various GST-fused proteins cloned into pGEX4T-1 (GST-E2-25K/Hip-2 [provided by S. Kang, Korea University, Seoul, Korea] and GSTE2D) were expressed in DH5α [provided by S. Kang, Korea University, Seoul, Korea] and GST-E2D) were expressed in DH5α [provided by S. Kang, Korea University, Seoul, Korea] and P46/78E1 (Macrogen), and 10 mM ATP. Proteins were preincubated with purified ubiquitin (Sigma-Aldrich), ubiquitin-activating enzyme E1 (Macrogen), and 10 mM ATP. Proteins were then incubated with 10 mM of substrate for 20 min at 37 °C. After washing twice with HBSS-EDTA, the fluorescence emitted by the cells was measured using a fluorescence microplate reader (FL-600; BioTek Instruments, Inc.). In the case of CMAC-(t-BOC-Leu-Met), B103 cells were incubated with 1 μM of substrate for 30 min and examined under a fluorescence microscope using a Hoescht filter.

Generation of E2-25K/Hip-2-deficient mice

E2-25K/Hip-2 gene-trapped embryonic stem cells (ES) (K109) were provided by BayGenomics of the International Gene Trap Consortium. Mice heterozygous for E2-25K/Hip-2 were generated by following the protocol provided by BayGenomics. Insertion of the gene-trap vector was confirmed by Southern blotting using NEBiot Plustopose (New England Biolabs, Inc.) with a β-gal probe recommended by BayGenomics. Genotypes were analyzed using genomic PCR with synthetic oligonucleotides for β-gal (β-gal-5’, 5‘-TTATCGATGAGCGTGGTGGTTATGC-3’; β-gal-3’, 5‘-GGCGCAGCATC- GCCCAAGATAATCT-3’) and β-actin. The expression of E2-25K/Hip-2 was examined using RTPCR with synthetic oligonucleotides derived from E2-25K/Hip-2.
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