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

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Amyloid-β (Aβ) neurotoxicity is believed to contribute to the pathogenesis of 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 calpainlike 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.

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; for review see Barnham et al., 2006). Substantial evidence suggests that Aβ-induced neuronal apoptosis is central to the progression of AD (Abad et al., 2006; Biswas et al., 2007). This has prompted investigation of the potential for caspase family cystein proteases, which are activated during apoptosis and execute various apoptotic processes to serve as therapeutic targets in the treatment of neurodegenerative diseases (Sanges et al., 2006; Emamaullee et al., 2007). Among the 14 caspase isoforms known to be expressed in mammals, the ER-resident caspase-12 was initially identified as a mediator of Aβ neurotoxicity, and its conserved pathway, termed "ER stress," has been implicated in the pathogenesis of such neurodegenerative ailments as Huntington's disease, prion disease, and AD (Nakagawa et al., 2000; Kouroku et al., 2002; Hetz et al., 2003, 2007; Hoozemans et al., 2007). However, the functional role of caspase-12 in humans remains to be further clarified. Human caspase-12 has no role in apoptotic pathways (Saleh et al., 2004) and it is still controversial that human caspase-12 acts as a functional counterpart of mouse caspase-12. Interestingly, the amino acid sequence of mouse caspase-12 has a 61% identity with human caspase-4, which is involved in apoptosis induced by ER stress (Hitomi et al., 2004) in the interleukin-1–converting enzyme homologous region. Thus, a fuller understanding of the mechanisms involved in regulating ER stress, caspase-12 activity, and their roles in Aβ neurotoxicity would be highly desirable.

The ubiquitin/proteasome system (UPS) is involved in many biological pathways, including regulation of the cell cycle and modulation of the degradation of short-lived and regulatory proteins (for review see Rubinsztein, 2006). Protein degradation by the UPS proceeds through two successive steps: (1) ubiquitination, i.e., conjugation of ubiquitin to a target protein

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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.

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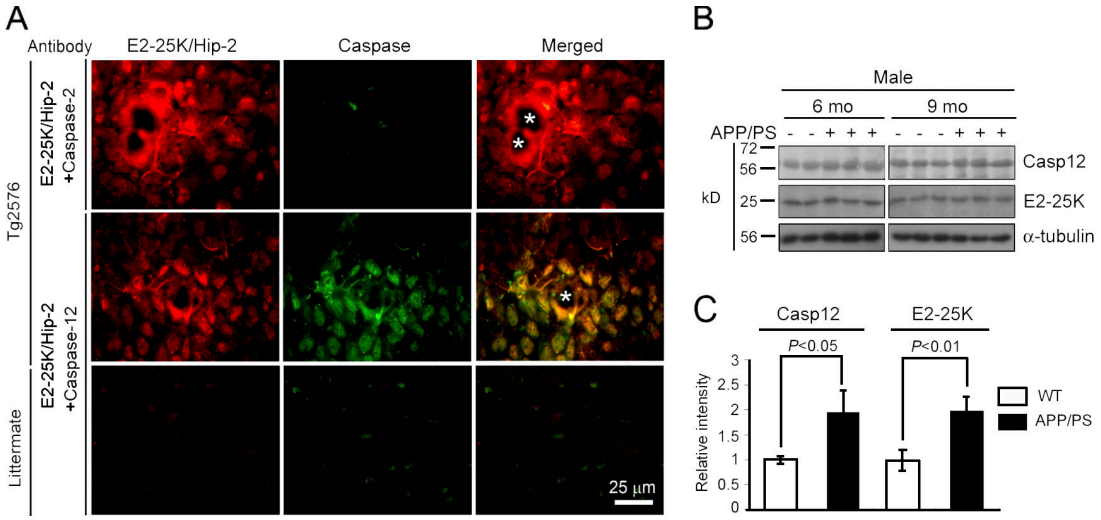

Figure 1. **Colocalization and increased expression of caspase-12 and E2-25K/Hip-2 in the brains of Tg2576 and APPswe/PS1dE9 mice.** (A) Hippocampal region of 21-mo-old Tg2576 mice and age-matched littermates were immunostained using anti–E2-25K/Hip-2, anticaspase-12 (middle and bottom), and anticaspase-2 (top) antibodies and examined under a fluorescence microscope. Asterisks indicate amyloid plaques. (B) Whole brains from APPswe/PS1dE9 (APP/PS) mice and wild-type (WT) littermates were harvested at the indicated ages and examined by Western blotting with anti–E2-25K/Hip-2, anticaspase-12, and anti–α-tubulin antibodies. (C) Caspase-12 and E2-25K/Hip-2 signals at 9 mo in B were quantified by densitometry using the histogram feature in Photoshop. Bars depict means ± SD (n = 3). P-values were calculated using t tests and are versus control.

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 Aβ 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;

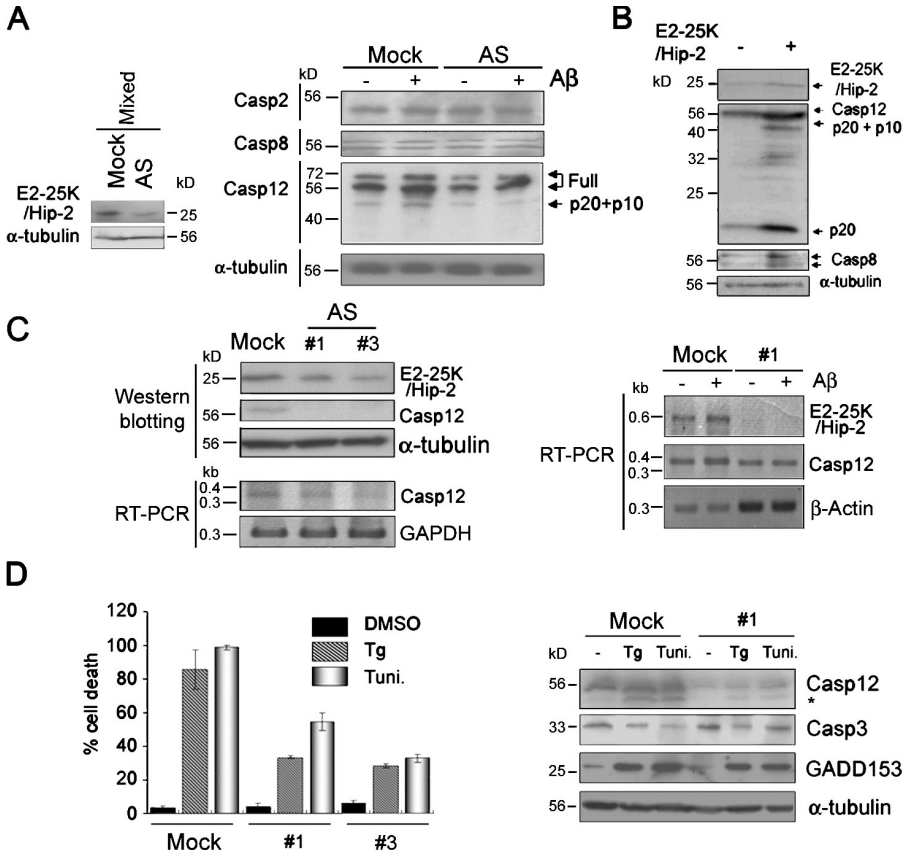

Figure 2. **E2-25K/Hip-2 is required for the expression and activation of caspase-12 during Aβ toxicity.** (A) Knockdown (KD) of E2-25K/Hip-2 expression reduces Aβ-induced expression and activation of caspase-12 protein. B103 cells were transfected with pcDNA3 (Mock) or antisense (AS) E2-25K/Hip-2 cDNA (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 $A\beta_{1-42}$ 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.

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.

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 D, left). Our Western analyses showed that thapsigargin and tunicamycin induced proteolytic activation of caspase-12 in control cells (Fig. 2 D, right), but levels of both procaspase-12 and activated caspase-12 were reduced in B103/E2-25K-AS cells. In contrast, GADD153/CHOP, an ER stress marker (Rao et al., 2002), was induced equally in both cell lines. E2-25K/Hip-2 thus appears to contribute to ER stress–induced cell death and the activation of caspase-12, but not the expression of at least some ER stress markers.

Given our finding that the expression and activation of caspase-12 is regulated by E2-25K/Hip-2, we were eager to know whether caspase-12 is a downstream mediator of E2-25K/Hip-2 in the cell death (Fig. 3). As compared with the relatively weak proapoptotic activities of E2-25K/Hip-2 (32%) and caspase-12 (26%) alone, coexpressed E2-25K/Hip-2 and caspase-12 acted synergistically to efficiently induce apoptosis (76%) in B103 cells (Fig. 3 A). In addition, ectopic expression of a caspase-12 active site mutant (Cys298→Ser) significantly suppressed apoptosis triggered by E2-25K/Hip-2. Also, caspase-12 knockout mouse embryonic fibroblasts (MEFs; C12 [−/−]) were resistant to cell death induced by the overexpression of E2-25K/Hip-2 but not to cell death induced by caspase-8, whereas wild-type and caspase-11 knockout MEFs (C11 [−/−]) remained sensitive to E2-25K/Hip-2 (Fig. 3 B). This suggests caspase-12 is a critical mediator of E2-25K/Hip-2–mediated cell death.

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

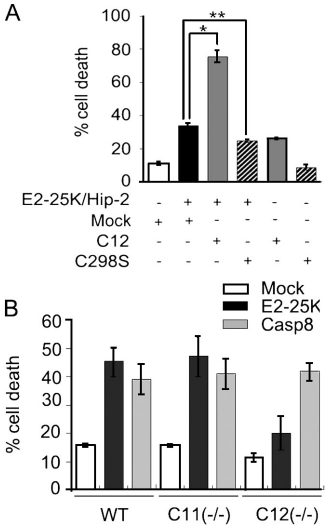

Figure 3. **Caspase-12 is a downstream molecule of E2-25K/Hip-2.** (A) Effects of wild-type caspase-12 and an active site mutant (C298S) on E2-25K/Hip-2–induced cell death. B103 cells were transiently transfected with pE2-25K/Hip-2, pEGFP (Mock), pCaspase-12–GFP (C12), or pCaspase-12(C298S)–GFP (C298S), as indicated. Total amounts of plasmid DNA used were equalized by addition of pcDNA3. After 48 h, cell viability was determined based on the morphology of GFP-positive cells under a fluorescence microscope (* and **, $P < 0.001$; *t* test). (B) Caspase-12 deficiency reduces E2-25K/Hip-2–mediated cell death. Wild-type (WT), C11 (−/−), and C12 (−/−) MEFs were transfected with pEGFP and either pcDNA3 (Mock), pE2-25K/Hip-2 (E2-25K), or pCaspase-8 (Casp8). After 48 h, cell viability was assessed as described in A. Bars depict means ± SD ($n = 3$).

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-dependant accumulation of caspase-12 protein in B103 cells incubated with the proteasome inhibitor MG132, but not with the lysosomal inhibitor NH_4Cl (Fig. 4 A and Fig. S3). In contrast, the level of caspase-12 mRNA was unchanged in cells exposed to MG132 (Fig. 4 A). Immunoprecipitation analysis using antiubiquitin antibody confirmed that the accumulated forms of caspase-12 protein in cells exposed to MG132 were ubiquitinated (Fig. 4 B). 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 the 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-LLY, 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 Bapta-AM, a calcium chelator, reduced Aβ-induced expression of E2-25K/Hip-2 and neurotoxicity (Fig. 6 A, top). Also, treatment

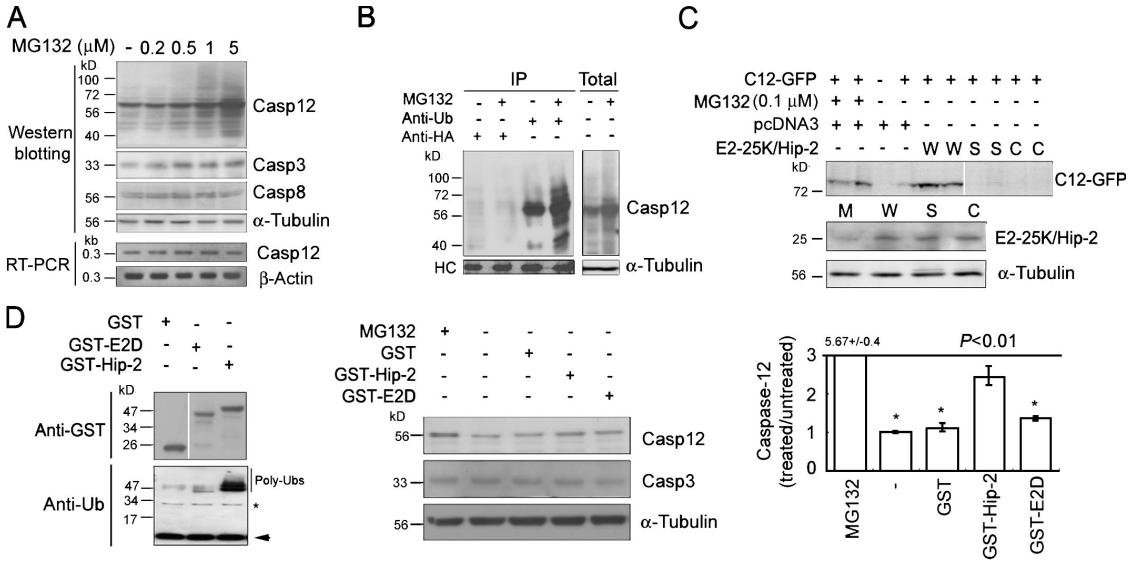

Figure 4. **Stabilization of caspase-12 protein by E2-25K/Hip-2 via the UPS.** (A) Proteasome-dependent degradation of caspase-12. B103 cells were incubated for 12 h with MG132, after which cell extracts were analyzed by Western blotting with anticaspase-3, -8, and -12 and anti–α-tubulin antibodies, and by RT-PCR using oligonucleotides specific for caspase-12 and β-actin. (B) Detection of ubiquitinated caspase-12. B103 cells were incubated with 5 μM MG132 for 12 h and cell extracts were prepared for Western blotting (Total) or subjected to immunoprecipitation (IP) using anti-HA (negative control) or antiubiquitin (Ub) antibody. The immunoprecipitates were analyzed by Western blotting with anticaspase-12 (top left) and anti–mouse IgG (bottom left) antibody; the latter showed the presence of equal amounts of IgG heavy chain (HC). (C) Stabilization of exogenous caspase-12–GFP (C12-GFP) fusion protein by ectopic expression of E2-25K/Hip-2, but not by its mutants. HEK293 cells were cotransfected for 24 h with pCaspase-12–GFP and either pcDNA3, pE2-25K/Hip-2 (W), or pE2-25K/Hip-2 mutant (S, S86Y; or C, C92S), and then cultured in the presence or absence of 0.1 μM MG132. Cell extracts were then analyzed by Western blotting with anti-GFP and anti–E2-25K/Hip-2 antibodies. (D) In vitro stabilization of caspase-12 protein by purified GST–E2-25K/Hip-2 protein. (Left) In vitro polyubiquitination by purified GST–E2-25K/Hip-2 protein. GST-fused proteins (GST, GST–E2-25K/Hip-2, and GST-E2D) were purified from *Escherichia coli* and preincubated with ubiquitin, E1, ATP, and an ATP regeneration system for 90 min at 37°C. The reaction products were analyzed by Western blotting with anti-GST antibody for the purified proteins (top) and antiubiquitin antibody for ubiquitination (bottom; see Materials and methods). (middle) GST–E2-25K/Hip-2–induced in vitro accumulation of caspase-12. B103 cell extracts were prepared, supplemented with ubiquitin, E1, ATP, and an ATP regeneration system, and left untreated or incubated with either 5 μM MG132, GST, GST–E2-25K/Hip-2, or GST-E2D for 2 h at 37°C. The reaction products were analyzed by Western blotting. (right) The caspase-12 signals on Western blots were quantified by densitometry using the histogram function in Photoshop. Bars represent means ± SD (n = 3; *, P < 0.01 vs. control). White lines indicate that intervening lanes have been spliced out.

with H_2O_2 induced the accumulation of E2-25K/Hip-2 (Fig. 6 A, bottom) and caspase-12 (Fig. 6 C). In addition, H_2O_2-induced accumulation and activation of caspase-12 were suppressed in B103/E2-25K/Hip-2-AS cells (Fig. 6 C), and there was a concomitant inhibition of cell death (Fig. 6 B). Further, H_2O_2-induced accumulation of GADD153 observed in control B103 cells was also suppressed in E2-25K/Hip-2-AS cells (Fig. 6 C). These results suggest that E2-25K/Hip-2 is required for cell death triggered by H_2O_2-derived reactive oxygen species (ROS), which are also generated by Aβ, further supporting our proposal that E2-25K/Hip-2 mediates ER stress responses.

E2-25K/Hip-2–deficient cortical neurons are resistant to Aβ neurotoxicity

To evaluate the role of E2-25K/Hip-2 in the regulation of caspase-12 and ER stress during Aβ neurotoxicity in vivo, we used a general protocol from BayGenomics to generate E2-25K/Hip-2–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 depletion of E2-25K/Hip-2 expression were confirmed using genomic DNA-PCR, RT-PCR, and Western analyses (Fig. 7 A). 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 (+/+) mice (Fig. 7 A), and that the levels of caspase-12 mRNA were reduced in the brains of 9-mo-old E2-25K/Hip-2–deficient mice (Fig. 7 B). 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 homozygotic (−/−) 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. 7 C). 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. 7 D), 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

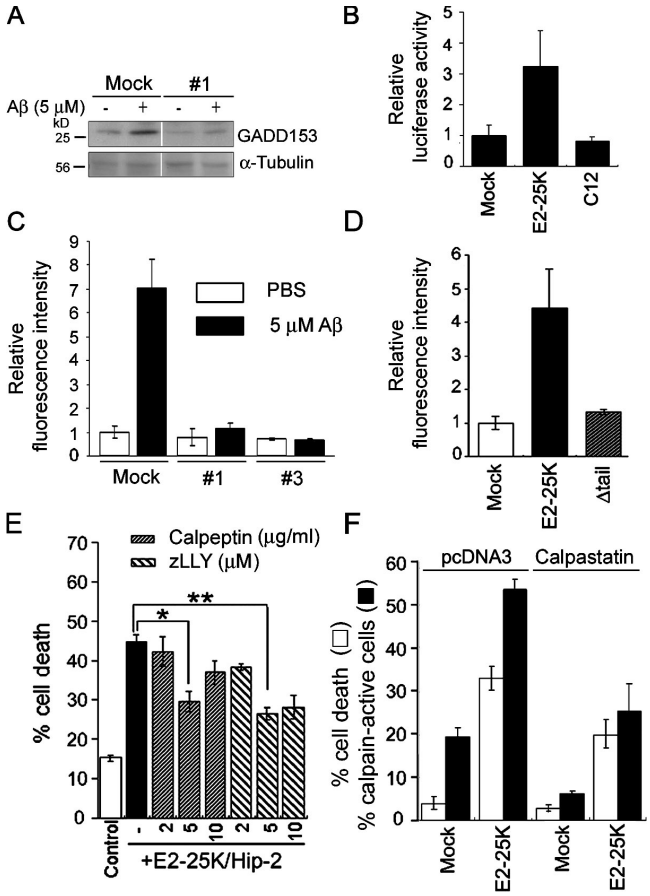

Figure 5. **Contribution of E2-25K/Hip-2 to Aβ-induced ER stress responses and calpainlike activity.** (A) Inhibition of Aβ-induced expression of GADD153 in E2-25K/Hip-2 KD cells. Mock and E2-25K/Hip-2-AS (#1) cells were incubated with Aβ for 48 h, after which cell extracts were examined by Western blotting with anti-GADD153 and anti–α-tubulin antibodies. (B) Induction of GRP78 promoter activity by the ectopic expression of E2-25K/Hip-2. B103 cells were cotransfected with pGrp78-luciferase reporter and pcDNA3 (Mock), pE2-25K/Hip-2 (E2-25K), or pCaspase-12–GFP (C12), and analyzed for luciferase activity as described in Materials and methods. (C and D) Induction of calpainlike protease activity by E2-25K/Hip-2. Calpainlike protease activity was measured using a fluorogenic substrate ([t-BOC-Leu-Me]2-R110; see Materials and methods) in Mock and E2-25K/Hip-2-AS cell lines (#1 and #3) after incubation with 5 μM Aβ for 48 h (C) and in B103 cells transfected for 24 h with pE2-25K/Hip-2 or pE2-25K/Hip-2–Δtail (D). (E) Suppression of E2-25K/Hip-2 neurotoxicity by calpain inhibitors. B103 cells were cotransfected with pEGFP and pE2-25K/Hip-2 for 48 h in the presence or absence of calpeptin or z-LLY, after which cell viability was determined based on the morphology of GFP-positive cells under a fluorescence microscope (* and **, $P < 0.001$). (F) Inhibition of E2-25K/Hip-2–induced cell death and calpain activation by calpastatin. B103 cells were transfected with pDsReD (Mock) or pDsRed-E2-25K/Hip-2 together with either pcDNA3 or pCalpastatin. After 48 h, cells were incubated with a fluoregenic calpain substrate, 7-amino-4-chloromethylcoumarin (t-BOC-Leu-Met; 1 μM), for 30 min. The cells showing shrinked morphology for dying cells (red filter; open bar) and/or calpain activity-positive fluorescence (Hoescht filter; closed bar) were counted under a fluorescence microscope as described in E ($n = 3$). Error bars represent SD.

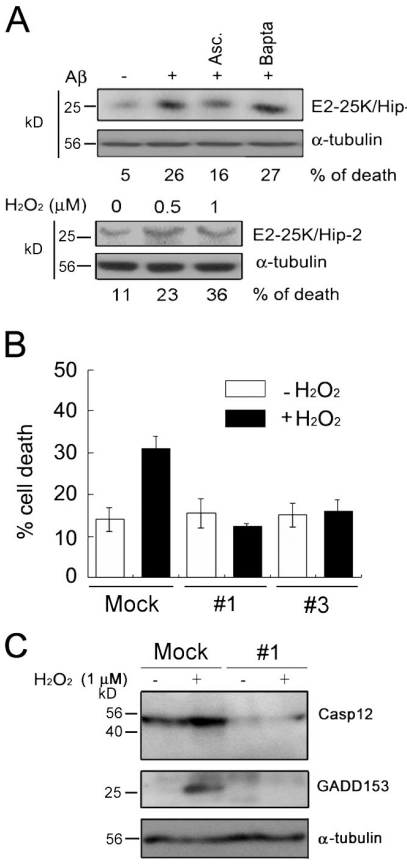

Figure 6. **ROS induce caspase-12 via E2-25K/Hip-2 during Aβ neurotoxicity.** (A) The induction of E2-25K/Hip-2 by Aβ is mediated via ROS. B103 cells were incubated with 5 μM $Aβ_{1-42}$ in the presence or absence of 300 μM ascorbic acid (Asc.) or 3 μM Bapta-AM (Bapta; top). B103 cells were incubated for 48 h with the indicated concentrations of H_2O_2 in serum-free DME and analyzed by Western blotting with anti–E2-25K/Hip-2 antibody (bottom). (B) E2-25K/Hip-2 KD cells are resistant to H_2O_2 toxicity. B103 cells stably transfected with pcDNA3 (Mock) or pAS-E2-25K/Hip-2 (#1 and #3) were left untreated or incubated for 48 h with 1 μM H_2O_2 in serum-free DME, after which cell viability was assessed by trypan blue exclusion ($n = 3$). Bars depict means ± SD. (C) Induction of caspase-12 and GADD153 by H_2O_2 is mediated by E2-25K/Hip-2. B103 cells stably transfected with pcDNA3 (Mock) or pAS-E2-25K/Hip-2 (#1) were incubated for 48 h with 1 μM H_2O_2 in serum-free DME, after which cell extracts were prepared and analyzed by Western blotting with anticaspase-12, anti-GADD153, and anti–α-tubulin antibodies.

functional interactions of E2-25K/Hip-2, caspase-12, and ER stress, and we report here that E2-25K/Hip-2 regulates the accumulation and activation of caspase-12 in vitro and in vivo as a necessary step leading to Aβ-induced neuronal cell death. It is also clear that E2-25K/Hip-2 plays a central role in ER stress–associated cell death triggered by Aβ and H_2O_2: E2-25K/Hip-2 mediates the induction of two ER stress markers, GADD153/CHOP and GRP78, as well as the activation of calpain (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-dependant 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

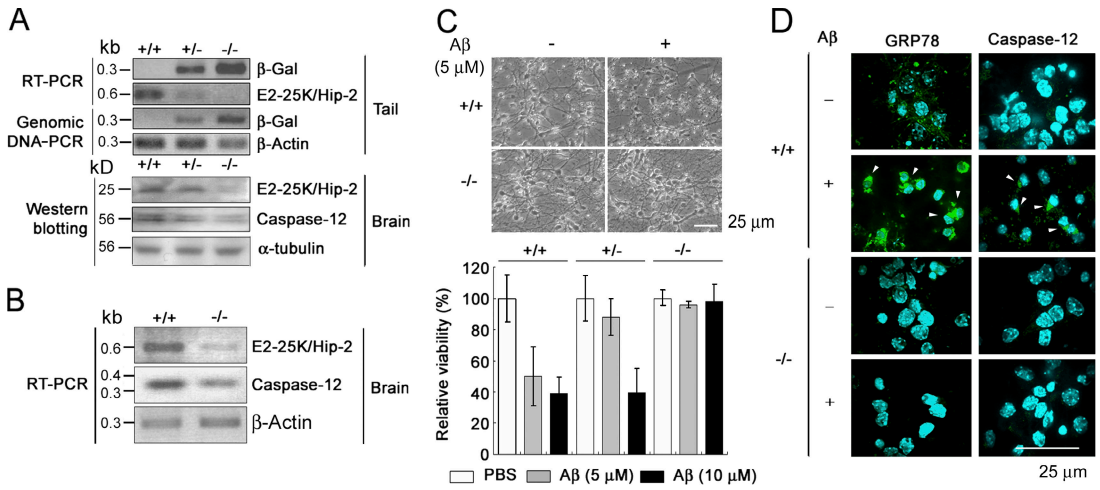

Figure 7. **Down-regulation of caspase-12 and resistance of E2-25K/Hip-2–deficient primary cortical neurons to Aβ toxicity.** (A) Generation of E2-25K/Hip-2–deficient mice. E2-25K/Hip-2–deficient mice were generated from XK109 embryonic stem cells manipulated using the gene-trap method. Genomic DNA and mRNA were purified from the tails of E2-25K/Hip-2–trapped mice and analyzed using genomic DNA-PCR and RT-PCR (Tail). Brain extracts were prepared from postnatal E2-25K/Hip-2 wild-type (+/+), heterozygotic (+/−), and homozygotic (−/−) mice and analyzed by Western blotting with anti–E2-25K/Hip-2 and anticaspase-12 antibodies (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. (C) Resistance of E2-25K/Hip-2–deficient cortical neurons to Aβ toxicity. Primary cortical neurons from E2-25K/Hip-2 wild-type (+/+), heterozygotic (+/−), and homozygotic (−/−) embryos at embryonic day 15 were cultured for 3 d and incubated with Aβ for 2 d. Cell morphology was observed (top) and 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 wild-type (+/+) and homozygotic (−/−) embryos at embryonic day 15 were left untreated or incubated for 48 h with Aβ, immunostained using anti-GRP78 (left) and anticaspase-12 (right) antibodies, and examined under a confocal microscope (see Materials and methods). Nuclei were stained with hoechst dye; arrowheads indicate the induction of each protein.

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 (Kouroku 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 calpainlike 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 calpainlike 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 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 UBB^{+1} (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

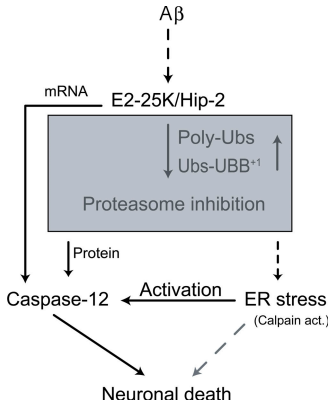

Figure 8. **Schematic diagram showing the regulation of caspase-12 and ER stress by E2-25K/Hip-2 during Aβ neurotoxicity.** The gray box depicts the inhibitory effect of E2-25K/Hip-2 on the proteasome activity via the accumulation of polyubiquitin (Poly Ub) and ubiquitinated UBB^{+1} ($Ubs\text{-}UBB^{+1}$; 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 $Ubs\text{-}UBB^{+1}$ (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 calpainlike activity, which is believed to activate caspase-12 during Aβ neurotoxicity.

unfolded proteins found in the case of Aβ neurotoxicity (Fig. 8; Nakagawa et al., 2000; Ferreiro et al., 2006).

It 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; S86Y, 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′-GCGAATTCTATGGCCAACATCGCG-GTG-3′) and mRed-Hip-2-3′ (5′-GCGGATCCTCAGTTACTCAGAAGCAA-3′). Human calpastatin cDNA cloned in pCMV-SPORT6 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 $A\beta_{1\text{-}42}$ peptide
Commercially available $A\beta_{1\text{-}42}$ was purchased from Sigma-Aldrich and dissolved to a concentration of 500 μM in phosphate-buffered saline. The $A\beta_{1\text{-}42}$ stock solution was incubated for 1 wk at 4°C and divided into small aliquots for storage at −70°C. Cells were incubated with $A\beta_{1\text{-}42}$ 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 effecter 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 (2×) containing 200 mM sodium phosphate, pH 7.3, 2 mM $MgCl_2$, 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.K67ON/M671L) found in a large Swedish family with early onset AD (Hsiao et al., 1996). Double transgenic mice expressing APPswe and PS1dE9 exon 9–deleted PS1dE9 (The Jackson Laboratory) were purchased and bred to generate transgenic mice and wild-type littermates.

Antibody generation, Western blotting, and immunocytochemistry
Generation of anti–E2-25K/Hip-2 and anticaspase-8 antibodies was described previously (Kim et al., 2002; Song et al., 2003). Anti–α-tubulin (Sigma-Aldrich), anticaspase-2 (Santa Cruz Biotechnology, Inc.), anticaspase-3 (Biomeda), 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

2% β-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 (huAPP695.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 coincubation 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

Immunohistochemical studies were photographed 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/DS-U2 digital camera and NIS-Elements F 2.20 program). Fluorescence (IX71S1F-3 with two objective lenses [LCPlanFl, 40×/0.60 Ph2 and 20×/0.45 Ph1]; Olympus) and confocal microscopes (UltraVIEW ERS FRET-EH with an oil immersion objective lens [DIC-H, 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 manager program [Olympus]; UltraVIEW LCI [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 GST-E2D) were expressed in DH5α cells and purified using glutathione–Sepharose 4B (GE Healthcare). For in vitro degradation assays, B103 cells (5×10^7) were harvested and lyzed in tris-based buffer (10 mM Tris-HCl, 0.05% NP-40, 150 mM NaCl, 3 mM $MgCl_2$, 0.5 mM PMSF, 1 mM EDTA, 1 mM ATP, and 1 mM DTT). Cell extract was incubated for 2 h at 37°C in the presence or absence of GST-fused proteins preincubated with purified ubiquitin (Sigma-Aldrich), ubiquitin-activating enzyme E1 (Macrogen), and 10 mM ATP. Protein degradation was then analyzed by Western blotting.

Measurement of calpainlike protease activity

The rhodamine 110-based substrate, (t-BOC-Leu-Met)2-R110, and chloromethylcoumarin-based substrate, CMAC-(t-BOC-Leu-Met; Invitrogen), were used as a calpain substrate to assess calpainlike protease activity in intact cells. When (t-BOC-Leu-Met)2-R110 was used, B103 cells were sedimented and resuspended in Hepes-buffered saline (5 mM Hepes and 0.15 NaCl, pH 7.35) containing 2 mM EDTA (Hepes-buffered saline–EDTA) to a density of $\sim 0.5-10^7$ cells/ml. Cells diluted by 100× were incubated with 10 μM 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 (XK109) were provided by BayGenomics of the International Gene Trap Consortium. Mice heterozygotic 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 NEBlot Phototope (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′-GCGCGTACATCGGGCAAATAATATC-3′) and β-actin. The expression of E2-25K/Hip-2 was examined using RT-PCR with synthetic oligonucleotides derived from E2-25K/Hip-2 (E2-25K/Hip-2-5′ and E2-25K/Hip-2-3′) and β-gal. The absence of E2-25K/Hip-2 expression in homozygous mice was confirmed by Western analysis of the proteins extracted from the brains of 5-d-old mice.

Primary culture of cortical neurons

E2-25K/Hip-2 heterozygous mice were interbred to generate homozygous, heterozygous, and wild-type embryos. Cortical tissues from embryonic day 14.5 embryonic brains were dissociated by incubation with 0.01% trypsin-EDTA (Invitrogen) and plated on culture dishes coated with poly-L-lysine (0.01% in 100 mM borate buffer, pH 8.5). Details of the procedures were described previously (Song et al., 2003). Cortical neurons from each embryo were plated separately and their genotypes were examined by Western blotting and genomic PCR. Neurons were cultured for 3 d before treatment with Aβ in the serum-free minimum essential medium.

Online supplemental material

In Fig. S1 the reporter assay showed that the promoter activity of caspase-12 in pGL3-0.8 was slightly increased by E2-25K/Hip-2. Western analysis showed that the higher molecular mass forms of caspase-12 protein accumulated in the brains of 9-mo-old APPswe/PS1dE9 double transgenic mice (Fig. S2), and that caspase-12 protein accumulated in B103 cells incubated with the proteasome inhibitor MG132 (Fig. S3). Further, in Fig. S4 DPro and p20 fragments of caspase-12 were highly stabilized in the cells exposed to MG132 (10- and sevenfold each). Fig. S5 shows the genomic locus of the gene-trap insertion in E2-25K/Hip-2–deficient mice. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200711066/DC1.

Supplemental materials and methods

Lysosomal inhibitor. Lysosome inhibitor ammonium chloride (NH_4Cl; Sigma-Aldrich) was purchased.

Plasmid construction. Serial deletions of caspase-12 were generated by PCR using synthetic oligonucleotides (C12full 5′KpnI [5′-GGTACCCGCATGGCGGCCAGGAGGACACAT-3′], C12full 3′KpnI [5′-GGTACCCGCATTCCCGGGAAAAAGGTAGAA-3′], C12Δpro 5′HindIII [5′-AAGCTTCGCATGTGTCCACGTGATCAGT-3′], C12Δpro 3′HindIII [5′-AAGCTTCGCATTCCCGGGAAAAAGGTAGAA-3′], C12pro 5′HindIII [5′-AAGCTTCGCATGGCGGCCAGGAGGACACAT-3′], C12pro 3′HindIII [5′-AAGCTTCAGTGTATCTTGGACTTCTGA-3′], and C12p20 3′KpnI [5′-GGTACCCGCATCAGCAGTGGCTATCCC-3′]), after which the PCR products were subcloned into pcDNA3-HA. Caspase-12 promoter constructs (pGL3-3.0 and pGL3-0.8) were previously described (Oubrahim et al., 2005).

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