Involvement of caspase-4 in endoplasmic reticulum stress-induced apoptosis and Aβ-induced cell death

Junichi Hitomi,1,6 Taiichi Katayama,1,6 Yutaka Eguchi,2,6,7 Takashi Kudo,3 Manabu Taniguchi,1,6 Yutaka Koyama,1,6 Takayuki Manabe,1,6 Satoru Yamagishi,1,6 Yoshio Bando,4 Kazunori Imaizumi,5 Yoshihide Tsujimoto,2,6,7 and Masaya Tohyama1,6

1Department of Anatomy and Neuroscience, 2Division of Molecular Genetics, and 3Division of Psychiatry and Behavioural Proteomics, Department of Post-Genomics and Diseases, Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871, Japan
4Department of Anatomy, Asahikawa Medical College, Midorigaoka Higashi, Asahikawa, Hokkaido, 078-8510, Japan
5Division of Structural Cell Biology, Nara Institute of Science and Technology, Takayama, Ikoma, Nara 630-0101, Japan
621st Century COE Program, Japan Society for the Promotion of Science, Chiyoda-ku, Tokyo 102-8471, Japan
7Solution Oriented Research for Science and Technology of Japan, Science and Technology Agency, Honcho 4-1-8, Kawaguchi, Saitama, 332-0012, Japan

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Introduction

Recently, it has been reported that some human diseases, such as Alzheimer’s disease (AD), Parkinson’s diseases, and cystic fibrosis, and neuronal damage by ischemia are related to stress acting on the ER, which leads to intraluminal accumulation of unfolded proteins (Katayama et al., 1999; Wigley et al., 1999; Imai et al., 2000, 2001; Nakagawa et al., 2000; Sato et al., 2001; Tamatani et al., 2001). Stress on the ER can be induced in vitro by depletion of calcium from the ER lumen, inhibition of asparagine N-linked glycosylation, reduction of disulfide bonds, expression of mutant proteins, and ischemia (Imai et al., 2001). ER stress induces three major cellular responses: unfolded protein response (UPR), ER-associated degradation, and apoptosis. Cells exposed to ER stress can up-regulate genes encoding chaperones that facilitate the protein folding process in the ER and reduce overall translation (UPR; Harding et al., 1999; Kaufman, 2002; Forman et al., 2003), or enhance proteasomal degradation of misfolded ER protein in cytosol (Bonifacino and Weissman, 1998; Travers et al., 2000), to reduce the accumulation and aggregation of misfolded proteins, and relieve cells from the stress (Kozutsumi et al., 1998). On the other hand, excessive or long-termed ER stress results in apoptotic cell death, involving nuclear fragmentation,
condensation of chromatin, and shrinkage of the cell body (Imazumi et al., 2001). Several mechanisms that activate apoptotic signaling pathways have been reported. For example, the UPR increases the transcription of CHOP/GADD153 (Brewer et al., 1997), which is closely associated with cell death (Zinszner et al., 1998), recruitment of tumor necrosis factor receptor–associated factor 2 (TRAF2) to activated IRE1α induces c-Jun NH2-terminal kinase activation (Urano et al., 2000), or calpain activates downstream caspase cascade (Nakagawa and Yano, 2000). However, little is known about the precise mechanisms to lead to ER stress-induced cell death in humans.

Activation of caspases, a family of cysteine proteases that cleave substrates at specific aspartate residues, is a central mechanism in the apoptotic cell death process (Salvesen and Dixit, 1997; Thornberry and Lazebnik, 1998). Most of apoptosis-inducing stimuli lead to release of cytochrome c from mitochondria, which binds to Apaf-1 to activate caspase-9 (Li et al., 1997; Zhou et al., 1997), one of initiator caspases with a long pro-domain, and then the activated caspase-9 cleaves effector caspases (Li et al., 1997), including caspases 3 and 7 with a relatively short pro-domain, to activate them. Antia apoptotic Bcl-2 family proteins can rescue cells from apoptosis by protecting mitochondria to prevent cytochrome c release (Kluck et al., 1997; Yang et al., 1997). Several initiator caspases are known to be activated upstream of the mitochondrial dysfunction by specific apoptotic stimuli. For example, Fas stimulation can activate caspase-8 (Fernandes-Alnemri et al., 1996; Muzio et al., 1996), which cannot be inhibited by Bcl-2 (Scaffidi et al., 1998). Among 14 known caspases, caspase-12 seems to be involved in signaling pathways specific to ER stress-induced apoptosis (Nakagawa et al., 2000). Pro–caspase-12 is predominantly localized to the ER, and specifically cleaved by ER stress. Furthermore, caspase-12–deficient mice show a reduced sensitivity to amyloid-β (Aβ), which is found in brains from Alzheimer’s patients (Selkoe, 1986) and shown to cause neuronal cytotoxicity (Yankner et al., 1989). Based on these findings, caspase-12 has been suggested to play an important role in the pathogenesis of AD and to represent a potential target of treatment. However, caspase-12 has only been cloned in the mouse and rat so far, and therefore it is controversial whether similar mechanisms operate in humans (Katayama et al., 1999; Rao et al., 2001; Fischer et al., 2002).

Human genome sequence that is highly homologous to mouse caspase-12 has been identified at the locus within the caspase-1/interleukin-1β converting enzyme (ICE) genes cluster on chromosome 11q22.3 (Fischer et al., 2002), but the gene is interrupted by frame shift and premature stop codon, and also has amino acid substitution in the critical site for caspase activity (Fischer et al., 2002). Therefore, human caspase-12 seems to be lost, and the caspases that substitute for caspase-12 to be activated specifically by ER stress have not been identified in humans so far. We described here that human caspase-4 located within the caspase-1/ICE genes cluster shows similar characteristics to mouse caspase-12. The role of the caspase-4 in ER stress-induced apoptosis and Aβ-induced cell death will be discussed.

### Results

#### Identification of caspase-4 as a gene homologous to caspase-12

To detect a caspase that was specifically involved in ER stress, we screened human colon cDNA libraries by the plaque hybridization method using the mouse caspase-12 gene as a probe. Human caspase-4 was cloned as the most homologous gene to mouse caspase-12, in agreement with the fact that both molecules belong to the caspase-1/ICE subfamily within the caspase family (Kamens et al., 1995; Lin et al., 2000). Although caspase-5, which has slightly less homology to caspase-12 (caspase-4: 48%; caspase-5: 45%), was also isolated, the screening process yielded much more caspase-4 clones than caspase-5. Because caspase-4 but not caspase-5 was expressed in the cell lines used in this work, which underwent apoptosis in response to ER stress, we assumed that human caspase-4 might functionally substitute for mouse caspase-12 in the human system, and further analyzed the possible role of caspase-4 as a mediator of ER stress-induced apoptosis.

#### Subcellular localization of caspase-4

First, we studied the subcellular localization of endogenous caspase-4 in SK-N-SH human neuroblastoma cells. Immunofluorescence microscopy showed that immunostaining pattern of caspase-4 strictly overlapped with that of ER markers such as GRP78 and GRP94 (Fig. 1, a–c). Immunoreactivity of caspase-4 was found to overlap only in part with fluorescence signals from Mitotracker (Fig. 1, d–f). These results suggest that caspase-4 was localized predominantly to the ER, and to the mitochondria in addition. The similar results were obtained using HeLa cells (Fig. 1, g–i). When caspase-4 fused with GFP at its COOH terminus was overexpressed in HeLa cells to see the subcellular localization in live cells, most of the fluorescent signals from caspase-4/GFP fusion protein overlapped with those from ER-tracker (Fig. 1, m–o), confirming predominant localization of caspase-4 to the ER by non-immunological method. The immunoelectron microscopic analysis showed that the immunoreactive signals for caspase-4 were found on the ER and mitochondria (Fig. 1, p–r), but much less signals on the nuclei (Fig. 1 r). We also performed biochemical fractionation analysis. Although we could not eliminate contamination of ER marker proteins in the mitochondria-enriched fraction using SK-N-SH cells, probably because we could not disrupt cells homogeneously as the cell line displays heterogeneity in cellular morphology, mitochondria-enriched fraction does not seem to contain mitochondria and cytosol (Fig. 1 s). Under these conditions, caspase-4 was recovered in both mitochondria-enriched fraction and microsome-enriched fraction, and in cytosolic fraction to a lesser extent (Fig. 1 s), indicating that caspase-4 was surely in mitochondria-enriched fraction. From these results, we concluded that caspase-4 was localized to the ER, and to the mitochondria in addition, in both SK-N-SH and HeLa cells.

#### Specific cleavage of caspase-4 by ER stress and Aβ treatments

To examine whether caspase-4 was specifically cleaved by ER stress, we analyzed the cleavage of pro–caspase-4 in re-
Response to several apoptotic stimuli (Fig. 2a). We found that cleavage of pro–caspase-4 was induced in SK-N-SH cells by treatment with tunicamycin and thapsigargin, both of which caused ER stress. In contrast, when cells were exposed to non-ER stress inducers such as etoposide, staurosporine, and UV at a dose providing similar extent of cell death to that by tunicamycin and thapsigargin, final cleavage products of pro–caspase-4 (Fig. 2a, cleaved-caspase-4, arrowhead) was not observed. Although the bands shown by the asterisks in Fig. 2a, which should be derived from pro–caspase-4 by unknown processing reaction, judging from the data below (Fig. 4b), were also increased, they were also observed in nontreated cells, so we speculated that these bands were not the final processed form of caspase-4. Under the same conditions, cleavage of caspases 3 and 7, the downstream caspases, was observed regardless of apoptotic stimulations (Fig. 2a). These results suggest that caspase-4 is specifically activated by apoptotic stimuli inducing ER stress, but not by other stimuli that do not cause ER stress.
To address the possibility that caspase-4 contributes to the mechanism of Aβ-induced cell death in humans, we examined the cleavage of caspase-4 in SK-N-SH cells after treatment with Aβ. When cells were incubated with 25 μM Aβ25-35 or 5 μM Aβ1-40, cleavage of caspase-4 was observed (Fig. 2 b). In contrast, treatment of cells with the reverse peptides (Aβ35-25 and Aβ40-1, respectively) did not induce the cleavage of caspase-4 (Fig. 2 c). These results suggest that caspase-4 is activated by neurotoxic Aβ treatment similar to ER stress-induced apoptosis.

**Caspase-4 expression in the presence of Bcl-2**

To confirm that cleavage of caspase-4 was not due to other caspases activated downstream of the mitochondrial pathway, we examined the effect of overexpression of Bcl-2 and Bcl-xL on apoptosis induced by tunicamycin. Apoptotic nuclear morphological changes were induced by treatment of vector transfectants of SK-N-SH and of HeLa cells with tunicamycin for 30 h, but such changes were completely suppressed by overexpression of Bcl-2 (Fig. 3) or Bcl-xL (not depicted), indicating that the apoptotic signaling pathway downstream of mitochondria was not operating in cells with overexpression of these antiapoptotic proteins. However, cleavage of caspase-4 after 16 h of tunicamycin treatment was only slightly affected by overexpression of Bcl-2 (Fig. 3) or Bcl-xL (not depicted). These results suggested that caspase-4 is largely activated before the activation of effector caspases during ER stress-induced cell death.

**Requirement of caspase-4 for ER stress- and Aβ-induced apoptosis**

To determine whether caspase-4 is required for ER stress-induced cell death, SK-N-SH cells that expressed endogenous caspase-4 were transfected with small interfering RNA (siRNA) to caspase-4 or GFP as a control. Immunofluorescence analysis showed that the amount of caspase-4 was substantially decreased by incubation for 60 h after transfection with siRNA directed against caspase-4, but immunoreactivity of caspase-4 was not affected by transfection with GFP-siRNA, when compared with nontransfected cells (Fig. 4 a). Western blot analysis also showed that the amount of caspase-4 was decreased by siRNA to caspase-4 (Fig. 4 b). These results suggest that the antiapoptotic signaling pathway downstream of mitochondria was not operating in cells with overexpression of these antiapoptotic proteins. However, cleavage of caspase-4 after 16 h of tunicamycin treatment was only slightly affected by overexpression of Bcl-2 (Fig. 3) or Bcl-xL (not depicted). These results suggested that caspase-4 is largely activated before the activation of effector caspases during ER stress-induced cell death.
results showed that the siRNA could diminish the amount of caspase-4, and that the antibody used here specifically recognized caspase-4 in immunohistochemical analysis. We next examined the effect of decrease in caspase-4 level by siRNA on ER stress-induced apoptosis. Assessment of cell death on the basis of morphological changes showed that ~60% of untransfected SK-N-SH cells were killed by treatment with thapsigargin for 40 h. The extent of cell death was unaffected by transfection with siRNA to GFP (Fig. 4 c). In contrast, only ~30% of the cells died after being transfected with caspase-4 siRNA and exposed to the same stimulation with thapsigargin (Fig. 4 c). As shown in Fig. 4 b, treatment with thapsigargin for 24 h yielded lower level of cleaved-caspase-4 in the cells transfected with caspase-4 siRNA than in the cells transfected with GFP-siRNA. Because the amount of cleaved caspase-4 shown in Fig. 4 b seemed to correlate with the extent of cell death in Fig. 4 c, incomplete inhibition of cell death by transfection with caspase-4 siRNA could be due to residual activity of caspase-4. These results indicate that cells with decreased expression of caspase-4 become more resistant to ER stress-induced cell death.

When cell death was examined by the MTS assay, treatment with caspase-4 siRNA, but not with GFP-siRNA, increased the resistance to ER stress-induced cell death (Fig. 4 d). The increase in the resistance to ER stress-induced cell death was also observed when siRNA to caspase-4 with a different sequence (caspase-4 siRNA-b) was used (Fig. 4 d), indicating that the effect was due to the decreased expression of caspase-4, but not by a specific side effect of caspase-4 siRNA that might affect the expression of other genes. On the other hand, the efficiency of cell death induced by etoposide treatment was not significantly affected by both caspase-4 siRNAs (Fig. 4 d). Therefore, caspase-4 is likely to be specifically involved in ER stress-induced cell death.

To know whether caspase-4 is involved in ER stress-induced cell death in other cell lines, we examined the effect of caspase-4 siRNA using HeLa cells. As shown in Fig. 4 e, treatment of HeLa cells with caspase-4 siRNA significantly increased the resistance to ER stress-induced cell death, although the extent of the increase in resistance was less than that observed for SK-N-SH cells. This is probably because some other apoptotic mechanisms might also operate simultaneously in HeLa cells. Therefore, we concluded that caspase-4 is likely to be involved in ER stress-induced cell death at least in part in HeLa cells.

We next examined whether caspase-4 is involved in Aβ-induced cell death. When treated with Aβ25-35, SK-N-SH cells transfected with caspase-4 siRNA showed significant reduction in cell death compared with the cells transfected with GFP-siRNA (Fig. 4 f). From these results presented here, we concluded that caspase-4 is involved in Aβ-induced cell death, as well as in ER stress-induced cell death.

Discussion

It has been known that apoptotic morphological changes are observed in cell death caused by ER stress (Imaiuzumi et al., 2001). Caspases are activated to transmit apoptotic signals transcending the difference in species (Alnemri et al., 1996). In rodents, caspase-12 mediates apoptosis specifically in response to ER stress (Nakagawa et al., 2000). Although human caspase-12 gene is transcribed into mRNA, mature caspase-12 protein would not be produced, because the gene is interrupted by frame shift and premature stop codon (Fischer et al., 2002). Furthermore, it contains amino acid substitution in the critical site, which leads to loss of function in several caspases (Fischer et al., 2002). Thus, human caspase-12 does not seem to function in ER stress-induced apoptosis, and some other caspases with similar structure might substitute functionally for caspase-12 in humans. The caspase-12 gene is located within a region where caspase-1/ICE subfamily genes cluster (caspases 1, 4, 5, 12 in human and caspases 1, 11, 12 in mouse). No locus with a comparably high homology to rodent caspase-12 could be found in the human genome. Caspases 4 and 5 are located between caspases 1 and 12 in human genome, whereas only caspase-11 is located between caspases 1 and 12 in mouse. Although it is not known why the region in human genome contains gene duplication, caspases 4 and 5 have been thought to function similarly to caspases 11 and 12. Mouse caspase-11...
is essential for the activation of caspase-1/ICE to promote pro–IL-1β (interleukin-1β) processing (Wang et al., 1996, 1998). On the other hand, caspase-5 is likely involved in processing of pro–IL-1β together with caspase-1/ICE (Martinon et al., 2002) and the caspase-5 gene resembles the mouse caspase-11 in its lipopolysaccharide inducibility (Lin et al., 2000). Therefore, caspase-5 should be the orthologue of caspase-11. Here, the screening process yielded the caspase-4 gene as the homologous gene to mouse caspase-12. Thus, caspase-4 is the best candidate that would function similarly to mouse caspase-12 in ER stress-induced cell death in humans.
Here, we examined the localization of human caspase-4 using several methods. The immunostaining analysis using anti–caspase-4 antibody and fluorescent analysis for caspase-4/GFP fusion protein in Fig. 1 showed the predominant localization of caspase-4 on the ER. On the other hand, the immuno-EM showed the nearly equal distribution of caspase-4 on the ER and mitochondria, and subcellular fractionation showed that caspase-4 was recovered in the microsome-enriched and mitochondria-enriched fractions, and also in cytosolic fraction. Although we could not eliminate contamination of ER marker proteins in the mitochondria-enriched fraction in subcellular fractionation using SK-N-SH cells, microsome-enriched fraction does not seem to contain mitochondria and cytosol. Under these conditions, caspase-4 was recovered in both mitochondria-enriched fraction and microsome-enriched fraction, and amounts of caspase-4 recovered in the microsome-enriched and mitochondria-enriched fractions were comparable to those of ER marker, presenilin-1. Therefore, considering all the results shown in Fig. 1, we concluded that caspase-4 was localized to the ER membrane, and probably to the mitochondria in addition.

Caspase-4 on the ER is supposed to function in ER stress-induced apoptosis similarly to caspase-12. In supporting this hypothesis, caspase-4 was cleaved specifically by ER stress and AΑβ-treatment, but not by other apoptotic stimuli including etoposide, staurosporine, and UV. Additionally because Bcl-2 that can completely inhibit the signaling pathway at least downstream from mitochondria, did not prevent the cleavage of caspase-4 by ER stress, it should be most probable that caspase-4 on the ER but not on mitochondria is primarily cleaved. We also showed that reduction of the level of caspase-4 by RNA interference (RNAi) resulted in decrease in ER stress-induced cell death and AΑβ-induced cell death, but did not affect cell death induced by etoposide. The characteristics of human caspase-4 shown here are very similar to those of mouse caspase-12 reported previously (Nakagawa et al., 2000), and therefore, caspase-4 is able to substitute the caspase-12 functions in ER stress-induced apoptosis and AΑβ-induced cell death. Because caspase-4 was also localized to the mitochondria in addition to the ER membrane, whereas caspase-12 was shown to localize predominantly to the ER, but not to the mitochondria (Nakagawa et al., 2000), caspase-4 might have additional function compared with caspase-12, although the function of caspase-4 on mitochondria is not clear.

Several mechanisms that activate caspase-12 have been proposed in mouse system. For example, calpain, a protease that can be activated by calcium released from ER upon ER stress, starts cleavage of caspase-12 (Nakagawa and Yuan, 2000), caspase-7 activates caspase-12 upon prolonged ER stress (Rao et al., 2001), or TRAF2 mediates caspase-12 activation, which is regulated by IRE1α (Yoneda et al., 2001). It is not clear which mechanism is involved in activation of caspase-4, but because final cleavage products were not observed in cells with activated caspase-7 (Fig. 2 a), activation of caspase-7 does not seem to be enough for full activation of caspase-4. To know the precise mechanism that activate caspase-4, it should be important to find proteins that interact with pro–caspase-4 during ER stress-induced apoptosis.

The inhibition of apoptosis induced by ER stress exposure by RNAi to caspase-4 was incomplete. It is possible that the residual activity of caspase-4 after RNAi would be responsible for the cell death. Alternatively, other apoptotic mechanisms might also operate simultaneously. Several possible pathways have been postulated for ER stress-induced apoptosis. ER stress is reported to activate ASK–c-Jun NH2-terminal kinase pathway through the IRE1–TRAF2–ASK1 complex formation (Nishitoh et al., 2002). Other signaling pathway is mediated by transcriptional activation of genes encoding proapoptotic function. Activation of stress transducer IRE1, PERK, or ATF6 leads to transcriptional activation of CHOP/GADD153, a bZIP transcription factor that potentiates apoptosis (Oyadomari et al., 2002). Operation of these mechanisms might account for incomplete inhibition of ER stress-induced apoptosis by knock out of caspase-12 and knockout of caspase-4 in mouse and humans, respectively. It is possible that caspase-dependent mechanism and other mechanisms function in parallel in initiating ER stress-induced apoptosis, and the mechanism that mainly operates could differ depending on cell types. We have shown that the extent to decrease in cell death by decreasing caspase-4 level of SK-N-SH cells was comparable to that reported for caspase-12 knockout mouse (Nakagawa et al., 2000), whereas that of HeLa cells was relatively less. We also find some cells, like HUVEC, in which decrease in caspase-4 did not affect the ER stress-induced apoptosis (unpublished data). Thus, caspase-4 has been shown to function in ER stress-induced apoptosis at least in several cell lines, including SK-N-SH and HeLa cells, but not all cells.

Cell death caused by AΑβ treatment was also partially inhibited by RNAi to caspase-4. Although it is controversial whether AΑβ-induced cell death involves ER stress-induced apoptosis, the results are consistent that these two types of cell death are mediated by common mechanism at least in part. Recent report described that the cell death induced by AΑβ was inhibited by the broad-spectrum caspase inhibitor z-VAD and more specifically by the down-regulation of caspase-2 with antisense oligonucleotides (Haviv et al., 1998). Neuronal culture derived from caspase-2 null mice was also shown to be partially resistant to AΑβ1-42 toxicity (Troy et al., 2000). Thus AΑβ1-42–induced cell death might be mediated by caspase-2 as well as caspase-12. It is possible that both caspases 2 and 4 are involved in AΑβ-induced cell death also in the human system.

Because caspase-4 seems to be responsible for cell death after AΑβ treatment, caspase-4 might be involved in pathogenesis of AD. Consistently to this hypothesis, our preliminary analysis showed an increase in cytoplasmic staining for caspase-4 in the pyramidal cell layer of the hippocampal CA1-2 region in AD patients, but not in control brains (unpublished data). All of the AD brains tested had stronger caspase-4 immunoreactivity than disease control brains from patients with other neurodegenerative disorders (n = 4 for AD and n = 3 for disease control), and the strong staining was remarkable in the pyramidal neurons around deposits of β-amyloid. Increased caspase-4 might elevate the vulnerability of neurons to apoptosis, and therefore may be involved in the pathogenesis of AD.
Bcl-2 family proteins play essential roles in regulating apoptosis. Although proapoptotic family members (Bcl-2, Bcl-xL) and multidomain proapoptotic members (Bak, Bax) are thought to function mainly on mitochondria, recent studies suggest that they may also function on the ER where they reside as well. Overexpression of Bcl-2 (Foyouzi-Youssefi et al., 2000; Pinton et al., 2000) or knockout of both Bak and Bax (Scorrano et al., 2003) are reported to reduce ER Ca\textsuperscript{2+} concentration, but other reports showed that Bcl-2 enhanced the retention of Ca\textsuperscript{2+} in the ER lumen (Distelhorst et al., 1996; He et al., 1997). Thus, although it is still controversial, Bcl-2 family members may contribute to regulating ER stress-induced apoptosis on the ER (Ferri and Kroemer, 2001; Scorrano et al., 2003; Zong et al., 2003) in addition to their main function on mitochondria. Our results demonstrate that overexpressed Bcl-2 and Bcl-xL, which can completely inhibit the signaling pathway at least downstream from mitochondria, did not prevent, but slightly affected, the cleavage of caspase-4 by ER stress. The slight decrease might be due to the absence of its feedback cleavage by downstream effector caspases, such as caspase-3. Therefore, without regard to the mitochondria or the ER where Bcl-2 family functions, activation of caspase-4 should be the primary reaction in ER stress-induced activation of caspases.

In this work, we discovered that caspase-4 plays a key role in ER stress-induced apoptosis in humans. Caspase-4 also seems to act in the β-amylod–induced cell death, suggesting that human caspase-4 corresponds to rodent caspase-12 to initiate cell death signaling pathway, and that the activation of caspase-4 would mediate neuronal cell death in neurodegenerative disorder. Caspase-4 could be the potential target to develop treatments for such diseases including AD.

Materials and methods

Chemicals and antibodies
We used the following antibodies: anti–caspase-4 mAb (4B9; MBL International Corporation), anti–caspase-4 pAb (Santa Cruz Biotechnology, Inc.), anti–KDEL mAb (10C3; StressGen Biotechnologies), anti–cytochrome c mAb (7H8.2C12; BD Biosciences), anti–glyceraldehyde-3-phosphate dehydrogenase mAb (6G7; Bio-genesis), anti–caspase-3 mAb (19; Transduction), anti–caspase-7 mAb (4G2; MBL International Corporation), anti–β-actin mAb (C4; Chemicon International Inc.), anti–Bcl-2 mAb (#100; BD Biosciences), Alexa 588–conjugated anti–mouse IgG antibody (Molecular Probes), FITC–conjugated anti–goat IgG antibody (Jackson ImmunoResearch Laboratories), gold-conjugated anti–goat IgG antibody (British Biocell), and HRP-conjugated anti–mouse IgG antibody (Cell Signaling). Anti–presenilin-1 polyclonal antibody was raised by immunizing rabbits with a synthetic peptide corresponding to residues 1–14 of human presenilin-1, and was affinity purified using ProtOn Kit1 (MultiplePeptide Systems). The chemical reagents used in this study were purchased from Sigma–Aldrich, and ER–tracker and Mitotracker (Molecular Probes). Cytotoxic peptides, Ap\textsubscript{1}B\textsubscript{25–35} and Ap\textsubscript{1b}B\textsubscript{25–40}, and their reverse peptides, Ap\textsubscript{1}B\textsubscript{25–21} and Ap\textsubscript{1b}B\textsubscript{25–15}, were purchased from Sigma–Aldrich.

Cell culture
Human neuroblastoma SK-N-SH cells and human carcinoma HeLa cells were respectively cultured in α-MEM (Invitrogen) and DMEM (Invitrogen) both containing 10% FBS, at 37°C under 5% CO\textsubscript{2}. For some experiments, these cells were stably transfected with pCAGGS–Hbl-2 (Iwashita et al., 1997) and pCAGGS–Hbl-xL (Tagami et al., 2000) to overexpress Bcl-2 and Bcl-xL, respectively.

cDNA cloning
A human colon cDNA library (Stratagene) was used for hybridization to isolated cDNA homologous to the partial sequence of mouse caspase-12 gene. The annealed double-stranded siRNAs listed below were obtained from Dharmacon, and were used to decrease expression of caspase-4. Caspase-4 siRNA-A: 5’–AAGUGCCGCUCCAGCAGUdTdT–3’ (sense), 5’–AAUAGACGUGUAGAGGCCCACdTdT–3’ (antisense); caspase-4 siRNA-B: 5’–AAAGAUUUCUCUCACUGGUUIdTdT–3’ (sense), 5’–AAAACAC–CAGTGAGGAAATCdTdT–3’ (antisense). These sequences were passed through a 25-gauge needle 13 times and centrifuged at 500 g for 10 min to collect a crude nuclear pellet. The supernatant was centrifuged at 1,200 g for 10 min to yield a mitochondria–enriched pellet, which contained mitochondria and microsome as shown in Fig. 1. When using Ap\textsubscript{1}B\textsubscript{25–35} and Ap\textsubscript{1b}B\textsubscript{25–40}, they were incubated with or without Mitotracker probe for 30 min, followed by observation under a fluorescence microscope (model IX71; Olympus).

Immunofluorescence microscopy
Cells were incubated with or without Mitotracker probes, and were fixed with 0.1 M phosphate buffer containing 4% PFA for 2 h at 4°C. Cells were incubated with anti–caspase-4 pAb with or without anti–KDEL mAb, followed by FITC– and Alexa 588–conjugated secondary antibodies, respectively. Stained cells were observed under a confocal microscope (model LSM510; Carl Zeiss MicroImaging, Inc.). To determine for the presence of caspase-4 in live cells, HeLa cells were transfected with caspase-4/GFP fusion gene subcloned into a pcDNA3.1 (Invitrogen) to produce caspase-4 fused with GFP at its COOH terminus, and after 24 h, cells were incubated with ER–tracker probe for 30 min, followed by observation under a fluorescence microscope (model IX71; Olympus).

Western blot analysis
Cells treated with the indicated reagents were washed with PBS, harvested, and lysed in TNE buffer (10 mM Tris–HCl, pH 7.8, 1 mM EDTA, 0.3 M sucrose, 0.1 mM PMSF) for 5 min on ice. The cells were homogenized in a Dounce homogenizer, and equal volume of each fraction was subjected to Western blotting as described below, using indicated antibodies.

Preparation and transfection of siRNAs
The annealed double-stranded siRNAs listed below were obtained from Dharmacon, and were used to decrease expression of caspase-4. Caspase-4 siRNA-A: 5’–AAGUGCCGCUCCAGCAGUdTdT–3’ (sense), 5’–AAUAGACGUGUAGAGGCCCACdTdT–3’ (antisense); caspase-4 siRNA-B: 5’–AAAGAUUUCUCUCACUGGUUIdTdT–3’ (sense), 5’–AAAACAC–CAGTGAGGAAATCdTdT–3’ (antisense). For control, siRNA to GFP was used. GFP siRNA: 5’–GAGCACGCUCCAGCAGUdTdT–3’ (antisense); caspase-4 siRNA-A: 5’–AAGAGUUUCUCUCACUGGUUIdTdT–3’ (sense), 5’–AAAACAC–CAGTGAGGAAATCdTdT–3’ (antisense). Cells were transfected with siRNAs at 50% confluence in 24-well plastic plates with 1.0 µg of each of the above siRNAs using Transmessenger transfection reagent (Qiagen) according to the manufacturer’s protocol. Transfected cells were incubated at 37°C for 60 h without changing the medium. siRNAs were introduced into HeLa cells by electroporation three times with 48–h intervals using Amaxa system according to the manufacturer’s protocol. Efficiency of RNAi was measured by immunoblotting and Western blot analysis using anti–caspase-4 antibody and anti–β–actin antibody.

Cell viability assay
Cells were cultured in 96-well plates, and after 24 h, the appropriate reagents were added and incubated for 24 h. Cell viability was evaluated using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay.
logical changes observed by phase-contrast microscopy or from nuclear morphological changes detected by fluorescence microscopy after staining the cells with 10 μM Hoechst 33342. At least 500 cells were counted, and the data was expressed as the mean ± SEM from three independent experiments and P values were calculated by t test. The MTs (13±4-5 dimethyl-\[\textit{S}-\textit{y}

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