An Endoplasmic Reticulum Stress-specific Caspase Cascade in Apoptosis

CYTOCHROME c-INDEPENDENT ACTIVATION OF CASPASE-9 BY CASPASE-12*

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Activation of caspase-12 from procaspase-12 is specifically induced by insult to the endoplasmic reticulum (ER) (Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, B. A., and Yuan, J. (2000) Nature 403, 98–103), yet the functional consequences of caspase-12 activation have been unclear. We have shown that recombinant caspase-12 specifically cleaves and activates procaspase-9 in cytosolic extracts. The activated caspase-9 catalyzes cleavage of procaspase-3, which is inhibitable by a caspase-9-specific inhibitor. Although cytochrome c released from mitochondria has been believed to be required for caspase-9 activation during apoptosis (Zou, H., Henzel, W. J., Liu, X., Lutschg, A., and Wang, X. (1997) Cell 90, 405–413, Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) Cell 91, 479–489), caspase-9 as well as caspase-12 and -3 are activated in cytochrome c-free cytosols in murine myoblast cells under ER stress. These results suggest that caspase-12 can activate caspase-9 without involvement of cytochrome c. To examine the role of caspase-12 in the activation of downstream caspases, we used a caspase-12-binding protein, which we identified in a yeast two-hybrid screen, for regulation of caspase-12 activation. The binding protein protects procaspase-12 from processing in vitro. Stable expression of the binding protein renders procaspase-12 insensitive to ER stress, thereby suppressing apoptosis and the activation of caspase-9 and -3. These data suggest that procaspase-9 is a substrate of caspase-12 and that ER stress triggers a specific cascade involving caspase-12, -9, and -3 in a cytochrome c-independent manner.

The caspase protease family plays a central role in the implementation of apoptosis in vertebrates (4, 5). Caspases are constitutively expressed in healthy cells, where they are synthesized as precursor proteins (procaspases). Caspases are activated upon processing of procaspases into ~20-kDa (p20) and 10-kDa (p10) mature fragments, in addition to the N-terminal prodomain. The caspase family is broadly divided into two groups: initiator caspases (caspase-8, -9, and -12) and effector caspases (caspase-3, -6, and -7). Initiator caspases undergo autoprocessing for activation in response to apoptotic stimuli. Active initiator caspases in turn process precursors of the effector caspases responsible for dismantling cellular structures.

Recent studies have suggested the existence of a novel apoptotic pathway in which caspase-12 functions as the initiator caspase in response to a toxic insult to the ER, such as by treatment with tunicamycin (an inhibitor of glycosylation), thapsigargin (an inhibitor of the ER-specific calcium ATPase), or calcium ionophores (1). Caspase-12 is specifically activated in cells subjected to ER stress. Furthermore, caspase-12-deficient cells are resistant to inducers of ER stress, suggesting that caspase-12 is significant in ER stress-induced apoptosis (1). ER stress has received growing attention because it is considered a cause of pathologically relevant apoptosis, and it is particularly implicated in neurodegenerative disorders (6). However, the mechanism of caspase-12-mediated apoptosis has been unknown, mainly due to the lack of identification of caspase-12 substrates. In this study, we have examined the susceptibility of procaspases to active caspase-12 and have shown that procaspase-9 can specifically be cleaved by caspase-12 in vitro.

Recent studies show that multiple death signals converge on the mitochondrion (7). Damaged mitochondria release cytochrome c, which facilitates conformational changes in Apaf-1, the specific activator of procaspase-9 (2, 3). The cytochrome c-Apaf-1 complex called an apoptosome (8, 9) is thought to recruit procaspase-9 through interaction between Apaf-1 and procaspase-9 and facilitate autoactivation of caspase-9. Active caspase-9 then activates caspase-3, the major effector caspase that is responsible for destruction of various substrates (4, 5). Cytochrome c release from mitochondria has also been observed in ER stress-induced apoptosis of several cell lines, including mouse embryonic fibroblast cells (10, 11). The in vitro cleavage of procaspase-9 by caspase-12 described above can be achieved in the absence of cytochrome c, suggesting the presence of the ER stress-specific caspase cascade, which comprises caspase-12, -9, and -3 in this order. For examination of the role of caspase-12 in activation of the caspase cascade in vivo, however, it would be desirable to use conditions in which cytochrome c is not released from mitochondria; otherwise,
caspase-9 could be activated by the cytochrome c-Apaf-1 mechanism, independent of caspase-12. We thus used a murine myoblast cell line, C2C12, to study caspase-12, because our preliminary data showed that ER stress induces the activation of caspase-12 and apoptosis in the cell line without the release of cytochrome c from mitochondria. This result suggests that cytochrome c release is not essential for ER stress-induced apoptosis. We took advantage of the fact that cytochrome c is not released to examine the mechanism of caspasecascade activation in the absence of mitochondrial damage, focusing on events that occur downstream of caspase-12 activation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**C2C12 cells (RIKEN Cell Bank, Tsukuba, Japan) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 50 units/ml penicillin, and 50 μg/ml streptomycin (Invitrogen) at 37 °C with 5% CO₂. Apoptosis was induced in cultured cells by adding the following reagents in culture medium unless otherwise stated: 2 mM glutathione (Sigma-Aldrich) for 4 h.

**Examination of Mitochondrial Transmembrane Potential**—Apoptosis was induced in C2C12 cells, and then the cells were stained with the MitoSensor reagent (CLONTECH) according to the manufacturer's protocol.

**Preparation of S-100 from C2C12 Cells**—The C2C12 cell 100,000 x g supernatant was prepared according to the method described in Liu and Wang (12). Briefly, cells were disrupted in buffer containing 250 mM sucrose by a Dounce homogenizer. The supernatant was centrifuged in a microcentrifuge for 10 min, and subsequently at 100,000 x g for 30 min in a tabletop ultracentrifuge (Beckman Coulter, Inc.).

**Western Blot Analysis**—Anti-MAGE-3 (melanoma-associated antigen-3) polyclonal antibody was generated by immunization of rabbit with a synthetic peptide (CHISYPPLHEWVLREGGE) as described previously (13). Primary antibodies for Western blot analysis were used at the following dilutions: anti-MAGE-3 polyclonal antibody, 1:400; anti-FLAG monoclonal antibody (Sigma-Aldrich), 1:1,000; anti-hexahistidine-tag monoclonal antibody (CLONTECH), 1:5,000; anti-caspase-12 rat monoclonal antibody (1), 1:100; anti-MAGE-3 monoclonal antibody (14), 1:2; anti-caspase-9 monoclonal antibody (Medical and Biological Laboratories, Nagoya, Japan), 1:1,000; anti-caspase-3 (cleaved form) antibody (Cell Signaling Technology Inc.), 1:1,000; anti-caspase-7 monoclonal antibody (BD Biosciences), 1:250; anti-cytocrome c monoclonal antibody (BD Biosciences), 1:500; anti-α-tubulin monoclonal antibody (Oncogene Science), 1:1,000; anti-BiP monoclonal antibody (BD Biosciences). GST-MAGE-3 was purified from E. coli expressing the fusion protein with glutathione Sepharose-4B beads (Amersham Biosciences). GST Fusion Protein Full-down Assay—GST-MAGE-3 protein (1 μg) and histidine-tagged caspase-12 (0.1 μg) were incubated with 10 μl of glutathione Sepharose-4B beads (Amersham Biosciences) for 1 h at room temperature in 150 μl of 20 mM phosphate buffer, pH 7.0, containing 200 mM NaCl and 0.02% Triton X-100. Anti-hexahistidine monoclonal antibody (CLONTECH) was used for the detection of caspase-12 p20. Proteins that bound glutathione resin were analyzed by Western blotting.

**In Vitro Cleavage of Radiolabeled Procaspases**—In vitro synthesis of 35S-labeled proteins and their detection by autoradiography were achieved as described previously (15). For mutant analysis, mutations at specific aspartic acid residues in procaspases were introduced by the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Examination of caspase-9 activation, the cytochrome c-free cytosol was incubated with 10 μM bovine cytochrome c (Sigma-Aldrich) and 1 mM ATP for 60 min at 37 °C. For inhibition of caspase-9 activity, LEHD-fluoromethylketone (BioVision, Palo Alto, CA) was added to the cytosol before the addition of caspase-12.

**Stable Cell Lines—**MAGE-3 stable cell lines of C2C12 were generated as follows. MAGE-3 cDNA was cloned into pcDNA3.1(+) vector (Invitrogen). The plasmid DNA was linearized by ScaI digestion before transfection. Transfection was performed with a Perfect transfection reagent (QIAGEN) according to the manufacturer's protocol. MAGE-3 cDNA cloned into the pcDNA3.1(+) vector (Invitrogen) was used for stable transfection. Stable transfectants were grown in medium containing 600 μg/ml G418 (Invitrogen) for 2 weeks before cloning.

**Indirect Immunofluorescence Microscopy**—Cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were incubated with either anti-caspase-12 monoclonal (1) or anti-MAGE-3 polyclonal antibody (this study). Primary antibodies were detected as described previously (15).

**RESULTS**

**Procaspa-9 Is a Substrate of Caspase-12**—We examined how caspase-12 processing is linked to the activation of other caspases. For an in vitro cleavage assay, we produced recombinant caspase-12 (p30) whose N-terminal prodomain had been removed and replaced with a hexahistidine tag. The p30 protein undergoes efficient autoprocessing into p20 and p10 peptides when overexpressed in E. coli (p30) (Fig. 1A). A mutant p30 (p30CS), whose active site Cys is substituted with Ser, is not processed in E. coli (Fig. 1A). The mature caspase-12 (p30*) exhibits proteolytic activity and cleaves procaspase-12 into 35- and 12-kDa fragments (Fig. 1B, lane 16). The cleavage site was located at Asp318, because a procaspase-12 mutant in which Asp318 was replaced with Ser was resistant to caspase-12 digestion (data not shown). Asp318 is also the cleavage site for autoprocessing in E. coli (Fig. 1A), as revealed by amino acid sequencing of p10 by the Edman degradation method (data not shown). p30* cleavage of caspase-12 is achieved by removing other caspase precursors (murine caspase-1 and -2, and human caspase-3, -6, and -8) under the experimental conditions. Note that processing site sequences between p20 and p10 are highly conserved between murine and human caspase-3, -6, -7, and -8. Mutation analysis of caspase-9 (Fig. 1C) indicates that caspase-12 cleaves at specific Asp residues in the linker region.
Several reports have demonstrated that ER stress
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between p20 and p10 in the procaspase-9 polypeptide (LDS349, SED353, and PEPD315 in the human caspase-9). Asp353 of the murine caspase-9 and Asp315 of human caspase-9 have been reported to be the cleavage sites for the activation of procaspase-9 (17, 18). The caspase-9 cleavage observed in vitro thus suggests the possibility that caspase-9 can be activated by caspase-12 during ER stress-induced apoptosis. Under the experimental conditions used, murine caspase-9 contains another cleavage site(s) for caspase-12 in vitro, cleavage at which generates 27- and 20-kDa fragments. We did not further analyze these additional cleavage site(s) because we could detect neither 27- nor 20-kDa caspase-9 fragments in apoptotic cells (described below; data not shown). Procaspase-7 seems to be only slightly processed by active caspase-12 (Fig. 1B, lane 10). The in vitro cleavage of procaspase-7 was not studied further because processing of procaspase-7 is undetectable in C2C12 cells subjected to ER stress (Fig. 1D).

Cytochrome c Is Not Essential for ER Stress-induced Caspase Activation—Several reports have demonstrated that ER stress causes mitochondrial damage, which results in the cytochrome c release from mitochondria (e.g., Refs. 10, 11). Cytochrome c in cytosol and Apaf-1 can induce activation of caspase-9 (2, 3). To examine whether there is an ER stress-specific caspase cascade that is initiated by caspase-12, we used a murine myoblast cell line, C2C12, because this cell line undergoes ER stress-induced apoptosis without cytochrome c release from mitochondria. Cytosolic extracts (S-100) of tunicamycin- or thapsigargin-treated C2C12 cells contain cytochrome c at the same level as that detected in S-100 fractions prepared from untreated cells (Fig. 2A). Nevertheless, more than 50% of the cells undergo apoptosis (see below). Cytochrome c release per se, however, is functional in C2C12 cells, because treatment of C2C12 cells with etoposide or serum deprivation induces apoptosis at a similar level of lethality and with a significant release of cytochrome c. After apoptosis was induced by ER stress inducers, the mitochondrial transmembrane potential was maintained in apoptotic cells (small cells with condensed nuclei), as in the case of untreated cells, which was exhibited by mitochondrial cytochrome c release. Therefore, it is unlikely that cytochrome c is involved in ER stress-induced apoptosis. Etoposide-treatment of C2C12 cells resulted in decrease in mitochondrial transmembrane potential, which was monitored by the green color of the fluorochromes in the cytosol (Fig. 2B). These results suggest that mitochondria in C2C12 cells do not suffer severe damages from ER stress, thus releasing little cytochrome c into cytosol.

Treatment of C2C12 cells with ER stress inducers, either tunicamycin or thapsigargin, results in the processing of procaspase-12 (48 kDa, Fig. 2C) and apoptosis. A 35-kDa fragment was detected by antibodies specific to the p20 region (1). Caspase-9 and caspase-3 are activated in C2C12 cells treated with ER stress inducers (Fig. 2C). The activation of caspase-3, one of the most downstream caspases, suggests that the ER stress-specific caspase cascade comprises caspase-12, -9, and -3. It has been suggested that calpain is involved in activation of caspases in cultured gliial cells after deprivation of oxygen and glucose (19). In the apoptotic C2C12 cells, however, cleavage of a calpain substrate, Bcl-XL, was not detected (Fig. 2D). The activation of caspase-9 has been reported to be independent of calpain. Direct Activation of Caspase-9 by Caspase-12 Wallace et al.

FIG. 1. Procaspase-9 is a substrate of caspase-12. A, purification of the caspase-12 p30 protein overexpressed in E. coli. Either wild-type p30 or the inactive mutant (C/S) protein was tagged with hexahistidine at the N terminus and purified by Ni-column affinity chromatography. Proteins were detected by Coomassie Brilliant Blue staining. B, procaspase-9 and procaspase-12 are specifically cleaved by active caspase-12. "S-Labeled procaspases were incubated with (+) or without (−) active caspase-12 at 37 °C for 4 h and analyzed by SDS-polyacrylamide gel electrophoresis as described previously (15). Arrowheads indicate cleavage products. C, cleavage sites within procaspase-9 are processing sites for activation. Mutation of specific Asp residues (Asp-349 and Asp-353 in murine procaspase-9 and Asp-315 in human procaspase-9, respectively) significantly reduces cleavage by caspase-12 (+). Arrowheads indicate cleavage fragments. D, caspase-7 is not activated in C2C12 cells under ER stress (TG, thapsigargin; TUN, tunicamycin). The Western blot was probed with an anti-caspase-7 monoclonal antibody.
orange mitochondria were stained in mitochondrial transmembrane potential using the MitoSensor reagent (CLONTECH). Apoptotic cells are indicated by potential during ER stress-induced apoptosis in C2C12. Cells were treated with either tunicamycin, thapsigargin, or etoposide and examined for cytosols.

**FIG. 2. Caspase activation in C2C12 occurs independently of cytochrome c release.** A, cytochrome c is not released in the cytosol of C2C12 cells treated with ER stress inducers. S-100 fractions of C2C12 cells after treatment with various apoptotic stimuli were immunoblotted and probed with antibody to cytochrome c. Actively growing C2C12 cells were treated with 2 μg/ml tunicamycin (TUN), 1 μM thapsigargin (TG) for 24 hr, or 100 μM etoposide for 48 h or subjected to serum deprivation for 48 h. UT, untreated cells. B, integrity of mitochondrial transmembrane potential during ER stress-induced apoptosis in C2C12. Cells were treated with either tunicamycin, thapsigargin, or etoposide and examined for mitochondrial transmembrane potential using the MitoSensor reagent (CLONTECH). Apoptotic cells are indicated by arrowheads. Intact mitochondria were stained in orange, whereas apoptotic cells containing damaged mitochondrial membrane is visualized by green fluorochrome in cytosols. C, caspase activation in apoptotic C2C12 cells. C2C12 cells were treated with either tunicamycin or thapsigargin for 24 h. Arrowheads indicate procaspases (pro) and their cleavage fragments.

**FIG. 3. Activation of caspase-9 by caspase-12.** A, caspase-9 cleavage by caspase-12. Caspase-9 in S-100 fractions was detected on a Western blot using anti-caspase-9 antibody. Lane 1, untreated S-100 prepared from C2C12 cells; lane 2, S-100 fractions treated with active caspase-12; lane 3, addition of cytochrome c and dATP to the S-100 fraction; lane 4, S-100 fraction prepared from tunicamycin-treated C2C12 cells. B, activation of the ER stress-specific caspase cascade in vitro. Incubation of S-100 fractions prepared from C2C12 cells with active caspase-12 results in the activation of the ER stress-specific caspase cascade in vitro. Lane 1, S-100 fractions were directly subjected to SDS-polyacrylamide gel electrophoresis without incubation. Lanes 2–4, S-100 was incubated at 37 °C for 4 h with or without reagents indicated in the figure. The Western blot was probed with anti-caspase-9 or anti-caspase-3 (cleaved form) antibody. An asterisk indicates a protein that nonspecifically reacts to the anti-caspase-3 antibody.

Control experiment showed that addition of cytochrome c and dATP to S-100 of untreated cells also caused processing of procaspase-9 into 35-kDa fragments that appeared as a doublet on the blot (Fig. 3B, lane 3). The lower band was less intense than the upper band in the case of caspase-12-induced processing (Fig. 3B, lane 2) and the apoptotic S-100 fractions (lane 4). The ratio of these 35-kDa fragments was different from that observed in the cytochrome c-treated S-100 (Fig. 3B, lane 3). It remains to be revealed whether the difference in the ratio of these fragments reflects a difference in mechanism of processing.

The cleavage of procaspase-9 is not suppressed in the presence of a caspase-9-specific inhibitor LEHD-fluoromethylketone (20), indicating that procaspase-9 is cleaved by active caspase-12, independent of the inherent autoprocessing activity of procaspase-9 (Fig. 3B, upper panel, lane 4). We then examined caspase-9 activation through detection of specific cleavage at Asp175 within the procaspase-3 polypeptide, the downstream target of caspase-9 (3). Incubation of the S-100 fraction with caspase-12 causes processing of caspase-3 at Asp175 of ITED175 (Fig. 3B, lower panel, lane 3), suggesting that the activated caspase-9 in the S-100 fraction cleaved procaspase-3. The specific cleavage of procaspase-3, but not procaspase-9, can be inhibited by LEHD-fluoromethylketone, (Fig. 3B, lane 4). This result indicates that cleavage of procaspase-3 is dependent on caspase-9, as already observed for apoptosis induced by various stimuli other than ER stress (3, 21–23).

**Suppression of Procaspase-12 Processing by Its Binding Protein—** We have recently isolated by yeast two-hybrid screening from a HeLa cell cDNA library a human cancer antigen, MAGE-3, as a protein that specifically binds the caspase-12 p10 fragment (see “Experimental Procedures”). Because MAGE-3 can suppress the activity of procaspase-12, as described below, we used the protein to examine the significance of caspase-12 activation in ER stress-induced apoptosis in C2C12 cells. MAGE-3 is a member of the MAGE gene family and is expressed in various types of tumor but not in normal tissues except for the testis (24). Although the specific interac-
tion between caspase-12 and MAGE-3 is intriguing, it remains unclear whether MAGE-3 plays any role in caspase regulation in human cells (see “Discussion”). MAGE-3 does not bind to other caspasas, such as caspase-9 (of either murine or human origin), as tested by the two-hybrid assay (results of murine caspase-1, -9, and -11 and human caspase-3, -6, and -7 are shown in Fig. 4A).

The MAGE-3 protein can also bind both the caspase-12 p10 fragment and procaspase-12 in mammalian cells. When MAGE-3 is expressed in COS-1 cells by transient transfection it can be co-precipitated with FLAG-tagged p10 (Fig. 4B, lane 3) or FLAG-tagged procaspase-12 (lane 7) using an anti-FLAG antibody. MAGE-3 was not co-precipitated with FLAG-tagged p10 fragments of murine caspase-2 and human caspase-8, whose binding ability could not be examined by the two-hybrid assay because of significant background activity (data not shown). Fig. 4C shows that p30C/S (unprocessed p30*) is not efficiently co-precipitated by GST-MAGE-3 (lanes 3 and 4). Under the same conditions, however, p30* (processed) is efficiently co-precipitated by GST-MAGE-3 (Fig. 4C, lanes 1 and 2), suggesting that MAGE-3 does not efficiently bind the p10 fragment in active caspase-12. It is possible that the p10 fragment within mature caspase-12 is not fully accessible to MAGE-3 because of steric hindrance by the p20 portion. X-ray crystallographic analyses of caspase-1 and caspase-3 have suggested that they undergo a conformational change upon maturation (25–27). This conformational change may occur in caspase-12 and result in the p10 fragment being less exposed for binding to MAGE-3.

Consistent with the binding of MAGE-3 to unprocessed caspase-12, MAGE-3 protects procaspase-12 from cleavage by active p30* in a dose-dependent manner (Fig. 4D, lanes 2–7). Substitution of MAGE-3 with bovine serum albumin fails to inhibit cleavage (Fig. 4D, lane 9). It is more likely that MAGE-3 protects procaspase-12 from processing by specifically binding the p10 portion of the precursor. This result is consistent with our observation that the affinity of MAGE-3 for p30C/S is much higher than that for active caspase-12 (Fig. 4C).

Suppression of Caspase-12 Activation Resulted in Suppression of Caspase-9 Activation and Apoptosis in Vivo—To examine the involvement of caspase-12 in the activation of the caspase cascade, we established stable transfectants (C2C12/MA21) of C2C12 cells that overexpress MAGE-3 (Fig. 5A). Colocalization of MAGE-3 with endogenous caspase-12, an ER-associated protein (1), in C2C12/MA21 was observed by double immunostaining (Fig. 5B), although signals of free MAGE-3 proteins (red color) were still evident in the merged image. This observation was supported by a cell fractionation experiment, where MAGE-3 was detected in the microsomal fraction as well as in S-100 (Fig. 5C). Treatment of either parental C2C12 cells
or a vector control line (C2C12/vec2) with ER stress inducers leads to morphological changes typical of apoptosis. Over 50% of C2C12/vec2 cells exhibit apoptotic morphology after 24 h treatment with tunicamycin or thapsigargin, as indicated by the small round shape of the cells (Fig. 5D). The nuclei of these round cells are fully condensed, as visualized by staining with Hoechst 33342 (data not shown). However, C2C12/MA21 cells undergo apoptosis at the same low background level (< 5%) observed in untreated cells under the same conditions (Fig. 5D). Activation of caspase-12 is almost completely suppressed in C2C12/MA21 cells treated with ER stress inducers (Fig. 5E).

Processing of caspase-9 and caspase-3 also does not take place in MAGE-3 overexpressing cells. Both C2C12/MA21 cells and C2C12/vec2 cells respond to ER stress and elicit the unfolded protein response (reviewed in Ref. 28), as demonstrated by the induction of BiP, an ER-specific heat shock protein (Fig. 5F). These data indicate that MAGE-3 overexpression renders cells resistant to ER stress by suppressing the activation of caspase-12. Therefore, caspase-12 is a critical component of the apoptotic machinery that responds to ER stress, confirming the previous observation (1) that caspase-12 null mice are resistant to the toxic effects of ER stress (e.g., intraperitoneal injection of tunicamycin). Furthermore, concomitant inhibition of the activation of other caspases (caspase-9 and -3) in stably transfected C2C12/MA21 cells strongly suggests that caspase-9 and -3 are located downstream of caspase-12 in the ER stress-specific caspase cascade. These results suggest that procaspase-9 is a substrate of caspase-12 in vivo as well as in vitro. Both C2C12/MA21 and C2C12/vec2 cells undergo apoptosis when treated with staurosporine, a protein kinase inhibitor (data not shown), indicating that the apoptotic machinery per se is functional. This result supports the idea that the suppressive effect of MAGE-3 is specific for the ER stress-induced apoptotic pathway mediated by caspase-12.

**DISCUSSION**

Our data suggest the following: 1) caspase-12 activation triggers the caspase cascade in response to ER stress; 2) procaspase-9 is a substrate of caspase-12 and caspase-9 activation can be achieved in cells without the release of cytochrome c from mitochondria; and 3) proteolytic signals in the cascade are transmitted from caspase-12 to an effector caspase (caspase-3) via caspase-9 (Fig. 6). An Apaf-1/cytochrome c-independent mechanism of caspase-9 activation has recently been reported for dexamethasone-induced apoptosis of multiple myeloma cells (29). Because recombinant caspase-9 prepared from E. coli exhibits protease activity (30), it is obvious that Apaf-1 (and cytochrome c) is not essential for the activation of caspase-9. However, the lack of cytochrome c release in C2C12 cells does not exclude the possibility that Apaf-1/cytochrome c is involved in other cell lines. Cytochrome c release has been observed in both mouse and rat embryonic fibroblast cells subjected to ER stress (10, 11). It is likely that caspase-9 activation can be achieved by caspase-12-dependent cleavage, by an Apaf-1/cytochrome c mechanism, or by both means (Fig. 6). A similarly complex mechanism by which apoptosis is triggered has been described previously for the death receptor mediated pathway.

![Fig. 5. Suppression of caspase-12 activation renders cells resistant to ER stress. A, stable expression of MAGE-3 in C2C12 cells. Cell lysates were probed with an anti-MAGE-3 monoclonal antibody (14). Lane 1, vector control (C2C12/vec2); lane 2, C2C12/MA21. B, Colocalization of caspase-12 and MAGE-3 in C2C12/MA21 cells detected by an anti-MAGE-3 rabbit polyclonal antibody and an anti-caspase-12 rat monoclonal antibody. Primary antibodies were detected with either Alexa594-conjugated anti-rabbit IgG antibody or a combination of biotin-labeled anti-rat IgG antibodies and Alexa488-conjugated avidin. C, intracellular localization of ectopically expressed MAGE-3. Either C2C12/MA21 or the vector control cell was fractionated into S-100 (M) IgG antibodies and Alexa488-conjugated avidin. C, intracellular localization of ectopically expressed MAGE-3. Either C2C12/MA21 or the vector control (C2C12/vec2) was incubated with 2 μg/ml tunicamycin or 1 μM thapsigargin (TG). UT, untreated cells. E, suppression of caspase-12 activation in stable cell lines. Stable C2C12/vec2 and C2C12/MA21 transfectants were incubated with 2 μg/ml tunicamycin or 1 μM thapsigargin. Cell extracts from these cell lines were analyzed by Western blot analysis. Arrowheads indicate caspase cleavage fragments. F, ER stress elicited the unfolded response in the MAGE-3 overexpressing cells. Cell lysates were probed with either anti-BiP antibody or anti-α-tubulin antibody.

![Fig. 6. Model of ER stress-induced caspase activation. See text for details.](http://www.jbc.org/Downloaded from http://www.jbc.org/)](http://www.jbc.org/Downloaded from http://www.jbc.org/)
Stimulation of death receptors (e.g., Fas) results in the activation of caspase-8, which in turn activates effector caspases in a direct manner. Alternatively, caspase-8 may cleave Bid, a pro-apoptotic member of the Bcl-2 family, and the cleaved Bid may in turn induce cytochrome c release through mitochondrial damage (32, 33). Our studies present another example of redundancy in the mechanisms by which apoptosis is executed. It is unclear, then, how cytochrome c release is induced by ER stress in cell lines other than C2C12 cells. ER stress induces cytochrome c release in rat fibroblast cells in a caspase-8- and Bid-independent manner (11). Possible mediators linking the ER to mitochondria, as suggested by recent studies, include the c-Ab1 tyrosine kinase (10) and calcium (34).

The present study reveals that C2C12 cells are useful for the study of the ER stress-specific caspase cascade because a simpler mechanism probably operates in these cells. Comparison of C2C12 cells with other cell lines would contribute to the dissection of the mechanism of ER stress-induced apoptosis.

To conclude that caspase-12 initiates the ER-specific caspase cascade in a direct manner, it should be critical to show that caspase-12 cleaves procaspase-9 at the processing site for activation, and the cleavage product (caspase-9) is active. We have demonstrated the specific cleavage and activation of procaspase-9 by purified caspase-12. Furthermore, we have shown direct correlation between suppression of caspase-12 activation and suppression of caspase-9 activation (and apoptosis) in vivo using the caspase-12 binding protein. These data strongly suggest that caspase-12, activated in response to ER stress, cleaves procaspase-9 to initiate the ER stress specific caspase cascade. During preparation of this article, Ellerby’s group reported that Apaf-1 cleaves procaspase-9 at the processing site for activation, and the cleavage product (caspase-9) is active. We have shown that murine caspase-12 ortholog. We have shown that murine caspase-12, although their cleavage site sequences are not identical (Fig. 1C) is interesting to note that the processing sites within procaspase-9 of both origins are functionally conserved so that they can be cleaved by caspase-12, implying the presence of a functional homolog of caspase-12 in human cells.

Prolonged ER stress contributes to cell death and is linked to the pathogenesis of several different neurodegenerative disorders (39). It is possible that suppression of caspase-12 activation by ER stress may provide a basis for the development of therapeutic agents against unwanted activation of caspases caused by ER stress.

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