Coupling Endoplasmic Reticulum Stress to the Cell Death Program

MECHANISM OF CASPASE ACTIVATION

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The endoplasmic reticulum (ER) is the site of assembly of polypeptide chains destined for secretion or routing into various subcellular compartments. It also regulates cellular responses to stress and intracellular Ca\(^{2+}\) levels. A variety of toxic insults can result in ER stress that ultimately leads to apoptosis. Apoptosis is initiated by the activation of members of the caspase family and serves as a central mechanism in the cell death process. The present study was carried out to determine the role of caspases in triggering ER stress-induced cell death. Treatment of cells with ER stress inducers such as brefeldin-A or thapsigargin induces the expression of caspase-12 protein and also leads to translocation of cytosolic caspase-7 to the ER surface. Caspase-12, like most other members of the caspase family, requires cleavage of the prodomain to generate active caspase-12, resulting in increased cell death. We propose that any cellular insult that causes prolonged ER stress may induce apoptosis through caspase-7-mediated caspase-12 activation. The data underscore the involvement of ER and caspases associated with it in the ER stress-induced apoptotic process.

The endoplasmic reticulum (ER) regulates protein synthesis, protein folding and trafficking, cellular responses to stress, and intracellular calcium (Ca\(^{2+}\)) levels (1–3). Alterations in Ca\(^{2+}\) homeostasis and accumulation of unfolded proteins in the ER cause ER stress (4, 5). Prolonged ER stress contributes to cell death and is linked to the pathogenesis of several different neurodegenerative disorders (6, 7). A variety of toxic insults, including Ca\(^{2+}\) ionophores, inhibitors of glycosylation, chemical toxicants, and oxidative stress, can all cause ER stress and ultimately lead to cell death (8–16). However, the central role of the ER in initiating cell death, following prolonged stress, is not well understood. Activation of caspases, a family of cysteine-dependent aspartate-directed proteases, is a central mechanism in the apoptotic cell death process (17). The localization of caspases and translocation of their active products appear to be critical for the development of the apoptotic process (18). Although studies have demonstrated the presence of caspase-7 as an unprocessed p35 proform both in the cytosolic and microsomal fractions (19, 20), caspase-12 in neurons, and other cells, is primarily associated with the endoplasmic reticulum (21, 22). Caspase-12 is specifically involved in apoptosis that results from stress in the endoplasmic reticulum (21–24). Caspase-12, whose activation may be mediated by calpain, participates in ER stress-induced apoptosis that is blocked by zVAD-fmk, a general caspase inhibitor (21, 22). Apoptosis triggered through pathways that do not involve the endoplasmic reticulum, such as serum deprivation or Fas activation, does not result in activation of caspase-12 (21, 22). The present study was performed to determine whether ER stress-induced cell death requires the interaction and subsequent activation of caspases, and if so, which ones. We demonstrate that ER stress not only induces the expression of caspase-12 but also causes translocation of cytosolic caspase-7 to the ER surface. We further show that caspase-7 is required for caspase-12 activation. Prolonged ER stress also facilitates movement of active caspase-12 into the cytoplasm where it interacts with caspase-9 and thus may set in motion the cytosolic component of the ER stress-induced apoptotic cascade. Our findings suggest that the ER plays a pivotal role in the activation of a subset of caspases during ER stress-mediated cell death that is induced by an array of insults.

EXPERIMENTAL PROCEDURES

Plasmids, Cells, and Culture Conditions—The human embryonic kidney 293T cell line was used for transient transfection using LipofectAMINE (Life Technologies, Inc.). Typically 2 \(\times\) 10\(^4\) and 4 \(\times\) 10\(^4\) cells were seeded in 10- or 15-cm dishes, respectively, and transfected 1 day later with 6 or 12 \(\mu\)g of the specified construct using a ratio of 1 \(\mu\)g:5 \(\mu\)l of DNA:LipofectAMINE. The transfection efficiency using these conditions was about 65–75%.

Mouse caspase-12 cDNA was amplified via PCR using primers flanking the start and stop codons (mouse caspase-12-F: aggcagcagacagcatcag and mouse caspase-12-R: gttagtggagctggtgta). PCR parameters were 2.5 \(\mu\)l of 4-fold-diluted cDNA reaction products, 25 pt primers, 0.5 mm dNTPs, and 2 units of Taq polymerase (Roche Molecular Biochemicals, Indianapolis, IN) with 2.5 \(\mu\)l of 10X buffer. Cycling conditions were 94 °C for 30 s, 60 °C for 30 s, and 65 °C for 1 min for 30 cycles. The 1.2-kb PCR product was gel-purified and ligated into the pCDNA 3.1 expression vector. Using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), the following caspase catalytic mutants were generated: caspase-7 (C186A) and caspase-9 (C287A) (25). Mutants of caspase-12 were obtained by a similar procedure. The sequences of all constructs were confirmed, and Western blot analyses
were performed to verify protein expression. His-tagged caspase-7 (gift of Dr. Guy Salvesen) was purified by nickel affinity chromatography as described previously (26, 27).

**Cell Fractionation and Limited Tryptic Digestion of Microsomal Proteins—**Fractionation was performed as described previously (28) with some modifications. Untreated and 2.5 μM thapsigargin-treated (24 h) 293T human renal epithelial cells were resuspended in ice-cold fractionation buffer, pH 7.4 (20 mM HEPES, 10 mM KCl, 250 mM sucrose, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and protease inhibitor mixture). After cell lysis, nuclei were pelleted by a 10-min, 750-g spin. The supernatant was centrifuged at 10,000 × g for 30 min. The mitochondrial pellet was resuspended in the above buffer, and the supernatant was re-centrifuged at 100,000 × g for 1 h. The resulting supernatant contained the soluble cytosolic fraction, and the pellet constituted the microsomal fraction. The purity of each fraction was assessed by the presence of specific marker proteins: PARP for nuclei, cytochrome c for mitochondria, and SREBP-1 for ER. Equal amounts of protein from normal and thapsigargin-treated cells were analyzed by electrophoresis. In our fractionation procedures, the low salt lysis buffer used for the isolation procedure did not lead to nonspecific binding of proteins to organelles based on the presence of the marker proteins only in their specific compartments.

Control, 2.5 μM thapsigargin-treated, and 2.5 μM brefeldin-A-treated cell extracts were subfractionated into microsomal fractions. The purity of the microsomal fraction was assessed by the presence of protein disulfide isomerase (PDI), an ER lumen protein. Isolated microsomes were digested by 0.05% trypsin-EDTA for 30 min at 35 °C. The resulting supernatant contained the soluble cytosolic fraction, and the pellet constituted the microsomal fraction. The purity of each fraction was assessed by the presence of specific marker proteins: PARP for nuclei, cytochrome c for mitochondria, and SREBP-1 for ER. Equal amounts of protein from normal and thapsigargin-treated cells were analyzed by Western blotting. In our fractionation procedures, the low salt lysis buffer used for the isolation procedure did not lead to nonspecific binding of proteins to organelles based on the presence of the marker proteins only in their specific compartments.

**Cell Free Extracts and Western Blotting—**Cell-free cytoplasmic extracts were prepared as previously described (29, 30). Briefly, cells were lifted gently, pelleted at 200 × g, and the resulting pellet was washed in 50 ml of ice-cold phosphate-buffered saline. The cell pellet was resuspended in a 15-ml conical centrifuge tube with 10 ml of hypotonic extraction buffer (HED; containing 50 mM PIPES, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride). The cells were centrifuged at 1000 × g (4 °C) to form a tight pellet, and the volume of the cell pellet was approximated. The supernatant was aspirated, and HEB was added to a volume between 0.5 and 1× the pellet volume. The cells were transferred to a 2-ml Dounce homogenizer and allowed to swell for 20–30 min on ice. Cells were lysed with 20–100 gentle strokes of a B-type pestle. The desired extent of lysis (>90%) was monitored under the microscope by trypan blue staining. The cell lysate was transferred to an Eppendorf tube and centrifuged for 30 min at 16,000 × g (4 °C). The clarified supernatant was removed carefully and either was used immediately or stored in aliquots at −84 °C. Reactions activated by cytochrome c and dATP, cell free extract (100–200 μg of protein) was incubated with cytochrome c (10 μM) and dATP (1 mM) at 37 °C for 1 h. Electrophoresis of equal amounts of total protein was performed on SDS-polyacrylamide gels. Separated proteins were transferred to polyvinylidene fluoride membranes at 4 °C for Western blot analysis. Membranes were probed with a 1:50 dilution of anti-caspase-12 antibody (gift of Dr. Junying Yuan), a 1:1000 dilution of the anti-CPP32 monoclonal antibody, 1:1000 dilution of anti-caspase-9 polyclonal antibody, 1:500 dilution of anti-caspase-7 monoclonal antibody (all from Transduction/PharMingen Laboratories, San Diego, CA). The anti-caspase-12 antibody was capable of recognizing human caspase-12 protein. The blots were incubated in a horseradish peroxidase-coupled secondary antibody for 1 h followed by enhanced chemiluminescence detection of the proteins with Hyperfilm ECL detection (Amersham Pharmacia Biotech, Arlington Heights, IL).

**Co-immunoprecipitation Assay—**Cell lysis and immunoprecipitation was performed as previously described (31, 32) to provide a qualitative assessment of caspase interaction before and after ER stress. Briefly, 293T cells treated with an ER stress inducer, were harvested, washed, and resuspended in cell lysis buffer (500 mM HEPES, pH 7.6, 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA). A total of 500–1000 μg of protein was subjected to immunoprecipitation. The lysate was first pre-cleared with an appropriate control IgG corresponding to the host species of the primary antibody. The pre-cleared cell lysate was incubated with 0.5–5 μg of primary antibody overnight at 4 °C. Later, 20 μl of resuspended volume of the appropriate agarose conjugate was added and incubated at 4 °C for 1 h to overnight. After extensive washes, proteins bound to protein A-Sepharose or protein A/G-Sepharose (Santa Cruz Biotechnology) were analyzed by immunoblotting. In a typical IP experiment we loaded 2.5-fold more starting protein in the IP lane than in the lysate lane. The presence of the interacting protein was detected by Western blot analysis using the respective antibody.

**Quantification of Apoptosis—**Control and treated cells were stained with Hoechst 33258 as previously described (29, 30) to assess nuclear changes associated with apoptotic cell death. Cells were scored as apoptotic if their nuclei exhibited margination and condensation of chromatin and/or nuclear fragmentation. A minimum of 100 cells from three different areas of each plate was evaluated for quantification of apoptosis.
Caspases-12 and -7 have different localizations during ER stress. Western blots of nuclear (N), mitochondrial (M), microsomal (ER), and cytoplasmic (S) fractions of 293T human renal epithelial cells before and after treatment with 2.5 μM thapsigargin for 24 h. Membranes were probed with anti-caspase-12, anti-caspase-8, anti-caspase-9, anti-caspase-7, and anti-caspase-3 antibodies. Cellular fractions were also probed for standard marker proteins, namely PARP (nuclei), cytochrome c (mitochondria), and SREBP (ER). The low salt lysis buffer that we used for the isolation procedure did not cause any leakage of proteins, nor did it lead to any nonspecific binding of proteins to organelles as indicated by the presence of SREBP, PARP, cytochrome c, and caspases-9, -8, and -3 that were present only in their specific compartments. Each Western blot is representative of three independent experiments.

RESULTS

Induction of Caspase-12 Expression and Localization of Caspases following ER Stress—Brefeldin-A, a fungal metabolite, blocks protein folding and export and induces ER stress and cell death (9, 15). Thapsigargin, an inhibitor of the ER Ca-ATPase, also causes ER stress and induces apoptosis in many cell types (13, 16). We used both of these compounds to induce ER stress and to investigate the role of caspases in mediating cell death. Cell extracts prepared from cells treated with 2.5 μM brefeldin or 2.5 μM thapsigargin showed increased caspase-12 protein expression (Fig. 1, a and b), with attenuation of expression at 48 h after thapsigargin treatment (Fig. 1b). Prolonged treatment of cells with brefeldin also caused a decrease in caspase-12 protein expression (data not shown). Such a regulated caspase-12 protein expression was not seen when cells were treated with tamoxifen (Fig. 1c), which causes apoptosis without inducing ER stress as confirmed by the GRP78 expression pattern. A high level of GRP78 protein expression is indicative of ER stress (8, 11, 33, 34). Similarly, no change in caspase-3, -8, or -9 protein expression was seen in cell extracts treated with brefeldin or thapsigargin (Fig. 1c), suggesting that ER stress specifically induces caspase-12 protein expression. Recent studies have demonstrated the association of caspase-7 and caspase-12 to the ER compartment (18–22). To determine the subcellular localization of caspase-7 and caspase-12 before and after ER stress induction, cell lysates from control and thapsigargin-treated 293T cells were fractionated into nuclear, mitochondrial, microsomal, and soluble fractions and analyzed by Western blotting. The quality of the fractionation experiments was controlled by assessing the distribution of standard marker proteins in the different fractions as described under “Experimental Procedures”. As shown in Fig. 2, caspase-12 was predominantly found in the microsomal fraction in control extracts. Thapsigargin treatment resulted in increased caspase-12 protein expression in the microsomal fraction. In addition, the active form of caspase-12 (42 kDa) was also detected in the soluble fraction after thapsigargin treatment (Fig. 2). Caspases-3, -8, and -9 were exclusively seen in the soluble fraction before and after treatment. In contrast, caspase-7, which was detected only in the soluble fraction before treatment, co-fractionated with the ER compartment following thapsigargin treatment. Similar results were also seen with cellular fractions from other cell types treated with brefeldin or thapsigargin (data not shown). This suggests that ER stress induction causes translocation of (a) cytosolic caspase-7 to the ER compartment and (b) active caspase-12 out of the ER.

Caspase-7 and Caspase-12 Are Localized on the Cytoplasmic Side of the ER—To understand the topology of caspases-7 and -12 in the ER compartment before and after ER stress, we isolated the microsomal fraction from untreated, brefeldin-A-treated, and thapsigargin-treated 293T cells and subjected them to limited trypsin digestion. The purity of microsomes was assessed by staining for PDI, an ER lumen protein. As shown in Fig. 3, caspase-7 is not detected in microsomes isolated from normal cells but is present in microsomes prepared from thapsigargin- and brefeldin-treated cells. Addition of trypsin resulted in complete digestion of caspase-7. Similarly, caspase-12 was completely digested by trypsin in microsomes isolated from control and treated cells. In contrast, PDI under similar conditions was resistant to trypsin treatment. These data indicate that, although caspase-12 is localized on the cytoplasmic side of the ER, ER stress also causes translocation of a subpopulation of cytosolic caspase-7 to the surface of the ER compartment. There was no evidence of localization of caspases-3, -8, and -9 on the cytoplasmic side of the ER before
Interaction of Caspase-12 with Caspases-7 and Caspase-9—
The subcellular location of caspase-12 and its active form has already been demonstrated and confirmed by us (21, 22) (Fig. 2). Similarly, caspase-7 is known to associate with the ER compartment following apoptotic stimuli (18–20) and ER stress (Fig. 2). Given these established subcellular locations for caspases-7 and -12, we proceeded to investigate possible interactions between them. The interaction of caspase-12 and caspase-7 was evaluated in cells treated with thapsigargin for 24 h, by which time caspase-7 was demonstrated to have translocated to the ER surface (Fig. 2). Immunoprecipitations were performed with anti-caspase-12 antibody, and the resulting immunoprecipitates were analyzed by immunoblotting using antisera specific for caspase-7. Association of caspase-7 and caspase-12 was seen only in cell extracts treated with thapsigargin (Fig. 4a). Because prolonged ER stress induces activation and movement of caspase-12 into the cytoplasm (Fig. 2), we sought to determine whether caspase-12 associates with any components of the apoptosome or other caspases. As shown in Fig. 4b, caspase-9 co-immunoprecipitated with caspase-12, providing evidence of interaction between caspase-9 and caspase-12. Immunoprecipitation of caspase-12 from thapsigargin-treated lysates did not reveal any association with Apaf-1, cytochrome c, caspase-8, or caspase-3 (data not shown), suggesting that the interaction of caspase-12 with caspase-7 and caspase-9 is specific. Similar results were seen following treatment with brefeldin (data not shown).

Overexpression of Caspase Catalytic Mutants Rescues Cells from ER Stress-induced Apoptosis—If the mechanism of ER stress-induced apoptosis does indeed involve caspase-7 and caspase-9 (in addition to the ER resident caspase-12), we reasoned that catalytic mutants of these caspases, which function as dominant negatives, should inhibit ER stress-induced apoptosis. This was found to be the case: 293T-cell survival, following exposure to thapsigargin for 36 h, was increased in cultures overexpressing a dominant negative catalytic mutant of caspase-7 (C7DN) and of caspase-9 (C9DN) (25, 31) (Fig. 4c). A mild stress is known to protect cells from a subsequent apoptosis signal. Although we do not rule out the induction of a mild stress response during transfection leading to some protection from a subsequent shock, we did not observe such protection in the pcDNA3-transfected cells treated with thapsigargin (Fig. 4c).

The Involvement of Caspase-7 in the Activation of Caspase-12—To determine whether caspase-7 might regulate caspase-12 activation, cell lysates from untreated and thapsigargin-treated cells were incubated with purified active caspase-7 protein. As shown in Fig. 5a, caspase-7 effectively cleaved caspase-12, giving rise to more of a 42-kDa cleavage product than in control lysates. In addition to this 42-kDa band, caspase-7-treated samples also showed a specific caspase-12 product in the 35-kDa range, which could arise from the cleavage of the prodomain and both the prodomain and the intersubunit region, respectively (see Fig. 6a).

To further elucidate the role of caspase-7 in the activation of caspase-12, we cloned caspase-12 from thapsigargin-treated mouse fibroblast cells. Overexpression of caspase-12 did not induce apoptosis in 293T cells (Fig. 5b). Western blot analysis of cell lysates transfected with caspase-12 revealed a 60-kDa procaspase-12 band and another band (45 kDa) that may represent an autocleavage product (23). Cells cotransfected with caspase-7 and caspase-12 expression vectors showed an additional 42- and a 35-kDa caspase-12 band. This was also associated with increased cell death suggesting that the 42-kDa band may represent the active form of caspase-12, which may cleave itself to generate the additional 35-kDa band. Caspase-7 was transfected in amounts that by themselves did not induce any significant cell death. Co-transfection of cells with caspase-12 and the catalytic mutant of caspase-7 (C7DN) not only blocked the cleavage and formation of the active 42- and 35-kDa caspase-12 products, but also attenuated the cell death process (Fig. 5b). This suggests that caspase-7 may be an upstream regulator of caspase-12. Because cytochrome c, dATP, and Apaf-1-mediated caspase-9 activation also result in activation of caspase-7 (35, 36), we sought to determine whether this process also activated caspase-12. When cell free extracts from caspase-12-transfected 293T cells were incubated with cytochrome c and dATP, caspase-12 was cleaved to its active 42-kDa band and 35-kDa products (Fig. 5c), suggesting that...
Caspase-12 has two predicted caspase cleavage sites: Asp-94 and Asp-341. Mutation of Caspase Cleavage Sites Prevents Processing of Caspase-12—Caspase-12 has two predicted caspase cleavage sites: Asp-94 and Asp-341. Caspase-12 can also be activated by the cytochrome c initiated caspase cascade involving active caspase-9 or active caspase-7.

Mutation of Caspase Cleavage Sites Prevents Processing of Caspase-12—Caspase-12 has two predicted caspase cleavage sites: Asp-94 and Asp-341. Caspase-12 can also be activated by the cytochrome c initiated caspase cascade involving active caspase-9 or active caspase-7. The VETD(341)F site lies downstream from the QACRG active site, presumably representing the COOH terminus of the prodomain. Asp-94 is closer to the N terminus and may represent the COOH terminus of the prodomain. Asp-341 is located downstream of the active site cysteine, possibly representing the COOH terminus of the prodomain. VETD(341)F site lies downstream from the QACRG active site, presumably representing the COOH terminus of the prodomain. Asp-341 is located downstream of the active site cysteine, possibly representing the COOH terminus of the prodomain.

DISCUSSION

The ER is a principal site for protein synthesis and folding and also serves as a cellular storage site for calcium (2, 3). Agents that block protein folding or export, inhibitors of protein glycosylation, and agents that affect calcium uptake and release from the ER can all lead to ER stress and ultimately cell death (8–15). Although the roles of the plasma membrane with its plethora of death receptors and the mitochondria in initiating cell death have been well studied (37), the involvement of the ER in the apoptotic process is still not clear. Our studies suggest that ER stress-induced cell death may proceed through caspase-7-mediated caspase-12 activation. Caspase-12, which belongs to the interleukin-1β converting enzyme subfamily of caspases (21), has some unique properties that are not shared by other family members. Apoptosis, triggered through pathways that do not involve the endoplasmic reticulum, does not
result in activation of caspase-12 (21, 22, 24). Our data, as shown in Fig. 1, confirm these earlier observations. Addition of tamoxifen, which induces cell death predominantly via the intrinsic pathway of apoptosis, did not up-regulate or activate caspase-12 protein (Fig. 1). Incidentally, translocation and activation of caspase-7 is seen in Fas and etoposide-induced cell death as well as following ER stress. Thus the specificity of the ER stress-induced cell death pathway is associated with the up-regulation and activation of only caspase-12 protein. Human caspase-12 exists on the cytoplasmic side of the ER, and this may be a mechanism to prevent its activation in normal cells (21). Activation of caspase-12 may therefore require its cleavage from the ER membrane surface by proteases that may be present on the ER surface or may warrant a translocation of cytoplasmic proteases to the ER surface. Recent studies have indicated that elevation of intracellular calcium levels causes activation and movement of calpain to the ER surface where it activates caspase-12 (22).

Our results describe an alternative mode of caspase-12 activation during ER stress that involves movement of caspase-7 to the ER surface. Our studies show that caspase-7 can cleave and activate caspase-12 resulting in increased cell death that is attenuated by the catalytic mutant of caspase-7. Further studies will also determine whether caspase-7 undergoes modifications during ER stress causing its translocation to the ER surface, because this type of a specific recruitment to the ER surface is not observed with other caspases. Caspase-12 activation by caspase-7 may also require additional ER storage components that serve as adaptor molecules whose expression may not be induced by non-ER stress signals. A possible mechanism may involve binding of caspases-7 and -12 to GRP78 at the ER surface resulting in their autoactivation. This would be similar to the activation of caspase-8 and -9, both of which are thought to be activated by “induced proximity” brought about by adaptor molecules. In support of this possibility, ER stress has recently been shown to cause clustering of caspase-12 with TRAP2 and IRE1 (23).

Once active, caspase-7 cleaves caspase-12 at Asp-94 and Asp-341 sites that are different from the calpain cleavage sites (22) to generate active caspase-12. Earlier studies (38) on caspase-9 suggest that cleavage site mutants would not act as dominant negative inhibitors because full-length caspase-9 is catalytically active. Cleavage of the caspase-12 prodomain either by calpain or by caspase-7 may somehow disrupt the membrane association of caspase-12, resulting in its activation. Although caspase-7 is highly related to caspase-3 and shows the same synthetic substrate specificity in vitro, it is possible that they may have distinct roles in ER-stress-induced apoptosis.

The significance of caspase-12 translocation to the cytosol and its downstream targets is yet to be understood. Our studies demonstrating the interaction of caspase-12 and caspase-9 and the attenuation of ER-stress-induced cell death by the catalytic mutant of caspase-9 (C9DN) suggest a role for caspase-12 in caspase-9-mediated processes. In conclusion, these studies highlight the relationship between caspases and ER stress responses leading to cell death and the role of the ER in modulating the cell death process through the activation of caspases.

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