Molecular Components of a Cell Death Pathway Activated by Endoplasmic Reticulum Stress*

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The endoplasmic reticulum (ER) is the principal organelle for proteins destined for the secretory pathway and also the site of biosynthesis for steroids and many lipids. Any alterations in the ER environment, such as perturbation of Ca2+ homeostasis, elevated secretory protein synthesis, deprivation of glucose or other sugars, altered glycosylation, and/or the accumulation of misfolded proteins, may cause ER stress and consequently cell death (1, 2). It has been shown that misfolded proteins lead to an ER stress response, whether the misfolded proteins build up within the ER or outside the ER (e.g. in the nucleus or cytosol) (3–5). This may be because interference with proteasomal degradation by misfolded proteins in any cellular compartment may inhibit proteasomal degradation of misfolded ER proteins that would otherwise be degraded by the proteasome following translocation out of the ER (4, 6, 7).

Apoptosis, a form of programmed cell death (8, 9), is triggered by ER stress by an unknown mechanism. Apoptotic pathways have been divided into an extrinsic pathway, which is triggered by cell death receptors such as Fas and mediated by the aspartyl-specific cysteine protease caspase-8 or caspase-10, and an intrinsic pathway, which is triggered by the mitochondrial release of cytochrome c and its subsequent complex formation with Apaf-1, dATP, and caspase-9 (10–12). In contrast, apoptosis triggered by ER stress proceeds via an alternative intrinsic pathway that does not require cytochrome c or Apaf-1 (13–15) but does require both caspase-12 and caspase-9 (13, 14, 16–19). All three of these pathways require the formation of molecular complexes that mediate caspase activation; in the case of the intrinsic pathway, this complex has been referred to as an apoptosome (10–12), and in the case of the extrinsic pathway, it has been referred to as a death-inducing signaling complex (10, 20). Although the molecular components that mediate ER stress-induced cell death have not been identified previously, recent studies (21, 22) have suggested that ER-based caspase-12zymogen is present as part of a high molecular weight (greater than 600 kDa) complex.

In order to discern the mechanism(s) by which ER stress triggers cell death pathways, we developed a cell-free system of ER stress-induced cell death that involves the addition of micromoles to a 400,000 × g supernatant (100,000 rpm) that lacks both Apaf-1 and cytochrome c. Initial studies indicated that microsomes isolated from thapsigargin-treated cell extracts are capable of mediating an Apaf-1 and mitochondrial-independent intrinsic cell death pathway (13). This system provided an opportunity to purify functional components and deplete candidate components that mediate ER stress-induced cell death.

In the present study, we have used a set of complementary approaches, including two-dimensional gel electrophoresis coupled with MALDI-TOF mass spectrometry and nano-LC-ESI-MS/MS, RNA interference, co-immunoprecipitation, immunodepletion of candidate proteins, and reconstitution studies, to identify mediators of the ER stress-induced cell death pathway. The present work describes the use of these approaches to...
identify two molecules, valosin-containing protein (VCP) and apoptosis-linked gene-2 (ALG-2), that appear to play a role in mediating ER stress-induced cell death in at least some paradigms.

**EXPERIMENTAL PROCEDURES**

**Cells and Culture Conditions—** Apaf-1-immortalized mouse embryonic fibroblasts and the human embryonic kidney 293T cell line were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. Apaf-1-immortalized mouse embryonic fibroblasts were derived by retrovirally driven T-antigen expression in Apaf-1-null mouse embryonic fibroblasts provided by Dr. Peter Greuss, Max Planck Institute for Biophysical Chemistry, Gottingen, Germany.

**Cell-free Extracts, Cell Fractionation, and Western Blotting—** Cell-free cytoplasmic extracts and microsomes were prepared as described previously (13, 18, 23). The 20,000 x g (14,000 rpm, Eppendorf Centrifuge, 5417R) supernatant that lacks whole cells, nuclei, and mitochondria was centrifuged at 400,000 x g (100,000 rpm Beckman Coulter Optima TLX Ultracentrifuge) for 1 h in the preparation of microsomes. The resulting supernatant contains the soluble cytosolic fraction (S), and the microsomal pellet (P) represents the ER membrane and lumen proteins as well as Golgi membranes. Cellular fractions were also probed with anti-protein disulfide isomerase (PDI) antibody and anti-
beta-tubulin as standard marker proteins for ER and cytosol, respectively. SDS-PAGE and Western blot analyses were performed as described earlier (13, 18, 19). Membranes were probed with a 1:500 dilution of caspase-3 polyclonal antibody or a mouse-specific anti-caspase-9 polyclonal antibody (both from Cell Signaling Laboratories), a 1:500 dilution of mouse anti-PDI, a 1:500 dilution of mouse anti-beta-tubulin, a 1:1000 dilution of mouse anti-VEGF monoclonal antibody (all from BD Pharmingen), a 1:750 dilution of rabbit anti-ALG-2 (Swant, Switzerland), 1:2500 dilution of rabbit anti-nm23-H1 (NDK, Santa Cruz Biotechnology), or a 1:1000 dilution of anti-caspase-12 antibody (gift of Dr. Junying Yuan), or a 1:500 dilution of caspase-12 antibody (Exalpsa Biologicals, Inc.)

**Purification Procedures—** The resuspended microsomal fraction from untreated and 0.5 μM thapsigargin-treated cells (24 h) was loaded onto a 5 x 100-cm Sepharose CL-6B gel filtration column (Bio-Rad). The fractions eluted from the column were analyzed for caspase cleaving activity. All fractions that were capable of cleaving procaspase-9 and procaspase-3 completely were pooled and subjected to one-dimensional SDS-PAGE or two-dimensional IEF/SDS-PAGE.

**Two-dimensional Gel Electrophoresis Protocol for the Identification of Microsomal Proteins—** Crude microsomes, partially purified microsomal fractions, or 20,000 x g supernatants from untreated and thapsigargin-treated Apaf-1-immortalized cells or 293T cells were prepared as described above. Samples were prepared for two-dimensional gel electrophoresis according to the manufacturer’s instructions (Bio-Rad). Protein spots of interest were manually excised from the gel and processed for trypsin digestion (Promega) at 37 °C for 4 h. The resulting tryptic peptides were then extracted from the gel by aqueous 10% formic acid extraction and analyzed by mass spectrometry. Mass spectra of digested gel spots were obtained by MALDI-TOF mass spectrometry on a Voyager DESTR TOF (Applied Biosystems, Framingham, MA). The obtained mass spectra were externally calibrated with an equimolar mixture of angiotensin I, ACTH (1–17), ACTH (18–39), and ACTH (7–38). In some cases, the proteolytic peptide mixtures were also analyzed by reverse-phase high-performance liquid chromatography–MS/MS. Mass spectrometric data were analyzed with the bioinformatics data base system RADARS (Genomic Solutions) (24) using the search engine for peptide Mass Fingerprint matching against peptides from known protein sequences entered in publicly available protein data bases (e.g. NCBI).

**Immunoprecipitations and Immunodepletions—** Transient transfection, cell lysis, and immunoprecipitation were performed as described previously (13, 19) to provide a qualitative assessment of protein-protein interactions in vivo and after ER stress. Briefly, Apaf-1 cells were precleared, transfected with 6 μg of pcDNA3, FLAG-pC9, or pC12. Twenty four hours later, ER stress was induced with 50 nM thapsigargin (Thaps). Cells were gently lifted 12 h after thapsigargin treatment, washed once with PBS at room temperature, 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 15 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 the appropriate resuspended agarose conjugate was added and incubated at 4 °C for 1–6 h. After extensive washes, proteins bound to protein A-Sepharose or protein A/G-Sepharose (Santa Cruz Biotechnology) were subjected to (a) one-dimensional SDS-PAGE followed by Western blot analysis or (b) two-dimensional IEF/SDS-PAGE for subsequent mass spectrometry analysis.

For immunodepletion and caspase-processing assays, microsomes were isolated from cell-free 20,000 x g (14,000 rpm, Eppendorf Centrifuge, 5417R) extracts prepared from thapsigargin-treated cells, as described above. The microsomal pellet was washed twice with 10 mM Tris–Cl/NaCl, pH 7.4, reconstituted in the same buffer, and briefly sonicated to disperse the pellet fraction. A total of 50–100 μg of protein was subjected to immunodepletion using either mouse or goat anti-VEGF antibody, anti-ALG-2 antibody, anti-nDk antibody, or anti-AIF monoclonal antibody conjugated to Sephadex beads (Bio-Rad). Immunodepletion of samples in all panels was also performed with control antibodies of the same type (Fig. 3, a and b, 3rd lanes). Following an overnight incubation at 4 °C with the antibodies, protein A/G-Sepharose was added to the samples and incubated at 4 °C for an additional 6 h. Samples were spun briefly to pellet the protein A/G-Sepharose conjugate. The supernatant was subjected to SDS-PAGE and probed for (a) the respective immunodepleted protein that would indicate the extent of immunodepletion of the candidate protein, and (b) probed for GRP78 or PDI as ER marker proteins to serve as loading controls. Additionally, caspase processing assays were carried out by incubating 50–100 μg of the supernatant protein at 36 °C for 5 h with untreated 400,000 x g supernatants isolated from untreated reactions, simultaneously subjected by SDS-PAGE and Western blotting. Membranes were probed with anti-caspase-9 antibody.

**siRNA Synthesis—** Small interfering RNAs (siRNAs) were generated by in vitro transcription using the Silencer siRNA Construction Kit from Ambion. siRNAs were designed to target two or more regions each for ALG-2, VCP, AIF, and NDK-A (nm23-H1) based on predicted accessible (loop) and unique (specific) regions. The following siRNA sequences were designed: ALG-2 (GenBank™ accession number AAB38108) regions 307–327 and 403–423; VCP (GenBank™ accession number NP_035529) regions 792–812 and 1320–1340; AIF (GenBank™ accession number AA539108) regions 305–325, 326–346, and 448–468; and NDK-A (nm23-H1) (GenBank™ accession number X73066) regions 91–111, 280–300, and 416–436.

**siRNA Transfection—** Apaf-1-immortalized cells (1 x 10⁵ cells per well in 6-well dishes or 2.5 x 10⁶ cells/10-cm dish) were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma) with no antibiotics for 16 h. Transfection of siRNA specific for each target transcript was carried out using Ambion’s siPORT Amine transfection reagent according to the manufacturer’s instructions. Briefly, 5 μl of siPORT amine was mixed with 95 μl of Opti-MEM (Invitrogen) and incubated at room temperature for 15 min. siRNA (80 nm) was added to the diluted siPORT transfection reagent and further incubated for 15 min. The final volume of the sample was diluted to 500 μl with Opti-MEM. siRNA transfections were performed in 30% confluent cells (1 g of protein) by measuring the release of AFC from the synthetic substrates using continuous recording instruments as described earlier (25). Enzyme activity was assayed 4 h after ALG-2, VCP, and NDK-A (nm23-H1) siRNA transfection, 72 h after AIF siRNA transfection, cell extracts were prepared and subjected to SDS-PAGE and Western blot analysis.

In order to study the effect of ER stress on cells after reducing the ALG-2, VCP, NDK, and AIF concentrations, cells were exposed to ER stress after transfection with ProPuri siRNA or brefeldin-A for 24 h after transfection with ALG-2, VCP, and NDK-A siRNA (48 h after AIF siRNA transfection). Cells were exposed to 50 nM Thaps or 2 μM brefeldin-A for 24 h. (These time points corresponded to the times when protein reduction could be demonstrated by immunoblotting.) Thapsigargin-brefeldin-A-induced cell death was quantified, and cell extracts were analyzed by SDS-PAGE and Western blotting. Immunochemistry

**Caspase Activity Assay and Evaluation of Apoptosis—** The synthetic fluorogenic substrates benzyloxycarbonyl-Asp–Glu–Val–Asp–7-amino-4-trifluoromethylcoumarin (Z-LEHD-AFC) were used. The synthetic substrates were dissolved in dimethyl sulfoxide and stored at –70 °C. The synthetic substrate, 400 μM, was added to cells 16 h after siRNA transfection. The synthesized substrates were used as controls. For the treatment of protein made from untreated or thapsigargin-treated cells were incubated with 100 μM peptide substrate. Caspase activity was determined by measuring the release of AFC from the synthetic substrates using continuous recording instruments as described earlier (25). Enzyme activities were assayed 4 h after ALG-2, VCP, and NDK-A (nm23-H1) siRNA transfection.
activities were analyzed using a SpectraMax 340 plate reader (Molecular Devices) at excitation and emission wavelengths of 444 and 538 nm, respectively.

Assessment of cell death was carried out by pelleting floating and adherent cells (after trypsinization) as described previously (26, 27). The pellet was resuspended in 1× PBS, 0.4% trypan blue, and cells were counted using a hemocytometer. Cell death was determined as the percentage of dead cells over the total number of cells. Statistical significance was determined by two-way analysis of variance analysis. Differences were considered statistically significant for p < 0.05.

**Plasmids, Transfection, and in Vitro Translation—**Transient transfections were performed as described earlier (13, 18). Typically, 1 × 10⁶ or 2 × 10⁶ cells were seeded into 6- or 10-cm dishes, respectively, and transfected a day later with 2 or 6 μg of the specified construct using a ratio of 1 μg of DNA, 5 μl of Superfect transfection reagent. The transfection efficiency using these conditions was about 30% for the Apaf-1−/− cells.

Plasmids pC12, pC7, pVCP, pALG-2, pcalreticulin, pC9, and pC3 were transcribed and translated (T7 polymerase) using the TNT system (Promega) for 2 h at 30 °C. The success of in vitro translations was monitored by PAGE. In vitro translated VCP (3 μl), ALG-2 (3 μl), caspase-7 (3 μl), and caspase-12 (3 μl) were incubated in 25 μl of in vitro translation buffer (2 mM HEPES, pH 7.4, 1 mM KCl, 0.15 mM MgCl₂, 0.1 mM phenylmethylsulfonyl fluoride) at 36 °C for 1 h with untreated 400,000 g supernatants isolated from untreated cells and assayed with the fluorogenic substrates Z-DEVD-AFC (to measure caspase-3-like activity) and Z-LEHD-AFC (to measure caspase-9-like activity) at 36 °C for 30 min and analyzed as described under “Experimental Procedures.”

**RESULTS**

**Identification of Candidates That Mediate ER Stress-induced Cell Death—**Thapsigargin, an inhibitor of the ER Ca²⁺-ATPase, has profound effects on the cell. Protein transport from the ER is blocked by depleting the ER of Ca²⁺, as is the folding of several proteins, resulting in the accumulation of misfolded proteins in the ER (28–32). Brefeldin-A, a fungal metabolite, and tunicamycin, an inhibitor of protein glycosylation, also block protein folding and export (33–36). Prolonged treatment with all three compounds ultimately leads to ER stress and cell death. We used these compounds to induce ER stress and identify the molecular components that mediate cell death. The overall approach included the following two steps: in the first step, a list of putative ER-apoptosomal components was generated, and in the second step, some of these components were validated or refuted by depletion and reconstitution experiments.

In the first step, we fractionated the caspase-activating extract from the microsomes of cells that had undergone ER stress induced by thapsigargin. These fractions were derived from Apaf-1-null cells so that the previously described mitochondrial (intrinsic) pathway of cell death was inactivated. Microsomes isolated from untreated and thapsigargin-treated Apaf-1−/− cells were briefly sonicated and passed through a Sephadex G-75 column. Fractions (0.1 ml) were collected and incubated at 36 °C for 1 h with 400,000 g (100,000 rpm) supernatants isolated from untreated cells to evaluate caspase processing. At the end of the reaction, samples were subjected to SDS-PAGE and Western blotting. Membranes were probed with anti-caspase-9 or anti-caspase-3 antibodies. As shown in Fig. 1, fractions 6–8 obtained from microsomes isolated from thapsigargin-treated cells (Fig. 1, b and d), but not from untreated cells (Fig. 1, a and c), were capable of activating the cleavage of caspase-9 and caspase-3 (fractions 5 and 9 were partially activating, Fig. 1, b and d), and these same fractions demonstrated caspase activity, based on the cleavage of LEHD-
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AFC and DEVD-AFC (Fig. 1e). These results are compatible with our earlier studies indicating caspase activation in Apaf-1"−/−" cells treated with thapsigargin (13).

In order to generate a list of putative ER-apoptosomal components, we utilized four complementary approaches. In the first approach (a), the active fractions from the Sephadex G-75 column were pooled and subjected to two-dimensional IEF SDS-PAGE coupled with MALDI-TOF MS or nano-LC-ESI-MS/MS, or to one-dimensional SDS-PAGE coupled with the same mass spectrometric analyses. In the second approach (b), one-dimensional SDS-PAGE or two-dimensional IEF/SDS-PAGE patterns from 20,000 × g (14,000 rpm) extracts or microsomes isolated from untreated cells were compared with those isolated from thapsigargin-treated cells. Protein spots that showed at least a 1.5-fold increase in ER-stressed cells were then identified by MALDI-TOF MS and nano-LC-ESI/MS/MS.

In the third approach (c), proteins co-immunoprecipitated with caspase-12 from ER-stressed cells were separated by two-dimensional gel electrophoresis and then identified by MALDI-TOF/nano-ESI-MS. In the fourth approach (d), simple internet-based literature searches were employed to identify proteins with potential involvement in ER stress and cell death. This fourth approach was used because of the inherent bias of the first three approaches against proteins of low abundance; it was felt that the fourth approach, although fraught with its own set of biases, may suggest testable candidates of both low and high abundance.

By using the above approaches, we generated a list of 40 putative ER-apoptosome components (data not shown) from which four potential candidates were chosen for further investigation. We then utilized a combination of four complementary techniques, RNAi, co-immunoprecipitation, immunodepletion, and reconstitution, to confirm or refute the involvement of these four potential candidates in ER stress-induced cell death. These are described further below.

Evaluation of Candidate Mediators of ER Stress-induced Apoptosis—Analysis of candidate mediators of ER stress-induced apoptosis began with the evaluation of the following four candidate proteins: valosin-containing protein (VCP), nucleoside diphosphate kinase-A (NDK-A), apoptosis-inducing factor (AIF), and ALG-2.

VCP, identified by the approaches a and b, is a member of the AAA (ATPases associated with diverse cellular activities) family of ATP-binding homo-oligomeric ATPase proteins. VCP participates in multiple cellular activities by binding to several target-specific adaptors. These include vesicle transport and fusion, 26 S proteasome function, assembly of peroxisomes, membrane transport processes, and ubiquitin-proteasome degradation (37–39). VCP also functions as a sensor of abnormally folded proteins and has been reported to act as a cell death effector in polyglutamine-induced cell death (40, 41). Recently, a Caenorhabditis elegans protein, Mac-1, that also belongs to the AAA family of proteins was shown to form a multiprotein complex with CED-3, CED-4, and CED-9 and thus regulate programmed cell death (42).

Nucleoside diphosphate kinase-A (nm23-H1, identified by approaches a and c) is encoded by the Nm23 gene and catalyzes the transfer of the γ-phosphate from nucleoside triphosphates to nucleoside diphosphates. Based on the analysis of multiple tumor types, the Nm23 gene has been suggested to be a tumor suppressor. Nm23 genes are expressed in different tumor types where their levels have been alternatively associated with growth, differentiation, and cancer promotion. NDK-A and NDK-B are identical to human Nm23 homologs, namely nm23-H1 and nm23-H2, respectively (43–46).

AIF, identified by approach d, is an NAD-dependent-oxidoreductase flavoprotein that translocates, in association with pro-apoptotic signals, from the mitochondrial intermembranous space to the nucleus (and, as described below, to the microsomal fraction) where it binds to DNA and provokes caspase-independent cell death (47, 48). It is perhaps noteworthy that another pro-apoptotic flavoprotein, named apoptosis-inducing protein, which was isolated from parasite-infected fish, was found to possess the ER retention signal (KDEL sequence) (49, 50). Because there is no known mammalian homolog for apoptosis-inducing protein, we focused on the possibility that the translocation of AIF may include an activity at the ER (see below).

Apoptosis-linked gene 2 (ALG-2, identified by approach d) is a low molecular weight (22 kDa) Ca2+-binding protein that possesses strong apoptosis inducing activity and is involved in T-cell receptor-, Fas-, and glucocorticoid-induced cell death. ALG-2 translocates from the membrane compartment to the cytosol during Fas-mediated apoptosis, and ALG-2 depletion blocks programmed cell death induced by several stimuli, such as synthetic glucocorticoid, T-cell receptor ligation, and Fas ligand (51–54).

To determine whether ALG-2, AIF, VCP, and NDK were localized to the ER compartment before and/or after ER stress, Apaf-1−/− cells were either left untreated or treated with 0.5 μM thapsigargin. Cell-free cytosolic extracts that lack whole cells, nuclei, and mitochondria were centrifuged at 400,000 × g for 1 h for the preparation of microsomes as described under “Experimental Procedures.” Microsomes (400,000 × g pellet (P)) and the 400,000 × g supernatant (S) were both subjected to SDS-PAGE and Western blot analysis. As shown in Fig. 2, although all four proteins were associated with the microsomal compartment, each displayed a different expression pattern. ALG-2 was present in both the supernatant and microsomal fractions and displayed decreased expression in both fractions with time of thapsigargin treatment. AIF is normally associated with the outer mitochondrial membrane, and upon induction of apoptosis, it translocates to the cytosol and nucleus where it initiates chromatin condensation. Because the 400,000 × g samples were prepared from 20,000 × g supernatants that lacked mitochondria, AIF was not detected in the untreated samples, as shown in Fig. 2; however, it was associated with microsomes as well as the soluble fraction from ER-stressed cells. This suggests that AIF translocates to the soluble and microsomal compartments with ER stress. NDK was associated with both cellular compartments from untreated and thapsigargin-treated cells and displayed a reduction, especially in the microsomal fraction, following thapsigargin treatment (36 h). VCP displayed yet another fourth pattern: it was identified only in the microsomal compartment and displayed a slightly increased expression with thapsigargin treatment. Similar patterns of expression were also seen in cellular extracts isolated from cells treated with 2.5 μM brefeldin or 1 μg/ml tunicamycin (data not shown).

To determine whether ALG-2, AIF, VCP, or NDK was required for the processing of caspases, we immunodepleted these proteins from fragmented (sonicated) microsomes isolated from thapsigargin-treated (24 h) Apaf-1−/− cells. These immunodepleted microsomal fractions were incubated with untreated cytosolic extracts, and their effects were compared with those of extracts that had not been immunodepleted. As shown in Fig. 3, a and b, although immunodepletion of AIF or NDK had no effect on the cleavage of procaspase-9 by the microsomal fractions, the immunodepletion of ALG-2 or VCP reduced the degree of cleavage of procaspase-9 by the activated microsomal fraction (Fig. 3, a and b) (note that the immunodepletion resulted in a decrease of all four proteins by 50–60%, and none
of the immunodepletions achieved complete depletion of the target). These results suggest that ALG-2 and VCP present in the microsomal fraction may play a role in mediating downstream caspase activation (at least in this model system).

**Effect of RNA Interference on ER Stress-induced Caspase Activation and Cell Death**—To complement the results obtained from immunodepletion studies and to evaluate the effects of reducing the ALG-2, VCP, NDK, and AIF concentrations in cells (as opposed to extracts), we used RNA interference (RNAi) to reduce the expression of these proteins in cells. Small interfering RNAs (siRNAs) were designed to target two or more regions for each gene based on predicted nucleotides 307 of the ALG-2 transcript failed to reduce the expression of the protein, siRNA targeting nucleotides 327 of the ALG-2 transcript failed to reduce the expression of the protein, siRNA targeting nucleotides 307 of the ALG-2 transcript failed to reduce the expression of the protein, siRNA targeting nucleotides 327 of the ALG-2 transcript failed to reduce the expression of the protein, siRNA targeting nucleotides 307 of the ALG-2 transcript failed to reduce the expression of the protein, siRNA targeting nucleotides 327 of the ALG-2 transcript failed to reduce the expression of the protein, siRNA targeting nucleotides 307 of the ALG-2 transcript failed to reduce the expression of the protein, siRNA targeting nucleotides 327 of the ALG-2 transcript failed to reduce the expression of the protein, siRNA targeting nucleotides 307 of the ALG-2 transcript failed to reduce the expression of the protein. As shown in Fig. 7, VCP co-immunoprecipitates with anti-PDI antibody and anti-β-tubulin as standard marker proteins for ER and cytosol, respectively. (Tubulin breakdown during ER stress may be the reason for the disappearance of the tubulin band after 36 h of thapsigargin treatment.)

**Interaction of ALG-2 and VCP with Vectorially Expressed Caspase-12 and Caspase-9**—To complement the functional studies of ALG-2 and VCP in ER stress-induced apoptosis, we asked whether these candidate mediators complex with the caspases shown previously to be required for ER stress-induced apoptosis, i.e. caspase-12 and caspase-9 (16, 18). Caspase-12 has been demonstrated to be associated with the endoplasmic reticulum (16–18). Caspase-9 has been shown to associate with caspase-12 following ER stress (18). We therefore investigated potential interactions of ALG-2 and VCP with these caspases. We did not observe co-immunoprecipitation of the endogenous proteins, but given the possibility that this failure was due to a lack of sensitivity, we repeated these studies following vector-driven expression of caspases-12 and -9. Apaf-1−/− cells were transfected with vector alone (pcDNA3), FLAG-pC9, or pC12. Twenty four hours later, ER stress was induced with Thaps. Immunoprecipitations were performed with anti-caspase-12 antibody and anti-FLAG monoclonal antibody (for FLAG-tagged caspase-9), and the resulting immunoprecipitates were analyzed by immunoblotting using antisera specific for ALG-2 or VCP (Fig. 7a), caspase-12, or FLAG-tagged caspase-9 (Fig. 7b). Immunoblotting of the latter two proteins was carried out to indicate the extent of immunoprecipitation of the candidate proteins. As shown in Fig. 7, VCP co-immunoprecipi-
tated weakly with caspase-12 in untreated cells and strongly in ER-stressed conditions, and with caspase-9 only following ER stress (Fig. 7a). ALG-2, on the other hand, co-immunoprecipitated with caspase-12 and caspase-9 only following ER stress (Fig. 7a). These results, taken together with the immunodepletion and siRNA results described above, suggest that ALG-2 and VCP present in the microsomal fraction may play a role in mediating a caspase-dependent ER stress-induced cell death.

Reconstitution Studies Support and Extend the Results Obtained from Immunodepletion and RNAi Studies—We reported previously that the combination of microsomes from thapsigargin-treated cells, in vitro translated caspase-9, and truncated in vitro translated caspase-12 is sufficient to induce caspase-9 cleavage. Control microsomes were not sufficient for caspase-9 cleavage in this reconstitution system (13). Based on the results presented in this work and those published previously, we carried out reconstitution experiments in which the cleavage of caspase-3 and caspase-9 was assayed. When we combined VCP, ALG-2, Ca\(^{2+}\), ATP, caspase-9, caspase-12, and caspase-7, the cleavage of caspase-9 and caspase-3 was observed, with the appearance of the smaller bands of the appropriate size for caspase (as opposed to calpain) cleavage (Fig. 8, b and c). However, if VCP, ALG-2, or Ca\(^{2+}\) and ATP were omitted, then caspase-9 and caspase-3 cleavage did not occur (Fig. 8, b and c). Addition of calreticulin, a Ca\(^{2+}\)-binding endoplasmic reticulum protein, in place of ALG-2 did not result in caspase-9 and caspase-3 cleavage (Fig. 8d), suggesting the requirement of ALG-2 specifically and not simply any Ca\(^{2+}\)-binding protein. Addition of microsomes isolated from untreated cells to the components listed above did not have any effect on caspase processing (data not shown). In contrast, the addition of microsomes isolated from early (8 h) thapsigargin-treated cells to the activating components listed above enhanced cleavage of caspase-9 (Fig. 8b, left panel, lane 7) and caspase-3 (Fig. 8b, right panel, lane 3). The 8-h treatment time was chosen because it was associated with a complete lack of cellular caspase activity, caspase processing, and cell death (13). Thus, the microsomes isolated from cells exposed to a brief period of stress apparently contribute additional stress-associated molecules that play a role in the cell death process. Moreover, it is noteworthy that despite the addition of various activating components in the presence or absence of stress-induced microsomes, caspase processing is still weak in comparison to the caspase processing seen in the 400,000 \(\times g\) supernatants incubated with microsomes isolated from 24-h thapsigargin-treated cells (Fig. 1). These observations suggest that although ALG-2 and VCP are necessary components in the present settings, they are not sufficient for optimal caspase processing, and additional factor(s) present in microsomes isolated from stress-induced cells are required to elicit complete caspase processing.

Because we observed processing of both caspases-9 and -3 to the appropriate sizes for caspase (not calpain) cleavage in this

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**Fig. 3.** VCP and ALG-2 are required for the processing of caspase-9 in response to thapsigargin-induced ER stress. Microsomes isolated from 24-h thapsigargin-treated cell extracts were immunodepleted of AIF or ALG-2 (a) and NDK or VCP (b), using the respective antibodies. Immunodepletion of samples in all panels was also performed with control antibodies of the same isotype (a and b, 3rd lane). Samples were spun briefly to pellet the protein A/G-Sepharose conjugate. The supernatant was collected, subjected to SDS-PAGE, and probed for 1) the respective immunodepleted protein that would indicate the extent of immunodepletion of the candidate protein, and 2) GRP78 or PDI as ER marker proteins to serve as loading controls. Additionally, caspase processing assays were carried out by incubating 100 \(\mu\)g of the supernatant protein at 36° C for 1 h with untreated 400,000 \(\times g\) supernatants isolated from untreated cells. Following the reactions, samples were analyzed by SDS-PAGE and Western blotting. Membranes were probed with anti-caspase-9 antibody. Note that the reduction of each of the four proteins (but not control protein) was ~50–60%.
system, it is unlikely that calpains present in the reticulocyte lysate account for this effect. It is also noteworthy that calpains are known to inactivate caspases by cleaving them at sites distinct from those of upstream caspases (58).

In our reconstitution experiments, Ca\(^{2+}\)/VCP was included because ALG-2 is a Ca\(^{2+}\)/VCP-binding protein; ATP was included because VCP is an ATPase; caspase-12 was included because previous immunodepletion studies demonstrated it to be required (13); and caspase-7 was included because previous studies (18) demonstrated its co-immunoprecipitation with caspase-12. Although ER stress-induced cell death may still proceed without the involvement of ALG-2 as suggested by the brefeldin-A and tunicamycin data, ALG-2 is needed when there is calcium involvement, involving extracellular flux or release from intracellular stores.

**DISCUSSION**

Misfolded proteins trigger cellular responses that include an ER stress response resulting in programmed cell death if the accumulation of misfolded proteins goes unchecked. Although a great deal of information has been published regarding the initial cellular steps taken in response to unfolded or misfolded proteins, the mechanism coupling ER stress to the cell death pathway is incompletely defined. Nakagawa *et al.* (16, 17) reported that caspase-12 is targeted to the cytoplasmic face of the ER and is activated by ER stressors. We found previously that caspase-7, caspase-12, and caspase-9 are involved in activating downstream effector caspases following ER stress and that GRP78 inhibits this caspase activation (13, 18, 19). We and others (13–15) found further that the ER stress-driven pathway does not require Apaf-1 or cytochrome c, unlike the mitochondrially driven intrinsic pathway of apoptosis. Finally, we found that cell-free reconstitution of ER stress-driven apoptosis requires activated microsomes (control microsomes did not trigger cell-free apoptosis), suggesting the existence of a factor or factors in microsomes from ER-stressed cells that trigger apoptosis. Recent work (21, 22) suggests that these postulated factors, together with procaspase-12, exist as a complex that exceeds 600 kDa in relative molecular mass.

Therefore, in the current studies, we have attempted to define the molecular components that mediate ER stress-induced cell death. Because Apaf-1 and cytochrome c are not required, it is clear that other molecules may be involved, distinct from those reported previously to comprise the apoptosome that mediates the mitochondria-dependent intrinsic pathway of apoptosis. Furthermore, because the microsomal fraction is required for cell death to proceed, the molecular component(s) are likely to be ER-associated.

In a first step, we generated a list of candidate components of this alternative ER-associated apoptotic complex. By using several complementary approaches, we identified VCP and ALG-2 to be mediators of ER stress-induced apoptosis (VCP for thapsigargin-induced, brefeldin-induced, and tunicamycin-induced death; ALG-2 only for thapsigargin-induced death), whereas two other candidates (NDK and AIF) were not found to be mediators of ER stress-induced cell death. The current results, coupled with results reported previously (13, 18), argue further that the induction of apoptosis by ER stress requires ATP, Ca\(^{2+}\), VCP, ALG-2 (for thapsigargin-induced death), caspase-12...

**FIG. 4.** Transfection of siRNA targeting ALG-2, VCP, AIF, and NDK. siRNAs were designed to target two regions each for ALG-2 and VCP and three regions each for AIF and NDK-A (nm23-H1). Apaf-1\(^{-/-}\) cells were first transfected with 80 nM siRNA as described under "Experimental Procedures." To estimate the efficiency of transfection, fluorescently labeled siRNA targeting the luciferase gene was used. The GAPDH siRNA served as a positive control. Furthermore, it was noted that none of the siRNAs cross-inhibited any of the non-targeted proteins. Cells were gently lifted 36 h after ALG-2, VCP, and NDK-A siRNA transfection and 72 h after AIF siRNA transfection (because AIF was noted to be more stable than the others, requiring longer to demonstrate reduction by Western following siRNA), and washed once with PBS at room temperature. Cell lysates were immunoblotted with anti-ALG-2 (a), anti-VCP (b), anti-AIF (c), and anti-NDK antibody (d). Cell extracts were also probed with anti-β-tubulin as a loading control. b, comb indicates co-transfection of the two siRNAs designed to target the VCP gene transcript (nucleotides 792–812 and 1320–1340).
12, caspase-9, and possibly caspase-7. Although studies of the other candidates are ongoing, the results reported here for VCP and ALG-2 argue that these represent mediators of ER stress-induced apoptosis in at least some paradigms.

Studies from multiple laboratories including ours point to the endoplasmic reticulum as a third subcellular compartment implicated in apoptotic execution. Like the mitochondria, the endoplasmic reticulum is a repository for both pro-apoptotic and anti-apoptotic molecules. In addition to caspase-12, the known pro-apoptotic molecules include p28Bap31 (59) and GADD153 (1), whereas the anti-apoptotic molecules identified to date include the ER chaperone proteins GRP78 (1), PDI (1), calreticulin (60), and ORP-150 (59, 61, 62), as well as DAD1 (63, 64). Recent studies have suggested that either a cytosolic pool of GRP78 or a subpopulation of GRP78 existing as an ER transmembrane protein may form a complex with caspases-7 and -12 at the ER surface and prevent their activation and release (19, 65), highlighting the importance of GRP78 as an anti-apoptotic protein.

In addition, a significant fraction of endogenous Bel-2 family members including Bel-2, Bel-x₁, Bax, Bak, and Bik have been shown to be associated with the endoplasmic reticulum, suggesting that Bcl-2 family proteins operate at the ER (at least in part) to regulate calcium homeostasis and apoptotic cell death (66–76). Bax/Bak doubly deficient mouse embryonic fibroblasts are resistant to both mitochondrial mediated apoptosis and ER stress stimuli, suggesting possible cross-talk between ER and mitochondrial cell death pathways or independent modulation of the two pathways (68–70). The potential ER-mitochondria cross-talk may be utilized for mitochondrial amplification of ER-initiated apoptotic pathways or for other pathways.

The previously reported intrinsic and extrinsic pathways of apoptosis feature caspase activation by induced proximity, the latter of which is achieved by recruitment to multimeric complexes (20, 77); the death receptors that mediate the extrinsic pathway typically form trimeric complexes, and the Apaf-1 mediator of the intrinsic pathway forms a homoheptamer (12, 78). Therefore, it is of interest that VCP undergoes homohexamerization and that this process is facilitated by ATP (37–39, 41, 79, 80). The requirement for ATP is also reminiscent of the finding reported previously (19) that GRP78 inhibition of ER

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**Fig. 5. Effect of RNA interference on ER stress-induced caspase activation and cell death.** siRNAs were designed to target ALG-2 and VCP. Apaf-1−/− cells were first transfected with 80 nm siRNA as described under “Experimental Procedures.” A combination of both siRNAs (regions 792–812 and 1320–1340) was transfected to optimize the decrease of expression of VCP (comb). Twenty four hours after transfection, cells were exposed to 50 nm thapsigargin (Thaps, a, d, and e) or 2.0 μmol brefeldin-A (b) for 24 h. None of the siRNAs had any effect on the expression of caspase-9 or caspase-3 proteins (c). Thapsigargin/brefeldin-induced cell death was quantified by the trypan blue procedure as described under “Experimental Procedures.” Cell extracts were also analyzed by SDS-PAGE and Western-blotted (WB) for thapsigargin-induced caspase-9 processing (d), and caspase-9-like activity was assayed with the fluorogenic substrate Z-LEHD-AFC (e). Asterisk, p < 0.05.
stress-induced caspase activation is reversed by ATP. It is of course possible that VCP plays an alternative role in ER stress-induced apoptosis that does not require its multimerization, and ongoing studies will address that issue.

It should be pointed out that caspase-12 was identified in the murine system, and its presence in human tissues is controversial. However, antibodies directed against mouse caspase-12 cross-react with a human cellular protein that is
of similar relative molecular mass to that of mouse caspase-12, is up-regulated during ER stress, and is proteolytically processed similarly to murine caspase-12 (16–18, 81). Furthermore, based on transfection studies, the human caspase-12-immunoreactive protein is not caspase-1–9.² Caspase-12 in combination with another ER stress-induced molecule(s) is required for caspase-9 processing and activation during ER stress-induced cell death (13–15). Although the human genome has not revealed a clear caspase-12 candidate, one group has reported a human caspase-12 sequence (82); however, this reported caspase-12 sequence is predicted to encode a protein that has a significant deletion, so that it would be unlikely to demonstrate a molecular mass similar to the murine caspase-12. Therefore, studies are ongoing to utilize a combination of genomics and mass spectrometric approaches to obtain sequence data on the putative human caspase-12.

In summary, the present work suggests that the activation of apoptosis by ER stress features an Apaf-1-independent pathway that requires ATP, calcium, VCP, ALG-2 (for thapsigargin-induced cell death), caspase-12 (in murine cells, and a similar as yet unidentified protein in human cells), caspase-9, possibly caspase-7, and an additional factor or factors present in microsomes. Further work will be required to determine what role each member plays in mediating ER stress-induced cell death. Overall, the requirements for a multimerizing ATPase, release and binding of an intra-organelar molecule, and caspase-complex formation are features common to both the mitochondrial and microsomal apoptotic pathways.

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REFERENCES
1. Kaufman, R. J. (1999) Genes Dev. 13, 1211–1233
2. Harding, H. P., Calton, M., Urano, F., Novoa, I., and Ron, D. (2002) Annu. Rev. Cell Dev. Biol. 18, 575–599
3. Kouroku, Y., Fujita, E., Jimbo, A., Kikuchi, T., Yamagata, T., Momoi, M. Y., Komnami, E., Kuida, K., Sakakura, K., Yonehara, S., and Momoi, T. (2002) Hum. Mol. Genet. 11, 1505–1515
4. Nishitoh, H., Matsuzawa, A., Tohume, K., Saegusa, K., Takeda, K., Inone, K., Hori, S., Kakizuka, A., and Ichijo, H. (2002) Genes Dev. 16, 1345–1355
5. Matsuzawa, A., Nishitoh, H., Tohume, K., Takeda, K., and Ichijo, H. (2002) Antioxid. Redox. Signal 4, 415–425
6. Drexler, H. C., Rosau, W., and Konoeding, M. A. (2000) FASEB J. 14, 65–77
7. Drexler, H. C. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 855–860
8. Lockshin, R. A., and Williams, C. M. (1984) J. Insect Physiol. 10, 643–649
9. Kerr, J. F., Wyllie, A. H., and Currie, R. (1972) Br. J. Cancer 26, 239–257
10. Earnshaw, W. C., Martinas, I. M., and Kaufmann, S. H. (1999) Annu. Rev. Biochem. 68, 383–424
11. Li, P., Nighawan, D., Bodhidarlo, I., Sinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) Cell 91, 479–489
12. Zou, H., Li, Y., Liu, X., and Wang, X. (1999) J. Biol. Chem. 274, 11549–11556
13. Rao, R. V., Castro-Orebro, S., Frankowski, H., Schuler, M., Stoka, V., Del Rio, 

² R. V. Rao, H. M. Ellerby, and D. E. Bredesen, unpublished data.
17. Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, B. A., and Kroemer, G. (1999) Nature 397, 441–446
18. Mirzam, M. D., Costantini, P., Ravaglin, L., Saravia, L. M., Haozoo, D., Brothers, G., Penninger, J. M., Polentarut, M. L., Kromer, G., and Susin, S. A. (2001) J. Biol. Chem. 276, 16391–16398
19. Murakawa, J., Jung, S. K., Iijima, K., and Yonehara, S. (2001) Cell Death Differ. 8, 298–307
20. Jung, S. K., Mai, A., Iwamoto, M., Arizono, N., Fujimoto, D., and Yonehara, S. (2000) J. Biol. Chem. 275, 1491–1497
21. Vito, P., Lacana, E., and D’Adamo, L. (1996) Science 271, 521–525
22. Vito, P., Pellegrini, L., Giusi, C., and D’Adamo, L. (1999) J. Biol. Chem. 274, 29819–29826
23. Lacana, E., Ganei, J. K., Vito, P., and D’Adamo, L. (1997) J. Immunol. 158, 5129–5135
24. Jurutka, M., Kim, S. K., Kim, K. D., Lim, J. S., Kim, J. W., and Kim, E. (2001) Biochem. Biophys. Res. Commun. 288, 420–426
25. Mathiasen, I. S., Sieger, I. N., Sallah, L., Norm, A. W., and Jaattela, M. (2002) J. Biol. Chem. 277, 30738–30745
26. Besser, S. P., Zhang, D., Oh, D., Fan, Z., Greer, E. L., Russo, M. L., Jaju, M., and Lieberman, J. (2001) J. Biol. Chem. 276, 43285–43293
27. Wolf, B. B., Gauldin, J. C., Stenrice, R. B., Heere, H., Amarante-Mendes, G. S., Salvesen, G. S., and Green, D. R. (1999) Blood 94, 1683–1692
28. Chua, B. T., Gan, K., and Li, P. (2000) J. Biol. Chem. 275, 5131–5135
29. Ng, F. W., Nguyen, M., Kwan, T., Branton, P. E., Nichelson, D. W., Crompton, J. A., and Shore, G. C. (1997) J. Cell Biol. 139, 327–338
30. Liu, H., Bowes, R. C., 111, van der Water, B., Sillence, D. G., Nagelkerke, J. F., and Stevens, J. L. (1997) J. Biol. Chem. 272, 21753–21759
31. Ozawa, K., Kuwabara, K., Tanamats, T., Takatsui, T., Teukamoto, Y., Kanesue, S., Yanagi, H., Stern, D. M., Eguchi, Y., Tsuboi, Y., Ogawa, S., and Tohyama, M. (1999) J. Biol. Chem. 274, 6397–6404
32. Tanaka, S., Uehara, T., and Nomura, Y. (2000) J. Biol. Chem. 275, 10388–10393
33. Brewster, J. L., Martin, S. L., Toms, J., Goss, D., Wang, K., Zachron, K., Davis, A., Carlsson, G., Hood, L., and Coffin, J. D. (2000) Genesis 26, 271–278
34. Hong, N. A., Planer, M., Hsueh, S. N., Cado, D., Pedersen, R., and Winoto, A. (2000) Dev. Biol. 229, 75–89
35. Reddy, R. K., Mao, C., Baumeister, P., Austin, R. C., Kaufman, R. J., and Lee, A. S. (2003) J. Biol. Chem. 278, 20915–20924
36. Kriegswein, D. G., Nguyen, M., Koppig, S., Roth, M., and Shore, G. C. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 4331–4336
37. Germain, M., Mathai, J. P., and Shore, G. C. (2002) J. Biol. Chem. 277, 18053–18060
38. Scorrano, L., Oates, S. A., Opferman, J. T., Cheng, E. H., Scurrin, M. D., Pozzan, T., and Korsmeyer, S. J. (2003) Science 300, 135–139
39. Wei, M. C., Zong, W. X., Cheng, E. H., Lindest, T., Panoutsakopoulou, V., Ross, A., Roth, K. A., and Gold, G. S., Thompson, C. B., and Korsmeyer, S. J. (2001) Science 292, 727–730
40. Zong, W. X., Li, C., Hatzevassiliou, G., Lindest, T., Yuan, J., and Thompson, C. B. (2003) J. Biol. Chem. 278, 59–69
41. Zhou, X., Wang, J., and Tohyama, K. (2002) J. Biol. Chem. 277, 27623–27630
42. Germain, M., and Shore, G. C. (2003) Science’s STKE http://www.stke.org/cgi/content/full/OC_sigtans;2003/;173,pe10
43. Adams, J. M., and Cory, S. (2001) Trends Biochem. Sci. 26, 61–66
44. Rudner, J., Jondrosvek, V., and Belka, C. (2002) Apoptosis 7, 441–447
45. Nutt, L. K., Pata, R., Pahler, J., Fong, B., Roth, J., McConkey, D. J., and Swisher, S. G. (2002) J. Biol. Chem. 277, 9219–9225
46. Nutt, L. K., Chandra, J., Pata, R., Fong, B., Roth, J. A., Swisher, S. G., O’Neil, R. G., and McConkey, D. J. (2002) J. Biol. Chem. 277, 20301–20308
47. Salvesen, G. S., and Dixit, V. M. (1997) Cell 91, 443–446
48. Saleh, A., Srinivasula, S. M., Acharya, S., Fishel, R., and Alnemri, E. S. (1999) J. Biol. Chem. 274, 17941–17945
49. Wang, Q., Song, C., and Li, C. C. (2003) Biochem. Biophys. Res. Commun. 300, 253–260
50. Rowe, T., and Balch, W. E. (1997) Nature 388, 20–21
51. Bitko, V., and Barik, S. (2001) J. Cell Biochem. 80, 441–454
52. Fischer, H., Koenig, U., Eckhart, L., and Tschachler, E. (2002) Biochem. Biophys. Res. Commun. 293, 722–726