Caspase-12 and Caspase-4 Are Not Required for Caspase-dependent Endoplasmic Reticulum Stress-induced Apoptosis*

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ALTERATIONS IN CELLULAR HOMEOSTASIS that affect protein folding in the endoplasmic reticulum (ER) trigger a signaling pathway known as the unfolded protein response (UPR). The initially cytoprotective UPR will trigger an apoptotic cascade if the cellular insult is not corrected; however, the proteins required to initiate this cell death pathway are poorly understood. In this study, we show that UPR gene expression is induced in cells treated with ER stress agents in the presence or absence of murine caspase-12 or human caspase-4 expression and in cells that overexpress Bel-2 or a dominant negative caspase-9. We further demonstrate that ER stress-induced apoptosis is a caspase-dependent process that does not require the expression of caspase-12 or caspase-4 but can be inhibited by overexpression of Bel-2 or a dominant negative caspase-9. Additionally, treatment of human and murine cells with ER stress agents led to the cleavage of the caspase-4 fluorogenic substrate, LEVD-7-amino-4-trifluoromethylcoumarin, in the presence or absence of caspase-12 or caspase-4 expression, whereas Bel-2 or a dominant negative caspase-9 overexpression inhibited LEVD-7-amino-4-trifluoromethylcoumarin cleavage. These data suggest that caspase-12 and caspase-4 are not required for the induction of ER stress-induced apoptosis and that caspase-4-like activity is not always associated with an initiating event.

The endoplasmic reticulum (ER) is the site for the proper folding and assembly of membrane-localized proteins and those destined for secretion as well as a regulator of intracellular calcium. Conditions that alter protein folding or calcium homeostasis in the ER, such as hypoxia or glucose starvation, trigger a signaling cascade known as the unfolded protein response (UPR) (1, 2). The UPR is also induced during secretory cell development (3–7) and leads to the increased expression of ER-resident chaperones and folding enzymes. These proteins prevent the aggregation of misfolded proteins and facilitate proper protein folding within the ER lumen. The UPR also leads to the induction of components of the protein degradative machinery that remove misfolded proteins from the ER and a transient decrease in general protein synthesis in order to decrease the client protein load on the ER (8, 9). This “physiological” UPR affords the cell time to survive a stressful insult or to develop into a mature secretory cell. However, excessive or prolonged ER stress leads to induction of the “terminal” UPR, which results in the initiation of a cell death cascade (10).

The ER is now being recognized as an organelle that can regulate programmed cell death, and ER stress-induced apoptosis is believed to be associated with several pathological processes, including diabetes (11), ischemia-reperfusion injury (12), Alzheimer disease (13, 14), and Parkinson disease (15). Additionally, ER stress-induced cell death may be involved in sensitizing malignant cells to certain drugs (16–18). However, the signal-transducing events that connect ER stress to the cell death machinery are not clearly understood. Specifically, it is unclear how ER stress results in the activation of caspases.

Caspases are a family of cysteine-dependent aspartate-specific proteases that play a major role in apoptosis induction (19, 20). Initiation of an apoptotic cascade involves the induced proximity and subsequent autoactivation of an initiator caspase (caspase-8, -9, or -10) (21), which then processes and activates effector caspases (caspase-3, -6, or -7) that cleave specific targets, which ultimately results in the morphological features of apoptosis (19). Extrinsic apoptotic stimuli activate caspase-8 and -10 through ligation of death receptors on the cell surface (22), whereas intrinsic apoptotic stimuli cause the release of apoptogenic factors, such as cytochrome c, from mitochondria, leading to apoptosis formation and the activation of caspase-9 (23). However, initiator caspases have been shown to be involved upstream of caspase-9 in several cell death pathways. For example, the release of mitochondrial components is required for death receptor signaling in many cell types. Therefore, caspase-8 can activate the BH3-only protein Bid to induce mitochondrial permeabilization (24, 25). Additionally, caspase-2 is involved in nuclear stress signaling (26–28), and caspase-12 and caspase-4 have been implicated in ER stress-induced apoptosis (29, 30).

Murine caspase-12 and human caspase-4 are members of the interleukin-1β-converting enzyme subfamily of caspases that are 48% identical at the amino acid level and have predicted structures that are consistent with initiator caspases (29, 31, 32). Although human caspase-12 expression has been shown to be limited to a subset of individuals of African descent and to play a role in inflammation and innate immunity (33), both murine caspase-12 and human caspase-4 have been reported to be localized to the ER and to be cleaved in cells treated with ER stress agents, but not in cells treated with death cytokines or various intrinsic apoptotic stimuli. Additionally, cells from caspase-12 knock-out mice and human cell lines treated with small interfering RNA to caspase-4 were shown to be resistant...
to ER stress-induced apoptosis triggered by known ER stress agents, and caspase-4 cleavage in treated cells was shown to be independent of Bcl-xL or Bcl-2 overexpression (29, 30, 34).

A recent study of caspase-12 protein expression in murine cell lines found that caspase-12 could not be detected in five different cell lines of hematopoietic origin (32). In the present study, we have examined one murine and one human cell line that lacks expression of murine caspase-12 or human caspase-4, respectively. We find that these cells are not protected from apoptosis induced by ER stress agents. Instead, these cells die in a caspase-dependent fashion, similar to caspase-12- or caspase-4-expressing cells. Additionally, we found that overexpression of the antiapoptotic Bcl-2 family member, Bel-xL, or a dominant negative caspase-9 were both capable of protecting cells from ER stress-induced cell death and inhibiting caspase-4-like activity within treated cells, indicating that ER stress-induced apoptosis proceeds through the intrinsic apoptotic pathway in these cell lines.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—The IL-3-dependent murine pro-B cell line FL5.12 was cultured and stably transfected with Bel-xL, or dominant negative caspase-9 constructs as previously described (35, 36). The MPC-11 murine myeloma cell line (American Type Culture Collection, Manassas, VA) was cultured in Dulbecco's modified Eagle's medium supplemented with 10% horse serum (Invitrogen) and 1% penicillin/streptomycin (Mediatech, Herndon, VA). The human multiple myeloma cell lines (8226/S, U266, MM.1S, KMS-11, and KMS-18) were cultured, and U266 cells were stably transfected with Bel-xL, as previously described (37, 38). To generate 8226/S pDNA3.1(+) (Invitrogen) and Bel-xL stable transfectants, 1 × 10⁷ cells were washed twice in phosphate-buffered saline and then resuspended in 100 µl of Nucleofector Solution V (Axama Inc., Cologne, Germany). After adding 8 µg of plasmid DNA, the cells were then nucleofected using program G-15 on the Nucleofector I machine. Stable transfectants were selected in 0.5 mg/ml G418 (Mediatech, Herndon, VA). Tunicamycin and melphalan were purchased from Sigma. Brefeldin A and thapsigargin were used as ER stress inducers.

Coincubation with the Amaxa Nucleofector I machine. Stable transfectants were selected in 0.5 mg/ml G418 (Mediatech, Herndon, VA). Tunicamycin and melphalan were purchased from Sigma. Brefeldin A and thapsigargin were used as ER stress inducers.

Generation of Caspase-12 eDNA Constructs—A wild-type caspase-12 construct was generated by PCR using IMAGE clone number 5375150 purchased from the American Type Culture Collection (Manassas, VA) with the following primers: 5'-CCGAGATCTCGACGAGCGCAGCCG-3' and 5'-CCGAAATTCCTTGCAAGCATGG-3'. The wild-type caspase-12 construct was sequenced and then cloned into the EcoRI site of pBluescript® SK (+) (Stratagene, La Jolla, CA). A dominant negative caspase-12 (12DN) was then generated in pBluescript® SK (+) mutating the catalytic cysteine into a serine using the Stratagene QuikChange® site-directed mutagenesis kit and the following primers: 5'-CATCATGCAGGCCAGCAAGGAGCATATGTTG-3' and 5'-CCATATATCTCTCTGAGCCGCGCTGATG-3'. The sequenced 12DN and wild-type caspase-12 constructs were then cut out of pBluescript® SK (+) with EcoRI and subcloned into the pSFFV-Neo expression vector (35).

Detection of Cell Death—For all cell death assays, cells (2.5 or 5.0 × 10⁶/ml) were treated with the indicated concentrations of drug or withdrawn from IL-3 for 12 or 24 h. Cells were washed twice in phosphate-buffered saline and stained with annexin V-fluorescein isothiocyanate (FITC) (Biovision, Mountainview, CA) and propidium iodide (Sigma) according to the manufacturer's instructions, and samples were acquired on a FACScan flow cytometer (BD Biosciences) to assess viability. Whole cell lysates (5.0 × 10⁶ cells) were prepared in modified radioimmunoprecipitation assay buffer containing protease inhibitors (170 µg/ml phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin, and 1 µg/ml leupeptin) (Sigma). Fifty micrograms of protein were resolved on an SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher and Schuell). The following primary antibodies were used: a rabbit polyclonal antibody against Bel-xL (39), monoclonal antibodies against caspase-4 and caspase-9 (Molecular and Biological Laboratories, Woburn, MA), a monoclonal antibody against GRP78/Bip (BD Biosciences), a rat monoclonal antibody against GRP94/p96 (Stressgen Bioreagents, Victoria, Canada), a rabbit polyclonal antibody against caspase-12 (Cell Signaling Technology, Beverly MA), and rabbit polyclonal antibodies against GADD153/CHOP (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and actin (Sigma). Sheep anti-mouse Ig-

FIG. 1. Identification of cell lines lacking ER-associated caspase expression. A, whole cell lysates were prepared from the murine myeloma cell line MPC-11 and from stable transfectants of the murine pro-B cell line, FL5.12, containing the empty vector, pSFFV-Neo (Neo), or overexpressing 12DN, 9DN, or Bel-xL. Expression of caspase-12, caspase-9, and Bel-xL was determined by immunoblot analysis. The blot was stripped and reprobed for actin to confirm equal protein loading. B, whole cell lysates were isolated from five different human multiple myeloma cell lines (8226/S, U266, MM.1S, KMS-11, and KMS-18) and from U266 cells stably transfected with Bel-xL. Caspase-4, Bel-xL, and actin expression were determined by Western blot analysis.

RESULTS

Identification of Cell Lines Lacking Caspase-12 or Caspase-4 Expression—Kalai et al. have reported that caspase-12 protein expression appears to be more restricted than expected and that they were unable to detect caspase-12 protein in five different murine hematopoietic cell lines (32). Similarly, we have determined that the FL5.12 murine pro-B cell line (Fig. 1A) and the human U266 multiple myeloma cell line (Fig. 1B) lack caspase-12 and caspase-4 expression, respectively, by Western blot analysis. Caspase-12 expression in the FL5.12 cell line was found to be more restricted that in the murine myeloma cell line, MPC-11. In order to examine the potential effects of inhibiting different points in the ER stress-induced apoptotic pathway, 12DN and dominant negative caspase-9 (9DN) constructs, in which the catalytic cysteine was mutated into a serine, and a Bel-xL construct were stably transfected into FL5.12 cells (Fig. 1A).

Caspase-4 expression in the U266 human multiple myeloma cell line was found to lack expression in four other human multiple myeloma cell lines: 8226/S, MM.1S, KMS-11, and KMS-18. U266 cells were also found to lack expression of caspase-4 mRNA by Northern blot and microarray analyses (data not shown). Similar to FL5.12 cells, U266 cells were also stably transfected with a Bel-xL construct.

ER Stress Agents Induce the UPR in Cell Lines Lacking Caspase-12 or Caspase-4 Expression—Conditions that alter ER homeostasis trigger a signaling pathway termed the UPR. The
UPR can be induced by chemical agents such as tunicamycin, an inhibitor of N-linked glycosylation, brefeldin A, an inhibitor of ER to Golgi transport, and thapsigargin, an inhibitor of the sarcoplasmic-endoplasmic reticulum Ca$^{2+}$-ATPase (1, 2). UPR induction is initially characterized by the up-regulation of ER-resident chaperones, such as GRP78/BiP and GRP94/gp96, which prevent the aggregation of misfolded proteins within the ER lumen (9, 40, 41). If the stress is persistent or excessive, the cell undergoes programmed cell death, and this has been associated with the induction of the transcription factor GADD153 (42, 43). As shown in Fig. 2, a functional UPR can be induced in vector only or 12DN-, 9DN-, or Bcl-xL-overexpressing FL5.12 cells. Additionally, GRP94, GRP78, and GADD153 were up-regulated in an ER stress-specific fashion, since growth factor withdrawal, an intrinsic apoptotic stimulus, did not cause these changes in gene expression.

Similarly, functional UPR induction by ER stress agents was detected in U266 cells regardless of Bcl-xL overexpression. Untreated U266 cells express high levels of the ER chaperones GRP94 and GRP78, and the levels of these proteins do not increase during any of the treatment regimens (Fig. 3). These ER chaperones are also constitutively expressed at high levels in the other human multiple myeloma cell lines (data not shown), and this expression pattern is consistent with published reports that certain survival or physiological components of the UPR must be constitutively active in secretory cells, such as plasma cells, in order for them to be able to produce and secrete large amounts of protein (3, 4, 6, 7). However, GADD153 is specifically induced by ER stress agents in U266 and U266 Bcl-xL cells. Consistent with this, the DNA-damaging agent melphalan did not induce the expression of GADD153 in U266 cells (Fig. 3) or in any of the other multiple myeloma cell lines (data not shown). Together these studies demonstrate that the lack of ER-associated caspase expression or the overexpression of antiapoptotic effector proteins has no effect on ER stress signaling.

**Lack of Caspase-12 or Caspase-4 Expression Does Not Protect FL5.12 or U266 Cells from ER Stress-induced Apoptosis, whereas Bcl-xL and Dominant Negative Caspase-9 Overexpression Are Protective**—Caspase-12 knockout mice and human cell lines with decreased caspase-4 expression via small interfering RNAs have been shown to be resistant to ER stress agent-induced apoptosis (29, 30). In order to determine the effect of a lack of caspase-12 or caspase-4 expression on the sensitivity of FL5.12 and U266 cells to ER stress agents, these cells were treated with increasing concentrations of tunicamycin, brefeldin A, and thapsigargin for 24 h. As shown in Fig. 4A, Neo and 12DN cells died in a dose-dependent fashion in response to all three ER stress agents. However, Bcl-xL or 9DN overexpression completely protected cells from brefeldin A- and thapsigargin-induced apoptosis and was partially protective against tunicamycin-induced apoptosis.

Similarly, U266 cells had levels of sensitivity to tunicamycin, brefeldin A, and thapsigargin that were comparable with other
human multiple myeloma cell lines (Fig. 4B) and Bcl-xL overexpression protected cells against brefeldin A- and thapsigargin-induced apoptosis and made cells more resistant to tunicamycin. These results suggest that the lack of caspase-12 or caspase-4 expression in FL5.12 and U266 cells, respectively, does not make these cells resistant to ER stress-induced apoptosis. Moreover, the Bcl-xL and 9DN overexpression experiments indicate that the classic intrinsic pathway of caspase activation is required for ER stress-induced apoptosis in these cells, although other death signaling pathways may be involved in tunicamycin-treated cells.

**ER Stress-induced Apoptosis in FL5.12 and U266 Cells Is Caspase-dependent**—One explanation for the sensitivity of cells lacking caspase-12 or caspase-4 to agents that are known to cause ER stress-induced cell death is that at the concentrations tested, these agents are inducing a caspase-independent necrosis. Therefore, in order to determine whether ER stress-induced cell death in caspase-12- and caspase-4-nonexpressing cells is a caspase-dependent process, FL5.12 (Fig. 5A) and U266 cells (Fig. 5B) were treated with low and high concentrations of tunicamycin, brefeldin A, and thapsigargin in the presence or absence of a 100 μM concentration of the broad-spectrum caspase inhibitor BocD-fmk. BocD-fmk restored cell viability for Neo, 12DN, and U266 cells under all treatment conditions except for high concentrations of tunicamycin. Whereas Bcl-xL or 9DN overexpression was generally protective against ER stress agent treatment, the addition of BocD-fmk restored cell viability in U266 Bcl-xL cells treated with a high concentration of thapsigargin. Similar to parental or vector control cells, cell viability could not be restored in Bcl-xL- or 9DN-overexpressing cells treated with 100 μM tunicamycin in the presence of BocD-fmk, consistent with the possibility that tunicamycin may induce other death-signaling pathways and suggesting that this concentration may induce a caspase-independent necrotic cell death. Taken together, these data suggest that ER stress-induced cell death is a caspase-dependent process at most of the drug concentrations examined, even in cells that do not express caspase-12 or caspase-4.

**Expressing Wild-type Caspase-12 in FL5.12 Cells Does Not Alter Sensitivity to ER Stress Agents**—Whereas comparing U266 cells to other myeloma cell lines that express caspase-4 suggests that cells lacking this caspase display similar sensi-
tivities to ER stress signals, we were unable to perform similar experiments with FL5.12 and MPC-11 cells. MPC-11 cells are more sensitive to ER stress- and proteasome inhibitor-induced apoptosis (data not shown). However, this myeloma cell line may have enhanced sensitivity to these agents due to its plasma cell phenotype. We have found that human multiple myeloma cell lines are also more sensitive to such signals when compared with other human cell lines, regardless of caspase-4 expression. Thus, in order to determine whether restoring caspase-12 expression would affect the sensitivity of FL5.12 cells to ER stress agents, wild-type caspase-12 was stably transfected into FL5.12 cells (Fig. 6A). Neo, wild-type caspase-12, and 12DN cells were then treated with increasing concentrations of tunicamycin, brefeldin A, or thapsigargin for 24 h before cell viability was assessed (Fig. 6B). The sensitivity of FL5.12 cells to treatment with any of the three ER stress agents was not altered by wild-type caspase-12 overexpression, suggesting that activation of the ER stress-induced apoptotic pathway occurs in an efficient manner in the absence of caspase-12 in these cells.

Caspase-4-like Activity Can Be Detected in Cells Treated with ER Stress Agents Regardless of Caspase-12 or Caspase-4 Expression, whereas It Is Inhibited in Cells Overexpressing Bcl-xL or a Dominant Negative Caspase-9—Processing of procaspase-12 and procaspase-4 in cells treated with ER stress agents has been reported to occur upstream of caspase-9 activation or mitochondrial events, respectively (30, 44–47). Rao et al. have shown caspase-12 processing occurs prior to caspase-9 processing (46), and Morishima et al. (44) have reported that inhibition of caspase-12 suppresses caspase-9 processing in cells treated with ER stress agents. Additionally, Hitomi et al. (30) demonstrated that caspase-4 cleavage in response to ER stress was only modestly affected by overexpression of Bcl-2 or Bcl-xL.

Since caspase processing is not always indicative of caspase activity (48–50), we chose to monitor caspase activity following ER stress by measuring the release of a fluorophore from a synthetic peptide target sequence. A caspase-12 fluorogenic substrate is not currently available; however, caspase-12 and caspase-4 are 48% identical (29, 32) and, similar to other human and murine caspase homologs, would be expected to be able to cleave similar targets in response to the same apoptotic

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iodide staining. Cell viability was determined by annexin V-FITC and propidium represent the mean protein loading. Analysis. The blot was stripped and reprobed for actin to confirm equal 12DN constructs. Caspase-12 expression was detected by Western blot cin (12DN cells were treated with the indicated concentrations of tunicamycin, brefeldin-A, or thapsigargin (Fig. 4), although they do not express caspase-12 or caspase-4 (Fig. 1). These data as well as a report describing that caspase-12 protein expression is more restricted in murine organs and cell lines than the mRNA expression patterns would indicate (32) suggest that other mechanisms for the initiation of ER stress-induced apoptosis must be present within cells.

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Increasing evidence indicates that ER stress can play an important role in the pathology of neurodegenerative diseases (13–15) and in the sensitivity of malignant cells to certain drugs (16–18), but the molecular connection between the ER stress response and the initiation of apoptosis is poorly understood. Murine caspase-12 and human caspase-4 have been reported to specifically initiate apoptosis in response to ER stress stimuli, and cells from caspase-12 null mice and cells where caspase-4 expression was decreased using small interfering RNA were shown to be partially protected from ER stress induced by chemical agents (29, 30). However, we have shown that murine FL5.12 and human U266 cells undergo apoptosis following induction of the UPR (Fig. 2) by the ER stress agents tunicamycin, brefeldin-A, or thapsigargin (Fig. 4), although they do not express caspase-12 or caspase-4 (Fig. 1).

**DISCUSSION**

Thus, FL5.12 cells were treated with ER stress agents or withdrawn from IL-3 for 12 or 24 h, and caspase-4-like activity was assessed with the caspase-4 fluorogenic substrate, LEVD-AFC. As shown in Fig. 7A, caspase-4-like activity can be induced over 60-fold in Neo and 12DN cells in response to ER stress agents as well as in response to IL-3 withdrawal, whereas caspase-4-like activity could not be detected in Bcl-xL or 9DN cells under any of the treatment conditions. Similarly (Fig. 7B), caspase-4-like activity could be detected in U266 cells in response to brefeldin A or thapsigargin treatment as well as in response to melphalan but was much lower in U266 cells overexpressing Bcl-xL. These studies show that caspase-4-like activity can be detected in cells that do not express caspase-12 or caspase-4 in response to ER stress and other intrinsic apoptotic stimuli. However, this activity is inhibited by Bcl-xL or dominant negative caspase-9 overexpression, suggesting that this caspase-4-like activity may be downstream of the mitochondria in a classical intrinsic apoptotic cascade.

**Bcl-xL Overexpression Can Inhibit ER Stress-induced Apoptosis and Caspase-4-like Activity in Caspase-4-expressing Cells—** In order to determine whether the effects of Bcl-xL overexpression on ER stress-induced apoptosis and caspase-4-like activity are dependent upon caspase-4 expression, the caspase-4-expressing 8226/S cell line (Fig. 1) was stably transfected with the empty pcDNA 3.1(+) vector or a Bcl-xL construct (Fig. 8A). Previous reports have demonstrated that overexpression of Bcl-2 (51–53) and Bcl-xL (54) can increase the cell viability of murine and rat cells treated with ER stress agents. Here we demonstrate that Bcl-xL overexpression has the same effect in myeloma cells treated with ER stress agents, regardless of caspase-4 expression. Namely, overexpression of Bcl-xL protected 8226/S cells from brefeldin A- and thapsigargin-induced apoptosis and made 8226/S cells more resistant to tunicamycin-induced cell death (Fig. 8B). Similar to what was observed in the U266 cell line, whereas caspase-4-like activity was induced by ER stress agents and melphalan in vector control 8226/S cells, it was greatly reduced in 8226/S Bcl-xL cells treated with ER stress agents (Fig. 8C). Interestingly, tunicamycin induces little or no caspase-4-like activity in the 8226/S and U266 myeloma cell lines. This pattern has also been observed in the MM.1S and KMS-11 myeloma cell lines (data not shown). Consistent with the fact that Bcl-xL overexpression is not as protective in tunicamycin-treated myeloma cells as it is in treated FL5.12 cells and the fact that tunicamycin induces a caspase-independent cell death at high concentrations, the lower levels of detectable caspase-4-like activity may indicate that the tunicamycin-induced cell death cascade proceeds through other pathways, especially in myeloma cells. However, the fact that Bcl-xL overexpression can inhibit ER stress-induced apoptosis and caspase-4-like activity in myeloma cells regardless of caspase-4 expression further suggests that the caspase-4-like activity detected in ER stress agent-treated cells is not the result of an initiating event but rather may occur further downstream in a postapoptosome apoptotic cascade.

**Caspase-12/Caspase-4-independent ER Stress-induced Apoptosis**

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Increasing evidence indicates that ER stress can play an important role in the pathology of neurodegenerative diseases (13–15) and in the sensitivity of malignant cells to certain drugs (16–18), but the molecular connection between the ER stress response and the initiation of apoptosis is poorly understood. Murine caspase-12 and human caspase-4 have been reported to specifically initiate apoptosis in response to ER stress stimuli, and cells from caspase-12 null mice and cells where caspase-4 expression was decreased using small interfering RNA were shown to be partially protected from ER stress induced by chemical agents (29, 30). However, we have shown that murine FL5.12 and human U266 cells undergo apoptosis following induction of the UPR (Fig. 2) by the ER stress agents tunicamycin, brefeldin-A, or thapsigargin (Fig. 4), although they do not express caspase-12 or caspase-4 (Fig. 1). These data as well as a report describing that caspase-12 protein expression is more restricted in murine organs and cell lines than the mRNA expression patterns would indicate (32) suggest that other mechanisms for the initiation of ER stress-induced apoptosis must be present within cells.

Consistent with the presence of alternative mechanisms for the initiation of ER stress-induced apoptosis, we found that U266 cells have a level of sensitivity to ER stress agents similar...
to other caspase-4-expressing multiple myeloma cell lines (Fig. 4B) and that introducing wild-type caspase-12 into FL5.12 cells does not make these cells any more sensitive to ER stress-inducing agents (Fig. 6B). This is concurrent with the finding that interferon-γ-induced caspase-12 expression does not alter the sensitivity of B16/B16 murine melanoma cells to thapsigargin-induced apoptosis (32). Additionally, we demonstrated that cells that do not express the ER-associated caspases generally die in a caspase-dependent fashion when treated with ER stress-inducing agents, although high concentrations of tunicamycin appear to induce a caspase-independent necrotic cell death (Fig. 5). These results are consistent with the finding that cells from caspase-12 null mice are not resistant to oxygen and glucose deprivation although oxygen and glucose deprivation induces an UPR, and the broad spectrum caspase inhibitor benzyloxycarbonyl-VAD-fmk can inhibit oxygen and glucose deprivation-induced cell death (34). Similarly, the data presented by Hitomi et al. (30) indicate that the effect of decreasing caspase-4 expression on the sensitivity of cells to ER stress agents may depend on the cell type. Although caspase-4 protein expression levels were significantly reduced in both the SK-N-SH and the HeLa cell lines with small interfering RNA, the sensitivity of SK-N-SH cells to thapsigargin was reduced by ~50%; however, the effect seen in HeLa cells was much less

Fig. 7. Cells overexpressing Bcl-xL or a dominant negative caspase-9 have decreased caspase-4-like activity. A, whole cell lysates were isolated from vector control (Neo−), 12DN−, 9DN−, and Bcl-xL-overexpressing FL5.12 cells cultured in medium alone, withdrawn from IL-3, or treated with 25 μM tunicamycin (TM), 2.5 μM brefeldin A (BfA), or 25 nM thapsigargin (TG) for 12 and 24 h. Caspase activity was measured in 200 μg of protein incubated with the caspase-4 fluorogenic substrate LEVD-AFC. B, U266 and U266 Bcl-xL cells were left untreated or treated for 12 and 24 h with 50 μM TM, 1 μM BfA, 5 μM TG, or 30 μM melphalan (Mel). The cells were lysed, and caspase-4-like activity was assessed as described above. The data are presented as the fold increase in fluorescence over untreated cells and the mean ± S.D. from three independent experiments.
dramatic. Additionally, the investigators observed that small interfering RNA to caspase-4 had no effect on ER stress-induced apoptosis in human umbilical vein endothelial cells (30). These data indicate that caspase-12 and caspase-4 expression are not required for the initiation of ER stress-induced apoptosis in all cell types and do not appear consistent with the idea that cells that lack these caspases have developed a compensatory mechanism for ER stress-induced cell death.

Whereas the lack of caspase-12 or caspase-4 expression did not protect FL5.12 or U266 cells from ER stress agent-induced cell death, Bcl-xL overexpression in either cell line and 9DN overexpression in FL5.12 cells inhibited brefeldin-A and thapsigargin-induced apoptosis (Fig. 4). Although these proteins could not completely protect these cell lines from tunicamycin-induced cell death, overexpression of Bcl-xL had some effect in U266 and 8226/S cells and overexpression of either Bcl-xL or 9DN resulted in FL5.12 cells becoming more

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**Fig. 8.** Bcl-xL overexpression is sufficient to inhibit ER stress-induced cell death and caspase-4-like activity in caspase-4-expressing cells. **A,** whole cell lysates were isolated from 8226/S cells stably transfected with the empty vector (pcDNA3) or a Bcl-xL expression vector. Bcl-xL expression was determined by Western blot analysis. The blot was stripped and reprobed for actin to confirm equal protein loading. **B,** vector control (pcDNA)- and Bcl-xL-overexpressing 8226/S cells were treated with increasing concentrations of tunicamycin (TM), brefeldin A (BfA), or thapsigargin (TG) for 24 h, and cell viability was assessed by annexin V-FITC and propidium iodide staining. The data represent the mean ± S.D. from three independent experiments. **C,** 8226/S pcDNA or Bcl-xL cells were cultured in medium alone or treated for 12 and 24 h with 50 μM tunicamycin, 1 μM brefeldin A, 5 μM thapsigargin, or 30 μM melphalan (Mel). Whole cell lysates were prepared, and caspase-4-like activity was assessed as described in the legend to Fig. 7. The data represent the mean ± S.D. from three independent experiments.
resistant to tunicamycin. The different levels of protection observed may reflect differences in the mechanism of action of these drugs. The regulation of ER calcium has been reported to be a control point in ER and mitochondrial cross-talk and apoptosis, and Bel-2 family members (Bel-2, Bax, and Bak) have been shown to have an effect on ER calcium (51, 52, 55–57). Therefore overexpression of Bel-xL, which can be localized on the ER or the mitochondria, or inhibition of caspase-9, which is thought to be activated downstream of mitochondrial permeabilization, may protect cells from apoptosis induced by thapsigargin, which causes the passive release of Ca²⁺ from the ER. Additionally, brefeldin A was shown to require ER Ca²⁺ and the mitochondria to induce cell death (55). Thus, regulating events such as Ca²⁺ mobilization from the ER or apoptosis formation in response to ER and/or mitochondrial signals can result in the inhibition of apoptosis. The inhibition of N-linked glycosylation by tunicamycin, however, may incorporate other death pathways. This is supported by the fact that 100 μM tunicamycin appears to induce a caspase-independent necrotic cell death in both FL5.12 and U266 cells (Fig. 5) and the fact that caspase-4-like activity is not greatly induced by tunicamycin in human multiple myeloma cell lines (Figs. 7B and 8C; data not shown). Additionally, Bel-xL and 9DN overexpression may have a greater effect in tunicamycin-treated FL5.12 cells than in myeloma cells, because FL5.12 is a pro-B cell line, whereas 8226/S secretes λ light chain and U266 secretes a complete IgE antibody. This may therefore indicate that in secretory cells, Bel-xL-independent cell death pathways may be activated by drugs that directly affect protein processing within the ER.

Published reports on the mechanism of caspase-12 initiated ER stress-induced cell death have proposed that caspase-12 can directly activate caspase-9 and therefore bypass the mitochondrial (cytochrome c, Apaf-1) apoptotic pathway (44–47). Similarly, caspase-4 was reported to be activated upstream of the mitochondria, since Bel-2 or Bel-xL overexpression was shown to have only a slight effect on caspase-4 cleavage in tunicamycin-treated SK-N-SH cells (30). Each of these studies used caspase processing as a measure of caspase activity. However, using a caspase-4-specific substrate, we were able to detect a caspase-4-like activity in cells treated with ER stress agents regardless of caspase-4 or caspase-12 expression (Figs. 7A and 8C). The fact that intrinsic apoptotic stimuli also induced some caspase-4-like activity may suggest that this activity is not completely specific to ER stress-induced apoptosis. Similarly, caspase-12 cleavage has been detected in L929aAltFas cells treated with FasL and B16/B16 cells treated with interferon-γ combined with tumor necrosis factor or lipopolysacchade (32), and we have observed a loss of the procaspase-12 band in wild-type caspase-12 overexpressing FL5.12 cells treated with ER stress agents or withdrawn from IL-3. Moreover, an increase in a cleavage product derived from procaspase-4 was detected in SK-N-SH cells treated with ER stress agents as well as other intrinsic apoptotic stimuli (30). The increase in caspase-4-like activity in treated cells was inhibited in FL5.12 and U266 cells by Bel-xL or 9DN overexpression (Fig. 7), suggesting that caspase-4 or caspase-12 activity can actually be downstream of the apoptosome or require mitochondrial amplification. This is further supported by the fact that Bel-xL overexpression could also decrease caspase-4-like activity in the caspase-4-expressing 8226/S myeloma cell line (Fig. 8C). Additionally, Bel-xL and 9DN overexpression inhibited apoptosis (Fig. 4A) and caspase-4-like activity (Fig. 7A) in ER stress-agent-treated FL5.12 cells to the same extent, indicating that in cells lacking caspase-12, ER stress-induced apoptosis proceeds through the classical pathway.

These findings are in agreement with those made in mouse embryonic fibroblasts, where caspase-12 processing was found to be downstream of the proapoptotic Bel-2 proteins Bax and Bak (53, 57) and downstream of caspase-9 (53).

These data strongly suggest that postmitochondrial caspase activation or mitochondrial amplification of caspase activation is involved in ER stress-induced apoptosis. This is supported by the finding that Bax/Bak double knock-out cells are resistant to ER stress-induced apoptosis (58). Concurrently, overexpression of Bel-2 (51–53) and Bel-xL (54) have been shown to protect murine and rat cells from thapsigargin-, brefeldin A-, and tunicamycin-induced cell death, and Bel-2 or Bel-xL overexpression was shown to protect human SK-N-SH cells from tunicamycin-induced apoptosis regardless of caspase-4 cleavage (30). Consistent with these findings, we have demonstrated that Bel-xL overexpression has the same protective effect on cell viability in the caspase-4-expressing 8226/S cell line (Fig. 8B) as it does in the caspase-4 null U266 cell line (Fig. 4B). Additionally, Bel-xL overexpression was shown to inhibit caspase-12 cleavage and tunicamycin- or thapsigargin-induced apoptosis in murine C2C12 cells (54). Although these cells do not release cytochrome c in response to ER stress agent treatment (44), other mitochondrial apoptogenic factors, such as Smac/Diablo and Omi/HtrA2, have been shown to be important in ER stress-induced apoptosis in certain cell types (59, 60).

Considering previous evidence together with the studies presented in this report, we conclude that ER stress-induced apoptosis can be initiated in the absence of caspase-12 or caspase-4 expression and can be inhibited by proteins that block the activation of the classical intrinsic apoptotic pathway. Additionally, the role of caspase-12, caspase-4, and Bel-2 family members and postapoptosome caspase activation in the ER stress-induced apoptotic cascade may depend on both the cell type and the mechanism of action of the ER stressor.

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