Translocation of Bim to the Endoplasmic Reticulum (ER) Mediates ER Stress Signaling for Activation of Caspase-12 during ER Stress-induced Apoptosis*

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Endoplasmic reticulum (ER) stress activates caspase-12 in murine cells, triggering the ER stress-specific cascade for implementation of apoptosis. In C2C12 murine myoblast cells, activation of the cascade occurs without release of cytochrome c from mitochondria, suggesting that the cascade is independent of mitochondrial damage. Stable overexpression of Bcl-xL in C2C12 cells suppressed activation of caspase-12 and apoptosis. In ER-stressed cells, but not in normal cells, Bcl-xL was co-immunoprecipitated with Bim, a pro-apoptotic member of the Bcl-2 family, suggesting that Bcl-xL sequesters Bim, thereby inhibiting the apoptotic signaling. Fractionation of C2C12 cells revealed that ER stress led to translocation of Bim from a dynein-rich compartment to the ER, while stable overexpression of Bcl-xL suppressed accumulation of Bim on the ER. Although the toxic effect of Bim had been previously observed only at the mitochondrial outer membrane, overexpression of a Bim derivative, Bim(ER), targeted at the surface of the ER led to apoptosis. A C2C12 transfectant overexpressing the caspase-12 suppressor protein was resistant to Bim(ER), suggesting that the toxic effect of Bim on the ER is dependent on activation of caspase-12. Knockdown of Bim by RNA interference provided cells resistant to ER stress. These results suggest that translocation of Bim to the ER in response to ER stress is an important step toward activation of caspase-12 and initiation of the ER stress-specific caspase cascade.

The caspase protease family plays a central role in the execution of apoptosis (1, 2). It is broadly divided into two groups: initiator caspases, which are activated first and initiate a caspase cascade, and effector caspases, which mainly dismantle cellular structures. Caspases are synthesized as precursor proteins (procaspases). Initiator caspases undergo autoprocessing in response to apoptotic stimuli, while effector caspases are cleaved by initiator caspases for activation. There are three major apoptotic cascades triggered by specific initiator caspases: the death receptor pathway, the mitochondrial pathway, and the endoplasmic reticulum (ER)3 pathway. These are activated by caspase-8, caspase-9, and caspase-12, respectively (3, 4).

ER stress is caused by the accumulation of unfolded proteins in the ER, which occurs under a variety of conditions (e.g. mutation in secretory proteins or disruption of calcium homeostasis). This type of cellular stress is receiving increased attention because it is considered a cause of pathologically relevant apoptosis (5) and is especially implicated in neurodegenerative disorders (6, 7). ER stress activates caspase-12 on the surface of the ER, and caspase-12-deficient cells are resistant to ER stress inducers (8), indicating that caspase-12 is significant in ER stress-induced apoptosis. However, the mechanism responsible for caspase-12 activation is largely unknown, unlike in the case of both caspase-8 and caspase-9 whose activation mechanisms have been revealed at the molecular level (9, 10).

The Bcl-2 family regulates activation of caspase-9. Proteins of the Bcl-2 family have either pro-apoptotic or anti-apoptotic functions (11, 12). The pro-apoptotic members are classified into two groups based on their structure, i.e. BH-3 (Bcl-2 homology domain-3) only proteins (e.g. Bad, Bid, and Bim), and multidomain pro-apoptotic members containing BH-1 through BH-3 (e.g. Bax and Bak). BH-3 only proteins can serve as intracellular death ligands proximal to multidomain pro-apoptotic members (13, 14). In response to apoptotic stimuli (e.g. death ligands, mitogen withdrawal), BH-3 only proteins translocate to mitochondria and induce conformational changes in Bax and Bak, which activate them. These activated multidomain proteins, in turn, cause an increase in mitochondrial membrane permeability leading to the release of cytochrome c from the mitochondria. The released cytochrome c, together with Apaf-1, activates caspase-9 (15, 16), resulting in the activation of downstream effector caspases, e.g. caspase-3. The lack of either Bax or Bak is not sufficient to prevent apoptosis, but double knock-out cells are highly resistant to a variety of apoptotic stimuli including ER stress (17). Evidently, Bax and Bak have mutually redundant functions. Anti-apoptotic members of the Bcl-2 family (e.g. Bcl-2 and Bcl-xL) exert their anti-apoptotic actions either by heterodimerization with pro-apoptotic members, especially BH-3 only proteins (18), or by directly protecting mitochondrial membrane integrity (19–21).

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1 The abbreviations used are: ER, endoplasmic reticulum; UPR, unfolded protein response; BH-3, Bcl-2 homology domain-3; RNAi, RNA interference; GFP, green fluorescent protein; MAGE-3, melanoma-associated antigen-3.
Although it had been thought that cytochrome c released from mitochondria is required for caspase-9 activation (15, 16), we have shown that caspase-12 can activate caspase-9 directly without cytochrome c release in C2C12 myoblast cells under ER stress (22). The active caspase-9, in turn, activates a downstream caspase such as caspase-3. We have proposed the existence of an ER stress-specific caspase cascade consisting of caspase-12, -9, and -3 (22). Other studies also show that caspase-9 activation can occur in an Apaf-1-independent, and thus cytochrome c-independent, manner (23). In some cell lines, however, ER stress induces cytochrome c release (24, 25) by an unknown pathway. Therefore, it is likely that the activation of caspase-9 and its downstream effector caspases can be achieved in ER stress-induced apoptosis either by caspase-12 or by the Apaf-1-cytochrome c complex (22). Since ER stress-induced caspase activation in C2C12 myoblast cells is independent of cytochrome c release and the mitochondrial pathway, this model provides the opportunity to analyze activation of caspase-12 without these complications. We have taken advantage of this to examine the possible involvement of pro-apoptotic members of the Bcl-2 family in the activation step. Our results suggest that ER stress induces translocation of Bim, which is associated with cytoplasmic dynein in healthy cells (26), to the ER, causing activation of caspase-12.

EXPERIMENTAL PROCEDURES

Reagents—Tunicamycin and thapsigargin were purchased from Calbiochem. Etoposide was obtained from Sigma. Cell Culture—C2C12 cells (RIKEN Cell Bank, Tsukuba, Japan) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Sigma), 50 units/ml penicillin, and 50 μg/ml streptomycin (Invitrogen) at 37 °C with 5% CO2. Adding either tunicamycin or thapsigargin induced apoptosis. Changes in mitochondrial membrane potential were assessed by fluorescence microscopic observation of cells stained with a MitoSensor reagent (Clontech), as described previously (22).

Cell Fractionation—C2C12 cells were scraped from culture dishes to obtain attached cells, and the culture medium was centrifuged at 1,000 × g for 10 min to obtain dying cells. All the following steps were carried out at either 0 °C or 4 °C. Cells were disrupted with a Dounce homogenizer in 10 mM Hepes/KOH (pH 7.6), 10 mM KCl, 1 mM MgCl2, 1 mM dithiothreitol, containing aprotinin (Sigma), 0.5 mM phenylmethylsulfonyl fluoride (Sigma), and Complete protease inhibitor mixture (Roche Applied Science). Immediately after homogenization, sucrose was added to 250 mM, nuclei and unbroken cells were removed by centrifugation at 3,000 × g for 5 min, and the heavy membrane (mitochondria-rich) fraction was sedimented by centrifugation at 9,000 × g for 20 min. The supernatant was split into the S-100 (cytosol) and the light membrane (ER) fraction by centrifugation at 100,000 × g for 60 min in a tabletop ultracentrifuge (Beckman Coulter, Inc.).

Western Blot Analysis—Denatured proteins were separated on an SDS-polyacrylamide gel (12 or 14% acrylamide) and transferred to a polyvinylidene difluoride membrane (Millipore). For immunoblotting we used antibodies against caspase-12 (8), cytochrome c (Pharmingen), caspase-3 (BD Transduction Laboratories), hemeoxygenase I (Stressgen), BiP (BD Transduction Laboratories), CHOP (Santa Cruz Biotechnology), caspase-9 (Medical and Biological Laboratory, Nagoya, Japan), Bcl-xL (BD Transduction Laboratory), Bcl-2 (Santa Cruz Biotechnology), Bid (R&D Systems), Bad (Santa Cruz Biotechnology), Bim (Calbiochem), Bnip3 (Chemicon), Bak (Oncogene), and Bax (Oncogene).

Plasmid Construction—We amplified the full-length human Bcl-xL lacking the C-terminal membrane-binding region (27) by PCR, compared it with that of the UPR-specific up-regulation. Treatment of C2C12 cells with tunicamycin, an inhibitor of glycosylation in the ER, caused induction of BiP, an ER resident molecular chaperon, and CHOP, an ER stress-specific transcription factor (31). Induction of these proteins started and persisted after no more than 4 h of tunicamycin treatment. After ~20 h of tunicamycin treatment, C2C12 cells started to show apoptotic morphology (Ref. 22 and data not shown). Around the same time, 20 h, activation of caspase-12 was detected by Western blot analysis, as the active form of caspase-12 (~35 kDa) (8), in addition to the 48-kDa precursor (Fig. 1A). These results suggest that although it takes about

FIG. 1. Activation of caspase-12 is preceded by the UPR. C2C12 cells were treated with 2 μg/ml tunicamycin for 0, 4, 8, 16, 20, and 24 h. Cell lysates were probed with anti-caspase-12 (A), anti-BiP (B), and anti-CHOP antibodies (C). A black arrowhead indicates the caspase-12 precursor. A white arrowhead shows the active form of caspase-12 (8, 22).
20 h for ER stress to initiate activation of caspase-12 under the conditions used, caspase-12, once activated, efficiently induces apoptosis within a relatively short time period, as observed in the cases of the death receptor and the mitochondrial pathways (15, 32). It is reasonable to assume that an intracellular event leading to the activation of caspase-12 takes place at around or just before 20 h of tunicamycin treatment.

**Bcl-xL Can Suppress ER Stress-induced Apoptosis**—To explore the regulation mechanism of caspase-12 activation, we tested whether an anti-apoptotic protein, Bcl-xL, could suppress ER stress-induced apoptosis in C2C12 cells. A recent study shows that Bcl-xL is predominantly localized on the mitochondrial outer membrane, due to the presence of the C-terminal signal sequence (33). However, Bcl-2, which lacks a strong localization signal of this kind, is distributed between several intracellular membranous structures and could presumably function in these different organelles. We anticipated that Bcl-xL might not be able to suppress ER stress-induced apoptosis, because ER stress-induced caspase activation in C2C12 occurs in the absence of cytochrome c release from mitochondria, being independent of the mitochondrial pathway (22). Consistent with the lack of cytochrome c release, the mitochondrial transmembrane potential was maintained in apoptotic cells after induction of apoptosis by ER stress inducers, tunicamycin and thapsigargin, an inhibitor of the ER-specific calcium ATPase. In contrast, a decrease in mitochondrial membrane potential was observed in apoptotic C2C12 cells under other conditions (Fig. 2A). Preliminary data showed, however, that transient overexpression of Bcl-xL provided resistance to ER stress (data not shown).

We therefore established stable transfectants (C2C12/Bcl-xL) that overexpressed Bcl-xL (Fig. 2B). Apoptosis of the parental C2C12 cells (Fig. 2C) or a vector-control line (C2C12/vec2 described in Ref. 22) was induced by the ER stress inducers, tunicamycin and thapsigargin. Approximately 40% of the C2C12 cells exhibited apoptotic morphology after 24 h exposure to the inducers (Fig. 2C) (22). Under the same conditions, C2C12/Bcl-xL cells displayed only the same low background level, <2%, of apoptosis observed in untreated cells (Fig. 2C). Western blot analysis showed that tunicamycin treatment did not activate caspase-12 in the C2C12/Bcl-xL cells (Fig. 2D), but it did in the parental C2C12 cells (Fig. 1A). This indicates that Bcl-xL on the mitochondrial outer membrane suppresses apoptosis by inhibiting procaspase-12 activation that presumably occurs on the surface of the ER (34). We hypothesized that mitochondrial Bcl-xL prevents pro-apoptotic ligands from translocating to the ER in response to ER stress for caspase-12 activation, as already suggested in connection with the inhibitory action of Bel-xL and Bcl-2 on the mitochondria-dependent apoptotic pathway (18).

To obtain supporting evidence that Bcl-xL can suppress ER stress-induced apoptosis not by a direct effect on the mitochondrial outer membrane but by sequestration of cytoplasmic proteins, we established and analyzed C2C12 transfectants stably overexpressing ER-targeted Bcl-xL (Bcl-xL/ER). For this purpose, we replaced the C-terminal membrane-associated portion of Bcl-xL with a potent ER-targeting signal sequence from cytochrome b5 (28). As controls, Bcl-xL mutants whose C-terminal portion was either deleted (Bcl-xLdel) or replaced with the mitochondria-targeting sequence of ActA protein (28) were also created. These ER-targeting and mitochondria-targeting signal sequences have been successfully used with Bel-2 (27, 28) and Bak (35).

As in the case of C2C12/Bcl-xL, a stable transfectant overexpressing the ER-targeted Bel-xL (Fig. 2B) was also resistant to ER stress inducers (Fig. 2C), and caspase-12 was again not activated (Fig. 2E). Induction of CHOP in these Bcl-xL-overexpressing cells suggests that ER stress is actually generated by ER stress inducers in these transfectants (Fig. 2F), while apoptotic signaling connecting ER stress to activation of caspase-12 is suppressed by Bcl-xL. Suppression of apoptosis and caspase-12 activation in the presence of ER stress was also observed with both the mitochondria-targeted and the deletion mutants of Bcl-xL (data not shown). These results indicate that Bcl-xL can probably suppress apoptosis regardless of its location provided that it can physically interact with the pro-apoptotic ligands.

**Bim Is Specifically Associated with Bel-xL in Cells under ER Stress**—To identify the pro-apoptotic ligands that are sequestered by Bcl-xL, we immunoprecipitated Bcl-xL proteins in stable C2C12 transfectants. For efficient recovery of the pro-apoptotic molecules involved in ER stress-specific caspase activation, we FLAG-tagged the N terminus of the ER-targeted Bel-xL and established a stable cell line overexpressing it.
were predominantly localized in the heavy membrane fraction, in which cytoplasmic dynein was efficiently recovered (Fig. 4B). The majority of Bad and Bid were detected in the S-100 fraction. The multidomain pro-apoptotic proteins Bak and Bak were mainly detected in the S-100 and the mitochondrial fractions, respectively, as has been observed in other cell lines (36). These results suggest that the pro-apoptotic members of the Bcl-2 family examined are preferentially localized in either mitochondria or the cytosol. Immunoblotting of higher amount of proteins, 60 μg, in the ER fraction, equivalent to \(-2 \times 10^6\) cells, showed that the subpopulation of these pro-apoptotic proteins, except for Bim, were localized in the ER of healthy cells (Fig. 4C (−)).

We examined whether ER stress induces either translocation of Bim to the ER or accumulation of other pro-apoptotic Bcl-2 family members on the ER, as had been suggested by our immunoprecipitation experiment. C2C12 cells were treated with tunicamycin for 20 h, and the ER fraction was isolated for Western blot analysis. The content of hemeoxygenase I in the ER fraction did not change after the tunicamycin treatment (Fig. 4C (− and +)). We did not detect an increase of either cytochrome c or caspase-3 in the ER fraction prepared from the apoptotic cells, suggesting that the ER fraction was not contaminated by proteins from other compartments. The amounts of Bad, Bid, Bak, and Bax were either unchanged or slightly increased after ER stress (Fig. 4C (+)). Only Bim showed a clear difference in ER localization between untreated and stressed cells. Bim specifically appeared in the ER fraction after the generation of ER stress (Fig. 4C).

To compare the time course of Bim translocation with that of caspase-12 activation, we prepared an ER fraction from C2C12 treated with tunicamycin for different durations. Fig. 4D shows translocation of Bim to the ER starting around a 20-h treatment of C2C12 cells with tunicamycin. The appearance of Bim on the ER coincided with caspase-12 activation (Fig. 1A). This result suggests the possibility that the Bim translocation mediates the apoptotic signaling, which possibly activates caspase-12 on the surface of ER (see “Discussion”). The translocation of Bim was not detected in the Bcl-xL-overexpressing cell line treated with tunicamycin (Fig. 4E), which is consistent with our hypothesis that Bcl-xL sequesters Bim released from the cytoplasmic dynein complex, thereby suppressing ER stress-induced apoptosis.

**Translocation of Bim Specifically Occurred in Apoptotic Cells**—Under the apoptotic conditions used (e.g. treatment with 2 μg/ml tunicamycin for 24 h), ER stress induces apoptosis in \(~40\%\) of C2C12 cells (22). To examine whether Bim translocation as well as caspase-12 activation specifically occurred in apoptotic cells, we prepared the ER fraction after separation of apoptotic cells from living cells, both of which had been treated with tunicamycin (see “Experimental Procedures”). The ER fractions prepared from apoptotic cells contained hemeoxygenase I at a level comparable with that in the ER fraction of living cells (Fig. 5A) and the both fractions were not contaminated by cytochrome c and caspase-3 (data not shown). Significant accumulation of Bim was observed in the ER fraction prepared from the apoptotic cells, but only a slight increase was detected in the ER in the living cells, demonstrating specific translocation of Bim to the ER in the apoptotic cells (Fig. 5A). Similarly, procaspase-12 (48 kDa) proved to be extensively (>\(90\%\)) processed into its active forms in the apoptotic cells, while it was largely unchanged in the live cells (Fig. 5B), suggesting that apoptosis is effectively executed once caspase-12 is activated (Fig. 1A). The correlation between Bim translocation and caspase-12 activation again implies that Bim on the ER may be a trigger of caspase-12 activation. Induction of the UPR ap-
Membrane fraction (L) was obtained by centrifugation of C2C12 cells. Subcellular fractionation of C2C12 cells was performed to obtain the heavy membrane (HM), cytosol (S-100), and the light membrane fraction (LM). Protein equivalent to ~7 x 10^6 cells was loaded in each lane and analyzed by Western blot analysis. Cyto-c, cytochrome c; HO, hemeoxygenase I.

B. Intracellular distribution of pro-apoptotic proteins. Western blots were probed with specific antibodies. C. Translocation of Bim to the ER. ER fractions were prepared as described in the legend to A. D. Time course of Bim translocation to the ER. ER fractions were prepared from C2C12 cells treated with 2 μg/ml tunicamycin for 20 h (+), and 60 μg of protein were loaded in each lane. E. The ER fraction of C2C12/Bcl-xL cells was prepared and analyzed as described in the legend to D.

To examine whether Bim on the ER membrane can induce caspase activation and apoptosis, we overexpressed an ER-targeted derivative of Bim (Bim(ER)) whose C terminus contained the signal sequence from cytochrome b_{6}. Transient transfection of C2C12 cells with the Bim(ER) cDNA considerably induced apoptosis (Fig. 6A), causing cell death in ~30% of the cells. It is possible that Bim(ER) causes apoptosis by activation of caspase-12, the ER resident caspase. To examine this possibility, we used a stable transfectant of C2C12 that overexpressed a suppressor of caspase-12. We previously identified a procaspase-12-binding protein, melanoma-associated antigen-3 (MAGE-3), which blocks activation of procaspase-12 (22). Stable overexpression of MAGE-3 rendered cells (C2C12/MA21) resistant to Bim(ER), as shown in Fig. 6A. These results suggest that the Bim(ER) predominantly exerts its apoptotic activity by activation of caspase-12. By comparing the action of BH-3 only proteins on the mitochondrial outer membrane, it is possible that Bim translocated to the ER induces conformational changes in Bax and Bak, activating caspase-12 in an indirect manner (see “Discussion”). The cytotoxicity of Bim(ER) was efficiently inhibited by the stable expression of Bcl-xL (Fig. 6A), which is consistent with the idea that mitochondrial Bcl-xL can trap Bim, preventing it from activating caspase-12.

**Suppression of Bim in C2C12 Cells Renders Cells Resistant to ER Stress**—We next examined whether the decrease in the level of Bim by RNAi affected ER stress-induced apoptosis. C2C12 cells were transfected with either a control vector or a Bim-specific siRNA expression plasmid. A GFP expression plasmid was also included in transfection for detection of transfectants. After 72 h of posttransfection, cells were either not treated or treated with tunicamycin for another 24 h. Observation of cell morphology showed that ~40% of GFP-positive cells transfected with the control vector underwent apoptosis (Fig. 6B). In contrast, RNAi to Bim partially (~30%) but considerably reduced tunicamycin-induced apoptosis in C2C12 cells (Fig. 6C). These results support the hypothesis that Bim is a major mediator of ER stress-induced apoptosis.
Induction of Apoptosis by Bim in the ER

Fig. 6. Bim localized to the ER can initiate apoptosis via caspase-12. A, Bim(ER) induces apoptosis, which is suppressed by the caspase-12-binding protein, MAGE-3 (22). C2C12 or its derivatives were grown in 6-well plates and transiently co-transfected with GFP (0.3 μg) and either pcDNA3.1 vector or Bim(ER) (1.8 μg). Over 200 GFP-positive cells were counted and assessed for apoptosis based on cell morphology (small and round cells). Typical images of GFP-positive cells are shown in the right panels (upper, live cell; lower, apoptotic cell). Each bar in the graph represents an average of three independent experiments. B, knockdown of Bim conferred cells resistant to ER stress. C2C12 cells were transfected with either a Bim-specific siRNA expression plasmid (1.2 μg) or control vector, in addition to a GFP expression plasmid (0.3 μg). After 72 h of posttransfection, cells were either treated with tunicamycin or left untreated for 24 h. Over 200 GFP-positive cells were randomly selected from each transfection experiment, and apoptosis was assessed by cell morphology. Each bar represents an average of three independent experiments. TUN, tunicamycin treated; UT, untreated.

Discussion

Our data show that ER stress induces translocation of Bim to the ER. It is likely that Bim accumulated on the ER acts as a trigger for caspase-12 activation causing ER stress-induced apoptosis. This model is supported by the following observations: (a) both translocation of Bim and the activation of caspase-12 started almost simultaneously, (b) Bim targeted to the ER (Bim(ER)) induced apoptosis, (c) ectopic expression of a caspase-12 suppressor protein inhibited Bim(ER)-induced apoptosis, and (d) silencing of Bim by RNAi provided cells resistant to ER stress. Interaction between BH-3 only proteins and caspase has not been reported, except for Bid cleavage by caspase-8 (37, 38). It is thus unlikely that Bim activates caspase-12 on the ER directly. It is more likely that the pro-apoptotic activity of Bim on the ER is manifested via the multidomain pro-apoptotic members of the Bcl-2 family (e.g., Bax and Bak), as happens in the case of mitochondrial translocation of BH-3 only proteins (13, 14). Recently, the involvement of Bax and Bak in ER stress-induced apoptosis has also been suggested (35, 39, 40). According to the model, conformational changes and oligomerization of Bax/Bak probably causes activation of caspase-12 (35), although a trigger for Bax/Bak activation has not been identified. Our data demonstrate that Bim is a candidate for the molecular link between ER stress and Bax/Bak activation. A remaining question is how active Bax/Bak activates caspase-12. It is unknown whether or not the effect of Bax/Bak on caspase-12 is direct.

Our results provide the first evidence that translocation of Bim to the ER can occur during ER stress-induced apoptosis. The success in detection of Bim translocation during ER stress-induced apoptosis possibly depends on the lack of cytochrome c release from mitochondria in C2C12 cells under ER stress conditions. Otherwise, cytochrome c release could result in full activation of the caspase family, and therefore the cells might complete the apoptotic process before Bim translocation to the ER can be detected. The molecular mechanism on how the release of Bim from the dynein complex is triggered by either UV irradiation or toxic drugs has not been elucidated (26). Our study suggests the presence of a common mediator for Bim translocation in both the mitochondria- and the ER-specific pathway.

Overexpression of Bim(ER) induced apoptosis in C2C12 cells, where the cell killing efficiency was around 30%, is shown in Fig. 6A. The relatively low efficiency of apoptotic induction by Bim(ER) may be due to the structure of the protein. Bim(ER) contains an artificial targeting sequence at its C terminus, which may affect the tertiary structure of the Bim molecule. Alternatively, or additionally, the extra region at the C terminus may interact with the binding region to anti-apoptotic proteins within the Bim molecule, thereby inhibiting the pro-apoptotic activity competitively. The mode of association to the ER would also be different between the authentic Bim and Bim(ER), because Bim(ER) is associated with the ER via its C-terminal sequence, which is absent in the authentic Bim. In addition to these, the quaternary structure of Bim or the absence of its associated protein may also affect the function of Bim. During apoptosis induced by either UV irradiation or drugs (e.g., staurosporine or doxorubicin), Bim and the dynein light chain (LC8) are both released from the dynein motor complex, and the Bim-LC8 complex is translocated to cytoplasmic membranes (26). Due to dimerization of LC8, the Bim-LC8 complex is dimeric in terms of the Bim molecule. It is possible that ligation of Bim in such a manner affects its activity and that LC8 may also be involved in the activation of Bax/Bak. In the case of Bim(ER) overexpression, however, the mutant Bim protein alone was supposed to move to the ER, probably acting as a monomer. The absence of the quaternary structure may result in the reduction of the pro-apoptotic activity of Bim.

We have shown that Bim is important in ER stress-induced apoptosis. However, our results do not necessarily indicate that BH-3 only proteins other than Bim do not play any role in ER stress-induced apoptosis. These BH-3 only proteins can work in concert, acting synergistically to induce apoptosis, because the BH-3 only proteins represent functional redundancy by antagonizing anti-apoptotic members of the Bcl-2 family such as Bcl-xL (12). We observed that other pro-apoptotic members of the Bcl-2 family in C2C12 cells could localize in the ER, although such ER localization appears to be unaffected under ER stress (Fig. 4C). It is possible that Bax/Bak activation is achieved in the case where the total amount of ER-resident BH-3 only proteins and translocated Bim reaches the level that is enough to antagonize anti-
apoptotic proteins and sufficient to activate Bax/Bak. Therefore, BH-3 only proteins on the ER may set the basal level of the pro-apoptotic activity or the sensitivity to ER stress. Although siRNA specific to Bim caused a significant reduction in ER stress-induced apoptosis, the suppression was incomplete (Fig. 6C). This may be due to the residual amount of Bim in the transfectants. Alternatively, other BH-3 only proteins, or proteins that can be regulated by Bcl-xL, may also activate caspase-12 in a Bim-independent manner.

Our study provides a clue as to why cytochrome c release from mitochondria is not detected during ER stress-induced apoptosis in C2C12 cells. Unlike ER stress, other apoptotic stimuli (e.g. serum deprivation or DNA damage) can trigger mitochondrial damage and cytochrome c release in these cells (Fig. 1B) showing that cytochrome c release per se can take place (22). The decision whether mitochondria release cytochrome c or not probably depends on the balance between the levels of anti-apoptotic proteins and those of BH-3 only proteins accumulated on the organelles (11, 12). Pro-apoptotic members of the Bcl-2 family in C2C12 cells can localize on mitochondria in addition to the ER. For instance, Bim and Bak are present in mitochondria under both normal and apoptotic conditions, while such mitochondrial localization is either unaffected or only slightly enhanced under ER stress (Fig. 4B and data not shown). It is probable that mitochondrial BH-3 only proteins surpass the anti-apoptotic proteins in C2C12 cells when cells suffer abuse such as DNA damages but not in the case of ER stress.

A decision mechanism similar to that of cytochrome c release could also operate for regulation of the ER stress-specific cascade, as discussed above. Therefore, it is not unreasonable to assume that the amounts of anti-apoptotic proteins in the ER and the mitochondria determine whether either or both of the ER and mitochondrial pathways are activated in ER-stress induced apoptosis. This model again illustrates the redundancy of ER stress-induced apoptotic pathways, as suggested by us (22) and more recently by others (33). A similar situation can be seen in the case of other apoptotic stimuli, where either parallel or branched apoptotic pathways are activated (32).

Such redundancy would be a basis of the compensatory effect observed in studies using caspase knock-out mice: the absence of a caspase family member can be compensated by other caspases (41, 42). Caspase-12 knock-out mice show no abnormality during development and adulthood (8), possibly because an alternative pathway dependent on the release of mitochondrial cytochrome c may activate other caspase family members.

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