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Review

Bcl-2 family on guard at the ER

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Szegezdi E, MacDonald DC, Ní Chonghaile T, Gupta S, Samali A. Bcl-2 family on guard at the ER. *Am J Physiol Cell Physiol* 296: C941–C953, 2009. First published March 11, 2009; doi:10.1152/ajpcell.00612.2008.—The endoplasmic reticulum (ER) is the main site for protein folding, lipid biosynthesis, and calcium storage in the cell. Disturbances of these critical cellular functions lead to ER stress. The ER responds to disturbances in its homeostasis by launching an adaptive signal transduction pathway, known as the unfolded protein response (UPR). The UPR strives to maintain ER function during stress; however, if the stress is not resolved, apoptotic responses are activated that involve cross talk between the ER and mitochondria. In addition, ER stress is also known to induce autophagy to counteract XBP-1-mediated ER expansion and assist in the degradation of unfolded proteins. One family of proteins involved in the regulation of apoptosis is that of B-cell lymphoma protein 2 (Bcl-2). Complex interactions among the three subgroups within the Bcl-2 family [the antiapoptotic, the multidomain proapoptotic, and the Bcl-2 homology domain 3 (BH3)-only members] control the signaling events of apoptosis upstream of mitochondrial outer membrane permeabilization. These proteins were found to have diverse subcellular locations to aid in the response to varied intrinsic and extrinsic stimuli. Of recent interest is the presence of the Bcl-2 family at the ER. Here, we review the involvement of proteins from each of the three Bcl-2 family subgroups in the maintenance of ER homeostasis and their participation in ER stress signal transduction pathways.

endoplasmic reticulum stress; unfolded protein response; inositol-requiring enzyme 1; apoptosis

EUKARYOTIC CELLS HAVE COMPARTMENTALIZED their functions to distinct intracellular organelles. The endoplasmic reticulum (ER) is the assembly line of the cell; it is primarily responsible for the synthesis and folding of secreted and membrane-bound proteins, as well as the biosynthesis of lipids and the storage of calcium (Ca^{2+}) (38, 76). The oxidative environment and the high Ca^{2+} concentration within the ER are necessary for optimal protein folding (36). Perturbation of these factors compromises the protein folding capacity of the ER and leads to the accumulation of unfolded proteins that aggregate within the ER lumen—a state broadly termed as ER stress. Numerous pathophysiological conditions are associated with ER stress, including ischemia, viral infections, and neurodegenerative diseases (127). Such diseases are associated with excessive loss of cell number due to apoptotic death of the affected cells.

Apoptosis initiated by ER stress is largely dependent on the release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol. This event is associated with the opening of the permeability transition pore (PTP) and a collapse in the mitochondrial transmembrane potential ($\Delta\Psi_m$) as a consequence of the intake of Ca^{2+} following its release into the cytosol from the ER. Once released, cytochrome *c* and procaspase-9 are recruited to apoptosis protease-activating factor 1 (APAF-1) to form the apoptosome holoenzyme complex, which catalyzes the allosteric conformational maturation of procaspase-9 to active caspase-9 (1, 161, 162). Caspase-9 then activates downstream effector caspases (109), which in turn act on various cellular substrates to facilitate cellular dismantling (110). Permeabilization of the mitochondrial outer membrane (MOM) and the consequent release of cytochrome *c* is regulated by a group of proteins known as the B-cell lymphoma protein 2 (Bcl-2) protein family. Collectively, the Bcl-2 protein family monitors incoming stress signals and orchestrates the initiation of the mitochondrial or intrinsic pathway to apoptosis. However, recent work has revealed that certain members of the Bcl-2 family are present on the ER where they seem to have a much broader and more comprehensive function. The emerging roles of the Bcl-2 family at the ER are currently under intense investigation within the cell death community with significant advances in our understanding being reported in the literature every year. This review will therefore discuss current opinions on how the Bcl-2 protein family couples the molecular events initiated by ER stress to the mitochondrial apoptotic pathway, their function on ER-mitochondrial Ca^{2+} signaling, the unfolded protein response (UPR), and autophagy.

The Bcl-2 Protein Family

The founding member of the Bcl-2 protein family, Bcl-2 itself, was discovered during molecular analysis of the t14–18 chromosomal translocation in B cell lymphoma (133). Since then, the family has grown to ~20 members. All Bcl-2 family

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proteins contain at least one of the four conserved α-helical motifs known as Bcl-2 homology (BH1–4) domains (95). According to function, the family is further classified into two groups, the antiapoptotic and the proapoptotic members. The antiapoptotic family members possess all four BH domains and include Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, and A1, while the proapoptotic proteins are divided into two distinct subgroups—the multidomain proteins containing BH domains 1–3 (Bax, Bak, and Bok; expression of the latter is restricted to reproductive tissues) and the BH3-only proteins (Bad, Bid, Bik/Nbk, Bim, Bmf, Nix/BNIP3, Hrk, Noxa, and Puma), which contain only the BH3 domain.

The three-dimensional structure of Bcl-X_L in complex with a BH3-containing peptide predicted that the BH1, BH2, and BH3 domains of the antiapoptotic members form a hydrophobic groove into which the amphipathic α-helical BH3 domain of the proapoptotic family members inserts (80, 111). This interaction between the antiapoptotic Bcl-2 proteins and the multidomain proapoptotic proteins neutralizes their proapoptotic function (21, 42, 59, 79, 81, 121, 143, 156). Bcl-X_L and Mcl-1, but not Bcl-2, were shown to target Bak, whereas all of the antiapoptotic members interact with Bax (reviewed in Refs. 34, 39, 41, and 148). Bax and Bak are essential components of the mitochondrial pathway of apoptosis (145). In response to apoptotic stimuli, Bax translocates to the MOM whereupon both Bax and Bak have been proposed to homooligomerize into pores (megachannels), facilitating the release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol (6, 7, 84, 145, 149).

The BH3-only proteins are regarded as the initial responders to incoming stress signals and developmental cues (2) and exhibit both stimulus and cell type-specific activities. Activation of the BH3-only proteins during stress can occur at the transcriptional level (for example, p53-mediated upregulation of Puma and Noxa) and/or via posttranslational alterations, such as phosphorylation (Bad and Bik/Nbk), dissociation from sequestering proteins (release of Bad from 14-3-3; release of Bim and Bmf from dynein light chain 1 and 2, respectively), or proteolytic cleavage (truncation of Bid by caspase-8) (61, 66, 83, 93, 102, 104, 137, 139, 154). How the BH3-only proteins promote apoptosis depends on their relative affinity for the various multidomain Bcl-2 family proteins. The "death agonists," such as Bid, Bim, and Puma, interact with and enhance the activity of multidomain proapoptotic Bcl-2 proteins, whereas the "survival antagonists," such as Bad, Bik/Nbk, Bmf, Hrk and Noxa, interact only with antiapoptotic Bcl-2 family proteins to displace sequestered Bax and Bak from the antiapoptotic Bcl-2 proteins (Fig. 1). However, this question is still under debate, and other results indicate that the BH3-only proteins with the exception of Bid function solely as sensitizers (survival antagonists) by neutralizing antiapoptotic Bcl-2 family proteins and do not directly activate Bax or Bak (3). Interestingly, although several BH3-only proteins (Bid, Bim, and Puma) interact with all antiapoptotic Bcl-2 family proteins, other BH3-only proteins exhibit marked selectivity in their binding, with Bad and Bmf having greater affinity for Bcl-2, Bcl-X_L, and Bcl-w, whereas Noxa binds only to Mcl-1 and A1 (23). Correspondingly, Bid, Bim, and Puma on their own are more potent inducers of apoptosis than other BH3-only proteins that require a combined effort to neutralize multiple antiapoptotic Bcl-2 family proteins. However, to commit to apoptosis, BH3-only proteins require either Bax or Bak, indicating that the multidomain proapoptotic proteins function as downstream effectors to the BH3-only proteins (145, 160).

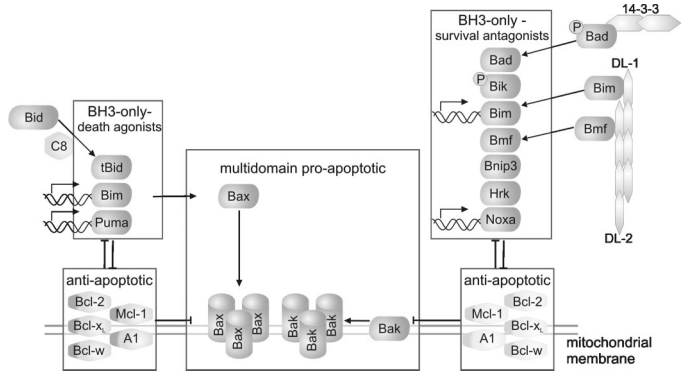

Fig. 1. Functional organization of the B-cell lymphoma protein 2 (Bcl-2) protein family. Various death stimuli trigger the transcriptional and/or posttranslational activation of Bcl-2 homology domain 3 (BH3)-only proteins, as indicated. BH3-only "death agonists" interact with the multidomain proapoptotic Bcl-2 family proteins leading to their activation and translocation through the cytosol to the mitochondrial outer membrane (MOM). BH3-only "survival antagonists" neutralize the protective influence of antiapoptotic Bcl-2 family proteins residing on the mitochondrial surface, facilitating the activation and the homooligomerization of Bak and Bax into proposed cytochrome *c*-conducting megachannels within the MOM. C8, active caspase-8; tBid, truncated Bid; DL, dynein light chain; P, phosphate; double helix with an arrow indicates transcriptional regulation; Bnip3 is also called Nix.

Subcellular Localization of the Bcl-2 Protein Family

Bcl-2 family proteins have diverse intracellular locations. BH3-only proteins are primarily localized in the cytosol while other Bcl-2 family members contain a carboxy-terminal hydrophobic tail that anchors them to intracellular membranes (128). Bcl-2 and Bcl-X_L are localized to the membrane surface of mitochondria, the ER, and the nucleus (4, 22, 57), whereas Bak has been detected on both the mitochondrial and ER membranes (25, 40, 144). Although Bax contains a hydrophobic COOH-terminal tail, inactive Bax is a cytosolic monomeric protein because its COOH-terminal anchor domain is internalized within a hydrophobic pocket formed by the BH1–3 domains (123). However, following an apoptotic stimulus, Bax changes conformation, leading to the exposure of the COOH-terminal tail and the translocation of active Bax to the MOM (7, 51, 149).

Although a vast body of research has focused on the actions of the Bcl-2 family at the mitochondria, it is long known that Bcl-2 has an antiapoptotic role at the ER (157). Since then, representatives from all three subclasses of the Bcl-2 family have been identified at the ER where they have been proposed to regulate induction of apoptotic pathways in response to a range of cellular stresses (24, 37, 72, 114, 147, 159; Table 1). Numerous recent studies have also identified the ER as a critical early checkpoint regulating the initiation of the mitochondria-dependent pathway to apoptosis in response to severe or prolonged ER stress (127). This has had a major impact on our understanding of how certain cellular stresses and disease states link into the apoptotic machinery and has illuminated the global regulation of stress-induced apoptosis by the Bcl-2 family. Furthermore, recent results indicate an even broader role of this protein family at the ER reaching beyond regulation

Table 1. *Proposed functions of known ER-localized Bcl-2 family proteins*

Member	Subclass	Proposed Functions		
		ER Ca^{2+} Homeostasis	UPR Signaling	ER Stress-Induced Autophagy
Bcl-2, Bcl-X_L	AA	Effect: attenuates proapoptotic Ca^{2+} signaling. Proposed mechanism(s): regulation of IP_3R Ca^{2+} channels lowering resting ER Ca^{2+} content or inhibiting release of ER Ca^{2+} into the cytosol upon stress.	No known function	Effect: suppress induction of autophagy. Proposed mechanism(s): direct inhibition of beclin-1.
Bax, Bak	MD-PA	Effect: increases ER Ca^{2+} load and enhances proapoptotic Ca^{2+} release. Proposed mechanism(s): inhibition of ER-localized Bcl-2 and Bcl-X_L.	Effect: promote IRE1-JNK signaling. Proposed mechanism(s): interaction with and stabilization of IRE1.	Effect: may promote induction of autophagy. Proposed mechanism(s): enhancement of IRE1-JNK signaling.
Bik	BH3-PA	Effect: promotes Ca^{2+} release from ER upon genotoxic stress. Proposed mechanism(s): activation of ER-localized Bax/ Bak.	No known function	No known function
Bad	BH3-PA	No known function	No known function	Effect: promotes autophagy. Proposed mechanism(s): inhibition of ER-localized Bcl-2 and Bcl-X_L.
Puma	BH3-PA	Effect: promotes ER Ca^{2+} pool depletion during thapsigargin-induced apoptosis. Proposed mechanism(s): Bax dependent.	Effect: initiates apoptosis in neuronal cells triggered by abnormal accumulation of malformed proteins. Proposed mechanism(s): unknown.	No known function
Nix/BNIP3	BH3-PA	Effect: increases resting ER Ca^{2+} load; required to induce Ca^{2+}-dependent PTP opening, $\Delta\Psi_m$ loss in cardiomyocytes. Proposed mechanism(s): unknown.	No known function	No known function
BI-1	AA	Effect: lowers resting ER Ca^{2+} load. Proposed mechanism(s): positive regulation of ER.-localized Bcl-2 and Bcl-X_L.	Effect: attenuates UPR signaling. Proposed mechanism(s): inhibition of PERK and IRE1 signaling.	No known function

ER, endoplasmic reticulum; Bcl-2, B-cell lymphoma protein 2; UPR, unfolded protein response; AA, antiapoptotic; MD-PA, multidomain, proapoptotic; BH3-PA, BH3-only, proapoptotic; IP_3R, inositol trisphosphate receptor; IRE1, inositol-requiring enzyme 1; PTP, permeability transition pore; PERK, PKR-like ER kinase; $\Delta\Psi_m$, mitochondrial transmembrane potential. BI-1, Bax inhibitor 1.

of apoptosis. The remainder of this review will discuss recent evidence demonstrating alternative roles for Bcl-2 family proteins in the regulation of critical ER functions and in the transmission of the apoptotic signal from the ER to the mitochondria during ER stress.

Modulation of ER Function and Stress Response by the Bcl-2 Protein Family

Regulation of ER Ca^{2+} signaling. High concentration of free Ca^{2+} in the cytosol can be toxic to cells (96). For this reason, and to maintain the necessary Ca^{2+} gradients required for optimal cell signaling, the majority of cellular Ca^{2+} is bound to specialized Ca^{2+}-binding proteins, such as calreticulin and calsequestrin, and compartmentalized in the ER. The movement of Ca^{2+} across the ER membrane is regulated by two main transporters: the sarcoplasmic/endoplasmic reticulum calcium-ATPase (SERCA) that actively imports Ca^{2+} from the cytosol into the ER lumen, and the inositol trisphosphate (IP_3) receptor (IP_3R), which mediates the transient release of Ca^{2+} into the cytosol according to physiological requirements (Fig. 2). Intracellular Ca^{2+} signaling is essential for orchestrating key events during cell division, differentiation, and metabolic processes occurring in mitochondria (12). A significant portion of Ca^{2+} released by the ER is taken up by mitochondria juxtaposed to ER Ca^{2+} release channels, and thus mitochondria play an important role in modulating Ca^{2+} signaling and recycling of Ca^{2+} into the ER (140).

However, depending on the amplitude of the Ca^{2+} signal as well as the condition of the cell, Ca^{2+} can also initiate apoptosis as a consequence of organelle disruption, free radical production, and the activation of Ca^{2+}-dependent phosphatases and proteases such as calcineurin and calpain (96).

The release of Ca^{2+} from the ER is a critical early event for the initiation of apoptosis induced by many apoptotic signals (115). The sensitivity of a cell to such apoptotic stimuli appears to be largely dependent on the ER Ca^{2+} load, with a high resting ER Ca^{2+} concentration sensitizing cells to apoptotic stimuli and a low ER Ca^{2+} concentration conferring resistance. Once released into the cytosol, Ca^{2+} is rapidly adsorbed by mitochondria that can trigger apoptotic responses by disrupting the mitochondrial respiratory chain and generating reactive oxygen species or by opening the PTP (122).

The original finding that Bcl-2 affected Ca^{2+} signaling was discovered over a decade ago. In these studies, Bcl-2 overex-

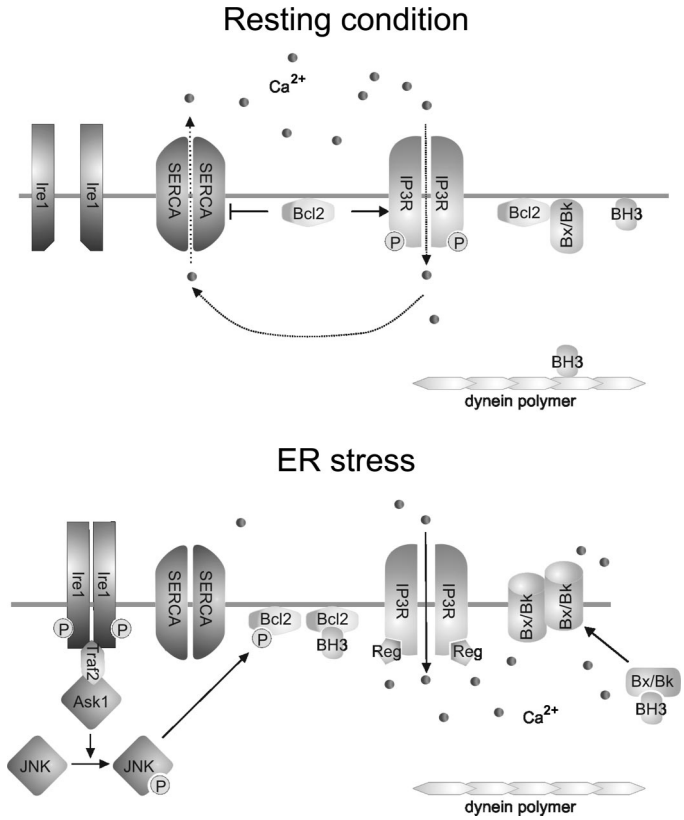

Fig. 2. Modulation of endoplasmic reticulum (ER) calcium by Bcl-2 family proteins under resting conditions and during ER stress. Functional interaction between the ER Ca^{2+} release channel inositol trisphosphate (IP_3) receptor (IP_3R) and the Bcl-2 family proteins Bcl-2 and Bcl-X_L is hypothesized to promote the leak of Ca^{2+} from the ER lumen into the cytosol. Parallel blockade of sacroplasmic/endoplasmic reticulum calcium-ATPase (SERCA) by Bcl-2 may attenuate active import of Ca^{2+} into the ER. Consequently, Bcl-2 and Bcl-X_L promote a lower resting state ER Ca^{2+} content and thus lower the cytosolic Ca^{2+} peak upon stress-mediated Ca^{2+} release. Bcl-2 has also been shown to block IP_3-mediated opening of the IP_3R and thus reduce Ca^{2+} release upon stress. During ER stress, the regulatory role of Bcl-2 and Bcl-X_L on IP_3R has been proposed to be compromised in a number of ways. First, interaction between Bcl-2/Bcl-X_L and IP_3R is inhibited by ER-localized Bax and Bak. ER-resident BH3-only protein Bik and the translocation of Bim and other BH3-only proteins may also contribute to this. Second, activation of JNK by the inositol-requiring enzyme 1 (IRE1)/apoptosis signal-regulating kinase 1 (ASK1) kinase arm of the unfolded protein response (UPR) leads to the phosphorylation of Bcl-2. Phosphorylated Bcl-2 is unable to regulate IP_3R and also becomes a target of proteosomal degradation. Finally, a number of other regulators (calcineurin, ATP, NADH, etc.) of IP_3R function can override the Bcl-2-mediated regulation or disrupt the interaction between Bcl-2 and IP_3R to trigger Ca^{2+} release. Bx, Bax; Bk, Bak; BH3, BH3-only Bcl-2 family member; Reg, IP_3R regulatory molecule.

pression reduced intracellular Ca^{2+} oscillations, thereby limiting the redistribution of Ca^{2+} from the ER to the mitochondria (8, 69). These early findings are in agreement with more recent data obtained from studies using ER-specific Ca^{2+} probes in HeLa cells (100), human prostate cancer cells (136), HEK-293 cells, and R6 fibroblasts (35) where Bcl-2 overexpression reduced ER Ca^{2+} content and thus conferred resistance against apoptosis. Moreover, an optimized Cameleon probe that permits the analysis of ER Ca^{2+} concentration at a single cell level demonstrated that Bcl-2 overexpression decreased resting ER Ca^{2+} levels specifically through increased leakage of Ca^{2+} into the cytosol (97). Subsequent studies found that Bcl-X_L was also able to enhance Ca^{2+} leak from the ER (147).

Several mechanisms have been proposed to explain how Bcl-2 and Bcl-X_L could augment ER ionic homeostasis. One early proposal was a direct release of ER Ca^{2+} through Bcl-2 and Bcl-X_L "ion channels" based on the discovery that the crystal structure of Bcl-X_L bore similarity to the pore-forming domains of the bacterial toxins colicins and diphteria toxin (80). Moreover, in addition to Bax, Bcl-2 and Bcl-X_L were shown to be capable of forming ion-conductive channels in synthetic lipid membranes (77, 112, 113). The Bcl-2 channel was shown to be selective for cations over anions; however, later studies showed selectivity for sodium, but not calcium, thus bringing an end to this particular line of reasoning (59, 112).

Efforts therefore turned to identifying Ca^{2+}-conducting channels on the ER surface that could be regulated by Bcl-2 and/or Bcl-X_L. Immunoprecipitation experiments identified a direct interaction between Bcl-2 and subunits 1 and 2b of SERCA resulting in its destabilization and inhibition (31, 32, 58). In a prostate cancer cell line, overexpression of Bcl-2 reduced the expression of SERCA2b, providing another possible explanation for the Bcl-2-mediated reduction in ER Ca^{2+} content (136). However, this finding contradicted an earlier study demonstrating that in a breast epithelial cell line, Bcl-2 overexpression led to increased SERCA2 expression (58, 136). These conflicting studies could be due to differences in the cell lines used or the expression levels of Bcl-2 induced.

Perhaps more convincing is the proposed direct and functional interaction between Bcl-2, Bcl-X_L, and the Ca^{2+} release channel, IP_3R (24, 44, 91, 147; Fig. 2). However, although there is consensus in the literature that the interaction between Bcl-2 and IP_3R limits the amplitude of Ca^{2+} signaling between the ER and mitochondria (91), the mechanism by which this is achieved is still being debated. Several groups have proposed that the association between IP_3R and either Bcl-2 or Bcl-X_L increases the phosphorylation and thus enhances its Ca^{2+} conductance (91, 147). This in turn supports the view that Bcl-2 and Bcl-X_L promote cell survival by maintaining a reduced ER Ca^{2+} concentration and consequently limiting the amount of Ca^{2+} that can be released upon cellular stress. An alternative hypothesis is that the functional interaction between Bcl-2 and IP_3R inhibits IP_3-mediated Ca^{2+} release upon a stress stimulus, leading to a decreased elevation of cytosolic Ca^{2+} levels and a similarly negative impact on mitochondrial Ca^{2+} uptake (24, 44). This latter model is convincingly supported by a recent report from the Distelhorst laboratory in which a peptide designed from an identified Bcl-2-binding region on IP_3R was able to displace Bcl-2 from IP_3R and reverse Bcl-2's inhibitory effect on IP_3R channel activity (107). The peptide also abrogated T-cell receptor (TCR)-induced Ca^{2+} elevation and apoptosis. If Bcl-2 and Bcl-X_L do indeed inhibit IP_3R channel activity, it raises the possibility that ER-localized Bax and Bak, through inhibiting Bcl-2 and Bcl-X_L function, could potentiate the release of ER Ca^{2+} during stress. However, this model does not provide an explanation for the widely observed ability of Bcl-2 and Bcl-X_L, to reduce the resting ER Ca^{2+} concentration. Nonetheless, that IP_3R channels represent the principle conduit through which Ca^{2+} is released from the ER to initiate apoptosis is also supported by a study demonstrating that cytochrome *c* released from mitochondria can translocate to the ER and, by directly binding to the carboxy-terminus of IP_3R channels, promote

IP_3R-mediated Ca^{2+} release (14). The apparent complexity and controversy of this field could be addressed by separately examining ER- versus mitochondrion-targeted Bcl-2 and thus distinguish between the direct and indirect effects (i.e., control of cytochrome *c* release). This question gets further emphasis when the wide range of IP_3R regulatory molecules, such as ATP or NADH, and their connection to the Bcl-2 family are taken into consideration.

Interestingly, it has recently been shown that the antiapoptotic function of Bcl-2 in protecting cells against Ca^{2+}-dependent death stimuli is dependent on its phosphorylation state. Phosphorylated Bcl-2, which is predominantly localized to the light membrane fraction rich in ER, is unable to interact with and neutralize proapoptotic Bcl-2 family proteins and is defective at lowering ER Ca^{2+} content (10, 91). Bcl-2 has been identified as a target of multiple kinases and is phosphorylated in response to various stimuli. JNK, known to phosphorylate Bcl-2 at the G_2/M stage of the cell cycle, is activated during ER stress and has recently been suggested to phosphorylate Bcl-2 on the ER surface (10, 91). How JNK-mediated phosphorylation of Bcl-2 affects its interaction with the IP_3R, or if it actually blocks the ability of Bcl-2 to inhibit SERCA, is still to be answered. Interestingly, a subsequent study, using sequential affinity chromatography to characterize Bcl-2-binding partners on the ER surface, identified a direct interaction between Bcl-2 and the serine/threonine protein phosphatase 2A (PP2A), which the authors suggested promoted the antiapoptotic function of Bcl-2 by protecting it from proteosomal degradation (64). Thus, posttranslational modifications, in addition to transcriptional control, may play a critical role in manipulating the balance between pro- and antiapoptotic signaling originating from the ER.

In contrast to the basal, physiological Ca^{2+} leak from the ER promoted by Bcl-2 and Bcl-X_L, it has been suggested that ER-localized Bax and Bak function to elevate the resting ER Ca^{2+} concentration and to trigger a large-scale efflux of the ER Ca^{2+} pool into the cytosol and its uptake by mitochondria during severe ER stress (91, 115). This view is supported by the observation that cells deficient in both Bax and Bak exhibit lower resting ER Ca^{2+} content and are protected from apoptotic stimuli that signal through Ca^{2+} (20, 115). Restoration of normal ER Ca^{2+} content could be achieved with re-expression of Bax, whereas an ER-targeted Bak mutant (but significantly neither Bax nor Bak targeted to the mitochondria) enhanced the depletion of ER Ca^{2+} stores during thapsigargin-induced ER stress in $Bax^{-/-}Bak^{-/-}$ mouse embryonic fibroblast (MEF) cells (115, 159).

Evidence that ER stress triggers the homooligomerization of Bax and Bak on the ER surface raised the possibility that Bax and Bak directly elicited the release of Ca^{2+} across the ER membrane by forming Ca^{2+}-conducting channels (90, 115, 159). However, in vitro studies demonstrated that the pores formed by Bax or Bak in lipid bilayers were selective for monovalent anions (113, 159). It waits to be examined what is the effect of BH3-only proteins on the type of Bax/Bak channels formed in the ER membrane. BH3-only proteins, similarly to their effect on the mitochondria, may promote the formation of Bax/Bak megachannels, large enough for the release of not only ions, but proteins up to the size of 50–60 kDa, thus allowing quick release of Ca^{2+} from the ER lumen. Furthermore, to date, no direct interaction between either Bax or Bak with an endogenous ER Ca^{2+} channel has been found. A recent report demonstrated an enhanced interaction between Bcl-2/Bcl-X_L and IP_3R leading to an increase in ER Ca^{2+} leak in the absence of Bax and Bak, suggesting that Bax and Bak manipulate ER Ca^{2+} signaling indirectly through inhibition of Bcl-2 and Bcl-X_L function on the ER surface (91; Fig. 2). Interestingly, modulation of ER Ca^{2+} signaling by Bax and Bak has also recently been shown to be essential for Ca^{2+}-dependent signaling in peripheral T cells in response to TCR stimulation (53). Thus, the presence of both pro- and antiapoptotic members of the Bcl-2 family at the ER appears to regulate not only Ca^{2+}-dependent death signal transduction but also diverse Ca^{2+}-dependent physiological functions ranging from mitochondrial energy metabolism to T-cell activation.

Several BH3-only proteins are localized at or translocate to the ER under conditions of cell stress (127). Although a specific role in regulating ER Ca^{2+} has been reported so far only for Bik/Nbk, Puma, and Nix/BNIP3 (67, 73), it is possible that all ER-targeting BH3-only proteins are potentially important regulators of ER Ca^{2+}-dependent apoptosis as a consequence of their modulating influences on the activities of pro- or antiapoptotic multidomain Bcl-2 family proteins at the ER surface.

In contrast to most other BH3-only proteins, Bik/Nbk is almost exclusively targeted to the ER membrane (72) where it interacts with Bax and Bak. Although Bik/Nbk does not appear to be induced by classical ER stress pathways resulting from protein malfolding (73), its privileged location at the ER nevertheless is utilized by other cell stress stimuli to relay a Ca^{2+}-dependent death signal from the ER to the mitochondrion. For example, in response to genotoxic stress simulated by ectopic expression of p53, ER-localized Bik/Nbk was shown to be required for the early release of ER Ca^{2+} in a Bax/Bak-dependent manner and the subsequent mitochondrial release of cytochrome *c* (73). Moreover, in the same study, overexpression of Bik/Nbk was shown to promote the recruitment and oligomerization of Bak at the ER membrane, suggesting that Bik/Nbk may play an equivalent role to that proposed for Bid at the mitochondria. Another BH3-only protein classically associated with p53-dependent apoptosis, Puma, is strongly upregulated during ER stress (106) and has recently been implicated in contributing to ER Ca^{2+} depletion-induced apoptosis (67). In this study, however, Puma was shown to be upregulated in response to thapsigargin treatment independently of p53 and to promote ER Ca^{2+} release through modulation of Bax activity.

Nix/BNIP3 is induced in cardiomyocytes during cardiac stress (30) and localizes to both mitochondria and the ER where it has recently been shown to play distinct roles in mediating Ca^{2+}-dependent apoptotic cell death (29). Nix/BNIP3 is known to target the mitochondria where it promotes the release of cytochrome *c* (153). In addition to this canonical role, Diwan and colleagues (29, 30) demonstrated that ER-localized Nix/BNIP3 was required to induce Ca^{2+}-dependent PTP opening and resultant loss of $\Delta\Psi_m$ in cardiomyocytes. However, the mechanism by which Nix modulates ER Ca^{2+} load is unknown.

Bax inhibitor-1 protein (BI-1) is an ER-localized, multitransmembrane protein initially discovered by a cDNA library screen in yeast for proteins that inhibited apoptosis induced by expression of mammalian Bax (151). BI-1 contains a BH3

domain and interacts directly with both Bcl-2 and Bcl-X_L; however, it does not appear to affect the function of Bax through direct interaction and it acts in an antiapoptotic manner (151). BI-1-deficient cells display an increased sensitivity to ER stress stimuli (9), whereas BI-1-overexpressing cells are resistant, exhibiting reduced translocation of Bax to the MOM (19). In addition, BI-1 has recently been implicated in regulating ER Ca^{2+} signaling (19, 146). Correlating with its antiapoptotic action, manipulating the expression levels of BI-1 alters ER Ca^{2+} content in a similar way as Bcl-2 or Bcl-X_L in several cell types (19, 146, 150). Suggested mechanisms by which BI-1 reduces ER Ca^{2+} content have included upregulation of IP_3R activity (perhaps by preventing displacement of Bcl-2/Bcl-X_L from IP_3R by proapoptotic Bcl-2 family proteins); modulation of other ER Ca^{2+} channels, such as SERCA (52); or formation of a homomeric transmembrane Ca^{2+}-conducting channel (146). A recent report by Xu and Reed has demonstrated that BI-1 is required for the ER Ca^{2+}-lowering activity of Bcl-X_L,; however, the molecular mechanism by which BI-1 affects Ca^{2+} storage has yet to be fully elucidated (150).

Regulation of the unfolded protein response. Cellular stresses leading to perturbed calcium homeostasis, redox state, or decreased ATP levels can interfere with protein folding. Disruption of ER function can be modeled pharmacologically through agents that selectively disturb calcium homeostasis (thapsigargin), redox balance (dithiotreitol; 68), inhibit protein modifications (tunicamycin; 33) or protein trafficking (brefeldin A; 78). All of these signals induce protein misfolding and activation of an adaptive stress response termed the unfolded protein response (UPR). The UPR attempts to increase the folding capacity of the ER through the induction of key proteins involved in chaperoning, protein folding, and degradation pathways (125). If, however, the stress is too great, an apoptotic program is activated.

Transduction of the UPR pathway occurs through three ER resident proteins that function as primary sensors of ER stress: PKR-like ER kinase (PERK, 46), inositol-requiring enzyme 1 (IRE1α and -β) (130, 131, 141), and activating transcription factor 6 (ATF6α and -β) (48, 142). All three of the stress sensors are transmembrane proteins containing luminal domains that sense the accumulation of unfolded proteins within the ER lumen. In the absence of stress, glucose-regulated protein 78 (GRP78) is bound to all three, maintaining the sensors in an inactive configuration (13, 116). Accumulation of unfolded proteins and the altered redox status within the ER lumen trigger the dissociation of GRP78 from all three sensors, leading to their initiation of UPR signal transduction pathways (82, 127).

The Bcl-2 protein family and UPR. Recent publications have brought to light a new function for the Bcl-2 family as potentially critical regulators of UPR induction (Fig. 3). First, both Bak and Bax have been shown to modulate UPR signaling. In $Bax^{-/-}Bak^{-/-}$ cells, the activation of the IRE1 arm of the UPR is impaired, leading to a decrease in splicing of XBP-1 and reduced phosphorylation of JNK (49; unpublished observations). Furthermore, Bax and Bak were shown to bind directly to the cytosolic face of IRE1 (49), suggesting that Bax and Bak can stabilize the active form of IRE1 and consequently enhance signal transduction. Furthermore, BI-1 inhibits the UPR, which contrasts with the opposite effect of Bax and Bak on this pathway, although the molecular mechanism underlying the effect of BI-1 on IRE1 signaling has not been elucidated. Furthermore, the ER-localized BH3-only member, Bik/Nbk (37), has been shown to influence ER calcium release. Thus, it is tempting to speculate that, depending on the cellular context and the stimuli, Bcl-2 and Bcl-X_L may influence the UPR by binding and modulating the function of Bax, Bak, Bik/Nbk, or BI-1 at the ER membrane. To support this idea, we found that the absence of Bcl-2 affected the upregulation of ER stress-induced UPR target genes. We found that induction of CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP), HERP, Hox1, ER degradation-enhancing α-mannosidase-like protein 1 (EDEM1), endoplasmic reticulum protein 72 (ERP72), tryptophanyl tRNA synthetase (WARS), and p58 inhibitor of interferon-induced double-stranded RNA-activated protein kinase (PKR) ($P58^{IPK}$) was increased in Bcl-2-deficient MEFs in response to thapsigargin and tunicamycin treatment (unpublished observations). It remains to be determined whether Bcl-2 affects the UPR signaling by binding to Bax, Bak, Bik/Nbk, or BI-1 at the ER or by an as yet unidentified mechanism. We propose a model in which the balance between the anti- and proapoptotic Bcl-2 members at the ER membrane controls IRE1 signaling and where disruption of the interaction between Bax/Bak and IRE1 will abrogate the prosurvival effects of this pathway.

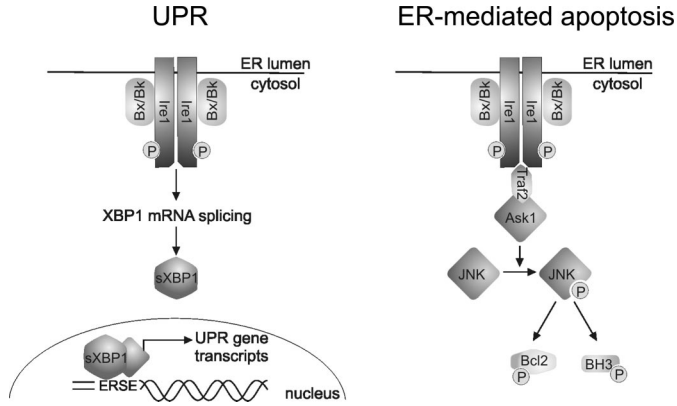

Fig. 3. Involvement of Bcl-2 family proteins in linking UPR to ER-mediated apoptosis. Activation of the IRE1 arm of the UPR is augmented by Bax and Bak. Upon activation of IRE1, IRE1 dimerizes and cross-autophosphorylation occurs by its serine/threonine kinase activity. The endoribonuclease activity of IRE1 then cleaves the mRNA of the transcription factor XBP-1, producing a frame shift spliced variant of XBP1 (sXBP1) with higher stability and transactivational activity. sXBP1 translocates to the nucleus, where it induces the transcription of ER stress response element (ERSE)-containing UPR genes. During prolonged ER stress, the UPR initiates apoptotic signaling pathways via differential modulation of Bcl-2 family proteins. Bax and Bak regulate activation of the IRE1/TNF receptor-associated factor 2 (TRAF2)/ASK1 arm of the UPR, leading to JNK activation. JNK positively regulates the proapoptotic BH3-only proteins Bim and Bad and negatively regulates Bcl-2. This shift in the balance toward proapoptotic members within the Bcl-2 family poises the cell for MOM permeabilization and activation of caspases.

Furthermore, Bak has been shown to have a unique function at the ER that is not shared by Bax. Coexpression of Bak and Bcl-X_L caused a prominent ER swelling and cytoplasmic vacuolization and ER remodeling (56). A Bak mutant lacking the BH1 domain lacked this activity, suggesting a role for the BH1 domain in this phenomenon (56). The Bak-induced ER structural changes were potentiated by dantrolene and inhibited by caffeine, pointing to a possible regulatory role of ryanodine receptor channels in this process (56). Considering the role of XBP-1 in ER expansion and remodeling in response to pro-

longed ER stress as well as secretory cell differentiation (60, 120, 132), further investigation may reveal interplay between Bak and XBP-1.

The Bcl-2 protein family and the commitment to apoptosis. During this phase, an attempt is made to rectify the protein-folding abilities of the ER. This is achieved by halting general protein translation to reduce the ER protein load and promoting the upregulation of ER chaperones and protein degradation pathways to aid protein folding and the clearance of malformed protein aggregates. However, in parallel with this adaptive phase, the cell prepares for an alternative situation in which ER homeostasis cannot be rectified and a threshold-dependent commitment is made to the initiation of apoptosis.

UPR signaling is known to lead to the activation and induction of several proapoptotic proteins (Fig. 3). In addition to the clear prosurvival function of IRE1 mediated by XBP-1, IRE1 can also activate a number of proapoptotic molecules (18, 135, 152). IRE1 recruits TNF receptor-associated factor 2 (TRAF2) that in turn recruits apoptosis signal-regulating kinase (ASK1) that activates c-Jun amino terminal kinase (JNK) (135). JNK is a major proapoptotic signaling kinase that targets several Bcl-2 family proteins and modulates their activity via phosphorylation. For example, JNK-directed phosphorylation of Bcl-2 inhibits its antiapoptotic activity and its ability to regulate ER Ca^{2+} release (10), whereas phosphorylation of Bax and Bim enhances their proapoptotic effects (98, 101). Corroborating the proapoptotic signaling by IRE1, overexpression of IRE1 triggered apoptosis (141). Similarly, overexpression of ASK1 induced apoptosis, whereas neuronal cells from $ASK1^{-/-}$ mice were resistant against lethal ER stress (47, 89). However, functional analysis of TRAF2 through genetic studies defies the model, because $TRAF2^{-/-}$ cells are more prone to apoptosis.

Although both the PERK and IRE1 arms of the UPR activate transcription factors that upregulate expression of the proapoptotic factor CHOP/GADD153, the PERK/eukaryotic initiation factor 2α (eIF2α)/ATF4 arm appears to be more important (45). $CHOP^{-/-}$ cells are partially resistant to ER stress-induced apoptosis, as are $C/EBP^{-/-}$ cells since C/EBP is a major dimerization partner for CHOP-mediated transcription (74, 158). CHOP deletion was also found to protect mice from dopaminergic neuron loss following exposure to 6-hydroxydopamine (119). CHOP, as a transcription factor, has been shown to repress Bcl-2 expression and induce Bim, thereby altering the balance between the pro- and the antiapoptotic Bcl-2 family members (75, 103). The involvement of Bim as a potential conduit for the transfer of the proapoptotic signal from the ER to the mitochondrion during ER stress has recently come to light (79, 126). In healthy cells, Bim is localized to the cytoskeleton, where it binds to the dynein motor complex. However, Bim has been reported to translocate to the ER following tunicamycin treatment and is subsequently maintained in a dephosphorylated state by PP2A, protecting it from ubiquitin-directed degradation during ER stress (103). Thus, the combined transcriptional upregulation of Bim by CHOP and its sustained activity during ER stress as a result of posttranslational modification could represent a principle mechanism through which the apoptotic cascade is set in motion during ER stress. On the other hand, although $CHOP^{-/-}$ cells are partially resistant to ER stress-mediated apoptosis, $CHOP^{-/-}$ mice develop normally, most likely due to the redundancy of the apoptosis pathway (158). It should be noted that cells from PERK knockout and phosphorylation mutant eIF2α (eIF2αS51A) knock-in mice are highly susceptible to ER stress, despite the fact that they are incapable of inducing CHOP upon ER stress.

Bim is not the only BH3-only protein that has been shown to play a role in ER stress-mediated apoptosis; both Puma and Noxa are also upregulated during ER stress in what appears to be a cell type-specific manner (62, 103, 106). Puma in particular has been shown to be critical for the initiation of apoptosis in cardiomyocytes during ischemia-reperfusion injury (94) and when treated with tunicamycin or thapsigargin (88), all of which are known to induce ER stress. Puma has also been shown to be required for the initiation of apoptosis in neuronal cells triggered by the abnormal accumulation of malformed proteins associated with several neurodegenerative diseases (for example, amyotrophic lateral sclerosis; 54) or as a result of disrupting cellular protein degradation pathways using proteasome inhibitors (26). Furthermore, Puma and Noxa can be induced by p53, and ER stress-induced apoptosis is partially suppressed in p53-deficient cells (62), illustrating that both p53-dependent and -independent proapoptotic pathways are active during ER stress. However, although both Puma and Noxa undoubtedly play a role in ER stress-induced apoptosis and in the case of Puma either in a p53-dependent or a p53-independent manner (67, 105), a direct molecular relationship linking them to components of the UPR has yet to be identified.

Bcl-2 proteins and relaying the ER stress signal to the mitochondria. Eventually, under conditions of prolonged and severe stress, the UPR is unable to retain ER homeostasis and apoptosis is triggered. Extensive research has demonstrated that, in most cases, ER stress-induced apoptosis is dependent on the mitochondrial pathway. For example, cells deficient in Bax and Bak are completely resistant to apoptosis triggered by ER stress-inducing stimuli (108, 145, 155). However, since this could be a consequence of Bax's and Bak's known function at the ER, more compelling evidence includes the observed attenuation of ER stress-induced apoptosis in APAF-1-deficient MEF and embryonic cortical cells (118) and as a result of ectopic expression of cytomegalovirus-encoded mitochondrial inhibitor of apoptosis, a specific inhibitor of the mitochondrial anterior nucleotide transporter (15). Overall, precisely how the apoptotic signal is transferred from the ER to the mitochondria remains controversial. As has been discussed, Ca^{2+} released from the ER during ER stress is known to promote the release of cytochrome *c* (15, 42, 155). Alternatively, ER stress is known to induce the translocation of Bax to the mitochondrial membrane (108, 145, 155), leading to the release of cytochrome *c* through Bax homooligomeric pores in the MOM.

Recent results brought another ER resident molecule in the forefront as the initiator of mitochondrial MOM permeabilization and cytochrome *c* release. B-cell receptor-associated protein 31 (Bap31, and its homologue, Bap29) is an ER transmembrane molecule and a cargo receptor for the ER quality control system and ER-associated protein degradation (5, 138). Bap31 contains a pseudo-death effector domain serving as a docking and activation platform for procaspase-8 (86). After binding and activation of caspase-8, caspase-8 cleaves off a 20-kDa fragment of Bap31 (16, 86). The p20 fragment induces Ca^{2+}-dependent mitochondrial fission, MOM permeabiliza-

tion, and cytochrome *c* release (17, 87). Interestingly, Bcl-2 and Bcl-X_L bind to Bap31 and prevent the activation of procaspase-8, the generation of the p20 fragment, and, ultimately, transmission of the death signal to the mitochondria (85, 86). Whether Bcl-2 also regulates the protein-sorting activity of Bap31 and this way maintaining and supporting ER function is to be answered.

Regulation of ER stress-induced autophagy by the Bcl-2 protein family. There is now consensus in the literature that ER stress is a potent inducer of autophagy (50); however, the precise molecular pathways linking ER stress to autophagy are still being worked out (an overview of how ER stress-induced autophagic pathways are thought to be regulated by Bcl-2 family proteins is depicted in Fig. 4). Furthermore, a persisting bone of contention is whether autophagy in this context is ultimately a cytoprotective mechanism or a precursor to a form of nonapoptotic cell death resembling necrosis (50, 134). In much the same way as a prolonged UPR leads to cell death via apoptosis, continued autophagy is detrimental to cell survival as a consequence of excess organelle and macromolecular catabolism. However, in a cytoprotective capacity, induction of autophagy plays an important role in counteracting ER expansion (11) and degrading protein aggregates during ER stress (129).

Beclin-1, the mammalian ortholog of yeast Atg6 and essential for the induction of autophagy, was initially identified as a Bcl-2-interacting protein in a yeast two-hybrid screen (63). The

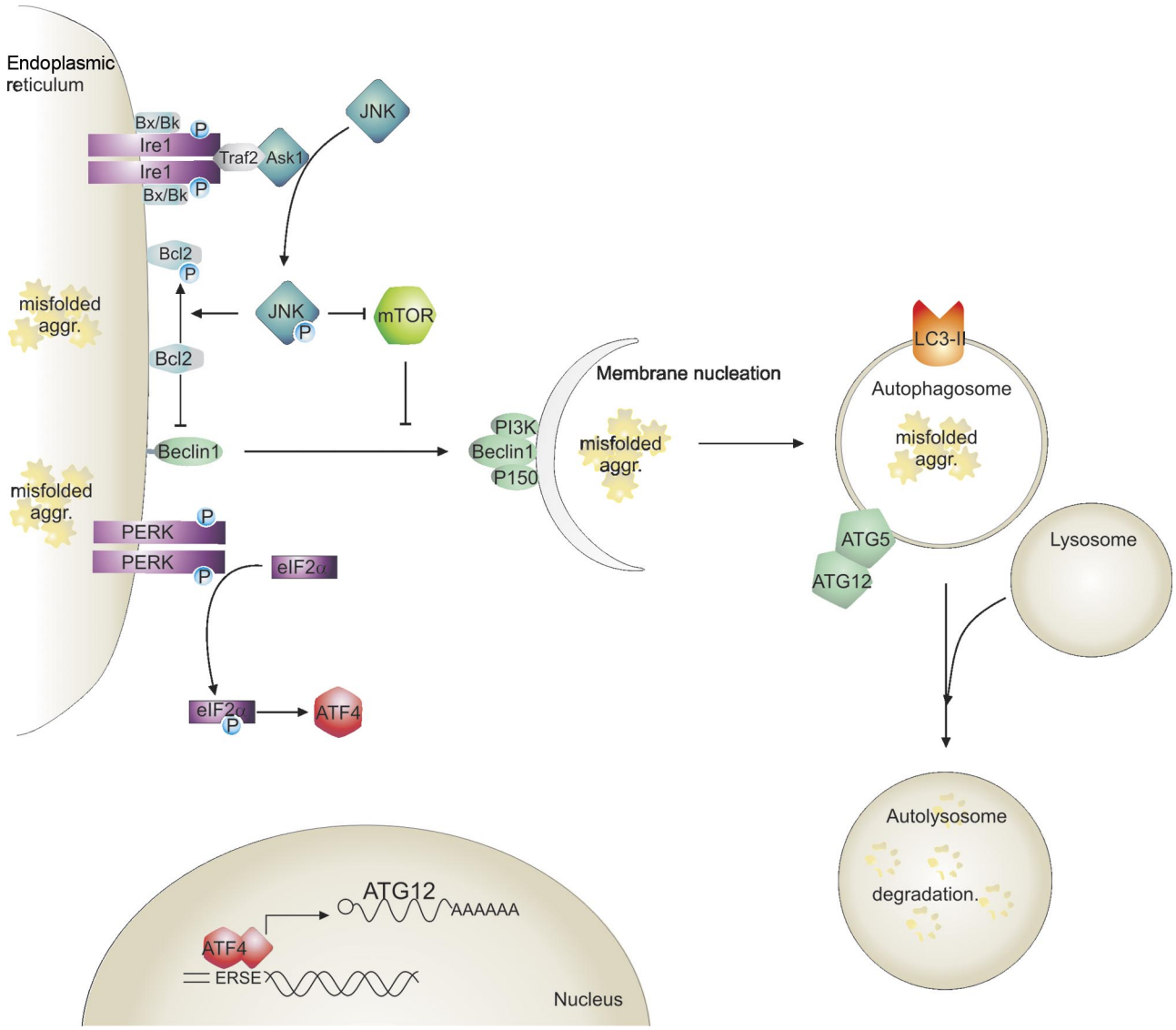

Fig. 4. Role of Bcl-2 family proteins in regulating ER stress-induced autophagy. In response to accumulation of malfolded protein aggregates within the ER lumen, the autophagic degradation pathway is initiated. Beclin-1 (also known as Atg6) is essential for the induction of autophagy and is sequestered by Bcl-2 on the ER membrane under resting conditions. Beclin-1 forms a complex with a class III phosphatidylinositol 3-kinase (PI3K) and P150 myristoylated kinase to initiate membrane nucleation and elongation around target cargo. Two ubiquitin-like conjugation systems (LC3I→LC3II and Atg5–Atg12) are required to form double membrane-walled autophagosomes. Fusion between autophagosomes and lysosomes forms autolysosomes within which sequestered material is degraded. Two arms of the UPR have been implicated in regulating induction of autophagy during ER stress. The IRE1/JNK pathway has been shown to enhance induction of autophagy; however, how JNK exerts this effect is unknown, as is the requirement of Bax and Bak in this pathway. PKR-like ER kinase (PERK) signaling is also thought to potentiate autophagy, possibly via transcriptional upregulation of Atg12. mTOR, mammalian target of rapamycin; aggr, aggregates; ATF4, activating transcription factor 4.

functional significance of this interaction was not revealed until 2005, when Pattingre and colleagues (99) reported that Bcl-2 suppressed starvation-induced, beclin-1-dependent autophagy in both yeast and mammalian cells. Thus, for the first time, direct evidence for a point of convergence in the regulation of apoptotic and autophagic pathways was provided. Significantly, although endogenous beclin-1 is found at mitochondria, the ER, and the *trans*-Golgi network (55, 63), the interaction between Bcl-2 and beclin-1 was found to take place exclusively on the surface of the ER (99). This suggests that the regulatory hold held by Bcl-2 over beclin-1 is particularly responsive to alterations in ER homeostasis.

Recently, Bcl-X_L has also been shown to directly interact with beclin-1 on the ER surface and to have a similar inhibitory influence on autophagy induction (71). Analysis of the crystal structure obtained from the interaction between Bcl-X_L and a peptide derived from beclin-1 demonstrated that beclin-1 contains a BH3 domain (92). This raises the intriguing possibility that BH3-only proteins may also contribute to the regulation of ER stress-induced autophagy, perhaps by displacing the beclin-1 BH3 domain from either Bcl-2 or Bcl-X_L. So far, only Bad, which is known to be induced during starvation (27), has been implicated in promoting autophagy via negative regulation of ER-localized Bcl-2/ Bcl-X_L (70). Whether other BH3-only proteins could participate in the induction of autophagy during ER stress in a similar way remains to be determined, although BNIP3, in light of its recent documented involvement in the induction of autophagy during myocardial ischemia-reperfusion injury, is a potential candidate (43, 124).

Although the functional contribution made by antiapoptotic multidomain Bcl-2 and Bcl-X_L to ER stress-induced autophagy is becoming established, defining the contribution made by ER-localized proapoptotic multidomain Bax and Bak is still some way off. Nevertheless, regulation of the IRE1 arm of the UPR by Bax/Bak is emerging as a potentially important regulatory checkpoint for the induction of autophagy during ER stress. IRE1 has recently been shown to be required for the induction of autophagy in cells treated with the ER stressors tunicamycin and thapsigargin (94), as well as in response to cellular protein overload as a consequence of proteasome inhibition (28). In particular, the IRE1-JNK branch of this signaling pathway was found to be important, because the inhibition of active JNK (but, significantly, not XBP-1) also attenuated induction of autophagy. It has been reported that ER-localized Bax and Bak are required for IRE1 signaling during ER stress (49; unpublished observation) and, in $Bax^{-/-}Bak^{-/-}$ cells, expression of phosphorylated JNK was significantly reduced. These findings therefore support the hypothesis that Bax and Bak are required for the induction of ER stress-induced autophagy via the IRE1-JNK signaling pathway and are in keeping with their counterbalancing influence on antiapoptotic Bcl-2 family protein activities. However, this hypothesis does not stand up when the induction of autophagy is monitored in $Bax^{-/-}Bak^{-/-}$ cells. In response to growth factor withdrawal, genotoxic stress as well as various ER stress-inducing agents, the rate at which autophagy is induced is comparable between wild-type and $Bax^{-/-}Bak^{-/-}$ cells (65, 117, 134), suggesting that neither the presence nor absence of Bax and Bak influences the induction of autophagy in these experimental systems. Further work is needed to elucidate the contribution made by ER-localized Bax and Bak to the induction of autophagy during ER stress and to determine the molecular mechanism through which JNK positively regulates autophagy.

Conclusion. Until relatively recently, the ER has been overlooked by the majority of researchers in the cell death community as an alternative site of convergence for Bcl-2 family proteins in the regulation of stress-induced apoptosis. Thanks to huge efforts by several laboratories, this oversight has been addressed and we now have a continuously growing understanding of the essential roles played by the Bcl-2 protein family in maintaining ER homeostasis and orchestrating ER stress-induced apoptosis. Although the mitochondrion remains the principle subcellular target of the Bcl-2 protein family during apoptosis, it could be argued that the activities of Bcl-2 family proteins at the ER are more relevant to sustaining cellular function and possibly setting the threshold of sensitivity to apoptotic stimuli. For instance, ER-localized Bcl-2 family proteins are engaged in regulating not just initiation of ER stress-induced apoptosis, but also in maintaining continued ER Ca^{2+} homeostasis, which is essential for intracellular Ca^{2+} signaling, induction of the UPR, and also induction of autophagy as adaptive responses to ER stress.

However, despite the huge volume of data obtained in the last few years of intensive research into the functions of Bcl-2 family proteins at the ER, pressing questions remain unanswered. For instance, does manipulating the kinetics of UPR activation affect the induction of apoptosis; are these two events intrinsically linked or completely independent signaling pathways; and, if linked, how is the proapoptotic message conveyed downstream of the ER? Recent data indicate that multiple transducers may be involved, such as Ca^{2+}, specific BH3-only proteins, and/or direct engagement of caspases. Furthermore, is the ability of the Bcl-2 family to potentiate or inhibit apoptosis from the ER wholly dependent on their modulation of ER Ca^{2+} levels? And we still do not know precisely by what mechanism Bax and Bak promote the release of Ca^{2+} across the ER—do they modulate the activity of Ca^{2+} channels such as IP_3R or does their reported oligomerization on the ER membrane mean that they are able to form Ca^{2+}-conducting pores? The momentum of current research in this field is such that answers to these questions (and others) are likely to emerge in the very near future. Ultimately, identifying new functions for the Bcl-2 protein family at the ER will provide a fresh perspective in the understanding of apoptosis regulation at the cellular level.

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