

Review

There is more to life and death than mitochondria: Bcl-2 proteins at the endoplasmic reticulum

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Abstract

Proteins of the Bcl-2 family are important regulators of cell fate. The role of these proteins in controlling mitochondrial apoptotic processes has been extensively investigated, although exact molecular mechanisms are incompletely understood. However, mounting evidence indicates that these proteins also function at the endoplasmic reticulum and other locations within the cell. Both pro- and anti-apoptotic Bcl-2 family members can regulate endoplasmic reticulum calcium, cellular pH and endoplasmic reticulum resident proteins. In this review, we discuss the activities and potential targets of Bcl-2 family members at the endoplasmic reticulum and other cellular locations. © 2003 Elsevier B.V. All rights reserved.

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1. Introduction

The Bcl-2 family of proteins can be separated into two functional categories, either pro- or anti-apoptotic (recently reviewed in Ref. [1]). Although cellular functions for these proteins are well recognized, the precise molecular mechanism by which these proteins regulate cell death (either positively or negatively) is controversial and the subject of many ongoing investigations. To be designated as a Bcl-2 family member, a protein must possess at least one of the four conserved Bcl-2 homology (BH) domains and have a demonstrable effect on apoptosis. Interactions between family members mediated by BH domains are critical for many aspects of their function. However, the sequence diversity of these proteins suggests a level of diversity in function that has not been fully explored: indeed, the number of both pro- and anti-apoptotic members exceeds those needed even in current models of multi-step regulation of apoptosis (e.g. Ref. [2]).

The three-dimensional structure of Bcl-X_L [3], Bid [4], Bax [5], Bcl-2 [6], and Bcl-w [7,8] are more similar than

predicted from primary sequence alignments. Each protein has two central hydrophobic helices surrounded by amphipathic helices in a structure that resembles the pore-forming domain of the bacterial diphtheria toxin. Consistent with the known structures, there is good in vitro evidence that some of these proteins can form pores or channels in lipid bilayers (reviewed in Ref. [9]). Thus, a combination of protein:protein interactions and pore/channel formation are postulated to constitute the main molecular mechanisms of Bcl-2 activity. Since many of the Bcl-2 family members have similar structures, the sequence diversity may determine subtle aspects of the regulation of these proteins through specific protein:protein interactions.

Bcl-2, the prototype for the Bcl-2 family, contains all four BH domains (BH1–4) and a carboxyl-terminal hydrophobic sequence called a tail-anchor. Unlike many other tail-anchor proteins which are targeted to a single subcellular location, Bcl-2 is localized to the endoplasmic reticulum (ER), nuclear envelope, and outer mitochondrial membrane in mammalian cells [10]. This broad localization allows Bcl-2 to function at spatially distinct regions of the cell, thereby enhancing its anti-apoptotic activity. The discovery that Bcl-2 targets via a tail-anchor sequence occurred at roughly the same time as the elucidation of the mechanisms that regulate targeting and integration of tail-anchor sequences in subcellular membranes [11,12]. These studies suggested that it

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Table 1
Extra-mitochondrial locations for selected Bcl-2 family members

<i>Anti-apoptotic Bcl-2 proteins</i>	
Bcl-2	ER and nuclear envelope [10]
Bcl-X _L	ER and cytosol [71]
Bcl-w	ER? [72]
Mcl-1	ER? [73]
Boo/Divia	ER? [74]
A1	?
<i>Pro-apoptotic Bcl-2 proteins</i>	
Bax	ER [35,37], cytosol [71]
Bak	ER [35,37]
Bim	Cytoskeleton [62]
Bad	Cytosol [75]
Bik	ER [55], cytosol
Bmf	Cytoskeleton [63]
Bid	Cytosol and membranes (ER?) [76]
Spike	ER [53]

would be possible to manipulate the location of Bcl-2 proteins by generating tail-anchor mutants. By using this approach, we demonstrated that Bcl-2 does not need to be located at both physiological sites (the ER and mitochondria) to function; in fact in stably transfected cells, Bcl-2 prevents apoptosis from either organelle with overlapping but non-identical specificity [13]. The use of organelle-targeted mutants has been very important for studies analyzing the contribution of the ER to the regulation of apoptosis. The general details of the construction of these mutants and their use are described in this review in the section titled Mutants of Bcl-2 with restricted subcellular localization.

Since Liu et al. [14] discovered the importance of cytochrome *c* in the induction of apoptosis, mitochondria have become the central focus for many investigations into the molecular mechanisms regulating apoptosis (reviewed in Ref. [15]). Many of these studies have shown that mitochondria are a point of convergence for extrinsic and intrinsic apoptotic pathways. Mitochondria also undergo extensive alterations during cell death, but the events leading up to mitochondrial dysfunction are poorly understood. Recently, a number of studies have highlighted the ER as a key upstream signaling organelle for the regulation of apoptosis, and consistent with this, several members of the Bcl-2 family are also located at the ER (Table 1). ER-specific functions for most of these proteins have yet to be discovered despite the emerging evidence that the ER is an important location for the regulation of apoptosis. One physiologically relevant candidate is control of ER Ca²⁺ levels. However, Bcl-2 family members also interact with proteins on the ER that are not involved in Ca²⁺ homeostasis.

2. ER Ca²⁺ and anti-apoptotic Bcl-2 proteins

The original observation that Bcl-2 regulates intracellular Ca²⁺ levels is now a decade old [16]. However, the effect of

Bcl-2 on intracellular and intra-organellar Ca²⁺ levels is still controversial and the literature contains conflicting data.

Overexpression of Bcl-2 in human breast epithelial cells results in increased [Ca²⁺]_{ER} [17], and in mouse lymphoma cells it increases Ca²⁺ uptake into the ER [18]. Although the mechanism by which Bcl-2 increases [Ca²⁺]_{ER} is not clear, breast cells expressing Bcl-2 have increased sarco(endo)plasmic reticulum Ca²⁺-ATPase (SERCA) pump expression [17]. Consistent with these results, increased expression of SERCA by transfection also increases [Ca²⁺]_{ER} [19]. Increased [Ca²⁺]_{ER}, or rather the maintenance of ER Ca²⁺ levels, promotes cell proliferation and growth, thus inhibiting cell death [18]. However, other studies indicate that Bcl-2 expression causes decreased [Ca²⁺]_{ER} in HeLa cells [20], human prostate cancer cells [21], HEK-293 (human embryonic kidney) cells and R6 fibroblasts [22]. These decreases in [Ca²⁺]_{ER} by Bcl-2 are postulated to protect cells from apoptosis by limiting the amount of Ca²⁺ released into the cytoplasm following an apoptotic stimulus. Reducing the amount of Ca²⁺ released into the cytoplasm in turn limits the uptake of Ca²⁺ into the mitochondria [23], which can lead to mitochondrial permeability transition and mitochondrial dysfunction (reviewed in Ref. [24]). In this case, lower [Ca²⁺]_{ER} is due to a Bcl-2 mediated increase in leakage of Ca²⁺ from the ER by an unidentified mechanism [20], which may involve the pore-forming capabilities of this molecule.

The above discrepancies between the reported effects of Bcl-2 on [Ca²⁺]_{ER} may be due to differences in cell lines, Bcl-2 expression levels, or the methodologies used to measure Ca²⁺. Intra-organellar Ca²⁺ levels have been measured by using either fluorescent dyes or Ca²⁺-binding proteins, both of which have important limitations.

Indirect measurements of [Ca²⁺]_{ER} using a cytoplasmic dye (e.g. Fura-2 or Indo-1) can detect the amount of Ca²⁺ released from the ER. The accumulation of Ca²⁺ in the cytoplasm is usually stimulated by the inhibition of the SERCA pump with either thapsigargin or tert-butyl-benzohydroquinone. Measurements of this kind require an even distribution of the fluorescent dye in the cytoplasm and are dependent on the amount of SERCA inhibitor added. When Bcl-2 was reported to increase [Ca²⁺]_{ER}, 1 μM thapsigargin was used [17]; by contrast, studies reporting decreased [Ca²⁺]_{ER} used ~ 100 nM thapsigargin [21,22]. As Bcl-2 interacts with SERCA directly [17], it is possible that this interaction makes SERCA more resistant to thapsigargin. If SERCA remains active following treatment with the lower amount of thapsigargin, it would cause [Ca²⁺]_{ER} to appear lower. In addition, dyes like Fura-2 may have unexpected effects on cell physiology when combined with toxic drugs.

Direct measurements of organellar Ca²⁺ have been made with targeted mutants of the photoprotein aequorin [25] or the fluorescent cameleon protein [26]. However, use of these organelle-targeted mutants requires genetic manipulation of cells and measurements cannot be taken until expression is sufficient (~ 24–36 h post-infection/transfec-

tion). Furthermore, expression of Ca^{2+} -binding proteins may alter the normal Ca^{2+} balance, thus complicating the interpretation of results.

Another disadvantage of ER-targeted aequorin is that ER Ca^{2+} depletion (usually accomplished by ionomycin) and refilling of ER with extracellular sources of Ca^{2+} is required. These manipulations may significantly alter mitochondrial function, cellular Ca^{2+} regulation and other aspects of cell physiology. Thus, the inherent limitations of current methods used to measure Ca^{2+} may influence the outcome of the studies on the role of Ca^{2+} in apoptotic signaling pathways. In addition, many investigations compare cells overexpressing Bcl-2 to control-transfected cell lines. Overexpressed Bcl-2 is certainly biologically relevant to several pathologies; however, transient overexpression of Bcl-2 in experimental systems can be toxic to cells due to membrane damage from rapid incorporation of large amounts of tail-anchor peptide into the bilayer [27]. Thus Bcl-2 expression levels (both the population average as well as variation between individual cells) may have unexpected effects on $[\text{Ca}^{2+}]_{\text{ER}}$ levels as well as membrane function.

Despite the limitations of current methods for measuring Ca^{2+} at steady state, it is clear that *changes* in cytoplasmic Ca^{2+} levels can trigger apoptosis. Indeed blocking Ca^{2+} signaling with cytoplasmic chelators inhibits cell death [28]. Release of Ca^{2+} from the ER initiates cell death by activating the Ca^{2+} -dependent protease calpain, caspases [29], and/or by directly causing mitochondrial dysfunction (reviewed in Ref. [24]). Crosstalk between the ER and mitochondria is a critical mechanism for determining cell fate [30]. Indeed, we have demonstrated that ceramide, a lipid second messenger, triggers apoptosis through the early loss of mitochondrial membrane potential [31], a process that is usually mediated by increased cytoplasmic Ca^{2+} [32]. Importantly, ceramide-induced cell death is inhibited by ER-localized Bcl-2 [31].

Bcl-2 is not the only anti-apoptotic family member that influences $[\text{Ca}^{2+}]_{\text{ER}}$: Bcl- X_L also decreases both the expression of the inositol-1,4,5-triphosphate receptor (IP_3R) and the amount of Ca^{2+} released following T cell stimulation [33]. In addition, in artificial lipid bilayers, luminal Ca^{2+} can inhibit cation-selective Bcl- X_L channels [34]. The effect of other anti-apoptotic Bcl-2 members (e.g. Mcl-1, Bcl-w or A1) on Ca^{2+} homeostasis has yet to be investigated.

3. ER Ca^{2+} and Pro-apoptotic Bcl-2 proteins

Consistent with the concept that Bcl-2 family members regulate similar aspects of cell physiology, recent investigation has demonstrated that the pro-apoptotic proteins Bax and Bak also modulate $[\text{Ca}^{2+}]_{\text{ER}}$ [35–37]. In human PC-3 prostate adenocarcinoma cells, ectopically expressed Bax or Bak is located in both mitochondria and the ER. The expression of these proteins causes a decrease in $[\text{Ca}^{2+}]_{\text{ER}}$, which can be inhibited by Bcl-2. In addition, the Bak/Bax-

induced decrease in $[\text{Ca}^{2+}]_{\text{ER}}$ causes a corresponding increase in uptake of Ca^{2+} into mitochondria. Furthermore, inhibition of mitochondrial Ca^{2+} uptake by treatment with RU360, an inhibitor of the Ca^{2+} uniporter, inhibits the release of cytochrome *c* and apoptosis induced by Bak/Bax overexpression [35]. It is interesting to note that in these cells, expression of Bcl-2 inhibits the release of ER Ca^{2+} induced by Bax expression. Whether this is the result of direct interaction between Bcl-2 and Bax or of Bcl-2 regulating Ca^{2+} levels independently of Bax remains to be determined.

In a human prostate cancer cell line (DU-145) that is deficient in Bax expression, ER Ca^{2+} is not released to the cytoplasm following staurosporine treatment. Ectopic expression of Bax in DU-145 cells restored the staurosporine-induced increase in cytoplasmic Ca^{2+} and cytochrome *c* release [36]. Prolonged overexpression of Bax in DU-145 cells caused a further decrease in $[\text{Ca}^{2+}]_{\text{ER}}$ with levels similar to those seen after staurosporine treatment. Thus, studies with human prostate cell lines demonstrate that overexpression of Bax or Bak results in decreased $[\text{Ca}^{2+}]_{\text{ER}}$ and indicate that Bax is required for staurosporine-induced release of Ca^{2+} from the ER.

The influence of Bax and Bak on intracellular Ca^{2+} has also been examined in double knock-outs of mouse embryonic fibroblasts (MEFs) where both the Bak and Bax alleles were deleted [37]. As measured by Fura-2 and ER-targeted aequorin, double knock-out cells have decreased $[\text{Ca}^{2+}]_{\text{ER}}$. As a consequence, these cells are more resistant to the lipid second messengers ceramide and arachidonic acid, which induce cell death by increasing cytoplasmic Ca^{2+} . Ectopic expression of HA-tagged-Bax restores $[\text{Ca}^{2+}]_{\text{ER}}$ to levels seen in wild-type MEFs and restores ceramide-induced cell death. Thus, in contrast to experiments with prostate cancer cells, the lack of Bax expression causes a decrease $[\text{Ca}^{2+}]_{\text{ER}}$ in MEFs. Aside from the different cell types studied, the apparently contradictory results may be due to differences in the time course and level of Bax expression. When expressed transiently at high levels, Bax causes the release of $[\text{Ca}^{2+}]_{\text{ER}}$; however, at low levels Bax appears to increase $[\text{Ca}^{2+}]_{\text{ER}}$.

In studies using the double knock-out MEFs, HA-tagged-Bax was targeted to mitochondria by adding the mitochondrial targeting sequence for inner membrane protein cytochrome *c* oxidase (subunit VIII) to the amino-terminus of HA-Bax. Unlike expression of wild-type Bax, expression of this mutant did not restore $[\text{Ca}^{2+}]_{\text{ER}}$. Therefore, overexpression of SERCA was used to increase $[\text{Ca}^{2+}]_{\text{ER}}$ to levels close to that of wild-type. By comparing double knock-out MEFs expressing either SERCA, mitochondrial-targeted Bax or both, the authors were able to separate the effects of alterations in $[\text{Ca}^{2+}]_{\text{ER}}$ from those of Bax on mitochondria. SERCA expression was required to restore cell death mediated by lipid second messengers; while tBid-induced apoptosis required the presence of Bax at the mitochondria. In contrast, induction of cell death in the double knock-outs

by etoposide, staurosporine or brefeldinA required the expression of both SERCA and the mutant Bax associated with mitochondria. Thus, Bax appears to function at both the ER and mitochondria in response to many death stimuli.

Although this is an elegant experimental system to investigate organelle-specific functions for Bax, several limitations are evident. Unlike Bcl-2 which is constitutively integrated into membranes, Bax is normally located in the cytoplasm or peripherally associated with membranes. Forcing Bax to be constitutively bound to membranes removes the translocation step that is important for regulation of Bax activity [38–42]. The carboxy-terminal tail of Bax is required for membrane association [5,39]; however, the amino-terminus of Bax may play a role in membrane localization and function [43–45]. Thus altering this region, by adding an HA-tag and/or a mitochondrial targeting sequence may further alter Bax function. It remains to be determined if more subtle mutants of Bax (e.g. BaxS184V [39,46]) that strongly enhance Bax-induced apoptosis also have effects on $[Ca^{2+}]_{ER}$. It would also be interesting to determine what effect regulated expression of ER-specific Bax mutants, reported to be too toxic to generate in the double knock-outs [37], have on the regulation of $[Ca^{2+}]_{ER}$.

Together, these studies suggest the following role for pro-apoptotic Bcl-2 members in the regulation of $[Ca^{2+}]_{ER}$: in healthy cells the pro-apoptotic proteins Bak and/or Bax maintain $[Ca^{2+}]_{ER}$. However, during apoptosis elicited by staurosporine or Bax overexpression, Bax and Bak cause the release of Ca^{2+} from the ER. This switch in activity could be due to a conformational change or an increased amount of protein integrated into the ER membrane. If this is true, then stable expression of Bak (which is constitutively integrated into membranes) in the double knock-out cells may not restore release of ER Ca^{2+} during apoptosis since induction of apoptosis would be unlikely to increase the amount of Bak at the membrane except perhaps by de novo synthesis. The results obtained after selective Bax relocalization suggest that Bak and Bax are not as functionally interchangeable as is usually proposed. Furthermore, translocation of Bax to the ER may be regulated differently than translocation to mitochondria.

Thus, the literature indicates that in different circumstances $[Ca^{2+}]_{ER}$ can be regulated in either direction by both pro- and anti-apoptotic Bcl-2 proteins. Bcl-2 can decrease $[Ca^{2+}]_{ER}$ [22,23] yet, depletion of $[Ca^{2+}]_{ER}$ can have detrimental effects on the cell [47]. Therefore, a delicate balance must exist between the activities of pro- and anti-apoptotic proteins in the regulation of $[Ca^{2+}]_{ER}$. The presence of a Ca^{2+} gradient between the ER (high) and the cytoplasm (low) indicates that regulation of $[Ca^{2+}]_{ER}$ by Bcl-2 proteins is unlikely to be explained solely by the ability of these proteins to function as ion selective pores: how would Bax/Bak ‘pump’ Ca^{2+} into the ER against a pre-existing concentration gradient? Since Bcl-2 interacts with SERCA and Bcl- X_L decreases IP_3R expression, perhaps Bax and/or Bak also directly interact with and modulate

existing Ca^{2+} channels/pumps to regulate $[Ca^{2+}]_{ER}$ independently of Bcl-2/Bcl- X_L . Alternatively, interactions between pro- and anti-apoptotic Bcl-2 family members via BH domains may be required to regulate $[Ca^{2+}]_{ER}$, or oligomerization and pore formation may be required. Clearly, these intriguing results suggest that many possibilities remain to be explored.

4. Other functions for Bcl-2 members at the ER

4.1. Bap31

Aside from the SERCA pump, another protein that interacts with Bcl-2 at the ER is the integral membrane protein Bap31 [48]. Bap31 forms a complex with pro-Caspase-8L and either Bcl-2 or Bcl- X_L at the ER [49]. When apoptosis is induced by activation of the Fas death receptor or by expression of adenoviral transforming protein E1A, Bap31 is cleaved by either Caspase-8 or Caspase-1 into a membrane integrated fragment (termed p20) that can efficiently induce apoptosis when expressed alone [48]. Expression of a mutant Bap31 resistant to cleavage by caspases inhibited cytoplasmic membrane blebbing, actin redistribution and cytochrome *c* release following Fas stimulation [50]. Expression of Bcl-2 inhibits the cleavage of Bap31 [49], and the pro-apoptotic activity of the p20 fragment [48]. Bap31 also interacts with myosin heavy chain and γ -actin. Fas stimulation disrupts both of these interactions, and the p20 cleavage fragment is unable to interact with γ -actin [51]. Thus the absence of specific morphological changes seen with the expression of caspase resistant Bap31 may be attributed to the prolonged tethering of Bap31 to actomyosin complexes. Ectopic expression of the p20 fragment of Bap31 induces the release of ER Ca^{2+} , leading to increased Ca^{2+} uptake by mitochondria and recruitment of dynamin-related protein (Drp1) to mitochondria causing mitochondrial fission [52].

Bap31 also interacts with the putative ion channel A4. Expression of caspase-resistant Bap31 in combination with A4 inhibits Fas-induced cell death [41]. Bap31 cleavage was not required for Bax translocation to mitochondria, but was required for Bax oligomerization. It is not clear how Bap31 cleavage at ER contributes to Bax oligomerization at mitochondria; however, it may be related to the Ca^{2+} signaling initiated by the p20 Bap31 fragment.

Spike is a BH3-only protein that is localized primarily to the ER [53]. Interactions between Spike and Bap31 displace the Bap31:Bcl- X_L interaction, suggesting that Spike regulates Bap31 activity. In addition, a mutant version of Spike that does not bind to Bap31 functioned as a dominant negative, inhibiting Fas-induced apoptosis as effectively as Bcl- X_L expression. Taken together these experiments suggest a compelling model for the regulation of apoptosis at the ER via the Bap31 complex (Fig. 1).

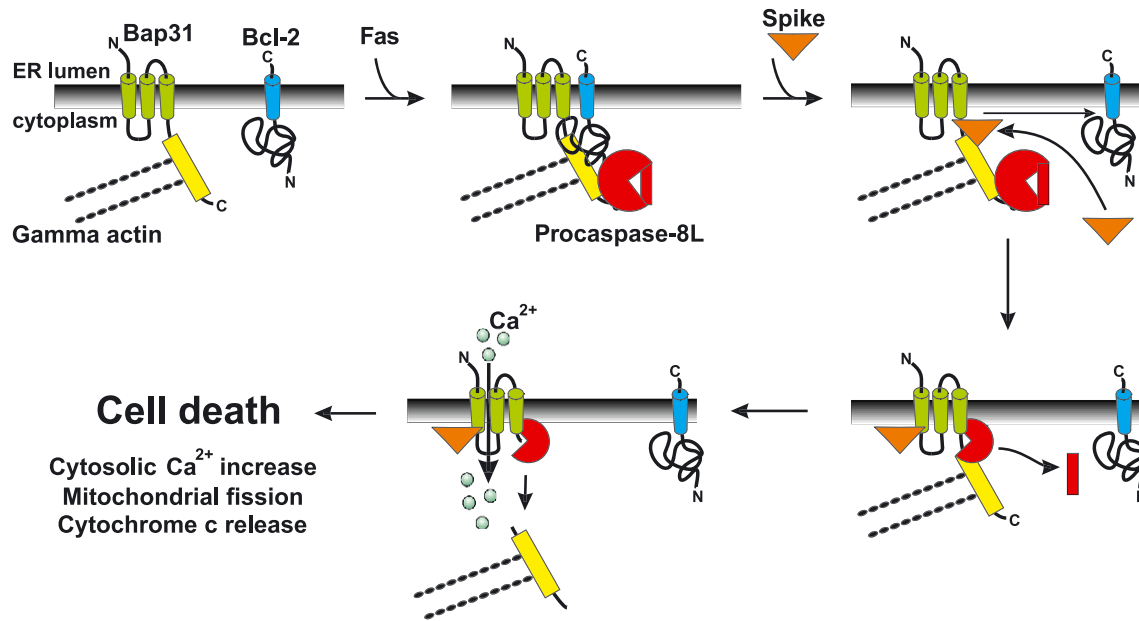


Fig. 1. Bap31 complex and apoptosis. In healthy cells, Bap31 exists as an integral ER membrane protein, possibly tethered to the cytoskeleton through interactions with γ -actin. Upon stimulation with the death receptor protein Fas or E1A, Bap31 forms a complex with Bcl-X_L and pro-Caspase-8L. In this state, a cell is protected from cell death. If the BH3 protein, Spike, is activated and can overcome the anti-apoptotic Bcl-X_L function, it then displaces the interaction between Bap31 and Bcl-X_L. This allows for the activation of pro-Caspase-8 to Caspase-8 (perhaps through multimerization of Bap31). Cleavage of Bap31 releases its tether on the cytoskeleton allowing its re-arrangement. In addition, the p20 membrane integrated fragment may also allow for the release of Ca^{2+} from the ER.

4.2. Binding of cytosolic pro-apoptotic factors by Bcl-2

Aside from interactions with ER resident proteins and membrane-bound pro-apoptotic family members that translocate to the ER; Bcl-2 may function by sequestering other pro-apoptotic factors. Consistent with this notion, Bad-induced cell death can be inhibited by ER-localized Bcl-2 [46]. Co-expression of Bad- and the ER-restricted Bcl-2 mutant, Bcl2-cb5, sequestered Bad to the ER. Furthermore, disruption of the Bad binding pocket on Bcl2-cb5 (BH1 mutant G145E) prevented the interaction of Bcl-2 with Bad and rendered Bcl-2 ineffective at inhibiting Bad-induced death [46]. The need for a direct interaction between Bcl2-cb5 and Bad to inhibit apoptosis argues that ER-localized Bcl-2 inhibits pro-apoptotic family members by sequestering them away from other intracellular targets. Other non-Bcl-2 cytosolic proteins that interact with Bcl-2 (e.g. calcineurin, raf-1, etc., reviewed in Ref. [54]) may be similarly regulated.

4.3. BIK

Pro-apoptotic Bcl-2 proteins may elicit cell death through mechanisms other than Ca^{2+} regulation. Replacing the tail-anchor of the pro-apoptotic BH3 protein BIK with the corresponding sequence from the ER-specific isoform of cytochrome *b*₅ to generate an ER-targeted BIK (BIK-cb5) protein. This BIK mutant causes the release of

mitochondrial cytochrome *c* independent of both Ca^{2+} release from the ER and the mitochondrial permeability transition pore [55]. These effects were also seen in vitro using cytosol, mitochondria and ER microsomes generated from Bax $-/-$ cells indicating that Bax is not required for BIK-cb5 to cause cytochrome *c* release. However, cytochrome *c* release required both a cytosolic component and the continued presence of ER microsomes, suggesting that ER signaling molecules either activate or are activated by cytoplasmic components to alter mitochondrial activity. It is unknown to what extent Bak can substitute for Bax, as these experiments were not performed in double knockout cells. Nevertheless, this system can be used to isolate and identify cytosolic and ER components required to trigger cytochrome *c* release, and may greatly increase our understanding of the role of ER in the regulation of cell death.

5. Other potential Bcl-2 targets in the ER

5.1. Cellular pH changes

Somatostatin induces apoptosis in susceptible cells via acidification of the cytoplasm. Acidification depends on the activity of Src homology 2 tyrosine phosphatase 1 (SHP-1) and can be inhibited by Bcl-2 [56]. However, if cells are artificially acidified by the proton ionophore, nigericin, and

low pH media, Bcl-2 is not effective in inhibiting apoptosis. Thus, Bcl-2 inhibits somatostatin-induced apoptosis upstream of acidification. Decreases in cellular pH can be prevented by Caspase-8 inhibition and may be mediated Na^+/K^+ proton exchangers at the ER, nuclear envelope and/or endosomes [57]. Because, Bcl2-cb5 inhibits somatostatin-induced apoptosis upstream of acidification (Coimbatore Srikant, personal communication) it is likely that inhibition of acidification is regulated by one or more ER proteins, perhaps by Bcl-2 directly modulating an ER-specific Na^+/K^+ proton exchanger. It would be interesting to determine if the Caspase-8 activity required for acidification is actually the ER-specific isoform, Caspase-8L; if so, Bap31 may be the relevant substrate. Alternately, Caspase-8L may cleave another protein that regulates acidification.

5.2. Unfolded protein response

ER stress, induced by brefeldinA or tunicamycin, activates the unfolded protein response (UPR) (recently reviewed in Ref. [58]). The accumulation of unfolded proteins in the lumen of the ER activates the transmembrane Ser/Thr Kinase, Ire1- α [59], which then activates the *c-Jun* NH2-terminal Kinase (JNK) pathway to induce apoptosis [60]. In addition to JNK activation, UPR also activates PKR-like ER kinase (PERK) causing it to phosphorylate the translation initiation factor eIF2- α , and thereby decreasing protein synthesis [61]. Expression of ER-restricted Bcl-2 can inhibit BrefeldinA- and tunicamycin-induced cell death [30]; however, the stage in the process and the targets that Bcl-2 regulates are not known.

6. Bcl-2 members at locations other than the ER or mitochondria

6.1. Cytoskeleton

Although this review has focused on Bcl-2 family members function at the ER, Bcl-2 family members have also been identified in other locations within the cell (Table 1). The BH3-only protein Bim has been shown to interact with the dynein motor complex of the cytoskeleton. In MCF-7 cells, induction of apoptosis with taxol releases Bim from the cytoskeleton to permit its translocation to heavy membranes and the nuclear membrane/ER [62]. Bim also interacts with the cytoskeleton [63]. Phosphorylation of Bim and Bim by JNK releases them from the cytoskeleton [64] and stimulates apoptosis. In addition, the pro-apoptotic protein Bad interacts with 14-3-3 scaffold proteins in its inactive phosphorylated state [65]. Localization at the cytoskeleton appears to sequester pro-apoptotic Bcl-2 proteins thereby preventing their activation and targeting the mitochondria and/or ER. As several different pro-apoptotic Bcl-2 proteins are located at different sites within the cytoskeleton, and full-length Bap31 also binds to the cytoskeleton this

suggests that Bcl-2 family members may actively participate in the rearrangement of the cytoskeleton during cell death.

6.2. Lysosomes

Inhibition of PI-3 kinase sensitizes vascular endothelial cells to cytokine-initiated apoptosis that involves release of cathepsins from lysosomes. In this form of programmed cell death, Cathepsin B translocates from lysosomes to the cytosol prior to the activation of caspases. While Bcl-2 has been shown to inhibit this form of cell death, the role of subcellular localization was not examined [66]. Oxidative stress has also been reported to induce apoptosis in part via lysosomal leakage that can be inhibited by Bcl-2. Unlike other Bcl-2 functions, it has been reported that phosphorylation of Bcl-2 is required for Bcl-2 to effectively inhibit lysosomal rupture in response to oxidative damage [67]. In most cells, phosphorylation of endogenous Bcl-2 is low (<10%). However, Bcl-2 mutants located in the cytoplasm due to removal of the tail-anchor are efficiently phosphorylated (~50%). Therefore, it may be that the small fraction of Bcl-2 that is phosphorylated and that prevents lysosomal rupture is not localized at the ER or mitochondria.

Thus, a wealth of data indicate that apoptosis is regulated at multiple sites within the cell. As additional roles for other organelles and subcellular locations in apoptosis [68] are more thoroughly investigated, other functions for the Bcl-2 family outside of the ER and mitochondria may yet be identified.

7. Mutants of Bcl-2 with restricted subcellular localization

One powerful method to determine the relative contribution of Bcl-2 family proteins at the ER or mitochondria is the use of mutants restricted to specific subcellular locations. This has been an important approach in our lab and in many others over the last decade. Using this approach, we generated a mutant Bcl-2 protein (Bcl2-cb5) restricted to the ER in mammalian cells [13]. This mutant was made by exchanging the carboxyl-terminal tail-anchor of wild-type Bcl-2 with the corresponding sequence from an ER-specific isoform of cytochrome *b₅*. A similar approach was used to restrict Bcl-2 to the mitochondria except that the tail-anchor of Bcl-2 was exchanged for the membrane anchor of the ActA from *L. monocytogenes* [13].

We and other investigators have demonstrated that Bcl2-cb5 can inhibit cell death induced by a variety of apoptotic stimuli including serum starvation [13], brefeldinA and tunicamycin [30], DNA damage [69], Bad expression [46], ceramide and staurosporine [31]. In MCF-7 cells, apoptosis induced by somatostatin analogues can only be blocked by Bcl-2 at the ER and not by Bcl-2-targeted exclusively to mitochondria (Coimbatore Srikant, personal communication). In these cells, doxorubicin-induced cell

death can be blocked by Bcl-2 at mitochondria but not the ER (Fiebig A., Zhu W., Leber B. and Andrews D.W., unpublished observations). These observations suggest that the relative importance of either the ER or mitochondria as the critical site in the regulation of cell death varies with the apoptotic stimuli.

In Rat-1mycER cells (in which myc activity is inducible with estrogen), Bcl2-cb5 prevents apoptosis induced by serum starvation, ceramide or staurosporine, but not etoposide or tertbutyl hydroperoxide [31]. Interestingly, apoptosis induced by either ceramide or serum starvation is characterized by early loss of mitochondrial membrane potential that is followed by the release of cytochrome *c*, whereas etoposide triggers translocation of Bax to mitochondria where it undergoes a conformational change and causes the release of cytochrome *c*. In this case, cytochrome *c* release precedes loss of mitochondrial membrane potential [31]. Thus, one difference between ER- and mitochondria-regulated apoptosis is the order of mitochondrial dysfunction events, indicating the presence of multiple pathways leading to cell death. As discussed previously, restoration of wild-type kinetics of etoposide-induced apoptosis to Bax/Bak double-knock-out cells required both ER Ca^{2+} and Bax at mitochondria [37]. Taken together, these observations suggest that either Bcl2-cb5 is not able to prevent ER Ca^{2+} release by etoposide, or that inhibition of Ca^{2+} release is not sufficient to inhibit etoposide-induced cell death. Consistent with the latter, expression of Bcl-2 does not appear to inhibit the release of Ca^{2+} from the ER in either doxorubicin- [70] or thapsigargin- [47] induced cell death. In addition, expression of SERCA to increase ER Ca^{2+} had a marginal effect on the kinetics of cell death following brefeldinA treatment that could be inhibited by Bcl2-cb5 expression [30]. In contrast, expression of mitochondrial-associated Bax in this system enhanced the kinetics of apoptosis considerably [37]. Thus, the use of organelle-targeted mutants (Bcl-2 and Bax) together with other studies suggest that Bcl-2 at the ER is regulating more than just Ca^{2+} and that Ca^{2+} may not be the critical signal for some death stimuli. As other locations within the cell are identified as important in the regulation of apoptosis, the organelle-specific contribution to cell death can be determined using a similar approach, but with different targeting sequences.

8. Summary

The Bcl-2 family of proteins regulates cell death at both the ER and mitochondria. The recent observation of pro- and anti-apoptotic Bcl-2 members at the ER in cells reaffirms the importance of the ER in the regulation of apoptosis. Bcl-2 family members modulate ER Ca^{2+} levels: in general, pro-apoptotic members decrease ER Ca^{2+} during cell death while anti-apoptotic Bcl-2 members have the opposite effect. At the ER, Bcl-2 members not only regulate Ca^{2+} homeostasis, but also interact with ER proteins such as

Bap31. As Bap31 and the pro-apoptotic family members Bim and Bmf interact with the cytoskeleton, this suggests a role for ER-associated Bcl-2 molecules in the rearrangement of the cytoskeleton during apoptosis. Examining the individual contributions of ER-associated Bcl-2 members suggests that the ER can act upstream of mitochondria in the initiation of apoptosis, and in some circumstances it can act independently of mitochondria. The use of organelle-restricted mutants has provided valuable insight into the importance and contribution of the ER in the regulation of apoptosis. These mutants will also be valuable in determining the critical sites in other apoptotic pathways where Bcl-2 family members function, and may thus aid in identifying new therapeutic targets.

References

- [1] S. Cory, J.M. Adams, The Bcl2 family: regulators of the cellular life-or-death switch, *Nat. Rev., Cancer* 2 (2002) 647–656.
- [2] A. Letai, M.C. Bassik, L.D. Walensky, M.D. Sorcinelli, S. Weiler, S.J. Korsmeyer, Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics, *Cancer Cells* 2 (2002) 183–192.
- [3] S.W. Muchmore, M. Sattler, H. Liang, R.P. Meadows, J.E. Harlan, H.S. Yoon, D. Nettlesheim, B.S. Chang, C.B. Thompson, S.L. Wong, S.L. Ng, S.W. Fesik, X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death, *Nature* 381 (1996) 335–341.
- [4] J.J. Chou, H. Li, G.S. Salvesen, J. Yuan, G. Wagner, Solution structure of BID, an intracellular amplifier of apoptotic signaling, *Cell* 96 (1999) 615–624.
- [5] M. Suzuki, R.J. Youle, N. Tjandra, Structure of Bax: coregulation of dimer formation and intracellular localization, *Cell* 103 (2000) 645–654.
- [6] A.M. Petros, A. Medek, D.G. Nettlesheim, D.H. Kim, H.S. Yoon, K. Swift, E.D. Matayoshi, T. Oltersdorf, S.W. Fesik, Solution structure of the antiapoptotic protein Bcl-2, *Proc. Natl. Acad. Sci. U. S. A.* 98 (2001) 3012–3017.
- [7] A.Y. Denisov, M.S. Madiraju, G. Chen, A. Khadir, P. Beauparlant, G. Attardo, G.C. Shore, K. Gehring, Solution structure of human BCL-w: modulation of ligand binding by the C-terminal helix, *J. Biol. Chem.* 278 (2003) 21124–21128.
- [8] M.G. Hinds, M. Lackmann, G.L. Skea, P.J. Harrison, D.C. Huang, C.L. Day, The structure of Bcl-w reveals a role for the C-terminal residues in modulating biological activity, *EMBO J.* 22 (2003) 1497–1507.
- [9] S. Desagher, J.C. Martinou, Mitochondria as the central control point of apoptosis, *Trends Cell Biol.* 10 (2000) 369–377.
- [10] S. Krajewski, S. Tanaka, S. Takayama, M.J. Schibler, W. Fenton, J.C. Reed, Investigation of the subcellular distribution of the Bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes, *Cancer Res.* 53 (1993) 4701–4714.
- [11] U. Kutay, E. Hartmann, T.A. Rapoport, A class of membrane proteins with a C-terminal anchor, *Trends Cell Biol.* 3 (1993) 72–75.
- [12] P.K. Kim, F. Janiak-Spens, W.S. Trimble, B. Leber, D.W. Andrews, Evidence for multiple mechanisms for membrane binding and integration via carboxyl-terminal insertion sequences, *Biochemistry* 36 (1997) 8873–8882.
- [13] W. Zhu, A. Cowie, G.W. Wasfy, L.Z. Penn, B. Leber, D.W. Andrews, Bcl-2 mutants with restricted subcellular location reveal spatially distinct pathways for apoptosis in different cell types, *EMBO J.* 15 (1996) 4130–4141.
- [14] X. Liu, C.N. Kim, J. Yang, R. Jemmerson, X. Wang, Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*, *Cell* 86 (1996) 147–157.

- [15] D.D. Newmeyer, S. Ferguson-Miller, Mitochondria. Releasing power for life and unleashing the machineries of death, *Cell* 112 (2003) 481–490.
- [16] G. Baffy, T. Miyashita, J.R. Williamson, J.C. Reed, Apoptosis induced by withdrawal of interleukin-3 (IL-3) from an IL-3-dependent hematopoietic cell line is associated with repartitioning of intracellular calcium and is blocked by enforced Bcl-2 oncoprotein production, *J. Biol. Chem.* 268 (1993) 6511–6519.
- [17] T.H. Kuo, H.R. Kim, L. Zhu, Y. Yu, H.M. Lin, W. Tsang, Modulation of endoplasmic reticulum calcium pump by Bcl-2, *Oncogene* 17 (1998) 1903–1910.
- [18] H. He, M. Lam, T.S. McCormick, C.W. Distelhorst, Maintenance of calcium homeostasis in the endoplasmic reticulum by Bcl-2, *J. Cell Biol.* 138 (1997) 1219–1228.
- [19] M. Brini, D. Bano, S. Manni, R. Rizzuto, E. Carafoli, Effects of PMCA and SERCA pump overexpression on the kinetics of cell Ca(2+) signalling, *EMBO J.* 19 (2000) 4926–4935.
- [20] P. Pinton, D. Ferrari, P. Magalhaes, K. Schulze-Osthoff, F. Di Virgilio, T. Pozzan, R. Rizzuto, Reduced loading of intracellular Ca(2+) stores and downregulation of capacitance Ca(2+) influx in Bcl-2-overexpressing cells, *J. Cell Biol.* 148 (2000) 857–862.
- [21] F. Vanden Abeele, R. Skryma, Y. Shuba, F. Van Coppenolle, C. Slomianny, M. Roudbaraki, B. Mauroy, F. Wuytack, N. Prevarskaya, Bcl-2-dependent modulation of Ca(2+) homeostasis and store-operated channels in prostate cancer cells, *Cancer Cell* 1 (2002) 169–179.
- [22] R. Foyouzi-Youssefi, S. Arnaudeau, C. Borner, W.L. Kelley, J. Tschopp, D.P. Lew, N. Demareux, K.H. Krause, Bcl-2 decreases the free Ca²⁺ concentration within the endoplasmic reticulum, *Proc. Natl. Acad. Sci. U. S. A.* 97 (2000) 5723–5728.
- [23] D. Ferrari, P. Pinton, G. Szabadkai, M. Chami, M. Campanella, T. Pozzan, R. Rizzuto, Endoplasmic reticulum, Bcl-2 and Ca(2+) handling in apoptosis, *Cell Calcium* 32 (2002) 413–420.
- [24] S.S. Smaili, Y.T. Hsu, A.C. Carvalho, T.R. Rosenstock, J.C. Sharpe, R.J. Youle, Mitochondria, calcium and pro-apoptotic proteins as mediators in cell death signaling, *Braz. J. Med. Biol. Res.* 36 (2003) 183–190.
- [25] M. Brini, R. Marsault, C. Bastianutto, J. Alvarez, T. Pozzan, R. Rizzuto, Transfected aequorin in the measurement of cytosolic Ca²⁺ concentration ([Ca²⁺]_c). A critical evaluation, *J. Biol. Chem.* 270 (1995) 9896–9903.
- [26] A. Miyawaki, J. Llopis, R. Heim, J.M. McCaffery, J.A. Adams, M. Ikura, R.Y. Tsien, Fluorescent indicators for Ca²⁺ based on green fluorescent proteins and calmodulin, *Nature* 388 (1997) 882–887.
- [27] N.S. Wang, M.T. Unkila, E.Z. Reineks, C.W. Distelhorst, Transient expression of wild-type or mitochondrially targeted Bcl-2 induces apoptosis, whereas transient expression of endoplasmic reticulum-targeted Bcl-2 is protective against Bax-induced cell death, *J. Biol. Chem.* 276 (2001) 44117–44128.
- [28] D.R. Dowd, P.N. MacDonald, B.S. Komm, M.R. Haussler, R.L. Miesfeld, Stable expression of the calbindin-D28K complementary DNA interferes with the apoptotic pathway in lymphocytes, *Mol. Endocrinol.* 6 (1992) 1843–1848.
- [29] T. Nakagawa, J. Yuan, Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis, *J. Cell Biol.* 150 (2000) 887–894.
- [30] J. Hacki, L. Egger, L. Monney, S. Conus, T. Rosse, I. Fellay, C. Borner, Apoptotic crosstalk between the endoplasmic reticulum and mitochondria controlled by Bcl-2, *Oncogene* 19 (2000) 2286–2295.
- [31] M.G. Annis, N. Zamzami, W. Zhu, L.Z. Penn, G. Kroemer, B. Leber, D.W. Andrews, Endoplasmic reticulum localized Bcl-2 prevents apoptosis when redistribution of cytochrome *c* is a late event, *Oncogene* 20 (2001) 1939–1952.
- [32] P. Pinton, D. Ferrari, E. Rappizzi, F.D. Di Virgilio, T. Pozzan, R. Rizzuto, The Ca²⁺ concentration of the endoplasmic reticulum is a key determinant of ceramide-induced apoptosis: significance for the molecular mechanism of Bcl-2 action, *EMBO J.* 20 (2001) 2690–2701.
- [33] C. Li, C.J. Fox, S.R. Master, V.P. Bindokas, L.A. Chodosh, C.B. Thompson, Bcl-X(L) affects Ca(2+) homeostasis by altering expression of inositol 1,4,5-trisphosphate receptors, *Proc. Natl. Acad. Sci. U. S. A.* 99 (2002) 9830–9835.
- [34] M. Lam, M.B. Bhat, G. Nunez, J. Ma, C.W. Distelhorst, Regulation of Bcl-xl channel activity by calcium, *J. Biol. Chem.* 273 (1998) 17307–17310.
- [35] L.K. Nutt, A. Pataer, J. Pahler, B. Fang, J. Roth, D.J. McConkey, S.G. Swisher, Bax and Bak promote apoptosis by modulating endoplasmic reticular and mitochondrial Ca²⁺ stores, *J. Biol. Chem.* 277 (2002) 9219–9225.
- [36] L.K. Nutt, J. Chandra, A. Pataer, B. Fang, J.A. Roth, S.G. Swisher, R.G. O'Neil, D.J. McConkey, Bax-mediated Ca²⁺ mobilization promotes cytochrome *c* release during apoptosis, *J. Biol. Chem.* 277 (2002) 20301–20308.
- [37] L. Scorrano, S.A. Oakes, J.T. Opferman, E.H. Cheng, M.D. Sorcinelli, T. Pozzan, S.J. Korsmeyer, BAX and BAK regulation of endoplasmic reticulum Ca²⁺: a control point for apoptosis, *Science* 300 (2003) 135–139.
- [38] K.G. Wolter, Y.T. Hsu, C.L. Smith, A. Nechushtan, X.G. Xi, R.J. Youle, Movement of Bax from the cytosol to mitochondria during apoptosis, *J. Cell Biol.* 139 (1997) 1281–1292.
- [39] A. Nechushtan, C.L. Smith, Y.T. Hsu, R.J. Youle, Conformation of the Bax C-terminus regulates subcellular location and cell death, *EMBO J.* 18 (1999) 2330–2341.
- [40] E.L. Soucie, M.G. Annis, J. Sedivy, J. Filmus, B. Leber, D.W. Andrews, L.Z. Penn, Myc potentiates apoptosis by stimulating Bax activity at the mitochondria, *Mol. Cell. Biol.* 21 (2001) 4725–4736.
- [41] B. Wang, M. Nguyen, D.G. Breckenridge, M. Stojanovic, P.A. Clemons, S. Kuppig, G.C. Shore, Uncleaved BAP31 in association with A4 protein at the endoplasmic reticulum is an inhibitor of Fas-initiated release of cytochrome *c* from mitochondria, *J. Biol. Chem.* 278 (2003) 14461–14468.
- [42] M. Sawada, W. Sun, P. Hayes, K. Leskov, D.A. Boothman, S. Matsuyama, Ku70 suppresses the apoptotic translocation of Bax to mitochondria, *Nat. Cell Biol.* 5 (2003) 320–329.
- [43] P.F. Cartron, C. Moreau, L. Oliver, E. Mayat, K. Meflah, F.M. Vallette, Involvement of the N-terminus of Bax in its intracellular localization and function, *FEBS Lett.* 512 (2002) 95–100.
- [44] P.F. Cartron, M. Priault, L. Oliver, K. Meflah, S. Manon, F.M. Vallette, The N-terminal end of bax contains a mitochondrial-targeting signal, *J. Biol. Chem.* 278 (2003) 11633–11641.
- [45] I.S. Goping, A. Gross, J.N. Lavoie, M. Nguyen, R. Jemmerson, K. Roth, S.J. Korsmeyer, G.C. Shore, Regulated targeting of BAX to mitochondria, *J. Cell Biol.* 143 (1998) 207–215.
- [46] M.J. Thomenius, N.S. Wang, E.Z. Reineks, Z. Wang, C.W. Distelhorst, Bcl-2 on the endoplasmic reticulum regulates bax activity by binding to BH3-only proteins, *J. Biol. Chem.* 278 (2003) 6243–6250.
- [47] R.K. Srivastava, S.J. Sollott, L. Khan, R. Hansford, E.G. Lakatta, D.L. Longo, Bcl-2 and Bcl-X(L) block thapsigargin-induced nitric oxide generation, *c-Jun* NH(2)-terminal kinase activity, and apoptosis, *Mol. Cell. Biol.* 19 (1999) 5659–5674.
- [48] F.W. Ng, M. Nguyen, T. Kwan, P.E. Branton, D.W. Nicholson, J.A. Cromlish, G.C. Shore, p28 Bap31, a Bcl-2/Bcl-XL- and procaspase-8-associated protein in the endoplasmic reticulum, *J. Cell Biol.* 20 (1997) 327–338.
- [49] D.G. Breckenridge, M. Nguyen, S. Kuppig, M. Reth, G.C. Shore, The procaspase-8 isoform, procaspase-8L, recruited to the BAP31 complex at the endoplasmic reticulum, *Proc. Natl. Acad. Sci. U. S. A.* 99 (2002) 4331–4336.
- [50] M. Nguyen, D.G. Breckenridge, A. Ducret, G.C. Shore, Caspase-resistant BAP31 inhibits fas-mediated apoptotic membrane fragmentation and release of cytochrome *c* from mitochondria, *Mol. Cell. Biol.* 20 (2000) 6731–6740.
- [51] A. Ducret, M. Nguyen, D.G. Breckenridge, G.C. Shore, The resident endoplasmic reticulum protein, BAP31, associates with gamma-actin and myosin B heavy chain, *Eur. J. Biochem.* 270 (2003) 342–349.

- [52] D.G. Breckenridge, M. Stojanovic, R.C. Marcellus, G.C. Shore, Caspase cleavage product of BAP31 induces mitochondrial fission through endoplasmic reticulum calcium signals, enhancing cytochrome *c* release to the cytosol, *J. Cell Biol.* 160 (2003) 1115–1127.
- [53] T. Mund, A. Gewies, N. Schoenfeld, M.K. Bauer, S. Grimm, Spike, a novel BH3-only protein, regulates apoptosis at the endoplasmic reticulum, *FASEB J.* 17 (2003) 696–698.
- [54] J.C. Reed, Double identity for proteins of the Bcl-2 family, *Nature* 387 (1997) 773–776.
- [55] M. Germain, J.P. Mathai, G.C. Shore, BH3-only BIK functions at the endoplasmic reticulum to stimulate cytochrome *c* release from mitochondria, *J. Biol. Chem.* 277 (2002) 18053–18060.
- [56] M. Thangaraju, K. Sharma, B. Leber, D.W. Andrews, S.H. Shen, C.B. Srikant, Regulation of acidification and apoptosis by SHP-1 and Bcl-2, *J. Biol. Chem.* 274 (1999) 29549–29557.
- [57] D. Liu, G. Martino, M. Thangaraju, M. Sharma, F. Halwani, S.H. Shen, Y.C. Patel, C.B. Srikant, Caspase-8-mediated intracellular acidification precedes mitochondrial dysfunction in somatostatin-induced apoptosis, *J. Biol. Chem.* 275 (2000) 9244–9250.
- [58] H.P. Harding, M. Calton, F. Urano, I. Novoa, D. Ron, Transcriptional and translational control in the mammalian unfolded protein response, *Annu. Rev. Cell Dev. Biol.* 18 (2002) 575–599.
- [59] C.E. Shamu, P. Walter, Oligomerization and phosphorylation of the Ire1p kinase during intracellular signaling from the endoplasmic reticulum to the nucleus, *EMBO J.* 15 (1996) 3028–3039.
- [60] F. Urano, X. Wang, A. Bertolotti, Y. Zhang, P. Chung, H.P. Harding, D. Ron, Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1, *Science* 287 (2000) 664–666.
- [61] Y. Shi, K.M. Vattam, R. Sood, J. An, J. Liang, L. Stramm, R.C. Wek, Identification and characterization of pancreatic eukaryotic initiation factor 2 alpha-subunit kinase, PEK, involved in translational control, *Mol. Cell. Biol.* 18 (1998) 7499–7509.
- [62] H. Puthalakath, D.C. Huang, L.A. O'Reilly, S.M. King, A. Strasser, The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex, *Mol. Cell* 3 (1999) 287–296.
- [63] H. Puthalakath, A. Villunger, L.A. O'Reilly, J.G. Beaumont, L. Coultas, R.E. Cheney, D.C. Huang, A. Strasser, Bmf: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis, *Science* 293 (2001) 1829–1832.
- [64] K. Lei, R.J. Davis, JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis, *Proc. Natl. Acad. Sci. U. S. A.* 100 (2003) 2432–2437.
- [65] J. Zha, H. Harada, E. Yang, J. Jockel, S.J. Korsmeyer, Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14–3–3 not BCL-X(L), *Cell* 87 (1996) 619–628.
- [66] L.A. Madge, J.H. Li, J. Choi, J.S. Pober, Inhibition of PI-3 kinase sensitizes vascular endothelial cells to cytokine-initiated cathepsin-dependent apoptosis, *J. Biol. Chem.* 278 (2003) 21296–21306.
- [67] M. Zhao, J.W. Eaton, U.T. Brunk, Bcl-2 phosphorylation is required for inhibition of oxidative stress-induced lysosomal leak and ensuing apoptosis, *FEBS Lett.* 509 (2001) 405–412.
- [68] K.F. Ferri, G. Kroemer, Organelle-specific initiation of cell death pathways, *Nat. Cell Biol.* 3 (2001) E255–E263.
- [69] J. Rudner, A. Lepple-Wienhues, W. Budach, J. Berschauer, B. Friedrich, S. Wesselborg, K. Schulze-Osthoff, C. Belka, Wild-type, mitochondrial and ER-restricted Bcl-2 inhibit DNA damage-induced apoptosis but do not affect death receptor-induced apoptosis, *J. Cell. Sci.* 114 (2001) 4161–4172.
- [70] M.C. Marin, A. Fernandez, R.J. Bick, S. Brisbay, L.M. Buja, M. Snuggs, D.J. McConkey, A.C. von Eschenbach, M.J. Keating, T.J. McDonnell, Apoptosis suppression by Bcl-2 is correlated with the regulation of nuclear and cytosolic Ca²⁺, *Oncogene* 12 (1996) 2259–2266.
- [71] Y.T. Hsu, R.J. Youle, Nonionic detergents induce dimerization among members of the Bcl-2 family, *J. Biol. Chem.* 272 (1997) 13829–13834.
- [72] L.A. O'Reilly, C. Print, G. Hausmann, K. Moriishi, S. Cory, D.C. Huang, A. Strasser, Tissue expression and subcellular localization of the pro-survival molecule Bcl-w, *Cell Death Differ.* 8 (2001) 486–494.
- [73] T. Yang, K.M. Kozopas, R.W. Craig, The intracellular distribution and pattern of expression of Mcl-1 overlap with, but are not identical to, those of Bcl-2, *J. Cell Biol.* 128 (1995) 1173–1184.
- [74] Q. Song, Y. Kuang, V.M. Dixit, C. Vincenz, Boo, a novel negative regulator of cell death, interacts with Apaf-1, *EMBO J.* 18 (1999) 167–178.
- [75] J. Zha, H. Harada, E. Yang, J. Jockel, S.J. Korsmeyer, Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14–3–3 not BCL-X(L), *Cell* 87 (1996) 619–628.
- [76] K. Wang, X.M. Yin, D.T. Chao, C.L. Milliman, S.J. Korsmeyer, BID: a novel BH3 domain-only death agonist, *Genes Dev.* 10 (1996) 2859–2869.