Review

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The daily job of night killers: alternative roles of the BCL-2 family in organelle physiology

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Apoptosis is essential for maintenance of tissue homeostasis and its deregulation underlies many disease conditions. The BCL-2 family of proteins is a group of evolutionarily conserved regulators of cell death, comprising both anti- and pro-apoptotic members, which operate at the mitochondrial membrane to control caspase activation. Different BCL-2-related proteins are also located in multiprotein complexes at the endoplasmic reticulum (ER), which are involved in the control of diverse cellular processes, including calcium homeostasis, autophagy, the unfolded protein response and ER morphogenesis. Here, we describe the emerging concept that BCL-2-related proteins have alternative functions beyond apoptosis to control the essential functions of the cell.

Introduction

Complex signaling responses mediate adaptation to organelle stress or initiation of cell-death processes when a threshold of damage has been reached. Execution of apoptosis depends on the activation of caspases, a process regulated tightly by the BCL-2 family of proteins. The BCL-2 family of proteins is comprised of pro- and anti-apoptotic members that are defined by the presence of up to four conserved domains. Antiapoptotic BCL-2 family members display sequence homology in four α-helical domains called BCL-2 homology (BH)1 to BH4 [1]. Proapoptotic BCL-2 members can be further subdivided into more highly conserved, 'multidomain' members displaying homology in the BH1, BH2 and BH3 (i.e. BAX and BAK) domains or the 'BH3-only' members (i.e. BIK, BIM, PUMA and NOXA), which contain a single domain crucial for the activation of apoptosis. Recently, a new subgroup of proapoptotic proteins, called BNip proteins, which have minimal sequence similarity in the BH3 domain, was identified (reviewed in [2]).

Each member of the BCL-2 family has distinct patterns of developmental expression, subcellular localization and differential responsiveness to specific death stimuli [3]. Some BH3-only proteins are thought to operate as sentinels of cellular damage [1], where, in response to various death stimuli (i.e. oxidative stress, DNA damage or death-receptor engagement), they are activated either by transcriptional upregulation or through post-translational modifications. BH3-only proteins then promote the activation of the core proapoptotic components BAX and/or BAK [4], resulting in mitochondrial-membrane permeabilization [1]. Released mitochondrial proteins, such as cytochrome *c*, then trigger caspase-mediated cell death [5].

Accumulating evidence indicates that members of all three subclasses of the BCL-2 family of proteins are also located at the endoplasmic reticulum (ER) membrane (see list in [6]). The ER is an organelle with multiple functions, including lipid synthesis and signaling. The two major roles of the ER are calcium storage and protein folding. Membrane-spanning and secreted proteins are synthesized and folded in the ER, undergoing post-translational modifications and oligomerization. Several conditions (such as proteasome inhibition, mutant-protein expression, ER-calcium depletion and redox changes) interfere with oxidative protein folding at the ER lumen [7], resulting in the accumulation of unfolded or misfolded intermediates, a cellular condition referred to as 'ER stress'.

To alleviate ER stress, cells activate a complex signaling pathway known as the 'unfolded protein response' (UPR). The UPR transmits information about the protein folding status in the ER lumen to the cytoplasm and the nucleus to decrease the unfolded protein load. Activation of the UPR affects the expression of proteins involved in nearly every aspect of the secretory pathway, including protein entry into the ER, folding, ER-associated degradation, ER biogenesis and vesicular trafficking [7]. However, if all these mechanisms of survival are insufficient to decrease the unfolded protein load, cells enter into apoptosis. Therefore, improper handling of ER stress constitutes a threat to the life of the cell. The UPR/ER-stress pathway has been implicated in many diseases, including neurodegenerative conditions, cancer and diabetes [7]. Increasing evidence suggests the BCL-2 family of proteins has key roles at the ER membrane where these proteins modulate the cellular responses to stress in addition to cell death. In this review, we focus on the alternative roles for the BCL-2 family of proteins at the ER membrane.

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The unfolded-protein response

In higher eukaryotes, the UPR is mediated by at least three distinct UPR signaling pathways initiated by the sensors IRE1α (inositol-requiring transmembrane kinase/endonuclease), PERK (PKR-like ER kinase) and ATF6 (activating transcription factor 6) (reviewed in [8]). IRE1α is a Ser/Thr protein kinase and endoribonuclease that, on activation, initiates the processing of the mRNA encoding XBP-1 (transcriptional factor X-Box-binding protein 1) [9–11]. A 26-nucleotide fragment of *xbp-1* mRNA is spliced out by activated IRE1α, shifting the coding-reading frame of XBP-1, and resulting in the production of a stable and potent transcriptional activator called XBP-1s that controls the upregulation of a broad spectrum of UPR-related genes [12]. Indeed, XBP-1 production is essential for the proper function of specialized secretory organs, such as liver, pancreas, salivary gland and plasma B lymphocytes, in which a high rate of protein synthesis constitutes an endogenous source of stress [13–16]. IRE1α activation also controls the activation of the c-Jun N-terminal kinase (JNK) [17,18], ERK [19] and NF-κB pathways [20], although their roles in the ER-stress response are not well understood.

The BCL-2 protein family and the UPR

We provided evidence recently for a possible function of the BCL-2 protein family in the UPR (Figure 1). BAX and BAK modulate the amplitude of IRE1α signaling by controlling its autophosphorylation and oligomerization [21]. BAX and BAK double-knockout (DKO) mice showed a decreased expression of IRE1α-downstream signals, including JNK phosphorylation and XBP-1s expression under experimental ER-stress conditions [21]. At the biochemical level, BAX and BAK form a protein complex with the cytosolic domain of IRE1α, which requires their conserved BH1 and BH3 domains [21]. These findings suggested a new role for proapoptotic family members to act as accessory factors for the instigation of certain UPR signaling events. This implies that, during early steps of UPR responses, the proapoptotic proteins BAX and BAK might have pro-survival effects by promoting adaptation to ER stress. Thus, the adaptive effects of BAX and BAK against ER stress contrast completely with their known proapoptotic effect at the mitochondria, suggesting a compartmentalization of their function.

BAX inhibitor-1 (BI-1) is related functionally to the BCL-2 family of proteins and is located in the ER membrane primarily [22]. BI-1 has no obvious homology with BCL-2-related proteins, yet it interacts with different members of the family such as BCL-X_L and BCL-2 [22,23]. Further studies revealed that BI-1 is well conserved in yeast, plants, viruses and many other organisms [24,25]. In mammalian cells, BI-1 is an antiapoptotic protein that affects ER-stress-dependent cell death partially, possibly by modulating calcium signaling [23] and oxidative-stress gene expression [26]. Interestingly, BI-1-deficient mice showed hyperactivation of the IRE1α pathway *in vivo* in a model of hepatic and renal ischemia [27]. These results were also recapitulated recently in cellular models of ER stress [26] in which BI-1 overexpressing cells showed decreased UPR activation as evidenced by decreased XBP-1s. These data suggested that BI-1 has an inhibitory activity on the UPR, which contrasts with the opposite effect of BAX and BAK on this pathway. An interaction between BI-1 and IRE1α remains to be determined. Based on these findings, we speculate that BI-1 has

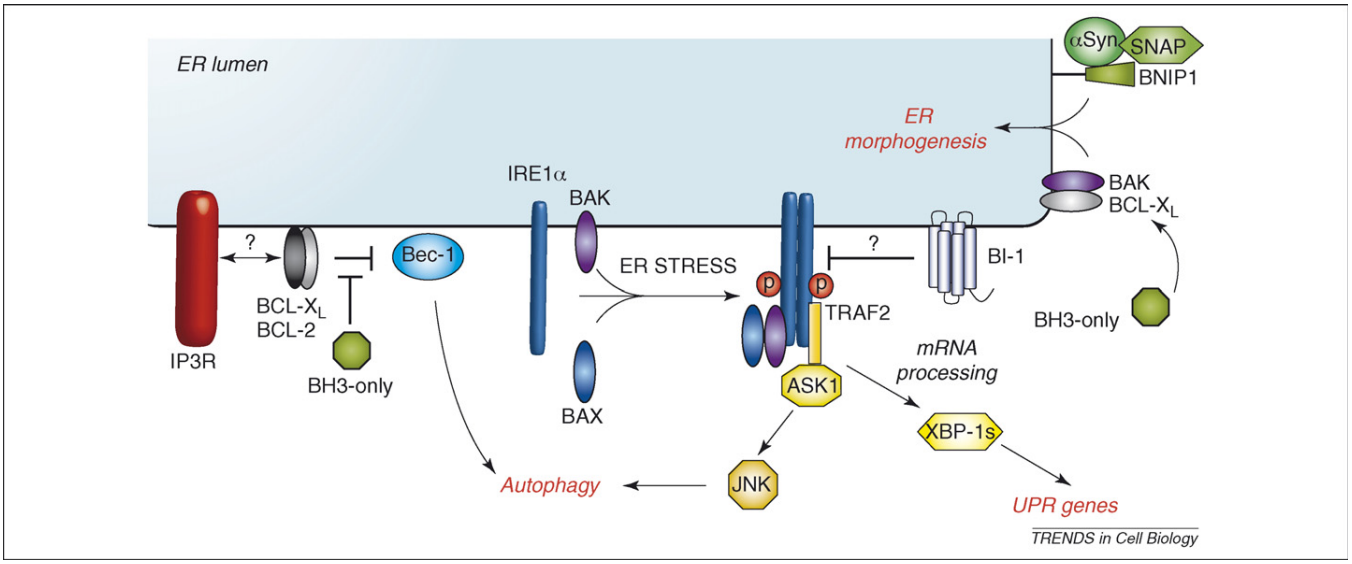

Figure 1. Modulation of the UPR, autophagy and ER-membrane remodeling by the BCL-2 family of proteins. Regulation of the UPR: under stress conditions, IRE1α dimerizes and autophosphorylates, leading to its activation. Phosphorylation of IRE1α triggers its endoribonuclease activity, which mediates the processing of the mRNA encoding *XBP-1*. Alternatively, activated IRE1α interacts with the adaptor protein TRAF2, leading to the activation of the JNK and NF-κB pathways. Activation of IRE1α requires the binding of accessory proteins BAX and BAK, which might stabilize the active form of IRE1α, augmenting its pro-survival signaling. The antiapoptotic protein BI-1 is located at the ER and has been suggested to be a negative regulator of the pathway. Regulation of ER remodeling: the interaction of BAK with BCL-X_L at the ER membrane leads to drastic changes in ER structure. These effects might be modulated by some BH3-only proteins. The BH3-only protein BNIP1 forms a protein complex with Syntaxin-18 (Syn) and α-SNARE, regulating ER-membrane remodeling and ER morphology. Autophagy: accumulation of misfolded proteins also triggers autophagy. Beclin-1 (Bec-1) is regulated negatively through an interaction with BCL-2 or BCL-X_L at the ER membrane and this interaction is antagonized by BH3-only proteins. Activation of IRE1α might increase the levels of autophagy through the activation of the JNK pathway. In addition, the IP3R controls autophagy that is dependent on BCL-2/BCL-XL expression.

an additional activity outside of the regulation of apoptosis at the ER, where it negatively controls IRE1α activation.

We envision a model in which a complex protein platform operates at the ER membrane to control IRE1α activity, an UPRosome, where multiple signaling responses are initiated (Figure 1). We predict that disruption of the interaction between BAX/BAK and IRE1α might shut down the pro-survival effects of this pathway, sensitizing cells to apoptosis. It remains to be determined whether or not BH3-only proteins modulate the association of BAX and BAK to IRE1α. Overall, this model proposes that the balance between anti- and pro-apoptotic members at the ER membrane might determine the ability of a cell to respond to ER stress by controlling the amplitude of UPR signaling.

Irreversible ER stress: the killers

Chronic or irreversible ER stress results in apoptosis. It is not clear what is the initial signal or sensing mechanism that activates apoptosis in ER-damaged cells. However, many different factors have been identified as mediating caspase-dependent cell death downstream of ER stress [28]. Special attention has been focused on the identification of possible BH3-only proteins as mediators of ER-stress-induced apoptosis because the upstream signals activating BAX and BAK under ER stress are unclear.

The activity of BH3-only proteins has been studied mostly at the mitochondria, where they trigger cytochrome *c* release. BH3-only proteins can be separated functionally into two subtypes: (i) activators (i.e. BID, BIM and PUMA) that activate BAX and BAK directly to trigger cytochrome *c* release but are sequestered by antiapoptotic BCL-2 molecules; and (ii) sensitizers (i.e. BAD and NOXA) that only bind to and antagonize antiapoptotic BCL-2 members to release activator BH3-only proteins [29–31]. Alternatively, differential binding to antiapoptotic proteins might explain the separation between activator and sensitizer or derepressor BH3-only proteins [32].

Two BH3-only proteins, PUMA and NOXA, are induced strongly at the transcriptional level in cells undergoing prolonged ER stress. In a pioneering study, cDNA microarray analysis showed that PUMA is one of the only BH3 members that are upregulated by ER stress [33]. A global RNA-interference screen for genes that regulate ER-stress-mediated apoptosis corroborated the functional role of PUMA in this process and identified NOXA additionally as part of the pathway [34]. These data are further supported by the fact that *puma*- or *noxa*-deficient cells are partially resistant to apoptosis induced by ER injuries [35]. Another BH3-only member, BIK, is localized primarily to the ER [36]. BAK becomes oligomerized at the ER following BIK expression and BIK requires BAX/BAK to trigger calcium release and apoptosis (see next section) [37]. Another interesting example of an ER-linked BH3-only family member is BIM. Three different mechanisms link BIM to ER stress. Under normal conditions, BIM is found in the dynein motor complex of the microtubule cytoskeleton, whereas BIM translocates to the ER following ER-stress induction, where it might promote caspase activation through an unknown mechanism [38]. In addition, dephosphorylation of BIM by the phosphatase 2A under ER stress increases BIM levels by preventing its ubiquitination and subsequent proteasomal degradation in different cell types [39]. Further, expression of the proapoptotic UPR transcription factor C/EBP homologous protein (CHOP) triggers the upregulation of BIM mRNA. These results provided a direct connection between activation of the UPR and activation of the core proapoptotic program, a phenomenon that has remained mostly obscure. Moreover, BIM-deficient mice are resistant to ER-stress-induced apoptosis *in vivo*, similar to the phenotype described for *chop*-deficient mice [40,41]. In summary, irreversible ER damage triggers the upregulation of proapoptotic BH3-only proteins, which then might converge on the activation of BAX and BAK at the mitochondria, thus leading to cell death.

In murine cells, the processing of the ER-resident caspase-12 depends on the production of BAX and BAK at the ER [42]. Caspase-12 has been suggested to be linked to the UPR pathway through an interaction with tumor necrosis factor (TNF) receptor-associated factor-2 (TRAF2) and possibly with active IRE1α [43], although a procaspase-12–TRAF2–IRE1α complex has not been described. TRAF2 is known to be involved in the activation of initiator caspase-8 and caspase-10 through the death-receptor pathway. Although caspase-12 processing is a well established ER-stress marker, its contribution to apoptosis is debated actively [44,45].

The BCL-2 protein family and the calcium rheostat

One of the main known functions described for the BCL-2 family of proteins at the ER is the control of calcium homeostasis (Figure 2). The balance between anti- and pro-apoptotic proteins at the ER determines the steady-state ER-calcium content and has a direct impact on the amount of calcium released after stimulation. For example, DKO cells for BAX and BAK show decreased ER-calcium content [42,46], similar to the phenotype of BCL-2 overexpressing cells [47]; overexpression of different BH3-only proteins triggers calcium release (reviewed in [6,48,49]). Thus, the BCL-2 family of proteins constitutes a rheostat for the fine tuning of calcium metabolism.

At the biochemical level, it has been documented by several groups that BCL-2 and BCL-X_L form a protein complex with the inositol triphosphate receptor (IP3R) [50–52], modulating its on–off status possibly through phosphorylation (Figure 2). The IP3R, together with the ryanodine receptor, are the main channel that controls ER-calcium release in cells. Phosphorylation of BCL-2 by JNK occurs at the ER membrane. This modification regulates the antiapoptotic activity of BCL-2 negatively, correlating with a decreased binding to BH3-only proteins and increased ER-calcium content [47]. Oakes *et al.* also suggested that the calcium defects observed in BAX and BAK DKO cells might be attributed to their effect on the interaction of BCL-2 and the IP3R [50]. The native protein complexes containing BCL-2 at the ER membrane were purified recently [53]. BCL-2 is regulated directly by the serine/threonine phosphatase, PP2A [53], which dephosphorylates the sites targeted by JNK, suggesting that the phosphorylation status of BCL-2 is key to its regulation at the ER. Finally, as described earlier, different BH3-only proteins are located at the ER or translocate to its membrane under stress conditions and

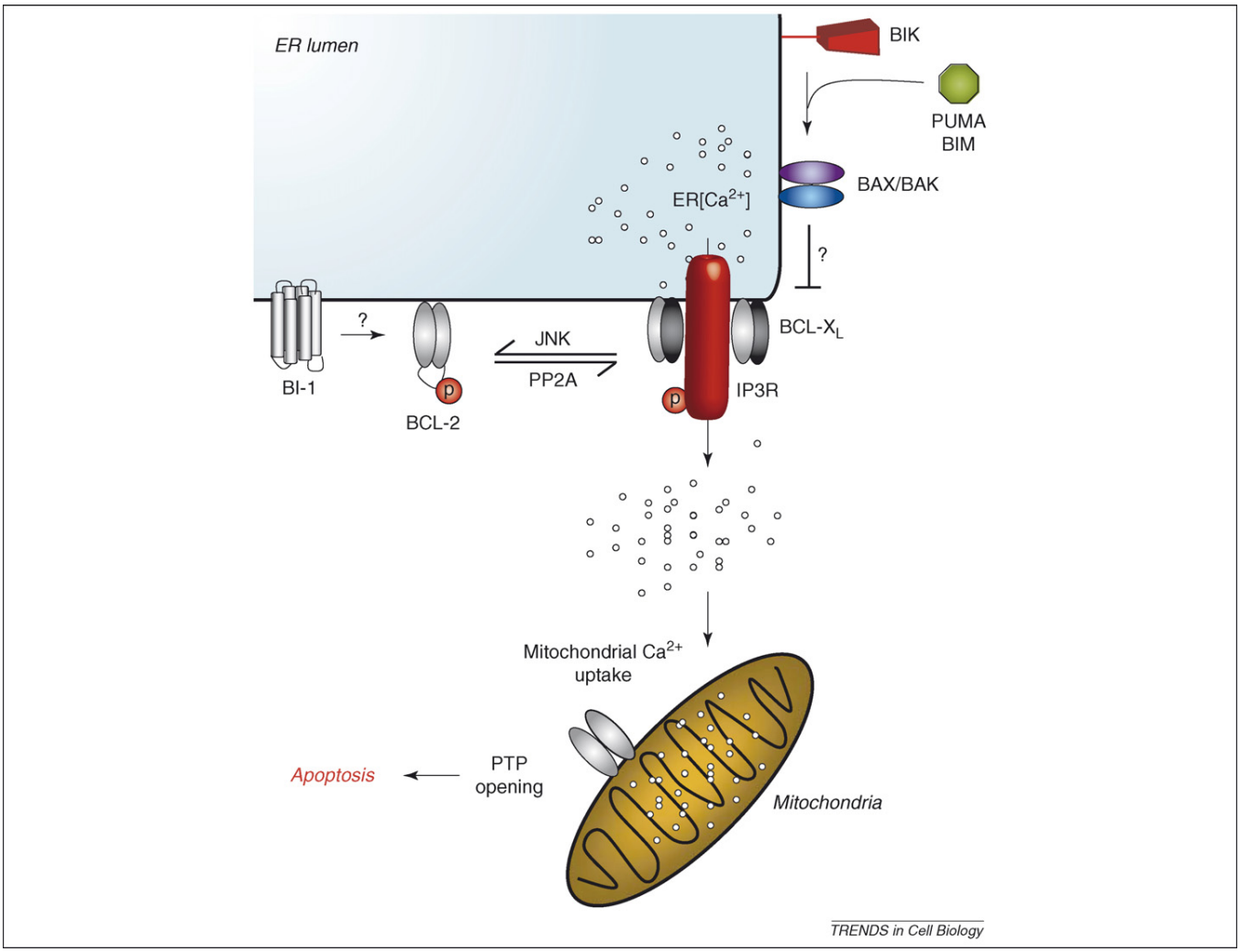

Figure 2. Regulation of ER-calcium homeostasis by the BCL-2 family of proteins. Different anti- and pro-apoptotic members of the BCL-2 family of proteins are located at the ER membrane, where they regulate ER-calcium content. BCL-2 and BCL-X_L interact with the IP3R calcium channel, modulating its activity. BCL-2 increases ER-calcium leak through the IP3 receptor (IP3R) owing to an increase of IP3R phosphorylation. By contrast, BAX and BAK have the opposite effect on ER-calcium content, a function that might be further modulated by BH3-only proteins (such as PUMA and BIK), possibly by affecting the interaction of BCL-2/BCL-X_L with the IP3R indirectly. In addition, the activity of BCL-2 at the ER membrane is regulated by phosphorylation. JNK phosphorylates BCL-2, decreasing its antiapoptotic activity and increasing ER-calcium content, whereas the phosphatase PP2A decreases this phosphorylation through a direct interaction. BI-1 is also located at the ER membrane, where it regulates calcium homeostasis by an unknown mechanism. However, calcium release from the ER influences mitochondrial-mediated apoptosis through calcium uptake followed by the opening of the mitochondrial permeability-transition pore (PTP).

impact calcium homeostasis (reviewed in [6]). Thus, one can speculate that, depending on the cellular context and the stimuli, different protein complexes among BCL-2 family members might exist at the ER membrane to control calcium signaling. Hence, the balance between pro- and antiapoptotic proteins at the ER determines the ER-calcium content and rate of calcium release. Calcium release by the ER can activate apoptosis under certain conditions. For example, calcium uptake by the mitochondria induces mitochondrial membrane permeabilization owing to the opening of the permeability-transition pore (PTP). This crosstalk between the ER and mitochondria affects calcium-dependent cell death (i.e. ceramides, arachidonic acid or oxidative stress) specifically but not ER-stress-mediated apoptosis [46] (Figure 2).

ER signaling, autophagy and the BCL-2 protein family

Autophagy refers to the global process by which intracellular components are recycled through lysosome degradation (reviewed in [54]). Autophagy acts as a crucial survival response under starvation conditions in which the degradation of intracellular proteins and organelles provides a source of amino acids during poor nutritional conditions. Intracellular components can be delivered to lysosomes for degradation by three different mechanisms known as macroautophagy, microautophagy and chaperone-mediated autophagy [54]. The best studied form of autophagy is macroautophagy, hereafter referred to as autophagy. The hallmark of autophagy is the formation of double-membrane-bounded autophagosomes. Autophagosomes fuse with lysosomes to form autophagolysosomes, in which intracellular components are degraded. Autophagy is a highly regulated process with complex steps that are controlled by a family of autophagic-related genes (termed *atg* genes) [54,55]. The generation of *atg*-deficient mice revealed the function of autophagy in diverse processes, including development, cell differentiation, tissue remodeling, immunity,

host-to-pathogen response and cell death or survival under stress conditions [54].

Beclin-1 (also known as Atg6) was the first identified mammalian autophagy gene product [56]. Beclin-1 was isolated originally as a BCL-2-interacting protein [57–59]. In fact, the BCL-2 was shown to negatively regulate autophagy through inhibiting Beclin-1 [60]. Surprisingly, this regulation was attributed specifically to the expression of BCL-2 at the ER membrane, suggesting that signaling events originating from the ER are crucial for autophagy. The formation of a BCL-2–Beclin-1 complex is also regulated by BH3-only proteins (i.e. BAD), revealing extensive crosstalk between apoptosis and autophagy [61,62]. More importantly, a functional BH3-like domain was identified in Beclin-1 and its mutation disrupted the interaction of Beclin-1 with BCL-X_L [61]. Experiments performed in *Caenorhabditis elegans* deficient in EGL-1, a BH3-containing protein, corroborated this model *in vivo*. Similarly, the pharmacological BH3 mimetic ABT-737 inhibited the interaction between Beclin-1 and Bcl-2/Bcl-X_L competitively, stimulating autophagy [61,62].

Along the same lines, a connection between ER-calcium homeostasis and autophagy was proposed to occur through the IP3R, a BCL-2 interactor as described earlier [63–65] (reviewed in [66]). IP3R-dependent autophagy was attributed to the activation of Beclin-1 and other autophagic-related genes, however, unexpectedly, this regulation was independent of calcium release. Finally, many laboratories have shown that ER stress triggers autophagy and this effect is also regulated by UPR stress sensors [63,67–72]. Autophagy might serve as a mechanism to eliminate damaged ER under stress conditions or to control the rate of ER expansion [70]. Surprisingly, the activation of autophagy by ER stress requires IRE1α and is not inhibited by BCL-2 overexpression, a strategy that blocks autophagy mediated by IP3R inhibition. Along the same lines, the IP3R regulates autophagy independently of IRE1α, suggesting that there are two independent pathways controlling autophagy from the ER [63]. It remains to be established whether or not a direct connection among the UPR, autophagy and the BCL-2 protein family exists.

The BCL-2 protein family and ER morphogenesis

The expression of some proapoptotic members of the BCL-2 family of proteins alters the structure of the ER. BAK regulates ER swelling and the remodeling of the reticular structure [73]. Co-expression of BAK and BCL-X_L or BAK mutants in the BH3 domain provoked extensive swelling and vacuolization of ER cisternae. Interestingly, the co-expression of upstream BH3-only activators (i.e. BIM or truncated BID) with BCL-X_L recapitulated ER swelling and vacuolization under conditions in which the ryanodine receptor-calcium channel is inhibited [73]. Surprisingly, the effects of BH3-only proteins on ER swelling were proposed to be dependent on BAK expression specifically but not on BAX, the closest BAK homologue. The physiological role of ER swelling in this context is not known.

The BH3-only member BNip1 is a component of a protein complex comprising syntaxin 18, an ER-located soluble N-ethylmaleimide-sensitive factor (NSF)-attachment protein receptor (SNARE) [74]. At the functional level, BNip1 participates in the formation and modeling of the ER network, being involved in ER membrane fusion but not membrane trafficking. In addition, the BH3 domain is important for the binding of BNip1 to the SNAREs [74]. These results unmasked a possible crosstalk between apparently independent cellular events, apoptosis and ER-membrane fusion (Figure 1). Alternatively, BAX and BAX might also regulate ER biogenesis through XBP-1 [21]. Similarly, expression of BAX and BAK are essential to mitochondria morphogenesis [75], suggesting that these proteins might have broad regulatory effects on organelle morphogenesis.

Concluding remarks

In this review, we have summarized evidence supporting an evolutionary process whereby key regulators of cell death also contribute to vital cellular functions. Accordingly, BCL-2-related proteins not only operate as upstream regulators of caspases but they also regulate specific cellular processes related to ER physiology actively. In support of this idea, there is growing evidence of nonapoptotic functions for the BCL-2 family, including cell-cycle regulation [76,77], participation in DNA-damage responses (i.e. BID) [78,79] and glucose or energy metabolism (i.e. BAD) [80]. In doing so, the BCL-2 protein family might perform as specialized stress sentinels that participate actively in crucial processes for the cell ('the day job'), enabling constant homeostatic quality control. Then the BCL-2 protein family could respond to irreversible cellular damage, activating apoptosis ('the killer properties').

Misfolding and accumulation of abnormal protein aggregates in the brain owing to mutations in certain genes are responsible for a variety of neurological disorders. In many of these diseases, alteration of ER homeostasis contributes to neuronal dysfunction. These diseases include Parkinson's disease [81,82], Alzheimer's disease [83], prion diseases [84–86], amyotrophic lateral sclerosis (ALS) [87], Huntington's disease [88,89] and many others (see list of diseases in [7]). Consequently, the first steps in the death pathways downstream of ER stress might represent important therapeutic targets. In addition, BH3-only proteins, such as BIM, have been implicated in Alzheimer's disease [90] and ALS [91] *in vivo*. Thus, pharmacological manipulation of the BCL-2 protein family activity might be beneficial in the treatment of these fatal diseases. Several small molecules and synthetic peptides are available currently with proven therapeutic applications in disease mouse models, including BCL-2 inhibitors [92], BAX-channel inhibitors [93], BAX/BAK-activator peptides [94,95] and many others (see reviews in [5,96]). These drugs might be used as pharmacological tools to manipulate the activity of stress-signaling pathways regulated by the BCL-2 protein family (i.e. autophagy, calcium, metabolism or the UPR) in pathological conditions.

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