Bcl-2-family proteins and the role of mitochondria in apoptosis

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Mitochondria are central to many forms of cell death, usually via the release of pro-apoptotic proteins from the mitochondrial intermembrane space. Some intermembrane space proteins, including cytochrome *c*, Smac/DIABLO, and Omi/Htra2, can induce or enhance caspase activation, whereas others, such as AIF and endonuclease G, might act in a caspase-independent manner. Intermembrane space protein release is often regulated by Bcl-2-family proteins. Recent evidence suggests that pro-apoptotic members of this family, by themselves, can permeabilize the outer mitochondrial membrane without otherwise damaging mitochondria. Mitochondria can contribute to cell death in other ways. For example, they can respond to calcium release from the endoplasmic reticulum by undergoing the mitochondrial permeability transition, which in turn causes outer membrane rupture and the release of intermembrane space proteins. Bcl-2-family proteins can influence the levels of releasable Ca^{2+} in the endoplasmic reticulum, and thus determine whether the released Ca^{2+} is sufficient to overload mitochondria and induce cell death.

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Current Opinion in Cell Biology 2003, **15**:1–9

This review comes from a themed issue on
Cell division, growth and death
Edited by Jonathon Pines and Sally Kornbluth

0955-0674/$ – see front matter

DOI 10.1016/j.ceb.2003.10.004

Abbreviations

$\Delta\Psi_m$	mitochondrial membrane potential
BH	Bcl-2 homology
IMS	intermembrane space
MOMP	mitochondrial outer membrane permeabilization
PT	permeability transition
ROS	reactive oxygen species
VDAC	voltage-dependent anion channel

Introduction

Mitochondria play an important role in apoptotic cell death by releasing key effector proteins [1], including cytochrome *c* and Smac/DIABLO, from the mitochondrial intermembrane space (IMS). IMS protein release can result from the remarkable event of mitochondrial outer membrane permeabilization (MOMP), which is thought to be regulated by proteins of the Bcl-2 family [2–6]. Of the 20 or so members of this family, some are constitutively associated with intracellular membranes, specifically the endoplasmic reticulum and outer mitochondrial and nuclear membranes. Other Bcl-2 relatives are soluble in the cytoplasm or, upon the engagement of an apoptotic pathway, can translocate from a soluble to a membrane-bound state.

How the Bcl-2-family proteins control MOMP, and therefore the release of IMS proteins, is a focus of intense study. Recent literature suggests that mitochondria can release IMS proteins through two different mechanisms (Figure 1): first, through a surgically precise permeabilization of the outer membrane mediated by Bcl-2 family members; and second, through induction of the mitochondrial permeability transition (PT), occurring mainly in response to the release of Ca^{2+} from endoplasmic reticulum (ER) stores. Bcl-2-family proteins also regulate this pathway through their localization at the ER.

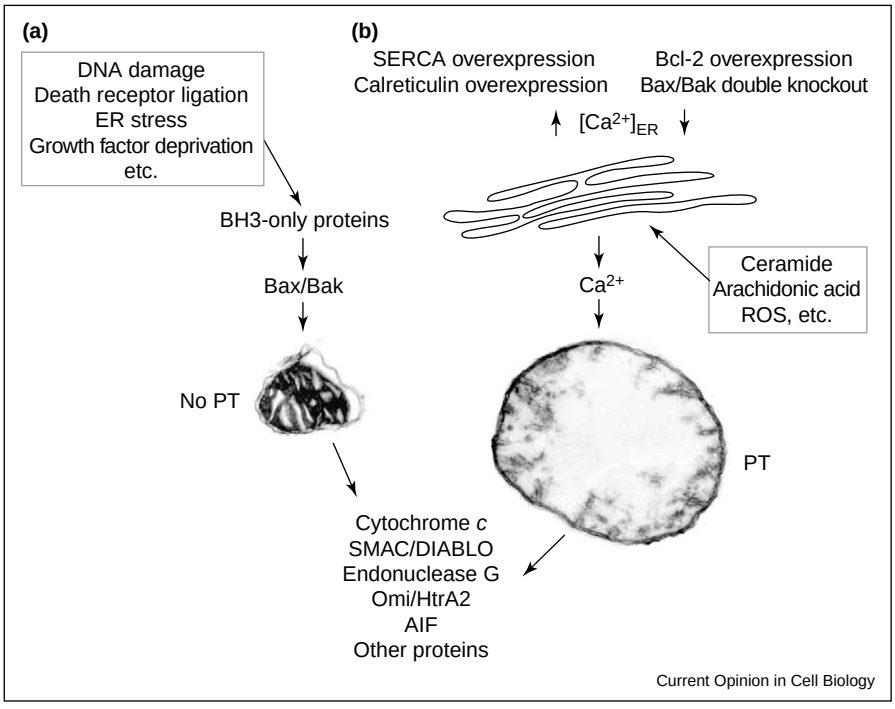
In this review we discuss how Bcl-2-family proteins regulate apoptotic cell death through pathways involving mitochondria. In particular, we highlight recent evidence for a direct action of these proteins on the outer mitochondrial membrane, as well as a more indirect pathway involving calcium release from the endoplasmic reticulum, leading to mitochondrial calcium overload.

Bcl-2 family proteins and apoptotic signaling

Cells can undergo apoptosis when they receive stimuli such as DNA damage (caused by irradiation or anti-cancer drugs), growth factor deprivation, ER stress, detachment from neighboring tissues, or the engagement of cell-surface 'death receptors' such as CD95 and the receptors for tumor necrosis factor α (TNFα) and TNF-related apoptosis-inducing ligand (TRAIL) [7,8]. Apoptotic cells have long been observed to undergo a characteristic series of morphological changes, regardless of the insult. This suggested the existence of a common pathway, which we now know involves the activation of a family of proteolytic enzymes known as caspases [9]. One pathway of caspase activation is triggered by cytochrome *c* following its release from mitochondria into the cytoplasm [10]. This mitochondria-dependent mechanism of caspase activation has been called the 'intrinsic' pathway of apoptosis. Bcl-2 blocks the release of IMS molecules from mitochondria [11,12], suggesting that mitochondria are a principal site for apoptotic regulation by the Bcl-2 family.

Another caspase cascade can be activated through a non-mitochondrial, or 'extrinsic', pathway initiated by ligation of death receptors and the recruitment and activation of

Figure 1



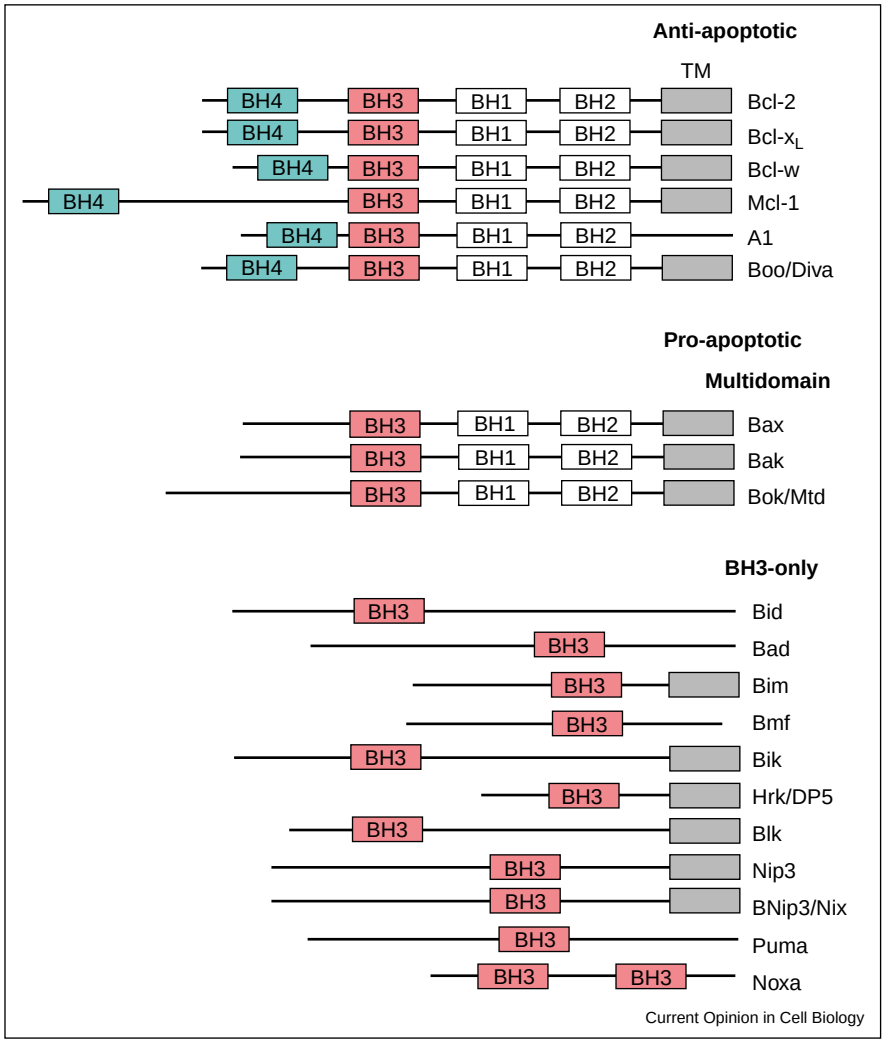
Pathways leading to mitochondrial apoptosis. **(a)** One mechanism involves the activation of BH3-only proteins, which activate or de-repress the BH1–3 proteins, such as Bax and Bak, producing MOMP. Consequently, pro-apoptotic proteins from the IMS are released into the cytoplasm, where they activate or de-repress caspases, leading to apoptosis. **(b)** In this pathway, certain agents produce a massive release of Ca^{2+} from the ER lumen. If enough Ca^{2+} is released, mitochondria can undergo permeability transition (PT), which produces swelling of the matrix. This swelling can be great enough to burst the outer membrane, releasing the soluble contents of the IMS into the cytoplasm. Bcl-2-family proteins can regulate the amount of releasable Ca^{2+}, thereby helping to determine whether mitochondria undergo the PT and hence death.

caspase-8 at the receptor complexes. However, activated caspase-8 can also engage the intrinsic pathway through cleavage of a Bcl-2-family protein, Bid [13,14]. Under certain circumstances in which the caspase-8 signal is weak or in which inhibitor of apoptosis (IAP) proteins inhibit the caspase cascade, this crosstalk between extrinsic and intrinsic mechanisms is needed to activate the death machinery effectively [15–19]. The contribution of the intrinsic pathway in such cases could be mediated by any of several IMS proteins, including cytochrome *c*, Smac/DIABLO [20,21], Omi/Htra2 [22–24], endonuclease G [25] and apoptosis-inducing factor (AIF) [26,27]. Smac/DIABLO and Omi/Htra2 can facilitate caspase activation, whereas endonuclease G and AIF might effect DNA fragmentation even in the absence of caspases.

Bcl-2-family members share one or more Bcl-2 homology (BH) domains and are divided into two main groups according to whether they promote or inhibit apoptosis. The pro-apoptotic family members are further classified according to whether they contain multiple BH domains or only BH3 (Figure 2). The BH3-only proteins are pro-apoptotic and require the co-operation of their multi-domain relatives to induce apoptosis [28–31]. More recently, a further subdivision of the BH3-only group was proposed [32•]. This was prompted by the observation that synthetic peptides corresponding to BH3 domains from Bid or Bim can activate Bax and Bak directly, whereas the Bad and Bik BH3 peptides act indirectly by inactivating Bcl-x_L or Bcl-2, thus allowing Bax and Bak to act unopposed. This 'de-repressor' class of BH3-only proteins (including Bik, Bad and presumably others) would not be expected to act alone but rather in concert with other BH3-only proteins such as Bid or Bim that can directly activate Bax and Bak.

The classification of Bcl-2 family members by sequence homology domains has proved to be consistent with the function of these molecules. However, when the structures of proteins belonging to the different functional categories were solved, including Bcl-x_L and Bcl-w (possessing homology domains BH1–4), Bax (BH1–3) and Bid (BH3-only), it came as a surprise that all have similar folds composed of seven or eight α helices, two of which are hydrophobic and presumably can become inserted within a membrane [33–38]. It is still not entirely clear what structural features determine whether these proteins function in a pro- or anti-apoptotic manner.

Figure 2



Schematic classification of some members of the Bcl-2 protein family. 'TM' refers to a hydrophobic region in the carboxyl terminus of several of these proteins that was originally assumed to be a transmembrane domain. It remains unclear whether these regions indeed facilitate membrane association in every case. The pro-apoptotic multidomain (BH1–3) proteins might contain a weak BH4 homology (not shown).

It is tempting to hypothesize that, in general, the response to each type of apoptotic stimulus is mediated through the activation of one or more BH3-only proteins, at least for forms of apoptosis involving MOMP. Each member of the BH3-only group might be activated in response to a particular set of stimuli and through a characteristic mechanism [39]. For example, Bid is cleaved by caspase-8 following death-receptor engagement [13,14], and after cleavage the potency of Bid becomes further enhanced by subsequent *N*-myristoylation on its carboxy-terminal fragment [40]. Bim is involved in UV-induced apoptosis, the death of autoreactive thymocytes (negative selection) [41] and in other forms of thymocyte death [42], as well as in the apoptosis of neurons deprived of nerve growth factor (NGF) [43]. Bmf can mediate death induced by cell detachment, known as anoikis [44]. Bim and Bmf are normally localized in specific cytoskeletal compartments — Bim on microtubules through interaction with dynein light chain 1 [45], and Bmf on myosin V motor complexes through dynein light chain 2 [44]. Perhaps because of their localization, Bim and Bmf may be poised to respond to certain death stimuli that can somehow cause the disengagement of these proteins from the cytoskeleton, allowing them to migrate to their sites of action. In contrast to these proteins that can be activated post-translationally, other members of the BH3-only group are activated only by transcriptional upregulation. Noxa [46] and Puma [47,48], for example, are upregulated following p53 activation in certain cell types, whereas Hrk/DP5 [49–51] is transcriptionally activated in neuronal cell death induced by NGF withdrawal or exposure to amyloid β.

Molecular mechanisms of outer membrane permeabilization by Bcl-2-family proteins

Structurally, Bcl-2-family proteins resemble the translocation domain of diphtheria toxin [33–36]. This observation inspired several studies showing that these proteins exhibit ion channel activities in synthetic lipid bilayers. However, in most cases, channel activities were recorded at non-physiological acidic pH [52–54], and the link, if any, between ion channel formation and apoptotic function remains unclear.

Other studies have examined the ability of Bcl-2 family proteins to make lipid bilayers permeable to proteins. On the one hand, Tsujimoto and colleagues [55] reported that Bcl-x_L, Bax and Bak interact with voltage-dependent anion channel (VDAC), an abundant protein in the outer membrane of mitochondria; moreover, these investigators observed that Bax stimulated the release of cytochrome *c* — but not larger proteins — from liposomes reconstituted with VDAC, apparently through a widening of the VDAC pore just wide enough to allow efflux of cytochrome *c*. On the other hand, Saito *et al.* [56] found that recombinant Bax alone was sufficient to release fluorescein-labeled cytochrome *c* from plain liposomes; calculations showed that the channel formed was composed of about four molecules of Bax and that the diameter was just wide enough to pass cytochrome *c*. Other investigators showed, using *in vitro* translated proteins, that Bax, but not Bcl-x_L, was able to rupture planar lipid bilayers, raising the possibility that Bcl-2-family proteins could destabilize membranes on a larger scale during apoptosis [57].

These systems, however, did not mimic *in vivo* observations such as the release of proteins larger than cytochrome *c* from mitochondria without gross disruption of the membrane, or the inhibition of membrane permeabilization by anti-apoptotic members of the Bcl-2 family. Recently, these hallmarks of mitochondrial apoptosis were reproduced *in vitro*, in a careful study employing cell-free systems of decreasing complexity, starting with isolated mitochondria, then resealed mitochondrial outer membrane vesicles, and culminating with the permeabilization of defined liposomes [58••]. The permeabilization effects were inhibited by Bcl-x_L (the same concentrations in each case) and occurred in each of the cell-free systems (mitochondria, outer membrane vesicles, and liposomes) at the same physiological concentrations of Bax. With liposomes, a mixture of tBid and Bax was required for permeabilization; neither tBid nor monomeric Bax alone was sufficient. This observation is consistent with the constitutive presence of monomeric Bax in the cytoplasm of living cells, as well as with genetic studies showing that the BH3-only proteins cannot trigger apoptosis in Bax/Bak double knockout cells [30,59]. The observations of Kuwana *et al.* [58••] show that Bcl-2-family proteins, by themselves, can permeabilize lipid bilayers, allowing the release of macromolecules considerably larger than cytochrome *c*. Moreover, this process appears not to require mitochondrial structures such as the inner membrane and matrix, nor the electron transport chain. This study also provided direct evidence that BH3 domains by themselves can activate Bax, resulting in membrane permeabilization. Thus, the role of BH3-only proteins is not merely to sequester anti-apoptotic proteins such as Bcl-x_L and Bcl-2.

These studies are consistent with a model in which Bcl-2-family members alone can regulate the formation of very large openings in the mitochondrial outer membrane. Although Kuwana *et al.* could not detect any morphological changes in the permeabilized membranes (presumably the Bax pores are easily closed under the conditions used for standard electron microscopy), another group was able to detect large pores formed by Bax in lipid bilayers (in the presence of supraphysiological Ca^{2+} concentrations), using atomic force microscopy [60]. Precisely how Bax permeabilizes membranes is unknown. Bax and Bak become oligomerized in the mitochondrial outer membrane both during apoptosis in cells [28,61,62] and *in vitro*, when recombinant Bid is added to isolated mitochondria [28,63•], and this oligomerization may be important for the permeabilization function. It is likely that the process of Bax activation, membrane binding and insertion, and oligomerization somehow increases the local curvature stress on membranes, resulting in the formation of lipidic pores. Consistent with this idea, Basanez *et al.* [64•] demonstrated that the ability of detergent-oligomerized Bax to produce pores in lipid membranes was affected by the addition of lipids that can alter the intrinsic curvature of the membrane, whereas these lipids had little effect on Bax binding or insertion into the membrane.

Kuwana *et al.* found that the signature mitochondrial lipid cardiolipin was required not merely for the targeting of Bid to mitochondrial membranes, as reported by Lutter *et al.* [65], but also for the membrane-permeabilizing activity of Bax. In analogy with the results of Basanez *et al.*, cardiolipin may increase the curvature stress within membranes, thus sensitizing them to Bax-induced pore formation. A difficulty here is that cardiolipin is much less abundant in the outer membrane than the inner membrane. Thus, an additional mechanism may be required either to concentrate cardiolipin in small domains, for example near contact sites between outer and inner membranes [66], or for membrane proteins to compensate somehow for a suboptimal concentration of cardiolipin. It will be important to determine whether other non-Bcl-2-family proteins in the outer membrane are required for MOMP in apoptosis, as proposed by Martinou and colleagues [63•].

It will also be important to discover how Bax and Bak oligomers are formed, how they alter the local structure of lipid bilayers, and how anti-apoptotic Bcl-2-family

members can interfere with oligomerization. It has been suggested that Bcl-x_L and Bcl-2 oppose the mitochondrial action of Bax and Bak by sequestering their cofactors, the BH3-only proteins [31,58••]. However, an elegant study by Ruffolo and Shore [67••] has highlighted a different mechanism. These investigators showed that Bcl-2 and Bcl-x_L can block the Bid-induced oligomerization of Bak, without inhibiting a Bid-induced conformational change in Bak that can be monitored by antibody binding. These results argue that Bcl-2 and Bcl-x_L can block Bak/Bax oligomerization without interfering with the ability of tBid to interact with Bak or Bax.

Intracellular Ca^{2+} mobilization and its effect on mitochondria

Recent studies have strengthened the case for an alternative pathway leading to mitochondrial apoptosis. In this scenario, certain apoptotic stimuli lead to the release of Ca^{2+} from ER stores, which in turn causes Ca^{2+} overload of the mitochondria, induction of the mitochondrial PT, and swelling of the mitochondrial matrix. Consequently, the outer mitochondrial membrane becomes ruptured, allowing pro-apoptotic IMS proteins to escape into the cytoplasm. Bcl-2-family members appear to regulate this process not by acting on mitochondria but through their localization at the ER [68••,69,70••]. For example, Bcl-2 can reduce the releasable pool of ER Ca^{2+}, desensitizing cells to death by C2-ceramide [69,71–73]. Bax and Bak act in the opposite manner, increasing the amount of Ca^{2+} releasable from the ER [68••,70••]. An issue raised by these results concerns how the pro- and anti-apoptotic Bcl-2-family members antagonize each other. Do they act in an independent but opposite manner on the ER Ca^{2+}-handling machinery? Or does one subfamily of Bcl-2 family proteins (e.g. Bcl-2 and Bcl-x_L) comprise the primary effectors, directly antagonized by the others (e.g. Bax and Bak)? And more questions arise: for example, how exactly do Bcl-2-family proteins regulate Ca^{2+} release from the ER? Is there a role for BH3-only proteins in this pathway? Finally, what are the relative contributions of the Ca^{2+} release and direct mitochondrial outer membrane permeabilization pathways in various apoptotic contexts?

A partial answer to this last question is that apoptotic messengers such as ceramide, arachidonic acid and reactive oxygen species (ROS) seem to rely significantly on calcium efflux from the ER, while other stimuli apparently do not [70••,74,75]. The importance of ER Ca^{2+} release in apoptosis may be both cell-type- and stimulus-specific; if so, this might help explain why some researchers observed evidence of PT in mitochondria before cytochrome *c* release in dying cells [76,77] while others did not [78–80]. Moreover, the response of mitochondria to the release of ER Ca^{2+} may also be subject to regulation, leading perhaps to further variation in the mechanisms of cell death. Indeed, in one study, when artificial calcium signaling was created in permeabilized cells, the mitochondria accumulated calcium, but underwent PT and cytochrome *c* release only if ceramide or staurosporine was added [81]. Furthermore, exogenously added recombinant Bid augmented the Ca^{2+} effect on mitochondria [82], suggesting a potential crosstalk between calcium signaling and the direct action of Bcl-2-family proteins on the outer mitochondrial membrane. In this regard, one might ask whether the Bid-induced changes in mitochondrial ultrastructure observed by Korsmeyer and colleagues [83] could in fact have been a response to Ca^{2+} that was somehow potentiated by Bid.

Aftermath of cytochrome *c* release from mitochondria and caspase-independent cell death

When PT is induced, the inner mitochondrial membrane potential ($\Delta\Psi_m$) becomes dissipated, leading to the loss of mitochondrial functions such as energy production and protein import into the organelle. In contexts in which MOMP is accomplished not through PT, but rather by the action of Bcl-2-family proteins on the outer membrane, mitochondrial respiration is affected only indirectly. Caspases that are activated by the released cytochrome *c* and Smac/DIABLO can apparently enter mitochondria to cleave key substrates in the mitochondrial electron transport chain, leading to a loss of respiration and an increased production of ROS [78,84,85•]. In these situations, intracellular ATP pools can maintain at least a small inner membrane potential for some period of time, through the reverse action of ATP synthase. Eventually, however, as ATP levels decline, the mitochondria lose mitochondrial membrane potential ($\Delta\Psi_m$) and protein import function, and are presumably degraded.

Under certain circumstances (in certain tumor cells, for example), caspase activation can be blocked by overexpressed IAPs, the loss of caspase expression, or the loss of IAP degradation pathways (e.g. [86–88]). In such cases, the cells nevertheless might die in a caspase-independent manner [86,87,89]. In caspase-inhibited cells, cytochrome-*c*-dependent respiration can continue for some time despite the dilution of cytochrome *c* into the whole cellular volume, because even the reduced concentrations of this protein are sufficient to support respiration [84,90,91]. In time, however, the pool of cytochrome *c* can diminish through degradation and reduced synthesis. Moreover, the conversion of apo-cytochrome *c* to the active holo form by the mitochondrial enzyme, heme lyase, could be slower following MOMP. As cytochrome *c* pools become depleted, respiration declines and eventually so does intracellular ATP concentrations. As a result, $\Delta\Psi_m$ cannot be maintained, and the mitochondria suffer a disruption of protein import and consequently a loss of other functions that depend on sustained protein import. This decline in mitochondrial

function may be a decisive factor in the late stages of caspase-independent death.

Caspase-independent cell death might also be aided, at least in theory, by the degradative effects of other IMS proteins released from mitochondria, such as AIF or endonuclease G. Caspase-independent cell death can also be induced by direct effects on mitochondria, for example by ROS, nitric oxide [92] or other substances that are directly toxic to mitochondria [93]. Potentially, caspase-independent pathways could be engineered to kill cancer cells, which often have defective apoptotic responses [94].

Conclusions

The past year has brought advances in our understanding of the actions of Bcl-2-family proteins and their effects on mitochondrial pathways of cell death. In one scenario, the pro-apoptotic proteins Bax and Bak can be activated by the BH3 domains of Bid or Bim, resulting in the formation of large lipidic pores in the outer mitochondrial membrane. Still to be resolved is whether other outer membrane proteins are required to activate or regulate this process in apoptotic cells. Another mechanism gaining attention involves the release of Ca^{2+} from ER stores, leading to the mitochondrial PT, rupture of the outer membrane, and release of pro-apoptotic IMS proteins. How Bcl-2-family proteins regulate this process, and the degree of importance of this mechanism in various forms of cell death, are open questions. Finally, the role of mitochondria in late stages of cell death, or in caspase-deficient cells, is just beginning to be understood.

Acknowledgements

The authors are grateful to Lisa Bouchier-Hayes, Christine Bonzon and Jean-Ehrland Ricci for helpful discussions.

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