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Bcl-2 family proteins and mitochondria

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Abstract

The Bcl-2 family of proteins plays a pivotal role in regulating cell life and death. Many of these proteins reside in the outer mitochondrial membrane, oriented towards the cytosol. Cytoprotective Bcl-2 family proteins such as Bcl-2 and Bcl-XL prevent mitochondrial permeability transition pore opening and release of apoptogenic proteins from mitochondria under many circumstances that would otherwise result in either apoptosis or necrosis. In contrast, some pro-apoptotic members of this family such as Bax can induce these destructive changes in mitochondria in both mammalian cells and when expressed exogenously in yeast. The mechanisms by which Bcl-2 family proteins control cell life and death remain elusive, but may include both the ability to form ion channels or pores in membranes and physical interactions with a variety of proteins implicated in apoptosis regulation.

Keywords: Mitochondrion; Bcl-2; Cytochrome *c*; Caspase; Apoptosis

1. Introduction

Ever since the discovery that the Bcl-2 protein resides in mitochondrial membranes, the worlds of mitochondrial physiology and apoptosis have been inextricably linked [1]. The Bcl-2 family consists of both cell death promoters and cell death preventers. Mammalian species appear to contain at least 14 members, including the anti-apoptotic proteins Bcl-2, Bcl-XL, Mcl-1, A1/Bfl-1, and Bcl-W [2–7], and the pro-apoptotic members Bax, Bcl-XS, Bak, Bad, Bik, Bim, Bid, Hrk, and Bok [3,8–17]. Homologs have also been found in avian species [18,19] and frogs [20], as well as some lower organisms (invertebrates), such as the anti-apoptotic protein CED-9 of *Caenorhabditis elegans* [21]. Several human herpesviruses also encode homologs of Bcl-2, including BHRF-1 in Epstein–Barr virus [22,23], ORF16 in herpesvirus Saimiri [24,25] and Kbcl-2 within the Kaposi sarcoma-associated virus, human herpesvirus-8 (HHV8) [26,27]. The African swine fever virus gene 5HL is also a Bcl-2 homolog [28]. In addition, the adenovirus E1b-19kDa protein serves as a functional homolog of Bcl-2, though sharing little amino acid sequence identity with it [29]. Many of the Bcl-2 family proteins can interact with each other, forming a complex network of homo- and heterodimers [8,30]. The relative ratios of anti-apoptotic and pro-apoptotic Bcl-2 family proteins play a major role in determining the ultimate sensitivity or resistance of cells to myriad stimuli and insults that induce apoptosis (reviewed in [31]).

Bcl-2 was first reported to be an inner mitochondrial membrane protein [1], but subsequent studies suggested an outer membrane location instead [32–36]. For example, during in vitro import studies, in

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vitro translated Bcl-2 appears to insert into the outer membrane due to a stretch of hydrophobic amino acids located at its carboxyl terminus but remains susceptible to proteolytic degradation unlike intramitochondria proteins. Immunoelectron microscopy studies have also provided evidence of a primarily outer membrane location. Interestingly, however, Bcl-2 appears to be enriched at the contact sites were the inner and outer membranes come into close apposition [33], suggesting a potential explanation for the association of some of the protein with mitoplasts which have been stripped of their outer membrane using digitonin [1].

Detailed knowledge about the locations of other Bcl-2 family proteins within mitochondrial membranes is relatively scant, but many members of the family do indeed appear to be associated with mitochondria. Interestingly, the association of some Bcl-2 family proteins with mitochondrial membranes appears to be inducible rather than constitutive. For example, typically about half the Bax protein found in cells resides in the cytosol until an apoptotic signal is delivered which causes its insertion into mitochondrial membranes [37,38]. The mechanisms responsible for this inducible association of Bax with mitochondria remain to be clarified, particularly since (similar to Bcl-2) it contains a hydrophobic domain at its carboxyl end that theoretically ought to target it constitutively into membranes. Not all Bcl-2 family proteins contain a membrane-anchoring domain near their carboxyl termini; however, most nevertheless associate with intracellular membranes perhaps via dimerization with other members of the family that are integral membrane proteins.

2. Mitochondrial functions regulated by Bcl-2 family proteins

Bcl-2 family proteins have been implicated in the regulation of two important aspects of mitochondria pathophysiology: (a) mitochondrial permeability transition (PT) pore opening; and (b) release of apoptogenic proteins from mitochondria into the cytosol. During apoptosis induced by myriad insults and stimuli, the electrochemical gradient ($\Delta\Psi$) across the inner mitochondrial membrane becomes dissipated (reviewed in [39]). This loss of $\Delta\Psi$ has been attributed to the opening of a large conductance inner membrane channel commonly referred to as the mitochondrial PT pore. Though the structure and biochemical composition of the PT pore remain poorly defined, its constituents are thought to include both inner membrane proteins such as the adenine nucleotide translocator (ANT) and outer membrane proteins such as porin (voltage dependent anion channel) which operate in concert, presumably at inner and outer membrane contact sites, to create a channel with approx. 1.5 kDa diameter (reviewed in [40]). The opening of this non-selective channel in the inner membrane allows for an equilibration of ions within the matrix and intermembrane space of mitochondria, thus dissipating the electrochemical gradient and uncoupling the respiratory chain.

Several potentially lethal events follow from mitochondrial PT pore opening. For example, electrons that normally would find their way through the respiratory chain to molecular oxygen are instead shunted into free-radical production. Mitochondrial ATP production also grinds to a halt, but presumably adequate intracellular stores typically persist since ATP depletion is not a general feature of apoptosis and indeed would usually preclude it. In addition, the volume dysregulation that occurs upon PT pore opening leads to entry of water into the protein-rich matrix of mitochondria, causing the matrix space to expand. Since the inner membrane with its folded cristae possesses a larger surface area than the outer membrane, this matrix volume expansion can eventually cause the outer membrane to rupture, releasing proteins located within the intermembrane space into the cytosol.

Two proteins have been described which can trigger apoptosis when released from mitochondria into the cytosol: cytochrome *c* and apoptosis inducing factor (AIF), a putative protease of 50 kDa [41–44]. Both cytochrome *c* and AIF share in common the ability to activate a group of intracellular cysteine proteinases called caspases. The caspases are the chief effectors of apoptosis (reviewed in [45]). Caspase activation represents the sine qua non of apoptosis. These proteins are not degradative proteases, but rather clip specific substrates at aspartic acid residues which appear within particular sequence contexts. Caspases lie in a latent state as inactive zymogens (pro-enzymes) in essentially all ani-

mal cells, becoming activated under conditions that culminate in apoptosis. It is the specific proteolytic events executed by caspases that account directly or indirectly for the morphological and biochemical characteristics of the apoptotic cell.

AIF appears to directly activate certain members of the caspase family, resulting in proteolytic processing of their proproteins and production of the mature enzymes [44]. It has been argued that AIF itself is possibly an aspartic acid-specific protease, based on experiments where AIF (which was purified to apparent homogeneity from the supernatants of mitochondria induced to undergo PT) was shown to cleave in vitro recombinant pro-caspase-3 (which was produced in bacteria and purified) through a mechanism that was suppressible by a peptidyl inhibitor of caspase family aspartyl proteases [46]. To date, however, cDNAs encoding AIF have not been cloned and thus it remains to be determined whether this protein shares similarity with caspase family proteases.

Cytochrome *c* activates caspases through it effects on a protein called Apaf-1, apoptosis protease activating factor-1 [47]. Apaf-1 lies in a latent state in the cytosol of cells. Upon binding cytochrome *c*, however, Apaf-1 becomes competent at binding the proform of a specific caspase, pro-caspase-9, presumably because of cytochrome *c*-induced conformational changes in the Apaf-1 protein [48]. This results in proteolytic activation of pro-caspase-9, possibly through an autoprocessing mechanism facilitated by the binding of the caspase-9 to the Apaf-1/cytochrome *c* complex. The redox state of cytochrome *c* is apparently unimportant for its function as a co-activator of caspases [49]. However, methylated cytochrome *c* from yeast is inactive, implying need for specific structural features of animal cytochrome *c* that allow binding to and activation of Apaf-1. Heme binding is also critical since apo-cytochrome *c* does not trigger caspase activation. The beauty of this system is that apo-cytochrome *c* can be produced in the cytosol by translation from a nuclear encoded gene and then transported into mitochondria for heme attachment, without killing the cell. Since cytochrome *c* is essential for execution of oxidative phosphorylation, cells cannot discard this molecule and still produce ATP through aerobic respiration. This dependence on cytochrome *c* for respiration, therefore, presumably ensures that nearly all cells retain the cytochrome *c*-based mechanism for apoptosis, perhaps explaining in part why all animals cells seem to be capable of undergoing apoptosis if stimulated appropriately or hit hard enough.

Where do Bcl-2 and its homologs fit into this story of mitochondrial PT pore opening and release of caspase activating proteins? Several groups have now reported that over-expression of Bcl-2 or similar cytoprotective members of the Bcl-2 family such as Bcl-XL confers protection upon mitochondria, making it more difficult for many stimuli to induce PT pore opening and release of AIF and cytochrome *c* [42–44,50,51]. Conversely, over-expression of the pro-apoptotic protein Bax has been demonstrated to induce loss of $\Delta\Psi$ and release of cytochrome *c* [52–54]. The mechanisms by which Bcl-2 prevents and Bax promotes these destructive changes in mitochondria, however, are far from clear.

It has been hypothesized that Bcl-2 family proteins directly participate in regulation of the PT pore. In support of this idea are experiments in which attempts were made to purify the multiprotein PT pore complex from mitochondria and reconstitute it in synthetic liposomes. During purification of the PT complex, Bax was reportedly co-purified, resulting in an approx. 50-fold enrichment of this protein and implying that Bax may be closely associated with proteins that constitute components of the PT pore [55]. Moreover, the partially purified PT pore complex retained many of its expected functional characteristics when reconstituted in liposomes, including suppression of pore opening by recombinant Bcl-2 protein but not by mutants of Bcl-2 which fail to suppress apoptosis in intact cells [55,56].

Some other observations, however, have suggested the possibility that Bcl-2 family proteins perform other functions in mitochondria which are perhaps only indirectly related to PT pore regulation. For example, comparisons of the time courses of cytochrome *c* release and mitochondrial $\Delta\Psi$ loss in cells undergoing apoptosis caused by growth factor deprivation or cytotoxic anticancer drugs have provided evidence that cytochrome *c* release can precede $\Delta\Psi$ loss in at least some types of cells [42,43,57]. In this scenario, it has been argued that only a portion of the total available cytochrome *c* needs to be released into the cytosol to result in caspase activation

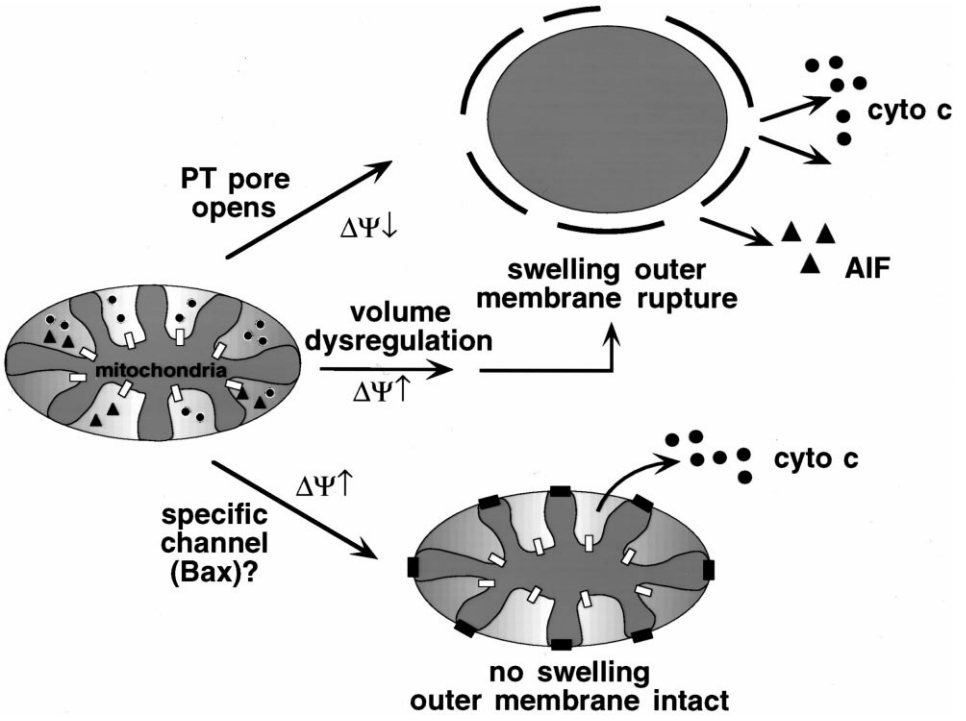

Fig. 1. Potential mechanisms of cytochrome *c* release from mitochondria. Available data suggest the existence of two principal pathways for release of cytochrome *c*: one in which the organelles swell and the outer membrane ruptures (top) and another in which the outer membrane remains largely intact but the permeability changes sufficiently to allow transit of cytochrome *c* (bottom). Depending on the stimulus for cell death, either pathway may predominate, but because active caspases can also induce mitochondrial PT pore opening [46,55], the opportunity for feedward loops exists in which release of cytochrome *c* through pathways that do not entail loss of $\Delta\Psi$ and outer membrane rupture can recruit additional mitochondria through caspase-dependent mechanisms that do involve loss of $\Delta\Psi$ and organellar swelling. It has been suggested that the pathway resulting in swelling and outer membrane rupture may occur either as a result of PT pore opening which is accompanied by loss of $\Delta\Psi$ [39,104] or through alternative mechanisms in which the inner membrane is largely intact and $\Delta\Psi$ is maintained [58]. The release of cytochrome *c* from mitochondria is caspase-independent, implying that this event is upstream from caspase activation at least in cell death scenarios where mitochondria play roles as initiators of apoptosis [54]. The induction of PT pore opening, as defined by loss of $\Delta\Psi$, however, may take place either upstream or downstream from caspase activation.

[54,58]. The idea is that sufficient cytochrome *c* remains inside mitochondria, bound to cytochrome *c* oxidase for handling electron transport from complex III to IV in the respiratory chain, so that respiration is unimpaired and $\Delta\Psi$ is maintained.

If true, then how does a protein the size of cytochrome *c* (approx. 12 kDa) transit from its normal place of residence in the intermembrane space across the outer membrane into the cytosol? One idea that has been proposed recently is that mitochondrial volume dysregulation with matrix swelling and outer membrane rupture can occur independent of PT pore opening, perhaps as a result of changes in the function of some of the various ion transporters found in the inner membrane [58]. Alternatively, it could be that pores are created de novo in the outer membrane that allow some of the cytochrome *c* to escape, while leaving the permeability of the inner membrane intact. In support of the latter, it has recently been shown that addition of recombinant Bax protein to isolated mitochondria can induce release of cytochrome *c* without attendant swelling of mitochondria, suggesting that PT pore opening is not necessarily required for escape of cytochrome *c* [54]. Moreover, evidence has been obtained suggesting that in some circumstances where cytochrome *c* release appears to precede PT pore opening, caspase activation may be required for the loss of $\Delta\Psi$ but not for the release of cytochrome *c* [57]. In this regard, certain caspases have been reported to induce PT pore opening when applied to isolated mitochondria in vitro [46,55]. Taken together, therefore, these ob-

servations imply the existence of a feed-forward mechanism by which cytochrome *c* release from a few mitochondria can induce mitochondrial PT pore opening, resulting in release of more cytochrome *c* and possibly other caspase activators such as AIF from mitochondria (Fig. 1).

3. Location, location, location

In the Real Estate business, location is everything. Is the intracellular location critical for the function of Bcl-2 family proteins? Though the mitochondrial location is typically emphasized, significant portions of the Bcl-2 protein are also integrated into the membranes of the endoplasmic reticulum (ER) and nuclear envelope [33–36]. Moreover, some members of the Bcl-2 family such as Mcl-1, Bak, and E1b-19kDa may be located preferentially if not exclusively in non-mitochondrial sites ([59,60]; R. Brown, personal communication). Attempts have been made to answer the question of whether association of Bcl-2 with mitochondria is absolutely required for its anti-apoptotic function through mutational analysis of this protein and a closely related homolog Bcl-XL. When the carboxyl approx. 20 amino acids constituting the membrane-anchoring domain is deleted from Bcl-2 or Bcl-XL, much of the protein is found in a soluble cytosolic fraction and cytoprotective function is often reduced but not completely abrogated [61]. In addition, an isoform of the Bcl-XL protein has been described which arises through alternative mRNA splicing mechanisms and that lacks the carboxyl transmembrane (TM) domain but which remains functional as a suppressor of cell death [62]. However, perhaps due in part to its ability to dimerize with endogenous Bcl-2 family proteins, substantial proportions of the Bcl-2 (ΔTM) and Bcl-XL (ΔTM) proteins are typically associated with membranes [63], making it difficult to interpret the significance of the findings.

Other experiments in which the carboxyl TM domain of Bcl-2 was substituted with analogous membrane-anchoring domains from either an outer mitochondrial protein or a type II ER membrane protein revealed that ER-targeted Bcl-2 was markedly impaired in its ability to protect against apoptosis induced by several stimuli, whereas mitochondria-targeted Bcl-2 was essentially as potent as the wild-type protein [64]. These observations support view that the major cytoprotective function of Bcl-2 is related to its effects on mitochondria. Nevertheless, ER-targeted Bcl-2 retained the ability to block apoptosis induced by serum deprivation from Myc-expressing cells, suggesting that Bcl-2 does indeed possess some non-mitochondrial mechanisms for promoting cell survival [64]. In this regard, Bcl-2 has also been reported to regulate Ca^{2+} homeostasis in the ER, where it evidently prevents or delays pathological leakage of Ca^{2+} from this organelle under some circumstances [65–68]. Moreover, Bcl-2 has been reported to bind two ER membrane proteins, Bap31 and BI-1, which participate in apoptosis regulation through undefined mechanisms [69,70].

In addition, Bcl-2 has been localized to the nuclear envelope and immunoelectron microscopic studies suggest potential association with nuclear pore complexes [32,34]. In some types of cells, over-expression of Bcl-2 prevents translocation of the apoptosis-inducing protein p53 from cytosol into nucleus [71,72], suggesting that Bcl-2 may snag selected proteins as they try to transit through nuclear pore complexes.

Taken together, therefore, it seems that Bcl-2's actions in cells are not relegated solely to protecting mitochondria during cellular confrontation with apoptosis-inducing insults and stimuli. Though mitochondria may be a major site of action of the Bcl-2 family proteins, clearly under some specific circumstances Bcl-2 can suppress potentially cytodestructive events at other locations.

4. Multi-functional Bcl-2 family proteins

Though a clear understanding of how Bcl-2 and its homologous proteins regulate cell life and death remains elusive, currently available data suggest that some members of this protein family are multifunctional, possessing more than one mechanism for modulating cell death (reviewed in [73]). Three general types of functions have been recognized: (a) dimerization with other Bcl-2 family proteins; (b) pore or ion channel activity; and (c) binding to non-homologous proteins. The dimerization among various members of the Bcl-2 family and its functional consequences will not be discussed here in detail.

5. Pore/channel formation

The first suspicion that some Bcl-2 family proteins might be capable of forming pores or ion channels in membranes came with the determination of the three-dimensional structure of Bcl-XL [74]. The Bcl-XL protein is comprised of seven α-helices joined by flexible loops, and shares striking similarity to the pore forming domains of some types of bacterial toxins, e.g., diphtheria toxin (DT) and the colicins. Molecular modeling studies suggest that anti-apoptotic proteins such as Bcl-2 and some types of pro-apoptotic proteins such as Bax share the same fold with Bcl-XL [75]. As predicted by their structures, Bcl-2, Bcl-XL and Bax have all been shown to form ion channels at least in vitro when added to synthetic membranes under conditions previously shown to be conducive for channel formation by DT and the bacterial colicins [76–79]. However, the relative conductances, ion selectivities and dynamic characteristics of these channels appear to differ. Though far more work needs to be done, the general suggestion is that the cytoprotective proteins Bcl-2 and Bcl-XL tend to form small channels that assume a mostly closed conformation, preferring cations, whereas the pro-apoptotic protein Bax tends to form larger channels, which assume a mostly open conformation and prefer anions (reviewed in [75]). Moreover, evidence has been obtained that under some in vitro conditions, Bcl-2 may be able to prevent channel formation by Bax [78], suggesting a potential mechanism for the antagonism displayed between these proteins.

How might channel forming activity explain the effects of Bcl-2 family proteins on mitochondria? Probably the simplest theory is that pro-apoptotic proteins such as Bax form large channels in the outer membrane of mitochondria which liberate cytochrome *c*. Though α-helical proteins in general form relatively small channels, diphtheria toxin is believed to create pores large enough to allow a polypeptide, namely the A-subunit of the toxin (ADP ribosylation factor), to escape the endosomal/lysosomal compartment and enter the cytosol where it inhibits translation and causes cell death [80]. Thus, precedence exists for protein transport by other pore-forming proteins which are predicted to share structural similarity with Bax. Indeed, the recent observation that recombinant Bax protein can induce specific release of cytochrome *c* from isolated mitochondria without attendant mitochondrial swelling lends support to this idea [54]. However, it still remains to be determined whether Bax directly forms pores in the outer membrane of sufficient size to liberate the approx. 12 kDa cytochrome *c* protein.

More difficult to envision is how a small conductance ion channel created in the outer membrane by Bcl-2 or Bcl-XL could impact mitochondrial physiology, given that the outer membrane is presently believed to be rather porous due to the presence of VDAC. Even in its 'closed' conformation, this β-barrel-type channel protein creates pores of estimated 1.5 nm diameter [81]. Thus, the outer membrane should be freely permeable to ions and most metabolites. One possibility is that Bcl-2 and Bcl-XL functionally communicate with inner membrane proteins that govern ion transport. These proteins could include components of the PT pore as suggested above [39,55,82], or ion transporting proteins that control volume regulation of the matrix space as implied by one recent report [58]. It has also been suggested that Bcl-2 and Bcl-XL somehow regulate the pH of the intermembrane space, reportedly causing increased rates of extrusion of protons from mitochondria [83]. Again, since pH regulation in mitochondria is governed by inner membrane transporters, this observation implies a mechanism for functional communication between Bcl-2 family proteins in the outer membrane and H^+ channels in the inner membrane. Alternatively, perhaps a small portion of the total mitochondria-associated pool of Bcl-2, Bcl-XL, or Bax protein is actually located in the inner membrane [84], an idea that deserves further scrutiny. Finally, though a heretical notion, it has also been argued by some that the outer membrane of mitochondria is not freely permeable to protons, prompting speculations that pore-forming Bcl-2 family proteins have a role to play as transporters of H^+ within the outer membrane [58]. The suspicion is that controlling proton transport across the outer membrane would contribute to $\Delta\Psi$ regulation by either increasing or decreasing the pH of the intermembrane space, and this in turn could translate somehow into either resistance or susceptibility to cell death stimuli.

Regardless of the mechanisms involved, studies of the effects of ectopic expression of human Bcl-2 family proteins in yeast have provided further indirect support for the hypothesis that at least some of these cell death-regulatory proteins may be capable of forming channels in membranes and have demonstrated evolutionary conservation of at least some aspects of the function of these proteins. For example, expression of the pro-apoptotic proteins Bax and Bak in either budding or fission yeast induces cell death, which can be prevented by co-expression of anti-apoptotic proteins such as Bcl-2 and Bcl-XL [30,85–88]. In contrast, deletion mutants of Bax and Bak that lack the putative pore-forming α5- and α6-helices are inactive when expressed in yeast ([87] and unpublished observations). Since yeast contain no recognizable Bcl-2 homologs, it is unlikely that Bax and Bak kill yeast by dimerizing and interfering with the functions of such proteins in yeast. In support of this argument, single amino acid substitution mutants of Bax have been described which fail to dimerize with Bcl-2 or Bcl-XL and yet retain their lethality in yeast as well as mammalian cells [89]. Moreover, Bcl-2 can still rescue both yeast and mammalian cells from death induced by dimerization-defective mutants of Bax, implying that Bcl-2 possesses intrinsic cytoprotective functions which can manifest themselves independent of heterodimerization with Bax. In addition, pro-apoptotic Bcl-2 family proteins such as Bcl-XS and Bik, which are not predicted to share the same structural fold with the pore-forming members of the family, fail to confer a lethal phenotype in yeast [30,87,88]. It has been speculated that these proteins like Bcl-XS and Bik are limited to counteracting the effects of Bcl-2, Bcl-XL, and other anti-apoptotic Bcl-2 family proteins with which they dimerize, lacking an intrinsic function as channel proteins, and thus have no phenotype when expressed in yeast [89]. While far more work needs to be done, the data available to date therefore strongly suggest that the phenotypes of certain Bcl-2 family proteins in yeast are a reflection of their ability to form channels in intracellular membranes. However, detailed mutagenesis experiments are needed to confirm this hypothesis by altering specific residues within the putative pore-forming α-helices of these proteins and correlating changes in their channel characteristics in vitro with their functions in yeast.

Where do mitochondria figure in the explanation for the cell death phenotype exhibited by certain Bcl-2 family proteins in yeast? When expressed in the budding yeast *Saccharomyces cerevisiae*, Bax is reportedly targeted to mitochondria. Deletion of the C-terminal TM domain from Bax results instead in a mostly cytosolic location of the protein and loss of its cytotoxic function in yeast [89], similar to the situation seen in some types of mammalian cells where Bax (ΔTM) truncation mutants have been studied [90]. Mitochondrial targeting and lethal function of Bax (ΔTM) can be restored in yeast by creating chimeric fusion protein which append the membrane-anchoring domain of the yeast outer mitochondrial membrane protein Mas70p to Bax (ΔTM), implying that integration of Bax into mitochondrial membranes is critical for its cell death-inducing activity in yeast. However, since no attempts have been made to force targeting of Bax to other organelles, it cannot necessarily be concluded that Bax's only site of action in yeast is mitochondria. Nevertheless, ectopic expression of Bax in *S. cerevisiae* has been reported to induce release of cytochrome *c* into the cytosol [91], paralleling the result obtained in mammalian cells where Bax has also been shown to induce cytochrome *c* release from mitochondria [52,54]. Furthermore, genetic analysis of yeast resulted in the discovery that certain subunits of the FoF1ATPase/proton pump of the inner membrane are required for Bax-induced death, thus further strengthening the functional connections between mitochondria and Bax [92]. Interestingly, a functional FoF1-ATPase/proton pump appears to also be required for optimal killing of mammalian cells by Bax, based on experiments where oligomycin was used to prevent H^+ transport by FoF1, resulting in reductions in Bax-induced apoptosis [92]. Why the FoF1-ATPase/proton pump is required for the cytotoxic activity of Bax is presently unknown, but possibilities include: (a) necessity of FoF1 for creating conditions of local pH that are conducive for channel formation by Bax; and (b) use of FoF1 as an effector of Bax-induced cell death, perhaps by running the pump 'backwards' so that protons are extruded from the matrix at the expense of ATP hydrolysis rather than the normal situation where protons flow down their concentration gradient from the intermembrane space into the matrix and ATP is gen-

erated. Alternatively, the FoF1-ATPase/proton pump may not be required for Bax-induced cell death, but its absence may have protective effects. For example, in the absence of the proton pump, less utilization of the proton gradient would occur and hence the ΔpH should be higher (i.e. lower pH in the intermembrane space). Perhaps this more acidic environment within the local space between the inner and outer membranes somehow precludes Bax from performing its functions. Interestingly, it has recently been suggested that Bcl-2 can increase the extrusion of protons through a mechanism that does not involve increased oxygen consumption [83]. If this enhanced extrusion of protons from the matrix of mitochondria is related to the mechanism by which Bcl-2 provides cytoprotection, then loss of the FoF1-ATPase/proton pump theoretically would mimic the actions of Bcl-2 by decreasing the local pH of the intermembrane space. Again, how this affects the state of Bax in the outer membrane remains unclear, but perhaps outer membrane proteins can be influenced by the local pH of the intermembrane space, particularly at the contact sites where the inner and outer membranes come into close apposition. Though loss of FoF1 may functionally mimic Bcl-2, circumstantial evidence suggests that Bcl-2 does not function by inhibiting FoF1 [83]. Bcl-2 also does not increase oxygen consumption or affect mitochondrial ATP levels, implying that it does not use the respiratory chain (complexes I, III, IV) to increase proton extrusion. Indeed, Bcl-2 can still protect from apoptosis under anaerobic conditions [93,94] and retains its activity in cells that are defective in oxidative phosphorylation due to a lack mitochondrial DNA [95].

Which of the effects of Bax on mitochondria is actually responsible for cell death? Bax induces cytochrome *c* release from mitochondria but yeast have no detectable caspases and fail to undergo apoptosis, as defined by morphological criteria. Thus, cytochrome *c*-mediated activation of cell death proteases seems an unlikely explanation. However, if cytochrome *c* loss from mitochondria reaches the point that electron transport halts, then clearly mitochondrial ATP synthesis would cease and electrons derived from NADH would likely be shunted into free radical generation, resulting in direct damage to membranes and macromolecules. The idea has therefore arisen that cytochrome *c* release from mitochondria has two ways of potentially killing cells: one involving caspase activation (at least in animal cells) and another resulting from the failure of electron chain transport in the inner membrane when cytochrome *c* becomes limiting (which presumably applies to both yeast and animal cells). In this regard, in mammalian cells, over-expression of Bax normally leads to $\Delta\Psi$ loss, cytochrome *c* release, caspase activation and apoptosis. However, if agents are employed to block caspases, then Bax still induces $\Delta\Psi$ loss and cytochrome *c* release but instead of apoptosis a necrosis-like cell death process ensues which (by electron microscopic analysis) shares remarkable ultrastructural similarity with Bax-mediated cell death in yeast [53,86–88]. These and other observations have provided evidence that 'commitment' to cell death can be regulated at a point upstream from caspases, at least when mitochondria play a pivotal role in the mechanism.

6. Pore-independent effects of Bcl-2 family proteins on mitochondria

In addition to forming channels in membranes, Bcl-2 or some of its anti-apoptotic homologs such as Bcl-XL can bind to several proteins located either in the cytosol or in intracellular membranes (reviewed in [73]). Perhaps the most interesting of these is Apaf-1, the cytosolic protein which cytochrome *c* uses to activate caspases. Apaf-1 contains an N-terminal domain that binds to caspase-9, and another domain that binds to Bcl-XL which is homologous to the nematode (*C. elegans*) death protein CED-4 [48,96]. Though no firm evidence yet exists that Bcl-XL can suppress the function of Apaf-1, studies of the analogous proteins in *C. elegans* have revealed that the Bcl-2/Bcl-XL homolog CED-9 can inhibit CED-4, preventing it from activating the caspase CED-3 [97,98]. Moreover, under some circumstances, it appears that Bcl-2 can suppress apoptosis induced by microinjection of cytochrome *c*, implying that it might interfere with Apaf-1. However, since this is not uniformly seen, cell context-dependent factors must influence whether Bcl-2 can suppress cell death downstream from cytochrome *c* [99,100].

By sitting in the outer membrane of mitochondria

oriented towards the cytosol, Bcl-2 is able to recruit cytosolic proteins with which it interacts to the mitochondrial surface. Examples of this phenomenon include the protein kinase Raf-1 and the phosphatase calcineurin, both of which can be co-immunoprecipitated with Bcl-2 and which are targeted from the cytosol to the organellar sites where Bcl-2 resides when Bcl-2 is over-expressed in cells [101,102]. A regulator of Hsp70/Hsc70 family molecular chaperones, BAG-1, can also be targeted to the surface of mitochondria through interactions with Bcl-2 [103]. This situation may create opportunities for Bcl-2 to bring signal transducing enzymes and chaperones into contact with mitochondrial proteins that they would not otherwise meet in cells. However, to date, the only recognized targets of such Bcl-2 interacting proteins are either Bcl-2 itself or other Bcl-2 family proteins. Interestingly, no known examples of Bax or other pro-apoptotic Bcl-2 family proteins binding to non-homologous proteins exist, implying a key functional difference between the death blockers and the death inducers. However, the dimerization of Bcl-2 or Bcl-XL with Bax or other pro-apoptotic Bcl-2 family proteins disrupts their interactions with proteins such as Apaf-1 and calcineurin [96,102], suggesting a mechanism by which proteins such as Bax, Bak, Bik, and BAD may antagonize the function of Bcl-2, Bcl-XL, and their anti-apoptotic homologs.

7. Summary

Though many details about mechanisms are lacking, it has become abundantly clear that Bcl-2 family proteins exert profound effects on mitochondria. The structural homology of some Bcl-2 family proteins to the pore forming domains of bacterial toxins raises the possibility that these molecules may act as channels for either ion or protein transport across the membranes of mitochondria. Mitochondria, long implicated in necrotic cell death through generation of reactive oxygen species during ischemia reperfusion injury, have now been tied to caspase activation and apoptosis through their ability to release proteins that trigger the cell death proteases into action. The intimate relation that mitochondria share with cell death stands in sharp contrast to the role that these organelles play as the engines of ATP production and sustainers of cell life. This dual role of mitochondria has served animal species well over evolution, but also creates opportunities for dysregulation that contributes to human diseases. Greater understanding of all facets of these complex organelles is needed if we are to ultimately tame their pathological tendencies in diseases characterized by inappropriate cell death such as myocardial infarction, stroke, AIDS, and neurodegeneration, or harness it for good purposes in disorders caused by insufficient cell death such as cancer, autoimmunity, and restenosis.

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