Review
Control of mitochondrial permeability by Bcl-2 family members
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Abstract
Programmed cell death (apoptosis) is regulated by the Bcl-2 family of proteins. Although it remains unclear how these family members control apoptosis, they clearly have the capacity to regulate the permeability of intracellular membranes to ions and proteins. Proapoptotic members of the Bcl-2 family, especially Bax and Bid, have been extensively analyzed for the ability to form channels in membranes and to regulate preexisting channels. Anti-apoptotic members of the family tend to have the opposing effects on membrane channel formation. The molecular mechanisms of the different models for the permeabilization of membranes by the Bcl-2 family members and the regulation of Bcl-2 family member subcellular localizations are discussed.

Keywords: Bax; Bid; Mitochondrion; Channel

1. Introduction
The Bcl-2 protein family members are potent regulators of apoptosis that can influence the permeability of the outer mitochondrial membrane [1]. Family members Bax, Bak and Bok promote apoptosis while Bcl-2, Bcl-xL, Bcl-w and Mcl-1 inhibit cell death. A third subfamily, called the BH3-only proteins, are more distantly related and promote apoptosis, perhaps by regulating the former family members. While some members of this family are constitutively localized on the outer mitochondrial membrane such as Bcl-2 and Bak, these proteins and other family members have been found in various other sub-cellular locations including the endoplasmic reticulum, the cytosol and bound to microtubules. The control over the subcellular localizations of different members of the Bcl-2 family occurs through heterodimerization, phosphorylation, proteolysis, or interaction with FKBP38. Regardless of the signaling pathway, Bcl-2 proteins appear ultimately to act at organelle membranes to tranduce cell survival and cell death signals. Permeability of the mitochondrial and ER membranes to proteins and ions is regulated by the Bcl-2 family and may be the mechanism of apoptosis control. How these family members control membrane permeability remains controversial and will be the focus of this review. The three dominant theories for how Bcl-2 family members control membrane permeability are: (1) they form de novo protein channels in membranes, (2) they interact with and regulate pre-existing mitochondrial membrane pores, and (3) they alter the membrane lipid order to produce lipidic pores.

2. Bax activation and translocation
In healthy cells Bax exists as a monomer either in the cytosol or loosely attached to the outer mitochondrial membrane. Upon induction of apoptosis, the cytosolic Bax translocates to mitochondria [2–4] and deeply inserts into the membrane [5]. Almost simultaneously with translocation, Bax oligomerizes into large complexes that can be detected biochemically by gel filtration or by chemical cross-linking [6,7]. Bax oligomerization is considered crucial for mitochondrial membrane permeabilization [8], at least for the proteinaceous pore model. The C-terminal membrane anchor folds into a hydrophobic pocket in the soluble monomeric form of Bax [9] and presumably becomes exposed and inserted into the lipid bilayer during apoptosis [10]. Although the domains of Bax that are involved in oligomer formation remain unknown, the C-
terminal tail does not appear essential [6]. The BH3 domain of Bax is important for bioactivity [11] and could fit into the empty hydrophobic pocket of another Bax molecule that would be revealed when the C-terminal tail disengages the pocket and inserts into membranes. However, a large conformational rearrangement of Bax would have to occur for the BH3 domain to become exposed and accessible to another Bax molecule’s pocket [9].

Confocal and electron microscopy reveal that Bax forms large clusters or complexes on the mitochondrial membrane that probably correspond to the biochemically detectable oligomers [12–14]. Although these foci are tethered to mitochondria, the Bax EM immunostaining can stretch some distance beyond the membrane. The activated Bax on mitochondria can be distinguished from the cytosolic and loosely attached Bax by a conformational change in the N-terminus that exposes the formerly buried 6A7 epitope comprising amino acids 13 to 19 [10,15,16]. Interestingly, in addition to the hydrophobic membrane anchor domain at the C-terminus that is established to be required for membrane docking, the N-terminus may also regulate mitochondrial targeting [17]. Thus, four events appear to occur in rapid succession upon Bax activation to promote apoptosis: exposure of the N-terminal epitope, mitochondrial translocation, oligomerization, and focal clustering on the mitochondrial membrane. These changes all occur prior to caspase activation downstream of mitochondria and correlate with the Bax activity that causes mitochondrial outer membrane permeabilization.

Further dissection of these steps in Bax activation suggests that Bax 6A7 epitope exposure is an initial, reversible event that occurs prior to oligomerization [71–73]. Loss of Myc expression also allows a separation of the Bax translocation step from downstream apoptosis events. In a model where Myc expression is required for apoptosis, the loss of Myc blocks cytochrome c release and cell death but not the cytosol to membrane Bax translocation step [8]. Interestingly, the absence of Myc also prevented exposure of the Bax 6A7 epitope [18]. Similarly, a cytosolic factor called IODA does not block E1A-induced Bax translocation to mitochondria but blocks cytochrome c release and apoptosis [19]. Bap31 is an ER protein that is normally cleaved after mitochondrial translocation and is resistant to Fas-mediated apoptosis although Bax still translocates and inserts into mitochondrial membranes. However, neither exposure of the Bax N-terminal epitope, Bax oligomerization nor cytochrome c release occurred [20]. Thus, multiple discrete steps occur during the Bax activation process. These recent reports suggest that Bax translocation occurs first, 6A7 epitope exposure occurs next and then, after passing an irreversible step, Bax oligomerizes and cytochrome c is released.

Genetically, Bax and Bak partially complement one another [21]. Unlike Bax, however, Bak constitutively resides on mitochondria [22]. Like Bax, during apoptosis Bak homo-oligomerizes [23,24], forms focal complexes on mitochondria that colocalize with Bax complexes [12] and exposes an N-terminal epitope [22]. Interestingly, upon binding Bid, the N-terminus of Bak becomes exposed prior to oligomerization [25]. Excess Bcl-2 appears to be able to bind the N-terminal exposed form of Bak and inhibits Bak oligomerization and thus inhibits mitochondrial membrane permeabilization [25]. These results argue against the model that Bcl-2 sequesters Bid to inhibit Bak oligomerization [26]. Similarly, E1A19K can bind selectively to a Bid-activated form of Bax and prevent Bax oligomerization and subsequent events of apoptosis [27]. Truncated Bid also homo-oligomerizes during apoptosis [28].

3. Bax permeabilization of the outer mitochondrial membrane

In 1997, two reports showed that Bcl-2 prevents cytochrome c release and apoptosis [29,30] indicating that Bcl-2 maintains the integrity of the mitochondrial outer membrane integrity to prevent cytochrome c release. Bax, opposing Bcl-2 bioactivity, was then shown to trigger cytochrome c release in cells [31]. Confirming this, elegant in vitro experiments showed that recombinant Bax induces cytochrome c release from isolated mitochondria independently of the origin of the mitochondria, i.e. mouse liver or cell lines [32–35]. The in vitro experiments also established that Bax must be in an oligomerized form to induce mitochondrial outer membrane permeabilization [8,16].

Incubation of isolated mitochondria with recombinant Bax showed that Bax also induces the release of other mitochondrial apoptogenic factors such as Smac/Diablo and HtrA2/Omi with the same kinetics as cytochrome c [36]. Interestingly, it was reported that recombinant Bax does not induce the release of the “caspase-independent mitochondrial cell death effectors” AIF and Endonuclease G [36–38].

Although Bax induces pore formation and cytochrome c release in many in vitro models, it has been difficult to show that this occurs in vivo. It has been reported that cytochrome c release occurs both following Bax translocation [39] and prior to Bax oligomerization [13] but this has not been studied in real time with endogenous cytochrome c due to technical limitations for the assessment of Bax activation and remains an important issue to establish.

4. Bid activation of Bax and Bak

A number of proteins have been reported to bind Bax and regulate Bax bioactivity. Bid, a BH3-only Bcl-2 family member, has been shown in several labs to activate Bax and is thought to promote apoptosis by this mechanism (Fig. 1). In healthy cells Bid is cytosolic. Stimulation of cell death through the Fas/Fas-L or TNF receptor pathway results in the
recruitment of the initiator caspase, caspase 8, to the death-inducing signaling complex. Caspase 8 is then activated and has the ability to cleave the amino portion of Bid to produce truncated or t-Bid [40,41]. This truncated protein can be further posttranslationally modified by the addition of a myristyl hydrocarbon chain [42]. This processing may result in the activation of Bid to interact directly with Bax or Bak. Although Bax alone can induce cytochrome c release from isolated mitochondria [32,34], Bid synergises with Bax to induce mitochondrial permeability [43]. Low concentrations of t-Bid alone also cause cytochrome c leakage from mitochondria isolated from mouse liver deficient in Bax [44]. However, the presence of Bak may account for the activity of t-Bid to induce cytochrome c release [23]. Bid also induces Bax N-terminal exposure [16], oligomerization [45] and Bak oligomerization [23].

In vivo results are similar, Bid induces Bax conformational change [16] and induces apoptosis in a Bax/Bak dependent way [21,46]. However, the in vivo results are inconclusive because many cellular insults induce Bax activation and it is difficult to know if Bid functions directly by binding Bax or indirectly by activating apoptosis. Complicating the interpretation that Bid acts directly on Bax is the difficulty in directly demonstrating Bid binding to Bax [28]. This difficulty has led to the kiss and run hypothesis [26,45] where Bid is proposed to bind to Bax, and thereby induce a conformational change to activate Bax and then cycle off the Bax forming only transitory complexes. The Bid knockout mouse [46] has more minor developmental defects than the Bax knockout mouse, indicating that other BH3 only proteins can substitute for Bid in activating the developmental cell death pathways controlled by Bax.

5. Bax may regulate the permeability transition pore via voltage-dependent anion channel (VDAC) or adenine nucleotide translocase (ANT)

One hypothesis proposes that permeabilization of the mitochondrial outer membrane to small proteins occurs through Bcl-2 family member interaction with the mitochondrial permeability transition (MPT) pore. The MPT was identified as a process characterized by massive swelling of the mitochondria, depolarization of the inner mitochondrial membrane and the uncoupling of oxidative phosphorylation...
that results in the loss of ATP synthesis [47]. The MPT is thought to be controlled by the permeability transition pore complex (PTPC). Although the complete molecular composition of this pore is still unclear, it does include the mitochondrial VDAC, the ANT and cyclophyllin D. This complex opens an ion channel in the inner mitochondrial membrane in response to calcium overload or oxidative stress that deflates the proton gradient and allows water to enter the matrix swelling the mitochondria.

Bax has been shown to induce the MPT in cells upon induction of apoptosis [48] and Bax has been reported to induce opening of VDAC in synthetic liposomes [49]. In addition, cyclosporine A and oligomycin, inhibitors of the MPT, were able to inhibit Bax-mediated VDAC opening [35]. Other evidence in support of this model comes from experiments showing that Bax is inefficient in inducing cytochrome c release in yeast VDAC-1 knockout cells [49].

It was recently reported that a minor isoform of VDAC, VDAC2, actually binds to Bak and not Bax. VDAC1 did not bind Bak. VDAC2 overexpression inhibits apoptosis and Bak oligomerization and may be one mechanism for the regulation of Bak activity, coupling the MPT pore to the Bcl-2 family [50].

The ANT, another candidate member of the PTP complex, is an inner mitochondrial membrane protein responsible for transporting ATP and ADP across the inner mitochondrial membrane. The ANT has been implicated in Bax-mediated apoptosis [51]. By yeast two-hybrid screening, Bax and Bcl-2 were shown to bind to peptides of the ANT and drugs that inhibit ANT activity also had an effect on apoptosis [48].

6. De novo Bax protein pores

The first evidence to suggest that Bcl-2 family members form pores in membranes came from structural data that showed that the anti-apoptotic Bcl-2 family member, Bcl-xL, is a highly alpha-helical protein with two central hydrophobic hairpin helices [52]. The structure is reminiscent of the domain of diphtheria toxin that forms pores in membranes [53]. Bcl-xL [54] and the C-terminally truncated form of Bax [55, 56] were shown to form pores in planar lipid bilayers at neutral pH, supporting the idea that pore formation is an important activity of these Bcl-2 family members. Bcl-xL can either form pores or inhibit Bax pore formation depending upon the conformation of the Bcl-xL or the experimental conditions.

7. Sizing the pore

The size and nature of the Bax pores are still a matter of debate. In experiments using reconstituted liposomes Saito et al. [57] found that C-terminally truncated Bax could form pores large enough to release cytochrome c and that these pores were either dimers or tetramers of Bax in dioleoylphosphatidic acid:dioleoylphosphatidyl-choline liposomes. Bax pores have been visualized in lipid bilayers, appearing as protein lined channels of different sizes depending on the bilayer lipid composition and requiring the presence of calcium [58]. Bax pores in dioleoylphosphatidylserine:dioleoylphosphatidylcholine LUV are predicted to have an inner diameter of ~20 nm and an oligomer number of 22 monomers [58]. Pavlov et al. [59] found by patch clamping of mitochondrial outer membranes pores of 4-nm diameter specifically in the presence of Bax. Kuwana et al. [43] showed that oligomerized Bax combined with a protease cleaved form of Bid forms very large pores that can release 2000-kDa size dextran molecules in the absence of membrane rupture. These megachannels were inhibited by Bcl-xL and occurred in vesicles derived from mitochondrial outer membranes.

8. Lipidic pores

Bax was also reported to form pores in planar lipid bilayer experiments that were unstable and resulted in membrane rupture [60]. The Bax antagonist Bcl-xl was able to form pores in these experiments, but these pores were stable and did not result in membrane rupture, indicating that the two proteins functioned differently. These data suggested the hypothesis that Bax forms lipidic pores. In this model Bax interacts specifically with lipids inducing a deformation of the bilayer structure and the formation of channels that contain both lipids and proteins. In support of this proposal, Basanez et al. [61] demonstrated that pore formation by Bax was more efficient in lipids with large head groups relative to hydrophobic acyl chains that induce a net positive (convex) curvature to membranes. In contrast, Bcl-xl prefers to make channels in lipids that induce a net negative curvature to membranes. In this model, Bcl-xl could inhibit Bax activity by altering the local curvature of the lipid resulting in decreased pore formation by Bax. However, in these experiments Bcl-xl was unable to inhibit pore formation by Bax [60,61]. Curiously, the truncated form of Bid has the opposite membrane curvature preference of Bax and was more efficient in permeabilizing liposomes containing lipids with relatively small head groups relative to the acyl groups that induce a net negative (concave) curvature to membranes [62]. Truncated Bid also promotes lipid transfer processes [62,63] and has the ability to destabilize planar lipid membranes. These results suggest an alternative model for the activity of Bid to activate Bax discussed previously. Bid activates Bax to permeabilize the outer mitochondrial membrane in vitro [16] but it is difficult to capture Bid–Bax complexes [28] and Bid does not colocalize with Bax in foci on mitochondria [12]. Alternative to the kiss and run hypothesis [45], Bid could alter the composition or curvature of the mitochondrial lipid bilayer to induce Bax to bind, intercalate and permeabilize the
membrane. In addition, cardiolipin has been found to be critical for Bax membrane pore formation [43]. As Bid interacts specifically with cardiolipin-containing membranes [64] and Bid can cause membrane lipid redistribution [65], cardiolipin redistribution induced by Bid could activate Bax. In these models no direct interaction between Bid and Bax need occur.

9. Bax inhibitors

Ku70 is a DNA repair enzyme found in the cytosol and nucleus but not associated with mitochondria. Ku70 was identified as a Bax binding protein using yeast to screen mammalian libraries for proteins that would inhibit the toxicity of Bax overexpressed in yeast [66]. Full-length Ku70 or the C-terminal 113 amino acids of Ku70 suppressed apoptosis in mammalian cells induced by Bax overexpression, staurosporine, and UV light. Elimination of Ku70 increased sensitivity to apoptosis. Ku70 was found by co-immunoprecipitation experiments to bind to the cytosolic form of Bax in HEK293T cells and the majority of the Bax was reported to co-immunoprecipitate with Ku70 in the presence of the detergent CHAPS. The authors suggest that Ku70 binds to Bax and retains it in an inactive conformation in the cytosol to inhibit apoptosis [66]. This is somewhat surprising as most of the Bax in the cytosol of healthy thymocytes [15] and HeLa cells (unpublished) migrate on gel filtration as a 21-kDa monomer and thus could not be bound to the 70-kDa version of Ku. Ku70 overexpression blocks Bax translocation but it is very difficult to know that this is not an indirect effect due to Ku70 inhibition of apoptosis. Similarly, inhibition of 6A7 antibody binding to Bax by Ku70 could result indirectly due to the inhibition of apoptosis by Ku70 instead of from a direct effect of Ku70 on the conformation of Bax. Ku70 clearly plays an interesting role in apoptosis independently of its DNA repair activity although it remains to be confirmed that this is due to direct Ku70 sequestration of Bax.

14-3-3 was also reported recently to bind and sequester Bax in the cytosol [67]. Using protein cross-linkers and co-immunoprecipitation, several isoforms of 14-3-3 can be found bound to Bax. However, the majority of the Bax from the cytosolic fraction of cells migrates at 21 kDa on gel filtration columns and thus is not tightly bound to the monomeric (28–33 kDa) or dimeric (56–66 kDa) forms of 14-3-3 nor can the bulk of the Bax be bound to both 14-3-3 and Ku70 simultaneously. Interestingly, the 14-3-3 Bax binding occurs independently of Bax phosphorylation, in contrast to the well-established Bad/14-3-3 interaction.

Humanin was recently found to bind to Bax and inhibit Bax activation [68]. Using a mutant form of Bax constitutively localized in the cytosol as bait in a yeast two-hybrid screen, Guo and colleagues identified the humanin peptide as a new Bax binding partner. This peptide binds to the cytosolic form of Bax and is thought to protect cells from apoptosis by inhibition of Bax activation and translocation to mitochondria. Interestingly, a second, virtual humanin peptide is encoded in the mitochondrial genome that, if expressed, could bind to Bax and inhibit apoptosis. It will be important to see if and where the mitochondrial encoded version of humanin is expressed in cells. In contrast to Ku70 and 14-3-3, the molecular weight of Humanin–Bax complexes in the cytosol is not inconsistent with the gel filtration molecular weight of cytosolic Bax.

10. Mitochondrial permeability in Drosophila

The dominant model today for the mechanism of Bax is that it permeabilizes the outer mitochondrial membrane allowing proteins in the intermembrane space such as cytochrome c, HtrA2/Omi and Smac/DIABLO to modulate downstream steps in apoptosis [69]. However, recent results show that cells from Drosophila melanogaster [70] undergo apoptosis without the release of cytochrome c from mitochondria. As these organisms express bona fide members of the Bcl-2 family, it seems plausible that Bcl-2 family members may also work independently of outer mitochondrial membrane permeabilization to control cell viability.

References


