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Review

Regulating cell death at, on, and in membranes

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ABSTRACT

Bcl-2 family proteins are central regulators of apoptosis. Various family members are located in the cytoplasm, endoplasmic reticulum, and mitochondrial outer membrane in healthy cells. However during apoptosis most of the interactions between family members that determine the fate of the cell occur at the membranes of intracellular organelles. It has become evident that interactions with membranes play an active role in the regulation of Bcl-2 family protein interactions. Here we provide an overview of various models proposed to explain how the Bcl-2 family regulates apoptosis and discuss how membrane binding affects the structure and function of each of the three categories of Bcl-2 proteins (pro-apoptotic, pore-forming, and anti-apoptotic). We also examine how the Bcl-2 family regulates other aspects of mitochondrial and ER physiology relevant to cell death.

1. Introduction: mitochondria, endoplasmic reticulum and apoptosis

The homeostasis of multi-cellular organisms is maintained by highly regulated mechanisms that ensure cells multiply in the correct context and are removed by programmed cell death when they pose a threat to the organism or are no longer needed. Apoptosis is a common type of programmed cell death which plays a fundamental role in eradicating old, excess, or dysfunctional cells [1]. Dysregulated apoptosis is at the heart of the pathophysiology of a wide variety of diseases: insufficient apoptosis leads to cancer and autoimmunity while hyperactive apoptosis is associated with neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's [2].

Apoptosis can be triggered from signals both extrinsic and intrinsic to the cell [1]. The extrinsic pathway is activated by extracellular death ligands, like tumor necrosis factor-α (TNF-α) binding their cognate death receptors; the intrinsic pathway is initiated by a wide variety of intracellular stressors (DNA damage, premature mitotic arrest, the unfolded protein response, etc.) that ultimately lead to the mechanical permeabilization of the mitochondrial outer membrane (MOM) [3–7]. Disruption of the MOM results in the release of apoptogenic factors such as cytochrome *c* and SMAC from the inter-membrane space (IMS) to the cytosol [8,9], which in turn activates the downstream "executioner" caspases that proteolytically cleave a wide variety of intracellular substrates thereby disrupting cellular physiology. Cleavage of a subset of these caspase targets in a coordinated fashion produces the phenotypic changes characteristic of an apoptotic cell [10].

The Bcl-2 family of proteins regulates both intrinsic and extrinsic pathways but is most intimately involved with the intrinsic pathway where they regulate Mitochondria Outer Membrane Permeabilization (MOMP) via a series of protein–protein and protein–membrane interactions [11,12]. Members in the Bcl-2 family are defined by the presence of one or more of the four conserved motifs known as Bcl-2 homology (BH) regions (termed BH1-4) [13,14]. The function of individual family members is largely determined by the specific combination of BH regions. The anti-apoptotic proteins (e.g. Bcl-2, Bcl-w, Bcl-XL, Mcl-1 and A1) contain all four BH regions whereas the pro-apoptotic proteins are comprised of the pore-forming multi-BH domain proteins (e.g. Bax, Bak, Bok) that contain BH regions 1–3 and a modified version of region 4 and the BH3 proteins (e.g. Bid, Bim, Bad, Bmf, Bik, Puma, Noxa, Hrk, Blk, Nip3, bNip3, Mule etc.) that are structurally distant from each other, sharing only the BH3 region [15]. During intrinsic apoptosis, BH3 proteins cause Bax/Bak to oligomerize within and permeabilize the MOM, whereas the anti-apoptotic family members inhibit this process at multiple steps [16,17].

Aside from the well-founded focus on mitochondrial dysfunction in apoptosis, other intracellular organelles are also involved with regulating cell death. The endoplasmic reticulum (ER) is a large and continuous membranous network that extends throughout the cytoplasm that

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functions primarily in protein production and transportation [18], calcium (Ca^{2+}) homeostasis, lipid and membrane biosynthesis and serves as both donor membrane and the initiating locus for autophagy. The disruption of ER function has been implicated in the pathophysiology of many cell-death related diseases [19–22]. The ER is both functionally and physically linked to mitochondria with implications for mitochondrial apoptosis [23]. The existence of ER Mitochondria-Associated Membranes (MAMs) was first detected by electron microscopy in 1960s [24] and velocity sedimentation experiments where ER components co-purified with mitochondria [25]. Furthermore MAM may be involved in regulating mitochondrial dynamics such as fission and fusion [26,27].

There are also distinct signals that regulate cell survival that uniquely arise from the ER. For example, ER stress will trigger a signaling pathway called unfolded protein response (UPR) which, aside from its primary function of slowing down protein translation and enhancing protein folding, can also promote cell death via both mitochondria-dependent and -independent pathways [19–21,27,28]. Several Bcl-2 family proteins are located at the ER membrane where they regulate cell death and autophagy such as Bcl-2, Bik, and Beclin-1 [29–31]. The death signals transmitted from the ER to mitochondria include both transcriptional up-regulation, and post-translational modification and activation of BH3 family members such as Bim, Puma and Noxa [32–34] as well as regulation of Ca^{2+} efflux via Bap31 and inositol-3-phosphate receptors [35]. Calcium efflux from the ER to the cytosol can result in an accumulation of Ca^{2+} in the mitochondrial matrix which triggers the opening of the mitochondrial Permeabilization Transition Pore (PTP) resulting in the permeabilization of the MOM and subsequent cell death [35–37].

Given the important roles mitochondria and ER play in cell survival and that Bcl-2 family proteins are targeted to these membranes, we will review the development of explanatory models for how these proteins regulate apoptosis emphasizing recent findings about how membrane binding modulates the structure and function of these proteins, and in turn how the Bcl-2 family regulates the dynamics and the integrity of these organelles.

2. Models of the mechanism of action of the Bcl-2 family

2.1. Initial models: rheostat, direct activation and de-repression

As the number of Bcl-2 family members increased beyond the original founding member, Bcl-2, the models explaining the functional significance of binding interactions increased as well (Fig. 1). The discovery of the second Bcl-2 family protein, Bax, in 1993, generated the first "Rheostat" model of Bcl-2 regulation that proposed that the numeric ratio between pro- and anti-apoptotic proteins determines cell fate [38,39]. Thus excess Bax compared to Bcl-2 would elicit apoptosis via activation of caspases, but how this occurred was not known [38,39]. The subsequent identification of other pro-apoptotic family members, the BH3 proteins, that (with the exception of Bid [40]) were structurally different from Bax by containing only the BH3 region elicited two competing models (De-repression and Direct Activation) that addressed the question of how Bax is activated, and how Bcl-2 prevents this.

The De-repression model postulates that Bax (and its closely related "cousin" Bak) are constitutively active and therefore need continuous binding to anti-apoptotic proteins to repress their activity. In this case BH3 proteins serve solely to displace Bax/Bak from anti-apoptotic proteins [41]. An important facet of this model is that the combinatorial specificity of BH3 proteins for other anti-apoptotic proteins discovered after the identification of Bcl-2 allows for the fine-tuning of cell death regulation [41]. For example Bid, Bim and Puma interact with all of the anti-apoptotic members; Bad only binds to Bcl-2, Bcl-XL, and Bcl-w; and NOXA specifically engages Mcl-1 and A1 [41]. Thus the model proposes that different sets of BH3 proteins, activated by specific stress signals, will neutralize the corresponding subsets of anti-apoptotic proteins to liberate the Bax/Bak.

In contrast to the De-repression model, the Direct Activation model states that Bax/Bak need to be activated to oligomerize and form pores in the MOM. The BH3 proteins perform this function in two distinct ways which divides them into functional sub-groups [42,43]. First, Bax/Bak can be activated by direct binding of "activator" BH3 proteins such as tBid (the truncated and active form of Bid), Bim and Puma [43–45]. Anti-apoptotic proteins such as Bcl-XL sequester the activators from Bax to prevent MOMP and cell death. This creates a scenario whereby "sensitizer" BH3 proteins such as Bad and Noxa displace BH3 activators from anti-apoptotic proteins, and thus activate Bax/Bak indirectly.

Therefore the De-repression and Direct Activation models propose different binding partners and functions for both the BH3 and anti-apoptotic family members. Both models cite evidence from a variety of assays to support their unique features, including co-immunoprecipitation assays in transfected cells, binding of peptides to truncated proteins in vitro, and gene knockout experiments in mice. However, certain inconsistencies persist: the De-repression model does not explain that in all cells, particularly growing ones, only a small fraction of Bax/Bak can be co-immunoprecipitated with anti-apoptotic proteins, in contrast to the claim that all Bax/Bak needs to be neutralized for survival [46–48]. The direct-activation model needed to be modified to acknowledge that anti-apoptotic proteins can also directly inhibit the auto-activation of Bax/Bak [49,50]. Both models propose that the interactions between the proteins are unidirectional rather than reversible equilibria.

2.2. The active role of membranes in apoptosis regulation and the Embedded Together model

To reconcile these differences concerning the proposed functions and binding partners of Bcl-2 family members an active role of membranes needs to be considered. This was not directly included in earlier models partly due to certain experimental limitations. For example, assays such as co-immunoprecipitations and immunoblotting from cell lysates require detergents to solubilize membranes. The detergents used in these experiments can affect the binding interactions between Bcl-2 family proteins: Triton X-100 artifactually promotes interactions between Bcl-2 family proteins (such as Bax and Bcl-2 or Bcl-XL), while CHAPS disrupts interactions between Bcl-2 members [51]. Furthermore, in vitro experiments, that do not require detergents, have classically used deletion mutants or peptides of Bcl-2 family members lacking the putative trans-membrane (TM) region due to difficulties in purifying the full length proteins [41]. In this circumstance, any effect of membrane binding on protein–protein interactions that might occur in vivo would be lost.

More recent evidence indicates that this is an important concern for Bcl-2 family members in all three functional groups. For example, Bax undergoes a series of conformation changes during activation when it transforms from a cytoplasmic or loosely membrane bound protein to a protein with helices 5, 6 & 9 integrated in the membrane before it oligomerizes with other Bax monomers to form a pore in the MOM, an event not observed with solution based assays [52]. Targeting of tBid to the membrane also causes a conformation change that allows it to both activate Bax [53], and facilitate the membrane insertion of cytoplasmic Bcl-XL [54]. These tBid membrane interactions are sensitive to membrane lipid composition [55] and catalyzed by the non-Bcl-2 family protein Mtch2 [53,56]. Furthermore recent reports suggest other novel roles for non-Bcl-2 family mitochondrial membrane proteins and lipids in the regulation of MOMP. For example, a GTPase of the dynamin superfamily named Drp1 was found to enhance the pore forming activity of Bax [57]. It has also been reported that tBid-Bax interaction on the membrane induces redistribution of membrane lipids [58].

Based on data obtained using recombinant full-length proteins and in vitro model systems free of detergents to assay Bcl-2 family protein–membrane binding and membrane permeabilization, the "Embedded

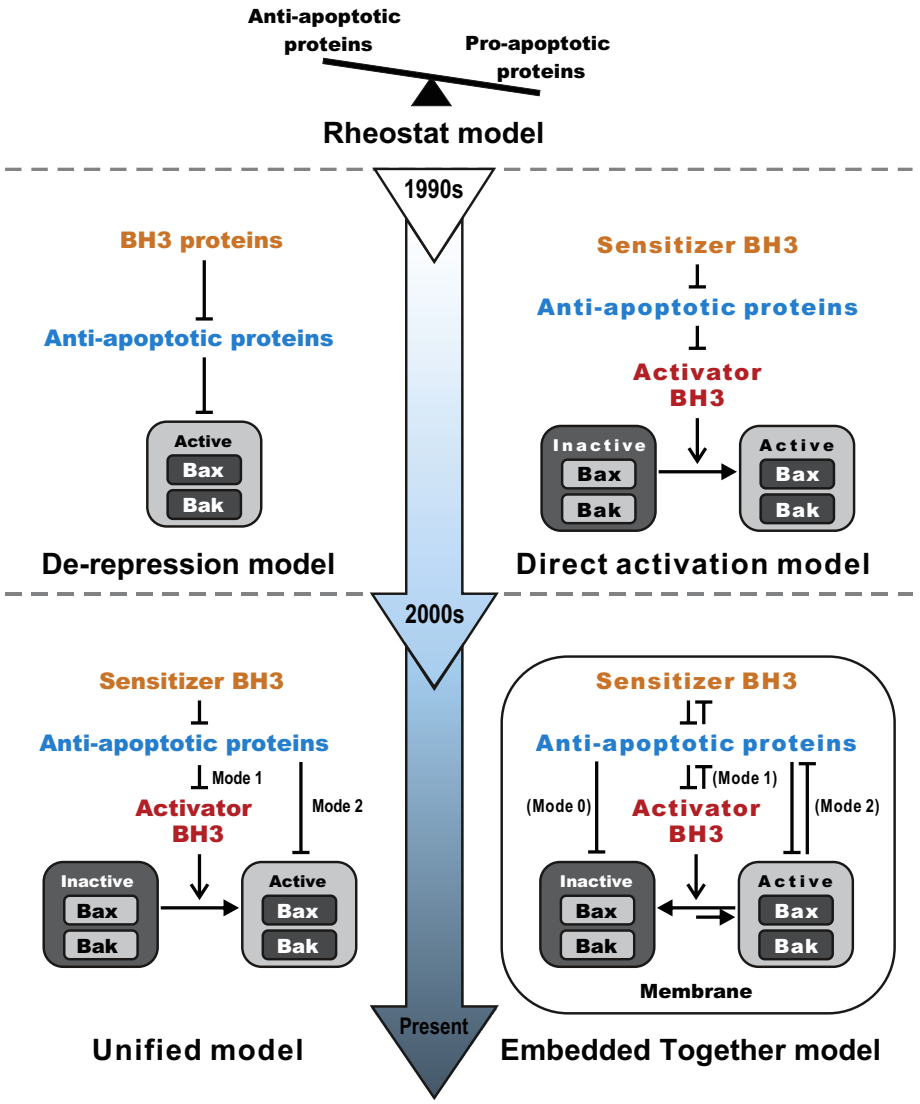

Fig. 1. Models illustrating mechanisms for the regulation of MOMP by Bcl-2 proteins. The earliest "Rheostat" model proposes that cell fate is determined by the ratio between pro- and anti-apoptotic proteins. Later models such as the De-repression and Direct Activation models hold different views on the role of BH3 proteins and anti-apoptotic proteins. The Embedded Together model and the newly proposed Unified model both agree that Bax/Bak need to be activated by BH3 proteins and that anti-apoptotic proteins inhibit MOMP through multiple interaction partners. The Unified model differs from the Embedded Together model chiefly by formalizing the relationship between mitochondrial dynamics and apoptotic regulation. However, the Embedded Together model distinguishes itself from other models by recognizing that: 1) The membrane is an active participant in the interactions between Bcl-2 family proteins. 2) Binding of anti-apoptotic proteins to BH3 or activated Bax/Bak leads to mutual sequestration. 3) MOMP is controlled by multiple reversible equilibria.

Together" model was proposed to explain the regulation of MOMP in apoptosis [11,12,59]. In addition to the interactions of some family members seen in solution (i.e. in the cytoplasm of cells), Bcl-2 family members were proposed and later demonstrated to adopt a different conformation after interaction with membranes. Most of these interactions were observed to be saturable and reversible. Thus the Embedded Together model incorporates protein–membrane and protein–protein interactions of Bcl-2 family proteins that are tightly regulated by multiple parallel equilibria. A focal point of the model describing the irreversible step in apoptosis, posits that the activation of Bax/Bak at the membrane is the rate-limiting step in MOMP, in part because it involves distinct stepwise but reversible conformational changes leading to formation of the oligomeric pore. Most activator BH3 proteins such as Bim, tBid and Puma can bind to membranes spontaneously where they recruit Bax (as well as anti-apoptotic proteins) to the membrane, initiating a series of conformational changes of Bax on the membrane leading to oligomerization of Bax. In the case of Bak, which is constitutively bound to the membrane, the first conformation change that leads to tight binding to membranes is not required. The model also suggests the involvement of accessory proteins such as Mtch2 and posits roles for post-translational modifications of the proteins [11,12]. Recent descriptions of the model made explicit that the molecular interactions are not one way. Thus binding of membrane bound anti-apoptotic proteins to activator BH3 proteins doesn't just lead to inhibition of the activator protein but, in this scenario the activator protein behaves like a sensitizer in that it inhibits the anti-apoptotic protein. This kind of inhibition was called mutual sequestration [60]. Furthermore, the anti-apoptotic proteins also bind to activated Bax/Bak on the membrane with high affinity, leading to mutual sequestration of this pair.

Sensitizer BH3 proteins such as Bad and Noxa have little binding affinity for Bax/Bak but can engage anti-apoptotic proteins with combinatorial specificity (e.g. Bad binding to Bcl-XL and Bcl-2, Noxa binding to Mcl-1, etc.), a scenario previously included in the De-repression model [41] but here complex formation inhibits both proteins. For example, when the sensitizer BH3 protein Bad recruits Bcl-XL into membranes the function of both proteins is inhibited via mutual sequestration [61]. Competition for binding from sensitizer BH3 proteins can disrupt the sequestration complex formed by activator BH3 and anti-apoptotic

proteins, as well as displace activated Bax/Bak from anti-apoptotic proteins, so that they are sensitizers for both mechanisms proposed by the De-repression and Direct Activation models [11,12,59]. The dual nature of the effect of these binding interactions makes the description of proteins as 'activators' or 'sensitizers' artificial and cumbersome.

A critical and previously under-appreciated feature of the Embedded Together model is that the target membrane plays an *active* role in mediating the interactions between Bcl-2 family proteins rather than being a "passive recipient" in the process. Thus MOMP is controlled by multiple reversible equilibria including protein–protein and protein–membrane interactions that initiate these conformational changes. For example, interaction between full length tBid and Bax is detected only when membranes are present [51]. Precisely measuring the rates and dissociation constants for these individual interactions will be required to permit the prediction of the direction and extent of a specific interaction when multiple interacting partners are present, as would occur in vivo.

The Embedded Together model also diverges from its predecessors by stating that anti-apoptotic proteins can be 'activated' in the sense that anti-apoptotic proteins undergo conformational changes that enhance or change their function in response to being recruited to the membrane by pro-apoptotic family members. The regulation of function by mutual sequestration proposes that the difference between activator and inhibitor is quantitative rather than qualitative as the balance is determined by the local concentrations of anti-apoptotic proteins and their binding partners. In support of this, Bcl-2 inserts helices 5 and 6 into membranes upon direct binding of BH3-activators, a conformation change critical for Bcl-2 mediated inhibition of Bax oligomerization [62,63]. This conformational change thus activates Bcl-2 even though in the absence of other changes the net effect of binding is inhibition of both the BH3-activator and Bcl-2 due to mutual sequestration. However, because binding is reversible and governed by an equilibrium binding constant the model predicts that additional interactions can release the activated Bcl-2 from the BH3-activator.

Finally the equilibrium binding underpinning the model suggests that another molecular mechanism by which membrane-bound anti-apoptotic proteins inhibit Bax/Bak is by functioning as a dominant negative Bax/Bak by capping the activated Bax/Bak monomer/oligomer and preventing it from further oligomerization. In keeping with the theme elaborated above, this is a form of mutual sequestration that arises because the anti-apoptotic proteins bind to one of the oligomerization surfaces of Bax/Bak but the anti-apoptotic proteins cannot further oligomerize. This function for Bcl-XL was demonstrated by the observation that Bcl-XL inhibited Bax-activation induced by tBid mt1 (a tBid mutant that no longer binds to Bcl-XL) in liposomes and mitochondria [64] and in live cells using FLIP (fluorescence loss in photobleaching) and FRAP (fluorescence recovery after photobleaching) [65]. Recently a new pro-survival mechanism was found for Bcl-XL in which it retro-translocates peripherally membrane-bound Bax to the cytosol [64–66]. This novel mechanism named MODE 0 will be discussed in detail in the following section.

2.3. Unified model

Another model has recently been proposed to account for how the anti-apoptotic proteins function by inhibiting both BH3 activators and Bax/Bak. This model also relates these two different modes of inhibition to the role of mitochondrial dynamics in apoptosis regulation [67]. In this model, sequestration of activators by the anti-apoptotic proteins is defined as MODE 1 and the inhibition of Bax/Bak by anti-apoptotic proteins is called MODE 2. While inhibition through either mode prevents MOMP, MODE 2 is considered more potent. In addition MODE 2 prevents mitochondrial fusion and promotes mitochondrial fragmentation.

By introducing the terms MODE 1 and MODE 2 the unified model more explicitly states the two ways anti-apoptotic proteins can inhibit apoptosis previously incorporated in the Embedded Together model. In this model, MODE 1 is equivalent to the inhibition of BH3 proteins and anti-apoptotic proteins by mutual sequestration, while MODE 2 reflects the dominant negative function of the anti-apoptotic proteins as inhibitors of Bax/Bak. The Embedded Together model predicts that both events can occur simultaneously and are controlled by both the relative abundance and the relative binding affinities of the Bcl-2 family members expressed in that particular cell. Thus unlike the Unified model, the Embedded Together model proposes that the dominance of MODE 1 vs MODE 2 depends on the extent to which the proteins are bound to membranes and the predominance of one mode over the other can change dynamically depending on both the amount of protein and post-translational modifications of the Bcl-2 proteins, such as phosphorylation, that affect binding to other family members and the membrane. The complexity of the inter-relationship of all of these events contributes to apoptosis occurring in a cell autonomous manner.

3. Importance of membranes: changes in conformation of Bcl-2 family proteins during apoptosis

A critical feature of the Embedded Together model is that for many Bcl-2 family members, membrane binding induces conformational changes that modify function [11,12,59]. Even for those family members like Bcl-2 and Bak which are constitutively integrated in the membrane, interacting with other membrane-bound binding partners causes distinct conformations that have functional consequences [62,68]. In this section we will review the details of these processes.

3.1. The role of specific membrane components in MOMP regulation

An initial hint of the importance of membranes in Bcl-2 family regulation came from reports of the importance of cardiolipin in regulating tBid-Bax mediated liposome permeabilization [69]. Cardiolipin (CL) is a mitochondria-specific membrane lipid synthesized within and localized predominantly to the inner mitochondrial membrane. However, a minor fraction of CL is located in the MOM [69]. This small pool of surface-exposed CL appears to impact the pro-apoptotic function of Bid and Bax [58,70] as removing CL from MOM-like liposomes abrogated pore formation by Bid and Bax in vitro [58]. In addition, CL binds to a truncated Bak mutant in liposomes and increases its sensitivity to Bid activation and subsequent membrane permeabilization [71]. Using high enough concentrations (μM range) of Bid BH3 peptides can also induce Bax pore formation in nanodisc lipid bilayers lacking CL [72]. The elimination of CL in yeast expressing human Bax also has no effect on Bax insertion into the MOM or induction of MOMP [73]. These reports indicate that CL is not absolutely required for Bax-dependent MOM permeabilization, but does not leave out the possibility of CL enhancing the activity or membrane binding of BH3 activators. It has been reported that CL promotes Bid translocation to liposomes, MOM and mitochondrial contact sites [54,55,74]. CL also promotes mitochondrial localization of caspase 8, providing a platform in cells for activation of Bid via cleavage by caspase 8 [75,76]. The BH3 sensitizer protein Bad also contains a lipid binding region that favors negatively charged lipids like cardiolipin [77]. Whether or not membrane binding by the anti-apoptotic proteins is affected by CL is currently unknown.

Cholesterol is the predominant component that controls the fluidity of membranes in mammalian cells. Although mitochondria only contain a restricted pool of cholesterol (3 ~ 5% of the total cellular cholesterol) [78], it plays a major role during apoptosis where it inhibits Bax-mediated MOMP by lowering membrane fluidity [79]. Correspondingly, enhanced mitochondrial cholesterol in cancer cells increases resistance to chemotherapy and Bax mediated MOMP, whereas lowering the cholesterol content of the cells induces p53 dependent activation of Bax [80,81]. The mechanism for this negative regulation of Bax by cholesterol is still poorly understood, although some evidence suggests high

concentrations of cholesterol hinders the insertion of Bax into the membrane [82] or the activation of Bax in the membrane [79].

The link between the sphingolipid ceramide and programmed cell death was discovered in 1993 when cells treated with C2-ceramide, a cell permeable ceramide analog, underwent apoptosis [83]. Additionally, cell death by C2-ceramide was shown to be Bax dependent [84]. Ceramide has been shown to act synergistically with Bax to promote MOMP [85]. Thus it is well established that C2-ceramide can regulate apoptosis. However, cellular ceramide is primarily generated by sphingomyelin hydrolysis via sphingomyelinase (SMase) or by de novo synthesis via Ceramide synthase (CerS) [86]. Increasing the amount of ceramide at the mitochondria by SMases or CerSs has been reported to promote Bax translocation to the MOM, subsequent MOMP and apoptosis, by a process that can be prevented by Bcl-2 or inhibitors of the aforementioned enzymes [87–91]. Nevertheless the molecular mechanism by which ceramide promotes apoptosis is still a mystery. It has been demonstrated that ceramide can form channels large enough to release IMS proteins from mitochondria [92–94] but it is not clear how this function could be regulated by Bax. Additionally a downstream metabolite of ceramide, hexadecenal, was shown to directly bind Bax and promote Bax activation and pore formation in large unilamellar vesicles, even when these vesicles lacked CL [88]. Clearly the field would benefit from a systematic examination of the quantitative effects of various lipids on the regulation of MOMP.

3.2. Bax/Bak change conformation in membranes

Bax and Bak are the effectors of MOMP that insert into the MOM and induce membrane permeabilization allowing the release of contents from the IMS that activate downstream effectors of apoptosis, such as cytochrome *c* and SMAC [95,96]. Both Bax and Bak contain 9 alpha-helices. The crystal structures of Bax and Bak suggest that they share a similar structure with each other and with their anti-apoptotic counterparts [97–99]. Helices 5 and 6 are in the center of the soluble version of the protein and are surrounded by amphipathic helices, that shield them from water and form a hydrophobic groove on the surface of Bax and Bak [97,98]. Notably, a similar hydrophobic groove is present in anti-apoptotic proteins and is the binding site for the BH3 region of pro-apoptotic Bcl-2 family members [100–103]. Based on this observation, it is likely that the hydrophobic groove plays a similar role in Bax/Bak. Indeed, a Bid BH3 peptide has been shown to bind to this site in Bax and Bak by NMR/X-Ray Crystallography [104,105]. Both Bax and Bak contain a carboxyl-terminal putative trans-membrane (TM) domain in helix 9 which targets the proteins to the membrane [104,106,107]. Soluble monomeric Bax, which is not active, is stable in the cytoplasm because the helix 9 TM domain binds in *cis* to the hydrophobic groove [108,109]; activation of Bax by binding to a BH3 protein causes a conformational change that displaces helix 9 such that it then inserts into membranes [52].

In the Embedded Together model, displacement of helix 9 from the hydrophobic groove is one of several distinct steps in Bax activation, but not the first (Fig. 2) [11,12,59]. The initial step is transient binding of Bax to the membrane without insertion of the TM into membranes; as with all the other steps, the cytoplasmic and peripherally-membrane-attached forms of Bax are in equilibrium. In growing cells, without active BH3 proteins present, the equilibrium is shifted toward the cytoplasmic form but some Bax is peripherally attached to the mitochondria membrane even in the absence of apoptotic stimuli [65,110–112]. Significantly, peripheral binding of Bax to liposomes leads to the exposure of an amino-terminal epitope recognized by the monoclonal antibody 6A7, which suggests a structural change that possibly facilitates Bax binding to membrane-bound BH3 proteins that leads to further conformational changes in Bax [113]. Studies using FLIP and FRAP reveal that Bax cycles on and off mitochondria in growing cells spontaneously [114] by a process that may be facilitated by an anti-apoptotic protein like Bcl-XL [65,66]. Thus the concentration of Bax at the membrane is kept low until an activator binds to Bax, triggering a conformational change that increases the affinity of Bax for the membrane shifting the equilibrium toward a membrane-bound state and effectively 'capturing' Bax within the membrane.

In contrast, Bak is constitutively inserted in the MOM via its helix 9 TM region, leaving the hydrophobic groove exposed, and bypassing the membrane targeting step(s) seen with Bax [115,116]. The high affinity of Bak for membrane insertion may be due to the increased hydrophobicity of the Bak TM region compared to that of Bax, which favors binding to membranes rather than to the hydrophobic groove in *cis* [115]. Consistent with this notion, mutations in the Bak TM that make its amino acid composition similar to Bax results in binding of the mutated helix 9 to the hydrophobic groove and, like Bax, shifts the Bak binding equilibrium to favor localization in the cytoplasm [68,115].

Bax and Bak can also be activated by various physical factors such as changes in pH [117] and heat [118] but in vitro these conditions are more extreme than found in cells. Exposure to MOM components like certain lipids that have been shown to activate Bax in vitro may be more physiologically relevant [88]. However, the most studied mode of activation is through activator BH3 proteins binding Bax/Bak [51,70,119,120]. The interaction between BH3 proteins and Bax/Bak has been described as a "hit and run" mechanism as the interaction is transient [121]. After binding to activator BH3 proteins, Bax/Bak undergo major conformational changes resulting in a complete structural rearrangement of the proteins including the amino-terminal region, BH3 region, and helices 5, 6 and 9 (the latter helix only in Bax) [52,120,122]. One or more of these conformation changes may reduce the affinity of Bax for BH3 proteins resulting in the observed 'hit-and-run' phenomenon. Recently a crystal structure of Bax (lacking helix 9) and BH3 peptides revealed that Bid BH3 and Bax BH3 bind to the canonical hydrophobic groove, resulting in a partial displacement of helix 2 [104]. This moves the helix 2/3 side of the Bax groove away from the bound BH3 peptide and further "opens up" the groove, which might weaken the contact between BH3 peptide and Bax [104]. This may be the structural basis of the transient nature of BH3 protein binding to Bax. The BH3 domain located in helix 2 exposed after activation may facilitate the homo-oligomerization of Bax/Bak by reducing the affinity for BH3 proteins and/or increasing the affinity for Bax/Bak and the anti-apoptotic proteins [120,123,124].

Bax spontaneously inserts into the membrane when helix 9 is displaced from the hydrophobic groove [104,119,120]. However, displacement of helix 9 also releases helices 5 and 6 from the hydrophobic core and they become tightly associated with the membrane, possibly in a hairpin fashion. In support of this schema, IASD labeling assays suggest that Bax inserts helices 5,6 and 9 and Bak inserts helix 5 and 6 into the into the MOM after activation [52,125]. Insertion of the hairpin, comprised of helix 5 and 6, and the intervening sequence into the MOM has been implicated in the pore-forming function of Bax, based on structural similarity to bacterial toxins such as Colicin A and Diphtheria toxin [99,126]. Another mechanism for dissociation of helix 5 and 6 from the Bax/Bak core was suggested by recent crystallography studies and termed "core/latch dissociation". In this process, the hairpin temporarily opens up, allowing helices 6–8 (the "latch") and helices 1–5 (the "core") to dissociate from each other [104]. Cross-linking of the cysteines between helices 5 and 6 inhibits Bax pro-apoptotic function, consistent with the functional requirement for dissociation [104]. By measuring these reactions simultaneously, it was revealed that at least for tBid and Bax they occur as an ordered series of events [51], although to date all of these measurements have been based on Bax activation by tBid therefore, the process may vary depending on the activator.

By using stabilized ("stapled") peptides from the BH3 region of the activator Bim a novel "rear pocket" interaction site in Bax was discovered. The rear pocket is located on the opposite side of Bax from the canonical BH3 hydrophobic groove and involves helices 1 and 6 [119]. Binding of the Bim BH3 to this site results in displacement of the helix 1, 2 loop which may facilitate the dispatch of helix 9 from the

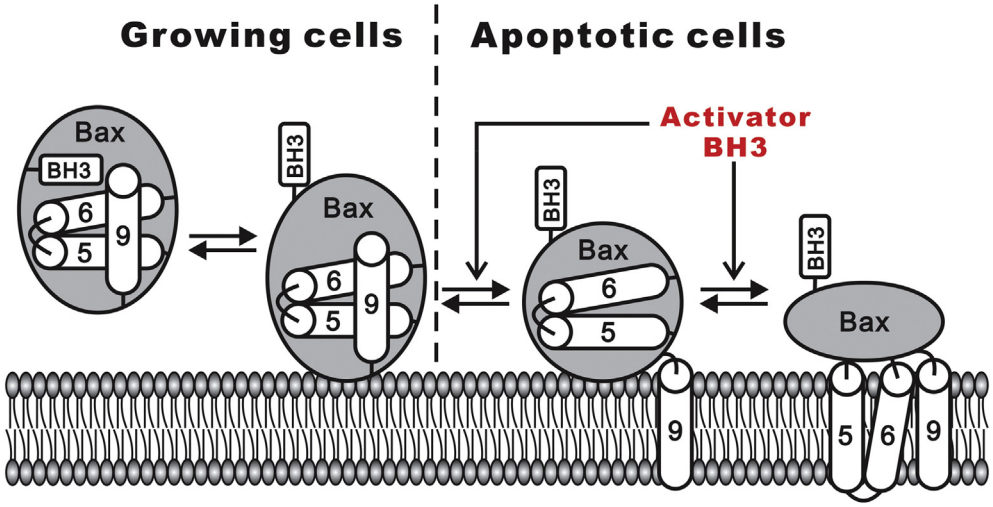

Fig. 2. Bax undergoes a step-wise activation mechanism that is tightly controlled by multiple equilibria. In growing cells, in the absence of apoptotic signals, cytoplasmic and peripheraly-bound Bax are in an equilibrium that greatly favors the cytoplasmic form. Upon activation by various physical factors and/or activator BH3 proteins, Bax undergoes further conformational changes involving unfolding of the protein and the insertion of helices 5, 6 and 9 into the membrane. Here Bax inserts helix 9 before inserting helices 5 and 6 into the membrane. This activated form of Bax then goes on to recruit more cytoplasmic Bax that oligomerizes and eventually permeabilizes the MOM.

hydrophobic groove, suggesting an additional activation mechanism of Bax by BH3 proteins [127]. However it is still unclear whether these stapled peptides faithfully recapitulate binding of the BH3 domain in full-length proteins, and whether this dimeric interaction measured in a solution-based assay also occurs on membranes, as would happen in vivo. An equivalent 'rear pocket' binding site has not yet been identified in Bak. However in Bak the helix 9 TM is constitutively inserted in the membrane, which by exposing the canonical groove on an ongoing basis may render rear-pocket activation irrelevant for Bak.

3.3. Bax/Bak pore formation on the MOM

Several models have been proposed describing the composition of the Bax/Bak pore (Fig. 3). Early models suggested that Bax and Bak function by modifying existing proteinaceous channels. For example, Bax may increase the permeability of the MOM by regulating VDAC1 channels [128]. However since Bax and Bak can activate, oligomerize and permeabilize MOM-like liposomes without any other membrane proteins, other models postulated that oligomerized Bax/Bak are the sole components of the pore. This has also been demonstrated in more physiologic membrane assays where Bax progressively forms a 5.5–6 nm sized pore composed of 9–12 monomers in mitochondria [129].

How are these oligomers assembled? It is clear that the BH3 region in helix 2 of Bax/Bak is essential for homodimerization [120,123,124]. Beyond this there are two interpretations for how this sequence (and potentially other binding sites) mediates oligomerization: The symmetrical model and the asymmetrical model. In the symmetrical model, Bax/Bak form dimers in which the BH3 regions bind reciprocally to the front hydrophobic groove of each other [123]. This resultant dimer can then bind to other dimers via the rear pocket allowing oligomers to grow in multiples of two. In support of this, a Bax "BH3-in-groove homodimer" crystal structure was obtained showing that a Bax BH3 binds to the front groove of BaxΔC [104]. Recent work using the site-directed spin labeling method of electron paramagnetic resonance (EPR) spectroscopy and chemical cross-linking have indicated that Bak disengages helices 1 and 6 and dimerizes with helices 2–5. This unit is structurally homologous to the Bax "BH3-in-groove homodimer". This method also identified a novel-interface involving the carboxyl-termini of helices 3 and 5 [104,130]. Extrapolation from the structure of the dimer indicated that Bak forms a lipidic pore with 4–6 homo-dimers aligned on the edge of the pore [130].

The asymmetrical model was proposed based on the discovery of the rear pocket in Bax; in this case, binding of a BH3 activator to the rear pocket is the initiator of a conformational change that permits the BH3 region of Bax to bind to the rear pocket of another Bax monomer, exposing the BH3 region of the latter, propagating elongation of the oligomer [131]. Consistent with this model, the NMR structure of a stapled Bax BH3 peptide/Bax complex showed that the BH3 peptide bound the rear pocket of Bax [127]. Recent modeling studies also propose that Bak oligomerizes to generate a pore-forming octamer in a similar manner, although a definitive rear pocket in Bak has not been discovered [132]. Though these two mechanisms identify slightly different BH3 region interaction sites, both agree that the BH3 region and the hydrophobic pockets are important for Bax/Bak oligomerization.

Whatever the exact alignment of Bax/Bak monomers in the oligomer, recent evidence indicates that lipids regulate pore formation. For example, lipids with either positive or negative intrinsic curvature modify Bax-mediated liposome permeabilization [58,133,134]. Reciprocally, Bax binding at very low concentrations can destabilize lipid bilayers, suggesting the pore may contain both a protein and a lipid component [135]. A structural model has been proposed in which Bax/Bak cooperate with mitochondrial lipids to form a toroidal protein–lipidic pore in which the two leaflets of the bilayer fuse to each other [58]. Mechanistically this may be mediated by Bax/Bak rearrangement of lipids within the MOM that helps to stabilize the pore [58]. Kinetic studies have been interpreted to suggest that Bax oligomerization is not the rate-limiting step of MOMP, indicating the possibility of lipid involvement in pore formation [136]. Additionally, experiments using an isolated helix 5 peptide of Bax support the formation of toroidal protein–lipidic pore [137].

Indeed the two views about pore composition may be complementary rather than mutually exclusive as there may be transitions between a proteinaceous pore and a protein–lipidic pore which depend on the equilibria of binding between lipids and Bax/Bak monomers and oligomers. For example, membrane permeabilization may start with a small proteinaceous pore. As the oligomer grows in size, the curvature tension of the membrane increases causing the intrinsically curved lipids to be re-arranged and participate in enlargement of the pore. The balance may be determined by local Bax/Bak concentration, and lipid composition and curvature. This model would be consistent with a Bax/Bak conformational change, which occurs on the membrane, being the rate-limiting step in MOMP [51]. The published data suggests that Bax/Bak activation and oligomerization are the crucial decision steps in the commitment to the execution phase of apoptosis, and that

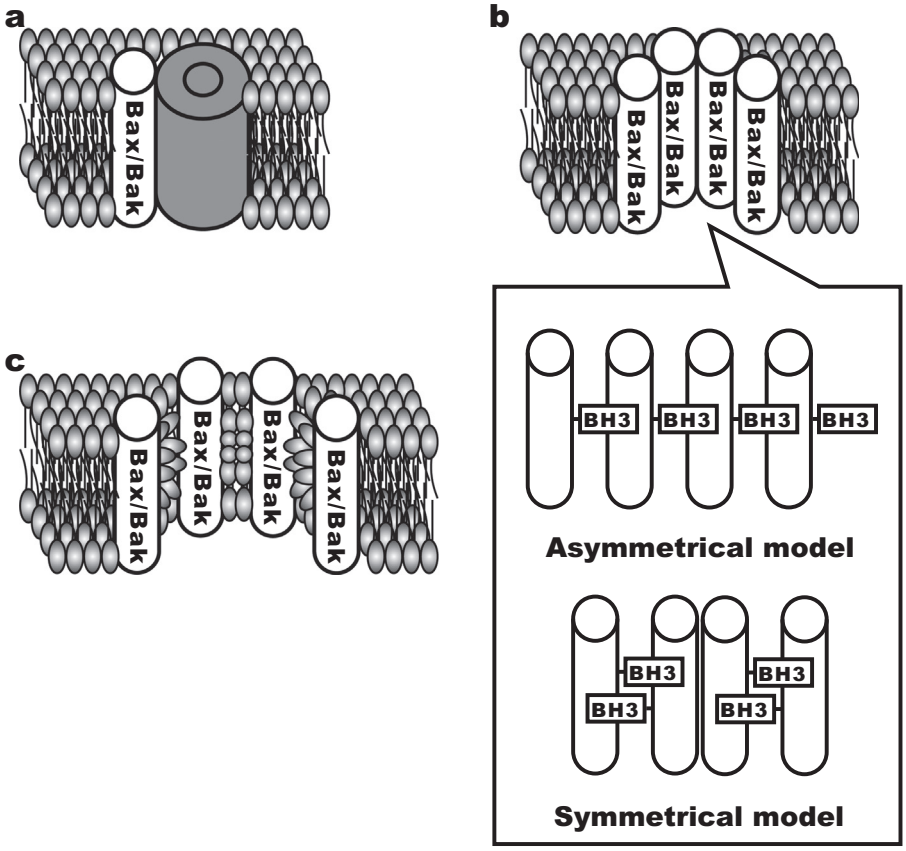

Fig. 3. Potential mechanisms of Bax/Bak mediated membrane permeabilization. a. Early models postulate that Bax/Bak regulate existing channels located within the MOM such as VDAC1. b. Other theories suggest that oligomerized Bax/Bak are the sole components of the pore. Two models, the symmetrical and asymmetrical models, propose potential explanations for the mechanism of Bax/Bak oligomerization. c. Recent evidence has suggested that lipids can regulate Bax/Bak pore formation, indicating the possible existence of a protein–lipidic pore.

lipids not only regulate this critical step but contribute to Bax/Bak pore structure and stability.

Bok, the third mammalian pro-apoptotic multi-domain protein, has been less intensively investigated. Bok was found to be highly expressed in germ tissues and may be associated with placental pathologies [138,139], however it was also found to be expressed in a variety of cell types [140]. Similar to Bax, it has been reported that Bok translocates from the cytoplasm to mitochondria during apoptosis [141] although it was recently found that both overexpressed and endogenous Bok mainly localizes to the membranes of the ER and Golgi apparatus [142]. Bok displays a high sequence homology to Bax/Bak, and as such it was predicted to function similarly by causing MOMP but recent evidence suggests that this may not be the case. Overexpression of Bok failed to kill Bax/Bak double knockout cells treated with apoptotic stimuli suggesting that Bok may not act in a similar manner to that of Bax and Bak [142]. Additionally this study showed that Bok induced apoptosis was largely Bax/Bak dependent, indicating that Bok acts upstream of Bax/Bak [142].

3.4. Targeting of BH3 proteins to membranes as a crucial step in regulating apoptosis: overview and context

After activation, BH3 proteins translocate to the MOM and/or the ER where they bind to pore-forming or anti-apoptotic proteins. Sequence analysis suggests that most BH3 proteins (Bid, Bim, Bmf, Bik and Puma) have a carboxyl-terminal tail-anchor region [143]. Other than Bik, which is constitutively localized to the ER, the listed BH3 proteins are found at the MOM only after activation [143]. In some cases the specific tail-anchor sequences are sufficient to determine the sub-cellular localization of proteins. For examples, the predicted tail-anchor regions of Bim and Puma are sufficient to target and insert GFP fusion proteins into the MOM [143]. Other BH3 proteins such Bad and Beclin-1 contain other types of hydrophobic sequences important for subcellular membrane localization [31,77] while Noxa contains a conserved mitochondrial targeting sequence found in non BH3 proteins at its carboxyl-terminus [144].

There are clear functional consequences of appropriate membrane targeting as BH3 peptides, derived from the sequence of specific BH3 proteins, bind to the appropriate Bcl2-family members but with markedly decreased affinity compared to the full-length proteins [145]. Moreover many full-length BH3 proteins such as Bim, Bmf and Bad lack a definitive structure in solution, but nevertheless show binding preferences in vivo implying that they attain a specific conformation when associated with the membrane [146]. The Embedded Together model would predict that these intrinsically unstructured proteins may adopt distinct conformations on the membrane which promote their function. We will discuss how these features are relevant for specific BH3 activator and sensitizer proteins.

3.4.1. BH3 proteins and membranes: Bim, Bid and Bad

Bim is an important mediator of apoptosis initiated by many types of intracellular stress including DNA damage and the ER-associated unfolded protein response [147–149]. Three major isoforms of Bim are formed by alternative splicing of mRNA: BimEL, BimL, and BimS [150]. BimEL and BimL are present in many different tissues and cell types [151] while BimS is almost never present in normal cells and has been detected only in HEK 293 cells [152]. Thus, BimEL and BimL are likely the most relevant isoforms mediating an apoptotic response for most cell types.

Although in vitro studies of protein function using full length proteins are lacking, structural studies using Bim BH3 peptides indicate that Bim binds to the hydrophobic groove of anti-apoptotic proteins [100,101]. It seems likely that binding anti-apoptotic proteins causes

the Bim BH3 region to adopt a defined alpha helical structure. As described above, NMR data with stapled peptides suggest that the Bim BH3 region binds directly to the Bax rear pocket to provoke Bax activation. It remains to be determined if the Bim BH3 region also binds the hydrophobic 'front pocket' on Bax and, if so, what the relative affinities are for the two pockets. However, a carboxyl-terminal deletion mutant of Bim lacking the membrane anchoring domain (BimΔC) failed activate Bax in vitro; this interaction can be partially restored if BimΔC is brought to the membrane by other mechanisms (e.g. by histidine-tag-Ni2 + chelating or Tom5 complex targeting sequence) [153]. Recent studies claim that Bim spontaneously inserts into the MOM via its putative tail-anchor, and that binding to the MOM is crucial for activating Bax [154]. Both of these results are consistent with Bim folding into a defined structure when it targets to membranes. However, it is unlikely that the mechanism is closely related to that of authentic tail-anchor proteins as the putative tail-anchor sequence of Bim contains charged residues which are normally incompatible with tail-anchor mediated insertion into the membrane. Thus, while the carboxyl-terminal 'hydrophobic region' of Bim is necessary and sufficient for binding to MOM [143] it is unlikely that it mediates membrane binding by a conventional tail-anchor insertion mechanism.

Because Bim binding to membranes occurs spontaneously and the unmodified protein is constitutively active there must be a control mechanism to prevent from Bim constantly provoking apoptosis. BimS appears to be regulated transcriptionally while BimL and BimEL are sequestered to the cytoskeleton via a short peptide motif (DKSTQTP) encoded by exon 4 that binds to dynein Light Chain LC1 [155]. The c-Jun NH2-terminal kinase (JNK), which is activated upon apoptosis stimuli such as cell stress, phosphorylates Bim at 2–3 conserved sites on and near the LC1 binding motif, specifically at Thr-56 and at least one of the adjacent serine residues, Ser-44 and/or Ser-58 (sequence positions refer to human BimL). Phosphorylation in this motif in BimL and BimEL causes the release of Bim from the motor complexes resulting in spontaneous relocalization to the MOM [156,157]. Thus phosphorylation can regulate BimL and BimEL activity in cells. Consistent with the complex regulation of apoptosis by many intracellular signaling pathways, Bim is also phosphorylated on serine-87 through PIP3/Akt pathway, although this has the opposite consequence to JNK phosphorylation as it leads to ubiquitination and degradation of the protein [158]. The absence of the dynein binding motif and the associated regulatory phosphorylation sites in BimS probably accounts for the extremely potent apoptotic activity of this isoform [156].

Bid is another activator BH3 protein that binds to and activates Bax in membranes [51]. It was first reported in 2000 that Bid induces the oligomerization and insertion of Bax into the MOM, inspiring much of the subsequent work on Bid using reconstituted systems [159]. The BH3 region of Bid also binds to Bak as demonstrated by a crystal structure of the dimer [105]. Unlike other BH3 proteins, in solution Bid has a distinct 3D-structure that is homologous to multi-BH domain family members like Bax and Bcl-XL. Functionally Bid serves as a link between the extrinsic pathway and the intrinsic pathways: at the onset of extrinsic apoptosis, Bid targets to membranes once it is cleaved into cBid by active caspase 8 which itself requires prior activation via death-receptor mediated cleavage. During the intrinsic pathway of apoptosis there are other mechanisms by which either caspase 8 is activated or Bid is cleaved. However in this case, cleavage of Bid generally occurs after MOMP and appears to provide amplification of the death signal. The cleaved version of Bid (cBid) consists of two fragments: an amino-terminal p7 fragment and a carboxyl-terminal p15 fragment (tBid). Using liposomes or isolated mitochondria with fluorescently labeled proteins, our lab has shown that membrane targeting of cBid is sufficient to cause the dissociation of the p7 fragment from tBid [53]. Thereafter a series of conformational changes ensue in which tBid first unfolds at the membrane such that α-helices 4, 5 and 8 interact with the membrane followed by insertion of α-helices 6 and 7 into the membrane [53]. This conformational change on the membrane constitutes the rate-limiting step for cBid activation. Interestingly the MOM protein Mtch2 facilitates tBid binding to membranes [56] and accelerates the conformational change of tBid thereby activating Bax [53]. However, Mtch2 is not essential for the cBid-mediated Bax permeabilization of all membranes, as liposomes or proteo-liposomes containing cardiolipin but lacking Mtch2 can be permeablized by cBid and Bax [53,160]. Numerous studies have indicated that the spontaneous membrane binding of tBid causes the migration of soluble Bax and Bcl-XL to membranes [51,54,64,158]. Recent studies have also shown that in many situations tBid preferably activates Bak while Bim preferably activates Bax [145].

The best characterized sensitizer BH3 protein is Bad. Two lipid binding regions have been identified in Bad, one of which confers binding to cholesterol and the other to negatively charged lipids [77]. Survival signals trigger the PI3K/Akt pathway and the phosphorylation of Bad at three conserved serine residues: S112, S136 and S155. Upon phosphorylation, Bad is sequestered by 14-3-3 chaperone proteins [161,162]. The phosphorylated Bad-14-3-3 complex has high affinity for cholesterol rich lipid membranes but a low affinity for the MOM, thus phosphorylation of Bad at these sites prevents the interaction of Bad with anti-apoptotic proteins [77]. When dephosphorylated, Bad localizes to the negatively charged MOM and exerts its pro-apoptotic effects by binding to anti-apoptotic proteins [77]. As mentioned above, while Bad binding inhibits Bcl-XL, it also activates Bcl-xl by causing the recruitment of Bcl-XL to the MOM because Bad binding to Bcl-XL is reversible. Thus, the outcome of mutual sequestration depends on the expression level, localization and post-translational modifications of Bad. Taken together these observations suggest that differential interactions with membranes and BH3 proteins govern structural changes that modify their function.

3.5. Anti-apoptotic proteins: multiple mechanisms to inhibit MOMP

Anti-apoptotic Bcl-2 family members are important guardians of cell survival [38]. Bcl-XL, Mcl-1 and Bcl-w are located in the cytoplasm in growing cells and localize to the mitochondria during apoptosis [108,163,164]. In contrast, Bcl-2 constitutively binds to both the MOM and the ER membrane [29,165] but undergoes a conformational change, after binding membranes, essential for it efficiently inhibit Bax mediated MOMP [62].

Detailed studies on the structure and function of some anti-apoptotic proteins are difficult for various technical reasons: it is challenging to purify recombinant Bcl-2 due to its marked tendency to aggregate in vitro while Mcl-1 is notoriously unstable both in vivo and in vitro. However, once purification of full length Bcl-XL (i.e. with the carboxyl-terminal targeting sequence intact) was possible, useful information concerning the mechanism(s) of apoptosis inhibition and how this is modified by binding membranes started to become available.

Despite opposite effects on apoptosis, there is a striking resemblance between Bcl-XL and Bax in terms of their structure and behavior. Both proteins contain hydrophobic grooves for binding BH3 regions of other proteins, and a carboxyl-terminal hydrophobic TM tail bound to this region in *cis* [100,103]. Both Bcl-XL and Bax remain mainly cytoplasmic or peripherally bound to membranes in growing cells and only bind tightly to the membrane after activation, by shifting the equilibrium to favor the membrane bound form [108]. Bcl-XL inhibits Bax/Bak activation and oligomerization on the MOM by several non-exclusive mechanisms [64]. Behaving as a dominant-negative Bax, Bcl-XL binds to BH3 proteins (MODE 1) or activated Bax/Bak (MODE 2). Both MODEs 1 and 2 lead to the membrane localization of Bcl-XL and mutual sequestration of Bcl-XL and its binding partners [64]. In both cases binding between Bcl-XL with its partners involves the interaction of the hydrophobic groove of Bcl-XL with the BH3 region of either Bax/Bak or BH3 proteins [100,103]. Recently studies have suggested that there are other sites outside of the BH3 region of Bim that mediate binding to Bcl-XL, as mutations that abrogate the binding of a Bim BH3 peptide

to Bcl-XL did not prevent binding of a similarly mutated full-length Bim to Bcl-XL in vivo [166].

It is now recognized that in addition to directly engaging and sequestering pro-apoptotic proteins, Bcl-XL also actively transports peripherally membrane-associated Bax to the cytoplasm [64–66]. This mechanism has been dubbed MODE 0 to complement the previously recognized MODEs 1 and 2 [167]. The mechanism of retro-translocation of Bax is still unclear, but there are hints that direct interaction is involved as mutations have been identified in the Bax BH3 domain, the Bcl-XL hydrophobic groove [65] and the BH4 region of Bcl-XL that inhibit this process [64]. A possible explanation for MODE 0 inhibition, consistent with the Embedded Together model, would involve MODE 0 acting principally on peripherally bound Bax. This form of Bax undergoes a limited conformational change in vitro, involving the exposure of an amino-terminal epitope recognized by the monoclonal 6A7 antibody with possible changes in the BH3 region [113]. An exposed site on Bax may interact transiently with either peripherally or integrally bound Bcl-XL, shifting the conformational equilibrium in Bax toward the cytoplasmic 6A7 negative form thereby reducing the affinity of the complex for the membrane and allowing Bax to retro-translocate back to the cytoplasm. The difference between Bax inactivation by anti-apoptotic proteins via MODE 0 and MODE 2 is the membrane status of Bax [67,113]. MODE 0 inhibition occurs in the absence of a BH3 activator, where Bcl-XL binds peripherally membrane bound Bax resulting in dissociation of both from the membrane. Reciprocally, MODE2 inhibition occurs when a BH3 activator promotes Bax conformation changes on the membrane and shifts the equilibrium toward membrane insertion. Then membrane-inserted Bax will recruit Bcl-XL from the cytoplasm where mutual sequestration results in MODE 2 inhibition. Thus, control of Bax subcellular localization clearly serves as a distinct mechanism governing survival in healthy cells. Other reports indicate that Bax can retro-translocate independent of anti-apoptotic proteins in mouse mammary epithelial cells [114]. However, in this study Bax was fully inserted into the MOM rather than peripherally bound to the membrane, indicating yet another poorly understood mechanism that regulates Bax localization (and hence function) in some cells.

Recently Mcl-1 has become a better recognized target for cancer treatment as it mediates chemotherapy resistance in many cancer cell lines [168–171]. Not surprisingly, Mcl-1 is over-expressed in many cancers and cancer cell lines [168–172]. A special role of Mcl-1 for cell survival is suggested by experiments in which genetic deletion of Mcl-1 induces cell death even when other anti-apoptotic proteins are present in these cells [170,171]. Among the anti-apoptotic proteins, Mcl-1 possesses several unique structural and functional features including a long and intrinsically unstructured amino-terminal region. The region contains interacting sites that target the protein for ubiquitin-dependent [173–176] and ubiquitin-independent [177] degradation via a multi-step mechanism that is partly responsible for the extremely short half-life of Mcl-1 [164]. When expressed in cells approximately 80% of the exogenous Mcl-1 is located in the cytoplasm of growing cells [178]. Partial proteolysis of the amino-terminus of Mcl-1 on mitochondria gives rise to three different species: 40 kD (full-length), 38 kD, and 36 kD [179]. Full-length 40 kD Mcl-1 is tightly bound to the MOM and resists alkaline extraction; 38 kD Mcl-1 is enriched at the MOM but has a lower binding affinity to membranes. Both 40 kD and 38 kD Mcl-1 fulfill their pro-survival function by mutually engaging specific pro-apoptotic proteins such as Bim [179]. The binding mechanism may be the same as other anti-apoptotic proteins like Bcl-XL, as the crystal structure of the stable core fragment of Mcl-1 in complex with Bim BH3 and Noxa BH3 shows great similarity with the Bcl-XL–BH3 complex [100,101,103,180].

The amino-terminal region of Mcl-1 has been shown to result in a fraction of the mitochondrial bound Mcl-1 inserting into the MOM with an opposite orientation. In this situation the amino-terminus of Mcl-1 enters the mitochondria matrix where it undergoes further cleavage to generate the 36 kD form. This novel matrix localized form of Mcl-1 regulates mitochondrial fission/fusion that, as noted above, modulates apoptotic function [179]. In the next section we will examine the regulation of these reciprocal processes by the Bcl-2 family in more detail.

4. Mitochondria dynamics and apoptosis

Mitochondria are highly dynamic organelles with major morphological differences seen between cell types. These differences in morphology are dictated by cytoskeletal transport and the relative rates of fission and fusion. By shifting the balance of these rates the morphology can range from a long filamentous network to highly fragmented uniformly shaped mitochondria.

Mitochondrial fission and fusion is regulated by large self-assembling dynamin-related GTPases. Fusion of the MOM is mediated by the partially redundant, membrane anchored mitofusins Mfn1 and Mfn2 which can form homotypic or heterotypic oligomers on the MOM [181,182]. When cells lack either Mfn1 or Mfn2, the mitochondria become highly fragmented resulting in a phenotype that can be rescued by overexpression of either Mfn1 or Mfn2 [182]. Fusion of the mitochondrial inner membrane (MIM) is mediated by Opa1 which is located in the IMS anchored to the MIM within cristae and cristae junctions [183,184]. Fission of mitochondria is carried out by a single protein Drp1 that is predominately cytoplasmic and localizes to sites of mitochondria fragmentation [185–187]; a process which may be facilitated by ER–mitochondria contact sites (discussed below) [188].

Mitochondrial fission and fusion are imperative for cells to respond to metabolic and environmental stress and play a prominent role in apoptosis [189]. In the majority of healthy cells mitochondria exist as a filamentous network and, upon transformation, cellular stress or apoptosis, undergo increased fission [190]. Generally, cells with highly fused networks of mitochondria are more resistant to apoptosis compared to cells with fragmented mitochondrial morphology. The overexpression of a dominant negative Drp1 results in decreased mitochondrial fission and impairs both MOMP and apoptotic cell death [187]. Furthermore, overexpression of Opa1 results in increased fusion and protection from apoptosis by preventing MOMP [191]. The mechanism of Opa1 protection may be due in part to control of cristae remodeling, which is independent of its role in mitochondrial fusion, whereby it prevents the redistribution and release of cytochrome *c* stored in cristae by forming tight cristae junctions [191,192]. Conversely, knockdown of Opa1 by siRNA results in fragmented mitochondria with unstructured cristae and spontaneous apoptosis that can be reduced by overexpression of Bcl-2, which presumably prevents Bax/Bak mediated MOMP [193]. Additionally, cells overexpressing Drp1 have highly fragmented mitochondria that are more sensitive to staurosporine induced apoptosis [194]. The exact mechanisms that regulate the interplay between mitochondrial dynamics and apoptosis are currently unclear however accumulating evidence suggests that the Bcl-2 family proteins can regulate fission and fusion.

During apoptosis Bax colocalizes to mitochondrial foci containing Drp1 where subsequent mitochondrial fission occurs, and Bax remains associated at both tips of the newly formed mitochondria [195,196]. Interestingly, Bax was found to also colocalize with Mfn2, a promoter of mitochondrial fusion, at the same fission sites [195]. To corroborate this finding, a soluble monomeric conformationally restrained Bax, which cannot insert into the MOM and thus does not promote MOMP, was found to associate with Mfn2 in healthy cells and promote mitochondrial fusion. This fusion was impaired by the addition of staurosporine or tBid [197]. Furthermore, during apoptosis Drp1 becomes stably associated with the MOM in a Bax/Bak dependent manner that occurs before MOMP [198]. The BH3 protein cBid plays a role in mitochondrial fission since addition of cBid to isolated mitochondria causes the disassembly of OPA1 complexes resulting in remodeled cristae that allow the mobilization and efficient release of cytochrome *c* from cristae during MOMP [191,199]. Cristae remodeling has also been observed for the BH3-only proteins BimS and Bnip3 [199,200]. cBid mediated cristae remodeling

transpires in a Bax/Bak dependent manner and can occur in the absence of MOMP [199]. Exactly how cBid, a cytosolic protein targeted to the OMM, results in the disassembly of Opa1 complexes located at the IMM, is currently unknown. There must be unidentified proteins responsible for transmitting a signal from the OMM to the IMM in order for Opa1 disassembly to occur. Typically cBid and Bax have similar effects within the cell, promoting apoptosis, so it is interesting to note that the addition of cBid has an opposing effect, promoting fission, to that of Bax, promoting fusion, in the aforementioned study [197]. The opposite effect observed is likely because a mutant Bax was used that is soluble and cannot insert into the MOM due to engineered disulphide bonds that prevent Bax conformation changes. These studies suggest that in growing cells cytoplasmic Bax works to promote fusion and upon the induction of apoptosis Bax inserts in and oligomerizes within the MOM where it can then promote the binding of Drp1 to the MOM thereby increasing fission leading to MOMP and mitochondrial fragmentation. It is to be noted that fission is not necessarily required for apoptosis to occur. When Drp1 was knocked down via RNAi, cytochrome c release was impaired however other apoptogenic IMS proteins such as Smac/DIABLO and Omi/Htra2 were released and the cells still underwent apoptosis [201,202]. This is in contrast to the studies that suggest fission is required for Bax/Bak mediated apoptosis [187,203]. All four studies suggest that mitochondrial fission is required for efficient cytochrome c release, potentially via cristae remodeling, and that the discrepancies between the studies may be explained by differences in cell types that require complete release of IMS localized apoptogenic factors in order to induce apoptosis.

The mechanism of how anti-apoptotic Bcl-2 family proteins regulate mitochondrial dynamics is less clear. As described above, post-translational proteolytic cleavage of Mcl-1 results in a truncated species that is tethered to the MIM, and is required for proper cristae structure and mitochondrial fusion [179]. Furthermore, Bcl-XL can stimulate mitochondrial fusion in vitro and in growing cells [197,204,205]. However, in response to apoptotic stimuli ectopically expressed Bcl-XL or Bcl-2 did not prevent Bax/Bak dependent mitochondrial fission but did prevent cytochrome *c* release suggesting that MOMP can be uncoupled from mitochondrial fragmentation [205]. It is unknown whether or not Bcl-XL or Bcl-2 affects mitochondrial dynamics by directly binding to fission or fusion machinery, or indirectly by sequestration of Bax/Bak and BH3-only proteins.

4.1. Cell death and the Endoplasmic Reticulum

The Bcl-2 family proteins regulate cell death at both the mitochondria and ER. This is evident by the fact that all three classes of the Bcl-2 family proteins target to the ER where they regulate both apoptosis and autophagy. Autophagy is primarily a cell survival mechanism activated by many stressors, most notably cellular starvation, and is mediated by the accumulation of autophagosomes that degrade and recycle intracellular contents such as damaged organelles and proteins [206]. Excessive autophagy results in cell death through degradation of the Golgi complex, the ER and finally the nucleus [207]. One important link between autophagy and apoptosis is through the dual-regulation of both processes via Bcl-2 and Bcl-XL. The BH3-only protein Beclin-1 has a central role in autophagy where it promotes autophagosome nucleation [208]. Bcl-2 localized to the ER can bind to and sequester Beclin-1 resulting in decreased autophagic cell death [209]. Furthermore cells expressing a Beclin-1 mutant which cannot bind Bcl-2 have increased levels of autophagic cell death suggesting that the anti-apoptotic Bcl-2 family proteins help regulate autophagy by keeping Beclin-1 activity in check [209]. The BH3 protein Bad is able to stimulate autophagy by disrupting the interaction between Bcl-2/Bcl-XL and Beclin-1, providing a link between autophagy and apoptosis [210]. Bim may represent another potential link between autophagic and apoptotic cell death processes as at least one report suggests that Bim can inhibit autophagy by recruiting Beclin1 to microtubules [211]. Nutrient starvation results in Bim phosphorylation by JNK causing Bim to release Beclin-1 and dissociate from microtubules, this results in the simultaneous induction of autophagy via Beclin-1 and apoptosis by Bim [211].

Another way cell death can arise from the ER is the initiation of the unfolded protein response (UPR). Generally UPR results from the accumulation of misfolded proteins within the ER as a result of a loss of ER homeostasis by physiological stress such as hypoxia and oxidative stress [212]. If UPR cannot reduce the amount of unfolded proteins within the ER lumen, thus relieving ER stress, then apoptosis is triggered [213]. ER stress is sensed by at least three ER-localized proteins, ATF6, IRE1α and PERK that are maintained in an inactive form in growing cells by binding to the chaperone Grp78. Increased unfolded proteins within the ER lumen results in the dissociation of Grp78 from ATF6, IRE1 α and PERK where they trigger UPR [214]. ATF6 and PERK activation during UPR results in upregulation of the transcription factor CHOP which upregulates the BH3 proteins Bim and Puma, in a p53 independent manner, and downregulates Bcl-2 resulting in apoptosis [33,215–217]. Moreover, overexpression of Bcl-2 or Bcl-XL protects cells from death triggered by ER stress [218]. It is unclear whether or not protection of apoptosis by Bcl-2 and Bcl-XL is mediated through prevention of MOMP at the mitochondria or by an ER specific cell death mechanism. However, there is some data that suggests that overexpression of Bcl-2 specifically targeted to the ER prevents cell death even after cytochrome *c* has been released in response to ER stress [219,220]. This implies that there are cell death pathways mediated through the ER by the Bcl-2 family proteins since MOMP, the point of no return in apoptosis, has already occurred within these cells. Additionally, some forms of cell death can be mediated at the ER that are not related to ER stress. Several agonists that induce cell-stress result in the post-translational modification of E-cadherin, preventing its trafficking from the ER to the plasma membrane, exacerbating apoptosis via the initiation of anoikis [221]. Clearly there is significant crosstalk between the ER and Mitochondria allowing for various forms of cell stress to be sensed and dealt with via apoptosis. This crosstalk may be facilitated, in part, by regions of the ER and Mitochondria that are in close proximity.

4.2. Integrating the organelles: mitochondrial associated membranes

The Mitochondria and ER have specific regions within the cell that are tightly associated termed “mitochondrial associated membranes” (MAMs) which may link cell death processes mediated by both the ER and mitochondria [222]. The ER has a very interconnected tubular structure and, much like mitochondria, the morphology of the ER is highly dynamic with tubules constantly undergoing fission and fusion [223]. It has even been suggested that specialized tubules of the ER wrap around and constrict the mitochondria facilitating mitochondrial fission [188]. Drp1 stably associates with mitochondria localized to these ER–mitochondria contact sites suggesting that the ER marks sites for mitochondrial division. Additionally, the formation of constriction sites was found to be independent of the mitochondrial fission machinery but may depend on Mfn2 homotypic complexes between the ER and MOM [188,224]. Furthermore, as a possible link between ER stress and mitochondrial dynamics and apoptosis, a recent study has identified that Mfn2 is phosphorylated by JNK during cellular stress. Phosphorylation increases ubiquitination and proteasomal degradation of Mfn2 leading to a decrease in the rate of fusion and thus extensive fragmentation of the mitochondrial network and cell death [225]. Additionally, autophagy is dependent on Mfn2 stabilization of MAMs since depletion of Mfn2 diminishes MAMs and severely compromises autophagy [226]. The enrichment of Mfn2 at MAMs, paired with the information that Bax colocalizes and potentially interacts with MFN2 suggests that MOMP is regulated at MAMs by Bcl-2 family proteins.

5. Conclusion

With the basic interaction network of Bcl-2 family members established, we are now at a stage where growing knowledge is yielding

practical results. The discovery that some cancer cells are "addicted" to one or more anti-apoptotic proteins for survival provides a way to categorize malignancies for target-specific treatment [227]. Small molecules mimicking the BH3 regions of Bad (e.g. ABT-737, ABT-263) bind to and inhibit Bcl-XL and Bcl-2 with high affinity [228] and have shown notable benefits in lymphoid malignancies as monotherapy or in combination with other chemotherapies [229,230]. A recent derivative ABT-199 that selectively targets Bcl-2 but not Bcl-XL has reduced thrombocytopenia [231] and is starting phase III clinical trials. We propose that membrane bound Bcl-2 family proteins may serve as a more precise target for drug screening. Our reasoning is that the Bcl-2 family proteins adopt distinct conformations at, on and in intracellular membranes altering their affinities for one another and potentially exposing new binding sites for targeted therapies.

Initially, regulation of the Bcl-2 family was thought of as a rheostat where the relative levels of pro- and anti-apoptotic proteins determines the fate of a cell. At present, it is clearly apparent that regulation of apoptosis is much more complicated. Whether the cell lives or dies is governed by a set of complex equilibria not only between the protein–protein interactions of the Bcl-2 family but also by their interaction with membranes. It is evident that intracellular membranes play an active role in apoptosis as the Bcl-2 family proteins undergo substantial conformational changes upon binding membranes that modifies their affinity for other Bcl-2 family proteins and other binding partners. Even more complexity is added via the localization of Bcl-2 family proteins at different intracellular membranes, where the Bcl-2 family proteins seem to regulate more than just MOMP. It is obvious that there is still much to learn about the Bcl-2 family proteins. It will be exciting to see additional studies that answer the mechanistic details of how Bcl-2 family proteins are regulated by each other, by their localization and by membranes, helping us to fully understand cell death regulation and aiding in the development of novel disease therapies.

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