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Apoptosis: embedded in membranes

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Many different signals for cell death converge on outer mitochondrial membrane permeabilization, a process controlled by the Bcl-2 family of proteins. Recent structural and biochemical analyses emphasize that key regulatory interactions between different classes of Bcl-2 family members occur at and in intracellular membranes, and insertion into membranes causes functionally important conformational changes in the proteins.

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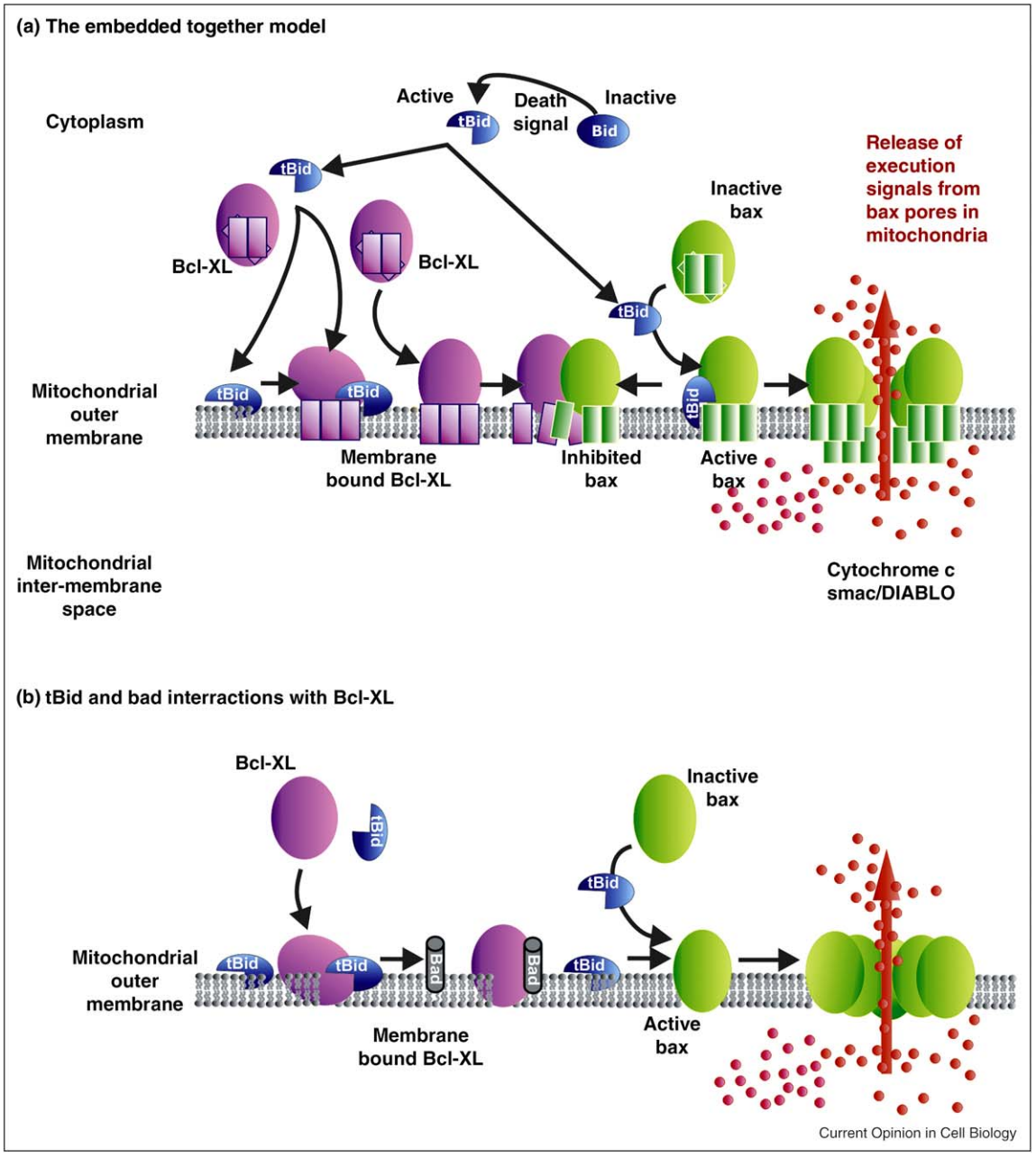
Introduction

In apoptosis that is initiated by stress internal to the cell, the process can be divided into an initiation phase and an execution phase where in most cell types the 'decision' to enter the execution phase is regulated by the Bcl-2 family of proteins. These proteins contain up to four regions of sequence homology (BH1–BH4) and can be divided into three structural and functional groups [1••]: anti-apoptotic proteins like Bcl-2 and Bcl-XL contain all 4 BH regions, the membrane permeabilizing pro-apoptotic proteins Bax and Bak contain BH regions 1–3, and the BH3-only proteins that respond to diverse forms of cellular stress. Thus different types of pro-apoptotic signals activate BH3-only proteins by several mechanisms including increased protein levels owing to increased transcription (PUMA [2]) or decreased turnover (Noxa [3]), post-translational modification (Bad [4]), proteolytic cleavage (Bid [5]), or a change in binding partner and subcellular localization (Bim [6]). The result of all these changes is that the activated BH3-only proteins and possibly other apoptosis regulators bind to one or more of the pro-apoptotic or anti-apoptotic multi-domain Bcl-2 family proteins. The balance between signaling from BH3 proteins and the anti-apoptotic Bcl-2 family members is integrated at the level of Bax or Bak, which when activated oligomerize in the outer mitochondrial membrane, thereby causing mitochondrial outer membrane permeabilization (MOMP) [7]. The direct consequence of MOMP is the release of inter-membrane space proteins such as cytochrome c and smac/DIABLO into the cytoplasm where they activate (or disinhibit) the cascade of proteases (caspases) that cleave multiple substrates thereby mediating the irreversible execution phase of apoptosis.

Bcl-2 family members are Embedded Together in membranes where they control apoptosis

In cells the Bcl-2 family 'interactome' is likely to be large, complex [8•,9,10,11,12••,13], and regulated by multiple post-translational modifications [1••,14], (Bcl-2 [15,16]), (Bax [17,18]), (Bad [19,20]), (Mcl-1 [21,22]). To study the interactions of the core mechanisms that form a basis for understanding the roles of these other factors, several groups [23•,24] have used full-length recombinant Bcl-2 family member proteins and purified organelles or a liposome system to recapitulate the events that regulate MOMP. One firm conclusion from these investigations is that the crucial interactions between the different classes of Bcl-2 family members happen at membranes, and can only be understood in the context of membrane interactions [25••,26•,27•,28]. Furthermore it appears that many BH3-only proteins are intrinsically disordered, and the BH3 region only assumes its secondary structure after it binds to its 'receptor' anti-apoptotic protein. Thus dynamic conformational control of function is noted in many contexts [29]. These insights have been incorporated into a model for apoptosis regulation called Embedded Together (Figure 1). In this model two unique features not previously included by two other major models (the Derepression model and the Direct Activation model, reviewed in [30•,31,32] and [23•,33,34••]) were introduced: BH3-only proteins can both inhibit and activate functions of anti-apoptotic Bcl-2 proteins and that many of the functional interactions between the proteins *occur only in membranes* because membrane binding induces the unique conformational changes required for the interactions. For example, by using an *in vitro* system, it was unequivocally demonstrated that Bcl-XL bound to both Bax and tBid (Figure 1a), but only on the membrane as the binding to either protein was extremely weak or undetectable when membranes were absent [26•]. To be fully active Bcl-XL must be triggered to bind to the membrane *after or concomitant with* binding to tBid and/or Bax that themselves

Figure 1



The Embedded Together Model. **(a)** Bcl-XL (purple) inhibits both tBid (blue) and Bax (green). Binding of Bcl-XL to tBid triggers insertion of Bcl-XL into membranes. Once inserted in membranes, Bcl-XL (or Bcl-XL/tBid complexes) recruits other Bcl-XL proteins to insert into membranes. From this location, Bcl-XL inhibits apoptosis through multiple mechanisms. 1) In membranes the affinity of Bcl-XL for tBid is higher than that between Bax and tBid therefore, Bcl-XL sequesters tBid from recruiting Bax to the membrane. 2) Membrane-bound Bcl-XL prevents the conformation change in Bax that occurs at the membrane that renders it 'competent' to bind to tBid. 3) Membrane-bound Bcl-XL binds to and inhibits the integral membrane (oligomerization competent) form of Bax. The hierarchy of interactions depends on the relative binding affinities of the various proteins that change when the proteins insert into membranes and in response to post-translational modifications (not shown). Apoptosis occurs when all available Bcl-XL is bound to tBid and/or Bax, and additional tBid molecules then activate unbound Bax. Activated, membrane integrated Bax monomers can then recruit more Bax proteins, leading to Bax oligomerization and permeabilization of the outer mitochondrial membrane. **(b)** Bcl-XL (purple) inhibits tBid (blue) from activating Bax by binding it on the membrane. Bad (gray) binds to Bcl-XL on the membrane and can displace a previously bound tBid molecule freeing it to bind to and activate Bax. The Embedded Together model proposes that BH3-only proteins can both inhibit and activate functions of anti-apoptotic Bcl-2 proteins and that many of the functional interactions between the proteins occur only in membranes because interactions with the membrane are required to induce the unique conformational changes required. A prediction of the Embedded Together model is that the conformation of Bcl-XL bound to tBid in the membrane may differ from that of Bcl-XL bound to Bad.

are already membrane bound. By using a variety of mutants that disrupt specific interactions between tBid or Bax and Bcl-XL, it is clear that both interactions occur independently. Furthermore when Bad binds to Bcl-XL, it can displace a previously bound tBid molecule such that this tBid is free to bind to and activate Bax, thereby causing membrane permeabilization. Bad by itself does not activate Bax [27•] (Figure 1b). This difference in the behavior of tBid and Bad is in concordance with the Direct Activation model in that the functional division of BH3-only proteins is real and crucial to understand apoptosis. However, the Embedded Together model goes beyond this distinction and proposes that BH3 proteins like tBid, as well as activated Bax can cause Bcl-XL to insert into membranes. Furthermore recombinant Bad spontaneously inserts into membranes where it can recruit Bcl-XL to insert into membranes [35]. However, unlike tBid, which is held in check by binding to Bcl-XL, direct binding of Bad is inhibitory to Bcl-XL function (Figure 1b). Therefore a prediction of the Embedded Together model is that the conformation of Bcl-XL bound to tBid in the membrane would be different from that of Bcl-XL bound to Bad (Figure 1b).

Bax conformational change is the rate-limiting step in MOMP

By using fluorescently labeled Bcl-2 family member proteins and lipids and measuring these interactions with Fluorescence Resonance Energy Transfer (FRET) and related techniques it is possible to examine in real time and provide an order for the various steps that lead to MOMP [27•]. These investigations demonstrated that when cleaved by caspase 8 (its physiologic activator) cleaved Bid binds to the membrane as the first step, which triggers dissociation of cleaved Bid into a p7 fragment that leaves the membrane, and a p15 fragment (tBid) that inserts into the membrane. This is followed by tBid binding to soluble Bax. These findings are consistent with elegant structural work demonstrating cleavage of Bid does not result in dissociation of the p7 and p15 fragments in aqueous solution [36]. From previous work [37], it is likely that Bax interacts transiently with the membrane to expose the 6A7 epitope as part of a conformational change that allows soluble Bax to bind to membrane-bound tBid. After binding to tBid, Bax inserts into the membrane (possibly by simultaneously inserting helices 5, 6, and 9 [38•]) and then oligomerizes to permeabilize membranes (Figure 1). By measuring these reactions simultaneously, it was determined that they occur as an ordered series of events that ultimately culminates in MOMP [27•]. Significantly Bax insertion into the membrane was identified to be the rate-limiting step in this process. These data confirm the importance of Bax insertion into the membrane and oligomerization as the *crucial decision node* in the commitment to the execution phase of apoptosis.

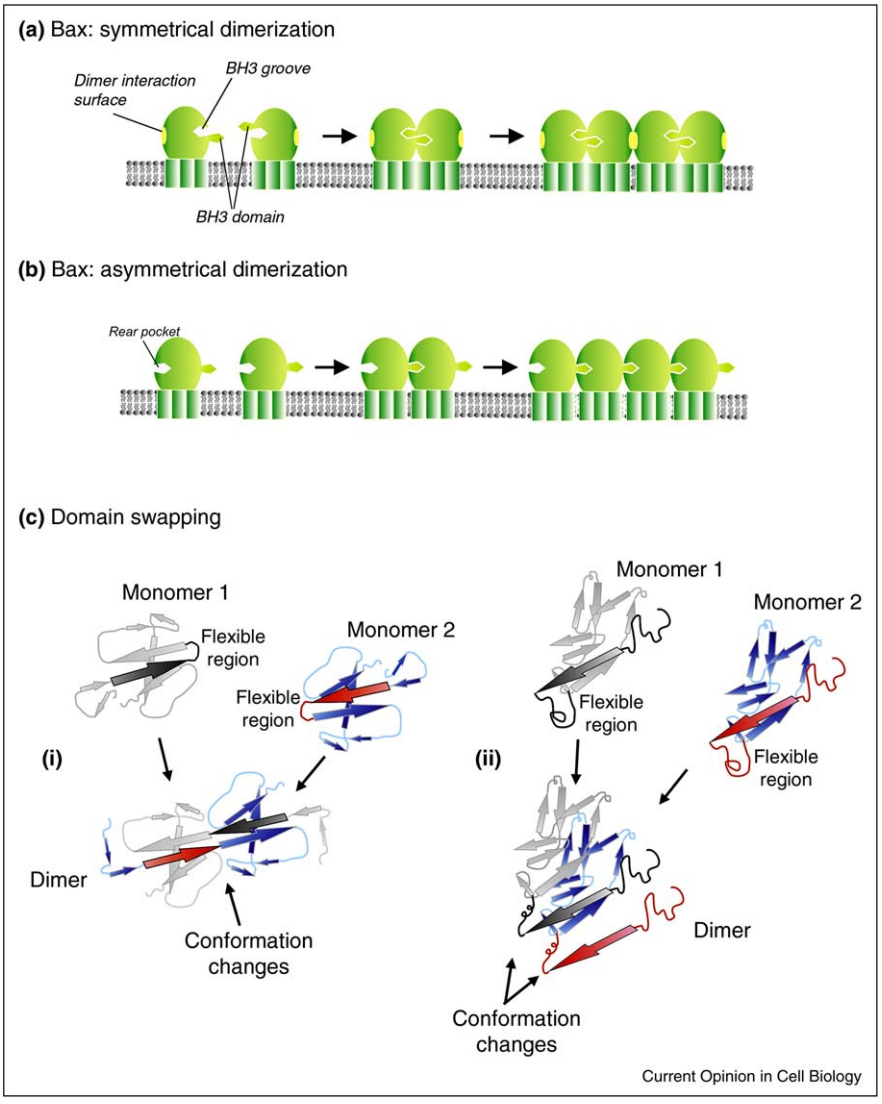
Bcl-XL inhibits apoptosis by acting as a dominant-negative Bax

Experiments using an *in vitro* system where the behavior of individual components of the core machinery can be investigated in detail have demonstrated significant parallels between the behavior of Bcl-XL and Bax [26•]. Both proteins are cytoplasmic or peripherally bound to membranes, and can be recruited in supra-stoichiometric excess into the membrane by tBid, indicating that tBid may function catalytically. Insertion into the membrane causes both proteins to change conformation (Figure 1a). In a similar fashion, Bax and Bcl-XL can be recruited into the membrane by activated Bax. However, for Bax and Bcl-XL the consequences of these 'behaviors' are different. Bcl-XL by binding to membrane-bound tBid sequesters it and prevents further activation of Bax. Yet when Bcl-XL is bound to activated, membrane-bound Bax it prevents further recruitment of soluble Bax and thereby halts the homo-oligomerization process necessary for membrane permeabilization (Figure 1a). This indicates that Bcl-XL functions in a manner conceptually similar to a dominant-negative Bax: Bcl-XL and Bax compete for the same binding partners, but Bcl-XL does not propagate oligomerization [26•].

The quaternary structure of Bcl-2 family member complexes regulates MOMP

Bax must bind to other Bax molecules to oligomerize and Bcl-XL binds to Bax to prevent this. Thus, determining the stoichiometry and quaternary structure of the multiprotein complexes that either promote or inhibit apoptosis is crucial to understanding mechanism. One mode of binding would be through an interaction between the BH3 region on one protein, and the BH3 binding pocket (formed by BH regions 1–3) on the second protein. This is the well-recognized mode of binding between BH3-only proteins, and Bcl-XL [39,40] and has also been described as the means by which Bak forms homo-dimers [41••]. This mode results in dimers that require a second binding surface to assemble into larger complexes. It has been proposed for Bak that the second binding surface involves interactions between helix 6 of adjacent 'symmetric dimers' [42] (Figure 2a). Recently another alternative mode of binding has been described that would also allow for oligomer propagation [43–45] (Figure 2b). By using a 'stapled' Bim BH3 peptide and Bax protein, NMR analysis of the complex revealed a binding site for the peptide located in a previously uncharacterized pocket in Bax formed by helices 1 and 6, and the unstructured loop connecting helices 1 and 2. The interaction of BH3 peptides with this 'rear pocket' caused several conformational changes in Bax including the flexible regions located N-terminal and C-terminal to the helix 1. Because each monomer in the Bax oligomer would use two different surfaces to interact with neighboring Bax proteins, we designate such a Bax oligomer as 'asymmetric' to distinguish it from the 'symmetric' oligomer proposed for

Figure 2



Bax Dimer Formation and Domain Swapping. **(a)** Symmetrical Dimers: The BH3 domain of one Bax interacts with the BH3 binding groove of another. Bax dimer formation causes a conformational change, which opens up a dimer interaction surface thereby permitting Bax dimers to join to each other and form higher order oligomers. **(b)** Asymmetrical Dimers: The BH3 domain of an activated Bax can interact with the 'rear pocket' of another Bax. This interaction causes a conformational change in Bax allowing the BH3 domain to be exposed such that it binds to the 'rear pocket' of an adjacent Bax. This leads to oligomerization of Bax dimers and results in MOMP. **(c)** Domain swapping as a potential mechanism to explain dimerization/ oligomerization of pro-apoptotic and anti-apoptotic BH multi-domain proteins. The structures and models illustrated here are speculative.

i. *Model of balanced domain swapping:* A conformational change in a flexible region allows the black arrow in monomer 1 to substitute for the red arrow in monomer 2. The red arrow substitutes for the black arrow in monomer 1. This would result in a symmetrical dimer.
ii. *Model of unbalanced domain swapping:* A conformation change in a flexible region allows the black arrow in monomer 1 to precisely substitute for the red arrow in monomer 2, forming an asymmetrical dimer. The displaced red arrow can now domain swap with a third monomer to form a trimer, and so on. Monomer 1 can also accept an arrow from another monomer thus allowing growth of the oligomer in both directions and permitting the formation of a complete circle that may function as a pore.

Bak [42,46]. An 'auto-activation' model of interaction with 'asymmetric' dimer formation (Figure 2b) would allow addition of a second Bax to the BH1-3 groove of the first Bax without dissociating the BH3-only protein, since these two interactions occur at opposite 'sides' of Bax. The second Bax would then bind to the rear pocket on a third Bax and thus propagate auto-activation to form the oligomer without requiring the initial BH3 protein to dissociate. While in both models the binding of the BH3-only protein is 'catalytic' in causing a conformation change in Bax, it is required for each step during oligomerization in the symmetric dimer model, but only to initiate oligomerization in

the asymmetric auto-activation model. However, recently published results suggest [47] that dimerization of Bax activated 'in solution' may involve additional surfaces compared to those involved with membrane associated dimerization.

Regardless of the binding surfaces involved, it is highly likely that Bcl-2 family members undergo major conformational changes in these complexes [48,49] and that it is possible for subunits to exchange as evidenced by the 25 nM apparent Kd for tBid binding to Bax in membranes [27•]. One type of conformational change for which *in vitro* evidence exists in this context is three-dimensional domain swapping (3DDS) between adjacent monomers in the complex (Figure 2ci). Dimerization of Bcl-XL mediated by three-dimensional domain swapping at alkaline pH has been well described [50]. Dimerization by α-helix swapping confirmed by nuclear magnetic resonance (NMR) spectroscopy has also been observed in a recent publication where heat was used to induce dimer formation [51•]. Bcl-XL 3DDS dimers exhibited some membrane permeabilizing activity compared to monomers in this artificial system, suggesting that similar 3DDS dimers may act as intermediates in membrane pore formation by Bax (shown schematically in Figure 2cii). Though dimerization and BH3-peptide binding take place at different sites of the Bcl-XL protein, the interconversion process (monomers to dimers) was found to be significantly slowed down by BH3-peptide binding [52,53], consistent with the allosteric regulation of multi-domain Bcl-2 family members by BH3-only proteins. Determining whether such structures occur in the more physiologic context of membrane-bound Bcl-2 family members is an important area of investigation.

Conclusions

The importance of apoptosis and the crucial role of the Bcl-2 family of proteins have been intensively investigated for more than 15 years [54•]. The first phase of investigating apoptosis in which the components of the core machinery are identified is at a mature, but not yet complete stage [1••,55]. The second phase that is currently underway, provided order to the list of components, categorized them into subgroups, and identified many (but not likely all) relevant interacting partners [1••]. Determining the relative affinities of these various interactions in a rigorous and quantitative fashion in the context of physiologic membranes is an important next step. The third phase of investigating apoptosis, which is in its infancy, will yield a detailed understanding of the structure of the components in functional complexes in membranes [41,38•,51•,56] and the molecular mechanisms that regulate the interactions among Bcl-2 family members. Deep understanding of the entire process of apoptosis will come with a quantitative analysis of these interactions, and how they interface with the signaling pathways that lead to their initiation [57].

Conflict of interest

The authors declare no conflict of interest.

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