DYNAMIC INTERACTION OF BID WITH LIPID MEMBRANES

A CHARACTERIZATION OF THE DYNAMIC INTERACTION BETWEEN THE PRO-APOPTOTIC PROTEIN BID AND THE MITOCHONDRIAL OUTER MEMBRANE

By

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Abstract

Bcl-2 family of proteins regulate apoptosis at the level of the mitochondrial outer membrane (MOM) through both protein-protein and protein-membrane interactions. While the role of the membrane as the "locus of action" has been recognized, the detailed molecular mechanisms and the consequences of the interactions of Bcl-2 family members with the membrane are yet to be fully understood. The findings presented here focus on the dynamic interactions of Bcl-2 proteins, most notably tBid with the MOM, and their functional significance on mitochondrial outer membrane permeabilization. We show that the activation of tBid is a multi-step process that is regulated by MOM lipids and proteins. The rate-limiting step in the activation of tBid is an elaborate conformational change that is facilitated by Mtch2, and is required for the activation and recruitment of Bax to the MOM. Furthermore, we demonstrate that binding of both tBid and Bax to the membrane is reversible and is governed by dynamic equilibria that potentially contribute to the propagation of the permeabilization signal within the cell for the regulation of apoptosis. We report that the transfer of tBid between membranes is accelerated by Bax and restricted by Bcl-XL, whereas the transfer of Bax between membranes is slower than and not influenced by tBid. Finally, by studying the effect of varying lipid composition on Bax-mediated permeabilization, we establish that electrostatic interactions mediate the binding of both tBid and Bim to the membrane. We demonstrate that while Bim does not exhibit any preference for a specific anionic lipid, tBid requires cardiolipin in order to undergo its conformational change at the membrane in the absence of Mtch2. Taken together, our work contributes to the growing understanding of the dynamic interactions and changes in conformation of Bcl-2 proteins at the MOM.

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List of Abbreviations

3D	Three-dimensional
ANT	Adenine nucleotide translocase
ANTS	8-Aminonaphthalene-1,3,6-Trisulfonic Acid
Bak	Bcl-2 antagonist/killer
Bax	Bcl-2 associated X protein
Bcl-2	B-cell CLL/lymphoma 2
Bcl-XL	B-cell lymphoma extra long
BH	Bcl-2 homology
Bid	BH3 interacting domain death agonist
cBid	Cleaved Bid
CHX	Cycloheximide
CL	Cardiolipin
DAC	N-(7-Dimethylamine-4-Methylcoumarin-3-yl)
DACM	N-(7-Dimethylamine-4-Methylcoumarin-3-yl) Maleimide
DiD	1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate Salt
DiI	1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate
DPA	Dipicolinic acid
DPX	*p*-Xylene-Bis-Pyridinium Bromide
ER	Endoplasmic reticulum
FCS	Fluorescence correlation spectroscopy
FIDA	Fluorescence intensity distribution analysis
FRET	Förster resonance energy transfer
K_d	Dissociation constant
KD	Knock down
KO	Knock out
IB	Immunoblot

IMS	Intermembrane space
IP3	Inositol 1,4,5 triphosphate
MAC	Mitochondrial apoptosis-induced channel
MBR	Membrane binding region
MCD	Mitochondrial carrier domain
MIM	Mitochondrial inner membrane
MLCL	Monolysocardiolipin
MOM	Mitochondrial outer membrane
MOMP	Mitochondrial outer membrane permeabilization
MTD	Mitochondrial targeting domain
NBD	*N*,*N'*-Dimethyl-*N*-(Iodoacetyl)-*N'*-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl) Ethylenediamine
NBD PE	1-palmitoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl) amino]dodecanoyl}-*sn*-glycero-3-phosphoethanolamine)
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PS	Phosphatidylserine
PTP	Permeability transition pore
SEM	Standard error of mean
Tb	Terbium III
tBid	Truncated Bid
TNF	Tumor necrosis factor
UPR	Unfolded protein response
VDAC	Voltage dependent anion channel
WT	Wild-type

INTRODUCTION

CHAPTER I

Mechanisms of Action of Bcl-2 Family Proteins

Preface

The work presented in this chapter has been previously published as a book chapter in:

Shamas-Din, A., Kale, J., Leber, B., and Andrews, D.W. (2013) Mechanisms of Action of Bcl-2 Family Proteins. *Cold Spring Harb Perspect Biol.* 5(4):a008714.

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Contribution of authors:

Shamas-Din, A wrote the sections on the four models of Bcl-2 family proteins in the introduction, the section 'The Models: Who binds to whom?', and the section on 'BH3 Members'. In addition, Shamas-Din, A prepared figures 1-2, and tables 1-2. Kale, J wrote the section on 'Multi-domain pro-apoptotic members' and prepared figure 3, and Leber, B wrote the first section of the introduction, the section on 'Anti-apoptotic members' and the conclusion section. Andrews, DW edited the manuscript and directed the layout of the book chapter.

Objective of the book chapter:

To provide a mechanistic overview of the different classes of Bcl-2 family proteins.

Highlights:

- The Bcl-2 family of proteins plays a crucial role in apoptosis by regulating mitochondrial outer membrane permeabilization.
- The family is divided into three classes:
  - The pro-apoptotic effector proteins, Bax/Bak, permeabilize the mitochondrial outer membrane.
  - The pro-apoptotic BH3 proteins come in two flavours: activator proteins, such as tBid and Bim, directly promote apoptosis by activating Bax/Bak, whereas sensitizer proteins, such as Bad, indirectly promote apoptosis by inhibiting the anti-apoptotic proteins.
  - The anti-apoptotic proteins, Bcl-2/Bcl-XL prevent apoptosis by sequestering either Bax/Bak or the BH3 proteins.
- Bcl-2 family proteins undergo structural conformational changes at the membrane that modify binding to other proteins and their apoptotic function.
- The relative binding affinities and the concentrations of the Bcl-2 family members govern the interactions between Bcl-2 family members and with the membrane.

Mechanisms of Action of Bcl-2 Family Proteins

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The Bcl-2 family of proteins controls a critical step in commitment to apoptosis by regulating permeabilization of the mitochondrial outer membrane (MOM). The family is divided into three classes: multiregion proapoptotic proteins that directly permeabilize the MOM; BH3 proteins that directly or indirectly activate the pore-forming class members; and the anti-apoptotic proteins that inhibit this process at several steps. Different experimental approaches have led to several models, each proposed to explain the interactions between Bcl-2 family proteins. The discovery that many of these interactions occur at or in membranes as well as in the cytoplasm, and are governed by the concentrations and relative binding affinities of the proteins, provides a new basis for rationalizing these models. Furthermore, these dynamic interactions cause conformational changes in the Bcl-2 proteins that modulate their apoptotic function, providing additional potential modes of regulation.

Apoptosis was formally described and named in 1972 as a unique morphological response to many different kinds of cell stress that was distinct from necrosis. However, despite the novelty and utility of the concept, little experimental work was performed during the following 20 years because no tools existed to manipulate the process. In the early 1990s, two seminal observations changed the landscape. First, as the complete developmental sequence of the nematode *Caenorhabditis elegans* was painstakingly elucidated at the single-cell level, it was noted that a fixed, predictable number of "intermediate" cells were destined to die, and that this process was positively and negatively regulated by specific genes. Second, a novel gene called B-cell CLL/lymphoma 2 (Bcl-2; encoded by *BCL2*) that was discovered as a partner in a reciprocal chromosomal translocation in a human tumor turned out to function not as a classic oncogene by driving cell division, but rather by preventing apoptosis. When it was discovered that the mammalian *BCL2* could substitute for *CED-9*, the *C. elegans* gene that inhibits cell death, the generality of the process was recognized, and the scientific literature exploded with now well over 10^5 publications on apoptosis. However, it is ironic to note that after a further 20 years of intensive investigation, it is clear that the mechanism of action of Bcl-2 is quite distinct from Ced-9, which sequesters the activator of the caspase protease that is the ultimate effector of apoptosis. In contrast, Bcl-2 works primarily by binding to other related proteins that regulate

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permeabilization of the mitochondrial outer membrane (MOM).

This review examines how apoptosis is regulated by the members of the (now very large) Bcl-2 family, composed of three groups related by structure and function (illustrated in Fig. 1): (1) the BH3 proteins that sense cellular stress and activate (either directly or indirectly); (2) the executioner proteins Bax or Bak that oligomerize in and permeabilize the MOM, thereby releasing components of the intermembrane space that activate the final, effector caspases of apoptosis; and (3) the antiapoptotic members like Bcl-2 that impede the overall process by inhibiting both the BH3 and the executioner proteins. To understand the consequence of the interactions among the three subgroups, several models have been proposed ("direct activation," "displacement," "embedded together," and "unified" models; illustrated in Fig. 2) that are briefly described here before a more detailed discussion of the Bcl-2 families.

DIRECT ACTIVATION MODEL

The distinctive feature of the direct activation model is that a BH3 protein is required to directly bind and to activate the Bcl-2 multihomology region proapoptotic proteins, Bax and Bak. The direct activation model classifies BH3 proteins as activators or sensitizers based on their affinities for binding to Bcl-2 multiregion proteins (see Table 1) (Letai et al. 2002). The activator BH3 proteins—tBid, Bim, and Puma—bind to both the proapoptotic and the antiapoptotic Bcl-2 multiregion proteins (Kim et al. 2006). The sensitizer BH3 proteins—Bad,

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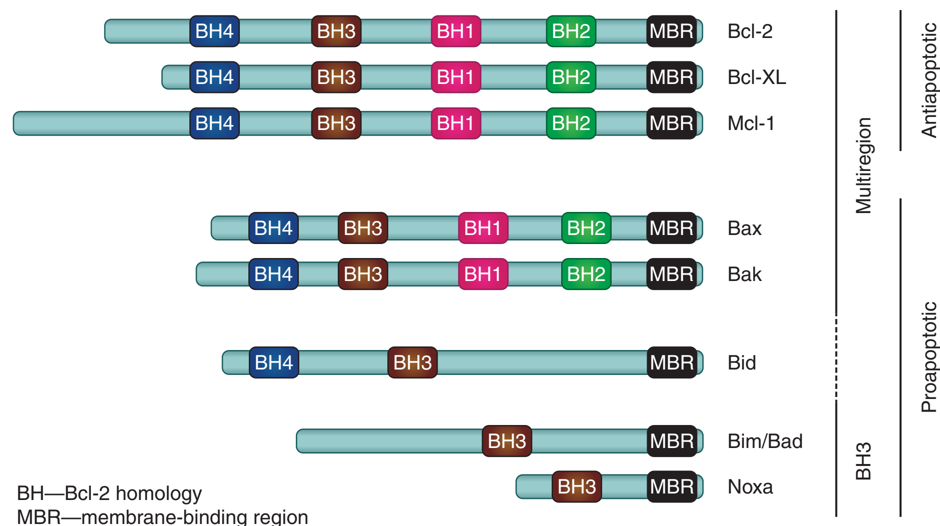

Figure 1. Schematic overview of the Bcl-2 family of proteins. The family is divided into two subgroups containing proteins that either inhibit apoptosis or promote apoptosis. The proapoptotic proteins are further subdivided functionally into those that oligomerize and permeabilize the MOM, such as Bax and Bak, or those that promote apoptosis through either activating Bax or Bak or inhibiting the antiapoptotic proteins, such as tBid, Bim, Bad, and Noxa. Proteins are included in the Bcl-2 family based on sequence homology to the founding member, Bcl-2, in one of the four Bcl-2 homology (BH) regions. All the antiapoptotic proteins, as well as Bax, Bak, and Bid, have multiple BH regions, are evolutionarily related, and share a three-dimensional (3D) structural fold. The BH3 proteins contain only the BH3 region, are evolutionarily distant from the multiregion proteins, and are intrinsically unstructured. Most members of the Bcl-2 family proteins contain a membrane-binding region (MBR) on their carboxyl termini in the form of a tail anchor, mitochondrial-targeting sequence, or as a hydrophobic amino acid sequence that facilitates binding and localization of these proteins to the MOM or to the endoplasmic reticulum (ER) membrane.

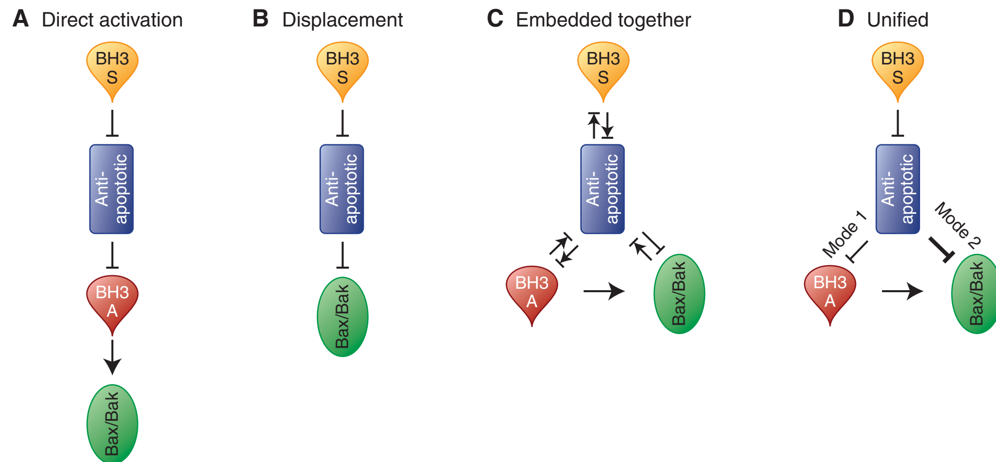

Figure 2. Schematics of the core mechanisms proposed by various models for the regulation of MOMP by Bcl-2 proteins. (↑) Activation; (⊥) inhibition; (⊥↑) mutual recruitment/sequestration. Paired forward and reverse symbols indicate the model makes explicit reference to equilibria. (*A*) The direct activation model divides the different BH3 proteins by qualitative differences in function. The BH3 proteins with high affinity for binding and activating Bax and Bak are termed as "activators," whereas those that only bind the antiapoptotic proteins are termed "sensitizers." The activator BH3 proteins directly interact with and activate Bax and Bak to promote MOMP. The antiapoptotic proteins inhibit MOMP by specifically sequestering the BH3 activators. The BH3 sensitizer proteins can compete for binding with the antiapoptotic proteins, thus releasing the BH3 activator proteins to avidly promote MOMP through activation and oligomerization of Bax and Bak. (*B*) The displacement model categorizes the BH3 proteins solely based on their affinities of binding for the antiapoptotic proteins (hence, does not recognize them as activators). In this model, Bax and Bak are constitutively active and oligomerize and induce MOMP unless held in check by the antiapoptotic proteins. Therefore, for a cell to undergo apoptosis, the correct combination of BH3 proteins must compete for binding for the different antiapoptotic proteins to liberate Bax and Bak and for MOMP to ensue. (*C*) The embedded together model introduces an active role for the membrane and combines the major aspects of the previous models. The interactions between members of the Bcl-2 family are governed by equlibria and therefore are contingent on the relative protein concentrations as well as their binding affinities. The latter are determined by posttranslational modifications, fraction of protein bound to the membrane, and cellular physiology. At membranes, the activator BH3 proteins directly activate Bax and Bak, which then oligomerize, inducing MOMP. Both activator and sensitizer BH3 proteins can recruit and sequester antiapoptotic proteins in the membrane. The antiapoptotic proteins inhibit apoptosis by sequestering the BH3 proteins and Bax and Bak in the membrane or by preventing their binding to membranes. At different intracellular membranes, the local concentrations of specific subsets of Bcl-2 family members alter the binding of Bcl-2 proteins to the membrane and the binding equilibria between family members. As a result, Bcl-2 family proteins have distinct but overlapping functions at different cellular locations. (*D*) The unified model builds on the embedded together model by proposing that the antiapoptotic proteins sequester the activator BH3 proteins (mode 1) and sequester Bax and Bak (mode 2). It differs in that in the unified model, inhibition of apoptosis through mode 1 is less efficient (smaller arrow in panel *D*) and therefore easier to overcome by sensitizer BH3 proteins. In addition, the unified model extends the role of Bcl-2 family proteins and the regulation of MOMP to mitochondria dynamics (not shown).

Noxa, Bik, Bmf, Hrk, and Bnip3—bind to the antiapoptotic proteins, thereby liberating activator BH3 proteins to promote mitochondrial outer membrane permeabilization (MOMP) (Letai et al. 2002; Kuwana et al. 2005; Certo et al. 2006). The antiapoptotic proteins bind to both the activator and the sensitizer BH3 proteins, but are unable to complex with Bax and Bak (Kim et al. 2006). Therefore, for a cell to evade apoptosis, antiapoptotic proteins must sequester the BH3 proteins to prevent Bax/Bak activation and apoptosis.

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Table 1. Binding profiles within Bcl-2 family members

Antiapoptotic protein	Antiapoptotic protein binds to		
	Bax/ Bak/Bid	BH3 proteins	
		Activator	Sensitizer
Bcl-2	Bax, Bid	Bim, Puma	Bmf, Bad
Bcl-XL	Bax, Bak, Bid	Bim, Puma	Bmf, Bad, Bik, Hrk
Bcl-w	Bax, Bak, Bid	Bim, Puma	Bmf, Bad, Bik, Hrk
Mcl-1	Bak, Bid	Bim, Puma	Noxa, Hrk
A1	Bak, Bid	Bim, Puma	Noxa, Bik, Hrk

Letai et al. (2002); Chen et al. (2005).

DISPLACEMENT MODEL

In the displacement model, BH3 proteins do not directly bind to Bax and Bak to cause their activation. Rather, Bax and Bak are constitutively active and therefore must be inhibited by the antiapoptotic proteins for the cell to survive. To initiate apoptosis, BH3 proteins displace Bax and Bak from the antiapoptotic proteins to promote Bax- or Bak-mediated MOMP. Because BH3 proteins selectively interact with a limited spectrum of antiapoptotic proteins, a combination of BH3 proteins is required to induce apoptosis in cells expressing multiple antiapoptotic Bcl-2 family members (see Table 1) (Chen et al. 2005). In support for this model, heterodimers of Bak with Mcl-1 and Bcl-XL are present in dividing cells, and overexpression of Noxa displaces Bak–Mcl-1 heterodimers, releasing Bak and forming Noxa–Mcl-1 complexes. In these cells, a combination of Bad and Noxa is required to neutralize the effects of both Bcl-XL and Mcl-1 to finally induce apoptosis (Willis et al. 2005).

EMBEDDED TOGETHER MODEL

The embedded together model incorporates the role of the membrane as the "locus of action" for most Bcl-2 family proteins because MOMP does not occur until Bax and Bak achieve their final active conformation in the membrane. The interactions with membranes result in distinct changes in conformations of the Bcl-2 family proteins that govern their affinity for the relative local concentrations of the binding partners (Leber et al. 2007, 2010; Garcia-Saez et al. 2009). For example, the cytoplasmic multiregion proteins Bax and Bcl-XL undergo large but reversible conformational changes after interacting with MOM (Edlich et al. 2011), which increase the affinity for binding to a BH3 protein, causing a further conformational change and allowing insertion in the membrane.

In this model, sensitizer BH3 proteins bind only to antiapoptotic proteins. However, the consequences of this interaction incorporate the features of both the displacement and direct activation models, because the sensitizer BH3 proteins neutralize the dual function of the antiapoptotic proteins by displacing *both* the activator BH3 proteins and Bax or Bak from the membrane-embedded conformers of the antiapoptotic proteins (Billen et al. 2008; Lovell et al. 2008). Because it is the activated forms of Bax and Bak that are bound to the membrane-embedded antiapoptotic proteins, sensitizer proteins release Bax and Bak conformers competent to oligomerize and permeabilize membranes.

Another distinguishing feature of this model is the dual role assigned to activator BH3 proteins, which directly activate proapoptotic proteins and also bind to antiapoptotic proteins. When activator BH3 proteins interact with Bax and Bak, they promote insertion into the membrane, whereupon Bax and Bak oligomerize and permeabilize cellular membranes. Similarly, interaction of activator BH3 proteins with antiapoptotic proteins promotes their insertion into membranes. However, in this case, the BH3 protein functions like a sensitizer, because the bound antiapoptotic protein is unable to bind Bax or Bak. However, sequestration goes both ways, and by binding the BH3 protein, the antiapoptotic protein inhibits it at the membrane. Moreover, because the interaction of the activator BH3 proteins with both the proapoptotic and the antiapoptotic Bcl-2 family proteins is reversible, it is therefore possible for a single BH3 protein to interact with both proapoptotic and antiapoptotic proteins (depend-

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ing on their relative expression levels), thereby changing their conformation at the membranes. Recently, many of the interactions proposed by this model have been measured directly in living cells (Aranovich et al. 2012).

UNIFIED MODEL

The unified model of Bcl-2 family function builds on the embedded together model (Llambi et al. 2011). This model distinguishes the known interactions of antiapoptotic Bcl-2 proteins to sequester the activator BH3 proteins as mode 1, and to sequester the active forms of Bax and Bak as mode 2 (Fig. 2D). Although in cells both modes of inhibition take place simultaneously, in the unified model, inhibition of apoptosis through mode 1 is less efficient and is easier to overcome by BH3 sensitizers to promote MOMP than inhibition through mode 2. Importantly, the unified model also incorporates the functions of Bax and Bak in mitochondrial fission and fusion and postulates that only mode 2 repression affects this process. This model is therefore the first to explicitly link modes of MOMP regulation and mitochondrial dynamics.

The dual function assigned to antiapoptotic proteins is thus shared by both embedded together and unified models. However, in the former, the interplay between members of the Bcl-2 family are determined by competing equilibria; therefore, the abundance of proteins and specific conditions of cell physiology including posttranslational modifications will determine the prevailing interactions. As a result, the embedded together model differs from the unified model in that it predicts that either mode 1 or mode 2 can be dominant depending on circumstances such as the particular form of stress and cell type. Further work to test the different predictions of the models with full-length, wild-type proteins in different cells is required to resolve these issues.

THE MODELS: WHO BINDS TO WHOM?

One aspect of many of the models that is potentially confusing is that if an activator BH3 protein binds to an antiapoptotic family member, which is being inhibited? Whether antiapoptotic proteins sequester the BH3 proteins or the BH3 proteins sequester the antiapoptotic proteins becomes a semantic argument. A more productive way of characterizing the interaction is as a mutual sequestration that prevents their respective activation or inhibition effects on Bax and Bak. Therefore, whether MOMP ensues is determined by the relative concentrations and affinities of the proapoptotic and antiapoptotic proteins at the membrane. This recasting of the players is reminiscent of the original rheostat model proposed by the Korsmeyer group (Oltvai et al. 1993); however, it extends that model in ways not originally envisioned. For example, the rheostat model did not anticipate autoactivation. If there is sufficient cytosolic antiapoptotic Bcl-XL, then those Bcl-XL molecules recruited to the membrane by a BH3 protein can recruit additional molecules of Bcl-XL to the membrane through "autoactivation," a process also observed for Bax. Because BH3 protein binding is reversible, autoactivation ensures recruitment of sufficient Bcl-XL to provide efficient inhibition of the BH3 protein.

Another recently recognized aspect that determines the nature and fate of the binding interactions is composition of different membrane organelles. As mentioned above, the unified model provides a mechanistic link between MOMP regulation and mitochondrial fission and fusion. The importance of membranes in modifying conformations and binding partners as proposed by the embedded together model accounts for the overlapping but distinct function of the Bcl-2 family at the mitochondria and endoplasmic reticulum (ER). It also explains how other membrane sites such as the Golgi can act as a reservoir for potentially activated Bax (Dumitru et al. 2012). Therefore, the roles of Bcl-2 family proteins in cell fate decisions and other processes such as mitochondrial fusion and autophagy appear to be primarily governed by the relative concentrations and affinities of the different binding partners available in that specific subcellular membrane.

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MULTIDOMAIN PROAPOPTOTIC MEMBERS

Bax (Bcl-2-associated X protein) was identified by coimmunoprecipitation with Bcl-2 (Oltvai et al. 1993). Unlike Bcl-2, overexpression of Bax promoted cell death, and the opposing functions suggested a rheostat model, whereby the relative concentrations of proapoptotic and antiapoptotic Bcl-2 family members determine cell fate. The discovery of Bcl-XL indicated that antiapoptotic function could be mediated by more than Bcl-2; shortly thereafter Bak (Bcl-2 antagonist/killer) was cloned and recognized as the second proapoptotic protein functioning similarly to Bax despite being more homologous to Bcl-2 than Bax (Chittenden et al. 1995; Farrow et al. 1995; Kiefer et al. 1995). Cells in which the gene encoding either Bax or Bak was knocked out were still susceptible to apoptosis. However, $Bax^{-/-}/Bak^{-/-}$ double-knockout cells were resistant to almost all death stimuli (Wei et al. 2001). These seminal studies placed Bax and Bak in the same prodeath pathway and indicated significant functional redundancy. Furthermore, the demonstration that they are jointly necessary for almost all types of apoptotic cell death (except for death receptor pathways, where effector caspases are directly activated by initiator caspases) provides the mechanism for integration of proapoptotic and antiapoptotic signals via the common mechanism of Bax- and Bak-mediated membrane permeabilization.

Both Bax and Bak mediate prodeath function at the MOM, where they oligomerize and permeabilize the MOM, resulting in the release of intermembrane space (IMS) proteins such as cytochrome *c*, OMI/HTRA2, SMAC/DIABLO, and endonuclease G (Kuwana and Newmeyer 2003). The solution structures of Bax and Bak reveal that both proteins are composed of nine α helices with a large hydrophobic pocket composed of helices 2–4 (Suzuki et al. 2000; Moldoveanu et al. 2006). Both Bax and Bak contain a carboxy-terminal transmembrane region, α helix 9, which targets the proteins to the MOM (see Table 2).

The differences between Bax and Bak are illuminating with respect to the common mechanism. Whereas Bax has a high affinity for the antiapoptotic proteins, Bcl-2 and Bcl-XL, Bak has a high affinity for the antiapoptotic proteins, Mcl-1 and Bcl-XL (Willis et al. 2005; Llambi et al. 2011). Another difference is that Bak is found constitutively bound to the MOM, whereas Bax is primarily cytosolic but migrates to the MOM after apoptotic stimuli (Hsu et al. 1997; Wolter et al. 1997; Griffiths et al. 1999). The difference in localization of Bax and Bak in nonstressed cells is a result of the position of helix 9. NMR studies indicate that in the initial step of activation, helix 9 of Bax is bound to the hydrophobic pocket in "*cis*," preventing helix 9 from inserting into the MOM (Suzuki et al. 2000). Disruption of the interaction of helix 9 with the hydrophobic pocket causes constitutive Bax targeting to the mitochondria, thus recapitulating the intracellular location of Bak (Nechushtan et al. 1999; Brock et al. 2010). Conversely, tethering helix 9 to the hydrophobic core of Bax abrogates Bax targeting to MOM and membrane permeabilization (Gavathiotis et al. 2010). Other portions of the protein involved in membrane binding (and MOMP) once helix 9 is displaced are described below. In contrast, it is presumed that helix 9 of Bak is positioned differently, because Bak bypasses the initial step of Bax activation and targets constitutively to mitochondrial membranes (Setoguchi et al. 2006).

Bax/Bak-Mediated MOMP

It was proposed that activated Bax would assemble a complex of proteins termed the permeability transition pore (PTP) to create an opening spanning through both membranes of the mitochondria, ultimately causing the MOM to rupture because of mitochondrial matrix swelling (Schwarz et al. 2007). The PTP is composed of the voltage-dependant anion channel (VDAC1) located within the MOM, adenine nucleotide translocase (ANT) located within the mitochondrial inner membrane (MIM), and cyclophilin D located within the mitochondrial matrix (Brenner and Grimm 2006). Opening of the pore would ensue after activated Bax bound to VDAC1, causing a conformational

Table 2. Localization, targeting mechanism, and nonapoptotic function of Bcl-2 family proteins

Bcl-2 protein	Targeting mechanism and location	Nonapoptotic function	References
Bax	Tail anchor Cystolic binds to MOM and ER membrane upon activation	Promotes mitochondria fusion in healthy cells and mitochondria fission in dying cells	Annis et al. 2005; Karbowski et al. 2006; Montessuit et al. 2010; Hoppins et al. 2011
Bak	Tail anchor Constitutively bound to MOM and ER membrane	Promotes mitochondria fusion in healthy cells, and mitochondria fission in dying cells	Griffiths et al. 1999; Karbowski et al. 2006; Brooks et al. 2007
Bid	Carboxy-terminal anchor? Helices 6 and 7 required for membrane binding Cytosolic and nuclear in healthy cells Localizes to MOM and ER upon cleavage by caspase-8 on the onset of apoptosis	Preserves genomic integrity and mediates intra-S-phase check point Interacts with and modulates NOD1 inflammatory response	Li et al. 1998; Luo et al. 1998; Hu et al. 2003; Kamer et al. 2005; Zinkel et al. 2005; Yeretssian et al. 2011
Bcl-2	Tail anchor Constitutively bound to MOM and/or ER membrane	Bcl-2 binds to the IP3 receptor at the ER, and inhibits the initiation phase of calcium-mediated apoptosis	Nguyen et al. 1993; Janiak et al. 1994; Hinds et al. 2003; Wilson-Annan et al. 2003; Chou et al. 2006; Dlugosz et al. 2006; Rong et al. 2009
Bcl-XL	Tail anchor Binds to MOM and ER membrane upon activation	Bcl-XL links apoptosis and metabolism via acetyl-CoA levels	Jeong et al. 2004; Brien et al. 2009; Yi et al. 2011
Mcl-1	Tail anchor Binds to MOM upon activation	Unknown Normally highly unstable protein	Zhong et al. 2005; Chou et al. 2006
Bcl-w	Noncanonical tail anchor Localization unknown	Unknown	Hinds et al. 2003; Wilson-Annan et al. 2003
A1/Bfl-1	Charged carboxyl terminus MOM (other membranes?) and perinuclear region	Unknown	Simmons et al. 2008
Bim	TOM complex-dependent carboxy-terminal hydrophobic segment—MOM	Associated with microtubules in healthy cells	O'Connor et al. 1998; Weber et al. 2007
Puma	Carboxy-terminal hydrophobic segment (?)—MOM	Unknown Transcriptionally regulated by p53	Nakano and Vousden 2001
Bad	Two lipid-binding domains at carboxyl terminus Cytosolic in healthy cells, mitochondrial in apoptotic cells	Regulation of glucose metabolism	Hekman et al. 2006; Danial 2008
Bik/Blk	Carboxy-terminal tail anchor Hydrophobic segment—ER	Unknown	Germain et al. 2002

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Table 2. *Continued*

Bcl-2 protein	Targeting mechanism and location	Nonapoptotic function	References
Noxa	Targeting region at carboxyl terminus and via BH3 region mediated interactions with Mcl-1—MOM	Unknown	Oda et al. 2000; Ploner et al. 2008
Bmf	By binding to Bcl-2 family members? MOM during apoptosis	Binds myosin V motors by association with dynein light chain 2 in healthy cells Function unknown	Puthalakath et al. 2001
Hrk/DP5	Tail anchor MOM	Role in hearing loss in response to gentamycin suggests function in inner ear	Inohara et al. 1997; Kalinec et al. 2005
Beclin-1	Carboxy-terminal dependent? ER, MOM, *trans*-Golgi network	Regulates autophagy	Sinha and Levine 2008

?, inconclusive data.

change in this preexisting channel, such that it is linked to ANT (Shimizu et al. 1999). However, by biochemical and gene knockout studies, all three components of the PTP have been shown to be dispensable for Bax-dependent MOMP (Tsujimoto and Shimizu 2007). Nevertheless, PTP formation by Bax/Bak-independent mechanisms does have a role in cell death by regulating necrosis in some circumstances (Nakagawa et al. 2005).

An alternative possibility is that activated Bax/Bak form pores directly in the MOM. Amphipathic α-helical peptides can porate membranes via two separate mechanisms termed barrel-stave or toroidal, leading to two distinct pores, proteinaceous or lipidic, respectively (Yang et al. 2001). In both models, the helices line the pore, perpendicular to the membrane. The barrel-stave model creates a proteinaceous pore devoid of lipids. Conversely, a toroidal pore is composed of protein and lipid components. Bax inserts three amphipathic helices (5, 6, and 9) into the MOM before oligomerization and MOMP (Annis et al. 2005). Currently, there is evidence for Bax and Bak generating both proteinaceous and lipidic pores. Electrophysiological studies using patch clamping identified a pore that was termed the mitochondrial apoptosis-induced channel (MAC) (Pavlov et al. 2001). The MAC contains oligomeric Bax or Bak, providing the first indication that these proteins can create a proteinaceous pore (Dejean et al. 2005). This complex is thought to be composed of nine Bax or Bak monomers yielding a pore diameter of ~5 nm, which should be sufficient to release cytochrome *c*, a 15-kDa protein. However, experiments investigating the core mechanism of Bax pore formation using liposomes or MOMs show that Bax can release high-molecular-weight dextrans up to 2000 kDa (Kuwana et al. 2002), suggesting that the pore is likely larger in vivo. Furthermore, Bax can create pores ranging in size from 25 to 100 nm, consistent with a lipidic pore, not a proteinaceous pore (Schafer et al. 2009). Additionally, peptides containing only helices 5 and 6 of Bax can cause pores to form in liposomes that resemble lipidic pores (Qian et al. 2008). Most of the evidence for proteinaceous pores has been observed with isolated mitochondria, whereas the evidence for lipidic pores is largely from experiments with liposomal-based systems. It is therefore conceivable that both mechanisms are operative at different steps in vivo: Bax or Bak may initially insert helices 5 and 6 (and 9) into the MOM, forming small pores that resemble proteinaceous pores, and after further oligomerization, the pores increase

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in size and alter the lipid structure of the membrane, facilitating the formation of a pore that can release larger IMS proteins such as OMI/HTRA2 (∼49 kDa) and SMAC/DIABLO (∼27 kDa).

Mechanism of Bax/Bak Activation and Pore Formation

A conformational change in the amino-terminal region of both Bax and Bak has been detected that correlates with activation (Hsu and Youle 1997; Griffiths et al. 1999; Dewson et al. 2009). When Bax interacts transiently with membranes, it exposes an amino-terminal epitope that can be detected using the 6A7 monoclonal antibody (Hsu and Youle 1997; Yethon et al. 2003). After interacting with BH3 proteins that cause membrane insertion of Bax, the epitope change detected by 6A7 is "locked in"; that is, it is no longer reversible. The exposure of the 6A7 epitope has been attributed to a conformational change in α helix 1 of Bax (Peyerl et al. 2007). The sequence of events is likely different for the amino-terminal conformational change in Bak because the protein is constitutively membrane bound.

After activation, the next step leading to oligomerization and pore formation is still under debate. Recently, a second hydrophobic pocket of Bax was identified through binding with the BH3 peptide of Bim (Gavathiotis et al. 2008, 2010). This new binding surface termed the "rear pocket" is composed of helices 1 and 6 and is located on the opposite side from the canonical "front" BH3-binding pocket of Bax (composed of helices 2–4). In the cytoplasmic form of Bax, the rear pocket is masked by an unstructured loop between helices 1 and 2, much as the front pocket is masked by helix 9. If the helix 1–2 loop is tethered to the rear pocket, Bax cannot expose the 6A7 epitope or release helix 9 from the front pocket, rendering Bax inactive. This suggests that Bax needs to undergo multiple conformational changes to bind to membranes and oligomerize to form pores. Bax and Bak also contain two more putatively transmembrane regions located in helices 5 and 6. After activation, Bax inserts helices 5, 6, and 9 into the MOM (Annis et al. 2005). In contrast, helix 9 of Bak is constitutively transmembrane, and Bak inserts (at least) α6 into the MOM after activation (Oh et al. 2010). Additionally, Bak lacking its carboxy-terminal transmembrane domain is still able to insert into membranes and oligomerize, causing pore formation after activation by BH3 proteins (Oh et al. 2010; Landeta et al. 2011). Thus, one or more domains of Bak in addition to helix 9 must be anchoring it within the membranes. Further studies are required to determine whether Bak inserts both helices 5 and 6, as appears to be the case for Bax.

Whether it binds to the "front" or the "rear" pocket, the BH3 region located in α helix 2 of both Bax and Bak is essential for homodimerization (George et al. 2007; Dewson et al. 2008). Two models of how the proapoptotic pore-forming proteins propagate the dimers into larger pore-forming oligomers have emerged. The asymmetric and symmetric dimer models both propose that Bax and Bak monomers interact via their BH3 regions and helix 6; however, they differ in which pockets the proteins use to oligomerize beyond the dimer (Fig. 3). The asymmetrical dimer model was proposed after the identification of the rear pocket (Gavathiotis et al. 2008, 2010). In this model, activator BH3 proteins initiate the activation of Bax by binding to the rear pocket, causing allosteric conformational changes that displace helix 9, which allows Bax to target to the MOM (Kim et al. 2009). Sequential oligomerization proceeds by the BH3 region of an activated Bax binding to the rear pocket of another Bax monomer, thereby exposing its BH3 region to further propagate oligomerization.

The second model proposes that Bax forms symmetrical dimers whereby the BH3 regions of two Bax monomers reciprocally bind the front pockets of each other (Dewson et al. 2009; Bleicken et al. 2010; Oh et al. 2010; Zhang et al. 2010). This dimerization changes the conformation of Bax such that the rear pockets interact with each other to facilitate oligomerization.

These contrasting models postulate different "units" that are joined to form the oligomer. However, it is clear that in each model both

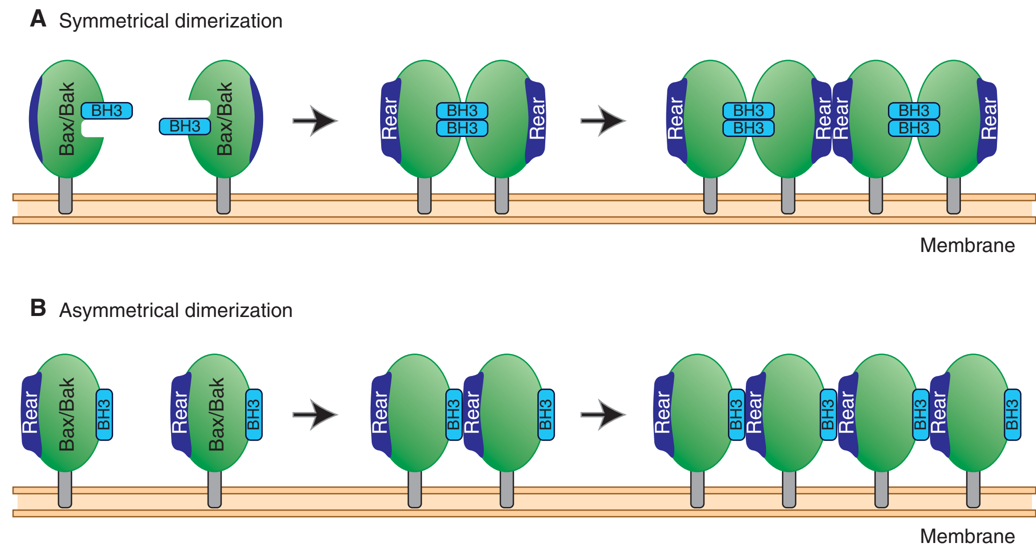

Figure 3. Models of Bax and Bak dimer formation. (*A*) Symmetrical dimers: Active Bax and Bak monomers with helices embedded within the MOM expose their BH3 regions, which, in turn, bind to the "front pocket" composed of the hydrophobic BH1-3 groove of an adjacent monomer. This binding changes the conformation of the "rear pocket" composed of helices 1 and 6, allowing homodimers to form tetramers and further propagate oligomerization. (*B*) Asymmetrical dimers: Active Bax and Bak expose their BH3 regions, which interact with the rear pocket on an adjacent monomer, forming an oligomer through subsequent rear pocket:BH3 region interactions.

hydrophobic pockets are important for the proapoptotic function of the proteins. Identification of the mechanism and dynamic binding surfaces that mediate oligomerization will be a great asset for assays testing small-molecule modulators of Bax and Bak function to allow this rate-limiting step in apoptosis to be selectively activated or inhibited as dictated by clinical need.

BH3 MEMBERS

Evolution and Structure of BH3 Proteins

BH3 proteins interact with and regulate multiregion proapoptotic and antiapoptotic Bcl-2 family members through the BH3 region, a shared homology region with other Bcl-2 family proteins. The specificity and affinity of the BH3 proteins for binding with their partners are determined by small differences in the amino acid sequence in the BH3 region (Table 1). Mutations in one or more of the key residues in the BH3 region of Bid and Bad can abolish binding with other Bcl-2 family proteins and impede their proapoptotic function (Wang et al. 1996; Zha et al. 1997).

The evolutionary relationship between multiregion Bcl-2 family members and BH3 proteins is distant, and BH3 proteins are thought to have originated after an expansion event during vertebrate evolution (Aouacheria et al. 2005). A subclass of BH3 proteins, Bnip proteins, has a different evolutionary history and likely originated independently. These Bnip proteins contain a less well-conserved BH3 region and may not require this to bind to other Bcl-2 family proteins (Chinnadurai et al. 2008).

NMR studies of Bim, Bad, and Bmf, and in silico predictions for other BH3 proteins indicate that they are intrinsically unstructured proteins in the absence of binding partners but undergo localized conformational changes in the BH3 region upon binding with antiapoptotic proteins (Hinds et al. 2007). Bid is an exception to this observation, because it shares

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phylogenetic, structural, and functional features with multiregion Bcl-2 family members (Billen et al. 2009). Bid was originally discovered through binding to both Bax and Bcl-2 and was classified as a proapoptotic "BH3-only" protein because it contained only a BH3 region. However, Bid shares a high degree of similarity in the overall three-dimensional (3D) fold of the structure with other multiregion Bcl-2 family proteins (Chou et al. 1999; McDonnell et al. 1999). Furthermore, the presence of a newly identified BH4 region (Kvansakul et al. 2008), phylogenetic evidence, and the mechanistic parallels between the activation of Bid and Bax suggest that Bid is more closely related to the multiregion family proteins than the BH3 proteins (Billen et al. 2009; Shamas-Din et al. 2011).

Structural Plasticity and Multiple Members Permits Diversity in Function

Although there are five major antiapoptotic and two main proapoptotic multiregion proteins, there are at least 10 different BH3 proteins in the vertebrate genome (Aouacheria et al. 2005). The amplification of the BH3 protein subgroup allows the organism to induce apoptosis selectively by monitoring many different types of cell stress that may be restricted to certain subcellular sites, specific cell types, or signaling pathways. Accordingly, there are many ways to turn on the different BH3 proteins, including transcriptional, translational, and posttranslational mechanisms. Furthermore, the consequence of turning on specific BH3 proteins differs according to the binding specificity of the BH3 region for its "target" (Table 1). According to the direct activation model, these quantitative differences in binding affinities lead to qualitative differences in function. Only a restricted subclass (so far only tBid, Bim, and Puma) has high enough affinity for the multiregion proapoptotic proteins Bax and Bak to allow direct binding and activation as discussed above. These BH3 proteins are thus designated "activators." In contrast, all other BH3 proteins have been proposed to act as "sensitizers" and displace activator BH3 proteins from binding to antiapoptotic members. This frees the activator BH3 proteins to then bind to Bax/Bak. Sensitizer binding also prevents antiapoptotic proteins binding activated Bax and Bak. Thus, sensitizer BH3 proteins specifically bind to antiapoptotic members and do not bind to Bax/Bak directly. In subclassifying the BH3 proteins, the role of Puma remains somewhat controversial, because it has been shown to be either an activator (Cartron et al. 2004; Kim et al. 2006, 2009; Gallenne et al. 2009) or a sensitizer in different studies (Kuwana et al. 2005; Certo et al. 2006; Chipuk et al. 2008; Jabbour et al. 2009). This controversy may be caused by the fact that BH3 activators can also act as sensitizers via mutual sequestration of antiapoptotic proteins (as discussed above).

The inherent structural plasticity of most BH3 proteins also facilitates interactions with multiple binding partners, including non-Bcl-2 proteins that govern their "day jobs" (i.e., BH3 proteins in their nonactivated state have roles independent of apoptosis). To further facilitate the "day jobs," constitutively expressed BH3 proteins are located in parts of the cell distant from their apoptosis target membrane(s), where they participate in various nonapoptotic cellular processes (Table 2). Thus, to switch the function of BH3 proteins from the "day jobs" to apoptosis, constitutively expressed proteins undergo posttranslational modifications, such as phosphorylation, myristoylation, ubiquitination, and proteolysis, that restrict the proteins to one of the alternative functions (Kutuk and Letai 2008). In addition, the function of BH3 proteins such as Puma and Noxa are controlled at the transcriptional level and are expressed only in the presence of death stimuli. Finally, the BH3 proteins can change their conformation at their target membrane(s) and upon binding to Bcl-2 family partner to change their function.

BH3 Proteins Binding to Membranes as a Critical Step in Regulating Apoptosis

For the amplification of death signals, BH3 proteins translocate to the MOM to activate Bax and Bak and promote MOMP. However, the exact

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mechanism by which different BH3 proteins migrate to and insert into membranes varies. Mitochondrial-targeting and tail-anchor sequences are used to target several of the BH3 proteins to the MOM (see Table 2) (Kuwana et al. 2002; Seo et al. 2003; Hekman et al. 2006; Lovell et al. 2008). Moreover, the presence of specific lipids such as cardiolipin and cholesterol (Lutter et al. 2000; Hekman et al. 2006; Lucken-Ardjomande et al. 2008) and protein receptors such as Mtch2 at the MOM have been shown to influence the targeting of other Bcl-2 family proteins to their target membranes (Zaltsman et al. 2010).

Once at the membrane, it is likely that BH3 proteins undergo extensive conformational changes that dictate their function. For example, after cleavage by activated caspase-8, initial association of cleaved Bid with the MOM causes separation of the two fragments, with subsequent insertion and structural rearrangement of the p15 fragment (tBid) that likely orients the BH3 region to bind to Bax or Bcl-XL. Furthermore, the other BH3 proteins that are intrinsically unstructured undergo localized conformational changes upon binding membranes and antiapoptotic proteins.

Despite strong evidence for the functional interaction and activation of Bax and Bak by activator BH3 proteins, demonstration of binding of the full-length protein (as opposed to peptides from the BH3 region) has only recently been reported: Strong reversible binding of tBid to Bax was observed in liposomal MOM-like membranes (apparent K_d ~25 nM) (Lovell et al. 2008). Furthermore, when synthesized by in vitro translation, full-length BH3 proteins tBid, Bim, and Puma induced Bax- and Bak-dependent MOMP and shifted monomeric Bax and Bak to higher-order complexes in mitochondria (Kim et al. 2006).

In vitro experiments clearly show that BH3 proteins recruit and sequester the antiapoptotic Bcl-2 family proteins at the membrane. BH3 proteins bind the antiapoptotic proteins by docking on the BH3 region in the hydrophobic groove made of BH1, BH2, and BH3 regions of the antiapoptotic family proteins (Sattler et al. 1997; Liu et al. 2003; Czabotar et al. 2007). Similar to the differential binding to proapoptotic family members, experiments in vitro suggest that each BH3 protein selectively binds a defined range of antiapoptotic proteins that is determined by differences in the structure and flexibility of the hydrophobic pocket of the antiapoptotic proteins, although, to date, these interactions have been measured only with peptides from the different BH3 regions rather than the full-length proteins (see Table 1).

ANTIAPOPTOTIC MEMBERS

Structure of Family Members Alone and in Complex with BH3 Peptides

Early observations that specific mutations in Bcl-2 abrogated both antiapoptotic function and binding to Bax and the presence of BH3 regions in both classes of the proapoptotic Bcl-2 families that bind Bcl-2 as “ligands” led to the concept of a receptor surface on Bcl-2. However, it was hard to confirm the details of this binding interaction using structural studies because of difficulties with purifying recombinant full-length Bcl-2. Initial success arose from NMR studies on Bcl-XL lacking its hydrophobic carboxyl terminus (Muchmore et al. 1996), which is similar to the structure obtained for Bax (Suzuki et al. 2000), was shown to contain two hydrophobic helices (5 and 6) forming a central hairpin structure surrounded by the remaining six amphipathic helices. Thereafter, cocrystals of “tail-less” Bcl-XL with BH3 peptides derived from Bak and Bim identified the BH3-binding surface as a hydrophobic cleft formed by noncontiguous residues in BH regions 1–3 (involving parts of helices 2, 7–8, and 4–6, respectively) (Sattler et al. 1997; Liu et al. 2003). These structural observations provided the platform for measurements of the many potential interactions between the binding pockets of different antiapoptotic members and the BH3 regions of the proapoptotic family members. These differing binding affinities cluster into functional groupings (e.g., binding to multiregion vs. BH3 proteins, or activators vs. sensitizers) (Table 1) with functional consequences as elucidated below.

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Multiple Mechanisms of Action of Bcl-XL: Evidence of Binding to Both Multiregion and BH3 Members

Measurements of the affinity of binding between individual pairs of antiapoptotic family members and BH3 peptides in solution provide valuable clues about functional relevance. However, in cells, most of these interactions occur at or within intracellular membranes, and, indeed, the final commitment step in apoptosis being regulated is MOMP. Thus, experiments using recombinant full-length proteins or proteins synthesized in vitro, and isolated mitochondria or liposomes, have been critical in translating these interactions into testable models. For practical reasons, such experiments are most feasible using recombinant Bcl-XL, because other antiapoptotic proteins are much more difficult to purify in sufficient quantities owing to problems with aggregation (e.g., Bcl-2) or marked protein instability (e.g., Mcl-1). Thus, details about the mechanism of action of Bcl-XL serve as a model for the other proteins, acknowledging that other members will differ in some aspects, as discussed below.

By examining membrane permeabilization in a system with recombinant Bcl-XL, Bax, and tBid (both wild type and a mutant form that is unable to bind to Bcl-XL, but still activates Bax), it was shown that Bcl-XL inhibits MOMP not only directly by binding to tBid but also by binding to membrane-bound Bax (Billen et al. 2008). Thus, both of the major interactions postulated by the competing direct activation and displacement models contribute to inhibition of apoptosis. Furthermore, other mechanisms of action of Bcl-XL independent of these binding interactions were also identified, including prevention of Bax insertion into membranes as perhaps the most potent mechanism. This initially contentious point has been recently supported by observations that Bax undergoes multiple conformational changes that ultimately lead to oligomerization and MOMP, but the first of these steps is the exposure of the amino terminus at the membrane in a reversible equilibrium (Edlich et al. 2011). Bcl-XL changes this equilibrium such that Bax is shifted out of the conformation that binds it loosely to membranes. Moreover, consistent with the postulation that dynamic conformational changes are a feature of all three Bcl-2 families, these investigators observed that Bcl-XL also undergoes reversible conformational changes that allow it to come on and off the MOM without being inserted. The structural basis of this mechanism is unclear, although it is speculated that sequestering of the opposite partner's carboxy-terminal helix 9 in the BH3-binding groove may mediate this effect. In essence, helix 9 of the other protein acts as an (inactive) BH3 mimetic.

Taken together, these observations have identified multiple mechanisms that contribute to the ultimate function of Bcl-XL. Using defined amounts of proteins with an in vitro system allows measurement of the stoichiometry of inhibition and indicates that one Bcl-XL can inhibit approximately four Bax molecules. Therefore, as a conceptual overview, the functions of Bcl-XL can be most simply summarized as a dominant-negative Bax, where it is able to undergo many of the binding interactions that Bax does but does not make the final conformational change that allows it to bind to other Bcl-XL/Bax molecules and oligomerize to form a pore. In accordance with the postulated models of oligomerization discussed above, this would imply that activated Bcl-XL cannot form a rear pocket in the analogous regions described for Bax/Bak.

Mediators of Multiple Mechanisms: Membrane Binding and Conformational Changes

Similar to Bax and Bak, there is evidence that the antiapoptotic Bcl-2 family proteins adopt multiple conformations in associating with membranes. Bcl-2 initially inserts helix 9 into the membrane, but after binding to tBid or a BH3 peptide derived from Bim, helix 5 moves to a hydrophobic environment consistent with insertion into the membrane (Kim et al. 2004). Therefore, it is plausible that Bcl-XL also adopts multiple conformations that are dictated by its interaction both with membranes and other

Bcl-2 family members that shift the dynamic equilibrium between the different forms. Specifically, the data suggest that there is a form that is loosely bound to membranes (form 1), another in which helix 9 is inserted into membranes but not other helices (form 2), and, finally, a form in which helix 9 as well as helices 5 and 6 are inserted into the membrane (form 3) (Fig. 4). It is possible that these different conformations independently mediate the different mechanisms of action of Bcl-XL in inhibiting the final process of pore formation by activated Bax. Such a scheme is also compatible with observations that mutations that do not affect the BH3-binding pocket can still enhance antiapoptotic function, either by forcing constitutive membrane insertion (into forms 2 or 3) by replacing the endogenous tail-anchor sequence (Fiebig et al. 2006), or by loosening intramolecular binding, thereby "freeing" helices 5 and 6 to insert into membranes (form 3) (Asoh et al. 2000).

Comparison of Different Antiapoptotic Members

In simpler organisms such as *Caenorhabditis elegans* and *Drosophila*, there is only one inhibitory Bcl-2 family member, whereas in vertebrates there are at least four. There are potentially multiple reasons for this redundancy. One that is firmly grounded on structural studies indicates that the different antiapoptotic family members bind to (and sequester) the multiple BH3 members differentially, including the multidomain proapoptotic members alluded to previously. Responding to multiple BH3 proteins allows fine-tuning of inhibitory responses in mammalian cells to different types of stress that "activate" specific BH3 proteins. Such a system provides multifactorial responses much more diverse than those in simpler eukaryote cells. Characterization of the differences in binding has received much attention and is conferred by the distinct sequence of each BH3 region that shares a propensity to form an amphipathic helix containing four hydrophobic residues, and the topology of the BH3-binding groove on the antiapoptotic "receptor." Peptides from certain BH3 regions like Bim bind with high affinity to all the antiapoptotic and apoptotic multiregion members, whereas others like Bad and Noxa are more selective (highly preferential binding to Bcl-2/Bcl-XL/Bcl-w or Mcl-1/Bfl-1, respectively). Some of this specificity is explained by well-defined requirements, for example, any amino acid at the fourth hydrophobic position in the BH3 region will bind to Mcl-1, which has a shallow, open pocket for this residue, as opposed to Bcl-XL, which does not accommodate charged or polar residues at this position (Lee et al. 2009; Fire et al. 2010). Other features also contribute; the higher global flexibility of Bcl-XL creates a pliable pocket for diverse BH3 mimetics compared with the deeper hydrophobic pocket with a rigid angle of entry in Mcl-1 that restricts binding to specific BH3 proteins (Lee et al. 2009).

As a consequence, no single antiapoptotic member binds to all BH3 proteins in vitro, as assessed by biophysical measurements (see Table 1). These measurements have been largely (although not entirely) confirmed by experiments in transfected cell lines where overexpression of single antiapoptotic proteins confers protection against apoptosis mediated by the BH3-binding partners identified in vitro. The discrepancy noted in a few experiments is likely because of the fact that in cells these interactions between full-length proteins occur on membranes rather than in the cytoplasm, and membrane binding may modify protein–protein interactions either allosterically or by post-translational modifications altering the binding surfaces (Feng et al. 2009) or affecting the orientation and proximity of the binding surfaces.

Multiple antiapoptotic proteins also allow differential control of processes relevant to cell death independent of BH3-binding-pocket interactions. The BH4 region of Bcl-2 binds to the regulatory and coupling domain of the inositol 1,4,5 triphosphate (IP3) receptor that controls calcium efflux from the ER, thereby inhibiting the initiation phase of calcium-mediated apoptosis (Rong et al. 2009). A residue critical for this binding interaction in Bcl-2 (Lys17) is not conserved in the BH4 domain of Bcl-XL (Asp11), rendering the latter ineffective at

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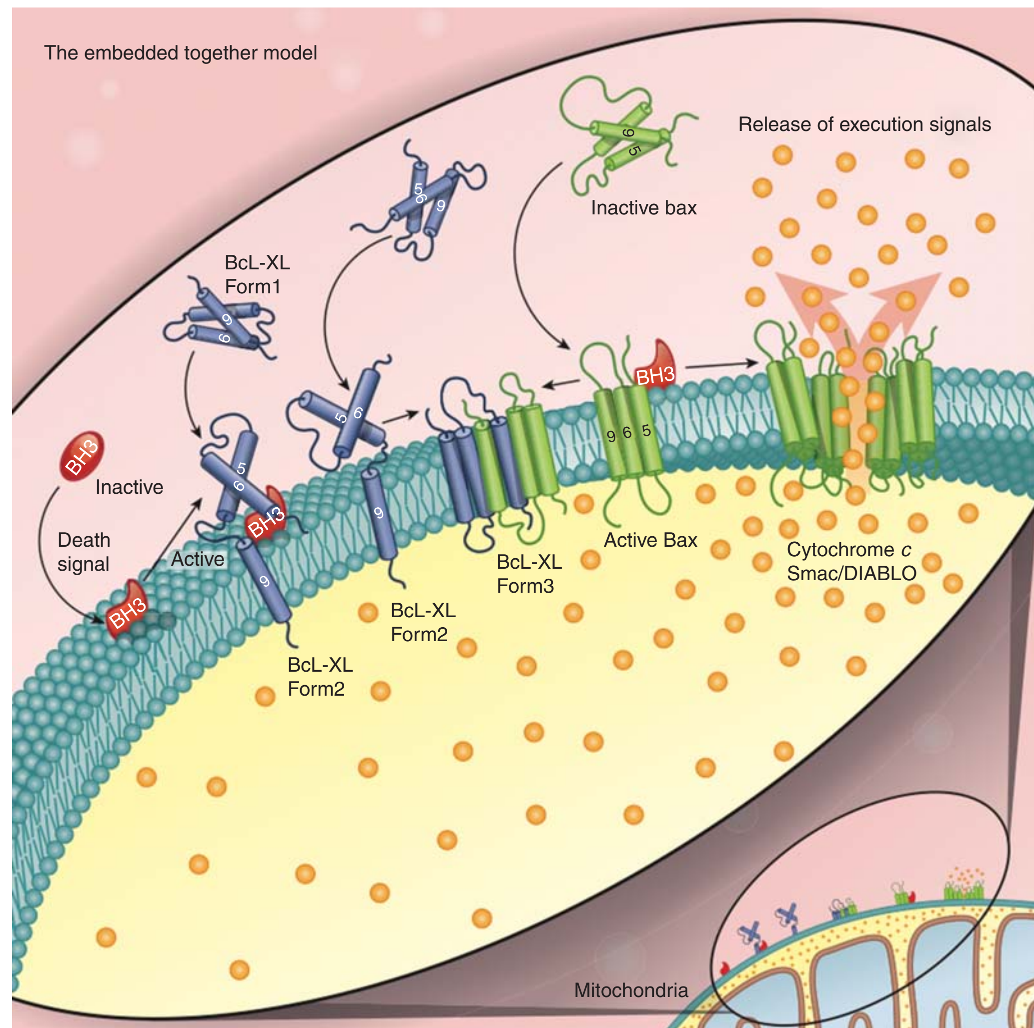

Figure 4. Schematic overview of the embedded together model. The role of the membrane is highlighted as the "locus of action" where the effects of the interactions between the Bcl-2 family members are manifest. After the cell receives a death signal, an activator BH3 protein migrates to and inserts into the MOM, where it recruits cytoplasmic Bax. Bax undergoes conformational changes at membranes that allow it to respond to chemical changes in the cell such as the generation of reactive oxygen species, ion concentration, and pH. Membrane-bound Bax or Bak changes its conformation such that they oligomerize, leading to MOMP and/or recruit other cytoplasmic Bax. Both the activator and the sensitizer BH3 proteins sequester the antiapoptotic proteins (such as Bcl-XL) by recruiting and strongly binding to them at the MOM, thereby preventing the inhibition of Bax and Bak. Bcl-XL changes its conformation depending on its binding partner. Upon binding to a BH3 protein or Bax/Bak, Bcl-XL changes from form 1 (cytoplasmic or loosely attached to the MOM) to form 2 (helix 9 inserted into MOM) or to form 3 (helices 5, 6, and 9 bound to or inserted into MOM), respectively. It is likely that form 2 binds primarily BH3 proteins but also recruits additional Bcl-XL to the membrane, whereas form 3 binds primarily Bax and Bak. No function has yet been ascribed to Bcl-XL form 1, although one is likely. Thus, by causing the proteins to adopt different conformations, the membrane regulates their function in determining the fate of the cell. Unlike other models that propose unidirectional interactions, in this model, all of the functional interactions are governed by dynamic equilibria of protein–membrane and protein–protein interactions.

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inhibiting IP3-mediated calcium release (Monaco et al. 2011).

Another reason for the diversity of antiapoptotic proteins beyond specificity conferred by different binding partners is the control of subcellular localization. In particular cell types, there may be a benefit to having Bcl-2 family members constitutively present on membranes such as is the case with Bcl-2, as opposed to Bcl-XL, Mcl-1, Bfl-1, and Bcl-w, all of which must undergo a conformational change before inserting into the membrane. In Bcl-2, it is presumed that the carboxy-terminal region that is necessary and sufficient for membrane insertion (Janiak et al. 1994) is not bound to other hydrophobic regions of the protein once it is synthesized and can therefore mediate direct membrane insertion. In the other antiapoptotic proteins, the carboxy-terminal tail is sequestered until the protein is activated. Even within this group, there are different strategies that control membrane localization. Unlike the other family members, Bfl-1 does not have a hydrophobic region at the carboxyl terminus that mediates membrane insertion but has an amphipathic helix (Brien et al. 2009). Bcl-XL is thought to exist as a homodimer in the cytoplasm, where the carboxy-terminal tail is bound reciprocally to a hydrophobic groove in the dimer partner (Jeong et al. 2004). The longer carboxy-terminal helix 8 of Bcl-w binds in its own BH3-binding pocket and can be displaced by BH3 peptides to allow membrane insertion (Hinds et al. 2003; Wilson-Annan et al. 2003), a mechanism reminiscent of Bax. Before apoptosis is elicited, Mcl-1 is constitutively loosely associated with mitochondria by an EELD motif in the amino-terminal portion, which can bind to the mitochondrial import receptor Tom70 (Chou et al. 2006). For all the antiapoptotic proteins, deletion of the carboxy-terminal α helix decreases function, presumably by preventing assumption of forms 2 and 3 on the membrane where many of the relevant binding partners are localized. Furthermore, attachment of the inhibitor to the membrane increases the probability of interaction by increasing local concentration and the viscosity of the membranes restricting diffusion. A third justification for diversity of antiapoptotic proteins is the benefit of varying regulation of protein abundance as a way of fine-tuning apoptosis. The *bcl-2* gene contains two estrogen response elements controlling expression in breast tissue. Bcl-2 is a long-lived protein whose expression does not change appreciably even during advanced stages of stress, partly because of the presence of an internal ribosome entry site (IRES) in the 5′ UTR that permits cap-independent translation (Willimott and Wagner 2010). The stability of the Bcl-2 transcript is positively regulated by the RNA-binding protein nucleolin, and negatively regulated by the microRNAs mi-R15a and 16-1 (Willimott and Wagner 2010). Bcl-XL protein levels are more variable and increase acutely in response to internal stress and extracellular signals, mediated by the Jak-STAT and rel/NF-κB pathways (Grad et al. 2000). In contrast, Mcl-1 is an extremely short-lived protein with rapid turnover tightly regulated by a complex cascade of phosphorylation-dependent deubiquitination by USPX9 (Schwickart et al. 2010) that reverses the ubiquitination and subsequent proteasomal degradation mediated by the BH3 protein E3 ubiquitin ligase MULE/ARF-BP1 (Zhong et al. 2005).

The consequences of these variations in the structure of binding pockets (control of subcellular localization and dynamic protein levels), is that despite sharing the core mechanism of inhibition, each antiapoptotic protein has a distinct personality. This is evident in the specific profile of expression of the proteins in different cell types and organs in whole animals, with the result that each protein has different physiological roles that are apparent in the phenotypes of the knockout mice with different antiapoptotic members (for review, see Hardwick and Soane 2013).

PERSPECTIVE AND FUTURE PROSPECTS

This brief overview illustrates the enormous growth in our understanding of the mechanisms behind the pivotal role that the Bcl-2 family plays in regulating apoptosis since the original identification of Bcl-2 as a chromosome translocation partner in human B-cell

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follicular lymphoma. We are now at a stage where this understanding is yielding practical results, as several drugs mimicking BH3 regions that bind to Bcl-2 and Bcl-XL are in late-stage clinical trials as cancer agents to elicit or enhance chemotherapy-induced apoptosis. The recognition that there are distinct binding profiles for each antiapoptotic protein that arose from fundamental studies has now motivated the search for other small molecules to expand the therapeutic tool kit (Stewart et al. 2010), so that in the future we will be able to target every antiapoptotic protein.

To date, most attention has been paid to the role of the Bcl-2 family in regulating MOMP because of the well-characterized consequences of releasing IMS proteins in activating caspases. However, it is increasingly apparent that the ER is the site of many important processes that determine cell death and survival in which the Bcl-2 family is intimately involved. Aside from controlling calcium flux (Rong et al. 2009) and regulating the activity of Beclin-1 to initiate autophagy (see Mah and Ryan 2012; Nixon and Yang 2012), other death pathways are also inhibited by Bcl-2 at the ER (Germain et al. 2002). Beyond this, there is also evidence that a portion of the antiapoptotic activity of Bcl-2/Bcl-XL does not depend on binding to and inhibiting the other two proapoptotic families (Minn et al. 1999). One recent study suggests that this mechanism involves regulation of cytoplasmic levels of acetyl-CoA as a substrate for protein α-acetylation (Yi et al. 2011). Elucidating potential binding partners that mediate this pathway is an important target of future research.

Our basic understanding of the core mechanism of the regulation of membrane permeabilization by Bcl-2 family members has passed from the stage of phenomenology to testable descriptions of mechanism. The next hurdle will be to extend quantitative measurements of the binding interactions that have been measured in vitro to what happens in organelles and in cells. This will allow further refinement and elaboration of exciting preliminary mathematical models of the control of apoptosis in whole cells (Spencer and Sorger 2011).

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CHAPTER II

BH3-only proteins: Orchestrators of apoptosis

Preface

The work presented in this chapter has been previously published as a review in:

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Contribution of authors:

Shamas-Din, A wrote the entire review and prepared all the figures and tables, with the exception that Leber, B and Brahmbhatt, H prepared sections 8 and 9. Leber, B and Andrews, DW edited the review and directed the layout of the sections.

Objective of the review:

To summarize the function and the known mechanisms of action in apoptosis of BH3-only proteins.

Highlights:

- BH3 proteins (other than Bid) are evolutionarily distant and structurally different from multi-region Bcl-2 proteins.
- The multiplicity of BH3 proteins allows the cells to respond to diverse damaging stimuli through activating different pathways.
- BH3 proteins contain a C-terminal membrane-binding region and undergo localized conformational changes at the membrane.
- In addition to mitochondria, BH3 proteins bind endoplasmic reticulum membranes to control cellular stress and autophagy.
- Chemical inhibitors of anti-apoptotic proteins Bcl-2 and Bcl-XL, ABT-737 and ABT-263 are BH3 mimetics and kill cells in a Bax/Bak dependent manner.

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Review

BH3-only proteins: Orchestrators of apoptosis ☆

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ABSTRACT

The BH3-only proteins of Bcl-2 family are essential initiators of apoptosis that propagate extrinsic and intrinsic cell death signals. The interaction of BH3-only proteins with other Bcl-2 family members is critical for understanding the core machinery that controls commitment to apoptosis by permeabilizing the mitochondrial outer membrane. BH3-only proteins promote apoptosis by both directly activating Bax and Bak and by suppressing the anti-apoptotic proteins at the mitochondria and the endoplasmic reticulum. To prevent constitutive cell death, BH3-only proteins are regulated by a variety of mechanisms including transcription and post-translational modifications that govern specific protein–protein interactions. Furthermore, BH3-only proteins also control the initiation of autophagy, another important pathway regulating cell survival and death. Emerging evidence indicates that the interaction of BH3-only proteins with membranes regulates binding to other Bcl-2 family members, thereby specifying function. Due to the important role of BH3-only proteins in the regulation of cell death, several promising BH3-mimetic drugs that are active in pre-clinical models are currently being tested as anti-cancer agents. This article is part of a Special Issue entitled Mitochondria: the deadly organelle.

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1. Introduction

Apoptosis is a form of programmed cell death that senses and purges developmentally excess, mutated and damaged cells. Apoptosis differs from other forms of programmed cell death by eliminating the target cells without eliciting inflammation. Studies in *Caenorhabditis elegans*, *Drosophila melanogaster* and mammals have shown that it is an evolutionary conserved process that is tightly regulated and required for the proper development and homeostasis of multicellular organisms. Accordingly, deregulation in apoptosis causes several important pathological processes. For example, tumour cells accumulate mutations that allow them to bypass apoptosis during clonal expansion, whereas the effects of stroke and many neurodegenerative diseases are mediated by neuronal apoptosis.

In mammals, apoptosis is initiated by two different pathways: one when cells receive *intrinsic* death stimuli such as excessive oncogene activation, DNA damage, or the unfolded protein response (UPR), and the other in response to *extrinsic* death stimuli such as the engagement of the Fas or TNFα ligands to their receptors on cell surface as part of the effector phase of an immune response. These pathways converge at the *m*itochondrial *o*uter *m*embrane (MOM) where the Bcl-2 protein family plays a pivotal role in the regulation of apoptosis. The family consists of more than 20 members with either pro-apoptotic or anti-apoptotic functions and is divided into three groups based on the presence of conserved *B*cl-2 *h*omology (BH) regions. The multi-region anti-apoptotic proteins Bcl-2, Bcl-XL, Bcl-W, Mcl-1 and A1 contain all four BH regions. The pro-apoptotic proteins are divided into two groups. The multi-region pro-apoptotic proteins, Bax, Bak and Bok were conventionally thought to share BH 1–3 regions, whereas the BH3-only proteins were proposed to share homology in the BH3 region only. Members of this diverse subset include Bad, Bim, Bid, Noxa, Puma, Bik/Blk, Bmf, Hrk/DP5, Beclin-1 and Mule (See Table 1) [1]. Recent results described below suggest this may be an over-simplified view.

Studies from *C. elegans*, knock-out mice and mammalian cell-lines have shown that BH3-only proteins are essential initiators of intrinsic apoptosis. BH3-only proteins monitor many cellular processes and transmit both intrinsic and extrinsic death signals to the multi-region Bcl-2 family proteins at the MOM. BH3-only proteins inhibit the anti-apoptotic proteins and activate the pro-apoptotic proteins to cause *m*itochondrial *o*uter *m*embrane *p*ermeabilization (MOMP) [2]. As a consequence of MOMP, mitochondrial inter membrane space proteins such as cytochrome *c* and SMAC are released into the cytoplasm. Once in the cytoplasm, these proteins activate or de-repress caspases which proteolyse a distinct cohort of proteins that cause the morphologic and functional features of apoptosis and eliminate the cell. As the main regulator of this final effector phase, MOMP represents the commitment step to apoptosis and thus is often the 'point-of-no-return' for a cell. Therefore studying the mechanisms of activation or inhibition of the membrane permeabilizing proteins Bax and Bak and

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Table 1
Localization and targeting mechanisms of BH3-only proteins.

BH3-only protein	Cellular localization	Targeting mechanism	Refs
Activator BH3-only proteins			
Bim	Associated with microtubules in healthy cells; localizes predominantly to mitochondria and also to intracellular membranes when over-expressed in apoptotic cells	C-terminal hydrophobic segment using TOM complex	[26,44]
Bid	Cytosolic and nuclear in healthy cells; localizes to Mitochondria and ER upon cleavage by caspase-8 on the onset of apoptosis	Membrane binding helix 6 and 7	[48,49,59]
Puma	MOM	Unknown; C-terminal hydrophobic segment (?)	[60]
Sensitizer BH3-only proteins			
Bad	Cytosolic in healthy cells, mitochondrial in apoptotic cells	Two lipid binding domains at C-terminus	[46]
Noxa	Mitochondria	Mitochondrial targeting region at C-terminus and BH3 region	[57,58]
Bik/Blk	ER	C-terminal hydrophobic segment	[45]
Bmf	Myosin V motors by association with dynein light chain 2 in healthy cells, and mitochondria upon induction of apoptosis.	Unknown; maybe through association with Bcl-2 members	[61]
Hrk/DP5	Predominantly localized to mitochondria	C-terminal hydrophobic segment	[62]
Beclin-1	ER, mitochondria, trans Golgi network	Unknown	[63]

the activator BH3-only proteins with the aim of developing small molecules that modify these processes is an important goal for researchers and pharmaceutical companies. Here we describe the structural and evolutionary relationship of BH3-only proteins to the Bcl-2 family and the biochemical function of BH3-only proteins as postulated by three different models of the regulation of apoptosis. We will discuss several topics that are the focus of ongoing research including: the role of membrane binding in modifying BH3-only protein structure and function, how BH3-only proteins activate the multi-region pro-apoptotic proteins, why there are so many BH3-only proteins, where they act and the emerging role of BH3 mimetic drugs as anti-cancer agents.

2. Evolution and structure of BH3-only proteins

The first evidence for the genetic basis for apoptosis came from *C. elegans*, in which four genes Egl-1, Ced-3, Ced-4 and Ced-9 altered the cell death phenotype prior to engulfment by neighbouring cells [3]. Shortly after, mammalian homologues of these genes were identified as part of a large network. While *C. elegans* has one homologue each of Ced-9 and Egl-1, human has 13 Ced-9 homologues (multi-region Bcl-2 proteins) and a number of highly divergent proteins that are analogous to Egl-1 (BH3-only protein). Within the Bcl-2 family, the evolutionary relationship between multi-region Bcl-2 family members and BH3-only proteins (other than Bid) is distant. The multi-region Bcl-2, Bcl-XL, Bax and Bid share a common origin, and other BH3-only proteins evolved later [4]. Bid may be an outlier in the BH3-only family, as it shares phylogenetic, structural and functional features with Bax [5].

Despite opposing functions and little shared sequence homology (other than the conserved BH regions), many Bcl-2 family proteins share a similar structure. The three-dimensional structures of Bcl-2 family proteins resemble membrane insertion domains of diphtheria toxins and colicins suggesting a role in pore formation in membranes [6]. However, of all the BH3-only proteins studied to date, only Bid shows a defined structure [7,8]. Other BH3-only proteins are intrinsically unstructured and only attain a structured BH3 region after binding to multi-region Bcl-2 partners [9]. This theme of dynamic conformation change with function will be examined in other contexts in subsequent sections.

3. Assigning a role: how do BH3-only proteins turn on apoptosis

It is now widely acknowledged that activation of either of the pro-apoptotic proteins, Bax and Bak leads to MOMP [10], and several different models have been proposed to explain the role of anti-apoptotic and BH3 proteins in this process. A significant point of contention between the original versions of the models is whether BH3-only proteins promote apoptosis by directly or indirectly activating Bax and Bak.

3.1. Direct activation model

The direct activation model postulates that BH3-only proteins directly bind to and interact with Bax and Bak to promote MOMP. Furthermore, BH3-only proteins can be classified as either activators or sensitizers [11] (Fig. 1A). Despite sharing sequence homology in the BH3 region and occupying the same hydrophobic pocket on anti-apoptotic proteins, each BH3-only protein selectively binds a defined range of anti-apoptotic proteins. The activators Bim, tBid and potentially Puma bind all five anti-apoptotic proteins. By contrast, the sensitizers Bad and Bmf bind Bcl-2, Bcl-XL and Bcl-W; Bik and Hrk bind Bcl-XL, Bcl-W and A1; and Noxa only binds to Mcl-1 and A1 [11,12]. In a healthy cell, BH3-only proteins are either inactive or are sequestered by anti-apoptotic proteins to keep apoptosis at bay. In response to an apoptotic signal, the activator BH3-only proteins are either activated through multiple mechanisms (transcriptional, post-transcriptional and post-translational), or are released from the anti-apoptotic proteins by being displaced by sensitizer BH3 proteins that bind to anti-apoptotic proteins with higher affinity. Once “freed”, these activators can bind to Bax or Bak [13].

Thus in this model, anti-apoptotic proteins function by binding to and sequestering the activator BH3-only proteins and not by binding directly to Bax or Bak [13], as activator BH3-only proteins Bim, tBid and Puma BH3 peptides and full-length versions bind to purified Bcl-2, Bcl-XL, Mcl-1, Bcl-W and A1 *in vitro* [11,13,14] (Fig. 1A). Both Bid and Bim bind to and are sequestered by anti-apoptotic proteins [15–17], and tBid induced permeabilization of liposomes and isolated mitochondria is inhibited by Bcl-2, Bcl-XL, Bcl-W, Mcl-1 and Bfl-1 [14,18–21].

Activator BH3-only proteins, Bid and Bim also bind to and induce conformational changes leading to the activation of Bax and Bak [13,22]. BH3 peptides of Bid and Bim induce Bax activation and membrane permeabilization in liposomes and mitochondria [11,23]. Recombinant tBid has been shown to cause insertion and oligomerization of Bax and Bak in artificial membranes and isolated mitochondria [20,24]. The role of recombinant Bim as an activator BH3-only protein comes from studies done using the different splice isoforms of Bim: BimS, BimL, BimEL and BimAD. While BimEL has been shown to activate Bax to release cytochrome *c* from isolated mitochondria, in the same study BimL was unable to do so [25]. BimS and BimAD isoforms have also been shown to promote membrane permeabilization by activating Bax [26,27]. While the role of Bid and Bim is well established in this model, the role of Puma

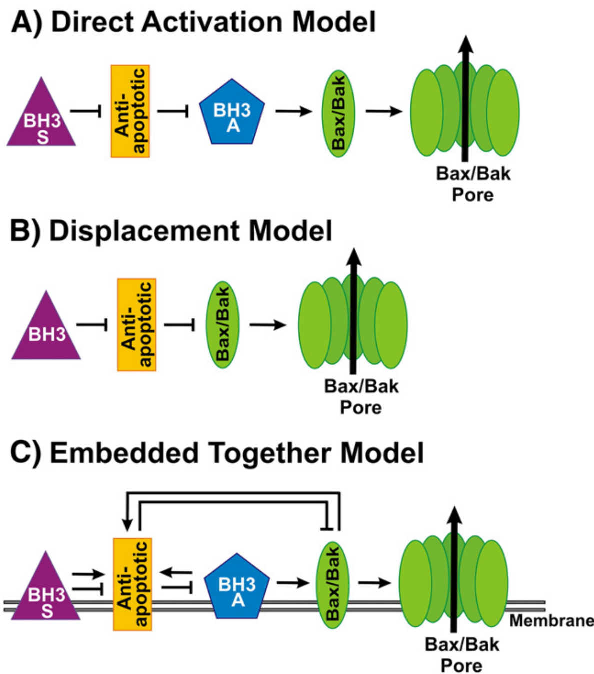

Fig. 1. Overview of the three models of regulation of apoptosis by Bcl-2 family proteins. BH3-S and BH3-A represent sensitizer and activator BH3-only proteins, respectively. A) The Direct activation model proposes that activator BH3-only proteins are required for activating Bax and Bak. Anti-apoptotic proteins inhibit the activator BH3-only proteins but not Bax and Bak to suppress apoptosis. Sensitizer BH3-only proteins displace the activator BH3-only proteins from the anti-apoptotic proteins to promote apoptosis. B) The Displacement model postulates that Bax and Bak are constitutively active in cells and must be sequestered by anti-apoptotic proteins for cell survival. BH3-only proteins only play the sensitizer role and inhibit their respective anti-apoptotic proteins to promote apoptosis. Both the direct activation and the displacement model do not define a role for the membrane. C) Embedded together model highlights the active role of the membrane where Bcl-2 family proteins insert into and change their conformations that dictate their functions. Cytoplasmic anti-apoptotic proteins are recruited to membranes and are activated by both sensitizer and activator BH3-only proteins as well as Bax/Bak. At the membrane, anti-apoptotic proteins inhibit the activator BH3-only proteins and Bax/Bak to prevent MOMP. Sensitizer BH3-only proteins displace the activator BH3-only proteins and Bax/Bak from the anti-apoptotic proteins to promote apoptosis. Activator BH3-only proteins recruit Bax to the membrane to induce MOMP and apoptosis. These interactions are reversible and are governed by equilibrium constants that are altered by the concentrations and interactions of the proteins with each other and with membranes.

as an activator is controversial. Puma has been shown to be an activator [13,28–30] and a sensitizer in different studies [14,31–33].

Sensitizer BH3-only proteins, such as Bad, Noxa, Bik, Bmf, Beclin-1, and Hrk function by liberating BH3-only activators from the anti-apoptotic proteins, and consequently promoting the activation of Bax and Bak. Therefore sensitizer BH3-only proteins indirectly promote MOMP, as they do not directly activate Bax or Bak [11,14,31] (Fig. 1A). Due to the distinct pattern of interaction with anti-apoptotic proteins, joint expression of Bad and Noxa is required for a cell to promote apoptosis in cells expressing Mcl-1 and Bcl-2.

3.2. Displacement model

The displacement model (or the indirect activation model) proposes that Bax and Bak are constitutively active and therefore must be inhibited by the anti-apoptotic proteins for the cell to survive. To initiate apoptosis, BH3-only proteins displace Bax and Bak from the anti-apoptotic proteins to promote Bax or Bak mediated MOMP (Fig. 1B). Since BH3-only proteins selectively interact with a specific spectrum of anti-apoptotic proteins, a combination of BH3-only proteins is required to induce apoptosis in cells expressing multiple anti-apoptotic Bcl-2 family members [12]. In the displacement model, anti-apoptotic proteins directly bind Bax and Bak for their inhibition, and BH3-only proteins do not directly bind to Bax and Bak to cause their activation.

In support of the displacement model, peptides derived from different BH3-only proteins display vastly different affinities for different anti-apoptotic proteins measured in solution or on a solid support [12]. In addition, BH3 peptides of multi-region pro-apoptotic proteins form complexes with anti-apoptotic proteins: Bak BH3 peptide and endogenous protein in cells selectively complex with Bcl-XL and Mcl-1, but not with Bcl-2, Bcl-W or A1 [34]. After apoptosis induction, Noxa binds to Mcl-1 thereby disrupting the Mcl-1–Bak complex and displacing Bak and promoting both Mcl-1 degradation and apoptosis [34]. Similarly, Bik displaces activated Bak from Mcl-1 and Bcl-XL [35]. Furthermore, Bax and Bak promote apoptosis in cells with no Bim or Bid and reduced Puma, leading the authors to conclude that the multi-region pro-apoptotic proteins are always active in the cells [36]. However, the activity of the residual Puma or the presence of other factors (such as p53 and Drp1) cannot be ruled out as possible activators of Bax or Bak in this context. Furthermore, the BH3 stapled peptide of Bim has been demonstrated to bind to Bax [37].

3.3. Embedded Together model

While the original versions of the direct activation and the displacement models proposed different functions for the anti-apoptotic proteins, both suggested that specificity was achieved via the differences in the affinity of BH3 proteins for anti-apoptotic proteins. The studies reporting the affinities of the various interactions were based on measurements of binding partners in solution or attached to a surface and thus in these models the membrane functions only as a passive recipient for the Bcl-2 proteins. We proposed a model termed “Embedded Together” to account for the consequences of these interactions occurring in and on membranes, which is the “locus of action” for apoptosis in cells. The ultimate outcome is therefore determined by the competing equilibria and the relative concentrations of binding partners [38–40] (Fig. 1C).

The most obvious consequence of the interactions between the different Bcl-2 family proteins taking place in the lipid bilayer is the inevitable change in the conformation of the proteins in a lipid environment. In an apoptotic cell, activator BH3-only proteins are required to interact with Bax and Bak at the MOM and the endoplasmic reticulum (ER). Both the activator and sensitizer BH3-only proteins recruit and activate anti-apoptotic proteins by changing their conformation at membranes. Once at the membrane, the anti-apoptotic proteins not only sequester the activator BH3-only proteins to prevent Bax and Bak activation but also bind and inhibit activated Bax and Bak in the membrane. Furthermore, sensitizer BH3-only proteins neutralize the function of the anti-apoptotic proteins by displacing both the activators and Bax or Bak from the membrane embedded conformers of the anti-apoptotic proteins. Because the protein conformations are critically modified by the presence of the membrane, the membrane is thus a partner rather than a “passive recipient” in the process (Fig. 1C).

Consistent with the Embedded Together model, the interaction of tBid with Bcl-2 in the MOM causes a conformational change in Bcl-2 (discussed later) [41] that strongly correlates with its anti-apoptotic activity [21]. In addition, only the membrane bound form of tBid binds to Bax [24], after which Bax undergoes at least two conformational changes: insertion into the membrane and exposure of the 6A7 epitope, before oligomerization and membrane permeabilization [22]. Using an *in vitro* system of purified proteins, liposomes and mitochondria, Bax activation has been shown to proceed via an ordered series of events: first tBid binds to the membrane which then recruits Bax to permeabilize the membrane. Bcl-XL inhibits recruitment of Bax to the membrane, whereas Bad neutralizes the effects of Bcl-XL and thereby promotes tBid and Bax mediated permeabilization

of the membrane [20,24]. Bcl-XL binds and sequesters both tBid and Bax equivalently in membranes [20], reconciling proposals of both the direct activation and displacement models. Recent experiments with knock-in mice in which the BH3 region of Bim was replaced with that of Bad, Noxa or Puma indicate that for the complete pro-apoptotic function of Bim, both engagement of all anti-apoptotic proteins, *as well as* activation of Bax is required [42]. This *in vivo* study confirms the multiple functions of BH3-only proteins as proposed by the Embedded Together model.

4. Getting to work: how do BH3-only proteins bind to membranes?

After activation, BH3-only proteins are located at mitochondrial and/or ER membranes. Targeting of BH3-only proteins to these membranes is necessary for the activation of Bax or Bak and the amplification of death signals. However, the exact mechanism by which specific BH3-only proteins migrate to and insert into membranes varies (Table 1). For example, Noxa contains a sequence of conserved amino acids found in non BH3-only proteins, that appears to be a mitochondrial targeting region at its C-terminus [43].

Some BH3-only proteins such as Bim, Puma, Bik and HRK have a C-terminal hydrophobic segment of amino acids known as a tail-anchor sequence. Tail-anchor sequences are necessary and sufficient in determining the sub-cellular location of proteins. The function of the tail-anchor sequence has been examined for Bim [26,44] and Bik [45]. Other BH3-only proteins, such as Bid, Bad and Beclin-1 lack an identifiable tail-anchor sequence but contain other types of hydrophobic sequences. These sequences presumably target to the MOM and/or ER by other mechanisms, including spontaneous partitioning into the lipid bilayer as we and others have observed using *in vitro* systems [19,24,46]. However, other factors may modulate this process *in vivo*. For example, various phosphorylation sites on Bad are known to influence sub-cellular localization [47], and cleavage of Bid by caspases is required before the p15 fragment (tBid) can bind to membranes [48,49].

Multiple Bcl-2 family proteins have been reported to interact with different lipids when targeted to membranes. Cardiolipin, a negatively charged lipid specific to mitochondria generated considerable interest when it was shown to mediate the specific targeting of tBid to MOM [19,50]. Furthermore, interaction of tBid with cardiolipin was proposed to occur at mitochondrial contact sites and cause mitochondrial cristae reorganization [51,52]. However, the role of cardiolipin for mediating targeting of tBid to the MOM has been disputed. Some experiments suggest that the overall negative charge of the lipid membrane, and not the individual lipids target tBid to liposomes [53]. Because a complete knock-down of cardiolipin would be fatal, *in vitro* studies are needed to examine the interaction between cardiolipin and tBid. Similarly, two lipid binding regions have been identified in Bad, one of which confers binding to cholesterol and the other to negatively charged lipids [46]. In its non-apoptotic phosphorylated form, Bad accumulates in raft microdomains of MOM, whereas when it is dephosphorylated, Bad translocates to the negatively charged MOM and exerts its pro-apoptotic effects. The authors suggested that binding of Bad is a prerequisite for the recruitment of Bcl-XL to the MOM.

In addition to different lipids facilitating the targeting of BH3-only proteins to membranes, protein factors that mediate binding of BH3-only proteins to mitochondria or ER are also being identified. Recently the membrane protein MTCH2/MIMP has been discovered as a major facilitator of tBid insertion into the MOM [54]. Using cell lines generated from knock-out mouse embryos and a conditional knock out mouse model, a marked decrease in tBid recruitment to the MOM was observed. MTCH2/MIMP shares considerable homology with the *m*itochondrial-*c*arrier *d*omain (MCD) of the mitochondrial nucleotide transporter ANT1 and in the predicted structure of the whole protein [55]. ANT1 is located in the mitochondrial inner membrane and has three MCDs, each of which binds to two molecules of cardiolipin. We speculate that MTCH2/MIMP, containing one MCD, binds to two molecules of cardiolipin at the MOM. Thus MTCH2/MIMP may enhance tBid function by facilitating interactions with cardiolipin rich regions. Alternatively, MTCH2/MIMP may act as a receptor (or help assemble a receptor) for tBid as its mechanism of action. Further studies in this area will be useful in suggesting candidates for cognate factors that may be involved in the membrane targeting of Noxa and Bad.

Numerous studies have reported that the spontaneous membrane binding of tBid causes the migration of soluble Bax and Bcl-XL to the membranes [19,20,24,56]. In these *in vitro* systems, the rate of spontaneous insertion of Bax and Bcl-XL into membranes is negligible, and tBid binds to both proteins very weakly in solution. Therefore, the most likely scenario is that soluble Bax and Bcl-XL interact with membrane-bound tBid and undergo conformational change(s) that facilitates their insertion into membranes. Similarly, Bad and Bim can cause the insertion of soluble Bcl-XL and Bax into membranes, respectively. It is possible that a reciprocal process occurs for other BH3-only proteins. Thus the multi-region, membrane resident Bcl-2 family proteins such as Bak, Bcl-2 and Mcl-1 may enhance or cause the binding of some BH3-only proteins to membranes. A few examples of the different possible scenarios that may be relevant for different BH3-only proteins include: first, by binding to membrane-resident proteins through the BH3 region, the BH3-only proteins may be “held in place” in close proximity (but not inserted into membranes) in the same conformation as the soluble form so that inefficient spontaneous insertion becomes more likely. Second, binding to multi-region Bcl-2 family members may induce a conformational change in the BH3-only protein thereby allowing membrane insertion. For example, Noxa requires both the MTD and the BH3 region for proper targeting to the MOM and cytochrome *c* release (reviewed in [57]). Mitochondrial localization of mouse Noxa was abolished by mutating the two BH3 regions, indicating that targeting of Noxa to the MOM is contingent on functional BH3 regions [58]. We speculate that the BH3 region of Noxa may first interact with Mcl-1 or A1 at the membrane, thereby changing the overall conformation of Noxa in way that may facilitate binding to the MOM through its MTD. In the absence of interaction with Mcl-1 and A1, this conformational change may not occur and perhaps the MTD of Noxa will not be properly oriented to insert in the membrane. There is considerable evidence for this scenario, as will be discussed in the following section. Third, BH3-only proteins may target independently to the MOM by as yet unknown mechanisms, and subsequently interact with their multi-region Bcl-2 partners. Finally, it is still unclear if all BH3 proteins are really inserted into membranes, or if some are peripheral membrane proteins and therefore are located at specific intracellular organelles by binding to other integral membrane proteins.

5. Bending into shape: does membrane binding cause functional conformational changes in BH3-only proteins?

It is well established that both soluble and membrane inserted multi-region Bcl-2 proteins change their three dimensional structure to a new conformation to exert anti- or pro-apoptotic functions [39]. Soluble Bax and Bcl-XL must fold their respective $\alpha 9$ helices out of the hydrophobic pocket of the protein so that the tail-anchor sequence contained therein is available to insert into the membrane. The membrane-resident members Bak and Bcl-2 change conformation within the membrane after activation, as detected by exposure of a neoantigen in the N-terminus of Bak [64,65], or a dramatic change in the hydrophobic helices $\alpha 5$ and $\alpha 6$, respectively [41]. Similarly, both Bak and Bax monomers must change conformation in the membrane to form oligomers that permeabilize the membrane [22], although the nature of this change is debated as will be discussed in a subsequent section. There is some evidence that dynamic conformational

regulation of function is a general feature of the entire Bcl-2 family. The BH3-only protein Bid has also been shown to change its conformation in the membrane after activation by caspase-8 mediated proteolysis [66].

Activation induced conformational changes may not be required for those BH3-only proteins which are expressed only in the presence of death stimuli. For example, after DNA damage, p53 induces the transcription of its primary target PUMA, as well as Noxa and Bik [60,67,68]. These proteins may be expressed in a "fully activated" conformation that allows them to target to membranes by the various mechanisms discussed in the previous section. However, constitutively expressed BH3-only proteins are usually located in parts of the cell distant from their target membrane(s) where they often participate in functions independent of apoptosis [69]. For example, functions in cell cycle regulation for Bid, and glucose metabolism for Bad have been identified (reviewed in [70,71]). Post-translational modifications may restrict the proteins to one of the alternative functions. Full length Bid is phosphorylated at positions S61 and S78 in murine Bid, and S78 in human and rat Bid by ATM and ATR kinases in response to DNA damage and replicative stress [72,73]. Casein Kinase I and II also phosphorylate mouse Bid at S61 and S64, which attenuates the caspase-8 mediated cleavage of Bid required for activation for apoptosis [74]. Impaired caspase-8 mediated cleavage of S61 phosphorylated Bid ensures that Bid remains locked in its 'non-apoptotic' conformation when it is regulating cell proliferation. Thus we propose that phosphorylation of Bid acts as a switch for the different functions of Bid. Although the structure of tBid solution showed only minor differences compared to the structure of full-length Bid [7], rearrangement of α6–8 helices was observed upon binding to membranes [66]. Furthermore, based on the structural similarities between Bid and multi-region Bcl-2 family proteins in solution (reviewed in [5]), tBid likely undergoes extensive conformational changes upon insertion into the membrane. The Embedded Together model [38,39] suggests that similar to Bax and Bcl-2, tBid may adopt additional discrete conformations depending upon whether it binds to pro-apoptotic or anti-apoptotic Bcl-2 proteins. We propose that these conformations would determine whether tBid promotes MOMP by directly activating Bax and Bak, or by sensitizing Bcl-2 and Bcl-XL.

Unlike Bid, other constitutively expressed BH3-only proteins Bim, Bad and Bmf are intrinsically unstructured in the absence of binding partners, but undergo localized conformational changes in the BH3 region upon binding with anti-apoptotic proteins [9]. This inherent structural plasticity may facilitate interactions with multiple binding partners that permit the proteins to have 'day jobs' and still function in the initiation of the apoptotic response. The most well characterized BH3-only protein with multiple binding partners is Bad. In response to survival factors, Bad is sequestered by 14-3-3 chaperone proteins [75]. Phosphorylation is the predominant molecular switch that modulates interactions of Bad with the pro-survival proteins or the 14-3-3 complex. Kinases whose activity mediates survival phosphorylate murine Bad at three evolutionary conserved serine residues, S112, S136 and S155 [75–78]. Residue S155 lies within the BH3 region and by altering charge and size, phosphorylation disrupts the interaction of Bad with the BH3-binding pocket of the anti-apoptotic proteins Bcl-2, Bcl-XL and Bcl-W [76]. Thus phosphorylation of Bad at multiple sites modifies the BH3 region of Bad such that it acquires a higher affinity for the ubiquitous 14-3-3 chaperone proteins. It is not clear in cells if Bad first translocates to the MOM spontaneously and then interacts with the anti-apoptotic proteins (as this sequence has been observed in cell free systems), or if interactions with the anti-apoptotic proteins mediate targeting to membrane. Our model predicts that if Bad targets to the MOM spontaneously (perhaps via a tail-anchor yet to be formally identified), then membrane binding will trigger at least the tail-anchor to become structured. Membrane binding may or may not trigger folding of the rest of the protein but ultimately the final conformation of Bad will be reached only after interacting with both the membrane and one of Bcl-2, Bcl-XL or Bcl-W.

A further level of complexity is present when studying Bim, which similar to tBid, promotes MOMP directly by activating Bax and Bak, and/or indirectly by inhibiting the anti-apoptotic proteins. We postulate that although on its own it is constitutively unstructured, Bim has four or more distinct conformations in the cell based on its different functions: one that confers binding to microtubules, a tail-anchored conformation that mediates insertion into the MOM prior to binding other proteins, and two further membrane-bound conformations that depend on whether its binding partner is a pro- or anti-apoptotic multi-region family member. There is intriguing circumstantial evidence for this proposition from several studies in the literature. For example, when attached to liposomes via binding to Ni^{2+} containing lipids, recombinant Bim-6xHis (without the endogenous C-terminal sequence) is much less effective than tBid at activating Bax [79]. This finding indicates that membrane binding is insufficient and a distinct conformation is required to properly orient the BH3 region to activate Bax. Another study that provided evidence for distinct modes of interaction of BH3-only molecules with pro- and anti-apoptotic proteins used a Bim stapled peptide. With this structured peptide, the authors identified a novel interaction site on Bax in an α1 and α6 helix containing region, distinct from the interaction site previously identified for pro-survival molecules like Bcl-XL (see below) [37]. As the stapled Bim peptide binds Bax but not Bcl-XL at this location, this result is consistent with our proposal that binding to the two classes of multi-region Bcl-2 proteins are mediated by discrete conformations of the BH3-only proteins. We further propose that Bim adopts these distinct conformations responsible for the different functions in response to binding to membranes.

6. Activation of multi-region Bcl-2 proteins by BH3-only proteins: kiss and run or kiss and stay?

Most experimental data suggests that BH3-only proteins bind the anti-apoptotic proteins by docking on the BH3 region in the hydrophobic groove made of BH1, BH2 and BH3 regions of the Bcl-2 like anti-apoptotic proteins [80]. Clear evidence of this interaction site comes from the crystal structures of the BH3-region of Bim bound to Bcl-XL [81] and Mcl-1 [82], the BH3-region of Bid bound to Mcl-1 [83], and BH3 regions of Puma, Bid and Bmf with A1 [84]. Despite strong evidence for the functional interaction and activation of Bax and Bak by activator BH3-only proteins, verification for binding has been minimal and inconsistent. Only the minor isoforms of Bim (BimS and BimAD but not the more common BimEL and BimL) bind Bax in the presence of detergents [27]. Although the hydrophobic groove that mediates BH3 region binding in the anti-apoptotic Bcl-2 proteins is also found in Bax and Bak, only one report has provided evidence for a weak interaction between tBid BH3 region peptide and the hydrophobic groove of Bak in solution [85]. In contrast, strong binding of tBid to Bax was observed for the full length proteins in membranes (apparent K_d ~25 nM) [24].Thus, the data from liposomes support a model in which there can be exchange of the subunits in the mitochondrial membrane but clearly at steady state most of the tBid is bound to Bax or Bak (or an anti-apoptotic protein). However, when both proteins are bound to membranes the hydrophobic groove observed in the soluble form of Bax may not be the authentic site of interaction. Therefore, other sites have been examined to determine whether they can mediate BH3-only protein binding.

Using bacterial two-hybrid assays and co-immunoprecipitation in human cell lines, the α1 helix in Bax was identified as an interaction site for the BH3 regions of Bid and Puma [28]. Mutations in the BH3 region of Bid or α1 helix of Bax abolished tBid and Puma mediated Bax activation suggesting that this interaction was necessary. The orientation of α1 helix of Bax is akin to the BH4 containing α1 helix

of Bcl-XL that stabilizes the overall structure of Bcl-XL [86]. The authors propose that the displacement of the α1 helix of Bax upon binding the BH3 region of Bid, leads to structural instability of Bax resulting in insertion in the membrane. One consequence of such a change in Bax could be a conformational change for nearby residues, accounting for the exposure of the 6A7 epitope during activation that is recognized as one of the preliminary steps in the oligomerization process.

As described above, Gavathiotis and coworkers reported the identification of a novel BH3 sequence binding site on Bax using a stapled BH3 region peptide from Bim. In contrast to the other proposals, this site encompassed α6, α1, α4 helices and the α1–α2 loop of Bax [37]. Since this newly identified BH3 region binding site on Bax is on the "opposite" side of the protein to the hydrophobic groove that is conventionally thought to bind to BH3 regions, we have termed this the 'rear' pocket. In the 'rear' pocket, binding of the BH3 region peptide allosterically induces a conformational change such as the exposure of the amino-terminal 6A7 motif of Bax. However, it is unclear if the BH3 stapled peptides recapitulate the physiological role of full-length BH3-only proteins, and if this interaction in solution reflects what happens in cells at membranes. Additionally, it is uncertain if binding of BH3 stapled peptides with Bak would occupy the corresponding 'rear' pocket. When the stapled BH3-peptide binds to Bax in solution it displaces the α9 helix of Bax allowing it to insert in membranes. In cells, Bak is already membrane-bound and the corresponding 'rear' pocket is obstructed by the residues E32, R36 and R156 making it difficult for a BH3 peptide to bind at that site. We also note that compared to the "floppy" α1–α2 loop in Bax, Bak contains a small helix which is more fixed due to nearby interactions, making it difficult to be displaced after binding to a BH3 peptide. Therefore, it is possible that binding of BH3 peptide to the 'rear pocket' is only relevant for Bax.

The identification of a 'rear' pocket binding site that initiates the activation of Bax (and possibly Bak) has profound implications for understanding the structure and process of Bax and Bak homo-oligomerization, a step essential for MOMP. Consistent with a role for the 'rear' pocket in Bax homo-oligomerization, a study using photoactive cross-linking demonstrated Bax–Bax interactions in the vicinity of both pockets when oligomerization was induced using non-ionic detergents [87].

In a model proposed by Dewson and colleagues, after activation by tBid, Bak everts its BH3 region revealing a hydrophobic groove which fits the exposed BH3 region of another Bak molecule to form a 'symmetrical' dimer [88]. Further evidence for direct BH3-BH3 contact in Bax homo-oligomerization has been provided by [89,90]. Because these Bak molecules would have "used up" their BH3 regions/binding pockets to form the dimer, they must use another binding surface to join with other dimers in forming oligomers. Through mutagenesis, these investigators identified a site on the α6 helix of Bak that would allow pre-formed adjacent dimers to join and extend the oligomer for MOMP [64] (Fig. 2A). Presumably each dimer would have to be individually assembled by tBid, another activator BH3 protein or an activated Bak to expose the oligomerization site on α6. In this model, the initiating BH3-only protein cannot be a stable part of the complex as it would block homo-dimer formation. Accordingly in this model BH3-only proteins function in a catalytic "kiss and run" fashion activating multiple Bak dimers.

If the activation of Bax and Bak is initiated by the binding of the BH3 protein to the 'rear' pocket, then by allosteric modification of Bax/Bak conformation, the same sequence may occur with eversion of the BH3 region and the opening of the BH3 binding groove to allow symmetric dimer formation. However, binding of a BH3 activator protein to the 'rear' pocket is also compatible with a completely different mode of oligomerization. Based on the auto-activation

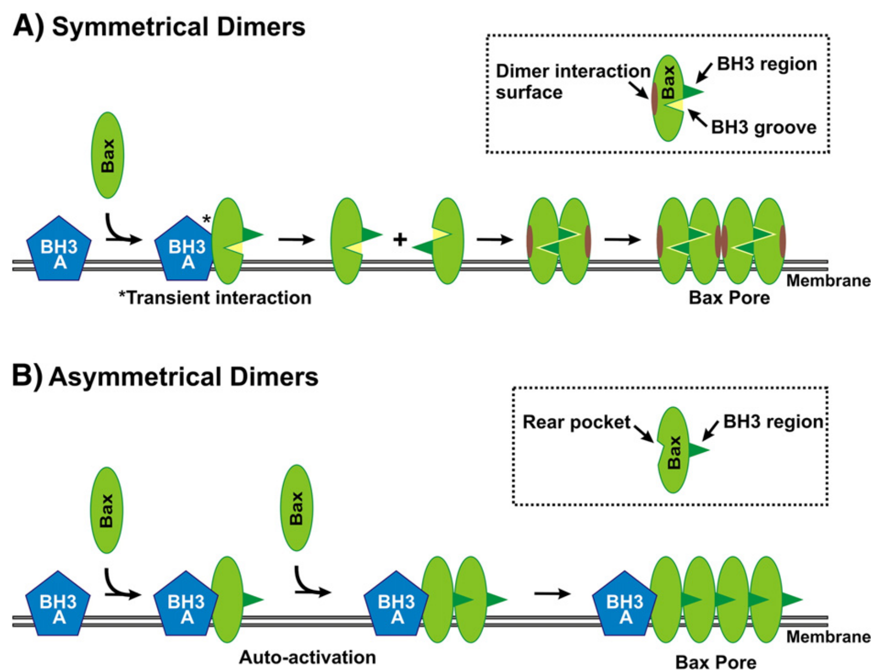

Fig. 2. Mechanisms of Bax/Bak oligomerization in membranes. Only Bax has been shown in the schematics for simplicity. A) The model for symmetrical dimers proposes that through transient interaction between an activator BH3-only protein and Bax/Bak, the BH3 region and a BH3 groove are exposed in Bax/Bak. Two monomers of Bax/Bak bind each other by binding of BH3 region of one to the BH3 groove of another, and vice versa. The activator BH3-only molecule falls off after either causing these conformational changes in the first Bax molecule or after assisting in the formation of the dimer. Since both these sites get occupied after forming a dimer, two dimers interact with each other through the exposure of a dimer interaction surface to form and subsequently enlarge the pore. B) In asymmetrical dimers, activator BH3-only proteins bind Bax/Bak on the 'rear' pocket to cause conformational changes in Bax/Bak to expose their BH3 regions. The exposed BH3 region of Bax/Bak can now act as an activator for other Bax/Bak molecules using auto-activation. The resulting Bax/Bak pore is asymmetrical because the BH3 region of one Bax molecule binds to the 'rear' pocket of the next Bax molecule. Of note, the activator BH3-only molecule can remain bound to the growing Bax pore in this model.

model of pro-apoptotic proteins [91,92], an activated Bax molecule with its BH3 region exposed can now act like a 'BH3-only molecule' and can bind the rear pocket of another Bax to dislodge the α9 helix and evert the BH3 region and groove. In this sense, the oligomer is propagated "asymmetrically", as each activated monomer has a BH3 region on one surface that binds to a 'rear' pocket of another monomer on the opposite side (Fig. 2B). By this mechanism, activated Bax molecules can undergo auto-activation to form Bax oligomers without having to dissociate the initiating activator BH3-only molecule (also reviewed in [38,40,93]. Additionally, oligomerization may reduce the affinity of the interaction between the initiating BH3-only molecule and the oligomer. This weaker binding may account for difficulty of detecting tBid-Bax complexes by immunoprecipitation of complexes from solubilised cells, as the detergents commonly used (e.g. CHAPS) have been shown to disrupt this interaction (Fig. 3 in [24]). Using fluorescence resonance energy transfer, a more direct assay that avoids any detergents, we have shown that at equilibrium tBid remains associated with Bax. Furthermore, this interaction can be abolished by titration with unlabelled Bax demonstrating that the subunits in the Bax pore are exchangeable and that tBid and Bax display a direct but reversible interaction in the membrane [24].

Further insight can be gained into the mechanism of BH3 region binding by examining the stoichiometry of activated Bax. Using defined concentrations of recombinant activator proteins, one molecule of tBid or Bim recruits up to 20 molecules of Bax to the membrane [20,24] (unpublished data). This result is most easily explained by transient binding of tBid or Bim to Bax, allowing one BH3-only activator to activate several pro-apoptotic molecules. However, due to the relatively tight binding of tBid with Bax in membranes, below a certain threshold all of the tBid is monopolized resulting in incomplete Bax activation and membrane permeabilization (unpublished data). Because the activated Bax in this circumstance apparently does not mediate auto-activation, these results suggest that there may be intermediate "activation complexes" of defined stoichiometry that are required to activate Bax.

A similar phenomenon has been reported for tBid and anti-apoptotic Bcl-XL. Supra-stoichiometric quantities of Bcl-XL can be recruited to the membrane by tBid or Bad — the ratio here is approximately 4:1 [20,56] (unpublished results). However, unlike the binding between tBid and Bax, stable complexes between membrane bound tBid and Bcl-XL can be detected by immunoprecipitation. Indeed, the stable binding of Bcl-XL to tBid to sequester it from binding to and activating Bax is one of the mechanisms of action of Bcl-XL. Thus in this case, the auto-activation of multiple Bcl-XL molecules may enhance the anti-apoptotic function — a process that we have described as tBid mediated activation of Bcl-XL.

Taken together, these results indicate that the interactions of BH3-only proteins with pro- and anti-apoptotic multi-region Bcl-2 family proteins can enhance or delay apoptosis depending on the relative stoichiometry and affinity of available partners in the membrane as well as the extent to which auto-activation amplifies each process. Furthermore, small changes in membrane fluidity are expected to have large effects on the apparent affinity of interactions within membrane protein complexes. Clearly further investigation is required to fully understand how signalling by BH3-only proteins is integrated at cellular membranes.

7. Too much of a bad thing: why are there so many BH3 proteins?

While there are approximately five anti-apoptotic and two main pro-apoptotic multi-region proteins, there are at least ten different BH3-only proteins in the vertebrate genome [4]. In *C. elegans*, there is only one anti-apoptotic Bcl-2/Bcl-XL homologue, Ced-9, and two BH3 proteins, Egl-1 and Ced-13 [94]. The function of Egl-1 and Ced-13 is to displace Ced-9 from Ced-4 so that the latter can activate Ced-3, the caspase analogue. There are no homologues to the multi-region pore forming members Bax or Bak, and therefore the sensitizer rather than the activator subgroup of mammalian BH3-only proteins is closer in function to invertebrate homologues. Egl-1 and Ced-13 have overlapping but not identical biological functions. While Egl-1 regulates developmentally programmed cell death in germ cells, as well as death related to infection by pathogens and DNA damage, Ced-13 is only involved in cell death in response to DNA damage [95]. Both are regulated by complex transcriptional networks. Consequently, the expansion of the BH3-only protein subgroup in vertebrates is likely due to many linked features including: more cell types and therefore more flexibility needed to monitor different specific types of cell damage; the presence of a complex and dynamic immune system that both respond to cell death during development, and elicits cell death in infected targets; the presence of multiple anti-apoptotic "targets" such as, Bcl-2 and Mcl-1 with different BH3 binding grooves (and therefore affinities); and finally the additional 'opportunity' to elicit cell death directly by activating Bax or Bak to permeabilize mitochondria.

The main consequence therefore, of the multiplicity of BH3-only proteins is that it bestows the cell with versatile control over the complex regulation of cell vitality in response to diverse damaging stimuli. However, the principal of partial redundancy of signals and responses to specific types of cellular damage seen with *C. elegans* is still present in the larger group of vertebrate BH3-only proteins. For example, Noxa and PUMA are both upregulated by p53 in response to DNA damage [68], Bad and Bim both respond to growth factor deprivation (reviewed in [71,96]), and Bim and Puma knock-out cells are both insensitive to apoptosis induced by ER stress [29]. In a healthy state, it appears that the cell employs transcriptional control and post-translational modifications to limit the apoptotic functions of BH3-only proteins, whereas receipt of diverse death signals release these controls.

How important are these redundant controls in the context of the whole organism? Bax/Bak double knock-out mice and mice in which one of the pro-survival molecules like Bcl-2, Bcl-XL, or Mcl-1 are knocked-out display various pathologic phenotypes or die as embryos. In contrast, knock-out mice of individual BH3-only proteins display more subtle abnormalities that can be specific to tissue-types and organs (reviewed in [2]). Moreover, in confirmation of the signalling pathways identified in cell biological models, mice that do not express individual BH3-only proteins have defects for some but not all death stimuli (reviewed in [97]). Moreover, mice that lack one of Bad, Bik, Hrk, Bmf or Noxa are normal in appearance and fertile. Bad knock-out mice have a high incidence of lymphoma, and some cell types display minor resistance to epidermal growth factor or insulin-like growth factor deprivation [98]. Knock-in mice whose genome encodes a mutant of Bad without the S112, S136 and S155 phosphorylation sites have defects in IL-7 dependent T-cell survival [99]. Bid knock-out mice are resistant to Fas induced hepatocellular apoptosis and fatal hepatitis [100]. However, Bid does not mediate all signalling from death receptors as Bax is activated after treatment with TNFα in Bid KO cells [101]. Loss of Noxa in fibroblasts confers a modest resistance to DNA damage induced apoptosis from etoposide and radiation [68]. These findings are consistent with functional redundancy but also demonstrate a certain degree of cell type/organ specificity for the BH3-only proteins.

Parallel to the multiplicity of death signals mediated by BH3-only proteins is the realization that these proteins in their "non-activated" state have roles independent of apoptosis (and in the case of Bad, an additional role in *non-apoptotic* cell death [102]). The specific role of Beclin-1 at the interface of apoptosis and autophagy will be discussed in more detail in the following section. The evolutionary logic and history of these "duality of functions" is unclear: did the non-apoptotic function precede the gain of a BH3 region that conferred a specific pro-apoptotic function, or was the sequence reversed? The evidence from specific knock-out mouse models has been only

partially informative in this regard. Studies with mice with knocked-out Bid show defects in cell proliferation and altered G_0–G_1 transition, consistent with its localization to both cytoplasm and the nucleus in dividing cells [70,103]. A role for Bid in sensing DNA damage and mediating cell cycle arrest has been proposed [72,73]. However, this role is controversial as other authors have shown Bid to be dispensable for DNA damage and replicative stress induced apoptosis and cell cycle arrest [104].

Bad localizes to the cytoplasm and *in vivo* regulates glucose-driven mitochondrial respiration, insulin secretion and glucose homeostasis [105]. Bad has also been shown to promote cycle–cycle progression during serum withdrawal or overexpression of anti-apoptotic proteins [106]. Noxa appears to play a role in the maintenance of memory CD4+ cells [107]. Bim and Bmf have been shown to localize to the microtubules and actin filaments, respectively [96]. However, it is not clear if these BH3-only proteins actually play a role in the maintenance of these processes as their 'day jobs', because knock-out mice show no abnormalities in these respective processes. Whether this indicates that all these non-apoptotic functions are dispensable, that adaptation obscures the effect of the knock-out or that observational studies with mice may not indicate the importance of these functions without specific stressors or circumstances will require further investigation.

In addition to the recognized activator BH3-only proteins, other types of proteins and even physical–chemical changes have been shown to activate Bax and Bak. For example, the p53 tumour suppressor has been shown to directly bind with Bax and activate it in response to DNA damage independent of its role in transcriptionally upregulating PUMA and Noxa [108]. Because it is possible that p53 activates Bax by binding to sites distinct from those of the known BH3-only activators; p53 may represent a class of activators that cannot really be classified as BH3-only proteins. It has also been reported that the CARD domain containing protein ASC acts as an adapter for Bax activation with p53 [109]. Finally, an increase in either acidity [110] or temperature [111] can induce the Bax conformational change associated with activation of the protein in cells. This diverse range of activators suggests that Bax (and Bak) are like coiled springs that can be 'released' to assume the pore-forming conformation by small perturbations at multiple trigger points. As such, it seems likely that there are more activators of Bax and Bak yet to be discovered. Given the short sequence length and the minimal essential sequence features it may also be that many more proteins contain functional BH3 regions.

8. Do BH3-only proteins have multiple workplaces in apoptosis?

It is well documented that many BH3-only proteins localize to MOM to promote apoptosis. However, Bcl-2 is normally targeted to multiple intracellular membranes, and a form of Bcl-2 targeted specifically to the ER efficiently inhibits selective forms of apoptosis [112,113]. At the ER, Bcl-2 can directly inhibit Bax and/or Bak, both of which have also been demonstrated to be present in this organelle [41]. However, consistent with our proposal that Bcl-XL inhibits mitochondrial permeabilization via multiple mechanisms including recruiting and binding to BH3-only proteins, many BH3 proteins bind to anti-apoptotic Bcl-2 family members at the ER. For example, in a variety of different cell lines, Bik, Bad and Beclin-1 have been identified as ER associated proteins. It is possible that these or other BH3 proteins sequestered by Bcl-XL at the ER are mediators of a cell death response that requires MOMP, and that therefore this site of action is still connected to, but upstream of the canonical apoptotic pathway. Alternatively when localized at the ER, BH3 proteins may mediate a pathway of cell death that does not require MOMP. There is evidence that different BH3-only proteins realize either or both these possibilities.

The ER lumen serves as a storage site for Ca^{2+} and as a site for the folding and modification of proteins. Depletion of ER calcium stalls protein folding by calcium dependent ER chaperons like calnexin and calreticulin, and prevents protein export from the ER. These changes (and others such as oxidative stress, and inhibition of glycosylation) increase the burden of misfolded proteins within the ER lumen and can elicit the *u*nfolded *p*rotein *r*esponse (UPR) [114]. Although the immediate effect of the various effector arms of the UPR (including activation of ATF-6, IRE-1 and PRK-1) is to decrease the protein folding burden by decreasing translation and increasing chaperone proteins, persistent UPR leads to apoptosis [115]. Cell lines lacking both Bax and Bak have higher than normal concentrations of intraluminal ER Ca^{2+}, and when Bax or Bak is specifically targeted to the ER, the cells die presumably because of abnormal Ca^{2+} flux [116]. It is most likely that the replacement of the tail-anchor sequence to localize Bax or Bak to the ER exclusively also results in spontaneous activation of the proteins. However, it is formally possible that over-expression in the ER generates an apoptotic signal that leads to activation via an interaction with a BH3-activator. At present it is unclear which activator BH3-only proteins are responsible for the oligomerization of Bax or Bak at the ER. Nevertheless, there are multiple examples of BH3-only proteins that potentially function at the ER. Puma and Noxa have been shown initiate Ca^{2+} release from the ER [117]. Bim can be upregulated by the ER-stress responsive transcription factor CHOP by virtue of a specific binding site in the promoter of Bim [118]. In this case, the ER stress related cell death would ultimately be mediated by MOMP. There is also a connection between ER and mitochondrial cell death pathways that is independent of ER calcium [45]. ER stress induces the activation of caspase-2 which cleaves and activates Bid [119]. In all these cases, the BH3-only proteins are not specifically localized to the ER. A special case is provided by Bik, a BH3-only protein with a tail-anchor sequence that mediates selective integration into the ER membrane [45,67]. Bik has been shown to induce ER resident Bax/Bak to release Ca^{2+} which can then lead to the release of cytochrome *c* [120].

Aside from these pathways in which ER stress leads to MOMP, the Bcl-2 family of proteins also regulate autophagy which functions as a separate cell death vs. survival pathway that is initiated at the ER. Autophagy is a catabolic pathway required for the degradation of proteins, cytoplasmic organelles and intracellular pathogens. The process of autophagy involves the sequestration of cytoplasmic constituents and intracellular organelles within newly generated double membrane vesicles called autophagosomes, which are then delivered to and fuse with lysosomes for degradation and recycling of their contents [121]. Cells maintain a low basal rate of autophagy to maintain homeostasis. In response to stress signals caused by decreased intracellular metabolite concentrations, autophagy prevents cell death by replenishing metabolites [122]; however, autophagy can also cause cell death.

During autophagy, the ER-associated platform for the initial formation of pre-autophagosomal vesicles is called the omegasome [123]. This platform is created when vesicles containing Vps34, a class III phosphatidylinositol 3-kinase, assemble in a complex isolated with Beclin-1. It is believed that Beclin-1 links autophagy and apoptosis because Beclin-1 was originally identified as a Bcl-2 binding protein in a yeast two-hybrid screen [124]. The BH3-region of Beclin-1 binds to Bcl-2 and Bcl-XL, thus Beclin-1 is a bona fide member of the BH3-only family [125–128]. Studies have shown that Bcl-2 targeted to the ER but not the MOM inhibits starvation-induced autophagy [126]. This suggests that binding of Bcl-2 to Beclin-1 prevents it from assembling the omegasome. It has also been shown that the BH3-only protein, Bad and the Bad mimetic drug ABT-737 can disrupt the Beclin-1–Bcl-2/Bcl-XL complex to restore autophagy [128]. This regulatory mechanism is consistent with the direct activation model, in which BH3 sensitizers (Bad) displace BH3 activators (Beclin-1) from the anti-apoptotic proteins. In this case the activator

is activating autophagy rather than Bax or Bak. In both cases, the binding affinity of the sensitizer Bad to Bcl-2 is greater than the respective activators, tBid or Beclin-1. The consequence of Beclin-1 displacement may be to change its conformation such that the inhibitory factor Rubicon no longer binds to the nascent omegasome complex, and the recruitment of proteins to membranes required to initiate autophagy can proceed. The binding of Bcl-2 to Beclin-1 is markedly enhanced in the presence of the recently characterized factor NAF-1 [129]. Furthermore the interaction between Bcl-2 and the IP_3 receptor that controls Ca^{2+} release from the ER is also dependent on NAF-1 [129,130].

Thus BH3-only proteins resident at the ER are critical regulators of multiple functions that determine cell fate, either upstream of their role in activating MOMP (Puma, Noxa, Bik, Bid) or in the case of Beclin-1 and autophagy, independent of other BH3 protein functions. That the regulation of autophagic cell death as opposed to autophagy mediated cell survival is biologically relevant is underscored by the observation that genetically Beclin-1 functions as a haplo-insufficient tumour suppressor [131]. How autophagy eventually kills the cell and the identity of the switch between autophagic survival and cell death still remains unknown.

9. Are BH3-only proteins the key to successful cancer therapy?

Inhibition of apoptosis is involved in the development of cancers as well as resistance to treatment thus, there is a great interest in developing agents that restore the process. Given the pedigree described here, it is not surprising that much effort has been expended to find small molecules that mimic BH3-only protein function as novel anti-cancer agents (also see [93]). The recognition of the control of the commitment step of apoptosis by the Bcl-2 family led to a highly useful scheme to identify three possible blocks that cancer cells can exploit: loss of BH3-only proteins (or inhibition of their activation), a reduction or elimination of multi-region pro-apoptotic proteins, and increased expression of an apoptosis inhibitor such as Bcl-2 or Mcl-1 [132]. Examples of each block have been noted in different cancer types.

Renal carcinoma cells transcriptionally repress the expression of Bik through DNA methylation [133]. Interestingly, failure to express Bim was also observed, suggesting that co-ordinate dysregulation of BH3-only proteins could nullify multiple death signals. B-cell non-Hodgkin lymphoma derived cell lines also showed inactivation of BH3 proteins through diverse mechanisms. In mantle cell lymphoma the *Bim* gene was deleted, in Burkitt lymphoma the *Bim* promoter was hypermethylated, and in diffuse large B-cell lymphoma the *Noxa* gene was both mutated and preferentially silenced [134]. The *Puma* gene was deleted in 3131 cancer specimens analysed by high resolution somatic copy-number alterations [135].

The *Bax* gene is inactivated by microsatellite instability and deletion in haemopoietic cell lines and colon cancer cell lines [136,137], and thus functions as a tumour suppressor. In addition, the *Bok* gene, another multi-region pro-apoptotic Bcl-2 family member similar to Bax and Bak, is deleted in many cancer specimens [135]. However, the most widespread block noted to date in human cancers is the overexpression of anti-apoptotic proteins [11,31,135]. Tumours are often described as being "primed" for death since they are dependent on or "addicted to" the presence of one or more anti-apoptotic protein for survival [14,15]. Directly inhibiting the interaction between the anti-apoptotic proteins and BH3 proteins is a strategy to initiate cell death that should be selective to these addicted cancer cells. A new technique called *BH3 profiling* has been introduced to specify the anti-apoptotic protein that is responsible for the block by exploiting the specificity of the binding pattern of BH3-only proteins with anti-apoptotic proteins [11,14]. Isolated mitochondria from the cancer cells are exposed to peptides derived from the BH3 region of BH3-only proteins, and the release of cytochrome *c* is measured as indication of the displacement of a BH3 activator protein by the peptide [11,14]. By using a combination of peptides that discriminate between different anti-apoptotic proteins the relevant one can be identified. For example, it has been shown that CLL samples depend on Bcl-2 rather than Mcl-1 [15].

Extending the concept of BH3 profiling of mitochondria with peptides to treating the whole cancer patient with drugs that mimic BH3 peptides is becoming a promising anti-cancer strategy. In tumours with the loss or inhibition of BH3-only proteins, BH3 mimetics replace the need to induce expression or activate BH3-only proteins to initiate death. On the other hand, in cancers over-expressing anti-apoptotic Bcl-2 family proteins, BH3 mimetics can compete with endogenous activator BH3-only proteins for binding to anti-apoptotic proteins. The released activator BH3-proteins can then initiate apoptosis via interactions with Bax and Bak. Since the BH3 regions are relatively small (14–24 amino acids), it has also been possible to design and synthesize or identify small molecules that can function as peptide mimics [138]. However, the limitations of low bioavailability, poor uptake, solubility and stability of peptides means that both chemical and peptide based peptide mimetics must be addressed [139]. Several advances have been made to improve the pharmacological properties of peptide based peptide mimetics. For example, chemically stapled BH3 peptides have increased stability, are protease resistant, and have better cellular uptake and increased affinity for binding to Bcl-2 family proteins [23]. The attachment of a fatty acid to a cell permeable Bad BH3 peptide can induce apoptosis *in vitro* [140].

The chemical inhibitor ABT-737 was developed by Abbott Laboratories using a nuclear magnetic resonance (NMR)-based screen to identify Bcl-2 inhibitors [141]. The hydrophobic binding groove of Bcl-XL was divided into two smaller binding sites, and each individually targeted by small molecules. The two small molecules were chemically linked and modified to form ABT-737. ABT-737 binds to Bcl-XL, Bcl-2 and Bcl-W but not to Mcl-1 and A1 [141]. The crystal structure of Bcl-XL and ABT-737 complex shows that ABT-737 most closely resembles the Bad BH3 region [142], and therefore displaces both Bad and Bim from the binding pocket of Bcl-2 [15]. ABT-737 also disrupts the interaction of Beclin-1 with Bcl-2 and Bcl-XL, thus resulting in autophagy [128]. Cancer cells with higher levels of Bcl-2 and Bcl-XL, but lower levels of Mcl-1 are sensitive to ABT-737 [15]. These features were also noted for the orally bioavailable derivative ABT-263 which is currently being tested in multiple clinical trials [143]. Because ABT-737 and ABT-263 do not bind Mcl-1, Mcl-1 expression is the determinant of resistance to ABT-737 in many different cell lines [15,132,144]. By using an RNAi-based screen Mcl-1 was confirmed as the most important source of resistance in small cell lung cancer [145]. Because resistance can be reversed by reducing Mcl-1 levels [144] it may be that combination of ABT-263 with an inhibitor of Mcl-1 will permit a selective kill of a wide variety of tumours.

Obatoclax is a synthetic indol bipyrrol derivate developed by Gemin X Biotechnologies. It was identified from a screen of small molecules that disrupt protein–protein interactions of the Bcl-2 family members. Obatoclax binds to Bcl-XL, Bcl-W, and Mcl-1 [146], and disrupts the interaction between Mcl-1 and Bak to overcome resistance to ABT-737 [147]. However, obatoclax can also kill Bax/Bak double knock out cells [148], suggesting that in some situations off-target effects may be responsible for cell death. Most recently, it has been shown that obatoclax disrupts the interaction between Mcl-1 and Beclin-1 and leads to Bax/Bak independent autophagic cell death in acute lymphoblastic leukemia cell lines and patient samples [149]. If this phenomenon is more general in cancer, it may be a way to exploit the non-mitochondrial cell death alluded to in the previous section for important therapeutic purposes.

The development of new BH3 mimetics that can target Mcl-1 and A1 to circumvent the observed resistance of many of the BH3 mimetic

drugs is an important priority. The scope of the problem is demonstrated by a recent structural analysis of the hydrophobic grooves of Mcl-1 compared to Bcl-XL. The hydrophobic pocket of Bcl-XL is flexible and therefore creates a pliable pocket for diverse BH3 mimetics, whereas the hydrophobic pocket of Mcl-1 is deeper with a rigid 'angle of entry'. This explains why Mcl-1 cannot adopt a conformation that binds to ABT-737 or related derivatives [150]. Therefore, rationally designing BH3 mimetics against Mcl-1 based on the specific binding requirements to the hydrophobic pocket of Mcl-1 is a likely way forward. As starting points the BH3 regions of Bid and Noxa bind to Mcl-1 and A1 [11,14,16]. As an alternative strategy, enhancing the already short half life of the Mcl-1 protein is being investigated by multiple approaches [151,152].

To date all BH3 mimetic drugs have been screened against soluble protein fragments of anti-apoptotic Bcl-2 family members. However, it is increasingly clear that both pro- and anti-apoptotic family members change conformation extensively after insertion into membranes — their physiologic site of action in cells [18,21,22,41]. Therefore, designing an activator with high binding affinity for Bax and Bak *in the membrane* would be a direct way to promote apoptosis in cancer cells that are already primed for death. Similarly, designing an inhibitor with high binding affinity for the membrane embedded forms of anti-apoptotic proteins may lead to more potent and selective therapy.

10. Summary

BH3-only proteins receive multiple death signals from inside and outside of the cell and relay this information to multi-region Bcl-2 proteins to induce apoptosis. Most BH3-only proteins are predicted to bind to MOM through their C-terminus using a tail-anchor or other sequences that target the protein to membranes. The diverse apoptotic and non-apoptotic functions of BH3-only proteins are regulated by transcriptional, post-transcriptional and post-translational modifications. After binding to the MOM, BH3-only proteins can bind to and thereby cause the insertion into membranes of both pro- or anti-apoptotic Bcl-2 family proteins. We speculate that BH3-only proteins mediate these opposing functions by multiple distinct conformational changes: first by binding to the membrane and subsequently while forming hetero-dimers with the different types of multi-region Bcl-2 family proteins. Interaction of the anti-apoptotic proteins with BH3-only protein in turn may result in two unique conformations of the anti-apoptotic proteins in the membrane, one that inhibits Bax/Bak activation and one that is inactivated for this function. Activation of Bax and Bak by activator BH3-only proteins also causes conformational changes that allow them to insert and permeabilize MOM. The large number of BH3-only proteins allows the cell to control initiation of apoptosis signalling at multiple "entry points". Furthermore, tissue specific expression and the balance between sensitivity to a limited number of stimuli with partial overlap allow BH3-only proteins to impart specific signals with minimal cross-talk. In addition to MOM, BH3-only proteins localize to ER and initiate ER mediated mitochondrial dependent and independent cell death. Since BH3-only proteins bind both the anti- and pro-multi-region Bcl-2 proteins, BH3-mimetics that selectively antagonize the anti-apoptotic proteins may prove to be successful in cancer therapy.

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Study Rationale and Thesis Outline

Bcl-2 proteins are characterized into different groups based on the presence of different Bcl-2 homology (BH) regions. The multi-region Bcl-2 family proteins contain more than one BH region, whereas BH3-only proteins contain only the BH3 region. Generally, the anti-apoptotic proteins and the pro-apoptotic effectors are multi-region proteins and the pro-apoptotic activators and sensitizers are the BH3-only proteins. Bid was originally classified as a BH3-only protein; however we proposed that Bid is more like a multi-region protein due to the discovery of its BH4 region, recent phylogenetic analysis and the fact that Bid has a three-dimensional structure. We have discussed this rationale in detail in the introduction of this thesis and here (Billen et al., 2009).

Due to the similarities between Bid and other multi-region Bcl-2 proteins, we asked questions regarding the activation mechanism of Bid and if that mechanism shared common features with Bax and Bcl-2. In addition, since Bid is cytoplasmic and becomes activated at the MOM, we asked questions about the nature of interaction of tBid with the membrane and if specific lipids played a role in the recruitment of tBid to the membrane. To answer these questions, I employed a combination of *in vitro* biochemical and fluorescence techniques consisting of purified proteins with liposomes or isolated mitochondria, and *in vivo* fluorescence techniques in cells.

To test the hypothesis that like Bax and Bcl-2, tBid undergoes a conformational change for activation, I created a series of single cysteine mutants of Bid and labeled them with fluorescent dyes to study the unfolding mechanism of tBid at the MOM. In chapter III, I used a range of biochemical and fluorescence techniques such as Förster Resonance Energy Transfer (FRET), and assays to monitor a change in the environment of a tBid residue labeled with a dye in liposomes and isolated mitochondria. I showed that the two cleaved fragments separate spontaneously in the presence of the MOM, and that activated tBid at the membrane undergoes a conformational change that is facilitated by the MOM protein, Mtch2. Furthermore, using the fluorescence of YFP-Bax and immunoblotting of Bid, I carried out an automated high-throughput imaging analysis of HeLa cells to show that the conformational change in tBid is required for the activation of

Bax. This works supports our hypothesis and strengthens our proposal that tBid is more similar to multi-region Bcl-2 proteins than the BH3-only proteins.

Bim and tBid both activate Bax and Bak, however a detailed comparison of their apoptotic function is currently lacking. In chapter IV, I focused on the role of different MOM lipids on the activation mechanism of tBid, Bim and Bax. I hypothesized that different lipids will be required for tBid and Bim activation, which can highlight the differences between the two activators. To test this hypothesis, I used purified proteins and liposomes system with a range of different fluorescence techniques. I showed that anionic lipids at the MOM are necessary to recruit tBid and Bim to the membrane, although tBid shows a higher dependence on the anionic lipids than Bim for the activation of Bax. In addition, cardiolipin is required by tBid to undergo the conformational change in liposomes. The presence of high levels of cholesterol inhibits membrane permeabilization by hindering tBid-Bax, Bim-Bax interaction and Bax insertion in the membrane. Therefore, I demonstrated that MOM lipids regulate MOMP by influencing the two rate-limiting steps of membrane permeabilization.

The interactions of Bcl-2 proteins with the membrane are dynamic and are modulated by equilibria that regulate these interactions. As a result, the binding of Bcl-2 proteins to membrane is expected to be reversible. In chapter V, I measure the on and the off rates of the proteins binding to the membrane to show that the binding of tBid and Bax to the membrane is reversible. I demonstrate that Bax and Bcl-XL regulate the rate of tBid binding to membranes. On the other hand, Bax binding to membranes is independent of tBid but the activation of Bax is dependent on the presence of an activator at the membrane. These results emphasize the dynamic binding of Bcl-2 proteins to the membrane, and suggest that the retro-translocation of tBid and Bax might play a role in the dissemination of the apoptotic signal throughout the cell.

MANUSCRIPTS

CHAPTER III

tBid Undergoes Multiple Conformational Changes at the Membrane Required for Bax Activation

Preface

The work presented in this chapter has been previously published as a research article in:

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Contribution of authors:

Shamas-Din, A carried out all the experiments, prepared the figures and the table, and wrote the manuscript that was edited by the Fradin, C, Leber, B, and Andrews, DW. Bindner, S and Campbell, C carried out preliminary experiments, Zhu, W prepared the cell lines, and Zaltsman, Y and Gross, A provided the isolated mitochondria used in this study. Andrews, DW, Fradin C and Leber B directed the research of the project.

Research Objective:

To study the detailed mechanism of fragment separation of caspase-8 cleaved Bid and the activation of tBid at the mitochondrial outer membrane.

Research Highlights:

- tBid binds tightly to mitochondria-like membranes with high affinity.
- The two fragments of cBid spontaneously separate in the presence of liposomes or isolated mitochondria.
- Activation of Bax with cBid is preceded by a lag phase, which is due to a conformational change in tBid at the membranes that is required for tBid-mediated activation of Bax.
- The conformational change in tBid entails an elaborate structural rearrangement of the protein that begins with a fast initial interaction of helices 4, 5 and 8 of Bid with the membrane and then a slow insertion of helices 6 and 7 in the membrane.
- Mtch2 facilitates the conformational change in tBid at the mitochondrial outer membrane that is required for the activation of Bax by tBid.
- The interaction of tBid and Mtch2 is a novel potential target for therapeutic regulation of Bid initiated apoptosis.

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tBid Undergoes Multiple Conformational Changes at the Membrane Required for Bax Activation*

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Background: tBid is a Bcl-2 family protein that promotes apoptosis at the mitochondria.
Results: tBid undergoes a reversible conformational change at membranes before activation that is accelerated by Mtch2.
Conclusion: The Mtch2 accelerated conformational change in membrane-bound tBid enables it to activate Bax.
Significance: The conformational change in tBid is a novel potential site of apoptosis regulation.

Bid is a Bcl-2 family protein that promotes apoptosis by activating Bax and eliciting mitochondrial outer membrane permeabilization (MOMP). Full-length Bid is cleaved in response to apoptotic stimuli into two fragments, p7 and tBid (p15), that are held together by strong hydrophobic interactions until the complex binds to membranes. The detailed mechanism(s) of fragment separation including tBid binding to membranes and release of the p7 fragment to the cytoplasm remain unclear. Using liposomes or isolated mitochondria with fluorescently labeled proteins at physiological concentrations as *in vitro* models, we report that the two components of the complex quickly separate upon interaction with a membrane. Once tBid binds to the membrane, it undergoes slow structural rearrangements that result in an equilibrium between two major tBid conformations on the membrane. The conformational change of tBid is a prerequisite for interaction with Bax and is, therefore, a novel step that can be modulated to promote or inhibit MOMP. Using automated high-throughput image analysis in cells, we show that down-regulation of Mtch2 causes a significant delay between tBid and Bax relocalization in cells. We propose that by promoting insertion of tBid via a conformational change at the mitochondrial outer membrane, Mtch2 accelerates tBid-mediated Bax activation and MOMP. Thus the interaction of Mtch2 and tBid is a potential target for therapeutic control of Bid initiated cell death.

Loss of integrity of the mitochondrial outer membrane (MOM)[4] is an event that commits a eukaryotic cell to undergo apoptosis (1, 2). The intrinsic and extrinsic pathways of apoptosis converge at the MOM where the Bcl-2 family of proteins plays a pivotal role in the regulation of apoptosis through protein-protein and protein-membrane interactions (3, 4). The Bcl-2 family includes more than 20 members with either pro-apoptotic or anti-apoptotic functions. It is divided into three groups based on function and the presence of conserved Bcl-2 homology (BH) regions. The pro-apoptotic proteins comprise two groups; those with all four homology regions, such as Bax and Bak, and those that contain only the BH3 region (5, 6). The multiregion family members Bax and Bak undergo complex conformational changes that mediate oligomerization and permeabilization of the MOM, thereby releasing apoptogenic factors such as cytochrome *c* and Smac (7, 8). These conformational changes are initiated by binding BH3 proteins such as tBid, Bim, and Puma, termed activators because they activate the latent pro-apoptotic activity of Bax and Bak (9, 10). The third group includes the anti-apoptotic proteins such as Bcl-2 and Bcl-XL that contain all four homology regions and function by binding either activated Bax/Bak or activator BH3 proteins. Bad and Noxa are examples of sensitizer BH3 proteins that promote apoptosis indirectly by competing for binding to Bcl-XL, thus displacing tBid and Bim to allow Bax/Bak activation (11). A summary of the properties of the Bcl-2 family proteins most relevant to this work is given in Table 1.

Binding to membranes has been shown to trigger structural rearrangements in both anti- and pro-apoptotic multi BH region members of the Bcl-2 family (8, 12–15). These conformational changes alter their affinity for different binding partners and consequently their role in apoptosis. On the other hand, the role of membrane binding in modifying the dynamic structure and function of BH3 proteins has not yet been studied (16).

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[4] The abbreviations used are: MOM, mitochondrial outer membrane; MOMP, MOM permeabilization; cBid, cleaved Bid; tBid, truncated Bid; BH, Bcl-2 homology; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt; DPX, *p*-xylene-bis-pyridinium bromide; PE, phosphatidylethanolamine; CHX, cycloheximide; KD, knockdown; DAC, *N*-(7-dimethylamino-4-methylcoumarin-3-yl); NBD, 12-(*N*-methyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)); DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate.

TABLE 1
Properties of Bcl-2 family proteins

Protein	Properties and function	Size
		kDa
Bid	Full-length Bid; α-helical; cannot activate Bax	22
cBid	Bid cleaved by caspase 8; N-terminal and C-terminal fragments remain bound due to hydrophobic interactions; activator to Bax	22
tBid (p15)	Truncated C-terminal fragment; activator to Bax	15
p7	Truncated N-terminal fragment; inhibitor to tBid	7
Bax	α-Helical, forms pores in the MOM	22
Bcl-XL	α-Helical, inhibits MOMP by sequestering tBid and Bax	27

Bid is a BH3 protein that is required for the transmission of many death signals originating outside the cell to the MOM (17). Bid is crucial for death receptor induced apoptosis in hepatocytes and pancreatic β cells. Binding of ligands to the cell surface death receptors or exposure to the contents of vesicles secreted from cytotoxic lymphocytes results in a single proteolytic cleavage of Bid by caspase 8, calpain, or granzyme B (18–20). Although the exact sites of cleavage are different, they are sufficiently close that the two fragments generated are generically referred to as p7 (7 kDa) and tBid (15 kDa, also called p15). In solution, the two cleaved fragments remain bound to one another in a complex termed cBid (cleaved Bid) due to tight non-covalent interactions (see Fig. 1) (21–24). In cells, the MOM protein Mtch2 facilitates the recruitment of tBid to the MOM by an unknown mechanism (25, 26), where tBid interacts with Bax and Bcl-XL to regulate MOM integrity. Mtch2 is a part of the mitochondrial carrier family proteins and has been recently identified as a novel binding partner of tBid on the MOM. Mtch2 anchors in the MOM by six transmembrane helices and is a structural homologue to the mitochondrial inner membrane protein ANT (26).

Unlike other well studied BH3 proteins that are unstructured, Bid shares a similar three-dimensional structural arrangement of its constituent α-helices with the multiregion family members Bcl-XL and Bax (27–29). Thus Bid contains eight α-helices, of which α6 and α7 are central hydrophobic helices necessary for binding to the membrane that are surrounded by the remaining amphipathic helices (21, 30–32). The BH3 region located within α3 is critical for binding to other Bcl-2 family members to regulate apoptosis (19, 33). In addition, a BH4 region is present close to the N terminus of Bid (6, 24); this feature as well as the specific arrangement of the core hydrophobic helices differentiates Bid from other BH3 proteins (34, 35). Additional clues from phylogenomic analysis and other aspects of the mechanism of action strongly suggest that Bid is not a *bona fide* BH3 protein and may function as a "pseudo-Bax" that is unable to oligomerize efficiently, and permeabilize the MOM itself but that functions to recruit Bax to the membrane and to activate it (for review, see Ref. 35).

Despite the critical role of Bid in apoptosis, the detailed mechanism of the events that follow the cleavage of Bid until it assumes its fully active form at the MOM, including how membrane binding may participate in this process, remains to be understood. To address these questions, we used fluorescence spectroscopy to investigate the mechanism of activation of Bid using a cell-free system with either isolated mitochondria or biomimetic liposomes. We used physiological nanomolar concentrations of recombinant proteins to accurately reflect cellular conditions (36). By labeling Bid at selected positions, the individual steps in this process were identified and studied separately. Our results indicate that the activation of cBid begins when binding to membranes triggers the separation of the two cleaved fragments. Then an elaborate conformational change results in the insertion of anchoring helices in the membrane to achieve the fully active form. Furthermore, we show that the conformational change in tBid after binding to the membrane is the specific step that is accelerated by Mtch2 on the MOM.

EXPERIMENTAL PROCEDURES

Protein Purification and Labeling—Different variants of recombinant His_6-tagged full-length murine Bid were obtained by site-directed mutagenesis and purified as described (15). All of the Bid proteins used in our studies contain an N-terminal His tag, except tBid, in which the His-tagged p7 fragment was removed. For clarity in the nomenclature, we have omitted the His prefix from the names of the proteins. Wild-type (WT) Bid has two endogenous cysteine residues at positions 30 and 126. Plasmids encoding single cysteine versions of Bid with a cysteine at one of the endogenous positions and a no-cysteine variant were generated through QuikChange mutagenesis (Stratagene). The endogenous cysteine residues in Bid were mutated to serine residues for the no-cysteine variant. Subsequently, Bid plasmids containing a single cysteine at various locations within the protein were generated from the no-cysteine Bid plasmid by using QuikChange mutagenesis. By convention, the Bid variants are identified by the location of the single cysteine residue used for labeling; thus, tBid 126C refers to the tBid fragment of Bid with a single cysteine at position 126. A plasmid was also generated in which all six lysine residues in Bid 126C were replaced with arginine residues (Bid ΔK 126C). All the lysine residues in murine Bid are located in close proximity; therefore, silent mutations were created to excise the coding region for Bid from amino acid Ala-130 to Val-175, and the sequence was replaced with an annealed pair of oligonucleotides containing the desired mutations.

All of the Bid proteins contained an N-terminal His tag and were purified using nickel-affinity chromatography (Qiagen) as described (23). At this stage, purified Bid mutants intended for labeling were dialyzed in storage buffer (10 mM Hepes, 7.3, 100 mM NaCl, 0.5 mM EDTA, 10% glycerol). The protein was labeled with the indicated dyes in the storage buffer in the presence of 0.5% CHAPS for 2–3 h at room temperature with rotation. For labeling, a 10–15-fold molar excess of dye dissolved in DMSO was added to the protein slowly while ensuring that the final DMSO content of the reaction did not exceed 10%. To remove the excess unreacted dye, Bid was subjected to nickel-affinity chromatography and washed with at least 25 ml of wash buffer (10 mM Hepes, pH 7.3, 300 mM NaCl, 0.5 mM EDTA, 10% glycerol, and 0.5% CHAPS). The labeled Bid was then eluted with elution buffer (wash buffer supplemented with 200 mM imidazole). The labeling efficiency of Bid at a given residue was measured by dividing the dye concentration (measured by absorbance) by the protein concentration (measured by Bradford assay). Only if the labeling efficiency was ~70% or higher was the protein used.

Purified unlabeled WT Bid or labeled Bid proteins (2–4 mg/ml) were incubated with 500 units of caspase 8 (Enzo Life Sciences) with a final concentration of 1 unit/μl for ~40 h at room temperature to generate cBid. The cleavage efficiency was checked by Coomassie Blue staining of an SDS-PAGE gel, and cBid was dialyzed against storage buffer. To generate recombinant tBid, cBid (labeled or otherwise) was subjected to nickel-affinity chromatography, tBid was isolated using 2% (w/v) octylglucoside (Sigma) in the wash buffer to remove tBid from the bound p7 fragment, and the eluent was dialyzed against storage buffer. WT Bax and Bcl-XL were purified as described previously (12, 37, 38).

Liposome Preparation—Liposomes 100 nm in diameter with a lipid composition made up of phosphatidylcholine (48%), phosphatidylethanolamine (28%), phosphatidylinositol (10%), dioleoyl phosphatidylserine (10%), and tetraoleoyl cardiolipin (4%) (all from Avanti Polar Lipids) were prepared as described previously (37), with the exception that the assay buffer used was 10 mM Hepes, pH 7.3, 200 mM KCl, and 1 mM $MgCl_2$. The liposome concentration was estimated as described (37) using the fact that there are ~84,000 lipids per liposome.

Mitochondria Preparation—Mitochondria were isolated from the livers of $bak^{-/-}$ (38) and $mtch2^{+/+}$ and $mtch2^{-/-}$ (25) mice and frozen in trehalose according to the literature (40). Briefly, mouse liver was prepared in AT buffer: 300 mM trehalose (Sigma), 10 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1 mM EGTA, 1 mM EDTA, 0.1% (w/v) BSA (Bioshop). The isolated mitochondria were frozen at 50 mg/ml final protein concentration (Bradford assay) in 10-μl aliquots and stored at −80 °C until use. When ready to use, mitochondria were rapidly thawed and washed in AT/KCl buffer at 1 mg/ml: 300 mM trehalose, 10 mM Hepes-KOH, pH 7.7, 80 mM KCl, 1 mM EGTA, 1 mM EDTA, 0.1% (w/v) BSA. The mitochondria were resuspended in regeneration buffer at 1 mg/ml final protein concentration (300 mM trehalose, 10 mM Hepes-KOH, pH 7.7, 80 mM KCl, 1 mM EGTA, 1 mM EDTA, 0.1% (w/v) BSA, 5 mM succinate, 2 mM ATP, 10 μM phosphocreatine, 10 μg/ml creatine kinase (added fresh)) and used immediately.

Membrane Permeabilization Assays—Membrane permeabilization assays with liposomes encapsulating ANTS (8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt) and DPX (*p*-xylene-bis-pyridinium bromide) were carried out as described previously (37, 38). Outer membrane permeabilization of mitochondria was assessed by measuring cytochrome *c* release after incubating the mitochondria with the proteins for 30 min at 37 °C. Mitochondria were isolated from the reaction by centrifugation, and cytochrome *c* was detected in the supernatant and pelleted fractions by immunoblotting. The cytochrome *c* polyclonal antibody was produced in our laboratory and was used at a dilution of 1:5000. The secondary antibody conjugated to horseradish peroxidase (Bio-Rad) was used at a dilution of 1:20,000. Immunoblots were analyzed using ImageJ. The curves were fit using Prism 5 (GraphPad).

Liposome Binding Assay—20 nM cBid 126C or tBid 126C labeled with Alexa Fluor 488 C_5 maleimide (Invitrogen) was incubated with different concentrations of liposomes labeled with 0.008 mol% DiD (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt; Invitrogen). After incubation for 15 min at 37 °C to reach binding equilibrium, the reactions were then transferred to 96-well Griener Senseoplates (Sigma), and fluorescence correlation spectroscopy measurements were performed on an Insight spectrometer (Evotec) as described (37). The fluorescence of cBid or tBid labeled with Alexa488 and DiD-labeled liposomes was excited at 488 and 635 nm respectively, and the emission was collected in separate channels. Measurements consisting of 8 individual 30-s runs were performed at room temperature.

The fraction of protein bound to liposomes was calculated by analyzing the autocorrelation functions for two independent species, the unbound protein and the protein bound to liposomes, as previously described (41). Then the dissociation constant, K_d, was extracted from the titration curve by assuming that the protein in solution (P) and the protein bound to membrane (P^*) were in equilibrium. The dissociation constant, K_d is,

$$K_d = \frac{[P][L]}{[P^*]} \quad \text{(Eq. 1)}$$

where L represents the available lipids. Because lipids were always present in excess, $[L]$ can be considered to be the total lipid concentration, so that the bound protein fraction is,

$$f = \frac{[P^*]}{[P] + [P^*]} = \frac{[L]}{K_d + [L]} \quad \text{(Eq. 2)}$$

To present the K_d in terms of liposomes, lipid concentration was converted to liposome concentration in Eq. 1, as explained above.

Separation of p7·tBid—To prepare double-labeled cBid, purified Bid ΔK 126C was first labeled with the acceptor Alexa Fluor 647 C_2 maleimide (Invitrogen) at position 126C and then with the donor Alexa Fluor 550 carboxylic acid, succinimidyl ester (Invitrogen), at the free amino group at the N terminus. As a control, Bid ΔK 126C was labeled with the donor only. The labeled proteins were then cleaved with caspase 8 to generate cBid ΔK 126C Alexa550-Alexa647 (+A) and cBid ΔK 126C Alexa550 (−A). The rate of separation of the two fragments was measured by recording the loss of fluorescent resonance energy transfer (FRET) signal between the two dyes at 37 °C with constant stirring. For this, the sample was excited at a wavelength of 550 nm (2-nm bandwidth), and the emission was recorded at a wavelength of 572 nm (10-nm bandwidth) to measure the intensity of the donor. Cleaved protein was added to assay buffer placed into a quartz cuvette coated with dioleoylphosphatidylglycerol to obtain a final concentration of 20 nM concentrations of protein. A small increase in the intensity of the donor was observed possibly due to fragment separation upon dilution. The intensity of the donor stabilized 1 min after adding the protein and was used as the initial donor fluorescence in the presence of the acceptor, F_{+A} $(t = 0)$. Liposomes were then added to the reaction, and the change in the intensity of the donor, F_{+A} (t), was recorded over time. A parallel reaction was prepared with cBid ΔK 126C Alexa555 where the fluorescence of the donor in the absence of the acceptor, F_{-A} (t) was recorded. In the end the change in % FRET Efficiency was assessed by considering the quantity $(1 - (F_{+A}(t)/F_{-A}(t)))$ $\times 100$.

The reactions with mitochondria with and without Mtch2 were carried out as described above with mitochondria resuspended in regeneration buffer at 1 mg/ml. However, in this case 2 nM cleaved protein was added directly to mitochondria, as no change in donor intensity was observed in the regeneration buffer.

Bid to Membrane FRET—For liposomes, the assay was carried out as described previously (11). Briefly, cBid and tBid single cysteine mutants were labeled with the donor, DACM (*N*-(7-dimethylamine-4-methylcoumarin-3-yl) maleimide (Invitrogen). Liposomes were prepared with 1 mol% of PE lipids labeled with the acceptor NBD either at the head (16:0 NBD PE 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2–1,3-benzoxadiazol-4-yl)) or at the tail (16:0–12:0 NBD PE 1-palmitoyl-2-{12-[(7-nitro-2–1,3-benzoxadiazol-4-yl) amino]-dodecanoyl}-*sn*-glycero-3-phosphoethanolamine) (Avanti Polar Lipids). Excitation at 380 nm (2 nm bandwidth) and emission at 460 nm (10 nm bandwidth) was used to measure the change in the intensity of the donor at 37 °C with constant stirring. The fluorescence intensity recorded in the sample containing cBid DAC and liposomes NBD was noted F_{+A} (t). A reaction with unlabeled liposomes was carried out in parallel to obtain the fluorescence intensity in the absence of acceptor, F_{-A} (t). The change in FRET was monitored by calculating % FRET Efficiency $(1 - (F_{+A}(t)/F_{-A}(t))) \times 100$.

For experiments containing mitochondria, cBid 126C was labeled with the donor Alexa Fluor 488 C_5 maleimide (Invitrogen). To label mitochondria with the acceptor, 10 μM DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; Invitrogen) was incubated with 1 mg/ml mitochondria suspended in regeneration buffer for 30 min at 37 °C. Mitochondria were pelleted to remove unincorporated DiI and resuspended in regeneration buffer. 4 nM cBid 126C Alexa488 was incubated with 1 mg/ml mitochondria with and without Mtch2 for 30 min at 37 °C. Excitation at 493 nm (5 nm bandwidth) and emission at 516 nm (5 nm bandwidth) was used to measure the change in the intensity of the donor. The fluorescence intensity recorded in the sample containing cBid 126C Alexa488 and mitochondria-DiI was noted F_{+A} (t). A reaction with unlabeled mitochondria was carried out in parallel to obtain the fluorescence intensity in the absence of acceptor, F_{-A} (t). The change in FRET was monitored by calculating % FRET Efficiency $(1 - (F_{+A}(t)/F_{-A}(t))) \times 100$.

Environment Change Measurements—Bid single cysteine mutants were labeled with the environment-sensitive dye, *N*,*N*′-dimethyl-*N*-(iodoacetyl)-*N*′-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine (IANBD) (Invitrogen). Changes in environment of the NBD dye were assessed by recording changes in either fluorescence intensity or lifetime.

For fluorescence intensity measurements, single cysteine cBid mutants labeled with NBD were added to either liposomes or mitochondria with and without Mtch2, and the change in the steady state intensity of NBD was recorded over time at 37 °C with constant stirring. The excitation wavelength used was 475 nm (2 nm bandwidth), and the emission wavelength used was 530 nm (10 nm bandwidth). The NBD fluorescence, $F(t)$, was normalized by the fluorescence at time $t = 0$, F_0. The quantity $F(t)/F_0$ is a measure of the hydrophobicity of the environment in the vicinity of the residue labeled with NBD.

The fluorescence lifetime experiments were conducted using frequency-domain lifetime measurements. Before the measurement, 20 nM cBid NBD was incubated with 7.2 nM liposomes for 15 min at 37 °C to reach binding equilibrium. A 470-nm laser with a 450/65-nm filter was used as the excitation source, and the emission was collected through a 525/40-nm filter. The excitation filter was used to block harmonics from the laser. Solutions of fluorescein in 0.1 N NaOH ($\tau = 4.1$ ns) and coumarin in 100% ethanol ($\tau = 2.5$ ns) were used as stan dards. The measurements were performed at room temperature. Uncertainty values of 0.2° for phase decay and 0.004 for modulation ratio were used for the fitting of the curves. The data were analyzed using a three-component model with the third lifetime to account for noise ($\tau = 0.2$–0.4 ns).

Mitochondria Binding Assay—2 nM cBid 30C or 126C labeled with Alexa Fluor 647 C_2 maleimide (Invitrogen) were incubated with 1 mg/ml mitochondria with and without Mtch2 for 30 min at 37 °C. The reactions were then transferred to 96-well Griener Senseoplates (Griener), and fluorescence intensity distribution analysis measurements were performed on an Insight spectrometer (Evotec). The sample was excited at 635 nm while circularly scanning the observation volume over a 45-μm radius circle with a frequency of 0.354 Hz to avoid photobleaching (37). For each sample, a series of 10 measurements, each lasting 30 s, were recorded.

The data were analyzed assuming the presence of two distinct protein species; one with a specific brightness fixed to that of a single cBid molecule (corresponding to unbound monomeric protein) and the other with a specific brightness left to vary and always found to be higher than that of a single cBid molecule (corresponding to mitochondria with several cBid bound to it). The percent bound protein to mitochondria was calculated as described in Satsoura *et al.* (37).

Relocalization of YFP-Bax and Bid in Wild-type and Mtch2 Knockdown HeLa Cells—HeLa cells expressing YFP-Bax under a doxycycline promoter were a gift from Dr. Richard Mosser (42). Using shRNA against human Mtch2 (a gift from Dr. Robert Screaton), we depleted Mtch2 protein from HeLa cells as verified by an immunoblot against Mtch2 (data not shown). For automated high throughput cell experiments, WT and Mtch2 knockdown (KD) cells were distributed in 384-well μClear-plates (Greiner), and YFP-Bax expression was induced. Two days after plating, cells were treated with either 10 μg/ml cycloheximide (CHX) alone or 1 ng/ml TNFα and 10 μg/ml CHX for different time periods as indicated. At the end of the treatment, the cells were fixed and immunostained for Bid using a primary anti-Bid antibody (3C5) (Abcam) at a 1:200 dilution and a secondary Alexa Fluor 594-AffiniPure Donkey Anti-Mouse IgG antibody (Jackson ImmunoResearch) at a 1:200 dilution. Thirty minutes before imaging the cells were stained with DRAQ5 (Biostatus) at a 5 μM final concentration. Images were acquired with an Opera High Content Screening System (PerkinElmer Life Sciences) using a 40× water immersion objective. For each well 15 different images were acquired, each containing ~25 cells. In each image, cells were identified by segmentation using nuclear and cytoplasmic DRAQ5 intensity. Cell features were

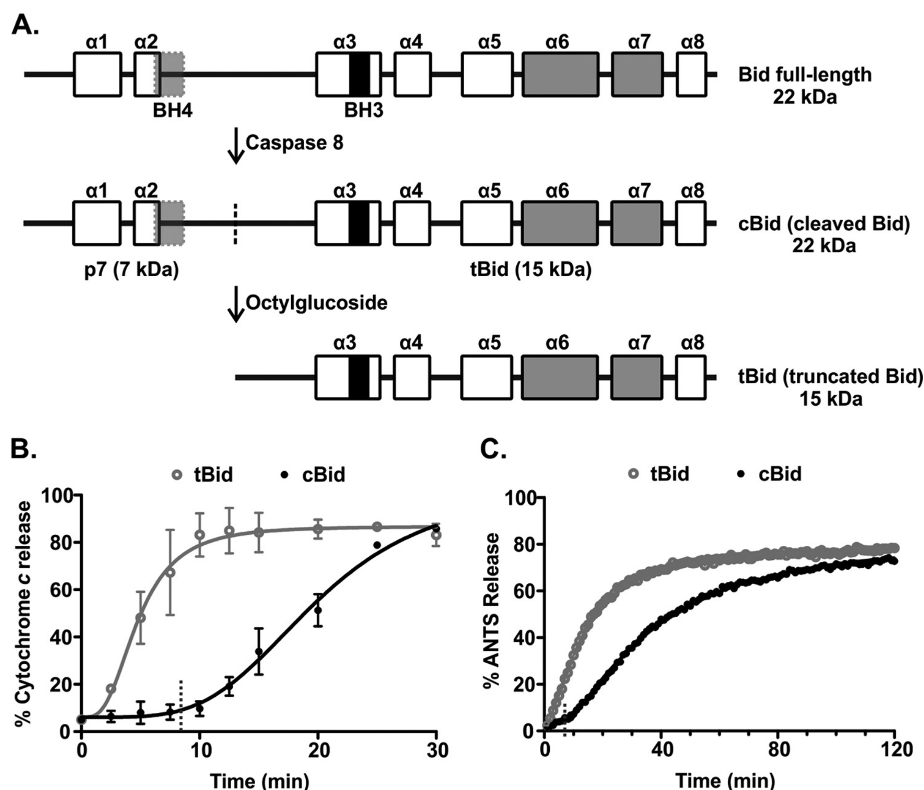

FIGURE 1. **There is a pronounced lag phase in membrane permeabilization by Bax when cBid is used as the activator.** *A*, shown is a schematic outlining the preparation of different forms of Bid. Full-length Bid was first subjected to cleavage by caspase 8 to obtain cBid (*cleaved Bid*). The cleavage site is indicated with a *dotted line*. Although cleaved, the two fragments of Bid remain in a complex due to strong hydrophobic interactions. To isolate the active, membrane binding tBid fragment (*truncated Bid*), cBid is treated with octylglucoside to remove the p7 fragment. The BH3 region (*black*), BH4 region (*light gray*), and the membrane binding helices (*gray*) are shown. The *boxes* indicate the individual α-helices (numbered α symbols *above*). *B*, shown is cytochrome *c* release from mitochondria isolated from $bak^{-/-}$ mice. Mitochondria (1 mg/ml) were incubated with 1 nM tBid (*gray, open circles*) or cBid (*black, closed circles*) and with 50 nM Bax. Cytochrome *c* release was quantified by immunoblotting the bound protein in the pellet and the free protein in the supernatant at the indicated times. The data (*circles*) are fit with a delayed single exponential function (*line*), (mean ± S.E., $n = 3$). *Dashed lines* indicate the value of the lag time obtained from the fit. *C*, permeabilization of liposomes encapsulating ANTS and DPX by incubation with 20 nM tBid (*gray, open circles*) or cBid (*black, closed circles*) and 100 nM Bax is shown. The increase in the fluorescence intensity of ANTS over time is due to membrane permeabilization of the liposomes. One representative series of measurements from three independent experiments is shown. The data (*circles*) are fit with a function accounting for a lag phase followed by a single exponential function (*line*). *Dashed lines* indicate the value of the lag time obtained from the fit.

extracted using a texture analysis script written for Acapella. The cells were then ranked by decreasing YFP-Bax intensity, and the top 200 cells per well were analyzed. Relocalization from a uniform cytoplasmic location to discrete foci representing mitochondria was measured using a Threshold Adjacency Statistics feature (43).

RESULTS

A Pronounced Initial Lag Phase in Bax-mediated Membrane Permeabilization Occurs with cBid but Not tBid—Cleavage of full-length Bid is necessary for the induction of its apoptotic function. Therefore, we generated cBid from purified full-length Bid through proteolysis by recombinant caspase 8. For comparative purposes, tBid was obtained by dissociating the two cBid fragments with the non-ionic detergent octylglucoside (Fig. 1*A*). Both cBid and tBid are pro-apoptotic and have been used in previously published mechanistic studies. Thus, to study the entire process of activation post cleavage, we compared the extent and kinetics of membrane permeabilization using mitochondria isolated from $bak^{-/-}$ mice. These mitochondria lack both Bak and Bax, as Bax is a cytoplasmic protein in mouse liver and does not detectably co-purify with mitochondria. To permeabilize these mitochondria, they were incubated with Bax and either tBid or cBid. Membrane permeabilization was measured by immunoblotting mitochondrial (pelleted) and released (supernatant) proteins to detect the release of cytochrome *c* at different time points (Fig. 1*B*). In a similar assay system that allows for control of lipid constituents, release of liposome-encapsulated fluorophore and quencher (ANTS and DPX) was measured during incubation with Bax and either tBid or cBid without stirring. Membrane permeabilization was followed by the increase in the ANTS fluorescence that occurs when the released fluorophore and quencher are diluted in the incubation buffer (Fig. 1*C*). Although ultimately achieving similar end points, membrane permeabilization by tBid-activated Bax was faster than that by cBid-activated Bax in both mitochondrial and liposomal systems (Fig. 1, *B* and *C*). The delay in membrane permeabilization using cBid-activated Bax was primarily due to a pronounced initial lag phase noted in both assays. To quantify the length of the initial lag phase and the kinetics of the following release phase in liposomes, we fit the data with a model where a no-release phase (lag phase) is followed by a single exponential kinetics release. In reactions

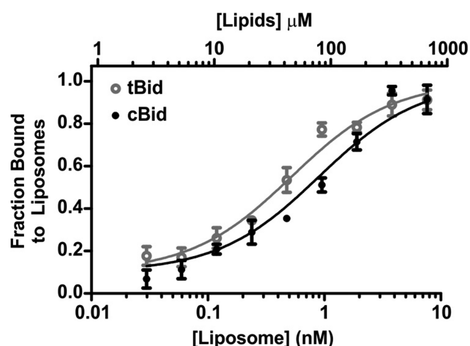

FIGURE 2. **The proteins tBid and cBid have a very similar apparent dissociation constant for binding to liposomes.** 20 nM tBid (*gray, open circles*) and cBid (*black, closed circles*) labeled with Alexa Fluor 488 were incubated with different concentrations of DiD-labeled liposomes for 15 min. The *x* axes indicates the concentration of liposomes in nM and the corresponding concentration of lipids in μM. The liposome-bound fractions as measured by fluo rescence correlation spectroscopy (8 measurements/experiment) are shown for tBid and cBid. The data (*dots*) are fit with Equation 2 (*line*), (mean ± S.E., $n = 3$ independent experiments).

using cBid, the initial lag phase lasted 8–10 min and was followed by an exponential phase that was slightly slower than that for tBid irrespective of whether liposomes or mitochondria were used as the target membrane.

Affinity of tBid and cBid for Liposomes—To characterize precisely any difference in the interaction between tBid and cBid with membranes, we measured the apparent dissociation constant (K_d) of tBid and cBid with liposomes. For these experiments tBid and cBid were labeled at the endogenous cysteine at position 126 with Alexa Fluor 488 and incubated with a range of liposome concentrations for analysis by fluorescence correlation spectroscopy. This method can distinguish between fluorescent species of different size based on their diffusion coefficient, and return their concentration (41). Free proteins in solution (~5-nm diameter) have a high diffusion coefficient compared with proteins bound to liposomes (~100-nm diameter). The percentage of tBid or cBid bound to liposomes could, therefore, be calculated from fluorescence correlation spectroscopy data (Fig. 2). Analysis of the data with a simple binding model gave an apparent K_d of 0.5 nM for the binding of tBid to liposomes (44 μM when considering the binding of tBid to lipids) and 0.9 nM for the binding of cBid to liposomes (76 μM for cBid and lipids), suggesting that tBid binds only slightly more avidly to the membrane than cBid.

The Initial Lag Phase Is Independent of Protein Concentration—To examine the difference in kinetics observed between tBid and cBid in more detail, we used the ANTS/DPX liposomal assay to determine the concentration dependence of permeabilization rates for tBid and cBid (Fig. 3, *Ai* and *Aii*). There was a clear dose dependence in membrane permeabilization for both proteins, and the reaction end points were similar (Fig. 3*B*). The curves did not reach 100% because we used detergent-solubilized liposomes as a reference for 100% release, and the ANTS fluorescence further increased in detergent due to a more hydrophobic environment. This resulted in an underestimate of the liposome permeabilization. Next, we obtained the duration of the lag phase and the half-time for the exponential permeabilization phase as a function of the concentration of tBid or cBid (Fig. 3, *C* and *D*, respectively) by fitting the data in Fig. 3, *Ai* and *Aii*. The duration of the initial lag phase displayed only a small decrease with increasing protein concentration (less than 5 min); therefore, the large difference in the lag phase durations observed for tBid and cBid remained throughout the concentration range studied (Fig. 3*C*). By contrast, the half-times for permeabilization decreased significantly with increasing protein concentration but remained comparable for the two proteins for the range of concentrations explored (Fig. 3*D*). The kinetics and the extent of membrane permeabilization both reached a plateau above a concentration of ~5 nM tBid or cBid. This concentration corresponds to a ratio of ~10 tBid/cBid molecules per liposome with 100 nM Bax. These results are consistent with the well known catalytic nature of Bax activation by substoichiometric amounts of Bid. Taken together, these data suggest that cBid can cause the same amount of Bax-mediated permeabilization as tBid but that this follows an initial inactive phase (lag phase) that is significantly longer for cBid than for tBid.

The Initial Lag Phase in Membrane Permeabilization with cBid Is Due to the Time Required for Its Activation at the Membrane—To investigate the lag phase further, we pre-incubated cBid for different periods of time with liposomes encapsulating ANTS/DPX before adding Bax at time $t = 0$ min (Fig. 3*E*). All reactions had a similar end-point independent of the preincubation time of cBid with the liposomes. Strikingly, reactions with longer cBid-liposome preincubation times displayed a shorter initial lag phase that approached the short duration of the lag phase observed with tBid (Fig. 3*F*). By contrast, the kinetics of the exponential phase of ANTS release remained constant over the explored range of preincubation periods (Fig. 3*G*). This is consistent with the lag phase perhaps being the result of tBid unfolding or changing conformation in some other way at the membrane. Thus, cBid needs to interact with lipid membranes before it activates Bax and causes membrane permeabilization. This rate-limiting step in cBid activation most likely involves the dissociation of the p7·tBid complex and/or the unfolding and insertion of the tBid fragment into the membrane.

Fast Separation of the Cleaved Fragments of Bid in the Presence of a Membrane—To determine if the dissociation of the p7·tBid complex contributes to the cBid lag phase, we examined the localization of the two fragments after contact with membranes using both enriched mitochondria and liposomal assays. For experiments with mitochondria, cBid or tBid was incubated with mitochondria isolated from the livers of $bak^{-/-}$ mice, and the membrane-bound protein fraction was separated from the unbound protein by pelleting. Proteins bound to mitochondria (pelleted) and unbound proteins in the supernatant were identified by immunoblotting with antibodies specific to each fragment (Fig. 4*A*). As expected, the tBid fragment was present in the membrane-bound fraction, whereas the p7 fragment was only seen in the free-protein fraction. Similar results were obtained when 20 nM cBid was incubated with liposomes, and the membrane-bound protein was separated from the free proteins using size-exclusion chromatography. Immunoblots of different fractions revealed that the tBid fragment eluted in the membrane-bound fractions, whereas the p7 fragment eluted in

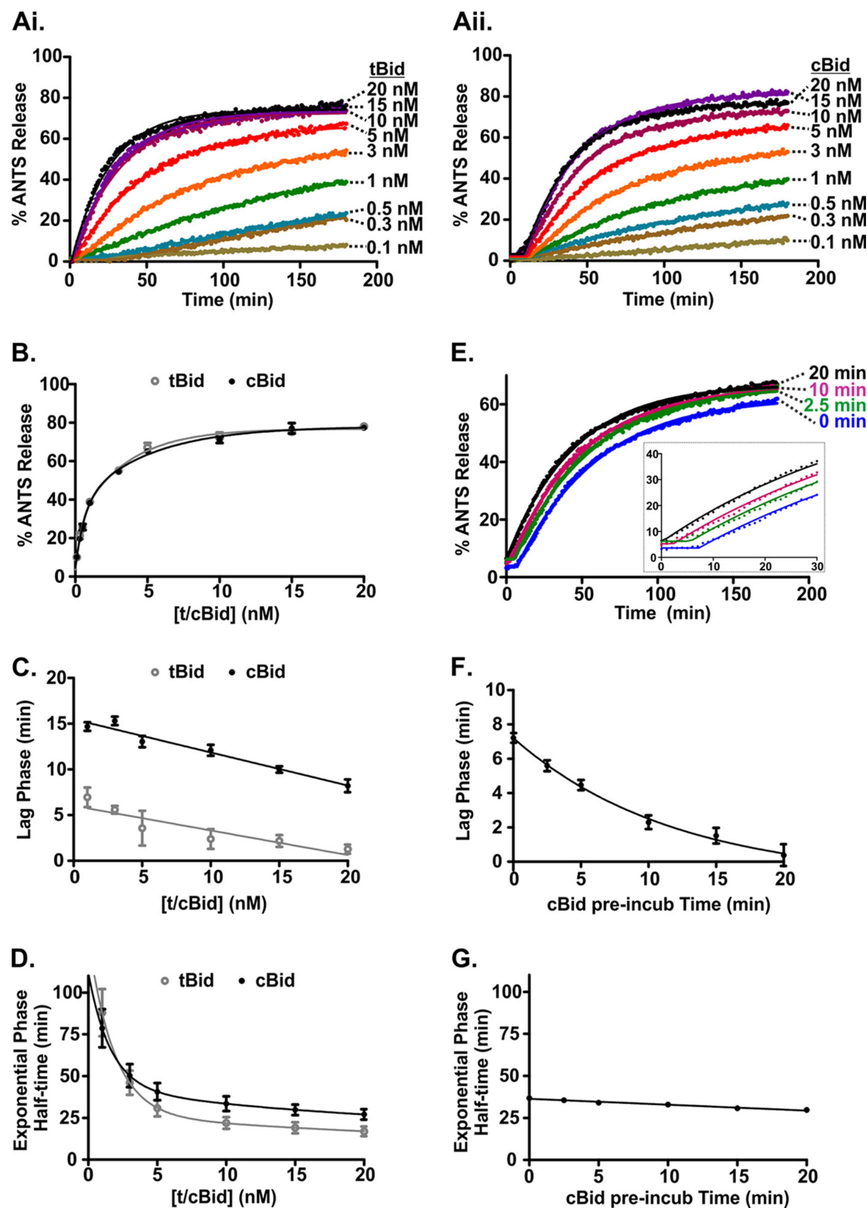

FIGURE 3. **The lag in membrane permeabilization is due to slow activation of cBid at the membrane.** *A*, ANTS release was observed when adding varying concentrations of (*i*) tBid or (*ii*) cBid to 0.5 nM liposomes containing ANTS/DPX and 100 nM Bax. One representative series of measurements from three independent experiments is shown. *Solid lines* are fit using a function accounting for a lag phase followed by a single exponential. *B*, ANTS release after 180 min for each reaction plotted against the concentration of tBid or cBid. Points are the mean ± S.E. $n = 3$. *Solid lines* are best fit using a single exponential. *t/cBid* indicates tBid or cBid. Shown are duration of the lag phase (*C*) and the half-time of the subsequent exponential phase obtained by fitting the data shown in *i* and *ii* (*lines*) as a function of tBid (*gray, open circles*) and cBid (*black, closed circles*) concentration (mean ± S.E., $n = 3$; *D*). Linear (*C*) and Exponential lines (*D*) of best fit are shown. *t/cBid* indicates tBid or cBid. *E*, shown is membrane permeabilization obtained after preincubating cBid with the liposomes for different lengths of time. Liposomes encapsulating ANTS/DPX (0.5 nM) were incubated with 20 nM cBid for the times indicated to the *right of the data points*, and then 100 nM Bax was added at time = 0 (mean, $n = 6$). Shown is duration of the lag phase (*F*) and the half-time of the subsequent exponential phase (*G*) as a function of the cBid preincubation time with liposomes (mean ± S.E., $n = 6$). Exponential (*F*) and linear lines (*G*) of best fit are shown.

the free-protein fractions (Fig. 4*B*). Thus there is complete dissociation of the p7·tBid complex after binding of the tBid fragment to 7.2 nM liposomes.

To examine if the separation of the two fragments was responsible for the initial lag phase associated with cBid activation, we designed a cBid mutant for use in an intramolecular FRET assay. To permit efficient and unambiguous labeling of the protein at two different and unique positions, we used a free thiol group for labeling with one dye and an amine group for labeling with the other dye. Therefore, we replaced the lysine residues in cBid 126C with arginine residues, generating Bid ΔK 126C. We labeled the cysteine at position 126 with the acceptor dye Alexa Fluor 647 and the free amino group of the N terminus with the donor dye Alexa Fluor 555. Cleavage by caspase 8 then produced cBid ΔK 126C Alexa555-Alexa647 (Fig. 4*C*). As a control for any change in the intensity of the donor not due to FRET, we also prepared cBid ΔK 126C Alexa555. Both cBid ΔK 126C Alexa555 and cBid ΔK 126C Alexa555-Alexa647 retained

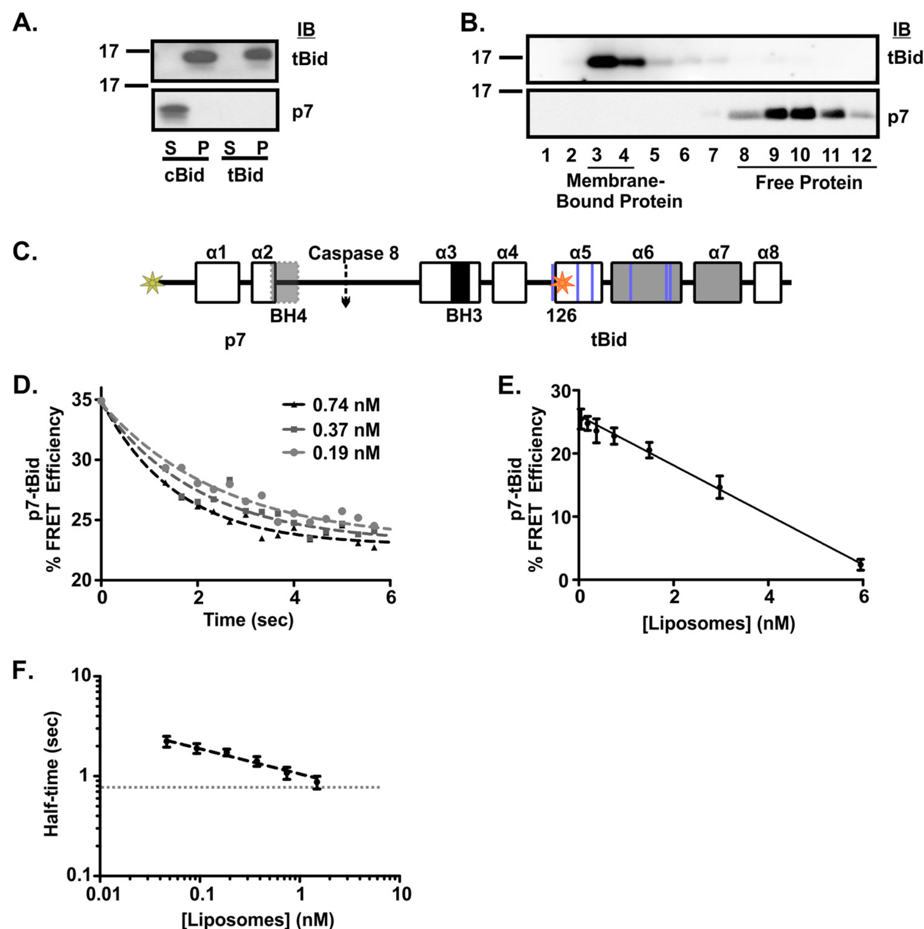

FIGURE 4. **The time required for separation of the p7 and tBid fragments of cBid does not account for the lag in membrane permeabilization.** *A*, localization of the p7 and tBid fragments of cBid is shown. Mitochondria lacking Bak (1 mg/ml) were incubated with 4 nM tBid or cBid, and separation of the fragments was assayed by pelleting the mitochondria and immunoblotting for the bound protein in the pellet (*P*) and free protein in the supernatant (*S*). The approximate position of the 17-kDa molecular mass marker is indicated on the *left*. The specificity of the antibody used to probe the immunoblots (*IB*) is indicated to the *right*. *B*, targeting of the fragments of cBid to liposomes is shown. Membrane-bound protein was separated from soluble protein by size exclusion chromatography. Membrane binding was assayed for 20 nM cBid incubated with 7.2 nM liposomes for 15 min by chromatography using CL2B resin. Elution fractions corresponding to the excluded volume containing liposomes (*fractions 3–4*) and the included fractions to the bed volume containing unbound proteins (*fractions 8–12*) were analyzed by immunoblotting. The approximate position of the 17-kDa molecular mass marker is indicated to the *left*. The specificity of the antibody used to probe the immunoblots (*IB*) is indicated to the *right*. *C*, shown is a schematic of cBid ΔK 126C labeled as in Fig. 1, highlighting the positions of the FRET donor (Alexa Fluor 555, *yellow-green star*) and acceptor (Alexa Fluor 647, *orange star*) dyes at the N terminus and the endogenous cysteine residue at position 126, respectively. The positions of the lysine residues converted to arginine are indicated with *blue lines*. *D*, representative data show that the p7 fragment rapidly separates from the tBid fragment in the presence of membranes. The decrease in % FRET efficiency indicates the separation of the p7·tBid fragments of cBid ΔK 126C in the presence of the indicated concentrations of liposomes (*black triangles*, 0.74 nM; *gray squares*, 0.37 nM; *gray circles*, 0.19 nM). % FRET Efficiency was calculated by: $(1 - (F_{+A}(t)/F_{-A}(t))) \times 100$. $F_{+A}(t)$ is the fluorescence of 20 nM cBid ΔK 126C labeled at the N terminus with Alexa Fluor 555 (donor) and at 126C with Alexa Fluor 647 (acceptor). $F_{-A}(t)$ is the fluorescence of a parallel reaction with 5 nM cBid ΔK 126C labeled only at the N terminus with Alexa Fluor 555 (mean, $n = 4$). A single exponential line of best fit used to determine the end point and half-time of fragment separation is shown. *E*, end points for % FRET efficiency data similar to that shown in *D* including the additional liposome concentrations tested indicates that at the end point fragment separation varies linearly with liposome concentration (mean ± S.E., $n = 4$). *F*, half-times associated with the separation of the two fragments (*black*) suggests that the rate of fragment separation (measured as decreased FRET) varies as a power of the liposome concentration. Half-times were obtained from fitting the % FRET efficiency data for each liposome concentration to a single exponential (mean ± S.E., $n = 4$). The minimum half-time detectable by the instrument is shown as a.

functional activity in the liposome based membrane permeabilization assay (data not shown).

To obtain the rate of separation of the two fragments after incubation with liposomes, we calculated % FRET efficiency as explained under "Experimental Procedures." Loss of FRET resulted from the separation of the two fragments (Fig. 4*D*). These data demonstrate that separation of the two fragments can be fit with a single-exponential function and require only a few seconds as shown for three sample concentrations (Fig. 4*D*). Therefore, this process (and by inference membrane binding as membranes are required for fragment separation) does not account for the delayed kinetics of activation of cBid at the membrane. By using additional liposome concentrations, we show that the extent of separation of the p7·tBid fragments increased linearly with increasing liposome concentration (Fig. 4*E*). This result suggests that the efficiency of fragment separation depends on the availability of a membrane. Furthermore, the FRET efficiency approaches 0 (indicating complete separation of the fragments) at ~6 nM liposomes. This value is in good agreement with the titration of cBid binding to liposomes data presented in Fig. 2.

Similarly, the half-time determined from the exponential phase of fragment separation decreased with increased concentrations of liposomes (Fig. 4*F*). The time it takes for sample addition and mixing may limit our ability to measure the rate of separation, suggesting that the rate of membrane-mediated fragment separation that we measured ($k \sim 0.25\ s^{-1}\ nM^{-1}$) is consistent with a diffusion-limited process ($k \sim 1\ s^{-1}\ nM^{-1}$) (44). Together, these results suggest that the separation of the two fragments of cBid is spontaneous and rapid, requiring only the presence of a membrane. Thus, fragment separation is not sufficient to account for the lag phase preceding membrane permeabilization (Fig. 3*F*). This indicates that there is one or several activation steps required for tBid on the membrane after fragment separation and before membrane permeabilization can occur.

The tBid Fragment Undergoes a Conformational Change after Binding to Membranes—Given that the difference in tBid and cBid activation of MOMP is not explained by the separation of the p7·tBid fragments, we used a protein to membrane FRET assay to measure the rate at which proteins integrated into liposome membranes (11). For these experiments, tBid and cBid were labeled with the donor fluorophore DAC at position 126C, and PE labeled with NBD at the tail was incorporated into the liposomes as the acceptor. An increase in % FRET efficiency was used to measure binding of tBid/cBid to liposome-containing NBD-PE. Using this approach, we previously reported a rapid reaction half-time of <5 s between tBid and liposomes (11); however, here we discovered that the reaction half-time of cBid and liposomes is significantly longer: ~50 s (Fig. 5*B*). We obtained similar 40–80 s half-times for dyes at other positions in the protein by using single cysteine mutants in protein to liposome FRET assays (data not shown). This result is consistent with the half-time measurements reflecting integration/binding of the protein into the membrane rather than a conformation change resulting in quenching of the dye at position 126.

The half-time of binding of the protein to membranes was greatly accelerated in these experiments compared with those shown in Figs. 1 and 3 because they were carried out with constant stirring. Stirring the sample improved the signal-to-noise ratio of the measurements but also resulted in faster kinetics observed compared with the previous experiments (Figs. 1 and 3). Therefore, to relate the kinetics of protein insertion in the membrane to the kinetics of the lag phase observed in membrane permeabilization (Fig. 3), we repeated the FRET without stirring and for the same liposome concentration as the membrane permeabilization assay. In these experiments the half-time for membrane insertion of cBid from the FRET assay was ~4 min instead of 50 s (data not shown), which is entirely consistent with the observed lag phase of ~7 min in membrane permeabilization, as the latter also incorporates the time required for Bax activation and oligomerization. Taken together, these data demonstrate that it is the insertion of tBid in membranes that causes the initial lag phase in cBid-Bax mediated membrane permeabilization.

It is likely that insertion into the membrane results in or from a conformational change in the tBid fragment of cBid. We, therefore, hypothesized that a slow structural conformation change in cBid occurs upon binding to the membrane, which is required for functional activity and is responsible for the lag phase we observed for cBid. To detect membrane dependent conformational changes in cBid, we created a series of single cysteine mutants spanning key regions of the protein (Fig. 5*A*). These mutants were labeled with the fluorophore NBD, a small uncharged dye that shows an increase in steady state emission intensity and lifetime in a hydrophobic environment. These features make NBD an excellent tool to study changes in the environment of specific residues of a labeled protein (11, 45). To ensure that the labeled protein accurately reflected the behavior of the WT protein, we used only the mutants that had a labeling efficiency of ~70% or higher and also retained membrane permeabilization activity (data not shown). Low labeling efficiencies prevented the analysis of additional residues spanning the membrane binding helices. To measure the kinetics of the conformational changes at or in the membrane, we added NBD-labeled single cysteine mutants of cBid to liposomes and recorded normalized fluorescence of NBD. Control experiments demonstrated that using the first measureable time point as F_0 was as accurate as, but less cumbersome than, using a separate sample without liposomes.

The time course for the change in the emission of NBD after membrane binding for selected, labeled cysteine residues in cBid is shown in Fig. 5*C*. The curve generated using tBid 163C NBD is shown for comparison (Fig. 5*C*, *black*). The endpoints of the change in the normalized fluorescence intensity of cBid NBD for the entire series of mutants studied are summarized in Fig. 5*D* and show a significant increase in hydrophobicity for residues situated in and around helices $\alpha 5$ and $\alpha 6$. For most cBid NBD mutants (cBid NBD 106, 111, 126, 136, 142, 163, and 165) the fluorescence intensity curves could be well fit with a single-exponential function, yielding a single half-time for the environmental change of the corresponding residue (Fig. 5*E*). For other mutants (cBid NBD 66, 186, and 190) a small but consistent non single-exponential behavior was observed that points to a progressive and complex conformational change. For these cBid mutants only the half-time corresponding to the faster exponential change in environment is shown (Fig. 5*E*). For mutants in the proximity of the BH3 region (cBid NBD 86, 91, and 93) the changes were too small to accurately determine the kinetics. The reaction half-time does not correlate with the extent of the change in NBD fluorescence for residues 163 and 165 (compare Fig. 5, *D* and *E*), demonstrating that longer reaction half-times are not necessarily related to the extent to which the residue becomes buried in the bilayer.

For residues in the BH3 region there was no measureable increase in hydrophobicity. Indeed for residue 91 there is a slight decrease in hydrophobicity. This result suggests that the BH3 region remains accessible to aqueous solution even after other regions of tBid have inserted into the lipid bilayer.

Due to the fast half-times of residues in the beginning of helices $\alpha 4$–5 and $\alpha 8$ of Bid, we propose that those regions of tBid interact with the membrane first, whereas the slow kinetics of the residues close to the end of helices $\alpha 4$–5 and the loop between helices 6–7 (L 6–7) indicate that this region is

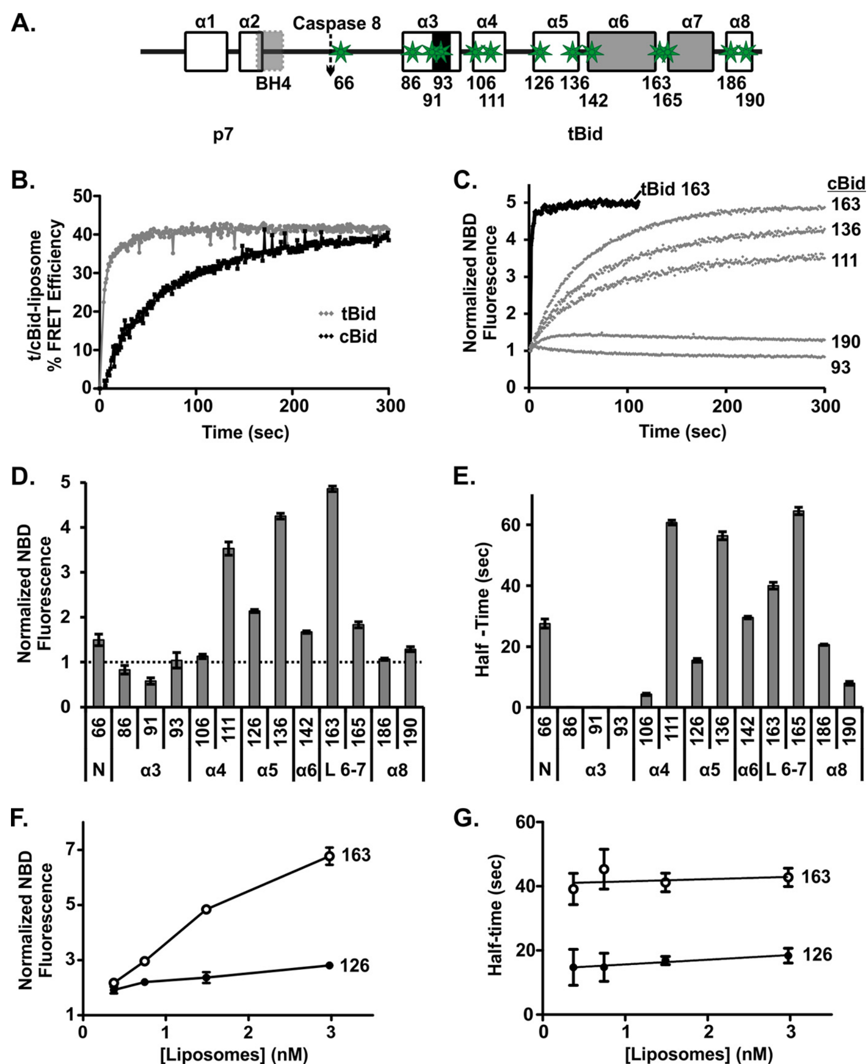

FIGURE 5. **tBid undergoes a conformational change after binding to membranes.** *A*, shown is a schematic labeled as in Fig. 1 illustrating the locations of the single-cysteine mutations (*green stars*) in Bid used for labeling with NBD as an environment-sensitive probe. Numbers *below the schematic* indicate the positions of the cysteine residues. *B*, shown is binding of tBid and cBid to membranes measured by FRET. An increase in % FRET efficiency indicates binding of protein to liposomes. 20 nM cBid 126C (*black*) or tBid 126C (*gray*) labeled with DAC (donor) was incubated with liposomes containing NBD-PE (acceptor), F_{+A} (t), and without NBD-PE, F_{-A} (t). As a measure of % FRET efficiency, $(1 - (F_{+A}(t)/F_{-A}(t))) \times 100$ was calculated (data points are the means, $n = 3$). Connecting lines are included as a visual guide only. *C*, shown are changes in NBD fluorescence reflecting changes in the environment for some representatives of the different residues of tBid (*black*) and cBid (*gray*) upon binding to membranes. An increase in the NBD fluorescence indicates the residue moving to a more hydrophobic environment. The *numbers to the right* indicate the residue labeled. Each experiment contained 20 nM labeled protein and 1.56 nM liposomes. NBD fluorescence, $F(t)$, was normalized by F_0, the fluorescence at time = 0 (mean ± S.E., $n = 3$). *Solid lines* are single-exponential fit. *D*, shown are end point fluorescence changes due to cBid binding to membranes for NBD fluorescence for all of the tested residues, assayed as in *C* ($t = 300$ s) (mean ± S.E., $n = 3$). The *numbers below the bars* indicate the residue number. The *labels at the bottom* indicate the region of the protein. Helices are indicated as in Fig. 5*A*. The N-terminal region (*N*) and the loop between helices 6 and 7 (*L6–7*) are also indicated. *E*, shown is the reaction half-times for the change in environment of each residue derived by the single-exponential fit from *C* upon binding to membranes (mean ± S.E., $n = 3$). *F*, shown is change in the end point fluorescence for cBid 163C and cBid 126C labeled with NBD as a function of liposome concentration ($t = 300$ s) (mean ± S.E., $n = 3$). *G*, half-times for the conformational changes in cBid 163C and cBid 126C labeled with NBD were not affected by liposome concentration (mean ± S.E., $n = 3$).

involved in the last step of the ordered conformational change (Fig. 5*D*).

tBid Conformational Change Occurs after Membrane Binding—Because the conformational change occurs in the membrane, we predicted the kinetics of the NBD fluorescence to be independent of liposome concentration. Therefore, we chose two NBD-labeled cBid mutants, one with faster kinetics (126C; a residue in α helix 5) and the other with slower kinetics (163C; a residue in the loop between helices 6 and 7), and incubated both with a range of liposome concentrations. As expected, the extent to which the fluorescence of the dye increased correlated with increasing liposome concentration (Fig. 5*F*) due to an increased fraction of the proteins binding to liposomes (Fig. 2). In contrast, the kinetics of the process captured by NBD fluorescence remained unchanged for the range of liposome concentrations explored (Fig. 5*G*). This result directly contrasts what was observed for the rate of separation of the fragments (Fig. 4*F*). These results support our hypothesis that the separation of the two fragments occurs concurrently with the initial binding of cBid to liposomes and is followed by a major confor-

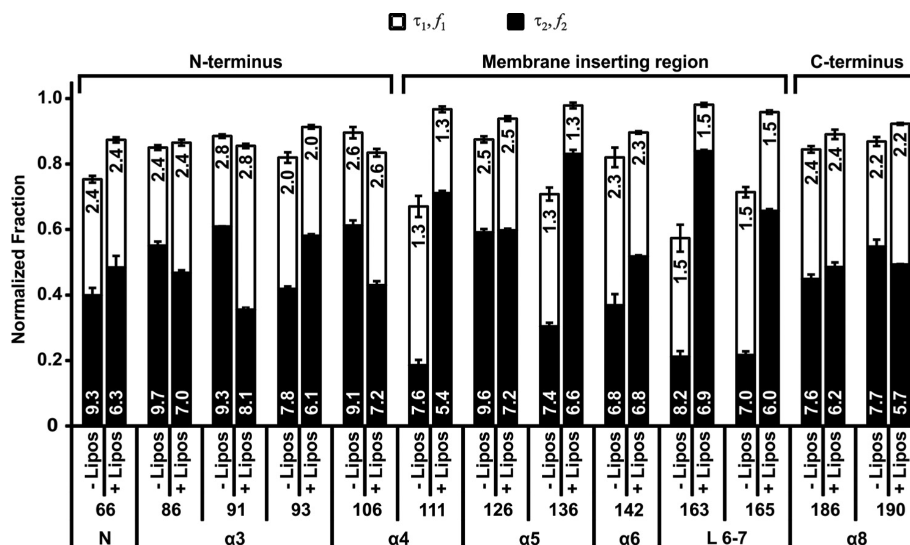

FIGURE 6. **tBid exists in two major conformations at the membrane.** The fluorescence lifetime of samples of 20 nM cBid labeled with NBD at different residues with and without 7.2 nM liposomes was measured. The data were analyzed using a model that included a hydrophilic (τ_1) and a hydrophobic lifetime (τ_2) and noise. The fractions corresponding to each lifetime (f_1 and f_2) are shown as *open* and *solid bars*, respectively, for the residues indicated *below the bars*, with (+) and without (−) liposomes (*Lipos*). The various regions probed are indicated at the *bottom* as in Fig. 5. The values of lifetimes (ns) are indicated on the *bars* (mean ± S.E., n = 3–4) and roughly divide the protein into the three regions (N terminus, membrane inserting, and C terminus) as indicated at the *top*.

mational change in tBid as the protein is inserting into the membrane. This latter event is, therefore, independent of liposome concentration as long as there are sufficient liposomes to provide binding sites for all of the cBid molecules in the reaction.

At the Membrane tBid Adopts Multiple Conformations—The changes measured in the fluorescence intensity of NBD for the different mutants after the addition to liposomes are relative to that observed for the initial environment of the dye on the protein. Therefore, to obtain a more complete picture of the hydrophobicity of the dye-labeled residues at different positions in cBid before and after the protein interacts with membranes, we measured the fluorescence lifetime of the probes on the proteins. Furthermore, lifetime measurements indicate whether the signal is composed of one, two, or more species on the membrane, which likely represent different conformers. To enhance our ability to detect multiple lifetimes of membrane-bound conformers, the signal from unbound protein was minimized by using a high liposome concentration (7.4 nM) at which most of the protein was bound to membranes (Fig. 2).

Most residues in soluble proteins are not in a single unique environment. Due to thermal and other motions of the protein, exposure to different environments can be modeled as a combination of hydrophilic and hydrophobic environments. The hydrophilic environment is dominated by exposure of the residue to the aqueous milieu, whereas the nature of the hydrophobic environment depends on interactions with hydrophobic residues in the protein, lipid molecules in a membrane, or both. Therefore, we fitted the curves generated from cBid NBD in solution (without liposomes) with a model that took into account the lifetime and the corresponding fraction of NBD in a hydrophilic (τ_1, f_1, *open bars*) or hydrophobic environment (τ_2, f_2, *solid bars*) (Fig. 6). Experimental noise ($\tau \sim 0.3$ ns) accounted for the remaining signal (typically less than 20%) and can be observed as the distance from the *top of the bars* to 1.0 on the scale.

As expected for cBid in solution, τ_1 was relatively constant between 1.3 and 2.8 ns, whereas τ_2 varied between 6.8 and 9.7 ns for all of the residues tested. These values of τ_1 and τ_2 are in general agreement with the lifetime of NBD in water and in chloroform, respectively. The fraction of protein associated with each lifetime, however, varied between different mutants, and the fraction of noise was higher in mutants that had a low hydrophobic fraction in solution (cysteine residues at positions 111, 136, 163, and 165) due to the increased uncertainty in measuring short lifetimes.

To analyze the curves from samples containing both cBid NBD and liposomes, we fixed the value of τ_1 to that obtained for each particular residue in solution. This accounts for changes in τ_1 due to local differences in amino acid sequence. We then obtained τ_2, f_1, and f_2 from the fit of the lifetime data of the sample with liposomes. The comparison of f_2 before and after the addition of liposomes is in general agreement with the changes observed in steady state intensities for NBD fluorescence (Figs. 5*D* and 6). For example, f_1 varied from >50% for residue 111, a residue that appears to be surface-exposed, to 28% for residues such as 126 in the hydrophobic region of the protein (Fig. 6).

Taken together, the results from the steady state intensity and lifetime measurements indicate that after binding to the liposome membranes, the N-terminal region from 66–106 becomes slightly more solvent-exposed, residues 111–165 enter a more hydrophobic environment except for 126 that is always in a hydrophobic environment, and the C-terminal region from 186–190 remains unchanged. Furthermore, f_2 is the predominant fraction for cBid mutants with a dye in the region found between residues 111 and 165, suggesting that this region is likely inserted into the membrane. By contrast, both the N- and the C-terminal

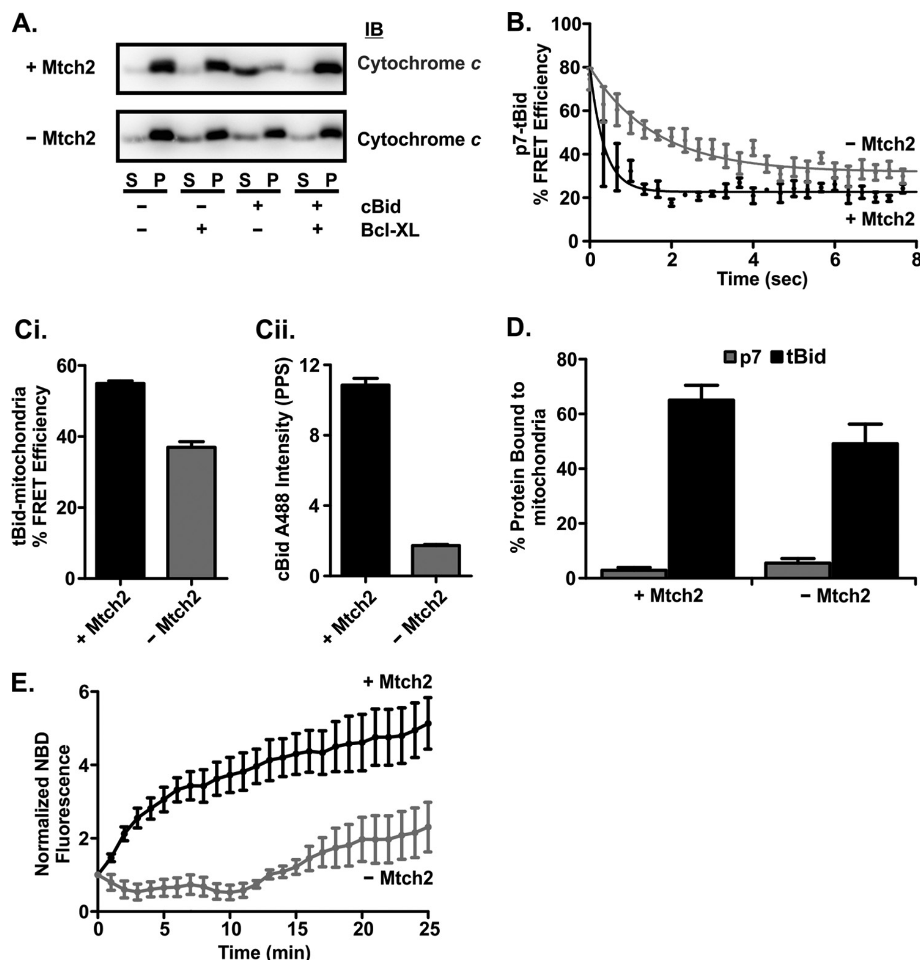

FIGURE 7. **Mtch2 facilitates the conformational change of tBid required for its activation upon binding to mitochondria.** *A*, mitochondria lacking Mtch2 are less susceptible to cBid-induced MOMP. cBid (1 nM) and/or 50 nM Bcl-XL (*indicated below the blots*) were incubated for 30 min at 37 °C with 1 mg/ml mitochondria with and without Mtch2 as indicated to the *left*. Cytochrome *c* release was assayed by isolating the mitochondria by centrifugation and immunoblotting (*IB*) the bound protein in the pellet (*P*) fractions and free protein in the supernatant (*S*) fractions. *B*, separation of the p7·tBid fragments is very rapid when incubated with mitochondria isolated from the livers of *mtch2*$^{+/+}$ or $^{-/-}$ mice. Mitochondria (1 mg/ml) with (*black*) and without (*gray*) Mtch2 were incubated with 2 nM cBid ΔK 126C labeled with Alexa Fluor 555 donor and Alexa Fluor 647 acceptor dyes. The decrease in % FRET Efficiency indicates the separation of the p7·tBid fragments of cBid ΔK 126C. % FRET efficiency was calculated by $(1 - (F_{+A}(t)/F_{-A}(t))) \times 100$. $F_{+A}(t)$ is the fluorescence of cBid ΔK 126C labeled with Alexa Fluor 555 (donor) and Alexa Fluor 647 (acceptor). $F_{-A}(t)$ is the fluorescence of a parallel reaction with cBid ΔK 126C labeled only with Alexa Fluor 555 (donor). *C*, FRET measurements suggest cBid binds to but does not insert into mitochondria from *mtch2*$^{-/-}$ mice. 4 nM cBid 126C labeled with Alexa Fluor 488 (donor) was added to 1 mg/ml mitochondria from *mtch2*$^{+/+}$ or *mtch2*$^{-/-}$ mice labeled with DiI (acceptor). An increase in % FRET efficiency indicates cBid binding to mitochondria. % FRET Efficiency was calculated by: $(1 - (F_{+A}(t)/F_{-A}(t))) \times 100$. $F_{+A}(t)$ is the fluorescence of cBid 126C Alexa Fluor 488 in the presence of mitochondria labeled with DiI. $F_{-A}(t)$ is the fluorescence of a parallel reaction with cBid 126C Alexa Fluor 488 in the presence of unlabeled mitochondria. *Ci*, end points for the % FRET efficiency of the reaction after 30 min are shown. *Cii*, after 30 min of incubation of cBid 126C Alexa Fluor 488 with mitochondria, the samples were centrifuged, and the pelleted mitochondria were resuspended. The fluorescence of cBid 126C Alexa Fluor 488 in resuspended mitochondria is shown (mean ± S.E., $n = 3$). *D*, shown is binding of p7 and tBid fragments to mitochondria from *mtch2*$^{+/+}$ and *mtch2*$^{-/-}$ mouse liver. 2 nM cBid 30C (p7, *white bars*) or 126C (tBid, *black bars*) labeled with Alexa Fluor 647 was incubated with 1 mg/ml mitochondria with or without Mtch2 (as indicated) for 20 min at 37 °C. The percentage of cBid bound to mitochondria was measured using fluorescence intensity distribution analysis by comparing the concentration of objects with a specific brightness equal to that of a single Bid molecule (presumably free protein), with the concentration of objects with a higher specific brightness (mitochondria with more than one bound fluorescent cBid molecule). Because a small number (1–4) of cBid molecules on a mitochondrion results in a specific brightness close to or equal to that of a single Bid and is considered as "free protein," this analysis inherently underestimates the amount of bound protein. *E*, the conformational change in cBid upon binding to mitochondria is delayed in the absence of Mtch2. cBid 163C NBD (2 nM) was added to mitochondria (1 mg/ml) from *mtch2*$^{+/+}$ or *mtch2*$^{-/-}$ mice, and the change in NBD fluorescence that occurs when the protein binds to membranes is expressed as $F(t)/F0$ (mean ± S.E., $n = 3$).

regions of tBid have intermediate fractions of protein with τ_1 and τ_2, indicating that these regions are in more than one conformation at the membrane. Taken together, these results indicate that the conformational change in tBid triggered by binding to membranes is a complex process that involves unfolding of the N- and the C- terminal regions of the protein and the insertion of helices α 4–7 into the membrane.

Mtch2 Facilitates tBid Binding to Membranes by Accelerating the Conformational Change—The MOM protein Mtch2 has emerged as an important factor regulating the ability of tBid to induce apoptosis (25, 46). Mtch2 is a novel protein integral to the MOM and is related to the members of the mitochondrial carrier protein family. Previous work suggested limited binding of tBid to mitochondria lacking Mtch2 when assessed by differ-

ential centrifugation (25). To determine the exact step that is facilitated by Mtch2, we compared cBid binding to mitochondria isolated from *mtch2*$^{+/+}$ and $^{-/-}$ mouse livers (40). As anticipated, the mitochondria lacking Mtch2 were less susceptible to tBid-mediated MOMP by a process that was inhibited by Bcl-XL (Fig. 7*A*).

To further elucidate the molecular mechanism involved, we used the fluorescence-based assays described above to compare the molecular mechanisms of cBid binding to and permeabilizing membranes from mitochondria with and without Mtch2. Potential differences in the separation of the two fragments of cBid in these mitochondria were assessed using the FRET assay described above (Fig. 4). Although the rate and the extent of fragment separation in mitochondria lacking Mtch2 was slightly compromised compared with mitochondria with Mtch2 (detected as higher % FRET efficiency between the dyes in the two fragments) (Fig. 7*B*), the separation of p7 and tBid was extended by only about ~10 s. Therefore, neither initial interaction with the membrane nor fragment separation accounts for the functional defect in MOMP observed in the absence of Mtch2 (Fig. 7*A*).

To observe binding of cBid to mitochondria directly rather than by using biochemical methods, we measured protein binding to mitochondria by FRET. As an acceptor, the MOM was labeled with the dye DiI, and cBid 126C labeled with Alexa Fluor 488 was used as a donor. cBid binding proceeded to a lesser extent to the MOMs without Mtch2 compared with Mtch2 (Fig. 7Ci), but the overall decrease in binding was relatively small compared with the decrease in MOMP (Fig. 7*A*). Furthermore, using fluorescence intensity distribution analysis to calculate protein binding to mitochondria, we observed ~50–70% binding of tBid and ~5% binding of the p7 fragment labeled with Alexa Fluor 647 to mitochondria with and without Mtch2 (Fig. 7*D*). However, when the mitochondria were pelleted and then resuspended, the amount of cBid 126 Alexa488 that remained bound to mitochondria lacking Mtch2 was very small (Fig. 7*Cii*). The simplest explanation for these data is that in the absence of Mtch2 cBid binds to but does not insert into the MOM to the same extent.

Therefore, we investigated the relative changes in the conformation of tBid upon binding to the MOM by adding cBid 163C NBD to mitochondria with and without Mtch2 and monitoring changes in NBD intensity over time. With mitochondria containing Mtch2, we observed an increase in cBid 163C NBD fluorescence indicative of the protein inserting residue 163 into the membrane. However, with mitochondria lacking Mtch2, there was a small initial decrease due to a decrease in the hydrophobicity of the residue, perhaps due to the separation of the p7 fragment. This is followed by fluctuations in the signal indicating that during the first 15 min, residue 163 of tBid did not move to a more hydrophobic environment (Fig. 7*E*). A slow increase in NBD fluorescence was visible after that, indicating tBid binding to mitochondria independent of Mtch2. Thus in mitochondria, Mtch2 greatly accelerates the conformational change in tBid shown here to be required for insertion into the MOM and essential for the activation of tBid.

Knockdown of Mtch2 Causes a Significant Delay between Bid and Bax Relocalization in Cells—Our data suggest that when cBid binds to membranes, the two fragments separate rapidly, but the Mtch2-facilitated conformational change that is required for tBid to recruit Bax is much slower. Thus, a clear prediction of our results is that in cells, knockdown of Mtch2 would delay recruitment of Bax to mitochondria without significantly affecting relocalization of Bid. To study the effect of Mtch2 on tBid and Bax relocalization in cells during apoptosis, we knocked down Mtch2 protein expression using an shRNA against Mtch2 in HeLa cells stably expressing YFP-Bax. Apoptosis was induced in WT and Mtch2 KD HeLa cells expressing YFP-Bax by treatment with TNFα and CHX for 8 h, as it is well established that this cell death pathway proceeds via activation of Bid by caspase 8-mediated cleavage. YFP-Bax can be imaged directly by fluorescence microscopy; however, to visualize endogenous Bid, treated cells were fixed and immunostained. Representative fluorescence images showing the localization of Bid and YFP-Bax in untreated (CHX alone) and treated (TNFα and CHX) cells are shown in Fig. 8, *Ai* and *Aii*. Using automated high content image acquisition in separate channels for YFP-Bax and Bid, >2000 cells per time point were analyzed to yield objective and quantitative measurements of changes in the distribution of the proteins over time. A threshold adjacency statistic feature that gave the largest relative change between untreated and treated WT cells while retaining equivalent background values for the untreated cell lines (WT and Mtch2 KD) was visually verified to report the relocalization of Bid and Bax in cells. The values of this Threshold Adjacency Statistics feature increase as the protein localization progresses from a uniform distribution (corresponding to a cytoplasmic protein) to a punctuated pattern (corresponding to mitochondria-bound protein) (Fig. 8*A*). Graphic representation of the relocalization of Bid and Bax in WT and Mtch2 KD HeLa cells is shown in Fig. 8, *B* and *C*, respectively. It is apparent in WT HeLa cells that as time proceeds, the Bid and Bax fluorescence signals become more punctuated indicative of mitochondrial localization simultaneously (Fig. 8*B*). This is more evident for YFP-Bax relocalization as indicated by the differences in the *y* axis scales shown in Fig. 8, *B* and *C*. Notably, in Mtch2 KD cells, Bid relocalization is as efficient as it is in the WT cells, whereas Bax relocalization is significantly delayed (Fig. 8*C*). This observation in intact cells is entirely consistent with results from isolated mitochondria (Fig. 7). When directly comparing the relocalization of Bid and Bax, the rate of change for both is synchronized in the presence of Mtch2 indicated by the linear positive correlation between Bid:Bax relocalization, whereas Bax relocalization is relatively delayed in the absence of Mtch2 (Fig. 8*D*). This supports the concept that the recruitment of tBid to membranes is not sufficient for the activation of tBid in cells, and that subsequent to cBid binding to MOM, Mtch2 accelerates tBid activation. Based on our *in vitro* results, we ascribe tBid activation to a conformational change in tBid.

DISCUSSION

We have examined the sequence of conformational changes that occurs when cBid interacts with membranes and propose a model for cBid activation subsequent to cleavage between the

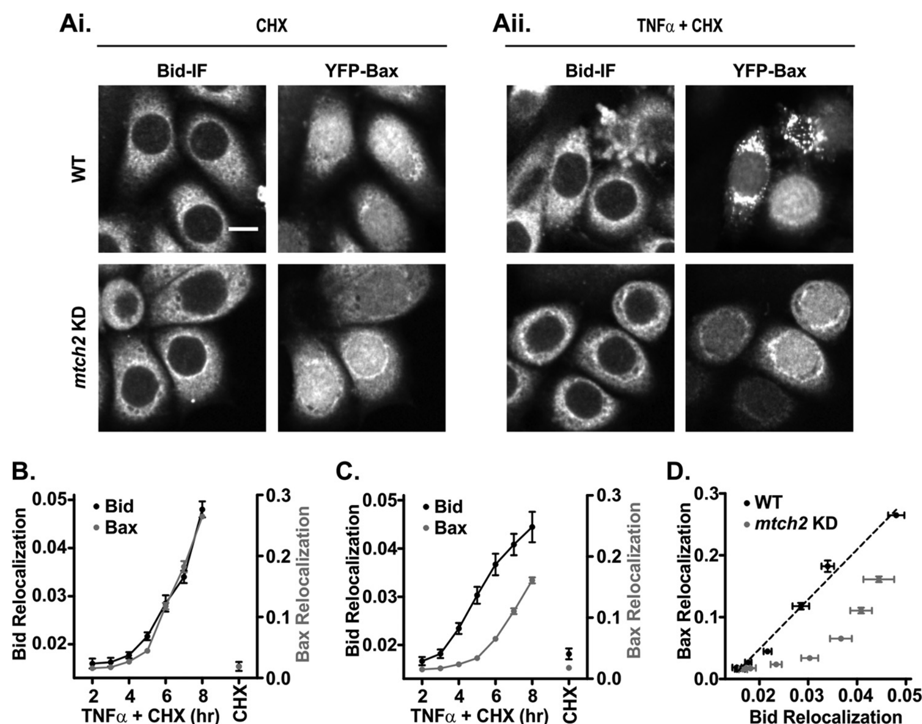

FIGURE 8. **Down-regulation of Mtch2 delays Bax relocalization subsequent to Bid relocalization in cells.** *A*, representative fluorescence images show the relocalization of Bid and YFP-Bax in WT and Mtch2 KD HeLa cells. Bid-mediated apoptosis was induced by treatment of the cells with TNFα and CHX in WT and Mtch2 KD cells. Treated cells were fixed at the end of the experiment and were immunostained for the endogenous Bid. *Panel Ai* shows the untreated cells (10 μg/ml CHX alone) at the 8-h time point, and panel *Aii* shows treated cells (1 ng/ml TNFα and 10 μg/ml CHX) at a 4-h time point. *Scale bar* = 10 μm. *B* and *C*. Relocalization of tBid was unaffected, but Bax relocalization was delayed in Mtch2 KD HeLa cells. WT (*B*) and Mtch2 KD HeLa cells (*C*) stably expressing YFP-Bax were treated as in *A*. Using high throughput image-based screening of images recorded in the YFP and the Alexa Fluor 595 channel of both cell lines, >2000 cells were analyzed for each TNFα + CHX treatment time. Cells were treated with CHX alone for 8 h. Relocalization of Bid (*black*) and Bax (*gray*) was measured by using a Threshold Adjacency Statistics feature that gave the largest relative change between the untreated and treated WT cells while retaining equivalent background values for both untreated cell lines (WT and Mtch2 KD). Connecting lines are included as a visual aid only. Each point is an average of 800 cells: 200 cells/independent experiment (*error bars*, S.E., $n = 4$). *D*, Bax relocalization is delayed relative to tBid relocalization in Mtch2 KD (*gray*) compared with WT (*black*) HeLa cells. The *black dotted line* is a linear line fit to the WT data.

p7 and the tBid fragments (Fig. 9). In the first step subsequent to cleavage, interaction with a membrane (liposome or mitochondrial) results in rapid separation of the fragments (Figs. 4, 7*B*, and 9*A*). After the initial binding to membranes, tBid undergoes an elaborate conformational change (Figs. 5, 6, 7*E*, and 9, *B* and *C*), facilitated by Mtch2 (Figs. 7 and 8) that results in the protein anchoring in the membrane. Although it is possible that tBid adopts a single unique conformation on the membrane, our data favor a situation in which at least two main conformers are in equilibrium on the membrane (Fig. 6). We show that the conformational change is a prerequisite for activation of Bax or Bak to elicit membrane permeabilization (including MOMP) (Figs. 1, 3, and 8). Therefore, Bax must be binding to the membrane-inserted conformation of tBid. This result is consistent with previous data showing that only the membrane-bound form of tBid binds Bax (apparent K_d 25 nM (10)).

The fast binding of cBid to the MOM (leading to the separation of the p7·tBid fragments) and the conformational change of tBid at the MOM recapitulated what we observed with liposomes (Fig. 7). Although cBid binding to mitochondria was independent of Mtch2 in enriched mitochondria and intact cells, the conformational change and tBid-dependent recruitment of Bax were delayed in the absence of Mtch2 (Figs. 7 and 8). Therefore, tBid undergoes a conformational change after binding to the MOM that is necessary for tBid activation and subsequent recruitment of Bax to the MOM leading to MOMP.

Previous studies have suggested that specific post-translational modifications are required for the activation of tBid, such as myristoylation of the tBid fragment (22) and "unconventional" ubiquitination and degradation of the p7 fragment (43). Using our *in vitro* approach with defined components, our results suggest that such modifications are not obligatory for the activation of tBid at the MOM. Furthermore, the very rapid spontaneous separation of the two fragments of cBid argues that post-translational modifications of tBid are unlikely to contribute to the separation of the fragments in a biologically significant manner.

Examining the kinetics with which individual cysteine residues at various locations in tBid interact with the lipid bilayer leads us to postulate that the unfolding of tBid at the membrane begins with the interaction of α-helices 4, 5, and 8 with the membrane (Fig. 9*B*) and is followed by insertion of α-helices 6 and 7 in the hydrophobic core of the membrane (Figs. 5*D* and 9*C*). Furthermore, the fluorescence lifetimes obtained for the NBD labels in the N- and C-terminal region of cBid with liposomes are well fit by a model containing substantial fractions of molecules with both an aqueous and a hydrophobic lifetime for NBD (Fig. 6). When the protein bound to membranes, the lon-

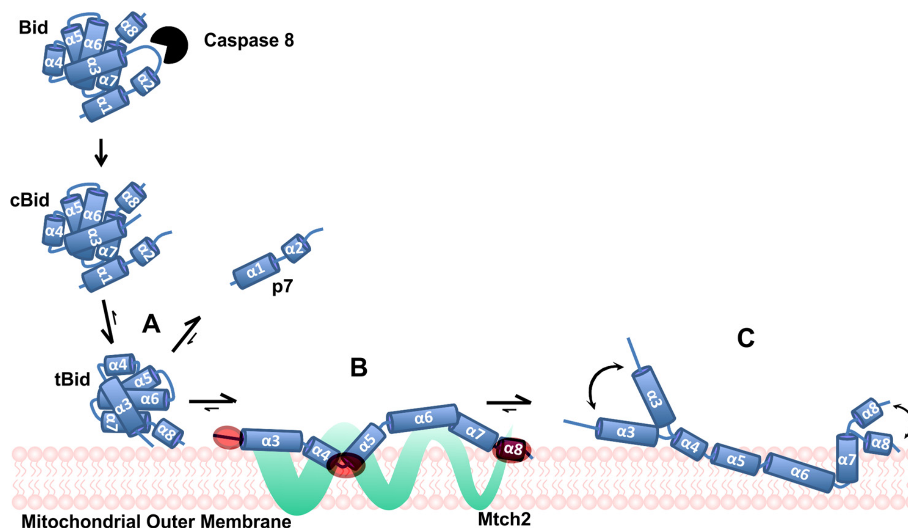

FIGURE 9. **Proposed activation mechanism for tBid.** Upon receiving an apoptotic signal via a death receptor, caspase 8 is activated and cleaves full-length Bid. In solution, the two fragments of cBid remain bound as a complex due to strong hydrophobic interactions. At the MOM, the p7 and the tBid fragments separate very quickly upon the initial binding of tBid to the MOM (*A*). Mtch2 acts as a receptor for tBid and catalyzes a conformational change in the protein (from the transient intermediate form *B* to the membrane-integrated form) (*C*) by anchoring the membrane binding helices in the membrane. As suggested by NBD lifetime data, two co-existing membrane-bound conformers are shown, although more different conformations may exist. The position of helices with respect to the membrane in *B* and *C* is putative but is in agreement with our data and with previously published studies (32, 39). The regions of tBid that are the first to interact with the membrane in the absence of Mtch2 (*highlighted in red*) also interact with Mtch2 (*B*). The conformational change in tBid leads to two plausible membrane-bound tBid conformers that are in equilibrium on the MOM, as suggested by the *curved arrows* (*C*). It is not clear whether Mtch2 remains bound to one or both conformers at the membrane.

ger lifetime and its corresponding fraction both dropped substantially for several of the residues in the N-terminal region consistent with this part of the protein unfolding (Fig. 6). However, under these conditions, our binding studies suggest that most of the protein is bound to the membrane (Fig. 2). Therefore, the simplest explanation for these data is that for these residues only half of the membrane-bound protein is in a hydrophobic environment that indicates fluctuations in the structure of the protein at the membrane. Together these results suggest that the labeled tBid exists as two different membrane-bound conformers. Although this is the simplest explanation, our data are also compatible with tBid having a large number of conformations on the membrane. However, we propose the simplest possible scenario where two membrane-inserted conformers of tBid are in equilibrium; one where the N- and C- terminal regions are present at the interface of the membrane (solvent-exposed), and the other where they interact with the membrane (hydrophobic) (Fig. 9*C*). Clearly, more extensive characterizations of this equilibrium will be required to understand the potential functional differences between the two different conformations of tBid at the membrane. Furthermore, the exact arrangement of the helices with respect to the membrane remains elusive.

One obvious possibility is that the fast membrane insertion of purified tBid is due to the incubation in octylglucoside, which is required for isolating tBid from the p7 fragment, substitutes for the time required for this transformation to occur *in situ*. Aptly, as a detergent, octylglucoside may mimic the environment of a membrane to facilitate the conformational change necessary for full activation of tBid.

Recently the regions of tBid and Mtch2 that mediate this protein-protein interaction have been identified (42). Peptides corresponding to residues close to the N terminus of tBid (59–73), surrounding the loop between α-helices 4 and 5 (111–125), and in α-helix 8 (181–191) bound to peptides from two separate regions of Mtch2 (Fig. 9, highlighted by *red* regions). Remarkably, the NBD kinetics of the single cysteine mutants (106, 126C, 186C, and 190C) close to these identified regions show the fastest half-times, suggesting that these sites are also the first to interact with the membrane. In addition, the finding that p13/p11 N-terminal cleaved product tBid is only generated in mtch2$^{+/+}$ but not in mtch2$^{-/-}$ cells (24) strongly supports that Mtch2 is essential for the conformational change in tBid during which the N terminus becomes solvent-exposed (Fig. 5) and accessible for cleavage (44). These results are completely consistent with our experiments in cells demonstrating that cBid migration to the MOM during apoptosis is not affected by Mtch2 knockdown, whereas Bax recruitment was significantly delayed. Taken together, our data indicate that the conformational change of tBid at the MOM is required for tBid activation and subsequent recruitment of Bax to the MOM. Whether Mtch2 binds to all tBid conformers or is reciprocal with binding of other Bcl-2 family proteins remains to be determined.

Our data suggest that α helices 4–7 of tBid become embedded in the membrane during the conformational change. We along with others have observed a similar change in the analogous helices of Bax that occurs during activation (7), further suggesting that Bid is more similar to Bax than to other BH3 proteins. Additionally, it is clear that tBid and Bim also recruit cytosolic anti-apoptotic proteins such as Bcl-XL to the MOM, where these proteins mutually sequester each other (37). Competitive binding between different members of the Bcl-2 family proteins, therefore, causes reciprocal changes in conformations between tBid and both other classes of Bcl-2 proteins. We pro-

pose that having multiple conformers is one way that a small protein can expose different sites to bind to different protein partners. Moreover, we propose that the effect of membrane binding on the conformation of tBid may extend to other Bcl-2 family proteins including those that are intrinsically disordered (*e.g.* Bim and Bad). For example, the unstructured BH3 region of Bim adopts a helical conformation upon binding to Bcl-XL (47). Studies to test these hypotheses and the functional consequences of the different conformations of tBid on the regulation of MOMP will be the subject of future detailed investigations.

We have identified three individual steps of activation of tBid on the MOM using two *in vitro* model systems (Fig. 9). Our results highlight the different steps that can potentially be regulated by post-translational modifications or small molecules during the initiation of MOMP by the activator tBid. After cleavage, the p7 and tBid fragments quickly separate at the MOM, where the conformational change in tBid is facilitated by its interaction with Mtch2. Because this conformational change is a prerequisite to Bax activation and MOMP, its modulation opens a new avenue for small molecule targeting to promote or inhibit MOMP.

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CHAPTER IV

Effect of lipid composition on membrane permeabilization by Bax when activated by tBid or Bim

Preface

The work presented in this chapter has been prepared for submission in *The Journal of Biological Chemistry* as a research article.

Shamas-Din, A., Bindner, S., Chi, X., Leber, B., Andrews, D. W. and Fradin, C. (2014) Effect of lipid composition on membrane permeabilization by Bax when activated by tBid or Bim.

Contribution of authors:

Shamas-Din, A carried out all the experiments, prepared the figures and tables, and wrote the manuscript that was edited by Fradin, C, Leber, B and Andrews, DW. Bindner, S carried out preliminary experiments and experiments summarized in Table 4. Chi, X provided purified Bim protein variants. Andrews, DW, Fradin C and Leber B directed the research of the project.

Research Objective:

To investigate the differences and similarities in the dependence on lipid composition of membrane permeabilization mediated by Bax when activated by either tBid or Bim.

Research Highlights:

- Both tBid and Bim activate Bax in liposomes and isolated mitochondria in a dose-dependent fashion.
- Cholesterol inhibits membrane permeabilization by inhibiting both Bax interaction with the activator proteins and Bax insertion in the membrane.
- Anionic lipids are necessary for the binding of tBid and Bim to the membrane.
- tBid requires cardiolipin at the membrane to undergo the conformational change that later allows Bax activation, whereas the activity of Bim is independent of cardiolipin.
- The distinct dependence of tBid and Bim on lipids is potentially a new target to modulate activator specific apoptosis.
- Lipids impact the two rate-limiting steps in membrane permeabilization: cardiolipin is required for the conformational change in tBid, whereas cholesterol hinders Bax binding to the activators and the insertion of Bax in membranes.

Effect of lipid composition on membrane permeabilization by Bax when activated by tBid or Bim*

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***Running title:** Distinct lipid requirements of tBid and Bim for Bax activation

Keywords: Apoptosis, Bcl-2 proteins, Bax, tBid, Bim, cardiolipin, cholesterol, fluorescence

CAPSULE

Background: tBid and Bim activate Bax to permeabilize mitochondria and induce apoptosis.

Results: Binding of tBid and Bim to membranes is regulated by electrostatic interactions, however tBid requires cardiolipin to adopt a membrane-inserted active conformation.

Conclusion: Both cardiolipin and Mtch2 facilitate the conformational change in tBid.

Significance: The distinct dependence of tBid and Bim on lipids is a novel avenue to modulate activator specific apoptosis.

ABSTRACT

After exposure to stressful stimuli, apoptotic signals are relayed to mitochondria through Bcl-2 pro-apoptotic activator proteins tBid and Bim, which activate Bax/Bak to induce mitochondrial outer membrane permeabilization (MOMP), an event that commits a cell to apoptosis. Thus protein-protein and protein-membrane interactions are both critical in mediating this process. While the distinct roles of tBid and Bim as sensors of different types of stress are well recognized, a comparison of the mechanisms through which they initiate MOMP is yet to be established. In this study we compare membrane permeabilization by Bax when activated by either tBid or Bim and we examine the role of mitochondrial outer membrane lipids in the recruitment and activation of these three Bcl-2 pro-apoptotic proteins. We employ fluorescently labeled proteins and liposomes, to study the effects of specific lipids on each of the well-characterized steps in Bax-mediated membrane permeabilization. First, we refine previous observations that high levels of cholesterol in the membrane inhibit permeabilization by categorically identifying the recruitment of Bax by the activators and Bax insertion in the membrane as the steps being hindered by cholesterol. Second, we show that binding of both tBid and Bim to membranes is regulated by electrostatic interactions with anionic phospholipids. While Bim does not require any particular anionic lipids, the conformational change leading to the membrane-inserted active form of tBid is specifically dependent on the presence of cardiolipin. This suggests that Mtch2 and cardiolipin engage in a common mechanism to facilitate this step. Thus, lipids modify multiple aspects of Bax mediated membrane permeabilization.

INTRODUCTION

Bcl-2 proteins play a pivotal role in apoptosis by regulating mitochondrial outer membrane permeabilization (MOMP)[6], the first event that commits a eukaryotic cell to undergo apoptosis and thus considered the "point-of-no-return" for cell survival (1-3). Bcl-2 proteins are sub-divided into anti- and pro-apoptotic based on the role they play in opposing or promoting cell death. Upon receiving an apoptotic stimulus, pro-apoptotic Bcl-2 activator proteins are post-translationally modified. For example, Bid is cleaved to produce tBid (truncated Bid) and Bim is phosphorylated. As a result, activators translocate to the mitochondrial outer membrane (MOM) (4-7). There they recruit the pore-forming pro-apoptotic proteins, Bax and Bak, which oligomerize to permeabilize the MOM to release cytochrome *c*, Smac and AIF in the cytoplasm (8-12). This process activates caspases that cleave multiple substrates resulting in cell death.

The interplay between the different Bcl-2 proteins regulating MOMP involves dynamic protein-protein and protein-membrane interactions (13-15). Many Bcl-2 proteins undergo conformational changes upon activation and binding to the MOM, which allow them to perform multiple cellular functions (16-21). Previous reports have suggested that different lipids regulate Bcl-2 proteins and MOMP (22,23). Mitochondrial phospholipid content is dominated by phosphatidylcholine (PC) and phosphatidylethanolamine (PE), which contribute 45-50% and 27-30% of the total lipid content, respectively. Phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidic acid each contribute 5-10%. Cardiolipin (CL) contributes up to 15% of the total lipid. The lipid composition of the MOM is very similar to that of the whole mitochondria, with the notable exception that the MOM contains less than 3% of CL (24,25).

The anionic phospholipid exclusive to mitochondria, CL has been implicated in the recruitment and activation of both tBid and Bax at the MOM (26-32). However, several reports have dismissed the obligatory role of CL at the MOM for the induction of MOMP as over 95% of CL is present in the mitochondrial inner membrane (24,33). In fact, the overall negative charge of the lipids in a liposome, rather than the individual lipids was reported to promote tBid targeting to the membrane (34) and the interaction of Bax C-terminus with the membrane (35). It was also proposed that tBid relocated to the MOM during apoptosis, despite a progressive loss of CL, by displaying enhanced binding to a metabolite of CL, monolysocardiolipin (MLCL) (36). It remains unclear if the small amount of CL present at the contact sites between the inner and outer mitochondrial membranes is sufficient and necessary to efficiently recruit tBid and Bax. Since targeting of tBid to the MOM must precede the assembly of Bax pores in the membrane (8,16), the specific interaction of tBid and CL at the MOM possibly influences a crucial checkpoint of apoptosis.

Although only ~5% of the total cellular cholesterol resides in the mitochondria, it plays a major role in regulating the integrity of the membranes during MOMP (25,37). At the mitochondria, cholesterol contributes 3-5% of total lipid mass, and most of it is present at the MOM (25). Enhanced mitochondrial cholesterol in cancer cells increases resistance to chemotherapy and Bax mediated MOMP. On the other hand, lowering the cellular cholesterol content induces p53 dependent activation of Bax and sensitizes these cancer cells to apoptosis (38,39). Cholesterol is well known to control the fluidity of lipid

membranes, and it is likely that this effect contributes to its inhibition of Bax-mediated MOMP. However it is not clear if cholesterol achieves MOMP inhibition by directly hindering the insertion of Bax in the membrane as was suggested in previous studies (40,41), or by influencing a step upstream of Bax insertion.

While the molecular mechanism of recruitment and activation of tBid and Bax at the MOM has been studied in considerable detail, not much is known about the interaction of Bim with different lipids present at the MOM. Both tBid and Bim induce Bax/Bak mediated apoptosis, however Bim preferentially binds to Bax, whereas tBid preferentially binds to Bak (42). In addition, whereas tBid shares its three-dimensional fold with Bax and Bcl-XL, Bim is intrinsically unstructured and evolutionary distant from tBid (43-45). We therefore asked whether this structural disparity resulted in different mechanisms of activation for these two proteins. To answer this question, we compared their interactions with membranes and with Bax, and we investigated whether modifying the lipid composition of the membrane could disrupt these interactions.

Complete knock down of essential lipids in the cells is not possible for cell survival, while experiments with incomplete knock down have been inconclusive for their role in MOMP (46). Therefore, to rigorously examine these issues, we employed a well-established system of purified full-length proteins and liposomes that authentically reproduces the core features of MOMP. Furthermore, we used fluorescence techniques to dissect the individual steps in the mechanism of pore formation in membranes containing different lipid compositions. See Table 1 for a summary of the properties of the lipids used in this study. We found that anionic phospholipids in the membrane are essential for binding of both tBid and Bim to the membrane. Interestingly, while CL is dispensable for the initial binding of tBid to the membrane, it is necessary to promote the conformational change in tBid. Furthermore, an increase in levels of cholesterol in the membrane impedes Bax activation by the activators at the membrane and Bax insertion in the membrane to inhibit membrane permeabilization.

TABLE 1. Physical properties of lipids used in assays.

Lipids	Source	Predominant Species	Charge	Curvature
PC	Egg	16:0/18:1	Neutral	Zero
PE	Egg	16:0/18:1	Neutral	Negative
PI	Bovine liver	18:0/20:4	1x Negative	Zero
PS	Synthetic	18:1/18:1	1x Negative	Negative
CL	Synthetic	18:1/18:1/18:1/18:1	2x Negative	Negative
MLCL	Bovine heart	18:2/18:2/18:2	2x Negative	Zero
PG	Egg	16:0/18:1	1x Negative	Zero
Cholesterol	Plant derived	NA	Zero	Negative

RESULTS

tBid and Bim induce Bax dependent membrane permeabilization in mitochondria and liposomes

To compare the activity of tBid and Bim in catalyzing Bax mediated membrane permeabilization, we tested the activators using *bak-/-* isolated mitochondria. There was a dose-dependent effect of sub nano-molar concentrations of tBid or Bim on the percentage of cytochrome *c* release in the presence of Bax, although tBid was active at lower concentrations (Fig. 1A). Next we carried out the titration of tBid or Bim with Bax using liposomes encapsulating both a fluorophore (ANTS) and a quencher (DPX) as a model system to study membrane permeabilization (47,48). As seen with isolated mitochondria, both tBid and Bim directly activated Bax to permeabilize the membrane to a comparable extent and in a dose-dependent manner (Fig. 1B).

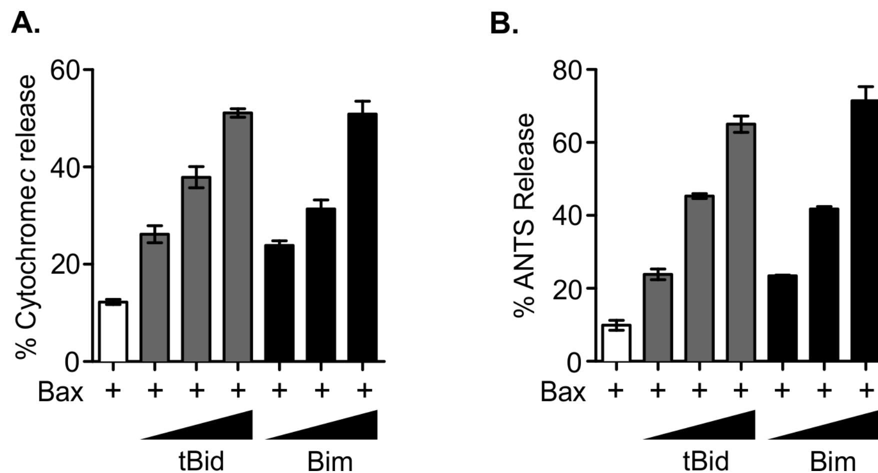

FIGURE 1. tBid and Bim directly activate Bax in a dose-dependent manner. (A) Membrane permeabilization assay in mitochondria isolated from *bak* -/- mice to quantify the release of cytochrome *c*. Mitochondria (1 mg/ml) were incubated with either 50 nM Bax alone or with increasing concentration of 20, 60 and 400 pM of tBid (Grey) or of 100, 300 and 2000 pM of Bim (Black). Cytochrome *c* release was measured by comparing the amount of protein in the pellet and the supernatant as quantified by immunoblotting (mean ± SEM, n = 3). **(B)** Membrane permeabilization in liposomes encapsulating ANTS and DPX. Liposomes were incubated with either 100 nM Bax alone or with increasing concentration of 1, 3 and 20 nM of tBid (Grey) or of Bim (Black), (mean ± SEM, n = 3).

TABLE 2. Nomenclature and lipid composition of liposomes used in assays.

Fig. No.	Name	Average negative charge/ Lipid	mol % composition							
			PC	PE	PI	PS	CL	ML CL	PG	Chol ester ol
Fig. 1	Mitochondria-like	0.28	48	28	10	10	4			
Fig. 2	8% Chol	0.28	40	28	10	10	4			8
	20% Chol	0.28	28	28	10	10	4			20
Fig. 3	Low charge	0.10	62	28	10					
	No charge	0	72	28						
Fig. 4	24% PS	0.24	48	28		24				
	14% PS	0.24	48	28	10	14				
	10% PS	0.20	52	28	10	10				
	0% PS, 4% CL	0.18	58	28	10		4			
Fig. 5	4% MLCL	0.28	48	28	10	10		4		
	14% PG	0.24	48	28	10				14	
	24% PI	0.24	48	28	24					

Cholesterol inhibits Bax membrane permeabilization by impeding the interaction of activators with Bax and its subsequent insertion in the membrane

Cholesterol-induced changes in membrane structure and/or dynamics affect the binding of tBid and Bax to liposomes and isolated mitochondria (38,40,41). To further characterize the mechanism by which cholesterol regulates the membrane permeabilization by Bcl-2 proteins, we prepared liposomes with two different amounts of cholesterol: 8% for moderate mitochondrial levels of cholesterol (8% Chol) and 20% for even higher mitochondrial levels of cholesterol (20% Chol) (See Table 2 for details on membrane composition) (37). In agreement with previous results, high cholesterol levels (20% Chol) caused a significant reduction in the final membrane permeabilization efficiency measured by ANTS release, when compared to liposomes with mitochondria-like composition, irrespective of whether Bax was activated by tBid or Bim (Fig. 2A).

To determine which step of Bax pore formation was influenced by cholesterol, we employed a dedicated fluorescence based assays to probe the efficiency of each step separately (8). First we tested to see if the presence of cholesterol had an effect on tBid or Bim binding to the membrane using a protein to membrane FRET assay. DAC labeled tBid or Bim was used as a donor, while liposomes were labeled with the acceptor fluoro-

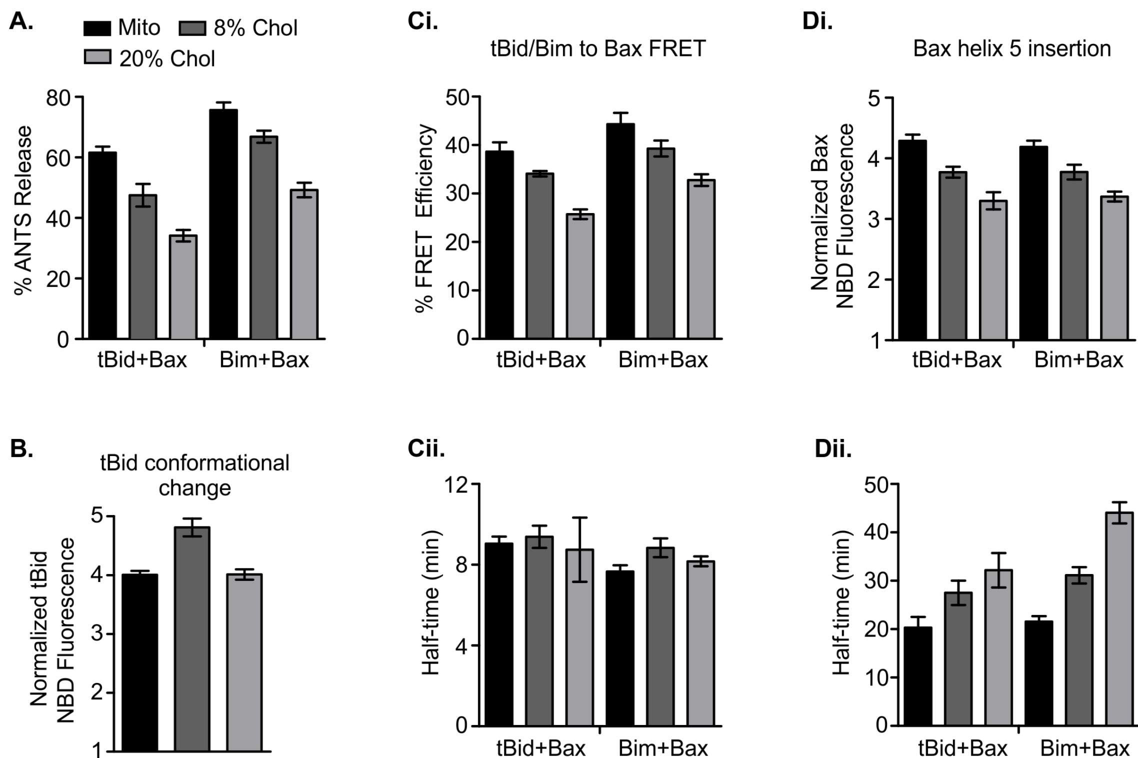

FIGURE 2. Cholesterol impedes membrane permeabilization by hindering Bax activation and insertion into the membrane. (A) Liposomes encapsulated with ANTS and DPX in mitochondria-like (Mito) and in 8% and 20% cholesterol (8% Chol and 20% Chol, respectively) lipid composition were used to assay membrane permeabilization using 80 nM Bax and 20 nM of either tBid or Bim, (mean ± SEM, n = 3). **(B)** tBid conformational change as assayed by an increase in the NBD fluorescence reflecting the residue moving to a more hydrophobic environment (membrane). 20 nM of tBid 163 NBD was added to 1.5 nM liposomes of indicated lipid composition, (mean ± SEM, n = 5). **(C)** Interaction of Bax to membrane-bound tBid or Bim using FRET. 20 nM of tBid 126 DAC or Bim 104 DAC (donor) with 80 nM Bax 126 NBD (acceptor) were added to 1.5 nM liposomes with indicated lipid composition. A high percentage of FRET efficiency indicates more interaction between tBid-Bax or Bim-Bax **(Ci)**, and the half-time obtained from the fit of the data with a single-exponential function is shown in **(Cii)**, (mean ± SEM, n = 5). **(D)** Insertion of Bax in the membrane after activation. The change in the environment of the NBD-labeled residue of 80 nM Bax 126 NBD activated with tBid or Bim from the same reaction as **(C)**. A decrease in NBD intensity signifies less insertion of Bax in the membrane. The end-points of the data are shown in **(Di)**, whereas the half-time obtained from the fit of the data with a single-exponential function is shown in **(Dii)**, (mean ± SEM, n = 5).

phore, NBD. Binding of either tBid or Bim to the membrane was not altered by the addition of cholesterol, as indicated by minor changes in the protein to membrane binding dissociation constant, K_D (Table 3). This result is in contrast to a previous report stating that cholesterol enhances tBid binding to membranes, as assessed using a targeting assay with isolated mitochondria (41). However, in our assays we observed a higher level of conformational change for tBid at 8% cholesterol liposomes, but not in 20% cholesterol liposomes indicating that a moderate level of cholesterol may help with tBid conformational change (Fig. 2B).

Next we employed a FRET based protein-protein binding assay to determine the effect of cholesterol on tBid-Bax and Bim-Bax interaction. DAC-labeled tBid or Bim was used as a donor and NBD-labeled Bax as an acceptor. The extent of tBid-Bax and Bim-Bax interaction was notably lower in liposomes containing higher amounts of cholesterol, although the kinetics of these interactions remained largely unaffected (Fig. 2C). We next tested the extent of Bax insertion into membranes using NBD-labeled Bax. As shown in Fig. 2D, both the end-points and the kinetics of Bax insertion were drastically hindered by increased amounts of cholesterol in the membrane. High levels of membrane cholesterol can therefore impede both Bax interaction with activator proteins and Bax insertion in the membrane leading to lower Bax oligomerization and membrane permeabilization.

TABLE 3. Dissociation constants (K_D) and partition coefficient (P) associated with the binding of tBid and Bim to liposomes with different lipid compositions. The K_D is reported in liposome concentration (nM).

Fig. No.	Liposomes	tBid		Bim	
		K_D (nM)	10^3 P	K_D (nM)	10^3 P
Fig. 2-5	Mitochondria-like	0.76 ± 0.17	17.3 ± 3.9	0.36 ± 0.10	36.5 ± 10
Fig. 2	8% Chol	1.35 ± 0.27	9.7 ± 2.0	0.80 ± 0.25	16.4 ± 5.2
	20% Chol	1.20 ± 0.15	11.0 ± 1.4	0.75 ± 0.15	17.5 ± 3.5
Fig. 3	No charge	> 40	< 0.3	8.24 ± 3.92	1.6 ± 0.8
Fig. 4	14% PS	1.06 ± 0.22	12.4 ±2.6	0.68 ± 0.08	19.3 ± 2.3
Fig. 5	4% MLCL	1.91 ± 0.30	6.9 ± 1.1	0.29 ± 0.06	45.4 ± 9.4
	14% PG	3.50 ± 0.81	3.8 ± 0.9	0.42 ± 0.10	31.3 ± 7.5
	24% PI	7.31 ± 2.0	1.8 ± 0.5	0.98 ± 0.13	13.4 ± 1.8

Anionic lipids are required for binding of Bcl-2 activators to membranes

We tested the hypothesis that electrostatic interactions play a crucial role in the recruitment of Bcl-2 activators and the activation of Bax. We used liposomes with a lipid composition resulting in either a low average negative surface charge of 0.10 per lipid

(Low charge) or no negative charge (No charge) (Table 2). In agreement with our hypothesis, membrane permeabilization with 'Low charge' and 'No charge' liposomes was drastically reduced. This reduction was greater when tBid was used as an activator (Fig. 3A).

To determine at which point electrostatic interactions played a major role in the Bax-mediated permeabilization process, we again investigated each step separately. To assess binding of tBid and Bim to membranes, we employed the protein-membrane FRET assay described above. We obtained a K_D of 0.36 nM for Bim, and a K_D of 0.76 nM for tBid for mito-like composition (a value that is very close to our previously reported K_D of 0.9 nM using a different technique (16)) (Table 3). With 'No charge' liposomes, the binding of both tBid and Bim to the membrane was drastically reduced: the K_D for Bim to membrane increased more than 20 fold and the K_D for tBid to membrane increased over 50 fold, indicating a stronger dependence of tBid on the negatively charged lipids for binding to the membrane (Fig. 3B and Table 3).

Since the binding of the activator proteins to membranes with low negative charge is significantly compromised, as expected we observed low level of tBid-Bax and Bim-Bax FRET and Bax helix five insertion (Fig. 3C and D). In fact, the observed higher than expected Bim-Bax FRET values in Fig. 3C are due an elevated background of the percentage of FRET efficiency signal of ~20% originating from Bim-Bax FRET taking place in solution without liposomes (data not shown).

Cardiolipin accelerates the conformational change in tBid to mediate membrane permeabilization

To determine the specific effect of CL on Bax activation by tBid or Bim, liposomes were prepared by varying the amount of the neutral lipid PC and three different anionic lipids PI, PS and CL (Table 2). Fig. 4A shows a comparison between Bax mediated membrane permeabilization of liposomes with different compositions and average negative charge per lipid. The level of membrane permeabilization was comparable for liposomes with similar average negative charge per lipid, irrespective of the presence of CL and for either tBid or Bim activation. However, once again reactions where Bax was activated by tBid showed a higher sensitivity towards liposomes with lower average negative charge of the membrane. To see this effect more clearly, we combined the data from Fig. 3A and 4A and plotted the endpoints of the percentage of membrane permeabilization as a function of average negative charge per lipid (Fig. 6A). The resulting graph demonstrates that the increase in the overall percentage of membrane permeabilization is directly correlated with the average negative charge per lipid of the different liposomes. In addition, the disparity in sensitivity of tBid and Bim towards the negative surface charge of the membrane is clearly highlighted.

Using the 14% PS liposome composition that is without CL but contains higher amount of PS, we observed a similar K_D for tBid and Bim binding to the mitochondria-like and 14% PS membranes (Table 3). Next, we determined the extent of tBid conformational change with membranes of similar average negative charge but lacking CL. Interestingly, only the liposomes containing CL facilitated the conformational change

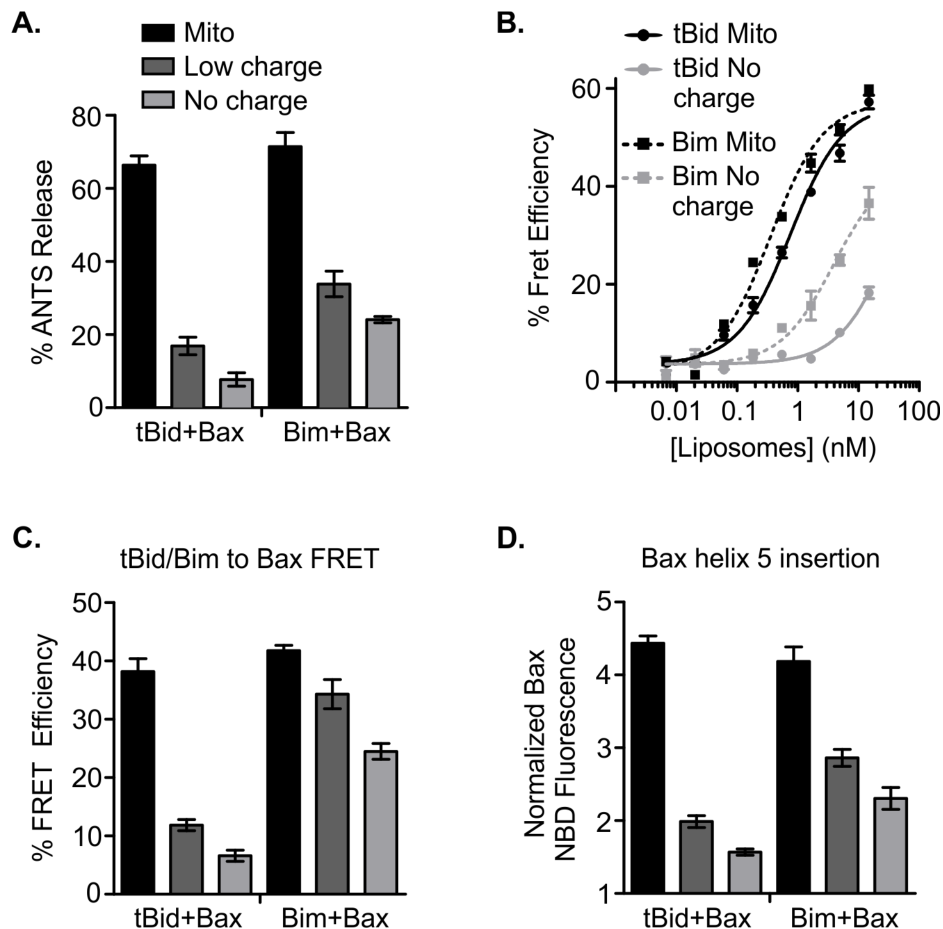

FIGURE 3. Binding of tBid and Bim to the membrane requires anionic lipids at the membrane. (A) Mitochondria-like (Mito), Low charge (with average negative charge/lipid of 0.10) and No charge (no negative charge/lipid) liposomes encapsulating ANTS and DPX were used to assay membrane permeabilization using 100 nM Bax and 20 nM of either tBid or Bim, (mean ± SEM, n = 3). **(B)** Binding of tBid and Bim to Mito (Black) and No charge (Grey) liposomes as measured by FRET. 20 nM of tBid 126 DAC (solid line) or Bim 104 DAC (dashed line) labeled with the donor dye were incubated with different concentrations of liposomes containing the acceptor, NBD-PE. An increase in the percentage of FRET efficiency indicates more binding of protein to liposomes. The data (dots/squares) are fit with an equation described in (16) (line) (mean ± SEM, n = 2) See Table 3 for the K_D values. **(C)** Interaction of Bax to membrane-bound tBid or Bim using FRET and **(D)** insertion of Bax in the membrane after activation. 20 nM of tBid 126 DAC or Bim 104 DAC (donor) with 100 nM Bax 126 NBD (acceptor) were added to 1.5 nM liposomes with indicated lipid composition. A high percentage of FRET efficiency indicates more interaction between tBid-Bax or Bim-Bax **(C)**, and a higher fold change in NBD intensity signifies more insertion of Bax in the membrane **(D)**, (mean ± SEM, n = 3).

in tBid (Fig. 4B) suggesting a direct role of CL in the conformational change step of tBid at the membrane.

Using the 14% PS liposome composition that is without CL but contains higher amount of PS, we observed a similar K_D for tBid and Bim binding to the mitochondria-like and 14% PS membranes (Table 3). Next, we determined the extent of tBid conformational change with membranes of similar average negative charge but lacking CL. Interestingly, only the liposomes containing CL facilitated the conformational change in tBid (Fig. 4B) suggesting a direct role of CL in the conformational change step of tBid at the membrane.

To measure the interaction between tBid-Bax and Bim-Bax, we used the protein-protein FRET assay and observed a correlation between lower endpoints and slower kinetics of the assay and lower average negative charge of the liposomes (Fig. 4C). This effect is due to inefficient binding of tBid and Bim to the membrane with lower average negative charge of the membrane, stressing the importance of anionic lipids for the initial steps of Bax mediated membrane permeabilization. When monitoring Bax helix five insertion, only the kinetics of the assay were slower and the end-point was not affected potentially due to the contribution of the auto-activation mechanism of Bax (Fig. 4D). Again, all the observed effects were inflated when Bax was activated with tBid, and subdued when it was activated with Bim.

Activation of Bax by Bim is independent of the identity of anionic phospholipids

To further investigate the mechanism of electrostatic interactions required for tBid and Bim binding to membranes, we prepared liposomes in which CL was replaced with either its metabolite MLCL, a predominant catabolic product of CL in mitochondrial membranes in healthy and apoptotic cells (36,49), or with PG or PI (Table 2). Using tBid or Bim to activate Bax, membrane permeabilization was assessed in these liposomes. Interestingly we found that membrane permeabilization was considerably compromised compared to mitochondria-like liposomes with tBid activated Bax, but only slightly impaired with Bim activated Bax in all of the liposome compositions tested (Fig. 5A).

To observe any change in tBid or Bim binding to membranes, we measured protein-membrane FRET and found that while the K_D of Bim binding to these membranes was not affected, the K_D of tBid binding to membranes with 14% PG and 24% PI increased four fold and ~ten fold, respectively (Table 3). As, the K_D of tBid binding to MLCL showed only a small increase, it was surprising that tBid did not undergo a conformational change with membranes containing MLCL, a lipid that is a metabolite of CL (Table 3 and Fig. 5B). In addition, tBid did not undergo the conformational change with liposomes containing PG or PI, thus displaying a very high specificity for CL (Fig. 5B).

Next, we tested the effect of MLCL, PG and PI on tBid-Bax and Bim-Bax FRET and observed minor differences in the end-points, but slower kinetics with membranes containing PG and PI for tBid-Bax, and with membranes containing PG for Bim-Bax (Fig. 5C). While the slower kinetics in tBid-Bax FRET for PI can be explained by inefficient binding of tBid to these liposomes, the slow kinetics with PG for both tBid and Bim may be due to a negative effect of PG on Bax. Interestingly, Bax helix five insertion

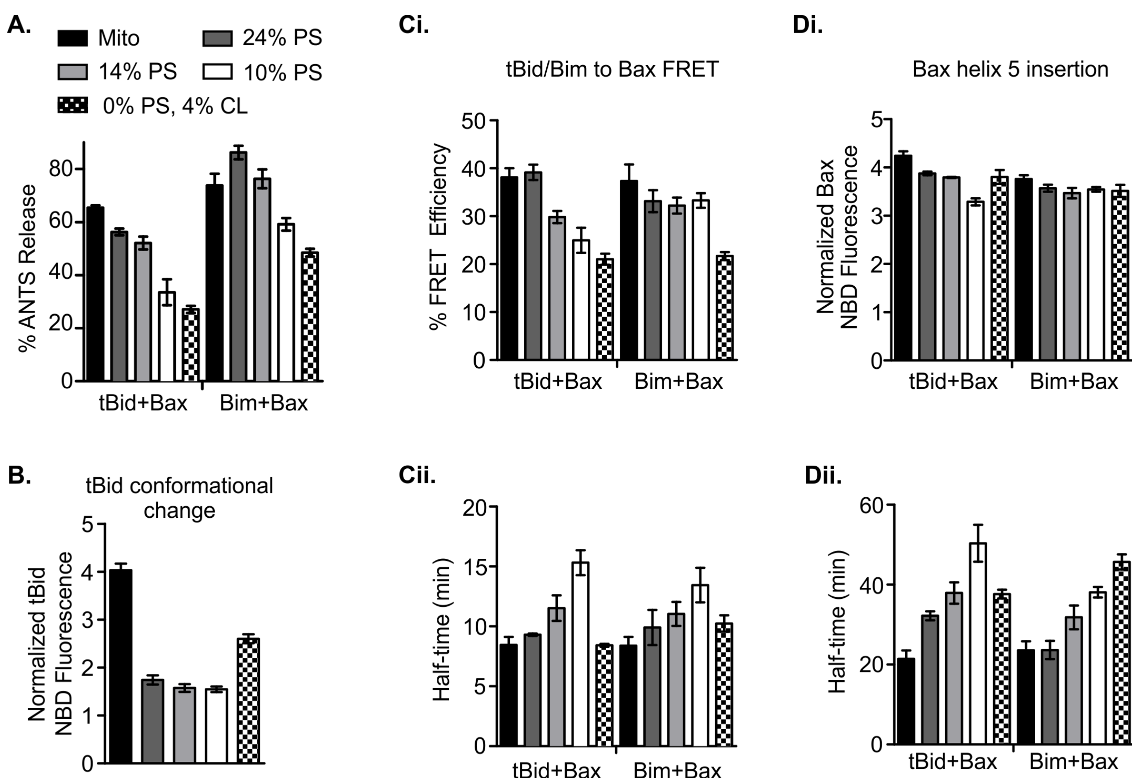

FIGURE 4. The effect of cardiolipin on membrane permeabilization is due to negative charge. (A) Liposomes encapsulating ANTS and DPX in mitochondria-like (Mito), 24% PS, 14% PS, 10% PS, and 0% PS 4% CL lipid compositions were used to assay membrane permeabilization using 100 nM Bax and 20 nM of either tBid or Bim, (mean ± SEM, n = 3). See Table 2 for the complete lipid composition. **(B)** tBid conformational change as assayed by an increase in the NBD fluorescence reflecting the residue moving to a more hydrophobic environment (membrane). 20 nM of tBid 163 NBD was added to 1.5 nM liposomes of indicated lipid composition, (mean ± SEM, n = 3). **(C)** Interaction of Bax to membrane-bound tBid or Bim using FRET. 20 nM of tBid 126 DAC or Bim 104 DAC (donor) with 100 nM Bax 126 NBD (acceptor) were added to 1.5 nM liposomes with indicated lipid composition. A high percentage of FRET efficiency indicates more interaction between tBid-Bax or Bim-Bax, and the half-time obtained from the fit of the data with a single-exponential function is shown in **(Cii)**, (mean ± SEM, n = 3). **(D)** Insertion of Bax in the membrane after activation. The change in the environment of the NBD-labeled residue of 100 nM Bax 126 NBD activated with tBid or Bim from the same reaction as **(C)**. A higher fold change in NBD intensity signifies more insertion of Bax in the membrane **(Di)**, whereas the half-time obtained from the fit of the data with a single-exponential function is shown in **(Dii)**, (mean ± SEM, n = 3).

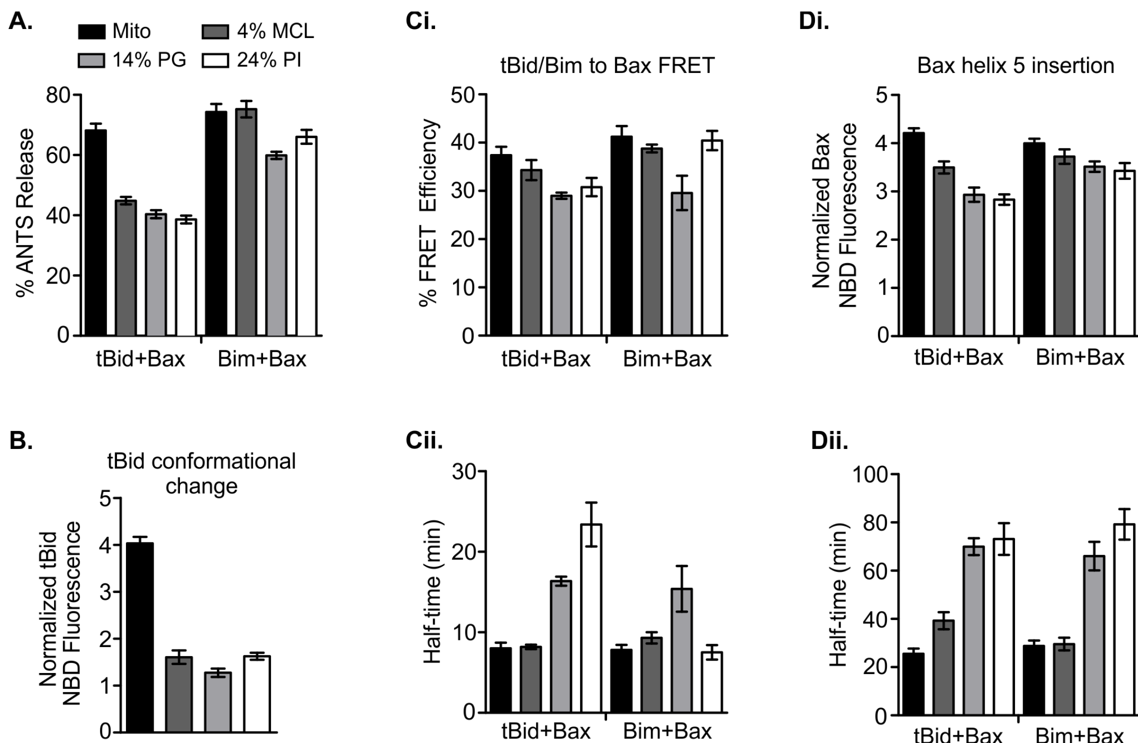

FIGURE 5. Specific anionic lipids are required to replace cardiolipin for efficient tBid mediated Bax membrane permeabilization. (A) Liposomes encapsulating ANTS and DPX in mitochondria-like (Mito), 4% MLCL, 14% PG and 24% PI lipid composition were used to assay membrane permeabilization using 100 nM Bax and 20 nM of either tBid or Bim, (mean ± SEM, n = 3). **(B)** tBid conformational change as assayed by an increase in the NBD fluorescence reflecting the residue moving to a more hydrophobic environment (membrane). 20 nM of tBid 163 NBD was added to 1.5 nM liposomes with the indicated lipid composition, (mean ± SEM, n = 3). **(C)** Interaction of Bax to membrane-bound tBid or Bim using FRET. 20 nM of tBid 126 DAC or Bim 104 DAC (donor) with 100 nM Bax 126 NBD (acceptor) were added to 1.5 nM liposomes with indicated lipid composition. A high percentage of FRET efficiency indicates more interaction between tBid-Bax or Bim-Bax *(Ci)*, and the half-time obtained from the fit of the data with a single-exponential function is shown in *(Cii)*, (mean ± SEM, n = 3). **(D)** Insertion of Bax in the membrane after activation. The change in the environment of the NBD-labeled residue of 100 nM Bax 126 NBD activated with tBid or Bim from the same reaction as *(C)*. A higher fold change in NBD intensity signifies more insertion of Bax in the membrane. The end-points of the data are shown in *(Di)*, whereas the half-time obtained from the fit of the data with a single-exponential function is shown in *(Dii)*, (mean ± SEM, n = 3).

also displayed lower endpoints and slower kinetics for liposomes with PG and PI for both tBid and Bim (Fig. 5D). This suggests that lower membrane permeabilization observed with PG and PI liposomes are due to the additive effect of the inefficiency in all the steps of the Bax mediated membrane permeabilization. On the other hand, MLCL specifically inhibits tBid-Bax mediated membrane permeabilization at the different steps.

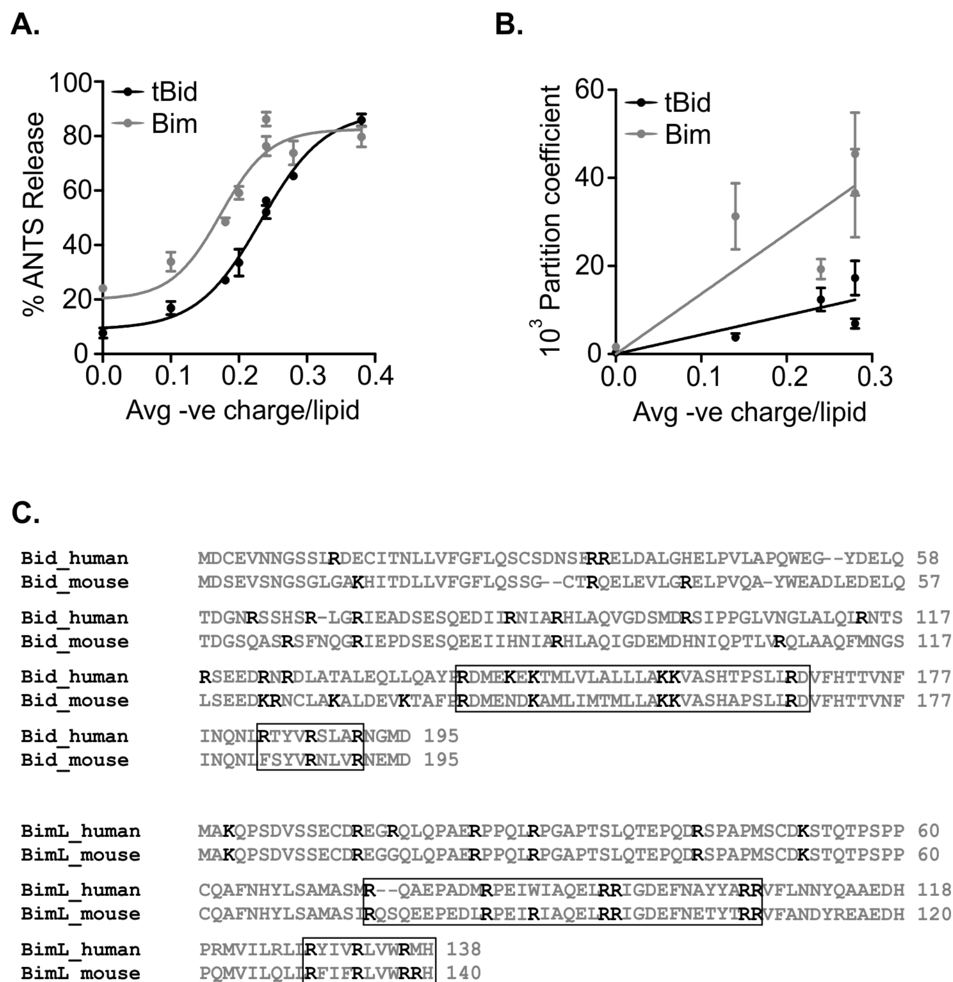

FIGURE 6. Electrostatic interactions mediate tBid and Bim binding to membranes. (A) Extent of liposome permeabilization as a function of average negative charge/lipid for tBid-Bax and Bim-Bax. **(B)** Partition coefficient, P, of tBid and Bim binding to membranes as a function of average negative charge/lipid. **(C)** Amino acid sequence alignment of human and mouse tBid and Bim. The positively charged residues are emphasized in black, and the known and potential membrane binding regions are identified in black boxes.

DISCUSSION

The main locus of the action of Bcl-2 proteins is the MOM, where protein-protein and protein-membrane interactions decide the fate of the cell. During MOMP, the membrane is an active participant that not only contains and recruits Bcl-2 proteins but also promotes conformational changes in Bcl-2 proteins to promote their apoptotic function (13,14). Therefore, it is essential to understand the interactions between Bcl-2 proteins and MOM lipids for the comprehensive knowledge of the mechanism of MOMP. In the present study, we show the similarities and differences in the dependence of MOM lipids for the Bcl-2 activator proteins, tBid and Bim. Both tBid and Bim require anionic phospholipids in the membrane to bind the membranes. While Bim activates and recruits Bax, tBid specifically requires CL at the membrane to undergo a conformational change for efficient activation of Bax. In addition, high levels of cholesterol inhibit membrane permeabilization by hindering the interaction of Bax with the activators and its subsequent insertion in the membrane (Fig. 7).

The requirement for the anionic phospholipids at the membrane strongly suggests that electrostatic interactions mediate the recruitment and binding of tBid and Bim to the membrane (Fig. 6B). We propose that the translocation of tBid and Bim to the MOM is governed by the interactions between the negatively charged lipids at the membrane and the cluster of positively charged residues between the membrane binding helices of tBid and the C-terminal region of Bim. In agreement to our findings, the role of electrostatic interactions in mediating the binding of tBid, and another Bcl-2 protein, Bad, to the membrane has been reported (34,50,51). In fact, the importance of electrostatic interactions for proteins binding to membranes has been documented for several membrane proteins, such as cytochrome *c* (52), diphtheria toxin (53), and colicin A (54). In our studies, a range of MOM lipids can contribute to the negative charge of the membrane. Furthermore, we observed an optimal level of average negative charge per lipid for efficient membrane permeabilization, since reduction of the average negative charge per lipid below ~0.2 impedes tBid and Bim binding to membranes and thereby suspends Bax mediated membrane permeabilization (Fig. 6A and B).

The two pro-apoptotic activator proteins tBid and Bim rely on different mechanisms to interact with the membrane. Bim is promiscuous in binding the membrane, whereas tBid displays a higher sensitivity towards the lipid makeup of the membrane. For example, binding of tBid to the membrane is reduced when solely PI provides the negative charge of the membrane (Fig. 5). One possible explanation for lower tBid binding to membranes containing PI or PG is inefficient electrostatic interactions with these lipids because of steric hindrance caused by the bulky inositol and glycerol groups on the respective lipid head groups. In addition, it is possible that a higher degree of saturation of the lipid tails plays a negative role in tBid binding with membranes. Thus, tBid shows favorable binding to membranes containing CL, MLCL and PS that have unsaturated tails, whereas lower binding to membranes with PG and PI that contain one saturated tail.

In addition, tBid depends on CL to undergo efficient change in its conformation after binding to the membrane. Surprisingly, MLCL was unable to induce the conformational change in tBid (Fig. 5) highlighting the specificity of the tBid-CL inter-

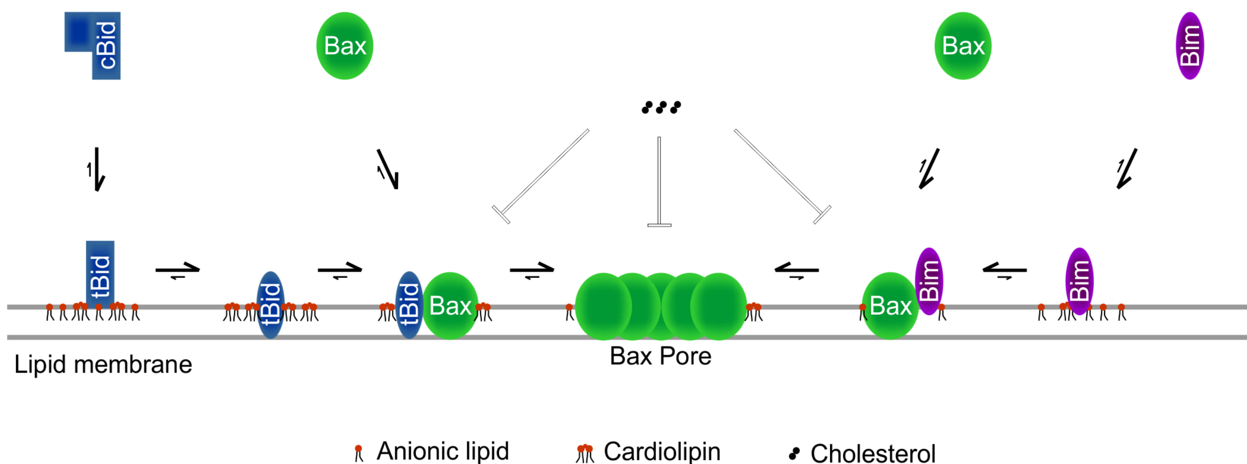

FIGURE 7. Summary of the control of lipids on membrane permeabilization by Bax when activated by tBid or Bim. Bax activated by tBid or Bim inserts and oligomerizes in membranes to permeabilize them. Initial binding of both tBid and Bim to the membrane is governed by electrostatic interactions between the positively charged residues on the proteins and the anionic lipids at the membrane. While specific lipids do not influence the activity of Bim, tBid requires cardiolipin at the membrane to undergo conformational change at the membranes before it can activate Bax. Cholesterol inhibits membrane permeabilization by impeding the interaction between Bax and the activator proteins and Bax insertion in the membrane. Therefore, the lipid content of the membrane can modulate membrane permeabilization at multiple steps.

an action. Binding to cardiolipin therefore provides an ideal situation that suits the requirements of tBid: it aids binding to membrane with electrostatic interactions due to its negative charges and it can assist with the conformational change. This observation is consistent with reports that the relocalization of CL to the MOM provides the signal for MOMP (33). However, liposomes that contained PS but not CL were also efficiently permeabilization by tBid and Bax (Fig. 4). This suggests that CL is not absolute requirement for membrane permeabilization by the pro-apoptotic proteins tBid and Bax in an *in vitro* setting. In cells, the MOM protein Mtch2 facilitates the conformational change in tBid that is required for the activation of Bax (16,55). The fact that both Mtch2 and CL mediate the conformational change in tBid strongly suggests that similar to Mtch2, CL is also a receptor for tBid at the MOM and that the function of CL may overlap or synergize with that of Mtch2. In addition, as we previously speculated (12), owing to its considerable structural homology to ANT1 and the presence of a mitochondrial-carrier domain, Mtch2 may bind CL molecules in the MOM and therefore facilitate the conformational change in tBid by providing a platform of CL-rich region in the MOM. However, this hypothesis remains speculative and will require future studies to be proven.

The observation that high levels of cholesterol in the membrane inhibit Bax insertion and therefore membrane permeabilization was previously reported (40,41). By

using fluorescence assays to monitor each step of the mechanism of MOMP, we were able to identify that cholesterol inhibits both the binding of Bax to the Bcl-2 activator proteins and the insertion of the hairpin helices of Bax in the membrane (Fig. 2). Therefore, inefficient initial steps in Bax activation cascade negatively impact the Bax oligomerization and membrane permeabilization. In agreement with previous reports, we propose that cholesterol inhibits MOMP due to changes in the physical properties of the membrane, thereby requiring more activation energy for proteins to partition the membrane.

Additional lipids found at the MOM, such as ceramide have been reported to induce membrane permeabilization (56). However we found that the addition of ceramide, sphingosine and sphingosine-1-phosphate in the membrane had no effect on tBid and Bax mediated membrane permeabilization (Table 4). In addition, an increase in PE to increase the negative curvature of the membranes had no effect of tBid and Bax mediated membrane permeabilization (Table 4).

Taken together, our data suggests that the lipid content of the membrane can modulate permeabilization by Bcl-2 pro-apoptotic proteins are several levels: recruitment of tBid and Bim to the membrane, the conformational change in tBid, binding of Bax to the activator proteins, and insertion of Bax in the membrane (Fig. 7). We have previously shown that the conformational change in tBid and Bax insertion in the membrane are the rate-limiting steps of each protein in their activation (8,16). It is therefore interesting to see that the lipid have "chosen" these rate-limiting steps to exert their control.

Using a system of liposomes and purified proteins to understand the core mechanism of Bcl-2 proteins has allowed us characterize the individual steps in the MOMP cascade. By using this system, we have uncovered mechanisms that can explain observations in cultured cells and in mice (8,9,16,57). Our results suggest that tBid and Bim employ different mechanisms of activating Bax, and therefore open avenues to modulate activator specific apoptosis. In addition, the dependence of tBid on CL and Mtch2 to undergo a conformational change at the membrane leads us to postulate that CL and Mtch2 may have a redundant role in the recruitment of tBid at the MOM. It will be interesting to examine tBid mediated MOMP in cells lacking CL and/or Mtch2.

TABLE 4. Summary of the effect of ceramide, sphingosine, sphingosine-1-phosphate and PE on membrane permeabilization by tBid and Bax.

Lipids	Membrane permeabilization
C16 Ceramide 18 – 70 nmoles titration	No effect
Sphingosine 70 nmoles	No effect
Sphingosine-1-phosphate 70 nmoles	No effect
PE 48 mol%	No effect

EXPERIMENTAL PROCEDURES

Protein purification and labeling

Recombinant full-length human wild-type (WT) Bax was purified, and the single-cysteine mutant, Bax 126C was purified and labeled with the environment sensitive dye *N*,*N'*-Dimethyl-*N*-(Iodoacetyl)-*N'*-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)Ethylenediamine (NBD) (Life Technologies) as described (8,58). Recombinant full-length N-terminal 6x-His-tagged murine WT Bid and the single-cysteine mutants, Bid 126C and 163C were purified, labeled and cleaved with caspase-8 as described (16). Bid 126C was labeled with N-(7-Dimethylamine-4-Methylcoumarin-3-yl) Maleimide (DAC), and Bid 163C was labeled with NBD (Life Technologies) as described (16). For simplicity, in the following caspase-8 cleaved Bid (cBid) is referred to as tBid. Recombinant full-length N-terminal 6x-His tagged human WT BimL and the single-cysteine mutant BimL 104C were purified as described (42). BimL 104C was labeled with DAC by following the same labeling protocol as used for labeling Bid (16). Briefly, the protein was incubated with a 10 to 15 molar excess of dye in the dialysis buffer for 2-3 hours at room temperature with rotation in the presence of 0.5% CHAPS. The dye was dissolved in DMSO, and the final % DMSO content of the reaction was kept below 10%. Then BimL was subjected to nickel-affinity chromatography and washed with buffer to remove the excess unreacted dye. The labeled BimL was then eluted, dialyzed and stored at -80°C until use. For simplicity, BimL is referred to as Bim.

Mitochondrial permeabilization assay

Mitochondria were isolated from the livers of *bak -/-* mice and frozen as described (16). MOMP was assessed by the release of cytochrome *c* release from mitochondria after incubation with indicated concentration of Bax with tBid or Bim for 30 min at 37°C. Detection of cytochrome *c* and quantification of the immunoblots was carried out as described (16).

Liposome preparation

Liposomes with mitochondria-like lipid composition were prepared as described previously, using the following lipids (all from Avanti Polar Lipids, Inc.) in the mol% ratio outlined in Table 2: PC (Cat. No. 840051), PE (Cat. No. 841118), PI (Cat. No. 840042), PS (Cat. No. 840035), CL (Cat. No. 710335) (16,58). This composition has been shown numerous times to support Bid and Bax-mediated pore formation (9,47,48). Other lipids were added to the membrane as required: MLCL (Cat. No. 850081), phosphatidylglycerol (PG) (Cat. No. 841138) and cholesterol (Cat. No. 700100). The surface charge of the membrane was determined for each different lipid composition based on the pK_a values of the functional groups at pH 7 (59). Liposome concentration was estimated using ~84,000 lipids per 100 nm-diameter liposome, see reference (58) for details.

Liposome permeabilization assay

Liposomes encapsulating the fluorophore, 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and the collisional quencher, *p*-xylene-bis-pyridinium bromide (DPX) were prepared as described (47,58). 0.5 nM of ANTS/DPX liposomes were incubated with the indicated concentration and combination of proteins, tBid, Bim and Bax for 3 hr at 37°C.

Membrane binding assay

Membrane binding was assessed using a protein-membrane Förster resonance energy transfer (FRET) assay described previously (8). Briefly, 20 nM of tBid 126C DAC or of Bim 104C DAC (donor) was incubated with different concentrations of unlabeled liposomes (no acceptor control) or of liposomes containing 1 mol% of PE labeled with the acceptor NBD at the tail (Avanti Polar Lipids, Inc. Cat. No. 810154) for 20 min at 37°C. The donor was excited at 380 nm (5 nm bandwidth) and its fluorescence emission monitored at 460 nm (20 nm bandwidth). The fluorescence intensity recorded in the sample containing both the donor and acceptor was denoted F_{+A} (t), and that recorded in the sample containing unlabeled liposomes was denoted F_{-A} (t). The FRET efficiency is given by: $E(t) = (1-(F_{+A}(t) / F_{-A}(t))) \times 100$. The final FRET efficiency, E (t = 20 min), is proportional to the fraction of proteins bound to the liposomes at equilibrium. Titration curves showing the final FRET efficiency as a function of liposome concentration were fit using the free ligand approximation to retrieve the dissociation constant associated with the binding of the protein to the liposomes, K_D, as described in (16). Alternatively the binding equilibrium of the protein to the lipid phase can be described by a partition coefficient, $P = 1/(V_L K_D)$, where V_L is the molar volume of the liposomes (volume of the lipid phase for 1 mole of liposomes), $V_L = 7.6 \times 10^4$ l/mol.

Protein-protein binding assay

Protein-protein binding was assessed using a FRET assay carried out as described previously (8). tBid 126 DAC or Bim 104 DAC (donor) was incubated with Bax 126 NBD (acceptor) or unlabeled Bax (no acceptor control) at the indicated concentration in the presence of liposomes for 2 hr at 37°C. The donor was excited at 380 nm (5 nm bandwidth) and its emission was monitored at 460 nm (20 nm bandwidth). The binding of the two proteins was assessed using the percentage of FRET efficiency, given as before by: $E = (1-(F_{+A}(t) / F_{-A}(t))) \times 100$.

Conformational change assay

The assay was carried out as described using the environment sensitive fluorescence of NBD (8,16). Liposomes were added to the indicated concentration of tBid 163 NBD or Bax 126 NBD and incubated for 20 min or 2 hr, respectively at 37°C. Measurements with Bax 126 NBD were obtained simultaneously with the protein-protein FRET assay described above. The change in the fluorescence intensity of NBD was monitored using excitation at 475 nm (5 nm bandwidth) and emission at 530 nm (20 nm bandwidth). Normalized change in NBD fluorescence was calculated as F/F_0, where F is the fluorescence of NBD at the reaction end-point, and F_0 is the fluorescence at time t = 0.

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FOOTNOTES

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[6]The abbreviations used are: MOMP, mitochondrial outer membrane permeabilization; MOM, mitochondrial outer membrane; CL, cardiolipin; MLCL, monolysocardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PG, phosphatidylglycerol; WT, wild-type; ANTS, 8-Aminonaphthalene-1,3,6-Trisulfonic Acid, Disodium Salt; DPX, *p*-Xylene-Bis-Pyridinium Bromide; DAC, N-(7-Dimethylamine-4-Methylcoumarin-3-yl) Maleimide; NBD, *N*,*N*'-Dimethyl-*N*-(Iodoacetyl)-*N*'-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)Ethylenediamine; FRET, Förster resonance energy transfer; K_D, Dissociation constant; P, Partition coefficient.

CHAPTER V

Multiple partners can kiss-and-run: Bax transfers between multiple membranes and permeabilizes those primed by tBid

Preface

The work presented in this chapter has been accepted by *Cell Death & Disease* as a research article.

Shamas-Din, A., Satsoura, D., Khan, O., Zhu, W., Leber, B., Fradin, C. and Andrews, D. W. (2014) Multiple partners can kiss-and-run: Bax transfers between multiple membranes and permeabilizes those primed by tBid.

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Contribution of authors:

Shamas-Din, A carried out all the experiments in figures 2-4, supplemental figure 2C, 3 and 4. Satsoura, D carried out preliminary experiments and experiments in supplemental figure 1, Zhu, W carried out experiments in figure 1, and Khan, O carried out experiments in supplemental figure 2A. Shamas-Din, A and Fradin, C prepared all the figures and wrote the manuscript and Leber, B and Andrews, DW edited the manuscript. Andrews, DW, Fradin C and Leber B directed the research of the project.

Research Objective:

To investigate the dynamic binding equilibria of tBid and Bax with membranes.

Research Highlights:

- Binding of tBid to membranes is reversible, and the rate of transfer between membranes is accelerated by Bax and inhibited by Bcl-XL.
- Mtch2 increases the retention of tBid at the recipient membrane.
- Binding of Bax to membranes is also reversible, but its unbinding from the membrane is slower than that of tBid, and is not influenced by the presence of tBid.
- At the recipient membrane, Bax requires tBid for oligomerization and membrane permeabilization.
- Interactions of tBid and Bax with membranes are governed by dynamic equilibria that potentially contribute to the propagation of the permeabilization signal within the cell during apoptosis.

Multiple partners can kiss-and-run: Bax transfers between multiple membranes and permeabilizes those primed by tBid

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Running Title
Dynamic interactions of Bax and tBid with membranes

ABSTRACT

During apoptosis Bid and Bax are sufficient for mitochondrial outer membrane permeabilization, releasing pro-apoptotic proteins such as cytochrome *c* and Smac/Diablo into the cytoplasm. In most cells both Bid and Bax are cytoplasmic but bind to mitochondrial outer membranes to exert pro-apoptotic functions. Binding to membranes is regulated by cleavage of Bid to tBid, by conformation changes in tBid and Bax and by interactions with other proteins. At least at the peripherally bound stage binding is reversible. Therefore, regulation of apoptosis is closely linked with the interactions of tBid and Bax with mitochondria. Here, we use fluorescence techniques and cell free systems containing mitochondria or liposomes that faithfully mimic tBid/Bax dependent membrane permeabilization to study the dynamic interactions of the proteins with membranes. We confirm that the binding of both proteins to the membrane is reversible by quantifying the binding affinity of proteins for the membrane. For Bax, both peripherally bound (inactive) and oligomerized (active) proteins migrate between membranes but much slower than and independent of tBid. When re-localized to a new membrane Bax inserts into and permeabilizes it only if primed by an activator. In the case of tBid, the process of transfer is synergetic with Bax in the sense that tBid "runs" faster if it has been "kissed" by Bax. Furthermore, Mtch2 accelerates the re-localization of tBid at mitochondria. In contrast, binding to Bcl-XL dramatically impedes tBid re-localization by lowering the off-rate three fold. Our results suggest that the transfer of activated tBid and Bax to different mitochondria is governed by dynamic equilibria and potentially contributes more than previously anticipated to the dissemination of the permeabilization signal within the cell.

KEYWORDS
Bcl-2 family, Bax, tBid, liposomes, mitochondria, fluorescence

ABBREVIATIONS

MOMP: mitochondrial outer membrane permeabilization; MOM: mitochondrial outer membrane; BH: Bcl-2 homology; cBid: cleaved Bid; tBid: truncated Bid; K_D: dissociation constant; FRET: Förster resonance energy transfer; CL: cardiolipin; WT: wild-type.

INTRODUCTION

Mitochondrial outer membrane permeabilization (MOMP) is widely regarded as the commitment step in apoptosis, and is controlled by the Bcl-2 family of proteins.[1, 2, 3] MOMP requires an activator such as tBid or Bim to relay the apoptotic signal to the effector members, Bax and Bak, which oligomerize in and permeabilize membranes. Anti-apoptotic proteins such as Bcl-2 or Bcl-XL counter this process by binding to both the activators and Bax/Bak.[4] In recent years, a consensus has emerged regarding the core MOMP mechanisms.[2, 5, 6] In particular, the crucial role played by membranes in modulating the complex network of binding interactions between Bcl-2 family proteins has been recognized.[7, 8, 9, 10] Moreover, it has been recently demonstrated that the interactions between key proteins in the family and the mitochondrial outer membrane (MOM) are reversible and dynamic.[11, 12] Intermittent interactions of Bcl-2 family proteins with membranes may set up transient loci from which apoptotic events are regulated. Furthermore, increasing evidence suggests that all the mitochondria in the cell need to be permeabilized for efficient apoptosis to occur.[13] Bcl-2 pro-apoptotic family members are heavily accumulated at sites of mitochondrial fission during apoptosis.[14] However, with limited pool of proteins in the cell, it is easy to imagine a situation after the onset of apoptosis where the available protein pool is quickly used up on limited number of mitochondria and the cell has to reuse the available protein to permeabilize all the mitochondria to proceed with apoptosis. Indeed in certain circumstances, it appears that MOMP is initiated at one precise location and then propagates through the rest of the cell as a wave over the ensuing five minutes.[15, 16] The diffusion of soluble apoptotic factors between mitochondria has been proposed as one potential mechanism to explain this phenomenon.[15]

Bax and Bak are composed of nine alpha helices, and contain a C-terminal tail-anchor region that targets them to the MOM and the endoplasmic reticulum.[17, 18] In the death receptor pathway, the apoptotic signal is transmitted to Bax and Bak via tBid,[19, 20] which like Bax is cytoplasmic in most dividing cells and localizes to target membranes only at the onset of apoptosis.[21, 22, 23] After the engagement of death receptors on the cell surface, Bid undergoes a proteolytic cleavage by caspase 8 and the larger C-terminal fragment of cleaved Bid (cBid) termed truncated Bid (tBid), binds the MOM and undergoes a conformational change facilitated by the MOM protein Mtch2, which renders tBid capable of activating Bax.[10, 24] Interaction of Bax with tBid at the membrane also causes a conformational change in Bax that is a necessary step for its activation.[25] As soluble Bid is structurally homologous to soluble Bax and both proteins require a conformational change at the N-terminus prior to activation, we have proposed that Bid should be considered a Bax-like protein distinct from other BH3 proteins.[26]

Another similarity between Bid and Bax that is relevant to the issue of propagation of an apoptotic signal is that both proteins interact dynamically with lipid membranes. Although tBid binds to liposomes rapidly,[8] it dissociates from them with an apparent dissociation constant (K_D) of 0.5 nM (lipids K_D 44 μM).[10] tBid has also been reported to have lipid-transfer activity.[27] Consistent with this observed capacity of tBid to "retro-translocate" from the membrane, electron paramagnetic resonance experiments,[24] NMR experiments,[28] and Monte Carlo simulations[29] all suggest that tBid binds only to the

outer leaflet of the membrane. By contrast, fully activated Bax adopts a transmembrane topology with three α-helices (5, 6 and 9) inserted in the membrane[30] that is carbonate resistant,[17] which suggests a tight and possibly non-reversible interaction with the membrane. However, other conformations with only α9 inserted into membranes are possible. In the absence of activator BH3 proteins, Bax binds reversibly to artificial membranes *in vitro*,[11, 31] a process which involves a reversible conformational change of its amino-terminal region that exposes an epitope recognized by the monoclonal antibody called 6A7.[11, 25] Accordingly, in growing cells, inactive Bax is normally in equilibrium between the cytoplasm and peripherally bound to the MOM.[12, 32] When Bcl-XL is present at the MOM, reversible binding of Bax favors the cytoplasmic form. This shift in localization has been referred to as retro-translocation and is an important aspect of the anti-apoptotic function of Bcl-XL.[4, 12]

Furthermore, the interaction between tBid and Bax is itself dynamic. It has been suggested that tBid activates Bax (and Bak) by a 'kiss-and-run' mechanism,[33, 34, 35] in which the interaction between the two proteins is transient allowing tBid to activate a series of different Bax (or Bak) molecules.[15] Consistent with this notion, we have shown that the tBid-Bax interaction, that occurs only in or on lipid membranes, is reversible.[8] Therefore, both tBid and/or Bax have the potential to propagate the apoptotic signal from one membrane to another.

Here we report that in an *in vitro* system consisting of fluorescently labeled tBid or Bax and mitochondria or liposomes, both activated and membrane bound tBid and Bax migrate between membranes. The transfer of activated Bax, however, proceeds very slowly, and an activator at the destination membrane is required for permeabilization to occur. Surprisingly, the transfer of tBid is enhanced by Bax and is repressed by Bcl-XL. Overall, our work suggests that by regulating tBid transfer, interaction with Bax and Bcl-XL provides an unexpected mechanism for regulating the intracellular propagation of apoptotic signals.

RESULTS

Mitochondria initially lacking tBid undergo Bax-mediated permeabilization in the presence of mitochondria pre-incubated with tBid

To determine whether tBid and Bax can mediate the transmission of apoptotic signaling between mitochondria without any other factors, we performed the experiment shown in Fig. 1. The first set of mitochondria (M) was isolated from *bax* -/- *bak* -/- BMK cells and incubated with tBid, and then the unbound tBid was removed by centrifugation. A second set of mitochondria ($M_{mCherry}$) was isolated from *bax* -/- *bak* -/- BMK cells that express a fluorescent Smac-mCherry fusion protein localized to the mitochondrial intermembrane space. The two sets of mitochondria were mixed in a 1:1 ratio, incubated with Bax and collected by centrifugation to evaluate the amount of MOMP in the $M_{mCherry}$ by comparing the quantities of Smac-mCherry in the supernatant and pellet fractions (Fig. 1). As expected, no MOMP was observed in control reactions lacking either tBid or Bax. Surprisingly, when the incubation contained $M_{mCherry}$ *not* pre-incubated with tBid, about

~50% of them underwent MOMP after addition of Bax, as long as mitochondria pre-incubated with tBid were also present. In a positive control incubation containing Bax and $M_{mCherry}$ pre-incubated with tBid, the amount of Smac-mCherry released was similar (~60%).

That Bax permeabilizes mitochondria with and without tBid pre-bound to the MOM demonstrates that apoptotic signaling is propagated between mitochondria in this simplified system. This transmission may be due to several mechanisms, either alone or in combination: (I) tBid originally bound to one mitochondrion can transfer to another mitochondrion, where it recruits soluble Bax; (II) After activation by tBid bound to a specific mitochondrion, activated Bax can insert into the membrane of another mitochondrion, irrespective of whether it contains tBid; (III) Only mitochondria with

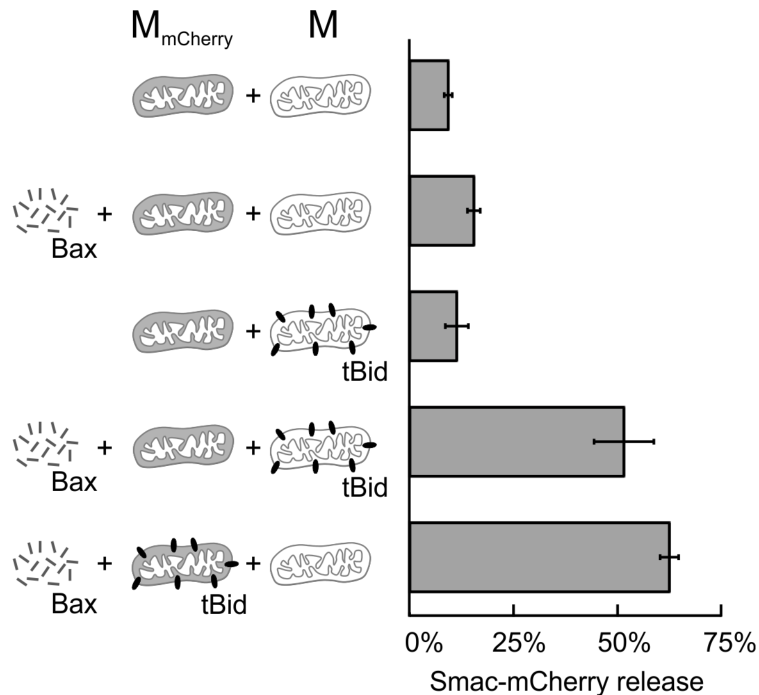

Figure 1. Bax releases Smac-mCherry from *bax*-/- *bak*-/- mitochondria in incubations containing mitochondria pre-incubated with tBid. Bars indicate the percentage of Smac-mCherry release upon addition of 20 nM Bax (as indicated) to incubations containing both *bax*-/- *bak*-/- mitochondria (M) and *bax*-/- *bak*-/- mitochondria expressing Smac-mCherry ($M_{mCherry}$), where one of the mitochondria populations has been pre-incubated with 2 nM tBid (as indicated). Release of Smac-mCherry was detected 2 hours after adding Bax by measuring the fluorescence in the supernatant after mitochondria were pelleted by centrifugation (mean ± s.e.m., n = 3). For each bar, the schematic on the left recapitulates the content of the incubation.

membrane-bound tBid are directly permeabilized by Bax, but these fuse to other mitochondria, leading to MOMP. As explained in the (Fig. S1), this third possibility was discarded in control experiments, and will not be discussed further.

Bax mediated permeabilization of membranes initially lacking tBid is as efficient as and only marginally slower than that of membranes pre-incubated with tBid

To determine which of the other two mechanisms mediate MOMP propagation, we used a liposome system that allowed precise measurements of reagent concentrations and reaction kinetics. Two distinct sets of liposomes containing spectrally distinct fluorophores ($_{ANTS}$liposomes and $_{Tb}$liposomes) made possible the concurrent evaluation of permeabilization of both sets after addition of Bax (Fig. 2A).

To reconstitute the observation with mitochondria in liposomes, we measured liposome permeabilization after mixing liposomes with or without membrane-bound tBid. First liposomes were incubated with tBid, and then unbound tBid was removed by gel filtration chromatography to obtain liposomes with bound tBid (confirmed by immunoblotting, data not shown), denoted $_{ANTS}$liposomes+tBid and $_{Tb}$liposomes+tBid. Exactly as observed with mitochondria, after the addition of Bax to a 1:1 mixture of $_{ANTS}$liposomes+tBid and $_{Tb}$liposomes, both sets of liposomes were permeabilized with a comparable efficiency (Fig. 2B, green symbols). To ensure that our observations were independent of the fluorescent molecules encapsulated in the liposomes, we performed the experiment with the reverse combination of fluorophores using 1:1 mixture of $_{Tb}$liposomes+tBid and $_{ANTS}$liposomes. We obtained the same results where both sets of liposomes were permeabilized to a similar extent (Fig. 2B, light blue symbols). As expected, in control incubations without Bax, co-incubation of the different liposomes did not lead to permeabilization even when external tBid was added (data not shown).

To more closely examine the possible differences between the permeabilization of liposomes with and without membrane-bound tBid, we evaluated the influence of varying Bax concentrations on permeabilization kinetics and end-points (Fig. 2C). Similar to the results above, $_{ANTS}$liposomes with or without pre-incubated tBid were permeabilized upon addition of Bax. However, the permeabilization half-time for liposomes pre-incubated with tBid was about half that of liposomes not pre-incubated with tBid (Fig. 2D) suggesting that the process is more complicated than simple diffusion. Nevertheless, permeabilization eventually proceeded to roughly the same extent in a Bax concentration-dependent manner for both types of liposomes at end point (Fig. 2E).

Transfer of tBid between membranes is efficient and is accelerated by Bax

For tBid to transfer between liposomes, tBid binding to membranes must be reversible. We therefore used gel filtration chromatography to confirm our previous observation that binding of tBid to the membrane is reversible (Fig. S2).[10] To determine the extent and the rate of tBid transfer between liposomes, we used a FRET (Förster Resonance Energy Transfer) based assay. For these experiments, tBid was fluorescently labeled with the donor DAC ($tBid_{DAC}$), and the membrane contained the acceptor NBD-PE ($liposomes_{NBD}$). FRET is observed only when $tBid_{DAC}$ migrates to $liposomes_{NBD}$.[8, 10]

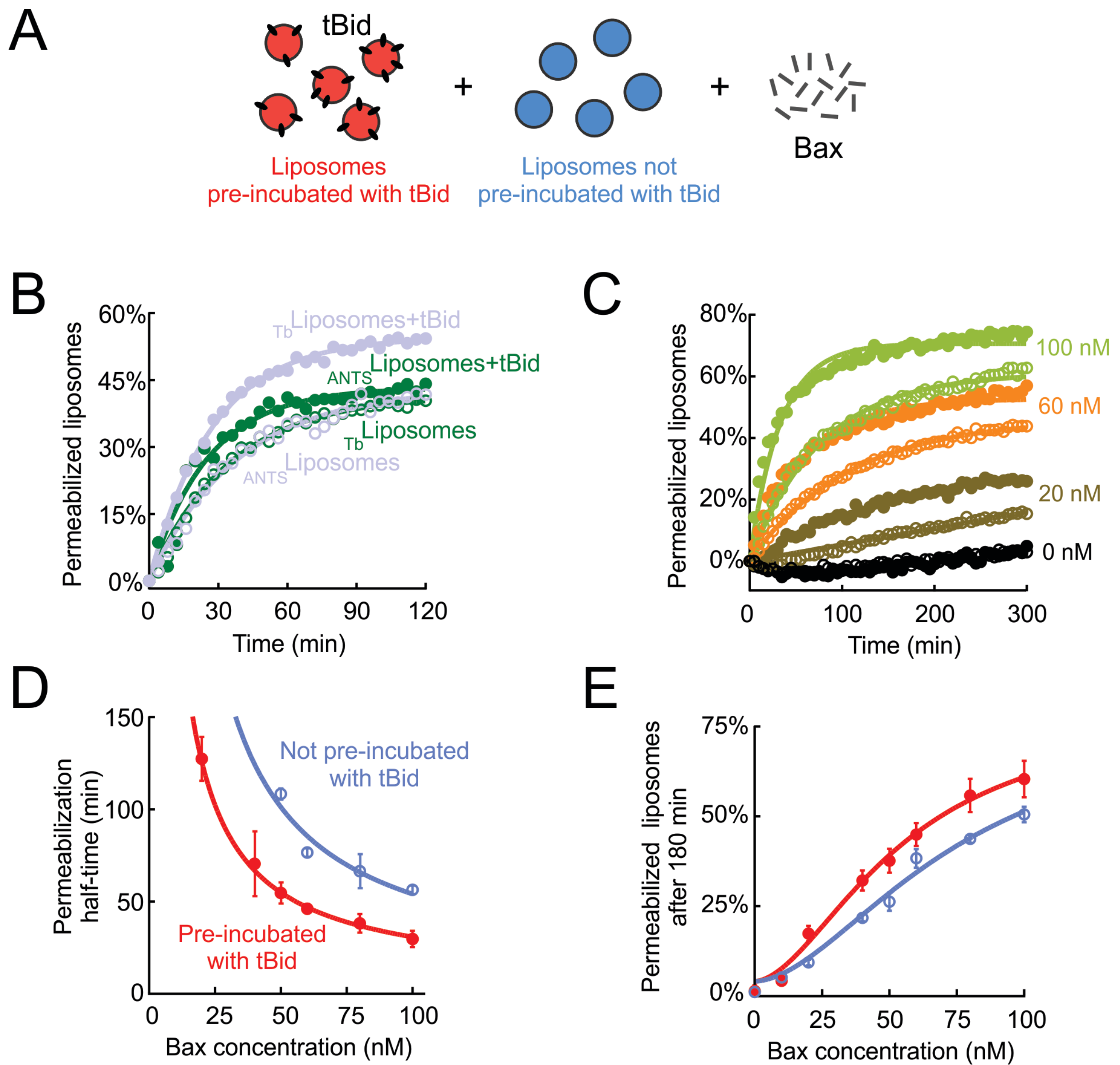

Figure 2. Bax permeabilizes liposomal membranes when liposomes pre-incubated with tBid are also present. (A) Principle of the protein transfer experiment: Liposomes pre-incubated with tBid (black ovals) and encapsulating one type of fluorophore (red – color arbitrary) are mixed with liposomes not pre-incubated with tBid and filled with a spectrally different fluorophore (blue – color arbitrary). Liposome permeabilization upon the addition of Bax can then be monitored separately. **(B)** Permeabilization of a 1:1 ratio of liposomes either pre-incubated with 20 nM tBid (filled symbols) or not (empty symbols) when mixed together and incubated with 100 nM Bax. Data represented by the same color symbols were recorded simultaneously in the same solution, using spectrally different encapsulated fluorophores (ANTS and Tb) as shown in A. **(C)** Permeabilization of liposomes either pre-incubated with tBid or mixed with an equal amount of liposomes not pre-incubated with tBid (filled symbols) or vice versa (empty symbols) when

incubated with the indicated amount of Bax. Curves with the same color have been recorded for the same Bax concentration but in two different solutions (one set of representative data). In (B) and (C), lines represent exponential fits of the data. **(D)** Permeabilization half-time as a function of Bax concentration and **(E)** percentage of permeabilized liposomes after 3 hr of incubation (mean ± s.e.m., n=3). In (D) and (E), the fit of the data with either a power law (D) or a sigmoidal function (E) is shown for both liposome populations.

As a positive control, we added $tBid_{DAC}$ to a 1:1 mixture of $liposomes_{NBD}$ and unlabeled liposomes (Fig. 3A), and observed rapid FRET between $tBid_{DAC}$ and $liposomes_{NBD}$ (Fig. 3C, grey symbols). A single-exponential fit of the data as predicted by the kinetic model obtained by considering a simple binding reaction between tBid and the membranes (Eq. 3), gives an average relaxation rate $k = (0.013 \pm 0.005)\ s^{-1}$ (mean ± stdev, $n = 3$) at this lipid concentration. Next, to determine the transfer of tBid between liposomes, we incubated $tBid_{DAC}$ with liposomes and used gel filtration to obtain a solution with liposomes+$tBid_{DAC}$, which was then added to $liposomes_{NBD}$ in a 1:1 ratio (Fig. 3B).

We observed efficient FRET between $tBid_{DAC}$ and $liposomes_{NBD}$ that reached the same level of completion as in the control experiment, indicating that $tBid_{DAC}$ transferred from one population of liposomes to the other such that at equilibrium it was equally distributed between the two populations of liposomes (Fig. 3C, black symbols). We fitted this data with a double exponential function (Eq. 4) to account for the fact that tBid is initially partitioned between the membrane of the liposomes and the solution, and that both these fractions eventually interact with the membrane of $liposomes_{NBD}$, but with different rates (see Materials and Methods). In this way, the retro-translocation rate of tBid was found to be on average: $k_{off} = (1.6 \pm 0.5)\ 10^{-3}\ s^{-1}$. This means that the half-time associated with the release of a molecule of $tBid_{DAC}$ from a liposome is ~7 min, considerably longer than average time necessary for a $tBid_{DAC}$ to bind to a liposome in our experimental conditions (~40 sec). These results demonstrate that tBid binding to mitochondrial-like membranes is reversible and the difference in rates is consistent with our previous observations that tBid co-fractionated with liposomes during gel filtration chromatography. Together our results suggest that tBid spontaneously transfers between two populations of liposomes, leading to permeabilization of the destination liposome.

Since tBid interacts with other Bcl-2 family members at the MOM, we next examined the effect of Bax and Bcl-XL on the dynamic equilibrium of tBid transfer between liposomes. We incubated liposomes+$tBid_{DAC}$ with $liposomes_{NBD}$ in a 1:1 ratio (Fig. 3B) with Bax or Bcl-XL. Consistent with their opposing functional roles in apoptosis, Bax and Bcl-XL had opposing effects on tBid transfer between liposomes. While Bax accelerated tBid transfer between liposomes, increasing the retro-translocation rate 3-fold to $k_{off} = (4.5 \pm 0.2)\ 10^{-3}\ s^{-1}$ (corresponding to a half-time of ~2.5 min), Bcl-XL reduced the retro-translocation rate 3-fold, to $k_{off} = (0.5 \pm 0.2)\ 10^{-3}\ s^{-1}$ (corresponding to a half-time of ~25 min) (Fig. 3D). This result suggests that regulation of retro-translocation may be a general property of Bcl-2 family proteins.

While these experiments demonstrate that no other proteins are required for tBid membrane binding and transfer, an integral MOM protein Mtch2 facilitates tBid binding to membranes and MOMP by accelerating the conformational change in tBid required for Bax activation in cells.[10, 36] Consistent with this function, Mtch2 at the recipient membrane increased the efficiency of tBid transfer (Fig. S3).

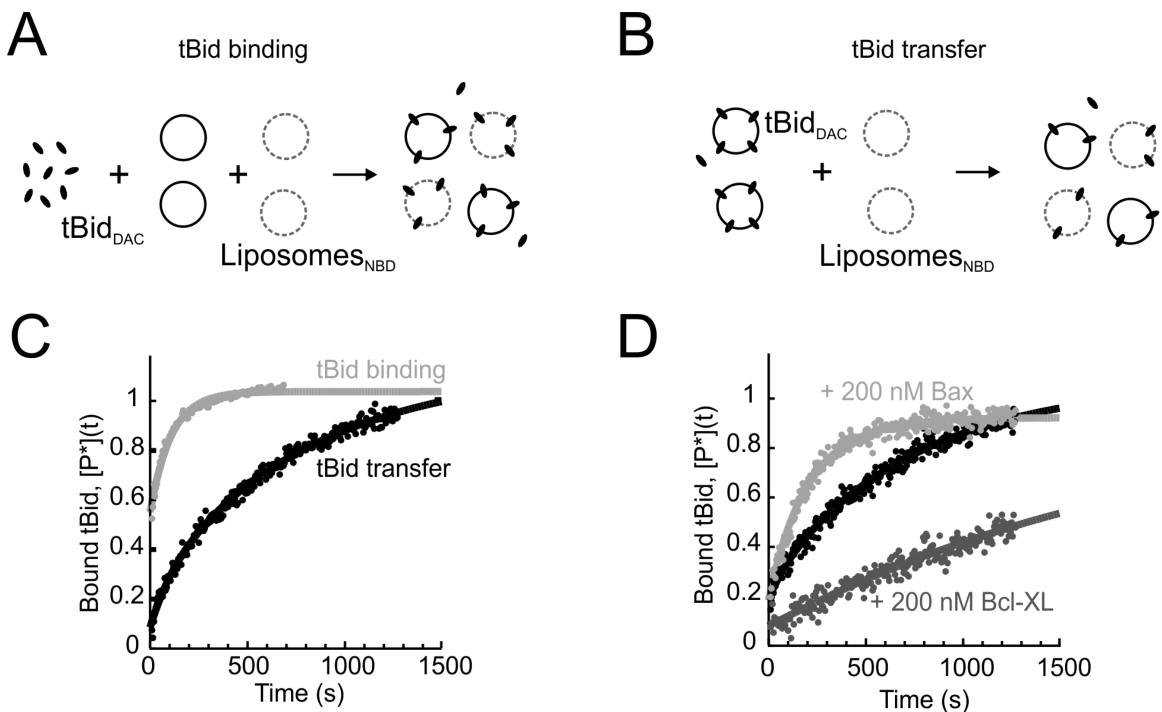

Figure 3. The binding of tBid to membranes is reversible. (A) Schematic of the kinetic experiments performed to measure the binding and unbinding rates of tBid to liposomes. tBid labeled with the donor DAC (tBid$_{DAC}$) is mixed with unlabeled liposomes (black ovals) and liposomes labeled with the acceptor NBD-PE (dotted ovals) in a 1:1 ratio. **(B)** Unlabeled liposomes pre-incubated with tBid$_{DAC}$ were mixed with liposomes labeled with the acceptor NBD-PE. **(C)** A tBid binding experiment (grey symbols, as shown in **A**) and a tBid transfer experiment (black symbols, as shown in **B**), showing the fraction of tBid bound to fluorescent liposomes as a function of time, as obtained by normalizing the signal obtained from the donor fluorescence. Lines are fit to the data according to Eq. 3 and Eq. 4. **(D)** Fraction of tBid transferred to fluorescent liposomes as a function of time in the presence of either 200 nM Bax (light grey symbols) or 200 nM Bcl-XL (dark grey symbols). Data in the absence of Bax or Bcl-XL is also shown for comparison (black symbols, as in **C**). Lines are fit to the data with Eq. 4.

Bax transfers between membranes but requires an activator at the recipient membrane for oligomerization

To determine whether activated Bax transfers between liposomes independent of tBid, we used liposomes that support Bax oligomerization but not tBid binding. We therefore compared a lipid composition without cardiolipin (–CL) to mitochondria-like liposomes with higher negative surface charge density (+CL). As expected, tBid was unable to bind to –CL liposomes (data not shown). Consequently, while tBid mediated Bax liposome permeabilization of +CL $_{ANTS}$liposomes was very efficient, permeabilization was greatly reduced in –CL $_{ANTS}$liposomes (Fig. 4A). However, when using another BH3 protein BimL as an activator for Bax, both sets of $_{ANTS}$liposomes were efficiently permeabilized, although –CL $_{ANTS}$liposomes were permeabilized to a lesser extent (Fig. 4A). Thus, once activated with BimL, Bax permeabilizes –CL $_{ANTS}$liposomes (>50% release) much more efficiently than when tBid is the activator (<25% release).

To exploit this difference, we examined whether Bax activated at one set of liposomes can permeabilize another by performing the experiment analogous to that shown in Fig. 2A. For this experiment tBid was incubated with +CL liposomes and the liposomes containing bound tBid were isolated by gel filtration, and mixed in an equal ratio to either +CL or –CL $_{ANTS}$liposomes. As expected, Bax efficiently permeabilized +CL $_{ANTS}$liposomes that promoted tBid transfer. However, –CL $_{ANTS}$liposomes were not permeabilized (Fig. 4B). Since Bax is able to permeabilize these membranes when activated by BimL, this indicates that Bax either cannot transfer between membranes or it cannot permeabilize membranes that are not "primed" with an activator after binding.

To investigate the transfer of soluble Bax between liposomes, we used a protein-liposome FRET assay similar to the one described above for tBid (Fig. 4C). When Bax_{DAC} (donor) was added to a reaction containing either +CL or –CL $liposomes_{NBD}$ (acceptor) the efficiency of FRET was not only similar in both cases (Fig. 4D, open circles), but was also independent of the presence of tBid (Fig. S4). This demonstrates that Bax binds to membranes independent of negative surface charge, and that FRET cannot distinguish between the peripheral binding of Bax to membranes (that occurs in the absence of tBid) and the membrane embedded form of Bax that mediates permeabilization (that occurs in the presence of tBid). Thus, there must be very little difference in the distance between the DAC dye on Bax and the NBD dye in the membrane for these two conformations. In addition, these results demonstrate that unlike tBid, the interaction of Bax with membranes is not dependent on the presence of cardiolipin in the membrane.

Next, we investigated whether the active, stably membrane bound Bax transfers between liposomes. Bax_{DAC} and tBid were incubated with liposomes and the liposomes containing membrane bound proteins (liposomes+tBid+Bax_{DAC}) were collected by gel filtration. These liposomes were then incubated with +CL or –CL $liposomes_{NBD}$ and the transfer of Bax_{DAC} was measured by FRET (Fig. 4C). Surprisingly, membrane bound Bax_{DAC} re-equilibrated between the two populations of liposomes, although at a slower rate than tBid, with an average k_{off} = (0.2 ± 0.1) 10^{-3} s^{-1} (binding half-time ~60 min) (Fig. 4D, filled circles) (Eq. 4). Furthermore, membrane bound Bax_{DAC} transferred to –CL liposomes as efficiently as to +CL liposomes, demonstrating that transfer of both the

active and the inactive conformers of Bax is independent of both tBid, and negative membrane surface charge.

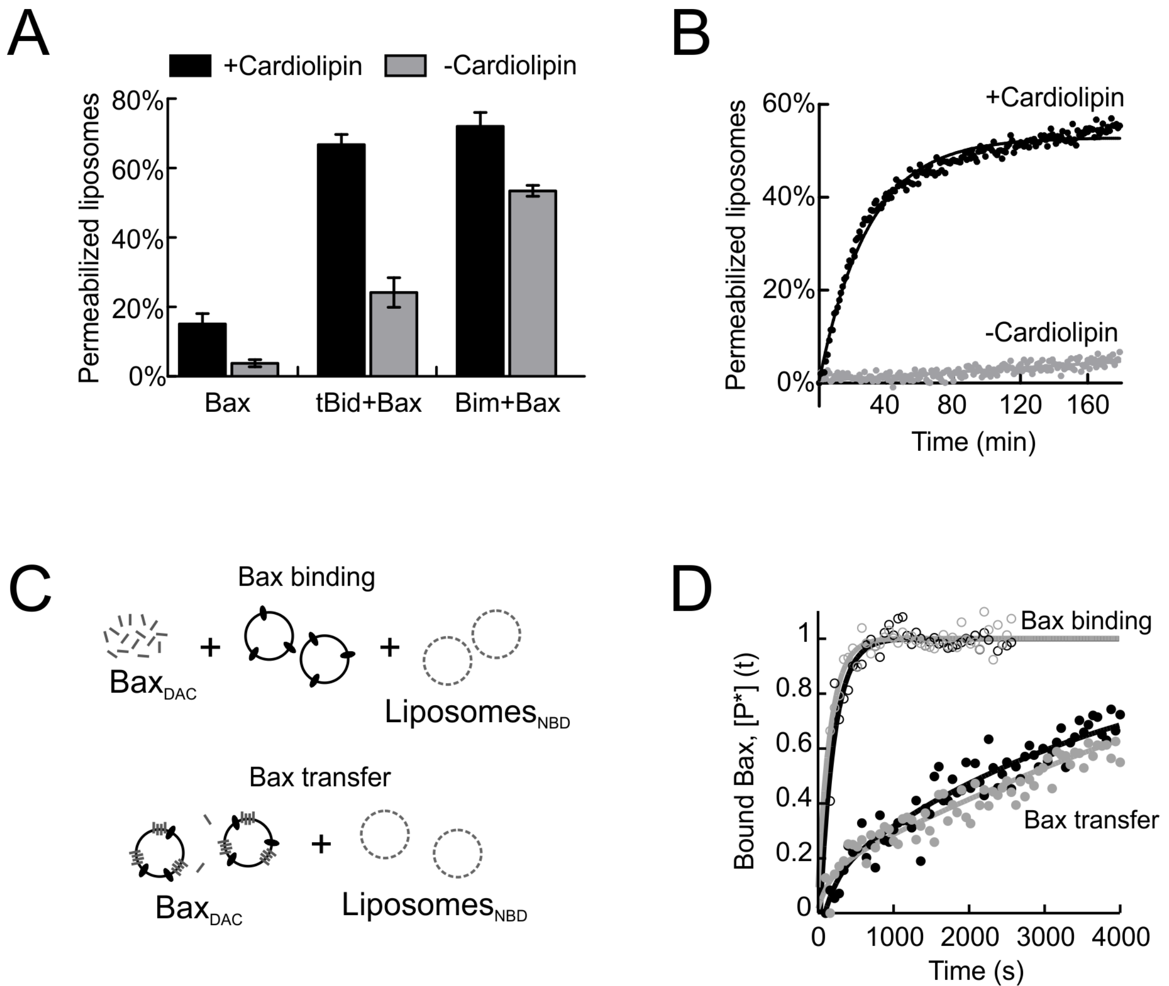

Figure 4. Bax transfers between membranes but requires an activator at the recipient membrane to form pores. Permeabilization of liposomes with either +CL or –CL lipid composition. **(A)** –CL liposomes were poorly permeabilized using 100 nM Bax and 20 nM tBid but were efficiently permeabilized by 100 nM Bax and 20 nM BimL. **(B)** Permeabilization of liposomes by Bax as a result of transfer of tBid between two liposome populations. Non-fluorescent +CL liposomes pre-incubated with 20 nM tBid were mixed with equal concentration of fluorophore-encapsulated liposomes with indicated lipid composition, followed by the addition of 100 nM Bax. **(C)** Illustration of the assays used to measure Bax binding and transfer. **(D)** *Bax binding:* 100 nM Bax

labeled with the donor, DAC, 20 nM tBid and liposomes containing the acceptor NBD-PE with the indicated lipid composition were incubated for 30 min. Line is fit to the data according to Eq. 3. Bax binds to both +CL (open symbols, black) and –CL (open symbols, grey) liposomes. Normalized FRET efficiency is indicative of binding of Bax_{DAC} to $liposomes_{NBD}$. *Bax transfer:* tBid activated, membrane bound Bax transfers to +CL (filled symbols, black) and –CL (filled symbols, grey). Unlabeled liposomes pre-incubated with 200 nM Bax_{DAC} and 20 nM tBid were passed over a gel-filtration column to remove free Bax and tBid. The fraction containing the liposomes and bound proteins was added to $liposomes_{NBD}$ in a 1:1 ratio to observe Bax transfer. Line is fit to the data according to Eq. 4. FRET efficiency was calculated as explained[10] (mean ± s.e.m., n = 3).

DISCUSSION

The fact that soluble and membrane bound forms of Bcl-2 family proteins are in reversible equilibrium is increasingly recognized as an important feature of these proteins.[10, 31] The retro-translocation of Bax from mitochondrial membranes in transformed cells that has been highlighted in several recent publications[12, 32] is a natural consequence of this equilibrium partition. The present study shows that the binding of both tBid and Bax to liposomal membranes is dynamic, with measurable off-rates on the order of 10^{-3} s^{-1} similar to that measured for Bax in cells,[12, 32] and that this permits redistribution of these proteins to other membranes. Surprisingly even though both Bax and tBid have multiple membrane conformations, binding was reversible in all cases, even for membrane-bound Bax resistant to gel-filtration. It remains speculative whether one Bax from an oligomer transfers or exclusively monomeric membrane-bound Bax retro-translocates, or if a combination of both processes occurs simultaneously. This result is consistent with the concept of multiple conformational changes between inactive and fully active tBid and Bax (Fig. 5).[8,10]

Consistent with redistribution of tBid being functionally important, it was modulated positively and negatively by pro- and anti- apoptotic Bax and Bcl-XL respectively. For Bax, oligomerization in the recipient membrane is strictly dependent on the presence of a BH3 activator (tBid or BimL) (Fig. 4). Bcl-2 family activators therefore ensure Bax permeabilizes the correct subcellular membrane, similar to the way that Mtch2 may act to trigger the activating conformational change in tBid, thereby restricting its activity to mitochondrial membranes.

One implication of the finding that an activator needs to be present on the recipient membrane for pore formation to occur is that retro-translocated Bax likely reverts to its inactive conformation. Our results also suggest that the physical migration of the activator throughout the cell may be required to ensure that all mitochondrial membranes are permeabilized, a requirement for apoptosis to kill the cell.[13] Consistent with this notion, in certain circumstances where the apoptosis signal is localized, a rapidly spreading wave is observed.[16] Moreover, quantitative kinetic analysis in model cellular systems indicates that the duration of the lag phase (initiation) of apoptosis is long and is variable between different cells, but the execution phase is rapid and with little variation

between cells,[15, 37] consistent with the diffusion of an apoptotic factor across the cell.[15] Our study suggests that BH3 activators play a bigger role in transmission of the apoptotic signal compared to effectors like Bax, since the k_{off} for tBid is more than eight times faster than that of Bax, in line with the different dissociation constants we previously measured for tBid (44 μM)[10] and for Bax in the presence of tBid (~ 1μM).[31] In addition, our results clearly demonstrate that both Bax and Bcl-XL regulate migration of tBid between membranes further illustrating total integration of the system.

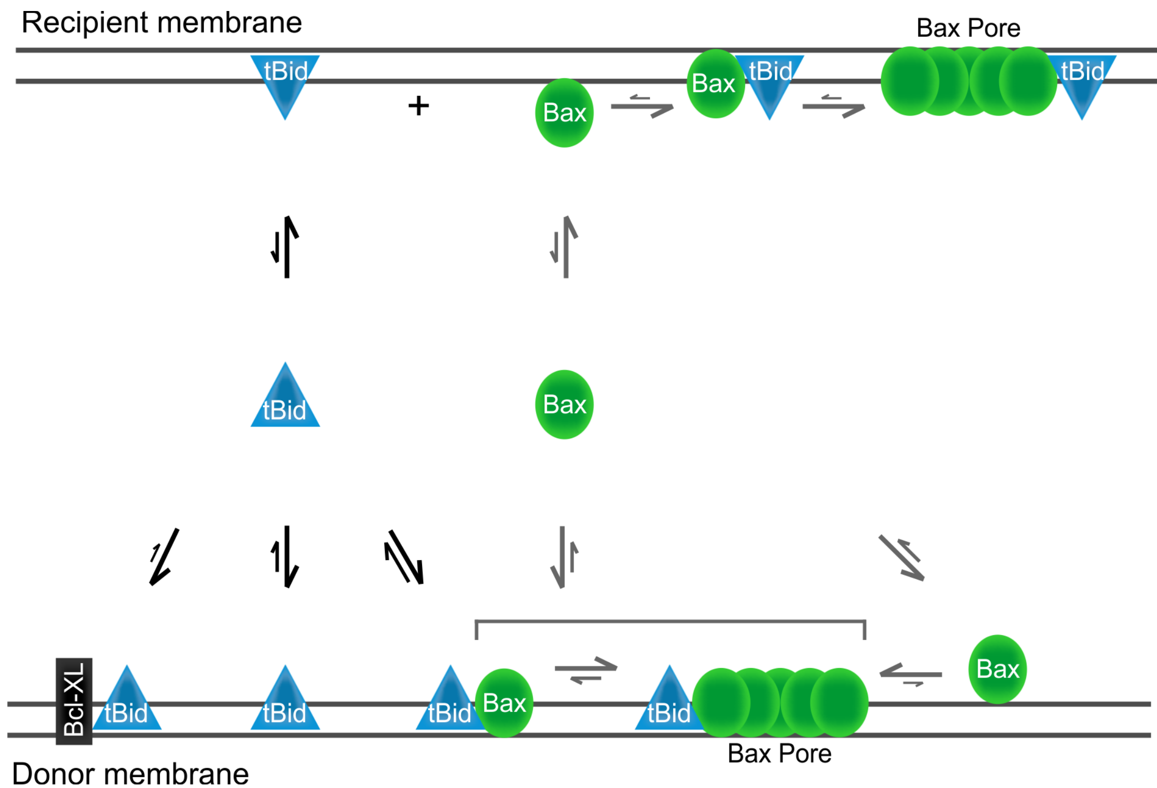

Figure 5. Bax and tBid transfer between membranes and Bax permeabilizes recipient membranes primed by tBid. Binding of tBid and Bax to membranes is reversible and is regulated by dynamic equilibria as indicated by equilibrium arrows. Binding of Bcl-XL to tBid decreases the transfer rate of tBid between the donor and the recipient membranes by decreasing the k_{off} three-fold. Interaction of tBid with Bax increases the transfer rate of tBid three-fold either by increasing the k_{off} or by decreasing the k_{on}. Both soluble and membrane-bound Bax are also constantly retro-translocating but the transfer rate of Bax is independent of tBid. Moreover, the k_{off} of membrane bound Bax is eight-fold less than that of membrane bound tBid. Once at the recipient membrane, Bax requires re-activation by tBid for insertion and pore formation suggesting that the active conformation of Bax is lost during retro-translocation.

The mechanism we propose here is a slight revision of the "kiss-and-run" model, in which tBid "kisses" not only Bax, but also a membrane, and then "runs" to other locations in order to propagate the apoptotic signal (Fig. 5). Such a mechanism would explain how cells can achieve rapid full-scale apoptosis activation since Bax continuously cycles on and off mitochondrial membranes and is primed to act, while requiring the signal provided by a fast moving activator before pore formation can occur. Furthermore, the migration of tBid and Bax between different mitochondria may also be involved in regulating mitochondrial dynamics possibly by promoting mitochondrial fragmentation.[38]

Our study has identified a number of factors that affect membrane binding by tBid, and which we propose should affect its rate of transfer between mitochondria and the propagation of apoptotic signals throughout the cell. First, Bcl-XL not only inhibits tBid binding to Bax, our results demonstrate that it also slows down tBid migration between membranes. This is a logical but not necessarily obvious result of mutual sequestration due to Bcl-XL binding to tBid in the membrane with higher affinity than in solution.[9] The p7 fragment of Bid slightly shifts the equilibrium in the other direction, towards the soluble form of tBid[10] through a similar mechanism: it binds tBid in solution exclusively and with a high affinity (K_D ~ 40 nM, Fig. S2). Second, and more surprising is our observation that Bax enhances tBid retro-translocation and therefore the rate of transfer of tBid between liposomes (Fig. 3D). The effect of Bax on tBid migration is harder to understand, as unlike the p7 fragment it binds tBid only in the membrane, and therefore would be expected to slow down retro-translocation. We speculate that the molecular mechanism by which Bax may accelerate retro-translocation of tBid is that it has an effect opposite to that of Mtch2. Mtch2 promotes tBid binding to MOM by enhancing a conformational change of tBid that leads to deeper membrane insertion and is required for its Bax activation capability (Fig. S3 and Ref. [10]), whereas Bax may be reversing this conformational change. In this way, after the "kiss" between tBid and Bax, tBid can "run" because the interaction with Bax (or the initiation of Bax oligomerization) pushes tBid out of the membrane. Regardless of the mechanism, it is interesting to note that the opposite effects of Bax and Bcl-XL on tBid retro-translocation are congruous with their opposite roles in apoptosis. This work therefore underlines the notion that a series of competitive equilibria between Bcl-2 family members and membranes lies at the heart of apoptotic regulation.

EXPERIMENTAL PROCEDURES

Protein purification and labeling

All experiments used recombinant full-length proteins or single cysteine mutants. The nomenclature for the single cysteine mutants is to indicate the residue number of the single cysteine introduced into variants without endogenous cysteine. Recombinant full-length human wild-type (WT) and single-cysteine mutant C126 of Bax were purified and labeled as described.[8, 31] Recombinant full-length N-terminal 6x-His-tagged murine WT and single-cysteine mutant 126C or 190C of Bid were purified, labeled and cleaved to isolate cBid or tBid as described.[10] Recombinant full-length WT Bcl-XL was purified as

described.[4] Recombinant full-length N-terminal 6x-His tagged human BimL mutant lacking all endogenous lysine and cysteine residues was purified as described.[39] Dyes for labeling, DACM ((N-(7-Dimethylamine-4-Methylcoumarin-3-yl) Maleimide) and Alexa Fluor 488 were purchased from Life Technologies (Waltham, MA, USA).

Mitochondria permeabilization assay

Mitochondria from *bax-/- bak-/-* BMK (baby mouse kidney) cells (either expressing Smac-mCherry or not) were isolated as described.[30] To assay MOMP, 0.2 mg/ml mitochondria (in 20 mM HEPES pH 7.5, 250 mM sucrose, 150 mM KCl, 1 mM EDTA) were incubated with tBid (2 nM) for 30 min at room temperature. Mitochondria with membrane-bound tBid were then isolated by centrifugation (13000 × *g* for 10 min at 4°C). To assess MOMP, mitochondria expressing Smac-mCherry were incubated with mitochondria pre-targeted with 2 nM tBid and with 20 nM Bax for 2 h at 37°C. Samples were then centrifuged at 13000 × *g* for 10 min. The relative amount of mCherry present in the supernatant and pellet fractions was measured by fluorescence. Samples were excited at 580 nm and monitored at 610 nm, with excitation and emission bandwidths of 5 nm. The percentage of Smac-mCherry release was calculated as the fraction of the total fluorescence coming from the supernatant.

Liposome preparation

Liposomes with a composition resembling that of mitochondrial outer membranes (PC, PE, PI, DOPS and TOCL in a 48:28:10:10:4 molar ratio, were prepared from lipids purchased from Avanti Polar Lipids, Alabaster, AL, USA), in assay buffer as described.[10] Liposomes indicated as –CL are with low surface charge and contain PC, PE and PI in a 62:28:10 molar ratio. –CL liposomes have a net negative charge/lipid of 0.10 compared to 0.28 for liposomes with a mitochondria-like composition. All liposome preps used in this work included a small fraction (0.008 mol%) of lipophilic dye (DiD (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate Salt), Life Technologies) to monitor the liposomes concentration. The fluorescence of DiD was assessed by excitation at 644 nm and emission at 665 nm with 5 nm bandwidths for both.

Liposome permeabilization assay

Liposomes containing the fluorophore ANTS and the quencher DPX ($_{ANTS}$liposomes) were prepared as described.[4] Permeabilization of $_{ANTS}$liposomes was measured by the increase in ANTS fluorescence that results from dilution of the liposome contents. The other set of liposomes contained terbium (Tb) and dipicolinic acid (DPA) ($_{Tb}$liposomes). $_{Tb}$Liposomes were prepared by adding $TbC1_3$ (0.8 mM) and DPA (2.4 mM) in the assay buffer (without EDTA). The Tb/DPA complex is highly fluorescent when encapsulated in liposomes but the fluorescence is reduced dramatically when the liposomes are permeabilized in a solution containing EDTA, due to chelation of Tb. Therefore, the permeabilization curves from $_{Tb}$liposomes were later normalized inversely to directly compare them with the curves generated from $_{ANTS}$liposomes. In both cases, non-encapsulated molecules were removed by gel filtration chromatography on a CL-2B Sepharose column prior to use in permeabilization assays.

ANTS and Tb/DPA were excited concurrently at 355 nm and 276 nm, respectively, with 5 nm bandwidths. In order to separate the short-lifetime fluorescence emission of ANTS from the long-lifetime emission of the Tb/DPA complex, ANTS emission was collected at 520 nm with a 12 nm bandwidth for 20 μs without any delay, while Tb/DPA emission was collected at 545 nm with a 12 nm bandwidth for 2 ms after a 40 μs delay. The extent of dye release was calculated as a percentage of maximum possible release: $P(t) = [F(t)-F_B(t)]/[F_M-F_B(t)]$, where $F(t)$ refers to either the ANTS or the Tb/DPA fluorescence signal, $F_B(t)$ refers to the background fluorescence of a control sample containing liposomes but no protein and F_M refers the fluorescence measured for each sample after lysis of the liposomes with 0.5 % w/v CHAPS or 0.2% v/v Triton X-100. $P(t)$ was then fitted with an exponential function, $P(t)= P_M-(P_M-P_0)\exp(-kt)$, which returned the percentage of dye release measured immediately after the addition of Bax at $t=0$ (P_0), the extrapolated final percentage of dye release (P_M) and the initial rate of release, k.

To prepare liposomes pre-incubated with tBid, 20 nM tBid was incubated with 0.3 mg/ml liposomes in assay buffer at 37°C for 30 min. To collect liposomes with bound tBid, the reaction was passed over a CL-2B Sepharose column. Liposomes without tBid with mixed in 1:1 ratio, and indicated amount of Bax was added. The reactions were carried at 37°C.

Liposome binding assay

For FRET experiments, 200 nM tBid 126C labeled with DAC was incubated with 0.3 mg/ml liposomes in assay buffer at 37°C for 30 min, or 200 nM Bax 126C labeled with DAC was incubated with 0.3 mg/ml liposomes at 37°C for 2 hr. Liposomes containing bound protein were isolated by gel-filtration on a CL-2B Sepharose column and mixed with 0.3 mg/ml liposomes containing the acceptor, NBD-PE (1-palmitoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl) amino]dodecanoyl}-*sn*-glycero-3-phosphoethanolamine)) at 1 mol% (Avanti) in 1:1 ratio. The detailed protocol for liposome preparation is previously published.[8, 10] The reactions were carried out at 37°C for 30 min to 2 hr without stirring.

For gel filtration experiments, 200 nM cBid 190C labeled with Alexa Fluor 488 was incubated with 0.3 mg/ml liposomes at 37°C for 30 min. Free protein was separated from liposome-bound protein using CL-2B Sepharose column and % bound protein was assessed by fluorescence (excitation at 495 nm, emission at 519 nm, 5 nm bandwidths) and by immunoblotting for Bid as described previously.[4]

Equilibrium binding of protein to liposomes

If a protein (e.g. tBid) binds reversibly to lipid membranes, the interaction can be represented by the equilibrium $P+L \leftrightarrow P^*$ (where P represents the protein in solution, P* the protein bound to the membrane and L the lipids), with a dissociation constant $K_D = [P][L]/[P^*]$. In the case where the lipids are in excess and the available lipid concentration is equal to the total lipid concentration, [L], the equilibrium fraction of bound protein becomes (free ligand approximation)

$$f_\infty = [L] / \left(K_D + [L] \right). \qquad \text{Eq. 1}$$

The K_D is the effective dissociation constant which may vary with the lipid composition.

In the presence of a competitor, such as the p7 fragment of Bid which can bind to the soluble protein to prevent membrane binding, then a second equilibrium needs to be taken into account, P+P'↔C, with the associated dissociation constant $K_D' = [P][P']/[C]$. Assuming that the lipids are in excess, and that $[P] = [P'] = c_P$ (i.e. that the concentration of both Bid fragments is the same), this simple competition model predicts that the equilibrium fraction of bound protein is:

$$f_\infty = \frac{[L]}{K_D + [L]} \frac{\sqrt{A^2\left(1+{[L]}/{K_D}\right)^2 + 4A\left(1+{[L]}/{K_D}\right)} - A\left(1+{[L]}/{K_D}\right)}{2} \qquad \text{Eq. 2}$$

where $A = K_D'/c_P$.

Kinetics of protein transfer between liposomes

We consider again the simple reaction P+L↔P* to analyze the kinetics of the reaction by considering the on-rate, k_{on}, and the off-rate, k_{off}. Thus d[P]/dt = k_{on}[P][L]-k_{off}[P*], leading to $K_D = k_{off}/k_{on}$ and to single exponential kinetics for the fraction of bound protein, $f(t) = f_\infty(1-e^{-kt})$ when soluble proteins are mixed with liposomes at time $t = 0$, where f_∞ is given by Eq. 1 and where the relaxation rate is $k = k_{off} + k_{on}[L]$. In the case when soluble proteins are incubated with a 1:1 mix of two types of liposomes (with total lipid concentration $[L]$) starting at $t = 0$, assuming that the affinity of the protein for both types of liposomes is the same, the fraction of bound protein to one type of liposome (a quantity accessible by FRET if the protein is labeled with a donor fluorophore and this set of liposomes is labeled with an acceptor) also increases with single exponential kinetics:

$$f(t) = \frac{1}{2} \frac{[L]}{K_D + [L]} \left[1 - e^{-kt} \right], \qquad \text{Eq. 3}$$

with a rate $k = k_{off} + k_{on}[L]$.

We now consider the more complicated case where protein is initially at equilibrium with a first population of liposomes (total lipid concentration $[L]/2$) and a second population of liposomes is introduced at time t = 0 (also with total lipid concentration $[L]/2$). The kinetics of the system is defined by a set of two differential equations, which can be solved to calculate the fraction of protein bound to the second set of liposomes (the quantity of which is measurable using FRET):

$$f(t) = \frac{1}{2} \frac{[L]}{K_D + [L]} \left[1 - \frac{1}{2} \frac{K_D}{K_D + [L]/2} e^{-kt} - \frac{1}{2} \frac{K_D + [L]}{K_D + [L]/2} e^{-k_{off} t} \right]. \qquad \text{Eq. 4}$$

This time the fraction of bound protein increases with double-exponential kinetics. The first exponential kinetics with rate $k = k_{off} + k_{on}[L]$ as before corresponds to the binding of soluble protein to the second set of liposomes, while the second exponential kinetics with a slower rate k_{off}, corresponds to the binding of the protein molecules originally bound to the first set of liposomes, and which have to detach before they can bind to the second set of liposomes.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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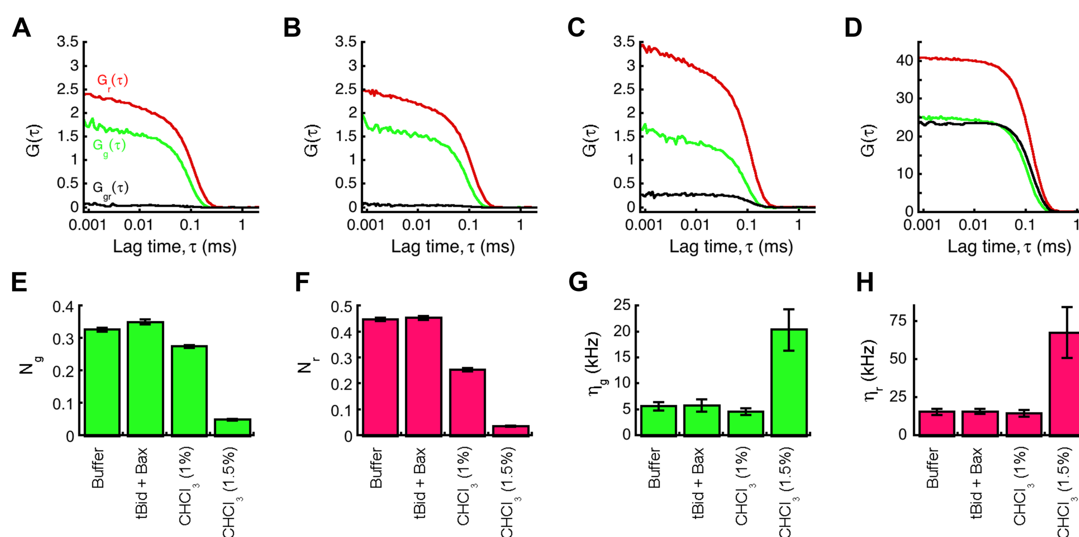
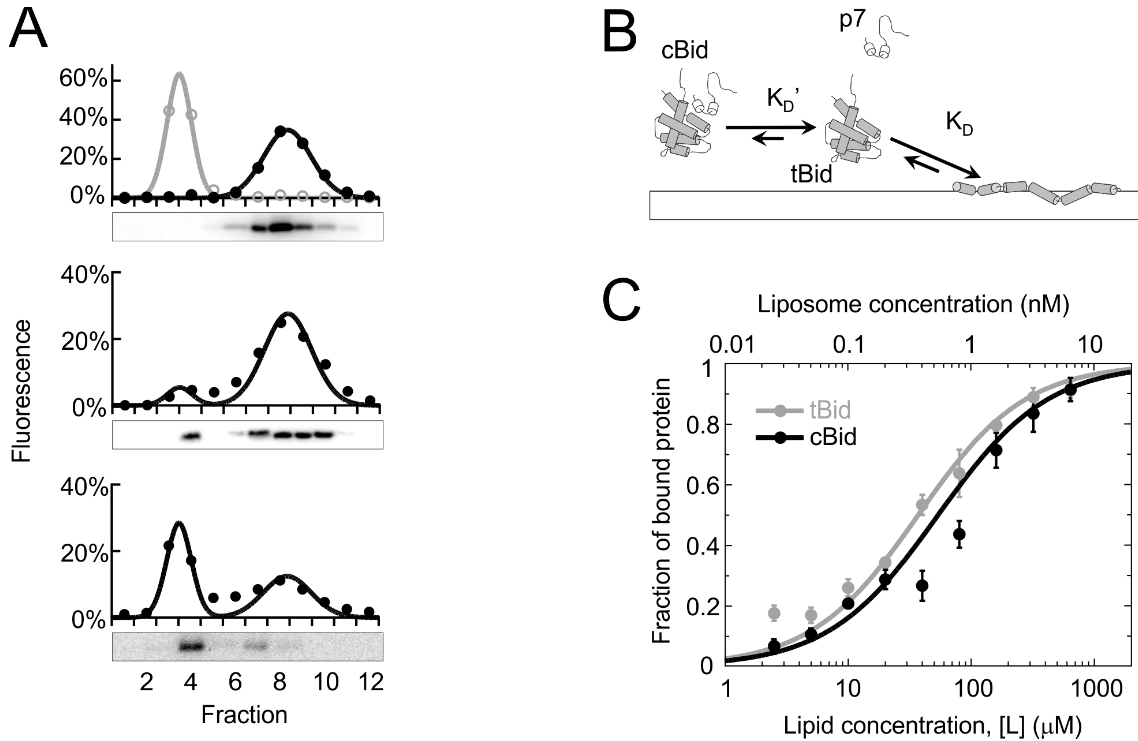
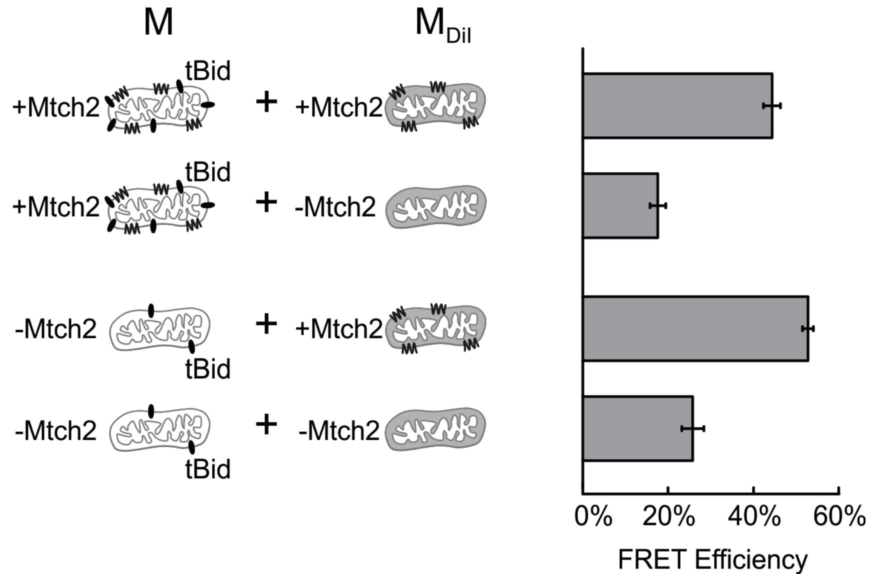
SUPPLEMENTARY MATERIAL



Supplemental Figure 1: tBid and Bax do not cause liposome fusion. Auto- and cross correlation functions obtained for a 1:1 mixture of green fluorescent liposomes (L_{DiO}, ~ 40 μg/ml lipid concentration) and red fluorescent liposomes (L_{DiD}, ~ 40 μg/ml lipid concentration) incubated for 1 hour with **(A)** buffer, **(B)** 60 nM tBid and 300 nM Bax, **(C)** 1 % chloroform or **(D)** 1.5 % chloroform. Auto-correlation functions obtained from the green and red channels are shown in green and red, respectively, and cross-correlation functions are shown in black. Each curve corresponds to an average of at least 9 experimental auto or cross-correlation curves. The concentration **(E,F)** and specific brightness **(G,H)** of the green **(E,G)** and red **(F,H)** liposomes in these incubations, as estimated using FIDA, is also shown (mean ± s.e.m., n = 10). These data show that whereas chloroform, as expected, causes liposome fusion (as indicated by a finite cross-correlation signal, accompanied by a decrease in liposome concentration and an increase in liposome specific brightness), incubation with tBid and Bax does not. Thus liposome fusion does not play any part in the Bax-mediated permeabilization of liposomes lacking tBid shown in Fig. 2, which occurs for lower tBid and Bax concentrations.

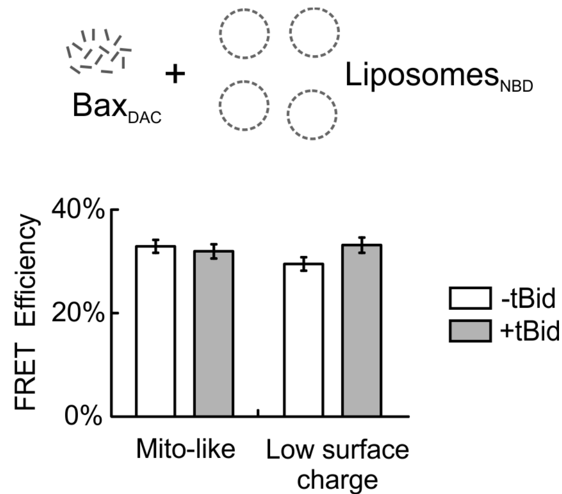


Supplemental Figure 2: Binding of tBid to membranes is reversible. (A) Results of gel-filtration experiments for mixture of tBid and liposomes, both fluorescently labeled. The top panel shows the percentage of liposomes (grey) and tBid (black) found in each fraction when run on the column alone. Lines are Gaussian fits of the data. The middle panel shows the percentage of 200 nM tBid found in each fraction after incubation with 4.75 nM liposomes. The lower panel shows the percentage of tBid in each fraction when the fraction containing the bound protein in the previous elution (fraction 4) is run on the column again. In the last two panels, lines are double Gaussian fit of the data fixing the position of the Gaussians to that found for the data shown in the upper panel. **(B)** Schematic outlining the binding equilibria between p7 and tBid, and tBid and the membrane. **(C)** Fraction of bound tBid or cBid measured by FCS when incubating 20 nM protein with the indicated amount of lipids. The data obtained for tBid was fit assuming a simple equilibrium for the binding of tBid to the membrane (Eq. 1, grey line), while the data obtained for cBid was fit assuming two equilibria, one regulating the binding of tBid to the membrane, and one regulating the binding of tBid to the p7 fragment (Eq. 2, black line), as shown in panel **B** (mean ± s.e.m., n=3).



Supplemental Figure 3: Reversible binding of tBid to mitochondria is facilitated by Mtch2. Mitochondria isolated from *mtch2* +/+ and -/- mice [1] were frozen in trehalose as described.[2] For protein to mitochondria FRET, labeling of mitochondria with DiI (Life Technologies) was carried out as described.[3] Briefly, 2 nM tBid 126C labeled with Alexa Fluor 488 ($tBid_{Alexa488}$) was incubated with 0.5 mg/ml unlabeled mitochondria (M) with or without Mtch2 for 20 min at room temperature. Mitochondria with bound $tBid_{Alexa488}$ were isolated by centrifugation (13000 × *g* for 10 min at 4°C). To observe transfer of $tBid_{Alexa488}$, mitochondria with pre-targeted $tBid_{Alexa488}$ were mixed with mitochondria labeled with DiI (M_{DiI}) with or without Mtch2 in a ~1:1 ratio for 30 min at 37°C. Bars indicate the percentage of FRET efficiency as a measure of tBid binding to mitochondria. Final FRET efficiency was calculated as in [3] (mean ± s.e.m., n = 3). For each bar, the schematic on the left recapitulates the content of the incubation.

It is clear that $tBid_{A488}$ originally bound to +Mtch2 mitochondria successfully migrated to +Mtch2 M_{DiI} (FRET efficiency of 45%), but had notably decreased transfer to –Mtch2 M_{DiI} (FRET efficiency of 18%). For –Mtch2 mitochondria, ten fold less $tBid_{A488}$ bound to the MOM compared to +Mtch2 mitochondria after incubation (shown previously in [3]). Therefore, when –Mtch2 mitochondria containing $tBid_{A488}$ were incubated with M_{DiI}, $tBid_{A488}$ had even more robust transfer to +Mtch2 M_{DiI} (FRET efficiency of 52%) but only limited transfer to –Mtch2 M_{DiI} (FRET efficiency of 25%). Thus, Mtch2 enhances tBid binding if it is in the "accepting" membrane.



Supplemental Figure 4: Binding of Bax to membranes is independent of tBid and cardiolipin. Percentage of FRET efficiency indicative of binding of Bax_{DAC} to $liposomes_{NBD}$. 100 nM Bax labeled with the donor, DAC and liposomes containing the acceptor NBD-PE with the indicated lipid composition (See Materials and Methods) were incubated for 30 min at 37°C. FRET efficiency was calculated as explained previously [3] (mean ± s.e.m., n = 3).

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CHAPTER VI

Concluding Remarks

With the discovery of Bcl-2 in 1984, immense and extensive research efforts have been made in the scientific community to understand the role of the Bcl-2 family of proteins in apoptosis. We now recognize that this family is composed of three distinct classes of proteins that are crucial regulators of apoptosis at the level of the MOM. The multi-region pro-apoptotic effector proteins, Bax and Bak, oligomerize in and permeabilize the MOM, a step that essentially commits a cell to apoptosis. The pro-apoptotic activator BH3 proteins, such as tBid and Bim, directly activate Bax and Bak, and the sensitizer BH3 proteins, such as Bad, indirectly promote apoptosis by neutralizing the anti-apoptotic proteins. The multi-region anti-apoptotic proteins, such as Bcl-2 and Bcl-XL, inhibit apoptosis by sequestering the effectors and the BH3 proteins.

The functional consequences of the protein-protein interactions within the Bcl-2 family proteins are well understood at a fundamental level, however, the role of the MOM as the "locus of the action" and the resulting complexity in the molecular mechanisms of these proteins has only recently been garnering attention. To incorporate the MOM in the functional interactions between the Bcl-2 family proteins, our lab introduced the embedded together model that has been described in the introduction chapters (chapter I and II). The embedded together model proposes that the membrane is an active participant in the MOMP process, and that Bcl-2 family members undergo conformational changes at the membrane to modulate their relative affinities for available binding partners to decide the fate of the cell.

The work presented in this thesis highlights the molecular mechanism of activation of the Bcl-2 pro-apoptotic protein, tBid, at the membrane. We show that the interaction of tBid with membranes is dynamic and results in conformational changes at the membrane that are necessary for tBid catalyzed activation of Bax. In addition, we demonstrate that lipids at the membrane can modulate different steps of membrane permeabilization by Bax activated by tBid and Bim. We employed both *in vivo* studies in cells and *in vitro* experiments conducted using isolated mitochondria, liposomes, and purified proteins to explore the molecular mechanisms of the interaction of Bcl-2 proteins with lipid membranes.

Bid, an unconventional BH3 protein

Bid was generally classified as a BH3 activator protein since it can potently activate Bax and Bak to commence MOMP. However, Bid is an outlier in the 'BH3-only' category, since it does not share features associated with the other members in this class. Rather, Bid shares phylogenetic, structural and functional features with the multi-region proteins, such as Bax and Bcl-XL (Billen et al., 2009). While other BH3 proteins evolved later, Bid along with Bcl-2, Bcl-XL and Bax share a common origin. In addition, Bid has a three-dimensional fold in solution that is similar to that of Bcl-XL and Bax, while all other BH3 proteins are intrinsically unstructured in solution. The mechanism of activation of Bid through the removal of its N-terminus, oligomerization of tBid in the membrane (Shivakumar et al., 2014) (Chapter III), and the presence of a BH4 region are analogous to that of Bax. Therefore, we proposed that Bid is a Bax-like protein that is unable to permeabilize the membrane.

Conformational change in tBid at the membrane

The Bcl-2 family of proteins consists of constitutively membrane-bound proteins, such as Bcl-2 and Bak, and of cytoplasmic proteins that translocate to the MOM during the onset of apoptosis, such as Bcl-XL, Bax, and Bid. Irrespective of the original localization, Bcl-2 and Bax embed their core membrane-binding helices (α5 and 6) in the membrane and undergo a structural change when activated (Annis et al., 2005; Dlugosz et al., 2006; Kim et al., 2004). Due to the similarities between Bid and the multi-region Bcl-2 proteins, we hypothesized that tBid also undergoes a conformational change after embedding in the membrane.

By fluorescently labeling the single-cysteine mutants of tBid that encompassed all the helices with an environment sensitive dye, we resolved the unfolding mechanism of tBid at the membrane that commences after the initial binding of tBid to the membrane (Chapter III). Our results demonstrate that helices 4, 5 and 8 of tBid are the first to

interact with the membrane, which is then followed by the insertion of the membrane-binding helices (α6 and 7) in the membrane. The BH3 region on helix 3 remained solvent-exposed and hence available for binding with other proteins, in agreement with the fact that this region of tBid is necessary for interactions with other Bcl-2 proteins (Desagher et al., 1999; Lovell et al., 2008; Luo et al., 1998).

In agreement with our results, others have proposed a similar structure of tBid in the membrane using computer modeling or by employing different techniques in lipid membranes (Oh et al., 2005; Veresov and Davidovskii, 2007). However their studies reported an average conformation of tBid in the membrane that was static. Based on the relative population of proteins with solvent exposed or membrane embedded environment, we proposed a model that suggests that the two conformers of tBid (with or without helices 6-7 inserted in the membrane) exist in equilibrium (Figure 9, Chapter III). We suspect that this equilibrium is significant in the activity of tBid, and perhaps interactions with other Bcl-2 proteins or MOM proteins perturb this equilibrium to modulate the function of tBid.

The progression of the conformational change and the final topology in the membrane appear to be a common hallmark of Bcl-2 proteins that share similar structural fold with Bax, Bcl-XL and Bid. For example, we expect Bak and Mcl-1 to undergo similar steps of activation as described above. However, whether intrinsically unstructured BH3 proteins, such as Bim and Bad, undertake a similar mechanism of activation at the membrane is yet to be determined. The BH3 region of Bim and Bad adopt a helical conformation upon binding to Bcl-XL (Liu et al., 2003; Petros et al., 2004), but it is not clear if membranes are required for such conformational change since Bim and Bad can bind to Bcl-XL in solution (unpublished data). Therefore, it is imperative to study the topology of BH3 proteins at the membrane, alone and with other Bcl-2 proteins to broaden our understanding of their molecular mechanism.

Mtch2 and cardiolipin: receptors of tBid at the membrane

For cytoplasmic Bcl-2 proteins, activation is dependent on post-translational modifications, i.e. cleavage or de/phosphorylation events. After activation, most Bcl-2 proteins are located at the MOM or at the ER membrane. The membrane-binding region, usually located on the C-terminus of the Bcl-2 proteins dictates their translocation to the membranes. However, different Bcl-2 proteins use different mechanisms to translocate to membranes: most multi-region proteins contain a tail-anchor, whereas the BH3 proteins contain either a tail-anchor or a hydrophobic targeting region that directs the proteins to the desired membrane (Chapter I and II). The requirement of receptor proteins or lipids at the preferred membrane adds another level of specificity in the recruitment process of Bcl-2 proteins to membranes, for example Bim interacts with the TOM complex to insert in the MOM (Weber et al., 2007).

Both cardiolipin, a negatively charged lipid exclusive to mitochondria, and Mtch2, a MOM resident protein, have been shown to facilitate the binding of tBid to the MOM (Gonzalvez et al., 2005; Lutter et al., 2000; Zaltsman et al., 2010). We showed that while the initial binding of tBid to membranes is not influenced by the lack of Mtch2 or cardiolipin, the change in conformation of tBid at the membrane is dependent on Mtch2 or cardiolipin in the absence of Mtch2 (Chapter III and V). The conformational change in tBid is the rate-limiting step in the activation mechanism and is required for Bax mediated cell death. Therefore, Mtch2 and cardiolipin act as beacons to accelerate tBid activation at the MOM and the resulting MOMP.

Furthermore, remarkably, the regions of tBid that first interact with the membrane (Chapter III) correspond to the regions of tBid identified to bind to Mtch2 in another study (Katz et al., 2012). The overlap in the function of Mtch2 and cardiolipin suggests a common or a synergistic mechanism of tBid activation. Mtch2 is a structural homologue of the mitochondrial protein, ANT1, which binds six molecules of cardiolipin in the MIM. Since Mtch2 is located on the MOM, we speculate that the role of Mtch2 is to bind to cardiolipin molecules on the MOM to create a ‘landing pad’ for tBid on cardiolipin

rich domains. However, this hypothesis remains speculative and will require future studies to be proven.

Effect of membrane lipids on tBid, Bim and Bax

To study the specific effects of different MOM lipids on membrane permeabilization mediated by Bax when activated by either tBid or Bim, we employed liposomes as a model system to have complete control over the constituents of the reaction. We demonstrated that the binding of tBid and Bim to the membrane is directly correlated with the anionic lipid content of a membrane. Binding to membranes is severely hampered when neutral lipids replace negatively charged lipids in the membrane. We further showed that different anionic lipids can recruit tBid and Bim to the membrane, and the initial binding of these proteins to the membrane is dependent on the overall negative charge of the membrane and not on the identity of individual lipids (Chapter V). These observations along with the fact that both tBid and Bim contain clusters of positively charged residues in their membrane-binding regions supports the idea that binding of tBid and Bim to the membrane is regulated by electrostatic interactions.

As described earlier, the rate-limiting step of the conformational change in tBid is dependent on cardiolipin in liposomes. Interestingly, the conformational change in tBid was only facilitated by cardiolipin, and not by the other negatively charged lipids although tBid binding to the membrane was not affected. Contrary to tBid, other than the requirement of a threshold of negatively charged lipids to assist in membrane binding, Bim activity was not affected by lipids. This highlights that while two major steps in the activation of tBid are dependent on lipids, only the first step of membrane binding is dependent on lipid charge for Bim. Presence of cholesterol increases membrane rigidity and has been shown to inhibit membrane permeabilization by Bax. We reported that cholesterol inhibits the interaction of Bax with the activator proteins and Bax insertion in the membrane, thereby impeding membrane permeabilization.

Our observations with the effects of different lipids on tBid, Bim and Bax support a model where lipids exert multiple levels of control over membrane permeabilization: the overall negative charge of the membrane modulates the binding of BH3 proteins to the membrane; whereas, cardiolipin and cholesterol influence the rate-limiting steps in the activation of tBid and Bax, respectively. Our model system faithfully recapitulates the core mechanism of Bcl-2 proteins and the effect of cardiolipin and cholesterol on MOMP, as observed *in vivo* by others (Lucken-Ardjomande et al., 2008; Lutter et al., 2000), is consistent with what we observed *in vitr*o. Our findings therefore highlight the multiple levels of control that are employed by cells to regulate MOMP. Furthermore, interaction of Bad with negatively charged lipids and cholesterol has been previously reported (Hekman et al., 2006), which leads us to propose that lipids of the MOM play a much wider role in the regulation of Bcl-2 family proteins than previously suggested. Whether the role of lipids extends to all Bcl-2 family proteins or only to the pro-apoptotic class remains unknown. Preliminary data from our lab suggests that anti-apoptotic proteins, such as Bcl-XL are not as influenced by the lipid composition of the membrane. Therefore, systematic studies to validate this observation will no doubt add to our understanding of the role of different membrane lipids in the regulation of MOMP.

Dynamic binding of tBid and Bax to membranes

In a healthy cell, Bcl-2 proteins perform functions to maintain cellular homeostasis in different subcellular locations. Upon apoptosis induction, both tBid and Bax bind to the MOM to promote membrane permeabilization. tBid and Bax rapidly bind to lipid membranes, and dissociate from them with measurable dissociation constants (Chapter III and V) (Lovell et al., 2008; Satsoura et al., 2012) indicating that the binding is reversible. In addition, both Bcl-XL and Bax have been reported to retro-translocate from the MOM in transformed cells (Edlich et al., 2011; Schellenberg et al., 2013). Our studies demonstrate that binding to membranes for both tBid and Bax (the loosely associated *and* the membrane-bound forms) is reversible.

The rate of transfer of tBid between membranes is accelerated by Bax, suggesting that tBid at the membrane is present in two pools: one that is bound to Bax with an apparent K_d of 25 nM (Lovell et al., 2008), and the other that transiently interacts with Bax and then unbinds from the membrane. Perhaps the first pool of tBid is necessary for the recruitment of more Bax molecules to the membrane (kiss and stay), whereas in the second pool, tBid is discarded to allow Bax homo-oligomerization and the extension of the pore (kiss and run). The rate of transfer of tBid between membranes is impeded by Bcl-XL, suggesting a mechanism in which Bcl-XL strongly binds to tBid and restrain it to a limited location. This may stop the spread of the apoptotic signal in the cell. The opposing effect of Bax and Bcl-XL on the rate of tBid transfer between membranes is analogous to the opposing functional roles of these proteins in apoptosis. On the other hand, the transfer of Bax between membranes does not depend on tBid. However, Bax requires an activator at the recipient membrane for membrane insertion and permeabilization. This finding emphasizes the necessity of activator Bcl-2 proteins for the direct activation of Bax (Chapter IV).

Our findings corroborate the hypothesis formulated in the embedded together model that the interactions between Bcl-2 proteins and the membrane are governed by dynamic equilibria. In addition, our results suggest that both tBid and Bax potentially play a role in the propagation of the apoptotic signal through the cell. Whether other Bcl-2 proteins, such as Bim and Bad participate in a similar mechanism remains to be tested. Based on the embedded together model and the dissociation constants of these proteins with the membrane, we expect them to also transfer between membranes.

Bcl-2 proteins: a therapeutic target

For most cells, MOMP is the commitment step to apoptosis and therefore modulating this step is a promising therapeutic target for the treatment of cancer. Many cancers have a defect in the apoptotic pathway for their development or for resistance to treatment. Over-expression of the anti-apoptotic proteins is a common mechanism for

tumours to evade apoptosis (Beroukhim et al., 2010). Consequently, chemical inhibitors of the anti-apoptotic proteins, known as BH3 mimetics for sharing their structure with the BH3 region of Bad are currently in clinical trials (van Delft et al., 2006; Varadarajan et al., 2013). Loss of expression of Bax and many BH3 proteins has also been observed in different cancers to escape apoptosis (Beroukhim et al., 2010; Brimmell et al., 1998; Rampino et al., 1997; Sturm et al., 2006). Therefore, promoting apoptosis by directly activating Bax/Bak or by activating the BH3 proteins will lead to novel avenues of therapy against cancer. Findings presented in this thesis emphasize that the active conformation of Bcl-2 proteins in apoptosis is at the membrane. Therefore, screening for small-molecule inhibitors and activators of Bcl-2 proteins at the membrane will yield promising results.

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