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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.

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

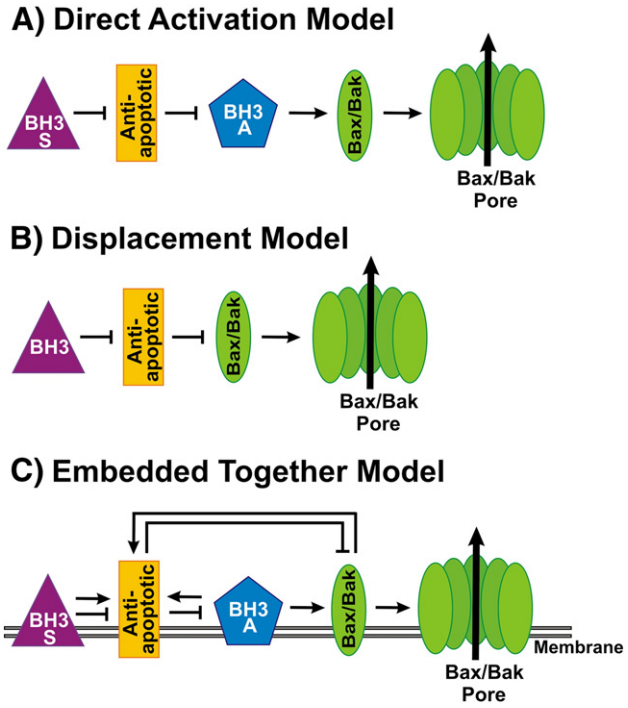

Fig. 1. Overview of the three models of regulation of apoptosis by Bcl-2 family proteins. BH3-S and BH3-A represent sensitizer and activator BH3-only proteins, respectively. A) The Direct activation model proposes that activator BH3-only proteins are required for activating Bax and Bak. Anti-apoptotic 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 [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 α9 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 α5 and α6, 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

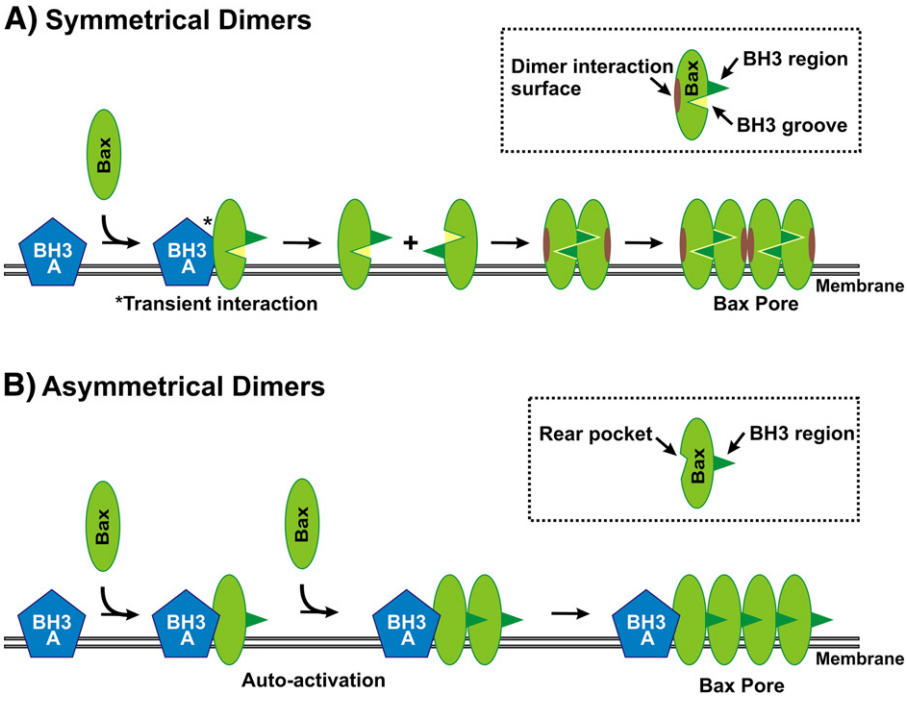

Fig. 2. Mechanisms of Bax/Bak oligomerization in membranes. Only Bax has been shown in the schematics for simplicity. A) The model for symmetrical dimers proposes that through transient interaction between an activator BH3-only protein and Bax/Bak, the BH3 region and a BH3 groove are exposed in Bax/Bak. 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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