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Review

Bcl-2 proteins and mitochondria—Specificity in membrane targeting for death ☆

Jennefer Lindsay [a,*], Mauro Degli Esposti [b], Andrew P. Gilmore [a]

[a] Wellcome Trust Centre for Cell Matrix Research, Faculty of Life Sciences The University of Manchester, UK
[b] Faculty of Life Sciences, The University of Manchester, UK

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ABSTRACT

The localization and control of Bcl-2 proteins on mitochondria is essential for the intrinsic pathway of apoptosis. Anti-apoptotic Bcl-2 proteins reside on the outer mitochondrial membrane (OMM) and prevent apoptosis by inhibiting the activation of the pro-apoptotic family members Bax and Bak. The Bcl-2 subfamily of BH3-only proteins can either inhibit the anti-apoptotic proteins or directly activate Bax or Bak. How these proteins interact with each other, the mitochondrial surface and within the OMM are complex processes we are only beginning to understand. However, these interactions are fundamental for the transduction of apoptotic signals to mitochondria and the subsequent release of caspase activating factors into the cytosol. In this review we will discuss our knowledge of how Bcl-2 proteins are directed to mitochondria in the first place, a crucial but poorly understood aspect of their regulation. This article is part of a Special Issue entitled Mitochondria: the deadly organelle.

1. Introduction: The mitochondria as an essential point for apoptosis regulation

Apoptosis is fundamental to multi-cellular organisms during both development and homeostasis. This process of controlled cell suicide ensures that cells grow and divide only when in the correct context and are removed when they become irreparably damaged or surplus to requirements. The absence of survival signals or cellular damage activates a highly ordered and controlled cell suicide program, which provides the most efficient and least damaging way to remove unwanted cells. The balance between survival and apoptotic signals must be tightly controlled, and the failure of these controls on apoptosis contributes to diseases such as cancer [1].

Apoptosis can be initiated by extrinsic factors, such as ligands for cell surface death receptors (not within the scope of this review) or intrinsically by responding to damage and stress [2]. In both cases, classical apoptosis results in the activation of cysteinyl aspartyl proteases (caspases) that then rapidly dismantle the cell [3]. Caspases are present as inactive pro-enzymes in healthy cells, poised to drive cellular destruction upon receipt of an activating signal. This destruction is not passive through to the degradation of cellular proteins, but rather is driven by signaling events where many caspase substrates are themselves activated by cleavage [4].

In most cases, cells undergo apoptosis *via* engagement of the intrinsic pathway, the regulation of which is centered at the mitochondria. This has as its critical point of no return mitochondrial outer membrane permeabilization (MOMP), a sudden and dramatic event resulting in the release of soluble factors from the inter membrane space [5]. The key factors released, cytochrome *c* and SMAC/DIABLO, cooperate with cytosolic factors to activate the caspases. The Bcl-2 family proteins are the essential regulators of MOMP, and the correct targeting of the Bcl-2 family to mitochondria is critical for apoptosis [6]. The purpose of this review is to discuss the mechanisms by which mitochondrial targeting of Bcl-2 proteins are achieved.

2. Bcl-2 protein function—The accepted models of cell death activation

The Bcl-2 family proteins are critical regulators of apoptosis, and their primary site of action is on the outer mitochondrial membrane (OMM). This is not to say that mitochondria are the only site of action of Bcl-2 proteins, and the literature has examples of other functions, such as on the ER for example [7]. Bcl-2 proteins can be classified as either pro-apoptotic or anti-apoptotic [6]. Initial characterization of Bcl-2 proteins, before any structural information became available, identified a number of regions of sequence homology between them

Abbreviations: BH, Bcl-2 Homology; CMC, Critical micellar concentration; CtTA, C-terminal tail anchor; ER, Endoplasmic reticulum; GFP, Green fluorescent protein; GIP, General import pore; LPG, lysophosphatidyl-glycerol; MTCH2, Mitochondrial carrier homologue 2; MOMP, Mitochondrial outer membrane permeabilization; OMM, Outer Mitochondrial Membrane; TIM, Transporter inner membrane; TOM, Transporter outer membrane; TMD, Transmembrane domain; SRP, Signal recognition particle; VDAC, Voltage dependent anion channel

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* Corresponding author. Tel.: +44 16 1275 5576.
E-mail address: Jennefer.Lindsay@manchester.ac.uk (J. Lindsay).

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that were termed Bcl-2 homology (BH) domains. These regions of sequence homology conveniently mapped onto the functional classification of Bcl-2 proteins, supporting the supposition that they did indeed represent structural domains within these molecules. The anti-apoptotic proteins, including Bcl-2, Bcl-X_L, Bcl-w and Mcl-1 contain BH domains 1–4. These anti-apoptotic proteins are generally found on the OMM, where they function to inhibit the pro-apoptotic Bcl-2 proteins. The pro-apoptotic Bcl-2 proteins are divided into the multi-domain effectors Bax, Bak and Bok (containing BH domains 1–3), or BH3-only proteins, which contain only the BH3 domain. However, care has to be taken when describing these regions of sequence homology as "domains". A protein domain is a conserved, 3-dimensional structure that is stably folded independent of the rest of the protein and can exist and evolve in isolation. The Bcl-2 family are all small, globular proteins. The 3-dimensional structures known for Bcl-2 proteins, including Bax, Bcl-w and Bcl-X_L suggest that the BH "domains" are not independently folded structural units, but instead are integral parts of the Bcl-2 proteins compact 3-dimensional structure [8–10]. However, two regions that do appear to have distinct structural and functional identities are the BH3 domain and the C-terminal tail anchor. The basis of Bcl-2 protein function in apoptosis is largely centered on these two regions.

The multi-domain pro-apoptotic proteins Bax and Bak are thought to promote MOMP by oligomerizing to form pores within the OMM [11]. The BH3-only proteins, such as Bid, Bim and Bad, act as either direct activators of pro-apoptotic Bax and Bak, or as de-repressors of anti-apoptotic Bcl-2 and Bcl-X_L [12,13]. Two conflicting models of how this occurs have been presented, reviewed in [14]. The first proposes that Bax and Bak are constantly held in check by the anti-apoptotic activity of the Bcl-2 like proteins. This repression is relieved by activated BH3-proteins, releasing Bax and Bak to drive MOMP. The alternative view is that some BH3-proteins (Bid, Bim and Puma) directly bind to and activate Bax and Bak, but that in a healthy cell their exposed BH3-domains bind to and are sequestered by anti-apoptotic Bcl-2 proteins. Bid has recently been found to be structurally and phylogenetically closer to multi-domain Bcl-2 proteins [6], but in this review we will maintain its historical definition as a classical BH3-only protein. The other BH3-proteins, such as Bad, compete for binding to the anti-apoptotic proteins, and displace Bid, Bim and Puma to allow them to activate Bax. These models share the common feature that the key interaction proposed is between the BH3-domain of the BH3-only proteins and the multi-domain proteins.

What is less clear is how survival or death signals lead to activation of the Bcl-2 family specifically on the *mitochondria*. In normal healthy cells, most anti-apoptotic multi-domain Bcl-2 family members are constitutively found in the OMM or the endoplasmic reticulum (Fig. 1, left). The multi-domain pro-apoptotic protein Bak is constitutively localized to the OMM. Conversely, Bax is predominantly found in the cytosol and is recruited to the OMM upon receipt of an apoptotic signal [15,16]. It should be noted, however, that Bax is not exclusively cytosolic prior to apoptosis, and that detectable amounts can be seen on mitochondria in healthy cells. Furthermore, Bax translocation to mitochondria does not commit a cell to undergo MOMP. Inducing Bax translocation, by either mutating amino acids within its targeting

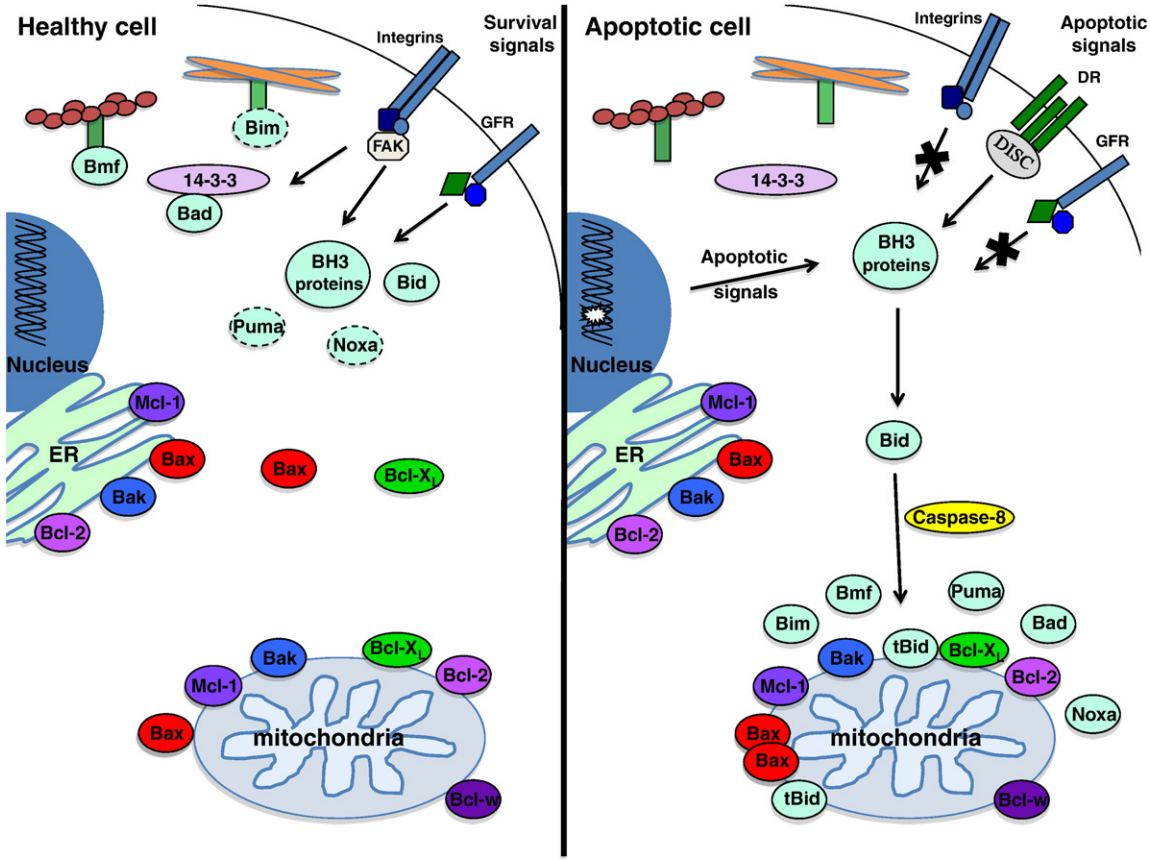

Fig. 1. Localization of Bcl-2 family proteins. In normal healthy cells Bcl-2 proteins are found both in the cytoplasm and on the OMM. It has been proposed that the anti-apoptotic proteins, such as Bcl-2, Bcl-X_L, Bcl-w and Mcl-1, are found on the OMM where they act to inhibit apoptosis through interaction and inhibition of the pro-apoptotic proteins Bax and Bak also on the OMM. Bcl-2 is always integrally inserted into the OMM, whereas the other anti-apoptotic members can be soluble, loosely attached or integrated into the membrane. Bak is constitutively found on the OMM whereas Bax is predominantly found as an inactive monomer in the cytoplasm, which then translocates to the mitochondria in apoptotic cells to form homo- and hetero-dimers with Bak. Some BH3-only proteins (Bim, Puma and Noxa) are expressed at low levels in healthy cells and are transcriptionally up-regulated following apoptotic signals, whereas others are post-translationally modified to control their localization (Bad, Bim, Bid). Once at the mitochondria the BH3-only proteins either directly activate the pro-apoptotic proteins Bax or Bak or inhibit the anti-apoptotic members such as Bcl-2 or Bcl-X_L.

domain [17] or by removing survival signals [18] can result in mitochondrial associated Bax without MOMP.

During apoptosis Bax and Bak both undergo conformational changes associated with MOMP [16,19]. Bax must undergo both conformational change and targeting to the OMM to activate apoptosis (Fig. 1, right). Members of the BH3-only proteins also target to the mitochondria to activate Bax or Bak or inhibit Bcl-2 or Bcl-X_L. These proteins are either transcriptionally induced (e.g. Bim, PUMA and Noxa) and/or post-translationally modified (e.g. Bim, Bid, Bad) prior to translocation [20]. For example Bad is phosphorylated by survival signals such as the PI3K/Akt pathway, which promotes binding to 14–3–3 scaffold proteins, sequestering it in the cytoplasm [21,22]. Loss of survival signal leads to Bad dephosphorylation and dissociation form 14–3–3, where it can translocate to the mitochondria and interact with Bcl-X_L. The BH3-only protein Bid is inactive in the cytosol in healthy cells and is predominantly regulated by cleavage by caspase 8 [23]. Following apoptotic signals, Bid or tBid translocates to the mitochondria and interacts with Bax/Bak on the OMM to promote cytochrome *c* release and apoptosis.

However, none of these models fully explains how these proteins get to the correct membrane in the first place. Although proper targeting of Bcl-2 family proteins to mitochondria is essential for their function, very little is actually known regarding how this is achieved. Because this represents a critical point in their regulation, it warrants some consideration.

3. Tail anchor mediated targeting of multi-domain Bcl-2 proteins to the OMM

3.1. Bcl-2 protein tail anchors

Out of the estimated 1000+ proteins found in mitochondria, only 13 are encoded for by the mitochondrial genome [24,25]. Thus, the vast majority of mitochondrial proteins, including the Bcl-2 family, need to be post-translationally targeted to the correct mitochondrial compartment [26,27]. A number of different types of mitochondrial addressing sequence are known that can achieve this, including N-terminal presequences, internal membrane spanning sequences, and C-terminal tail anchors. Each utilizes distinct mechanisms for mitochondrial import, some of which are better understood than others. For example, much is known about the mechanisms by which mitochondrial proteins containing an N-terminal presequence are imported through the general import pore (GIP), consisting of the TOM and TIM translocase complexes [28]. The TOM complex acts as both a receptor and the import machinery for proteins with N-terminal presequences. These are unfolded, translocated through the pore to traverse the membrane, and subsequently refolded following arrival at the correct mitochondrial compartment. The nature of the N-terminal presequence plays a role in the final destination.

C-terminal tail anchors are another class of targeting sequence, but are not restricted to mitochondrial proteins [29]. These sequences target to other organelles as well, notably the endoplasmic reticulum (ER), the organelle where this mechanism has been most extensively studied [30]. Unlike N-terminal targeting sequences, a C-terminal tail anchor sequence (CtTA) requires the protein to have completed translation before targeting can occur. Tail-anchored proteins do not show exact sequence conservation in the tail region, but instead share some common characteristics that determine CtTA function. There is a hydrophobic transmembrane domain (TMD) of approximately 20 amino acids, enough to span the target membrane once, flanked by charged amino acids. Variations in hydrophobicity and the flanking sequence contribute to targeting specificity [31–33]. All CtTA proteins, whatever their subcellular localization, are positioned such that the proteins N-terminus, containing the proteins functional domain, is cytosolic. The C-terminus is usually very short, usually only a few amino acids, and is always within the lumen of the target organelle. The addition of a large protein domain C-terminal to a CtTA blocks targeting and insertion [34].

Almost all the multi-domain Bcl-2 proteins, both pro- and anti-apoptotic, contain a functional CtTA. The multi-domain Bcl-2 proteins Bak, Bcl-2, Bcl-X_L, Bcl-w and Mcl-1 have C-terminal sequences that contain all the classical features of a CtTA [34–38] (Fig. 2). In most cases, the CtTA of each has been validated experimentally. Deletion of the CtTA domain prevents targeting, and attachment of the CtTA to a heterologous protein (such as GFP) directs targeting, two key tests to show a putative CtTA is functional [34,39–41]. Bcl-2 has flanking sequences that are not as polar as in Bcl-X_L, and this explains its distribution between both the ER and the mitochondria. Bcl-X_L, in contrast, targets mitochondria. Manipulation of the TMD flanking sequences of either Bcl-2 or Bcl-X_L altered their targeting specificity [32].

Not all multi-domain Bcl-2 proteins have CtTA sequences that fit the classical criteria. Bax is unusual in that its Ct-TA is not preceded by any polar amino acids (Fig. 2). From this, some studies suggested that Bax does not have a functional Ct-TA, and is instead targeted to mitochondria *via* an N-terminal presequence [42,43]. The Bax N-terminus was proposed to direct Bax to the GIP through an interaction with TOM22, a model that might explain the N-terminal conformational change in Bax associated with apoptosis [44]. However, the data supporting a role for TOM proteins in Bax targeting is conflicting, and will be discussed further below. Furthermore, a number of other studies have presented convincing evidence that Bax does indeed have a functional CtTA, which is both necessary and sufficient for mitochondrial targeting [34,45]. Bax is unusual, however, in that its tail anchor does not constitutively target. Instead, Bax is predominantly cytosolic until the cell receives an apoptotic stimulus [17,18,46]. The differences between the Bax and other mitochondrial CtTA sequences appear to be important for this regulated insertion of Bax compared with the constitutive insertion of other proteins. The region where Bcl-X_L has acidic residues N-terminal to the TMD is replaced in Bax by a proline at residue 168. In the NMR structure of human Bax, the *trans* conformation of this proline enables the CtTA to be folded back along a groove on the surface of Bax, explaining why targeting is not constitutive [8]. Experimental manipulation of Pro168 or sequences within the TMD itself, result in either constitutively targeted Bax or versions that neither target nor induce apoptosis [17,45]. However, as mentioned earlier, expression of constitutively targeted Bax does not result in uncontrolled cell death and instead it now resembles Bak in function [17]. Thus, once on the OMM, Bax is

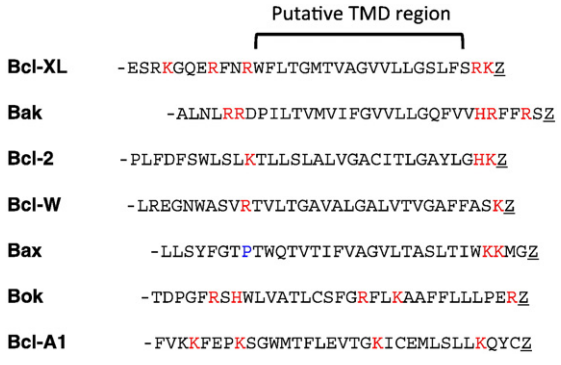

Fig. 2. Bcl-2 family tail anchor sequences. Comparison of putative c-terminal tail anchor sequences in multi-domain Bcl-2 family members. The underlined Z represents the stop codon in the coding sequence, highlighting that the C-terminal portion of the protein within the mitochondria is extremely short (between 1 and 6 amino acids). Most contain a readily identifiable transmembrane domain (TMD), apart from Bok and Bcl-A1, which contain polar amino acids within the TMD. These might be predicted to prevent membrane insertion, but to date no data is available for the function of these putative tail anchor sequences. As can be seen, there is considerable variation in the TMD region (both in terms of length and hydrophobicity) and the flanking sequences. Bcl-X_L and Bak have the most polar flanking sequences (polar amino acids are highlighted in red). The regulatory proline in Bax (P168) is highlighted in blue. Although this Bax CtTA does not have any N-terminal charged residues, it has been verified as a functional mitochondrial targeting sequence (see references in the text).

still subject to some regulation, and therefore the function of this unusual CtTA remains unclear.

How the Bax CtTA is exposed to direct targeting is largely speculative, as there is no structure available for the membrane-inserted conformation. However, the regulation is not intrinsic to the Bax CtTA itself or its unusual N-terminal flanking sequence, and does require the context of the rest of Bax. The Bax CtTA, including Pro168, constitutively targets GFP to mitochondria [34]. The current model is that binding of activated BH3-only proteins induces the conformational exposure of the Bax CtTA. The Bim BH3 helix has been shown to bind on the opposite face of Bax, and that this might loosen the association of the CtTA to allow targeting [47]. However, activated Bid has been shown to only bind to Bax once both Bid and Bax have already associated with a membrane, suggesting that targeting must precede the BH3-domain interaction [48]. It is not clear if Bim and Bid activate Bax through different mechanisms, or if the use of isolated BH3-peptides alters the way they interact with multi-domain Bcl-2 proteins.

Bok, the third mammalian pro-apoptotic multi-domain protein, is less understood, has a restricted expression and may be associated with placental pathologies [49,50]. Bok is variously described as having a nuclear or mitochondrial distribution. The C-terminus of Bok does not fit the consensus for a functional CtTA, as it contains lysine and arginine residues within what should be the hydrophobic TMD (Fig. 2). This would be predicted to prevent membrane insertion, but to date there is no published data on the ability of the Bok C-terminus to target any organelle. Whether or not Bok has a functional CtTA remains to be determined.

3.2. Mechanisms of tail anchor import

Given that the vast majority of mitochondrial proteins are imported from the cytosol, it should be no surprise that the mechanisms for their import have been an intensive area of study. However, although a lot is known about mitochondrial import of N-terminally tagged proteins, which occurs *via* the GIP, much less is known about how mitochondrial CtTA proteins are imported and what, if any, co-factors are required. Fortunately, rather more is known about ER CtTA import, and from this some general concepts can be extracted that may apply to mitochondria. At least three distinct pathways have been characterized at a molecular level for ER CtTA import [30]. These can involve both chaperones and receptors, and some are dependent upon nucleotide hydrolysis. The functions of some chaperones, like SRP, appear to involve masking the more hydrophobic CtTAs as they emerges from the ribosome, preventing aggregation, and then interacting with a receptor on the ER membrane. There is also evidence of a further protein required for driving the membrane insertion of the TMD. Another chaperone, involving Hsp40 and Hsc70, appears to target less hydrophobic CtTA proteins, such as cytochrome *b*5, and these do not require proteins present on the ER for membrane integration [51]. Thus, the level of hydrophobicity of the CtTA itself contributes to the requirement for different chaperones. Moderately hydrophobic TMDs, such as in cytochrome *b*5, do not require chaperones to insert into membranes following *in vitro* translation, whereas more hydrophobic sequences, such as that found in synaptobrevin, do. However, the key finding is that there is no universal mechanism for the biogenesis of CtTA proteins on the ER. It would not, therefore, be surprising if multiple mechanisms were seen for mitochondrial CtTA proteins as well. Indeed, it has been shown that the CtTA proteins of the TOM complex are targeted by a mechanism distinct from CtTA proteins that are dispersed throughout the OMM [52].

So what is known about factors required for targeting mitochondrial CtTA sequences, and specifically Bcl-2 proteins? The published data here are often conflicting, but there is evidence that other mitochondrial proteins do contribute to Bcl-2 protein targeting. An interaction between Bcl-2 and TOM-20 has been suggested to be required for its mitochondrial insertion in yeast [53]. However, other studies showed that trypsin treatment of mitochondria did not block Bcl-2 association and insertion into the OMM [36]. Bcl-2 has a moderately hydrophobic CtTA and, like cytochrome *b*5, may well be able to undergo unassisted membrane insertion. A recent study has shown that the targeting of Bak and Bcl-X_L to mitochondria is TOM independent [54]. The cytoplasmic portion of the protein may also be important, and the requirement for a mitochondrial protein for targeting and insertion was dependent on the presence of the intact, full-length Bak. The Bak CtTA alone was able to target and insert into mitochondria in which the voltage dependent anion channel, VDAC2, had been depleted. However, in the context of full-length Bak, loss of VDAC2 prevented targeting and insertion. VDAC2 has been shown in other studies to be able to interact with Bak, with a role in preventing Bak activation [55,56]. Another study has also indicated a role for VDAC in Bak mitochondrial targeting, suggesting that biogenesis of at least some Bcl-2 proteins requires a mitochondrial receptor [57].

Whether or not Bax requires a mitochondrial receptor is also unclear, with a number of studies indicating that TOM proteins may play a role [44,58]. Again, if we compare with ER-CtTA import, we see that SRP is best known as a mediator of classical signal-sequence driven, co-translational import [30]. Thus, it is not unprecedented for components of an N-terminal import sequence pathway to direct the insertion of CtTA containing proteins as well. Thus, it cannot be ruled out that components of the GIP can act as receptors for Bcl-2 proteins. However, the published data on Bax targeting and the GIP are confusing and contradictory. Direct binding between Bax and TOM22 has been shown [44]. However, studies in yeast have provided conflicting data. Using a temperature sensitive yeast mutant, Ott et al. demonstrated a requirement for the TOM40 channel for tBid induced Bax mitochondrial membrane insertion and cytochrome *c* release [58]. However, in a similar study not only was TOM40 not required for Bax induced cytochrome *c* release, but the three TOM complex receptors, TOM20, TOM22 and TOM 70 were also dispensable [59]. Bax can insert into and permeabilize protein-free liposomes prepared from extracted mitochondrial lipids [60]. This would imply that Bax does not absolutely require a receptor protein for membrane insertion. However, whether this is the case *in vivo*, or if different multi-domain Bcl-2 proteins use different mechanisms, has yet to be clarified.

A number of studies have suggested that cytoplasmic Bax may interact with chaperones, such as 14–3–3 proteins [61,62]. These interactions have been proposed to control its distribution by maintaining Bax in the cytoplasm in healthy cells. Some ER CtTA proteins do interact with chaperones in the cytoplasm, as discussed above. These chaperones both mask the hydrophobic TMD to maintain its integration competence and to direct interactions with receptors on the target membrane. However, the structure of Bax suggests that it can act as a chaperone for its own CtTA, which is folded back along the surface groove [8]. Whether or not other Bcl-2 family proteins interact with chaperones is not completely clear. It has been suggested that cytoplasmic proteins may not be required for Bcl-X_L import, but were required for full-length Bak [54]. At present our knowledge of Bcl-2 protein targeting to mitochondria is still incomplete.

4. Non-tail anchor mediated interactions with mitochondria

Most BH3-only proteins present a different set of issues with regard to mitochondrial targeting. Many studies have focused on the interactions of these *via* their BH3-domains, and the contribution of the rest of the molecule is poorly understood in most cases. However, in those cases where it has been examined, it is clear that BH3-only proteins contain specific targeting regions that are vital for their normal regulation. Fundamentally, mitochondrial targeting of Bcl-2 family proteins may depend upon three types of interactions: 1) with

other members of the Bcl-2 family; 2) with lipid constituents of mitochondria; 3) with proteins of other families, including enzymes that produce post-translational modifications. Intriguingly, the first two types of interactions can be mimicked *in vitro* by certain detergents. Therefore, we will first survey how detergents influence the oligomerization and reciprocal interaction of Bcl-2 proteins.

4.1. Bcl-2 proteins are sticky and react with detergents

One basic fact underlies both the process of activation and that of mitochondrial targeting: Bcl-2 proteins with multiple BH domains are rather sticky and therefore can dynamically associate with small hydrophobic molecules like detergents and lipids. Besides the hydrophobic C-terminal tail already discussed here, Bcl-2 proteins possess a prominent hydrophobic groove at their surface, in which the BH3 domain of other Bcl-2 proteins can fit. This property has provided a model for building potent antagonist drugs such as ABT-737 [63,64]. The structural design of these drugs revealed that the surface groove of Bcl-X_L is likely to accommodate a variety of hydrophobic molecules, besides the amphipatic alpha-helix forming the BH3 domain of BH3-only proteins such as Bad [64]. Indeed, the same groove of Bcl-X_L has been reported to bind diverse detergents at concentrations in which they are monomeric in solution [65].

Above their critical micellar concentration (CMC), the same detergents produce large conformational changes in Bcl-X_L [65] as well as Bax [8], due to complex interactions with the micelles that drive protein oligomerization [66–69]. Because in most instances cell extracts have been made with concentrations of non-ionic detergents (such as Triton-X-100) that are well above their CMC, spurious homo- and hetero-oligomerization of Bcl-2 proteins have been detected solely due to the multimeric effect of interacting with detergent micelles [68]. This problem is sometimes overlooked, especially in recent literature. Considering also the lack of specific reviews on the interactions between Bcl-2 proteins and detergents, we need to stress their important implications, in experimental studies as well as in the understanding of the activation and membrane interaction of Bax.

Detergents are amphipatic molecules that mimic natural lipids, in particular the mono-acyl derivatives of phospholipids such as lysophosphatidyl-glycerol (LPG). They can be ionic and negatively charged like natural LPG, e.g. SDS, or non-ionic, as most detergents used in cell biology (Triton, Tween, octyl-glucoside etc.). There also are a few detergents with zwitterionic properties, i.e. having both positive and negative charges. Perhaps the most popular of these detergents is CHAPS, a cholesterol derivative, which has been considered the only detergent capable of maintaining Bax monomeric in micelles [66,69]. However, CHAPS may not be ideal for extracting mitochondrial Bax in its native quaternary structure [70].

Bax interactions with detergents have received most attention. However, it remains unclear whether the protein is truly oligomeric in detergent micelles or is merely embedded in multiple copies within the same micelles, without forming directly bound oligomers. Indeed, it has been recently claimed that Bax (without its C-terminal helix) may be monomeric within the micelles of non-ionic detergents [71]. The intriguing proposal, which would not explain the established evidence of detergent-induced Bax oligomerization [66], has been subsequently criticized [72] with reference, once more, to the importance of the C-terminal helix of Bax. When the C-terminal helix of Bax is missing, the surface groove is free and may be filled with detergent molecules after artificial activation with micelles of non-ionic detergents, as documented with truncated Bcl-X_L [65]. Perhaps the C-terminal helix of Bax can effectively work like a detergent in driving the conformational changes associated with activation and then binding to the OMM. In this respect, the interactions of Bcl-2 proteins with detergents may mimic protein–protein interactions, besides their most obvious mimic of protein–lipid interactions.

4.2. Mitochondrial lipids: Cardiolipin

The interaction of Bid with mitochondrial lipids has provided new insights to explain how Bcl-2 proteins target mitochondria and then elicit MOMP [98]. Although joined at contact sites, the two membranes of mitochondria are very different in their lipid composition. While the outer membrane is rather similar to those of the ER compartment, with which it is often contiguous, the inner membrane contains the smallest complement of glycolipids and cholesterol, as well as the highest concentration of the glycerol-based phospholipid cardiolipin of any intracellular membrane. Cardiolipin is indeed synthesized and re-modeled within mitochondria and is present in discrete amounts also on the outer surface. It is this small pool of surface-exposed cardiolipin that can justify the role that cardiolipin has been shown to play in the integrated pro-apoptotic action of Bid and Bax [73]. Here we discuss cardiolipin only in the context of mitochondrial targeting of Bcl-2 proteins. Except for the subgroup of BNIP3, BH3-only proteins do not contain a tail-anchor sequence for insertion into mitochondrial membranes but may contain C-terminal lipid-binding motifs as Bid does [74–80]. For example, the C-terminal region of Bad contains two lipid binding motifs required for binding to negatively charged lipids, such as cardiolipin, and for targeting to the mitochondria. This binding of Bad to membranes was shown to also facilitate the translocation of Bcl-X_L to membranes. The targeting of Bcl-X_L to mitochondria and its insertion into the OMM was inhibited by lipid binding mutants of Bad that could still interact with Bcl-X_L but not membranes.

However, hard evidence for a direct interaction with cardiolipin (and its metabolites) is available only for Bid, and especially for its protease-cleaved form tBid. The interaction of tBid with cardiolipin has been shown to involve helix αH6 of the protein, in particular its lysines 157 and 158 [76]. However, recent work in our lab has shown that although this interaction is important for Bid:lipid binding *in vitro*, mutation of one of these residues did not affect the ability of Bid or tBid to localize in mitochondria and induce cytochrome *c* release and apoptosis *in vivo* [81]. Multiple interpretations of these results are possible [81] but it is probable that the interaction of Bid with cardiolipin has three-dimensional connotations that alterations in individual amino acids may not disrupt. Although some studies have reported that a lack of cardiolipin has no effect on cytochrome *c* release or targeting of tBid to mitochondria [58,82–84], recent evidence has established that cardiolipin does have a role in promoting MOMP (see review by Crimi and Degli Esposti in this special issue).

In essence, it seems increasingly likely that the interactions of Bid (and other Bcl-2 proteins) with cardiolipin documented *in vitro* reflect the existence of proteo-lipid domains that are involved *in vivo* in the process of MOMP. Specific proteins may therefore be either part of these domains or modulate the insertion of Bcl-2 proteins into them.

4.3. Interaction with other proteins

In the context of protein–protein interactions within the Bcl-2 family, the BH3 domain has received the most attention. However there is clear evidence that although the BH3 domain is required for the apoptotic function of these proteins it is not essential for targeting [74,79,85]. Bid translocates to mitochondria following apoptotic stimuli as either the full-length protein or caspase cleaved p15Bid [85–87]. However, the targeting of Bid to the mitochondria is independent of the BH3 domain [85,88]. A truncated form of tBid, lacking the BH3 domain, constitutively targets to mitochondria but does not kill the cells [81]. This suggests that targeting of Bid to mitochondria does not require interaction with multi-domain Bcl-2 proteins. Bid may, however, bind to other proteins on the mitochondria that specifically regulate its targeting. Mitochondrial carrier homologue 2 (Mtch2) is a recently characterized OMM protein that

has been shown to form a complex with Bid and Bax [89,90]. Mtch2 knockout mice die at embryonic day E7.5 mainly due to multiple defects in gastrulation [89]. Conditional knockout MEFs show decreased sensitivity to tBid-induced apoptosis due to decreased recruitment of tBid to the mitochondria, and subsequent Bax activation and cytochrome *c* release. This decreased tBid recruitment was also shown *in vivo* in purified intact liver mitochondria prepared from liver-specific knockout mice. These results suggest a direct link between the levels of an OMM protein, Mtch2, and the level of recruitment of tBid to the OMM. Mtch2 deficiency did not affect the lipid composition of the OMM [89], suggesting a direct protein:protein recruitment model. However, tBid recruitment is not completely blocked suggesting an additional level of control, such as another protein or the lipid composition of the OMM as discussed above.

Bad is perhaps the best understood BH3-protein in terms of its interactions with other proteins, and demonstrates a remarkable complexity. The interactions of Bcl-2 proteins with other proteins encompass the issue of post-translational modification that can drive activation. For example, in healthy cells Bad is phosphorylated on serine residues leading to its sequestration in the cytosol through interactions with 14–3–3 scaffold proteins. The function of Bad can also be controlled on the mitochondria. In healthy liver cells, a portion of Bad resides in a functional holoenzyme complex on mitochondria that includes Protein Kinase A, Protein Phosphatase 1, WAVE-1 as an A-kinase anchoring protein (AKAP) and glucokinase [21,91]. This protein scaffold allows PKA to specifically phosphorylate Bad on serine 122 following IL-3 induction [21]. In addition, in PC12 cells, the AKAP protein AKAP121 targets PKA to the mitochondria, which stimulates BAD phosphorylation on serine 155 [92]. Disruption of this scaffold impairs BAD phosphorylation and sensitizes cells to apoptosis even in the presence of survival factors [21,92]. Bad can also be cleaved by caspases following apoptotic stimuli [93–95]. Bad lacking the N-terminal region has a higher affinity for the OMM and Bcl-X_L and is a more potent inducer of apoptosis. This is analogous to Bid, which translocates to the mitochondria and is more active following caspase cleavage [87,96,97]. One could speculate that these proteins contain an N-terminal inhibitory domain, that when released allows the protein to interact with a mitochondrial receptor.

5. Conclusions and perspectives

An essential part of the role of Bcl-2 family proteins is that they are directed to the correct intracellular membranes. Although recent work on these apoptosis regulators has provided much data on the interaction between family members, our knowledge of how they correctly target to mitochondria is still fragmentary. Given that the molecular mechanisms for correct mitochondrial targeting are likely to be central to the proper regulation of this important protein family, an understanding of this process and the interactions required is essential. This review has outlined the various interactions that may drive the targeting of Bcl-2 proteins to mitochondria and their subsequent activity in apoptosis. Better understanding of these targeting mechanisms is important if we are to clarify how apoptosis is regulated.

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