

# Bcl-2 family members: intracellular targeting, membrane-insertion, and changes in subcellular localization

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## Abstract

The members of the Bcl-2 family of proteins are crucial regulators of apoptosis. In order to determine cell fate, these proteins must be targeted to distinct intracellular membranes, including the mitochondrial outer membrane (MOM), the membrane of the endoplasmic reticulum (ER) and its associated nuclear envelope. The targeting sequences and mechanisms that mediate the specificity of these proteins for a particular cellular membrane remain poorly defined. Several Bcl-2 family members have been reported to be tail-anchored via their predicted hydrophobic COOH-terminal transmembrane domains (TMDs). Tail-anchoring imposes a posttranslational mechanism of membrane insertion on the already folded protein, suggesting that the transient binding of cytosolic chaperone proteins to the hydrophobic TMD may be an important regulatory event in the targeting process. The TMD of certain family members is initially concealed and only becomes available for targeting and membrane insertion in response to apoptotic stimuli. These proteins either undergo a conformational change, posttranslational modification or a combination of these events enabling them to translocate to sites at which they are functional. Some Bcl-2 family members lack a TMD, but nevertheless localize to the MOM or the ER membrane during apoptosis where they execute their functions. In this review, we will focus on the intracellular targeting of Bcl-2 family members and the mechanisms by which they translocate to their sites of action. Furthermore, we will discuss the posttranslational modifications which regulate these events.

*Keywords:* Bcl-2 family; Subcellular localization; Tail-anchoring; Membrane insertion; Posttranslational modification; Translocation

## 1. Membrane association via COOH-terminal anchors (tail-anchoring)

### 1.1. General mechanisms of tail-anchoring into the membrane of the endoplasmic reticulum (ER) and/or the mitochondrial outer membrane (MOM)

Apoptosis is a tightly regulated physiological process with important implications during development and in the progression of disease. The Bcl-2 family of proteins are important regulators of apoptosis in mammalian cells [1,2].

*Abbreviations:* MOM, mitochondrial outer membrane; ER, endoplasmic reticulum; *cyt.b5*, ER specific isoform of cytochrome *b5*; TMD, transmembrane domain; BH, Bcl-2 homology domain; tBid, truncated Bid; aa, amino acid

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There is growing evidence indicating that all of the members recognized so far act exclusively on the cytoplasmic face of mitochondria and/or the ER. Many of the Bcl-2 family proteins are tail-anchored to these membranes via a hydrophobic COOH-terminal transmembrane domain (TMD) [3,4]. The members carrying a COOH-terminal TMD belong to a growing class of tail-anchored proteins, which include a variety of proteins that have different functions (for review see Ref. [3]). Tail-anchored proteins are specifically targeted to and inserted into either the MOM or the ER membrane via the hydrophobic TMD, which varies in length between 15 and 22 aa and is suggested to span the membrane once in an  $\alpha$ -helical conformation [3,5]. Once inserted into the membrane, the N-terminal bulk of the protein faces the cytosol. Tail-anchoring therefore imposes a posttranslational mechanism of membrane insertion on these proteins as the N-terminal bulk of the protein is folded before the hydrophobic COOH-terminus is released from the ribosome. The release of this hydrophobic sequence implies a requirement for mechanisms that prevent aggregation, such as the presence

of cytosolic chaperone proteins or translation within the vicinity of the membrane. Several interactions with molecular chaperones or other cytoplasmic factors have been described for the delivery of nuclear encoded polypeptides to the MOM, which may also be required for the targeting of tail-anchored proteins [6].

Little is known about the targeting signals and mechanisms by which tail-anchored proteins reach their final destination. Much of the research on tail-anchoring is derived from studies in yeast in which 55 tail-anchored proteins were identified in a recent sequence analysis study [7]. The vast majority of the proteins studied, including several SNARE proteins, are initially targeted to the ER and only six proteins, including the trans outer membrane proteins TOM-5, -6, -7 and -22 are targeted directly to the MOM [7–12]. The mechanisms of targeting of the mammalian VAMP/SNARE proteins, the two isoforms of cytochrome *b5* (ER-specific and MOM-specific *cyt.b5*) and the Bcl-2 family members have also been investigated [5,11–21]. Among these studies, saturable, ATP- and receptor-dependent, as well as non-saturable, ATP- and receptor-independent mechanisms have been described using *in vitro* assays.

A common feature of tail-anchored proteins is that all of the necessary targeting information is located in, directly up- or downstream of the TMD itself. Generally, the COOH-terminal stretch of the protein, which includes the TMD and its flanking residues, is sufficient for the insertion of a reporter protein into the membrane with the same specificity and stability as the wild-type protein. Recent reports have highlighted the role of the flanking regions in defining the membrane-specificity of the protein [8,12,22]. Investigations of the targeting mechanisms of the TOM-proteins in yeast have determined that both the internal characteristics of the TMD (mainly its hydrophobicity) as well as the basicity of the flanking regions are required for specific MOM- or ER-targeting [8]. Highly hydrophobic TMDs (e.g. as calculated using the algorithm of Kyte and Doolittle [23]) direct the protein to the ER whereas less hydrophobic TMDs show a higher tendency for the MOM. Additionally, the presence of basic aa downstream of the TMD increases the specificity for the MOM [8]. The recent resolution of the three-dimensional structure of TOM-20 has determined its affinity for the hydrophobic face of the amphipathic signal sequence of proteins to be imported into the mitochondrial matrix or to be inserted into the inner mitochondrial membrane. In contrast, the associated TOM-22 receptor has been reported to display a preference for the hydrophilic face of these signal sequences [10,24,25]. For the above-mentioned reasons, TOM-20 may also act as a receptor for MOM-targeted tail-anchored proteins, although as yet unidentified receptors may also play a role [7,11,26]. Recent evidence for a role of the TOM complex as a receptor for Bcl-2 family members is derived from an *in vitro* study describing the insertion of Bcl-2 into the yeast MOM. This study reports the involvement of a TOM-22-independent interaction of the hydrophobic TMD

of Bcl-2 with TOM-20. Interestingly, this interaction is dependent on the presence of two positively charged lysine-residues flanking the TMD of Bcl-2 [27].

The mechanisms by which putative receptors on the ER recognize the signal sequences of tail-anchored proteins and mediate their membrane insertion are not well understood. One of the best candidates is the posttranslational Sec-machinery; however, the exact composition of the complex as well as the mechanisms by which it interacts with the TMD of targeted proteins remain unclear [28–33]. Furthermore, Sec-independent mechanisms mediating ER-membrane tail-anchoring have been implicated in these processes [34].

### *1.2. Organelle specificity of the various Bcl-2 family members*

Bcl-2 family members that have a COOH-terminal hydrophobic TMD are targeted to the appropriate membrane either immediately following their synthesis or in response to an apoptotic stimulus. It is believed that the main function of these proteins is restricted to these sites within the cell [1,2]. It has been suggested that the anti-apoptotic Bcl-2 family members, such as Bcl-2, Bcl-x<sub>L</sub>, Bcl-w and Mcl-1, reside on several intracellular membranes in order to control apoptosis. Whilst the majority of endogenous Bcl-2 (about two thirds) is located on the ER membrane and associated nuclear envelope, the remaining fraction (about one third) is located on the MOM [19,35–37]. Supporting evidence comes from a study in which GFP fused to the last 39 aa of Bcl-2 is mainly located on the ER membranes when overexpressed in yeast. In a similar fashion, the MOM protein TOM-22 is redirected to the ER when carrying the last 33 aa of Bcl-2 [12]. Bcl-2 differs from its “colleagues” in that it is always integrally inserted into membranes, whereas Bcl-x<sub>L</sub> and Bcl-w are both found in a soluble, loosely attached form as well as an integrally membrane-inserted protein [16,22,38–40].

In contrast to Bcl-2, Bcl-x<sub>L</sub> shows high specificity for the MOM, which may be also true for other Bcl-2 family members such as Bcl-B and Bcl-w [22,41]. When comparing the TMDs of Bcl-2 and Bcl-x<sub>L</sub>, no obvious differences in length (19 aa for both) or in mean hydrophobicity (hydropathy scores were calculated using the algorithm of Kyte and Doolittle, as reported by Beilharz et al. [7]) were detected (Fig. 1a). However, some tendencies can be observed when looking at the shape of the hydropathy plots. MOM-targeted proteins such as Bcl-x<sub>L</sub>, myxoma virus M11L protein, hNIP3 and hNIPL all show a similar plot with an increased hydrophobicity in the C-terminal half of their TMD (Fig. 1d). This also holds true for three of the four tail-anchored TOM-proteins in yeast (TOM-5, -6, -7, -22), in which only the TMD of TOM-5 differs in the shape of its plot (Fig. 1c). On the other hand, proteins that are also targeted to the ER/nuclear envelope, such as Bcl-2 and Mcl-1, are more hydrophobic in the N-terminal half of their

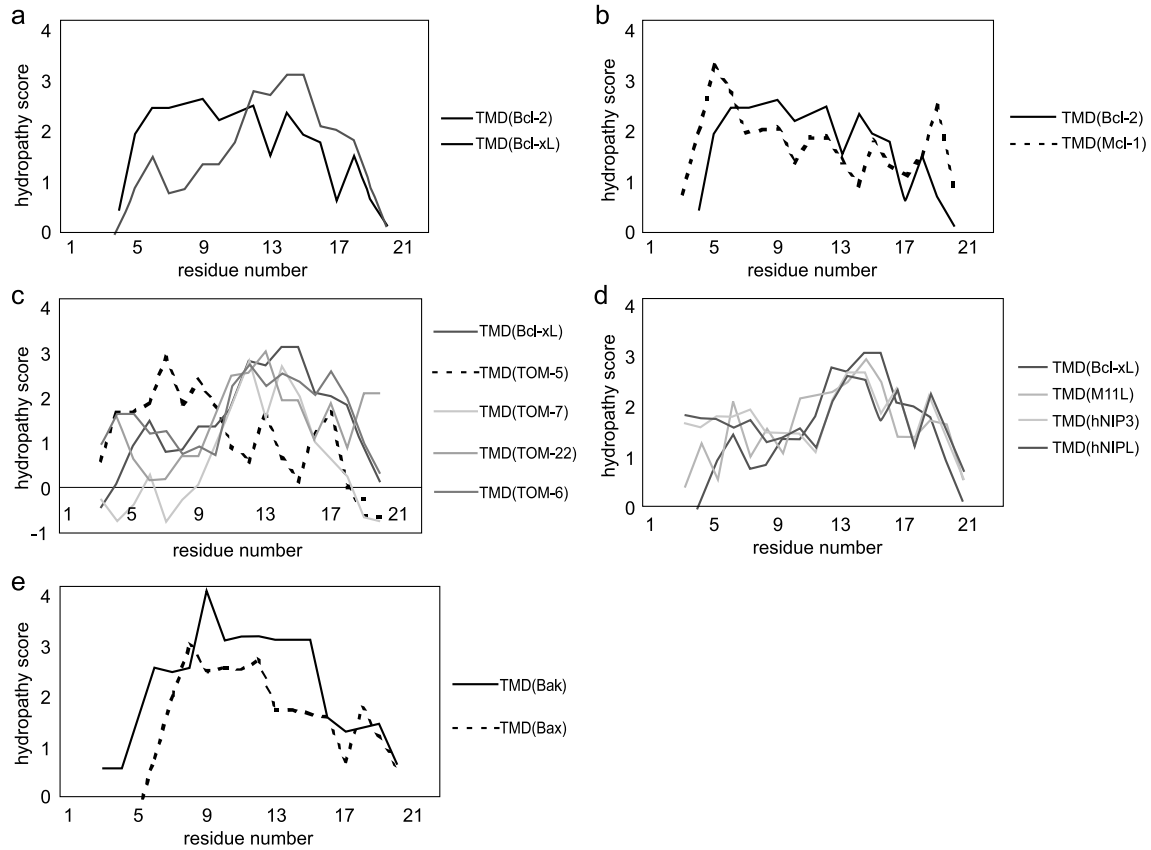


Fig. 1. Differences between MOM- and ER-specific tail-anchored proteins in the hydrophobic distribution within the TMD. Hydrophobicity plots of the TMDs of several tail-anchored proteins. Amino acids are represented on the X-axis, the relative hydrophobic score on the Y-axis. Hydrophobicity analysis was performed as described in Beilharz et al. [7], using the ProtScale site at <http://www.expasy.org/cgi-bin/protscale.pl>. Scores were obtained by applying the Kyte and Doolittle algorithm on the TMD sequence (22 aa) using a window size of 5 aa. (a) Although the mean hydrophobicity of the TMDs does not significantly differ, the TMD of the specifically MOM-targeted Bcl-x<sub>L</sub> shows another plot shape than the one from the ER/MOM targeted Bcl-2. (b) Similar plots for ER/MOM targeted Bcl-2 and Mcl-1. (c) The TMDs of TOM-6, -7 and -22 look similar to Bcl-x<sub>L</sub>, TOM-5 being an exception. (d) Other MOM-specific proteins (M11L, NIPL and NIP3) have also a TMD with a similar plot shape as for Bcl-x<sub>L</sub>. (e) The hydrophobicity plots for the TMDs of Bax and Bak look similar.

TMDs (Fig. 1b). However, there are exceptions to this “rule” and it remains to be determined whether the hydrophobic character and distribution of hydrophobic residues within the TMD play an important role in targeting specificity. Taken together, it is rather unlikely that the hydrophobicity of the TMD is the only determinant for ER- or MOM-specific targeting in these proteins.

There are, however, indications that the flanking regions, and especially the number of positively charged residues (arginine and lysine), play an important role in the membrane specific targeting of tail-anchored proteins. High basicity surrounding the TMD usually leads to a more MOM-specific targeting, whereas TMDs surrounded by fewer basic residues have a higher tendency to be targeted to the ER-membrane. This observation also holds true for tail-anchored proteins other than Bcl-2 family members [8,17,22,42]. Bcl-2 can be specifically targeted to the MOM by increasing the net positive charge at the very COOH-terminus of the protein. Furthermore, a decrease in the number of basic residues at the very COOH-terminus in Bcl-x<sub>L</sub> or in a GFP-TOM-5 fusion protein causes a marked decrease in MOM-specificity, leading to an accumulation of these pro-

teins on the ER-/nuclear outer membrane. These experiments also support the idea that the TMD itself does not solely determine the membrane specificity. Furthermore, whilst the number of basic charges is critical, the nature of the basic residues as well as the spacing between them do not appear to be relevant (Lys can be exchanged by Arg and vice versa without diminishing the specificity and efficiency of targeting) (Refs. [8,22], and own unpublished observations).

There is yet little published data describing cytosolic binding proteins or putative membrane receptors for the antiapoptotic Bcl-2 family members. One very recent paper by Shirane and Nakayama [43] shows that the mitochondrial FK506-binding protein 38 (FKBP38) interacts with both Bcl-2 and Bcl-x<sub>L</sub> and may play an important role in targeting these proteins to the mitochondrial membrane. FKBP38 would therefore act as a mitochondrial receptor for Bcl-2 and Bcl-x<sub>L</sub>, probably by associating with members of the TOM machinery [44]. However, these findings do not explain how and why Bcl-2 also localizes to the ER- and nuclear outer membrane.

The Bax-like proapoptotic members (Bax, Bak and Bok) were initially described to be targeted to the MOM where

they are thought to exert their function. Recently, ER-localization and, in consequence, ER-specific functions have been reported for these proteins [4,45,46]. Most of the targeting studies have been performed in vitro or on over-expressed proteins. In addition to its membrane attached form, Bax is found up to 60% as a soluble protein in the cytosol in healthy cells and it is proposed to translocate to and insert into the MOM during apoptosis [39,47]. The possible mechanisms by which Bax translocation occurs are discussed below. In contrast to Bax, Bak is inserted into the MOM and to a lesser extent into the ER membrane. When comparing the hydropathy plots of the TMDs of Bax and Bak, a similar curve is seen (which interestingly differs from those of Bcl-x<sub>L</sub>, M11L, hNIP3 and hNIP1), supporting the concept that they are both targeted to and functional on the same intracellular membranes (Fig. 1e) [1].

## 2. Translocation of Bcl-2 family members to intracellular membranes during the onset of apoptosis

Although some Bcl-2 family members are targeted directly to the membrane on which they execute their function via

specific targeting sequences, others require a secondary level of regulation allowing them to translocate in response to an apoptotic stimulus. This secondary level of regulation may act as a ‘safety switch’ preventing premature activation or inactivation of these proteins. Translocation is initiated by a variety of mechanisms, which include transcriptional regulation, posttranslational modification and/or conformational change [2,48,49]. In the case of the multidomain Bcl-2 family members Bax and Bcl-w, their TMDs are folded back into a hydrophobic pocket formed by the protein [40,50,51]. In response to an apoptotic stimulus, the TMD has been suggested to become available for MOM targeting and/or insertion. Several BH3-only proteins are sequestered to subcellular locations other than those on which they function and require modification before they can translocate to the mitochondria where they interact with multidomain Bcl-2 family members. Whilst Bid requires proteolytic cleavage to expose residues that are subsequently modified to allow its translocation, BH3-only proteins such as Bad, Bmf and Bim are sequestered by binding proteins and translocate to the mitochondria following specific apoptotic stimuli (Fig. 2). Certain BH3-only proteins including Bik, Hrk, Blk, and Bim have TMDs, which may become exposed and available for target-

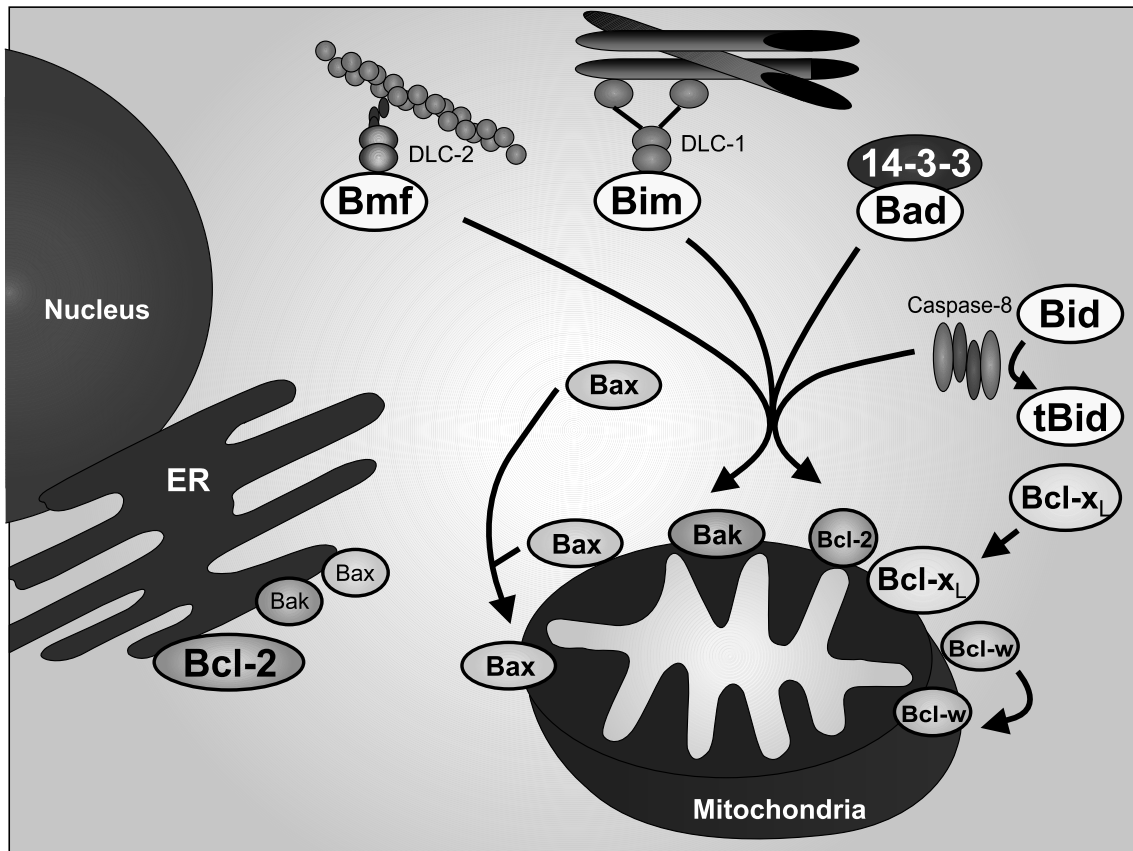


Fig. 2. Posttranslational modification is required for the translocation of certain multidomain and BH3-only Bcl-2 family members, whilst some proteins are directly targeted to and inserted into the MOM and/or the ER membrane (Bak, Bcl-x<sub>L</sub> and Bcl-2). In response to an apoptotic stimulus, BH3-only proteins (yellow) translocate to interact with multidomain Bcl-2 family members on the MOM. Bim and Bmf dissociate from cytoskeletal structures, Bad from 14-3-3 and Bid is proteolytically cleaved. Bax and Bcl-w undergo a conformational change, which allows them to translocate and/or insert into the MOM.

ing during apoptosis. However, these proteins may also depend on chaperone proteins or their affinity for multidomain Bcl-2 family members for efficient translocation [1].

### 2.1. Bax and Bcl-w: intra-molecular folding conceals the mitochondrial signal sequence

The structures of the multidomain Bcl-2 family members Bcl-x<sub>L</sub>, Bcl-2, Bcl-w and Bax closely resemble each other despite their different roles in regulating the apoptotic process [40,50–53]. As previously discussed, Bak and Bcl-2 constitutively localize to the MOM and/or ER membrane where they are inserted, whilst Bax and Bcl-w require regulatory mechanisms which induce their translocation and insertion into the MOM [19,22,54]. In non-apoptotic cells, Bax is located in the cytosol and loosely attached to the MOM, whilst Bcl-w is loosely attached to the MOM [38,55]. Whether MOM receptors or lipids mediate the loose attachment of Bax and Bcl-w remains undefined. In response to certain apoptotic stimuli, Bcl-w and Bax translocate and have been suggested to insert into the MOM via their TMDs [38,47,56,57]. Bcl-x<sub>L</sub> on the other hand is distributed between the cytosol and the MOM in different ratios, which depend on the cell line analyzed [38,39,47].

The solution structures of Bcl-2 and Bcl-x<sub>L</sub> were determined in the absence of their C-terminal TMDs, as the presence of these hydrophobic sequences would render them insoluble [52,53]. This suggests that the TMDs of these proteins may not be intra-molecularly folded, but become exposed following synthesis and therefore require chaperone-like molecules to prevent aggregation. These chaperones may also guide Bcl-2 and Bcl-x<sub>L</sub> to their respective membranes and be released upon association with receptors on the target membrane. Bacterially synthesized recombinant proteins may not contain these chaperones and can therefore only be purified and crystallized in high quantities when the hydrophobic C-terminal TMD is removed [52,58]. In contrast, the structures of the soluble forms of Bax and Bcl-w have revealed that their TMDs are folded back into their hydrophobic pockets thereby increasing the solubility of these proteins [40,50,51].

Bax is primarily located in the cytosol and loosely attached to the MOM in non-apoptotic cells. In response to an apoptotic stimulus, Bax has been suggested to undergo a conformational change during which an N-terminal epitope is revealed, the TMD is released and the hydrophobic pocket becomes available for oligomerization [50,55,59,60]. Both the N- and the C-termini of Bax have been implicated in its targeting to the MOM. Recently, an N-terminal targeting sequence composed of amino acids 20–37 has been reported as a targeting signal when fused to GFP [61]. However, deletion of the C-terminus of Bax prevents its translocation, highlighting its importance as a targeting sequence in this process [47,59]. Furthermore, our recent data show that the last 23 aa of Bax, which constitute its TMD, are necessary and sufficient to target Bax to the

MOM. In its cytosolic conformation, the TMD of Bax is bound back into a hydrophobic pocket formed by its BH1, BH2 and BH3 domains [50]. This conformation influences the solubility of Bax by reducing its exposed hydrophobic surface, preventing release of its TMD and exposure of the hydrophobic pocket. Suzuki et al. [50] have reported that the interactions between the pocket and the TMD are primarily hydrophobic, except for a hydrogen bond, which is formed between Asp<sub>98</sub> in  $\alpha$ -helix 4 and Ser<sub>184</sub> in the TMD. Mutation of Ser<sub>184</sub> to Valine, Alanine or its deletion in a GFP-Bax fusion protein causes Bax to become constitutively located on the mitochondria, suggesting that this interaction plays an essential role in retaining the TMD in the hydrophobic pocket [50,59].

The factors that initiate a conformational change in Bax or cause its retention in the cytosol remain unknown. Suzuki et al. [50] have suggested that the BH3 domain of other Bcl-2 family members could not compete with the C-terminus of Bax for the hydrophobic pocket and therefore an independent energy-driven process would be required to disengage the TMD. One possibility is that additional cytosolic proteins bind to the TMD and act as a clamp to prevent its release. A second possibility is that cytosolic binding proteins associate with the N-terminus of Bax, which somehow inhibit its conformational change. It has become apparent that the N-terminus of Bax plays a role in its retention in the cytosol. The structure of Bax shows that its N-terminus is flexible and solvent-exposed; however, inside cells, the N-terminal epitope of cytosolic Bax is not accessible for antibody binding [39,50,59,62,63]. In this respect, the N-terminus of Bax may act as an inhibitor of mitochondrial translocation. Indeed, removal of the N-terminal 20 aa ART (apoptosis regulation of targeting) domain allows Bax to be more effectively targeted to mitochondria [56,64]. Furthermore, it has been reported that the N-terminal cleavage of Bax by calpain results in the generation of a highly apoptogenic 18-kDa fragment [65–68]. Therefore, the binding proteins associated with the N- or C-termini of Bax would have to be degraded or released before the TMD can be released from the hydrophobic pocket.

Several candidate proteins, which may assume this role, have recently been described. Cuddeback et al. [69] have reported that Bif (Bax-interacting factor) promotes the translocation of Bax from the cytosol to the mitochondria following an apoptotic stimulus possibly by inducing a conformational change in Bax. However, the exact molecular mechanism causing this event remains undefined. Furthermore, Nomura et al. [70] have recently proposed that the cytosolic chaperone 14-3-3 interacts with Bax. Interestingly, they have reported that the protein makes contacts with the N- and C-termini of Bax and therefore may act as a bridge between these extremities. This would not only prevent the TMD from being released, but also prevent a change in the cytosolic conformation of Bax, which may be reflected by the opening and accessibility of its N-terminus. How would N-terminal opening be initiated inside cells? The 14-3-3

protein is cleaved by a caspase in apoptotic cells, releases Bax and may initiate N-terminal opening and release of the TMD. Recently, Sawada et al. [71] have found that Ku70, a protein known so far for its function in DNA repair within the nucleus, binds to the N-terminus of cytosolic Bax and prevents its translocation to mitochondria following staurosporine or UVC treatment. A peptide of 5 aa encompassing the binding domain in Ku70 has a similar inhibitory effect [72]. The mechanism by which Ku70 is thought to act is by inhibiting N-terminal opening and thereby preventing conformational change. However, the forced dissociation of Ku70 is not sufficient to activate Bax, suggesting that another event is required to make Bax competent for mitochondrial translocation [71]. Another cytosolic retention factor, which has recently been described, is the peptide Humanin. Humanin binds to the C-terminus of Bax and has been shown to prevent Bax translocation following transfection into human cells [73]. However, as for Ku70, reducing endogenous levels of Humanin only sensitized cells to apoptotic stimuli, and therefore a secondary step would be required for the activation of Bax [73].

Two recent reports have described the structure of Bcl-w [40,51]. The C-terminus of Bcl-w, like that of Bax, is folded back into a hydrophobic pocket giving it the attributes of a compact globular molecule. Bcl-w is found loosely attached to mitochondria in healthy cells and becomes integrated into the MOM in response to an apoptotic stimulus [38,40,51]. Like Bax, Bcl-w has been suggested to undergo a conformational change, during which its C-terminal helix is displaced from the pocket, allowing the insertion of Bcl-w into the MOM [40,51]. The intra-molecular interactions between the C-terminus and the hydrophobic pocket in Bcl-w appear to be weaker than in Bax, making its C-terminus more mobile than that of Bax. However, the C-terminus still restricts access to the hydrophobic pocket as truncation of the C-terminus of Bcl-w resulted in a higher affinity of certain BH3-only proteins for Bcl-w [40,51]. Therefore, contrary to the suggestions made for Bax, BH3-only proteins appear to be able to displace the C-terminus of Bcl-w from the hydrophobic groove without any additional energetic input [50,51]. Hinds et al. [40] have shown that Bim and Bmf can bind to Bcl-w and that a conserved leucine residue in their BH3 domains may be necessary for the displacement of the C-terminus from the hydrophobic pocket. A similar mechanism in which the C-terminus restricts access to the hydrophobic pocket has been suggested to occur in Bcl-x<sub>L</sub> [40].

## 2.2. BH3-onlies: subcellular localization mediated by posttranslational modification

As the sensors of apoptotic stress, BH3-only proteins are sequestered in the cytoplasm, but are suggested to function in association with the multidomain Bcl-2 family members, which are located predominantly on the MOM, ER membrane and its associated nuclear envelope. Certain BH3-only

proteins including Noxa, Puma/Bbc3, Bim, Hrk/Dp5 are transcriptionally induced in response to apoptotic stimuli; whilst others, including Bid, Bim, Bmf and Bad, have been reported to be posttranslationally modified and/or proteolytically processed in response to apoptotic stimuli [2,48,49]. These modifications may induce the exposure of targeting sequences, allow for interactions with chaperones and/or increase the affinity of BH3-only proteins for multidomain Bcl-2 family members. Although some BH3-only proteins bind to multidomain Bcl-2 family members with high affinity and may therefore be attached to the MOM and ER membrane via these proteins, others such as Bik, Hrk, Blk, Bim, and Bim contain their own C-terminal TMD regions. As for Bax and Bcl-w, the TMDs of these BH3-only proteins may be unavailable in healthy cells and only become available for targeting in response to an apoptotic stimulus [1].

### 2.2.1. Bid

In healthy cells Bid is located in the cytosol in an inactive form. In response to death receptor activation, Bid is cleaved by caspase-8 to form truncated Bid (tBid). tBid can then translocate to the mitochondria to interact with multidomain Bcl-2 family members [74–76]. Targeting of tBid to the mitochondria following proteolytic cleavage is facilitated by posttranslational *N*-myristoylation of a glycine residue, which becomes exposed following the cleavage of Bid by caspase-8 [77]. Furthermore, Bid has been reported to be targeted to the mitochondria via its high affinity for the lipid cardiolipin [78,79]. The phosphorylation of Bid by casein kinases I and II has been reported to prevent its translocation. This phosphorylation event occurs in the vicinity of the recognition site for caspase-8 and consequentially renders Bid resistant to cleavage [76]. Granzyme B has also been reported to cleave Bid to generate a 14-kDa granzyme-truncated Bid (gtBid), which translocates to mitochondria and associates with Bax [80].

### 2.2.2. Bad

In vitro and in cell culture, Bad is regulated by a variety of survival promoting kinases, which phosphorylate it on multiple serine residues. The phosphorylation of these residues promotes its interaction with 14-3-3 scaffold proteins and its sequestration in the cytosol [81–84]. Several kinases have been reported to phosphorylate Bad on serines 112 and 135. These include: AKT/PKB, which is a transducer of survival signals of growth factors within the PI3-kinase pathway, and Raf-1, which couples growth factor receptors to the MAPK pathway. [81,82,85]. PKA phosphorylates serine 155 of Bad, which is located in its BH3 domain. This phosphorylation event has been reported to decrease the affinity of Bad for Bcl-2 [83,86]. The importance of Bad phosphorylation has been emphasized by the phenotype of Bad ‘knock-in’ mice, which express a constitutively active Bad mutant, in which serine residues at positions 112, 136 and 155 are replaced by alanines. These mice display no obvious abnormalities but are hypersensi-

tive to a variety of apoptotic stresses [87]. The kinases responsible for the phosphorylation of Bad are located both in the cytosol and on the mitochondria. The mechanisms by which Bad is phosphorylated by mitochondrial kinases without associating with Bcl-2 family members has not been defined. In a recent review, Puthalakath and Strasser [49] have suggested that Bad may either have a higher affinity for these kinases than for Bcl-2 family members or may rely on chaperone proteins to guide it to Bcl-x<sub>L</sub>, its putative binding partner on the MOM. Growth factor withdrawal has been shown to lead to the dephosphorylation of Bad and its dissociation from 14-3-3, hence promoting its pro-apoptotic activity by its association with Bcl-x<sub>L</sub> [88]. In calcium-induced apoptosis, calcineurin has been shown to dephosphorylate Bad allowing its translocation and interaction with Bcl-x<sub>L</sub> [89]. Other events have also been suggested to lead to the disassociation of Bad from 14-3-3, for example, the cleavage of 14-3-3 by caspase-3 has been reported to facilitate Bad activation [90].

### 2.2.3. Bim and Bmf

In healthy cells, Bim and Bmf are bound to cytoskeletal structures. In response to an apoptotic stimulus, these proteins are released and translocate to interact with multi-domain Bcl-2 family members on the mitochondria [91]. As well as being transcriptionally regulated, Bim is also regulated by alternative splicing during which three major isoforms are formed, Bim<sub>EL</sub>, Bim<sub>L</sub> and Bim<sub>S</sub> [92]. Bim<sub>EL</sub> and

Bim<sub>L</sub> have been shown to bind to DLC1/LC8 of the microtubular dynein motor complex [91]. Treatment with taxol, cytokine deprivation and abnormal calcium flux induce the dissociation of Bim<sub>L</sub>, still bound to DLC-1/L8, from the dynein motor complex. This dissociation event allows Bim<sub>L</sub> to translocate to the MOM, ER membrane and its associated nuclear envelope where it interacts with Bcl-2 family members [91,93]. Little is known about the mechanisms by which Bim translocates, although enzymes such as CDK5, which are known to affect the dynein motor function, have been suggested [94,95]. Withdrawal of NGF has also been shown to promote Bim phosphorylation mediated by the MAPK pathway, possibly regulating the association of Bim with an unknown partner [96]. Bmf is bound to DLC2 on the actin cytoskeleton-based myosin V motor complex in healthy cells and translocates to the mitochondria to bind Bcl-2 family members in response to apoptotic stimuli such as anoikis and actin depolymerising drugs [49,94]. Upon exposure to environmental stress, JNK has been suggested to phosphorylate both Bim and Bmf causing their release from the dynein motor complexes [96].

## 3. Conclusions and perspectives

Members of the Bcl-2 family are crucial regulators of apoptosis, which are located on different intracellular membranes and activated by a variety of different mechanisms.

Table 1  
Overview on the intracellular localizations, translocations and posttranslational modifications of some Bcl-2 family members

Bcl-2 Family Member	COOH-terminal TMD	Localization in healthy cells	Localization in apoptotic cells	Binding proteins or receptors in healthy cells	Binding proteins or receptors in apoptotic cells	Posttranslational modification
Bcl-2	Yes	MOM-, ER-, nuclear envelope inserted	MOM-, ER-, nuclear envelope inserted	FKB38, BAP-31, Pro-apoptotic, Bax-like proteins (Bax, Bak) ?	BH3-onlies, others?	?
Mcl-1	Yes	MOM-, ER-, nuclear envelope inserted (?)	MOM-, ER-, nuclear envelope inserted (?)	Pro-apoptotic, Bax-like proteins (Bax, Bak) ?	BH3-onlies, others?	?
Bcl-x <sub>L</sub>	Yes	Cytosolic, MOM-inserted and loosely attached	MOM-inserted	FKBP38, Pro-apoptotic, Bax-like proteins (Bax, Bak) ?	BH3-onlies others?	Conformational change (?)
Bcl-w	Yes	MOM—loosely attached	MOM-inserted	Pro-apoptotic, Bax-like proteins (Bax, Bak)	BH3-onlies others?	Conformational change
Bax	Yes	Cytosolic, ER and MOM—loosely attached	MOM-inserted, ER-inserted (?)	14-3-3, Bif-1, Ku70, humanin	tBid (transient interaction ?)	Conformational change
Bak	Yes	MOM-, ER-inserted	MOM-inserted, ER-inserted (?)	?	tBid (transient interaction ?)	?
Bad	No	Cytosolic	MOM	14-3-3	Bcl-x <sub>L</sub>	Phosphorylation
Bid	No	Cytosolic	MOM	?	Bax, Bak (transient interaction ?)	Proteolytic processing, phosphorylation, myristoylation
Bim/Bod	Yes	DLC1-associated (microtubules)	MOM inserted ?	DLC1	Anti-apoptotic Bcl-2 family members	Phosphorylation
Bmf	No	DLC-2 associated (myosinV/actin-cytoskeleton)	MOM inserted ?	DLC2	Anti-apoptotic Bcl-2 family members	Phosphorylation

An overview of some of their features is given in Table 1. It has become increasingly clear that the multidomain members of the Bcl-2 family carry a COOH-terminal TMD and therefore behave like other tail-anchored proteins. In contrast to the ‘classical’ membrane insertion pathways, the research describing the targeting and insertion of tail-anchored proteins is recent and the details of these mechanisms remain undefined. Following synthesis, tail-anchored proteins are targeted either to the ER membrane and its associated nuclear envelope or to the MOM where the TMD becomes inserted in a stable fashion and the bulk of the protein faces the cytosol. Membrane-bound receptors appear to be involved in this process, and it may eventually become evident that some of the receptors and mechanisms required for ‘classical’ insertion or translocation pathways may also play a role in tail-anchoring.

The sequences and chaperones necessary for intracellular targeting, the mechanism of activation and the exact function of many individual Bcl-2 family members in promoting or preventing apoptosis remain to be defined. The majority of studies, which have defined the subcellular location, targeting sequences, modifications and interactions of these proteins, have been performed *in vitro* or in cell culture models, often using fusion proteins or overexpressed wild-type proteins. However, little is known about whether these proteins are indeed targeted to and have specific functions on particular intracellular organelles in tissues of multicellular organisms. It remains to be determined whether these events are of physiological relevance. For example, does Bcl-2, which is reported to reside to a major extent on the ER membrane, have an ER-specific function? Is Bcl-x<sub>L</sub> only functional when it resides on the MOM? What influence do chaperone proteins have on the subcellular distribution of Bcl-2 and Bcl-x<sub>L</sub>? Are these two proteins equally distributed throughout the various tissues of an organism? Do the low levels of Bax and Bak located on the ER membrane control membrane permeability of the ER in a similar way as they do on the MOM? Or is there an alternative ER-specific function for these proteins?

There is little evidence describing the mechanisms by which BH3-only proteins, which do not have a TMD, reach and remain on the appropriate intracellular membrane in response to apoptotic stimuli. One possibility is that BH3-only proteins interact with multidomain Bcl-2 family members, which are already present on the MOM or the ER membrane at the time of their activation. It appears that BH3-only proteins interact with antiapoptotic proteins such as Bcl-2 and Bcl-x<sub>L</sub>, neutralizing their antiapoptotic function. The only known exception so far is tBid, which has been shown to favor the binding to and activation of proapoptotic Bax and Bak. It may also be possible that BH3-only proteins bind to unknown proteins on the MOM and ER-membranes and this could very well be the case for multidomain Bcl-2 family members as well.

Bcl-2 family members, which are sequestered in the cytosol or on intracellular structures and are not directly

targeted to the membrane on which they function, require secondary mechanisms of regulation for their translocation. These posttranslational mechanisms may be dependent on the apoptotic stress and cell type. Therefore, in different tissues distinct posttranslational mechanisms could activate specific Bcl-2 family members in response to various apoptotic stimuli. Generation of mice in which one or more Bcl-2 family members have been ‘knocked out’, conditional and tissue-specific knockouts as well as ‘knock in’ mice will provide further insight into the role of posttranslational mechanisms in the activation of Bcl-2 family members *in vivo*.

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