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The mitochondrial outer membrane protein import machinery: a new player in apoptosis?

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1. ABSTRACT

The proteins of the BCL-2 family (pBCL-2s) are involved in the sequence of events that culminates in apoptosis, a type I cell death program. The mitochondrion is the primary site of action of pBCL-2s and represents the cross roads between the initiation and the execution phases of apoptosis. pBCL-2s are either constitutively associated with the mitochondria outer membrane (MOM) or targeted to this membrane at the onset of apoptosis. The mechanisms implicated in their targeting and activation during apoptosis is largely unknown. Recently, several pBCL-2s have been shown to interact with components of the translocase of the outer membrane (TOM), a complex responsible for the import into and across the MOM of nuclear encoded mitochondrial proteins. Here we will review the available data and discuss the possible implications of this interaction in the apoptotic programme.

2. INTRODUCTION

The mitochondria are central to the control of cell life and death mainly through the production of ATP or reactive oxygen species (ROS) and the imprisonment of proteins that trigger cell death upon the induction of apoptosis (1). Most of these functions involve a strict separation of the organelle from other cellular cytoplasmic components. This separation is reinforced by a tight control of molecular exchanges through the mitochondrial outer and inner membranes. At least, three different forms of mitochondrial permeability can be ascribed to the organelle:

1. Protein import machinery: More than 90% mitochondrial proteins are nuclear encoded and have thus to be imported by a complex array of proteins that facilitate both the membrane(s) translocation and the functional

addressing of the proteins. Two distinct, yet cooperative, translocation machineries have been extensively described: the TOM (translocase of the outer membrane) and the TIM (translocase of inner membrane) complexes.

2. Mitochondrial permeability transition (MPT): MPT is responsible for the increase in the permeability of the mitochondrial membranes to molecules of less than 1500 Daltons. MPT has two primary functions: firstly, regulation of the oxidative phosphorylation functions through the ADP/ATP exchange between mitochondria and the cytosol and secondly, the opening of the MPT is associated with certain forms of cell death when it is converted into an unspecific channel (1).

3. Apoptotic mitochondrial outer membrane permeability (AMOMP): This type of permeability occurs early during apoptosis and represents, in most cases, the point of no return in the cell death program. AMOMP is under the control of proteins of the BCL-2 family, which either form the AMOMP (pro-apoptotic proteins, mainly Bax and Bak) or control its formation (Bid, Puma, Bcl-2, Bcl-Xl…). This protein family can be divided into anti-apoptotic (Bcl-2, Bcl-Xl, Bcl-w, Mcl-1, A1) and pro-apoptotic (Bax, Bak, Bok/Mtd, Bcl-Xs, Bid, Bad, Bik/Nbk, Bim, Blk) members (2). These proteins contain conserved domains, referred to as Bcl-2 homology domains (BH1-4). The number of these domains varies between anti- and pro-apoptotic proteins (usually four domains in anti-apoptotic proteins and a maximum of 3 in pro-apoptotic proteins) and from one class of pro-apoptotic protein to another (three domains in the multidomain pro-apoptotic proteins and one in the so-called "BH3-only proteins") (2). The activity of these proteins appears to be regulated, at least in part, by formation of homo- and hetero-complexes through the conserved BH domains (2). In the pro-apoptotic proteins, Bax and Bak, the BH3 domain is essential for complex formation and in their killing activity (2, 3). Also present in most of the pBCL-2s, a hydrophobic C-terminal domain that has been implicated in the targeting of the proteins to intracellular membranes (see discussion below).

If the association between the MTP and apoptosis is the topic of intense debate (4, 5), very little data are available on the importance of the TOM and TOM-related complexes and apoptosis. Others and we have observed that the TOM components are involved in the docking and the function of anti-apoptotic proteins such as Bcl-2 and Mcl-1 or pro-apoptotic Bax. Thus, the interaction of the proteins of the BCL-2 family and TOM could represent an important new constitutive/regulatory pathway in AMOMP.

3. ACTIVATION AND MITOCHONDRIAL TARGETING OF PROTEINS OF THE BCL-2 FAMILY

The abnormal regulation of apoptosis is involved in much major pathology and results in either an escalation (e.g. neurodegenerative disorders or autoimmune diseases) or a diminution of cell death (e.g. cancer). Proteins belonging to the BCL-2 family function as regulators of 'life-or-death' decisions in response to various intrinsic or extrinsic stimuli. Bak and Bax, pro-apoptotic members of the BCL-2 family are essential for the conclusion of apoptosis in the majority of higher vertebrate tissues (1). Structural and functional homologues of the BCL-2 protein family also exist in lower eukaryotes, such as flies but not in fungi such as yeast. In the latter case, the lack of endogenous pBCL-2s has been used in many studies to analyze the molecular mechanisms causal to mammalian pBCL-2s interaction with mitochondria in the absence of cell death (3).

In mammals, upon induction of apoptosis, Bax and Bak undergo several changes in conformation, which results in their incorporation into the mitochondrial outer membrane and to the ensuing modifications in the AMOMP (1-3). However, although the latter step is crucial for apoptosis, the precise molecular partners and mechanisms involved in this process are still largely unknown (1-3). During apoptosis the degree of intervention of the MPT, a multi-component protein complex that involves both inner and outer mitochondrial membrane components as well as matrix proteins, has been controversial for quite some time (4, 5). It has been antagonistically proposed that the opening of the MPT and/or one of its outer membrane components, the voltage dependent anionic channel (VDAC) is either instrumental or an ancillary signal of cell death or merely a consequence of apoptosis (4, 5).

3.1 Activation and mitochondrial targeting of the multidomain pro-apoptotic proteins Bax and Bak

We have recently described in detail our view of the process involved in the activation and targeting of Bax to the mitochondria (6, 7). Originally, the mitochondria targeting signal of Bax has been located in its C-terminal region although in many instances Bax mutants lacking this sequence were as efficient as the wild type in its mitochondrial targeting (6, 7). As the illustration of the difficulty to determine whether the C-terminal end of Bax is involved in its mitochondrial addressing, is that, contrary to the C-terminal of Bcl-Xl, which is a *bona fide* targeting signal, a point mutation (i.e. Ser184Val) in Bax C-terminal is mandatory to induce its non-apoptotic mitochondrial localization (6, 7). We have located an alternative addressing signal (but not its membrane anchoring domain) in the N-terminal extremity of Bax (6, 7). This implies that the control and the mechanisms of the translocation of Bax to the mitochondria appear to be quite unique in the pBCL-2s.

Several steps in Bax translocation are described in Figure 1.

Step 1: in resting healthy cells, the conformation of Bax is blocked in a configuration that does not allow the exposition of the N-terminal helix (i.e. Hα1) or that of the C-terminal helix (Hα9) (2). The maintenance of this conformation is under the control of two Prolines: Pro13 in the N-terminus (8) and Pro168 in the C-terminus (9). The Bax molecule in this configuration, that we have called the Cytosolic locked In Conformation (CLIC), is monomeric and does not seem to interact with any other protein (6, 7). The Hα1 is in close contact with the BH3/Hα2 through an

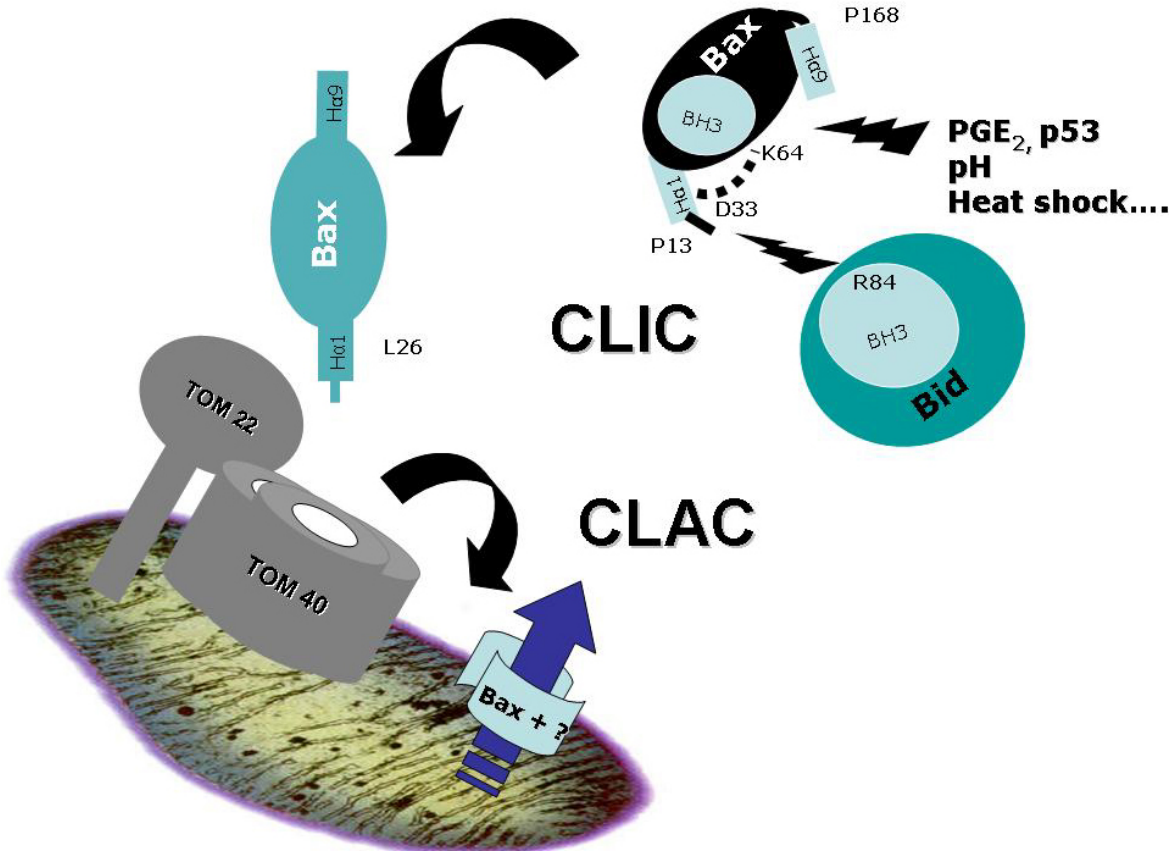

Figure 1. Bax cytosolic and mitochondrial changes during apoptosis. *Step 1:* In resting healthy cells, Bax is blocked under an inactive conformation (CLIC) that does not allow the exposition of the addressing signal located in its Hα1 and membrane/oligomerization domains: Hα1 is in close contact with BH3/Hα2 through a salt bridge and Hα9 occludes from the Hα5-Hα6 pocket. *Step 2:* The interaction of Bax with a BH3-only protein such as Bid; which would allow the exposition of Hα1 that can then interact with mitochondrial receptor(s) while the Hα5-Hα6 is liberated from its interaction with Hα9 and triggers the integration of Bax into the membrane. Other stimuli such as bioactive lipids (i.e. PGE_2), p53 or a change in pH or temperature also induce similar but not identical changes (see references 6 & 7 for details). *Step 3*: After interacting with TOM22 and TOM40, Bax inserts into MOM through Hα5-Hα6 and possibly Hα9. Both Hα2 (BH3) and Hα9 participate in the oligomerization process (CLAC), which is associated with the Bax-induced liberation of apoptogenic proteins from the inner compartment of the organelle through a pore that might be composed of different elements.

electrostatic interaction, which involves Asp33 and Lys64 (10). Hα9 precludes the Hα5-Hα6 pocket (11) thereby preventing the cytosolic exposition of this putative membrane insertion domain (10).

Step 2: An apoptotic stimulus permits the exposure of the Hα1 that can then interact with mitochondrial receptor(s), which may be of different natures (proteins or lipids). Concurrently, the Hα5-Hα6 is liberated from its interaction with the Hα9 and triggers the integration of Bax into the mitochondrial outer membrane (6, 7). Of note, Arg84 in the BH3-only protein, Bid has been shown to interact with Asp33 in Bax, which possibly, induces the rupture of the Asp33/Lys64 bond thereby facilitating its mitochondrial association (12).

Step 3: This step involves the insertion of the Hα5-Hα6 and Hα9 into the outer mitochondrial membrane. Both Hα2 (BH3) and Hα9 have been suggested to participate in the oligomerization process (13). Once inserted into the mitochondria, Bax adopts another conformation (cytochrome c liberation associated conformation, CLAC) that is associated with its oligomerization and results in the liberation of apoptogenic proteins from the inner compartment of the organelle (1, 2). Of note, it has been demonstrated by Annis *et al*. that Bax oligomerization takes place in the MOM from transmembrane monomers rather than from a cytosolic preformed oligomer (14). Thus, the insertion of Bax monomers into the mitochondria can be dissociated from its pro-apoptotic activity and differs significantly from that of most pore-forming proteins (14).

In contrast, considerably less information is available on the mitochondrial targeting and the activation of Bak (1, 2). By structural analogy with other pBCL-2s, the C-terminal region of Bak has been suggested to be its membrane anchor domain (2). Bak is a mitochondrial

resident and thus must be activated "on site" upon the induction of apoptosis (15). This activation of Bak is supposed to be similar to that described for Bax either by a direct stimulation by certain BH3-only proteins or by a release of constraints of anti-apoptotic mechanisms (2). In fact, in healthy cells, Bak forms a complex with VDAC-2 present in MOM and this strong interaction is mandatory for its mitochondrial import and to sustain its inactive conformation (16, 17). It has also been suggested that the MOM-targeting signal of Bak is occluded within the protein and is activated or unmasked upon the interaction of the cytoplasmic domain of Bak with VDAC-2 before MOM targeting (17). Bak is also kept inactive by its interaction at the mitochondrial level with the multidomain anti-apoptotic BCL-2 family members such as Mcl-1 and is activated upon its displacement by BH3-only proteins (18, 19).

3.2 Activation and mitochondrial targeting of the multidomain pro-apoptotic proteins Bcl-2 and Bcl-Xl

Bcl-2 and Bcl-Xl are thought to be largely redundant in their function as both inhibit apoptosis by blocking BH3-only proteins and Bax/Bak induction of MOM permeability (1, 2). However, these proteins exhibit obvious differences in their sub-cellular localization as Bcl-2 has multiple membrane attachments (i.e. nuclear envelopes, endoplasmic reticulum and mitochondria) while Bcl-Xl is mainly cytosolic in healthy cells and becomes mitochondrial bound upon the induction of apoptosis (2). Both proteins are tail-anchored proteins, which imply that their C-terminal end is a transmembrane domain and a targeting signal (2). However, there is very little information available on the nature of the signals that trigger the targeting of these anti-apoptotic proteins to mitochondria or other intracellular membrane.

Nguyen *et al.* have shown that the role of the C-terminal transmembrane domain of human Bcl-2 is to target and then insert the protein into the outer membrane in a "cytosolic N-terminal and a MOM anchored C-terminal" orientation, signifying that most of the protein is located in the cytosol (20). It is interesting to note that in this study, the authors show that a synthetic peptide comprising the signal anchoring sequence of a mitochondrial outer membrane protein (i.e. TOM70) effectively competed for the insertion of Bcl-2 into the outer membrane but had no effect on its association with the endoplasmic reticulum suggesting the existence of a finite amount of its receptor(s) (20).

Kaufman *et al.* have shown that the C-terminus of Bcl-Xl is a *bona fide* mitochondrial-targeting signal as it interacts with specific subcellular localization contrary to that of Bcl-2, which can be addressed to several membranes (21). Bcl-Xl is activated and directed towards mitochondria at the onset of apoptosis (2). In healthy cells Bcl-Xl forms homodimers that are disrupted by BH3-only proteins such as Bad, triggering its association with mitochondria (22). Interestingly, the C-terminal end of Bcl-Xl folds into the BH3-binding hydrophobic cleft of the protein, in a fashion similar to that of many C-terminal transmembrane helices of the pBCL-2s (2).

Another Bcl-2 homologue, Mcl-1 plays a similar role in blocking apoptosis but is also involved in the regulation of the cell cycle. Although it is thought to associate with intracellular membranes through its C-terminus similar to Bcl-2 (23), recent results suggest that the first 79 amino acids of Mcl-1 may also regulate its subcellular localization by promoting its association with mitochondria (24). Thus a feature common to some pBCL-2s responsible for their mitochondrial localization could be the association of a specific N-terminal mitochondrial signal with less specific domains located in the C-terminal of the protein involved in membrane anchoring.

4. PROTEIN IMPORT ACROSS MOM

Mitochondria have probably arisen from an endosymbiotic relationship where redundant genes were loss and essential genes were relocated to the host nucleus. The majority of the mitochondrial proteins are nuclear encoded, synthesized in the cytosol then imported into the mitochondria. Different types of signals are responsible for the addressing of mitochondrial preproteins. In eukaryotes the importation of the mitochondrial precursors is directed by different translocation complexes such as the translocase of the outer membrane (called the TOM complex) and the translocase of the inner membrane (called the TIM complex), as well as the sorting and the assembly machinery (the SAM complex). Almost all the proteins that have to be addressed to the mitochondria must traverse the TOM complex. These different complexes recognize the targeting signal in the mitochondrial preproteins that have to be imported into the mitochondria and help their translocation through the mitochondrial membranes. (25). The TOM complex is composed of the general insertion pore (GIP) that consists of TOM40, TOM22 and the small TOM proteins (TOM5, TOM6 and TOM7), to which are dynamically associated TOM20 and TOM70 upon their interaction with nuclear-encoded mitochondrial proteins (25). TOM40 forms the channel through which proteins are transported across the membrane and TOM22, the central receptor, connects the TOM complex to the translocase of the inner membrane (TIM) (25). The SAM complex in yeast consists of three subunits: SAM35, SAM37 and SAM50 that can associate with other proteins such as Mdm10 and possibly, in mammals, with metaxin-1 and metaxin-2 (25). Outer membrane β-barrel proteins that are usually MOM pore-forming proteins such as VDAC-1 and TOM40, utilize TOM and SAM complexes in a sequential manner (25). Of note, Mdm10 was initially identified for its role in the maintenance of mitochondrial morphology and as such could provide a link between the two functions (26). Originally, the TOM complex has been shown to accommodate proteins with a well-defined mitochondrial-addressing signal, however, it has become increasingly apparent that mitochondrial proteins possess numerous, not yet identified, topogenic signals, which can be used to associate with the organelle (25). For example, the mechanism by which the group of C-terminal or tail-anchored proteins, to which most of the pBCL-2s belong, integrate the various intracellular membranes is currently unknown (27)

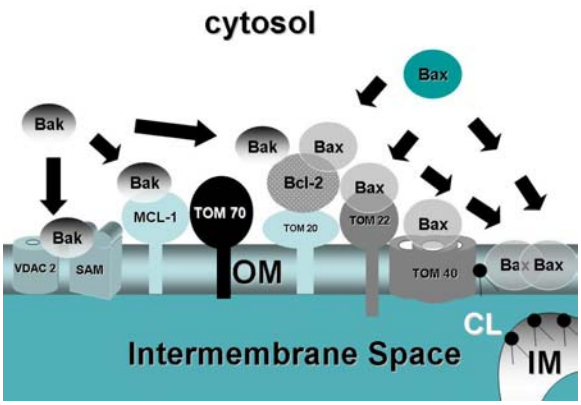

Figure 2. Schematic representation of TOM/pBCL-2s interaction. Recent data from the literature suggest that several members of the BCL-2 family interact with members of the TOM/SAM complexes. Bax interacts sequentially with TOM22 (this interaction is rate limiting and its inhibition impairs Bax-dependent apoptosis) and then with TOM40 before insertion/ oligomerization into the MOM possibly though a co-operation with cardiolipin (CL). Bcl-2 interacts with TOM20 but also with Bax and Bak. In healthy cells, Bak associates with VDAC2, Mcl-1 (and Bcl-Xl) and possibly with some component of the SAM complex. OM: outer membrane, IM: inner membrane.

5. INTERACTION BETWEEN MEMBERS OF THE BCL-2 FAMILY AND TOM PROTEINS

Kuwana *et al.* have raised the possibility that mitochondrial proteins are dispensable for Bax insertion into mitochondrial membrane and that Bax-mediated outer membrane permeabilization could be mimicked in simplified systems in which only lipids are present (28). The only requirement would be the presence of cardiolipin, a lipid specific of the inner membrane of the mitochondria (28). The latter feature appears to be also important for insertion into mitochondrial membranes of Bid, a BH3-only protein (29), in conjunction with the interaction with a mitochondrial inner membrane transporter (30). However, as stated by the authors, this work was designed to define minimal conditions required for Bax membrane permeabilization but does not rule out the participation of proteins that could modulate and/or enhance this function (31). In agreement with this view, mitochondrial protein precursors, which otherwise interact with TOM complex, have a specific affinity for cardiolipin, through the interaction of their signal peptides and the polar head of the cardiolipin molecules (32).

Several groups have postulated the existence of mitochondrial proteins involved in the docking and/or the insertion of Bax into mitochondria (33, 34). We have recently shown that the interaction of Bax with a mitochondrial receptor occurs through its Hα1, which does not exhibit the features of a classical mitochondrial addressing signal (35). To date, several proteins of the BCL-2 family have been shown to interact with proteins of the TOM complex (Figure 2): namely Bcl-2 with TOM20 (36, 37), Mcl-1 with TOM70 (38) and Bax with TOM22 (39) and TOM40 (40, 41) (Figure 2). It should be noted that an unnatural C–terminal mutant of Bax (i.e. Bax Ser184Val) associates constitutively with mitochondria and does not interact with the TOM complex (39). This result suggests that either the forced localization of Bax through this C-terminal mutant is artificial or that depending on the nature of the apoptotic signal the TOM complex is dispensable for Bax targeting to mitochondria. It should be added that the interaction of Bax with TOM has been recently challenged (42). However, in the latter study, the addition of Bax to yeast mitochondria induces not only a TOM-independent release of cytochrome c but also that of numerous proteins of high molecular weight from different sub-mitochondrial compartments, raising doubts about the physiological relevance of this observation to the mammalian situation.

The precise implication of the TOM complex in the function of these proteins in healthy cells and during apoptosis remains to be defined. Indeed, there are several differences between the "traditional" mitochondrial outer membrane pathway and that described for the above-mentioned pBCL-2s; firstly, the integration of Mcl-1 with TOM70 occurs through a negatively charged internal sequence (EELD), which differs from the canonical mitochondrial-addressing sequences and seems to be constitutive and not transient as in most nuclear-encoded mitochondria protein (38). Secondly, the interaction of Bax with TOM occurs through a non-conventional addressing sequence located in the N-terminus of Bax (35) and a region in TOM22 that differs from that used by other matrix mitochondrial preproteins (Cartron *et al.,* unpublished results). Thirdly, the interaction of Bcl-2 with TOM20 is not followed by an interaction with TOM40 (37).

The situation could be complicated by the fact that monomers and not dimers of Bax require the presence of TOM to integrate into mitochondria and that those Bax dimers could interact directly with lipids (40, 41). In addition, we have shown that once inserted into the mitochondrial membrane through TOM22, monomeric Bax can act as a receptor for cytosolic Bax (41). These results suggest that depending of the type of apoptotic stimulus and/or the kinetics studied, the TOM / Bax interaction, which is transient (39), could be elusive. We have also recently determined that metaxin-1 and metaxin-2, as stated above, two proteins belonging to the SAM (43) are involved in the docking and the control of the activity of Bak in healthy and apoptotic cells (Cartron *et al.,* unpublished results). This result is consistent with the observation that metaxin-1 is involved in TNF-induced apoptosis (44) and raised the possibility that the SAM complex is also involved in apoptotic mitochondrial permeability.

These features suggest mechanisms distinct from those described for other mitochondrial proteins including tail-anchored proteins (25). It remains to be ascertained whether other pBCL-2s can interact with mitochondria through different TOM components and the significance of

the diversity of the receptors in the modification of mitochondrial permeability during apoptosis. It would be very tempting to postulate that the pBCL-2s convert the normal import activity of TOM into an export activity (as delineated by the release of apoptogenic proteins) during apoptosis. It is interesting to note that the channel activity of the TOM complex is not drastically modified during apoptosis (45, 46). The latter result implies that mitochondrial channels formed during apoptosis are not likely to derive from the TOM40 channel activity.

6. CONCLUDING REMARKS

Others and we have found a new form of regulation of apoptosis through the interaction of pBCL-2s with the translocation machineries of the mitochondrial outer membrane (36-41). It remains to be seen whether this interaction is responsible for the specificity of the interaction of pBCL-2s with mitochondria that in turn could affect the efficiency of apoptosis (47). The relationship between pBCL-2s and the TOM and SAM complexes could also provide the link between these proteins and some important mitochondrial functions such as the control of mitochondrial shape. Similarly, it would be important to understand the importance of the cardiolipin interaction with pBCL-2s and especially the role of this interaction (and possibly components of the inner membrane) in the mitochondrial phase of apoptosis. In this respect, it is conspicuous that the degradation of TIM23 (a pore conducting protein of the inner membrane translocase) is instrumental in the completion of caspase-independent cell death (48).

The future direction of research would be to decipher the nature of the interaction of pBCL-2s with the TOM and SAM complexes, to identify their crucial steps in not only the assembly of the Bax/Bak networks but also possible points of regulation of their activities. These studies could provide new insights into apoptosis and propel further developments for the comprehension and possibly the manipulation of apoptosis.

7. ACKNOWLEDGEMENTS

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Abbreviations: AMOMP: Apoptotic mitochondrial outer membrane permeability; pBCL-2s: BCL-2 family; MOM: mitochondria outer membrane; MPT: Mitochondrial permeability transition; SAM: sorting and assembly machinery; TOM: translocase of the outer membrane; TIM: translocase of the inner membrane; VDAC: Voltage-dependent anion-selective channel.

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