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Review

Mitochondria as the target of the pro-apoptotic protein Bax

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Abstract

During apoptosis, engagement of the mitochondrial pathway involves the permeabilization of the outer mitochondrial membrane (OMM), which leads to the release of cytochrome *c* and other apoptogenic proteins such as Smac/DIABLO, AIF, EndoG, Omi/HtraA2 and DDP/TIMM8a. OMM permeabilization depends on activation, translocation and oligomerization of multidomain Bcl-2 family proteins such as Bax or Bak. Factors involved in Bax conformational change and the function(s) of the distinct domains controlling the addressing and the insertion of Bax into mitochondria are described in this review. We also discuss our current knowledge on Bax oligomerization and on the molecular mechanisms underlying the different models accounting for OMM permeabilization during apoptosis.

Keywords: Apoptosis; Bax; Activation; Conformational change; Addressing/targeting; Permeabilization; Mitochondrion

"Life is pleasant, death is peaceful. It's the transition that's troublesome"
(Isaac Asimov)

Apoptosis is a cell death program that is central to cellular and tissue homeostasis, involved in many physiological and pathological processes [1]. The proteins of the Bcl-2 family are key regulators of this program and their main function is to control mitochondrial permeability and particularly, the release of apoptogenic proteins from this organelle [2].

The Bcl-2 family of proteins can be divided into three groups based on their structure and their role in apoptosis (Fig. 1):

- The anti-apoptotic proteins like Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1/Bfl-1, NR-13, Boo/Diva/Bcl-2-L-10, Bcl-B, E1B 19K (adenovirus), BHFR1 (Epstein Barr virus), CED-9 (*C. elegans*). These proteins are characterized by the presence of four Bcl-2 homology domains (BH: BH1, BH2, BH3 and BH4) (Fig. 1).
- The pro-apoptotic proteins such as Bax, Bak, Bok/Mtd and Bcl-Xs, which contain three homology domains BH1, BH2 and BH3.
- The BH3-only proteins (BOP), which include Bid, Bad, Noxa, Puma, Bmf, BimL/Bod, Bik/Nbk, Blk, Hrk/DP5, Bnip3 and Bnip3 L. These proteins induce apoptosis by activating pro-apoptotic proteins like Bax or by inhibiting anti-apoptotic proteins like Bcl-2.

Most of these proteins contain a C-terminal hydrophobic α-helix, which is a potential transmembrane domain involved in their localization to the membranes of organelles such as the mitochondria, the endoplasmic reticulum and the nucleus [3,4]. These proteins can form homo-dimers and/or hetero-dimers [5,6], essentially through the interaction of their BH3 domain [7]. The BH4 domain that corresponds to the first α helix (Hα1)

Abbreviations: ANT, adenosine nucleotide translocator; AIF, Apoptosis inducing factor; ART, Apoptosis Regulation of Targeting; BdGBM, Bax-deficient Glioblastoma Multiforme; Bif-1, Bax interacting factor 1; BH, Bcl-2 homology; BOPs, BH3-only proteins; BimEL, extra long isoform of Bim; GFP, green fluorescent protein; Hα, α helix;Hn, Humanin; MAC, Mitochondrial Apoptosis-induced Channel; Δψm, mitochondrial change in potential; mPTP, mitochondrial permeability Transition Pore; OMM, outer mitochondrial membrane; PI3, phosphatidylinositol 3; tBid, truncated form of Bid; VDAC, voltage dependent anionic channel

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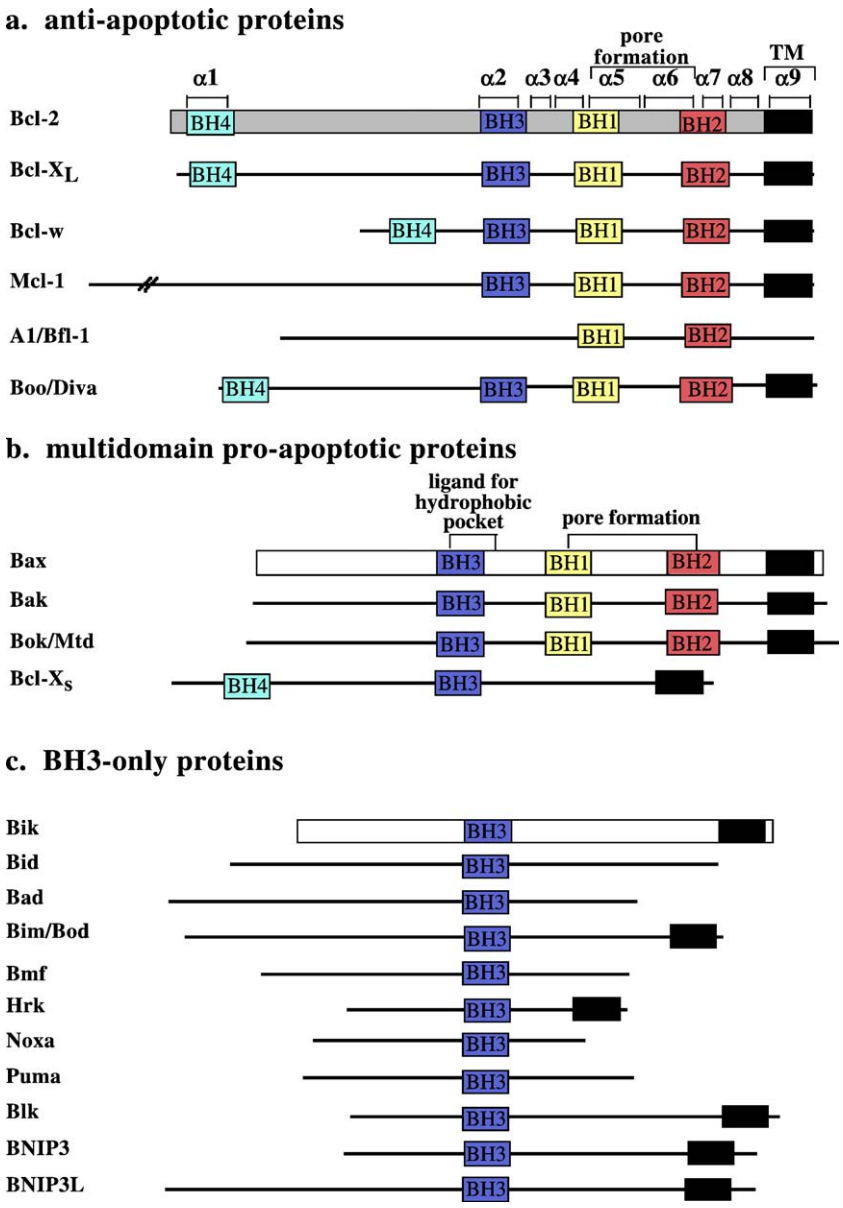

Fig. 1. Representation of all the known mammalian Bcl-2 family members. Bcl-2 homology regions 1–4 (BH1–4) are indicated. TM indicates a putative transmembrane region that mediates localization to intracellular membranes. α: Hα. The Hα5–Hα6 overlapping region containing the BH1 domain corresponds to the pore-forming region based on structural homology with bacterial toxins. The BH3 domain in the pro-apoptotic members is a ligand for the hydrophobic pocket formed by the BH1–BH3 domains of the anti-apoptotic members.

of anti-apoptotic proteins (Fig. 1) is implicated in the control of their anti-death functions [8,9].

1. Cellular localization of Bax

The activation of Bax during apoptosis usually does not require an increase transcription of its gene. Cell fractionation and confocal microscopy showed a preferential cytosolic localization of Bax in most cells in spite of the presence of the C-terminal putative transmembrane domain similar to that of Bcl-2 [10–12]. NMR data on the 3-D structure of soluble Bax showed that the C-terminal hydrophobic Hα9 is bent in a hydrophobic groove. Although Hα9 is essentially composed of hydrophobic amino acids, it also contains several polar amino acids (Thr172, Thr174, Thr182, Ser184, Thr186). Except Ser184, all are exposed to the solvent and thus may contribute to the solubility of Bax in this conformation [13]. On the other hand, the N-terminus is also essential to keep the cytosolic conformation of Bax as a deletion of the first 20 amino acids results in a constitutive mitochondrial localization [14,15]. However, the cytosolic localization of Bax has not been unanimously observed and some groups describe a constitutive mitochondrial localization of Bax in healthy cells [16]. Of note, the activation of mitochondria-bound Bax requires additional partners such as c-myc-regulated elements [17,18], and thus can be dissociated from the mitochondrial addressing step, at least in some cells.

1.1. Induction of apoptosis causes the translocation of Bax to the OMM

The mitochondrial form of Bax found in non-apoptotic cells is a 21 kDa monomer weakly associated with the OMM or soluble in the cytosol. Upon the induction of apoptosis, this monomer evolves into a high molecular complex (96 to 260 kDa) inserting into the OMM, suggesting that an oligomerization accompanies this insertion, see, for example [19–21]). These complexes appear to be homo-oligomers of 6 to 8 molecules of Bax as observed in gel-filtration assays on isolated mitochondria. This oligomerization seems to be an important step in the activation of Bax. Electron microscopy indicates that Bax translocation to the OMM is the first step in Bax activation and that the mitochondria-associated oligomer is the biologically active pro-apoptotic structure [22].

1.2. Bax conformational changes, addressing to mitochondria and insertion into the OMM

Data have shown that the BH3 domain of inactive/soluble Bax is inaccessible for the oligomerization events [13]. Indeed, Bax translocation to the OMM during apoptosis assumes the unfolding of the Hα9 from its hydrophobic groove and a change in the conformation in the N-terminus of the protein [23]. Thus, Bax would undergo one or more conformational change(s) during its activation leading to its anchoring in the OMM and its oligomerization. Two models have been proposed to explain the activation of Bax during apoptosis. The first model suggests that Bax, constitutively associated with the OMM, undergoes a conformational change upon induction of apoptosis leading to oligomer formation and anchoring into the OMM [16]. The second model suggests that a conformational change in Bax occurs in the cytosol resulting in the opening of the C-terminal Hα9 causing the exposure of the pore forming domain Hα5–Hα6 and probably also the accessibility of the BH3 domain and only then Bax is addressed to the mitochondria through its N-terminus [24] or its C-terminus [25,26]. Recently, Annis et al. [27] showed that Bax is actually inserted into membranes as a monomer implying that the insertion step occurs prior to oligomerization.

The hydropathy profile of Hα9 suggests that it could be a putative anchoring domain. The role of these 21 amino acids in the C-terminal domain was first studied by deletion mutagenesis. Studies based on the cellular localization of the fusion protein green fluorescent protein (GFP)-BaxΔC showed that a deletion of 21, 10 or 5 amino acids from the C-terminal generates a protein that is strictly cytosolic under apoptotic conditions [11,23]. These data suggest that the Hα9 plays an essential role in anchoring Bax into the OMM. The crucial role of the conformation of Hα9 was investigated by analyzing different substitutions at Ser184, the polar residue in Hα9 facing the protein core. A substitution by Asp, Gln or Lys generates a protein exclusively cytosolic whereas the deletion of Ser184 or a substitution by Val or Ala produces to a cytotoxic protein exclusively localized to the OMM [23]. Moreover, the BaxΔSer184 mutant fused to GFP exhibited a higher affinity for mitochondria. In order to determine if the C-terminus of Bax is a mitochondrial insertion sequence, cytosolic proteins were fused to this domain. It was showed that GFP fused to the last 21 amino acids of Bax (GFP-21) does not co-localize to the OMM even during apoptosis whereas the same construct with a Ser184 deletion (GFP-20) inserted into the OMM [23]. The central role of the conformation of Hα9 has been further underlined by substitution of Pro168 located in the loop preceding the Hα9 (Fig. 2). Data by Borner's group suggested that this residue was essential for the targeting of Bax to the mitochondria [25,26]. However, we have recently observed that this Pro was more likely involved in the control of Bax membrane-integration by occluding the Hα5 and Hα6 "pore forming" domain [24,28].

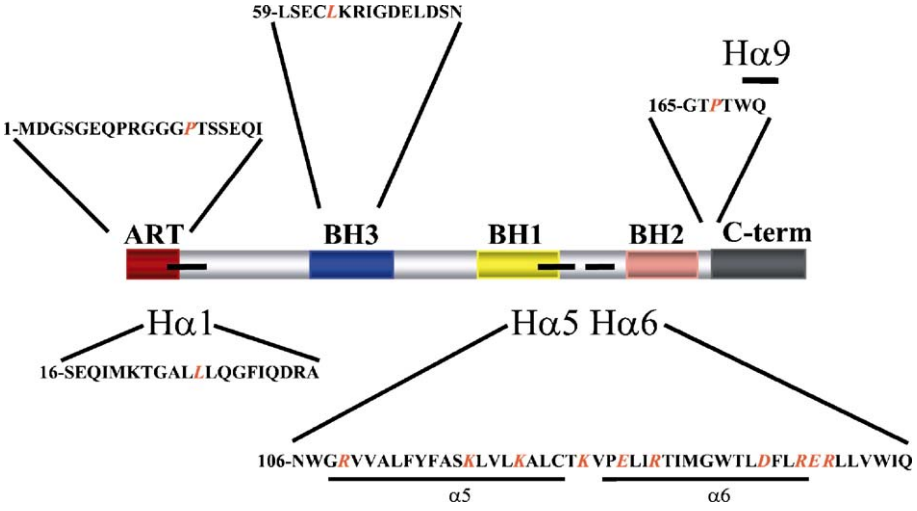

Fig. 2. Crucial amino acids and domains involved in Bax functions and/or mitochondrial targeting. Bcl-2 homology regions are indicated based on their structural homology with Bcl-2. C-term indicates the terminal alpha helix which has been suggested to be the transmembrane/addressing signal (see ref 25). The amino acid sequences of the alpha helices Hα1, Hα5, Hα6, Hα9 are represented in the single letter code. Crucial amino acids involved in Bax translocation and/or activation are labeled in red.

In addition to the conformational changes in the C-terminus, the control of Bax-addressing and insertion also involves the N-terminus. The implication of the N-terminal domain was suggested by experiments on epitope exposure after Bax activation. This region shows a differential affinity to N-terminal specific antibodies before and after Bax activation implying that this domain undergoes crucial changes during apoptosis. The 6A7 antibody does not recognize the soluble cytosolic form of Bax but interacts with the mitochondrial or oligomeric forms [22,23]. Numerous studies have revealed that Bax deleted of 19 amino acids in its N-terminus (BaxΔART, Apoptosis Regulation of Targeting) constitutively inserts into the OMM [14,15,24,29–31]. The role of the Pro residues located at position 8 and 13 within the ART might be crucial. Indeed, site-directed mutagenesis experiments have shown that Pro13 was decisive for the maintenance of the cytosolic Bax conformation and that a substitution for Val increased its apoptogenecity [32]. This substitution Pro13Val caused conformational changes in soluble Bax, a strong binding of Bax to mitochondria and an enhancement of its oligomerization. Similarly, the simultaneous replacement of both Pro residues by Gly stimulates Bax translocation to the OMM in *S. cerevisiae* [28].

The association of Bax with microsomal or mitochondrial membranes using a cell-free system showed that contrary to Bcl-2, Bax bound poorly to these organelles [29]. Deletion of the C-terminus of Bax (BaxΔC) or exchanging the C-terminus of Bax and Bcl-xL shows that, unlike that of Bxl-xL, the C-terminus of Bax is not an addressing/anchoring signal. Furthermore, the consequence of a deletion of the C-terminus was investigated in yeast strains that co-expressed truncated forms of Bax (i.e., BaxΔC) and Bcl-xL (i.e., Bcl-xLΔC) and showed that BaxΔC was as efficient as full-length Bax in promoting cytochrome *c* release [30]. Moreover, it was reported that a substitution of the C-terminus of Bax by that of Bcl-xL (BaxΔCxL) does not modify its sub-cellular localization and its association with mitochondria in human and rat cells or in *S. cerevisiae* but abrogated its pro-apoptotic properties [33]. Overall, these results suggest that the C-terminus of Bax plays an important role in apoptosis but not in the addressing/targeting to the mitochondria. Moreover, Bax or BaxΔC over-expression in BdGBM (Bax-deficient Glioblastoma Multiforme) cells enhanced their sensitivity to apoptosis, which was enhanced by that of BaxΔART or the combined deletion of the 2 extremities (BaxΔARTΔC) [24]. Likewise, there is a natural variant of Bax α, Bax Ψ, which lacks the N-terminus that is constitutively localized to mitochondria and is a more powerful inducer of apoptosis than Bax α [34]. Taken together these results indicate that both the N- and the C-termini functionally interact to control the activity of Bax and thus constitute two distinct ART domains as shown in yeast model by Arokium et al. [28].

Pro-apoptotic activity assays and localization studies by confocal microscopy of several N-terminal mutants of Bax α and Bax Ψ and chimera constructs indicated that the Hα1 corresponding to amino acids 20–37, is implicated in Bax targeting to mitochondria. Deletion of Hα1 impairs the binding of Bax α to mitochondria whereas a fusion of the N-terminus of Bax α with the cytosolic protein RFP (Red Fluorescent Protein), results in the binding of the chimera proteins to mitochondria both in cell-free assays and in vitro. In addition, a fusion protein Hα1-RFP has a mitochondrial localization, whereas the RFP-Hα9 remained cytosolic. The Hα1-RFP construct associated with mitochondria but was sensitive to an alkali treatment, suggesting that Hα1 was not an anchoring domain but an addressing sequence. Furthermore, a Leu26Gly substitution within the Hα1 significantly decreased the acellular association of in vitro translated Bax α and Bax Ψ mutants with mitochondria and also affected their in vivo mitochondrial localization [34]. Mutations in the Hα1 also blocked the subsequent activation of the execution phase of apoptosis. Conversely, a deletion of the C-terminus does not appear to influence Bax α and Bax Ψ mitochondrial addressing. It can thus be concluded that the N-terminal domain determines the addressing of Bax to mitochondria, while the C-terminal domain is involved in the maintenance of the inactive form of Bax. The pro-apoptotic activity of Bax is thus regulated by the conformation of both extremities.

The third important domain in the process of Bax-translocation to the OMM is formed by the central amphipathic helices Hα5 and Hα6, which are the putative pore forming domain of Bax as shown by Nouraini et al. [35]. Bax seems to insert into the OMM following a model already described for bacterial toxins, with the hydrophilic surfaces of Hα5 and Hα6 facing each other and the hydrophobic sides facing the liquid phase [13,36]. A recent study demonstrated the role of Hα5 and Hα6 as a transmembrane domain of Bax [37]. All these data suggest that Hα9 might not be the only domain involved in the membrane insertion of Bax (see ref. [25] for an alternative view).

1.3. Regulation of Bax by phosphorylation

Both anti- and pro-apoptotic Bcl-2 family members may be controlled by phosphorylation [38–40]. Several studies have reported a MEK1 or Akt regulation of Bax activation in diverse cellular systems [41–44]. A recent study has indicated that Akt kinase directly prevented Bax translocation to mitochondria via a phosphorylation of Ser184 [45]. It was reported that Bax conformational changes were sensitive to glucose metabolism and that Akt could prevent Bax activation and apoptosis by promoting glycolysis through multiple post-transcriptional mechanisms [43]. This phosphorylation required the PI3 kinase/Akt-pathway activation and appeared to be mediated by Akt itself. Akt-dependent phosphorylation of Bax promoted its sequestration to the cytoplasm, as phosphorylated Bax could not be immunoprecipitated with the conformation-specific antibody 6A7, indicating that phosphorylation maintained Bax in an inactive form. This phosphorylated Bax was shown to hetero-dimerize with Mcl-1, Bcl-xL and A1 in the cytoplasm of neutrophils [45]. Another recent study reported that nicotine could inactivate the pro-apoptotic function of Bax also through phosphorylation of Ser184 [46]. In contrast, Linseman et al. [47] demonstrated that a phosphorylation of Ser163 stimulated

apoptosis in neurons by promoting the translocation of Bax to the mitochondria.

Thus depending on the nature of the stimulus and/or site of phosphorylation (i.e., Ser163 or Ser184), Bax could be activated or inactivated. These results could have a very significant impact on the design of therapeutic strategies modulating apoptosis.

1.4. How apoptotic signals trigger Bax mobilisation/ activation?

1.4.1. pH and temperature

It was reported that Bax activation and variations in the pH occurred in parallel during apoptosis [48,49]. For example, staurosporine or TNFα treatment of HeLa cells was accompanied by intracellular pH variations and Bax activation [48]. Furthermore, it was shown that a low pH promoted dimerization among Bcl-2 family proteins, suggesting that a conformational change occurred [50]. A recent study by our group showed that pH could induce a conformational change in Bax resulting in the N-terminal and BH3 domain being exposed leading to mitochondrial insertion and oligomerization [51]. Cell-free experiments have shown that in the pH range of 6.8 to 7.8, Bax bound poorly to mitochondria whereas at pH 8.2 or 6, Bax binding to mitochondria was significantly increased [52]. Immunoprecipitation analyses have suggested that pH changes could induce two major alterations in the conformation of Bax. The first occurs at a pH between 6 and 6.8 or between 7.6 and 8, resulting in exposure of the BH3 domain. The second occurs at a pH <6 or >8, causing the N-terminus, which contains the addressing signal to be exposed [52]. These results suggest that high or low pH can induce a change in the conformation of Bax leading to its activation.

Several recent studies reported that heat could also induce Bax conformational changes and apoptosis [53–55]. It was shown that high temperatures (42–45 °C) induced apoptosis whereas mild heat stress-induced thermotolerance in CHO and human adenocarcinoma cells [54]. Heat shock-induced apoptosis was associated with mitochondrial membrane potential decrease, Bax translocation to mitochondria, cytochrome *c* release and caspase 3 activation in normal cells whereas thermotolerant cells, that over-expressed heat shock proteins (Hsps) 27, 32, 70 and 90 were resistant. It was also reported that heat treatment of cells for 1 h at 43 °C resulted in Bax activation, as demonstrated by immunofluorescence analyses using a conformation specific anti-Bax antibody (6A7) [53]. These events are inhibited in cells over-expressing Hsp70. However, Hsp70 was unable to prevent cell death after mitochondrial disruption and subsequent caspase 3 activation, indicating that Hsp70 acted upstream of mitochondria by inhibiting events leading to Bax activation and thereby preventing the release of pro-apoptotic proteins from mitochondria. Of note, a recent study reported that Bax and Bak could be directly activated by heat [55]. Taken together, these results suggest that heat can induce conformational changes in Bax leading to its translocation to mitochondria.

1.4.2. BH3-only proteins

The BH3-only proteins (BOPs) have an essential role in the initiation of apoptosis in *C. elegans* [56], mice [57] and human cells [58]. A study of the phenotypes of transgenic mouse combined with genetic analyses support the idea that BOPs are specific sensors of different signals of apoptosis and convey the apoptotic signal to the different multidomain pro-apoptotic proteins [57,59–69]. For example, Bim and Bad are activated after cytokines deprivation [60–62], Noxa and Puma are activated after genomic DNA damage [63,64], Bmf is activated by anoikis and UV-irradiation [65] and Bid is activated by the death receptor Fas [16,66].

BOPs mediate the Bax-dependent cell death pathway by acting upstream as sensors of different apoptotic stimuli [67–69]. Recent studies with BH3 peptides have suggested that there are two subgroups in the BOPs, leading to the classification of BOPs as either death agonists or survival antagonists: the Bid-like proteins (Bid, Bim) that can directly activate Bax and Bak and the Bad-like proteins (Bad, Bik) that sensitize cells to apoptosis by inhibiting the Bcl-2/Bax interaction [70,71].

Some BOPs activate apoptosis by inhibiting anti-apoptotic members of the Bcl-2 family by formation of mitochondrial hetero-dimers Bcl-2 (or Bcl-xL)/BOP, leading to the activation of Bax [57,72]. NMR studies have shown a possible interaction of Bcl-2 and Bcl-xL with Bad in vitro [73] and several studies have shown a Bim/Bcl-2 and Bim/Bcl-xL interactions [61,74].

On the other hand, some proteins of the BOP group, like Bid, were shown to directly interact with Bax [75]. However, although binding of BOPs to their anti-apoptotic relatives has been well documented, the direct interaction of BOPs such as Bid or Bim with Bax has only recently been reported [32,76,77]. Bid could interact with the Hα1 of Bax, leading to Bax activation as measured by the binding of the conformation-specific antibody 6A7 and the release of cytochrome *c*. Furthermore, a mutation in the Bid–BH3 domain, Arg84Gly, was found to abrogate these effects and this effect can be mirrored by a Asp33Ala mutation in the Hα1 of Bax [76]. Of note, the latter mutation has been found to disrupt the salt bridge formed between the Hα1 of Bax and BH3 domain suggesting that the Bid–BH3 domain could induce Bax conformational changes, at least partially, by disrupting this endogenous salt bridge by competition for the Hα1 of Bax [24].

1.4.3. Inhibitors and activators of Bax

To identify proteins that would selectively bind to Bax, numerous yeast two-hybrid screens were performed.

1.4.4. Humanin

Humanin (Hn), a 3 kDa peptide, was first identified as an anti-apoptotic peptide that inhibited presenilin-induced apoptosis in neuronal cells [78]. Hn was shown to impede the translocation of Bax from the cytosol to the mitochondria thereby protecting cells from apoptosis-inducing signals that target the mitochondrial but not the non-mitochondrial, cell death pathway [79]. Indeed, a knock-down in Hn expression augmented the sensitivity of cells to apoptosis by increasing the translocation of Bax to mitochondria. Surprisingly, there is a

mitochondrial gene coding for the same peptide suggesting a possible gene transfer from the mitochondrial to the nuclear genome during evolution [79].

Two studies by Reed's group have shown that Hn was able to bind to the extra long isoform of Bim (BimEL) and Bid thereby abrogating their activation of Bax and Bak [80,81]. In vitro protein–protein interaction assays, immunoprecipitation assays and fluorescence polarization assays have been used to demonstrate the binding of Hn to Bid. Moreover, Hn conserved a protective activity in *bax*−/− mitochondria, indicating that Hn could inhibit the tBid-induced release of apoptogenic factors from this organelle in a Bax-independent manner. In the same study, it was shown that the first 12 amino acids of Hn were necessary and sufficient to bind and suppress tBid activity in vitro and in vivo.

1.4.5. Ku70

Yeast two-hybrid screening allowed the identification of Ku70 as an inhibitor of Bax [82]. Ku70 is a ubiquitous protein present in the cytoplasm and nucleus and is a subunit of the Ku antigen implicated in the reparation of non-homologous double-stranded DNA. Independently, Ku70 appears to inhibit Bax. Ku70 deficient mice are extremely sensitive to anti-cancer drugs without having any effect on DNA repair and an increased concentration of Ku70 has been detected in some cancers [83]. An over-expression of Ku70 inhibited the Bax-dependent signaling pathway whereas the suppression of Ku70 increased Bax-dependent apoptosis. This interaction of Ku70 and Bax occurs via the C-terminus of Ku70 and the N-terminus of Bax resulting in the cytosolic sequestration of Bax. Minimal peptides inhibiting Bax were designed from the C-terminal domain of Ku70 [84,85].

1.4.6. 14-3-3 proteins

Some isoforms of the cytosolic and highly conserved protein 14-3-3 (θ, ε, ζ) seem to regulate Bax function. Numerous cellular processes (cell cycle, cell death, intracellular traffic) are under the control of these proteins, which sequestrate regulatory proteins by hetero-dimerization. The 14-3-3θ isoform can interact directly with Bax and sequestrate Bax in the cytosol [39]. This interaction requires both the N- and C-terminal domains of Bax but does not require its phosphorylation. The over-expression of 14-3-3θ decreases apoptosis induced by the over-expression of Bax in 293T cells or by an agonist of death receptors in HeLa cells. Conversely, during apoptosis, Bax is liberated from 14-3-3θ by caspase-dependent mechanisms and is translocated to the mitochondria.

1.4.7. Bif-1

By yeast two-hybrid using Bax as bait, a new interacting protein was identified and called Bif-1 (Bax interacting factor 1) [86,87]. Bif-1, a member of the endophilin B protein family is a protein of 365 amino acids and its C-terminal domain (amino acids 308 to 364) contains a region with a high similarity to the SH3 domain (Src homology 3 domain) implicated in signal transduction pathways and cellular communication. Bif-1 mRNA is expressed in several tissues such as heart, skeletal muscle, kidneys and placenta [86]. There are controversial results about Bif-1 function and effect on Bax.

Heim's group showed that Bif-1 could interact with Bax via their N-terminal domains and co-localized with Bax in the cytosol. However, the over-expression of Bif-1 had no influence on Bax-dependent apoptosis in 293T and HeLa cells [87]. Wang's group suggested that Bif-1 was partially co-localized with Bax and was involved in the activation of Bax-dependent pathways. The interaction of Bif-1 with Bax via the N-terminal domain induced a conformational change in Bax and its translocalization to mitochondria [86].

Bif-1 was also shown to play a regulatory role in apoptotic activation, not only of Bax, but also Bak [88]. Inhibition of the endogenous expression of Bif-1 by siRNA abrogated Bax conformational change, cytochrome *c* release and caspase 3 activation induced by various intrinsic death signals (adriamycin, staurosporine, actinomycin D, tunicamycin). Bif-1 was shown to interact with Bax on mitochondria but this association decreased 24 h after tunicamycin treatment of MEFs, suggesting the formation of a transitory Bif-1/Bax complex in response to apoptotic stimuli. Furthermore, the suppression of Bif-1 expression had no effect on the cell death induced by BaxΔS184, a conformational changed and mitochondria-bound mutant, suggesting that Bif-1 was involved in Bax-mediated apoptosis by controlling its recruitment to mitochondria. Immunoprecipitation assays using an anti-Bak antibody that recognized only the conformational altered Bak protein showed that loss of Bif-1 prevented Bak activation induced by tunicamycin or staurosporine treatment. Moreover, suppression of Bif-1 expression was associated with an enhanced ability of HeLa cells to form colonies in soft agar and tumors in nude mice after implantation. These results suggest that Bif-1 may function as a tumor suppressor.

1.4.8. Clusterin

Clusterin, also named apolipoprotein J or TRPM-2 (testosterone-repressed prostate message 2), is a glycoprotein that has diverse functions, notably apoptosis regulation and is over-expressed in several human cancers such as prostate cancers, breast cancers and squamous cell carcinoma [89,90]. There are two forms of clusterin: the cytoplasmic form of 60 kDa and the secreted form of 40 kDa, which is a hetero-dimer consisting of α and β chains. Unlike other inhibitors of Bax, clusterin specifically interacts with conformation-altered Bax in response to chemotherapeutic drugs, thus inhibiting the oligomerization of Bax [91]. Indeed, immunocytochemistry and cell fractionation experiments showed that clusterin was mainly mitochondrial. Transfection experiments showed that clusterin provided protection against apoptosis induced by etoposide or camptothecin. Moreover, the 6A7 antibody that specifically detects activated Bax co-immunoprecipitated clusterin in etoposide-treated cells. Cross-linking experiments showed that this interaction inhibited Bax oligomerization. Moreover, mutation analyses showed that the α chain and not the β chain, of clusterin interacted with Bax. Given that clusterin is widely over-expressed in human cancers and induced by chemotherapeutic drugs, these results suggest that disrupting the

clusterin–Bax interaction may be an attractive strategy for anti-cancer therapy.

1.5. Bax oligomerization, mitochondrial permeabilization and cytochrome c release

Activation of the pro-apoptotic proteins Bax and Bak lead to their oligomerization and the permeabilization of the OMM. Two models of permeabilization are proposed: the non-specific rupture of the OMM or the formation of specific channels in the OMM [92].

The first model implicates the rupture of the OMM after the swelling of the mitochondrial matrix. This model is based on different observations: the fall of $\Delta\psi m$ and cytochrome *c* release induced by the addition of Bax on isolated mitochondria are inhibited by the addition of cyclosporine A, bongkrekic acid and the calcium depletion, which inhibit the mitochondrial permeability Transition Pore (mPTP) [93–95]. The opening of this non-specific megachannel induces the fall of $\Delta\psi m$ and the swelling of the mitochondria that causes the rupture of the OMM and the massive release of cytochrome *c*. However, several studies show that swelling is not an absolute prerequisite during the in vivo apoptosis [93,96] and cytochrome *c* release has been shown to occur in the absence of mitochondrial depolarization [97].

Bcl-2 family proteins have been proposed to be regulatory elements of the mPTP by interaction between Bax/ANT (adenosine nucleotide translocator) and Bax/VDAC (voltage dependent anionic channel) thanks to the BH3 domain [98,99]. It was shown that only VDAC is essential for the apoptotic function of Bax [98,100]. Bax, localized to mitochondria after apoptotic stimuli, directly interacts with VDAC thus leading to its opening and the release of cytochrome *c* whereas Bcl-xL interaction with VDAC leads to its closure. However, the diameter of VDAC is not enough large to permit cytochrome *c* passage. It was then suggested that Bax could induce a conformational change in the VDAC or, another possibility was that Bax and VDAC together formed a megachannel. On the flip side, Kroemer's group showed the importance of Bax/ANT interaction [99,101]. Two-hybrid and co-immunoprecipitation experiments suggest that these two proteins can interact. Moreover, purified Bax and ANT are sufficient to induce liposome permeabilization after atractyloside-treatment. The oligomerization (homo-dimerization or multimerization) of Bax seems to be needed to form channels. In this case, ANT regulates the insertion and the oligomerization of Bax in the OMM and cytochrome *c* release is dependent on the channel characteristics of Bax.

Other studies have shown that VDAC or ANT do not participate in mitochondrial clustering formed by Bax oligomerization [20,102,103]. An alternative model is based on intrinsic characteristics of Bcl-2 protein family. 3-D structural analyses of Bcl-xL revealed structural and functional similarities with the diphtheria toxin and the bacterial colicine, which can form channels [36]. Moreover this channel activity was shown in lipidic membranes [104]. Later, it was shown that Bax, tBid and Bcl-2 have similar structures and were able to form channels in lipidic membranes [105–108]. Channel

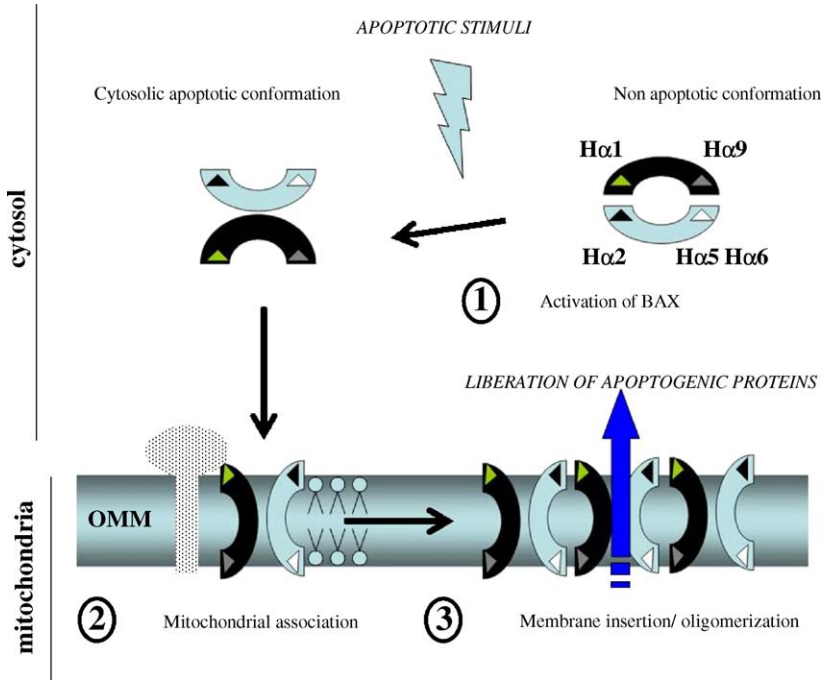

Fig. 3. Schematic representation of Bax activation and its mitochondrial membrane integration during apoptosis. *Step 1*: the conformation of ART is blocked in a conformation (with the participation of cytosolic proteins such as Ku70) that does not allow the exposition of Hα1. The latter helix is in close contact with BH3/Hα2, while Hα9 occludes from the Hα5–Hα6 pocket. *Steps 2*: the movement of ART around the flexible zone between Pro8 and Pro13 allows the exposition of Hα1, which can interact with putative mitochondrial receptor(s) that may be of different natures (proteins, lipids) while the Hα5–Hα6, which is liberated from its interaction with Hα9 and triggers the integration of Bax into the membrane. *Steps 3*: Hα5–Hα6 and Hα9 are inserted in the OMM. Both Hα2 (BH3) and Hα9 might participate in the oligomerization process. The insertion of Bax into mitochondria is associated with the liberation of apoptogenic proteins from the intermembrane space.

formation by bacterial toxins has been described and is realized in three steps: association with membranes and orientation (with decrease in pH and negatively charged lipids). Then the amphipathic Hα5 and Hα6 are perpendicularly integrated into the lipidic bilayer and the neighboring helices are folded like an opened umbrella. Finally, the channel is formed by homo-dimerization or oligomerization of the bacterial toxin [107]. Similarly, Bcl-2 family proteins form channels with their central Hα5 and Hα6 at acidic non-physiologic pH. However, only Bax has a channel activity in liposomes at neutral pH and must undergo, like bacterial toxins, conformational changes in order to be translocated to OMM. Antonsson et al. [20] showed that Bax (BaxΔC) induced the release of a fluorescent probe from liposomes and at neutral pH this event could be inhibited by the addition of Bcl-2. Therefore, the channel characteristics of Bax are different from those of Bcl-2. Besides, destabilization of phospholipid membranes by Bax and especially by oligomers of Bax, in the presence of calcium [109], seems to generate channels with a diameter of 22 Å, which might be sufficient for cytochrome *c* release [110,111]. The last model could explain how oligomers of Bax permeabilize the OMM and induce the release of apoptogenic factors from the intermembrane space to the cytosol. Although the existence of such channels has not been shown in vivo, patch-clamp experiments have suggested a correlation between the apparition of a new ionic channel on proteoliposomes made from OMM obtained from mammalian apoptotic cells or from Bax-expressing yeast [112]. This channel, named MAC (Mitochondrial Apoptosis-induced Channel) is linked to Bax on mitochondria, its activity is sensitive to the anti-apoptotic protein Bcl-2 and its largest conductance implies a diameter enough large (≥4 nm) for the diffusion of cytochrome *c* and molecules with higher molecular weight. Alternatively, Bax and tBid can generate lipidic channels enough large to permit the release of cytochrome *c* [110,113]. It has been suggested that membrane permeabilization needs the cooperation between Bax, tBid and lipids, essentially cardiolipids, in order to permit Bax activity [102]. Models based on channel formation in the OMM allow for the conservation of integrity and functionality of the inner mitochondrial membrane [114].

2. Conclusion

Of the several hundreds of nuclear encoded proteins, which compose the mitochondrion, Bax is somewhat special, as it becomes mitochondrial only when a death sentence is passed on the cell. This feature might not be restricted only to Bax as an increasing number of proteins have been described to translocate to this organelle during the first stages of apoptosis and in due course, mitochondria take revenge by exporting intrinsic proteins to the cytosol. Bax appears to be the orchestra-conductor of this lethal exchange together with Bak, a constitutive mitochondrial protein, with which Bax shares many but not all functions. Bax is a fascinating protein as it undergoes probably several distinct changes in conformations at the onset of apoptosis before becoming fully integrated into the OMM. As depicted in Fig. 3, several steps can be considered. First, the activation of Bax by various stimuli of different natures (proteins, lipids, pH, heat…), which causes a conformational change in Bax "*en route*" to the mitochondria; second, the interaction of Bax with putative receptors (lipids, proteins) in the OMM resulting in the association and presumable integration into mitochondria; third, the acquisition of a membrane embedded conformation, which is associated with the oligomerization of Bax and the release of mitochondrial proteins such as cytochrome *c* or AIF. Many questions remain open from this simplistic model. What are the partners of Bax during this journey? What kind of changes in conformation does Bax undergo, and where and when do they take place? The answers of these questions are of importance not only for the comprehension of apoptosis but may be also in other functions in which Bax could be implicated such as the mitochondrial fission or fusion. More importantly, they may be useful in the design of new therapeutic tools as apoptosis is involved in many pathologies (cancer, neurodegenerative diseases such as Parkinson, Huntington, Alzheimer's disease, amyotrophic lateral sclerosis).

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