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Review

Bax: Addressed to kill

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ABSTRACT

The pro-apoptototic protein Bax (Bcl-2 Associated protein X) plays a central role in the mitochondria-dependent apoptotic pathway. In healthy mammalian cells, Bax is essentially cytosolic and inactive. Following a death signal, the protein is translocated to the outer mitochondrial membrane, where it promotes a permeabilization that favors the release of different apoptogenic factors, such as cytochrome c. The regulation of Bax translocation is associated to conformational changes that are under the control of different factors. The evidences showing the involvement of different Bax domains in its mitochondrial localization are presented. The interactions between Bax and its different partners are described in relation to their ability to promote (or prevent) Bax conformational changes leading to mitochondrial addressing and to the acquisition of the capacity to permeabilize the outer mitochondrial membrane.

1. Introduction

Apoptosis, the main and more studied form of programmed cell death, plays a central role in tissue homeostasis during development ([1] for review) and all along the life of multicellular organisms ([2] for review). Alterations of apoptosis are involved in tumorogenesis ([3,4] for reviews), as well as in the cellular response to anti-tumor treatments ([5] for review). Thus, apoptosis has been one of the most studied field of cell biology over the last 10 years. Among the numerous regulators of apoptosis, proteins of the Bcl-2 family are major players ([4,6,7] for reviews). Their main function is to regulate the permeability of the outer mitochondrial membrane (OMM) to different apoptogenic factors, namely cytochrome c [8,9], Smac/Diablo [10,11], Omi/HtrA2 [12], endonuclease G [13], and AIF [14]. This permeabilization is generally considered as a "point-of-no-return" of apoptosis. Two proteins of the Bcl-2 family, namely Bax [15] and Bak [16,17], are pivotal effectors in this process. These two proteins, although expressed in non-apoptotic cells, remain inactive. Upon a cell death signal, they undergo a conformational change that ultimately leads to their insertion, their oligomerization and the formation (or the activation) of large pores through the OMM, through which the different apoptogenic factors are released.

Bax and Bak seem to be functionally redundant:indeed, the inactivation of both of them is required to fully impair apoptosis in most cells [18]. However, Bax is generally more strongly expressed than Bak, and Bak is overexpressed (and active) when Bax has been previously inactivated [19]. Consequently, Bax has been more studied than Bak, even though recent elegant studies on Bak have enlighted both the differences and similarities between the function and the regulation of the two proteins (see e.g. [20]).

Since the identification of Bcl-2 as an anti-apoptotic protein [21], the other members of the family have been identified on the basis of primary structure homologies in the so-called "Bcl-2 Homology" domains (BH1 to BH4) (Fig. 1). These domains are conserved through the whole animal reign [22]. A remarkable fact about this family is the structural homology of the whole proteins. It is particularly striking that proteins having opposite functions (such as pro-apoptotic Bax and anti-apoptotic Bcl-2, for example) share a common tertiary structure, even though their primary structures are somewhat distant (except, of course, in the BH domains). However, it should be noted that most structural data on proteins of the Bcl-2 family was obtained on engineered proteins that were modified (namely through the deletion of the C-terminal hydrophobic α-helix) to make them more soluble in aqueous solvents [23–25]. This might be particularly critical for Bax, of which the localization is changing, during apoptosis, from cytosol to OMM [26,27]. Available data thus provide informations on the inactive conformation of the protein (cytosolic or weakly bound to mitochondria), but not on its active conformation (inserted

Abbreviations: AIF, apoptosis-inducing factor; ANT, adenine nucleotides translocator; ART, apoptotic regulation of targeting; BHx, Bcl-2 homology domain; IMM, inner mitochondrial membrane; MAC, mitochondria apoptosis-induced channel; mPTP, mitochondrial permeability transition pore; OMM, outer mitochondrial membrane; SAM, sorting and assembly machinery; TOM, translocase of outer membrane; VDAC, voltage-dependent anion channel.

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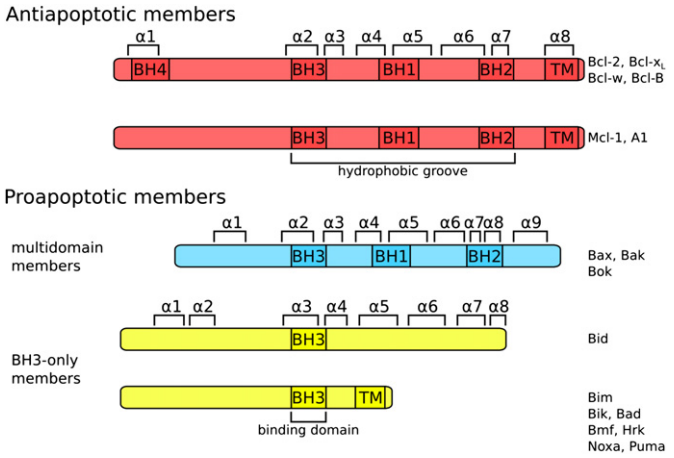

Fig. 1. Primary alignment of mammalian Bcl-2 family members. The positions of the Bcl-2 homology domains (BH) and of the α-helices are indicated (adapted from [4]).

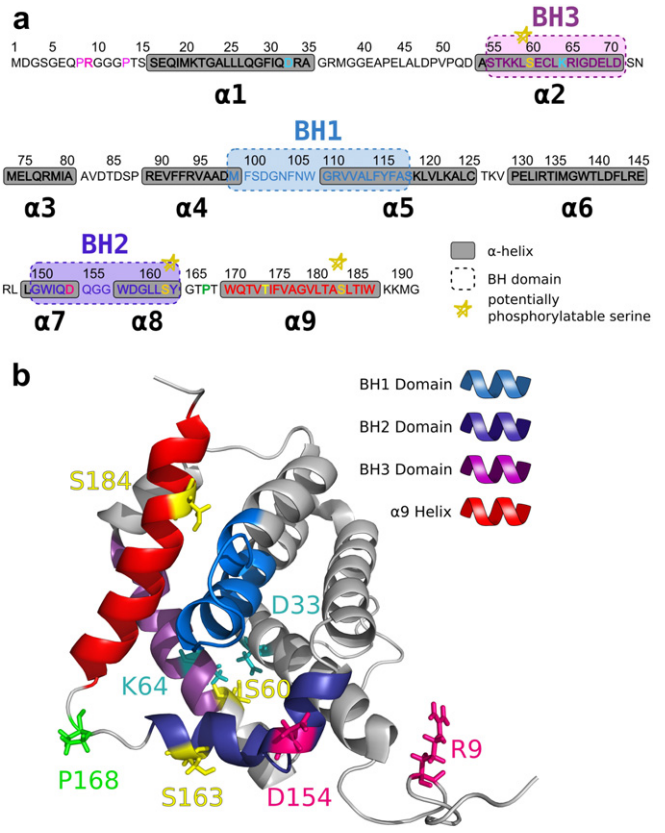

Fig. 2. Primary (A) and tertiary (B) structures of human Bax. (A) Colored residues indicate mutations that are described in this review. (B) The structure shown is based on the lowest energy conformer in PDB file with accession number 1F16 [28]. The same color code as for (A) is used, but some residues were omitted for clarity.

in the OMM). In the absence of structural informations on the OMM-inserted protein, indirect methods have been used to identify the domains and the residues of Bax that are important for its localization, its function and its interactions with different partners. The avaibility of a NMR structure of soluble full-length Bax [28] made site-directed mutagenesis followed by functional studies easier, as investigators could target helices and loops potentially involved in conformational changes.

This review aims to give an overview of present knowledge on Bax structure/function relationships, and to provide some clues for further studies on molecular mechanisms underlying the function of this unique protein.

2. Bax conformational changes

2.1. Structure of cytosolic Bax

In healthy cells, Bax is essentially a cytosolic protein, although a fraction of the protein is generally found in mitochondria [26,27]. This mitochondrial fraction is loosely attached to the OMM, as it can generally be removed by a chaotropic treatment. A small portion of Bax has also been found in ER, although the function of this subpopulation remains undetermined [29]. The NMR structure of Bax in an aqueous environment has been determined [28], and this structure is still an essential basis for investigators interested in Bax structure/function relationships (Fig. 2). The protein is formed of 9 α-helices connected by short loops. Three of these helices, namely α5, α6 and α9 are likely involved in the interaction of Bax with OMM: α5 and α6 are amphipathic helices organized as a hairpin, in a way similar to bacterial toxins known to form pores in lipid bilayers [30,31]. α9 is a hydrophobic helix that masks the hydrophobic groove formed by the BH domains. Although this helix has all the required characteristics of a transmembrane helix [32], experimental data suggest that it is not a typical transmembrane anchor homolog to the C-terminal α-helices of anti-apoptotic proteins (see below).

2.2. Conformational changes of the N-terminal end of Bax

Although the 3 helices cited above have been under particular scrutiny by investigators due to their potential ability to insert the protein into membranes, another domain of the protein has provided a definitive tool to follow Bax translocation. The 19 first residues of Bax, that precedes the α1-helix, are very mobile, as suggested by NMR data [28]. It had been shown, rather incidently, that removing these 19 residues, by starting the translation at Met20 residue, created a protein (BaxΔN) that had a strong capacity to be inserted into the outer mitochondrial membrane [33]. Interestingly, a natural Bax variant found in low grade glioblastoma lacked the 20 first residues (including the Met20) [34]. This variant, called BaxΨ, had a stronger capacity to be translocated to mitochondria and, consequently, a higher pro-apoptotic activity when assayed in mammalian cells [35,36], and a higher capacity to release cytochrome c, when heterologously expressed in yeast [37]. These data suggest that the N-terminal domain of Bax, has the ability to lock the protein under a soluble inactive conformation and that a movement of this domain is required to favor the mitochondrial translocation of Bax: this domain has been called “Apoptotic Regulation of Targeting” (ART) [27].

The function of ART has been further investigated by introducing point mutations. The ART of human Bax contains 2 proline residues (in position 8 and 13) flanking a Arg–Gly–Gly–Gly sequence (Fig. 3a): Pro residues are known to form rigid square angles in peptidic chains while Gly residues are very mobile. This peculiar structure might therefore help transitions between different positions of ART. Indeed, the replacement of Pro8 and Pro13 (or Pro13 alone) by Gly favored the mitochondrial translocation of Bax both in human [35] and in yeast cells [38], and apoptosis in human cells [36].

The replacement of the Arg9 residue by Glu, that introduces an electrostatic repulsion between this residue and Asp154 also activated Bax mitochondrial tranlocation in yeast; this was completely abolished if Asp154 was converted to Lys [39]. This suggests that

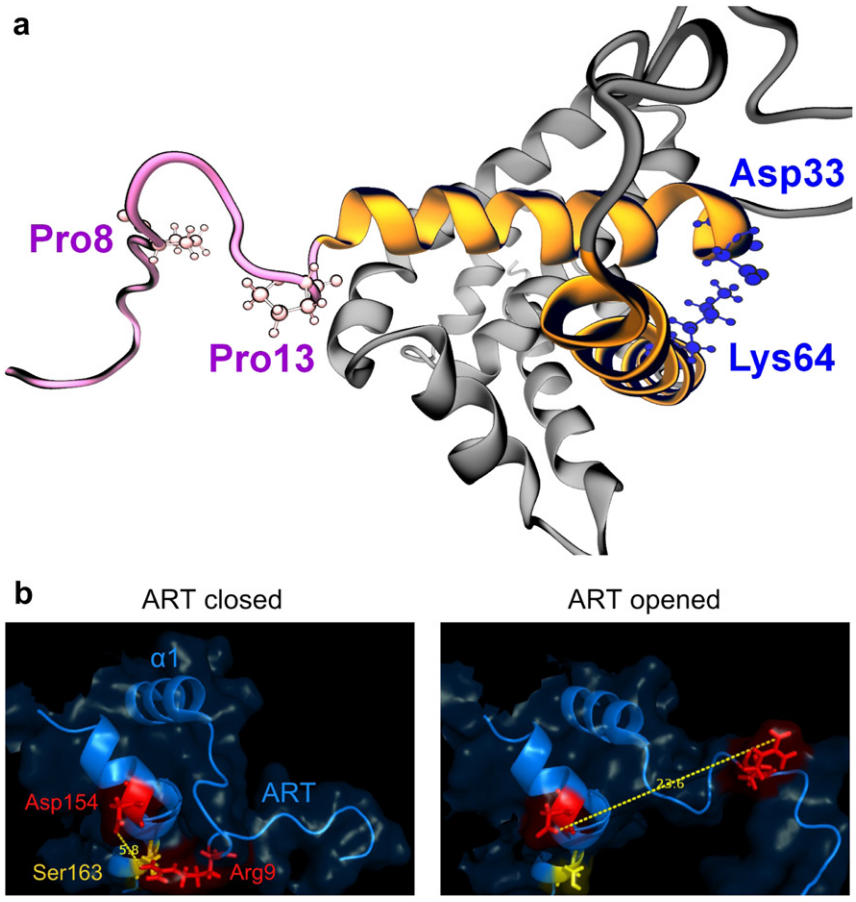

Fig. 3. The N-terminal part of human Bax. (A) Magnification of the N-terminal part of human Bax showing the ART with the positions of the 2 proline residues 8 and 13, and the helices α1 and α2 with the residues Asp33 and Lys64 forming a salt bridge. (B) View of two positions of ART found in PDB structure 1F16, showing the position of Arg9 relative to Asp154, and the position of phosphorylatable Ser163. These structures are 2 of the 20 lowest energy conformers (among 50) submitted by Suzuki et al. [28].

interactions between ART and residues localized in other domains of Bax (in this case, α8-helix) are central regulators of ART movements and, subsequently, of Bax mitochondrial translocation (Fig. 3b).

Even though the structure of ART is undefined, an immunological tool depicting this region of Bax, (together with a part of α1 helix), has been used for long by investigators: a monoclonal antibody targeted at residues 12–24 of human Bax, 6A7, is able to detect specifically the active form of Bax, while another antibody, 2D2, targeted at the same residues, is able to detect all the cellular Bax [40]. Structurally, it has been shown that such a conformation specificity requires a large movement of ART [41], thus confirming the extent of the conformational change between cytosolic Bax and OMM-inserted Bax.

2.3. The helices α1 and α2

Once ART has moved, Bax can be activated. Experimental data suggest that the relative movement between α1 and α2 is involved in Bax activation. The examination of these helices suggest that a salt bridge might exist between Asp33 (α1) and Lys64 (α2) (Fig. 3a) contributing to the stabilization of this region. Both the mutants Asp33Ala and Lys64Glu (or Lys64Asp) are spontaneously able to localize to the OMM in mammalian cells, isolated mitochondria and yeast cells [39,42]. However, although both mutants are active when heterologously expressed in yeast cells [39], they are not in mammalian cells [42]. Since α2 corresponds to the BH3 domain, and Asp33 is involved in the binding of Bax with BH3-only proteins such as tBid [42] and Puma [43], additional regulations are likely required for these mutants to be fully activated in mammalian cells, whereas these putative regulations are not required in the more simple yeast cells.

The helix α1 by itself has been shown to be involved in the translocation of Bax to the OMM. The introduction of mutation Leu26Gly prevented the constitutive mitochondrial localization of Baxψ and impaired the translocation of wild-type Bax following apoptosis in human cells [36]. Mutations Ala24Arg and Leu26Gly/Leu27Val had a similar effect [36]. Interestingly, these two later mutants also impaired the mitochondrial localization of Baxψ heterologously expressed in yeast cells [36,37], suggesting that this role of helix α1 was an intrinsic property of Bax. We will discuss below the possible involvement of the mitochondrial receptor Tom22 in this role of the α1-helix of Bax.

2.4. The C-terminal α9-helix

Although the conformational change of the N-terminal end of Bax is associated to its translocation, it is not sufficient to promote the full activation and insertion of the protein, when human Bax is expressed alone (*i.e.* in the absence of any other Bcl-2 family members) in yeast: for example, an analysis of the capacity of different point mutants of Bax to promote the release of cytochrome c from yeast mitochondria showed that the double mutant Pro8Gly/Pro13Gly did not induce a complete release [38].

Like most Bcl-2 family members (with the noticeable exception of Bid), Bax has a C-terminal hydrophobic α-helix (α9) that has the properties of a transmembrane anchor [32]. The homologous helices

in anti-apoptotic Bcl-2 and Bcl-xL have been clearly shown to be transmembrane anchors: their suppression completely prevents the membrane-insertion of these proteins, and only residual loosely-bound Bcl-2 remains attached to mitochondrial and ER membranes [44,45]. The behavior of Bax deprived of its α9 (BaxΔC) is more ambiguous. Early production and purification assays of Bax were done with BaxΔC, since the presence of hydrophobic α9 tended to induce the aggregation of the protein. However, BaxΔC was able to be inserted in liposomes and to permeabilize them, suggesting that the absence of α9 did not impair the capacity of the protein to be inserted in a lipid bilayer [46]. Furthermore, in the same study, it was shown that BaxΔC had almost the same activity as Bax full-length when expressed in neurons. On the opposite, many cell biology studies made with a fusion protein GFP-Bax showed that the absence of α9 prevented the ability of the fusion protein to be translocated to the OMM following an apoptotic signal (see e.g. [47]). Experiments made on isolated rat liver mitochondria showed that, while full-length Bax is not able to be inserted in OMM, a chimeric protein formed of BaxΔC fused to the C-terminal α-helix of Bcl-xL (BaxΔC-CxL) was inserted. Conversely, while full-length Bcl-xL was inserted, a chimeric protein formed of Bcl-xLΔC and the C-terminal α-helix of Bax was not [48]. This suggests that, opposite to the C-terminal α-helix of Bcl-xL that is a "true" transmembrane anchor, the C-terminal α-helix of Bax is not. Bax-α9 alone is not able to drive the insertion of a reporter protein (unrelated to the Bcl-2 family) in a bilayer, unless one residue located in the middle of the helix, Ser184, is deleted [28]. This suggests that α9 is "almost" a transmembrane anchor, but that it requires a significant conformational change to be able to act as such. It must be noted that Ser184 is a critical residue for Bax conformational changes: indeed, it is a target for phosphorylation by different kinases playing a role in the regulation of Bax (see the discussion on phosphorylation below).

When expressed in yeast, human full-length Bax is not localized in mitochondria, but the addition of a *c-myc* tag at the C-terminal end, or the replacement of a part of α9 by a random sequence are sufficient to promote the mitochondrial localization [37]. When expressed in mammalian or yeast cells, the chimeric protein BaxΔC-CxL is localized to the OMM but is inactive [49]. Taken together, these data suggest that, although involved in Bax mitochondrial translocation, α9-helix is not a simple membrane anchor similar to that of Bcl-2 or Bcl-xL, but that its role is "integrated" in a more general conformational change affecting a larger domain of the protein.

As already discussed above for certain mutants, the heterologous expression of human Bax in yeast is a powerful tool to study the cellular behavior of Bax independently from other Bcl-2 family members. Different point mutations of residues in α9 have been studies with this model (*note*: although Ser184 is in α9, it will not be discussed in this section, but in the section about Bax phosphorylation). In the NMR structure of soluble Bax, Thr174 is located on the inner face of α9, facing the core of the protein (Fig. 2b). A point mutation Thr174Asp induces a dramatic stimulation of Bax mitochondrial translocation [38]. The position 174 faces the residue Glu69 of α2, that is part of the highly conserved BH3-domain. It is probable that the close proximity of the two negative charges of Asp174 and Glu69 induces an electrostatic repulsion of α9, moving it further apart from the core of the protein. Other studies were done with Pro168, that is located just upstream the beginning of α9 (Fig. 2b). This residue forms a rigid right angle positionning α9 along the hydrophobic groove formed by the BH domains (Fig. 4). The effect of a mutation of this residue to Val has been studied in 2 models: the native full-length protein, or a N-terminal GFP fusion both expressed in mammalian cells. The native Pro168Val mutant is constitutively localized to mitochondria [42], while the GFP-fused Pro168Val is not, and is nor translocated following an apoptotic signal [50]. This points again to the differences that may exist between the behavior of native Bax and

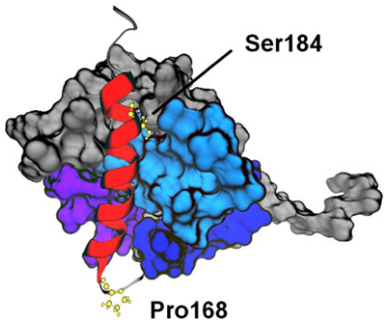

Fig. 4. The C-terminal part of human Bax. Surface view of the 3 BH domains forming the hydrophobic groove, in which the helix α9 is positioned in inactive (cytosolic) Bax. The positions of residues Pro168 and Ser184 are indicated.

of the fusion protein GFP-Bax. Interestingly, a Pro168Ala mutant of native Bax has been expressed in yeast: like the Pro168Val mutant expressed in mammalian cells, this mutant is strongly localized to the OMM [38]. This suggests that, like for the Thr174Asp mutant, the movement of α9 resulting from the higher flexibility of the loop between α8 and α9 is sufficient to destabilize the interaction between α9 and the hydrophobic groove formed by BH domains. The movement of α9 seems to be a crucial event in Bax translocation, not because it might be a membrane anchor, but more because this movement unmasks the BH domains and the hydrophobic hairpin α5/α6, that are crucial for the subsequent events of dimerization and oligomerization [51,52].

2.5. The helices α5 and α6

Following the partial resolution of the structures of proteins of the Bcl-2 family, and the observation that, when inserted in lipid bilayers, these proteins could form ionic channels, it has been proposed that the hairpin structure found in most proteins of the Bcl-2 family, including Bax, could form pores, when inserted in membranes [30,31]. Considering the similarity of this hairpin structure with that of bacterial toxins forming "killer" pores, it was obviously tempting to speculate that these helices could directly participate to the permeabilization process induced by Bax, and this capacity has been demonstrated in synthetic models [53]. Mutations in Bax α5/α6 actually alter the capacity of the protein to promote membrane permeabilization and to trigger apoptosis ([54]; see however [55]). Furthermore, fusion proteins of GFP with Bax α5 and/or Bax α6 are specifically localized in mitochondria, and are abel to promote the release of cytochrome c, when expressed in mouse embryonic fibroblasts, while fusions with α1 or α9 do not have any effect [56].

However, the fact that anti-apoptotic proteins Bcl-2 and Bcl-xL also exhibit this hairpin structure seems to contradict this hypothesis, since these proteins are not supposed to form high-conductance channels in membranes. It should be noted, however, that Bcl-2 and Bcl-xL are able to create low-conductance channels, similar to those created by Bax [30,31]. The difference between anti- and pro-antiapoptotic proteins could be the further capacity of Bax to convert low-conductance channels into high-conductance channels, but this capacity may not be based on α5 and α6, but on the process of Bax-oligomerization, that would depend on other domains of the protein.

2.6. The BH domains

The molecular basis of the interactions between proteins of the Bcl-2 family, is the interaction between the BH3 domain of a protein

and the BH1/BH2 domains of its partner. For example, the BH3 domain of Bax is able to interact with the BH1/BH1 domain of Bcl-2. This asymmetric interaction is one of the basis of the regulation of the apoptotic network. Indeed, a BH3-only protein, such as Bad, is able to compete with Bax for the binding to Bcl-2, thus releasing Bax from this inhibitory interaction to trigger its pro-apoptotic function. It is therefore crucial to understand the molecular mechanisms underlying this interaction. The BH3-domain of Bax roughly corresponds to the α2-helix of the protein. Indeed, it has been shown that the deletion of four highly conserved residues Ile66-Gly67-Asp68-Glu69 in this helix completely impaired the capacity of Bax to promote apoptosis [57]. It is now well-accepted that the BH3 domain of Bax is exposed during the conformational change leading to the activation of Bax. As discussed above, it has been suggested that two residues, Asp33 (in α1 helix) and Lys64 (in α2 helix) may form a salt bridge stabilizing the BH3 under a closed (non-exposed) conformation [39,58]. Interestingly, Asp33 is also involved in the interaction between Bax and the BH3-only protein PUMA [43,58].

There is still a debate about the reality of the interaction between Bax and PUMA and whether this interaction (if it actually exists) plays any role in the activation of Bax [59]. Additional experiments are still required to solve this issue. In addition to Puma, BH3-only proteins BimEL and tBid have also been shown to promote Bax conformational change by interacting at the level of α1 [60]. Interestingly, a mutation Leu63Glu in Bax (close to the Lys64 involved in the interaction with Asp33) was able to bypass this activation step, and to overcome the inability of a BH1 mutant Gly108Glu mutant to interact with these 3 BH3-only proteins.

Another poorly resolved issue is whether the interaction between the BH3 domain of Bax and the BH1/BH2 of another Bax is relevant to Bax activation. A recent study suggests that such a dimer can stably exist after the BH1/BH2 loop interacts, with Bim leading to the opening of the structure, and the exposure of Bax-BH3 [61,62]. Furthermore, this interaction favors the movement of α9, that can be blocked by creating an artificial disulfide bond between residues in α9 (Val177Cys) and α5 (Ala112Cys). Interestingly, the mutation Pro168Gly prevented Bax oligomerization, and prevented Bax pro-apoptotic function. This might seem contradictory with observations showing that the mutation Pro168 to Ala or Val activated Bax both in mammalian and yeast cells [38,42]. However, this might indicate that, because of the freedom of movements of Gly, the interactions between α9 and the hydrophobic groove overcome the capacity of α9 to move from its inactive position; such a stabilization might not occur with Ala or Val, forcing α9 to stabilize under an "open" conformation. Whatsoever, the study by Gavathiotis et al. [62] suggests that a simple movement of the loop between α1 and α2 is enough to induce the cascade of events that initiates the pathway leading to full Bax activation.

3. Bax interaction with OMM components

3.1. Bax and VDAC

In healthy cells, wild-type Bax is cytosolic or loosely bound to the OMM. Bax is hardly present in other membrane fractions, such as the ER. When expressed in yeast, full-length and untagged Bax is also mainly localized in the cytosol [37,38]. Conformational changes induced by mutations described above (for example, Pro13 to Gly, Pro168 to Ala, addition of a C-terminal *c-myc* tag, …) trigger the translocation of the protein to the OMM, and not to other membranes, such as the ER [37,38; Renault et al., unpublished data]. It is therefore likely that some OMM components interfere with Bax to provide this selective membrane tropism. Compared to other membranes, the OMM is relatively poor in proteins. Main protein components are the mitochondrial Porin (also termed Voltage-Dependent Anion Channel or VDAC) ([63] for a review) and complexes involved in the translocation of nuclear-encoded mitochondrial proteins to the different mitochondrial compartments: these are essentially the TOM (Translocase of Outer Membrane) and the SAM (Sorting and Assembly Machinery) complexes ([64] for a review). Because of the particular organization of the outer and inner mitochondrial membranes, the contact sites between the two membranes might represent a "hot spot" for Bax-targeting. These contact sites are also rich in VDAC and in ANT (Adenine Nucleotides Translocator), a carrier localized in the inner membrane, that has been tentatively identified as the main component of the so-called mitochondrial Permeability Transition Pore (see below).

Early studies in yeast had shown that VDAC did not participate to Bax-induced release of cytochrome c [65,66]. On the contrary, a study suggested that VDAC was involved in Bax-dependent permeability of the OMM in mammalian cells [67]. This feeded several contradictory papers on the role of VDAC, until definitive arguments against the role of VDAC1 in Bax effects have been published [68,69].

3.2. Bax and Tom22

In 2002, Motz et al. have reported that Bcl-2 could interact with one of the receptors present in the TOM complex, namely Tom20 [70]. This was quite unexpected since the interaction between mitochondria-targeted proteins and TOM generally involves a consensus targeting sequence that is absent from proteins of the Bcl-2 family. A later study, based on a two-hybrid screen in *Escherichia coli* identified Tom22 as a physical partner of Bax [71]. Further investigations showed that Bax and Tom22 also interacted in human cells and that manipulating Tom22 both on isolated mitochondria and in whole cells decreased the addressing of Bax and its capacity to trigger apoptosis [72]. Interestingly, human Bax could also interact with yeast Tom22, despite of the poor sequence conservation between human and yeast Tom22. This suggested that the interaction between Bax and Tom22 was more likely dependent on a general structural organization of the two proteins (that is conserved between human and yeast Tom22) rather than on a selective interaction through specific residues. Another study made in yeast showed that Tom22 was involved in the addressing of human Bax to yeast mitochondria [72]. An *in vivo* study in Drosophila also pointed out the role of DmTom22 in the effect of human Bax expressed in flies [73].

In the same study by Bellot et al. [71], it was reported that the mitochondrial targeting of the mutant of Bax lacking the Ser184, was not dependent on Tom22. As reported above, the deletion of this residue turns the α9 helix of Bax into a genuine transmembrane anchor, that is able to drive the addressing of a reporter protein, such as RFP, while the native α9 helix of Bax is not [28]. This is important since two later studies questionned the role of Tom22 in the mitochondrial localization of Bax, but these two studies were performed with a Bax mutant carrying a mutation on Ser184 (ser184 to Val) [74,75]. Another study questionning the role of Tom22 was based on studies made with a HA-tagged recombinant Bax [76], under conditions where a cleaved version of Bax (p18) was also present [77] that was shown to play the role of a Bax-activating BH3-only protein [78]: consequently, this study did not accurately reflect the behavior of native Bax alone. It should be noted that the role of Tom22 in the mitochondrial localization of Bax might be transient: a Bax mutant carrying point mutations in its α1 helix (see above Section 2.3) was unable to be addressed alone to mitochondria; however, when co-expressed with wild-type Bax, this mutant was targeted to mitochondria, suggesting that membrane Bax could act as a receptor for cytosolic Bax [79].

3.3. Bax and mitochondrial lipids

The role of specific lipids of OMM (or of contact sites between OMM and IMM) in the mitochondrial localization of Bax has also been investigated. In eukaryotic cells, cardiolipin molecules (diphosphatidylglycerol) are specifically localized in the IMM but were also found in OMM fractions, because of contact sites between the two membranes [80–82]. In fact, it has been proposed that cardiolipin molecules could play a crucial role in the stabilization of contact sites, because of their capacity to assemble into inverted micelles (Hexagonal structure H_{II}). The inactivation of the enzyme leading to the synthesis of cardiolipin induces the accumulation of its substrate, phosphatidylglycerol, both in yeast and mammalian mitochondria [83,84]. It is noteworthy that, if phosphatidylglycerol can provide the same surface charge density as cardiolipin (since it is twice as abundant), it is unable to stabilize the H_{II} structure and then cannot help the formation of contact sites [80]. Cardiolipin-containing and phosphatidylglycerol-containing mitochondria therefore have very different structural properties. Human Bax has been expressed in yeast mutant unable to synthesize cardiolipin, without significant changes in the capacity of the protein to be localized to mitochondria and to release cytochrome c, suggesting that cardiolipin does not play a crucial role in the mitochondrial localization of Bax [85]. However, cardiolipin is crucial for the mitochondrial localization of tBid [86], a BH3-only protein that is considered as a major positive regulator of Bax-activation ([87] and see above). The presence of cardiolipin in artificial membranes strongly modulates the binding of cytochrome c [88,89]. Consequently, cardiolipin is crucial for the binding of cytochrome c to the intermembrane face of the IMM [90], and cardiolipin conversion to lysocardiolipin due to the activation of phospholipases A during apoptosis might help the release of cytochrome c [91]. Cardiolipin deficiency has been shown to favor Bax-dependent release of cytochrome c in yeast cells [92], on isolated mammalian mitochondria [93], and in mammalian cells [84]. Further, it has been shown that cardiolipin provided a platform for the activating cleavage of Bid by Caspase 8 [94]. In addition to participating to the activation of Bid and to the destabilization of the interaction of cytochrome c with the IMM, cardiolipin might participate more directly in the effects of Bax: it has been shown recently that cardiolipin was required for Drp1-dependent membrane hemifusion, that would help Bax-oligomerization [95].

These different observations support the view that cardiolipin may indirectly participate to the regulation of Bax-addressing and Bax-activity, through their role in membrane dynamics.

Another family of lipid has recently emerged as a possible regulator of the function of Bcl-2 family. Ceramides are well-established second messengers in a variety of cellular functions, including cell death ([96,97] for reviews). Biochemical studies have suggested that ceramides could form pores of varying size in biological membranes [98] and that Bcl-2 family members could regulate the formation and/or the conductance of these pores [99]. Namely, Bcl-2 was shown to destabilize ceramides channels [100] and, conversely, Bax could stabilize them [101]. In addition, Bak was shown to favor the formation of long-chain ceramides able to form large channels [102]. It is not clear, however, if ceramides participate directly to the targeting of proteins of the Bcl-2 family to membranes. It should be noted that, since ceramides are modulators of the protein kinase AKT which is able to phosphorylate Bax (see below §4.2.), the effect of ceramides on Bax might be indirect.

3.4. Activation of Bax by other members of the Bcl-2 family

The network of interactions between Bcl-2 family members is complex: Fig. 5 is an attempt to list the known interactions converging toward the activation of Bax. The two main partners of Bax are obviously the anti-apoptotic proteins Bcl-2 and Bcl-xL. It is well-established that both Bcl-2 and Bcl-xL inhibit Bax activation. However, after 15 years of intensive studies by many groups, the exact molecular mechanism underlying this inhibition remains debated, with somewhat contradictory observations. The early "rheostat" model assumed that stable heterodimers Bax/Bcl-2 (or Bax/Bcl-xL) could form and prevent, by a simple competing effect, the formation of homodimers (or homooligomers) Bax/Bax, responsible for Bax proapoptotic properties [103]. In this model, heterodimers Bax/Bcl-2 (or Bax/Bcl-xL) are destabilized by BH3-only proteins such as Bad, that compete with Bax for the binding to anti-apoptotic proteins (Fig. 6a). Other BH3-proteins, such as tBid, may eventually help the targeting and/or the oligomerization of Bax in the OMM.

However, this simple view is difficult to conciliate with the different sub-cellular localization of these proteins. Indeed, while inactive Bax is essentially cytosolic in healthy cells, Bcl-2 and Bcl-xL are constitutive membrane proteins, that are mostly (although not exclusively) located in the OMM. Considering that Bax can interact with Bcl-2 and Bcl-xL, it is reasonable to hypothesize that these anti-apoptotic proteins could work as mitochondrial receptors for Bax *en route* toward its active conformation (Fig. 6b). This is somewhat counter-intuitive since Bcl-2 and Bcl-xL are antiapoptotic proteins, but recent evidences suggest that both proteins could help Bax-addressing

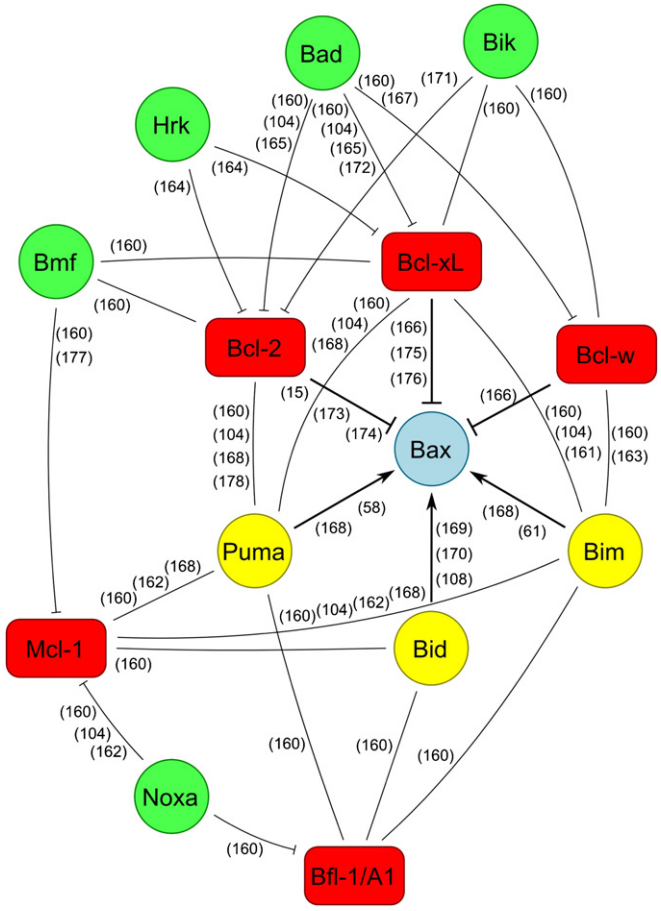

Fig. 5. The Bcl-2 family network. Interactions between Bcl-2 family members and converging toward the activation of Bax. Only interactions depicted by biochemical methods (co-immunoprecipitation, plasmon surface resonnance, functional assays, …) are listed, this excluding indirect methods such as, e.g., two-hybrid assays. When clearly established, the direction and the function (inhibitory or activatory) of the interaction is indicated. Numbers indicate the references where the interactions are described [160–178].

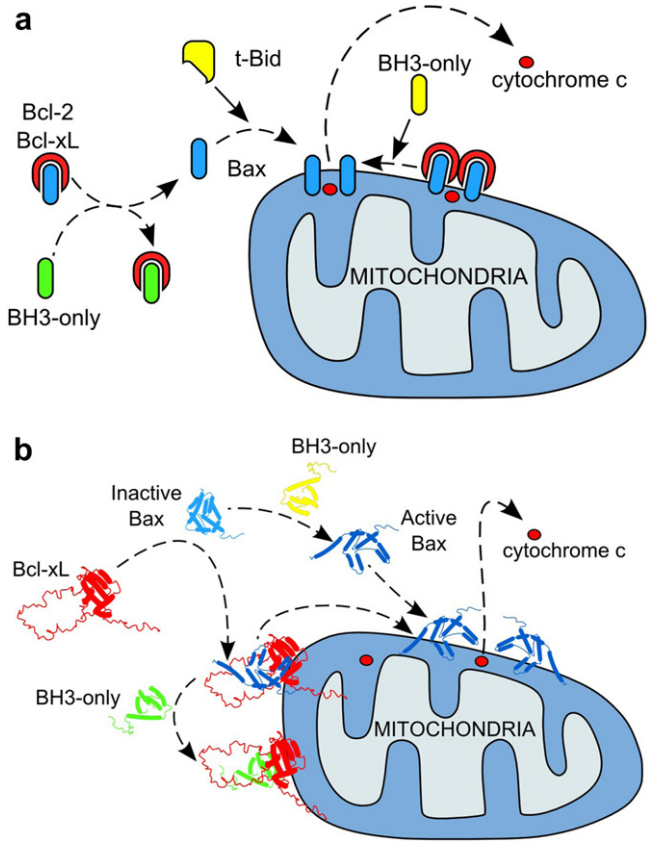

Fig. 6. Models of Bax activation by other Bcl-2 family members. (A) The "classical" model: Bax (blue) is retained away from mitochondria through its interaction with anti-apoptotic proteins (red). Following a death signal, BH3-only inhibitor proteins (such as Bad, green) trap anti-apoptotic proteins, thus inducing the liberation of Bax. BH3-only activator proteins (such as tBid, yellow) help the addressing/insertion/oligomerization of Bax into the OMM. (B) The "priming" model: the interaction between Bax and anti-apoptotic proteins in the OMM allows to "prime" Bax under a pre-active conformation. Once Bax is released, after the interaction of anti-apoptotic proteins with Bad, it is already activated and ready to permeabilize the OMM. Alternatively, Bax can be activated by BH3-only activator proteins, like in the "classical" model. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

under non-apoptotic conditions. Interestingly, this process could "prime" Bax for further activation by an apoptotic signal [104]. For example, it has been shown that BH3-mimetic molecules such as ABT-737 or Terphenyl-K were more efficient to induce apoptosis in cells co-expressing Bax and Bcl-xL than in cells expressing Bax alone [105]. Under physiological conditions, BH3-only proteins that do not interact with Bax, such as Bad, might have the same function, as it was shown for the Bad-BH3 domain.

The other group of proteins helping the addressing of Bax are BH3-only proteins. There is a large consensus about the role of tBid, the active form of Bid, that is able to interact with Bax. Since tBid is constitutively localized in the OMM, it could serve as a "receptor" for Bax, with anti-apoptotic proteins such as Bcl-xL, competing with Bax for this binding [106,107]. Furthermore, the Bax/tBid interaction was shown to favor Bax oligomerization both *in vitro* and *in vivo* [108]. This does not preclude other possible roles of tBid in apoptosis, such as the capacity to rearrange lipids to help the formation of Bax-dependent lipidic pores by itself [109,110].

Another BH3-only proteins, PUMA, has recently emerged as a possible Bax activator. It has been shown that, like tBid, the co-expression of PUMA helped the addressing of Bax and the Bax-dependent release of cytochrome c both in mammalian and yeast mitochondria [43,60]. There are some disagreements in the literature about the further capacity of PUMA to favor Bax-dependent apoptosis [59], that may depend on the presence of other BH3-only proteins [111].

As reported above, Bim has been shown to help the conformational change of the α1/α2 loop of Bax, that seems to be repercuted until the C-terminus of the protein [61,62]. Bim effect thus represents another way to induce Bax conformational changes, distinct from the effect of tBid and, probably, from that of PUMA.

4. Relationships between conformational changes of Bax and interactions with partners

4.1. Cytosolic retention by interacting partners

In non-apoptotic cells, Bax remained under a closed conformation, making it poorly able to interact with other partners. It has been suggested that several proteins were able to retain Bax under this conformation. For example, Ku70 had been proposed to inhibit Bax-activation by preventing the movement associated to the exposure of the epitope 6A7; however, the results showing a direct physical interaction between Bax and Ku70 have been retracted, and it seems that the effect of Ku70 is indirect, by helping Bax ubiquitinylation [112]. A similar capacity to bind and retain Bax in the cytosol has been suggested for other antiapoptotic proteins such as Humanin [113]. Also, it has been shown that 14-3-3ζ was able to interact with Bax through both the N- and the C-terminal ends of the protein [114] However, these proteins require to be overexpressed (that may occur in cancer cells) to counteract efficiently Bax activation, and it is not clear if they participate to Bax inhibition under normal conditions in healthy cells.

4.2. Phosphorylation and dephosphorylation

A large number of evidences have been published, showing that proteins of the Bcl-2 family can be regulated by post-translational modifications, the most common being obviously phosphorylation. A particularly well-described model was proposed for the BH3-only protein Bad [115]. Bad is a strictly indirect activator of apoptosis, that interacts with Bcl-2 and Bcl-xL, preventing them to display their antiapoptotic function. Bad can be phosphorylated by the survival protein-kinase Akt1: this phosphorylation favors another interaction of Bad with 14-3-3 proteins, preventing its interaction with Bcl-2 and Bcl-xL. Bcl-2, Bcl-xL, Bid, Mcl-1,… are all phosphorylatable, but the role of phosphorylations on these proteins remains somewhat elusive ([116] for review). Considering Bax, it was observed that the phosphorylation on Ser184 prevented its translocation to mitochondria, whereas non-phosphorylated Bax was mitochondrial [117]. Site-directed mutagenesis on Ser184 also supported this view, since a Ser184Ala mutant was active while a Ser184Asp was not. Later, the effect of nicotin allowed to draw a correlation between the phosphorylation of Bax and the regulation of Bax-dependent apoptosis [118]. Nicotin was shown to favor Akt-dependent phosphorylation of the Ser184 of Bax and, at the same time, to inhibit Bax-dependent apoptosis. Conversely, it was observed that the dephosphorylation of Phospho-Ser184 by PP2A could lead to Bax activation [119]. As discussed above (Section 2.4), the role of this Ser184 residue might be crucial. It is located in the middle of α9, facing the core of the protein [28]. When it is deleted, the α9 behaves like a true transmembrane anchor; this suggests that the phosphorylation of Ser184 could readily play a role in the way α9 interacts with the OMM.

Another possible target for phosphorylation is Ser163. This residue is the first of the S-X-X-X-S/T motif, that is a consensus site for a phosphorylation by GSK3β (provided the last residue of this

motif is already phosphorylated). Indeed, it has been shown that GSK3β could phosphorylate Ser163, then inducing the activation of Bax [120]. Since GSK3β can be itself phosphorylated, and inactivated, by Akt, it follows that the survival kinase Akt would inactivate Bax through two different modes: directly by phosphorylating Ser184, and indirectly by preventing the phosphorylation of Ser163.

Phosphorylations on Ser163 and Ser184 may participate to the regulation of Bax conformational changes ([39]; Renault et al., unpublished data). The localization of Ser184 in the middle of α9 and facing the BH3 might be crucial for displacing this helix. As shown above (Section 2.4), a mutation Thr174 to Asp, that introduces a negative charge in front of another negative charge (Glu69), strongly stimulates Bax addressing, insertion and capacity to release cytochrome c when expressed in yeast cells (*i.e.* in the absence of any other Bcl-2 family members) [38]. As this indicates that Bax is activated when α9 is forced to move away from BH3, it would be expected that the phosphorylation of Ser184 introduced a size constraint that would also help the movement of α9: however, the phosphorylation prevents the targeting of Bax. The substitution Ser184 to Asp, that introduces a negative charge but a moderate size constraint does not lead to an increase of the addressing of Bax (as compared to the wild-type protein) but induced an increase of its activity: this indicates that any movement of α9 favors a stabilization of Bax under an active conformation, but does not necessarily lead to an increase of membrane targeting. This suggests that there is not an uniquivocal link between the addressing of Bax and its activity. Another example of this distinction between Bax addressing and activity has been provided by the effect of kinase p38MapK. During anoikis, Bax is translocated to the OMM but is not activated until Bax is phosphorylated by this kinase [121]; however the residue targeted by p38MapK was not determined in this study.

The phosphorylation of Ser163 was shown to stimulate Bax-dependent apoptosis [120]. When expressed in yeast (in the absence of any other Bcl-2 family members), the phosphomimetic mutant Ser163 to Asp did not exhibit any significant increase of mitochondrial localization and activity, as compared to Bax wild-type. However, when associated to a double-mutant Pro168Ala/Ser60Ala that is inactive but more subject to conformational changes (since the helix α9 is more susceptible to move away from the hydrophobic groove), the mutation Ser163Asp induced a strong stimulation of both addressing and activity of Bax. This suggests that the phosphorylation of Ser163 is not able, alone, to stimulate Bax, but may participate to the stimulation once other conformational changes (for example, the movement of α9) have occurred [38]. It may be noted that the Ser163 residue is relatively close to the Arg9/Asp154 couple that may be involved in the stability of ART (Fig. 3b). The introduction of the negative charge of phosphate on Ser163 might therefore help the movement of the N-terminal end of Bax.

PKC isoforms are also able to regulate Bax. PKCζ was shown to phosphorylate Bax on Ser184, like Akt [122]. When co-expressed in yeast with Bax, human PKCα increased Bax content, suggesting that it was able to stabilize Bax [123]. However, this later effect did not seem to entirely depend on the kinase activity. Other residues of Bax might be subjected to phosphorylations: for example, the Ser60 residue is located in close proximity to the Asp33/Lys64 couple, that was shown to be involved in the maintenance of the geometry of α1 and α2 (Section 2.3). Ser60 is part of a Lys-Lys-Leu-Ser sequence that might be recognized by PKA. A mutant Ser60Ala can be addressed to OMM in yeast but cannot be activated, even when associated to activating mutations such as Pro168Ala or Pro13Gly. It can be hypothesized that a putative phosphorylation of Ser60 would help the separation of α1 and α2 by destabilizing a salt bridge between Asp33 and Lys64, but such a phosphorylation has not been yet evidenced to date.

5. Bax-dependent permeabilization of the OMM

5.1. Bax alone is able to induce the release of cytochrome c

The discovery that mitochondria were able to release apoptogenic factors during apoptosis was a major breakthrough in the understanding of the regulation of this process. Among these factors, the release of cytochrome c has been intensively studied, since this protein was already well-known for its function in the mitochondrial electron transport chain. The fact that cytochrome c was released from mitochondria and that this release was a crucial step of apoptosis was established in 1996 by two groups [8,9]. At the same time, structural studies on Bcl-2 family proteins suggested that these proteins could form pores, in a similar way as some bacterial toxins [124,125]. More specifically, it had been shown that Bax was forming channels that could be inhibited by Bcl-2 [46]. It was therefore tempting to hypothesize that Bcl-2 family proteins, and namely proapoptotic proteins Bax and Bak, could be directly responsible for the release of cytochrome c from mitochondria. The heterologous expression of Bax in yeast, in the absence of any apoptotic network, indeed established that Bax alone was able to induce the release of cytochrome c from mitochondria [126], and this observation was further confirmed in mammalian cells [127].

5.2. The permeability transition pore

Simultaneously, an alternative hypothesis suggested that Bcl-2 family members could also regulate a pre-existing mitochondrial pore, the Permeability Transition Pore. This system was functionally described since the mid-seventies as the capacity of isolated mitochondria placed under peculiar conditions (high concentrations of Ca^{2+}, oxidant conditions, …) to initiate the formation of a large-sized pore able to release molecules having a size below 1500 Da ([128] for review). The collapse of bioenergetic properties following the opening of this pore induces a swelling of the mitochondrial matrix that may ultimately lead to a rupture of the OMM. It had been proposed that the pore was negatively regulated by antiapoptotic proteins and that proapoptotic proteins could relieve this inhibition by trapping antiapoptotic proteins [129]. This model was obviously contradictory with the observations that Bax alone was able to induce the release of cytochrome c, including from artificial vesicles devoid of any mitochondrial components. An additional complexity was introduced through reports that putative components of the pore, such as the Adenine Nucleotide Translocator (ANT), the mitochondrial Porin, and the Cyclophilin D, were required for apoptosis and that Bax was able to interact with ANT [130]. However, the roles of ANT and of Porin in the pore formation were severely questionned following studies of the effects of Bax in yeast mutants deleted of these proteins [64,65,131,132] and, even more, after studies on apoptosis in mammalian cells where the genes encoding these proteins had been knocked-out [133,134]. Furthermore, alterations of the regulation of Pore in Cyclophilin D-less mice did not have any consequence on Bax-dependent apoptosis, but were required for necrosis, the other major form of cell death [135,136]. Although there is still some debates on the implication of the pore in the late apoptotic events (sometimes called “secondary necrosis”), it now appears that Bcl-2 family members are able to regulate the permeability of OMM to apoptogenic factors, independently from the presence of any pre-existing pore.

5.3. Bax channels and MAC

The first evidences that Bcl-2 family members, namely Bax, were indeed able to permeabilize membranes were obtained with purified proteins reconstituted in liposomes or in black lipid bilayers

[46]. However, the size of the channels that were formed under these conditions did not correlate with the hypothesis that a protein like cytochrome c (12.5 kDa) could go through these channels. It may be noted that, at the time, the technique of production of recombinant Bcl-2 family members did not permit the synthesis of full-length proteins; consequently, these experiments were done with proteins devoid of the C-terminal hydrophobic α-helix.

A major advance was obtained in 2001, when electrophysiological studies on OMM reconstituted in liposomes demonstrated that apoptotic cells contained a large channel, called MAC, that was not active in the presence of antiapoptotic protein Bcl-2 [137]. Furthermore, an identical channel was observed in the OMM of yeast cells expressing human Bax (activated by the introduction of a C-terminal *c-myc* tag). This was the demonstration that Bax was indeed responsible for the formation of a large channel having a size compatible with the release of cytochrome c. Further studies demonstrated that the ionic conductance of MAC was decreased in the presence of cytochrome c (12.5 kDa) or of RNase (14 kDa), but not in the presence of haemoglobin (68 kDa), showing that MAC has an upper limit size [138]. Also cytochrome c has no effect on the conductance of other OMM channels, namely VDAC, confirming that MAC is a *de novo* channel, independent from pre-existing structures. From comparison of the properties of MAC with those of channels created by recombinant Bax, it has been calculated that MAC contained about 10 monomers of Bax [139].

It is still unknown whether MAC is the only system responsible for the release of all apoptogenic factors, or if it is specific for cytochrome c. Many data suggest that apoptogenic factors are not released alltogether but sequentially; however, the sequence of events is different from study to study. For example, cytochrome c was released alone before smac/diablo and AIF in the experiments reported by Adrain et al. [140], but cytochrome c and smac/diablo were released together and before AIF in the study by Arnoult et al. [141]. However, in general, small proteins like cytochrome c and smac/diablo are released before bigger proteins like endonuclease G and AIF. This would agree with the view that MAC may increase in size with time, but also that MAC is only responsible for the release of small proteins while another later process (PTP?) would be responsible for the release of bigger proteins. It should be noted that some reports have been confusing since they used fusion proteins like cytochrome c-GFP (see e.g. [142]), that has been shown to behave more like a big protein than like native cytochrome c [143]. More precise investigations based on careful measurement of the kinetics of release for each apoptogenic factor are required to resolve this issue.

5.4. Bax and lipidic pores

As suggested above (Section 3.3), the interaction of Bax with membranes might favor a reorganization of lipid molecules that would favor the formation of lipidic pores. It has been shown that curvature changes induced by lysophospholipids helped the formation of Bax-dependent pore [144,145].

Ceramides, which are produced following the activation of death-signalling pathways, are naturally able to form pores in lipid bilayer and natural membranes. It has been established that the anti-apoptotic protein Bcl-2 was able to inhibit the formation of these pores, although the molecular mechanism underlying this inhibition remains unknown. Conversely, a recent work suggested that oligomerized Bax was able to stabilize large ceramides channels having a size sufficient for the passage of cytochrome c, thus providing a link between the “ceramides pore hypothesis” and the requirement of Bax oligomerization for membrane permeabilization [106].

5.5. Bax and the fusion/fission machinery

Mitochondria are dynamic organelles that can form a complex filamentous network, or a collection of apparently disconnected dots ([146] for review). The regulation between these two forms is still a matter of debate, and is out of the scope of this discussion ([147–149] for reviews). However, it has been established that the mitochondrial step of apoptosis is generally associated to a disruption of the network, but whether this disruption actually has a function in apoptosis or is a consequence of it remains an open question [150]. Indeed, the studies of the consequences of the manipulation of the mitochondrial fission/fusion machinery on apoptosis led to contradictory reports: inhibiting fission generally delayed apoptosis [151], but a clear correlation with an effect on the release of apoptogenic factors could not be definitely established [152]. It has even been observed that preventing mitochondrial fission by altering two different genes led to opposite effects on cell death [153,154]. The model gained even more complexity recently when it was reported that, in non-apoptotic cells, Bax and Bak could be regulators of the fusion machinery [155–157], a function that was obviously lost in apoptotic cells.

6. Conclusion

Since it has been identified as a homolog of Bcl-2 in 1993, Bax has been the subject of many biochemical studies aiming at identifying its function at a molecular level. It is now ascertained that Bax plays a key role in apoptosis by acting on mitochondria permeability. However, the regulation of this process remains poorly defined. Early hypotheses often implied that Bax-addressing and further activation were spontaneous processes, and that cell survival relied on the capacity of antiapoptotic Bcl-2 family members or other proteins to block them. Consequently, modulation of apoptosis could be achieved more easily by acting on anti-apoptotic proteins rather than Bax (or its closely related homolog Bak). This view is certainly not completely erroneous, since alterations of apoptosis, namely in cancer cells, are often associated to the overexpression of antiapoptotic proteins Bcl-2 and Bcl-xL. However, this is incomplete. During the last 10 years, the proapoptotic function of BH3-only proteins tBid, Bim and Puma has been found to rely, at least partly, on direct Bax activation. Even more recently, the possible role of antiapoptotic proteins Bcl-2 and Bcl-xL in “priming” Bax for further activation is more and more studied.

One of the major challenge for a complete description of Bax function is to determine its active structure. The avaibility of a NMR structure of soluble (inactive) Bax [28] has been a major step toward the understanding of molecular changes underlying Bax activation: it has provided the investigators a molecular support for structure/function studies. However, 10 years later, it is still not known how Bax permeabilize the OMM. Of course, our knowledge of this mechanism has expanded: the hypothesis that Bax was a simple positive effector of the mPTP has progressively been ruled out, and replaced by hypotheses based on the direct pore-forming activity of Bax. But the exact structure of this pore remains elusive: Bax oligomers probably form the core of these pores, but it is still not elucidated whether Bax oligomers themselves would form the “walls” of the pores or whether other components (proteins or lipids) are reorganized around Bax oligomers to form pores. It should be noted that, since pores of different size have been observed, the two hypotheses are not mutually exclusive. Structural data on active Bax are now required for a better understanding of how Bax is forming a pore. Since active Bax is, of course, a membrane protein, the purification of Bax oligomers from apoptotic cells, would be challenging. Recombinant Bax produced in bacteria would be a good alternative,

but how to ensure that Bax is under an active conformation? As shown in this non-exhaustive review, several constitutively active mutants of Bax have been characterized by us and others. Production, purification and structural studies of these mutants are an exciting route for further understanding of Bax function.

In addition to the underestimation of Bax function as a target of regulatory pathway, another factor has probably contributed to the relatively low number of studies on Bax function (as compared to other proteins involved in apoptosis): opposite to antiapoptotic proteins, Bax is not conserved in simplified models [22]. Indeed, the worm *C.elegans* has clear homologs of antiapoptotic proteins (*ced9*) and of BH3-only proteins (*Egl1*) but not of Bax. The interaction between *Ced9* and *Egl1* is sufficient to relieve the inhibitory effect of *Ced9* on the downstream apoptotic pathway formed by *Ced4* and *Ced3*. The fly *Drosophila* contains a protein, called *Debcl* that has been considered as a Bax homolog. Interestingly, *Debcl* is required for the proapoptotic function of mammalian Bax heterologously expressed in *Drosophila*; however, *Debcl* does not appear to be a strict Bax homolog since it does not promote, by itself, any alteration of the permeability of the OMM. It is noteworthy that, in these two apoptotic models where Bax is absent, the permeabilization of the OMM, namely to cytochrome c, is not a required event for the setting up of apoptosis, although mitochondria are still required ([158,159] for reviews): this suggests that the appearance of Bax in the course of evolution would be closely linked to the involvement of the release of mitochondrial apoptogenic factors, namely cytochrome c, in apoptosis.

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