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Multistep and multitask Bax activation

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ABSTRACT

Bax is a pro-apoptotic protein allowing apoptosis to occur through the intrinsic, damage-induced pathway, and amplifying that one occurring *via* the extrinsic, receptor mediated pathway. Bax is present in viable cells and activated by pro-apoptotic stimuli. Activation implies structural changes, consisting of exposure of the N terminus and hydrophobic domains; changes in localization, consisting in migration from cytosol to mitochondria and endoplasmic reticulum membranes; changes in the aggregation status, from monomer to dimer and multimer. Bax has multiple critical domains, namely the N terminus exposed after activation; two hydrophobic stretches exposed for membrane anchorage; two reactive cysteines allowing multimerization; the BH3 domain for interactions with the Bcl-2 family members; alpha helix 1 for t-Bid interaction. Bax has also multiple functions: it releases different mitochondrial factors such as cytochrome c, SMAC/diablo; it regulates mitochondrial fission, the mitochondrial permeability transition pore; it promotes Ca^{2+} leakage through ER membrane. Altogether, Bax activation is a complex multi-step phenomenon. Here, we analyze these events as logically separable or alternative steps, attempting to assess their role, timing and reciprocal relation.

1. Introduction

1.1. Historical perspective

In 1993 a protein cognate of Bcl-2 with pro-apoptotic functions (Oltvai et al., 1993) was identified (Bcl-2 Homolog X, Bax); it soon became evident that the molar ratio between Bax and the anti-apoptotic Bcl-2 was the main molecular switch between apoptosis and survival (Korsmeyer et al., 1993) to a given insult.

The mechanisms through which apoptosis is favored by Bax remained obscure until much later, when it was found that Bax translocates to mitochondria in reconstituted sub-cellular systems as well as in whole cells undergoing apoptosis (Wolter et al., 1997). Later, it was shown that the pro-apoptotic activity of mitochondrial Bax consists of forming/favoring membrane protein channels (Schlesinger et al., 1997) allowing release of pro-apoptotic factors such as cytochrome c (Jurgensmeier et al., 1998) and SMAC/diablo (Deng et al., 2002) thereby activating the caspase cascade (Kirsch et al., 1999).

The major anti-apoptotic function of Bcl-2 was then clarified as that to heterodimerize with Bax, preventing oligomerization and pore assembly (Yang et al., 1997).

1.2. Mitochondria permeabilization

The role of mitochondria as principal crossroad of the apoptotic process had emerged since the mid '90s, when it was shown that mitochondria of apoptosing cells lose their inter-membrane potential (Shidoji et al., 1997) and that cytochrome c is released from mitochondria to cytosol acquiring pro-apoptotic functions (Liu et al., 1996) Both phenomena (Hirsch et al., 1997) were attributed to the permeability transition pore (PTP), a multi-ion channel that opens during mitochondrial stress (Bernardi et al., 1992). Soon topological features and size concerns questioned cytochrome c release *via* PTP. A channel linking the inter-membrane mitochondrial space to the cytosol was sought to explain release of cytochrome c. The term mitochondrial outer membrane permeabilization (MOMP) was coined (Green and Kroemer, 2004), which indicates release of inter-membrane proteins rather than ion passage. Indeed in healthy cells, the presence of porin channels in the outer mitochondrial membrane constitutively allows osmotic equilibrium between cytosol and trans-membrane mitochondrial space (Benz, 1985). During PTP instead, ionic communication between the mitochondrial matrix and the cytosol, which is normally banned, is established; this process is also referred to as MIMP (mitochondrial inner membrane permeabilization).

Abbreviations: AIF, apoptosis inducing factor; ANT, adenine nucleotide translocator; AP-1, activator protein-1; Bax, Bcl-2 homolog X; Bcl-2, B-cell lymphoma-2; BH, Bcl-2-homology; DISC, death inducing signaling complex; ER, endoplasmic reticulum; IAP, inhibitor of apoptosis proteins; IP3r, inositol-3-phosphate receptors; JNK, c-Jun N-terminal kinase; MAC, mitochondrial apoptosis channels; MIMP, mitochondrial inner membrane permeabilization; MOMP, mitochondrial outer membrane permeabilization; PTP, permeability transition pore; t-Bid, truncated Bid.

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1.3. Bcl-2 family

After Bax identification, a large number of proteins of the Bcl-2 family have been described. They all belong to three sub-families according to the number of Bcl-2-Homology (BH) domains present in these proteins (Kelekar and Thompson, 1998). Members possessing 3 domains (BH1 to 3) such as Bax and Bak exert a pro-apoptotic function, whereas those expressing 4 domains (BH1 to 4), such as Bcl-2, Bcl-Xl are anti-apoptotic (another anti-apoptotic member, Mcl-1 has a different anti-apoptotic domain (Day et al., 2005)). The BH3-only group consists of proteins possessing only one (BH3) of the BH domains (Giam et al., 2008), and exerting regulatory roles.

The proteins of this family are only partially redundant, and behave as a cohort, establishing interactions *via* the BH3 domain (Kelekar and Thompson, 1998). Any of the anti-apoptotic proteins can inhibit Bax or Bak, even though some preferences have been reported, e.g., Bak is the historic inter-actor of Bcl-Xl (Sattler et al., 1997). The final result is the finely tuned regulation of Bax (or Bak) activation.

The pro-apoptotic action of the BH1-3 proteins is the promotion of MOMP, whereas the anti-apoptotic activity of the BH1-4 counterparts consists in binding to the pro-apoptotic members, inhibiting their membrane pore-forming ability *via* the BH4 domain. Interestingly, Bcl-2 can naturally turn into a pro-apoptotic protein upon elimination of the BH4 domain (Cheng et al., 1997; Kirsch et al., 1999).

The BH3-only proteins regulate Bax activation *via* a complex pattern of interactions with the pro- or the anti-apoptotic members. They belong to two distinct functional groups of activators or sensitizers of apoptosis. Though all ultimately lead to the activation of Bax (or Bak), the activators (i.e., Bid (Desagher et al., 1999), Bim (Czabotar et al., 2009)) directly activate Bax (or Bak) through physical binding, whereas the sensitizers bind to Bcl-2 (or another anti-apoptotic member) displacing and liberating Bax (Antignani and Youle, 2006) thus stoichiometrically inhibiting the anti-apoptotic action of the anti-apoptotic family members. Each of the BH3-only proteins has its peculiar mechanism of activation, including up-regulation, molecular activation by proteolysis, liberation from cytoskeleton sequestration and phosphorylation. Altogether, these proteins provide multiple sensors of cell alterations that all converge into Bax activation.

1.4. Apoptotic pathways

The commitment phase of apoptosis consists of signal transduction steps occurring prior to the cellular dismantling by caspases; it occurs *via* multiple pathways, and the extrinsic and the intrinsic cell death pathway are the ones better characterized in molecular terms (Fulda and Debatin, 2006).

The term “extrinsic” evokes signaling from the extracellular milieu, consisting of cell-to-cell or ligand-receptor-mediated interactions. The prototypical extrinsic pathway is induced by Fas ligand, which trimerizes and stimulates the death receptor to form a multi-protein complex (Death Inducing Signaling Complex, DISC) recruiting and activating the upstream caspase 8 (Wajant, 2002).

The intrinsic pathway is instead activated by internal sensors of damage or physico-chemical alterations produced by cell stress, which activate Bax to translocate to mitochondria and release cytochrome c. Once in the cytosol, cytochrome c nucleates the assembly of a multi-protein complex, the apoptosome, functionally analog to the DISC, which recruits and activates the other upstream caspase 9 (Zou et al., 1997). Caspase 8 and caspase 9 converge into the proteolytic activation of caspase 3, leading to the execution phase of apoptosis and cell dismantling.

Molecular cross talks between the two pathways create amplification loops that allow or speed up finalization of the apoptotic process. It was observed that upon Fas stimulation, finalization of apoptosis through caspase 8–caspase 3 activation occurred only in some cells (type I cells), whereas other (type II) cells required recruitment of mitochondria to activate caspase 3 (Scaffidi et al., 1998, 1999). The molecular mechanisms of such differences include the proteolytic activation of Bid by caspase 8, which produces truncated Bid (t-Bid), a potent activator of Bax (Li et al., 1998) and the consequent intrinsic mitochondrial pathway. Summarizing, Bax acts as the initiator of the intrinsic, and also as the amplifier of the extrinsic pathway (Fig. 1).

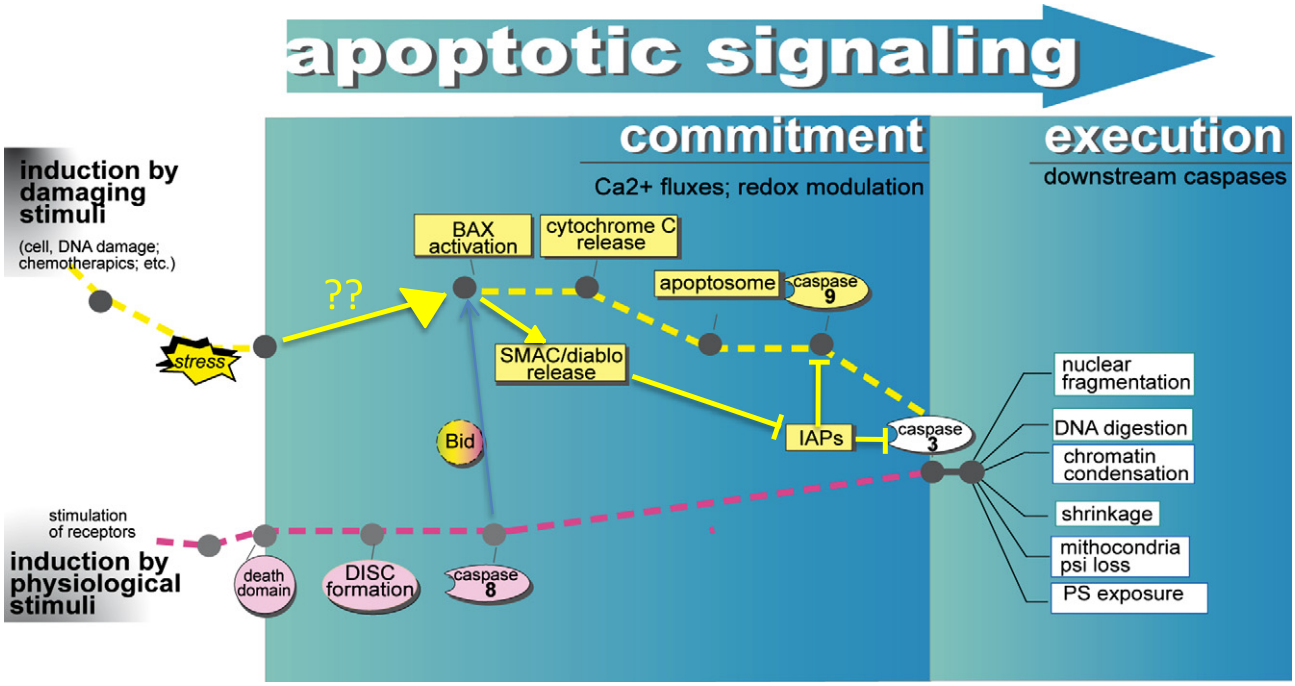

Fig. 1. Apoptotic Pathways. Schematic representation of the intrinsic (yellow) and extrinsic (pink) apoptotic pathways. Bax triggers the intrinsic pathway by releasing cytochrome c; it reinforces the extrinsic pathway when caspase 8 generates t-Bid; it allows to bypass the IAPs-induced caspase inhibition by releasing SMAC/diablo (that inhibits IAPs) thus restoring caspase function and apoptosis. The mechanisms of Bax activation in response to cell damage and physico-chemical alterations still need further clarifications.

The expression of a set of proteins called Inhibitor of Apoptosis Proteins (IAP) (Deveraux and Reed, 1999) tightly controls apoptosis, especially in tumor cells (LaCasse et al., 2008). IAPs possess ubiquitin-ligase activity that leads to the degradation of mature caspase 3 and 9 (Yang and Li, 2000), thus blocking both apoptotic pathways. The inhibition of apoptosis *via* IAPs can be overridden by SMAC/diablo, a protein that inhibits the functions of IAPs (Du et al., 2000). Then, caspase 3 and 9 are liberated, allowing apoptosis. Interestingly, SMAC/diablo is a mitochondrial protein in healthy cells, which is released during apoptosis through Bax channels (Deng et al., 2002). This observation highlights an additional function of Bax: allowing finalization of both intrinsic and extrinsic pathways bypassing the blockage *via* IAPs. The apoptotic pathways are illustrated in Fig. 1.

1.5. Bax activation

Under some circumstances, pro-apoptotic stimuli promote c-Jun N-Terminal kinase (JNK) activator protein (AP)-1/p53 regulated signal transduction pathways; these transcription factor families up-regulate the Bax promoter, leading to protein-synthesis-dependent apoptosis by increasing Bax levels and the Bax/Bcl-2 ratio (Roos and Kaina, 2006). However, apoptotic stimuli typically activate, rather than up-regulate Bax protein. Bax is present in the cytosol of viable cells, kept silent by chaperones like Ku70 (Mancinelli et al., 2006) and 14-3-3 (Nomura et al., 2003). Apoptotic stimuli liberate Bax *via* acetylation of Ku70 (Cohen et al., 2004) or JNK-dependent phosphorylation of 14-3-3 (Tsuruta et al., 2004). Bax liberation is necessary but not sufficient for activation, and specific additional events are required.

Bax can be activated by various stimuli, through specific mechanisms that target different domains of the protein, and may lead to different final results. These complex phenomena are the main topic of this review and will be discussed in detail here.

2. The functions of Bax

2.1. Mitochondria fission

Mitochondria dynamics consists of coordinated fission and fusion events that regulate the mitochondrial network in living cells (Polyakov et al., 2003). During apoptosis, the mitochondrial network collapses, due to excess of fission and inhibition of fusion (Polyakov et al., 2003). Bax is strongly implicated in this phenomenon; it is present at fission sites in apoptosis (Karbowski et al., 2002); its over-expression or re-introduction into Bax null cells accelerates (Scott and Youle, 2010) mitochondrial collapse, and activated Bax in apoptosis binds to proteins of the mitochondrial fission machinery (Suen et al., 2008). Kinetics and spatial evidences link mitochondrial fission in apoptosis with the release of cytochrome c, but there is no consensus as to whether these events are causally linked; in fact, recent evidences dissociate the two phenomena, suggesting that they are due to different Bax functions (Parone et al., 2006; Sheridan et al., 2008). An unsolved question is whether or not the low amounts of active Bax that are often detectable in healthy cells may play a role in the physiological events of mitochondria fission of viable cells (Autret and Martin, 2009), or if Bax intervention leads to an irreversible fission cascade, mitochondria collapse and cell death.

2.2. Release of mitochondrial factors

Activated Bax typically promotes apoptosis by allowing the release of cytochrome c, SMAC/diablo, omi, endo G or Apoptosis Inducing Factor (AIF) from mitochondria.

Cytochrome c is a ~15 kD protein acting in healthy cells as an intermediate of the electron transport chain, bound *via* cardiolipin to the outer face of the internal mitochondrial membrane, mostly trapped within the cristae, structures that depend on multimeric OPA1 complexes to preserve the functional closed structure (Yamaguchi and Perkins, 2009). Accordingly, at least three events must take place to allow export from mitochondria (Pellegrini and Scorrano, 2007). Cytochrome c must be freed from cardiolipin anchorage; cristae junctions must be opened; and Bax pores must form through which cytochrome c may translocate to cytosol. In cell-free experiments, Bax addition to mitochondria is sufficient to trigger cytochrome c release (Kuwana et al., 2002), implying that not only a pore has formed, but also that cardiolipin anchorage is lost, and cristae junctions opened. Bax plays a key role in pore formation, and the details of Bax pores in the outer mitochondrial membrane will be discussed later. Strong evidence indicate that Bax may be responsible also for cristae loosening; indeed, Bax was found able to disassemble OPA1 complexes, thus creating a spatial continuity between cristae and the inter-membrane space required for cytochrome c release; loosening of the cristae structure is achieved independently on pore formation, and requires an intact BH3 domain (Yamaguchi et al., 2008).

Bax may also be involved in breaking cardiolipin anchorage, which is sensitive to high Ca^{2+} (Peng and Jou, 2010). Indeed mitochondria are juxtaposed to endoplasmic reticulum (ER), especially close to areas rich in inositol-3-phosphate receptors (IP3r), (Rizzuto et al., 1998) and take up much of the IP3-induced Ca^{2+} effluxes (Landolfi et al., 1998), when present in ER membranes (Zong et al., 2003), Bax increases the extent of such effluxes, promoting very high Ca^{2+} levels in mitochondrial micro-domains (Wiswedel et al., 2010), compatible with a disturbance of cardiolipin anchorage.

SMAC/diablo is a mitochondrial dimer of about 40 kD (Verhagen et al., 2000). It is released into the cytosol upon apoptogenic stimuli through Bax pores (Du et al., 2000), and has the function of liberating active caspases when they are inhibited by IAPs expression (Du et al., 2000). Since SMAC/diablo floats in the mitochondrial inter-membrane space (Verhagen et al., 2000), the presence of Bax pores is sufficient to allow its migration to the cytosol. The mechanisms of release of omi as well as its functions once in the cytosol are quite similar to SMAC/diablo, also sharing homology for IAPs (Hegde et al., 2002).

Cytochrome c and SMAC/diablo are released independently during apoptosis despite the fact that both require Bax (Kandasamy et al., 2003): many cells release only cytochrome c (Lim et al., 2006) or only SMAC (Deng et al., 2003), or both (Rehm et al., 2003; Usuda et al., 2002); in the last instance, they may be released with different kinetics (Gorka et al., 2004). This, together with the different size and mitochondrial steady state location of the two proteins, leads to believe that they are released by different mechanisms.

The scenario is different for AIF release. AIF is a large protein located in the inter-membrane space, tightly bound to the inner mitochondrial membrane (Du et al., 2000). Some studies report requirement of caspase activation (Arnoult et al., 2003) or other proteolytic events (Norberg et al., 2010) to break anchorage and allow release. AIF possibly leaks through outer membrane ruptures following PTP, and Bax may be involved (Scharstuhl et al., 2009) *via* its amplification effects of PTP via VDAC binding. Once in the cytosol, AIF elicits a caspase-independent apoptotic mechanism leading nevertheless to typical apoptotic features. Endo G is an endonuclease that is released from the mitochondrial inter-membrane space with similar kinetics (Arnoult et al., 2003), possibly providing the DNAse function during AIF-induced apoptosis.

2.3. Bax at the endoplasmic reticulum membrane

The ER membrane is a major Bcl-2 localization in healthy cells. This protein acts as an anti-apoptotic protein interfering with stimuli leading to ER Ca^{2+} depletion, thus helping to keep the luminal Ca^{2+} concentration at physiological levels (Lam et al.,

1994). Bax translocates to the ER membrane after apoptogenic stimuli (Zong et al., 2003) causing a decrease in ER luminal Ca^{2+} (Wang et al., 2010), and exerting a complex pro-apoptotic regulatory activity (Nutt et al., 2002a) thus maintaining its antithetic role with Bcl-2 also in the control of Ca^{2+} mobilization (Chami et al., 2004). In lipid vesicles, Bax channel formation allows slight anion, but no cation, passage (Schlesinger et al., 1997), indicating that Bax-induced decrease of ER Ca^{2+} is hardly attributable to ionic Bax pores. Instead, strong evidence indicates that Bax and Bcl-2 act on the IP3 receptor, by controlling its phosphorylation state and hence its functions (Oakes et al., 2005). In fact, Bcl-2 physically interacts with IP3r (Rong et al., 2009), reducing its activation in response to IP3 challenge (Chen et al., 2004); in the presence of Bax or Bak, this interaction is loosened, suggesting that in this instance Bax may interact with, and sequester, Bcl-2, thus interfering with its pro-survival effect at the ER level (Scorrano et al., 2003). Bax-mediated promotion of IP3-mediated efflux increases Ca^{2+} concentration of vicinal mitochondria, favoring PTP and cardiolipin oxidation and promoting cytochrome c release (Wiswedel et al., 2010). Interestingly, the released cytochrome c may physically interact with IP3r, and this prevents closure of the IP3 channel after the initial Ca^{2+} efflux, thus transforming a transient into a sustained efflux (Boehning et al., 2003). Altogether, these events stimulate further cytochrome c release, creating a feed-forward loop that amplifies the initial signal (Nutt et al., 2002b).

The Bcl-2 family plays an additional apoptotic control function at the ER membrane; Bcl-2 promotes a slight ER Ca^{2+} decrease, whereas Bax favors Ca^{2+} intake from cytosol (Chami et al., 2004). Though apparently contradictory with previous findings, this indicates a potential of a highly Ca^{2+} charged ER to promote apoptosis, whereas a partially emptied ER blunts the apoptotic signal (Cerella et al., 2007; Chami et al., 2004). The Bax domain required for this ER functions does not involve the alpha5/alpha6 putative mitochondria pore-forming domain, thus possibly individuating two different Bax pro-apoptotic regions (Chami et al., 2004).

Very recently, it was shown that Bax translocation to ER may occur *via* t-Bid activation, which results in Bcl-Xl-sensitive pore formation and release of ER luminal proteins (Wang et al., 2010). These observations suggest a Bcl-2 family interplay (and functions) in the ER analogous to what occurs in mitochondria.

3. Bax pores

3.1. Cooperation with the mitochondrial permeability transition pore

Stress conditions such as Ca^{2+} overload or oxidative stress promote the interaction between the inner mitochondrial membrane complex adenine nucleotide translocator (ANT) (Belzacq et al., 2002) and the outer mitochondrial membrane complex voltage-dependent anion channels (VDAC, also known as porin (De Pinto and Palmieri, 1992)), leading to the formation of PTP, also known as mega-channel, which spans the double mitochondrial membrane (Szabo and Zoratti, 1992). PTP-dependent cytochrome c release was historically the first mechanism proposed (Marchetti et al., 1996). In fact this release cannot occur as a simple passage, because PTP spans the two membranes, creating communications between cytosol and the mitochondrial matrix, but not with the inter-membrane space, where cytochrome c resides. Moreover, molecules larger than 1.5 kD cannot pass through PTP (Bernardi et al., 1994). The current view is that cytochrome *c* release *via* PTP occurs by indirect mechanisms. PTP may produce matrix swelling due to ions and solutes intake. Subsequent ruptures of the outer mitochondrial membrane then causes a generalized leakage of inter-membrane proteins including cytochrome *c* (Scarlett and Murphy, 1997). In this way, PTP may allow cytochrome *c* (and other factors) to leak rather than be specifically released.

Bax can promote PTP: in cell-free systems, low doses of purified Bax directly activate PTP and mitochondrial protein release; at higher Bax doses, mitochondrial swelling also occurs (Pastorino et al., 1999). Such effects of Bax on mitochondria can be prevented by the PTP inhibitor cyclosporin A (18771651).

PTP may also help Bax pore-forming activity (Narita et al., 1998): it has been reported that whenever PTP is open, Bax recruitment from the cytosol to the mitochondrial membrane is facilitated (Precht et al., 2005); in addition, PTP facilitates the acquisition of the correct pore-forming supra-molecular assembly of membrane-bound Bax (De Giorgi et al., 2002).

3.2. Cooperation with VDAC/porin

VDAC (or porin) is the major protein of the outer mitochondrial membrane, forming pores that allow passage of molecules <5 kD and ensure the uptake of cytosolic molecules for mitochondrial functions and ionic communication with the cytosol (De Pinto and Palmieri, 1992). VDAC pore is regulated by physico-chemical mechanisms such as voltage, which is maintained by trans-membrane potential, and by molecular mechanisms including phosphorylation and binding by cytosolic proteins (Chen et al., 2010; Gincel et al., 2001). An important regulatory function is exerted by hexokinase (Arzoine et al., 2009); the Bcl-2 family exert complex effects: the BH4 domain of the anti-apoptotic members behaves as an inhibitor (Shimizu et al., 2000), whereas Bax and Bak act as activators keeping VDAC in an open configuration (Chandra et al., 2005), indicating VDAC as a major route for mitochondrial release of pro-apoptotic factors (Kumarswamy and Chandna, 2009). All these inter-actors modify the oligomeric state of VDAC (Mader et al., 2010), possibly regulating pore size. As Bax and Bak can also form pores, this leads to the intriguing situation of interaction between two different pore-forming proteins. Bax binding may enlarge VDAC pores to a size compatible with cytochrome c passage (Banerjee and Ghosh, 2004); additionally, VDAC-only pores for cytochrome c release may form (Shoshan-Barmatz et al., 2010). A model of VDAC organization is shown in Fig. 2. Although less investigated, it was hypothesized that VDAC-dependent channels allow also AIF (Scharstuhl et al., 2009) and SMAC/diablo (Shoshan-Barmatz et al., 2010) release upon damage-induced apoptosis. At variance with these findings, it was reported by (Rostovtseva et al., 2004) that Bax does not interact with VDAC channels, which rather respond to Bid.

3.3. Mitochondrial apoptosis channels (MAC)

Unlike VDAC, MAC is voltage independent and forms only during apoptosis; MAC is assembled by Bax and/or Bak molecules that interact by electrostatic binding to form high oligomeric complexes possibly including other proteins (Martinez-Caballero et al., 2009). Bak is a protein of the outer mitochondrial membrane, kept inactive by binding to VDAC2, a mammalian isoform of VDAC/porin (Cheng et al., 2003), and/or by the Bcl-Xl or Mlc-1 (Willis et al., 2005); upon VDAC2 (Cheng et al., 2003) or Bcl-Xl/Mlc-1 (Willis et al., 2005) displacement by BH3-only proteins, Bak is liberated and interacts with either other Bak molecules or Bax, forming MAC pores (Willis et al., 2005).

Upon t-Bid induction, Bax and Bak pores sequentially form within minutes; these oligomeric structures are independent of VDAC, and consist of 9–10 monomers (Martinez-Caballero et al., 2009), sufficient for cytochrome c passage. Most of the studies focus on cytochrome c release, whereas the evidences of a MAC involvement in SMAC/diablo release are less clear. A simplified model is shown in Fig. 2.

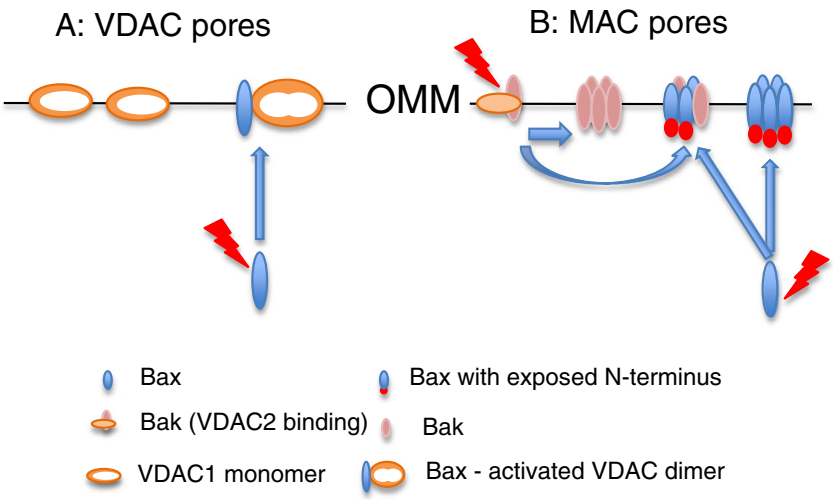

Fig. 2. Bax-dependent membrane pores. Bax can mediate pore formation in the outer mitochondrial membrane (OMM) by at least two mechanisms. In A, we show the dimerization of porin (VDAC) induced by Bax binding, which should increase the pore size allowing cytochrome c release. In B, the MAC pore is depicted, where Bax (blue) or Bak (pink) monomers assemble to create a pore of a size compatible with cytochrome c release (9–10 monomers). Bax activation consists in molecular changes linked to translocation to mitochondria, whereas activation of Bak, a resident mitochondrial protein, consists in its liberation from the VDAC2 protein anchorage.

4. The structure of Bax

4.1. Critical functional domains

Bax is a 21 kD protein of 192 amino acids, whose three-dimensional crystal structure was described back in 2000 (Suzuki et al., 2000). As shown in Fig. 3, Bax possesses 9 alpha helices, an unstructured N-terminus, two exposed and reactive cysteines and a number of critical phosphorylation sites. Alpha helix 9 and the alpha helices 5/6 are hydrophobic regions, buried in the cytosolic form of inactive Bax.

The functionality of the different Bax domains has been extensively studied. Many functions of Bax could be attributed to specific domains by using mutagenesis approaches including point mutations, domain deletions or domain insertions into homolog proteins. This approach is very important, and is especially useful when the tri-dimensional structure of the resulting mutant proteins is verified by crystallography or by *in silico* modeling: it needs to be ascertained that no artifactual alteration of the final structure is achieved, which may provide false indications.

The BH3 domain resides in the alpha 2 helix, and is involved in the hetero-dimerization with other Bcl-2 family members (Kelekar and Thompson, 1998).

The hydrophobic helix 9 and helices 5/6 are involved in membrane insertion; any of them allow translocation to membrane, and possibly the type of apoptotic stimulus may determine which part of the protein is used in different activation contexts. Helices 5/6 are widely recognized as the putative mitochondria pore-forming domain (Heimlich et al., 2004), however, they are not involved in ER-dependent Ca^{2+} uptake by ER or ER-dependent apoptosis (Chami et al., 2004).

Bax oligomerization, the event leading to pore formation, only marginally requires the BH3 domain. Deletion experiments showed that fragments expressing helices 2 to 5 are sufficient for full Bax oligomerization, whereas helix 5 is necessary; in fact, it confers oligomerization ability when introduced into the anti-apoptotic protein Bcl-Xl (George et al., 2007).

Helix 1 is the site of interaction with t-Bid and the other BH3-only protein Puma (Cartron et al., 2004).

The N-terminal region of Bax is exposed after Bax activation; the use of antibodies specific for this epitope (e.g., the 6A7 antibody) allow discriminating between the active and inactive conformations of the native Bax proteins and are useful for *in situ* and immuno-

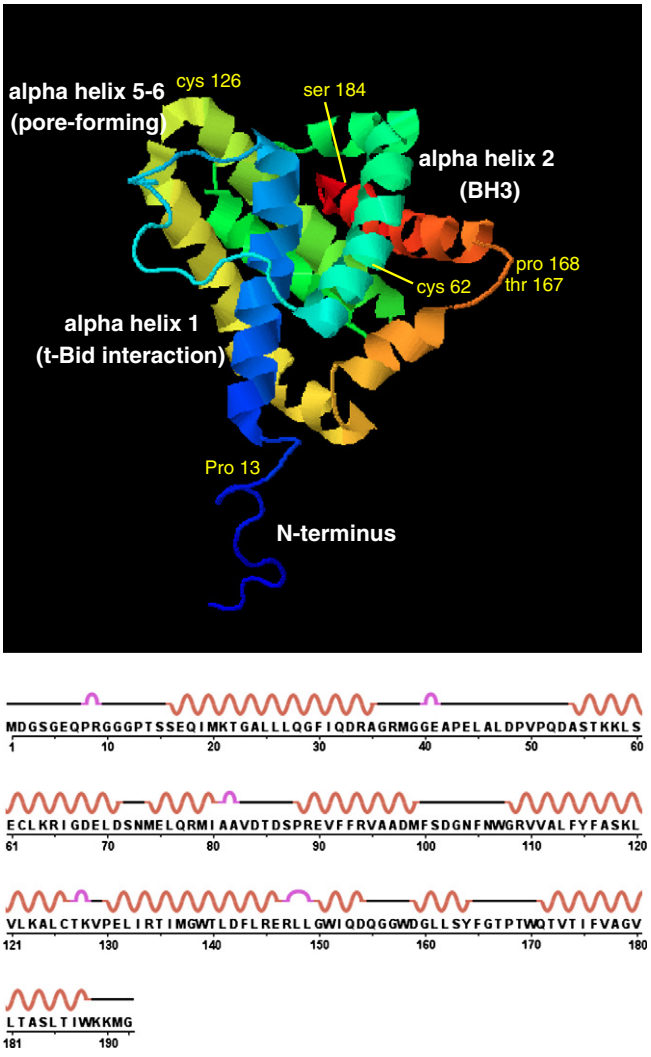

Fig. 3. Critical Bax domains. The Bax 3D structure (PDB code: 1f16) is shown in the top panel with the critical residue positions highlighted in yellow; the 3D representation has been obtained with Jmol. Bax secondary structure is displayed in the bottom panel as calculated from the same source (Suzuki et al., 2000) (Jmol: an open-source Java viewer for chemical structures in 3D. http://www.jmol.org/).

precipitation analysis. N-terminus exposure was found to occur in any instances of Bax activation, but the exact role of this conformational change in Bax activation is still elusive. It is interesting to note that, in dormant Bax, the N-terminus is close to and hides, the alpha 1 helix, which is the site of Bax activation by t-Bid (Cartron et al., 2004): this observation implies that one of its activity is possibly to maintain Bax inactive in healthy cells, whereas its displacement liberates a reactive domain. In line with this observation, is the finding that deletion of the N-terminus leads to constitutive Bax activation (Gao and Dou, 2000), and that N-terminus exposure may occur in the cytosol (prior to mitochondrial translocation), e.g., where a putative interaction with t-Bid may occur. However, there are also evidences of an active role played by the N-terminus in mitochondrial targeting (Cartron et al., 2005). Interestingly, in some circumstances Bax translocates without N-terminus exposure, leading to inactive mitochondrial Bax; further signals are required to expose the N-terminus, after which activation of Bax is achieved (Owens et al., 2009). Thus, if N-terminus exposure is always associated with Bax activation, being in fact the most reliable activation marker available so far, it is not necessarily associated to Bax translocation to mitochondria.

4.2. Critical amino acid residues

Bax has two cysteines, the first one at position 62 within the alpha 2 helix, close to the BH3 domain and the second at position 126, between the alpha 5 and alpha 6 helix within the pore-forming region. Both cysteines are exposed and potentially reactive to form disulfide bridges for either homo- or hetero-dimerization (D'Alessio et al., 2005); *in silico* models propose that homodimers *via* disulfide bonds between cysteine 62 and cysteine 126 expose the hydrophobic alpha helix 9 promoting membrane insertion (D'Alessio et al., 2005).

Two critical phosphorylation sites have been mapped. Serine 184 is at the end of the hydrophobic C-terminus; its phosphorylation by protein kinase C (PKC) zeta (Xin et al., 2007) or AKT (Xin and Deng, 2005) inactivates Bax, and conversely its de-phosphorylation by protein phosphatase 2A activates Bax by promoting exposure of the N-terminus (Xin and Deng, 2006). Ser 184 plays a key role in controlling Bax sub-cellular localization (Nechushtan et al., 1999). Threonine 167 is in the un-structured linker region between helix 8 and helix 9; its phosphorylation by p38 and JNK is required for Bax translocation to mitochondria after stress-induced apoptosis in HepG2 cells (Kim et al., 2006).

Proline 13 in the N-terminus region confer ability to progress in the activation of mitochondrial Bax (Upton et al., 2007), whereas proline 168, which is located in the unstructured region upstream to the hydrophobic helix 9, is required for Bax localization to mitochondria (Cartron et al., 2005). Moreover, glycine 67 was found to determine the ability of the BH3 domain to interact with Bcl-2 and Bcl-Xl (Meijerink et al., 1998).

These amino acid residues are highlighted in Fig. 3.

5. Bax activation

5.1. Signals from the extrinsic pathway

In the amplification branch linking the extrinsic to the intrinsic pathway, caspase 8 proteolyses Bid resulting in truncated Bid that is a potent Bax activator (Li et al., 1998). t-Bid allows amplification of apoptosis by recruitment of the cytochrome c/apoptosome/caspase 9 signals and, in case of cells over-expressing the IAP proteins, allows finalization of apoptosis by promoting Bax-dependent SMAC/diablo release and IAP degradation.

t-Bid-dependent Bax activation is by far the best studied, both in examples of apoptosis in whole cells, and in reconstituted sub-cellular or lipid systems. Molecular analysis in a purified proteins/lipids system showed that t-Bid does not participate to the mitochondrial pore, but recruits Bax in the cytosol, promotes N-terminal exposure and mitochondria localization, after which it detaches and is thus free to recruit new Bax molecules, possibly acting *via* a catalytic mechanism rather than stoichiometric mechanism (Bleicken et al., 2010). In other studies however it was observed that t-Bid inserts into the outer mitochondrial membrane (Guillemin et al., 2010). Recruitment of Bax by t-Bid leads to MAC pores formation (Martinez-Caballero et al., 2009), but also interaction with VDAC (Rostovtseva et al., 2004) was reported.

5.2. Damage signals from the intrinsic pathway: kinases and calpains

The intrinsic pathway is typically activated by cell damage and physico-chemical alterations. Many distinct sensors for various damage and environmental alterations activate signals that converge into Bax activation, which is the most upstream molecular event of the intrinsic apoptotic pathway. This implies that Bax must respond to many different activation stimuli, being an indirect sensor of damage and alterations, and accounting for the large number of critical domains of the Bax protein.

Oxidative stress activates many responses including two MAP kinases such as JNK and p38, which are implicated in both survival and apoptotic pathways in response to stress. Bax phosphorylation at threonine 167 by JNK/p38 is required for mitochondrial translocation (Kim et al., 2006), thus allowing Bax to respond to oxidative stress (Fig. 4).

Deregulated increase of cytosolic Ca^{2+} may develop in cell stress and damage, and many sensors of Ca^{2+} alterations activate either cell-protective or pro-apoptotic responses (Cerella et al., 2010). Calpains

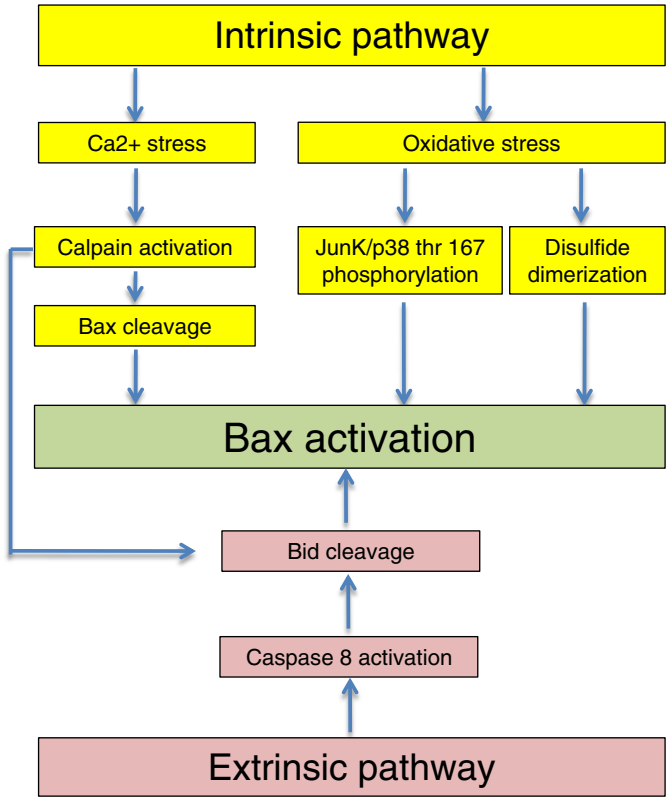

Fig. 4. Mechanisms of direct Bax activation. Bax activation is caspase-dependent in the extrinsic-, but caspase-independent in the intrinsic pathway. The molecular mechanisms through which cell damage or environmental cell alterations activate Bax are still poorly defined. Here three independent mechanisms are reported, where Bax can be a direct sensor of oxidative stress, or can be activated by molecular signals coming from sensor proteins (i.e., the Ca^{2+} sensor calpain). The mechanisms of indirect Bax activation, i.e., by BH3-only proteins that sense cell alterations and act *via* binding to anti-apoptotic Bcl-2 proteins, are not included in this scheme.

are a set of Ca^{2+} sensitive cysteine proteases activated by micromolar (micro-calpains) or millimolar (milli-calpains) cytosolic Ca^{2+} levels (Yoshimura et al., 1983). Among the pro-apoptotic responses, calpains have been shown to proteolytically activate Bax by cleaving its N-terminal region (Gao and Dou, 2000). This truncated Bax is highly active, possibly because a negative regulation signal has been removed (Toyota et al., 2003). In addition, calpain were also shown to cleave Bid to a cleavage site distinct from caspase 8 (Chen et al., 2001); this calpain-dependent t-Bid shares similar pro-apoptotic activity with caspase 8-cleaved t-Bid (Mandic et al., 2002), including Bax recruitment. Thus Ca^{2+} alterations may elicit at least two pro-apoptotic signals *via* calpain activation (Fig. 4), activating Bax by direct cleavage or through processing of Bid.

5.3. Bax as a sensor of physico-chemical alterations

In an oxidative environment, the two exposed cysteines of Bax may theoretically react to produce disulfides. It was shown that after treatment with H_2O_2 at low concentrations, or after glutathione depletion in U937 and HepG2 cells, cytochrome c is released in the absence of apoptosis (Ghibelli et al., 1999); at the same time, Bax translocates to mitochondria, and undergo dimerization, as detected in non-reducing but denaturing electrophoresis (SDS-PAGE) (D'Alessio et al., 2005). Cell-free oxidation of cytosolic extracts with H_2O_2 leads to disulfide dimerization. Oxidized Bax dimers then acquire the ability to translocate to purified mitochondria fractions (D'Alessio et al., 2005). *In silico* models predict that homodimerization between cysteine 62 and cysteine 126 allows exposure of the hydrophobic helix 9 (D'Alessio et al., 2005), possibly allowing membrane insertion; this would provide a functional role to oxidative dimerization. In colon adenocarcinoma cells, substitution of cysteine 62, but not 126, abolishes pro-apoptotic activity of Bax in response to H_2O_2-induced stress, but not to non-oxidative damage (Nie et al., 2008). Interestingly, in colorectal cancer cells both cysteines are required for Bax activation in selenite-induced apoptosis (Huang et al., 2009). Altogether results indicate that oxidative Bax activation may be an alternative way of Bax activation, and that Bax can be a direct sensor of oxidations (Fig. 4).

6. Multistep Bax activation

Despite many evidences attributing a role to the N-terminus region of Bax for mitochondrial targeting (Cartron et al., 2002), it has been described that Bax can migrate to the mitochondria without exposing the N-terminal domain. In this case, membrane integration does not automatically lead to release of apoptotic mitochondrial factors, but other events must take place in order to expose the N-terminus, activate Bax, and release cytochrome c. This was very well described in models of anoikis, a mechanism of apoptosis induction where cells activate cell death by apoptosis after the rupture of integrin interactions with neighboring cells. This cell death mechanism is likely to kill cells that detached including migrating cells in order to avoid metastasis (Frisch and Screaton, 2001). After experimental cell detachment, Bax migrates to mitochondria in a t-Bid-independent manner (Valentijn and Gilmore, 2004). At this point, apoptotic factors are not released and cells can be still be rescued (Gilmore et al., 2000).

Afterwards, Bax molecules form clusters, the N-terminal domain is exposed, and cytochrome c is released (Valentijn et al., 2003). This mechanism of Bax activation within mitochondria requires p38 signaling (Owens et al., 2009), and an intact Bax N-terminus, since proline 13 substitution abolishes this regulation (Upton et al., 2007). Bax activation in mitochondria occurs in response to c-myc deregulation (Soucie et al., 2001). c-myc is an oncogene that immortalizes cells and stimulates their proliferation, actively contributing to tumor progression when over-expressed or deregulated (Garte, 1993). Moreover, as an independent function, c-myc also induces apoptosis by promoting strictly Bax-dependent mitochondria damage (Juin et al., 2002): c-myc does not modify Bax protein abundance or localization, but promotes Bax activation once Bax is already inserted in the mitochondrial membrane (Cao et al., 2008).

Another example of mitochondria localization of inactive Bax was reported in cells rescued by melatonin from stress-induced apoptosis: also in this case, cytochrome c is not released, nor Bax N-terminus is exposed, nor it migrates as a disulfide in non-reducing electrophoresis (Radogna et al., 2008).

7. Conclusions

Bax is the unique entry point for the intrinsic apoptotic signaling, the key amplifier of the extrinsic apoptosis, and the molecule that allows bypassing the IAP blockage. Due to the importance of these processes in the resistance to anti-tumor therapies, many structural and functional studies on Bax have been published. It is clear that many different, often hardly compatible results are reported. Many factors contribute to this situation, including the complex pattern of proteins interacting with Bax, the different types of activation, and the different functions that contribute to apoptosis. Many studies will be necessary to shed light on the Bax-governed signaling network. As an example, in the past, it was long debated why in some cases Bax activation was caspase dependent, whereas in other situations it was prevented by caspase inhibition: after that, the intrinsic and extrinsic apoptotic signal transduction pathways were logically separated. The answer to this question became clear, implying that in the intrinsic pathway, Bax is activated in a caspase-independent manner, whereas caspase 8 is necessary for recruiting Bax from the extrinsic pathway. Likewise, we expect that other apparent paradoxes may be solved by increasing the knowledge about the mechanisms of Bax activation. Likely, we expect that the multiple alternative pathways of Bax activation may be individually explained, and (possibly) linked to an alternative outcome. Most mechanistic studies have focused on t-Bid as the trigger, and cytochrome c as the outcome of Bax activation.

Thus, many key questions remain: what is the role of the different Bax domains in the various mechanisms of Bax recruitment? Also, the different types of proteins released from mitochondria remain to be further investigated.

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