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Mitochondria in Apoptosis: Bcl-2 family Members and Mitochondrial Dynamics

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Abstract

Mitochondria participate in apoptosis through a range of mechanisms that vary between vertebrates and invertebrates. In vertebrates, they release intermembrane space proteins, such as cytochrome c, to promote caspase activation in the cytosol. This process is the result of the loss of integrity of the outer mitochondrial membrane caused by proapoptotic members of the Bcl-2 family. This event is always accompanied by a fissioning of the organelle. Fission of mitochondria has also been reported to participate in apoptosis in *Drosophila* and *Caenorhabditis elegans*. However, in these organisms, mitochondrial membrane permeabilization does not occur and the mechanism by which mitochondrial dynamics participates in cell death remains elusive.

Introduction

Mitochondria are essential organelles because they supply the cell with metabolic energy in the form of ATP generated by oxidative phosphorylation. In addition they perform a number of other key metabolic reactions. Their shape, from spherical to elongated, is continually remodeled by fusion and fission events that link all the organelles within a cell into a continuum over time. Although why mitochondria are so dynamic is not known, the process is essential for mitochondrial maintenance in yeast (Hoppins et al., 2007) and mammals (Chen et al., 2003). Recently, mitochondrial fission has been linked to the cellular death program of apoptosis. Mitochondria are involved in the so-called intrinsic pathway of apoptosis where they release soluble proteins, including cytochrome c, from the intermembrane space to initiate caspase activation in the cytosol (Kroemer et al., 2007; Vaux, 2011). The release of these proteins is a consequence of the integrity of the mitochondrial outer membrane (OMM) being compromised, a process called mitochondrial outer membrane permeabilization (MOMP). So far, this process has only been validated in vertebrates; it does not seem to be required in *C. elegans* and is debated in *Drosophila*. As reviewed here, in these invertebrates, mitochondria may be involved in apoptosis through distinct mechanisms. In vertebrates, MOMP is under the control of the proapoptotic Bcl-2 family members. Here, we will focus on the mechanisms by which Bcl-2 family members

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elicit MOMP during apoptosis. We will also review the link that connects some Bcl-2 family proteins with fission and fusion of the organelle in apoptosis and in healthy cells.

Bcl-2 family members

Bcl-2 family proteins are subdivided into three groups on the basis of their pro- or antiapoptotic action and the Bcl-2 Homology (BH) domains they possess (Figure 1A) (Schinzel et al., 2004; Youle and Strasser, 2008). Antiapoptotic Bcl-2-like proteins (e.g. Bcl-2, Bcl-x_L, Bcl-w, Mcl-1 and A1/Bfl-1) and proapoptotic Bax-like proteins (e.g. Bax, Bak and Bok/Mtd) display 4 BH domains (Kvansakul et al., 2008). The proapoptotic BH3-only proteins (e.g. Bid, Bim/Bod, Bad, Bmf, Bik/Nbk, Blk, Noxa, Puma/Bbc3 and Hrk/DP5), on the other hand, possess only a short motif called the BH3 domain as their name indicates. BH3 proteins integrate and transmit death signals that emanate from defective cellular processes to other Bcl-2 family members. Through their BH3 domain, these proteins either interact with antiapoptotic proteins to inhibit their function and/or to interact directly with multidomain proteins such as Bax or Bak to stimulate their activity. The former are often referred to as ‘sensitizers’, whereas the later are classified as ‘activators’ (reviewed in Giam et al., 2008). The multidomain proapoptotic proteins, Bax and Bak, and perhaps Bok in some tissues, are responsible for MOMP and are the master effectors of apoptosis as cells lacking Bax and Bak fail to undergo MOMP and apoptosis in response to many death stimuli (Wei et al., 2001).

Mechanisms of activation of proapoptotic Bcl-2 family members

Activation of the proapoptotic Bcl-2 family protein Bax results from a highly regulated multistep process involving its translocation from the cytosol to the OMM where it inserts and oligomerizes. In contrast to Bax, Bak is constitutively inserted in the OMM by a C-terminal transmembrane domain. Its insertion can be facilitated by the voltage-dependent anion channel isoform 2 with which it was found to interact (Cheng et al., 2003; Lazarou et al. 2010; Roy et al., 2009). In this section, we will describe what maintains Bax in the cytosol in healthy cells and how BH3-only proteins, together with the lipid bilayer, cooperate to promote oligomerization of Bax and Bak in the OMM during apoptosis.

Bax travels back and forth from the cytosol to the OMM in healthy cells

In healthy cells, Bax is mainly cytosolic with a minor fraction loosely attached to mitochondria. In contrast to what is observed in cultured cells, the amount of membrane-bound Bax is negligible in tissues such as liver or brain. This suggests that the mitochondrial sub-population of Bax at the mitochondria could be a measure of the stress experienced by the cells, which increases with in vitro culture. Using fluorescence loss in photobleaching, Edlich et al. (2011) observed that Bax constantly travels back and forth from the cytoplasm to mitochondria (Figure 2). Once attached to the OMM, it can be retrotranslocated to the cytosol by Bcl-x_L by a mechanism that is not yet fully understood. Bax retrotranslocation by Bcl-x_L and possibly by other prosurvival Bcl-2 proteins, would ensure that Bax does not chronically accumulate at the OMM to reach a critical level that could promote its autoactivation. It is still unclear what allows Bax to attach to the OMM specifically in healthy cells. The most likely explanation is that Bax undergoes discrete and reversible conformational changes that would expose not only its BH3 domain but also its N- and C-terminal domains. Consistently, it was previously observed that during detachment of cells from the extracellular matrix, the N-terminus of Bax undergoes a conformational change that can be reversed upon re-attachment of cells to substrate (Gilmore et al., 2000). Understanding what triggers and controls changes in Bax conformation in healthy cells requires further investigations. A reversible conformational change in Bax could be triggered by a simple contact of the protein with the lipid bilayer of the OMM, as suggested

by experiments performed with liposomes (Yethon et al., 2003). Other triggers, including BH3-only proteins, Prostaglandins (Lalier et al.), p53 (Chipuk et al., 2004), pH variations (Khaled et al., 1999), or post-translational modifications (Kutuk and Letai, 2008), all could also be responsible for sending Bax to the OMM. But what would prevent a complete conformational change in Bax in healthy cells? The structure of the OMM may counteract Bax activation. Its cholesterol content has been shown to prevent Bax insertion or oligomerization in the OMM (Christenson et al., 2008; Lucken-Ardjomande et al., 2008). Moreover, previous data have shown that stress-induced mitochondrial hyperfusion, which leads to highly elongated organelles, delays Bax activation upon exposure of cells to apoptotic stimuli (Tondera et al., 2009). Thus, the lipid content and the shape of the OMM may be refractory to complete Bax activation in healthy cells.

The role of BH3-only proteins and of the lipid bilayer

The protein tBid has been originally described as a direct activator of Bax, able to induce a conformational change of the Bax N-terminus, as well as modulate Bax insertion and oligomerization in the OMM (Desagher et al., 1999; Eskes et al., 2000). Many groups have confirmed and extended these observations to other BH3-only proteins, including Bim and Puma (Gallenne et al., 2009; Kim et al., 2009; Kuwana et al., 2005; Merino et al., 2009; Ren et al. 2010). Importantly, these studies have also provided details about the mechanisms by which BH3-only proteins trigger conformational changes in Bax, leading to its insertion and oligomerization in the OMM. Most BH3-only proteins, except Bid, appear to be intrinsically unstructured proteins. However, their BH3 domain is known to form an alpha-helix upon binding to the hydrophobic groove that is on the surface of antiapoptotic proteins. In Bax, this hypdrophobic groove is occupied by alpha-helix 9 (the transmembrane domain) (Figure 1B). Until recently, it was unclear where BH3 activators impact Bax to induce the structural changes required for its insertion and assembly in the OMM. To answer this question, Walenski and colleagues used peptides whose alpha-helicity was stabilized by a chemical modification termed ‘hydrocarbon stapling’. They derived staple peptides from the BH3 domain of Bid or Bim and reported the first evidence of a direct interaction between the BH3 domain of Bim and Bid with Bax (Gavathiotis et al., 2008; Walensky et al., 2006). Moreover, consistent with previous data (Cartron et al., 2004), their studies localized the binding site of the BH3 domain of Bim to the junction of alpha-helices 1 and 6 on the N-terminal side of Bax, i.e. on the opposite side of the hydrophobic groove (Gavathiotis et al., 2008) (Figure 1B). Thus, BH3-only proteins appear to interact with different sites in Bax and in prosurvival Bcl-2 proteins in which the hydrophobic groove is the receiving domain. According to a model derived from recent NMR studies, upon interaction with Bim, Bax structural metamorphosis begins with the displacement of its alpha-helices 1 and 2 from a closed to an open position, which leads to the release of the alpha-helix 9 from the hydrophobic groove and exposure of the BH3 domain (Gavathiotis et al. 2010). Whether the results obtained with peptides in solution in these studies faithfully reflect what occurs in the cell, at the OMM, will require further investigation (discussed in Czabotar et al., 2009). Moreover, it will be important to clarify where BH3-only proteins impact Bak, as a previous report defined the hydrophobic groove as the receiving domain of the tBid BH3 domain (Moldoveanu et al., 2006).

In contrast to the stapled Bim peptide, tBid is unable to interact with full length Bax or Bcl-x_L in solution (Lovell et al., 2008) begging the question of where in the cell do interactions between BH3-only proteins and Bax or Bak occur. Following its cleavage by caspase 8, the N-terminus of Bid (N-Bid) remains attached to the C-terminal portion of the protein (C-Bid), possibly masking the BH3 domain in C-Bid. However, in the presence of a lipid bilayer with a phospholipid composition mimicking that of the OMM, it was shown that only C-Bid inserts while N-Bid remains in solution (Lovell et al., 2008). Thus, upon

interaction with the membrane, tBid is fully operational to recruit Bax to the OMM as shown by fluorescence resonance energy transfer (Lovell et al., 2008). These results emphasize the importance of the lipid environment for activation of these Bcl-2 proteins and prompted Andrews and colleagues to propose the 'membrane-embedded together' model. According to this model, tBid first inserts in the membrane, then recruits Bax that in turn inserts and oligomerizes. According to these authors, Bcl-x_L and other antiapoptotic proteins such as Bcl-w that are in part cytosolic, are also recruited and activated at the membrane by both the activator and sensitizer BH3-only proteins (Shamas-Din et al. 2011). This part of the model confers on BH3-only proteins an ambiguous role since, in theory, they could recruit both pro- or antiapoptotic proteins. If this is the case, and if the role of antiapoptotic Bcl-2 proteins in the OMM is to prevent Bax or Bak oligomerization, then BH3-only proteins should be classified as antiapoptotic proteins, which is not consistent with their known function. Therefore, it is unlikely that the BH3-only proteins recruit antiapoptotic Bcl-2 family members into the OMM to inhibit Bax or Bak function. Rather, BH3-only proteins may recruit antiapoptotic proteins and remain tightly bound to them to inhibit their function. Soluble and membrane bound antiapoptotic Bcl-2 proteins could therefore be inhibited either in the cytosol or at the membrane level by soluble or membrane-associated BH3-only proteins, respectively. Moreover, antiapoptotic Bcl-2 proteins, whether constitutively present in membranes (such as Bcl-2), or cytosolic (such as Bcl-x_L and Bcl-w), have the possibility to directly interact with the BH3-only domain of Bax or Bak during the process of their oligomerization. This implies that the affinity of antiapoptotic proteins for the BH3 domain of Bax or Bak must be very high to compete with other Bax or Bak molecules and prevent their homo-oligomerization.

Is the lipid composition of the membrane crucial for tBid-induced Bax oligomerization?

Using liposomes containing phosphatidylcholine and phosphatidylglycerol, it was previously found that tBid can recruit Bax to the membrane and drive formation of small pores that could allow passage of small molecules (such as carboxyfluorescein) but were impermeable to cytochrome c (Roucou et al., 2002). Analysis of the quaternary structure of Bax revealed that the protein was monomeric under this condition. At the same time, Kuwana et al. (2002) and later others (Lucken-Ardjomande et al., 2008; Terrones et al., 2004), reported that cardiolipin is required for the formation of tBid-induced Bax oligomers and of giant pores. Thus the presence of cardiolipin in the membrane appears to promote tBid-induced Bax oligomerization and formation of cytochrome c permeable pores. Cardiolipin is a negatively charged phospholipid, exclusively found in mitochondria, mainly in the IMM where it is important for the activity of several complexes of the respiratory chain and of some transporters (Hasler, 2011; Schlame et al., 2000; Schug and Gottlieb, 2009). Subfractionation of the mitochondrial membranes, together with the accessibility of cardiolipin to enzymatic degradation by Phospholipase 2 added to isolated mitochondria, indicate that small amounts of this phospholipid are also present in the OMM, probably enriched at contact sites between the OMM and the IMM (Ardail et al., 1990; Daum, 1985; Epand et al., 2007; Gebert et al., 2009; Hovius et al., 1990). tBid was found to bind cardiolipin through central alpha-helices, which would explain its recruitment to mitochondria, principally at contact sites between the IMM and the OMM (Kim et al., 2004; Lutter et al., 2000). In addition to promoting membrane insertion of tBid, cardiolipin could be involved in the Bax oligomerization process itself as suggested by the experiment described above (Roucou et al., 2002). Besides cardiolipin, Gross and colleagues recently identified MTCH2/MIMP, a protein similar to mitochondrial carriers of the adenine nucleotide translocator family, as a receptor for tBid (Zaltsman et al. 2010). In the absence of this protein, tBid is recruited less efficiently to the OMM and apoptosis is delayed when cells are exposed to tBid-dependent death stimuli. Thus MTCH2/MIMP seems to accelerate the recruitment of tBid to the OMM, and may cooperate with cardiolipin to provide the

OMM with an optimal concentration of tBid. Further experiments, in particular using proteoliposomes prepared with recombinant MTCH2/MIMP, are required to determine how tBid binds this carrier protein. Moreover, the requirement of cardiolipin for Bax activation and MOMP in the cell requires further investigation as several reports have shown that this phospholipid can be dispensable for Bax activation (Gonzalvez et al., 2008; Iverson et al., 2003; Polcic et al., 2005). Importantly, in most studies using liposomes, the process of Bax oligomerization induced by tBid does not appear to be optimal as only a small fraction of Bax oligomerizes, even when high concentrations of tBid are used and despite the presence of cardiolipin in membranes (Kuwana et al., 2002; Schafer et al., 2009). This suggests that, in vitro, either important components that facilitate Bax insertion and/or oligomerization are missing or that the lipid bilayer structure in these experimental se-ups is not optimal to allow efficient Bax oligomerization.

The role of mitochondrial fission

Mitochondria are dynamic organelles that fuse and divide continuously. The core components of the fusion and fission machineries have been identified as the dynamin-related proteins Drp1, mitofusins (Mfn) 1 and 2 and Opa1 (Box 1) (reviewed in Westermann, 2010). Fission of mitochondria is a constant in apoptosis (Figure 3)(Martinou and Youle, 2006). The mechanism underlying this event appears to involve Drp1, the most downstream component of the fission machinery (Frank et al., 2001). The precise mechanism by which Drp1 is recruited to the mitochondria during apoptosis remains vague. Under normal conditions, Drp1 moves back and forth from the cytosol to the OMM. However, following Bax activation, Drp1 stably associates with the OMM due to Bax/Bak-dependent SUMO modification (Wasiak et al., 2007). However, the mechanistic link between activation of Bax or Bak and Drp1 SUMOylation is still missing. Importantly, it should be mentioned that inhibition of Drp1, delays, but does not completely prevent, mitochondrial fission (Ishihara et al., 2009). This suggests that a Drp1-independent mechanism also seems to participate in the fission of the organelle during apoptosis. A link between mitochondrial fission and Bax activation was suggested by the detection of active Bax in discrete foci at mitochondrial fission sites (Karbowski et al., 2002). Bak, which initially surrounds the organelle, also coalesces into foci at mitochondrial fission sites during apoptosis. In most cases, (although not always), these foci appear to be located in proximity to Drp1 and Mfn2 and form independently of Drp1 activity (Frank et al., 2001; Ishihara et al., 2009; Montessuit et al. 2010). By inhibiting Drp1 and delaying mitochondrial fission, Karbowski et al. (2002) could observe these Bax spots at the surface of elongated mitochondria. Interestingly, some of them were located at constriction sites that the authors described as ‘aberrant mitochondrial scission attempts’. This observation further supports the existence of a Drp1-independent mechanism responsible for constricting, and possibly fissioning, the organelle during apoptosis. Such constriction sites have also been observed in healthy Drp1-deficient cells, not only on mitochondria (Ishihara et al., 2009), but also on peroxisomes, which rely on Drp1 for division (Koch et al., 2004). These sites may provide the appropriate membrane curvature, or lipid composition, for the assembly of Drp1 rings and the formation of Bax foci during apoptosis.

It was recently found that the formation of membrane hemifusion intermediates is a process able to promote tBid-induced Bax oligomerization (Montessuit et al. 2010). This membrane remodeling can be promoted by Drp1 in a cell free system and may recapitulate the membrane remodeling that occurs at mitochondrial fission sites in cells undergoing apoptosis (Figure 3). When membranes divide or fuse, contacting leaflets fuse to form a stalk. The stalk then expands to form a metastable non-bilayer membrane intermediate also called hemifission or hemifusion intermediate for membrane fission and fusion respectively (Chernomordik and Kozlov, 2008; Kozlovsky and Kozlov, 2003). For fusion, this transient

state would evolve towards the formation of a fusion pore, while for membrane fission, it would decay spontaneously into two separate membranes, thereby completing the fission process. The mechanism by which these membrane intermediates promote Bax oligomerization is still unclear. Hemifusion or hemifission intermediates are expected to occur during fusion or fission of the organelle, respectively. These structures may also possibly form at contact sites between the OMM and the IMM, which are enriched in cardiolipin and phosphatidylethanolamine, two phospholipids that have a propensity to form non-bilayer structures (Figure 3). The limited number of such membrane structures at the surface of mitochondria may explain why Bax or Bax oligomers are not randomly distributed in the OMM in apoptotic cells.

How does Bax or Bak permeabilize the outer mitochondrial membrane?

The mechanism by which Bax, or Bak, permeabilizes membranes to allow efflux of large proteins is still unclear, although several models having been proposed and challenged. These models predict that Bax, alone or combined with other proteins, forms channels large enough to allow passage of cytochrome c and other proteins. Alternatively, Bax or Bak could modulate the opening of existing channels such as the so-called permeability transition pore, to induce MOMP. However, opening of the permeability transition pore has been questioned by a number of genetic studies that have excluded the requirement of some of its major components (reviewed in Tait and Green 2010; Westphal et al. 2011). It is therefore unlikely that the permeability transition pore plays a role in Bax and Bak-induced MOMP.

Proteinaceous or lipidic pores

The model of Bax or Bak pore formation finds its roots in the determination of the 3D structure of Bcl-x_L (Muchmore et al., 1996). This protein exhibits a structural similarity to bacterial pore-forming toxins - certain colicins and the translocation domain of diphtheria toxin-. Other members of the Bcl-2 family were later determined to have a similar structure. Consistent with this structural resemblance, Bax and Bcl-2 were found to form pores in planar lipid bilayers (Antonsson et al., 1997; Schlesinger et al., 1997). The ability of Bax and Bak to induce MOMP seems to reside at least in part in the nature of their central alpha-helices 5 and 6. Thus, replacing the alpha-helix 5 of Bcl-x_L by the equivalent in Bax is sufficient to turn Bcl-x_L into a ‘killer’ protein (George et al., 2007). In addition, differences in the ability of pro- and anti-survival proteins to oligomerize could explain their capacity to induce MOMP. Biochemical data support a model in which Bak and Bax oligomers are formed by two interfaces involving the BH3:groove and alpha-helix 6: alpha-helix 6 interfaces, while alpha-helices 5, 6 and 9 are embedded in the membrane (Dewson et al., 2008; Zhang et al. 2010). Although a model for Bax or Bak oligomerization is beginning to emerge, the atomic details of membrane-embedded Bax and Bak oligomer are still missing. In particular the number of units present in the Bax or Bak oligomers remains elusive and estimates vary considerably depending on the approaches used. Assessing the minimal size of the Bax or Bak oligomer that is responsible for MOMP is critical, but difficult. Indeed, once oligomerization has been initiated, self-oligomerization proceeds which may lead to the recruitment of hundreds of Bax molecules, that then protrude out of the OMM (Nechushtan et al., 2001b). Whether all these molecules are required for pore-formation in the membrane is questionable. Such heterogeneity of oligomers may hamper resolution of the 3D structure of these proteins within a membrane. Moreover the nature of the pore formed by Bax -- whether proteinaceous or lipidic (Box 2) -- is still under debate. Although some reports favor formation of a proteinaceous channel (Dejean et al., 2005; Epand et al., 2002), other results are consistent with formation of a lipidic pore (Basanez et al., 1999; Garcia-Saez et al., 2007; Garcia-Saez et al., 2006). In favor of the latter model, alpha-helix 5 of Bax, which is sufficient to perforate membranes, was found to form lipidic pores in a

synthetic membrane by X-ray diffraction (Qian et al., 2008). However, one must be cautious with overall structural predictions of Bax based on data obtained with only minimal domains of the protein. Nevertheless, there are other reasons to favor the lipidic pore model for Bax and Bak. Recently, analysis of Bax-permeabilized liposomes by cryo-electron microscopy revealed large openings of the membrane, from 25 to 100 nm, compatible with the release of megadalton dextrans. Importantly, the edges of these pores were devoid of protein (Bleicken et al. 2010; Schafer et al., 2009). Whether pores of this type are formed in the OMM of mitochondria undergoing MOMP remains now to be shown.

Mechanistic link between mitochondrial dynamics and MOMP: the role of membrane hemifusion/hemifission intermediates

The notion that MOMP and mitochondrial fission are mechanistically linked remains controversial. In some reports, inhibition of mitochondrial fission or stimulation of fusion, significantly delayed apoptosis (Cassidy-Stone et al., 2008; Frank et al., 2001; Germain et al., 2005; Merrill et al. 2011). Puzzlingly, cells from the neural crest failed to die in Drp1-knockout mouse embryos (Wakabayashi et al., 2009), whereas mouse embryonic fibroblast cell lines generated from these Drp1-deficient mice displayed little (Wakabayashi et al., 2009) or partial (Ishihara et al., 2009) resistance to apoptosis. In other studies, inhibiting mitochondrial fission in HeLa cells had no or only a minor impact on the kinetics of MOMP and cell death (Estaquier and Arnoult, 2007; Ishihara et al., 2009; Parone et al., 2006; Sheridan et al., 2008). Moreover, some studies showed that it was possible to dissociate mitochondrial fission from MOMP. James et al. (2003) reported that Bcl-x_L could inhibit MOMP and apoptosis due to overexpression of the putative Drp1 receptor hFis1, without preventing Fis-1-induced fission. On the other hand, Sheridan et al. (2008) observed that Bcl-x_L, as well as other members of the apoptosis-inhibitory subset of the Bcl-2 family, antagonized Bax and/or Bak-induced cytochrome c release but failed to block mitochondrial fragmentation associated with Bax/Bak activation. Several studies also reported that inhibition of mitochondrial fission did not prevent the efflux of Smac/DIABLO, a protein present in the intermembrane space of mitochondria, although it could delay cytochrome c release (Estaquier and Arnoult, 2007; Parone et al., 2006). These data favor a model in which mitochondrial fission inhibition could retard cytochrome c release by preventing the remodeling of mitochondria cristae, a process that is thought to be required for efficient cytochrome c mobilization (Cipolat et al., 2006; Yamaguchi et al., 2008). Re-evaluation of these conflicting data, in particular in light of the new results by Montessuit et al. (2010) on the role of mitochondrial membrane hemifusion/hemifission intermediates in Bax oligomerization, provides at least two explanations for why inhibiting Drp1 can only delay cytochrome c release and cell death. First, this is because the inhibition of known components of the mitochondrial fission machinery cannot completely prevent mitochondrial fission as previously mentioned. Second this is because it appears that rather the fission of mitochondria *per se*, it is the formation of membrane hemifission intermediates that may play a central role in Bax oligomerization and MOMP. According to theoretical studies, a place where the cost in energy to form a membrane hole would be minimal is at the edge of a membrane hemifusion structure (Katsov et al., 2004). The observation that Bax is confined at discrete foci at the surface of mitochondria, some of which coincide with mitochondrial fission sites, together with the new role of membrane hemifusion or hemifission intermediates in the oligomerization of Bax during apoptosis, raise the possibility that Bax may opportunistically target those sites that are optimally designed for it to oligomerize and to perforate the membrane (Figure 3).

The role of mitochondria in Drosophila cell apoptosis

Steps of apoptosis upstream of caspases are regulated differently in flies and mammals (Salvesen and Abrams, 2004). Indeed, the role of Bcl-2 family members in *Drosophila* in apoptosis is relatively minor (Sevrioukov et al., 2007). In contrast, the Hid/Grim/Reaper gene products that derepress the IAP proteins that block caspases through the ubiquitin proteosome pathway, are potent inducers of fly cell apoptosis (Bader and Steller, 2009). Recent work in flies links mitochondrial fission and fusion processes to Hid, Grim and Reaper, as well as to the Bcl-2 family members Buffy and DEBCL and to apoptosis. As derepressors of cytosolic IAP, there had been no explanation why Hid (Haining et al., 1999), Grim (Claveria et al., 2002) and Reaper (Olson et al., 2003) localize to mitochondria until recent work has linked Reaper to the mitochondrial fusion machinery. Although caution should be taken in interpreting these results due to the expression in heterologous cells of fly proteins that lack known mammalian homologues, Reaper expressed in HeLa cells localizes to the OMM and inhibits mitochondrial fusion inducing mitochondrial fragmentation. Reminiscent of mammalian proapoptotic Bcl-2 family members (Nechushtan et al., 2001a), Reaper forms concentrated foci on mitochondria (Thomenius et al., 2011). Overexpression in HeLa cells of Mfn2, a dynamin family member that mediates mitochondrial fusion, changes Reaper localization from foci to more evenly coat mitochondria suggesting they interact, consistent with data that a peptide of Reaper can co-immunoprecipitate with *Xenopus* Mfn2 (Thomenius et al., 2011). Mitochondrial localization, mitochondrial fragmentation activity and Mfn2 interaction required the GH3 domain of Reaper indicating that Reaper binds Mfn2 on mitochondria through the GH3 domain that is required to induce apoptosis (Thomenius et al., 2011). Alternatively or additionally, mitochondrial Hid may recruit Reaper to mitochondria (Sandu et al. 2010). Interestingly, mitochondrial Reaper and Hid can recruit IAPs to mitochondria where they may induce IAP autoubiquitination and proteosomal degradation. Mitochondria fragment during apoptosis in Drosophila (Goyal et al., 2007) (Abdelwahid et al., 2007) possibly through Reaper interaction with mitofusin and inhibition of mitochondria fusion (Thomenius et al., 2011). Preventing this mitochondrial fragmentation by inhibition of Drp1 prevents death of fly cells in vitro and in vivo (Abdelwahid et al., 2007; Goyal et al., 2007).

Apoptosis in the worm

CED-9, the sole multidomain Bcl-2 family member in *C. elegans*, is localized to the mitochondria (Chen et al., 2000). Although it primarily inhibits apoptosis through the binding and sequestration of the caspase activator, CED-4, CED-9 can promote apoptosis in weak loss of function CED-3 caspase mutants (Hengartner and Horvitz, 1994). As in mammalian and *Drosophila* cells, mitochondria fragment in the worm during apoptosis and this fragmentation is dependent on CED-9 and the BH3-only protein, EGL-1 (Jagasia et al., 2005). Although there is no evidence of cytochrome c release or MOMP in the worm, inhibition of mitochondrial fragmentation by expression of a dominant negative inhibitor of Drp1 prevents 20% of the normal developmental cell death in *C. elegans* (Jagasia et al., 2005).

Bcl-2 family proteins and mitochondrial dynamics

The link between mitochondrial fission and apoptosis has been furthered strengthened by several studies that show Bcl-2 family members alter mitochondrial dynamics even in healthy cells (Delivani et al., 2006; Karbowski et al., 2006; Rolland et al., 2009; Tan et al., 2008). Overexpression of Bcl-2 family proteins CED-9 and Bcl-x_L can induce mitochondrial fusion in mammalian cells (Delivani et al., 2006) and in *C. elegans* (Rolland et al., 2009; Yamaguchi et al., 2008). Bcl-x_L can also accelerate mitochondrial fission in mammalian

neurons thereby, accelerating mitochondrial dynamics (Berman et al., 2009). The proapoptotic Bax and Bak, although having the opposite effect on cell viability relative to Bcl-x_L, are required for the normal rate of mitochondrial fusion in cells (Karbowski et al., 2006). Recent work also shows that in a cell free system of mitochondrial fusion, recombinant Bax can stimulate the process specifically through Mfn2 and not Mfn1 (Hoppins et al., 2011). Tethering Bax with disulfides to hinder conformational change abolishes the mitochondrial fusion activity indicating that at least subtle conformational changes are involved. Promotion of mitochondrial fusion may be a consequence of direct interaction between Bcl-2 family members and Mfn1/2 (Brooks et al., 2007; Delivani et al., 2006; Rolland et al., 2009; Cleland et al., 2010) although how mitofusins may be activated remains unclear. Recent work links Bcl-2 family members to mitochondrial dynamics also in Drosophila, during fly oogenesis. Loss of the fly Bcl-2 family members, Buffy and/or DEBCL, disrupts nurse cell death, which occurs after the nurse cells transfer some of their cytosol and organelles into the developing oocyte. Buffy and/or DEBCL loss also increase mitochondrial network formation and mitochondrial clumping in the surviving cells, suggesting that in certain tissues in the fly, Bcl-2 family members affect mitochondrial dynamics upstream of apoptosis (Tanner et al., 2011). Thus, links between Bcl-2 family members and mitochondrial dynamics have been identified in mammals, insects and roundworms. How the effects of the Bcl-2 family members on mitochondrial dynamics in healthy cells may relate to mitochondrial fragmentation during apoptosis (subsequent to Bcl-2 family member conformational change and insertion into mitochondrial membranes) is an important question that remains to be resolved.

Concluding remarks

Over the past decade, our understanding of the mechanism of action of Bcl-2 family members has expanded significantly. The 3D structure of many members of the family, in solution, has been solved and the mechanism by which these proteins interact is better understood, even though some controversies remain as to how BH3-only proteins regulate multidomain Bcl-2 family members. Importantly, the lipid composition and structural organization of the OMM has emerged as one of the central players in the regulation of Bcl-2 family member activity. However, the precise 3D structure that many Bcl-2 proteins adopt in this membrane is still unknown. Without this essential information, it is impossible to understand precisely how MOMP occurs. The challenge for the coming years will be to solve the structure of membrane-embedded key Bcl-2 family members and to understand how the lipid composition and structure of the OMM controls activation of Bcl-2 family members and MOMP.

Recent work from several laboratories has also unraveled new functions for many pro-and antiapoptotic members of the Bcl-2 family, besides their role in apoptosis. Although our review has been focused on the involvement of Bcl-2 family members in mitochondrial dynamics, it is clear that these proteins play additional roles in other cellular processes. These functions, and new ones that may be discovered in the future, will certainly help decipher the complex role Bcl-2 family members play in life and death of the cell.

Box 1: Dynamin- related proteins involved in the fusion and fission mitochondrial machineries

1. Drp1 is a large GTPase of the dynamin family that is required for mitochondrial fission. The protein is cytosolic and translocates to the mitochondria where it binds to receptors such as Mff and Fis1. It assembles into rings around mitochondria and constricts and breaks down their membrane by a mechanism that is not completely understood.

2. Mitofusins: two mitofusins (Mfn), Mfn1 and Mfn2, are required in the fusion of the outer mitochondrial membrane. These proteins are integrated in the OMM, facing the cytosol, and mediate the docking of mitochondria to one another during fusion by engaging in trans-homo-oligomeric and hetero-oligomeric complexes.
3. Opa1 (Optic atrophy 1): As its name implies this protein was found to be mutated in the disease optic atrophy, which leads to degeneration of the optic nerve. Opa1 is required for fusion of the inner mitochondrial membrane. Different isoforms of this protein that form a complex integrated in the IMM, facing the intermembrane space of mitochondria, have been identified.

Box 2: Proteinaceous *vs* lipidic pores

Many toxins are known to spontaneously induce transmembrane pores in lipid bilayers under certain conditions. Depending on their structure, they can either form proteinaceous or lipidic pores (Yang et al., 2001).

Proteinaceous pores (also called barrel-stave pores): toxins form a barrel-like pore that spans the membrane. The pore lumen is lined by peptides that are perpendicularly inserted in the membrane

*Lipidic pore*s (also called toroidal pores): insertion of the peptides in the membrane triggers lipid monolayer bending such that the outer and inner leaflets of the membrane are continuous. The pore is lined by both the peptides and the lipid head-groups.

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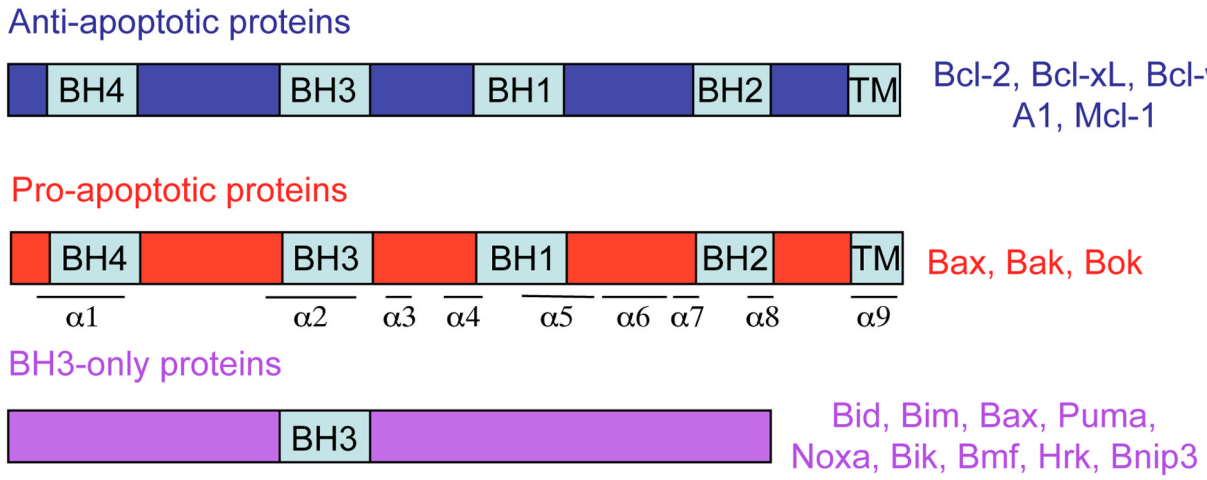
A.



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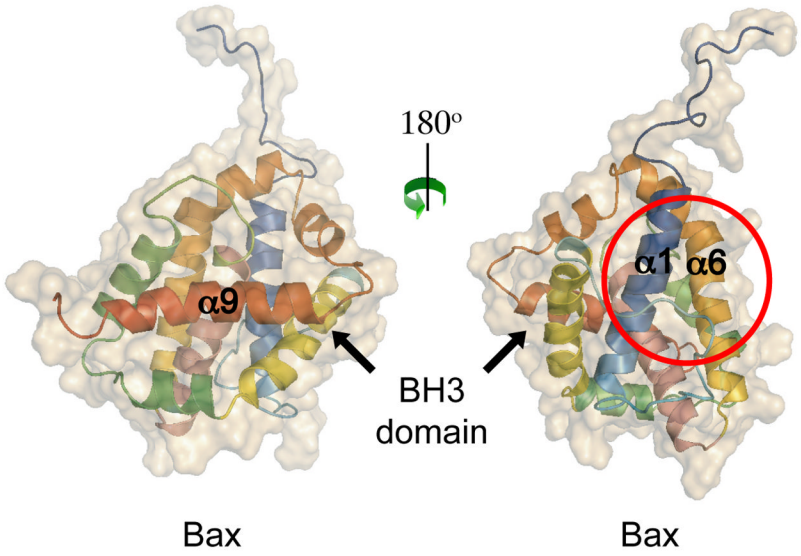

Figure 1. Structure of Bcl-2 family proteins

A) The Bcl-2 family is divided into three groups based on their Bcl-2 Homology domains (BH). Pro- and antiapoptotic proteins contain 4 BH domains, while BH3-only proteins, as their name indicates, contain only the BH3 domain. B) Structure of Bax showing the alpha-helix 9 (transmembrane domain) embedded in the hydrophobic groove (left structure). A 180° rotation (structure on the right) shows the alpha-helices 1 and 6. The red circle represents the binding site for the Bim BH3 domain.

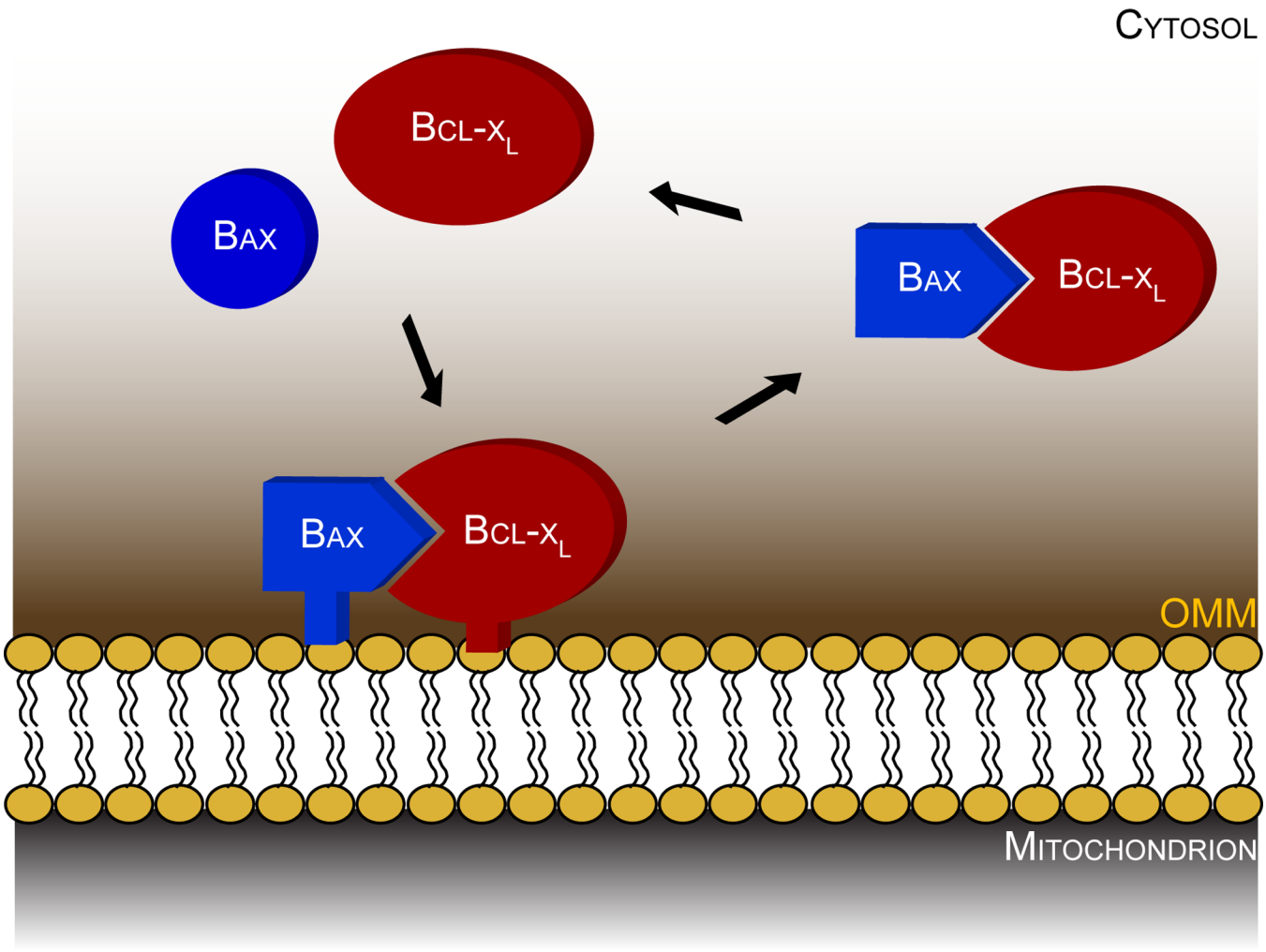

Figure 2. Bax moves back and forth from the cytosol to the mitochondrial outer membrane

In healthy cells, Bax (blue) and Bclx-L (red) are translocated from the cytosol to the outer mitochondrial membrane (OMM) where they attach loosely. The triangle in Bax represents its BH3 domain, which needs to be exposed in order for Bcl-x_L-mediated retrotranslocation to occur. OMM: outer mitochondrial membrane.

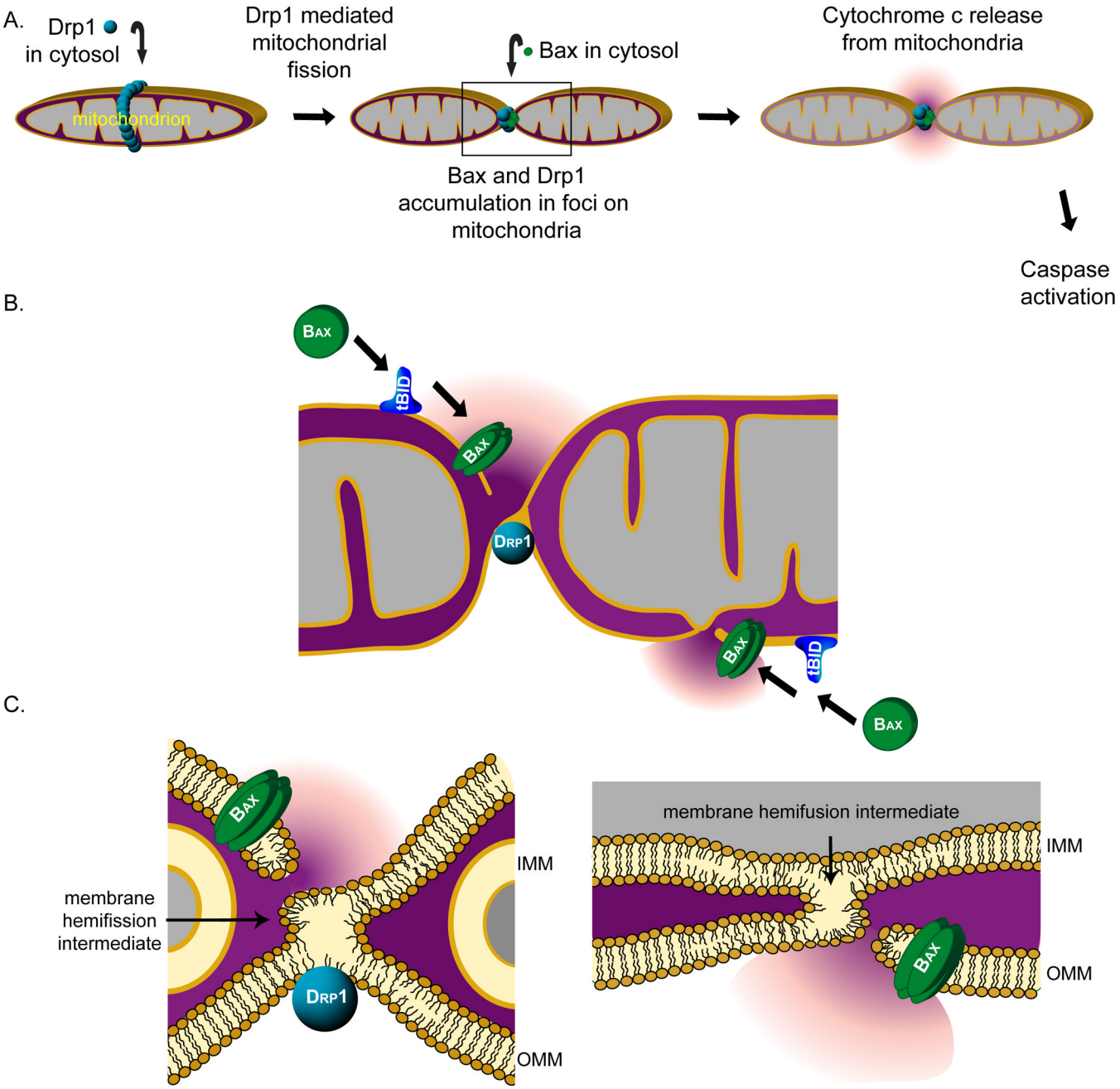

Figure 3. Model for Bax activation and mitochondrial outer membrane permeabilization: the role of membrane hemifission and hemifusion intermediates

A) During apoptosis, Drp1 and Bax are recruited to mitochondrial foci. As a result MOMP occurs concomitantly with mitochondrial fission. The release of cytochrome c promotes caspase activation in the cytosol. A magnification of what occurs at the level of mitochondrial fission sites is shown in (B). According to the 'embeded together model', tBid first inserts in the outer mitochondrial membrane (OMM) to expose its BH3 domain. Upon interaction with the Bid BH3 domain, Bax is in turn recruited to the OMM where it inserts and oligomerizes. This process does not occur randomly at the OMM surface. Bax mainly oligomerizes at mitochondrial fission sites and at contact sites between the OMM and the

IMM. C) (Left) A detailed structure of the hemifission intermediate that forms before fission of the organelle. The membrane forms a non bilayer structure that represents a privileged site for the formation of a membrane hole. Upon its oligomerization in the OMM, Bax forms a lipidic pore or triggers the formation of a hole at the edge of the hemifission intermediate. (Right) A detailed structure of the membrane at OMM and inner mitochondrial membrane (IMM) contact sites. The model postulates that Bax forms a pore in the membrane at the edges of membrane hemifusion intermediates that may form between the OMM and the IMM.