Newcomers in the process of mitochondrial permeabilization

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Summary
Under stress conditions, apoptogenic factors normally sequestered in the mitochondrial intermembrane space are released into the cytosol, caspases are activated and cells die by apoptosis. Although the precise mechanism that leads to the permeabilization of mitochondria is still unclear, the activation of multidomain pro-apoptotic proteins of the Bcl-2 family, such as Bax and Bak, is evidently crucial. Regulation of Bax and Bak by other members of the family has been known for a long time, but recent evidence suggests that additional unrelated proteins participate in the process, both as inhibitors and activators. The important rearrangements mitochondrial lipids undergo during apoptosis play a role in the permeabilization process and this role is probably more central than first envisioned.

Key words: Apoptosis, Mitochondria, Bcl-2, Bax, Bak, Lipids, Cardiolipin

Introduction
Programmed cell death is essential for the development of multicellular organisms, for tissue homeostasis, and to eliminate damaged and infected cells. Apoptosis is one form of programmed cell death. It is defined by specific biochemical and morphological changes, including DNA fragmentation, compaction of the chromatin into dense structures, blebbing of the plasma membrane, exposure of phosphatidylserine in the outer leaflet of the plasma membrane, and engulfment of the apoptotic bodies by phagocytes. These changes follow the activation of the cysteinyl aspartate-specific proteinases known as caspases (Fischer et al., 2003; Nicholson, 1999).

Two main pathways leading to the activation of caspases have been identified (Zimmermann et al., 2001). The first, known as the extrinsic pathway, is activated when ligands bind to death receptors on the plasma membrane. The second, the intrinsic pathway, is activated by various stress signals, such as DNA damage, growth factor withdrawal, or anoikis (cell detachment), and involves the permeabilization of mitochondria, which then release apoptogenic factors (e.g. cytochrome c, Smac/DIABLO and Omi/HtrA2) normally sequestered in the mitochondrial intermembrane space. Crosstalk can occur between these two pathways, as found in some cell types in which death-receptor-induced apoptosis involves mitochondria (Scaffidi et al., 1998). However, these are not the only pathways; recent work has identified alternative mechanisms for the activation of caspase-2 (Lassus et al., 2002), caspase-4 (Hitomi et al., 2004) and caspase-12 (Morishima et al., 2002; Nakagawa et al., 2000).

Permeabilization of mitochondria is regulated by proteins of the Bcl-2 family, which are defined by the presence of at least one of four Bcl-2-homology domains (BH1 to BH4) (Borner, 2003; Cory et al., 2003; Willis et al., 2003). Bcl-2 family proteins have been subdivided into three groups on the basis of their pro- or anti-apoptotic action and the BH domains they possess. Anti-apoptotic Bcl-2-like proteins (e.g. Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and A1/Bfl-1) have the four BH domains; pro-apoptotic multidomain proteins (e.g. Bax, Bak and Bok/Mtd) lack BH4; and, as their name indicates, pro-apoptotic BH3-only proteins (e.g. Bid, Bim/Bod, Bad, Bmf, Bik/Nbk, Blk, Noxa, Puma/Bbc3 and Hrk/DP5) possess only a BH3 domain. Some proteins of the Bcl-2 family have in addition a hydrophobic C-terminal tail that allows their association with membranes (Schinzel et al., 2004a). The level of expression of each member of the family is tightly regulated and post-translational modifications modulate their activities (Borner, 2003; Willis et al., 2003). Although their mechanisms of action are incompletely understood, heterodimerization between pro- and anti-apoptotic Bcl-2-like proteins is known to be important. Structural studies have revealed that a hydrophobic groove on the surface of anti-apoptotic Bcl-2 family members is the binding site for the BH3 domain of their pro-apoptotic counterparts (Petros et al., 2004).

Pro-apoptotic multidomain proteins seem to be the active members of the Bcl-2 family that trigger the release of mitochondrial apoptogenic factors into the cytosol. The presence of Bak or Bax is known to be crucial for numerous stress signals to induce cell death (Wei et al., 2001). By contrast, Bok/Mtd has been less examined, perhaps because of its restricted tissue distribution and its small number of known binding partners among Bcl-2 family members (Hsu et al., 1997a; Inohara et al., 1998). From extensive studies on the sequence of conformational changes that Bak and Bax undergo when they are activated, the following model has emerged (Fig. 1). In resting cells, Bak is an integral protein of the mitochondrial outer membrane, whereas Bax is cytosolic or loosely associated with the mitochondria in a form that can be detached from membranes by alkali treatment (Desagher et al.,...
Fig. 1. Model for the mechanism of activation of Bak and Bax. In resting cells, Bak is a tail-anchored protein of the mitochondrial outer membrane (MOM) and associates with VDAC2, whereas Bax is loosely attached to mitochondria or sequestered in the cytosol, probably through interactions with retention factors (14-3-3 isoforms σ, θ, ε, ζ; humanin; Ku70; αA- and αB-crystallin; Hsp70-dj1 and Hsp70-dj2). Pro168 plays a crucial role both in preventing the inappropriate exposure of the N-terminal domain of Bax and in unleashing the C-terminal tail of Bax from its hydrophobic pocket as it occurs when Bax is activated (Schinzel et al., 2004b). After apoptosis induction, the conformations of Bak and Bax change, leading to exposure of their N-terminal domains and to oligomerization. Apoptogenic factors are then released from the mitochondrial intermembrane space. Several proteins have been suggested to participate in the activation of Bak and Bax, although the mechanisms through which they contribute remain unclear. In addition, some proteins promote apoptosis by inhibiting Bcl-2-like proteins.

In response to certain apoptotic signals, the conformations of Bak and Bax change. Their N-terminal domains, which are not accessible to antibodies in the inactive proteins, become exposed, and Bak translocates to the mitochondria and integrates into the outer membrane as an alkali-resistant form (Desagher et al., 1999; Goping et al., 1998; Hsu et al., 1997b; Hsu and Youle, 1997; Makin et al., 2001). The subsequent oligomerization of Bak and/or Bax is thought to result in the permeabilization of the mitochondrial outer membrane (Antonsson et al., 2000).

Insertion of Bax into the mitochondria requires the release of its C-terminal hydrophobic tail from the hydrophobic pocket formed by its BH1, BH2 and BH3 domains (Nechushtan et al., 1999; Schinzel et al., 2004b). Deletion of proline 168 (Pro168) abolishes the capacity of Bax to translocate after a cytotoxic insult (Schinzel et al., 2004b). Isomerization or post-translational modification of Pro168 might therefore be required to disrupt the intramolecular interactions that maintain Bax as a soluble protein. Alternatively, Pro168 could be essential for the association of Bax with specific regulators (Schinzel et al., 2004b).

BH3-only proteins are crucial intermediates that link specific apoptotic stimuli to the permeabilization of mitochondria (Puthalakath and Strasser, 2002). Even though killing by BH3-only proteins requires Bax or Bak, most of them seem to promote apoptosis by binding to and inhibiting pro-survival Bcl-2 proteins (Fig. 2A) (Cheng et al., 2001). Only Bid (Desagher et al., 1999; Eskes et al., 2000; Wei et al., 2000), some Bim isoforms (Marani et al., 2002) and the distantly related protein MAP-1 (Tan et al., 2001) have been suggested to bind to Bax and Bak and induce their activation. Incubation of isolated mitochondria with recombinant Bax and tBid, which is the active form of Bid generated by proteolytic cleavage, leads to the oligomerization of Bax and to cytochrome c release (Desagher et al., 1999; Eskes et al., 2000). However, Bax cannot be activated if mitochondria are pre-incubated with proteinase K, which indicates that additional factors are required for tBid to activate Bax (Roucou et al., 2002). Analysis of the 3D structure of soluble Bax has revealed that binding of the BH3 domain of another Bcl-2 family member would not be enough to disrupt the interactions between the C-terminal tail of Bax and its hydrophobic pocket (Suzuki et al., 2000). However, some reports suggest that, in the presence of pure synthetic liposomes, tBid could be sufficient (Kuwana et al., 2002; Terrones et al., 2004).

Every mammalian cell type seems to require the expression of at least one anti-apoptotic Bcl-2-like protein to survive (Ranger et al., 2001). These proteins can protect cells from apoptosis at multiple levels (Cory et al., 2003; Gross et al., 1999). They can sequester BH3-only factors (Fig. 2B) (Cheng et al., 2001), and even though they do not appear to bind to inactive Bax and Bak, they can neutralize them if the latter have undergone conformational changes resulting in the exposure of their N-terminal domains, preventing their oligomerization and the release of apoptogenic factors from mitochondria (Desagher et al., 1999; Hsu and Youle, 1997; Perez and White, 2000; Ruffolo and Shore, 2003). Indeed, heterodimerization only occurs when non-ionic detergents, which artificially promote rearrangements that Bax and Bak normally undergo during the intrinsic pathway of apoptosis, are included in the extracts (Hsu and Youle, 1997). The pro-survival actions of Bcl-2-like proteins extend beyond the direct inhibition of pro-apoptotic members of the family, as they seem to control endoplasmic reticulum (ER) and mitochondrial homeostasis, probably by regulating Ca2+ fluxes (Distelhorst and Shore, 2004) and by mechanisms that defend against oxidative stress (Jang and Surh, 2003).

In addition to members of the Bcl-2 family, numerous proteins have been identified as Bax- and/or Bak-interacting proteins that prevent or promote the conformational
rarrangements that Bax and Bak undergo during apoptosis. Below, we focus on these new actors and discuss the roles that lipids have recently been suggested to play in the permeabilization of mitochondria.

**Activation of Bax and Bak**

Proteins that inhibit the activation of Bax and Bak

Several proteins have been reported to interact with Bax under resting conditions. Gel filtration analysis suggests that the soluble form of Bax is monomeric (Antonsson et al., 2000), but it might associate in vivo with cytosolic retention factors (Fig. 1). 14-3-3 isoforms (σ, θ, ε, ζ), the small peptide hominin, Ku70 and the apoptosis repressor with caspase-recruitment domain (ARC) were identified as Bax-interacting proteins whose overexpression inhibits Bax-dependent apoptosis, preventing its translocation to mitochondria (Guo et al., 2003; Gustafsson et al., 2004; Nam et al., 2004; Nomura et al., 2003; Samuel et al., 2001; Sawada et al., 2003). Similarly, Bax is retained in the cytosol when cells stably transfected with the Bax-interacting heat shock proteins (Hsp)s ααA- and αB-crystallin or the chaperone pairs Hsp70-dj1 and Hsp70-dj2 are exposed to stimuli that normally promote the intrinsic pathway of apoptosis (Gotoh et al., 2004; Mao et al., 2004). Knocking down hominin or Ku70 by RNA interference (RNAi) and knocking out 14-3-3σ only sensitizes cells to Bax-dependent apoptosis (Guo et al., 2003; Samuel et al., 2001; Sawada et al., 2003), whereas downregulation of ARC results in spontaneous exposure of the N-terminal domain of Bax and perhaps to increased cell death (Nam et al., 2004).

A more thorough examination of the properties of these proteins will be required to determine whether the binding of Bax is important for their anti-apoptotic actions. Proteins of the 14-3-3 family are adaptors that modulate the localization and activities of a wide array of targets and are known to have other anti-apoptotic activities – for example, through the sequestration of the BH3-only protein Bad – and to inhibit cell-cycle progression following DNA damage (Dougherty and Morrison, 2004). Therefore, modulating 14-3-3 levels will certainly affect several mechanisms that contribute to cell death and the effects observed cannot be unequivocally associated with Bax binding. Hominin was initially identified as a secreted peptide that has a neuroprotective effect, but under certain conditions it might bind to Bax in the cytosol and prevent its translocation to mitochondria (Hashimoto et al., 2001). The Ku70-Ku80 complex is involved in DNA double-strand repair. Although overexpression of Ku80 does not suppress Bax-mediated apoptosis (Sawada et al., 2003), the phenotypes of Ku70−/− mice and of Ku80−/− mice are similar (Gu et al., 1997). This suggests that, even though a cytosolic fraction of Ku70 associates with Bax, it might not be relevant under physiological conditions.

ααA- and αB-crystallin protect lens epithelial cells from cell death induced by a variety of apoptotic insults (Andley et al., 2000). However, in addition to Bax sequestration, two other protection mechanisms have been proposed: inhibition of pro-caspase-3 processing and inhibition of the upregulation of some pro-apoptotic genes (Kamradt et al., 2002; Mao et al., 2004). Because ααA- and αB-crystallin are primarily expressed in the lens and the phenotypes of knockouts do not suggest abnormal activation of apoptotic pathways (Brady et al., 1997; Brady et al., 2001), their Bax-binding activity does not appear to be a general mechanism of regulation of Bax. Hsp70-dj1 and Hsp70-dj2 complexes prevent Bax translocating to mitochondria after ER-stress-induced apoptosis. Hsp70 has been proposed to inhibit cell death at multiple levels and Hsp70 knockouts are characterized by genomic instability and increased radiosensitivity (Hunt et al., 2004). Therefore, the importance of the sequestration of Bax by these complexes will need to be examined further.

ARC is the only Bax-interacting protein whose downregulation results in exposure of the N-terminus of Bax and increased cell death (Nam et al., 2004). However, the insertion of Bax into the mitochondria and its oligomerization status have not been assessed, and cell death was only monitored by trypan blue exclusion in these experiments. Because ARC interacts with other proteins involved in apoptosis regulation, such as death receptors and adaptors,
pro-caspase-2 and pro-caspase-8, the relevance of its association with Bax for its anti-apoptotic actions still needs to be confirmed (Koseki et al., 1998; Nam et al., 2004).

Another potential mechanism of inhibition of Bax mitochondrial translocation involves hexokinase II. Overexpression of hexokinase II, a glycolytic enzyme associated with the mitochondrial outer membrane, also prevents the translocation of Bax to mitochondria (Pastorino et al., 2002). The overexpressed hexokinase might block access of Bax to contact sites that are already highly enriched in proteins (Majewski et al., 2004).

Voltage-dependent anion channel 2 (VDAC2), which is a relative of the VDAC protein perhaps implicated in mitochondrial permeabilization (see below), was recently identified as a Bak-interacting protein that stabilizes Bak in an inactive conformation (Cheng et al., 2003). Downregulation of VDAC2 sensitizes cells to multiple apoptotic stimuli, and VDAC2−/− mouse embryonic fibroblasts are more susceptible to apoptosis than are wild-type cells (Cheng et al., 2003).

Proteins that stimulate the activation of Bax and Bak

In addition to the proteins that might stabilize Bax and Bak in inactive conformations, others have been proposed to promote their activation (Fig. 1). Bax associates with endophilin B1a (also known as Bif-I) in FL5.12 cells, and the degree of interaction increases after withdrawal of interleukin 3 (Cuddeback et al., 2001). Endophilin B1a translocates to mitochondria during apoptosis (Karbowski et al., 2004) and its overexpression accelerates apoptosis under cytotoxic conditions (Cuddeback et al., 2001)). Endophilin B1a is similar to endophilin A1, a protein involved in the regulation of synaptic vesicle endocytosis that binds to components of the endocytosis machinery and can modify phospholipids in the bilayer (Huttner and Schmidt, 2002). Similarly, downregulation of endophilin B1a by RNAi alters mitochondrial morphology (Karbowski et al., 2004).

Endophilin B1a might contribute to the permeabilization of mitochondria by modifying the properties of the lipid bilayer or by bridging Bax to components of the mitochondrial fission machinery. Indeed, during apoptosis, mitochondria fragment and cluster in the perinuclear region (Desagher and Martinou, 2000; Frank et al., 2001), and components of the mitochondrial fusion and fission apparatus are thought to modulate their permeabilization (Karbowski and Youle, 2003).

The p53 tumor suppressor accumulates in cells exposed to various stress conditions and can either promote growth arrest or apoptosis (Slee et al., 2004). In addition to its effects on gene expression, which sometimes seem to be essential for apoptosis (Haupt et al., 2003), p53 can also promote cell death through transcription-independent mechanisms (Fig. 3) (Chipuk and Green, 2003; Chipuk et al., 2004; Slee et al., 2004). Following certain cytotoxic signals, a small fraction of p53 translocates to mitochondria (Erster et al., 2004; Marchenko et al., 2000). Interaction of p53 with Bcl-2 and Bcl-xL (Chipuk et al., 2004; Mihara et al., 2003), and with Bak (Leu et al., 2004), has been reported. However, although cytochrome c release has been observed by some groups when they incubate isolated mitochondria with purified p53 (Leu et al., 2004; Mihara et al., 2003), another group failed to see any release unless Bax is also included (Chipuk et al., 2004). Therefore, it is unclear whether p53 promotes the permeabilization of mitochondria by inhibiting pro-survival Bcl-2-like proteins and/or by activating Bak or Bax. p53 might also lead to caspase activation through mitochondria-independent pathways (Ding et al., 1998), perhaps by promoting the activation of caspase-2 (Lin et al., 2000; Tinel and Tschopp, 2004).

Upon DNA damage, p53 also upregulates the expression of several pro-apoptotic proteins that localize to mitochondria (Fig. 3). Among these, p53-regulated apoptosis-inducing protein 1 (p53AIP1) can interact with Bcl-2 (Matsuda et al., 2002), and the apoptosis-associated speck-like protein (ASC) can interact with Bax (Ohtsuka et al., 2004). Overexpression of either protein leads to cell death, and their downregulation inhibits apoptosis following DNA damage (Matsuda et al., 2002; Oda et al., 2000). ASC can also lead to caspase activation and apoptosis independently of Bax and Bak (Ohtsuka et al., 2004) and was recently shown to be important for maturation of the inflammatory caspase-1 (Mariathasan et al., 2004). ASC-deficient cells will need to be further tested in order to analyze their sensitivity to DNA-damage-induced cell death.

Under certain apoptotic conditions, pro-survival proteins of the Bcl-2 family seem not only to be neutralized but also even to become pro-apoptotic. For example, removal of the BH4 domains of Bcl-2 and Bcl-xL by caspase cleavage makes them apoptogenic (Cheng et al., 1997; Clem et al., 1998). Following certain apoptotic signals, the nuclear orphan receptor Nur77/TR3 translocates from the nucleus to mitochondria and promotes cytochrome c release (Li et al., 2000). It binds to Bcl-2, inducing the exposure of its BH3 domain and reducing the accessibility of its hydrophobic pocket (Lin et al., 2004). By interacting with Nur77/TR3, Bcl-2 becomes pro-apoptotic, but still requires Bak to lead to cell death (Lin et al., 2004). Even though Nur77 knockouts have a minimal phenotype (Lee et al., 1995), Nur77/TR3 could participate in T-cell apoptosis (Calnan et al., 1995). Recently, Kim et al. reported that Bcl-2 changes its membrane topology during apoptosis (Kim et al., 2004a). Although this could be required for the anti-apoptotic activity of Bcl-2, it might represent a mechanism through which Bcl-2 is converted to a pro-apoptotic factor (Puthalakath and Strasser, 2002).

Several other proteins can promote the permeabilization of mitochondria, but it is still unclear whether they also regulate members of the Bcl-2 family. The linker histone H1 is released into the cytosol when cells are treated with DNA-damaging agents that cause double-strand breaks (Konishi et al., 2003). The subtype H1.2 induces the activation of Bak and release of cytochrome c, but no direct interaction with Bcl-2 proteins has been detected (Konishi et al., 2003). After apoptosis induction, the actin-depolymerizing factor cofilin rapidly translocates from the cytosol to mitochondria (Chua et al., 2003). Downregulation of cofilin prevents apoptosis, whereas the overexpression of a cofilin mutant that constitutively localizes to mitochondria leads to cell death (Chua et al., 2003). Finally, the expression level of the chloride intracellular channel mtCLIC/CLIC4 increases under several apoptotic conditions (Fernandez-Salas et al., 1999; Fernandez-Salas et al., 2002). Cell death that follows the overexpression of mtCLIC/CLIC4 is independent of Bax but is inhibited by Bcl-2 (Suh et al., 2004), and downregulation of mtCLIC/CLIC4 inhibits p53-induced cell death (Fernandez-Salas et al., 2002). mtCLIC/CLIC4 could promote classical intrinsic apoptotic
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pathways by modifying intra-organelle Cl" levels, but it remains possible that at the mitochondria it directly promotes cytochrome c release.

Relevance of these interactions
Most of the putative Bax and Bak regulators have been identified by co-immunoprecipitation, and their relevance to cell death has been proposed from studies showing modulation of the sensitivity to apoptotic insults following their overexpression or their downregulation by RNAi. Such results are important, but should be interpreted with caution. For example, the multiplicity of Bcl-2-binding partners could be explained by the stickiness of the Bcl-2-like proteins. It is also difficult to understand how downregulation of each putative Bax-binding protein always results in approximately 30% inhibition of, or sensitization to, apoptosis. Indeed, it is conceivable that downregulation of a bona fide Bax-binding protein might have no impact on cell death, but such results have so far never been reported. Even if they do not allow the resolution of all the issues regarding the roles of these Bax regulators, a thorough analysis of knockouts and their sensitivity to apoptotic insults, together with a detailed study of the conformations of Bax and Bak, will be required to determine precisely which of these proteins, if any, are relevant apoptosis regulators.

Permeabilization of mitochondria
The precise mechanism that leads to the permeabilization of mitochondria is still unclear, despite several models having been extensively debated (Crompton et al., 2002; Desagher and Martinou, 2000; Martinou and Green, 2001; Waterhouse et al., 2002). The first invokes opening of permeability transition...
PT) pores at contact sites between the inner and outer mitochondrial membranes, subsequent swelling of the matrix and rupture of the outer membrane, which has a smaller surface area than the inner membrane. VDAC, the adenine-nucleotide translocator (ANT) and the matrix chaperone cyclophilin D were initially proposed to be central components of these pores (Crompton et al., 1998; Halestrap et al., 2002), and Bcl-2 family proteins were proposed to regulate their opening (Zamzami and Kroemer, 2001). Inhibition of cytochrome c release by the cyclophilin-D-binding drug cyclosporin A (CsA) supports this model. However, CsA is not as specific as initially thought, and binds not only to all the members of the cyclophilin family but also to other proteins (Fruman et al., 1992; Moss et al., 1992). Moreover, it partitions in membranes and could affect their permeability indirectly by modifying the properties of the lipid bilayer (Epand et al., 2002a; McGuinness et al., 1990). Furthermore, this model has recently been challenged by the observation that ANT-deficient mitochondria can still undergo PTs and ANT-deficient cells can die by apoptosis (Kokoszka et al., 2004).

An alternative model is that modulation of the properties of VDAC by Bcl-2 proteins is sufficient to control the permeability of mitochondria (Tsujimoto and Shimizu, 2000). Finally, given their structural similarity to some bacterial toxins, Bcl-2 family proteins might form proteinaceous channels themselves (Epand et al., 2002c; Saito et al., 2000). These models might reflect the events that occur during apoptosis but are incomplete, because it is increasingly clear that lipids have an important role in the permeabilization of mitochondria.

Lipids and apoptosis
During apoptosis, lipid membranes undergo important rearrangements (Cristea and Degli Esposti, 2004; Wright et al., 2004). Exposure of phosphatidylserine on the external face of the plasma membrane and plasma membrane blebbing were the first of these to be discovered. More recently, several groups reported that the levels of cardiolipin diminish when cell death is induced by a variety of conditions (Matsko et al., 2001;
Cardiolipin is a negatively charged phospholipid that mainly resides in the mitochondrial inner membrane, where it is required for the activities of several proteins of the electron transport chain, including cytochrome c, and of several mitochondrial carriers, including ANT (McMillin and Dowhan, 2002). Several groups have reported that peroxidation of cardiolipin occurs during apoptosis (Garcia Fernandez et al., 2002; Nomura et al., 2000) and is required for detachment of cytochrome c from the mitochondrial inner membrane and its complete release (Nomura et al., 2000; Ott et al., 2002; Petrosillo et al., 2001; Shidoji et al., 1999).

Cardiolipin also appears to be present in the mitochondrial outer membrane, enriched at contact sites with the inner membrane, where it could interact with Bcl-2 family proteins (Ardail et al., 1990; Hovius et al., 1993; Qi et al., 2003). Indeed, activated Bax seems to permeabilize synthetic liposomes only if cardiolipin is present (Kuwana et al., 2002; Terrones et al., 2004). Interestingly, the number and the surface area of contact sites increase in rod photoreceptors of mice exposed to toxic insults (He et al., 2003), and oligomeric Bax lacking its C-terminal tail (BaxΔC) does not permeabilize outer membrane vesicles if the contact sites have been removed (Wieckowski et al., 2001). These results highlight the significance that the specific lipid and/or protein composition of contact sites might have for the action of Bax and Bak.

Upon activation, tBid is recruited to mitochondria, in particular to contact sites, where it binds to cardiolipin or its derivative monolysocardiolipin, produced during Fas-induced cell death (Lutter et al., 2000; Sorice et al., 2004). When mouse liver mitochondria are incubated with tBid and when cells undergo apoptosis, mitochondrial cristae remodel (Scorrano et al., 2002). They become more interconnected and cristae junctions widen. CsA inhibits remodeling (Scorrano et al., 2002), either because CsA targets a protein involved in the process or because of its direct effects on membrane curvature and stability (Epand et al., 2002a; Epand et al., 1987). Pre-incubation of mitochondria with the cardiolipin-specific dye 10-N-nonyl acridine orange (NAO) prevents the binding of tBid to contact sites, and cristae remodeling and cytochrome c release are abolished, even though Bak still oligomerizes (Kim et al., 2004b). Such cristae opening might increase the pool of cytochrome c available for release. However, association of tBid with contact sites might also be directly necessary for the complete permeabilization of mitochondria by activated Bax or Bak.

Oligomeric Bax, which stimulates transbilayer lipid diffusion (Epand et al., 2003; Terrones et al., 2004), and tBid, which possesses a lipid transfer activity (Degli Esposti, 2002; Esposti et al., 2001), might also contribute to the redistribution of cardiolipin between the mitochondrial membranes, to intracellular organelles and to the plasma membrane, as seems to occur during apoptosis (Qi et al., 2003; Sorice et al., 2004). A rat liver fatty-acid-binding protein released from isolated mitochondria after incubation with tBid could also contribute (Van Loo et al., 2002).

Other studies have focused on the influence of the composition of liposomes on their permeabilization by activated Bak. Although a particular lipidic composition might be required only for oligomeric Bax to adopt an active conformation, lipids seem to play an active part in the permeabilization process. Basañez and coworkers reported that this is potentiated by lipids that have a positive intrinsic curvature, such as lysophospholipids [oleoyl-phosphatidylcholine (LPC) and oleoyl-phosphatidylethanolamine (LPE)], and is inhibited by lipids that have a negative intrinsic curvature, such as dioleoylglycerol (DOG) or dioleoylphosphatidylethanolamine (DOPE) (Basañez et al., 2002; Terrones et al., 2004) (Fig. 4). Moreover, oligomeric Bax destabilizes planar lipid bilayers, such that they ultimately rupture, and this effect is similarly modulated by the presence of the above-mentioned lipids (Basañez et al., 1999; Basañez et al., 2002). In addition, both LPC and LPG increase the amount of cytochrome c that is released upon incubation of mouse liver mitochondria with tBid (Degli Esposti, 2002; Esposti et al., 2003). This behavior is reminiscent of the bacterial pore-forming peptide magainin (Matsuzaki et al., 1998) and suggests that the mechanism of Bax-induced permeabilization of synthetic liposomes involves lipid pores (Fig. 4). The Bax-interacting protein endophilin B1a binds to fatty acids and possesses a lysophosphaticid acid acyltransferase activity similar to that of endophilin A1, which participates in synaptic vesicle formation by increasing membrane positive curvature (Modregger et al., 2003). Endophilin B1a could similarly help Bax create lipid pores in the mitochondrial outer membrane.

Epand and colleagues have reported that the permeabilization and destabilization of membranes that are induced by tBid are instead potentiated by lipids that promote negative membrane curvature and a lamellar-to-hexagonal II phase transition (Fig. 4), and are inhibited by compounds, such as CsA, that favor positive curvature (Epand et al., 2002a). Formation of non-lamellar structures is known to be important for membrane fusion and fission events (Melikyan and Chernomordik, 1997; Ortiz et al., 1999) and has also been suggested to occur in the plasma membranes of cells undergoing apoptosis or necrosis (Aguilar et al., 1999). Interestingly, cardiolipin tends to adopt a hexagonal II phase in the presence of divalent cations that reduce the electrostatic repulsion between its negatively charged headgroups (Ortiz et al., 1999), and an increase in cytosolic Ca²⁺ levels is often observed during apoptosis. Epand and colleagues have also reported that permeabilization of liposomes by oligomeric BaxAC requires Ca²⁺ and/or Mg²⁺ (Epand et al., 2002b; Epand et al., 2004). However, they also observed that it is activated by fatty acids and inhibited by LPC and dilsocardiolipin, even if all these lipids promote positive membrane curvature (Epand et al., 2004). Therefore, changing membrane curvature might not be the only mechanism through which lipids can influence the permeabilization of liposomes by activated Bax.

The results obtained in studies of synthetic liposomes must, however, be considered with caution, because the structure adopted by lipids is profoundly influenced by the experimental conditions, including salt concentration, pH, presence of divalent cations, temperature, transmembrane potential and nature of the fatty acyl chains of the phospholipids (Lewis and McElhaney, 2000; Ortiz et al., 1999). Moreover, artificial membranes do not reflect some of the important properties of biological membranes, in particular their mosaic nature, with organized protein and lipid microdomains. Understanding the importance of the lipidic composition of mitochondria for their permeabilization by Bax and Bak will certainly require
working with cells and knocking down of enzymes involved in the biogenesis and the metabolism of mitochondrial lipids.

Conclusions
The number of molecules suggested to be involved in the regulation of apoptosis is increasing daily, yet important doubts remain concerning the mechanism of permeabilization of the mitochondrial outer membrane. The multiple binding partners reported for proteins of the Bcl-2 family could reflect the need to integrate information about the complex state of a cell. However, care must be taken, because Bcl-2 proteins are sticky and their conformation is influenced by the experimental conditions, in particular the presence of detergents. Most studies are carried out with cancer cell lines, in which components of the death pathways are altered. We must therefore emphasize the need to confirm observations in several cell types and ideally in primary cultures. Finally, even though lipids certainly play an important role in mitochondrial permeabilization, it remains very difficult to analyze their contributions in vivo. Therefore indirect evidence (e.g. analysis of Bax/Bak activation and cytochrome c release after changing the expression levels of lipid-modifying enzymes) will be needed, and innovative methods will have to be developed.

Apoptosis and necrosis are only extremes of an important array of mechanisms by which cells can die (Leist and Jaattela, 2001; Lockshin and Zakeri, 2002). Indeed, proteolytic degradation and autophagy can share some of the characteristics of the death pathways. Understanding the relationship between these pathways will certainly prove crucial to the development of new therapeutic agents to provoke the death of cancer cells, in which one or several of these pathways may be inactivated.

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