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Mechanisms of cytochrome *c* release by proapoptotic BCL-2 family members

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Abstract

A crucial amplificatory event in several apoptotic cascades is the nearly complete release of cytochrome *c* from mitochondria. Proteins of the BCL-2 family which include both anti- and proapoptotic members control this step. Here, we review the proposed mechanisms by which proapoptotic BCL-2 family members induce cytochrome *c* release. Data support a model in which the apoptotic pathway bifurcates following activation of a "BH3 only" family member. BH3 only molecules induce the activation of the multidomain proapoptotics BAX and BAK, resulting in the permeabilization of the outer mitochondrial membrane and the efflux of cytochrome *c*. This is coordinated with the activation of a distinct pathway characterized by profound changes of the inner mitochondrial membrane morphology and organization. This mitochondrial remodelling insures complete release of cytochrome *c* and the onset of mitochondrial dysfunction that is a typical feature of many apoptotic deaths.

Programmed cell death is a well-conserved pathway whose basic tenets appear common to all metazoans. Key components identified by genetic and biochemical approaches regulate the commitment step and/or participate in effecting cell demise [1]. Intracellular organelles are key participants in apoptosis, of which mitochondria are the most well documented [2]. The manifold aspects of mitochondrial involvement in apoptosis include two crucial events, the release of proteins normally stored in the intermembrane space, including cytochrome *c* [3] and the onset of multiple parameters of mitochondrial dysfunction [4]. Cytochrome *c* triggers a post-mitochondrial pathway forming an oligomeric complex of cytochrome *c*/Apaf-1/caspase-9, the "apoptosome," which activates the initiator caspase-9 to subsequently cleave the effector caspases-3 and -7 [5]. Cells deficient for cytochrome *c* [6], Apaf-1 [7], caspase-9 [8] or caspase-3 [9] display defects in apoptosis following intrinsic signals providing further testimony to the importance of this pathway.

The BCL-2 family proteins are critical death regulators that reside immediately upstream of mitochondria and consist of both anti- and proapoptotic members. BCL-2 family members possess conserved α-helices with sequence conservation clustered in BCL-2 homology (BH) domains. Antiapoptotic members exhibit the homology in all segments BH1 to 4, while proapoptotic molecules lack stringent sequence conservation of the first α-helical BH4 domain and can be further subdivided into "multidomain" and "BH3-only" proteins (Fig. 1). Multidomain proapoptotic members such as BAX and BAK display sequence conservation in BH1-3 domains. BH3-only members display sequence conservation only in the amphipathic α-helical BH3 region [10]. The BH3-only proteins studied to date reside upstream in the pathway. Death signals trigger their activation by transcriptional regulation or post-translational modification to connect proximal signals with the core apoptotic pathway [11]. While considerable detail has been revealed, uncertainties still exist concerning the precise mechanism(s) by which proapoptotic BCL-2 family members regulate the release of cytochrome *c* from mitochondria. Here, we review data that support the existence of a bifurcated cytochrome *c* release pathway controlled in concert by BH3-only and

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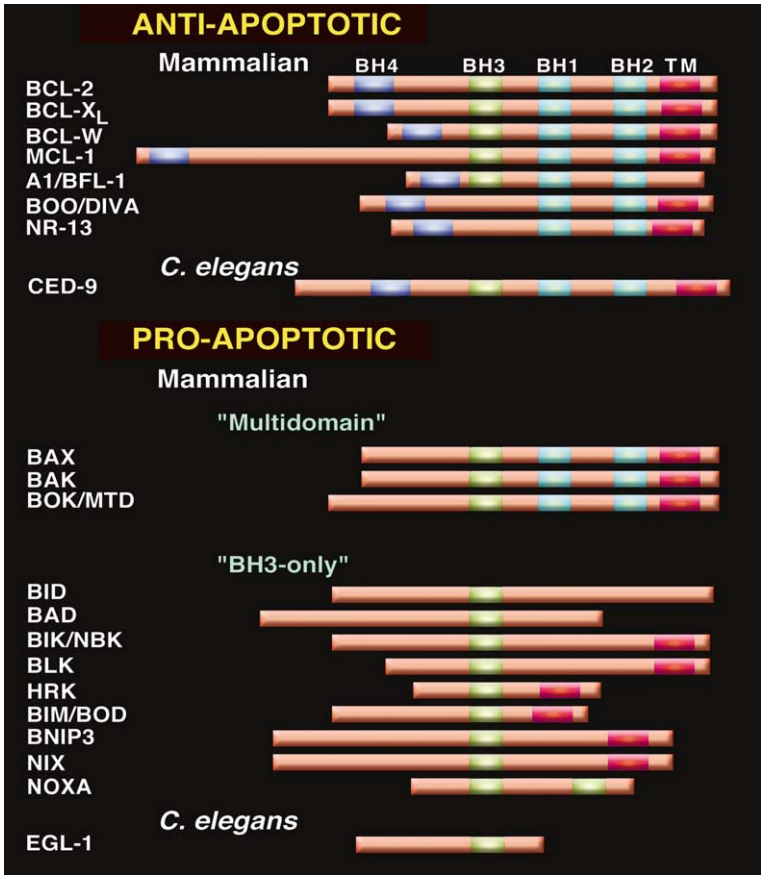

Fig. 1. Summary of anti- and proapoptotic BCL-2 family members. BCL-2 homology domains are highlighted.

multidomain proapoptotic BCL-2 family members. One arm activates the multidomain proapoptotics and grants a physical pathway for cytochrome *c* release across the outer mitochondrial membrane; the other, independent of BAX, BAK, orchestrates changes of mitochondrial inner membrane necessary to grant complete release of cytochrome *c* and mitochondrial dysfunction.

Cytochrome *c* release: activation of multidomain BAX, BAK

Activation

Following a variety of intrinsic and extrinsic death signals, BH3 only proteins undergo post-translational modifications that range from dephosphorylation [12] to cleavage [1,10,13]. Such modifications result in activation of BH3-only proteins and often in their translocation to mitochondria, where they exert their biological function. The efficiency of the translocation process can be enhanced for example by modifications such as the N-terminal myristoylation of tBID that follows its cleavage by caspase-8 [14]. Alternative hypotheses have been proposed for the mechanism by which BH3 only proteins exert their biological actions. At least four main different models have been put forth: (i) BH3 only proteins autonomously induce cytochrome *c* release, without the participation of BCL-2 family or intrinsic mitochondrial proteins but with a marked requirement for negatively charged lipids such as cardiolipin [15,16]; (ii) they interact and inhibit antiapoptotic BCL-2 family members [17–21]; (iii) they activate the multidomain proapoptotic BAX and BAK to mediate cytochrome *c* release [5,22,23]; (iv) they interact with intrinsic mitochondrial proteins such as the adenine nucleotide exchanger (ANT) or the voltage dependent anion channel (VDAC) to induce mitochondrial dysfunction and cytochrome *c* release [24,25]. All BH3-only molecules tested, including BID, BIM, BAD, and NOXA, require BAX, BAK to exert their mitochondrial pro-apoptotic activity, as evidenced by the lack of cytochrome *c* release and apoptosis in *Bax*, *Bak* doubly deficient cells [5,22]. Yet, most BH3-only proteins, including BAD, BIM, NOXA, and BIK, display a marked binding preference for antiapoptotic members BCL-2, BCL-X_L versus the multidomain proapoptotics in multiple interaction assays of yeast two-hybrid, pulldown, or coimmunoprecipitation from detergent-solubilized lysates [26,27]. Moreover, functional and mutational analysis suggests

that BAD promotes death only when it binds to BCL-X_L or BCL-2 [20,26,28]. Insight into this apparent dichotomy came from the functional analysis of BH3 peptides from the different BH3 only proteins. Using synthetic peptides it first of all has been elucidated that the BH3 regions are in and of themselves functional domains. BH3 domains of BID and BIM when added directly to mitochondria activate BAX and BAK. Conversely, the BH3 domains of BAD and BIK cannot directly trigger BAX, BAK, but instead preferentially bind to antiapoptotic BCL-2 members. This in turn enables a displacement reaction in which lower affinity BID-like activator BH3-only proteins are displaced from the antiapoptotic pockets [29]. Thus, "sensitizing," BAD-like BH3 domains can preferentially act on antiapoptotic members and thus enable subliminal concentrations of BID-like activators to induce activation of the multidomain proapoptotics BAX, BAK. Studies of *Bad*-deficient as well as a constitutively active non-phosphorylatable Bad^{3SA} knockin mouse are consistent with this "sensitizer" role for BAD in response to the presence or absence of survival factors in which BAD sets the threshold of susceptibility to apoptotic stimuli [30]. We believe that these convergent genetic and functional analyses indicate that BH3-only molecules require the multidomain proapoptotics to induce cytochrome *c* release and death.

What is the activation event for the multidomain proapoptotic BAX and BAK? The measurable hallmark of BAX, BAK activation is an exposure of new epitopes and their homo-oligomerization in the outer mitochondrial membrane [31–33]. BAX and BAK differ in their intracellular localization prior to death stimuli. While BAK is already an integral protein of the mitochondrial outer membrane, BAX in viable cells resides as a monomer in the cytosol or loosely attached to intracellular membranes. Approximately 10–15% of BAX or BAK is also localized at the endoplasmic reticulum [34]. Activation of BAX requires the further step of translocation as well as integration into mitochondrial membranes. The structure of monomeric, soluble BAX reflecting its inactive conformation indicates that its C-terminal α9 helix, essential for mitochondrial targeting, is bound in the hydrophobic pocket formed by the BH1, 2, 3 domains of the molecule. Following activation, a structural change occurs and α9 is somehow displaced from the BH3 pocket [35]. The structural similarity between multidomain BAX and BCL-X_L and BCL-2 suggests that the BH3 domains of BH3 only proteins could occupy the BH3 pocket of BAX. However, the pouch is normally occupied by α9 and hydrophobic interactions stabilize this intramolecular association. Whether the BID-like activator BH3 domains can in and of themselves drive the disengagement of α9 by competition for the binding pocket of BAX is uncertain [29,35]. It is possible that further molecules, lipid or protein, may be involved in regulating the conformation of BAX and BAK. Since BAK is already integrated in the outer mitochondrial membrane, its C-terminal α helix is unavailable, suggesting that it will prove to utilize other resident mitochondrial molecules to regulate its conformation. BAX or BAK activation results in higher order homo-oligomers within the mitochondrial membrane. Gel filtration and crosslinking experiments substantiate this in that BAX oligomers were found only in the mitochondrial membranes after apoptotic stimulus or BAX activation by BID [33,36]. Protein crosslinking reveals a predominance of multimeric species with the size of BAX homodimers, trimers, and tetramers [36]. The mass of oligomers assessed by size exclusion chromatography in the presence of detergent, approximately 96–260 kDa, was consistent with even larger complexes [33]. Following activation by BID [31], BIM [22] or by BH3 domains of BH3-only proteins [29], BAK undergoes a similar homo-oligomerization generating distinct complexes revealed by crosslinking, again consistent with dimers, trimers, and prominently tetramers. A global change in the conformation of the protein accompanies BAK oligomerization, as shown by the change in the sensitivity of BAK to digestion by proteases [31]. The combination of genetic analysis and functional studies in reconstituted systems substantiate the physiological role of activated BAX and BAK in the permeabilization of the outer mitochondrial membrane and the release of IMS proteins, including cytochrome *c*. The development of tools to analyze BAX, BAK and their homo-interaction in real time at the single cell level will also help decipher the serial timecourse of these events.

Function

Activation of the multidomain proapoptotics results in the release of cytochrome *c* from mitochondria to effect caspase activation and a series of other molecules, including SMAC, endonuclease G, and AIF, which augment cell death. Genetic analysis confirmed that BAX, BAK are required to mediate cytochrome *c* release and death in response to a panoply of intrinsic stimuli [23], including response to BH3-only proteins BID, BAD, BIM, and NOXA [22]. Type II cells that require a mitochondrial amplification loop [37] following extrinsic death receptor signals (Fas and TNFR1) [23,38] also required BAX, BAK [23]. The ability of BAX, BAK to release cytochrome *c* coincides with their oligomerization. Enforced dimerization of FKBP-BAX chimera in intact cells results in translocation of FKBP-BAX to mitochondria insertion of multimers and release of cytochrome *c* [39]. Purified mouse liver mitochondria that lack BAX, release cytochrome *c* across the outer membrane in response to tBID only if BAK is present and is allowed to oligomerize [31,40]. Moreover, tBID

will activate recombinant BAX and result in release of cytochrome *c* from *Bak*-deficient mitochondria [23]. A major question is how oligomerized multidomain BAX, BAK affect the release of proapoptotic activators from mitochondria. Current models include (i) a global effect on the permeability of the OM, this includes a number of possibilities, including a concept of "lipid" channels in the bilayer [15,16]; (ii) interactions between BAX and resident mitochondrial proteins such as VDAC [41] or ANT [42] which are proposed to release cytochrome *c* directly or trigger the mitochondrial permeability transition (PT); and (iii) that BAX, BAK oligomers generate a pore in the outer mitochondrial membrane permeable to cytochrome *c* and possibly to other proapoptotic proteins.

The interaction of multidomain proapoptotics with VDAC and ANT, proposed components of the PT pore (PTP) a high conductance, non-selective inner mitochondrial membrane channel whose opening increases inner membrane permeability to solutes up to 1500 Da [43], has been pursued. First of all, it is uncertain whether ANT or VDAC will prove to be primary constituents of the PTP, an issue that awaits definitive biochemical and genetic evidence (see [43] for a discussion). Contrasting results have emerged from studies that attempted to measure evidence of PT, such as mitochondrial swelling and depolarization in response to activated BAX. While under some experimental conditions active BAX (and BAK) was noted to induce PT [42,44,45], others reported that BAX was unable to promote it [46,47]. Some of this discrepancy may reflect (i) studies in isolated mitochondria do not completely reproduce what occurs in situ, where the cellular milieu and the complexity of mitochondrial architecture differs and (ii) many of the techniques used in isolated mitochondria lack sensitivity and are not able to measure "transient" PT. Openings of the PTP can be transient [48–50], as originally noted at the single channel level in patch-clamp experiments [51], in that the PTP rapidly flickers over msecs between open and closed states. These openings are not associated with detectable mitochondrial swelling and/or depolarization, yet can be measured following the release of fluorescent dyes of appropriate molecular mass entrapped in the mitochondrial matrix [48]. Further insight was provided by the groups of Farber and Orrenius who, respectively, showed that depending on the experimental condition, active BAX could either induce a "full" PT, accompanied by swelling and depolarization, or a transient one, lacking all of the above, but associated with the release of calcein, a $\sim$680 Da fluorophore previously entrapped in the matrix [52]. Oligomeric BAX appears to release cytochrome *c* in the absence of a PT, while monomeric BAX induces the PT [53].

Activated BAX, BAK may form pores in the OMM that directly or indirectly allow the efflux of cytochrome *c* and perhaps of the other IMS proteins that drive death effector pathways. Supporting evidence is provided by recombinant BAX which forms homo-oligomeric pores in liposomes which in a concentration dependent fashion will release cytochrome *c* from liposomes. A Hill plot of the kinetics of release indicates a slope that progresses from 2 to 4 participating molecules of BAX. Release through a putative pore at a BAX molecularity of 4 can be competed by blocking molecules that size the pore at 22 Å, slightly bigger than soluble cytochrome *c* [54]. Electrophysiological analysis of outer mitochondrial membrane patches isolated from apoptotic cells has reported novel, high conductance channels. Their properties are comparable to channels recorded in yeast mitochondria after treatment with recombinant BAX. Interestingly, these channels were also noted in mitochondria from VDAC defined strains, suggesting that this intrinsic OMM protein is not required [55].

Irrespective of the exact mechanism by which active BAX, BAK release cytochrome *c*, it appears clear that these multidomain proapoptotics act as the essential gateway to the mitochondria, serving as the critical step of their engagement. Recent data indicate that while this step is requisite there is an additional complex process, resulting in a massive remodelling of mitochondrial ultrastructure.

A second mitochondrial pathway remodels the inner membrane and mobilizes cytochrome *c*

Analysis of cytochrome *c* release has argued that its kinetics are rapid and its extent is remarkably complete [56,57]. In vivo observations substantiate this, in that following Fas receptor activation most cytochrome *c* is released from liver mitochondria within 60 min and irreversible damage to mitochondria follows [58]. Discrete levels of cytochrome *c* are required in the cytosol to activate the death response [59]. In certain cells the amount of cytochrome *c* released is critical to overcoming protection by the IAP caspase inhibitors [60–62]. This may be of particular importance in neoplastic cells, where IAPs are elevated.

The complete release of cytochrome *c* must be put into the context of our current understanding of mitochondrial ultrastructure. The availability of electron microscopic techniques, in particular of high-voltage electron microscopic (HVEM) tomography, led to a remarkable change in our view of the internal structure of mitochondria. Three-dimensional reconstruction of mitochondria after HVEM tomography revealed that the intermembrane space is very narrow, as the average distance between the outer and inner boundary membranes is only $\sim$20 nm. The pleomorphic, tubular *cristae* constitute highly sequestered compartments separated from the IMS by narrow *cristae* junctions, with average

diameters of only ~20–25 nm [63]. The majority of oxidative phosphorylation complexes preferentially localize to the *cristae* [64], as do most (~85%) of the cytochrome *c* stores [65]. This is consistent with functional estimates that only 15–20% of total cytochrome *c* is available in the IMS [66]. Computer models indicate that this subcompartmentalization has a functional counterpart in the generation of ion and ADP diffusion gradients across the narrow cristae junctions [67]. We therefore need to explain how the stores of cytochrome *c* that appear to be so heavily subcompartmentalized can be fully released in the absence of mitochondrial swelling, as is frequently observed in the course of apoptosis [31,36,68].

Our recent data indicate that such full cytochrome *c* release reflects a pathway of mitochondrial remodelling, that can be followed, occurs in isolated mitochondria in response to tBID and in situ after several apoptotic stimuli (Fig. 2) [40]. A genetic dissection indicated that in response to tBID mitochondria initially release only the small fraction of cytochrome *c* (~15%) in the IMS, and this release across the OMM depends on BAK. The bulk of cytochrome *c*, which by functional measurements we found normally stored in the cristae, must be mobilized before it can be released. This is accomplished by a remarkable degree of remodelling of the inner mitochondrial membrane (IMM), as revealed by serial morphometry and electron microscopic tomography coupled to three dimensional reconstructions. We recognized distinguishable stages of inner membrane morphology, which we entitled Class I–IV. Morphological parameters that, respectively, characterize these classes are summarized in Table 1. Class II mitochondria represent the crucial stage that occurs early after the administration of the apoptotic stimulus in vitro and in situ. These mitochondria possess previously undescribed characteristics. There are no substantial changes to the relative volume occupied by the matrix or the cristae space. However, the cristae are markedly interconnected and appear to be fused into a handful or perhaps a quasi-single compartment. Fusion is accompanied by shortening of the elongated tubular junction that connects the cristae compartment with the IMS and by a remarkable widening of the narrow cristae junction. This morphologic change, including the widening of the narrow tubular cristae junctions is accompanied by the complete availability of the cytochrome *c* stores for release across the outer membrane. Mitochondrial remodelling, while triggered by tBID, occurs independent of its BH3 domain and does not require multidomain BAX, BAK. The observed remodelling of the inner membrane associated with mobilization of cytochrome *c* is generally consistent with previous reports of mitochondrial ultrastructural changes in early stages of apoptosis [69–72]. Class II mitochondrial morphology characterizes early stages of apoptosis following Fas activation of hepatocytes in vivo [58], TNF receptor engagement [73,74], or treatment of target cells with granzyme B [75]. Moreover, additional events are also critical for complete release of cytochrome *c* including

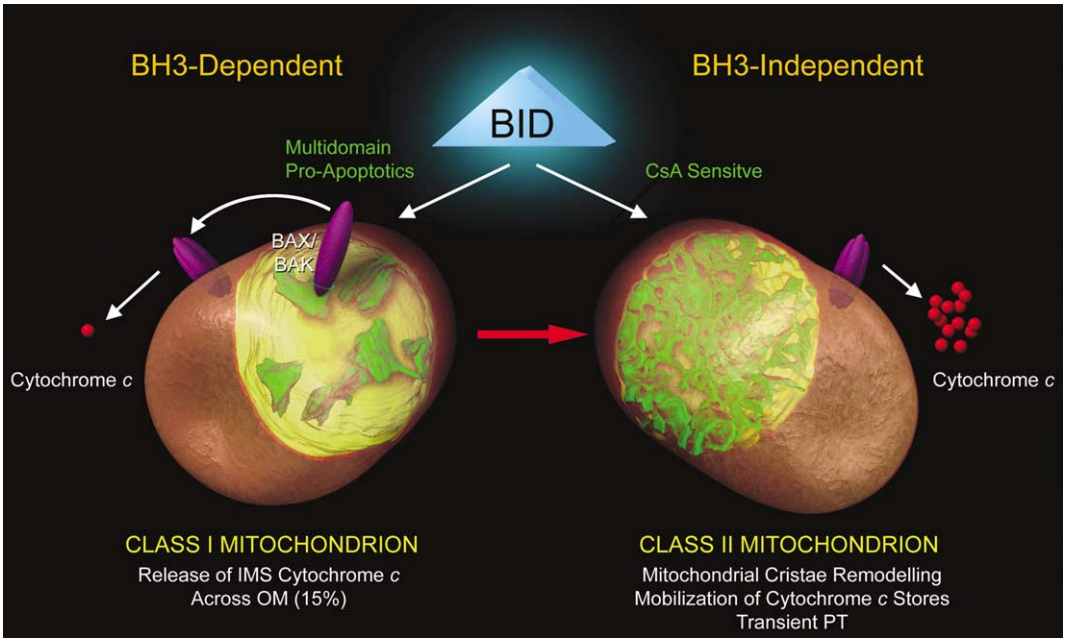

Fig. 2. Schematic of the two apoptotic pathways triggered to reach complete cytochrome *c* release. Following a BH-3 only proapoptotic stimulus, BAX and BAK oligomerize in the outer membrane, generating the physical pathway for cytochrome *c* release that grants the release of the initial 15% stored in the intermembrane space. A second pathway of inner membrane remodelling occurs independently from the multidomain proapoptotics but sensitive to cyclosporine A and results in the occurrence of Class II mitochondria which mobilize the cytochrome *c* (85%) stored in the cristae.

Table 1
Characteristics of Class I–IV mitochondria

Feature	Class I	Class II	Class III	Class IV
Occurrence	Before apoptotic stimulus	Early after apoptotic stimulus	Rare, later in the progression of apoptosis	Rarest, terminal mitochondrial deregulation
Outer membrane	Intact	Intact	Asymmetric rupture	Disrupted
Inner boundary membrane	Intact	Intact	Asymmetric blebbing trough the outer membrane	Disrupted
Matrix	Normal volume and electron density	Normal volume and electron density	Asymmetric swelling	Swollen
Cristae	Individual	Interconnected, fused	Fused in the conserved part, absent in the blebbed portion	Disrupted
Cristae junction	Tubular, elongated, narrow with an average diameter of 18 nm	Enlarged, shortened, widened with an average diameter of 58 nm (up to 70 nm)	Where present, widened with an average diameter of 28 nm	NA

the demonstrations by Orrenius and colleagues that cytochrome *c* detachment from its high affinity cardiolipin binding sites on the inner mitochondrial membrane is crucial [76]. Attardi and co-workers noted that only cells primed for apoptosis by Fas activation released all stores of cytochrome *c* upon selective permeabilization of the OM by digitonin [77]. We also found that the mobilization process, is accompanied by an increase in "soluble" vs. membrane bound cytochrome *c* prior to its release across the outer membrane [40]. Therefore, this pathway of mitochondrial remodelling may represent a crucial commitment step of mitochondria during early stages of apoptosis.

The mechanism(s) that regulate cristae remodelling during apoptosis warrant their exploration. We noted that transient PT accompanied the remodelling process and that the PT inhibitor CsA was capable of blocking the mobilization of cytochrome *c* as well. Other reports also noted that CsA could inhibit a substantial part of tBID-induced death [24]. Transient PT has been recorded in isolated mitochondria and intact cells and is not associated with mitochondrial swelling or depolarization [48–50]. This indicated that transient PT and mitochondrial remodelling share of at least one component, although it is uncertain whether transient PT and the remodelling process are identical. It has been suggested that components of the PTP reside at contact points between IM and OM [78]. One possibility for further testing is whether alterations at OM/IM contacts could affect the opening of *cristae* junctions and the changes to IM curvature noted in Class II mitochondria. In this context, mathematical modelling studies have recently proposed that the formation of complex cristae junction structures requires the outer membrane [79]. The capacity of CsA to block this process suggests that its mitochondrial target, cyclophilin D [80], could be a functional component of this remodelling process. Alternatively, a CsA/cyclophilin D complex might affect another mitochondrial protein by analogy with the mechanism by which CsA inhibits cytosolic calcineurin [81]. Another possibility is that CsA blocks remodelling by acting intrinsically on the curvature of the inner mitochondrial membrane. It has been proposed that tBid promotes negative curvature of membranes and as a result it destabilizes them [82]. Conversely, CsA induces positive membrane curvature, counteracting tBID effects [82]. The possibility that intrinsic membrane curvature plays a crucial role in regulating cristae remodelling is an attractive one. Lipid monolayers composed by most naturally occurring lipids tend to have a positive curvature. The inner membrane contains high concentrations of phosphatidylethanolamine, a so-called nonbilayer lipid. Nonbilayer lipids tend to form the hexagonal phase when purified and facilitate membrane fusion and exocytosis [83]. They may also facilitate the formation of the sharp bends found at the level of cristae junctions [79]. If BID possesses lipid binding, or even lipid transfer activity, as has been recently proposed [84], it might prove to be involved in changing intrinsic curvature by altering lipid composition. Mitochondrial remodelling during apoptosis may also represent an interface between the cell death pathway and the normal mechanisms that maintain mitochondrial shape and regulate their division. The shape of the mitochondrial network and of the individual mitochondrion results from a net balance of fusion–fission processes regulated by a family of proteins collectively named dynamins [85]. Dynamins are large, ubiquitous mechanoenzymatic GTPases that control the dynamics of membrane fusion, tubulation, budding, and of vesicle formation [86]. Most of the mitochondrial dynamins identified so far are cytosolic proteins that interact with mitochondria when their function is needed through specific adapters [85], or are loosely attached to the mitochondrial outer membrane [87]. Recently, an intrinsic mitochondrial dynamin, associated with the IM

has been identified [87]. This protein, mgm1p, has been suggested to be crucial in maintaining the shape of the mitochondrial cristae [87]. A dominant negative of Drp1, a dynamin family protein normally involved in the fission of the mitochondrial reticulum, has been shown to alter mitochondrial apoptosis [88]. Proteins that control mitochondrial shape are therefore candidates for the dramatic reorganization of the IM that occurs in Class II mitochondria during apoptosis.

In summary, our recent data indicate that the apoptotic pathway bifurcates following activation of a BH3-only molecule. The BH3 domains of such triggers induce BAX, BAK activation resulting in permeabilization of the OMM and cytosolic release of the cytochrome *c* within the IMS. Other domains of tBID trigger a second path of mitochondrial remodelling that is BAX, BAK independent, but CsA sensitive, correlates with transient PT and results in a dramatic remodelling of the IM which mobilizes the cristae stores of cytochrome *c* for release.

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