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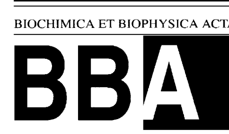

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Review

Mitochondrial membrane dynamics, cristae remodelling and apoptosis

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Abstract

Mitochondria form a highly dynamic reticular network in living cells, and undergo continuous fusion/fission events and changes in ultrastructural architecture. Although significant progress has been made in elucidating the molecular events underlying these processes, their relevance to normal cell function remains largely unexplored. Emerging evidence, however, suggests an important role for mitochondrial dynamics in cellular apoptosis. The mitochondria is at the core of the intrinsic apoptosis pathway, and provides a reservoir for protein factors that induce caspase activation and chromosome fragmentation. Additionally, mitochondria modulate Ca^{2+} homeostasis and are a source of various metabolites, including reactive oxygen species, that have the potential to function as second messengers in response to apoptotic stimuli. One of the mitochondrial factors required for activation of caspases in most intrinsic apoptotic pathways, cytochrome *c*, is largely sequestered within the intracristae compartment, and must migrate into the boundary intermembrane space in order to allow passage across the outer membrane to the cytosol. Recent evidence argues that inner mitochondrial membrane dynamics regulate this process. Here, we review the contribution of mitochondrial dynamics to the intrinsic apoptosis pathway, with emphasis on the inner membrane.
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Keywords: Fusion; Fission; Caspase; BCL-2; Calcium

1. Introduction

Mitochondria form an interconnected tubular network, whose steady-state morphology is derived in part from frequent fission and fusion events, as well as from the metabolic status of the organelle. The outer membrane (outer mitochondrial membrane, OMM) surrounds an inner membrane (inner mitochondrial membrane, IMM), which encloses a protein-rich matrix containing the mitochondrial DNA (mtDNA). The large surface area of the IMM in relation to that of the OMM necessitates organization of the IMM into numerous invaginations, or cristae. Cristae are connected to the boundary intermembrane space (IMS) between the OMM and IMM by narrow tubular junctions. The IMM/cristae compartments are highly flexible, and undergo morphological changes in response to alteration of the metabolic state and/or matrix volume. Mixing of contents between the intra-cristae and boundary intermembrane space plays a role in regulation of both metabolic and apoptotic pathways [1–3]. Despite a growing understanding of the mechanisms behind the dynamics of mitochondrial structure and morphology, much remains to be learned about the relationship between these dynamics and mitochondrial function, in both normal cell physiology and during apoptosis.

Mitochondria have emerged as the central regulators of the apoptotic program in mammalian cells, except in those situations in which the extrinsic death receptor pathway activates sufficient initiator caspase-8 for direct processing of downstream effector procaspases. The core mitochondrial apoptotic pathway is both executed and regulated by members of the BCL-2 protein family (see Fig. 1) and has been the subject of several recent in-depth reviews [4–11]. Members of the BCL-2 family also function at the surface of the endoplasmic reticulum (ER) where they regulate both the homeostasis of Ca^{2+} stores, and their release in response to apoptotic stresses [12–18]. Regulation/release of ER Ca^{2+} stores mediates Ca^{2+} signal transmission to mitochondria, which in turn regulates both metabolic and apoptotic functions within the organelle [12,14,15,17,19–21]. Curiously, the picture

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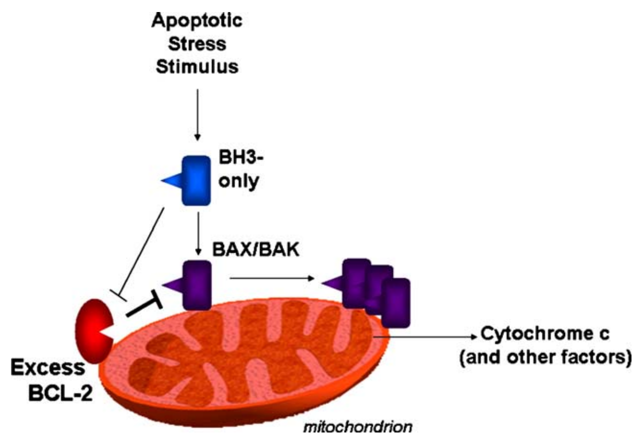

Fig. 1. Regulation of the mitochondrial apoptosis pathway by BCL-2 family proteins. The BCL-2 family is characterized by the presence of one or more BCL-2 homology (BH) domains, and can be subdivided into three groups: BH3-only proteins, which couple upstream death stimuli to downstream regulation of the multi-BH family members; the pro-apoptotic multi-BH proteins BAX and BAK, which are directly activated by certain BH3-only family members, allowing mitochondrial release of factors that stimulate activation of caspases and chromosome fragmentation; and pro-survival BCL-2 proteins (BCL-2, BCL-X_L, MCL-1, BCL-w, and A1), which antagonize BAX and BAK and inhibit stimulus-induced release of pro-apoptotic factors from mitochondria. Pro-survival BCL-2 family members, in turn, can be antagonized by BH3-only proteins. The outcome of the mitochondrial apoptosis pathway is therefore dependent on a complex 3-way ratio between interacting sub-groups of the BCL-2 family, the nature of which remains controversial and incompletely understood.

that has emerged from in vitro reconstitution of the BCL-2 family-regulated mitochondrial apoptotic pathway (where Ca^{2+} is unlikely to play a significant role) suggests a far simpler mechanism than that suggested by studies employing intact cells [9,22–32]. A potential clue as to the reason for this apparent disparity, however, was provided through a ground-breaking in vitro study by Scorrano et al. [33]. In addition to a requirement for a mechanism to release cytochrome *c* across the OMM (mediated by oligomers of BAX and BAK), this study also revealed a requirement for opening of cristae junctions to permit access of cytochrome *c*, which is largely sequestered within cristae, to the IMS. It is not yet known to what extent mitochondrial dynamics influence the release of other pro-apoptotic factors from the IMS, including Smac/DIABLO and Omi/Htra2 (which contribute to caspase activation), and endonuclease G and AIF (which contribute to DNA fragmentation) [34]. Moreover, the influences of mitochondrial fusion–fission and the mitochondrial association with a dynamic cytoskeletal network are lost in in vitro reconstitution experiments, and can therefore only be studied in living cells. Here we review the emerging field pertaining to the relationship between mitochondrial dynamics and apoptosis in intact cells, with an emphasis on the regulation and role of inner membrane dynamics in the mitochondrial pathway of apoptosis.

1.1. Pitfalls and caveats

A number of studies have been reported in which the normal balance of mitochondrial fusion and fission has been disrupted either by over-expression or by knock-down of individual components of the fusion–fission machinery [35–44]. This influences the structural, metabolic, ionic, electrochemical, etc status of the organelle, and therefore may or may not reflect the regulated influences of physiological apoptotic stimuli on the fusion–fission machinery or the mitochondrial apoptotic pathway. Additionally, such manipulations may trigger events that are out of sequence with respect to physiological death pathways. For example, excessive mitochondrial fragmentation triggered by over-expression of a fission enzyme has been reported to inhibit subsequent mitochondrial uptake of Ca^{2+}, and, in consequence, to protect against certain apoptotic stimuli [40]. A very different outcome, however, might be expected if Ca^{2+} was taken up by the mitochondria prior to fragmentation. Induction of apoptosis using non-specific cellular stress stimuli (which influence many signalling pathways), such as the pan-kinase inhibitor staurosporine, might likewise lead to disordered apoptotic responses. Care may therefore be required in interpreting studies that rely on initiation of apoptosis via methods others than manipulation of specific components of a physiological extrinsic or intrinsic death pathway.

2. Mitochondrial fusion and fission

Studies of mitochondrial fusion/fission are complicated by the fact that mitochondria are double membrane bound organelles and, as such, must maintain both outer/inner membrane integrity and cristae structure during any dynamic process. Recent reviews [1–3,45–47] have addressed these issues in detail, but are briefly summarized below from the perspective of their relevance to apoptosis in mammalian cells.

2.1. Mitochondrial fusion machinery

In mammalian cells, mitochondrial fusion is dependent on the large GTPases mitofusin (MFN) 1, MFN2 and optic atrophy protein 1 (OPA1) [2,35,45,48–54]. MFN1 and 2 mediate the initial docking of apposing mitochondria [55]. Both proteins are anchored in the OMM via two transmembrane domains connected by an IMS loop, with the N-terminal GTPase domain and the C-terminal coiled coil domain facing the cytosol [52,54]. In spite of their similarities, MFN1 and 2 do not appear to be functionally redundant [48,50]. Detailed structural studies have shown that the cytosolic heptad repeats formed by the C-terminal region of MFN1 mediate trans-mitochondrial tethering [55]. MFN2, which displays a lower rate of GTP hydrolysis than MFN1, has less efficacy with respect to tethering [56], and has recently been shown to act as a signalling GTPase [57].

OPA1 is an IMM associated intermembrane space dynamin related protein, and is required for fusion, but not for mitochondrial docking [35,49–51,58–61]. Although both OPA1 and MFN1/2 are required for mitochondrial fusion, no direct interaction between the two has been observed. The yeast orthologs of OPA1 and MFN1/2 (mitochondrial genome maintenance/Mgm1p and fuzzy onion/Fzo1p), however, interact both directly and indirectly [61,62]. The indirect interaction between Mgm1p and Fzo1p occurs via the OMM spanning

adaptor protein Ugo1p [61–64]. To date, no functional equivalent of Ugo1p has been identified in mammals, but it is conceivable that a similar mammalian membrane spanning adaptor exists.

2.2. Mechanisms and coordination of OMM and IMM fusion

Recent studies have demonstrated that fusion of the OMM and IMM are separable events in both yeast and mammalian systems [65–67]. In vitro studies have indicated that mitochondrial fusion requires initial physical proximity, which may be mediated in vivo by cytoskeletal elements, followed by docking and tethering via Fzo1p/MFN1/2. This initial OMM interaction requires GTP and (at least in yeast) the proton gradient component of the IMM electrochemical potential ($\Delta\Psi$). OMM fusion can be uncoupled from IMM fusion (i.e., fusion of the OMM can be seen in the absence of IMM fusion), and in contrast to IMM fusion is independent of the electrical component of $\Delta\Psi$ [65–67]. In a mammalian system, IMM fusion was also sensitive to inhibition of glycolysis [67], and in a yeast system IMM fusion was dependent on elevated GTP levels [65]. OPA1/Mgm1p is required for both OMM and IMM fusion, and has been implicated in regulation of cristae morphology [35,49,50,58,61,62,68,69]. In addition, subcellular localization studies have shown Mgm1p/OPA1 in association with cristae [59], and potentially in regions of IMM/OMM apposition [61]. The requirement for high levels of GTP during IMM fusion likely reflects activity of Mgm1p/OPA1, and sensitivity of this process to inhibition of glycolysis may indicate alteration in ultrastructure secondary to increased respiration and/or decreased levels of GTP [66,67]. OPA1 may therefore be required both for OMM/IMM fusion (possibly for coordination of the two), and for maintenance of cristae structure during this process. It should be noted that, although OPA1 plays a role in mitochondrial fusion, and OPA1 knockdown leads to increased fission [35,51,58], overexpression of the protein also results in mitochondrial fragmentation [51,58]. The reason for this phenotype is unknown, but may indicate a need for balanced levels of different OPA1 isoforms (which are known to exist) [69,70], or for a balance between levels of OPA1 and other members of the fusion machinery [2].

Although fusion of the IMM and OMM are coordinated under normal conditions, the above evidence suggests that they are regulated separately. To date, however, the processes involved in OMM/IMM fusion are poorly understood, as is communication between the two membranes.

2.3. Mitochondrial fission machinery

In mammalian cells, the core mitochondrial fission machinery consists of the proteins hFIS (*fiss*ion) and DRP1 (dynamin related protein 1) [38,45,47,71–74]. hFIS has an N-terminal transmembrane domain and six C-terminal α-helices (α1–6), of which α2–3 and α4–5 form tetratricopeptide repeat (TPR) folds [75,76]. DRP1 is a soluble protein, with an N-terminal GTPase domain and a C-terminal GTPase effector domain (GED), both of which are necessary for fission [71,73,74,77–79]. DRP1 recruitment to the OMM is thought to occur via transient interaction with the TPR motifs of hFIS [80,81]. hFIS α1 is not part of a TPR fold and has been shown to destabilize DRP1 binding [81]. hFIS α1 may therefore act as a regulatory element during fission, allowing release of DRP1 from hFIS. Release of DRP1 may be followed by direct binding of DRP1 to the OMM, and subsequent fission. Alternatively, release of DRP1 could occur following fission, allowing regeneration of the GTP bound form [81].

Little is known about the mechanisms behind DRP1 recruitment to hFIS, or about regulation of the interaction. Recently, however, several interesting observations have been made. First, the OMM associated pool of DRP1 is stabilized by sumoylation, which subsequently increases mitochondrial fission [82]. Secondly, the retrograde motor protein complex dynein–dynactin plays a role in mitochondrial fission, possibly through DRP1 recruitment [83]. Cytoskeletal components may also influence DRP1 recruitment, as disruption of F-actin inhibits both OMM DRP1 accumulation and fission [84]. Finally, the overexpression of a mutant form of the small GTPase Rab32, which is unable to hydrolyse GTP, results in apparent fission defects. It is therefore possible that Rab32 also plays a role in DRP1 regulation [85].

2.4. Mechanisms and coordination of OMM and IMM fission

In mammalian systems, cytosolic DRP1 is recruited to the mitochondria prior to fission [39,71,73]. DRP1 displays both intramolecular (between the GTPase/middle and the GED domains) and intermolecular (between GED domains) interactions [78]. Cytosolic DRP1 exists primarily as tetramers or dimers, but can assemble into higher order rings/spirals on the OMM [73,78,86,87]. Once assembled on the OMM, DRP1 rings/spirals are thought to drive mitochondrial fission. This process is reminiscent of dynamin-mediated endocytosis (for review, see [88,89]), although it is unclear at this point whether DRP1 acts as a regulatory GTPase or as a mechanoenzyme (or as a combination of both) [2,3,73,78,88–91]. Similarity of the mitochondrial fission process to dynamin-mediated endocytosis is further suggested by the involvement of endophilin B1 in mammalian mitochondrial morphology [92,93]. The endophilin family of fatty acyl transferases acts to regulate membrane curvature, and endophilin 1 is a necessary cofactor for dynamin-mediated endocytosis [92,94]. Knockdown of endophilin B1 leads to dissociation of the OMM and IMM, possibly through inhibition of OMM fission [93]. Endophilin B1 acts downstream of DRP1, and, like DRP1, cycles on and off of the OMM [93]. Intriguingly, a mitochondria localized synaptojanin (synaptojanin 2A) has also been implicated in mitochondrial morphology [95]. Synaptojanin 1, along with endophilin 1, participates in dynamin 1-mediated synaptic vesicle recycling [92,94], and the involvement of a synaptojanin in mitochondrial fission would therefore be consistent with a “dynamin-mediated endocytosis-like” mechanism. Although endophilin B1 [93], and likely synaptojanin 2A, act downstream of DRP1, no direct interaction between the proteins has been observed, and it is likely that other cofactors are involved.

Although DRP1 is required for mitochondrial fission, yeast studies have shown that Dnm1p (the yeast homolog of DRP1) recruitment occurs after membrane constriction [96]. In support of a role for DRP1/Dnm1p as a "pinchase", self assembled Dnm1p spirals and mitochondrial constriction sites have similar dimensions [97]. However, Dnm1p cycles on and off of the OMM without inducing fission, and sites of mitochondrial constriction are seen in the absence of Dnm1p recruitment [96]. It therefore seems that Dnm1p (and likely DRP1)-mediated scission occurs only when recruitment coincides with a site of constriction, and that constriction is mediated by separate (unknown) elements [96]. As mitochondrial organization is dependent on components of the cytoskeletal machinery [2,98], it has been suggested that tension along microtubules may act to produce areas of constriction, which would then act as sites of fission [96]. Alternatively, or in conjunction, IMM fission/constriction may occur prior to OMM fission, giving rise to areas of mitochondrial narrowing [2,71,99,100].

Coupling of IMM to OMM fission is necessary under normal circumstances, however, it is possible to separate the two processes (i.e., IMM fission can be observed in the absence of OMM fission). In *C. elegans*, introduction of dominant negative DRP1 results in blebs of matrix connected by thin tubules of OMM, indicating that DRP1-mediated constriction is not solely responsible for scission of both membranes [71]. A similar phenotype was seen in budding yeast lacking Fis1p (the yeast homolog of hFIS) or Dnm1p [99], and, although neither dominant negative DRP1 nor knockdown of hFIS have been reported to lead to OMM/IMM separation in mammalian cells, knockdown of endophilin B1 produces a phenotypically similar effect [93]. As DRP1/Dnm1p, hFIS/Fis1p, and endophilin B1 are necessary for OMM fission, but do not appear to act in IMM fission, additional IMM fission associated IMM/IMS factors must exist. One candidate for induction of IMM fission is the mammalian protein MTP18 [101]. MTP18 is an IMS integral membrane protein which is required for hFIS induced fission, and, when overexpressed, leads to DRP1-mediated fission. It has been proposed that MTP18 may be essential for mitochondrial fission, possibly acting to coordinate IMM and OMM processes, or to induce IMM fission [101]. In yeast, the IMM protein Mdm33 may function in IMM fission [102], possibly in a manner similar to that of MTP18. In yeast lacking Mdm33 fission is inhibited, while Mdm33 overexpression leads to apparent enhancement of IMM (but not OMM) fission [102]. Based on the occurrence of IMM fission upstream of OMM fission, it is possible that initiation/regulation of this process occurs primarily at the level of the IMM.

2.5. Maintenance and biogenesis of cristae structure

Maintenance of normal cristae structure is essential for mitochondrial function, but is poorly understood [1,47]. In yeast systems cristae organization requires Mgm1p [61,62], as well as both the OMM Mmm1p/Mdm10p/Mdm12p complex [103–105] and the IMM Mdm31/Mdm32 complex [106]. The two complexes may act in cooperation, possibly at contact sites between the OMM/IMM [45,106]. In mammalian systems, both OPA1 and the IMM protein mitofilin 1 have been implicated in cristae organization. Downregulation of mitofilin 1 affects cristae morphology without a concomitant effect on fission/fusion [107], while OPA1 is involved in both maintenance of IMM structure and in the fusion process [35,49,50,58,61,62,68,69].

Recent studies have revealed an essential role for the ATP synthase complex in cristae biogenesis [68,108–114]. Dimerization of ATP synthase (which spans the IMM, and is enriched in cristal membranes [1,115]) is required for normal cristae morphology, likely due to induction of membrane curvature [108,113]. Mitochondrial morphology is linked to energy demand [1], and ATP synthase is responsible for the majority of cellular energy production [116]. Regulation of cristae biogenesis by ATP synthase may therefore link cellular energetics to IMM morphology [1,109].

Finally, Mgm1p has recently been shown to regulate ATP synthase assembly, in addition to cristae organization and mitochondrial fusion [68]. This finding may represent a link between mitochondrial OMM fusion, IMM fusion, cristae morphology and ATP synthase. Alternatively, it may indicate a dual role for Mgm1p, via separate regulation of OMM/IMM fusion and IMM structure, likely mediated by different forms of the protein [68].

3. Apoptosis and mitochondrial dynamics

3.1. Apoptosis and mitochondrial fission

As described in the Introduction, recent studies have indicated that BAX/BAK-mediated OMM permeabilization alone may not be sufficient for cytochrome *c* release during apoptosis. In addition, mitochondrial fragmentation in response to a variety of apoptotic stimuli has been reported [3,39,117–125], and inhibition/deletion of components of the fission machinery, including both hFIS and DRP1, inhibits or delays cell death [36,38,39,44,81,117,124,125]. Mitochondrial fragmentation appears to occur early in the apoptotic process, prior to either cytochrome *c* release or BAX activation, and independent of caspase activity [39,124,125]. It has also been shown that a dominant-negative form of DRP1 delays apoptosis at a point prior to mitochondrial fragmentation, cytochrome *c* release and loss of $\Delta\Psi$, but does not affect translocation/activation of BAX [36,39,117,124]. DRP1 recruitment to mitochondria, and foci formation at scission sites and tips, has been observed during apoptosis, as has BAX colocalization with these sites [117]. Depletion of hFIS also leads to inhibition of mitochondrial fragmentation and cytochrome *c* release following apoptotic stimuli, but acts prior to BAX recruitment [36,39]. Interestingly, BAX colocalization with BAK and DRP1, but not with hFIS, has been observed [117]. This observation supports a model wherein a transient interaction of hFIS with DRP1 is followed by stable (hFIS independent) DRP1 association with the OMM (or OMM associated factors) [80,81].

Although mitochondrial fission may play a role in apoptosis, the same process is seen under normal conditions, without pathological consequences [1–3,45,46]. The link between

fission and apoptosis may therefore depend on the involvement of other "apoptosis-specific" factors/stimuli in addition to the core fission machinery. As previously mentioned, BAX colocalizes with DRP1 during apoptosis associated fission [117]. BAX overexpression itself induces apoptosis, and during this process promotes mitochondrial fission, which is blocked by dominant negative DRP1 [39]. BAX has also been shown to interact with endophilin B1, and it has been suggested that endophilin B1 may play a role in BAX/BAK activation [126,127]. Loss of endophilin B1 delays apoptosis, BAX/BAK activation and cytochrome *c* release [127]. As endophilin B1 is involved in mitochondrial fission, its role in BAX/BAK recruitment/activation represents another potential link between apoptosis and fission [93]. Apoptosis induced by excessive fission (i.e., via overexpression of hFIS [38,81]) can, in some cases, be inhibited by the antiapoptotic BCL-2 family members [38,42]. It is unclear, however, whether this inhibition occurs prior to [42] or following [38] the fission process, and it is likely that the role of the antiapoptotic BCL-2 family members depends on the system, the apoptotic stimulus, and the level of expression.

Further support for the involvement of fission in apoptosis has been provided by the identification and characterization of Death Associated Protein 3 (DAP3) [128]. DAP3 is a mitochondrial ribosome associated matrix protein, and regulates mitochondrial fragmentation only under conditions of cellular stress. Overexpression of DAP3, which presumably mimics the activated state induced by cellular stress, leads to mitochondrial fission and loss of $\Delta\Psi$. In addition, knockdown of DAP3 leads to reduced fission and decreased sensitivity to staurosporine induced apoptosis [128]. Apoptosis associated fission, as well as physiological fission, must involve coordinated fragmentation of the inner and outer membranes. As DRP1 and hFIS are likely not sufficient for IMM as well as OMM scission, there must be other factors involved. The matrix localization of DAP3 suggests that it may play a role in IMM scission, specifically in response to apoptotic stimuli [128]. Other IMM proteins may be involved as well (MTP18, for example, is a potential candidate [101]), however, DAP3 is particularly interesting due to its involvement only under stress conditions [128].

The above findings suggest that mitochondrial fission is triggered during apoptosis in response to a variety of stimuli, and that it may be required for release of cytochrome *c* (and potentially other proapoptotic factors). Mitochondrial fission itself does not normally lead to apoptosis, and it therefore seems likely that it acts as a "sensitizing" process, facilitating cytochrome *c* release mediated by additional apoptosis associated events [2,3,10,11,19,90,91,129,130]. For example, the transient pore opening seen during fission may be potentiated by BAX/BAK during apoptosis, thus allowing cytochrome *c* release [46,90]. Alternatively, modification of membrane curvature and/or IMM/OMM contacts may render the OMM more sensitive to BAK/BAX-mediated permeabilization [10,21,43,90,129,131]. It is also possible that fission is linked to IMM remodelling, leading to mobilization of intramitochondrial cytochrome *c* stores [21,47,125], a possibility which is discussed in some detail further in this report. It should be noted, however, that fission is not always required for cytochrome *c* release/apoptosis [43,129,132–134], and that the relationship between fission and apoptosis and/or OMM permeabilization is very poorly understood. Further elucidation of both the processes underlying physiological fission, and of mechanisms involved in OMM permeabilization, will likely be necessary in order to understand the role/importance of fission in apoptosis.

3.2. Apoptosis and mitochondrial fusion

The increase in mitochondrial fragmentation seen during apoptosis is likely due to a decrease in fusion, as well as an increase in fission [2,45–47]. Inhibition of mitochondrial fusion has been temporally linked to BAX activation and OMM permeabilization [100], and BAX foci formed during apoptosis contain MFN2, as well as DRP1 and BAK [117]. In addition, inactivation of the core components of the mitochondrial fusion machinery, MFN1/2 [43] and OPA1 [35,36,134], has been linked to the apoptotic process. Silencing of MFN1/2 leads to increased mitochondrial fragmentation and increased sensitivity to apoptotic stimuli [43], and a dominant active form of MFN2 (which increases mitochondrial fusion) inhibits BAX activation and cytochrome *c* release, and protects against free-radical induced permeability transition [57]. Overexpression of the rat homologue of MFN1/2 also leads to decreased mitochondrial fragmentation and delayed activation of BAX/BAK/cytochrome *c* release in response to apoptotic stimuli [43]. Knockdown of OPA1 increases mitochondrial fragmentation and sensitivity to apoptosis [36,134], and loss of OPA1 has also been reported to lead to apoptosis in the absence of other stimuli [35,36]. Apoptosis induced by loss of OPA1 is accompanied by loss of $\Delta\Psi$ and cytochrome *c* release and can be inhibited by BCL-2 (downstream of fragmentation) [35]. Notably, depletion of hFIS as well as OPA1 provides protection against both spontaneous and induced apoptosis, suggesting that OPA1 may act to inhibit the proapoptotic function of hFIS [36]. Finally, release of OPA1 itself has recently been reported to occur during apoptosis, at the same time as the release of cytochrome *c* [134].

Although the role of mitochondrial fusion in resistance to apoptosis remains to be articulated, it may reflect the buffering of potentially damaging factors, the sharing of protective factors, or inhibition of excessive fission [1,3,36,57].

3.3. Apoptosis and cristae remodelling

Under normal conditions selective permeabilization of the OMM results in release of only about 15% of the total pool of cytochrome *c*, due to sequestration of the remaining amount within the mitochondrial cristae [33,135]. The complete release of cytochrome *c* seen during apoptosis therefore appears to involve cristae remodelling/opening, in addition to OMM permeabilization [3,33,47]. Using isolated mitochondria, Scorrano et al. have shown that tBID, the proapoptotic caspase cleavage product of the BH3 only protein BID, induces cytochrome *c* mobilization into the IMS, dependent on cristae remodelling but independent of BAX/BAK [33]. Fusion of

individual cristae and opening of the junctions between the cristae and the IMS were observed in response to tBID, dependent on transient opening of the permeability transition pore (PTP), but not on permeability transition (PT) [33] (for review of the contribution of the PTP/PT to cell death see [7,8,14,136]). Under physiological conditions, tBID is generated through cleavage by caspase 8 following death receptor engagement [137–140], and induces cytochrome *c* release dependent on BAX/BAK activation/oligomerization [141–143]. It now appears that tBID induced cristae remodelling is necessary for complete cytochrome *c* release following BAX/BAK-mediated OMM permeabilization [33].

The concept of cristae remodelling as a primer for complete release of cytochrome *c* following OMM permeabilization is further supported through a recent study by Germain et al. [125]. In this case it was shown that the BH3 only protein BIK, acting at the ER, mobilized cytochrome *c* for subsequent release by stimulating opening of the cristae junctions. Mitochondria isolated from BIK treated cells showed increased availability of cytochrome *c* to the IMS, consistent with a role for cristae remodelling in cytochrome *c* mobilization. Cristae remodelling, in this case, was dependent on Ca^{2+} release from the ER and uptake by the mitochondria, as well as on DRP1 activity and PTP opening. Of note, the involvement of DRP1 in cristae remodelling was independent of its role in mitochondrial fission. Mitochondrial fragmentation, dependent on Ca^{2+} signalling and DRP1, was also observed in response to BIK, and was preceded by loss of $\Delta\Psi$. BIK alone eventually induced BAX activation, cytochrome *c* release and caspase activation, however, this process was greatly accelerated by co-expression of the weak BH3 only protein NOXA. NOXA alone induces caspase activation only after an extended period of time, and does not lead to either ER Ca^{2+} release or mitochondrial fission [125]. The cooperation observed between BIK and NOXA with respect to cytochrome *c* release supports a "two-hit" model in which cristae remodelling leads to increased availability of cytochrome *c* to the IMS, and thus increased cytochrome *c* release once the OMM is permeabilized by activated BAX/BAK (Fig. 2) [125].

Additional evidence for alteration of mitochondrial morphology prior to cytochrome *c* release was provided through a study by Breckenridge et al. [124]. In this case, p20, the ER localized caspase cleavage product of BAP31, was shown to induce mitochondrial fragmentation, dependent on ER Ca^{2+} release and mitochondrial Ca^{2+} uptake. Mitochondrial fragmentation preceded BAX activation and cytochrome *c* release, and dominant negative DRP1 prevented cytochrome *c* release and caspase activation, as well as mitochondrial fission. p20 itself induced apoptosis only after a significant delay, but greatly enhanced caspase 8/tBID-mediated cytochrome *c* release [124]. Although cristae remodelling was not examined in this study, other features strongly parallel the pathway seen during BIK/NOXA induced apoptosis, and further support the above described "two-hit" hypothesis.

3.4. Initiation of cristae remodelling during apoptosis

Although cristae remodelling may be required for cytochrome *c* release, the mechanisms behind this process are not understood. A number of different signals, acting through different mediators, may be able to trigger the same end result (i.e., cytochrome *c* mobilization via opening of cristae), reflecting the diversity of the signalling pathways that lead to stress induced apoptosis.

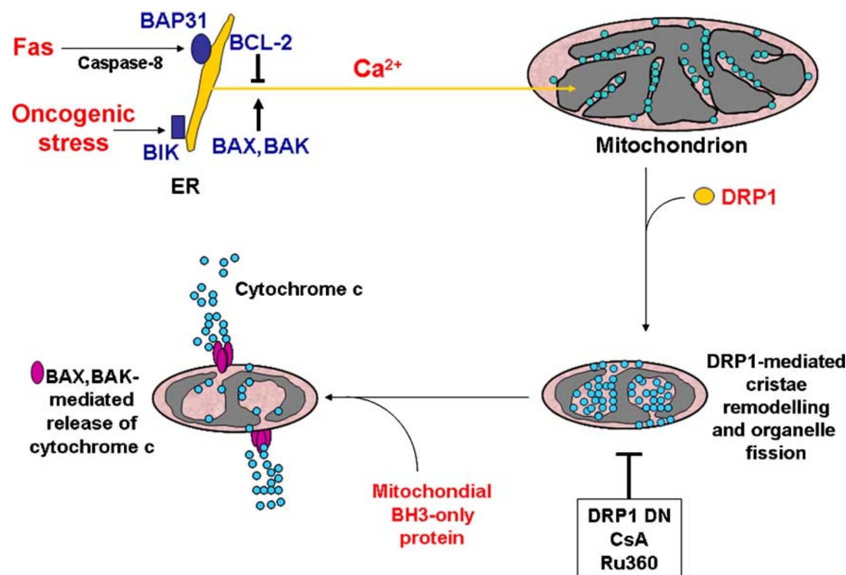

Fig. 2. Endoplasmic reticulum Ca^{2+} signalling and the "two-hit model" for cytochrome *c* release from mitochondria. Induction of pro-apoptotic proteins at the ER, such as BIK or the caspase-8 cleavage product of BAP31, results in Ca^{2+} release from ER stores and subsequent Ca^{2+} uptake by mitochondria. This in turn triggers mitochondrial accumulation of DRP1, leading to cristae remodelling, mobilization of intra-cristae cytochrome *c*, and excess organelle fission. Upon activation and oligomerization of BAX/BAK at the OMM, mobilized cytochrome *c* is released from the organelle. Stimulus-induced opening of cristae can be restrained by an inhibitor of mitochondrial Ca^{2+} uptake (Ru360), dominant negative (DN) DRP1, or cyclosporine A (CsA). Of note, release of Ca^{2+} from ER stores can be influenced by the status of pro-survival and pro-death members of the BCL-2 family acting at this site. Details are provided in the text.

First, direct activity of BH3 only proteins at the mitochondria, as exemplified by tBID, can lead to cristae reorganization as well as to OMM permeabilization [33]. tBID-mediated OMM permeabilization is dependent on activation of BAK/BAX, and requires the tBID BH3 domain [141–143]. Recent evidence has suggested that tBID and BAX are recruited to hFIS and VDAC containing microdomains, indicating that tBID activation of BAX may be regulated by lipid rafts, and that components of the fission machinery may play a role in tBID induced apoptosis [144]. tBID-induced cristae reorganization, in contrast, is independent of the BH3 domain [33], and could be triggered by tBID binding to the acidic phospholipid cardiolipin [145–149]. Cardiolipin is present primarily in the IMM, but is highly enriched at "contact sites", where the IMM and OMM are in close proximity [150–152]. Targeting of tBID to these regions may therefore aid in transducing signals that affect cristae opening [3,147–149].

tBID-induced cristae remodelling has also been suggested to be dependent on the production of reactive oxygen species (ROS) [153], which have been implicated in a variety of apoptotic pathways, and may either be generated in response to, and/or trigger, PTP opening [15,130,154–158]. As both ROS production [153] and transient PTP opening [33] precede cristae remodelling, it may be that ROS production is stimulated by PTP opening. The precise role of ROS in tBID induced cristae remodelling is unknown, however, one of the observed effects of ROS is lipid peroxidation [158–161], which could conceivably modulate membrane curvature, leading to cristae remodelling. In addition, tBID also displays lipid transferase activity [162], and has been shown to directly modulate membrane curvature [145]. Finally, loss of $\Delta\Psi$, which is an integral feature of many apoptotic pathways, can be triggered by ROS [155,163–165] and/or the PTP [166–168]. Loss of $\Delta\Psi$ has been shown to result in matrix remodelling to a condensed state, leading to cristae opening and increased availability of cytochrome *c* to the IMS [169].

Cytochrome *c* release is impeded not only by sequestration within cristae, but also by stable interaction with cardiolipin [157,170]. In order for complete cytochrome *c* release to occur, it is likely that both cristae remodelling and disruption of the cytochrome *c*–cardiolipin interaction are required. Oxidative modification of cardiolipin has been shown to disrupt binding to cytochrome *c* [159,160,171–174], and tBID induced ROS production may therefore play a dual role in cytochrome *c* release, initiating both cristae remodelling and cardiolipin peroxidation [153,157,171]. Alternatively, the lipid transferase activity of tBID may also play a direct role in cardiolipin modification [162]. Interestingly, a recent study has shown that mitochondrial ROS production in response to complex 1 (NADH/ubiquinone oxidoreductase) deficiency sensitizes isolated mitochondria to BAX-mediated cytochrome *c* release. This sensitization is based on increased availability of cytochrome *c* to the IMS and was correlated with cardiolipin peroxidation [175]. Although it was not examined in this study, it is not unreasonable to hypothesize that cristae remodelling may also (in addition to cardiolipin peroxidation) have been responsible for the observed mobilization of cytochrome *c* stores. The above results, with respect to both tBID and complex 1 deficiency, indicate that ROS production, leading to cristae remodelling and/or cardiolipin peroxidation, may play an important role in cytochrome *c* release during apoptosis, potentially in response to a wide variety of stimuli.

The BH3 only protein BIK also induces cristae remodelling, but, unlike tBID, does so through Ca^{2+} signalling between the ER and mitochondria [125,176]. Ca^{2+} uptake by the mitochondria has been shown to trigger both opening of the PTP and ROS production (for review, see [177]). BIK may therefore trigger cristae remodelling though a ROS dependent mechanism similar to that proposed for tBID; i.e., through PTP induced ROS production. Whatever "second messenger" is induced upon BIK-initiated uptake of Ca^{2+} by mitochondria, it is intriguing that this signal leads to cristae remodelling dependent upon functional DRP1 at the OMM [124]. Cristae remodelling dependent on DRP1 represents a novel finding, and points to an as yet unknown DRP1-dependent signalling pathway [124].

It should be noted that mitochondrial uptake of sufficiently high Ca^{2+} levels can result in PTP opening, swelling of the matrix, and subsequent rupture of the OMM (reviewed in [15,20,130,156]). This, or a similar pathway, however, does not appear to operate in the case of either tBID or BIK, as mitochondrial swelling and OMM rupture are not observed, and, in the case of tBID, $\Delta\Psi$ is not disrupted [33,124,125]. It has also been reported that mitochondrial fission protects against Ca^{2+}-mediated apoptosis [40]. This result was likely due to the induction of mitochondrial fragmentation prior to Ca^{2+} signalling [40], which would have impeded signal propagation. During BIK-mediated apoptosis, however, Ca^{2+} uptake precedes mitochondrial fragmentation, and signal propagation would therefore not be affected by the fission process [125,176].

Several other factors may also control cristae remodelling in response to tBID and BIK, as well as to other stimuli. First, release of OPA1 has recently been observed in temporal proximity to release of cytochrome *c* [134]. Loss of OPA1 is associated with cristae reorganization, mitochondrial fragmentation, loss of membrane potential, and sensitization to tBID induced cytochrome *c* release [35,36,58,134]. Disassociation of OPA1 from the IMM may therefore lead to cristae reorganization, and could conceivably be triggered by ROS induced signalling (although there is as yet no evidence for this) and/or DRP1-mediated signalling from the OMM. It has also been proposed that OPA1 may function to counteract hFIS, and that loss of OPA1 would result in unopposed hFIS-mediated fission [36]. Cristae remodelling could therefore also be a result of unopposed hFIS activity or of DRP1 activity downstream of hFIS. It should be noted that OMM permeabilization and concomitant release of cytochrome *c* and OPA1 were observed prior to mitochondrial fragmentation [134]. Although this result was not consistent with those of several other studies, which demonstrated mitochondrial fission prior to cytochrome *c* release [39,124,125], it could simply reflect differences between experimental systems. It is, however, consistent with the notion that DRP1-dependent cristae opening occurs prior to fission in the BIK pathway[125].

It is also possible that other IMM/IMS proteins contribute to cristae remodelling during apoptosis. Potential candidates include the IMM protein mitofilin 1 [107] and the matrix protein DAP3 [128], as well as components of the ATP synthase complex [178,179]. Correct assembly of ATP synthase is required for normal cristae morphology [108–113], and cellular respiration/ATP synthesis is known to be altered during apoptosis [130,148,158]. In addition, Mgm1p (the yeast homologue of OPA1) has been shown to play a role in ATP synthase assembly [68]. It is therefore possible that disruption of ATP synthase, potentially through loss of OPA1- or ROS-mediated damage, leads to cristae remodelling during apoptosis.

4. Summary and future directions

Based on the above evidence, it appears that robust cytochrome *c* release during apoptosis can occur via a "two-hit" process, where mobilization of intramitochondrial cytochrome *c* stores via cristae remodelling and cardiolipin peroxidation is required for subsequent transport of cytochrome *c* across the OMM. Both events may be initiated by the same effector protein (as in the case of tBID [33]) or may require combined effectors, one mediating a "sensitizing" signal such as transmission of ER Ca^{2+} to mitochondria, and the other contributing to activation of BAX/BAK (as in the case of BIK/NOXA[125] and p20/tBID [124]). The underlying signals and mechanisms leading to opening of cristae junctions, however, remain to be determined. Candidates include ROS production, likely involving the PTP, with the initial stimulus being either lipid modification/direct interaction with the PTP (for tBID), or Ca^{2+} signalling (for BIK). ROS production could modify membrane curvature, lead to cardiolipin peroxidation, disrupt proteins essential for cristae organization, such as OPA1 and/or components of the ATP synthase complex, or even stimulate accumulation of DRP1 at the mitochondrial surface.

Following cristae reorganization, permeabilization of the OMM results in rapid and complete release of cytochrome *c* [33,125]. This event is mediated by a second stimulus leading to recruitment and activation of BAX/BAK [33]. The increased fission and decreased fusion often observed during apoptosis may function to facilitate this "second hit". It has been proposed that mitochondrial fission may lead to alteration in membrane curvature such that the OMM is rendered more susceptible to permeabilization, or, alternatively, that transient pore opening during fission may be potentiated by BAX/BAK [10,21,43,46,90,129,131].

The above "two-hit" hypothesis represents one potential pathway for cytochrome *c* release during stress induced apoptosis. This model may not be universal (e.g., depending on the metabolic status of the cell, cristae opening may not require a specific stimulus), and is certainly not complete. Many questions remain with respect to the mechanisms behind both cristae remodelling and mitochondrial fission during apoptosis, as well as to their relative importance and their relationship to each other. We have only recently begun to recognize the role of mitochondrial morphology and dynamics under both apoptotic and normal conditions, and a great deal remains to be discovered. Further investigation into mitochondrial dynamics and structural biogenesis is currently underway, and will provide us with new and exciting information pertaining to both pathological and physiological processes.

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