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News and Commentary

Outer mitochondrial membrane permeabilization: an open-and-shut case?

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Extensive evidence indicates that during apoptosis the outer mitochondrial membrane (OMM) becomes permeable to numerous intermembrane space proteins, including (but not limited to) cytochrome *c*, Smac/DIABLO, and Omi/HtrA2.[1] Once released, cytochrome *c* promotes the activation of procaspase-9 directly within the apoptosome complex, whereas Smac/DIABLO indirectly triggers caspase activity by relieving the caspase-inhibitory properties of cytosolic inhibitor of apoptosis proteins. Permeabilization of the OMM is modulated by members of the Bcl-2 family of proteins.[2] Antiapoptotic members, such as Bcl-2 and Bcl-X_L, inhibit protein release, whereas 'BH1-3' proapoptotic members, such as Bax and Bak, stimulate this release. 'BH3-only' proteins, such as Bid and Bim, contribute to the proapoptotic function of Bax or Bak by inducing homo-oligomerization of these proteins. However, the precise mechanisms whereby mitochondrial proteins cross the OMM during apoptosis, and how Bcl-2 family proteins regulate this process are less certain. Here, we summarize some recent findings on this topic and evaluate their significance within the context of what we already know.

There are currently two recognized mechanisms for OMM permeabilization (Figure 1a, b). The first, which may be operable during both necrotic and apoptotic cell death, involves the induction of permeability transition (PT) because of opening of the PT pore followed by measurable osmotic swelling of the mitochondrial matrix, rupture of the OMM, and the release of cytochrome *c*. However, transient pore opening may also occur whereby a small fraction of mitochondria have open pores at a given time; in this case, mitochondrial protein release occurs without observable large-amplitude swelling or drops in membrane potential in the entire population.[3] This model of OMM permeabilization may be most relevant during instances of ischemia–reperfusion injury or in response to cytotoxic stimuli resulting in localized mitochondrial Ca^{2+} overload. The second mechanism of OMM permeabilization involves members of the Bcl-2 family. As mentioned above, the proapoptotic 'BH3-only' subset of Bcl-2 family members requires the presence of a 'BH1-3' member, notably Bax or Bak, to induce cytochrome *c* release. In fact, Bax/Bak double knockout MEFs are refractory to most intrinsic death stimuli, despite the presence of 'BH3-only' proteins.[4] Moreover, antiapoptotic Bcl-2 and Bcl-X_L operate, at least in part, to sequester 'BH3-only' proteins into stable complexes and thus prevent activation of Bax or Bak.[5]

Cytochrome *c* is normally bound to the inner mitochondrial membrane (IMM) by an association with the anionic phospholipid cardiolipin.[6] Cardiolipin is unique to mitochondria and is present predominantly, if not exclusively, in the IMM.[7] Evidence suggests that dissociation of cytochrome *c* from cardiolipin is a critical first step for cytochrome *c* release into the cytosol and the induction of apoptosis.[8–11] In particular, it was demonstrated that exposing submitochondrial particles to ROS produced by the mitochondrial electron transport chain stimulates a pronounced mobilization of cytochrome *c* and a concomitant loss of cardiolipin.[8] Similarly, other studies showed that lowering mitochondrial cardiolipin content correlates not only with a decrease in respiration[9] but also with a stoichiometric increase in cytochrome *c* release.[10] Further, a recent study from this laboratory demonstrated that simple permeabilization of the OMM by oligomeric Bax (Figure 1b) is insufficient for cytochrome *c* release, and that peroxidation of cardiolipin is a critical first step in order to mobilize cytochrome *c* from the IMM (Figure 1d).[11] Combined, these findings indicate that cardiolipin plays an important role in the structure and function of the respiratory chain, as well as in the retention of cytochrome *c* within the intermembrane space.

A different role for cardiolipin was proposed in a recent paper by Kuwana *et al.*,[12] suggesting that pore formation in the OMM generated by mixtures of tBid and monomeric Bax requires cardiolipin. (This finding differs from that of Lutter *et al.*,[13] who demonstrated previously that although tBid and cardiolipin do not bind *in vitro*, cardiolipin is nonetheless important for tBid targeting to mitochondria.) Perhaps the most surprising result from Kuwana *et al.* is the apparent size of this membrane pore, which allegedly permits the passage of 10 or 2000 kDa dextran molecules equally well, and in the absence of any gross ultrastructural membrane alterations (Figure 1c). Finally, it is notable that neither stable Bax oligomerization nor increased Bax membrane association was necessary for permeabilization of mitochondrial (M) or ER liposomes.

Together, these results evidently describe a novel role for cardiolipin, and at the same time beg the question of how this is supposed to work. In particular, how does an IMM phospholipid, that is, cardiolipin, regulate not only tBid/Bax targeting to the OMM but also Bax function? Do these proteins translocate to the IMM of intact mitochondria? If, on the other

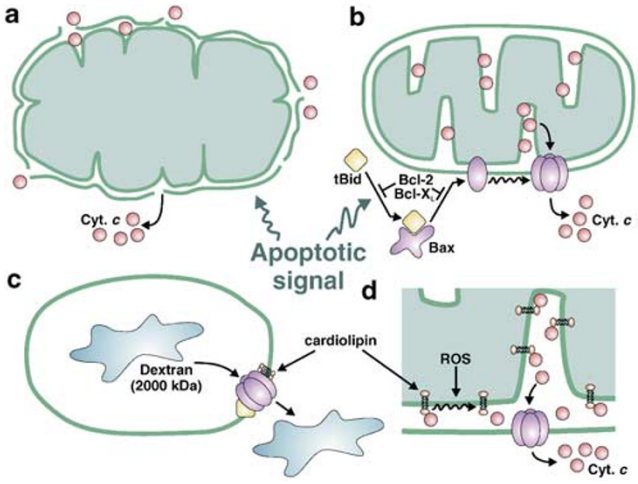

Figure 1 Schematic representation of mechanisms accounting for outer mitochondrial membrane (OMM) permeabilization and the release of cytochrome *c*. (**a**) Induction of permeability transition (PT) because of PT pore activation, leading to matrix expansion and rupture of the OMM. (**b**) Bax-mediated permeabilization of the OMM, involving tBid-induced Bax insertion and homo-oligomerization that can be inhibited by Bcl-2 or Bcl-X_L. (**c**) OMM permeabilization promoted by an interaction among tBid, Bax, and cardiolipin to form supramolecular openings allowing the passage of very large dextran molecules. (**d**) Peroxidation of cardiolipin is a key first step to mobilize cytochrome *c* from the IMM prior to Bax-induced (**b**) permeabilization of the OMM

hand, cardiolipin *is* present in the OMM (as speculated by the authors), how much is there? Moreover, considering cytochrome *c*'s association with cardiolipin in the IMM, how appropriate is it to study OMM permeabilization and protein efflux in the absence of the IMM?

Another point of contention involves the purported size and regulation of the supramolecular pores formed by tBid, Bax, and cardiolipin. In particular, considerable evidence that protein release from mitochondria may be differentially regulated is hard to integrate with these findings. That is, if the tBid/Bax/cardiolipin triumvirate truly forms a membrane pore allowing the passage of very large 2000 kDa molecules, then how, for instance, could the release of cytochrome *c* ever precede that of Smac/DIABLO as described previously?[14,15] A possible explanation is that these proteins are located in different intramitochondrial compartments and that mitochondrial remodeling, especially that of cristae, is needed for their release.[16] Concerning the size of the supramolecular openings, the nonselective nature of 10–2000 kDa dextran passage is more suggestive of large breaks in the OMM than the formation of discrete, tightly regulated pores.[17] Can resealing or fusion of outer (mitochondrial) membrane vesicles (OMVs) following dextran release be ruled out as an explanation for the absence of ultrastructural changes?

Kuwana *et al.* also examine Bax oligomerization and not surprisingly find that tBid stimulates oligomerization of nearly all Bax into detergent-stable, higher-order complexes[18] when these proteins are added to intact mitochondria or OMVs – an effect that correlated strongly with dextran release from OMVs. In contrast, however, tBid was unable to invoke massive Bax oligomerization when added to M liposomes, despite the release of a large percentage ($\sim$50%) of dextran. Since the level of cardiolipin in M liposomes is presumably the same or even greater than that found in OMVs, what accounts for the fact that dextran release from OMVs is far greater than that which is released from M liposomes? This apparent discrepancy between the two experimental models suggests to us that other molecules may exist in the OMM that are important for its permeabilization.

Evidence for such a notion is presented in a recent paper by Roucou *et al.*[19] The authors suggest that while tBid is required for Bax oligomerization, by itself it is not sufficient and a mitochondrial protein must also be present. In particular, their findings, which clash with those of Kuwana *et al.*, indicate that oligomerization of Bax occurs neither spontaneously when monomeric Bax is added to isolated mitochondria nor when mixtures of monomeric Bax and tBid are added to liposomes consisting of either 30% cardiolipin or lipids isolated from mitochondria. Perhaps these authors' most significant finding is that tBid-induced oligomerization of Bax in isolated mitochondria was inhibited when these organelles were pretreated with protease K, an agent used for the general digestion of proteins. Taken together, these findings suggest that an OMM protein, and not cardiolipin, is required for pore formation and protein efflux induced by mixtures of tBid and monomeric Bax.

Previously, it was reported that voltage-dependent anion channel (VDAC), a protein in the OMM, is required for Bax-induced cytochrome *c* release.[20] To test whether VDAC was involved in their system, Roucou *et al.* performed experiments using VDAC-deficient or wild-type yeast mitochondria and determined that Bax oligomerized and induced cytochrome *c* release equally well in both types of mitochondria. Interestingly, both Bax oligomerization and Bax-induced cytochrome *c* release occurred without tBid in these mitochondria, indicating that Bax-induced OMM permeabilization of yeast mitochondria may occur by a different mechanism than that of mammalian mitochondria. Therefore, one should be careful not to misread the generality of these findings, since an absolute requirement for Bid during Bax-induced cytochrome *c* release in mammalian systems was demonstrated previously.[21] The authors also performed experiments in which soluble proteins from mitochondria were reconstituted in M liposomes and demonstrated that mitochondrial proteins, in fact, sensitize M liposomes to mixtures of tBid and monomeric Bax.

But if the protein is not VDAC, then what is it? A role for a dynamin-related protein, Drp1, has been demonstrated during Bax-induced permeabilization of the OMM. Drp1 normally mediates OMM fission, and cells expressing dominant-negative Drp1 are deficient in this process. A paper by Frank *et al.*[22] examined a role for this protein during apoptosis because of morphological similarities between mitochondria of apoptotic cells and those undergoing fission. Their findings indicate that Drp1 is normally present in the cytosol and localizes to mitochondria during apoptosis. Expression of mutant Drp1 effectively prevented mitochondrial fragmentation, loss of membrane potential, and cytochrome *c* release induced by Bax overexpression but did not prevent Bax insertion into mitochondria. Moreover, the authors show that mutant Drp1 inhibits apoptosis induced by either anti-Fas or etoposide. Together, it appears that Drp1 is a critical modulator of OMM permeabilization and mitochondrial

remodeling[16] during apoptosis. In fact, its potential role in mitochondrial remodeling is attractive since it might bolster our understanding of mechanisms regulating differential protein release from the intermembrane space following OMM permeabilization.[14,15] However, considering Drp1's cytosolic localization prior to apoptosis induction, it is difficult to envisage how Drp1 represents the *only* missing link for OMM permeabilization induced by mixtures of tBid and monomeric Bax, although trace amounts of this protein may be constitutively present in the OMM and all that is needed to promote tBid/Bax-induced cytochrome *c* release. However, because novel Drp1 homologues that regulate mitochondrial fission in yeast were described recently, it seems likely that other dynamin-related proteins may also modulate mitochondrial morphology, remodeling, or permeabilization during apoptosis.[23]

It is worth noting that other recent findings suggest that various proteases may also be critical modulators of OMM permeabilization or mitochondrial dysfunction. In particular, accumulating evidence indicates that certain proteases, such as granzyme B[24] and caspase-2,[25,26] are activated upstream of cytochrome *c* release and, in fact, can target the OMM directly or at least without using Bid or other Bcl-2 family proteins, such as Bax and Bak. Further evidence for such a mechanism indicates that overexpressing Bcl-2 is more effective than eliminating either Apaf-1 or procaspase-9 at preventing caspase activity.[27] In addition, other studies indicate that while caspase activity may not be required for cytochrome *c* release in response to different proapoptotic stimuli, it is necessary for the subsequent loss of membrane potential that is often observed during apoptosis – although the underlying mechanism is unknown.[28,29] Together, these findings indicate that apoptosome formation may operate more as a caspase-amplifying mechanism than as an apical trigger of a caspase cascade.

In conclusion, many lines of research have focused on achieving an emergent understanding of the mechanisms whereby mitochondria release their proteins during apoptosis. Unfortunately, however, it may be that these recent papers raise more questions concerning OMM permeabilization than they answer. An obvious question is whether it is cardiolipin or a mitochondrial protein that is the critical modulator of OMM permeabilization induced by mixtures of tBid and monomeric Bax? Also, how physiological is a pore that permits the passage of dextran molecules up to 2000 kDa in size? And finally, what are potential OMM substrates for proteases that allegedly act directly on mitochondria? Taken together, while the findings presented in these papers are intriguing and describe novel phenomena, they also seem to underscore a need for future careful examination of the elusive mechanisms accounting for OMM permeabilization and cytochrome *c* release.

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