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Apoptosis: Mitochondrial Membrane Permeabilization — The (W)hole Story?

Dispatch

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One critical step of apoptosis is the release of mitochondrial proteins through the outer mitochondrial membrane. Recent work shows that two pro-apoptotic Bcl-2 family proteins, Bax and Bid, as well as the mitochondrion-specific lipid cardiolipin may cooperate in chemically defined liposomes to generate a protein-permeable conduit, relaunching the debate on the identity of the pore responsible for mitochondrial membrane permeabilization during apoptosis.

The point-of-no-return of apoptotic cell death is mostly determined by two intertwined phenomena, namely mitochondrial membrane permeabilization (MMP) and caspase activation [1]. MMP culminates in the complete loss of the barrier function of the outer mitochondrial membrane and the consequent release of potentially toxic proteins that are normally secured in the intermembrane space between the inner and outer mitochondrial membranes. Such cytotoxic proteins include caspase-independent death effectors (nucleases and proteases) as well as caspase activators, namely cytochrome *c* (which activates the so-called apoptosome, a caspase activation complex containing Apaf-1 and procaspase-9), and Smac/DIABLO and Omi/Htr2 (which both block the AIP family of caspase inhibitors).

A plethora of different pro-apoptotic signals trigger MMP. One such stimulus is provided by caspase-8, which mediates the proteolytic maturation of the pro-apoptotic Bcl-2 family protein Bid. Caspase-8-digested Bid (also called 'truncated Bid', t-Bid) translocates from the cytosol to mitochondrial membranes, attracted by the mitochondrion-specific lipid cardiolipin [2]. Bid causes MMP in a manner that is strictly dependent on the presence of either of two pro-apoptotic Bcl-2 proteins, namely Bax or Bak, as indicated by mouse knock-out studies [3]. The t-Bid-triggered Bax-dependent MMP correlates with the full insertion of Bax in the membrane, linked to a conformational change with exposure of the amino terminus, and the formation of Bax oligomers [4]. The apoptosis-inhibitory oncoproteins Bcl-2 and Bcl-x_L, which are predominantly present in mitochondrial membranes, inhibit apoptosis by locally antagonizing Bax or Bak [5]. It thus appears that pro- and anti-apoptotic members of the Bcl-2 family locally engage in a battle of molecular interactions to regulate MMP. A recent paper published in *Cell* [6] confirms the above scenario and provides new insights into what might be the minimum requirements for MMP, at least as far as the outer membrane is concerned.

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The Hole Story

Based on a stepwise reductionist approach using isolated mitochondria, outer mitochondrial membrane vesicles (OMVs), or chemically defined liposomes, as well as purified recombinant proteins, Don Newmeyer and coworkers defined the minimal conditions for the formation of a Bcl-2-regulated protein-permeable pore [6]. One particularly important issue concerned the question of why t-Bid and Bax permeabilize mitochondria yet do no affect other organelles. According to Newmeyer's scenario (Figure 1), the mere presence of cardiolipin (a lipid which is only present in mitochondria, and in particular in the inner mitochondrial membrane as well as in the inner–outer membrane contact sites) would be both sufficient and necessary for t-Bid/Bax-mediated permeabilization reactions [6]. Thus, t-Bid and Bax fail to permeabilize cardiolipin-free endoplasmic reticulum membranes, yet act on cardiolipin-containing protein-free liposomes. Mitochondrial proteins would therefore not be required to confer any specificity to this organelle with regard to t-Bid/Bax-mediated MMP.

Together, t-Bid and Bax (but neither of two proteins alone) were found to permeabilize cardiolipin-containing liposomes *in vitro* to fluorescent dextran of up to 2,000 kDa. This correlates nicely with the observation that apoptotic MMP occurring in true cells is non-selective and thus affects all soluble intermembrane proteins, irrespective of their size [7]. Previous studies involving cardiolipin-free liposomes had suggested that Bax alone [8] (or Bax plus the voltage-dependent anion channel, VDAC [9]) would form a conduit selective for small proteins such as cytochrome *c* (14.5 kDa) or, alternatively, that t-Bid/Bax channels would be electrophysiologically active, yet cytochrome *c*-impermeant [10]. As a result, the reported t-Bid/Bax-elicited channel formed in cardiolipin-containing liposomes [6] reproduces apoptotic MMP more accurately than other experimental systems. Intriguingly, the apoptotic release of mitochondrial intermembrane proteins is not usually accompanied by signs of membrane rupture, and similarly t-Bid/Bax-permeabilized cardiolipin-containing liposomes do not manifest any (permanent) membrane discontinuities at the ultrastructural level.

Not The Whole Story

The data discussed above suggest that t-Bid, Bax, and a defined lipid environment including cardiolipin can mimic many properties of apoptotic MMP (Figure 1). However, as the authors admit [6], this experimental system does not recapitulate all properties of apoptotic MMP. Thus, the inner membrane of mitochondria or the membrane of mitoplasts (mitochondria which lack the outer membrane) is not completely permeabilized by t-Bid/Bax, in spite of the fact that the inner membrane contains large amounts of cardiolipin. Indeed, t-Bid and/or Bax added to mitochondria can cause the formation of ion-permeable channels in the inner

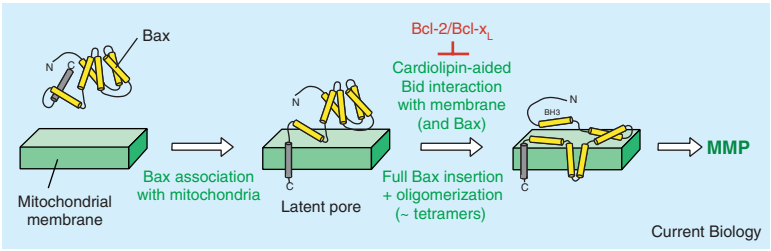

Figure 1. Hypothetical sequence of events involved in MMP mediated by Bax and t-Bid.

Bax is either monomeric in the cytosol or loosely attached to the outer mitochondrial membrane. After interaction with t-Bid it becomes fully inserted, adopts the apoptotic conformation, and oligomerizes to at least tetramers, resulting in MMP. Bcl-2 and Bcl-x_L would inhibit the action of Bid on Bax, while the pro-apoptotic proteins Bad and Bik would inhibit Bcl-2 or Bcl-x_L.

membrane (which in hypotonic media causes mitochondrial swelling), yet does not lead to the release of matrix proteins [11–15]. This clearly illustrates that intact mitochondria manifest a more complex behavior than liposomes and implies the existence of regulatory molecules that prevent the formation of protein-permeable pores in the inner mitochondrial membrane.

Another inconsistency between the reconstituted system and the physiology of apoptosis concerns the oligomerization of Bax. In whole cells stimulated to undergo apoptosis, Bax forms an oligomeric complex whose estimated molecular mass exceeds 200 kDa [16]. This higher-order complex is also formed when t-Bid and Bax are added to intact mitochondria [16] and OMVs [6]. However, in cardiolipin-containing liposomes devoid of mitochondrial proteins, only smaller Bax oligomers (~100 kDa) are formed [6]. The capacity of OMVs to induce the formation of higher-order complexes is destroyed by treatment with proteinase K [16], indicating that one or several yet-to-be-identified outer membrane proteins contribute to the higher order oligomerization of Bax. Whether such a protein or protein complex also facilitates the t-Bid/Bax-triggered permeabilization reaction has not been determined in detail. However, unlike chemically defined liposomes, OMVs loaded with dextran and treated with t-Bid/Bax completely release their content [6], suggesting that some mitochondrial protein(s) can indeed facilitate the permeabilization reaction (Figure 2A).

Additional Elements in the Scenario

Several previous studies have demonstrated that cyclosporin A, an inhibitor of the so-called permeability transition pore complex (PTPC), can prevent or reduce the MMP induced by Bax and/or t-Bid *in vitro*, in isolated mitochondria [11–15]. In addition to cyclophilin D, the mitochondrial target of cyclosporin A, the PTPC contains the adenine nucleotide translocater (ANT) of the inner membrane, the VDAC of the outer membrane, as well as the peripheral benzodiazepin receptor [17]. Liposome-based assays indeed revealed that Bax may cooperate with VDAC to form cytochrome c-permeable conduits in the outer membrane [9]. Moreover, Bax may interact with ANT to form composite ion channels in the inner membrane [11]. According to one study [15], Bid added to purified mitochondria causes a partial release (~15%) of cytochrome *c* in the presence of cyclosporin A. However, the release of the remaining ~85% cyto-chrome *c* would depend on the (PTPC-dependent) structural reorganization of the junctions between the intercristae and the intermembrane space of mitochondria. Thus, one possible scenario involves two independent steps of MMP, one which occurs in a PTPC-independent fashion and is recapitulated in the paper by Newmeyer and colleagues [6], and a second one which involves physical or functional interactions of pro-apoptotic proteins with PTPC proteins (Figure 2B).

Unresolved Conundra

The exploration of MMP mechanisms is still plagued with several major enigmas. One conundrum concerns the specific lipid environment in its interaction with apoptosis-regulatory proteins. Are lipid microdomains important for the insertion/oligomerization of Bax? Does a regulated change in the submitochondrial

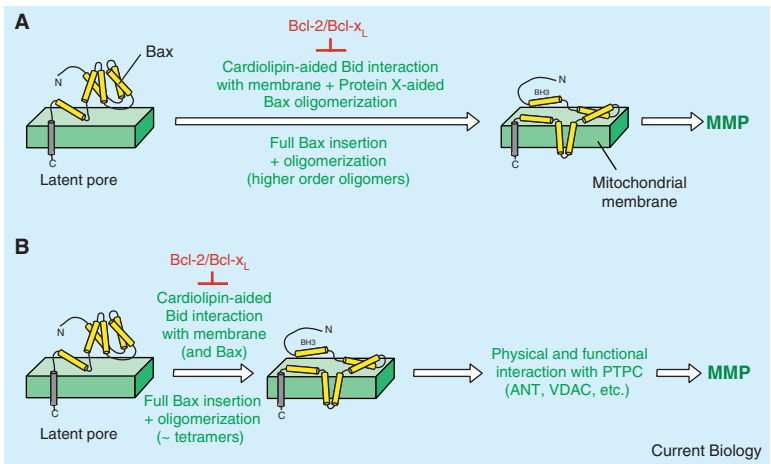

Figure 2. Additional elements in the cascade of events leading to MMP.

(A) Implication of an elusive mitochondrial outer membrane protein or protein complex (protein X) in the insertion/oligomerization reaction. (B) Implication of Bax interactions with PTPC components. After Bid-mediated insertion in mitochondrial membranes, Bax would engage in physical and/or functional interactions with PTPC proteins, which would be necessary for full MMP. Note that the two scenarios may be compatible.

distribution of lipids, including a putative flipping out of cardiolipin from the inner to the outer leaflet of the inner membrane (and perhaps to the contact site?) [18] contribute to apoptosis? How does this relate to the reported phospholipid transferase activity of Bid [19]? How does the oxidation of cardiolipin, which apparently contributes to the release of cytochrome c [20], fit into the scenario? Another series of questions concerns the mechanisms of the permeabilization process itself. Is the possible breakage of the outer membrane a transient process? Does it relate to the regulation of membrane curvature [15]? And last but not least, is there only one MMP mechanism involving pro-apoptotic members of the Bcl-2 family, or are there different modes of MMP, depending on the apoptosis-initiating stimulus?

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