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The channel of death

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The proapoptotic members of the Bcl-2 family have been proposed to participate in the formation of a channel that releases these apoptogenic factors when mitochondria receive apoptotic signals. A recent study provides the first direct, biophysical measurement of a potentially apoptosis-specific mitochondrial channel, which is regulated by Bcl-2 family members and may play a primary role in the release of the proapoptotic factors.

Apoptosis is a highly regulated cellular suicide program present in every metazoan cell. Diverse toxic signals (e.g., genotoxic drugs, UV and gamma irradiation, endoplasmic reticulum stress, etc.) trigger the activation of a universal apoptosis response, which eliminates damaged cells from the organism in a highly ordered and controlled fashion. Mitochondria play a pivotal role in apoptosis by acting as a sensor and an integrator to receive as well as to amplify signals from diverse upstream signaling pathways, and to initiate downstream execution steps.

Mitochondria consist of an outer membrane (OMM),* which is readily permeable to solutes with sizes <1.5 kD, and an essentially impermeable inner membrane (IMM). The integrity of the latter is critical for maintaining an electrochemical potential ($\Delta\psi_m$) across the IMM, which is required for oxidative phosphorylation. Recent studies showed that a number of apoptogenic factors, including cytochrome c, apoptosis inducing factor, endonuclease G, SMAC/DIABLO and procaspases, are safely sequestered in the mitochondrial intermembrane space between the OMM and IMM in healthy cells, but are released into cytosol under apoptotic conditions (Jacobson and Duchen, 2001; Li et al., 2001). Since the size of these factors exceeds the permeability barrier of the OMM, permeabilization of the OMM is expected to be required for their release.

Cytochrome c is the best characterized factor released from mitochondria during apoptosis. Upon cytosolic entry, it serves as a cofactor in the formation of the "apoptosome," a complex consisting of the adaptor protein Apaf-1 and procaspase-9, which in turn causes the activation of caspase-9 and downstream caspases, such as caspase-3 (Chinnaiyan, 1999). Two major models have been put forward to explain the molecular mechanism by which cytochrome c is released during apoptosis. One model proposes that proapoptotic members of the Bcl-2 protein family directly form pores in the OMM, which can selectively mediate cytochrome c release without major effects on mitochondrial function (Harris and Thompson, 2000; Korsmeyer et al., 2000; Waterhouse et al., 2001) (Fig. 1). The second model argues that cytochrome c is released as a result of mitochondrial membrane rupture in apoptosis (Harris and Thompson, 2000; Tsujimoto and Shimizu, 2000; Zamzami and Kroemer, 2001) (Fig. 1). According to this model, disruption of the OMM is the result of the opening of the mitochondrial megapore called the permeability transition pore (PTP), which is formed at the contact sites between the IMM and OMM. The core components of the PTP are the adenine nucleotide translocator (found in the IMM) and the voltage-dependent anion channel (VDAC, located in the OMM). Opening of the PTP during apoptosis is postulated to result in the loss of $\Delta\psi_m$ and swelling of the mitochondrial matrix, which causes eventual rupture and nonselective permeabilization of the OMM. In either case, the direct characterization of an apoptosis-specific mitochondrial channel(s) is extremely important for understanding of the mechanism of OMM permeabilization. Until now, however, no one has been able to directly demonstrate the existence of such channel(s) despite intensive studies of mitochondria. The previously published studies primarily used in vitro electrophysiological analyses of the channels formed in artificial lipid bilayers by various putative apoptotic factors.

Kinnally and colleagues (Pavlov et al., 2001, this issue) use patch clamping techniques to obtain the first direct biophysical evidence for the existence of the apoptotic mitochondrial channel. The authors of this paper unambiguously show the appearance of a new channel in the OMM upon induction of apoptosis in response to IL-3 deprivation of murine FL5.12 cells. They further find that proteoliposomes prepared from the fragments of the OMM of apoptotic cells, but not from normal cells, lose encapsulated exogenous cytochrome c, demonstrating that the ability to release cytochrome c can be reproduced in reconstitution experiments and thereby arguing against the release of cytochrome c through nonspecific OMM rupture. Using patch clamping techniques, Kinnally and coworkers further show that increased OMM permeability

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*Abbreviations used in this paper: BH, Bcl-2 homology; IMM, inner mitochondrial membrane; MAC, mitochondria apoptosis-induced channel; OMM, outer mitochondrial membrane; PTP, permeability transition pore; VDAC, voltage-dependent anion channel.

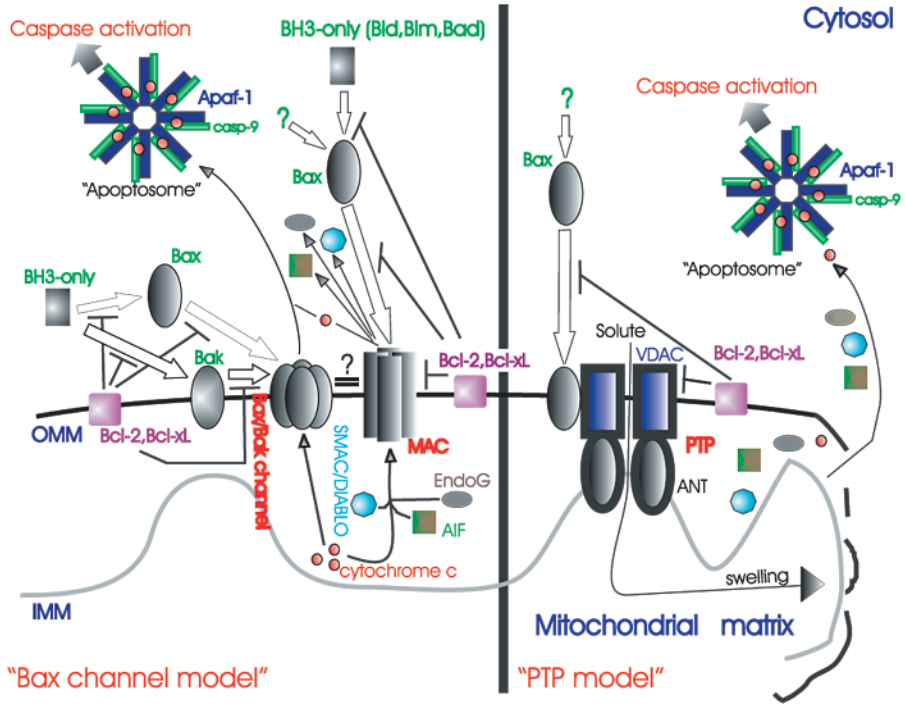

Figure 1. **The molecular mechanisms of the mitochondrial regulation during apoptosis.** (Left, Bax channel model) According to this model, the release of apoptogenic factors from the mitochondrial intermembrane space is mediated by tetrameric channels formed by proapoptotic Bcl-2 family members Bax and Bak. Bax and Bak are activated by BH3-only factors (Bid, Bad, Bim, etc.). Formation of these channels is blocked by antiapoptotic Bcl-2 family members (Bcl-2, Bcl-xL, etc.) at multiple steps. MAC, described by Kinnally and coworkers (Pavlov et al., 2001), may represent a channel similar to the Bax/Bak channel, but may contain additional components. (Right, PTP model) According to this model, Bax binds to the PTP complex and causes its opening, resulting in the swelling of the mitochondrial matrix and rupture of the OMM. Antiapoptotic Bcl-2 family members close the PTP channel and block translocation of Bax from cytosol to mitochondria.

in apoptotic cells can be attributed to a specific channel, which they term mitochondria apoptosis-induced channel (MAC). Analysis of MAC shows that it is voltage-independent and displays multiple conductance levels, with a peak single channel opening of 2.5 ± 0.6 nS, corresponding to a pore diameter of 4.0 ± 0.5 nm. A pore of this size would allow the passage of cytochrome c and other proteins across the OMM. Such large conductance channel activity is rare under normal conditions, and MAC properties are readily distinguishable from that of VDAC (Colombini et al., 1996) and the protein import channel (TOM) (Hill et al., 1998) that are not altered in IL-3 deprived apoptotic cells.

Mitochondria are the primary sites of action of the Bcl-2 family of proteins in the regulation of apoptosis. The exact mechanisms by which it induces or protects against mitochondrial damage, however, are the subjects of intensive studies and are highly controversial (Gross et al., 1999; Harris and Thompson, 2000; Tsujimoto and Shimizu, 2000). The Bcl-2 family is characterized by the presence of Bcl-2 homology (BH) domains. It consists of both proapoptotic (Bid, Bax, Bak, Bim, Bad, etc.) and antiapoptotic (Bcl-2, Bcl-xL, etc.) members, which affect mitochondria in antagonistic fashion. Proapoptotic Bcl-2 family members can be further subdivided into multidomain family members (Bax, Bak), containing multiple BH domains also characteristic for the antiapoptotic factors (BH1–BH4), and BH3-only proteins (Bid, Bad, and Bim). Initial characterization of Bcl-2 family members suggested that they possess two major functions: BH3-domain–mediated homodimerization and heterodimerization and in vitro channel-forming activity. In the latter case, both Bcl-2 and Bax have been shown to form channels in artificial liposomes (Antonsson et al., 1997; Saito et al., 2000).

Genetic knockout analyses of Bcl-2 family recently performed in the laboratories of Stanley Korsmeyer and Craig Thompson suggest that Bax and Bak may serve as the major mitochondrial sensors of upstream apoptotic signaling, and cells missing both factors are resistant at the mitochondrial level to killing by a wide variety of stimuli (Wei et al., 2001). Killing by Bax and Bak involves posttranslational activation of these factors (Gross et al., 1999; Korsmeyer et al., 2000), since these two proteins are abundantly present in the live cells. Several observations suggest that Bax is a monomeric soluble cytosolic factor in normal cells, but, upon induction of apoptosis, it multimerizes, translocates and inserts into the OMM. Nuclear magnetic resonance analyses showed that the Bax COOH-terminal tail masks the BH3 domain, maintaining the protein in the inactive form (Suzuki et al., 2000). Activation of Bax can be accomplished by high concentration of detergents or binding of BH3-only Bcl-2 family members. The resulting oligomeric form of Bax possesses a greatly increased channel-forming and cytochrome c releasing activity. In contrast to Bax, Bak usually resides in the OMM. However, it also undergoes BH3-dependent oligomerization and activation brought about by BH3-only factors.

One obvious possibility, therefore, is that MAC represents the channel formed by Bax in mitochondria (Fig. 1). The authors find that although MAC does share a number of similarities with Bax channels formed in artificial liposomes, such as that they both display multiple conductance levels and a slight cation selectivity. The former is much larger than Bax channel, which has its typical peak conductance of only ~0.5 nS (Pavlov et al., 2001; Saito et al., 2000). Thus, MAC is not equivalent to the Bax channel. On the other hand, the authors demonstrate that Bax is able to induce the formation of MAC in yeast, as MAC opening can be detected in Bax overexpressing yeast cells that lack endogenous Bcl-2 family of proteins. In addition, the authors report that although no novel channel activity can be found in mammalian cells overexpressing Bcl-2, MAC was not detected in these cells, sug-

gesting that pore formation by Bcl-2 is not required for the inhibition of MAC. From these results, the authors conclude that Bax induces and may be a direct component of MAC, which can be inhibited by Bcl-2, but that MAC is not equivalent to the in vitro tetrameric Bax pore (Pavlov et al., 2001).

One possible explanation for the observed differences in MAC and Bax channel properties is that although Bax or Bak are integral components of MAC, initial activation by BH3-only factors may trigger further posttranslational modification(s) and/or recruitment of additional factors that are missing in vitro. The lipid composition of the endogenous OMM can also have substantial effect on the pore formation. Alternatively, Bax and Bak may only serve to induce the formation of MAC without being directly involved in the channel itself. Future experiments are needed to distinguish between these possibilities.

One of the previous arguments against the "Bax channel" model is that mitochondria are known to release factors, such as SMAC/DIABLO, with sizes exceeding the inner diameter of in vitro generated Bax channel (Saito et al., 2000) (Fig. 1). This is not expected to be a problem for MAC: the calculated pore size of MAC is significantly larger than that of the Bax channel. The large conductance of MAC, however, does pose the question of selectivity. Does MAC selectively release cytochrome c and a few other proteins or does it essentially open the door in the OMM for everything in the intermembrane space? Although mitochondria do seem to release components nonspecifically sooner or later during the course of apoptosis (Zamzami and Kroemer, 2001), it is still possible that some kind of gating system may allow the passage of only selected proteins, even when the conductance is large.

Although the results of Pavlov et al. (2001) provide a boost for the Bax channel hypothesis, the role of VDAC in mitochondrial damage and release of cytochrome c cannot be ignored. Shimizu et al. have recently suggested that Bax and Bak, but not BH3-only factors, can cause $\Delta\psi_m$ loss in isolated mitochondria (Shimizu and Tsujimoto, 2000). Even though Kinnally and coworkers' yeast data argues against a contribution of VDAC to MAC (Pavlov et al., 2001), it is quite intriguing that the in vitro VDAC-Bax channel reported by Shimizu et al. (Shimizu et al., 2000) appears to be very similar in size to that of MAC and also was found to possess a second state with lower conductivity. The use of VDAC-targeting antibodies and small molecule inhibitors may help clarify this issue.

Interestingly, in cells lacking a downstream caspase activation mechanism (caspase-$3^{-/-}$, caspase-$9^{-/-}$ or Apaf-$1^{-/-}$), a delayed Bax/Bak-mediated $\Delta\psi_m$ loss can still be detected (Cheng et al., 2001). Thus, in the absence of caspases, Bax/Bak may be able to induce the opening of VDAC, which results in the swelling of mitochondrial matrix, rupture of OMM, and death by a caspase-independent pathway. Therefore, Bax/Bak-mediated channel activity may have multiple ways to induce cell death; the exact pathways to be activated may vary in a cell type– and stimulus-dependent manner.

Overall, the approach developed by Kinnally and coworkers (Pavlov et al., 2001) provides researchers with a valuable and exciting possibility to directly address the mechanism of the mitochondrial regulation of apoptosis. It can be coupled with other available research tools, such as using mitochondria lacking a particular putative regulatory factor from genetic knockout mice and specific small molecule– and antibody-based inhibitors of such factors, to further characterize the mechanism by which mitochondria regulate apoptosis.

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