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Mitochondria: Releasing Power for Life and Unleashing the Machineries of Death

Review

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The mitochondrion has long been known both as a chemical powerplant and as a cellular compartment housing various biosynthetic pathways. However, studies on the function of mitochondria in apoptotic cell death have revealed a versatility and complexity of these organelles previously unsuspected. The mechanisms proposed for mitochondrial involvement in cell death are diverse and highly controversial. In one model, mitochondria are seen as passive containers that can be made to leak out cytotoxic proteins. In other scenarios, however, certain more or less familiar aspects of mitochondrial physiology, such as oxidative phosphorylation, generation of oxygen radicals, dynamic morphological rearrangements, calcium overload, and permeability transition, are proposed to play crucial roles. In this review, we examine a few promising mechanisms that have been gaining attention recently.

Introduction

After the first reports connecting mitochondria with apoptotic cell death (Marchetti et al., 1996a; Newmeyer et al., 1994; Petit et al., 1995; Zamzami et al., 1995), it was natural to wonder whether the life-supporting functions of mitochondria were somehow linked to their death-promoting activity. Early evidence showed that cultured cells whose organelles lacked mitochondrial DNA and were therefore deficient in respiration nevertheless could undergo normal apoptosis (Jacobson et al., 1993; Marchetti et al., 1996b). This suggested that mitochondrial physiology may not play a major role in apoptotic cell death, at least in cultured cells.

Indeed, early discoveries suggested that the pro-death and pro-survival functions of mitochondria are quite distinct. In particular, it was observed that in apoptosis, mitochondria release proteins, such as cytochrome c (Liu et al., 1996) and AIF (Susin et al., 1999), which display cryptic cytotoxic activity after they escape from the mitochondrial intermembrane space into the cytoplasm (Figure 1). Other such proteins, including Smac/DIABLO, Endo G, and Htra2/Omi, were identified later (reviewed by Green and Evan, 2002). As cytochrome c clearly illustrates, these proteins can have functions in cell death that are unrelated to their normal action, if known, within mitochondria. It was further shown that the antiapoptotic Bcl-2 protein, which was already known to be located on mitochondrial and ER membranes, could block apoptosis by inhibiting the release of cytochrome c and other proteins of the intermembrane space (Kluck et al., 1997; Yang et al., 1997).

These first observations suggested a simple and transparent explanation for mitochondrial involvement in cell death. However, the complexity of normal mitochondrial function may hint that the role of mitochondria in apoptosis could be similarly complicated. Indeed, in recent years, multiple mechanisms have been proposed to explain mitochondrial function in cell death (Figure 2). Some of these involve aspects of mitochondrial physiology (see below), such as the production of reactive oxygen species (ROS), opening of the permeability transition (PT) pore, respiration (Chandra et al., 2002; McClintock et al., 2002), or ATP synthase (Shchepina et al., 2002), to give just a few examples. On the other hand, growing evidence also supports the simple view of mitochondria as passive structures, acted upon by proteins that permeabilize the outer membrane to release apoptogenic proteins.

A challenge we now face is to determine which of these various proposed mechanisms are employed in specific cellular contexts, bearing in mind that in some settings several mechanisms could occur in parallel. Thus, one of our highest priorities must now be to tease apart apoptotic pathways by selective loss-of-function approaches. With new techniques for ablation or "knock-down" of specific genes, this goal is coming within reach, at least for those proteins whose function in non-dying cells is dispensable, or for which mutant forms exist that dissociate the normal and apoptotic activities. Unfortunately, however, as many mitochondrial functions are essential for cellular housekeeping, these genetic approaches may sometimes be difficult or impractical.

Mitochondria: The Cell's Powerhouse, and What Else?

Mitochondria have long been considered to play a straightforward but critical role in the life of the cell; namely, to carry out energy-yielding oxidative reactions that create the vast majority of ATP necessary to support all cellular functions. Interruption of this mitochondrial function in vivo leads to death, as dramatically illustrated by such poisons as cyanide. Indeed, a major advance in the last 20 years has been the recognition of many mitochondria-related diseases that result from severely compromised energy generation, often due to genetic defects in the mitochondrial genome (Wallace, 1999b). Although it is well established that mitochondria carry out numerous metabolic reactions, involving amino acids, lipids, and ketone bodies, the role of mitochondria in calcium signaling and their contribution to the production of damaging oxygen radicals has not been clear. A much more significant role than previously thought in both these key processes (Lenaz et al., 2002; Pozzan et al., 2000) is now established.

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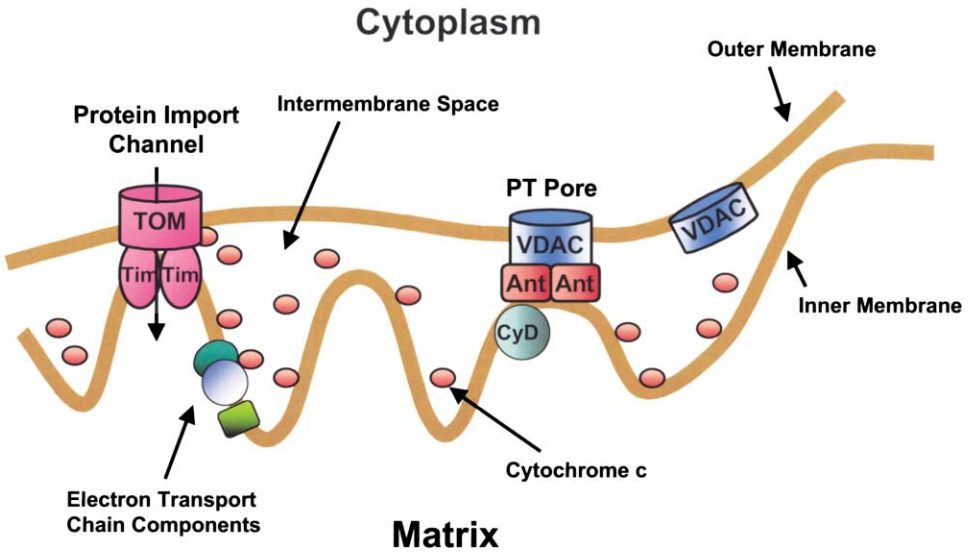

Figure 1. Mitochondrial Architecture

Between the outer and inner membranes lies the intermembrane space, where proapoptotic proteins such as cytochrome c are located. The constituents of the electron transport chain, except for cytochrome c, are embedded in the inner membrane. VDAC (voltage-dependent anion channel) is found exclusively in the outer membrane; this channel allows diffusion of metabolites and ions across the outer membrane. In contrast, the inner membrane is impermeable, allowing for the maintenance of a transmembrane potential, $\Delta\Psi_m$. The inner membrane contains transport molecules, e.g., the adenine nucleotide transporter (ANT), responsible for the exchange of specific small molecules. Within the inner membrane is the matrix. Most mitochondrial proteins are encoded by nuclear genes and imported from the cytoplasm, through one or both membranes, via transport complexes Tom and Tim.

That mitochondria have their own genome does not liberate them from control by nuclear genes, as only a few of the mitochondrial proteins are encoded internally. Most of the proteins from which mitochondria are built, as well as those forming the machinery for building them, are imported from the cytosol. These nuclear-encoded gene products account for many mitochondrial diseases (Melov et al., 1999; Wallace, 1999a). Mitochondrial protein import involves elaborate molecular mechanisms that are also dependent on energy, in the form of a potential across the inner membrane. In fact, all the proteins known to be released during the induction of cell death (e.g., cytochrome c, AIF, and Smac/DIABLO) are originally imported from the cytosol. Interestingly, cells lacking mitochondrial DNA still contain mitochondria that maintain a membrane potential and protein import function, even though they cannot carry out oxidative phosphorylation.

It was recognized early on that apoptotic death was an energy-dependent process, and might be inhibited, rather than promoted, in the absence of a significant level of mitochondrial function. Therefore, the situation is not as simple as implied by many mitochondrial disease states, where deprivation of energy in the longer term is the key to destruction, perhaps by necrotic as well as apoptotic mechanisms. But how much energy is required for apoptosis? And can low energy, in the short term, be a stimulus for apoptotic cell death? Answers to these questions have eluded us, mainly because our ability to quantify and time-resolve mitochondrial activities inside a cell are not yet as sophisticated as we might wish.

It is evident that the loss of cytochrome c, an essential player in the respiratory chain, can inhibit the electron transfer process and thus lower ATP levels. However, the degree of inhibition is dependent on how much of the cytochrome c is lost and what proportion of the cell's mitochondrial population is involved. Therefore, it is important to know whether cytochrome c is rate-limiting for the electron transfer process (Mootha et al., 2001; von Ahsen et al., 2000; Waterhouse et al., 2001), as well as how homogeneous the mitochondrial response is to apoptotic signals (D'Herde et al., 2000; Goldstein et al., 2000; Krysko et al., 2001).

Electron transport is carried out in the mitochondrial inner membrane through a series of membrane-embedded proteins (Figure 3) that communicate via several smaller molecules, the lipid-soluble ubiquinone, and the water-soluble protein cytochrome c. Both these molecules are players in aspects of programmed cell death. While the inner membrane is only permeable to ions via specific carrier systems, the mitochondria also possess an outer membrane that is readily permeable to small charged molecules because it contains pores formed by the protein VDAC (voltage-dependent anion channel, or porin). Normally, the outer membrane is not permeable to proteins that reside in the intermembrane space, such as cytochrome c.

Electrons derived from foodstuffs are fed into the respiratory chain at a high potential energy through the NADH:ubiquinone oxidoreductase (complex I) or via $FADH_2$-containing enzymes in the inner membrane such as succinate dehydrogenase (complex II) and glycerolphosphate dehydrogenase. The electrons are passed sequentially to ubiquinone, cytochrome bc_1, cytochrome c, and ultimately delivered to oxygen, the final electron sink, via cytochrome c oxidase. The potential energy of the electrons is converted into a charge gradi-

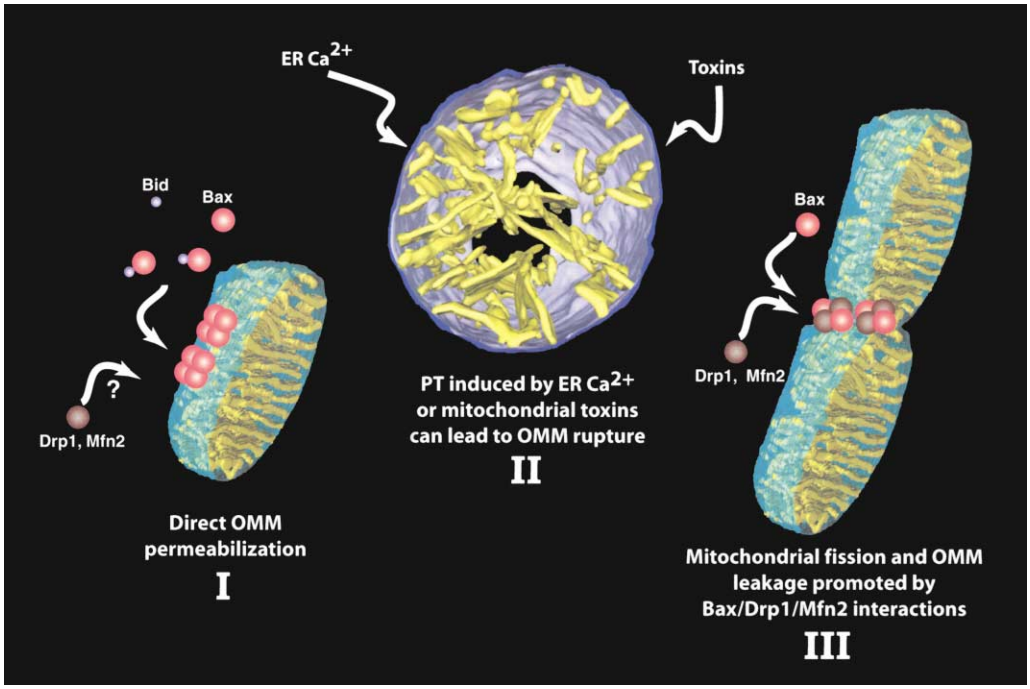

Figure 2. Three Mechanisms of Apoptotic Outer Mitochondrial Membrane (OMM) Permeabilization Gaining Support.

Mechanism I: BH3-only proteins (e.g., Bid or Bim) induce direct OMM permeabilization via Bax/Bak·lipid interactions. (The activation of BH3-only proteins is a crucial subject, which however is beyond the scope of this article.) In one hypothetical model, tBid interacts transiently via its BH3 domains with Bax, displacing the C-terminal α helix from the BH3 binding groove. This in turn allows unstable Bax·Bax intermediates (displacing tBid) to form in solution; these are stabilized by insertion into the membrane. Alternatively, Bax complexes could assemble in the membrane. Stable Bax tetramers in the membrane act in concert with cardiolipin to increase curvature stress, leading to the formation of lipidic pores. Possibly other proteins, e.g., Drp1, Mfn2, or VDAC, facilitate this process through cardiolipin redistribution or by otherwise destabilizing the membrane.

Mechanism II: Bax helps mobilize ER Ca^{2+} stores, leading to mitochondrial PT, matrix swelling, and rupture of the OMM. Agents such as mitochondrial toxins can also induce PT, perhaps directly or by stimulating ROS production by the respiratory chain.

Mechanism III: Bax/Drp1/Mfn2 interactions could hypothetically promote mitochondrial fission and destabilize the OMM.

Note that mechanisms I and III do not necessarily exclude one another.

ent across the inner membrane, positive outside, by the proton pumping activity of the respiratory chain. The energy potential across the inner membrane, $\Delta\Psi_m$, drives ATP synthesis via the F_0F_1-ATPase (ATP synthase) or is used directly for transport processes, e.g., calcium uptake and protein import.

Blockage of electron transfer, e.g., by antimycin or cyanide, or a temporary lack of the final acceptor, oxygen, can lead to the increased reduction of ubiquinone and increased levels of partially reduced ubisemiquinone bound to complex I or to cytochrome bc_1 (Staniek et al., 2002). Some of these accumulated quinone species appear to react directly with oxygen, once it is available, and to be a major source of oxygen radicals (Liu et al., 2002). This can cause, for instance, reperfusion injury following a heart attack, as a result of the ability of oxygen radicals to induce cell death. Since cytochrome c delivers electrons from cytochrome bc_1 to cytochrome c oxidase, the complete loss of cytochrome c could similarly lead to accumulation of electrons in the chain and oxygen radical formation. However, a less substantial loss of cytochrome c might generate little ROS, since cytochrome c may not become limiting.

In apoptosis, coincident with the permeabilization of the outer mitochondrial membrane, there is typically a rapid reduction in the mitochondrial membrane potential (Goldstein et al., 2000). Recent studies have shown that this loss of $\Delta\Psi_m$ reflects a block of respiratory function which, interestingly, is not due to reduced concentrations of cytochrome c, as this protein is present in such functional excess that it is able to support respiration even after dispersal in the cell (Mootha et al., 2001; Waterhouse et al., 2001). Rather, the loss of membrane potential is due to the cleavage and inactivation of electron transport chain constituents by activated caspases (Bossy-Wetzel et al., 1998; Ricci et al., 2003; von Ahsen et al., 2000).

Along with their role in ROS production, mitochondria are now understood to be a significant player in the regulation and response to calcium changes in the cell. It has become clear in the past few years, as a result of elegant localization experiments using targeted fluorescent proteins (Magalhaes and Rizzuto, 2001), that mitochondria in situ respond to calcium in signaling processes in a much more dramatic way than previously predicted (Rizzuto et al., 2000). Local gradients of calcium cause local, transitory and massive calcium uptake by mitochondria (Rizzuto et al., 1999). The studies illustrate the profound inhomogeneity of certain mitochon-

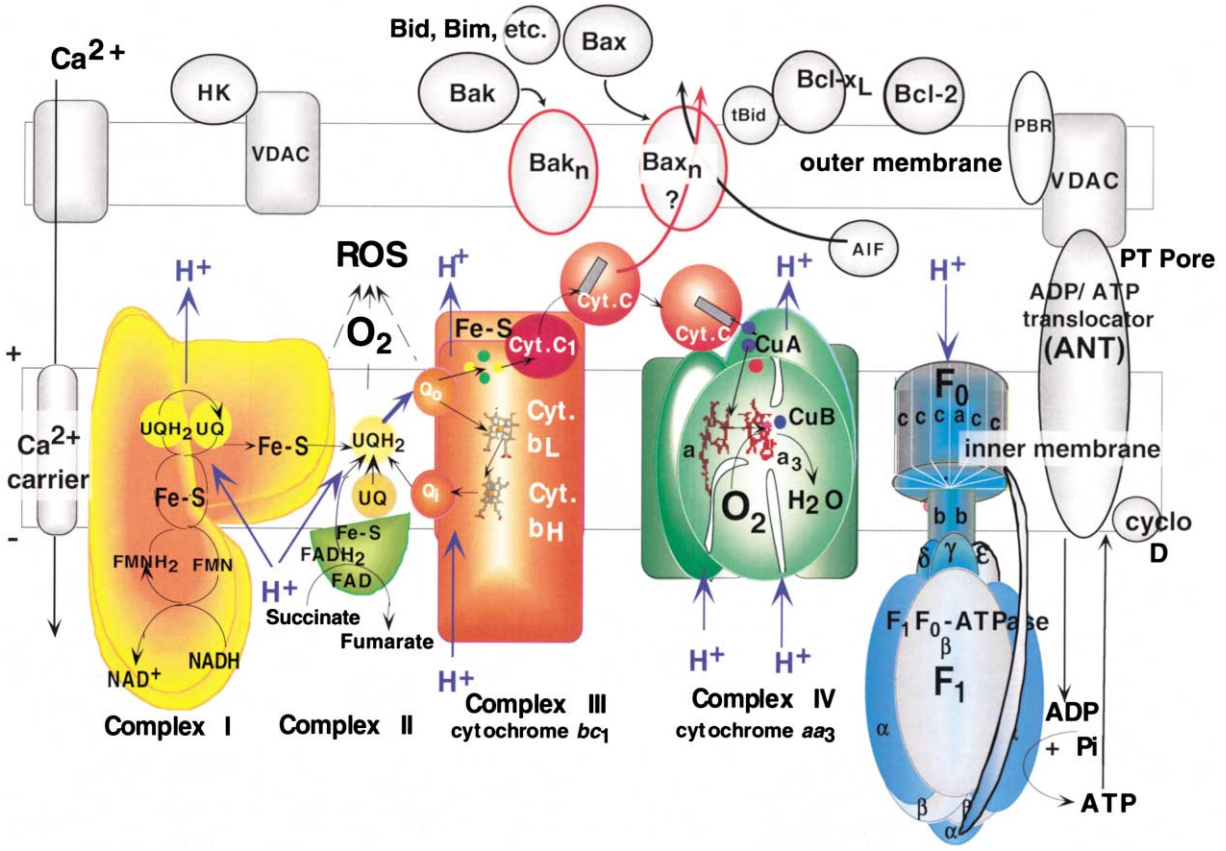

Figure 3. The Mitochondrial Electron Transport Chain, Represented in the Context of Processes and Proteins that May Play a Role in Apoptosis

In the inner membrane of the double membrane system of mitochondria, the four respiratory chain components are shown: complexes I (yellow), II (light green), III (brown), and IV (green), with their substrates, cofactors, and the paths of electron flow (black arrows). Proton paths involved in generation and utilization of the membrane potential are indicated with blue arrows and blue protons. The ATP synthase (complex V, blue) is shown using the proton gradient to drive ATP synthesis. Radical oxygen species (ROS) are indicated to be produced at the level of complexes I and III where ubiquinol (UQH_2), and ubisemiquinol are formed. In the outer membrane, the pore protein, VDAC (voltage dependent anion channel) is shown interacting with various cytosolic and mitochondrial proteins (see text), including the ADP/ADP translocator (or ANT). The latter interaction is represented as forming the permeability transition pore (PT pore) sometimes associated with loss of the inner membrane potential during apoptosis (see text). Abbreviations: UQ = ubiquinone, or coenzyme Q; NAD+ = nicotinamide adenine dinucleotide; FMN = flavin mononucleotide; Fe-S = iron sulfur center; FAD = flavin adenine dinucleotide; Qo = outer quinone binding site; Qi = inner quinone binding site; Cyt.b_L, Cyt. b_H = low and high potential cytochrome b; Cyt.c1, Cyt.c = cytochrome c1, c; CuA, CuB = copper site A and B; a, a_3 = hemes a and a_3; Fo and F_1 = membrane and soluble domains of ATP synthase, with subunits designated; cyclo-D = cyclophilin D; PBR = peripheral benzodiazepine receptor; HK = hexokinase; Bax_n = polymeric form of Bax, inserted in the outer membrane, and similarly for Bak_n. Bax, Bak Bcl-x_L, Bcl-2, tBID, AIF: see text.

drial responses within the cell as well as their complex kinetics. In theory, mitochondria could respond to death signals in a similarly localized and transitory way, making it difficult to accurately assess the changes in energy, ions, and radicals that are contributing to the mechanism of apoptosis.

Indeed, mitochondria within single cells were found to undergo permeability transition asynchronously following treatment with TNFα (Lemasters et al., 1999). On the other hand, studies in HeLa cells expressing mitochondrially localized GFP-cytochrome c observed a much more uniform response to apoptotic stimuli. When these cells are treated with staurosporine, for example, the cells initiate apoptosis stochastically, at various times after the insult. However, once a given cell has begun to undergo apoptosis, within ~5 min all of its mitochondria become permeabilized and release all of their GFP-cytochrome c into the cytoplasm (Goldstein et al., 2000). It is unknown whether this synchronization reflects a global activation of a mitochondrial permeabilizing signal, or instead a rapid cascade in which one mitochondrion triggers the permeabilization of its neighbors.

The Bcl-2 Family of Proteins Are Critical Regulators of Mitochondrial Apoptosis

Bcl-2 was discovered as an oncoprotein that supports neoplastic growth, not by stimulating cellular proliferation, but rather by blocking cell death (Hockenbery et al., 1990). In recent years, a number of proteins functionally and structurally related to Bcl-2 have been discovered (reviewed recently by Borner, 2003). Some Bcl-2-family proteins, like Bcl-2 itself, Bcl-x_L, and Mcl-1, inhibit cell death, whereas others promote apoptosis. The anti-apoptotic proteins generally contain four domains of sequence similarity, designated BH1 through BH4. The

proapoptotic members of the family, on the other hand, are classified according to whether they contain one homology region (the "BH3-only" proteins, e.g., Bid, Bad, Bim, Noxa, and Puma) or three (the "multidomain" or "BH1-3" proteins, including Bax, Bak, and Bok).

A striking simplification in the apoptosis field came with the discovery that many forms of cell death require the BH1-3 proteins Bax and Bak, and by analogy, perhaps Bok, in tissues expressing this related protein (Lindsten et al., 2000; Wei et al., 2001). Mice that are deficient in both *bax* and *bak* genes display a striking phenotype of supernumerary cells; furthermore, cultured cells derived from these animals are resistant to a wide range of apoptosis-inducing treatments.

How does this observation make things easier? We can now begin to classify instances of cell death according to whether they require BH1-3 proteins like Bax or Bak. Moreover, except for those unusual forms of cell deaths that do not involve Bax, Bak, and Bok, our investigations can now focus mostly on discovering the activation pathways and effector functions of these BH1-3 proteins. The problem, of course, is that we cannot claim to know all the ways in which Bax-type proteins act. However, clues are provided by the association of these proteins with the endoplasmic reticulum and outer mitochondrial membranes. That the antiapoptotic members of the Bcl-2 family (including Bcl-2 and Bcl-x_L) are also typically found at mitochondria and the ER suggests that cell death may be regulated both positively and negatively in a localized, organelle-specific fashion. A vital but partly unresolved issue concerns whether the pro- and antiapoptotic Bcl-2-family proteins act in an opposite manner on the same effector molecules, whether they neutralize one another through direct binding, or whether they exert their opposing functions through separate effectors. The answer to these questions will likely depend on the particular sites and mechanisms of action of Bcl-2-family proteins in specific instances of cell death.

Bcl-2-Family Proteins Can Act Directly on the Outer Mitochondrial Membrane

Experiments in which Bcl-2 was targeted exclusively either to mitochondria or to the ER revealed that this protein can block apoptosis by acting specifically at one or the other location, depending on the death stimulus (Annis et al., 2001; Lee et al., 1999; Zhu et al., 1996). Analogous studies with organelle-targeted Bax and Bak have yet to be reported. However, a number of investigations have focused on the ability of Bcl-2-family proteins, both pro- and antiapoptotic, to act directly on isolated mitochondria, where they promote or inhibit the release of apoptogenic proteins from the intermembrane space.

Mechanisms of Membrane Permeabilization by Bax: Lipidic Pores

Several laboratories have investigated the direct actions of Bcl-2-family proteins on membranes, ultimately in an attempt to define the minimum set of proteins needed to permeabilize mitochondrial outer membranes during apoptosis (Basanez et al., 1999, 2001, 2002; Epand et al., 2002a, 2002b, 2002c, 2002d; Kuwana et al., 2002; Roucou et al., 2002b; Saito et al., 2000; Shimizu et al., 1999). There is still disagreement over whether Bcl-2-family proteins alone are sufficient, or whether other outer membrane proteins, such as VDAC or other unidentified molecules, are required. Much of the confusion may be due to the technical difficulties inherent in membrane reconstitution. However, a recent report (Kuwana et al., 2002) showed that the process of Bax-mediated outer membrane permeabilization could be reproduced authentically in three progressively simplified vesicular systems, which consisted either of purified outer membrane ghosts, liposomes composed of extracted mitochondrial lipids, or ultimately liposomes of defined composition. Protease-activated Bid cooperated with recombinant monomeric Bax, in the absence of any other proteins, to permeabilize membrane vesicles, allowing the passage of extremely large macromolecules. Some investigators have reported that Bid alone can permeabilize membranes (Epand et al., 2002a, 2002b; Kudla et al., 2000); however, Kuwana et al. (2002) were unable to observe any such effects, but rather saw a requirement for both cleaved Bid (or a Bid BH3 domain peptide) and Bax, which is consistent with the finding that constitutively active forms of the BH3-only proteins, Bim and Bad, are unable to induce apoptosis in Bax/Bak knockout cells (Zong et al., 2001).

Other investigators also reported that detergent-oligomerized Bax (Epand et al., 2002c) or a cleaved form of Bcl-x_L (Basanez et al., 2001) can form large openings in lipid bilayers. Interestingly, atomic force microscopy was used successfully to visualize large Bax pores (in this case formed in vesicles of nonphysiological lipid content and in the presence of very high Ca^{2+} concentrations; Epand et al., 2002d). In disagreement with these results and those of Kuwana et al., Martinou and colleagues (Roucou et al., 2002b) found that Bid and Bax added together could not produce large pores in liposomes and concluded that at least one other mitochondrial protein is required. Because Bax has been observed to form high-molecular-weight CHAPS-stable oligomers in mitochondrial outer membranes, when activated either by detergent treatment or the addition of a BH3-only proteins such as cut Bid, some have hypothesized that this oligomerization is a mechanism underlying Bax-induced membrane alterations. However, cleaved Bid and Bax alone are not sufficient to form large-scale Bax oligomers in liposomes; other proteins of the outer mitochondrial membrane appear to be required to stabilize or facilitate oligomerization (Kuwana et al., 2002; Roucou et al., 2002a). Because Kuwana and colleagues (Kuwana et al., 2002) found that Bax could nevertheless permeabilize liposomes, they concluded that stable massive oligomerization of Bax (other than the formation of tetramers) is not required for membrane permeabilization. The discrepancies between some of these liposome studies can perhaps be reconciled if one supposes that, while Bid and Bax together can permeabilize membranes under optimal conditions, the efficiency or stability of pore formation may be enhanced by other proteins present in the outer mitochondrial membrane.

Indeed, while the studies of Kuwana et al. define the minimal conditions needed for membrane permeabilization, they do not rule out the participation of other molecules that could modulate this function. For example, the potency of Bax could be regulated by posttranslational

modifications. Alternatively, other proteins, either by binding to Bax or by interacting independently with the membrane, could regulate the translocation of Bax to mitochondrial membranes or potentiate pore formation. In particular, it is possible that the machinery of mitochondrial fission (discussed below) may facilitate Bax-mediated membrane pore formation.

A Role for Mitochondrial Fusion- and Fission-Related Proteins?

Mitochondria exist in normal interphase cells as a tubular network, but prior to mitosis, the mitochondria become highly fragmented to facilitate their segregation between the daughter cells. Following cell division, the tubular mitochondrial network is re-established. These morphological transitions are controlled by a balance between fission and fusion processes (reviewed in Frank et al., 2001; Osteryoung, 2001; Westermann, 2002). In yeast and mammals, a number of proteins involved in these processes have been identified.

Youle and colleagues have demonstrated a spatial and functional connection between Bax and the mitochondrial fission process (Frank et al., 2001; Karbowski et al., 2002; Nechushtan et al., 2001). These investigators observed that during apoptosis, the mitochondrial network becomes fragmented, and Bax co-localizes with the fission- and fusion-related proteins, Drp1 and Mfn2, at fission sites in the outer mitochondrial membrane. Overexpression of Bax stimulates fission. Furthermore, a dominant-negative form of Drp1 inhibits apoptosis, raising the possibility that this co-localization may be important for Bax function in apoptosis. It appears likely that Bax has something to do with the process of mitochondrial fission that occurs during apoptosis. However, the converse, namely that fission processes are important in Bax-mediated permeabilization of the outer membrane, is not yet established, especially in light of the finding that Bax can permeabilize liposomes effectively in the absence of Drp1 (Kuwana et al., 2002). An alternative explanation for the effect of dominant-negative Drp1 is that this protein, merely by interacting in an abnormal way with Bax (directly or indirectly), interferes with its outer membrane permeabilization function. Hence, we cannot yet conclude that wild-type Drp1 is required for the process of outer membrane permeabilization. Nevertheless, these observations are intriguing, and it will be important to determine unambiguously if Drp1 or Mfn2 are somehow involved in the membrane recruitment or permeabilization function of Bax (e.g., by stabilizing Bax oligomers or Bax-induced membrane pores).

A Role for the Mitochondrial Membrane Lipid, Cardiolipin

Still another potential mechanism of apoptotic regulation stems from the observation that the Bax-mediated permeabilization of liposomes is dependent on the signature mitochondrial lipid, cardiolipin (Epand et al., 2002c, 2002d; Kuwana et al., 2002). While cardiolipin is known to be present in high amounts in the inner membrane, its abundance in the outer membrane is disputed. Conceivably, however, cardiolipin could be heterogeneously distributed in the membrane and, in particular, may be concentrated near the sites of contact between inner and outer membranes, where Bid and Bcl-2 appear also to cluster (Krajewski et al., 1993; Lutter et al., 2000, 2001). Bid has a lipid transfer activity (Degli Esposti, 2002b), and there could possibly be other proteins that modulate the concentration or distribution of cardiolipin or covalently modify this lipid. Moreover, it is conceivable that the organelle fission process discussed above could cause a redistribution of cardiolipin within the outer membrane, or from the inner to the outer membrane, facilitating membrane permeabilization by Bax. Lipid peroxidation might also affect the ability of Bax to permeabilize the outer membrane and may be one means through which oxygen radicals can directly regulate cell death (Asumendi et al., 2002; Nomura et al., 2000). Degli Esposti (2002a) has reviewed some possible roles for lipid metabolism in apoptosis. Surprisingly little is known about the contributions of this potentially important class of molecules to cell death, but there is now a great impetus for future investigations.

Mitochondrial Permeability Transition (PT) and Apoptosis

Another mechanism through which mitochondrial outer membranes can become permeabilized is through the mitochondrial permeability transition (PT), reviewed in detail elsewhere (Zamzami and Kroemer, 2001). PT involves the opening of a proteinaceous channel, known as the PT pore, in the inner membrane (Figure 3), which is thought to be comprised of complexes between cyclophilin D and the adenine nucleotide translocator (ANT) protein in the inner membrane, associated with VDAC and the peripheral benzodiazepine receptor in the outer membrane. Sustained opening of the PT pore allows the equilibration of ions between the matrix and cytoplasm, which implies a dissipation of the inner membrane potential, $\Delta\Psi_m$. This ionic redistribution leads to an osmotic swelling of the matrix and a consequent rupture of the outer membrane, because it cannot expand as much as the highly invaginated inner membrane.

Unfortunately, gene knockout approaches testing the importance of PT in vivo or in cell culture have not been reported, presumably because the principal PT pore constituents, VDAC and ANT, are essential proteins. Although several articles have reported that Bax can regulate PT (Brenner et al., 2000; Marzo et al., 1998; Narita et al., 1998; Pastorino et al., 1998, 1999), it appears that Ca^{2+}-induced PT can occur efficiently in mitochondria from Bax/Bak-deficient cells (Scorrano et al., 2002). Thus, any role for Bax in activating PT may be upstream of mitochondria (see below). Some investigators have proposed a role for Bid (a "BH3-only" member of the Bcl-2 family) in facilitating PT, perhaps by increasing the flow of Ca^{2+} from ER to mitochondria through permeabilization of the outer mitochondrial membrane (Csordas et al., 2002). Bid has also been proposed to activate another PT-like mechanism that produces a cyclosporin A-sensitive remodeling of mitochondrial cristae (mobilizing remote internal stores of cytochrome c) without concomitant swelling or loss of membrane potential (Scorrano et al., 2002). However, this latter phenomenon did not occur in studies by another group (von Ahsen et al., 2000) and also seems incompatible

with observations that GFP-cytochrome c is released rapidly and completely from mitochondria in pre-apoptotic HeLa cells (Goldstein et al., 2000).

Several laboratories have questioned the importance of PT in the majority of cell deaths, because protein release from mitochondria can often occur in the absence of any of the hallmarks of PT, which include loss of the inner membrane potential, large-amplitude swelling of the mitochondrial matrix, and mechanical rupture of the outer membrane. Instead, these groups point to the ability of Bcl-2-family proteins to regulate permeability of the outer membrane specifically, while leaving the inner membrane intact (Bossy-Wetzel et al., 1998; Desagher et al., 1999; Eskes et al., 1998; Gao et al., 2001; Kluck et al., 1997, 1999; von Ahsen et al., 2000).

An alternative view of mitochondrial protein release that combines elements of both PT and outer membrane-specific Bax action was recently offered by Ichas and colleagues (De Giorgi et al., 2002). These authors suggested that in the cellular milieu, as opposed to the buffers typically used with isolated mitochondria, PT pore opening may not lead to mitochondrial swelling, because swelling depends on an osmotic imbalance between the matrix and the cytoplasm that may not exist in all cells. Instead, De Giorgi et al. assert that PT indirectly promotes permeabilization of the outer membrane through the recruitment and activation of Bax, which then acts directly on the outer membrane as described above. It remains to be seen whether this kind of hybrid model will satisfy both the proponents and skeptics of PT.

Instances of Cell Death Clearly Involving PT; The Roles of ROS and Ca^{2+} Release from the ER

Despite the long-standing controversy concerning whether PT or a direct outer membrane event is responsible for the release of proapoptotic mitochondrial proteins, there are some situations in which PT is clearly important: for example, some instances of death involving massive generation of reactive oxygen species (ROS), which can in particular be mediated or exacerbated by NO (reviewed, e.g., in Fleury et al., 2002; Moncada and Erusalimsky, 2002; Ueda et al., 2002). There are also toxins whose effects can be traced to a direct or indirect activation of PT, including the potential chemotherapeutic F16 (Fantin et al., 2002). Also, certain compounds induce an immediate upregulation of mitochondrial respiratory proteins and subsequently lead to cell death, suggesting that respiratory activation can predispose mitochondria to undergo PT via an increased production of ROS (Chandra et al., 2002; Tang et al., 1998).

Conversely, the importance of redox metabolism in ameliorating the effects of normal mitochondrial ROS production, thereby preventing cell death, has been revealed through experiments using cells deficient in mitochondrial thioredoxin-2 (Tanaka et al., 2002) (which surprisingly do not appear to undergo death via the PT) or in mice with the *harlequin* mutation, in which AIF is downregulated (Klein et al., 2002). Like cytochrome c, AIF now appears to have both a life-supporting role within mitochondria and a death-inducing role elsewhere in the cell. Perhaps the notion of selective pressure favoring the maintenance of pro-death molecules through their essential pro-survival functions could be more widely relevant than previously supposed.

Finally, there is mounting evidence (e.g., Baffy et al., 1993; Pu and Chang, 2001; Szalai et al., 1999; Vanden Abeele et al., 2002) that some forms of cell death can be mediated by Ca^{2+} release from ER stores, perhaps accompanied by capacitative Ca^{2+} entry from the extracellular milieu. This redistribution of Ca^{2+} leads finally to a decisive induction of PT through mitochondrial Ca^{2+} overload. (There could also be other Ca^{2+}-dependent mechanisms that may or may not involve mitochondria, such as calpain activation.) The interorganelle flow of Ca^{2+} is facilitated by direct physical connections between mitochondria and the ER. Interestingly, VDAC appears to be rate-limiting for Ca^{2+} flux, as overepression of VDAC increases mitochondrial Ca^{2+} uptake from the ER and sensitizes cells to ceramide-induced death (Rapizzi et al., 2002). Bcl-2 is known to regulate this redistribution of Ca^{2+}, and in particular reduces the amount of Ca^{2+} releasable from the ER, thus preventing mitochondrial calcium overload and ensuring cell survival (Vanden Abeele et al., 2002). Recent studies using Bax-deficient cells have now shown that Bax can produce the opposite effect, making more Ca^{2+} available for inducing PT in mitochondria (Nutt et al., 2002a, 2002b). Bcl-2 controls ER Ca^{2+} levels by several mechanisms, including an increase in the rate of Ca^{2+} leak, changes in expression of Ca^{2+}-regulatory proteins, and by affecting store-operated channels that drive capacitative Ca^{2+} entry (Vanden Abeele et al., 2002). This prompts the question of whether Bax acts at the ER to counter all of these functions, or instead at the mitochondria, by lowering the Ca^{2+} uptake threshold needed to induce PT. Based on observations that Bax/Bak-deficient mitochondria are capable of undergoing Ca^{2+}-mediated PT (Scorrano et al., 2002), the ER seems a likely site of Bax action in this context. However, because ER membranes lack significant amounts of cardiolipin, Bax probably does not form lipidic pores in the ER membrane, as it does with the outer mitochondrial membrane (Kuwana et al., 2002).

Conclusions

Despite the contentiousness that has beset research on mitochondrial apoptosis, there is hope that the field is entering a phase of clarification and simplification, as a few basic mechanisms are gaining credibility. However, as Albert Einstein once said, "Everything should be made as simple as possible, but not simpler." Paradoxically, some of the confusion in the cell death literature has come from oversimplification and dogmatic assertions that apoptosis always occurs by this or that mechanism. It may now be possible to design incisive experimental approaches that show definitively which pathways are employed in specific cell death situations. In particular, gene loss-of-function approaches may be most fruitful. For example, the Bax/Bak double knockout mouse has shown decisively that BH1-3 proteins are essential in many forms of apoptosis. In contrast, with regard to PT, we are forced to rely on pharmacological inhibitors like cyclosporin A, which like all drugs can never be proven absolutely specific. But if, for example,

a mutant of ANT could be found that prevents PT pore formation but allows the essential exchange of metabolites across the inner mitochondrial membrane, a targeted gene replacement strategy in mice might allow us to discover just how critical PT is for a variety of cell deaths in the animal. Of course, these genetic approaches typically depend on prior knowledge of the molecules involved. It is also likely that new and unexpected mechanisms of mitochondrial function will be discovered. As Einstein also remarked, "If we knew what it was we were doing, it would not be called research, would it?"

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