Mitochondria—the suicide organelles

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Summary
One of the near-to-invariant hallmarks of early apoptosis (programmed cell death) is mitochondrial membrane permeabilization (MMP). It appears that mitochondria fulfill a dual role during the apoptotic process. On the one hand, they integrate multiple different pro-apoptotic signal transducing cascades into a common pathway initiated by MMP. On the other hand, they coordinate the catabolic reactions accompanying late apoptosis by releasing soluble proteins that are normally sequestered within the intermembrane space. In a recent study, Li et al. described a nuclear transcription factor (Nur77/TR1/NGFI-B) that can translocate to mitochondrial membranes to induce MMP. Moreover, two groups identified a novel intermembrane protein (Smac/DIABLO) that specifically neutralizes the inhibitor of apoptosis (IAP) proteins, thereby facilitating the activation of caspases, a class of proteases activated during apoptosis. These findings refine our knowledge how MMP connects to the cellular suicide machinery.

Introduction
Recently, mitochondria have come to be regarded as a key players in the regulation of cell death. These organelles collect information on various aspects of cellular metabolism and signal transduction cascades, process this information, decide on the cell’s fate and participate in the execution of the death sentence. Apoptosis (programmed cell death) can be viewed as a triphasic process: initiation, decision, and degradation. During the initiation phase, pro-apoptotic second messengers, which will finally act on mitochondria to increase the permeability of their membranes, accumulate in the cell. The permeabilizing agents produced depends on the death-inducing stimulus and are, therefore, rather heterogeneous. During the decision phase, mitochondrial membrane permeabilization (MMP) occurs, presumably via a limited set of mechanisms. Finally, the degradation phase is triggered by the activation of catabolic hydrolases, mainly caspases (apoptosis-specific cysteine proteases) and nucleases. Such hydrolases are activated due to the release of proteins normally confined to the mitochondrial intermembrane space. Here, we summarize recent progress in the identification of proteins involved in the mitochondrial regulation of apoptosis.

The mitochondrion as an integrator of cell death pathways
The mitochondrion is the target of numerous pro-apoptotic signal transducing molecules that can induce MMP. This applies to common second messengers of stress responses including ceramide and its product ganglioside GD3, fatty acids (e.g. palmitate) and their oxidation products (e.g. 4-hydroxynonenal), reactive oxygen species (e.g. superoxide anion which may be formed by the respiratory chain), nitric oxide, and Ca²⁺ ions. Both Ca²⁺ elevations in the cytosol and local Ca²⁺ spikes elicited via IP3-receptors in the proximity of the endoplasmic reticulum may play a major role, either in the transient regulatory permeabilization of the inner mitochondrial membrane, or in the induction of the apoptotic response. Local concentrations of essential metabolites (ATP, ADP, NADH, NADPH, creatine, carnitine etc.) as well as ions (e.g. Mg²⁺, protons) modulate the susceptibility of mitochondria to undero MMP. As a result, mitochondria continuously monitor the overall bioenergetic state of the cell as well as non-specific cellular stress responses mediated by small (non-proteaceous) molecules (Fig. 1A).

Numerous proteins with apoptosis-regulatory functions are known to be localized in mitochondria in normal circumstances, before apoptosis induction. This applies to a heterogeneous collection of proteins including potentially apoptosis-regulatory proteins such as protein kinase A (which is tethered to a mitochondrial A-kinase anchoring protein), A-RAF-1 kinase (which binds to proteins from the mitochondrial protein import machinery, see Ref. 5), Grb10 (which interacts with mitochondrial Raf-1), CIDE-B (which is a homologue of ICAD, the inhibitor of caspase-activated DNAse, CAD), the voltage-dependent anion channel (VDAC), and the adenine nucleotide translocase (ANT). The protein DAP-3, a nucleotide-binding protein whose expression is required for apoptosis induced by interferon-γ, CD95 and tumor necrosis factor, is confined to the mitochondrial matrix. Moreover, the anti-apoptotic members of the Bcl2 family, in particular Bcl2 and Bcl-XL, are also normally present in mitochondrial membranes where they exert an MMP-inhibitory effect. Some pro-apoptotic Bcl2 homologues, such as BNIP3 or Bak, may also be loosely associated with mitochondrial membranes and, in response to pro-apoptotic signaling, can fully insert in the membrane to promote MMP.

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Numerous proteins involved in lethal signal transduction translocate to mitochondria to cause MMP. Redistribution of proteins from a cytosolic to a mitochondrial localization has been reported for pro-apoptotic proteins from the Bcl2/Bax/Bid family. Thus, cytosolic Bax has been shown to insert into the outer mitochondrial membrane, a process that is accompanied (or triggered?) by conformational changes and oligomerization of these proteins. In addition, cytosolic Bid can be activated by proteolytic cleavage, for instance by caspase 8 (a signal-transducing caspase activated upon interaction with the plasma membrane death receptors Fas/CD95 and TRAIL) or by granzyme B (a component of the granules of cytotoxic T lymphocytes).\(^{(10)}\) The cleavage product of this reaction (truncated Bid, t-Bid) then inserts into mitochondrial membranes, presumably via interaction with Bax or Bak.\(^{(9,11)}\) It appears that cardiolipin, a lipid confined to the inner mitochondrial membrane, confers specificity to the subcellular relocalization of t-Bid.\(^{(12)}\) Upon dephosphorylation by calcineurin or protein phosphatase 1, the protein Bad can move to mitochondria, where it inactivates the anti-apoptotic protein Bcl-X\(_L\). Another example is provided by Bim, a pro-apoptotic protein from the Bcl2/Bax/Bid that normally interacts with the microtubule-associated dynein complex and which translocates to mitochondria (where it neutralizes the anti-apoptotic protein Bcl2) upon perturbation of the microtubular network.\(^{(13)}\) Bax, Bak, t-Bid, and Bim all cause MMP when added to purified mitochondria in vitro.\(^{(14)}\) Some proteins relocalizing to mitochondria covalently modify proteins from the Bcl2/Bax/Bid family, thereby affecting their local apoptosis-regulatory potential and/or their subcellular localization. For example, c-Jun kinase (JNK, also called stress-activated protein kinase, SAPK), phosphorylates and inactivates the anti-apoptotic protein Bcl-X\(_L\).\(^{(15,16)}\)

As recently discovered,\(^{(1)}\) TR3 (also called Nur77 or NGFI-B), a transcription factor from the steroid/thyroid receptor superfamily normally present in the nucleus, also moves to mitochondria to trigger MMP. TR3 is an “orphan” receptor in the sense that its endogenous ligand (if any) is not known. Under normal circumstances, TR3 is confined to the nucleus where it exerts a transactivating function, via specific interaction with a DNA octamer sequence, the Nur77/NGFI-B-binding response element. In addition, when heterodimerized with another protein from the same family of proteins, the 9-cis-retinoic acid receptor (RXR), TR3 also interacts with the

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**Figure 1.** Dual implications of mitochondrial membrane permeabilization (MMP) in the apoptotic process. In the upper part of the figure, pro-apoptotic pathways converging on mitochondria are shown. Different apoptogenic molecules act on a variety of tentatively identified mitochondrial receptors (arrows), which in turn regulate MMP. Execution pathways triggered by MMP are depicted in the lower part. The relative contribution of these pathways to cell death may depend on the concentration of inhibitors specific for caspases or caspase-independent effectors as well as the relative speed of outer membrane permeabilization and inner membrane permeabilization, the latter being particularly deleterious for the bioenergetic state of the cell. GSG, glutathione; ROS, reactive oxygen species.
retinoic acid response element. In several apoptosis-inducing conditions, TR3 is overexpressed and translocates from the nucleus to mitochondrial membranes, presumably through an active export mechanism. Recombinant TR3 induces the release of cytochrome c when added to purified mitochondria in a cell-free system, suggesting that TR3 permeabilizes mitochondrial membranes on its own, without the concourse of additional factors. p53, a transcription factor generally thought to induce apoptosis via the induction of pro-apoptotic genes including pro-apoptotic genes from the Bcl2 family, has also recently been reported to translocate to mitochondria. Indeed, part of the MMP-inducing potential of p53 can be uncoupled from its transactivation potential. p53 also transcriptionally activates several genes whose products can translocate to the mitochondrial outer membrane (e.g. the two pro-apoptotic Bcl2 family members Bax and Noxa) or to an unknown submitochondrial compartment, presumably to the mitochondrial matrix (p53AIP1). Overexpression of each of these genes suffices to induce MMP.

In addition, viral or bacterial proteins can regulate MMP via direct effects on mitochondrial membranes. This has been shown to apply to a growing number of viral Bcl2 homologues, as well as a number of proteins without any structural similarity with Bcl2. Pro-apoptotic proteins acting on mitochondria include viral protein R (Vpr) encoded by human immunodeficiency virus 1, which acts on ANT, and hepatitis virus protein X (HVP-X), which interacts with VDAC3.

Altogether these findings indicate that an extremely heterogeneous collection of factors (non-proteaceous, Bcl2-like proteins, kinases, transcription factors, and viral proteins) can act on mitochondria to regulate MMP. The mitochondrion is definitively a major integrator of death pathways (Fig. 1A).

The mitochondrion as a death executioner

The release of mitochondrial intermembrane proteins through the outer mitochondrial membrane results in the activation of catabolic reactions culminating in the degradation of essential macromolecules as well as the acquisition of the apoptotic morphology. One characteristic feature of apoptosis is the release of mitochondrial intermembrane proteins, Smac/DIABLO to exert its killer function.

It has recently become clear that proteins other than cytochrome c may facilitate caspase activation. A significant portion of pro-caspase 9 and pro-caspase 3 may themselves be localized within the mitochondrial intermembrane space, depending on the cell type. Heat-shock proteins (hsp) 10 (from the intermembrane space) and 60 (from the matrix) facilitate the cytochrome c-triggered activation of caspase 3 in cytosolic extracts. In addition, a recently discovered intermembrane protein (Smac/DIABLO) facilitates activation of caspases indirectly, by eliminating their inhibition by inhibitor of apoptosis proteins (IAPs). From its crystal structure, it can be deduced that Smac/DIABLO binds to IAPs through an extensive hydrophobic interface. Importantly, although it appears that its N-terminal 10 residues are disordered in solution, the apoptogenic activity can be attributed to a heptapeptide corresponding to the extreme N terminus of the molecule, which can activate pro-caspase 3 in a cell-free system. The apoptogenic mode of action of Smac/DIABLO appears analogous to that of three proteins from Drosophila (Reaper, Hid, and Grim), which also interact with IAPs, presumably through their N terminus. Moreover, transfection experiments in which Hid or Grim are overexpressed in mammalian cells suggest that they act on mitochondria. Another fascinating aspect of the biology of Smac/DIABLO concerns the tight regulation of its bioactivity. Similar to other intermembrane proteins, Smac/DIABLO is synthesized in the cytoplasm as a precursor carrying an N-terminal mitochondrial localization sequence (MLS). Upon import into mitochondria, this MLS is cleaved off, yielding the mature Smac/DIABLO molecule. Only the mature Smac/DIABLO molecule (not its precursor) is apoptogenic, implying that only Smac/DIABLO that has been successfully imported into mitochondria can participate in the apoptotic process. At difference with Reaper, Hid and Grim, which lack an MLS, the simple transcriptional upregulation of Smac/DIABLO thus is unlikely to be apoptogenic. Rather, MMP is a pre-requisite for Smac/DIABLO to exert its killer function.

In addition to pro-caspases and caspase activators, the mitochondrion contains a cell-widespread death executioners. One such effector is apoptosis-inducing factor (AIF), a flavoprotein with significant homology to plant ascorbate reductases and bacterial NADH oxidases. In contrast to cytochrome c and Smac/DIABLO, which are released into the cytosol (and mostly spare the nucleus), AIF translocates from the mitochondrial intermembrane space via the cytosol to nucleus. AIF has been shown to induce chromatin condensation and large-scale DNA fragmentation in purified nuclei in vitro. The intracellular neutralization of extramitochondrial AIF by microinjection of an AIF-specific antibody has clarified the contribution of AIF to the apoptotic process. In mouse embryonic fibroblasts, AIF neutralization only abolishes nuclear apoptosis when the apoptosis/caspase 3/CAD pathway is simultaneously blocked by genetic
The presence of catabolic enzymes (e.g., arginase 1, glycine cleavage system protein, lysozyme homologue AT 2, soluble branes has not been elucidated. According to one model, the invalidation of Apaf1 or caspase 3, addition of the pan-caspase inhibitor Z-VAD.fmk, or microinjection of ICAD.(31) In contrast, nant AIF causes the permeabilization of the outer mitochondrial membrane, leading to the release of cytochrome c and subsequent caspase activation (while cytochrome c neutralization and caspase inhibition do not affect the release of AIF), placing AIF upstream of the cytochrome c/Apaf1/caspase/CAD pathway.(30,32) Accordingly, when added to purified mitochondria in combination with cytosolic extract, recombinant AIF causes the permeabilization of the outer mitochondrial membrane, leading to the release of cytochrome c and subsequent caspase activation.(30) Moreover, microinjection of recombinant AIF into HeLa syncytia(32) can trigger the release of cytochrome c from mitochondria. As a result, AIF may act as a facultative signaling molecule upstream (or at the level) of mitochondria, as well as a death effector downstream of mitochondria.

In addition to the above apoptosis effectors, mitochondria can release a minimum of 60 intermembrane proteins into the cytosol.(33) It remains to be established whether the ectopic presence of catabolic enzymes (e.g., arginase 1, glycine cleavage system protein, lysozyme homologue AT 2, soluble epoxide hydrolase and sulfite oxidase) or the removal of antioxidant enzymes (e.g., glutathione peroxidase, thioredoxin-dependent peroxide reductase) from the site at which most reactive oxygen species are generated, the respiratory chain, may have fatal (presumably caspase-independent) consequences for cellular metabolism (Fig. 1B).

Open questions and perspectives
The exact mode of permeabilization of mitochondrial membranes has not been elucidated. According to one model, the so-called permeability transition pore complex (PTPC which contains ANT and VDAC) constitutes a major integrator of proapoptotic signaling.(14) According to another model, proapoptotic members of the Bcl2 family may cause MMP without the help of sessile mitochondrial proteins.(19,11,34) Moreover, the relative contribution of caspase-dependent and caspase-independent post-mitochondrial events to cell death remains a matter of debate. Resolving these issues maybe of the utmost importance for the comprehension of the apoptotic process as well as for the design of apoptosis-modulatory drugs.

References