# Mitochondria and Apoptosis

Douglas R. Green and John C. Reed

REVIEW

A variety of key events in apoptosis focus on mitochondria, including the release of caspase activators (such as cytochrome c), changes in electron transport, loss of mitochondrial transmembrane potential, altered cellular oxidation-reduction, and participation of pro- and antiapoptotic Bcl-2 family proteins. The different signals that converge on mitochondria to trigger or inhibit these events and their downstream effects delineate several major pathways in physiological cell death.

About 2 billion years ago, the cells destined to become the ancestors of all eukaryotes entered into a partnership with an ancestor of today's purple bacteria. This partnership promised huge benefits to both parties: it allowed them to exploit the energy opportunities inherent in the emerging oxygen atmosphere, which was toxic to most other life forms. The result was a protoeukaryotic cell, and its new endosymbiotic bacteria were to become mitochondria (*1*).

This alliance was probably a shaky one, and catastrophic conflicts in selection between the two genomes undoubtedly occurred (*2*). Once the new symbiotic organism moved into the aerobic world, life and death were controlled by the protomitochondria, which provided not only critical antioxidants but also a source of reactive oxygen species (ROS) as a by-product of oxidative phosphorylation. Conditions that favored the protomitochondria over the host cell would lead to death of the cell and release of the free-living endosymbiont. Therefore, the symbiosis was perilously unstable until essential genes for mitochondrial metabolism and biogenesis transferred to the nuclear genome, resulting in an obligate symbiosis.

Several investigators have hypothesized that the endosymbiotic origins of mitochondria and the evolution of aerobic metabolism in eukaryotes formed the basis for evolution of active cell death, which manifests predominantly as apoptosis in metazoans (*3, 4*). Although apoptosis is independent of oxidative phosphorylation, lacking a requirement even for mitochondrial DNA (*5*), central roles for mitochondria as the orchestrators of apoptosis have been firmly established in many systems.

## Mitochondria and Commitment to Cell Death

When is a dying cell actually dead and when is a cell irrevocably committed to death? In recent years, we have come to understand that the effectors of apoptosis are represented by a family of intracellular cysteine proteases known as caspases. Inhibiting caspases, however, does not always inhibit cell death induced by proapoptotic stimuli. Although caspase inhibitors block some or all of the apoptotic morphology induced by growth factor withdrawal, etoposide, actinomycin D, ultraviolet (UV) radiation, staurosporine, enforced c-Myc expression, or glucocorticoids, they do not necessarily maintain replicative or clonogenic potential; ultimately, the cells die despite inactivation of caspases by way of a slower, nonapoptotic cell death (*6–9*). In contrast, antiapoptotic proteins such as Bcl-2, Bcl-$x_L$, and oncogenic Abl can maintain survival and clonogenicity in the face of these treatments. Conversely, some proapoptotic proteins such as Bax, a mammalian cell death protein that targets mitochondrial membranes, can induce mitochondrial damage and cell death even when caspases are inactivated (*10*). Such experimental observations argue that a caspase-independent mechanism for commitment to death exists. This mechanism is likely to involve mitochondria, as we will see.

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## Understudy for the Executioner: How Mitochondria Trigger Apoptotic and Nonapoptotic Cell Death

If mitochondria are pivotal in controlling cell life and death, then how do these organelles kill? At least three general mechanisms are known, and their effects may be interrelated, including (i) disruption of electron transport, oxidative phosphorylation, and adenosine triphosphate (ATP) production; (ii) release of proteins that trigger activation of caspase family proteases; and (iii) alteration of cellular reduction-oxidation (redox) potential.

*Disruption of electron transport and energy metabolism.* For decades disruption of electron transport has been recognized as an early feature of cell death. γ-Irradiation induces apoptosis in thymocytes and a disruption in the electron transport chain, probably at the cytochrome b–$c_1$/cytochrome c (cyto c) step (*11*). Ceramide (a "second messenger" implicated in apoptosis signaling) disrupts electron transport at the same step in cells as well as in isolated mitochondria (*12*). Ligation of Fas also leads to a disruption in cyto c function in electron transport (*13*).

One consequence of the loss of electron transport should be a drop in ATP production. Although such a drop has been observed during apoptosis, it often occurs relatively late in the process (*14*). Indeed, ATP appears to be required for downstream events in apoptosis (*15*). Thus, although loss of mitochondrial ATP production can kill a cell, it is unlikely that this is a mechanism for induction of apoptosis.

*Release of caspase-activating proteins.* The importance of mitochondria in apoptosis was suggested by studies with a cell-free system in which spontaneous, Bcl-2–inhibitable nuclear condensation and DNA fragmentation were found to be dependent on the presence of mitochondria (*16*). Subsequently, studies in another cell-free system showed that induction of caspase activation by addition of deoxyadenosine triphosphate depended on the presence of cyto c released from mitochondria during extract preparation (*17*). During apoptosis (in vitro and in vivo) cyto c is released from mitochondria and this is inhibited by the presence of Bcl-2 on these organelles (*18, 19*). Cytosolic cyto c forms an essential part of the vertebrate "apoptosome," which is composed of cyto c, Apaf-1, and procaspase-9 (*20*). The result is activation of caspase-9, which then processes and activates other caspases to orchestrate the biochemical execution of cells.

Significantly, caspase inhibitors do not prevent cyto c release induced by several apoptogenic agents, including UV irradiation, staurosporine, and overexpression of Bax (*14, 21, 22*). An exception is cyto c release from mitochondria induced by the tumor necrosis factor receptor family member Fas, in which cyto c release is prevented by inhibition of caspases (primarily caspase-8) recruited to the cytosolic domain of ligated Fas (*21*). Nevertheless, cyto c release can sometimes contribute to Fas-mediated apoptosis by amplifying the effects of caspase-8 on activation of downstream caspases (*23*).

The emergent view is that once cyto c is released, this commits the cell to die by either a rapid apoptotic mechanism involving Apaf-1–mediated caspase activation or a slower necrotic process due to collapse of electron transport, which occurs when cyto c is depleted from mitochondria, resulting in a variety of deleterious sequelae including generation of oxygen free radicals and decreased production

of ATP (Fig. 1).

The consequences of cyto c release may depend on the cell type. In those cells where cyto c is available in excess, caspases can be activated and enough cyto c may remain docked by its high-affinity binding sites to cytochrome b–$c_1$ and cyto c oxidase to maintain electron transport. In this case, oxygen consumption and ATP production may continue unabated while caspases unleash their attack on cytosolic and nuclear substrates, resulting in apoptosis. Alternatively, in cells that contain large quantities of endogenous caspase inhibitors, release of cyto c may fail to induce caspase-dependent apoptosis and instead the eventual loss of electron chain transport may drive the cell toward a necrotic demise (Fig. 1).

Other apoptosis mediators are also released from mitochondria. Mitochondria of some cells contain procaspase-3 (*24*) that is liberated into the cytosol during apoptosis, although it remains unclear whether it becomes activated before release. The possibility that an intramitochondrial pool of procaspase-3 participates in apoptosis of some primary cells is intriguing and, if prevalent in neurons, could provide an explanation for the profound impact of murine caspase-3 gene disruption on neuronal but not other forms of apoptosis (*25*).

Another caspase-activating protein that is released from mitochondria is apoptosis-inducing factor (AIF), which apparently processes purified procaspase-3 in vitro (*26, 27*). Its activity is blocked by zVAD-fmk, a general caspase inhibitor, raising the possibility that AIF is another caspase.

*Reactive oxygen species and cellular redox*. Mitochondria are the major source of superoxide anion production in cells. During transfer of electrons to molecular oxygen, an estimated 1 to 5% of electrons in the respiratory chain lose their way, most participating in formation of $O_2^-$. Anything that decreases the coupling efficiency of electron chain transport can therefore increase production of superoxides.

Superoxides and lipid peroxidation are increased during apoptosis induced by myriad stimuli (*28*). However, generation of ROS may be a relatively late event, occurring after cells have embarked on a process of caspase activation. In this regard, attempts to study apoptosis under conditions of anoxia have demonstrated that at least some proapoptotic stimuli function in the absence or near absence of oxygen, which implies that ROSs are not the sine qua non of apoptosis (*29, 30*). However, ROSs can be generated under conditions of virtual anaerobiosis (*31*), and thus their role in apoptosis cannot be excluded solely on this basis.

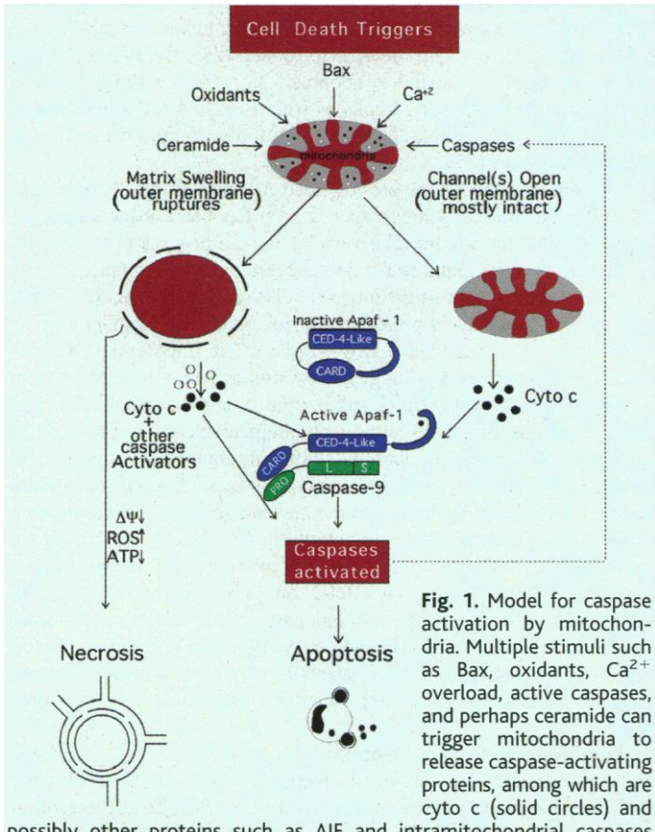


**Fig. 1.** Model for caspase activation by mitochondria. Multiple stimuli such as Bax, oxidants, $Ca^{2+}$ overload, active caspases, and perhaps ceramide can trigger mitochondria to release caspase-activating proteins, among which are cyto c (solid circles) and possibly other proteins such as AIF and intramitochondrial caspases (open circles). Two general mechanisms for release of caspase-activating proteins from mitochondria have been proposed: one involves osmotic disequilibrium leading to an expansion of the matrix space, organellar swelling, and subsequent rupture of the outer membrane (left); the other envisions opening of channels in the outer membrane (without concomitant organellar swelling), thus releasing cyto c from the intermembrane space of mitochondria into the cytosol. Cyto c activates caspases by binding to Apaf-1, inducing it to associate with procaspase-9, thereby triggering caspase-9 activation and initiating the proteolytic cascade that culminates in apoptosis. Cells in which mitochondria have ruptured are at risk for death through a slower nonapoptotic mechanism resembling necrosis because of loss of the electrochemical gradient across the inner membrane ($\Delta\Psi$m), production of ROS, and declining ATP production.

## PT Pore: Assassin or Accomplice?

In many apoptosis scenarios, the mitochondrial inner transmembrane potential ($\Delta\Psi$m) collapses (*32*), indicating the opening of a large conductance channel known as the mitochondrial PT pore (*33*) (Fig. 2). The structure and composition of the PT pore remain only partially defined, but its constituents include both inner membrane proteins, such as the adenine nucleotide translocator (ANT), and outer membrane proteins, such as porin (voltage-dependent anion channel; VDAC), which operate in concert, presumably at inner and outer membrane contact sites, and create a channel through which molecules ≤1.5 kD pass (*32, 34*). Opening of this nonselective channel in the inner membrane allows for an equilibration of ions within the matrix and intermembrane space of mitochondria, thus dissipating the $H^+$ gradient across the inner membrane and uncoupling the respiratory chain. Perhaps more importantly, PT pore opening results in a volume dysregulation of mitochondria due to the hyperosmolality of the matrix, which causes the matrix space to expand. Because the inner membrane with its folded cristae possesses a larger surface area than the outer membrane, this matrix volume expansion can eventually cause outer membrane rupture, releasing caspase-activating proteins located within the intermembrane space into the cytosol (Fig. 1).

Inhibitors of PT pore opening, including cyclosporins (which

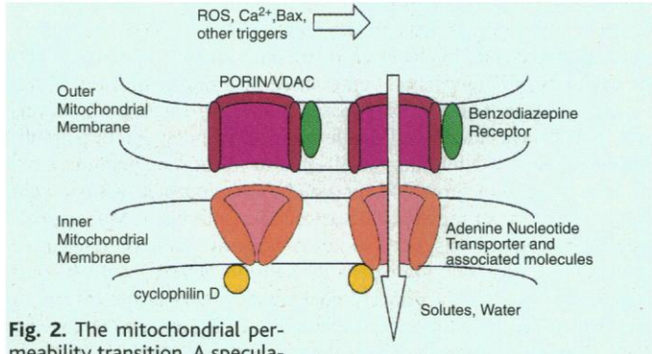


**Fig. 2.** The mitochondrial permeability transition. A speculative model showing some of the components of the permeability transition pore. The roles of porin and the benzodiazepine receptor remain circumstantial. In the open configuration, water and solutes enter the matrix, causing matrix swelling and outer membrane disruption (see Fig. 1), leading to release of cyto c and other proteins.

bind cyclophilin D, associated with the ANT; Fig. 2) and bongkrekic acid (which also inhibits the ANT), appear to block apoptosis in some systems, thus providing support for the idea that the PT is central to apoptotic processes (*35*). Bcl-2 can prevent the PT (*36*), whereas the ANT-activator atractyloside and Bax induce both apoptosis and PT (*36, 37*). Other stimuli that affect the PT pore directly, such as oxidants and pathological elevations in cytosolic $Ca^{2+}$, can induce rupture of the outer membrane of mitochondria and release of caspase-activating proteins (*26, 27*). Thus, in many cases, the PT appears to be the mastermind that orchestrates apoptosis.

Pharmacological inhibitors of the PT pore reportedly prevent apoptosis induction by several direct stimuli (*35*), implying a general role. However, some studies have provided evidence that cyto c release and caspase activation can occur before any detectable loss of $\Delta\Psi m$, implying that PT pore opening may occur downstream of apoptosome-mediated caspase activation (*14, 21*). The ability of caspases to induce PT pore opening (*27, 38*), which in turn can induce caspase activation (by release of cyto c and AIF), creates opportunities for a feedforward amplification loop (Fig. 1) and complicates attempts to dissect the normal sequence of cell death events.

The release of cyto c before or in the absence of a drop in $\Delta\Psi m$ in some cells suggests that different regulatory events control permeability of the inner and outer mitochondrial membranes. A rapid opening and closing of the PT pore at its reversible low conductance state may allow a repeated, respiration-driven reestablishment of $\Delta\Psi m$ (*39*) so that outer membrane disruption and cyto c release can occur before $\Delta\Psi m$ collapse.

## More Than One Way to Skin an Organelle

Another mechanism of outer membrane disruption has recently been suggested, and it involves hyperpolarization of the mitochondrial inner membrane (in sharp contrast to the hypopolarization associated with PT pore opening). This transient hyperpolarization has been observed in several cell types under a variety of conditions (*21*) and is suppressed by Bcl-$x_L$. How the polarization occurs is obscure; nevertheless, increased export of protons into the intermembrane space may result in protonation of weak acids. These can then freely diffuse across the inner membrane and are trapped when the protons are lost (and exported). As these metabolites accumulate, osmolality increases and water enters, resulting in matrix space expansion and eventually rupture of the outer membrane, thereby releasing all contents of the intermembrane space.

However, nearly all studies of $\Delta\Psi m$ regulation have relied exclusively on cationic lipophilic dyes such as rhodamine 123, $DiOC_6$, and others that partition across the inner membrane based on charge. These are fraught with potential artifacts, including autoquenching of their fluorescence when the intramitochondrial concentrations are too high, oxidative inactivation of their fluorescence, and alterations in uptake (and hence total fluorescence) caused by changes in mitochondrial volume rather than $\Delta\Psi m$. For example, rhodamine 123 can indicate a hyperpolarization of the inner membrane after ligation of Fas, whereas other dyes simultaneously reveal a hypopolarization of this membrane, suggesting that the apparent hyperpolarization may be an artifact (*40*).

Although rupture of the outer membrane causes release of cyto c, caspases, AIF, and sometimes generation of ROSs, only a subset of mitochondria appear to be affected (*21*). In contrast, studies have suggested that all (or most) of the cyto c in mitochondria can be released during apoptosis (*18, 19*), which suggests either that the methods for detecting disruption of the outer membrane are insensitive or that release of cyto c involves other mechanisms.

An alternative to gross disruption of the outer membrane theoretically could involve opening a large outer membrane channel capable of liberating cyto c or other proteins from the intermembrane space. Although speculative, the idea of a protein channel is attractive because it obviates the need to postulate mitochondrial swelling—fitting better with intact mitochondrial morphology in most apoptosis in vivo (*41*).

## Holey Mitochondria: Bcl-2 Family Proteins As Pores

Another link between apoptosis and mitochondrial physiology is suggested by the presence of Bcl-2 family proteins in mitochondrial membranes (*42*). The *Caenorhabditis elegans* Bcl-2 homolog CED-9 is expressed from a bicistronic mRNA that encodes both CED-9 and cytochrome b (*43*). This suggests a functional connection between Bcl-2 family proteins and these organelles and implies that CED-9 may have originated from the genome of protomitochondrial symbionts, transferred along with other mitochondrial genes to the nuclear genome.

Many (but not all) Bcl-2 family proteins reside in the mitochondrial outer membrane, anchored by a hydrophobic stretch of amino acids located within their COOH-termini, with the proteins orientated toward the cytosol (*42*). A wide variety of mitochondrial events have been reported to be modulated by Bcl-2 and its homologs. These include some that directly affect mitochondria such as oligomycin, which inhibits the $F_0F_1$–adenosine triphosphatase (ATPase) proton pump of the mitochondrial inner membrane; cyanide, which poisons oxidative phosphorylation; and BSO, which inhibits glutathione synthesis (*21, 35, 44–48*). Bcl-2 and Bcl-$x_L$ suppress release of sequestered matrix $Ca^{2+}$ induced by uncouplers of respiration (*49*). In isolated mitochondria, Bcl-2 or Bcl-$x_L$ enhances proton extrusion from mitochondria and increases mitochondrial $Ca^{2+}$ buffering capacity (*26, 50*).

One clue to how Bcl-2 family proteins exert their mitochondrial effects has come from determination of the three-dimensional structure of the Bcl-$x_L$ protein (*51*). The Bcl-$x_L$ protein is composed of seven $\alpha$ helices joined by flexible loops and shares striking similarity to the pore-forming domains of some types of bacterial toxins—for example, diphtheria toxin and the colicins. As predicted by their structures, Bcl-2, Bcl-$x_L$, and Bax can form ion channels when they are added to synthetic membranes (*52–55*). Deletion of the predicted pore-forming $\alpha5$ and $\alpha6$ helices abolishes channel formation by Bcl-2 and Bax in synthetic membranes (*52*).

How can a small conductance ion channel created in the outer membrane by Bcl-2 or Bcl-$x_L$ influence mitochondrial physiology? Even in its closed conformation, VDAC creates pores of an estimated 1.5-nm diameter (*56*); thus, the outer membrane should be freely permeable to ions and most metabolites. Bcl-2 and Bcl-$x_L$ may communicate functionally or physically with inner membrane proteins that govern ion transport, such as components of the PT pore, or ion-transporting proteins that control volume regulation of the matrix space independently of the PT (*21*). Bcl-2 and Bcl-$x_L$ may also somehow regulate the pH of the intermembrane space, causing increased rates of proton extrusion from mitochondria (*50*).

Bax-mediated cytotoxicity in yeast and Bax-induced apoptosis in mammalian cells requires a functional $F_0F_1$-ATPase proton pump in the inner membrane of mitochondria (*57*). In vitro evidence suggests that the Bax channel is pH- and voltage-dependent (*54, 55*). Although the outer membrane of mitochondria apparently is not polarized, its close apposition to the inner membrane at the junctional complexes may affect proteins located there.

Whatever the biochemical mechanism of Bcl-2 family proteins, their effects cannot be reduced merely to models that envision anti-apoptotic Bcl-2 homologs as suppressors of caspase-activating proteins such as CED-4 family members, because (i) Bcl-2 family proteins regulate a variety of mitochondrial events even in the presence of broad-spectrum caspase inhibitors (zVAD-fmk), (ii) Bcl-2 can inhibit not only caspase-dependent apoptosis but also oxidant and hypoxia-induced necrosis, and (iii) Bax can induce cyto c release and

cell death (inhibited by Bcl-2/Bcl-$x_L$) even in yeast, which lack caspases (*10, 22, 48*).

## Conclusions

Although mitochondrial involvement and cyto c release may not be universal aspects of apoptosis, they appear to be frequently involved in vertebrates. In contrast, there is currently no evidence that cyto c plays roles in apoptosis in either nematodes or insects. In the former, caspase activation through interaction with Ced-4 (whose role presumably is similar to that of Apaf-1) proceeds without a requirement for cyto c. A more extensive phyletic survey will help establish whether the involvement of cyto c is a relatively late evolutionary innovation or whether it was secondarily lost from some lineages.

Nevertheless, the role of mitochondria as stress sensors and executioners makes intuitive sense for maintenance of the process of apoptosis in multicellular organisms. That is, cells cannot gain a growth advantage simply by losing mitochondria, because these organelles are also needed for efficient energy metabolism, production of membrane lipids, and cell growth. Defects in the apoptotic pathway downstream of mitochondria (for example, caspases) might also fail to provide a growth advantage, because cells may nevertheless die because of the mitochondrial effects (albeit more slowly), and thus caspase mutations may not promote oncogenesis.

Most intriguing is the structural similarity of Bcl-2 family proteins to channel-forming proteins such as the colicins, which are used as weapons by bacteria to kill competing bacteria. This ancient system involving secreted channel-forming proteins and opposing immunity proteins that bind colicins, preventing channel formation, is highly reminiscent of the scenario in mammalian cells where apoptotic stimuli induce translocation of cytosolic Bax to mitochondrial membranes, thereby killing the cell, unless opposed by Bcl-2. Perhaps a few hundred million years ago, either convergent or divergent evolutionary processes allowed the same fundamental framework for bacterial warfare to be incorporated into the cell death mechanisms used by animal cells, thereby establishing mitochondria as important participants not only in animal cell life but also in active cell death.

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# Caspases: Enemies Within

**Nancy A. Thornberry and Yuri Lazebnik**

REVIEW

**Apoptosis, an evolutionarily conserved form of cell suicide, requires specialized machinery. The central component of this machinery is a proteolytic system involving a family of proteases called caspases. These enzymes participate in a cascade that is triggered in response to proapoptotic signals and culminates in cleavage of a set of proteins, resulting in disassembly of the cell. Understanding caspase regulation is intimately linked to the ability to rationally manipulate apoptosis for therapeutic gain.**

Apoptosis is a type of cell death that is accomplished by a specialized cellular machinery. That this machinery exists and is highly conserved was predicted from observing a stereotypical morphology of cells dying either under physiological conditions or after mild injury (*1*). These changes reflect complex biochemical events carried out by a family of cysteine proteases called caspases. In this review, we describe properties of caspases, how they kill a cell, how they are regulated, and discuss the potential therapeutic utility of caspase modulation. Because relatively little is known about caspase regulation, it is instructive to first review lessons learned from well-studied proteolytic systems, which provide a framework for understanding the biology of caspases and can serve as guiding principles for ongoing research in this area.

*Proteolysis is irreversible, unlike most other posttranslational modifications.* This implies that regulation of proteases is limited to control of their activity and availability of substrate since the only known way of "correcting" a cleaved protein is to make it afresh. Considering this feature of proteolysis, it is not surprising