REVIEW

The expanding role of mitochondria in apoptosis

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The initial insight into the genetic basis of apoptosis, or programmed cell death, was gained from ingenious studies of the roundworm *Caenorhabditis elegans* (for review, see Horvitz 1999). These studies revealed a linear pathway whereby the products of two genes, designated *Ced-3* and *Ced-4*, were necessary and sufficient to trigger the perfectly timed and orchestrated death of 131 preordained cells during development. The relevance of this pathway to higher animals was established by the discovery of apparent mammalian orthologs of these genes and the demonstration that the mammalian *Ced-3*-related genes encode proteases (designated caspases) whose activities are responsible for the morphological changes characteristic of apoptosis (for review, see Hengartner 2000).

The complexity of the apoptotic program began to increase with the discovery of *Bcl-2*, a gene whose product causes resistance to apoptosis in lymphocytes (Vaux et al. 1988; McDonnell et al. 1989). *Bcl-2* was shown to correct partially the phenotype of a *C. elegans* mutation in *Ced-9*, a cell survival gene that functions upstream of *Ced-4* and *Ced-3* (Vaux et al. 1992). This finding suggested an apparent one-for-one correlation between the *C. elegans* and mammalian pro- and antiapoptotic pathways. However, this correlation did not explain two observations made in mammalian cells. First, the Bcl-2 protein was found on the membrane of mitochondria, which were not implicated in *C. elegans* apoptosis; and second, apoptotic changes could be produced in *Xenopus laevis* oocyte extracts only when a membrane fraction enriched in mitochondria was present (Hockenberry et al. 1990; Newmeyer et al. 1994).

The complex role of mitochondria in mammalian cell apoptosis came into focus when biochemical studies identified several mitochondrial proteins that are able to activate cellular apoptotic programs directly (Liu et al. 1996; Susin et al. 1999; Du et al. 2000; Verhagen et al. 2000; Li et al. 2001). Normally, these proteins reside in the intermembrane space of mitochondria. In response to a variety of apoptotic stimuli, they are released to the cytosol and/or the nucleus. They promote apoptosis either by activating caspases and nucleases or by neutralizing cytosolic inhibitors of this process. A complex picture has emerged in which mitochondrial and cytosolic proapoptotic proteins interact with antiapoptotic proteins with each cell's life or death hanging in the balance. This review summarizes the recent data on the expanding and complex role of mitochondria in apoptosis.

Execution of mitochondrial apoptotic signals

Release of cytochrome c

Cytochrome *c*, a component of the mitochondrial electron transfer chain, initiates caspase activation when released from mitochondria during apoptosis (Liu et al. 1996). As illustrated in Figure 1, cytosolic cytochrome *c* binds to Apaf-1, a cytosolic protein containing a caspase-recruitment domain (CARD), a nucleotide-binding domain, and multiple WD-40 repeats (Zou et al. 1997). Apaf-1 alone binds the nucleotide dATP or ATP poorly, despite the presence of Walker's consensus nucleotide binding sequences. However, the binding of cytochrome *c*, which is not dependent on the presence of nucleotide, increases Apaf-1 affinity for dATP/ATP by about 10-fold, perhaps by opening up the nucleotide binding site or stabilizing the bound nucleotide to Apaf-1 (Jiang and Wang 2000). The binding of nucleotide to the Apaf-1/cytochrome *c* complex triggers its oligomerization to form the apoptosome, a multimeric Apaf-1 and cytochrome *c* complex (Zou et al. 1999). The CARD domains of Apaf-1 become exposed in the apoptosome, which subsequently recruit multiple procaspase-9 molecules to the complex and facilitate their autoactivation. Only the caspase-9 bound to the apoptosome is able to efficiently cleave and activate downstream executioner caspases such as caspase-3 (Rodriguez and Lazebnik 1999). These executioner caspases subsequently cleave many important intracellular substrates, leading to characteristic morphological changes in apoptosis such as chromatin condensation, nucleosomal DNA fragmentation, nuclear membrane breakdown, externalization of phosphatidylserine, and formation of apoptotic bodies (for review, see Hengartner 2000).

Results from gene knockout (KO) experiments underscore the importance of each component of the apopto-

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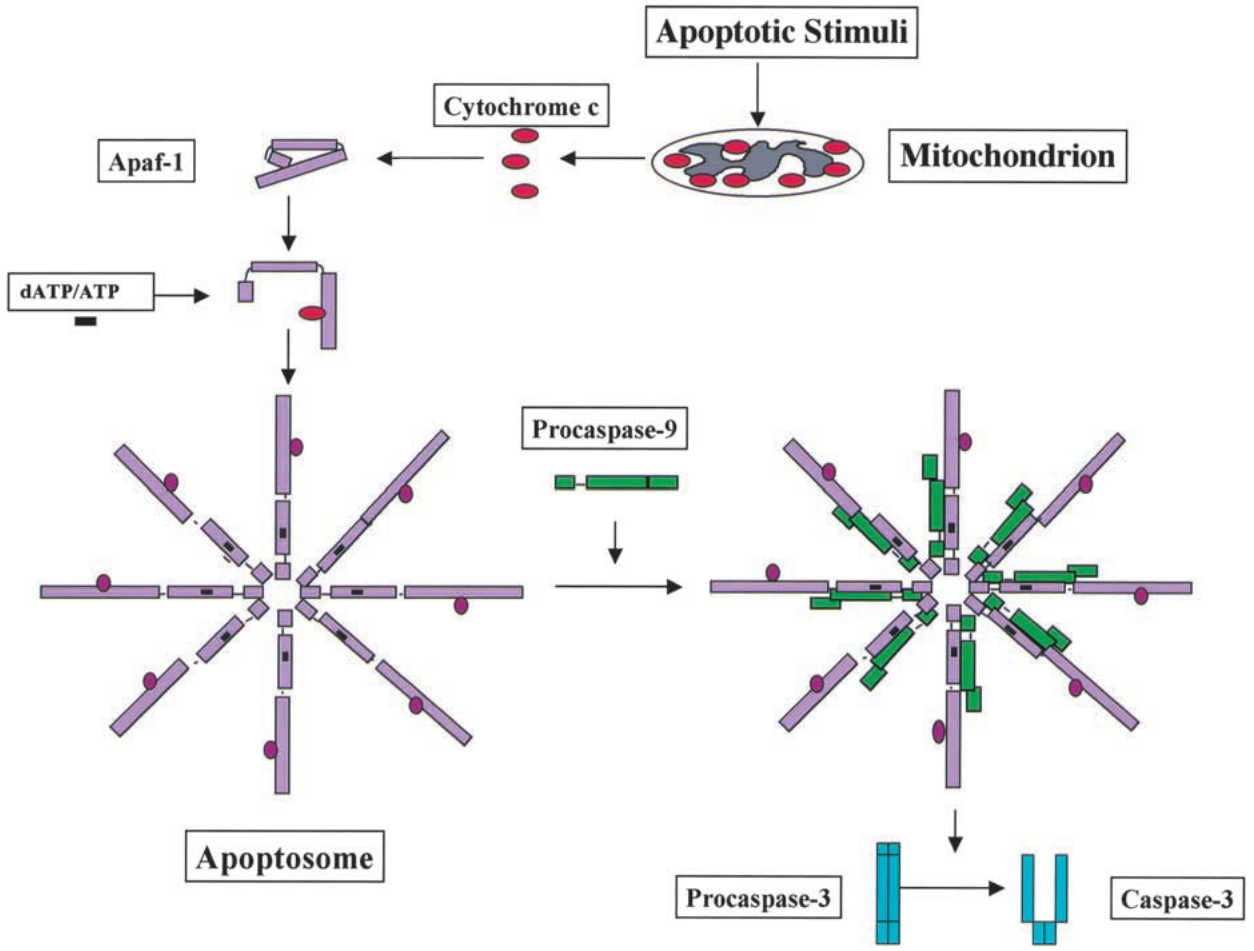

Figure 1. The cytochrome *c*-induced caspase activation pathway. Apoptotic stimuli exert their effects on mitochondria to cause the release of cytochrome *c*. Cytochrome *c* in turn binds to Apaf-1, a cytosolic protein that normally exists as an inactive monomer. The binding of cytochrome *c* induces a conformational change in Apaf-1, allowing it to bind the nucleotide dATP or ATP. The nucleotide binding to the Apaf-1–cytochrome *c* complex triggers its oligomerization to form the apoptosome, which recruits procaspase-9. The binding of procaspase-9 to the apoptosome forms the caspase-9 holoenzyme that cleaves and activates the downstream caspases, such as caspase-3.

some in apoptosis. Eliminating Apaf-1, caspase-9, or caspase-3 led to the inhibition of most neuronal cell death during normal development, resulting in exencephaly, craniosches is, and spina bifida (Kuida et al. 1996, 1998; Cecconi et al. 1998; Yoshida et al. 1998; Honarpour et al. 2000, 2001). Embryonic stem cells (ES) and fibroblasts (MEF) from these mice failed to activate caspases in response to damage signals such as UV, γ-irradiation, and treatment with chemotherapeutic drugs. Cells from the cytochrome-c-deficient mouse embryos that survived up to embryonic day 8.5 showed a similar deficiency in response to various apoptotic stimuli (Li et al. 2000).

These knockout experiments also verify the linearity of the cytochrome-c–Apaf-1–caspase-9–caspase-3 pathway. The Apaf-1 protein from the *cytochrome c* KO cells remained in the monomeric state in the presence of apoptotic stimuli (Li et al. 2000). In *Apaf-1* or *caspase-9* KO cells, no caspase-3 activation was detected in the presence of apoptotic stimuli even though cytochrome *c* was released into the cytosol (Hakem et al. 1998; Yoshida et al. 1998).

Another remarkable revelation by these knockout experiments is that despite crippled caspase activation, most organs in the null mice developed normally except for an excessive number of neurons and neuron progenitor cells. Some of the *Apaf-1*-null mice even survived to adulthood without apparent defects other than male sterility (Honarpour et al. 2000). These results contrast genetic studies in *C. elegans* (Horvitz 1999), in which loss-of-function mutation of *Ced-3* (the *C. elegans* caspase) or *Ced-4* (an *Apaf-1* homolog), blocks all developmental cell death in this organism.

One obvious explanation for this difference, in the case of *Apaf-1*, is that redundant pathways compensate for the loss of *Apaf-1* in mammals. However, examination of the human genome revealed that *Apaf-1* is the only protein that shows extensive homology to the worm *Ced-4* (Aravind et al. 2001). More importantly, when the ES and MEF cells from the *Apaf-1* KO mice

were treated with serum withdrawal and other stress signals, no caspase activation was detected but the cells still died, albeit at a slower rate than wild-type or heterozygous cells (Honarpour et al. 2000; Haraguchi et al. 2000). Consistent with this observation, in *Apaf-1* KO mice, the recession of the interdigital webbing still occurs with a one-day delay (Yoshida et al. 1998). When the cells in the interdigital web area were examined, no caspase activity was observed and the dying cells showed morphology resembling necrosis (Chautan et al. 1999).

A major difference between apoptosis in worm and mammals is probably the greater role of mitochondria in the latter but not the former. Unlike the worm system in which the activation of caspase is possibly triggered by the disassociation of CED9 from a CED3/CED4 complex (Chen et al. 2000), in mammals mitochondria control apoptosis by sequestering the apoptogenic proteins in their intermembrane space and releasing them when apoptotic signals are sensed. Recent studies in mammalian cells have uncovered three mitochondrial proteins, in addition to cytochrome *c*, whose release from mitochondria may contribute to apoptosis.

Release of Smac

Concurrent with cytochrome *c*, Smac/Diablo, a 25-kD mitochondrial protein, is released from mitochondria into the cytosol during apoptosis (Du et al. 2000; Verhagen et al. 2000). Smac is a bona fide nuclei-encoded mitochondrial protein containing a 55-amino-acid mitochondrial targeting sequence at its N terminus. This sequence is removed on import into the mitochondria (Du et al. 2000). As shown in Figure 2, the removal of this targeting sequence generates a new N terminus in the mature Smac protein. The first four amino acids of the mature Smac, Ala-Val-Pro-Ile (AVPI), binds to the BIR (baculovirus IAP [inhibitor of apoptosis protein] repeat) domain of IAPs (Chai et al. 2000). IAPs are a family of

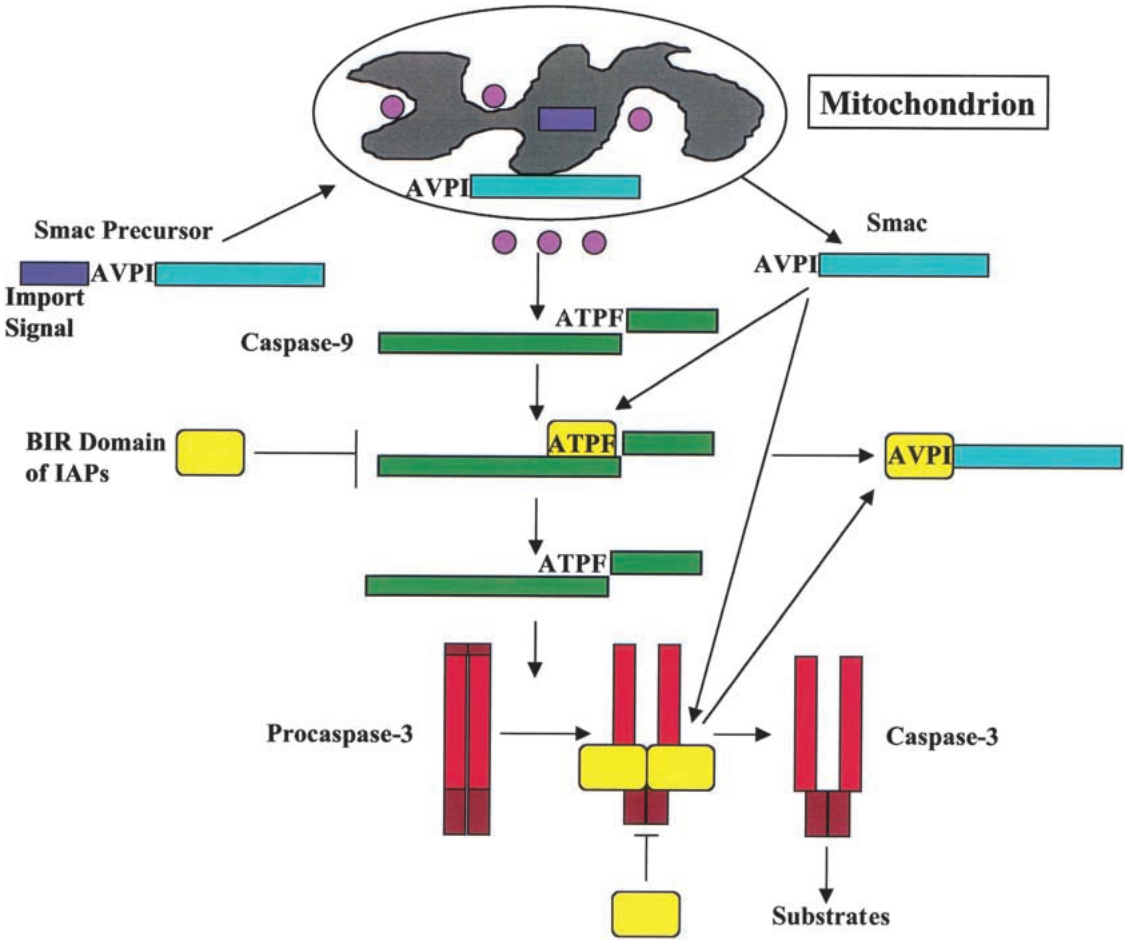

Figure 2. Displacement of IAPs from caspases by Smac/Diablo. The precursor of Smac/Diablo is synthesized in the cytosol and transported to the mitochondria. After mitochondrial entry, the mitochondrial targeting sequence of Smac (dark purple rectangle) is cleaved, exposing the four amino acid residues Ala-Val-Pro-Ile through which Smac binds to the BIR domain of IAPs. The mature Smac (aqua rectangle) is normally located in the mitochondrial intermembrane space. During apoptosis, cytochrome *c* (light purple circles) and Smac are released from the mitochondria. Cytochrome *c* triggers the activation of caspase-9 (green rectangles) and caspase-3 (red rectangles). The IAP molecules bind and inhibit active caspase-9 and caspase-3 via their BIR domains (yellow boxes). The IAP inhibition is relieved by Smac that competitively binds to the BIR domains of IAP molecules and excludes them from binding caspases.

intracellular proteins that contain one or multiple BIR domains, which are known to inhibit active caspases (for review, see Deveraux and Reed 1999). The exposed N-terminal Ala of mature Smac is absolutely required for the binding of IAPs (Chai et al. 2000; Liu et al. 2000; Wu et al. 2000). Because this Ala becomes exposed only when the signal peptide gets cleaved after mitochondrial entry, mitochondrial targeting becomes a critical step for Smac function. This arrangement also ensures that Smac and other mitochondrial apoptogenic proteins do not trigger premature apoptosis before their entry into mitochondria. (Wu et al. 2000).

The four amino acid residues (Ala-Val-Pro-Ile) of Smac that bind to the BIR3 domain of XIAP (X chromosome-encoded IAP) is similar to the XIAP-binding sequence of active caspase-9 (Ala-Thr-Pro-Phe) (Srinivasula et al. 2001). This IAP-binding motif is exposed only after the processing of procaspase-9 into its mature form. IAP binding results in the inhibition of caspase-9 activity, which is relieved when Smac competes off caspase-9 (Srinivasula et al. 2001).

In addition to the BIR3 domain of XIAP, Smac/Diablo has also been shown to form a stable complex with the BIR2 domain of XIAP (Chai et al. 2001). The linker sequence immediately preceding the BIR2 domain is involved in XIAP-mediated binding and inhibition of caspase-3 and caspase-7 (Sun et al. 1999; Chai et al. 2001; Huang 2001; Riedl et al. 2001). Smac/Diablo binds to the BIR2 domain and presumably disrupts its inhibition of the active caspase-3 and caspase-7 by steric hindrance (Chai et al. 2001). The ability of Smac/Diablo to counter the inhibition of IAP at multiple levels provides a powerful way for mitochondria to ensure rapid execution of apoptosis.

The direct competition and mutual exclusion between Smac and activated caspases suggest an interesting feedback system in cells. When released from mitochondria, cytochrome *c* binds to Apaf-1 with high affinity and triggers apoptosome formation and caspase activation (Purring et al. 1999). However, in the presence of high levels of IAPs, this pathway will be aborted when IAPs bind and inhibit the active caspases in the apoptosome (Bratton et al. 2001; Srinivasula et al. 2001). The inhibition could become permanent because many IAPs also contain a RING finger domain that may target the bound caspases for proteasome degradation (Yang et al. 2000; Suzuki et al. 2001). Such a system provides a safety net for the transient or incidental mitochondria leakage of cytochrome *c*, a much smaller molecule than Smac (Chai et al. 2000). If the damage to mitochondria is severe and persistent, more Smac will be released, together with cytochrome *c*, to remove IAP inhibition and allow apoptosis to proceed.

The regulation provided by the Smac and IAPs interaction may not be limited to the cytochrome *c* pathway (Green 2000; Srinivasula et al. 2000). Cell surface death receptors and their ligands, such as Fas/FasL and the Trail/trail receptor, are able to initiate caspase activation independent of mitochondria (for review, see Krammer 2000). However, because this pathway and the cytochrome *c* pathway converge at the step of caspase-3 activation, high levels of IAP molecules such as XIAP are able to abort the receptor pathway by inhibiting caspase-3. For apoptosis to proceed, the receptor pathway may rely on the activation of Bid, a BH3-only protein that is activated by the initiator caspase in the receptor pathway, caspase-8 (Li et al. 1998; Luo et al. 1998; Gross et al. 1999). Activated Bid induces the release of apoptogenic proteins including Smac from the mitochondria to counter XIAP inhibition. As caspase-8 is not sensitive to XIAP inhibition (Riedl et al. 2001), persistent activation of the cell surface death receptor should eventually overcome IAP inhibition through the mitochondrial apoptotic pathway.

The finding that Smac interacts with IAPs mainly through a few amino acid residues at the N terminus of Smac provides a plausible explanation for a puzzling observation in the field. In *Drosophila*, it has long been recognized that three proteins, Reaper, Grim, and Hid, promote apoptosis by antagonizing the two *Drosophila* IAPs, DIAP-1 and DIAP-2 (Vucic et al. 1998; Wang et al. 1999; Goyal et al. 2000). Despite similar biochemical activity, Reaper, Grim, and Hid share little sequence homology other than a few amino acids at their N termini; no mammalian homologs for any of those three proteins have been found. However, if the comparison is restricted to the N-terminal four amino acids of mature Smac and the few amino acid residues of Reaper, Grim, and Hid after their initiator methionines, obvious homology is noted (Liu et al. 2000, Wu et al. 2000). Structural analysis using a BIR domain of DIAP-1 and the N-terminal peptides of Grim and Hid (minus the initiator Met) confirmed that the Smac–IAP interaction is conserved between mammals and *Drosophila* (Wu et al. 2001).

The presence of Smac does not explain why those knockout cells deficient in *Apaf-1*, *cytochrome c*, or *caspase-9* still die without apparent caspase activation. It is likely that other caspase-independent pathways emanating from the mitochondria are able to kill cells, a scenario that is played out by several recent studies discussed below.

Release of apoptosis-inducing factor

Apoptosis-inducing factor (AIF) is a 57-kD flavoprotein that resembles bacterial oxidoreductase and resides in the mitochondrial intermembrane space (Susin et al. 1999). Upon induction of apoptosis, AIF translocates from the mitochondria to the nucleus and causes chromatin condensation and large-scale DNA fragmentation (Susin et al. 1999). These effects are independent of caspases and the oxidoreductase activity of AIF (Miramar et al. 2001).

Deficiency of AIF has profound effects in animal development. Disruption of *AIF* in mice prevents the normal apoptosis necessary for the cavitation of embryoid bodies in the embryo (Joza et al. 2001). This very early apoptotic event is essential for mouse morphogenesis. Moreover, embryonic stem cells lacking AIF are re-

sistant to cell death after vitamin K3 treatment and serum starvation (Joza et al. 2001). Because AIF is also an oxidoreductase that may play an important role in normal mitochondrial physiology, it is not clear whether the observed phenotype in the AIF KO mouse embryos is caused entirely by the elimination of the apoptotic activity of AIF or because of the loss of oxidoreductase function of AIF as well.

What remains to be worked out is the biochemical mechanism by which AIF induces large-scale DNA fragmentation and chromatin condensation. Inasmuch as AIF itself has no measurable DNase activity (Susin et al. 1999), it is possible that AIF may work with another protein to cause such an effect.

Release of endonuclease G

Endonuclease G (EndoG), a known 30-kD nuclease in the mitochondria, was purified recently from the supernatant of mouse mitochondria that had been treated with caspase-8-activated Bid (tBid), a condition that mimicked the initiation of cell death after activation of the cell surface death receptor (Li et al. 2001). EndoG is encoded by a nuclear gene, translated in the cytosol, and imported subsequently into the mitochondria (Cote et al. 1993). It has been proposed that it participates in mitochondrial replication by eliminating RNA primers for the initiation of mitochondrial DNA synthesis (Cote et al. 1993; Tiranti et al. 1995). The proposed function of EndoG in mitochondrial DNA replication was based on its location and substrate specificity, as EndoG prefers GC-rich substrates, which resemble the DNA sequences in the mitochondrial DNA replication origin (Cote et al. 1993). However, this proposal has been challenged by three experimental observations. First, another endonuclease, RNase MRP, has also been shown to cleave the RNA primers in a site-specific manner needed for initiation of mitochondrial DNA replication (Clayton 1987). Second, a yeast mitochondrial nuclease that shows ~40% similarity to human EndoG has been knocked out and no mitochondrial defect was observed in the mutant yeast (Zassenhaus et al. 1988). Third, EndoG is specifically and quantitatively released from the mitochondria together with the other apoptogenic proteins in the intermembrane space. Under the same condition, the mitochondrial matrix protein mtHsp70 remains in the mitochondria (Li et al. 2001). Therefore, at least a substantial portion of EndoG must be located in the mitochondrial intermembrane space, not in the matrix where DNA replication takes place.

Once released, EndoG is able to induce nucleosomal DNA fragmentation. Unlike DFF40/CAD, the well-characterized apoptotic nuclease whose activation requires caspase-3 cleavage of the chaperone/inhibitor DFF45/ICAD, EndoG activity is independent of caspase activation (Liu et al. 1997; Enari et al. 1998; Liu et al. 1998; Li et al. 2001). Furthermore, it has been shown that EndoG activity may be responsible for DNA fragmentation observed in *DFF45*-deficient MEF cells after induction of apoptosis by UV-irradiation and TNF treatment (Zhang et al. 1998; Li et al. 2001). The identification of AIF and EndoG indicates that apoptosis can proceed in the absence of caspase activity when the mitochondria are damaged. In this case, release of AIF and EndoG from mitochondria starts an apoptotic program parallel to caspase activation.

The role of EndoG in apoptosis is apparently conserved from worms to mammals. Using a suppressor screen for the active *C. elegans* caspase CED-3, Ding Xue's group identified a mutant named *cps-6* (CED-3 protease suppressors), which shows delayed progression of apoptosis and abnormal DNA fragmentation (Parrish et al. 2001). CPS-6 protein is localized in the mitochondria and exhibits striking similarity in sequence and biochemical properties to the mammalian EndoG (Parrish et al. 2001). The *cps-6* mutant is also phenocopied when the worm EndoG was eliminated functionally by RNA interference (RNAi) and can be rescued by mouse *EndoG* (Parrish et al. 2001). These results indicate EndoG might represent an ancient evolutionarily conserved pathway. It is not clear whether EndoG in worms is working downstream or in parallel with CED-3. Additional studies are needed to clarify this issue.

The release of cytochrome *c* and other apoptogenic proteins from mitochondria is known to be regulated by the Bcl-2 family of proteins. The pro-death members of this group of protein promote the release of these apoptogenic factors whereas the anti-death members prevent it (for review, see Korsmeyer et al. 2000).

Regulation of mitochondrial apoptotic signals

Translocation of the BH3-only family of proteins to mitochondria

The BH3-only family of proteins share sequence homology with Bcl-2 only in the BH3 domain, an amphipathic helix required to interact with other Bcl-2 family members (Huang and Strasser 2000). These proteins are normally located in other cellular compartments and translocate to the mitochondria in response to apoptotic stimuli. Once translocated to the mitochondria, they cause mitochondrial damage and release of apoptogenic proteins by interacting with other members of the Bcl-2 family.

Cleavage of Bid Cell surface death receptors are a family of transmembrane proteins that belong to the tumor necrosis factor receptor (TNF-R) superfamily, including Fas/APO-1/CD95, TNFR1, DR-3, DR-4/TRAIL-R1, and DR5/TRAIL-R2 (for review, see Ashkenazi and Dixit 1998). These receptors share a cysteine-rich repeat in their extracellular domains, and a 'death domain,' in their cytoplasmic tail that is required for apoptotic signaling. The activating ligands for these death receptors are structurally related molecules that also belong to the TNF gene superfamily. Fas/CD95 ligand (FasL) binds to Fas, TNF binds to TNFR1, Apo3 ligand (Apo3L) binds to DR3, and Apo2 ligand (Apo2L or TRAIL) binds to DR4 and DR5 (for review, see Ashkenazi and Dixit 1998).

One of the well-characterized death-signaling pathways involves Fas/CD95 receptor and its ligand. This pathway is initiated by a series of sequential steps: ligand-induced receptor trimerization, formation of DISC (death-inducing signaling complex) involving adaptor proteins such as FADD (Fas-associating protein with death domain), and recruitment of procaspase-8 and its subsequent proteolytic activation (for review, see Krammer 2000). Activated caspase-8 then initiates a cascade of caspase activation by directly cleaving and activating downstream caspases. In certain types of cells (type I), enough caspase-8 can be activated by the activation of the death receptor to cause apoptosis (Scaffidi et al 1998). In this type of cells, caspase inhibitor, but not the overexpression of Bcl-2, can usually prevent apoptosis. In other cell types such as hepatocytes (type II), caspase-8 activation cannot achieve such a high level and the apoptotic signal needs to be amplified by the mitochondrial pathway. The signal from caspase-8 is relayed to mitochondria by Bid, a BH3-only protein.

Bid is exclusively cytosolic in living cells. Upon activation of cell surface receptors, Bid is cleaved by caspase-8; the truncated Bid (tBid) translocates from cytosol to mitochondria and induces cytochrome *c* release (Li et al. 1998; Luo et al. 1998). tBid targets mitochondria through its helices 4–6; the targeting specificity is provided by the binding to the mitochondria-specific lipid, cardiolipin (Lutter et al. 2000). The targeting efficiency is dramatically enhanced by myristoylation at the N-terminal glycine residue of tBid that becomes exposed after caspase-8 cleavage (Zha et al. 2000).

The importance of this cross-talk between the cell surface and the mitochondria is illustrated by *Bid* KO mice. Hepatocytes from Bid-deficient mice are resistant to Fas- and TNF-induced apoptosis, although cells from these mice remain susceptible to apoptosis after treatment with other apoptosis-inducing agents that do not activate death receptors (Yin et al. 1999; Zhao et al. 2001). It is worth noting that there has not been any report on other defects in the Bid-knockout mice. It is possible that other BH3-only proteins such as Bad can also be activated through caspase-8 cleavage (Condorelli et al. 2001).

The ability to cleave and activate Bid may not be limited to caspase-8. Other caspases, such as caspase-3, as well as other proteases, such as granzyme B and lysosomal proteases, have been shown to cleave and activate Bid (Li et al. 1998; Heibein et al. 2000; Sutton et al. 2000; Alimonti et al. 2001; Stoka et al. 2001). Bid (and proteins that function similarly) may therefore serve as a general integrator and amplifier for many apoptotic signals.

Phosphorylation of Bad Bad, another BH3-only protein, is regulated primarily by phosphorylation and dephosphorylation (Zha et al. 1997). In the absence of survival signals, Bad is dephosphorylated. The BH3 domain of Bad binds to and inactivates the antiapoptotic members of the Bcl-2 family at the outer mitochondrial membrane, thereby promoting cell death. Conversely, in the presence of trophic factors, Akt and mitochondria-anchored PKA phosphorylate Bad, allowing it to bind 14-3-3 protein and to remain in the cytosol (Datta et al. 1997; Harada et al. 1999). Phosphorylation of Bad also dissociates its interaction with antiapoptotic Bcl-2 family of proteins, allowing these proteins to promote survival.

The critical site of phosphorylation induced by survival factors occurs at Ser 155 within the BH3 domain of Bad (Datta et al. 2000; Lizcano et al. 2000; Virdee et al. 2000; Zhou et al. 2000). This phosphorylation requires prior phosphorylation of Ser 112 and Ser 136, which recruits 14-3-3 proteins to the Bad/Bcl-xL complex (Datta et al. 1997; Zha et al. 1997; Harada et al. 1999). 14-3-3 proteins effectively increase the accessibility of Bad to Ser-155 kinases, which then phosphorylate Bad within its BH3 domain. Phosphorylation of this domain permanently blocks the ability of Bad to bind to Bcl-xL because of electrostatic and steric constraints and consequently inhibits Bad-mediated death (Datta et al. 2000).

Several phosphatases, including calcineurin, protein phosphatase 1α, and protein phosphatase 2A, have been shown to dephosphorylate Bad in vitro (Wang et al. 1999; Ayllon et al. 2000; Chiang et al. 2001). How these phosphatases are regulated in vivo by apoptotic signals remains to be investigated.

Disassociation of Bim Bim, another BH3-only protein, has been found to associate with cellular microtubule complexes by binding to dynein light chain LC8 (Puthalakath et al. 1999). Early during apoptosis, Bim/LC8 dissociates from the microtubule complex and translocates to the mitochondria. Recombinant Bim alone is as efficient as tBid in releasing cytochrome *c* and EndoG when incubated with mitochondria in vitro (Li et al. 2001). Mice lacking Bim show defects in apoptotic response in their immune system (Bouillet et al. 1999). How the disassociation of Bim with the dynein complex is regulated during apoptosis remains unclear. Because *Bim*-null lymphocytes are refractory to some apoptotic stimuli, such as cytokine deprivation, calcium ion flux, and microtubule perturbation, but not to others, it is plausible that these stimuli trigger the dissociation of Bim from the microtubules. Moreover, the abundance of Bim also seems to be critically regulated at the level of transcription during apoptosis.

Transcriptional regulation of BH3-only proteins Transcription regulation of the BH3-only protein may be important for apoptosis that requires new protein synthesis. In this case, it is conceivable that the newly generated protein will target directly to the mitochondria. Cytokine withdrawal in cultured neurons or hematopoietic progenitors drastically increases the mRNA and protein level for Bim (Dijkers et al. 2000; Putcha et al. 2001; Shinjyo et al. 2001). Forkhead transcription factor FKHR-L1, whose activity is suppressed by AKT phosphorylation, induces Bim transcription (Dijkers et al. 2000). Dominant-negative c-Jun is also able to block Bim upregulation after NGF withdrawal in cultured neurons, suggesting that c-Jun may also participate in the transcriptional regulation of Bim (Whitfield et al. 2001). In

addition to Bim, another BH3-only protein, HRK, is also up-regulated during cytokine withdrawal in hematopoietic progenitor cells (Sanz et al. 2000, 2001).

Transcriptional regulation of BH3-only protein seems to play a major role in DNA-damage induced apoptosis. Two proapoptotic gene products, Noxa and Puma, were identified recently as BH3-only members of the Bcl-2 family that are induced in a p53-dependent manner in primary mouse cells exposed to X-ray irradiation (E. Oda et al. 2000; Nakano and Vousden 2001; Yu et al. 2001). The BH3 domain is important for these two proteins to trigger cytochrome *c* release and caspase activation.

Translocation of other proteins to mitochondria during apoptosis

In addition to the pro-death member of the Bcl-2 family, several proteins, including p53 itself, an orphan receptor TR3, the p53-target gene p53AIP, and the *Peutz-Jegher* gene product LKB1 have been shown to migrate to the mitochondria during apoptosis and to cause mitochondrial damage (Li et al. 2000; Marchenko et al. 2000; K. Oda et al. 2000; Karuman et al. 2001). How these proteins, and possibly others, migrate to the mitochondria and release the contents of their intermembrane space is not known. Nevertheless, these studies suggested an exciting new direction for future research that may reveal other unknown biochemical pathways.

Mitochondrial response to apoptotic signals

Bax and Bak

Once the BH3-only proteins reach the mitochondria, they need to cooperate with other mitochondrial proteins to induce the release of apoptogenic proteins. The proapoptotic members of the Bcl-2 family that contain BH1–BH3 but not BH4, such as Bax and Bak, are the likely mediators for the BH3-only proteins (Korsmeyer et al. 2000). The important role of these proteins in apoptosis has been demonstrated dramatically in the *Bax* and *Bak* double KO mice (Lindsten et al. 2000). Most of these mice die during embryonic development; but the few survivors showed a persistence of interdigital webs, an imperforate vaginal canal, and an accumulation of excess cells within the nervous and hematopoietic systems (Lindsten et al. 2000). Moreover, MEF cells lacking both *Bax* and *Bak* are resistant to multiple apoptotic stimuli, including overexpression of the BH3-only proteins tBid, Bim, and Bad (Wei et al. 2001; Zong et al. 2001).

Concurrent with the translocation of the BH3-only proteins to the mitochondria, Bax and Bak undergo conformational changes and oligomerization, presumably induced by transient interaction with the BH3-only protein (Korsmeyer et al. 2000; Nechushtan et al. 2001). The oligomerized Bax and Bak may form a pore big enough for the apoptogenic proteins to pass through or else destabilize the mitochondrial outer membrane through an unknown mechanism.

Bcl-2 and other antiapoptotic members

The activities of the BH3-only proteins and proteins such as Bax and Bak can be neutralized by the antiapoptotic member of the family such as Bcl-2 and Bcl-xL. These proteins do not seem to affect the translocation of the BH3-only proteins to the mitochondria. Instead, they block the oligomerization of Bax and Bak and abort the apoptotic program at this stage (Nechushtan et al. 2001; Sundarajan and White 2001; Wei et al. 2001).

VDAC and ANT

VDAC and ANT, two of the most abundant proteins of the outer and inner membranes of the mitochondria, have also been shown to interact with the Bcl-2 family of proteins and to mediate mitochondrial damage during apoptosis (Marzo et al. 1998; Narita et al. 1998; Shimuzu et al. 1999). It has been hypothesized that the interaction between Bax and VDAC causes a change of VDAC permeability to allow proteins such as cytochrome *c* to pass through (Shimuzu et al. 2000). Similarly, Bax and ANT may also form some kind of protein pore (Marzo et al. 1998). Additionally, since these two proteins play important roles in facilitating the transport of small metabolites and nucleotides across the mitochondrial membrane, the binding of Bax may also contribute to the observed blockage of ATP/ADP exchange and the export of creatine phosphate during apoptosis induced by cytokine withdrawal (Vander Heiden et al. 1999).

Loss of mitochondria functions during apoptosis

Mitochondria are the bioenergetic and metabolic centers of eukaryotic cells. During apoptosis, mitochondria suffer specific damages that result in loss of their function. For example, release of cytochrome *c*, the sole water-soluble component of the electron transfer chain, can potentially halt the electron transfer, leading to failure in maintaining the mitochondrial membrane potential and ATP synthesis. Moreover, because cytochrome *c* carries electrons from cytochrome *c* reductase (complex III) to cytochrome *c* oxidase (complex IV), by which oxygen molecules are reduced to water, a blockade at this step would increase the production of reactive oxygen species with subsequent lipid peroxidation (Hockenbery et al. 1993; Cai and Jones 2000).

Loss of mitochondrial ATP synthesis has been reported in cells after growth-factor deprivation (Vander Heiden et al. 1999). In these apoptotic cells, cellular ATP content decreases whereas cytosolic ADP and intermembrane creatine phosphate concentrations increase. This phenomenon, which is presumably triggered by the translocation of proapoptotic BH3-only proteins to the mitochondria, can be reversed by Bcl-xL overexpression (Vander Heiden et al. 1999). Therefore, it is likely that one of the critical antiapoptotic roles of the Bcl-2 family proteins relies on their ability to protect mitochondrial homeostasis. For example, Bcl-xL can promote survival following apoptotic induction by inhibiting VDAC clo-

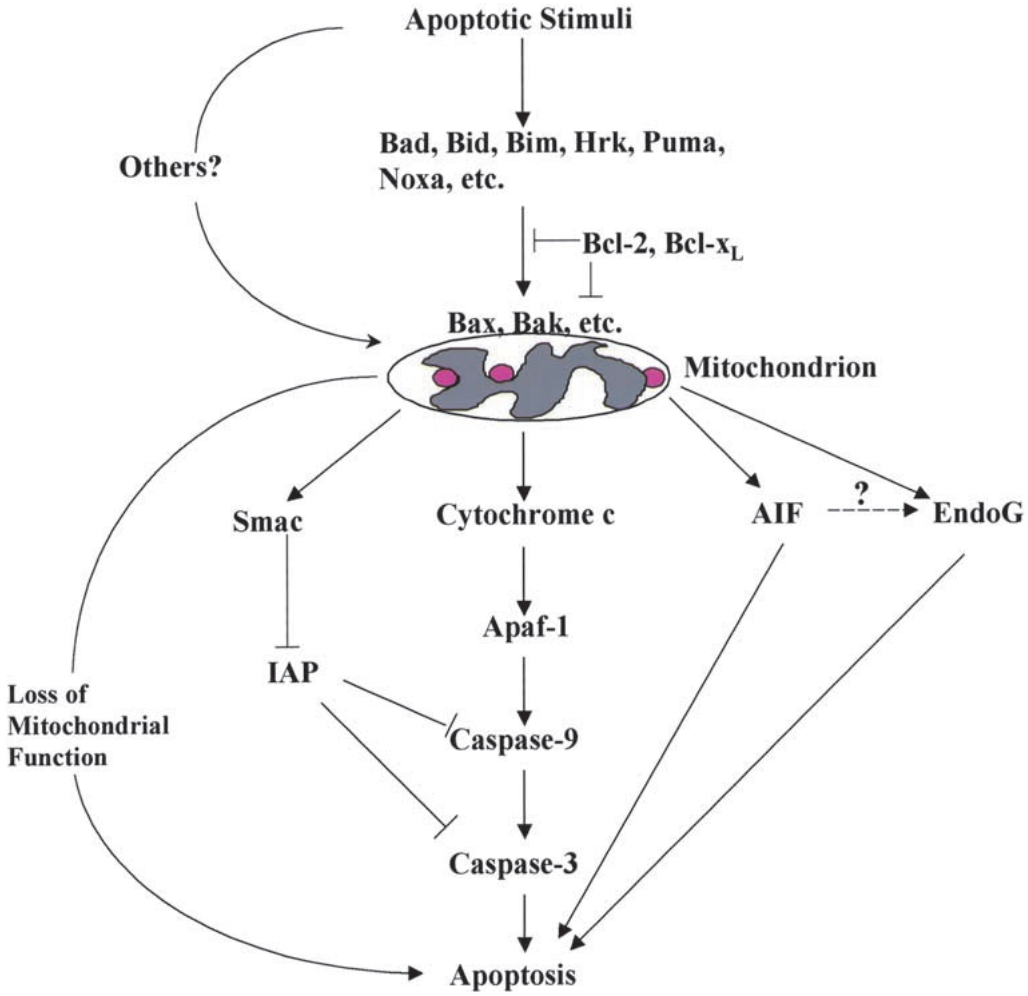

Figure 3. Multiple apoptotic pathways emanate from the mitochondria. Apoptotic stimuli are transduced to mitochondria by the BH3-only proteins and possibly by additional pathways. The signal from the BH3-only protein can be either neutralized by the antiapoptotic protein, such as Bcl-2 or Bcl-xL, or further transduced to mitochondria by the proapoptotic protein such as Bax or Bak. The mitochondrial damage caused by apoptotic stimuli triggers the release of apoptogenic proteins including cytochrome *c*, Smac, AIF, and EndoG. Cytochrome *c* triggers caspase activation through Apaf-1, and Smac relieves IAP inhibition of caspases. AIF and EndoG cause chromatin condensation and fragmentation in a caspase-independent manner. The mitochondrial damage may also passively lead to cell death due to loss of mitochondrial function.

sure, therefore, maintaining metabolite exchange across the outer mitochondrial membrane (Vander Heiden et al. 2001).

Isolated liver mitochondria are known to have respiration coupled tightly to oxidative phosphorylation; that is, the rate of oxygen consumption depends on the availability of ADP for the synthesis of ATP. In the presence of ADP, the mitochondrial respiratory activity transits from state 2 (resting state in which ADP is not available to stimulate respiration and oxygen consumption is minimal) to state 3 (active state in which the addition of ADP drives ATP synthase and oxygen consumption increases; Chance and Williams 1955).

The treatment of isolated mitochondria with tBid triggers the release of cytochrome *c* (Li et al. 1998; Luo et al. 1998; Gross et al. 1999). Intially, however, the basal level (state 2) of electron transfer does not appear to be affected grossly, suggesting that the amount of cytochrome *c* released is not sufficient to halt the electron transfer chain. In contrast, the coupling of electron transfer with oxidative phosphorylation is blocked completely, as shown by the inhibition of ADP-stimulated oxygen consumption (I. Budihardjo and X. Wang, unpubl.). This inhibition can be reversed by the presence of Bcl-xL. In addition, we also observed that tBid treatment abolished the ability of mitochondria to buffer calcium, another important mitochondrial function for cellular homeostasis (I. Budihardjo and X. Wang, unpubl.). These phenomena have also been observed in vivo using wild-type and *Bid*-null mice (Mootha et al. 2000). In the early stage, the inhibition of mitochondrial respiratory transitions can be rescued partially by adding exogenous cytochrome *c*. However, at a later stage, the inhibition was irreversible (Mootha et al. 2001).

The findings that tBid influences the coupling of electron transfer and oxidative phosphorylation have important implications. It is known that inhibition of caspases by a pan-caspase inhibitor eliminates the typical morphological changes seen in cells undergoing apoptosis (Xiong et al. 1997). However, elimination of caspase activities, either by small molecule inhibitors or by gene knockout, often fails to rescue cells from inevitable death for most of the apoptotic stimuli (Kuida et al. 1996, 1998; Yoshida et al. 1998; Haraguchi et al. 2000; Honarpour et al. 2000; Li et al. 2000). This suggests that cytochrome *c* release and the associated caspase activation is sufficient but not necessary for cell death. Other cellular events following apoptotic stimuli are able to kill cells even in the absence of caspase activation. In addition to the caspase-independent pathways mediated by AIF and EndoG, interruption of the reactions associated with ATP synthesis by mitochondria may be one such event.

Early in the course of apoptosis, mitochondria also undergo metabolic changes that can potentially alter the enzymatic reactions crucial for mitochondrial function. These changes include alkalinization of the mitochondrial matrix (Matsuyama et al. 2000) and up-regulation of proteins involved in controlling intracellular redox potential, such as glutathione S-transferase, fructose-1,6 biphosphate and fatty acid binding proteins, uncoupler protein-2 (UCP-2), and VDAC (Voehringer et al. 2000).

In summary, mitochondria-initiated apoptosis has three important features. First, as illustrated in Figure 3, multiple factors function in conjunction and in parallel to trigger cell death. The release of cytochrome *c* activates caspases, the release of Smac removes IAP inhibition on caspases, and the release of EndoG and AIF induces DNA fragmentation and chromatin condensation. Second, the pathway is able to feed-forward and amplify the apoptotic signal. Active caspases can cleave the Bcl-2 family of proteins to cause more mitochondrial damage (Cheng et al. 1997); and active DNases will generate DNA breaks that are signals for mitochondrial damage. Third, even when caspase-dependent and caspase-independent pathways cannot function properly, mitochondrial dysfunction caused by apoptotic stimuli may lead passively to cell death, owing to compromised energy production.

The logical conclusion from studying mitochondria-initiated apoptosis is that the best way to prevent cell death is to block apoptotic signals before mitochondrial damage occurs.

Perspectives

Despite a breathtaking rate of progress, many questions regarding the mitochondria-mediated cell-death pathways remain unanswered. One of the most elusive is the biochemical mechanism for the release of the apoptogenic proteins from the mitochondria. A related question is the relationship between the release of apoptogenic proteins and the loss of mitochondrial functions such as matrix alkalinization, uncoupling of oxidative phosphorylation, and defects in ATP/ADP exchange.

In comparison to the execution phase of the mitochondrial apoptotic pathway, we know relatively little about how the upstream signaling pathways to the mitochondria are regulated. In particular, how are the signals from either developmental cues or damage signals transduced to and integrated in the mitochondria? Are the BH-3-only proteins the major signal transducers? Or are they only part of a more complicated network of proteins? To answer these questions, we will need to develop more sophisticated strategies to identify other players, either through biochemical assays or genetic screens. Only then will we begin to see the big picture of what is happening when cells decide whether to live or die.

Acknowledgments

I thank Dr. Imawati Budihardjo for helpful discussions regarding this article and Drs. Yigong Shi, Joe Goldstein, and Mike Brown for reading the manuscript critically. The author is grateful for support from the HHMI, the NIH, and the Welch Foundation.

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The expanding role of mitochondria in apoptosis

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Genes Dev. 2001, **15:**