Mitochondrial intermembrane proteins in cell death[☆]

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Abstract

Apoptosis is a form of programmed cell death important in the development and tissue homeostasis of multicellular organisms. Mitochondria have, next to their function in respiration, an important role in the apoptotic-signaling pathway. Malfunctioning at any level of the cell is eventually translated in the release of apoptogenic factors from the mitochondrial intermembrane space resulting in the organized demise of the cell. Some of these factors, such as AIF and endonuclease G, appear to be highly conserved during evolution. Other factors, like cytochrome *c*, have gained their apoptogenic function later during evolution. In this review, we focus on the role of cytochrome *c*, AIF, endonuclease G, Smac/DIABLO, Omi/HtrA2, Acyl-CoA-binding protein, and polypyrimidine tract-binding protein in the initiation and modulation of cell death in different model organisms. These mitochondrial factors may contribute to both caspase-dependent and caspase-independent processes in apoptotic cell death.

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Programmed cell death (PCD) is a genetically encoded form of cell suicide that is central to the development and homeostasis of multicellular organisms. The basic machinery of PCD is conserved in yeast, sponge, *Hydra vulgaris*, *Caenorhabditis elegans*, *Drosophila melanogaster*, zebrafish, mice, and humans [1]. All

these organisms share the genes encoding the basic cell death machinery and the morphological and biochemical features of apoptosis [1]. Three main morphologies of PCD have been described in the context of embryonic development [2]. In brief, type I apoptotic cell death includes morphological changes such as cell shrinkage

[☆] **Abbreviations:** ACBP, acyl-CoA-binding protein; AIF, apoptosis inducing factor; AMID, AIF-homologous mitochondrion-associated inducer of cell death; ANT, adenine nucleotide translocator; Apaf-1, apoptotic protease activating factor-1; ATP, adenosine triphosphate; Bad, Bcl-2-antagonist of cell death; Bak, Bcl-2-antagonist/killer; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; BH, Bcl-2 homology domain; Bid, BH3 interacting domain death agonist; Bim, Bcl-2 interacting mediator of cell death; BIR, baculovirus IAP repeats; BLAST, basic local alignment search tool; *C. elegans*, *Caenorhabditis elegans*; CAD, caspase-activated DNA nuclease; CARD, caspase activating and recruitment domain; Ced, *C. elegans* death gene; cIAP, cellular inhibitor of apoptosis; CKI, casein kinase I; CKII, casein kinase II; Complex I, ubiquinone oxidoreductase; Complex II, succinate-ubiquinone reductase; Complex III, cytochrome *bc1*; Complex IV, cytochrome oxidase; Complex V, F₁F₀ ATPase or ATP synthase; CPS-6, Ced-3 protease suppressor; cyt *c*, cytochrome *c*; *D. melanogaster*, *Drosophila melanogaster*; Dark, *Drosophila* Apaf-1-related-killer; dATP, deoxyadenosine 5'-triphosphate; DFF, DNA fragmentation factor; DIABLO, direct IAP binding protein with low pI; DNA, deoxynucleic acid; *E. coli*, *Escherichia coli*; Egl-1, egg laying defective; ES cells, embryonic stem cells; FADH₂, flavin-adenine dinucleotide (reduced); Hsp, heat shock protein; HtrA2, high temperature requirement protein A2; IAP, inhibitor of apoptosis protein; IBM, IAP binding motif; ICAD, inhibitor of caspase-activated DNA nuclease; IMM, inner mitochondrial membrane; IMS, intermembrane space; IRES, internal ribosome entry site; kbp, kilo basepairs; kDa, kiloDalton; MPT, mitochondrial permeability transition; NADH, nicotinamide-adenine dinucleotide (reduced); OMM, outer mitochondrial membrane; PARP, poly(ADP-ribose) polymerase; PCD, programmed cell death; PTB, polypyrimidine tract-binding protein; PTP, permeability transition pore; Q, ubiquinone; RNAi, RNA interference; ROS, reactive oxygen species; Smac, second mitochondria-derived activator of caspase; tBid, truncated Bid; UV, ultraviolet; VDAC, voltage-dependent anion channel; WAH-1, worm AIF homolog.

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and extensive chromatin condensation. Type II autophagic cell death is characterized by the formation of autophagic vacuoles inside the dying cell, whereas rapid loss of plasma membrane integrity and spillage of the intracellular content distinguish type III PCD. Depending on the stimulus and the cellular context one distinct cell death program will become apparent, most probably because every cell death program is a result of self-propagating signals and signals that suppress the other cell death programs [2]. In this review we focus on apoptotic cell death with knowledge of the existence of other cell death programs.

Mitochondria and oxidative phosphorylation

A crucial step in the understanding of the molecular mechanism of apoptotic cell death was the observation by the research group of Xiadong Wang that mitochondrial factors contribute to the activation of caspases and the consecutive internucleosomal cleavage of DNA [3]. Typically, mitochondria from rat liver parenchymal cells are 0.45 μm in diameter and 4.42 μm in length, 1312 per cell, and 17% of the cellular volume [4]. The organelle is surrounded by a double membrane that divides it into two submitochondrial compartments: the intermembrane space (IMS) between the two membranes and the inner compartment called the matrix. A transmembrane channel, called the permeability transition pore or PTP, is formed at the contact sites between the inner mitochondrial (IMM) and outer mitochondrial membrane (OMM). The core components of PTP are the voltage-dependent anion channel (VDAC) in the OMM and the adenine nucleotide translocator (ANT) in

the IMM. VDAC makes the OMM permeable to most small molecules ($<5\text{ kDa}$), allowing free exchange of respiratory chain substrates [5]. Main constituent of the IMM is cardiolipin that makes the membrane impermeable to all but a few selected ions and metabolites. This is essential if the mitochondrion is to maintain an electrochemical potential required to drive mitochondrial ATP synthesis by oxidative phosphorylation, providing the cell with ATP. During normal mitochondrial respiration, five protein supercomplexes situated in the mitochondrial IMM mediate the electron transfer from NADH or FADH₂ to oxygen as final acceptor (Fig. 1). Cytochrome *c* (cyt *c*) resides in the IMS and functions as an electron shuttle between Complex III and IV. The electron transport is coupled to the generation of a proton-motive force across the IMM. This proton-motive force is a combination of the proton concentration gradient (pH) and the membrane electric potential ($\Delta\Psi_m$). The movement of protons back into the matrix, down their concentration gradient, is coupled to the synthesis of ATP from ADP and Pi. The ANT mediates the exchange of ADP for ATP. Mitochondrial ATP production is absolutely dependent on the integrity of the IMM. However, it has become apparent that mitochondria possess a latent mechanism called mitochondrial permeability transition (MPT) that, when activated, destroys this permeability barrier and disrupts normal mitochondrial function [5]. An important consequence of an activated MPT is uncoupling of oxidative phosphorylation meaning that the oxidation of the metabolites by O₂ still occurs with concomitant electron flux, but this flux is no longer coupled to proton pumping. Consequently, no transmembrane proton gradient can be maintained abolishing the production of

Fig. 1. Mitochondrial respiration. The flow of electrons through the electron transport chain is coupled to the translocation of protons from the mitochondrial matrix to the IMS. This process generates a proton gradient across the IMM, the proton-motive force. The movement of protons back into the matrix, down their concentration gradient, is coupled to the synthesis of ATP from ADP and Pi. The exchange of ADP for ATP is mediated by the ANT, which is, next to VDAC, a core component of the transmembrane channel formed at the contact sites between IMM and OMM. VDAC makes the OMM permeable to most small molecules ($<5\text{ kDa}$), allowing free exchange of respiratory chain substrates. Complex I, ubiquinone oxidoreductase; Complex II, succinate-ubiquinone reductase; Complex III, cytochrome *bc*₁; Complex IV, cytochrome oxidase; Complex V, F₁F₀ ATPase or ATP synthase; cyt *c*, cytochrome *c*; FADH₂, flavin-adenine dinucleotide (reduced); NADH, nicotinamide-adenine dinucleotide (reduced); and Q, ubiquinone.

ATP. Under these conditions, electrons can escape from the electron transport chain to form reactive oxygen species (ROS).

Release of apoptogenic factors from the intermembrane space of mitochondria

Different models have been proposed to explain how mitochondria release apoptogenic factors. One model argues that existing pores, viz. the permeability transition pore (PTP), functioning within the context of the energy metabolism are actively opened. In favor of this model, propagating a role of PTP in apoptogenic factor release, is that pro- and anti-apoptotic Bcl-2 proteins were shown to interact with the PTP complex proteins [6,7]. A direct consequence of the loss of integrity of the inner mitochondrial membrane by PTP formation is the dissipation of the transmembrane potential. Many reports have indeed reported the loss of the transmembrane potential as a primary event in the apoptotic process [7]. However, there are also numerous reports that have described the release of intermembrane space proteins such as cyt c without a detectable decrease in the transmembrane potential [8,9]. A second model by which mitochondria release apoptogenic factors is the direct pore forming properties of some pro-apoptotic Bcl-2 proteins in the outer mitochondrial membrane. The Bcl-2 family members are characterized by at least one conserved Bcl homology (BH) domain. Bax, a monomeric soluble cytosolic factor, oligomerizes, translocates, and inserts in the mitochondrial outer membrane upon induction of apoptosis [10,11]. Together with Bak, present in the OMM, Bax is thought to form tetrameric channels through which cyt c may escape [12]. BH3-only proteins such as truncated Bid induce the conformational change of Bax, needed to form pores in the mitochondrial membrane. BH3-only proteins function as sensors for cellular integrity and functionality, e.g., Bim as sensor for cytoskeleton integrity [13], Bad for growth factor withdrawal [14], and Bid as a sensor for death domain receptor signaling [15]. Following their activation, BH3-only proteins relocate to the mitochondria. Bid, originally discovered as a binding partner of Bax and Bcl-2 [15,16], can be cleaved by caspase-8 [15,17], granzyme B [15,18], cathepsin [15,19], and calpain [20] generating proteolytically activated Bid (truncated Bid or tBid). Although tBid has been reported to be involved in channel formation [17,21], genetic studies place Bax and Bak downstream of tBid as Bax and Bak double knockout cells are completely resistant to mitochondrial cyt c release during apoptosis [12]. The conformational change and/or oligomerization of Bax and tBid can be inhibited by Bcl-2 and Bcl-X_L [21,22].

Besides this function in apoptosis induction, Bid is reported to have a lipid transferase activity, reflecting its possible role in dynamic remodeling of intracellular membranes [15]. Phosphorylation of Bid by the casein kinase I (CKI) and casein kinase II (CKII) can be a mechanism by which cells control apoptosis induced by Bid activation [23]. Inhibition of CKI and CKII accelerated Fas-mediated apoptosis and Bid cleavage. Hyperactivity of the kinases resulted in a delay in apoptosis. In vitro, phosphorylated Bid was insensitive to caspase-8 cleavage. Phosphorylation also modulates the activity of other Bcl-2 family members. Phosphorylation of the proapoptotic protein BAD by PKB/AKT survival signaling leads to inactivation of Bad [14]. Bcl-2 can also be regulated via phosphorylation. Phosphorylation of serine residue 70 in the region between the BH3 and BH4 domain of Bcl-2 has been shown to be essential for full apoptotic activity [24]. On the other hand, Bax and other proapoptotic members, such as Noxa and Puma, are regulated at the transcriptional level by p53 [25,26]. After the identification of cyt c as apoptogenic factor [3], we performed a proteomic approach to identify the proteins that are released from isolated liver mitochondria treated with recombinant tBid as compared to non-treated mitochondria [27]. We identified cyt c, adenylate kinase 2, endonuclease G, Smac/DIABLO, and HtrA2/Omi (Fig. 2), which were also reported by other groups. We also identified some other proteins such as acyl-CoA-binding protein, polypyrimidine tract-binding protein (PTB), and proteins associated with fatty acid metabolism or protein transport such as fatty acid binding protein and a translocase of the inner mitochondrial membrane. In a similar approach the release of mitochondrial proteins from atractyloside-treated mitochondria has been determined [28,29]. Atractyloside is a ligand of the ANT that induces permeability transition [7]. Compared to the mitochondrial proteins identified by treatment with tBid (16) the number of released mitochondrial proteins after atractyloside exposure is much higher (almost hundred). While tBid specifically targets Bax and Bak in the OMM [12], the effect of high concentration of atractyloside (5 mM, [28]) far above the affinity for ANT (30–50 μ M) may explain the vast release of mitochondrial proteins due to the hydrophobic and steroid-like nature of the molecule [30]. In the following sessions we discuss the apoptogenic factors that are clearly from mitochondrial IMS origin and compare their role in different model organisms (Table 1).

Cytochrome c

(Holo)cytochrome c, first described in 1930 by Keilin [31], is synthesized in the cytoplasm as apocytochrome c and translocated through the OMM. During or after

Fig. 2. Many death signals converge onto mitochondria and release multiple intermembrane space proteins. Black arrows represent caspase-dependent cell death pathways; dashed arrows represent caspase-independent cell death pathways; grey arrows represent the convergence of death signals on the mitochondria and the subsequent release of intermembrane space proteins. A variety of apoptotic stimuli (death domain receptors, chemotherapeutics, DNA-damaging agents, growth-factor withdrawal, and irradiation) trigger mitochondria, which results in the release of apoptotic proteins including cytochrome *c*, AIF, endonuclease G, Smac/DIABLO, Omi/HtrA2, and ACBP. Cytochrome *c* induces caspase activation by binding to Apaf-1. Smac/DIABLO and Omi/HtrA2 can neutralize IAP inhibition of caspases. AIF and endonuclease G are involved in caspase-independent nuclear DNA degradation. ACBP can promote the activation of m-calpain, which belongs to a family of cysteine proteinases. The release of PTB from mitochondria is not clear, since it lacks a mitochondrial localization signal.

Table 1
Mitochondrial apoptogenic factors, overview of homologs, and their function in apoptosis

Function	<i>C. elegans</i>	<i>D. melanogaster</i>	<i>Mus musculus</i>	<i>Homo sapiens</i>
DNA condensation	wah-1	c	AIF	AIF
DNA degradation	cps-6	c	Endonuclease G	Endonuclease G
Apoptosome formation	b	b*	Cytochrome <i>c</i>	Cytochrome <i>c</i>
	a	a	DIABLO	Smac
IAP binding	a	c	Omi/HtrA2	Omi/HtrA2
	a	Reaper, Hid, Grim, Sickel	a	a

a, No known homolog.
b, A homolog is present but has no function in apoptosis.
c, A homolog is present but no function in apoptosis is known at this moment.

import in the IMS, heme is covalently attached to apocytochrome *c* via stereospecific thioether linkages to two cysteine residues in the protein. This complex is refolded to a more compact structure called holocytochrome *c* (referred to as cytochrome *c* or cyt *c*), which has an important role in the oxidative phosphorylation as electron shuttle between Complex III (cytochrome *c* reductase) and IV (cytochrome *c* oxidase). For more than 60 years, this was the only known function of cyt *c*, although the “cytochrome *c* effect,” i.e., translocation of cytochrome *c* to the cytosol and reduced oxidative phosphorylation after γ -irradiation of cancer cells, has already been reported in the 1950s and 1960s [32]. In the

1990s, it became clear that cyt *c* is involved in the execution of programmed cell death in more complex organisms as *Xenopus laevis*, mice, and human [1,3]. Cellular stress can induce the release of cyt *c* from the IMS, which will eventually lead to caspase activation [3]. Cyt *c* isolated from bovine, rat, pigeon, tuna heart and, to a lesser extent, the insects *Manduca sexta* and *D. melanogaster*, were able to activate DEVD-cleaving caspases (DEVDases) in *X. laevis* egg or mammalian cytosols, while yeast cyt *c* cannot [33]. In *C. elegans*, cyt *c* does not interfere with cell death. Moreover CED-4 lacks the WD-40 repeats domain, although it does contain a caspase recruitment domain, also called

CARD and a nucleotide-binding domain [34]. A role for mitochondria beyond cyt *c* release in nematode cell death is provided by Egl-1, the BH3-only Bcl-2 ortholog that targets the mitochondria and induces the release of WAH-1, the worm AIF ortholog. WAH-1 and CPS-6, the worm endonuclease G ortholog, both contribute to caspase-independent mechanisms of apoptotic cell death such as DNA condensation (see below).

In mammals, cyt *c* triggers the assembly of the apoptosome. The apoptosome is a complex composed of cyt *c*, Apaf-1, and dATP. Cyt *c* binds Apaf-1 at WD-40 repeats domain in the presence of ATP or dATP. Successive binding of cyt *c* and dATP converts Apaf-1 from a closed monomeric configuration to an open heptameric platform for procaspase-9 assembly [35]. Procaspase-9 binds Apaf-1 homotypically at a conserved amino acid sequence called the caspase recruitment domain or CARD, leading to the activation of procaspase-9 [35]. The importance of cyt *c* [36], Apaf-1 [37], and caspase-9 [37] for the execution of apoptotic cell death in mammals has been confirmed by genetic studies in mice in which the corresponding gene was disrupted. Cytochrome *c* deficient animals have a prominent overgrowth of neural structures based on a failure of developmentally mediated apoptosis, resulting in prenatal or perinatal death [36]. Cells derived from these cyt *c* deficient E8–E9 mouse embryos have reduced caspase-3 activity and behave differently in response to a variety of apoptotic stimuli. Nevertheless, despite the profound developmental delay of the cyt *c* knockout embryos, they did develop differentiated tissues derived from the three germ layers in the early gastrula stage, indicating that cyt *c* and its release, unlike AIF (see below), may not be essential for cavitation [36,38].

Apoptosome formation is not essential for hematopoietic homeostasis [39]. The absence of Apaf-1 or caspase-9 does not affect hematopoietic development and homeostasis as evidenced from *in vivo* experiments transferring fetal liver stem cells from Apaf-1 or caspase-9 null mice in a wild type host context [39]. In contrast, the same transfer experiment using cells from Bcl-2 transgenic mice specific for the hematopoietic system results in outgrowth of the progenitor cells in the wild type host context. Moreover, the donor-derived lymphocytes in these transfer experiments exhibit enhanced resistance to many apoptotic stimuli [40]. Bcl-2 may regulate caspase activation independently of the cyt *c*/Apaf-1/caspase-9 apoptosome. The cyt *c*-dependent formation of the apoptosome is not an essential trigger but may be rather an amplifier of the caspase cascade. These conclusions are also supported by the fact that in evolution cyt *c*-dependent activation of the apoptosome complex does not occur in *C. elegans* and presumably has a minor role in *Drosophila* where other ways of mitochondria-dependent caspase activation are operating (see below). The development of a modified yeast cyt

c, unable to activate the apoptotic cascade but is competent in electron transfer and antioxidant activities [41], opens the way to knockin studies to elucidate the physiological role of cytochrome *c*-mediated cell death.

The role of cyt *c* in caspase activation in *D. melanogaster* is less clear. Dark (Hac-1/Dapaf-1/Ark) is the ortholog of mammalian apoptotic protease activating factor-1 (Apaf-1) and the nematode Ced-4. In favor of a role for cytochrome *c* is the fact that Dark contains, like Apaf-1 but in contrast to Ced-4, a carboxy-terminal WD-40 repeat region, which is required though not sufficient for the binding of cytochrome *c* to Apaf-1 (see references in [37]). Binding of cytochrome *c* to Dark has been reported [42] and the addition of cytochrome *c* and dATP to S100 extracts from *Drosophila* BG2 cells enhances the spontaneous formation at 27°C of a >670 kDa complex in which DRONC, the fly caspase-9 ortholog, is recruited [43]. Whether Dark is also present in this complex is not mentioned in the publication [43]. On the other hand, there is no evidence during apoptosis in *Drosophila* cells for relocation of mitochondrial cyt *c* to the cytosol. Moreover, immunolocalization studies suggest that cyt *c* remains associated with the OMM in *Drosophila* cells undergoing apoptosis or may undergo a conformational change [43,44]. Cell fractionation and immunolocalization studies show that a significant proportion of DRONC is associated with the mitochondria [43]. Therefore, it is conceivable that in the fly an apoptosome-like complex is formed on or in the vicinity of the mitochondrial membrane. Further studies are necessary to identify both the composition and the molecular mechanism of action of this *Drosophila* apoptosome.

Apoptosis inducing factor

The mammalian mitochondrial protein AIF [45] was identified as a flavoprotein, which shares similarity with bacterial, plant, and fungal oxidoreductases [1], although *C. elegans* AIF lacks this activity. AIF has an N-terminal mitochondrial localization signal and is confined to the mitochondria in normal conditions. After import into the mitochondrial IMS, the mitochondrial localization signal is cleaved off to generate mature 57 kDa AIF. Mature AIF translocates to the nucleus in response to apoptogenic stimuli, e.g., poly(ADP-ribose) polymerase-1 (PARP) activation in response to DNA damage [46]. Overexpression of AIF induces peripheral chromatin condensation, dissipation of the mitochondrial transmembrane potential, exposure of phosphatidylserine on the plasma membrane, and high molecular weight (50 kbp) DNA fragmentation [47]. Heat-shock protein 70 (Hsp70), a cytoprotective factor, protects cells against the apoptogenic effects of AIF by binding and neutralizing AIF [48]. The exact function of AIF

under normal physiological conditions is not clear. AIF has been reported to function as a free radical scavenger acting on hydrogen peroxide [49] or to function in a redox cycling or coupling with NAD(P)H as the redox partner [50]. The molecular mechanism as to how AIF exerts its cytotoxic activity is unknown. It has no intrinsic nuclease activity and its oxidoreductase activity is not required for its apoptogenic function [51], which is in accordance with the findings in *C. elegans* where WAH-1, the AIF ortholog, lacks oxidoreductase activity [52]. Moreover, EGL-1, the BH3-only domain protein in *C. elegans*, induces the release of WAH-1 from mitochondria [52], resembling the tBid-dependent release of AIF in isolated mitochondria [27,53].

In contrast to mammals where the human AIF pathway is essentially caspase-independent [47], the nematode homolog WAH-1 requires functional CED-3 to be fully active. Worms in which the expression of the *wah-1* gene was inhibited using RNA interference (RNAi) were viable but had a slower growth rate and a smaller brood size than wild type animals [52]. Results in *C. elegans* demonstrate that WAH-1 acts in a synergistic way with CPS-6, the ortholog of endonuclease G, in DNA fragmentation. AIF appears to be involved in PCD very early in mammalian development. AIF deficient mice die at a very early stage during embryogenesis, since they lack the formation of the proamniotic cavity normally induced by death of the central core of ectodermal cells [38]. This cavitation-associated cell death is a caspase-independent process, since it occurs in the presence of the pancaspase inhibitor zVAD.fmk [38]. Embryonic stem (ES) cells from AIF deficient mice remain sensitive to most conventional apoptotic stimuli, including staurosporin, etoposide, and UV irradiation, while AIF^{-/-} ES cells are less sensitive to cell death after growth factor deprivation [38].

AMID (AIF-homologous mitochondrion-associated inducer of cell death) is an AIF-homologous flavoprotein [54]. Using immunofluorescent staining it was found that AMID, which unlike AIF lacks a mitochondrial localization signal, co-localizes with mitochondria and is also present in the cytoplasm. Presumably, AMID is associated with the OMM. Overexpression of AMID in 293T cells induced apoptotic cell death in a dose-dependent manner. Bcl-2 was not able to inhibit AMID-induced cell death. Also CrmA did not inhibit AMID-induced apoptosis, nor did the pancaspase inhibitor z-VAD, suggesting that caspases are not involved. Despite these data, the mechanism by which AMID induces apoptosis is unclear and requires further investigation.

Endonuclease G

Endonuclease G is a non-specific mitochondrial nuclease that is highly conserved in the eukaryotic kingdom.

Endonuclease G is encoded by a nuclear gene and was originally thought to be involved in the replication of the mitochondrial genome. Recently it was shown that endonuclease G resides in the mitochondrial IMS and therefore is less likely to participate in mitochondrial DNA replication [55]. Its precise role in mitochondrial nucleic acid metabolism remains unclear. During apoptosis in mammalian cells, endonuclease G is released from the mitochondria and translocates to the nucleus. Endonuclease G digests nuclear DNA in the absence of caspase activity or the caspase-activated deoxyribonuclease CAD/DFF [56,57]. Although Wang and co-workers [58] showed endonuclease G-dependent internucleosomal 'DNA-laddering' of isolated nuclei, we only observed higher order DNA degradation [57]. Probably endonuclease G acts in concert with both exonucleases and DNase I in the nucleus to generate DNA cleavage products [58]. The finding that endonuclease G is released from mitochondria upon an apoptotic assault and translocates to the nucleus sheds some light on previous findings from genetic knockout studies. DFF40/CAD is sequestered in the cytosol by its chaperone and inhibitor DFF45/ICAD [59]. During apoptosis, DFF45/ICAD is cleaved by caspase-3 allowing delocalization of DFF40/CAD to the nucleus [59]. DFF45/ICAD deficient mice or transgenic mice lacking the ICAD-caspase-3 cleavage site are phenotypically normal but display impaired DNA fragmentation in in vitro cultured thymocytes in response to DNA damaging agents [60]. Surprisingly, in vivo these mutant thymocytes underwent chromosomal DNA degradation when phagocytosed by macrophages, most likely by the lysosomal DNase II from the phagocyte [60]. By generating a CAD deficient cell line it was shown that CAD is dispensable for the early stage chromatin condensation or high molecular weight DNA fragmentation [61]. These results indicate that CAD/ICAD is not required during murine development and that internucleosomal DNA cleavage may constitute a terminal stage of nuclear dismantling during apoptosis in the absence of phagocytes.

Unlike DFF40/CAD, endonuclease G- and AIF-induced DNA degradation is essentially caspase-independent [56,62]. Genetic studies in *C. elegans* also provide evidence for a role of the endonuclease G ortholog *cps-6* in the breakdown of DNA during developmental cell death. Downregulation of *cps-6* expression using RNAi or reduction of *cps-6* activity caused by a genetic mutation impairs the extent of apoptosis associated DNA degradation and results in delayed appearance of cell corpses during development in *C. elegans* [63]. More recently, CPS-6 was shown to act synergistically with WAH-1 [52]. In contrast to the mammalian situation with AIF, WAH-1 acts in concert with CED-3. Taken together, these results support the paradigm that AIF and endonuclease G, whether or not synergistically, may be important for the execution of

PCD in situations where caspase activation is limited or compromised. This may occur during viral infection or in conditions where caspase inactivating mechanisms are induced such as NO and ROS [64]. Since both endonuclease G and AIF are evolutionary conserved they may also participate in DNA degradation associated with PCD in fungi or plants [1].

Smac/DIABLO

Formation of the apoptosome upon release of cyt c into the cytoplasm does not always suffice to spark the caspase cascade. In insects and vertebrates endogenous inhibitor of apoptosis proteins (IAPs) are present that prevent the activation of procaspases and inhibit the activity of mature caspases [65]. The family of IAP proteins is characterized by the presence of one or more baculovirus IAP repeat (BIR) domains, an approximately 70 amino acid motif conserved from yeast to human. Murine Smac and its human ortholog DIABLO are 29 kDa mitochondrial precursor proteins, proteolytically processed in mitochondria to a 23 kDa mature form, and released from the IMS after an apoptotic trigger [27,66,67]. Smac/DIABLO acts as a dimer and contributes to caspase activation by sequestering IAPs. Smac/DIABLO binds to several IAP proteins including XIAP, c-IAP1 and c-IAP2, baculoviral Op-IAP, and survivin, thwarting their caspase-inhibitory functions [66,67]. IAP binding requires the amino terminal four residues of mature Smac/DIABLO (Ala–Val–Pro–Ile) that recognize a hydrophobic groove in the BIR3 domain of XIAP [68]. A similar recognition motif is present in the linker sequence of caspase-9 small subunit (Ala–Thr–Pro–Phe). Smac/DIABLO is able to compete with caspase-9 for binding to the BIR3 domain of IAPs [68,69]. The same amino terminal sequence of Smac/DIABLO binds to the BIR2 motif of XIAP, allowing competition with the XIAP-dependent inhibition of caspase-3 and -7 [70].

The physiological mitochondrial function of Smac/DIABLO is unknown and Smac/DIABLO knockout mice are apparently normal. In addition, a splice variant of Smac/DIABLO, Smac- β , which lacks the mitochondrial targeting sequence and the IAP-binding domain, is proapoptotic [71]. Furthermore, overexpression of a truncated Smac/DIABLO mutant lacking the IAP-binding motif (IBM) induces cell death to the same extent as full-length Smac/DIABLO and Smac- β , suggesting an alternative, unknown cytotoxic mechanism of Smac/DIABLO not depending on its IBM [71]. In *Drosophila* DIAP-1 and -2 bind the proform of DRONC to block its activation and therefore play a more profound way in controlling cell death [72]. The proapoptotic proteins Hid, Grim, Reaper, and Sickle bind to and antagonize DIAP-1 and -2, thereby promoting

apoptosis [73–77]. Although Smac/DIABLO does not resemble Hid, Grim or Reaper, the four amino terminal residues of Smac/DIABLO and caspase-9 that interact with the BIR3 domain of XIAP share significant homology [69,76]. The resemblance of the IAP interacting motif in a different protein context is an example of convergent evolution in the regulation of caspase inhibitors.

Omi/HtrA2

The mammalian serine protease Omi, also known as HtrA2 [78,79], was identified as a 49 kDa serine protease, homologous to the bacterial endoprotease HtrA (high-temperature requirement). Phylogenetic analysis of the HtrA2 family supports the mitochondrial endosymbiosis theory and a mitochondrial origin of the eukaryotic HtrA-like proteases [1]. The *Escherichia coli* DegP/HtrA endoprotease is necessary for bacterial oxidative, thermo-, and osmotic tolerance. Bacterial HtrA functions as a protease at high temperature and as a chaperone at normal temperature [80]. Whether Omi/HtrA2 exerts a similar function in mammals is unclear although it does seem to be upregulated in conditions of cellular stress such as ischemia-reperfusion, heat shock, and endoplasmic reticulum stress [78,79]. The proapoptotic feature of Omi/HtrA2 was first identified through its ability to bind and antagonize IAPs [81,82]. Unlike Smac/DIABLO however, Omi/HtrA2 does not interact with survivin [81,82]. Although reported as being associated with the endoplasmic reticulum [79] or the nucleus [83], Omi/HtrA2 is localized in the mitochondria [81]. Omi/HtrA2 is synthesized as a 49 kDa precursor carrying an amino terminal mitochondrial localization signal (MLS). Upon mitochondrial transport an amino terminal presequence is cleaved off generating the mature 37 kDa protein. Overexpression of Omi in *E. coli* indicated that this maturation is dependent on its catalytic activity but whether processing during mitochondrial translocation is also due to autocatalysis is not clear [84]. Omi/HtrA2 is released from mitochondria to the cytoplasm during apoptosis where it contributes both to caspase-dependent and caspase-independent PCD [81,82,85]. In the cytoplasm, Omi/HtrA2 interacts with cytosolic IAP proteins via its IBM similar to Smac/DIABLO [81,82]. This feature of Omi/HtrA2 explains its caspase-dependent proapoptotic mode of action. Analogous to the N-terminus of the small subunit of mature caspase-9, mature Smac/DIABLO, and the *Drosophila* proteins Hid, Grim, Reaper, and Sickle, the free amino terminus of mature Omi/HtrA2 exposes the conserved IBM AVPA and AVPS, respectively, in mouse and human [86].

Using the mature Omi/HtrA2 amino acid sequence to BLAST public genomic databases, the following

organisms were found to contain putative orthologs of Omi/HtrA2: the bacteria *E. coli* (Htra, 34% identity), the plant *Arabidopsis thaliana* (At5g27660.1, 43% identity), the fruit fly *D. melanogaster* (CG8464 gene product, 51% identity), the malaria mosquito *Anopheles gambiae* (agCP13350, 55% identity), and the pufferfish *Fugu rubripes* (SINFRUP00000077310, 70% identity). Surprisingly, this approach did not reveal clear Omi/HtrA2 orthologs in *C. elegans* or *Saccharomyces cerevisiae*. Nevertheless, the presence of Omi/HtrA2 likes in the organisms mentioned above contrasts sharply with Smac/DIABLO, which apparently is confined to the vertebrates [1].

Omi/HtrA2 and Smac/DIABLO are redundant in many features: both are mitochondrial proteins, both are released from the IMS of mitochondria, and both interact with cytosolic IAPs via a similar IBM. What could be the functional difference between these two proteins? First, Smac/DIABLO and Omi/HtrA2 display a different tissue distribution pattern. Northern blot analysis showed that Smac/DIABLO is most abundant in heart, liver, kidney, and testis with little or no expression detected in skeletal muscle, lung, thymus, and the brain [66,67]. Omi/HtrA2, however, is expressed ubiquitously [78,79]. Furthermore, Omi/HtrA2 has a carboxyterminal PDZ domain [79], a motif generally involved in protein–protein interactions, and an amino terminal serine protease catalytic domain [81]. Cytoplasmic overexpression of mature Omi/HtrA2 induces cell death independent of caspase activation or IAP interaction but solely relying on the catalytic activity of Omi/HtrA2 [81,82]. The crystal structure of Omi/HtrA2 revealed a trimeric structure with the PDZ domains oriented inwards and covering the catalytic center of the protease [87]. Mutational studies demonstrated that the PDZ domain tempers the proteolytic and cytotoxic potential of Omi/HtrA2 [87]. Thus, unlike Smac/DIABLO, we exclude for a while the Smac- β splice form discussed above [71], Omi/HtrA2 seems to have a dual function: one as an inhibitor of XIAP, propagating caspase cascades, and one as a serine protease, propagating atypical caspase-independent cell death. In this respect the PDZ domain may provide a targeting signal for Omi/HtrA2 once released from the mitochondria.

Controversial and miscellaneous mitochondrial factors

Acyl-Co-A-binding protein (ACBP) and pyrimidine tract-binding protein (PTB) are two other proteins released from the IMS when isolated mitochondria were exposed to recombinant tBid [27]. ACBP is a 20 kDa homodimer that can promote the activation of m-calpain by decreasing the Ca^{2+} -concentration required for m-calpain activity [88]. The calpains constitute a family of structurally related

intracellular multidomain cysteine proteinases containing a papain-related catalytic domain, whose activity depends on calcium. Calpain activation has been documented in caspase-dependent as well as caspase-independent cell death [89]. The proteolytic activation of Bid by calpain suggests an alternative pathway can affect mitochondrial integrity in the absence of caspase activity [20].

Although we identified PTB as a protein that is released from isolated mitochondria treated with tBid, this apparent mitochondrial localization of PTB can be questioned, since it lacks a mitochondrial localization signal and has never been reported to be associated with mitochondria. PTB is an RNA binding protein required for efficient translation of some mRNAs containing internal ribosomal entry sites (IRES), for example, PTB stimulates the initiation of translation by internal ribosome entry from hepatitis C and A virus RNA in vivo [90]. It has been reported to shuttle between the nucleus and the cytoplasm [91]. Interestingly, PTB is involved in Apaf-1 IRES activation [92].

Our proteomic approach did not reveal the presence of AIF or caspases in the supernatant of tBid-treated mitochondria. However, release of AIF was confirmed by Western blotting, but neither caspase immunoreactivity nor caspase enzymatic activity could be revealed in the supernatant of tBid-treated mitochondria. The absence of caspases in mitochondria was confirmed in three independent cellular systems (anti-Fas-induced hepatotoxicity, Fas ligand induced Jurkat cells apoptosis, and apoptosis in Ba/F3 cells induced by growth factor withdrawal). Neither in the control nor in the apoptotic conditions caspases were detectable in mitochondria [53].

Conclusions

The mitochondrial apoptogenic factors can be divided into two classes: those that function independent of caspases and those that activate caspases directly or indirectly (Fig. 2). AIF and endonuclease G belong to the first group. They operate, at least in mammals, independent of caspases and have been conserved during evolution. Therefore they may be compounds of an ancestral cell death pathway. Omi/HtrA2, being involved in both caspase-dependent as caspase-independent cell death, may be a step further in evolution integrating both caspase-dependent and caspase-independent phenomena after release from mitochondria. Smac/DIABLO sharing the IAP-interacting function with Omi/HtrA2 is another caspase-dependent apoptogenic factor. Cyt c triggers a caspase-dependent phenomenon, i.e., the assembly of the apoptosome. This role of cyt c in *D. melanogaster* apoptosis is not well defined and no function has been reported for cyt c in

the execution of PCD in *C. elegans*. Presumably, the cyt c-dependent activation of caspases may have evolved later in evolution. Besides AIF, endonuclease G, Omi/HtrA2, Smac/DIABLO, and cyt c, other factors, such as acyl-CoA-binding protein and polypyrimidine tract-binding protein are released after tBid treatment of isolated mitochondria [27]. However, it remains to be determined if this release can be confirmed by cellular and in vivo data. One could conceive that the release of caspase-independent factors such as AIF, endonuclease G or Omi/HtrA2 may also operate during caspase-independent cell death processes such as necrosis. However, we could not find any evidence for the release of these factors from mitochondria in early stages of necrotic cell death, i.e., before cells have lost membrane integrity [53,93]. Therefore, although the release of mitochondrial factors that mediate caspase-independent mechanisms may represent an ancestral form of a cell death program, they seem not to participate in necrotic cell death. In conclusion, the release of mitochondrial factors during apoptotic cell death can both propagate caspase-dependent as well as caspase-independent processes, suggesting that postmitochondrial events during apoptosis are not an exclusive caspase-dependent phenomenon.

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