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Review

Mechanisms of cytochrome *c* release from mitochondria

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Abstract

In healthy cells, cytochrome *c* (Cyt *c*) is located in the mitochondrial intermembrane/intercristae spaces, where it functions as an electron shuttle in the respiratory chain and interacts with cardiolipin (CL). Several proapoptotic stimuli induce the permeabilization of the outer membrane, facilitate the communication between intermembrane and intercristae spaces and promote the mobilization of Cyt *c* from CL, allowing for Cyt *c* release. In the cytosol, Cyt *c* mediates the allosteric activation of apoptosis-protease activating factor 1, which is required for the proteolytic maturation of caspase-9 and caspase-3. Activated caspases ultimately lead to apoptotic cell dismantling. Nevertheless, cytosolic Cyt *c* has been associated also to vital cell functions (i.e. differentiation), suggesting that its release not always occurs in an all-or-nothing fashion and that mitochondrial outer membrane permeabilization may not invariably lead to cell death. This review deals with the events involved in Cyt *c* release from mitochondria, with special attention to its regulation and final consequences.

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Abbreviations: ADD70, AIF-derived decoy of HSP70; AIF, apoptosis-inducing factor; ANT, adenine nucleotide translocator; Apaf-1, apoptosis-protease activating factor 1; CL, cardiolipin; Cyt *c*, cytochrome *c*; cypD, cyclophilin D; $\Delta\Psi$m, mitochondrial transmembrane potential; ER, endoplasmic reticulum; IAP, inhibitor of apoptosis protein; IM, mitochondrial inner membrane; IMS, intermembrane space; IP3R, inositol 1,4,5-trisphosphate receptor; MOMP, mitochondrial outer membrane permeabilization; MPT, mitochondrial permeability transition; OM, mitochondrial outer membrane; PTPC, permeability transition pore complex; ROS, reactive oxygen species; tBID, truncated BID; VDAC, voltage-dependent anion channel

Introduction

Cytochrome *c* (Cyt *c*) is undoubtedly one of the most prominent actors in the apoptotic scene. Although it is usually a quiet worker on the respiratory chain, it can escape from the cell's power plant, the mitochondrion, when this organelle is damaged or when it receives instructions to break down its outer membrane. Once unleashed from its usual context, Cyt *c* can be recruited into the death squad and contribute to the apoptotic dismantling of the cell. The group directed by the late Korsmeyer has contributed seminal insights into the mechanisms through which BCL-2-like proteins regulate the mitochondrial release of Cyt *c*, and this is the topic of the present review.

Mitochondrial Cyt *c*: a Vital Protein

Cyt *c*, a peripheral protein of the mitochondrial inner membrane (IM), functions as an electron shuttle between complex III and complex IV of the respiratory chain and its activity is necessary for life. In mice, the disruption of the unique somatic Cyt *c* gene causes embryonic lethality.[1] Cyt *c* is synthesized in the cytosol as an apoprotein and, upon translocation to the mitochondria, it associates with the heme prosthetic group. Thus, functional Cyt *c*, or holocytochrome *c*, is composed of a single polypeptide chain of 104 amino acid residues covalently bound to the heme group. According to crystallographic data, Cyt *c* appears roughly as a sphere with the diameter of 3.4 nm. At physiological pH, Cyt *c* is mostly protonated meaning that most Cyt *c* binds via electrostatic bonds to acidic phospholipids, which are abundantly present in IM.[2] In mitochondria, hence the majority of Cyt *c* is membrane-bound. At least 15% of mitochondrial Cyt *c* is tightly bound via both electrostatic and hydrophobic interactions. The remaining Cyt *c* is loosely attached to IM, as a result of weak electrostatic interactions, and can be readily mobilized. Loosely and tightly bound Cyt *c* pools have been implicated in different functions. The former participates in electron transport, inhibits reactive oxygen species (ROS) formation and prevents oxidative stress. The latter is probably bound to cardiolipin (CL), an unusual lipid largely confined to the IM. Indeed, CL appears to be necessary for the insertion of Cyt *c* into mitochondrial membranes. CL-bound Cyt *c*, probably does not participate in electron shuttling of the respiratory chain, but may account for the peroxidase activity recently attributed Cyt *c*.[2]

Cytosolic Cyt *c*: a Lethal Protein

The mitochondrial intermembrane space (IMS) contains a heterogeneous class of proteins whose release promotes cell death. The first molecule belonging to this class of proapoptotic proteins that has been identified at the molecular level is Cyt *c*.[3] Upon apoptotic stimuli, Cyt *c* is released into the cytosol where, in the presence of ATP (and more efficiently in the presence of deoxyATP, dATP), it mediates the allosteric activation and hepta-oligomerization of the adaptor molecule apoptosis-protease activating factor 1 (Apaf-1), generating the complex known as apoptosome. Each apoptosome can recruit seven dimers of caspase-9 and favor their activation leading to proteolytic self-processing.[4] These events are tightly regulated by several heat shock proteins (HSPs)[5,6] (Figure 1) and finally allow for the catalytic maturation of caspase-3 and other caspases that eventually mediate the biochemical and morphological features of apoptosis. Additional soluble proteins released from mitochondria upon apoptosis induction facilitate the caspase cascade by neutralizing caspase-inhibitory proteins known as IAPs (inhibitor of apoptosis proteins). One example is provided by the second mitochondria-derived activator of caspases/direct inhibitor of apoptosis-binding protein with a low isoelectric point (Smac/DIABLO), whose N-terminus is exposed upon proteolytic maturation when the precursor polypeptide is imported in the mitochondria. This N-terminus can interact with and neutralize IAPs when the protein is released into the cytosol.[7] Another example is provided by the high temperature requirement protein A2 (HtrA2, also known as Omi), a serine protease that, similarly to Smac/DIABLO, contains an IAP-binding N-terminus.[8]

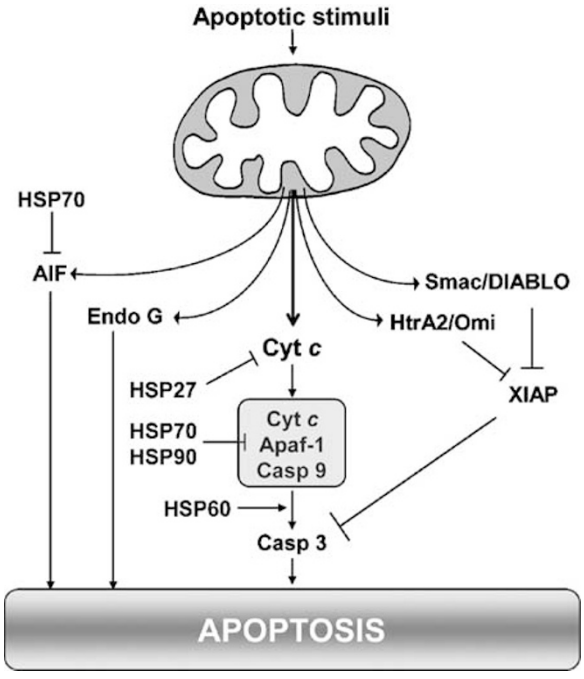


Figure 1 Mitochondrial release of proapoptotic molecules. Soluble molecules from the IMS include Cyt *c* which, once in the cytosol, is able to interact with Apaf-1 to generate the apoptosome, a molecular platform for caspase-9 activation. The catalytic maturation of caspase-9 activates the caspase cascade and, ultimately, favors the acquisition of the apoptotic morphology of cell death. These events are controlled by several HSPs at different levels along the apoptotic cascade, and by the X-linked inhibitor of apoptosis protein (XIAP), that negatively regulates caspase-3 and -9. Smac/DIABLO and HtrA2/Omi neutralize XIAP. AIF and Endo G translocate to the nucleus where they trigger DNA fragmentation and chromatin condensation in a caspase-independent fashion. Casp, caspase

Depending on the cell type, moreover, mitochondria contain procaspases in the intermembrane space. The proteolytic activation of such procaspases occurs upon release into the cytosol and may be facilitated by the simultaneous liberation of HSPs[9] and requires the denitrosylation of the catalytic cystein residue. Although activated caspases are important mediators of cell death and essential for the acquisition of several facets of the apoptotic morphology, their inhibition protects cells only transiently against cell death. It is believed that once mitochondria are irreversibly permeabilized, cell death proceeds regardless of caspase activity, although it can be delayed if caspases fail to come into action. This caspase-independent cell death may result from the loss of essential mitochondrial functions and/or from the apoptogenic function of additional molecules translocating from the IMS to the cytosol: the flavoprotein apoptosis-inducing factor (AIF) and endonuclease G (Endo G). Once in the cytosol, both proteins are able to translocate to the nucleus where they promote DNA fragmentation and apoptotic cell death in a caspase independent fashion[10] (Figure 1).

The two functions of Cyt *c* (caspase activation and electron transport) reside in distinct domains of the protein. The substitution of the iron atom within the heme prosthetic group abolishes Cyt *c*-mediated electron transfer, yet does not affect its potential to mediate caspase activation. This implies that the redox and the proapoptotic activity of Cyt *c* obey to distinct biochemical principles.[2] Accordingly, Cyt *c* from *Saccharomyces cerevisiae* is able to substitute for its mammalian counterpart in the respiratory chain, but does not induce apoptosome formation in mammalian cells. Site-directed mutagenesis studies have confirmed the crucial role of lysine 72 in the proapoptotic, but not respiratory, activity of Cyt *c*.[11] Hao *et al*.[12] have recently generated knocking mice expressing a mutant Cyt *c* able of sustaining mitochondrial respiration but lacking Apaf-1 activation potential. The abnormalities exhibited by these animals were comparable to those described in mice deficient for caspase-9 or Apaf-1. Bone marrow from mutant mice transplanted into wild-type recipients gave rise to a pronounced B- and T-cell hyperplasia, splenomegaly and lymphoadenopathy. Taken altogether, these observations suggest the existence of an apoptotic pathway efficiently activated in lymphocytes from wild type, but not mutant, animals. A recent study in which a truncated form of Apaf-1 (lacking its WD-40 domain) was used, demonstrates that its nucleotide-binding site is essential for caspase activation.[13] ATP/dATP-binding and hydrolysis catalyze the conformational changes that are required to expose the caspase recruitment domain (CARD) and for the apoptosome assembly. WD-40 repeats are although to be responsible for Cyt *c* binding and to have regulatory roles in Apaf-1 function. Accordingly, the full-length Apaf-1 efficiently binds to caspase-9 only in the presence of both Cyt *c* and ATP/dATP. It remains to be demonstrated whether ATP/dATP are able to gain access to their binding site on Apaf-1

independently of Cyt *c* and whether, as a consequence, a small amount of Apaf-1 is constitutively activated. This occurs in *Drosophila*, where the constitutively activated Apaf-1 is kept under control by an IAP (DIAP-1).[14]

It is a matter of controversy whether all proteins from IMS are released at the same time or with different kinetics. In some models, Cyt *c* mobilization has been reported as an early event, preceding the release of other IMS proteins.[15] It has been recently shown that release of AIF or Endo G from the mitochondria may require caspase-independent events following mitochondrial outer membrane permeabilization (MOMP) such as AIF processing in the IMS[16] or calpain-mediated cleavage.[17] However, in other models AIF is released before Cyt *c*.[18] In the presence of an heat-labile cytosolic factor, AIF can promote MOMP, as demonstrated *in vitro* by the release of *Cyt c* from purified mitochondria induced by the combination of AIF and cytosolic fractions, but not by the recombinant protein alone or when the cytosolic extracts were previously heat inactivated.[19] Thus, once in the cytosol AIF is able to sustain the release of Cyt *c*.[20,21] This may result from the fact that Cyt *c* is tightly associated with IM, meaning that in some cases in which AIF is released via MOMP, Cyt *c* is still tethered to IM. This scenario has also been invoked to explain that in some cases MOMP results in the release of Smac/DIABLO before that of Cyt *c*.[17]

Nonapoptotic Functions of Mitochondrial Cyt *c* Release

The activation of caspases via a mitochondrion-dependent pathway, has been implied in nonapoptotic differentiation programs, that affect nucleated as well as enucleated cells.[22–24] Thus, in some instances, activated caspases lead to the cleavage of proteins of the nuclear envelope (such as lamin B) without inducing cell death.[22]

Mitochondrial Cyt *c* release occurs during the fragmentation of the mature megakaryocyte, the process required for the formation of platelets. Platelets are enucleated bodies arising from the development of thin, long, cytoplasmic extensions of the megakaryocyte called proplatelets. In differentiation megakaryocytes, caspase-3 activation occurs in a spatially restricted fashion, within the perinuclear area of the cell. This caspase-3 activation correlated with the release of Cyt *c* from local mitochondria and can be prevented by BCL-2 overexpression. BCL-2 overexpression or caspase inhibition inhibits the generation of proplatelets and mature thrombocytes.[25]

Several caspases, including caspase-9 and -3, are activated in peripheral blood monocytes undergoing differentiation towards the macrophagic lineage, when cultured in the presence of macrophage colony stimulating factor (M-CSF). On the contrary, no active caspases are detectable when monocytes differentiate into dendritic cells. Again, caspase activation accompanying the monocyte-macrophage differentiation involves the mitochondrial release of Cyt *c* and results in the cleavage of specific proteins, one of which is acinus, a protein that may be involved in apoptotic chromatin condensation. In contrast, other well-characterized caspase targets, such as poly(ADP-ribose) polymerase (PARP), remain uncleaved. In this model, overexpression of BCL-2 and inhibition of caspases by z-VAD-fmk or by the baculoviral inhibitory protein p35 block cellular differentiation.[26]

Cyt *c* has also been implicated in *Drosophila* sperm cell differentiation. The *cyt-c-d* gene (which is expressed at lower levels than the alternative *Drosophila* Cyt *c* gene, *cyt-c-p*) does not seem to have an essential role in mitochondrial respiration but is crucial for caspase-dependent spermatide differentiation. *Cyt-c-d* mutant males are viable but sterile. An IAP, *Drosophila* Bruce (dBruce, homolog of human Bruce/Apollon), protects differentiating fly spermatids from the destruction by caspases activated during the process.[27]

Cyt *c*-induced caspase activation may have a function also in B-cell proliferation. The activation of caspase-3 has been described during B-cell cycle progression. Mice deficient in caspase-3 have an increased number of splenic B cells that exhibit normal apoptosis but enhanced proliferation, both *in vivo* and after mitogenic stimulation *in vitro*.[28]

Thus, Cyt *c* release induces caspase activation, which in turn may promote either cell death or vital processes like differentiation and proliferation. These opposite outcomes may derive from different subsets of substrates, which are cleaved by the caspases in different situations. Various mechanisms could account for such a selective cleavage, including post-translational modifications of caspases and/or of their substrates, subcellular compartmentalization of the caspases, protection of substrates by scaffold molecules, activation of antiapoptotic factors and recruitment of antagonistic proteins at the level of caspase activation platforms (for a review see Garrido and Kroemer[23] and Launay *et al*.[24]).

What are the mechanisms underlying Cyt *c* release that is not followed by cell death? MOMP is mandatory for apoptotic Cyt *c* release and it has been considered as the 'point of no-return' in the cell death process. One possibility is that a low amount of Cyt *c* can exit the mitochondria without an important and irreversible MOMP. According to this model, in response to a partial Cyt *c* release, a limited activation of caspases leads to the cleavage of only a subset of the substrates.[29] Thus, Cyt *c* might be released through a selective mechanism without major permeabilization of the mitochondrial outer membrane (OM), and without the release of other proapoptotic factors. On the other hand, some reports suggest that MOMP does not necessary result in immediate cell death. For example, lymphoid cells from mice expressing a Cyt *c* mutant unable to bind Apaf-1 undergo MOMP independently from apoptosis.[12] Whether such post-MOMP cells demonstrate an increased long-term survival as compared to normal cells, however, has not been determined.

Mitochondria and BCL-2 Family Proteins

Mitochondria are vital intracellular organelles whose functions encompass, but are not limited to, ATP production, apoptosis regulation and biosynthesis of several metabolites. They are also the main cellular source and immediate target of ROS. Mitochondrial size, shape and number vary dramatically according to cell type and physiological, environmental or pathological conditions. Under normal circumstances, mitochondrial dynamics is dictated by the equilibrium between fusion and fission (or division). Both processes are subjected to a complex regulation involving multiple proteins that,

among the other roles, maintain the integrity of mitochondrial compartments (membranes, IMS and matrix).

BCL-2 family proteins regulate MOMP.[30–33] They can be divided into three main groups on the basis of their BCL-2-homology (BH) domains and the resulting functions. The antiapoptotic BCL-2 members, such as BCL-2 itself, BCL-X_L or MCL-1, contain four BH domains (BH1–BH4); the proapoptotic multidomain members (like BAX, BAK or BOK) possess three BH domains; finally, the BH3-only proapoptotic proteins, such as BAD, BID or BIM, share homology only within the BH3 domain.[33] These molecules are able to associate via their BH domains to form homo- and/or hetero-complexes and play distinct roles in governing MOMP.

BAX and BAK are essential for MOMP. Double-deficient cells are resistant to Cyt *c* release and apoptosis.[32] BH3-only proteins function upstream of BAX and BAK because their heterologous expression do not restore Cyt *c* release and apoptosis in $BAK^{-/-}$-$BAX^{-/-}$ cells.[34] On the other hand, overexpression of BCL-2 or BCL-X_L is able to block MOMP induced by ectopically expressed BH3-only proteins and mediated by BAX and/or BAK.[35] In other words, different BCL-2 family members are crucial in determining the ultimate mitochondrial response to diverse proapoptotic stimuli.

BID is commonly considered as the most important link between the extrinsic and intrinsic apoptotic pathways. Upon the transduction of extracellular proapoptotic signals through death receptors, BID undergoes proteolytic activation mediated by caspase-8.[36] The C-terminal fragment of BID (tBID, for truncated BID) then translocates to the mitochondria and promotes the release of Cyt *c*. Moreover, the native, uncleaved form of BID has been reported to insert into mitochondrial membranes and function as a lipid transferase between mitochondria and other intracellular membranes.[37] BID can activate the BAX/BAK channel, as described above. In addition, Bid may trigger activation of the PTPC leading to MOMP[38,39] and/or to cristae remodeling[40] and maximal Cyt *c* release. As a matter of fact, three-dimensional reconstructions of mitochondrial membranes suggest that the intercristae space, which contains most of Cyt *c*,[41] is separated from the intermembrane space by openings whose size impedes the passage of proteins like Cyt *c*, under normal circumstances. During cristae remodeling, a process entailing a profound structural reorganization of the IM, such openings would increase in size and lose their diffusional barrier function, thereby allowing the access of Cyt *c* to the intermembrane space.[40]

During apoptosis, OM is affected by caspase-independent changes which enhance BID binding and 'prime' the OM for the leakage of proapoptotic factors.[42] One such priming event may be the translocation of CL from IM to OM, within the contact sites between the membranes. tBID preferentially associates with liposomes containing CL levels comparable to those found in mitochondrial membranes. Moreover, the absolute requirement of CL for tBID binding has been recently suggested by studies involving CL-deficient yeast strains.[43] A plethora of apoptotic stimuli induce the degradation of CL presumably through the activation of lipases and/or oxidative mechanisms. For instance, in FasL-induced apoptosis a CL loss reportedly is an early event. Recently, the association between the antiapoptotic BCL-2 family member MCL-1 and tBID has been reported to regulate tBID-induced mitochondrial apoptosis. While depletion of MCL-1 by RNA interference (RNAi) sensitizes Hela cells to death receptor-triggered tBID-mediated apoptosis, overexpression of MCL-1 confers apoptosis resistance.[44]

Altogether, the available evidence strongly underscores the notion that BCL-2 proteins play a major role in the control of MOMP.

Molecular Mechanisms of MOMP

Lethal signals (or the absence of vital signals), originated from the extracellular environment and/or from intracellular compartments such as the nucleus, lysosomes, endoplasmic reticulum (ER) or autophagic vacuoles, induce MOMP. MOMP generally occurs in a concerted and rapid fashion affecting most, if not all, mitochondria within the cell and leads to the release of IMS proteins including Cyt *c*. As previously mentioned, MOMP is a decisive event in the process of Cyt *c* release and it has been proposed as a 'point of no return' of the mitochondrial apoptotic pathway.[45] So far, the mechanisms of MOMP have not been fully elucidated. Several alternatives, nonexclusive models explaining how MOMP is induced have been proposed (Figure 2).

It has been suggested that MOMP might depend on the activation of the molecular machinery involved in mitochondrial fission.[46] Before Cyt *c* release, mitochondrial membranes undergo morphological changes that closely resemble the events associated with fission. In addition, the proapoptotic protein BAX has been shown to interact with endophilin 1, one of main regulators of mitochondrial division.[47] Thus, BAX might stimulate mitochondrial fission, which in turn would favor MOMP.[46] In yeast, a link between the fission machinery and apoptosis has been demonstrated.[48] The same link, however, has not been totally validated in mammalian cells.[46]

An alternative model proposes MOMP to be initiated at the level of the IM, which would undergo a sudden increase in permeability to solutes of low molecular weight. Many signals, like increased cytosolic Ca^{2+} or ROS, promote this phenomenon. As a result, osmotic forces drive water into the matrix, which undergoes swelling and causes distension of the IM and eventually rupture of the OM. This process has been dubbed mitochondrial permeability transition (MPT).[49,50] The permeability transition pore complex (PTPC) is a large high-conductance multiprotein complex constituted of several components. The exact molecular composition of the PTPC has not yet been determined. However, it is currently believed that the major PTPC components are the voltage-dependent anion channel (VDAC), an OM protein, the adenine nucleotide translocator (ANT), resident in the IM, and cyclophilin D (CypD), resident in the matrix.[50] PTPC may include several supplementary proteins, whose interactions with the main constituents vary during apoptosis.[51] It is possible that some of the PTPC components are dispensable for its function. Accordingly, it has been reported that ANT is not always required for the induction of MPT and consequent apoptosis.[52] Moreover, mitochondria from CypD-deficient mice are relatively resistant to the induction of MPT by agents such as Ca^{2+} and ROS, yet are normally permeabilized by BAX and

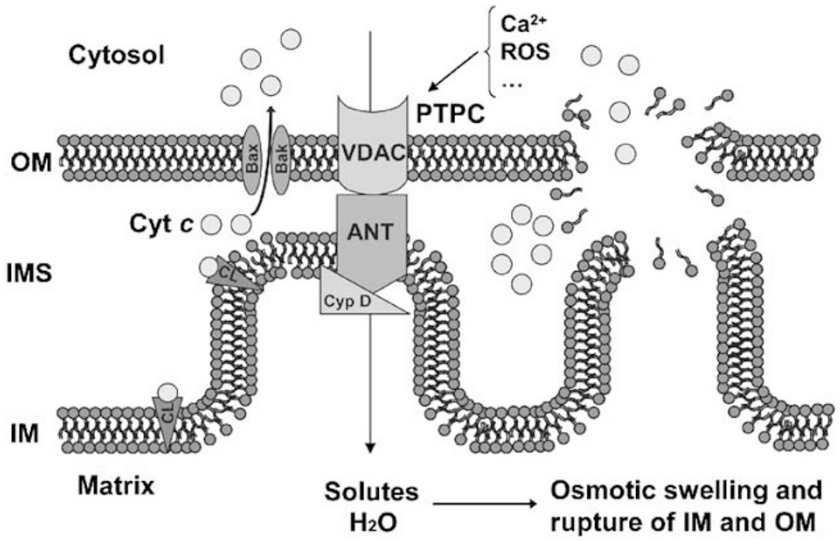


Figure 2 Two nonexclusive models for MOMP. According to one model, multidomain proapoptotic members of the BCL-2 family, such as BAX and BAK, act to create pore-like structures in the OM without affecting directly the IM and/or the matrix. The assembly of these pores results in MOMP and consequent release of the IMS proapoptotic proteins, including Cyt *c*, AIF and Endo G. An alternative model (MPT) suggests that the opening of the PTPC allows small solutes and water to enter the mitochondrial matrix. The resulting osmotic swelling eventually leads to the rupture of both membranes and MOMP. For further details please refer to the main text

BID.[53,54] Thus, ANT and CypD may be superfluous for the induction of MOMP and apoptosis, in some settings. In contrast to apoptosis, some pathways leading to necrosis are blocked in CypD-deficient cells, highlighting a role for CypD in the mitochondrial necrotic pathway.[54]

According to yet another model, MOMP is initiated by multidomain proapoptotic members of the BCL-2 family. These proteins (such as BAX or BAK) would create protein-permeable 'pores' in the OM, through which soluble IMS proteins are free to translocate to the cytosol, without directly affecting the function of the IM and/or of the matrix. In support of this model, BCL-2, BCL-X_L and BAX share structural similarities with the pore-forming domain of bacterial toxins.[55] According to a simple model of MOMP, BH3-only proteins, activated by distinct apoptotic signals, would induce an allosteric conformational change in BAX/BAK, which triggers their homo-oligomerization into large multimeric pores in the OM. BH3-only proteins are typically activated either by post-translational modifications (i.e., caspase-8 mediated cleavage of BID into the active truncated form tBID) or by transcriptional upregulation.[56] In the BAX/BAK 'rheostat' model, the balance between the intracellular levels of BAX/BAK and those of antiapoptotic BCL-2 family members determines the ultimate fate of the cells. The levels and/or activity of the antiapoptotic BCL-2 like proteins are controlled by specific interactions with the BH3-only proteins. An obvious specificity of interaction has been demonstrated: only some BH3-only proteins are able to associate with any given subset of antiapoptotic BCL-2 family members.

Mitochondrial apoptosis is significantly inhibited when BAX and BAK activation is prevented. For instance, $BAK^{-/-}$-$BAX^{-/-}$ cells survive for a long-time starvation conditions which efficiently induce apoptosis in their normal counterparts. In this model, metabolic needs are met by the activation of the autophagic program.[57] Inactive BAX exists mainly as a cytosolic monomer. Its activation results in a conformational change allowing its insertion into the OM, and is required for MOMP induction. Presumably, the same applies to BAK, although the latter resides in the OM also in its inactive state.[58] It has been proposed that BAK is maintained in a monomeric inactive conformation thanks to interactions with VDAC2, a scarcely expressed VDAC isoform.[59] BIM and BID are able to activate BAX in a direct fashion.[60] Other BH3-only proteins (including BAD, Noxa or PUMA) do not interact directly with BAX, but may play a role in its activation by binding to and neutralizing antiapoptotic BCL-2 proteins. BCL-2 like proteins exert their antiapoptotic function by sequestering BAX and BAK into inactive dimers, thus impeding their oligomerization. Recently, it has been reported that heat can directly activate BAX and BAK, in a process under the regulation by both the antiapoptotic and BH3-only members of the BCL-2 family.[61] In summary, the activation of BAX and BAK and the blockade of antiapoptotic BCL-2 proteins are pivotal steps in the control of the mitochondrial pathway of apoptosis.

BAX oligomers on their own, or tBID and BAX together, are able to form openings in reconstituted liposomes allowing the passage of large (2 MDa) dextran molecules.[60] The formation of these pores requires the presence of CL. As previously mentioned, CL is prominently found in the IM, within cristae, but it has been suggested to reside also at the contact sites between OM and IM.[62] The exact submitochondrial localization of CL, however, has not yet been accurately determined. Thus, it remains unclear whether CL is located also in the OM and whether it is truly accessible by cytosolic proteins from its IM localization, through the contact sites. Interestingly, also tBID and BCL-2 have been shown to cluster at the same contact points.[63] Moreover, the PTPC spans the OM and the

IM in close proximity to these sites, suggesting that the two proposed mechanisms for MOMP may be connected and work in a coordinate manner.[64] Accordingly, BAX and BAK may cooperate with the PTPC to form a channel in the OM whose activity or assembly is inhibited by BCL-2.[49] In an alternative or concomitant scenario, BCL-2 family members may also play a role in reorganizing the cristae of mitochondria in a process that involves the action of PTPC components such as CypD.[40,63]

There has been a long-standing debate whether MOMP is mainly mediated by the PTPC and/or the pore-forming function of BAX/BAK. Based on knockout experiments (that affect CypD as an essential PTPC component or BAX and BAK), as well as on pharmacological experiments, it appears likely that both models are correct for distinct subsets of cell death pathways. Whether MOMP results from PTPC opening, rather than from BAX/BAK activation (or both), depends on the cellular model, on the apoptotic stimulus and the experimental conditions.[40,49] Moreover, it is formally possible that both mechanisms of MOMP may cooperate to some extent in a simultaneous or sequential fashion as will be discussed in the following section.

Biphasic Release of Cyt *c*

Cumulative data suggest that Cyt *c* release does not always take place in an all-or-nothing manner as previously believed, but rather follows a biphasic kinetics. According to this model, a first wave of Cyt *c* release would affect only a small part of the Cyt *c* pool, most likely the soluble and loosely bound fractions, which would escape the IMS as a result of a partial MOMP. A second, larger pool of Cyt *c* would only be released upon cristae remodeling (see above). This model of biphasic Cyt *c* release has been first proposed by Scorrano *et al.*,[40] from the group of the late Korsmeyer. Alternatively, or in addition, one fraction of Cyt *c* may be relatively soluble while a second, quantitatively more relevant portion may be sequestered by interactions with CL, as discussed above.[3,65]

The first pool of Cyt *c* that is released from mitochondria may boost several different amplification loops, which allow for the complete release of Cyt *c*, as discussed in the following paragraphs (Figure 3). It remains an ongoing conundrum, which among these putative amplification loops decisively contributes to cell death in which particular paradigm of physiological or pathological cell demise. As a matter of speculation, the absence or inhibition of such amplification loops, might explain, at least partially, the nonapoptotic functions of cytosolic Cyt *c*.

Amplification Loop 1: ROS and CL Oxidation

Experimental evidence indicates that ROS play a crucial role in provoking lethal cell injury. Mitochondria are a major source of ROS, mainly at the level of complexes I and III of the respiratory chain. Given that ROS are highly reactive and short-lived molecules, it can be safely assumed that their effect is maximal in the immediately vicinity of their site of generation. CL is particularly rich in unsaturated fatty acids and therefore represents a suitable target for ROS reactivity. CL is the only phospholipid in mitochondrial membranes that undergoes early oxidation during apoptosis. This results from the CL-specific peroxidase activity of CL-bound Cyt *c*.[66]

Some apoptotic signals directed to mitochondria and provoking MOMP and Cyt *c* release, are able to disrupt the electron transport, thus leading to an increased generation of ROS, which, in turn, readily induce extensive CL peroxidation in the IM. Oxidized CL has a reduced affinity for Cyt *c*, which hence dissociates from the IM and enriches in the IMS.[65] In addition, CL oxidation may favor the assembly and function of the PTPC.[2,67] Taken altogether, these observations explain the rapid and massive Cyt *c* release induced by superoxide anion. Indeed, CL is the preferential docking partner for tBID translocating to mitochondria upon apoptosis.[43] CL-bound Cyt *c* is able to catalyze the peroxidation of the phospholipid, thus transducing and amplifying different proapoptotic signals.[66] CL deficiency leads to alterations in the stability and permeability of mitochondrial membranes, to decreased respiratory rates and to the complete functional impairment of the organelle[68] (Figure 3a).

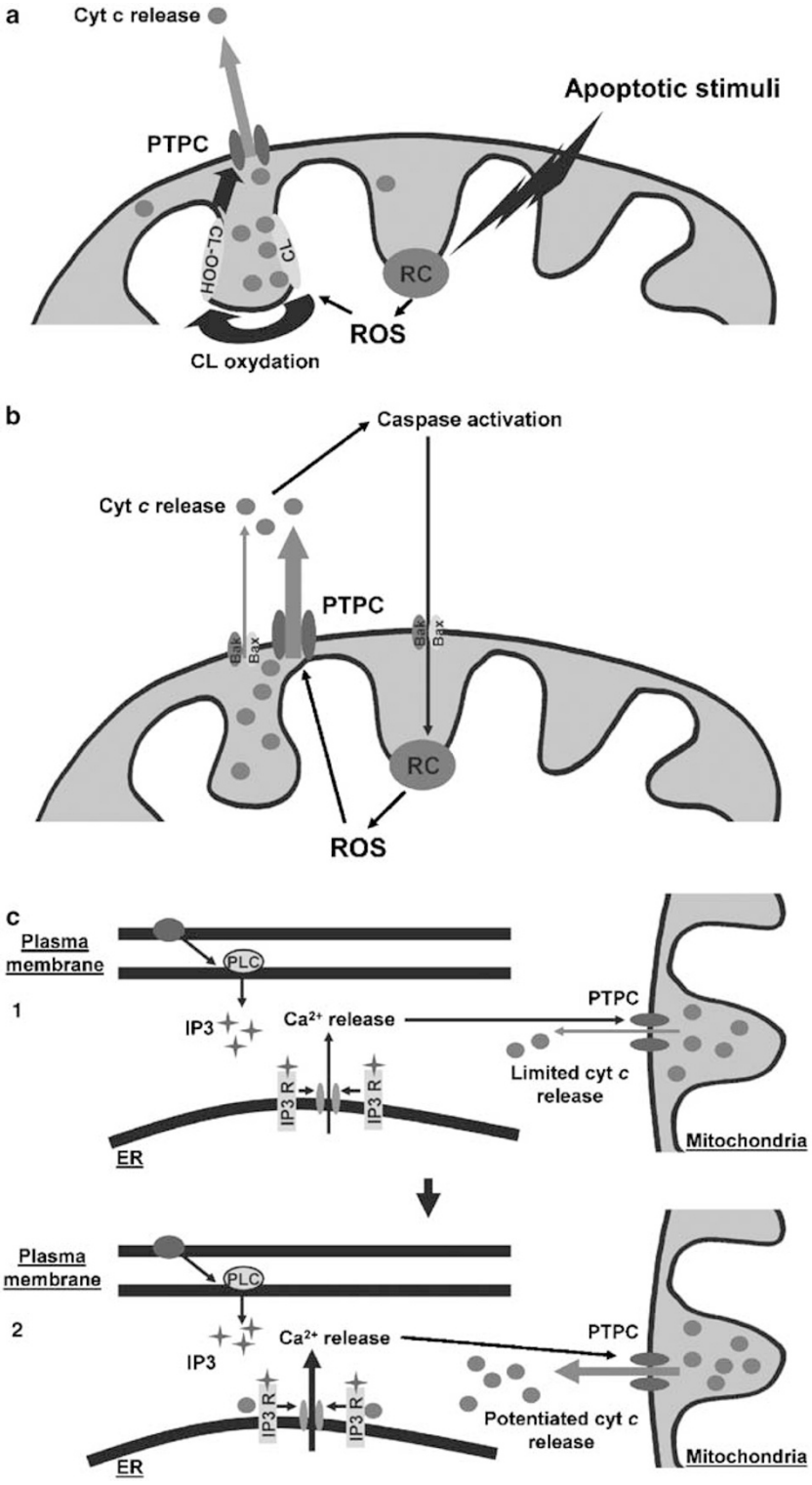
Amplification Loop 2: Caspase Activation

Although it is well established that Cyt *c* release results in caspase activation, it is less clear whether caspases are necessary for its release, via putative regulatory loops. In the extrinsic apoptotic pathway mediated by death receptors, the initiator caspases, such as caspase-8, induce MOMP and Cyt *c* release by the proteolytic activation of BID.[36] In the case of intrinsic apoptotic signals, for instance due to genotoxic stress, several studies indicate that Cyt *c* is released also in the presence of caspase inhibitors.[69] However, it should be noted that the caspase inhibitors most frequently employed (such as Z-VAD-fmk) are not efficient against caspase-2. Moreover, it has been shown that caspase-2 knockdown by small interfering RNAs (siRNAs) blocks Cyt *c* release from mitochondria upon genotoxic stress.[70] In addition, it has been

Figure 3 Amplification loops underlying biphasic Cyt *c* release. (**a**) Feed forward loop involving ROS production and CL oxidation. A limited release of Cyt *c* results in enhanced ROS generation at the level of complexes I and III of the respiratory chain. ROS, in turn, induce CL peroxidation, thus decreasing its affinity for Cyt *c*, which is released into the IMS. ROS have also a direct effect on the PTPC, whose opening favors MOMP and/or cristae remodeling and the release of Cyt *c* into the cytosol. RC, respiratory chain. (**b**) Loop involving caspase activation. Caspase-3 that is activated by the initially released Cyt *c*, enters the mitochondria through the OM partially permeabilized by BAX/BAK and cleaves a 75-kDa component of the respiratory complex I. This provokes the disruption of the respiratory chain and intense production of ROS, which in turn promote CL oxidation and PTPC opening. (**c**) Loop involving Ca^{2+} stores of the ER. Upper Panel: the binding of neurotransmitters or hormones to transmembrane receptors activate PLC, which catalyzes the cleavage of membrane phospholipids to produce IP3. IP3 then acts on specific receptors (IP3R) at the ER surface, where it modulates Ca^{2+} stores mobilization. Ca^{2+} induces PTPC opening and Cyt *c* release. Lower panel: cytosolic Cyt *c* is able to interact with the IP3R at the ER membranes and to block a negative feedback system in which Ca^{2+} limits its own release. Low amounts of Cyt *c* are sufficient to inactivate this regulatory system, and result in unrestrained Ca^{2+} mobilization. As a consequence, released Ca^{2+} promotes protracted Cyt *c* release

reported that caspase-2-induced Cyt *c* release in isolated mitochondria, but that its enzymatic activity was dispensable for MOMP.[71] Nevertheless, since caspase-2 knockdown does not lead to significant defects in apoptosis during mouse development[72] and given the results of recent works performed with efficient caspase-2 inhibitors,[73] it can be concluded that Cyt *c* release occurs also in the absence of caspase activity.

It has been demonstrated that caspases cause mitochondrial respiratory dysfunction, when a limited MOMP is induced by BAX/BAK.[74] Upon access to the IMS, indeed, caspase-3 is able to cleave the 75-kDa subunit of the respiratory complex I,



thus provoking the impairment of the electron flow from complex I to complex II, loss of the mitochondrial transmembrane potential ($\Delta\Psi m$) and increased ROS generation. All these events, but not Cyt *c* release, are prevented by a caspase-resistant p75 mutant. These results suggest that there may be an initial caspase-independent Cyt *c* release not followed by the total disruption of respiration, which therefore would ensure the supply of ATP needed for Cyt *c*-induced caspase activation in the cytosol. Activated caspases could subsequently enter the mitochondria through the partially permeabilized OM and induce the complete block of the respiratory chain, eventually resulting in cell death. Such a model would explain the intense ROS generation, lipid peroxidation, mitochondrial swelling and morphological alterations usually associated with Cyt *c* release and ensuing apoptosis[58,74] (Figure 3b).

Amplification Loop 3: Cyt *c* and Calcium Mobilization from the ER

Molecules such as neurotransmitters or hormones act at the cell surface by binding to receptors which are able to transduce the received signal across the membrane and activate intracellular pathways including phospholipases. One activated, phospholipase C cleaves membrane phospholipids and generates diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3), two of the main second messengers in the cell. Specific receptors for IP3 (inositol 1,4,5-trisphosphate receptor (IP3R)) are found on the ER membranes, where they regulate the mobilization of Ca^{2+} stores.[75] Ca^{2+} released from the ER may activate the PTPC, ultimately inducing Cyt *c* release. Recent studies indicate an intimate link between Cyt *c* and IP3-elicited Ca^{2+} mobilization.[76] As a matter of fact, Cyt *c* released at early stages of apoptosis has been shown to bind to IP3R at the ER, whose membranes are in close proximity to mitochondria. Nanomolar concentrations of Cyt *c* are able to interrupt a negative feedback loop in which Ca^{2+} released from the ER inhibits the ability of IP3 to sustain further Ca^{2+} release. As a result of the binding of Cyt *c* to IP3R, hence, the ER Ca^{2+} release becomes unrestrained and increasing cytosolic Ca^{2+} then can stimulate mitochondrial Cyt *c* release. By means of such a feed-forward system the proapoptotic wave may spread throughout the cell, leading to the cell-wide coordinated release of Cyt *c*.[77] A cell permeable peptide derived from the IP3R domain involved in the interaction with Cyt *c* acts as a competitive inhibitor of the Cyt *c*-IP3R interaction and prevents apoptosis in various cell lines.[76] BCL-2 family proteins, such as BAX, BAK and BCL-2 itself, localize also to the ER membranes and affect Ca^{2+} release as well as its subsequent uptake by mitochondria, two important steps in the regulation of PTPC opening and Cyt *c* release. Furthermore, BCL-2 has been shown to interact with IP3R and to modulate its phosphorylation state.[78] These data support an important role for IP3R in the regulation of apoptosis (Figure 3c).

HSP70: a Decisive Regulator of Cyt *c* Release and Mitochondrial Apoptosis

Mice expressing a Cyt *c* mutant able of supporting mitochondrial respiration but lacking Apaf-1 activation potential, exhibit some defects in the apoptotic process, yet no dramatic phenotypical alterations.[12] This underscores that although Cyt *c*-dependent caspase activation is important in the mitochondrial apoptotic pathway, the critical events determining cell death lie upstream of Cyt *c* release, and most likely at the level of MOMP. Therefore, the modulation of endogenous MOMP regulators appears as a promising approach in anticancer therapy, in order to sensitize cancer cells to conventional chemotherapy and circumvent resistance. Excluding members of the BCL-2 family, which have been extensively proposed as targets for chemosensitization, HSP70 appears an interesting target for cancer chemotherapy.

HSP70 is an evolutionarily conserved protein whose expression is induced by an panoply of stimuli, including anticancer agents. HSP70 blocks the apoptotic pathway at different levels. At the premitochondrial level it inhibits p53,[79] stress-activated kinases[80] and lysosomal membrane permeabilization.[81] At the mitochondrial level, it prevents MOMP by blocking BAX translocation.[82] This HSP70 function depends on both the chaperone and ATP hydrolytic domains.[83] Finally, at the postmitochondrial stage, it inhibits AIF[84] and Apaf-1[6] (Figure 4). In tumors, HSP70 overexpression occurs frequently and is associated with a negative prognostic impact, especially in breast and renal cancer.[85] Transgene-enforced overexpression of HSP70 suffices to induce lymphomas in mice,[86] and antisense constructs directed against HSP70 have been shown to increase sensitivity to apoptosis and to eradicate tumors in several experimental paradigms.[87] In addition, the subcellular localization of HSP70 differs between tumor cells and their normal counterparts. In the former, an enrichment of HSP70 in the lysosomal membranes as well as its expression on the cell surface have been demonstrated.[88]

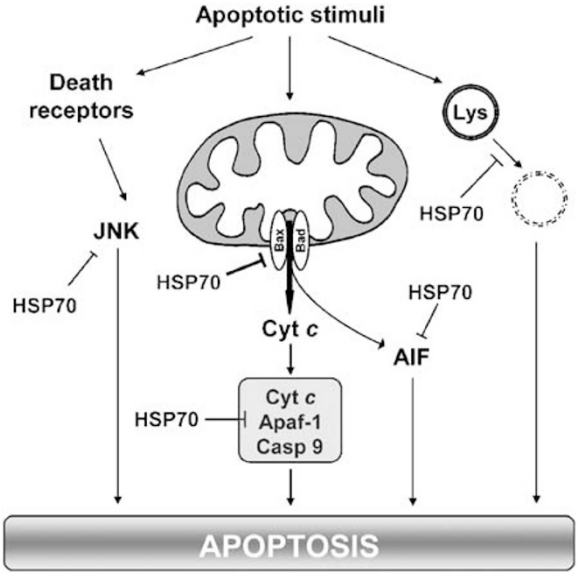


Figure 4 HSP70 modulation of apoptosis. HSP70 controls the apoptotic pathway at different levels: at a premitochondrial stage by inhibiting stress-induced signalling (for instance mediated by JNK kinases) or by stabilizing lysosomal membranes; at the mitochondrial level, by preventing MOMP although the blockade of BAX translocation; finally, at the postmitochondrial stage by interacting with AIF and Apaf-1. Casp, caspase; Lys, lysosome

For all these reasons, HSP70 is a promising therapeutic target, but, in contrast to HSP90 (which is specifically inhibited by the geldanamycin derivative 17-AAG), so far no specific small-molecule HSP70 inhibitors have been described. We have constructed an AIF-derived decoy of HSP70 (ADD70), which selectively inhibits HSP70 and prevents its interaction with AIF and other molecules. ADD70 is an effective chemosensitizing agent in multiple human tumor cell lines. This effect depends specifically on the interaction between ADD70 and HSP70, since it is lost in HSP70-deficient cells.[89] ADD70 has recently been validated for its chemosensitizing effect *in vivo*, in mice xenografted with human tumors expressing active ADD70 or inactive ADD70 mutants.[90] Moreover, we have generated a panel of high-affinity aptamers (8–13 amino acids), interacting with HSP70 *in vitro*, which wait for evaluation of their capacity to inhibit HSP70 in living cells. Such short polypeptides will allow us to determine the exact HSP70 domain that should be targeted by small-molecule inhibitors to achieve an optimal chemosensitization. Future will tell whether this approach intended to favor MOMP in malignant cells is useful for anticancer therapy.

Conclusions

A vast panel of distinct proapoptotic stimuli converge on mitochondria to induce MOMP and Cyt *c* release. In many instances, this seems to occur in a biphasic fashion that develops according to the following hypothetical scenario. At a first level, an incomplete MOMP is induced and only a small fraction of Cyt *c* exits to the cytosol. Thereafter, a total, irreversible MOMP may take place, leading to sustained Cyt *c* release and eventually apoptosis. The first wave may occur independently from caspase activity and does not significantly affect the mitochondrial respiration. The initial release of Cyt *c* induces the activation of caspases that, in turn, may be implicated in the second wave of MOMP. Moreover, during the first stages a slight increase in ROS results in the peroxidation of CL. Oxidized CL, together with the structural alterations of the mitochondrial cristae in which most Cyt *c* is sequestered, allows for the complete release of Cyt *c* from mitochondria. In this setting, Ca^{2+} released from the ER promotes a cell-wide coordinated release of Cyt *c*. At the same time, mitochondrial functions are progressively lost. Although cell fate may be decided at earlier stages, these last events are likely to represent the mechanisms that account for rapid apoptotic cell death. New strategies designed to pharmacologically manipulate MOMP in order to control cell death, for instance via the pharmacological modulation of BCL-2 proteins, PTPC components, HSP70 or IP3R, may open new perspectives for the therapeutic correction of deficient or excessive cell death.

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