The Modulation of Inter-Organelle Cross-Talk to Control Apoptosis

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Abstract: Mitochondria fulfill a wide array of functions dedicated to the energetic metabolism as well as the control of cell death. These functions imply that mitochondria can be activated by a variety of signals and can integrate them to trigger a process called mitochondrial membrane permeabilization (MMP), which induces the ultimate events of apoptosis. MMP consists in a sudden increase in the permeability of mitochondrial membrane that results in the release of critical proapoptotic intermembrane space effectors into the cytosol such as cytochrome *c*, apoptosis-inducing factor (AIF), Smac/Diablo, Endo G, and pro-caspases. In many models of apoptosis, mitochondrial translocation of proteins and/or lipids concomitantly with alterations of the intracellular milieu has been shown to activate MMP. This applies to tumor suppressors of the Bax/Bcl-2 family (Bax, Bad, Bid, Bim), several protein kinases (Akt, ASK1, hexokinase), p53, NF-κB, and nuclear orphan receptors such as TR3/Nur77. After mitochondrial membrane association, these proteins target constitutive mitochondrial proteins including the permeability transition pore complex (PTPC), Bcl-X_L, HSP70, and/or the lipid interphase. Subsequently, they switch their vital function into a lethal function to promote membrane permeabilization and protein release. In this review, we will describe some general rules of inter-organelle cross-talk activating MMP and will review selected examples of pro-apoptotic protein translocation. Finally, we will propose new pharmacological strategies to modulate this process in a therapeutic perspective.

Key Words: Cell death, endoplasmic reticulum, mitochondria, nucleus.

I. INTER-ORGANELLE CROSS-TALK: MUTUAL COMPARTMENT INFLUENCE

Apoptosis is a genetically controlled cell death program, composed of several essential phases, that represent critical check-points, as well as non-essential phases dependent on the cell type, the physio-pathological context or the pro-apoptotic stimuli. Apoptosis can be elicited *via* two main pathways: the cell death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway (Fig. **1A**). On the one hand, the death receptor pathway of apoptosis is initiated through the ligation of death receptor (CD95, Fas, APO-1) and the association of the death-inducing signaling complex (DISC), followed by activation of caspase-8, thus downstream effector caspases, like caspase-3, leading to DNA fragmentation (Fig. **1A**). On the other hand, the mitochondrial pathway is induced *via* growth factor deprivation, DNA damage or chemotherapeutic drugs and results in the release of apoptogenic factors in the cytosol, accompanied or followed by the loss of transmembrane potential ($\Delta\Psi m$). Subsequently, cytochrome *c* oligomerizes with APAF-1 and ATP. The resulting complex, termed apoptosome, facilitates the recruitment and activation of caspase-9, triggering effector caspases activation, such as caspase-3 or caspase-7 (Fig. **2**).

Although these two pathways can be interconnected, another level of complexity can be distinguished. Thus, the induction of apoptosis can be elicited from various organelles as reviewed by Ferri and Kroemer, [1], suggesting the existence of a permanent cross-talk between intracellular organelles to modulate the cell fate (Fig. **1B**).

How might an organelle elicit apoptosis? Irrespective to the specific functions of each organelle, they are surrounded by a membrane that defines the sub-cellular compartmentation, and ensures the permeability barrier necessary to intracellular homeostasis. Thus, inter-organelle cross-talk relies on (**i**) the release of soluble factors (proteins, peptides, ions…) through the various membranes. For instance, the release of proteins has been reported to occur from mitochondria (e.g. cytochrome *c*, apoptosis inducing factor (AIF), Endo G) as well as from endoplasmic reticulum (ER) (e.g. c-Abl), from the nucleus (e.g. TR3/Nur77) as well as from lysosomes (e.g. cathepsin), (**ii**) the transport of the released factor to an heterologous organelle *via* a close apposition of membranes, the involvement of cytoskeleton and/or association with cargo proteins, and (**iii**) the import of the factor into an heterologous organelle, its activation and its association with a resident protein to acquire a novel lethal function. The resident protein acts as a receptor/anchor and for example might be a member of the mitochondrial permeability transition pore complex (PTPC), such as the voltage-dependent anion channel (VDAC) or the adenine nucleotide translocase (ANT), an heat shock protein (e.g. HSP70), a member of the Bcl-2 family, as well as the inositol 1, 4, 5 triphosphate receptor (IP3R). Thus, in many models of apoptosis, protein translocation triggers the mitochondrial membrane permeabilization (MMP) process that ultimately leads to the cell death.

Defect in inter-organelle cross-talk may have important physio-pathological and therapeutic implications, as shown

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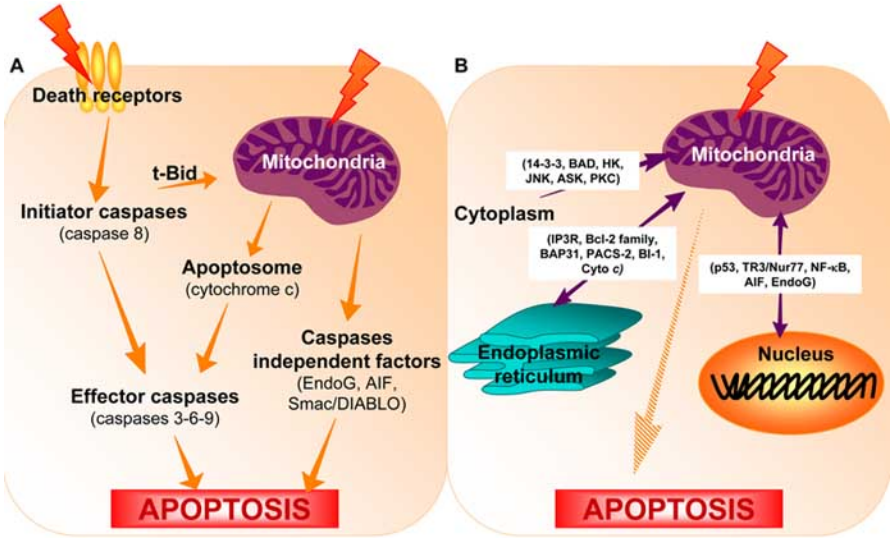

Fig. (1). Main pathways and cellular proteins involved in apoptosis regulation. A: Exogenous and endogenous signals can be integrated respectively by membrane death receptors and mitochondria to activate downstream regulators and then induce apoptotic cell death. **B**: Organelle cross-talk can regulate apoptotic mitochondrial activation *via* protein translocation. The selected interconnections between cytoplasm, endoplasmic reticulum, nucleus and mitochondria are discussed in this review.

by cells that have been genetically inactivated for key inducers of the mitochondrial pathway of apoptosis and that failed to dye in response to chemotherapeutic agents targeting the intrinsic death pathway [2].

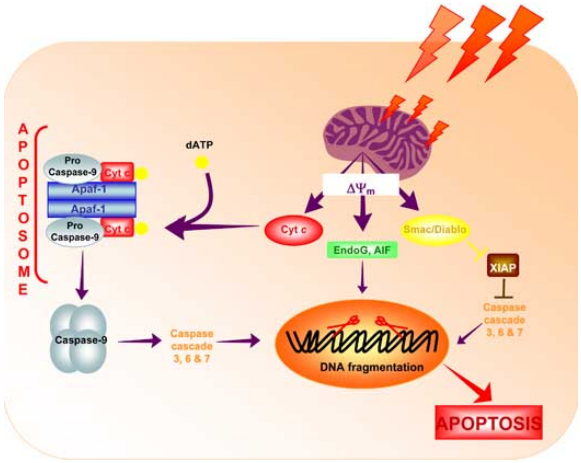

Fig. (2). Apoptogenic factors release and cell death regulation. Stress signals transduced at mitochondrial sites can induce pro-apoptotic molecules release. Then, apoptosis can be induced by apoptosome complex formation (composed of cytochrome *c*, Apaf-1, procaspase-9 and ATP), nuclear translocation of EndoG and AIF proteins, or, XIAP inhibition through Smac/Diablo mitochondrial release.

1. Cross-Talk Between Cytosol and Mitochondria

A large number of interconnections exist between cytosol and mitochondria, evolving under the influence of numerous pro- and anti-apoptotic factors. Among these events, phosphorylation and dephosphorylation of intracellular molecules are the most common and important regulatory mechanisms in signal transduction. Moreover, this post-traductional mechanism controls a variety of cellular events ranging from cell growth to apoptosis.

A. Akt-Dependent Cross-Talk

One of the key players of the process of phosphorylation and dephosphorylation is the protein Akt, a downstream component of the ras-PI3 kinase pathway [2, 3]. This cytoplasmic serine threonine kinase can transduce signals that lead to cell proliferation or inhibition of programmed cell death by activating transcription factors, by deregulating the cell cycle control or by inhibiting pro-apoptotic molecules related to intrinsic cell death machinery.

14-3-3 / Bad Pathway

The first component of the apoptotic machinery found to be phosphorylated by Akt was the Bcl-2 family member, Bad. The pro-apoptotic protein Bad interferes with the anti-apoptotic function of Bcl-2 and Bcl-X_L in the mitochondria unless it is bound to 14-3-3 in the cytosol of cells [4, 5]. 14-3-3 binds to murine Bad that it is phosphorylated on serine 112 or 136 by anti-apoptotic kinases. Phosphorylation of serine 136 by Akt is associated with the translocation of Bad from the mitochondrial membrane to the cytosol [6]. Mutation of serine 112 and 136 of Bad leads to an increased pro-apoptotic activity and an enhanced binding to Bcl-2 and Bcl-X_L that is mediated by a BH3 domain [4, 5]. Interestingly, the BH3 domain of Bad is located closely to serine 136 at amino acids 143–170 [7] and mutation within this domain prevents BAD phosphorylation [8]. Therefore, 14-3-3 interferes with the ability of Bad to bind to Bcl-2 and Bcl-X_L because it obscures the BH3 domain of Bad.

Moreover, regulation of this inhibitory interaction has been identified, with the cleavage of 14-3-3 protein on Asp238 residue by caspase-3, facilitating Bad interaction with Bcl-X_L and promotion of cell death [9].

Hexokinases

Hexokinase activity has been implicated in glucose transport and regulation of ATP synthesis. In cancer cells, increased level of cytosolic glucose is correlated with higher hexokinase activity, leading to aerobic glycolysis [10]. More recently, hexokinases have been identified playing a role in the control of mitochondrial phase of apoptosis [12, 13]. Some hexokinase isozymes (HKI and HKII) present a porin-binding domain and are able to bind to the mitochondrial PTPC member, VDAC, inhibiting its apoptotic function [14, 15]. This mitochondrial translocation is finely modulated by the anti-apoptotic serine/threonine kinase Akt that is required to maintain hexokinase association with mitochondria [15, 16]. This suggests that, in cancer cells, elevated levels of mitochondria-bound isoforms of hexokinase can result in apoptotic evasion, allowing the cells to proliferate.

B. ASK/MAPK Kinases -Dependent Cross-Talk

Among the numerous proteins able to translocate to the mitochondria, some protein kinases from the ASK/MAPK cascade have been identified to play a critical role.

c-Jun N-Terminal Kinases, JNK

The c-Jun N-terminal kinases (JNKs), a subfamily of the mitogen-activated protein (MAP) kinases, are considered as essential signalling molecules for neurodegeneration in the mammalian brain [17].

Recently, increasing evidence indicated the involvement of JNKs at different functional levels leading to mitochondrial dysfunctions with subsequent initiation of apoptosis. First, JNKs associate with the mitochondria, following cellular stress [18-21]. Second, JNKs inactivate anti-apoptotic and activate pro-apoptotic proteins of the Bcl-2 family such as Bcl-2, Bcl-X_L, Bad, Bim, or Drp5 [21-28]. Third, JNKs release inhibitors of anti-apoptotic proteins, such as Smac/Diablo from the mitochondria [29]. Finally, JNKs trigger the expression of the pro-apoptotic protein, Bim in the nucleus [22, 30]. It is worth noting that JNK apoptotic functions can depend on the protein isoform considered. Thus, Eminel *et al.* showed that differences in activation and translocation of JNK isoforms, suggesting the existence of separate apoptotic JNK pathways [31].

Apoptosis Signal-Regulating Kinase 1, ASK1

Apoptosis signal-regulating kinase 1 (ASK1) is a member of the mitogen-activated protein MAP3 kinase family. ASK1 protein can mediate response to pro-inflammatory stimuli, reactive oxygen species (ROS), and cellular stress leading to activation of MAP2K-JNK/p38 cascade in a mitochondria-dependent pathway [32]. Several cellular inhibitors including thioredoxin-1 (Trx1) have been shown to bind to and inhibit ASK1 apoptotic activity in the cytosol of resting cells [33]. ASK1 can also be localized in mitochondria where it binds to thioredoxin 2 (Trx2), and, recently, Zhang *et al.* showed that mitochondria-specific expression of a constitutively active ASK1 strongly induces apoptosis without JNK activation, Bid cleavage, and Bax mitochondrial translocation [34]. Thus, the sub-cellular location of ASK1 (cytoplasm *vs.* mitochondria) signals for two distinct apoptotic pathways.

Protein Kinases C

The protein kinase C (PKC) family of serine-threonine kinase is activated by diverse stimuli and participates in cellular process such as cell growth, differentiation and apoptosis. While most of PKC isozymes are associated with cell survival, novel PKCs subfamilies are involved in cell death [35].

In response to different stimuli, PKCδ is activated, leading to apoptosis induction [35-38]. Activity of PKCδ is controlled by phosphorylation [39], proteolytic cleavage by caspases [40-42], as well as by translocation from the cytosol to sub-cellular compartments [38, 43] and notably to mitochondria [44-46]. This translocation can induce large modifications in mitochondrial functions, such as decrease in mitochondrial membrane potential and release of cytochrome *c* [44, 45]. PKCδ can also induce apoptosis, *via* phosphorylation of cellular targets. For example, the post-translational modifications of the p53 homolog, p73β, lead to p73β transactivation and apoptotic functions [47]. More recently, Murriel and colleagues showed that PKCδ mediates the accumulation and dephosphorylation of the pro-apoptotic BAD, cytochrome *c* release and cell death [48].

Another member of PKC family, PKCε can regulate apoptosis through modulation of Bax/Bcl2 family members' proteins levels. PKCε activation was found to induce the phosphorylation of BAD indirectly, which renders BAD unable to participate to apoptosis [49]. PKCε activity may also regulate the expression of anti-apoptotic protein Bcl-2 [50]. More recently, PKCε has been identified to translocate to mitochondria, to interact with and to inhibit the PTPC, *via* direct or indirect phosphorylation of VDAC [51].

C. Non-Kinase-Dependent Pathways

Caspases

From the discovery of the first mammalian caspase ICE/caspase-1 sixteen years ago [52], twelve mammalian caspases are presently known, numbered in the chronological order of their identification. Caspases are synthesized as single-chain inactive zymogens, which become activated after proteolytic cleavage [53]. Based on their apoptotic functions, the caspase family has been divided into two groups. The first group represents initiator caspases (e.g. caspases-2, -8, -9, -10 and probably –11) able to activate the second group of effector caspases (e.g. caspases-3, -6, -7). Caspase activation in mammals is mediated by two main routes: as indicated previously, the extrinsic pathway induced by TNF cell membrane receptor, *via* the formation of DISC complex and activation of caspase-8; and the intrinsic pathway induced by mitochondrial sensors, *via* the recruitment of the apoptosome complex (for review, see

[54]). Effector caspases can directly degrade multiple substrates including the structural and regulatory proteins in the cell nucleus, cytoplasm and cytoskeleton. In some cases, initiator caspases can also function as effector caspases, to amplify the cell death signal. Furthermore, the activation of effector caspases can not only be due to initiator caspases but also by other, non-caspase proteases, including cathepsins, calpains and granzymes (for review, see [55]).

As described above, caspases can play a role in apoptosis induction upstream and downstream the mitochondria. For example, caspase-2, which is unable to initiate the processing of pro-caspases on its own, is activated early in apoptotic response and can stimulate the efflux of cytochrome *c* from mitochondria, by increasing activity of the pro-apoptotic protein Bid [56]. Moreover, effector caspases can be activated after mitochondria signal integration, *via* other mitochondrial apoptogenic factors release, such as Smac/DIABLO or Omi/Htra2. These proteins play their role through downregulation of caspases specific inhibitors (e.g. XIAP, c-IAPs), allowing the proteases to target their cellular substrates [57-60]. The mitochondrion contains also pro-caspases like activities. Indeed, pro-caspases-2 and -9 are released into the cytosol upon induction of mitochondrial transition permeability and apoptosis after the rupture of the outer membrane [61].This redistribution can be inhibited by Bcl-2 [61].

Cytochrome c

The cytoplasmic efflux of cytochrome *c* is one of the key events in the activation of mitochondria-dependant apoptotic pathway (see Fig. **2**). After outer membrane permeabilization, released cytochrome *c* promotes the activation of effector pro-caspase-9, facilitated by Apaf-1 in presence of dATP or ATP. Altogether, these molecules generate a supramolecular complex with a molecular weight between 700 and 1400 kDa, referred to the apoptosome. Upon formation of this complex, caspase-9 triggers the processing and activation of the downstream caspases-3 and -7 that culminates in apoptotic cell death [62]. Using green fluorescent protein (GFP)-tagged cytochrome *c*, Green *et al.* found that the release of cytochrome-*c*-GFP always precedes exposure of phosphatidylserine and the loss of plasma-membrane integrity. Once initiated, the process continued until all of the protein is released from all mitochondria in individual cells, within about 5 min [63].

2. Cross-Talk Between the Nucleus and the Mitochondria

A. Transcription Factors

p53

The product of the p53 tumor suppressor gene is involved in multiple responses to various and numerous cellular stimuli, such as genotoxic stress, oncogene activation or hypoxia. Constitutively located in the cytoplasm where its level is maintained low *via* MDM2-mediated proteasome degradation, p53 transcription factor translocates to nucleus after cellular damage, leading to cell cycle arrest or apoptosis **(**Fig. **3)**. p53 implication in apoptotic response may depend on its transactivation abilities. Among p53-target genes involved in transcription-dependant apoptosis, Killer/DR5, CD95 (Fas/Apo-1) and PERP encoded membrane proteins induced in response to DNA-damage [64-66]. Some other p53 target genes encode cytoplasmic proteins, such as PIG or PIDD [67, 68] or mitochondrial proteins such as Bax, Noxa, PUMA, Apaf-1 and p53 AIP1 [69-71]. Bax, NOXA and PUMA proteins belong to the Bcl-2 family and their functional role and interaction will be developed in this review below. p53-dependent apoptosis can also rely on its transcription-independent functions through its ability to translocate to mitochondria in a rapid first wave of response to a cellular stress [72].

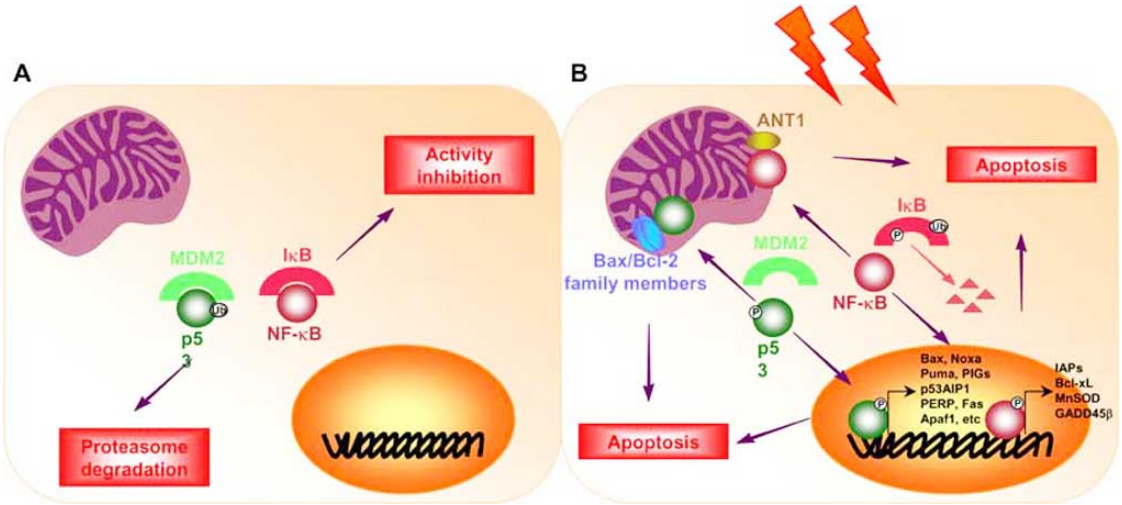

Fig. (3). Release of hijacked proteins. **A:** Prior to apoptotic stimuli being applied, the NF−κB: IκB and p53: MDM2 complexes are cytosolic and remained low through proteasome degradation. **B:** After appropriate stimulation, post-translational modifications of p53 or IκB disrupt inhibitory interactions, allowing p53 and NF-κB released to the nucleus, where they transactivate/repress their specific target genes. Apoptosis can also be induced by mitochondrial translocation of these proteins, where p53 has been shown to interact with Bax or Bak and NF-κB to interact with the inner membrane protein ANT1.

TR3/Nur77

TR3/Nur77 is an orphan transcription factor from the steroid/thyroid receptor superfamily with unknown ligand. TR3/Nur77 was originally recognized for its role in cell proliferation and differentiation. Paradoxically, Nur77 was later found to be a potent pro-apoptotic molecule [73, 74]. The expression of Nur77 was rapidly induced during the apoptosis of immature thymocytes and T-cell hybridomas, as well as various types of cancer cells. Overexpression of a dominant-negative Nur77 protein or inhibition of Nur77 expression by anti-sense Nur77 inhibited apoptosis, whereas constitutive expression of Nur77 resulted in massive apoptosis [75-78].

Recent studies have demonstrated that Nur77 can also act outside the nucleus to mediate several important biological functions, including apoptosis and differentiation. Under normal conditions, TR3/Nur77 is located in the nucleus exerting its transactivation functions. Under several apoptosis-inducing conditions, TR3/Nur77 is overexpressed and translocates from nucleus to the cytoplasm where it targets mitochondria, triggering cytochrome *c* release and apoptosis [75]. Furthermore, Nur77 targets mitochondria through its interaction with Bcl-2, resulting in conversion of Bcl-2 from an anti-apoptotic to a pro-apoptotic molecule [81]. Nur77 targets mitochondria in prostate cancer [75, 82], lung cancer [83, 84], colon cancer [82]), ovarian cancer and gastric cancer cells [85], and its mitochondrial localization might be involved in Sindbis virus-induced apoptosis [86]. Thus, the diverse biological activities of Nur77 depend strongly on its sub-cellular localization.

NF-κB

NF-κB is one of the first nuclear encoded transcription factors described to be localized to the mitochondria. The NF-κB family (RelA/p65, c-Rel, RelB, p50 and p52) form heterodimers that act as transcription factors and upregulate anti-apoptotic genes. The inactive form of NF-κB is retained into the cytosol, sequestered by members of the IκB family (Fig. **3**). Malignant transformation, viral infection or cytokine signaling have been associated with the phosphorylation of IκB and degradation *via* the ubiquitin proteasome pathway of the inhibitor. NF-κB can then translocate to the nucleus and activate transcription of various genes including IAPs [87, 88], anti-apoptotic genes Bcl-X_L [89, 90], Mn-superoxide dismutase (MnSOD) [91] and GADD45β [92, 93]. Protein interaction between NF-κB and mitochondria will be developed in details as a potential therapeutic target.

B. Apoptogenic Factors

During apoptosis, the mitochondrion releases AIF and endonuclease G (EndoG). Subsequently, both proteins translocate to the nucleus and are implicated in apoptotic nuclear changes that occur in a caspase-independent manner.

Apoptosis Inducing Factor, AIF

In normal cells, AIF is strictly confined to the mitochondrial inter-membrane space, and thus colocalizes with heat shock protein 60 (Hsp60). Upon induction of apoptosis by several agents, AIF translocates to the nucleus, where it can play its pro-apoptotic role, inducing chromatin condensation and DNA fragmentation. The mitochondrio-nuclear redistribution of AIF is prevented by a Bcl-2 protein specifically targeted to mitochondrial membranes [94, 95]. This translocation involves a cytoplasmic transition finely controlled by HSP70 proteins whose endogenous levels are sufficient to inhibit and prevent AIF nuclear translocation, retaining the apoptotic factor into cytosol [96].

Endo G

A hallmark of apoptosis is the fragmentation of nuclear DNA, resulting from the activation of nucleases in cells undergoing cell death. One such nuclease is endonuclease G (EndoG), a mitochondrion-specific nuclease that translocates to the nucleus during apoptosis. Once released from mitochondria, EndoG cleaves chromatin DNA into nucleosomal fragments [97]. Activation of EndoG is still controversial: some authors demonstrated that the nuclease is able to play its role in response to truncated Bid, independently of the caspases [97, 98], whereas others showed the requirement of caspase activation [99]. These results suggest a hierarchical ordering of the effectors involved in cell death induction, supported by phylogenetic studies showing a high conservation through evolution [100]. EndoG-null mice phenotype is also controversial and several groups have published different phenotypes suggesting, on one hand, implication of EndoG in early embryogenesis, and on the other hand, no obvious anatomical or histological consequences [101, 102].

3. Cross-talk Between Endoplasmic Reticulum and Mitochondria

Mounting evidence suggests that calcium (Ca^{2+}) released from internal stores such as the endoplasmic reticulum (ER) plays a critical role in the progression of apoptosis. Indeed, in several models of apoptosis, a release of calcium from ER to the mitochondria leading to the activation of the apoptotic intrinsic pathway has been reported. Protein involved in this cross-talk might control the apposition of ER to mitochondria and the flux of calcium from ER to mitochondria (Table **1**).

A. IP3R

The primary calcium release channel on ER membranes is the inositol 1, 4, 5-triphosphate receptor (IP3R) **(**Fig. **4**). Deletion of the gene for IP3R results in defects in apoptosis in response to multiple stimuli. Conversely, augmented IP3R levels are associated with increased cell death.

IP3R-deficient cells are resistant to T-cell receptor (TCR)-induced apoptosis due to lack of Ca^{2+} release from ER and calcineurin activation [103, 104]. Caspase-9 and -3 are not activated in IP3R1-deficient cells after TCR stimulation, consistent with the resistance of these cells to apoptosis. Bcl-2 expression in IP3R-deficient cells is similar to control cell, excluding the possibility of an inhibition of apoptosis by Bcl-2. Taken together, these results strongly suggest that IP3R-mediated Ca^{2+} release plays a critical role in regulating the activity of caspases-3 and -9 independent of Bcl-2 [105].

Table 1. Proteins Involved in the Interconnections Between RE and Mitochondria

Protein	Localization	Role of in apoptosis	Mechanism	References
IP3R	ER membrane	Pro-apoptotic	Release of calcium from the ER upon apoptotic stimuli	[103-105]
Bcl-2	ER, nucleus and mitochondria membrane	Anti-apoptotic	ER Bcl-2 binds to IP3R and induces permeable, hyperphosphorylated state of IP3R. Mitochondrial Bcl-2 stabilizes mitochondrial membrane.	[108]
Bax and Bax	ER, nucleus and mitochondria membrane	Pro-apoptotic	ER Bax inhibits between IP3R -Bcl-2 interaction. Mitochondrial Bax permeabilizes mitochondrial membrane.	[108, 111]
p20 (cleavage product of BAP 31)	ER membrane	Pro-apoptotic	Release of calcium from the ER	[112-114]
PACS-2	Colocalizes with ER	Pro-apoptotic	Control apposition of mitochondria and ER membrane.	[115]
BI-1	ER membrane	Anti-apoptotic	Inhibition of Bax activation and translocation to mitochondria Reduction of the calcium content of the ER	[116, 117]

B and T lymphocytes undergoing apoptosis in response to anti-immunoglobulin M antibodies and dexamethasone, respectively, were found to have increased amounts of IP3R, at the levels of mRNA and protein. Expression of IP3R3 antisense constructs in S49 T cells blocked dexamethasone-induced apoptosis. Thus, increase in IP3R3 levels have been causally related to apoptosis [106]. B cells in which a single type of IP3R has been deleted still mobilize calcium in response to B-cell receptor (BCR) stimulation, whereas this Ca^{2+} mobilization is abrogated in B cells lacking all three types of IP3R. Ca^{2+} mobilization by a transfected G protein-coupled receptor (muscarinic M1 receptor) was also abolished in only triple-deficient cells. Capacitative Ca^{2+} entry, stimulated by the pharmacological inhibitor of SERCA pump, thapsigargin, remains unaffected by loss of all three types of IP3R. These data established that IP3R-induced Ca^{2+} fluxes are mediators for both BCR- and muscarinic receptor-induced apoptosis [107].

B. Bcl-2 Family

Proapoptotic Bcl-2 family members Bax and Bak are required for the initiation of mitochondrial dysfunction during apoptosis, but also for maintaining the ER Ca^{2+} stores necessary for Ca^{2+} -dependent cell death. Thus, Bax and Bak might regulate the IP3R-1 and calcium leak from the ER [108]. Conversely, anti-apoptotic Bcl-2 might decrease Ca^{2+} concentration in the ER. Bax (-/-)Bak(-/-) double-knockout (DKO) cells harbored reduced resting ER Ca^{2+} levels because of increased Ca^{2+} leak and an increase in the Ca^{2+}-permeable, hyperphosphorylated state of the IP3R-1. The ER Ca^{2+}- defect of DKO cells is rescued by RNA interference reduction of IP3R-1, supporting the argument that this channel regulates the increased Ca^{2+} leak in these cells.

Moreover, agents that initiate ER stress responses can induce conformational changes and oligomerization of Bax on the ER as well as on mitochondria [109]. In wild-type cells, ER stress is associated with caspase-12 cleavage that is abolished in DKO cells. Moreover, in these cells, introduction of Bak mutants selectively targeted to either mitochondria or the ER can induce apoptosis. However, ER-targeted, but not mitochondria-targeted, Bak leads to progressive depletion of ER Ca^{2+} and induces caspase-12 cleavage. In contrast, mitochondria-targeted Bak leads to enhanced caspase-7 and PARP cleavage in comparison with the ER-targeted Bak. These findings demonstrate that in addition to their functions in mitochondria, Bax and Bak also localize to the ER and function to initiate a parallel pathway of caspase activation and apoptosis.

In the absence of Bax and Bak, the physical binding of Bcl-2 and IP3R-1 at the ER membrane is increased and the genetic invalidation of Bcl-2 decreases IP3R-1 phosphorylation and ER Ca^{2+} leak rate. Thus, Bcl-2 family members might regulate IP3R-1 phosphorylation to control the rate of ER Ca^{2+} leak from intracellular stores, and consequently, might decrease the Ca^{2+} uptake by mitochondria [110].

Moreover, Bcl-2 would be able to intercept some models of mitochondrial apoptosis induced by ER stress agents at the level of ER membrane [111]. For example, cytochrome *c* release induced by brefeldin A was blocked by Bcl-2 engineered to be located exclusively at the ER membrane.

C. BAP 31

BAP 31 is a 28kDa integral membrane protein of the ER whose cytosolic domain contains two identical caspase recognition sites (AAVDG) [112]. After the activation of the Fas/CD95 pathway or after the induction of an ER stress, BAP 31 is cleaved by caspase-8 to generate a p20 fragment **(**Fig. **4**) that remains integrated in the membrane [112, 113]. BAP 31 cleavage is important for Fas-mediated release of cytochrome *c* from mitochondria and for the decrease of the $\Delta\Psi m$. Furthermore, p20 caused an early release of Ca^{2+} from the ER **(**Fig. **4**), concomitant uptake of Ca^{2+} into

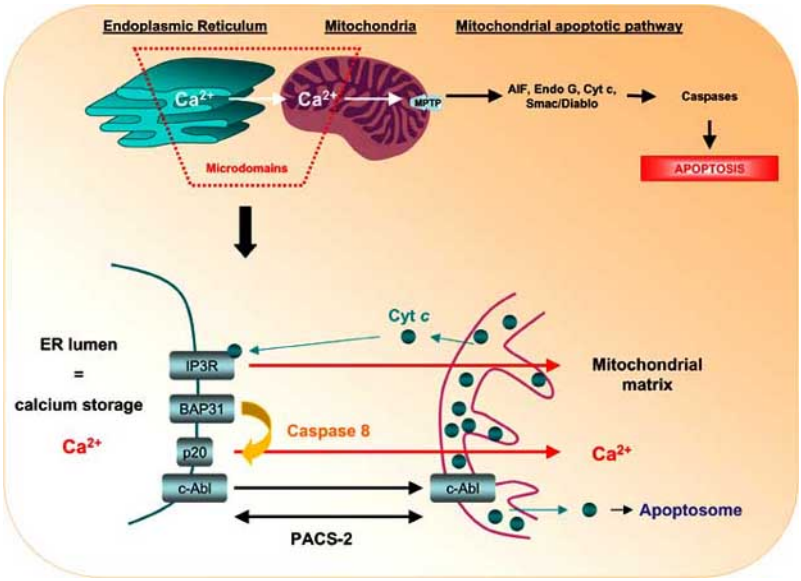

Fig. (4). Schematic representation of interconnections between mitochondria and RE during apoptosis. In several forms of apoptosis, calcium is released from ER and enters into mitochondria owing to contacts sites between these two organelles. Once into mitochondrial matrix, calcium activates opening of the Permeability Transition Pore Complex (PTPC) leading to swelling of the mitochondrial matrix, rupture of the outer membrane and releasing of apoptogenic factors from the mitochondrial inter-membrane space into the cytosol. Several proteins permit close contacts between ER and mitochondria (PACS-2), release of calcium from ER (IP3R, p20 product of cleavage of BAP 31) and regulation of flux of calcium between ER and mitochondria (proteins from Bcl-2 family and cytochrome *c*). Other proteins from ER, such as c-Abl, can induce cytochrome *c* releasing in a calcium independent manner.

mitochondria, and a mitochondrial recruitment of Drp1, a dynamin-related protein that mediate scission of the outer mitochondrial membrane, resulting in dramatic fragmentation and fission of the mitochondrial network [114]. Thus, p20 enhances the release of cytochrome *c* in response to this initiator caspase.

D. PACS-2

PACS-2 is a sorting protein that might control the apposition of mitochondria with the ER **(**Fig. **4**), as depletion of PACS-2 by mRNA interference causes BAP31-dependent mitochondria fragmentation and uncoupling from the ER [115]. However, PACS-2 depletion induced both ER stress and the p20-mediated mitochondrial fragmentation but not apoptosis, suggested that PACS-2 is somehow required for p20-mediated apoptotic induction.

E. BI-1

Bax inhibitor-1 (BI-1) is an evolutionarily conserved ER protein that suppresses cell death in both animal and plant cells. BI-1 overexpression protects against apoptosis induced by ER stress [116]. BI-1 mediated protection from apoptosis induced by ER stress correlated with inhibition of Bax activation and translocation to mitochondria, preservation of $\Delta\Psi m$, and suppression of caspase activation. BI-1 over-expression also reduces releasable Ca^{2+} from the ER and then protects the cells. BI-1 protects cells by reducing the calcium content of the ER in models of ligand-induced, oxygen/glucose deprivation-induced and probably Ca^{2+} - apoptosis models [117]. Presumably, a reduction in ER calcium leads to cell survival by lowering the mitochondrial Ca^{2+} accumulation that leads to irreversible opening of the PTPC. A plausible hypothesis would be that BI-1 regulates the number or activity of ER calcium channels like the IP3R. However, as BI-1 possesses channel-like structural features, one could propose that it might constitute a homomeric channel itself that enhances the Ca^{2+} permeability of the ER resulting in a leaky ER. Alternatively, it could form a heteromeric channel together with Bcl-2 or Bcl-X_L that have also channel-like properties.

II. TRANSLOCATION/IMPORT OF POTENTIAL THERAPEUTIC TARGETS

Protein translocation as a central mechanism of inter-organelle cross-talk plays a crucial role to determine the cell fate and then, modulation of this process might constitute a promising therapeutic strategy. Some selected examples of proteins that are susceptible to intracellular translocation will be detailed below.

A. Translocation from Mitochondria

Cytochrome **c***: from Mitochondria to RE*

Cytochrome *c* is released from the mitochondrial intermembrane space during apoptosis into the cytosol, but also targeted to the ER membrane to bind to IP3R and to amplify calcium-dependent apoptosis [118, 119]. In turn, this interaction blocks the self-inhibition of IP3R, resulting in increased Ca^{2+} release from internal stores **(**Fig. **4**). The resultant cytoplasmic and mitochondrial Ca^{2+} overload culminates in cell-wide cytochrome *c* release and maximal

caspase activation. Interestingly, a peptide inhibitor of cytochrome *c* / IP3R binding blocks intrinsic and extrinsic cell death pathways [120]. Therefore, small molecule inhibitors of cytochrome *c* - IP3R interaction may prove useful in treating disorders associated with inappropriate intrinsic and extrinsic apoptotic signaling.

AIF: from Mitochondria to Nucleus

Mammalian AIF is a 67-kDa protein with both NAD(P)H oxidase and monodehydroascorbate reductase activity but oxidoreductase activity of AIF is not required for its apoptogenic property [121-123]. AIF translocates from the mitochondria to the nucleus where it mediates chromatin condensation and large-scale fragmentation of DNA, possibly by binding to DNA [124, 125]. In addition to caspase activation [99], disruption of mitochondrial membrane potential, activation and oligomerization of Bax and Bak, mitochondrial permeability transition and mitochondrial fission might induce the release of AIF. Therefore, AIF release from mitochondria can be independent and dependent on caspases [95, 125]. For instance, there are no known inhibitors of AIF. The broad-spectrum cytoprotective molecules (Bcl-2 and a member of the heat shock protein 70 family [128]) can delay or prevent AIF-mediated toxicity. Moreover, AIF nuclear translocation can depend on the presence of p53 and its transcriptional target Bax [127]. The mechanism of AIF-mediated chromatin condensation and DNA fragmentation in cell death is unclear, but AIF might bind to DNA and recruit proteases and nucleases that cause chromatin condensation. Another possibility is that AIF could have a concealed nuclease activity. In *Caenorhabditis elegans*, WAH1, a homolog of AIF, associates with CPS-6, the homolog of mammalian endonuclease G. This association enhances the nuclease activity of CPS-6 and results in apoptotic DNA degradation [128]. Recently, WAH1 and CPS-6 have been shown to join with nucleases such as CRN1 in a nuclear complex (the degradasome) to influence nuclease activity [129]. Then it clearly appears that pharmacological agents that hinder the translocation of AIF or inhibit the promotion of DNA fragmentation induced by AIF might have tremendous therapeutic potential.

B. Translocation to Mitochondria

c-abl: from ER to Mitochondria

The only protein known to date to translocate between these two organelles during apoptosis is c-Abl. It is a tyrosine kinase ubiquitously expressed which localizes mainly to the nucleus and cytoplasm. The nuclear c-Abl is involved in response to genotoxic stress whereas the cytoplasmic c-Abl is involved in response to oxidative stress. It have been demonstrated that 20% of c-Abl also localize in the ER [130]. Reportedly, induction of ER stress with calcium ionophore A23187, brefeldin A, or tunicamycin is associated with translocation of ER-associated c-Abl to mitochondria (Fig. **4**). Concomitantly, the import of c-Abl to mitochondria triggers the cytochrome *c* release. The mechanism appeared to depend on c-Abl, because apoptosis is significantly attenuated in c-Abl-deficient cells.

The mechanism by which c-Abl exerts its action at this site is unclear, but it may function in concert with JNK kinases, which are recruited and activated by Ire1 during ER stress, and are essential for mediating cytochrome *c* release in other death pathways.

Bcl-2 Family

Bax, Bad, Bak, Bid are members of the Bcl-2 family that play key roles in the regulation of apoptosis. As potent tumor suppressors, they are frequently down-regulated or mutated

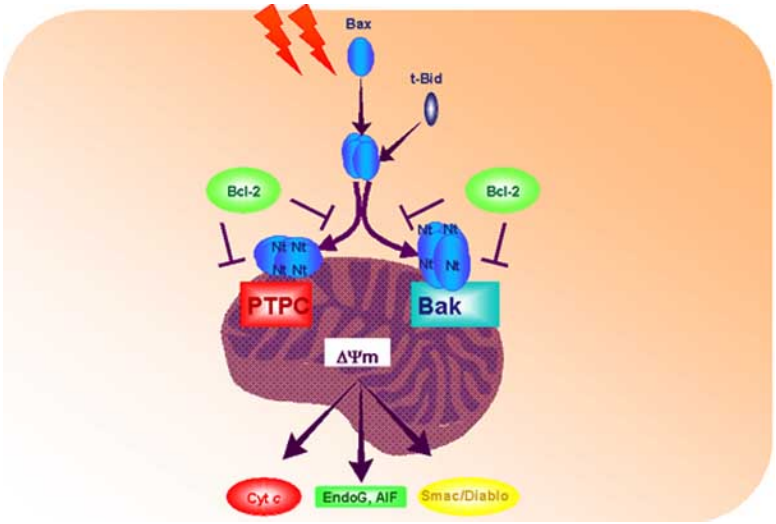

Fig. (5). Bcl-2 family members and mitochondria-induced apoptosis. Bax is a monomeric cytosolic protein in resting conditions. Upon apoptosis induction, it oligomerizes and translocates to mitochondrial membranes where it might interact with the permeability pore transition complex (PTPC) or with the resident protein Bak to induce mitochondrial membrane permeabilization, loss of the electrochemical potential (ΔΨm) and the release of pro-apoptotic proteins into the cytosol (e.g. AIF, cytochrome c, and Smac/Diablo). Anti-apoptotic Bcl-2 might intercept the cascade of mitochondrial activation at the level of Bax import and at the level of the PTPC.

in many cancers. Unlike anti-apoptotic members such as Bcl-2 and Bcl-X_L that are membrane bound, these proteins are believed to be cytosolic and upon apoptotic stimulation, and to translocate to mitochondrial membrane [131, 132]. Using the fusion of the green fluorescent protein (GFP) to the N-terminus of Bax, Wolter *et al*. showed that in resting cells, the fluorescence is diffuse indicating a cytosolic localization of Bax, whereas upon induction of apoptosis, GFP-Bax moved into the cell to a punctuated distribution that partially colocalizes with mitochondria. Once initiated, Bax movement is complete within 30 min, before cellular shrinkage or nuclear condensation [131, 132]. Once incorporated into mitochondrial membrane, Bax undergoes activation, consisting in the exposure of its N-terminus, its oligomerization with Bid and/or Bak [133] and the capacity to form composite channels to allow the cytochrome *c* release [134]. Another possible mechanism is the integration of Bax or Bid into mitochondrial membrane to interact and cooperate with PTP members such as ANT [95, 135] and VDAC [136] to induce MMP, $\Delta\Psi$m loss and cytochrome *c* release.

Bcl-2 and Bcl-X_L inhibit Bax-induced apoptosis by complex mechanisms involving heterodimerization process, membrane stabilization and channel inhibition (for review see: [137]).

p53 Family Proteins

Under stress conditions, low cytoplasmic p53 level is quickly increased *via* p53-MDM2 disruption, due to p53 post-translational modifications. Then, p53 protein can play its transcription factor's role and transactivate its targets genes. This scheme has been revised the last years with the discovery of a mitochondrial translocation for p53, first by Ute Moll's team [72]. The authors demonstrate p53 mitochondrial targeting in response to a wide spectrum of stress signals, including DNA damage and hypoxia (camptothecin, etoposide, actinomycin D, desferoxamine and TNF). More recently, other work proved the biological relevance of such mitochondrial p53 accumulation *in vivo* conditions after gamma radiation of wt or p53-null mice [138] or induction of endogenous p53 using E1A/H-rasG12V–transformed wild-type p53, Bax–/–, and p53–/– mouse embryonic fibroblasts (MEF) [139]. New data on the transcription-independent functions of p53 emerged with recent results [139-141]. First, Mihara and colleagues showed that p53 can bind to Bcl-X_L *via* a conserved domain in the DNA binding domain (residues 239-248), highly mutated in cancers. This interaction is so strong that only high amount of Bax or Bid can disrupt the association [140]. Second, Dumont and al identified that variant with an arginine at codon 72 (R72) instead of a proline (P72) exert an increase of p53 mitochondrial localization and dependant apoptosis, connecting this with previous characterization of these polymorphic variants [142, 143]. Third, p53 has been shown to release pro-apoptotic Bcl-2 proteins sequestered by Bcl-X_L and directly activate the pro-apoptotic Bcl-2 protein Bax, allowing for mitochondrial membrane permeabilization and apoptosis [139]. Taken together, these data suggest that cytoplasmic p53 can function analogously to the BH3-only proteins, a subset of pro-apoptotic Bcl-2 proteins. Mitochondrial p53 maybe involved in mitochondrial transcription/replication [144, 145] or mitochondrial DNA repair machinery by Base Excision Repair (BER) system [146].

The involvement of p53 homologs in apoptosis induction, such as p73 family proteins, renders the situation more complex. p73α is able to transactivate to some extend p53 target genes [147-150] and to induce cell death *via* Bcl-2 family members [151]. Whereas p73 is not regulated by p53 inducing stimuli, it can be targeted and modified by c-Abl kinase [152, 153], which can also translocate to the mitochondria (see above). Moreover, even if no evidence exists for a mitochondrial translocation of p73 protein, some lipid binding properties of its Sterile Alpha Motif (SAM) domain could allow the protein to target membranes [154] and based on the sequence homologies between p53 and p73 (especially in the domain from residues 239 and 248 on p53), we can not exclude a possible interaction with Bcl-2 family members.

Even if some unanswered questions remains concerning the regulation of the intracellular movement of p53 and the role of transcription-independent apoptosis in the tumor suppressor function of p53, this biological function could become a new therapeutical strategy to modulate apoptotic cell death.

NF-κB/IκB

Recently, the importance of the physical interaction of NF-κB regulatory loop with mitochondria has been emphasized. Indeed, the localization of NF-κB to the mitochondria raises interesting questions concerning a potential role in regulating apoptosis. It has been shown that NF-κB activates several gene products (see above) to inhibit the caspase cascade and to block cytochrome *c* release from mitochondria [155, 156]. It is interesting to speculate that mitochondrial NF-κB may play a role in the suppression of apoptosis through transactivation of mitochondrial genes [157] or through protein-protein interactions. Concerning this latter aspect, mitochondrial NF-κB might physically interact with glucocorticoids receptor to regulate key processes involved in cell growth and apoptosis [158].

In parallel, Bottero and co-workers identified IκB regulator in mitochondrial intermembrane space, interacting with the adenine nucleotide transporter, ANT, involved with apoptosis through its ability to participate to the PTPC [159]. More recently, Zamora *et al.* correlated the decrease of nuclear anti-apoptotic transactivation of NF-κB by its recruitment to mitochondria and sequestration by ANT1, explaining the pro-apoptotic effect of ANT1 overexpression [160].

III. THERAPEUTIC IMPLICATIONS

Apoptosis deregulation can be involved in numerous human pathologies as diverse as cancer, neurodegeneration, viral/bacterial infection, auto-immune diseases, as well as ischemia-reperfusion. For instance, an apoptosis excess can be the cause of the selective depletion of a peculiar cell type as for example in HIV infection that manifests as a depletion of CD4+ T lymphocytes. Conversely, in cancer, cells frequently exhibit an altered capacity to die and an increased

resistance to the induction of death by chemotherapy. One major challenge to the future is to define new strategies to modulate cell death [161]. As presented in this review, communications between heterologous organelles appear to be central to the determination of the cell fate and modulation of the inter-organelle cross-talk might be a mean to influence the cell balance between life and death. Determination of the fine mechanisms of inter-organelle cross-talk allowed identifying novel intra-cellular targets that potentially might serve in a therapeutic perspective. This applies to p53 and its relative p73 and p63, PTPC members, AIF, NF-κB protein, kinases, as well as ER proteins that regulate Ca^{2+} fluxes. Future studies will have to decipher whether the right strategy is to correct the function of these candidates at the genetic or at the protein level using small molecules.

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ABBREVIATIONS

AIF = Apoptosis inducing factor

ANT = Adenine nucleotide translocase

HK = Hexokinase

IP3R = Inositol, 1, 4, 5, triphosphate receptor

MMP = Mitochondrial membrane permeabilization

PTPC = Permeability transition pore complex

VDAC = Voltage-dependent anion channel.

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