Mitochondria play an important role in both the life and death of cells. The past 7-8 years have seen an intense surge in research devoted toward understanding the critical role of mitochondria in the regulation of cell death. Mitochondria have, next to their function in respiration, an important role in apoptotic signaling pathway. Apoptosis is a form of programmed cell death important in the development and tissue homeostasis of multicellular organisms. Apoptosis can be initiated by a wide array of stimuli, including multiple signaling pathways that, for the most part, converge at the mitochondria. Although classically considered the powerhouses of the cell, it is now understood that mitochondria are also "gatekeepers" that ultimately determine the fate of the cell. Malfunctioning at any level of the cell is eventually translated in the release of apoptotic factors from the mitochondrial intermembrane space resulting in the organized demise of the cell. These mitochondrial factors may contribute to both caspase-dependent and caspase-independent processes in apoptotic cell death. In addition, several Bcl-2 family members and other upstream proteins also contribute to and regulate the apoptosis. In this review, we attempt to summarize our current view of the mechanism that leads to the influx and efflux of many proteins from/to mitochondria during apoptosis.

Keywords: c-Abl, AIF, Apaf1, Apoptosis, Bcl-2, Caspases, Cell death, Cytochrome c, DIABLO, JNK, Mitochondria, PKCδ, p53, Smac, Translocation, TR3

Programmed cell death or apoptosis plays an integral role in a variety of biological events, including morphogenesis, tissue homeostasis, and removal of unwanted or harmful cells. Apoptosis is a continuous physiologic process and is one of today's most active fields of biomedical research. Failure to accurately undergo apoptosis can cause severe anomalies in humans, either due to the accumulation or due to the deficiency of a particular cell type. Abnormal inhibition of apoptosis is a hallmark of cancer and autoimmune diseases, whereas excessive cell death has been implicated in a number of neurodegenerative disorders. Extensive studies performed over a decade have revealed a large part of the molecular basis of cell death. It is now apparent that mitochondria are the central regulators of cell death and survival.

Cell death via apoptosis follows the activation of effector proteases called caspases, which participate in enzymic cascades that terminate in cellular disassembly. Effector caspases, such as caspase-3 and -7, are activated by initiator caspases, such as caspase-9, through proteolytic cleavage. Once activated, effector caspases are responsible for the proteolytic cleavage of a diverse array of structural and regulatory proteins, resulting in an apoptotic phenotype. Death by the terminal pathways of apoptosis is frequently compared with death by necrosis, but it is distinguished from necrotic death in that apoptosis is a closely regulated process induced by a specific stimulus, and occurs without the release of inflammatory mediators. Death by necrosis occurs because of failure to control cellular homeostasis after undergoing damage. Although apoptosis can be initiated via a plethora of stimuli - including ultraviolet light, oxidative stress, viruses, chemicals, drugs, cytokines, and ligands. Most of the pathways ultimately converge at the mitochondria, which then converts these signals into a pro-apoptotic response. The complex role of mitochondria came into focus when biochemical studies identified several mitochondrial proteins that are able to activate cellular apoptotic programs directly. In response to apoptotic stimuli, they are released to the cytosol and/or nucleus. They promote apoptosis either by activating caspases and nucleases or by neutralizing cytosolic inhibitors of this process. This review summarizes the current understanding as to how the
mitochondrion deciphers pro-apoptotic signals and how it responds to these signals to initiate the final executioner stage of apoptosis.

**Cell death cascades**

Mitochondria play an essential role as a power plant of the cell, providing energy for specialized cell functions. In the context of cell death, they play a central role in apoptotic pathways. Apoptosis is often referred to as caspase-dependent process. Caspases are a family of cysteine proteases and are expressed as inactive zymogens (procaspases). Activation of the proenzyme requires autocleavage or cleavage by other caspases at specific aspartate residues. Two pathways leading to caspase activation and proapoptotic cell death have been well characterized. One is initiated from death receptors at the cell surface, and other is triggered by mitochondria. Death receptors belong to the tumor necrosis factor α (TNF-α) superfamily and include, for example, the Fas/CD95 receptor. Binding of the death ligand to its receptor is followed by the recruitment of procaspase-8 to the plasma membrane and autoproteolytic activation. Once activated, caspase-8 activates procaspase-3 and other downstream procaspases. Caspase-8 knockout cells are therefore resistant to TNF and Fas-mediated apoptosis.

DNA damage, genotoxic agents, oxidative stress, ultraviolet radiation are some of the apoptotic signals channeled through the mitochondria to activate caspases. This path is independent of the cell membrane receptor-mediated activation of cell death. Recently published articles, however, contradicted this view of stress-induced mitochondrial-only apoptosis. Lassus and coworkers demonstrated that stress-induced cytokines can directly activate caspase-2. Therefore, mitochondrial permeabilization may function as amplification of caspase activation. The final steps to cell death signal transduction to the nucleus and subsequent DNA fragmentation by specific nucleases, however, are shared by both conduits, the receptor-mediated and mitochondrial-initiated apoptosis.

**Mitochondrial release of apoptogenic factors**

*Cytochrome c*—Holo-cytochrome c (cytochrome c with attached heme) remains sequestered in the mitochondrial intermembrane space where it serves as an electron shuttle between complex III and IV of the mitochondrial respiratory chain. Xiodong Wang and co-workers have reported the surprising observation that holo-cytochrome c (but not apo-cytochrome c) is required for the activation of caspase-3 in a cell free system. Following cell exposure to apoptotic stimuli, cytochrome c was shown to be released into the cytosol. Today it is an established fact that cytochrome c, once present in the cytosol, drives the assembly of a high molecular weight caspase activating complex termed “apoptosome”. Cytochrome c binds to apoptotic protein activating factor-1 (Apaf-1) in the presence of ATP/ADP, leading to the formation of apoptosome. The cytosolic protein containing a caspase-recruitment domain (CARD) of Apaf-1 become exposed in the apoptosome, which subsequently recruit multiple procaspase-9 molecules to the complex and facilitate their autoactivation. Only the caspase-9 bound to the apoptosome is able to cleave and activate downstream caspases such as caspase-3. The knockout experiments also verify the linearity of the cytochrome c–Apaf-1–caspase-9–caspase-3 pathway.

**DIABLO/Smac**—DIABLO (direct IAP-binding protein with a low isoelectric point)/Smac (second mitochondrial-derived activator of caspase) is another protein released from mitochondria during apoptosis. DIABLO/Smac, a 25 kD protein normally localizes to the mitochondrial intermembrane space and is released to the cytosol only during apoptosis. In response to various apoptotic stimuli, DIABLO/Smac is released into the cytosol where it inhibits the inhibitor of apoptotic proteins (IAPs). By binding to IAP, Smac displaces active caspases or prevents IAPs binding active caspases and thus promote death of the cell. When Smac binds to X chromosome-encoded IAP (XIAP) it prevents it from binding caspase-9 and thus promotes death following UV irradiation. However, caspase activation facilitated by Smac may involve additional mechanisms that are independent from its interaction with XIAP.

**HtrA2/Omi**—Mammalian mitochondria also releases a protein called high-temperature requirement A2 (HtrA2)/Omi, which like DIABLO/Smac binds to IAPs during apoptosis. The Omi precursor protein possesses an amino-terminal mitochondrial translocation sequence that directs the protein into mitochondria. Once in mitochondria, the translocation sequence is cleaved to generate a mature 36 kD protein. During apoptosis, mitochondria release Omi together with cytochrome c and Smac. Omi has an IAP-binding motif that allows it to bind IAPs and suppress...
their caspase-inhibitory activity. Interestingly, deletion of the IAP-binding motif prevents its interaction with IAPs but does not abolish its apoptosis-inducing activity, suggesting that Omi has additional effects on apoptotic cascade. Omi also has a trypsin-like serine protease domain, indicating that it may induce apoptosis in a caspase-independent manner through its protease activity. Recently, it has been reported that the protease activity of HtrA2/Omi contributes to its ability to potentiate caspase activation and apoptosis via at least two different mechanisms. It cleaves and/or degrades IAPs and an unidentified substrate, resulting in inactivation of IAPs and permeabilization of the outer mitochondrial membrane followed by cytochrome c-dependent caspase activation, respectively.29

Apoptosis-inducing factor—The apoptosis-inducing factor (AIF) is a 57 kD flavoprotein located in the mitochondrial intermembrane space.20 AIF is one of the first evidenced mitochondrial apoptotic activity. Upon induction of apoptosis, AIF translocates to the nucleus where it induces condensation of chromatin. Unlike cytochrome c, AIF activation of apoptosis is independent of caspases.32 However, cytochrome c usually accompanies AIF release from mitochondria, resulting in caspase activation and subsequent DNA fragmentation.33 Because the molecular weight of AIF is much greater than cytochrome c, it still remains unclear whether these proteins are released from mitochondria via the same mechanism.

Endonuclease G—Endonuclease G (EndoG) has been shown to be required for early embryogenesis and normal apoptosis.36 EndoG is a nuclear-encoded mitochondrial protein reported to be important for nuclear DNA fragmentation during apoptosis. Like AIF, EndoG in mammalian cells is released from mitochondria during apoptosis and translocate to the nucleus to cleave DNA into nucleosomal fragments independent of caspases.15 Mitochondria release EndoG together with other apoptogenic proteins, indicating that EndoG may be located in the mitochondrial intermembrane space. The identification of AIF and EndoG indicates that apoptosis can proceed in the absence of caspase activity when the mitochondria are damaged. In this case, release of AIF and EndoG from mitochondria starts an apoptotic program parallel to caspase activation.

The release of cytochrome c and other apoptogenic proteins from mitochondria is known to be regulated by the Bcl-2 family of proteins. The pro-death members of this group of protein promote the release of these apoptogenic factors whereas the anti-death members prevent it.37,38

Regulation of mitochondrial apoptotic signals

One striking feature of apoptosis signaling is protein translocation of signal and effector molecules between three major cellular compartments. This includes translocation to and from mitochondria, the cytoplasm and the nucleus. Of particular interest are a growing list of pro-apoptotic proteins that undergo translocation to mitochondria, where they exert their pro-apoptotic functions by inducing organellar dysfunction (Fig. 1).

Translocation of Bcl-2 family (BH3-domain containing) proteins

A wide variety of mitochondrial events have been reported to be modulated by Bcl-2 and its homologs. The release of cytochrome-c and other apoptogenic proteins from the mitochondria is known to be regulated by Bcl-2 family members. It is widely accepted that the pro-apoptotic members of Bcl-2 family promote the release of the apoptogenic factors whereas the anti-apoptotic members prevent it. Anti-apoptotic members of Bcl-2 family (such as Bcl-2 and Bcl-XL) reside mainly, but not exclusively, in mitochondrial membrane, where they locally inhibit mitochondrial membrane permeabilization (MMP).

Proapoptotic members of the Bcl-2 family such as Bax can translocate to the mitochondria while undergoing a conformational change; they then oligomerize within mitochondrial membranes and facilitate MMP. This translocation and oligomerization reaction is inhibited by anti-apoptotic members of Bcl-2 family and is stimulated by pro-apoptotic BH3-only members of Bcl-2 family (such as Bid). Bid is exclusively cytosolic in living cells and upon activation of cell surface receptor, it is cleaved by caspase-8. The truncated Bid translocates to the mitochondria and induces cytochrome-c release.40,41

Bad, another BH3-only protein, is regulated by phosphorylation and dephosphorylation.42 The BH3 domain of Bad binds to and inactivates the anti-apoptotic members of the Bcl-2 family at the outer mitochondrial membrane, thereby promoting cell death. Conversely, in the presence of tropic factors, Akt and mitochondrial-anchored PKA phosphorylate Bad, allowing it to bind 14-3-3 protein and to remain in the cytosol.43,44 Phosphorylation of Bad also disso-
Apoptotic Stimuli

Bad, Bid, Bax etc.

\[ \downarrow \]

Bcl-2, Bcl-xl

Mitochondrion

Loss of Mitochondrial Functions

AIФ

Cytochrome c

Smac

EndoG

Apaf-1

Caspase-9

IAP

Caspase-3

Apoptosis

Fig. 1—Multiple apoptotic pathways originate from the mitochondria. Apoptotic signals are transduced to mitochondria by the BH3-only proteins and possibly by additional pathways. These signals can be neutralized by the anti-apoptotic proteins, such as Bcl-2 or Bcl-xl. The mitochondrial damage, in apoptotic response, triggers the release of apoptogenic proteins including cytochrome c, Smac, AIФ, and EndoG. Cytochrome c triggers caspase activation through Apaf-1, and Smac relieves IAP inhibition of caspases. AIФ and EndoG cause apoptosis through chromatin condensation and fragmentation, independent of caspases activation. The mitochondrial damage may also lead to cell death due to loss of mitochondrial functions.

ciates its interaction with anti-apoptotic Bcl-2 family of proteins, allowing these proteins to promote survival.

**Translocation of other proteins (non-BH3 containing) to the mitochondria**

In addition to Bcl-2 family members, several other proteins have been shown to migrate to the mitochondria in response to certain stimuli and regulate the apoptosis by causing mitochondrial damage. These proteins include p53\(^{45,46}\), an orphan receptor TR\(^3\)\(^{47}\), c-Jun N-terminal kinase\(^{48,49}\), c-Abl tyrosine kinase\(^{50-52}\) and protein kinase C\(^\alpha\)\(^{53-55}\). How these proteins, and possibly others, translocate to the mitochondria and regulate its functions is now fully known. In this section, we summarize the recent reports on the following proteins, which translocate to mitochondria in response to specific stimuli.

\(p53\)—p53 is a nuclear phosphoprotein and a transcription factor\(^{56}\). p53 is a multi-functional protein involved in the control of cell cycle progression, apoptosis and genomic integrity in cells exposed to DNA-damaging agents. Evidence suggests that p53 induces cell death by a dual mode of action involving activation of target genes and transcriptionally-independent direct signaling. p53 is present predominantly in the cytoplasm of primary cultured cells and various cancer cells. p53 translocates to the nucleus to initiate gene activation and DNA synthesis for cell proliferation\(^{37}\).

Recent studies have indicated that p53 has a direct signalling role in the induction of apoptosis\(^{58,59}\), although the mechanisms involved are not completely understood. Marchenko and coworkers\(^{45}\) have demonstrated that a fraction of stress-activated p53 translocates to mitochondria after an apoptotic stimulus, but not during p53-independent apoptosis or p53-mediated cell cycle arrest. The translocation of p53 to mitochondria is rapid (within 1 hr after p53 activation) and precedes changes in MMP, cytochrome-c release and procaspase-3 activation. In contrast, p53 does not translocate during p53-independent apoptosis or p53-mediated cell cycle arrest. They have further identified that p53 protein can directly induce permeabilization of the outer mito-
Protein kinase induce translocation of JNK to mitochondria, which generally, JNK has been implicated in the regulation of DNA-damaging agents including 1-D-arabinofuransose, ionizing radiation (IR) and phorbol ester 67. JNK is also activated in response to cell stress induced by certain cytokines, growth factor withdrawal 64, and treatment with chemotherapy drugs including paclitaxel 65,66. JNKs are potently and preferentially activated by virtue of subcellular targeting, appear to be capable of inducing apoptosis.

TR3—The nuclear orphan steroid receptor TR3 (also called Nur77 or NGFI-B) is a member of the steroid/thyroid receptor family is a bonafide transcription factor with a zinc finger DNA-binding domain flanked by transactivation domains and a binding domain for an as yet unknown ligand. TR3 is induced and acts as a transcription factor in response to epidermal growth factor and all-trans-retinoic acid. TR3 mediates apoptosis in different cell types in vivo, e.g. in neurons, T-cells and human cancer cells 67. Unexpectedly, when TR3 works as an apoptotic factor, its transcriptional activation function is turned off. Instead, in response to wide variety of apoptotic stimuli, TR3 translocates from the nucleus to mitochondria to induce cytochrome-c release and apoptosis. The TR3 DNA-binding domain, required for transcriptional activity, is not required for mitochondrial targeting. Its mitochondrial action is also blocked by Bcl-2 47. Thus, both p53 and TR3 (nuclear transcription factors) by virtue of subcellular targeting, appear to be capable of inducing apoptosis.

c-Jun N-terminal kinase—The c-Jun N-terminal kinases (JNKs) are classic stress-activated protein kinases 61. JNKs are potently and preferentially activated following various cell stress applications including UV irradiation, heat and osmotic shock 62, treatment with a protein synthesis inhibitor 63, inflammatory cytokines 64, growth factor withdrawal 44, and treatment with chemotherapy drugs including paclitaxel 65,66. JNK is also activated in response to cell stress induced by certain DNA-damaging agents including 1-D-arabinofuranosylcytosine 65, cis-platinum, and mitomycin C 66. Generally, JNK has been implicated in the regulation of apoptosis 60,67. Recently, we and others have reported that ionizing radiation (IR) and phorbol ester induce translocation of JNK to mitochondria, which in turn causes phosphorylation, and inactivation of anti-apoptotic Bcl-xL 48,49. Deng and coworkers 44 have reported that JNK also work as Bcl-2 kinase. JNK has been found to phosphorylate and co-localize with Bcl-2 in the mitochondria where it plays an important role in regulating apoptosis 21. On the other hand, report suggests that activated JNK promotes Bax translocation to mitochondria through phosphorylation of 14-3-3, a cytoplasmic anchor of Bax. Phosphorylation of 14-3-3 led to dissociation of Rap from this protein. Expression of phosphorylation-defective mutants of 14-3-3 blocked JNK-induced Bax translocation to mitochondria, cytochrome-c release and apoptosis 72. These reports open up a new exciting field for the identification of JNK-mediated apoptotic signaling.

c-Abl tyrosine kinase—The c-Abl protein tyrosine kinase, product of the cellular Abelson (c-abl) gene is ubiquitously expressed and localized mainly in the nucleus and cytoplasm. c-Abl has been shown to mediate inhibition of cell cycle progression and apoptosis when cells are exposed to genotoxic stress. Moreover, it has also been shown that c-Abl regulates the G1 growth arrest in response to DNA damage by p53 and its analog p73 dependent mechanism. Recently, we have demonstrated that c-Abl exerts apoptotic effects not only in response to DNA damaging agents but also in response to other stimuli such as oxidative stress, ER and microtubule-stress. In addition, we found that c-Abl in response to certain apoptosis-inducing agents translocates from cytoplasm and/or other organelles to the mitochondria. These include oxidative stress 50,51, ER-stress 52 and microtubule-stress (unpublished observation). Its translocation induces change in MMP, cytochrome-c release, caspase-3 activation and apoptosis 52. ROS-induced localization of c-Abl to mitochondria is dependent on activation of protein kinase c delta and the c-Abl kinase function 51,52. These reports further demonstrate that stress-induced cytochrome-c release is apparently dependent on the c-Abl tyrosine kinase, because c-Abl null mouse embryonic fibroblasts are resistant to ER and oxidative stress-induced cytochrome-c release and apoptosis 52,73,50. These studies enhance the understanding of the mechanism by which c-Abl exerts apoptotic effects by regulating mitochondrial functions.

Protein kinase Cδ—Protein kinase Cδ (PKCδ) belongs to the novel PKC subfamily and is, therefore, activated by DAG/phorbol esters in calcium-independent manner. Following its activation, PKCδ...
PKCδ undergoes proteolytic degradation or down regulation via the ubiquitin-proteasome system. PKCδ has been reported to play a critical role in the control of cell growth. Loss of PKCδ leads to cell transformation in fibroblasts, whereas, its overexpression results in G2/M arrest of the cell cycle. PKCδ has been reported to translocate to nearly all subcellular organelles, including nucleus, mitochondria, Golgi complex, ER and plasma membrane. At each subcellular organelle, PKCδ phosphorylates different substrates inducing various responses that eventually lead to cell death. It has been known for a long time that upon activation, full-length PKCδ translocates from the cytoplasm to the plasma membrane. One of the main targets of PKCδ is the mitochondria. Indeed, PKCδ has been reported to localize to the mitochondria in response to PMA in mouse keratinocytes and U-937 myeloid leukemia cells. The translocation can also be induced by oxidative stress. In addition, these studies have demonstrated that over-expression and activation of PKCδ exert change in mitochondrial functions such as a decrease in MMP and release of cytochrome c.

Fig. 2—Regulation of mitochondrial apoptotic signals. Many death signals converge onto mitochondria and are mediated through members of the Bcl-2 protein family (BH3-domain containing) and other proteins which are having role in specific apoptotic pathways. In some cells, binding of Fas ligand to its receptor Fas leads to the caspase-8 activation, which proteolytically cleaves Bid, whose C-terminal fragment (t-Bid) translocates to mitochondria, where it activates Bax or Bax-like proteins and results in cytochrome-c (cyt c) release. Once in the cytosol, cyt c activates caspase-9 by binding to Apaf-1 and dATP. Caspase-8 can also initiate a direct signaling pathway that is independent of mitochondria by activating downstream caspases. Apoptotic stimuli (death receptor independent) and growth-factor deprivation can trigger apoptosis by inducing translocation of bax or Bad to mitochondria. In normal cells, Bad can be phosphorylated by AKT, PKA and other enzymes which results its sequestration in cytosol through its binding to 14-3-3. Bad is also dephosphorylated by the phosphatases and translocates to mitochondria, where it binds to Bcl-xL. This displaces Bcl-xL from Bcl-xL-Bax heterodimers, thereby inhibiting the death-repressor activity of Bcl-xL. In certain cells, in response to apoptotic stimuli p53 translocates to mitochondria and permeabilize the outer mitochondrial membrane by forming complexes with the protective Bcl-2 and Bcl-2 proteins, resulting in cyt c release. TR3, another transcription factor also translocates to mitochondria from the nucleus and releases cyt c. In response to ionizing radiation and phorbol ester, c-jun-N-terminal kinase (JNK) translocates to mitochondria, where it causes phosphorylation and inactivation of anti-apoptotic Bcl-XL and Bcl-2. JNK promotes Bax translocation to mitochondria through phosphorylation of 14-3-3. Other kinases like c-Abl tyrosine kinase (in ROS and ER-stress response) and Protein kinase Cδ (in ROS and PMA response) translocates to mitochondria and facilitate apoptosis by causing loss of mitochondrial transmembrane potential and release of cyt c and caspase-3 activation.
cytochrome c. In contrast, cytosine arabinoside (Ara-C) and etoposide (VP-16) induced nuclear translocation of PKCζ preceded its cleavage by caspase-3. However, the catalytic fragment of PKCζ, generated following treatment with cisplatin or UV radiation has been found to be localized to the mitochondrial fraction. Recently, Kajimoto and coworkers have reported that activation and translocation of PKCζ to the Golgi complex is critical for the ceramide-induced apoptosis. These studies suggest that depending on the cell types and apoptotic stimuli, the targets of PKCζ may resides in the plasma membrane, nucleus or mitochondria.

The evolving theme here is that these mitochondrially translocating proteins belong to unrelated biochemical classes of molecules, most of which are previously not associated with mitochondrial functions. However, these studies suggest a new exciting direction for future research that ultimately may reveal other unidentified apoptotic pathways.

Concluding remarks

Existence of multiple programs of cell death is strongly supported by the vast amount of information disclosed in recent years. It has now become apparent that mitochondria act as integrators of pro-apoptotic signals, transducing these to the final execution machinery of apoptosis. In summary, mitochondrial-initiated apoptosis has two important signaling events. First, as illustrated in Figure 1, multiple factors function work together to trigger apoptosis. The release of cytochrome c activates caspases, and the release of Smac removes IAP inhibition of caspases, and the release of EndoG and AIF induces DNA fragmentation and chromatin condensation. Second, as illustrated in Figure 2, the regulation of mitochondrial apoptotic signals. These signals are executed by translocation of cytosolic BH3-only (Bax, Bad, t-Bid) and other non-BH3 proteins (p53, TR3, JNK, c-Abl and PKCζ) to mitochondria, thereby releasing pro-apoptotic factors required for the cell death. Unraveling the precise mechanism of action of these proteins will undoubtedly help our understanding of how signals from and to mitochondria regulates cell death.

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