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CYTOCHROME *C*-MEDIATED APOPTOSIS

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■ **Abstract** Apoptosis, or programmed cell death, is involved in development, elimination of damaged cells, and maintenance of cell homeostasis. Deregulation of apoptosis may cause diseases, such as cancers, immune diseases, and neurodegenerative disorders. Apoptosis is executed by a subfamily of cysteine proteases known as caspases. In mammalian cells, a major caspase activation pathway is the cytochrome *c*-initiated pathway. In this pathway, a variety of apoptotic stimuli cause cytochrome *c* release from mitochondria, which in turn induces a series of biochemical reactions that result in caspase activation and subsequent cell death. In this review, we focus on the recent progress in understanding the biochemical mechanisms and regulation of the pathway, the roles of the pathway in physiology and disease, and their potential therapeutic values.

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INTRODUCTION

In the middle of the last century, the concept of apoptosis, or programmed cell death, emerged with its unique and dynamic morphological features that are distinguishable from senescence or necrosis, such as cell shrinkage, plasma

membrane blebbing, chromatin condensation, nuclear membrane breakdown, and formation of small vesicles from the cell surface also known as apoptotic bodies (1). After apoptosis, the apoptotic bodies are rapidly engulfed by phagocytes, and thus a potential inflammatory response is avoided (1). This deliberate physiological cell suicide concept was proved molecularly in the 1990s by Horvitz and colleagues by showing that in *Caenorhabditis elegans*, there is an intrinsic signaling pathway controlling the cell death of a group of specific neuronal cells during development (2).

In the *C. elegans* apoptosis pathway, there are both positive and negative regulators of cell death (3–5). The pathway is initiated by Egl-1, which functions to antagonize the negative regulator Ced-9. Egl-1 causes Ced-9 to release its inhibition on Ced-4, which in turn recruits and activates Ced-3, a cysteine protease and the executioner of apoptosis. Interestingly, Ced-9 is homologous to human Bcl-2 (6), which was originally identified as an oncogene product, because translocation and subsequent overexpression of the gene causes B-cell lymphoma (7–11). Later, it was found that the oncogenic property of Bcl-2 is due to its activity to protect cells from death (12, 13). Therefore apoptosis and oncogenesis are linked together: Cancer can develop as a result of not only overproliferation of cells but also inhibition of normal physiological cell death. In addition to Ced-9, the executing molecule of the *C. elegans* apoptosis pathway, Ced-3 also has many homologs in mammals. The homology of Ced-9 and Ced-3 with mammalian apoptotic proteins indicates a highly conserved cell death mechanism utilized by worms and mammals.

Ced-3 and its mammalian homologs are cysteine proteases called caspases (14, 15). Caspases are normally inactive in their zymogen form or proform. During apoptosis, a procaspase is proteolytically cleaved to generate a small subunit and a large subunit, and two cleaved caspase molecules form a heterotetramer, which is the active form of the enzyme. On the basis of structural studies of many caspases associated with specific peptide inhibitors (16–20), and a more recent study on the structure of free caspase-7, in both proform and activated form (21), a general mechanism for caspase activation becomes clear. The proteolytic cleavage of a caspase can induce a dramatic conformational change that exposes the catalytic pocket of the enzyme, and therefore results in its activation. The proteolytic activation of caspases can be achieved either by autocatalysis or by an upstream protease. A caspase that cleaves and activates itself is called an initiator caspase. Once an initiator caspase is activated, it can trigger a cascade to activate downstream executioner caspases. Subsequently, the activated executioner caspases cleave numerous cellular targets to destroy normal cellular functions, activate other apoptotic factors, inactivate antiapoptotic proteins, and eventually lead to apoptotic cell death (14, 15). The central role of caspase activity in apoptosis is further underscored by the observation that inhibition of caspase activity can block apoptosis and all classical morphological changes associated with the process (14, 15). Therefore, understanding caspase

activation is essential for apoptosis research and development of therapies for apoptosis-related diseases.

THE DEATH RECEPTOR-MEDIATED CASPASE ACTIVATION PATHWAY

One of the caspase activation pathways being characterized in mammals is the cell surface death receptor-mediated pathway (22). This pathway is initiated by extracellular hormones or agonists that belong to the tumor necrosis factor (TNF) superfamily, including TNFα, Fas/CD95 ligand, and Apo2 ligand/TRAIL. These agonists recognize and activate their corresponding receptors, members of TNF/NGF receptor family, such as TNFR1, Fas/CD95, and Apo2. Then, via a series of protein-protein interactions involving domains, which include the death domain and the death effector domain, the receptors will recruit specific adaptor proteins to form a complex called the death-inducing signaling complex (DISC). DISC recruits and activates the initiator caspases, caspase-8 or caspase-10, probably by bringing the procaspases close enough in proximity so that they can cleave each other. These activated initiator caspases trigger a caspase cascade and subsequent cell death by activating downstream executioner caspases, such as caspase-3 and caspase-7.

Genetic evidence indicates that the cell surface death receptor-mediated apoptosis is critical for normal immune system function. For example, mutations on Fas and Fas ligand in humans can lead to a complicated immune disorder known as the autoimmune lymphoproliferative syndrome (ALPS) (23, 24), a resemblance of murine lymphoproliferation (lpr) and generalized lymphoproliferative disorder (gld) caused by Fas and Fas ligand mutations, respectively (25–27).

Identification of caspase-8/caspase-10 activation by the TNF pathway revealed an important apoptosis mechanism. However, this pathway could not answer many outstanding questions in the field. First, this receptor-mediated pathway does not explain the involvement of the Bcl-2 family members in apoptosis, whose worm counterpart Ced-9 negatively regulates the worm caspase, Ced-3. Second, there are numerous cases showing non-receptor-mediated caspase activation. Third, molecular cloning has identified many putative initiator caspases in addition to caspase-8 and caspase-10. All these questions set the stage for the cytochrome *c*-initiated caspase activation pathway, a pathway with multiple components homologous to the players in the *C. elegans* apoptosis pathway.

THE CYTOCHROME *C*-INITIATED CASPASE ACTIVATION PATHWAY

In 1995, the laboratory of Xiaodong Wang set out to study the mechanisms of caspase activation using an in vitro biochemical approach. Initially, using a cell-free system to study apoptosis seemed unfeasible because the programmed

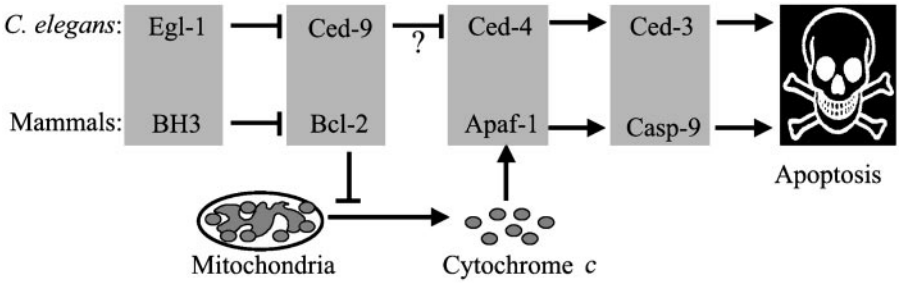

Figure 1 A comparison of the *C. elegans* programmed cell death pathway and the mammalian cytochrome *c*-mediated death pathway. The homologous molecules of the two pathways are labeled with shaded boxes. The question mark indicates that the mechanism by which Ced-9 inhibits Ced-4, whether via a direct interaction or not, has not yet been directly demonstrated.

cell death requires intact cellular architecture, and processing of cells to a cell-free state virtually kills them. However, apoptosis is unique. Caspase activation is one of its hallmarks, and preparation of a cell-free system from naïve, healthy cells does not activate caspases even though it kills all the cells (28). Therefore, it is theoretically possible to study caspase activation in vitro.

Then the question became how to initiate the caspase cascade in the cell-free system. The laboratory found that addition of the nucleotide dATP, or the less potent ATP, induced activation of caspase-3, a major executioner caspase in HeLa cell cytosolic extracts. This assay led to purification of the first protein required for dATP-triggered caspase-3 activation, which turned out to be cytochrome *c* (28). Subsequently, the other two components required for dATP-initiated caspase-3 activation were identified as Apaf-1, which is the binding partner of dATP and homologous to the *C. elegans* protein Ced-4 (29), and caspase-9, an initiator caspase homologous to *C. elegans* caspase Ced-3 (30).

The role of cytochrome *c* in activating apoptosis seemed puzzling at first glance because it is an essential protein in energy production and is located inside the mitochondria. But the pivotal role of cytochrome *c* in apoptosis was quickly confirmed in large by two results. The first one was the identification of its downstream binding partner, Apaf-1, a homolog of *C. elegans* Ced-4 (29). The second was the demonstration that Bcl-2 inhibits cell death by preventing cytochrome *c* release from mitochondria (31). Therefore, the discovery of a cytochrome *c*-mediated caspase activation pathway delineated a mammalian caspase activation pathway that is the counterpart of the *C. elegans* cell death pathway (Figure 1), and it led to identification of the mechanism by which the oncogene product Bcl-2 prevents apoptosis. As illustrated in Figure 1, in the mammalian pathway, the Egl-1 homologs are BH3-only proteins, such as Bim, Bid, Bad, Box, Noxa, and Puma. The Ced-9 homologs are the antideath members of the Bcl-2 family, such as Bcl-2, Bcl-XL, and Mcl-1. The Ced-4 homolog is

Apaf-1, and the Ced-3 homolog is caspase-9. An obvious difference between these two pathways is the function of Bcl-2 in mammalian cells and Ced-9 in the worms. Although these proteins are homologous to each other, Bcl-2 functions to inhibit cytochrome *c* release from mitochondria and thereby prevent downstream caspase activation, whereas Ced-9 is believed to directly inhibit the activity of Ced-4 to recruit and activate the worm caspase, Ced-3 (3–5).

The physiologic roles of the cytochrome *c*-mediated caspase activation pathway are intriguing. Much knowledge was gained from targeted gene disruption studies in mice, and the gene for every component of the pathway downstream of mitochondria, including cytochrome *c*, has been knocked out (32–37). In vitro studies showed that the embryonic fibroblast cells with Apaf-1, caspase-9, caspase-3, or cytochrome *c* knocked-out are resistant to various apoptotic stimuli. At the whole-animal level, the predominant phenotype shared by the knockout of Apaf-1, caspase-9, and caspase-3 genes is a severe developmental defect in the central nervous system (CNS) that results in the protrusion of brain tissue from the forehead and perinatal lethality. These results indicate an essential role of this apoptotic pathway in brain development. However, it is peculiar to see that this is the only predominant phenotype because apoptosis has been demonstrated to be involved in development of other body processes, such as the immune system. Therefore, there must be other tissue-specific pathways involved in the development of these organs, for example, the cell surface receptor-mediated death pathway.

On the other hand, the cytochrome *c*-mediated pathway is still likely to be involved in other biological events, including immune system homeostasis and elimination of damaged or harmful cells, during the normal life span after birth. It is unfortunate that conventional gene disruption of Apaf-1, caspase-9, and caspase-3 all result in lethality upon birth, raising the necessity to engineer more sophisticated, tissue and time-specific conditioning-knockout or transgenic animal models. Until now, most supporting evidence for the critical roles of the pathway in after-birth life is from tissue culture studies. For example, numerous experiments revealed that the pathway is essential for stress-induced and genotoxic-induced cell death, indicating the pathway plays a pivotal role to protect the organism from deadly diseases such as cancer (33, 34, 38, 39). This notion is supported by the finding that many malignant human melanoma cells, from both cancer patients and established cell lines, lose expression of Apaf-1 protein and are resistant to chemotherapy or P53-induced apoptosis (40).

Besides its essential role in CNS development and mediating stress-induced apoptosis, another function of the cytochrome *c*-mediated caspase activation pathway is that it can always serve as an amplifying/accelerating route for other apoptotic pathways, such as the death receptor pathway (15, 41, 42) and the cytotoxic T lymphocyte-mediated pathway (43, 44). In these pathways, although downstream executioner caspases can be directly activated by upstream proteases, i.e., caspase-8/10 for the death receptor pathway (41, 42) and Granzyme B for cytotoxic T lymphocyte pathway (43), these upstream proteases can also

cleave and activate Bid, a prodeath member of the Bcl-2 family. The truncated Bid (t-Bid) is targeted to mitochondria to induce cytochrome *c* release and the subsequent cytochrome *c*-mediated caspase activation pathway (41). This amplification process is particularly important in certain cells known as type II cells. In these cells, the death receptor-mediated apoptosis has to be mediated by the mitochondria pathway, and as a result it can be blocked by antiapoptotic members of the Bcl-2 family (45). Mechanistically, the death receptor-mediated caspase cascade in type II cells is inhibited by inhibitor of apoptosis (IAP) proteins, which are inhibitors of caspases, and the inhibitory activity of IAPs needs to be antagonized by a protein released from mitochondria, SMAC/DIABLO, to ensure progression of programmed cell death (46, 47). (IAP and SMAC/DIABLO will be discussed in detail below.) The amplification role of the cytochrome *c* pathway for other apoptosis pathways has profound therapeutic implications: When drugs targeted to both the cytochrome *c* pathway and other pathways are combined, a synergistic effect could be achieved, even though separate usages only have poor effect. This cocktail therapy might have huge benefits, especially for cancers with type II cell origin.

MITOCHONDRIA, AN ORGANELLE FOR LIFE AND DEATH

Many cellular structures and organelles are damaged or destroyed as a consequence of apoptosis (1). However, mitochondria, the organelle essential for life, is not only affected passively but is also actively involved in promoting apoptosis, as revealed by the cytochrome *c*-mediated caspase activation pathway. More mitochondrial proapoptotic proteins have been discovered. As was the case in the discovery of the apoptotic function of cytochrome *c*, the other proteins were not necessarily found from studies designed to target a potential apoptotic function of mitochondria. For example, another proapoptotic activity, SMAC/DIABLO, was not realized to be a mitochondrial protein until its identification and characterization (48, 49).

The IAP family of proteins (50) preluded discovery of SMAC/DIABLO. IAP proteins inhibit caspase activity by directly binding to the active enzymes (51, 52). These proteins contain single or multiple baculovirus IAP repeat (BIR) domains, which are responsible for the caspase inhibitory activity (50). It is likely that IAP proteins serve to inhibit residual or unwanted caspase activity in healthy cells. But then the question is, When cells are committed to apoptosis, is there a specific mechanism to antagonize the inhibitory role of IAP and thereby render more potent caspase activity? Such a mechanism was identified by two independent groups that applied distinct approaches. Du et al. (48) observed that dATP-initiated caspase-3 activation in HeLa cell cytosolic extracts could be greatly enhanced by a HeLa cell membrane fraction solubilized with detergent. The protein responsible for the enhancement was purified and found to be, again,

a mitochondrial protein. This novel mitochondrial protein was called the second mitochondria-derived activator of caspase (SMAC), and it was found that SMAC enhances caspase activation via antagonizing IAP function. At about the same time, Vaux and colleagues (49) made the same discovery by directly searching for IAP antagonists using a coimmunoprecipitation approach. They named the protein DIABLO (direct IAP binding protein with low pI).

There is documentation that expression of various IAP proteins is aberrantly upregulated in certain cancer tissues. For example, Survivin, a member of the IAP family, is overexpressed in most cancer cells (53); another member, ML-IAP/Livin, was originally identified because of its overexpression in human melanoma (54); cIAP1 is overexpressed in esophageal squamous cell sarcoma (55); and the cIAP2 locus is translocated and results in a fusion in mucosa-associate lymphoid lymphoma (56). Therefore, upregulation of IAPs might contribute to oncogenesis, and they can be categorized as oncogenes just like antiapoptotic members of the Bcl-2 family. However, it should be noted that the oncogenecity of Survivin might be due to its function in cytokinesis rather than that in apoptosis (57).

The oncogenic nature of IAP proteins makes them potential drug targets in IAP-overexpressing cancers. A mechanism-based drug design strategy is to develop drugs mimicking SMAC/DIABLO function. Structural studies of IAP-caspase complexes (58–60), SMAC (61), and SMAC-IAP complexes (62, 63) provide valuable insight for this purpose. It was found that the first four amino acids of mature SMAC, AVPI interact tightly with the BIR3 domain of xIAP, and the first residue alanine fits perfectly into a groove of the BIR3 domain. Consistently, a single mutation of this N-terminal alanine of SMAC to any other tested residue completely abolishes the ability of SMAC to interact with and suppress IAP activity. Similarly, addition of a single residue in front of this critical alanine does the same thing. More strikingly, the small peptide AVPI and other synthetic peptides with conserved alterations of the three later residues can also interact and suppress IAP, though with less potency than the SMAC protein (61). This result provides a promising approach for designing drug leads to attack IAP proteins. Practically, to make a feasible AVPI-like drug lead, cell permeability, peptide stability, and many other parameters should all be taken into account.

Discoveries of the mitochondrial protein SMAC/DIABLO in addition to cytochrome *c* as a proapoptotic player further suggest a central role of mitochondria in programmed cell death. And the story does not end here. Another mitochondrial protein, Omi/HtrA2, can also function as SMAC/DIABLO to antagonize the caspase inhibitory activity of IAP (64–68). A difference between SMAC and Omi/HtrA2 is that the latter is also a serine protease that can proteolytically cleave and inactivate IAP proteins, and therefore it is presumably a more efficient IAP suppressor than SMAC (69, 70). In addition to proteins that can trigger or enhance caspase activation, mitochondria also release proapoptotic proteins with functions unrelated to caspase activation. Such proteins include

apoptosis inducing factor (AIF) (71) and endonuclease G (Endo G) (72). These proteins are involved in DNA fragmentation and subsequent chromosomal condensation, a hallmark morphological feature of programmed cell death.

Why do cells utilize so many mitochondrial proteins as apoptotic factors? Strategically, it is an efficient and safe mechanism. In normal cells, these proteins are all in mitochondria, but their targets are in either cytoplasm (e.g., Apaf-1 for cytochrome *c* and IAP for SMAC/DIABLO) or the nucleus (e.g., genomic DNA for Endo G). This spatial segregation ensures that the proteins perform their killing functions only when they are deliberately released from the organelle during apoptosis. Although these proteins are transiently exposed to the cytoplasm when newly synthesized, their apoptotic functions require them to be processed inside of mitochondria. For example, cytochrome *c* has to be folded into the mature, heme-bound form to activate Apaf-1 (28), and the mitochondria target sequence of SMAC/DIABLO has to be removed in order to antagonize IAP (48, 49). Another advantage of spatial separation is that these proteins can be multifunctional, i.e., they can have other functions inside mitochondria in normal cells. For example, cytochrome *c* is an essential component of the mitochondrial electron transfer chain, Omi/HtrA2 can function as molecular chaperone and degrade denatured proteins (73–75), and AIF, with a pyridine nucleotide-disulphide oxidoreductase domain, can protect cells from oxidative stress (76). Whether SMAC/DIABLO and Endo G also have nonapoptotic functions is not clear.

In summary, a central role of mitochondria in mammalian apoptosis has been firmly established, though more detailed work is needed for better understanding. The organelle is involved in both caspase-dependent (cytochrome *c*, SMAC/DIABLO, and Omi/HtrA2) and caspase-independent (Endo G and AIF) cell death mechanisms, and release of the mitochondrial death proteins is closely regulated by the Bcl-2 family of proteins. The apoptotic function of mitochondria is probably not limited to vertebrates. Originally, it was believed that mitochondria did not have apoptotic function in *C. elegans* because the Bcl-2 homolog Ced-9 was thought to directly inhibit Ced-4 rather than inhibit mitochondria-releasing events (2). However, this proposed mechanism for Ced-9 still lacks direct biochemical demonstration, and the mitochondrial localization of Ced-4 and Ced-9 (77) suggests a connection of the worm death pathway with the organelle. Furthermore, recent studies strongly support an apoptotic role for mitochondria in *C. elegans*, carried out by the worm counterparts of Endo G and AIF, two mitochondrial proteins (78–80).

REGULATION OF MITOCHONDRIAL FUNCTIONS BY THE Bcl-2 FAMILY

Retrospectively, it is now clear why protein release from the mitochondria has to be precisely regulated by a big family of proteins, the Bcl-2 family members, and why malfunction of these proteins has severe consequences, such as B-cell

lymphoma caused by translocation of Bcl-2 locus. Mechanistically, although a complete picture has been elusive, considerable progress has been made on regulation of mitochondrial protein release by Bcl-2 proteins, summarized in Figure 2.

The Bcl-2 family can be divided into two subgroups, prodeath members and antideath members (81, 82). Within the same subgroup, the exact function of individual members can still be quite different. Among the prodeath members, Bak and Bax have been categorized as the last gateway of cytochrome *c* release, and their homooligomerization on the mitochondrial membrane is essential for release (83, 84). Bak/Bax function appears to be regulated by mitochondria-specific lipids and proteins, such as cardiolipin (85, 86) and VDAC2 (87), but whether any mitochondrial protein, such as VDAC (88–90), adenine nucleotide translocase (ANT) (91, 92), or components of permeability transition pore (91, 93, 94) are indispensable for Bak/Bax-induced protein release is still under debate. Other prodeath members, mainly BH3-only proteins, are thought to directly induce Bak/Bax oligomerization or to antagonize the antideath Bcl-2 members, and their regulation is very different. For example, Bid can be activated by caspase cleavage as discussed earlier. In response to growth hormones, Bad is phosphorylated by the PI3 K-Akt survival pathway and is thus inactive (95–97). Puma (98) and Noxa (99) are transcriptionally upregulated by p53 after DNA damage, and Bim is regulated both by phosphorylation (100–102) and by transcription (103).

The antiapoptotic Bcl-2 members prevent mitochondrial protein release by interacting with and inhibiting both Bak/Bax and BH3-only proteins. Interestingly, recent evidence from the laboratory of Xiaodong Wang indicates an apical function for the antideath member Mcl-1 that distinguishes it from Bcl-2 or Bcl-XL (104). Mcl-1 is a quick-turnover protein, and it can be degraded by the ubiquitination-proteasome pathway. When cells are treated with various apoptotic signals, Mcl-1 protein level decreases dramatically, due to a blockage of its synthesis as well as a possible acceleration of its degradation. And Mcl-1 disappearance is a prerequisite for downstream apoptotic events, such as Bcl-XL inactivation, Bim dephosphorylation, Bax translocation, Bax/Bak oligomerization, and subsequent cell death (Figure 2).

The function of the Bcl-2 family may be more than just regulation of mitochondria. New observations emerge suggesting that the Bcl-2 family can also regulate endoplasmic reticulum integrity and that this regulation is also important for apoptosis (105, 106). Moreover, a model suggesting an inhibitory role of Bcl-2 on caspase(s) upstream of cytochrome *c* release, via interaction with a yet-to-be-identified mammalian Ced-4 homolog other than Apaf-1, is still viable (107). This model of Bcl-2 function, rooted from its *C. elegans* homolog Ced-9, has, however, no direct experimental support. Yet evidence has been presented that in human fibroblasts transformed with the adenoviral oncogene E1A, cytotoxic stress can induce caspase-2 activation, which is upstream of and is required for cytochrome *c* release in this specific context (108).

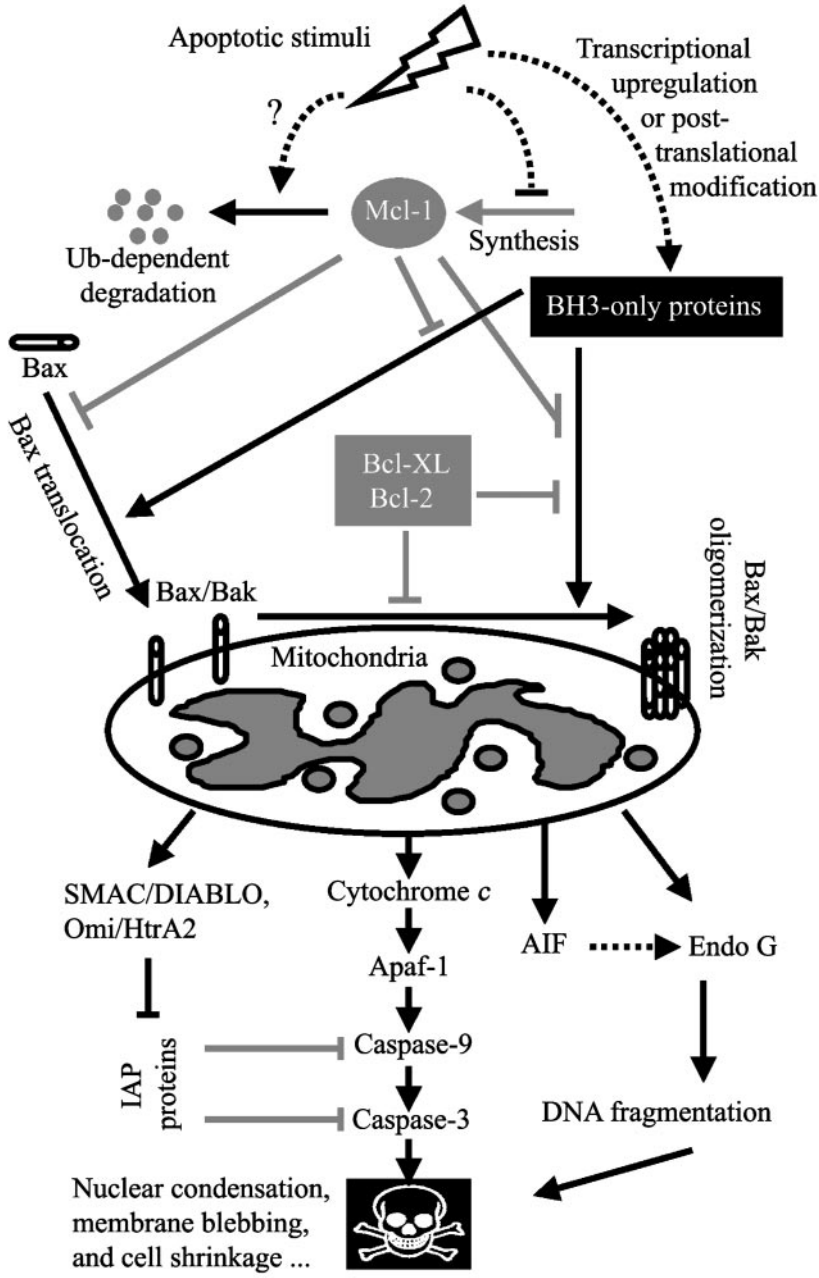

Figure 2 Regulation of the mitochondria apoptotic pathway by the Bcl-2 family members. Regulation by the Bcl-2 family members and the events downstream of mitochondria are shown.

THE APOPTOSOME, A CASPASE ACTIVATION MACHINERY

After release from mitochondria, the biochemistry of how cytochrome *c* triggers caspase activation is very complex. It was found that cytochrome *c* can interact with the C-terminal WD40 repeats of Apaf-1 and that this interaction is required for activation of the pathway (109). Further, a de novo reconstitution was achieved by using purified recombinant Apaf-1, procaspase-9, procaspase-3, and highly purified horse cytochrome *c* (110). When all the proteins are incubated together in the presence of nucleotide dATP/ATP, caspase-3 is activated. A striking phenomenon observed in this in vitro system is that Apaf-1 and cytochrome *c* are induced into a huge complex in a dATP/ATP-dependent manner. This complex was termed the apoptosome (110).

Binding of nucleotide to Apaf-1 is critical for apoptosome formation and is stimulated by cytochrome *c* (111). In the Ced-4 homologous domain of Apaf-1, there are classical Walker's A and B boxes, and they are believed to be the dATP/ATP binding and hydrolysis motifs (29). Using a rapid filtration assay, it was shown that cytochrome *c* stimulated dATP binding to Apaf-1 (111). This finding provides a biochemical explanation of how this essential protein for life can function as a death inducer. A surprising finding from this study is that nucleotide hydrolysis by Apaf-1 is not required for caspase activation because dATP, but not dADP, is associated with the mature apoptosome. There is no correlation between dATP hydrolysis and caspase activation, and a nonhydrolyzable ATP analog, AMPPCP, can also trigger apoptosome formation and caspase-3 activation (111).

Walker's boxes are also present in the Apaf-1 homologs, Ced-4 in *C. elegans*, and DARK in *Drosophila* (112). Presumably, nucleotide binding is also required for the ability of these two proteins to activate caspase, though formal experimental tests are needed for a final conclusion. Interestingly, sequence alignment of DARK predicts that its Walker's boxes have only nucleotide binding activity but not hydrolysis activity (112). Again, this prediction lacks experimental demonstration. But if this is true, it confirms the result that nucleotide hydrolysis by Apaf-1 is not required for caspase activation. Yet it also raises the question—why does mammalian Apaf-1 but not its *Drosophila* homolog possess nucleotide hydrolysis activity? Speculatively, the nucleotide hydrolysis activity of mammalian Apaf-1 can provide a safeguard mechanism because dATP or ATP, but not their hydrolyzed products, can drive the formation of a functional apoptosome complex. Thus, in a healthy cell, low levels of unwanted apoptosome complexes can be discharged by Apaf-1 nucleotide hydrolysis activity before they can reach their targets, such as initiator caspases, to cause irreversible damages.

The apoptosome machinery also provides a unique mechanism for caspase-9 activation. Unlike the conventional caspase activation mechanism in which a proper proteolytic cleavage of caspase is both necessary and sufficient for its activation, activation of caspase-9 by the apoptosome requires a constant association of the enzyme with the oligomeric death machinery (111, 113). As a

matter of fact, as long as the caspase is in this million-dalton complex, proteolytic processing is not really required for its activity (114). This observation raised the question—why is caspase-9 processed during apoptosis? A potential answer came from a biochemical-structural study (114). It was found that the caspase inhibitory protein XIAP can only interact with processed caspases (51, 52). For caspase-9, autocleavage of human caspase-9 at D315 exposes a new N terminus that starts with ATPF, similar to the N terminus of SMAC, AVPI. It was then confirmed by mutagenesis that this newly exposed sequence is required for interaction of the processed caspase-9 with XIAP (114). On the basis of this work, it is reasonable to assume the purpose of autocleavage of caspase-9 is to ensure that leaky, unwanted apoptosome-caspase-9 activity can be blocked by IAP proteins. In addition to the D315 autocleavage site, human caspase-9 also has a D330 site that can be recognized and cleaved by caspase-3, an executioner caspase in the downstream of caspase-9 (115). Kinetic studies showed that once this site is cleaved by caspase-3, the apoptosome-caspase-9 holoenzyme is eightfold more active than the D330A caspase-9 mutant associated with the apoptosome (autocleavage at D315 does not have this effect), suggesting this cleavage functions as a positive feedback loop (116). Although this is a reasonable hypotheses, physiological relevance of these two cleavage events needs to be addressed experimentally. The only apparent approach, although difficult, might be gene knock-in experiments.

Recently, a 27-Å three-dimensional structure of the apoptosome complex has been solved using cryo-electron microscopy (EM) technology (117). The structure gives insight on how the apoptosome assembles, how it activates caspase-9, and why activation of the caspase is distinct from the conventional caspase activation mechanism. The structure revealed that the apoptosome is composed of seven molecules of Apaf-1, and they form a symmetrical wheel-like structure. In the apoptosome complex, Apaf-1 interacts with the adjacent Apaf-1 molecules via their N-terminal CARD domains to form a central hub region, and the C-terminal WD40 repeats are extended to form the outside ring. On the basis of electron density, it was proposed that there was only one cytochrome *c* associated with each Apaf-1, though an early kinetic study suggested that there were two (118, 119). The central hub region is also the location for caspase-9 recruitment based on a cryo-EM study of the apoptosome when complexed with procaspase-9. Because there are seven Apaf-1 CARD domains in each apoptosome hub, caspase-9 can be highly enriched locally. Furthermore, it is likely that the CARD domain interaction between Apaf-1 and caspase-9 induces the enzyme to a fully extended, active conformation that cannot be achieved by proteolytic cleavage alone. It has been suggested that like other caspases, caspase-9 also needs to form a heterotetramer in the apoptosome to be active (117, 120, 121). However, direct evidence is required to confirm this assumption.

Incorporating all the biochemical and structural studies, a model illustrating the detailed biochemical mechanism of cytochrome *c*-induced caspase activation is presented in Figure 3. Upon sensing a variety of apoptotic stimuli, cytochrome *c* is released from mitochondria and associates with the apoptotic mediator Apaf-1 in its

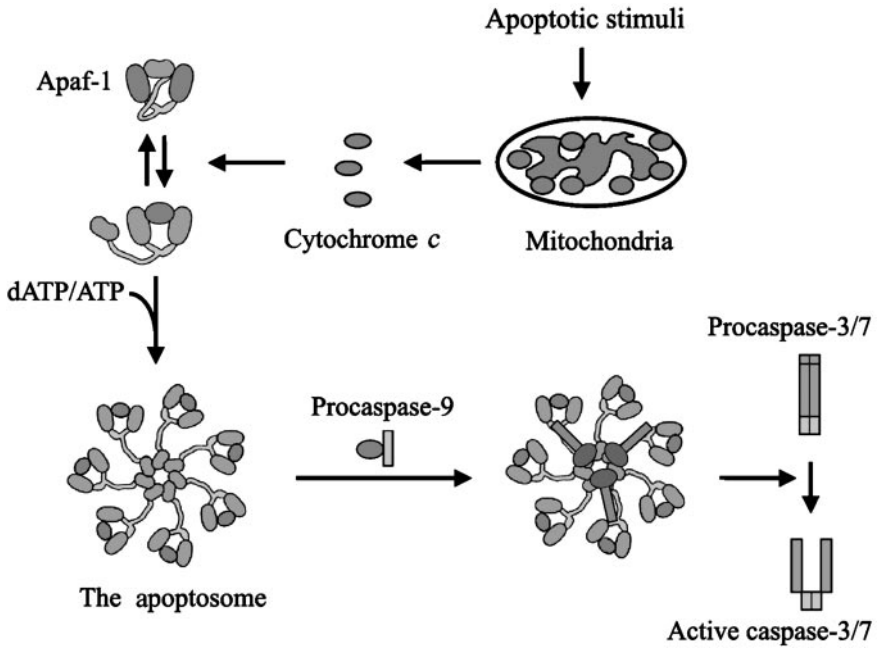

Figure 3 The mechanisms of apoptosome formation and caspase activation initiated by cytochrome *c* release.

C-terminal WD40 repeats. After association with cytochrome *c*, Apaf-1 switches from a rigid conformation to a more flexible one such that the nucleotide dATP/ATP binding activity of its Walker's motif is greatly facilitated. Binding of dATP/ATP in turn triggers formation of the active, seven-span symmetrical complex, the apoptosome, via interaction among the N-terminal CARD domains of the individual Apaf-1 molecules. The apoptosome subsequently recruits procaspase-9 into its central hub region through CARD domain interaction, and a conformational change of the enzyme is induced. Therefore caspase-9 and the apoptosome form an active holoenzyme to activate downstream executioner caspases, such as caspase-3 and caspase-7, which eventually lead to programmed cell death. In this pathway, IAP proteins function to inhibit caspase activity, and they can be overcome by mitochondrial proteins SMAC/DIABLO and Omi/HtrA2, as discussed above.

ADDITIONAL REGULATIONS OF THE CYTOCHROME *C* PATHWAY

It was long suspected that cytochrome *c*-mediated caspase activation has additional regulatory mechanisms based upon certain observations. For example, in the initial dATP-triggered caspase-3 activation assay, up to 1-mM dATP is

required to activate apoptosome formation and subsequent caspase activation in HeLa cell extracts, whereas in the final reconstitution system, micromolar levels of dATP are sufficient to activate these events (28, 110, 111). More importantly, the cellular concentration of dATP is ~10 micromoles, and it does not increase during apoptosis (122). This discrepancy of nucleotide concentration points to a potential regulation at the level of dATP binding to Apaf-1. Furthermore, the fact that the nucleotide binding motif is within the Ced-4 domains of Apaf-1 and its *C. elegans* and *Drosophila* counterparts also indicates the conservation and significance of nucleotide binding in this pathway. Another observation is from studies on an ovarian cancer cell line SKOV-3. Cytochrome *c* and dATP failed to activate caspase-3 in SKOV-3 cell extracts, even though the expression levels of Apaf-1, caspase-9, caspase-3, and IAP were all normal (123). It was found that apoptosome formation in the cell extracts could still be induced, but subsequent recruitment and activation of caspase-9 by the complex was defective (123). These results suggest a novel regulation at the level of caspase-9 activation by the apoptosome complex, and this regulation is repressed in the ovarian cancer cells. In addition, it was reported that multiple heat shock proteins, such as Hsp90, Hsp70, and Hsp27, could inhibit cytochrome *c*-initiated caspase activation by directly interacting with Apaf-1 or other players in the pathway (124–128). However, the physiological relevance of these in vitro experiments is not yet clear. Also, mounting evidence indicates a regulatory role of the signaling lipid ceramide in apoptosis, and under specific conditions, ceramide might function in the upstream of mitochondria via Bax activation, although the detailed molecular mechanism is still obscure (128a, 128b).

A recent chemical-biological study, initiated by a high-throughput screen to search for chemicals that can activate caspase-3 in HeLa cell extracts, sheds light on understanding the additional regulation of the cytochrome *c* pathway (129). An interesting analogy is that the high-throughput screen performed in this study is very similar to the original dATP assay (28), which can be viewed as the result of a primitive, infant form of chemical screen. These two screens share a common feature strategically: unlike many screens with defined targets, these screens were designed to study a biochemical event without knowing the direct targets and were therefore likely to identify new pathways. As a result, the earlier screen led to the discovery of the cytochrome *c*-initiated caspase activation pathway (28). The later screen fished out PETCM (α-(trichloromethyl)-4-pyridineethanol) as a caspase-3 activator (Figure 4), and further studies using PETCM revealed a regulatory pathway controlling the apoptosome machinery (129).

Biochemical studies of PETCM-triggered caspase-3 activation showed that the compound can activate apoptosome formation by suppressing the inhibitory effect of prothymosin-α (ProT), an oncogene product. After apoptosome formation, caspase-9 recruitment and activation can be enhanced by PHAP proteins, which are putative tumor suppressors. Previously, although these two proteins

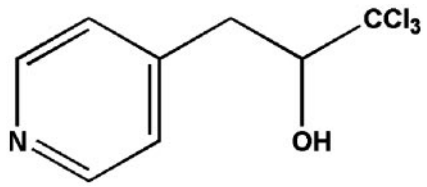

Figure 4 Covalent structure of PETCM (α-(trichloromethyl)-4-pyridineethanol).

were characterized as an oncoprotein and tumor suppressor, the biochemical activities responsible for these properties were not known (130–133). Therefore, the activities of these proteins in apoptosis might contribute to their functions in oncogenesis. Also, this provides an explanation of why a high amount of nucleotide is required for caspase activation in the crude system. It is likely that in cells, a PETCM-like signal is required to suppress inhibition of dATP binding to Apaf-1 by ProT during apoptosis, whereas in the cell extracts prepared from naïve HeLa cells, high amounts of dATP have to be used to overcome this suppression.

The oncogenic and tumor suppressive property of ProT and PHAP suggest that malfunction of this death regulatory pathway might result in tumorigenesis. This hypothesis is consistent with many reports showing overexpression of ProT in tumors, which include breast cancer, colon cancer, lung cancer, and liver cancer (134–137). Therefore, this new death regulatory pathway presents a potential target for cancer chemotherapy. In fact, development of cancer therapy was the original purpose of the chemical screen that uncovered PETCM. However, to make PETCM a realistic drug lead from the current form with an apparent EC50 of 0.05 mM (129), the compound has to be modified to get better potency as well as cell permeability. Additionally, its direct target needs to be identified in order to perform structure-based modification.

It is also possible that this pathway is involved in brain development and the neurodegenerative disorder spinocerebella ataxia type-1 (SCA1) because PHAP is upregulated in the early brain developmental stage characterized by massive apoptosis (138–140), and the protein has been reported to interact with Ataxin-1 protein, whose mutation by insertion of polyglutamine tract is the genetic cause of SCA1 (141).

Further studies of this pathway will focus on (*a*) identification of new components of the pathway (which include the direct PETCM target and the physiological signals whose function is mimicked by PETCM), (*b*) investigation of the physiological roles of the pathway, and (*c*) clinical applications. Overall, this new pathway revealed how complicated regulation of the cytochrome *c*-mediated apoptosis pathway can be and how it functionally interacts with multiple proteins involved in oncogenesis.

PERSPECTIVES

To date, the cytochrome *c*-mediated caspase activation pathway is well established, and its physiological and pathological significance is overwhelmingly supported by studies at biochemical, genetic, and cellular levels. The pathway is under precise regulation in a time- and spatial-specific manner, both before and after cytochrome *c* release. However, there are still many important questions. A few examples include, Mechanistically, how do the Bcl-2 family members, Bax and Bak in particular, interact with mitochondria to control release of cytochrome *c* and other proteins? Developmentally, how is this pathway regulated in a tissue-specific manner, especially in the nervous system? In terms of regulation, interaction of this pathway with other signaling networks, especially those functioning in oncogenesis, needs to be extensively investigated. And clinically, although this pathway is a promising cancer therapy target, the theoretical value is yet to be translated into medicine. Taking all of these into account, this complex and important pathway still presents an enormous challenge for both basic biological research and therapeutic exploration in the future.

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