

BIOCHEMICAL PATHWAYS OF CASPASE ACTIVATION DURING APOPTOSIS

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Key Words cell surface death receptor, mitochondria, Bcl-2, cytochrome *c*,
Apaf-1

■ **Abstract** Caspase activation plays a central role in the execution of apoptosis. The key components of the biochemical pathways of caspase activation have been recently elucidated. In this review, we focus on the two most well-studied pathways of caspase activation: the cell surface death receptor pathway and the mitochondria-initiated pathway. In the cell surface death receptor pathway, activation of caspase-8 following its recruitment to the death-inducing signaling complex (DISC) is the critical event that transmits the death signal. This event is regulated at several different levels by various viral and mammalian proteins. Activated caspase-8 can activate downstream caspases by direct cleavage or indirectly by cleaving Bid and inducing cytochrome *c* release from the mitochondria.

In the mitochondrial-initiated pathway, caspase activation is triggered by the formation of a multimeric Apaf-1/cytochrome *c* complex that is fully functional in recruiting and activating procaspase-9. Activated caspase-9 will then cleave and activate downstream caspases such as caspase-3, -6, and -7. This pathway is regulated at several steps, including the release of cytochrome *c* from the mitochondria, the binding and hydrolysis of dATP/ATP by Apaf-1, and the inhibition of caspase activation by the proteins that belong to the inhibitors of apoptosis (IAP).

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INTRODUCTION

Apoptosis, or cell suicide, is a form of cell death that is morphologically and biochemically distinct from necrosis. Although the concept of apoptosis was introduced 26 years ago (Kerr et al 1972), the mechanisms of how apoptosis is initiated and executed remained unclear until recently. During the past five years, tremendous progress has been made in understanding apoptosis as a result of molecular identification of the key components of this intracellular suicide program (reviewed by Ellis et al 1991, Steller 1995, Nagata 1997). The core of this cell suicide program is evolutionarily conserved from worm to human. It consists of three major components: the Bcl-2 family proteins; the caspases, which belong to a family of cysteine proteases that cleaves after aspartic acid residues; and the Apaf-1/CED-4 protein that relays the signals integrated by Bcl-2 family proteins to caspase (Adams & Cory 1998). Biochemical activation of these key components of the cell death program is responsible for the morphological changes observed in apoptosis, including mitochondrial damage, nuclear membrane breakdown, DNA fragmentation, chromatin condensation, and the formation of apoptotic bodies (Thornberry & Lazebnik 1998).

In this review, we focus on the biochemical pathways that control caspase activations, particularly the activation pathways that are initiated by cell surface death receptors and mitochondria.

CASPASES AND THEIR ACTIVATION DURING APOPTOSIS

The first caspase initially discovered as a cytokine-processing enzyme was designated interleukin-1 β -converting enzyme (ICE). Due to the rapid expansion of the expressed sequence tag (EST) database and the presence of the conserved pentapeptide sequences QACR(N/Q)G at the caspase active site, over 14 new caspases have been cloned in a short period of time (Thornberry & Lazebnik 1998) (Table 1). Caspase-1 and caspase-11 have been shown to function mainly in cytokine processing (Li et al 1995, Kuida et al 1995, Wang S et al 1998). On the other hand, caspase-2, -3, -6, -7, -8, -9, -10 are involved in the regulation and execution of apoptosis (Kuida et al 1996, 1998; Hakem et al 1998; Varfolomeev et al 1998; Bergeron et al 1998). The functions of the remaining caspases are largely unknown at this moment.

As the number of the cloned caspases and the volume of the sequenced genome increase, more proteins related to the caspases are being identified with divergence

even at the most highly conserved pentapeptide active site. Although CED-3 is the only caspase found to genetically promote apoptosis in *Caenorhabditis elegans*, three caspase-related proteins, the CSP genes, with divergent active sites have been found in the recently completed genome of *C. elegans*. For instance, CSP-1 has been shown to have protease activity despite the SACRG active site (Shaham 1998). Similarly, the Dredd caspase has been cloned from *Drosophila* with a QACQE active site and uncharacterized protease specificity (Chen et al 1998). Furthermore, sequencing the region encoding the Mediterranean fever gene revealed a human caspase-like gene with unknown function (Centola et al 1998). Some of these genes may turn out to be pseudogenes; however, others may represent novel classes of the caspase family with either atypical protease activities or an expansion of the family of caspase decoys such as FLAME, MRIT, and CASH (Hu et al 1997, Han et al 1997, Goltsev et al 1997).

All apoptotic caspases exist in normal cells as inactive enzymes analogous to the zymogens involved in the regulation of blood clotting. When cells undergo apoptosis, these caspases become activated through one or two sequential proteolytic events that cleave the single peptide precursor into the large and small fragments that constitute the active enzyme (Thornberry & Lazebnik 1998). There are currently two well characterized caspase-activating cascades that regulate apoptosis: one is initiated from the cell surface death receptor and the other is triggered by changes in mitochondrial integrity.

CASPASE ACTIVATION BY CELL SURFACE DEATH RECEPTORS

One pathway that leads to caspase activation is initiated by the engagement of cell surface death receptors with their specific ligands. Cell surface death receptors are a family of transmembrane proteins that belong to the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily. Mammalian death receptors include Fas/APO-1/CD95, TNFR1, DR-3/Apo-3/WSL-1/TRAMP, and the TRAIL receptors DR4/TRAIL-R1 and DR5/TRAIL-R2/TRICK2/KILLER (Ashkenazi & Dixit 1998). These receptors share a conserved cysteine-rich repeat at their extracellular domains. Although the regions of greatest sequence homology between superfamily members are extracellular, Fas and TNFR1 share a region of homology at the cytoplasmic face (68 amino acids) termed the death domain. This domain, which is discussed below, is required for apoptotic signaling by both Fas and TNFR1. The activating ligands for these death receptors are structurally related molecules that belong to the TNF gene superfamily (reviewed in Nagata 1997). Fas/CD95 ligand (FasL) binds to Fas, TNF and lymphotoxin α bind to TNFR1, Apo3 ligand (Apo3L) binds to DR3, and Apo2 ligand (Apo2L, or TRAIL) binds to DR4 and DR5 (reviewed in Ashkenazi & Dixit 1998).

When the Fas receptor binds its ligand, this recognition event is translated into intracellular signals that eventually lead to caspase activation. In particular, there

TABLE 1 Known caspase family proteases

	Synonyms	Prodomain module	Adapter protein	Comments
Mammalian large prodomain				
Caspase 2	Nedd2, ICH1	CARD	RAIDD	CASP2 -/- mice have female germ cell hyperplasia; decreased facial nuclei neurons attributed to loss of inhibitory short form of the protein; B cells resistant to granzyme B
Caspase 8	FLICE, MACH	DED	FADD	CASP8 -/- mice embryonic lethal, resistant to Fas, DR3, and TNFR; cardiac defects and MCH5 loss of hematopoietic precursor cells
Caspase 9	MCH6, ICELAP6	CARD	APAF1	CASP9 -/- mice have neuronal hyperplasia; thymocytes and MEFs resist etoposide, staurosporine, UV, and dexamethasone-induced cell death; sensitive to Fas and CD3 activation; lack of cytochrome <i>c</i> activation of caspases
Caspase 10	FLICE2, MCH4	DED	FADD	
Cytokine processing are related				
Caspase 1	ICE	—	—	CASP1 -/- mice resistant to LPS; deficient in IL-1 beta production
Caspase 4	TX, ICH2, ICErII	—	—	Cleaved by CASP8
Caspase 5	TY, ICErIII	—	—	
mCaspase 11		—	—	CASP11 -/- mice resistant to LPS; production of IL-1 alpha and beta blocked
mCaspase 12		—	—	
Caspase 13	ERICE	—	—	CASP13 cleaved by CASP8 and granzyme B ^a

are three distinct steps: ligand-induced receptor trimerization, the recruitment of intracellular receptor-associated proteins, and the initiation of caspase activation (Figure 1).

The binding of FasL to Fas receptor induces trimerization of Fas. The cytoplasmic region of Fas, which contains a death domain (DD), recruits a DD-containing adaptor molecule designated FADD (Fas-associating protein with death domain). FADD also contains a death domain at its C terminus and binds to Fas via interactions between the death domains. A single point mutation in this domain abrogates the apoptotic signal, suggesting that the death domain is required for initiating the signal inside the cell (Boldin et al 1995, Chinnaiyan et al 1995). Several other novel proteins that contain homologous death domains have subsequently been identified, including TRADD (TNF-receptor associated death domain), RIP (receptor interacting protein), RAIDD, and MADD (reviewed in Cryns & Yuan 1998).

Whereas the death domain of FADD is necessary for physical association with the ligand bound-death receptor complex (the death-inducing signaling complex, or DISC), the N terminus of FADD, which is termed the death effector domain (DED), is critical for recruiting the upstream procaspases such as procaspase-8 and/or procaspase-10. Procaspase-8 contains two DED domains at the N-terminal region through which it binds FADD. The C-terminal domain of procaspase-8 contains a caspase homology region. Immediately after recruitment, procaspase-8 is proteolytically processed to the active forms that consists of large and small catalytic subunits (Boldin et al 1996, Muzio et al 1996, Srinivasula et al 1996).

Several lines of evidence suggest that procaspase-8 can be proteolytically activated by oligomerization following its recruitment to the DISC. First, chemically

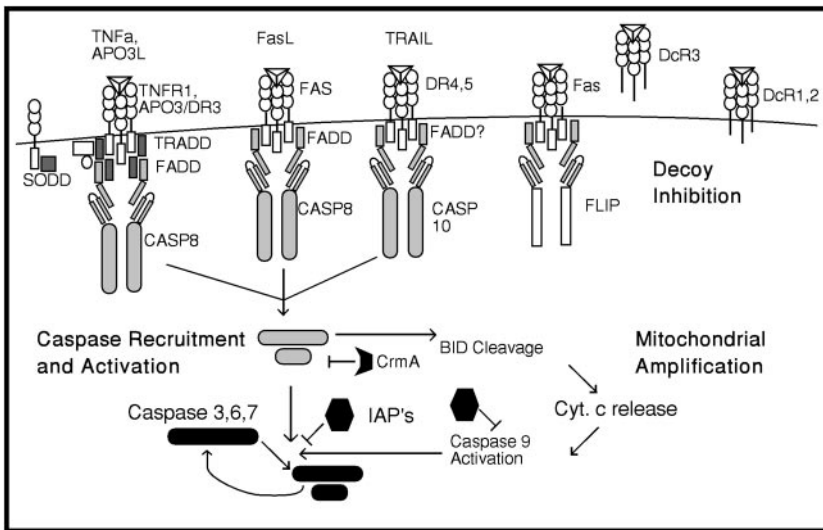


Figure 1 Caspase activation by cell surface death receptors.

induced dimerization of membrane-targeted procaspase-8 resulted in its proteolytic autoactivation and subsequent activation (Muzio et al 1998). Likewise, transfecting cells with a chimeric caspase-8 construct in which its prodomain had been replaced with an N-terminal CD8 dimerization domain resulted in caspase-8 autoactivation and apoptosis (Martin et al 1998). Recently, by using two inducible oligomerization systems, Yang et al showed that oligomerization activates autoproteolysis of procaspase-8, which in turn activates their cell death activity. This study further demonstrated that the prodomain of procaspase-8 is first separated from the protease domain, followed by the separation of the large and small protease subunits (Yang et al 1998), suggesting that procaspases may have weak proteolytic activity and cleave one another when they are brought into close proximity.

REGULATION OF CELL SURFACE DEATH RECEPTOR ACTIVATION

There are three distinct mechanisms involved in regulation of death receptor activity. The first mechanism prevents procaspase recruitment and/or activation at the DISC. Recently, several endogenous inhibitors of death-receptor-induced caspase activation have been identified (reviewed by Cryns & Yuan 1998). One group of these inhibitors belong to a family of viral proteins, FADD-like ICE inhibitory proteins (vFLIPs), that contain two DEDs (Hu et al 1997, Thome et al 1997). The presence of DEDs in these proteins prevents procaspases recruitment to the DISC by competing with the procaspases for binding to the DED of FADD.

A mammalian homologue of viral FLIP (cFLIP) subsequently identified by several laboratories is also known as Casper, I-FLICE, FLAME, CASH or MERIT (Srinivasula et al 1997, Inohara et al 1997b, Hu et al 1997, Goltsev et al 1997, Han et al 1997). There are two alternatively spliced forms of FLIP, FLIP-long and FLIP-short. Interestingly, in addition to the two N-terminal DEDs, FLIP-long possesses a C-terminal domain that resembles caspase-8, although it lacks protease activity due to the absence of several conserved residues at the caspase active sites. As expected, both isoforms of cellular FLIP bind to FADD, procaspase-8, and procaspase-10 via DED interactions (Irmeler et al 1997) and block the processing of procaspases at the DISC due to the competition for DED (Irmeler et al 1997, Goltsev et al 1997). Consistent with this mechanism, cells transfected with FLIP became resistant to death-receptor-inducing stimuli, but not to other apoptotic stimuli such as staurosporine or UV-irradiation (Irmeler et al 1997).

The second mechanism for inhibiting death-receptor-induced apoptosis is through the expression of decoy receptors for TRAIL. Decoy receptors are closely related to the TRAIL receptors DR4 and DR5 (Golstein 1997). However, this receptor lacks the cytoplasmic domain (DcR1) or contains a cytoplasmic region with a truncated death domain (DcR2), thereby specifically inhibiting TRAIL-induced apoptosis by sequestering the TRAIL ligand away from the death receptors DR4 and DR5 (Marsters et al 1997). Interestingly, normal human tissues express these

decoy receptors more abundantly than tumor tissues (reviewed in Ashkenazi & Dixit 1998), raising the possibility that the increased sensitivity to apoptosis in tumors is partly due to the decreased expression of decoy receptors.

Recently, the identification of a different type of decoy receptor has been reported. Unlike decoy receptors 1 and 2, which are specific for TRAIL ligand, decoy receptors 3 (DcR3) can bind Fas ligand and block its binding to Fas receptor (Pitti et al 1998). In addition, DcR3 is amplified in lung and colon cancer cells. Although its significance is not yet clear, it is intriguing to speculate that the overexpression of DcR3 may provide a mechanism for tumor cells to evade the immune surveillance by cytotoxic lymphocytes.

Finally, the third mechanism for preventing death-receptor-inducing stimuli is by directly inhibiting the proteolytic activation of the initiator procaspases such as procaspase-8 or procaspase-10. An example of this class of inhibitor is the viral protein crmA, a member of the serpin family that is a potent inhibitor of procaspase-8 (Ray et al 1992, Komiyama et al 1994). CrmA can inhibit both autoproteolytic activation of procaspase-8, as well as the ability of caspase-8 to cleave Bid (discussed below), which then leads to cytochrome *c* release and the activation of the downstream caspases (Luo et al 1998, Li et al 1998).

Most recently, a 60-kDa protein, silencer of death domains (SODD), has been identified (Jiang et al 1999). In the absence of TNF treatment, SODD is associated with the death domain of tumor necrosis factor receptor type 1 (TNF-R1), thereby preventing the spontaneous signaling by death domain-containing receptors.

Despite recent advances in understanding how ligand binding to cell surface death receptors initiates caspase-8 activation, one puzzle still remains. In some cell types, caspase-8 is activated within minutes of Fas activation. However, in other cell types, caspase-8 activation proceeds much slower. The activation step often occurs within several hours and can be inhibited by overexpression of Bcl-2 on the mitochondria. Interestingly, the levels of FADD and procaspase-8 are indistinguishable between these two cell types. Instead, the rate of the DISC formation is very different (Scaffidi et al 1998). The explanation for this phenomenon is currently unknown. Unlike the Apaf-1 pathway (discussed below), the *in vitro* system for caspase-8 activation is not available; therefore, it is still unclear whether there are other factors involved in addition to FasL, Fas, FADD, and SODD.

CASPASE ACTIVATION BY MITOCHONDRIA

Another caspase-activating pathway was discovered by the observation that the addition of ATP, or preferably dATP, to cell extracts prepared from normally growing cells initiates an apoptotic program, as measured by caspase-3 activation and DNA fragmentation (Liu et al 1996). Biochemical fractionation and reconstitution experiments have led to the identified of three proteins that are necessary and sufficient to activate caspase-3 *in vitro*.

Absorbance spectrum, protein sequencing and immunoreactivity identified the first protein factor as human cytochrome *c*. Cytochrome *c* isolated from other mammalian sources can substitute for human cytochrome *c* in the caspase-3 activating activity (Liu et al 1996). Interestingly, only holocytochrome *c*, and not apocytochrome *c* that is newly translated in the cytosol, is functional in this in vitro assay. In addition, the apoptosis-inducing activity of cytochrome *c* seems to be independent of its redox status (Liu et al 1996, Yang et al 1997, Kluck et al 1997, Bossy-Wetzel et al 1998). Consistent with these observations, it has been shown that cytochrome *c* is indeed released from mitochondria in cells undergoing apoptosis induced by a variety of stimuli, including DNA damaging agents, kinase inhibitors, and activation of cell surface death receptors (Yang et al 1997, Scaffidi et al 1998). Once released from the mitochondria, cytochrome *c* works together with the other two cytosolic protein factors, Apaf-1 and procaspase-9, to activate caspase-3 (Li et al 1997) (Figure 2).

Apaf-1 is a 130-kDa protein consisting of three distinctive domains. The N-terminal 85 amino acids shows homology with the prodomain of several

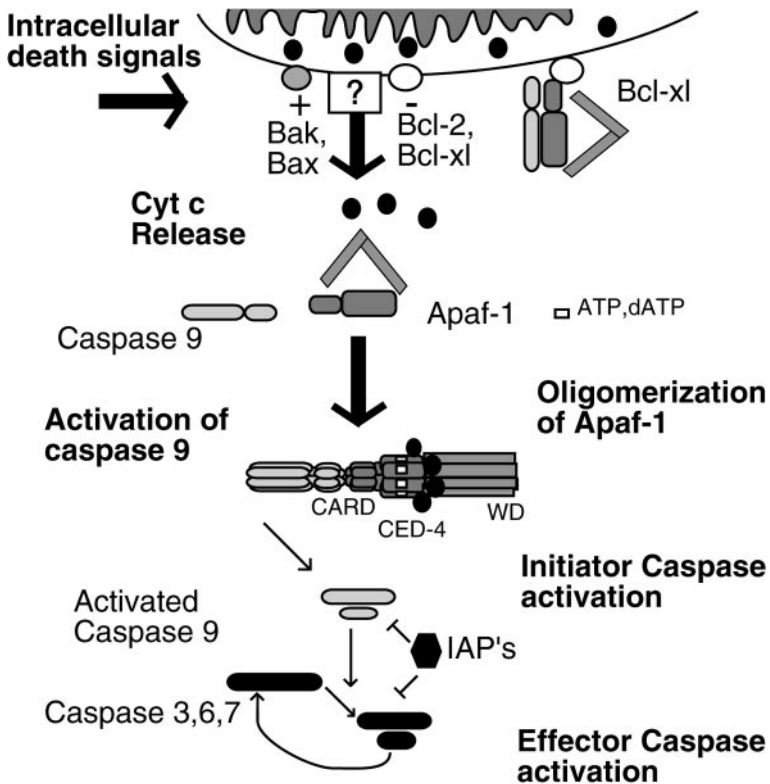


Figure 2 Caspase activation by mitochondria.

caspases such as caspase-1, caspase-2, and caspase-9. This domain is proposed to function as the caspase recruitment domain (CARD) that binds caspases with a similar CARD (Hofmann et al 1997). Of all the CARD-carrying caspases, only procaspase-9 is activated by Apaf-1 (Hu et al 1998). Following the CARD, Apaf-1 contains a stretch of 310 amino acids that shows 50% similarity in primary amino acid sequence to the *C. elegans* death-promoting protein CED-4. The most noticeably conserved regions of this domain are the Walker's A and B boxes believed to be required for nucleotide binding (Zou et al 1997). Mutations in this nucleotide-binding site abolish both Apaf-1 and CED-4 function (Seshagiri & Miller 1997a, Zou et al 1999). The C-terminal half of Apaf-1 is composed of 12–13 WD-40 repeats (from different spliced forms), a motif that mediates protein-protein interactions. Deletion of the WD-40 repeats renders Apaf-1 constitutively active in vitro, independent of ATP/dATP and cytochrome *c* (Srinivasula et al 1998). However, the activated caspase-9 cannot be released from Apaf-1 when the WD-40 repeats are truncated, indicating that this domain normally has dual functions that inhibit Apaf-1 activity and to help release the activated caspase-9 (Srinivasula et al 1998, Zou et al 1999).

Procaspase-3 activation by Apaf-1 and caspase-9 has been characterized using highly purified recombinant Apaf-1 and procaspase-9. Biochemical analysis reveals a multistep reaction leading to caspase-3 activation. First, Apaf-1 binds ATP/dATP and hydrolyzes it to ADP and dADP, respectively. This hydrolysis, however, does not have any functional consequence if cytochrome *c* is absent. Likewise, cytochrome *c* will bind Apaf-1 in the absence of ATP. This complex, however, is unstable and inactive. In contrast, in the presence of cytochrome *c*, the binding and hydrolysis of ATP/dATP promote the formation of a multimeric Apaf-1/cytochrome *c* complex. This multimeric complex is fully functional in recruiting and activating procaspase-9 (Zou et al 1999). Therefore, the formation of this multimeric complex of Apaf-1/cytochrome *c* represents the commitment step in caspase activation. Once this complex is formed, procaspase-9 is recruited to the complex at approximately 1:1 ratio to Apaf-1 and becomes activated through proteolysis. The active site mutant of procaspase-9 cannot be activated even though it can be recruited to the complex. This finding suggests that Apaf-1-mediated procaspase-9 activation is through autocatalysis (Zou et al 1999).

Finally, activated caspase-9 is subsequently released from this complex to cleave and activate downstream caspases such as caspase-3, -6, and -7. The formation of this multimeric Apaf-1/cytochrome *c* complex may serve two purposes: first, to increase the local concentration of procaspase for intermolecular cleavage and, second, to set the threshold of caspase activation relatively high so that occasional leakage of cytochrome *c* will not cause cells to commit to apoptosis. The linear caspase activation pathway that begins with mitochondrial damage followed by cytochrome *c* release and Apaf-1 activation has been confirmed in vivo, as shown by the recent results from the gene knockout experiments. First caspase-3, caspase-9, and Apaf-1 knockout mice show remarkably similar phenotypes. All these knockout mice display excessive neuronal cells, both progenitors and mature neurons,

in their brains. These mice die within one or two days postnatal. Furthermore, in Apaf-1 knockout mice, caspase-9 and caspase-3 cannot be activated in response to various apoptotic stimuli, even though cytochrome *c* release still occurs. Likewise, caspase-3 activation is abolished in caspase-9 knockout mice (Hakem et al 1998, Kuida et al 1998, Yoshida et al 1998, Cecconi et al 1998).

REGULATION OF MITOCHONDRIAL-INITIATED CASPASE ACTIVATION

The primary regulatory step for mitochondria-mediated caspase activation might be at the level of cytochrome *c* release. Cytochrome *c* normally resides exclusively in the intermembrane space of mitochondria, whereas its cofactors, Apaf-1 and procaspase-9, are both cytosolic proteins. Microinjection or electroporation of cytochrome *c* induces apoptosis in certain cell types (Srinivasan et al 1998, Garland & Rudin 1998), indicating that in these cells cytochrome *c* release might be the key regulatory step. The known regulators of cytochrome *c* release are Bcl-2 family proteins. Overexpression of Bcl-2 or Bcl-xL blocks cytochrome *c* release in response to a variety of apoptotic stimuli (Yang et al 1997, Kluck et al 1997, Vander Heiden et al 1997, Scaffidi et al 1998). On the contrary, the proapoptotic members of Bcl-2 family proteins such as Bax (Rosse et al 1998, Juergensmeier et al 1998) and Bid (Luo et al 1998, Li et al 1998, Kuwana et al 1998, Gross et al 1999) promote cytochrome *c* release from the mitochondria. The precise biochemical mechanisms of cytochrome *c* release and its regulation by Bcl-2 family proteins remain elusive. Currently, three theories have been proposed: the permeability transition pore theory of the Kroemer group (Kroemer et al 1997), the ion flow model of the Thompson group (Vander Heiden et al 1997), and the BH3-containing protein model (Cosulich et al 1997)

Bcl-2 As a Regulator of the Permeability Transition Pore

Bcl-2 family members are present on the cytoplasmic surface of various organelles, including the mitochondria, endoplasmic reticulum, and nucleus (Park & Hockenbery 1996). Initial work on the role of mitochondria in apoptosis revealed that certain signs of mitochondrial damage, such as loss of membrane potential, were early markers of a commitment to cell death (Zamzami et al 1996, Marchetti et al 1996). Researchers drew upon previous work that described an activity designated as the permeability transition pore (PTP), which regulates the potential of the inner mitochondrial membrane. It has been speculated that the PTP is regulated by the Bcl-2 family (Kroemer et al 1997, Marzo et al 1998). Accordingly, pharmacological inhibitors of the PTP, such as cyclosporin A, were shown to inhibit certain types of apoptotic stimuli, such as Bax-mediated cell death (Juergensmeier et al 1998, Pastorino et al 1998). Whereas the identity of the PTP remains elusive, cyclophilin D (Crompton et al 1998), the adenine nucleotide transporter (ANT)

(Marzo et al 1998) on the inner membrane and porin on the outer membrane of the mitochondria are known to be involved (Narita et al 1998). It is currently unknown how the opening of the pore leads to loss of outer membrane integrity, but it is speculated the disruption of electrostatic and osmotic gradients leads to swelling of the mitochondria and release of calcium and intermembrane proteins such as cytochrome *c* and apoptosis-inducing factor (AIF) (Kroemer et al 1997).

Bcl-2 As an Ion Channel

The possibility that Bcl-2 possesses ion channel activity was suggested by the three-dimensional structure (NMR) of Bcl-xL, which resembles the structure of bacterial toxins such as diphtheria (Muchmore et al 1996) (Figure 3). These toxins are known to insert into lipid bilayers and form channels capable of conducting ions (Schendel et al 1997, Senzel et al 1998). In accordance with this model, the Bcl-2 homologue Bcl-xL was shown to form a cation-specific channel in both vesicles and planar lipid bilayers (Minn et al 1997, Schendel 1997, Schlesinger 1997), whereas Bax, the proapoptotic counterpart of Bcl-2, formed an anion-selective channel (Antonsson et al 1997).

The Thompson group further demonstrated that mitochondrial swelling and outer mitochondrial membrane rupture are early events in many forms of apoptotic death (Vander Heiden et al 1997). Interestingly, Bcl-xL can protect mitochondria from this damage, suggesting that mitochondrial damage may be the committed step in apoptosis. Whereas a direct link between ion flow and mitochondrial

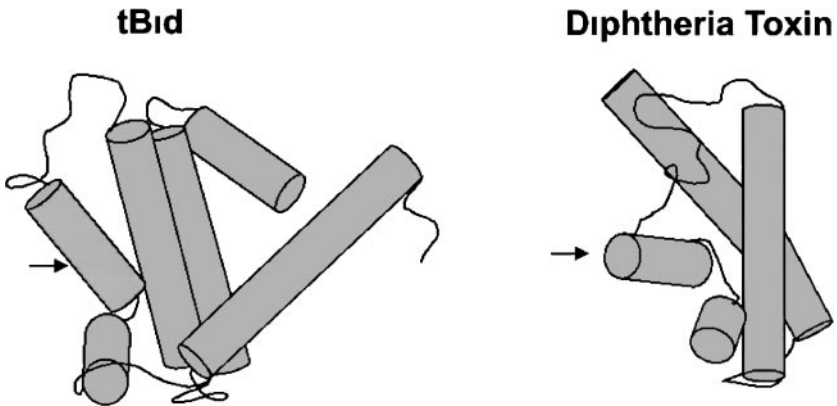


Figure 3 Comparison of truncated Bid (*left*) with the pore-forming helices of Diphtheria toxin (*right*). The predicted structure of truncated Bid (tBid) contains a hydrophobic helical hairpin flanked by a pair of amphipathic helices. Similarities have been noted with the insertion helices of Diphtheria toxin. *Arrows* indicate amphipathic helices adjacent to hairpin for comparison.

homeostasis has not been established, Thompson and colleagues speculate that swelling of the inner membrane results in the rupture of the outer membrane and subsequent release of cytochrome *c*. Therefore, relative ratios of proapoptotic and anti-apoptotic proteins could influence the flow of ions and, subsequently, the flow of water (Shimizu et al 1998). Alternatively, the channels may influence the opening of another pore, such as the permeability transition pore (PTP), which could regulate the volume of the mitochondria.

The BH3-Containing Protein Model

Despite the intriguing finding that some Bcl-2 family members can act as channels, not all Bcl-2 homologues possess this feature. The pore-forming helices that are conserved in two domains, designated BH1 and BH2, are not present in many proapoptotic proteins, including Bid and Bad. Mutagenesis experiments have demonstrated that a 9–16 amino acid stretch in the BH3 domain is necessary for the functioning of these members (K Wang et al 1998). In fact, high levels of BH3 domain peptides are capable of inducing cytochrome *c* release (Cosulich et al 1997). Because not all apoptotic stimuli are inhibited by PTP inhibitors, it is tempting to speculate that BH3-containing proteins alone act through a separate, undefined pathway in the mitochondria. The validity of this hypothesis remains to be determined.

Recent findings have revealed that Bid, a member of the BH3-containing proteins (Wang et al 1996), mediates cytochrome *c* release from the mitochondria after its cleavage by caspase-8 (Luo et al 1998, Li et al 1998). Bid contains a single BH3 domain that is also shared by proapoptotic members of Bcl-2 family including Bad, Bik, Bim, Blk, and Harakiri (Zha et al 1996, Han et al 1996, Inohara et al 1997a, O'Connor et al 1998). It has been shown that the BH3 domain of Bid is important for its proapoptotic activity, as well as for its ability to interact with other members of Bcl-2 family proteins (Wang et al 1996). Upon cleavage by caspase-8, the C terminus of Bid, which contains the BH3 domain, translocates to the mitochondria and triggers cytochrome *c* release. Interestingly, mutation in the BH3 domain dramatically reduces the cytochrome *c*-releasing activity of Bid and abolishes its interaction with Bcl-2 or Bax (Wang et al 1996), although it does not alter its ability to translocate to the mitochondria (Luo et al 1998). These observations raise the possibility that Bid may interact with a yet unidentified target at the outer membrane of the mitochondria. However, this interaction is not sufficient to trigger cytochrome *c* release in the absence of a functional BH3 domain.

How does Bid mediate communication between activated caspase-8 and the mitochondrial death machinery? Caspase-8 activation can initiate two pathways leading to the activation of downstream caspases. Caspase-8 can activate downstream caspases such as caspase-3, -6, and -7 by direct cleavage (Muzio et al 1996, Srinivasula et al 1996). This pathway is predominant when the caspase-8 concentration is high (Kuwana et al 1998). On the other hand, caspase-8 can activate the

downstream caspases indirectly by inducing cytochrome *c* release from the mitochondria that triggers caspase activation through Apaf-1. The indirect pathway, mediated by Bid and dependent on cytochrome *c* release, represents an important amplification step in the presence of low caspase-8 concentration. Although both pathways can be blocked by caspase-8 inhibitor such as CrmA, only the latter pathway is sensitive to inhibition by Bcl-2 or Bcl-xL (Vander Heiden et al 1997, Medema et al 1997, Srinivasula et al 1998).

In addition to caspase-8, other caspases such as caspase-3 can also cleave Bid. Bid cleaved as a result of proteolysis by caspases other than caspase-8 is equally potent in inducing cytochrome *c* release from the mitochondria (Luo et al 1998). This observation suggests that Bid may also play a role in amplifying various apoptotic signals in addition to the signals that come from the cell surface death receptor.

Recently, a protein, Egl-1, has been identified in *C. elegans* as a component of the cell death pathway upstream from Ced-3 and Ced-4. Mutation of the *egl-1* gene prevents somatic cell death during nematode development (Conradt & Horvitz 1998). Interestingly, Egl-1 contains a BH3 domain, a hallmark of the proapoptotic members of Bcl-2 family of proteins. Perhaps Egl-1 plays a role in transducing the upstream signal to activate the death machinery in *C. elegans*.

Another new member of proapoptotic Bcl-2 homologue, Diva, has recently been identified (Inohara et al 1998). Unlike other proapoptotic members, Diva lacks critical residues in the BH3 domain that are involved in the interaction with the antiapoptotic members of Bcl-2 family proteins. Furthermore, mutagenesis studies indicated that Diva-induced apoptosis is independent of the BH3 domain. Instead, immunoprecipitation assays suggest that Diva directly interacts with Apaf-1 and inhibits the binding of Bcl-xL to Apaf-1 (Inohara et al 1998). Consistent with this model, other investigators have shown that Bcl-xL binds to Apaf-1 and caspase-9 to form a ternary complex called the apoptosome (Pan et al 1998).

While Bcl-2 family proteins regulate caspase activation through the release of cytochrome *c* from mitochondria, another regulatory step in this pathway could be at the level of ATP/dATP binding and hydrolysis by Apaf-1. The CED-4 homologous domain of Apaf-1 contains a conserved Walker's A and B box that is critical for its function (Zou et al 1997). Apaf-1 is able to bind and hydrolyze ATP or dATP, and it has been shown that non-hydrolyzable ATP analogs potently inhibit Apaf-1 activity (Zou et al 1999). Although there is no direct evidence that Apaf-1 activity is regulated at the levels of nucleotide binding and hydrolysis under physiological conditions, several lines of evidence suggest that such regulation does occur under certain pathological and pharmacological conditions. Patients with adenosine deaminase (ADA) deficiency tend to accumulate high levels of dATP (up to mM levels) in their lymphocytes, which results in the massive death of CD⁸ low transitional and CD⁴ CD⁸ double positives thymocytes by apoptosis (Benveniste & Cohen 1995, Cohen et al 1978, Goday et al 1985). Therefore, it is possible that high levels of dATP in ADA cells lower the apoptosis threshold so much that a

small leakage of cytochrome *c* will be sufficient to trigger apoptosis. Consistent with this hypothesis, chemotherapeutic drugs such as 2-chloro-2'-deoxyadenosine (2 CdA or Cladribine) and 9- β -D-arabinofuranosyl-2-fluoroadenine (Fludarabine) are potent inducers of apoptosis in non-dividing lymphocytes (Carson et al 1983, Beutler 1992). The cytotoxicity of these drugs depends mainly on the selective and progressive accumulation of their 5'-triphosphate metabolites (Carson et al 1983, Kawasaki 1993). These metabolites can substitute for dATP or ATP in activating Apaf-1 in vitro (Leoni et al 1998), suggesting that high levels of these nucleotides can trigger apoptosis in patients with lymphoid malignancy.

Role of IAPs

Other important negative regulators of apoptosis are the inhibitors of apoptosis (IAP) family of proteins. To date, two IAPs have been discovered in baculoviruses (Cp-IAP and Op-IAP), two in *Drosophila* (DIAP-1 and DIAP-2), and five in humans (c-IAP-1, c-IAP-2, XIAP, survivin, and NAIP). These proteins share a common ~ 70 amino acid motif termed BIR (baculovirus IAP repeat). Most IAPs contain two or three copies of the BIR repeats, except for survivin which has only one repeat (Ambrosini et al 1997). In addition to BIR, most IAPs (except for NAIP and survivin) also contain a RING finger zinc-binding domain C terminal to the BIR repeats (Liston et al 1996). Furthermore, c-IAP-1 and c-IAP-2 also contain a caspase recruitment domain (CARD) (Hofmann et al 1997), raising the possibility that they may interfere with the procaspase-9 activation by Apaf-1/cytochrome *c* complex.

Overexpression of IAPs renders the cell resistant to a wide variety of apoptotic stimuli. Structural and functional studies reveal that the BIR repeat in these IAPs is required for their protective activity (Duckett et al 1996, Liston et al 1996, Ambrosini et al 1997). The exact target of inhibition by IAPs is currently unknown. At least two modes of action have been proposed for IAPs. On the one hand, they inhibit apoptosis by interfering directly with the catalytic activity of certain caspases (Roy et al 1997, Deveraux et al 1997). On the other hand, IAPs can also prevent the processing or activation of procaspases upon overexpression (Seshagiri & Miller 1997b), raising the possibility that IAPs may inhibit the procaspases or other proteins that are necessary to activate procaspases (Takahashi et al 1998).

Recent studies on the mammalian IAPs provide important clues to the molecular mechanisms of the action of these proteins. Mammalian IAPs, especially XIAP, are potent active-site inhibitors of the catalytically active, death effector caspase-3 and -7. These IAPs do not, however, inhibit caspase-1, -6, -8, or -10 (Deveraux 1997). They inhibit by directly and specifically binding to the active forms, but not to the precursors of these caspases. These mammalian IAPs were found to interfere with the function of caspase-9 via an essentially different mechanism. In the latter case, the IAPs bind to inactive procaspase-9, thereby interfering with the processing of procaspase-9 (Deveraux et al 1998, Takahashi et al 1998). These differential

effects of IAPs suggest that IAPs function in both major pathways of apoptosis, the cell surface death receptor and the cytochrome *c*-dependent (Apaf) pathway. In the cell surface death receptor pathway, IAPs block the effector caspase-3 and -7, therefore arresting caspase-8-initiated apoptosis. In the cytochrome *c*-dependent (Apaf) pathway, IAPs exert their effects on three distinct steps: (a) through direct interaction with procaspase-9, thereby interfering with its processing; (b) through competing for Apaf-1 binding by their CARDS; and (c) through directly inhibiting active caspases. With the availability of the recombinant Apaf system (Zou et al 1999), it is now possible to distinguish which pathway is predominantly used.

The IAPs apparently provide a safeguard mechanism against minimal activation of the apoptosis program. In other words, they set up an endogenous threshold level for caspase activation. The cellular levels of IAPs may determine the difference in sensitivities to apoptosis-inducing stimuli in various cell types. For this reason, the regulation of IAPs levels becomes an important issue in apoptosis. It has been shown that the *c*-IAPs are the direct targets of transcriptional regulation by NF- κ B (Chu et al 1997). More detailed and complete studies on the transcriptional and post-transcriptional regulation of IAPs should provide important insights into the regulation of apoptosis.

PERSPECTIVES

Regulation of caspase activation is one of the key events in apoptosis. Despite rapid progress in the identification of the molecules that are critical for caspase activation, many questions remain, particularly those related to how apoptosis-inducing stimuli signal the activation of the death machinery inside the cells. For example, what are the roles of mitochondria in sensing the apoptosis-inducing stimuli? Are there other pathways involved in caspase activation?

The challenge ahead is to map the functions of newly found apoptotic proteins in the biochemical pathways of apoptosis that will allow us to better understand how cells make the decision between life and death. An equally important task is to study how these pathways are modified in human diseases such as cancer or neurodegenerative diseases in which apoptosis dysregulation contributes to the pathogenesis. During the course of this pursuit, novel proteins that currently do not belong to the known family members of apoptosis regulatory proteins may be identified. Finally, it is possible that these basic scientific discoveries on apoptosis may reveal logical strategies for the discovery of new therapeutic drugs for the treatment of either cancer or neurodegenerative disease.

ACKNOWLEDGMENTS

We thank the members of Xiaodong Wang's laboratory for the helpful comments and suggestions on this manuscript. I.B is supported by the Postdoctoral Fellowship from the Leukemia Society of America. The work in our laboratory is supported

by grants from Howard Hughes Medical Institute, the American Cancer Society, and the National Institutes of Health (to XW).

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