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CELLULAR MECHANISMS FOR THE REPRESSION OF APOPTOSIS*

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■ **Abstract** Apoptosis, also known as programmed cell death, is a ubiquitous mode of cell death known to play an important role during embryogenesis, development, and adult cellular homeostasis. Disruption of this normal physiological cell death process can result in either excessive or insufficient apoptosis, which can lead to various disease states and pathology. Since most cells contain the machinery that brings about apoptosis, it is clear that living cells must contain inherent repressive mechanisms to keep the death process in check. In this review, we examine several modes of repression of apoptosis that exist in cells.

INTRODUCTION

Apoptosis is a physiological cell death process in which individual cells are eliminated or removed from the body in a temporal manner or in response to a specific signal without affecting neighboring cells or eliciting an inflammatory response. It has long been assumed that cells must be continuously lost from the body to balance cell division. It was not until the concept of a programmed cell death was introduced approximately 30 years ago, when the term apoptosis was coined by Kerr et al., that mechanisms to account for homeostasis were recognized (1). Kerr and coworkers first described the morphological features of apoptosis as a cell deletion process that was complementary but opposite to the cell proliferation process known as mitosis. It is interesting that, while this original report has become one of the most widely cited papers in the field of apoptosis, its impact was not immediately appreciated. Intensive study of the biochemistry and signal transduction pathways of apoptosis only really began approximately 10 to 15 years after this initial observation. When genetic work in the nematode *Caenorhabditis*

elegans suggested a specific and conserved pathway for programmed cell death, the study of apoptosis began in earnest (2–4).

Apoptosis is defined by a distinct set of morphological and biochemical characteristics observed exclusively with this mode of cell death (5–7). These apoptotic features include the loss of cell volume or cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation, and the formation of apoptotic bodies that are then phagocytized by macrophages or neighboring cells to rid the body of these dying cells, thus avoiding an inflammatory response such as occurs during necrosis. Recently, depolarization of the mitochondrial membrane potential and release of cytochrome c, as well as the activation of specific proteases known as caspases, have also been suggested to play critical roles in the apoptotic process. Additionally, apoptosis is a highly conserved cell death process, as several mammalian apoptotic genes have been identified that encode proteins with sequence similarity to *C. elegans*. Furthermore, this organism has become a simple genetic model system in which the process of cell death can be observed during development and has provided invaluable insight into our understanding of apoptosis (8–10). However, apoptosis in vertebrates appears to be more complex as numerous related genes serve a similar function, which may reflect differences in various cell types or developmental stages. This permits specific groups of cells to undergo apoptosis at appropriate times. From nematodes to vertebrates, the ability of cells to undergo programmed cell death appears to be an inherent, normal, physiological process.

While it has been generally accepted that apoptosis plays an important role during development and maintenance of tissue homeostasis, recently it has become increasingly clear that this mode of cell death also plays a critical role in many pathological conditions. A balance between cell death and cell proliferation is required to maintain a cellular homeostatic state, and deviation from this cellular balance disrupts the normal state and can lead to or contribute to human disease (11, 12). Cell accumulation via insufficient apoptosis can contribute to conditions such as cancer, inflammation, and autoimmune disease. In contrast, excessive apoptosis can play an important role in neurodegeneration, AIDS, osteoporosis, and heart failure to name a few (13). Thus, an understanding of this complex physiological cell death process can be of great benefit in maintaining human health.

Because all cells contain the apoptotic machinery required for cell death, it is generally accepted that each cell has an internally controlled suicide program, i.e., the ability to undergo its own cell death. Therefore, the cell must control for inappropriate activation of apoptosis to ensure survival. As our understanding of apoptosis has increased over the past several years, cellular mechanisms and proteins have been identified that function at various points in the cell death process to prevent or inhibit errant activation of the death program. In this review, we describe the current understanding of inhibition of the apoptotic process at three critical points: the caspases, the mitochondria, and the plasma membrane. In concert, these data strongly support the theory that keeping apoptosis in check is a necessary and highly controlled cellular process.

INHIBITION OF APOPTOSIS AT THE LEVEL OF CASPASES

The degradation or cleavage of various intracellular proteins during apoptosis is a common feature of this physiological cell death process. Over the past several years, important insights into the nature of this proteolytic degradation have been described. Based largely on studies using the nematode *C. elegans*, a family of cysteine-activated aspartate-specific proteins known as caspases has been identified (14–17). These enzymes play an important role in both the initiation and execution phases of apoptosis. Caspases are synthesized as relatively inactive zymogens that become activated either upon self-association or by upstream proteases in a cascade-like manner. Although some low-level caspase activity is associated with the zymogen state, complete processing is required for maximal proteolytic activity. Thus, regulation of caspase activity can be a central point in the cell death process. In the following section, we describe the known mechanisms cells use to inhibit the activation and activity of caspases during apoptosis.

IAP Family Members

In the mid 1990s, a screen for viral-encoded suppressers of apoptosis led to the identification of two death-suppressing genes from *Cydai pomonella granulovirus* (CpGV) and *Orgyia pseudotsugata nucleopolyhedrovirus* (OpNPV), each with a high degree of similarity to the other (18, 19). These viral proteins, Cp-IAP and Op-IAP, defined the first members of a family of inhibitors of apoptosis proteins (IAP) containing one or more baculoviral IAP repeats (BIR). This BIR domain consists of approximately 70 amino acids with a conserved spacing of cysteine and histidine residues, which suggests that this structure represents a novel zinc-binding fold (20). Homology to this BIR domain has been used to isolate a number of cellular proteins that inhibit apoptosis. Additional members of the IAP family include XIAP, c-IAP1, c-IAP2, NAIP, and Survivin in mammals; DIAP1, DIAP2, and Deterin in Drosophila, and CeBIR1 and CeBIR2 in *C. elegans*; TnIAP and SfIAP in Lepidopteran; and SpIAP and ScIAP in yeast (20, 21). Various members of this family of inhibitory proteins can contain one to three BIR domains.

The criteria for membership in this IAP family is the presence of a BIR domain, along with the ability to inhibit apoptosis, such that not all BIR-containing proteins are considered IAPs (21, 22). While BIR domains are known to be critical for the inhibitory activity of IAP proteins by providing an interaction site for caspases, the presence of only one BIR domain in the protein Survivin suggests that redundancy of this key domain may not be required (23). Indeed, the antiapoptotic activity of XIAP has been localized specifically to the second BIR repeat, while the first and third BIR domains lack caspase-binding capability (24); however, this does not exclude other regulatory functions for these domains. The human c-IAP1 and c-IAP2 proteins are unique in that they contain a caspase recruitment domain (CARD) near their C-terminus, along with several BIR domains.

However, deletion of the CARD domains from these proteins does not prevent inhibition of apoptosis, which suggests that a CARD domain is not an essential component for the functional activity of IAPs (25). Several IAPs also contain a RING domain located near their C-termini, but the necessity of this domain in suppressing apoptosis appears to be dependent on the cellular context (20). Under some circumstances, removal of this RING domain actually enhanced the antiapoptotic function of these proteins (24–27). Additionally, an interesting region found in the murine IAP BRUCE (28) and in the human homologue Apollon (29) is a functionally intact ubiquitin-conjugating (UBC) domain, which suggests a connection between apoptotic proteins and the ubiquitin proteasome pathway for protein degradation.

Overexpression of IAPs prevents apoptosis induced with a wide variety of stimuli, including TNF, Fas, staurosporin, etoposide, and growth factor withdrawal (20, 30). However, in primary cerebellar granule cell neurons transfected with either NAIP, XIAP, c-IAP1 or c-IAP2, cell death induced by potassium depolarization or serum deprivation was only delayed but not prevented (31), which suggests that the ability of various IAPs to prevent apoptosis is dependent on both cell type and apoptotic stimulus. As diagrammed in Figure 1, various IAPs are thought to function as direct inhibitors of either initiator and effector caspases during apoptosis. Several IAPs, including XIAP, c-IAP1, and c-IAP2, bind directly to and inhibit caspases 3, 7, and 9 (25, 27), whereas these IAPs did not inhibit caspases 1, 6, 8, and 10 or the *C. elegans* caspase CED3. Recently, the structural basis of caspase-7 and caspase-3 inhibition by XIAP was described (32–34). The crystal structure of the second BIR domain of XIAP in complex with caspase-3 revealed that inhibition is due to a steric blockade on the enzyme's active site, distinct from the mechanism utilized by synthetic substrate inhibitors where the substrate binding sites are fully occupied. In contrast, the crystal structure of XIAP in complex with caspase-7 closely resembled that observed with the synthetic tetrapeptide inhibitor DEVD-CHO but in the opposite orientation, thus preventing caspase activity. This suggests that the ratio of caspase to IAP is likely to be the key point in determining if inhibition of apoptosis will occur. Binding of IAPs to caspase-3 or caspase-7 requires that these caspases be in an active configuration, while IAPs can inhibit caspase-9 in either its active or inactive (proform) state. This suggests differences exist in their activation mechanisms (20).

IAPs interact with members of the TNF-receptor family. Figure 1 shows that both the human c-IAP1 and c-IAP2 can be recruited by and bind to the TRAF-1 and TRAF-2 receptor complexes through their amino-terminal BIR domain (35). This interaction appears to be specific, as other IAPs do not bind to these receptor complexes, and c-IAP1 and c-IAP2 do not bind to other TRAF receptors. Some of the protection against apoptosis afforded by IAPs in response to TNF signaling has also been attributed to the ability of these receptors to activate NF-κB (36). This suggests that IAPs can regulate apoptosis at various points in different pathways, or at multiple levels of a single pathway, to protect the cell from aberrant cell death.

FLICE-Inhibitory Protein (FLIP); CrmA; p35

A subgroup of membrane receptors of the TNF superfamily, collectively known as the death receptors, induces apoptosis upon ligand binding (37). CD95 (Fas) is the most extensively studied member of the death receptor family. Upon ligation of the Fas receptor with its ligand (FasL), receptor clustering at the cell membrane results in conformational changes, enabling recruitment of an adaptor molecule FADD, and procaspase-8 (also known as FLICE) to the membrane-bound receptor in a complex termed the death inducing signaling complex (DISC). Recruitment of procaspase-8 to this complex permits its activation, initiating a caspase cascade that ultimately leads to cell death. Therefore, a level of control can also occur at the level of this receptor complex. A family of FLICE-inhibitory proteins (FLIPs) has recently been identified, with both viral and mammalian members (38–42). The viral proteins (v-FLIPs) contain two tandem death effector domains (DED), while the human homolog (c-$FLIP_L$) contains two DED regions along with an inactive caspase-8–like catalytic domain at the C-terminus. The short form of this protein (c-$FLIP_S$) is similar in structure to the viral FLIPs. FLIPs are thought to be recruited to the DISC through their DED domains, thus preventing procaspase-8 recruitment and activation (Figure 1). Therefore, receptor-mediated cell death can be regulated at the level of the most proximal caspase, caspase-8. Inhibition of apoptosis by FLIPs through the inactivation of caspase-8 occurs in several apoptotic model systems, including germinal center B-cells, human peripheral blood T-lymphocytes, and mast cells (43–45).

CrmA, a 38-kD cytokine response modifier protein of the cowpox virus, is a protease inhibitor of the serpin family initially shown to inhibit interleukin-1 beta converting enzyme (ICE) and cytokine-induced apoptosis (46–48). CrmA is a unique member of the serpin family in that it can inhibit both cysteine proteases (caspases) and serine proteases, such as granzyme B (49). CrmA is a potent inhibitor of diverse apoptotic model systems such as Fas- and TNF-induced apoptosis (48), serum withdrawal (50), and hypoxia-mediated apoptosis (51). While crmA is most effective at inhibiting caspase-1 (ICE) and caspase-8, it can also inhibit with decreasing effectiveness caspase-10, -6, -3, and -7 (52). In general, crmA rescues cells primarily from apoptotic signals involving death receptors, or other mechanisms in which caspase-8 (or caspase-10) acts as the apical protease (Figure 1; 53). In contrast, crmA is ineffective at preventing cell death owing to stress or genotoxic damage, which suggests that apoptotic stimuli that act directly at the level of mitochondria are relatively resistant to crmA inhibition. Thus, apoptotic stimuli activating caspases, such as caspase-9, as the initial protease are essentially crmA-insensitive.

Another caspase inhibitor identified from a novel gene product of the baculovirus *Autographa californica* nuclear polyhedrosis virus, designated p35, prevents cell death in various apoptotic model systems from insects to mammals (54–56). p35 also blocks cell death induced with a wide variety of apoptotic

induction signals including ionizing radiation, Fas, and TNF (57, 58). In contrast to crmA, which has a differential ability to inhibit the activity of specific caspases, Figure 1 depicts the broad range of caspase inhibition of p35 at near equal efficiency (52). p35 prevents caspase activity by binding to various target enzymes, is cleaved at specific sites within the protein, and remains bound to the active caspase after cleavage (54, 59). Thus, the ability of p35 to inhibit caspase activity occurs in a stoichiometric fashion (59). The crystal structure of the caspase-8/p35 complex was recently solved and revealed that caspase inhibition occurs owing to a covalent thioester linkage between p35 and the active site of caspase-8 (60). It is interesting to note, p35 inhibits the activity of a number of different caspases; however, it was recently reported that p35 does not prevent caspase-9 activity in a cell-free system of mammalian caspase activation (61). Thus similar to crmA, p35 appears to be ineffective at preventing cell death from apoptotic stimuli that act directly at the level of the mitochondria.

In summary, proteins such as IAPs, FLIPs, crmA, and p35 prevent apoptosis at the level of caspases to inhibit aberrant cell death. This variety of different regulating proteins used to inhibit specific caspase activities depends on the particular stimuli employed to initiate apoptosis. One advantage of this diverse capacity of various caspase inhibitors to prevent apoptosis is the ability to use these proteins to define a specific caspase cascade that a particular stimuli engages to commit the cell to death.

Additionally, structural studies examining the interactions between caspase inhibitors and specific initiator or effector caspases offers new insights into the design of synthetic proteins with increased selectivity for their use in therapeutic approaches for controlling apoptosis.

INHIBITION OF APOPTOSIS AT THE LEVEL OF THE MITOCHONDRIA

The mitochondria plays a notable role in the regulation of apoptosis, at least in organisms evolutionarily higher than *Drosophila* and *C. elegans*. Loss of the mitochondrial membrane potential, or mitochondrial depolarization, occurs in numerous apoptotic model systems (62–65), although it should be mentioned that altered mitochondrial function has also been associated with necrosis. On an interesting note, a number of cell death proteins have also been localized to the mitochondria, from which they are released upon apoptotic stimulation to activate downstream effectors of the death process (66–69). These studies suggest that a critical point of control during apoptosis may exist at the level of the mitochondria. Additionally, several proteins have been identified that prevent apoptosis specifically at the level of the mitochondria. Details of how antiapoptotic proteins function at the level of the mitochondria to prevent and thus control apoptosis are described below.

Bcl-2 Family Members

Bcl-2 is the product of the *bcl-2* oncogene identified by two groups in the mid 1980s as a novel gene product at the breakpoint of translocations between chromosomes 14 and 18 (70, 71). This 26-kDa protein prevents cell death induced with a variety of apoptotic agents, thus acting as a key regulator of the apoptotic process (72, 73). Bcl-2 is the founding member of a family of proteins with similar sequence identity belonging to a subgroup of antiapoptotic proteins that includes Bcl-X_L, Bcl-w, Mcl-1, and A-1 (74). It is interesting that this family also includes a subgroup of proapoptotic proteins such as Bax, Bak, Bok, Bad, Bid, and Bik (74). Together these proteins function primarily at the level of the mitochondria to either prevent or enhance apoptosis.

A defining feature of these Bcl-2 family proteins is the presence of Bcl-2 homology (BH) domains. Members of the Bcl-2 family contain at least one of four conserved BH domains. Recently, Kelekar & Thompson (74) classified members of the Bcl-2 family based on their function and BH domain organization. The antiapoptotic family members all contain conserved BH-1 and BH-2 motifs, while those most closely related to Bcl-2 contain all four BH domains. One class of the proapoptotic members of the Bcl-2 family contains BH-1, BH-2, and BH-3 motifs, while a specific subset of proapoptotic Bcl-2 proteins contain only the BH-3 domain. Bcl-X_S is a unique member of this family in that this proapoptotic protein contains only the BH-3 and BH-4 domains. These BH domains play a role in the ability of various family members to interact with each other (75, 76). However, the significance of these interactions has not been clearly defined (77). Many Bcl-2 family proteins, both anti- and proapoptotic, contain a C-terminal membrane anchoring region that targets these proteins to the outer mitochondrial membrane, endoplasmic reticulum membrane, and the outer nuclear envelope (78–81). This membrane association is thought to be of critical significance, as mutant Bcl-2 molecules lacking the membrane anchoring domain show a decreased capacity to prevent apoptosis in some model systems (80, 82, 83).

A single mechanism by which members of the Bcl-2 family regulate apoptosis has not been completely elaborated. However, a direct interaction between pro- and antiapoptotic Bcl-2 family members is one of several ways in which control of the cell death process is thought to occur (84, 85). Specific regions within the antiapoptotic Bcl-2 protein are required for binding to the proapoptotic protein Bax (86). Recently, it has been shown that the BH3 domain of Bak itself is capable of inducing apoptosis in cells, possibly by antagonizing the function of antiapoptotic Bcl-2 proteins (87). Thus, competition between homodimerization and heterodimerization of Bcl-2 family members may play a crucial role in their capacity to induce or prevent cell death by regulating the release of proapoptotic factors, such as cytochrome c, from the mitochondria (Figure 2). However, mutations in Bcl-X_L that prevent heterodimerization with Bax or Bak did not limit the ability of Bcl-X_L to protect cells from apoptosis. This suggests that

antiapoptotic proteins of this family can also function independently to promote cell survival (77).

In addition to direct interactions between pro- and antiapoptotic members of the Bcl-2 family to prevent apoptosis, Figure 2 shows a number of other models that have been proposed to explain the protective nature of these antiapoptotic proteins; this includes prevention of mitochondrial membrane swelling, maintenance of mitochondrial metabolism, limiting the effect of reactive oxygen species, and regulation of the mitochondrial permeability transition pore (Figure 1; 88). Each of these proposed mechanisms may involve direct interaction between pro- and antiapoptotic Bcl-2 family members as described above; however, an underlying component of each proposed model is the ability of antiapoptotic Bcl-2 proteins to prevent the release of proapoptotic factors from the mitochondria.

Cellular metabolic changes in response to apoptotic stimulation have been suggested to result in swelling of the mitochondria and rupture of the outer mitochondrial membrane, releasing proapoptotic factors such as cytochrome c and an apoptotic-inducing factor (AIF) to initiate the death program (89, 90). While mitochondrial swelling has been observed following numerous apoptotic insults including growth factor withdrawal, heat shock, and TNF treatment (91–93), swelling of this organelle has classically been associated with the accidental cell death process known as necrosis. Additionally, the initial studies on the morphology of apoptosis suggested that intracellular organelles, such as the mitochondria, remained unchanged (94, 95). However, given the controversial state of mitochondrial morphology during apoptosis, it has been suggested that antiapoptotic members of the Bcl-2 family may simply function to strengthen the outer mitochondrial membrane to withstand the stress of mitochondrial swelling (Figure 2; 88).

Central to mitochondrial metabolism is the ability of this organelle to generate ATP (96). Key to this process is maintenance of exchange of ATP for ADP across the mitochondrial matrix and the cytosol. This nucleotide exchange is dependent upon the activity of the adenine nucleotide translocator (ANT) (97), which in conjunction with other mitochondrial proteins is also involved in the movement of other mitochondrial factors. As shown in Figure 2, antiapoptotic Bcl-2 family members, by maintaining ANT in an open state, may sustain ATP/ADP exchange, therefore inhibiting disruption of mitochondrial function and preventing eventual stress and rupture of the mitochondrial membrane that may result during inappropriate anion exchange. Although it is unclear how antiapoptotic Bcl-2 proteins facilitate channel opening, it has been suggested that ANT and Bcl-2 (or Bax) proteins may form composite channels in the mitochondrial membrane (98). In contrast, other studies have suggested that antiapoptotic Bcl-2 family proteins may interact independently of ANT, and directly with the voltage dependent anion channel (VDAC) also found in the mitochondrial membrane, through its insertion into and/or alteration of the local lipid environment of the membrane (99). Additionally, antiapoptotic Bcl-2 proteins alone may act as ion channels (discussed in detail later), thus affecting ANT activity potentially though redistribution of the charge across the outer mitochondrial membrane (88).

The mitochondria is also the major site for the generation of reactive oxygen species (ROS), such as superoxide anions, hydrogen, peroxides, and radicals (100). Disruption of the mitochondrial respiratory chain can result in an overproduction of ROS leading to oxidative stress and activation of apoptotic mediators. Changes in the redox state of a cell undergoing apoptosis occur with a variety of apoptotic stimuli (100–102). Antiapoptotic members of the Bcl-2 family may act to maintain the normal mitochondrial physiology and events associated with the respiratory chain to prevent the generation and eventual release of ROS (Figure 2). Gottlieb et al. (103) showed that Bcl-X_L prevents loss of the mitochondrial membrane potential and induction of ROS, but does not act as a ROS scavenger. Additionally, it was shown that ROS are important mediators of mitochondrial depolarization and that ROS scavengers fail to prevent this change in mitochondrial membrane potential, which suggests that the two events are independent (103). The release of calcium from the mitochondria by oxidants also occurs in some models of apoptosis (104). This increase in intracellular calcium may result in stimulation of calcium-dependent enzymes, such as nucleases and proteases, to trigger apoptosis. Antiapoptotic Bcl-2 proteins prevent the ROS-mediated release of calcium from the mitochondria, but this mechanism of the inhibition is currently undefined. It is interesting to note that the importance of ROS generation during apoptosis was questioned in several studies where antiapoptotic Bcl-2 family members prevented apoptosis but not ROS generation (105, 106). This suggests that the specificity of the apoptotic signal may dictate the various physiological events that occur during cell death.

Recently, specific changes in the mitochondrial membrane potential due to the opening of the mitochondrial permeability transition (PT) pore have been observed during apoptosis (107, 108). Figure 2 shows the PT pore is a large multiprotein complex that spans the mitochondrial membranes, creating a channel between the cytosol and the mitochondrial matrix through which proteins up to 1.5 kD in size are thought capable of passing (88, 109). The proposed components of the PT pore include VDAC, ANT, the benzodiazapine receptor, hexokinase, creatine kinase, and cyclophilin D (109). Opening of the PT pore is predicted to depolarize the mitochondrial membrane potential, thus compromising the integrity of the outer mitochondrial membrane to permit the release of proapoptotic factors, such as cytochrome c and AIF (110, 111). As shown in Figure 2, antiapoptotic Bcl-2 proteins may regulate the PT pore, thus preventing the release of proapoptotic factors from the mitochondria (102). However, Bcl-2 does not prevent the PT pore in purified mouse liver mitochondria induced to undergo apoptosis with antiFas antibody (112). While the opening of the PT pore can promote the release of cytochrome c and other proapoptotic factors, further studies are still required to determine the exact role antiapoptotic Bcl-2 family members play in modulating the PT pore during cell death.

Interesting insight into the mechanism of Bcl-2 family proteins came from initial X-ray crystallographic and NMR examination of the structure of Bcl-X_L (113). This antiapoptotic member of the Bcl-2 family has structural similarity

to the pore-forming domains of bacterial toxins. Subsequent electrophysiological studies demonstrated that Bcl-X_L can form a pH-sensitive cation-selective ion channel in synthetic lipid membranes (114). Additional studies on other members of the Bcl-2 family of proteins, such as Bax and Bcl-2, also revealed ion flux or channel-like characteristics for these family members (115). It is interesting that Bax displays Cl^- selectivity while Bcl-2 displays K^+ selectivity. Therefore, the counter-ion selectivity of these pro- and antiapoptotic proteins and their ability to form pores in lipid membranes strongly suggests that a level of control during programmed cell death may involve the movement of ions (Figure 2). Gilbert et al. (116) provided further evidence for ionic regulation of apoptosis, which demonstrates that overexpression of Bcl-2 in a human B-cell lymphoma cell line and in HL-60 cells affects the cellular membrane potential. Cells expressing Bcl-2 were hyperpolarized relative to control cells, with a correlation between this more negative membrane potential and increased radioresistance.

In summary, release of proapoptotic factors from the mitochondria can occur by various mechanisms, including rupture of the mitochondrial membrane, formation of specific channels by various mitochondrial proteins, and through the action of both pro- and antiapoptotic proteins, which by themselves may form specific mitochondrial channels. Since there appear to be numerous ways in which proapoptotic factors can be released from the mitochondria to initiate the cell death program, it is reasonable to assume that antiapoptotic Bcl-2 family proteins may also function in multiple ways to prevent cell death. Additionally, if a single apoptotic stimulus activates the release of pro-apoptotic factors through multiple mechanisms, a single approach to inhibit apoptosis induced by any given apoptotic agent would not be achievable.

INHIBITION OF APOPTOSIS AT THE LEVEL OF THE PLASMA MEMBRANE

Maintenance of ionic gradients is a requirement of all living cells, and it has been suggested that cells may use up to 60% of their energy (ATP) to maintain a homeostatic ionic environment. Throughout evolution, cells have developed an inherent ability to survive in response to changes in their extracellular environment through a movement of ions. These ion movements known collectively as volume regulatory responses permit cells to retain a near normal cell size and thus function in the presence of adverse conditions. During apoptosis however, cells shrink and lose cell volume as part of the cell death process. This universal characteristic of apoptosis is in sharp contrast to the cellular swelling that occurs during accidental cell death known as necrosis. While the characteristic cell shrinkage associated with apoptosis was relatively unexplored for many years, several studies have recently focused on the movement of ions and the role these ions play during apoptosis. In the following section, we summarize the current understanding of ions and apoptosis, as well as discuss the protective role the plasma membrane plays during the cell death process.

Volume Regulatory Responses

Cellular survival depends on the ability of the cell to maintain a specific set of intracellular and extracellular ionic conditions to preserve a natural state of homeostasis. Most mammalian cells contain a high intracellular concentration of potassium and a low intracellular concentration of sodium, opposite of the concentrations observed in the extracellular environment. The intracellular and extracellular contents of the cell should remain electrically neutral, such that every positive charge on the inside or outside of the cell must be counter-balanced by a negative charge (117). However, a small unbalanced charge ratio exists across the plasma membrane owing to the difference in ionic concentrations of potassium and sodium, and to the electrogenic nature of the Na^+/K^+-ATPase, which plays an important role in setting and maintaining the plasma membrane potential. Balance of osmotic pressure across the plasma membrane is also important to cellular homeostasis, such that the total concentration of solute particles inside and outside the cell must be equal, permitting no net movement of water into or out of the cell.

While the loss of cell volume, or cell shrinkage, is a unique and distinctive characteristic of the programmed cell death process, this feature is not limited specifically to apoptosis. Under both normal and pathological conditions, cells may be exposed to a hypertonic environment with increased extracellular osmotic strength. This hypertonicity results in loss of intracellular water and cell shrinkage. However, most cells can subsequently respond to this loss in cell volume through the activation of specific ionic transport mechanisms known collectively as a regulatory volume increase (RVI) response (118–121). Figure 3 shows the various ionic transport mechanisms that a cell may activate during an RVI response, including the $Na^+/K^+/2Cl^-$ cotransporter, the Na^+-coupled amino acid transporters, or the Na^+/H^+ exchanger coupled to the Cl^-/HCO_3^- exchanger. Activation of each of these ionic mechanisms results in an increase in intracellular ions. During this RVI response, the initial influx of Na^+ and Cl^- upregulates the Na^+/K^+-ATPase, which exchanges Na^+ for K^+, thus permitting a net increase in intracellular potassium and brings the cell back to a normal homeostatic balance. The increase in intracellular ions in turn leads to an influx of water into the cell, which allows the cell to achieve a near normal cell size.

Conversely, cellular swelling results from exposure to a hypotonic environment. However, the cell responds to decreased extracellular ionic strength with the activation of a regulatory volume decrease (RVD) response. Several ionic transport mechanisms are shown in Figure 3 and include individual K^+ and Cl^- channels, K^+/Cl^- cotransporter, and the K^+/H^+ exchanger coupled to the Cl^-/HCO_3^- exchanger. Opposite to what is observed during an RVI response, activation of these ionic transporters results in an efflux of intracellular ions. The RVD response, mediated by the loss of intracellular ions, permits the concomitant movement of water out of the cell to again achieve a near-normal cell size. While different cell types use different ionic transport mechanisms to achieve this response, it is generally

accepted that most mammalian cells have the ability to control their cell volume and thus protect themselves from changes in their extracellular environment.

An interesting exception to volume regulatory responsiveness under anisiotonic conditions has been shown for T lymphocytes, which respond to hypotonic stress as described above, but lack the ability to generate an RVI response under hypertonic stress (122–124). T lymphocytes have been an excellent model system for studying apoptosis because of their marked susceptibility to undergo cell death with a variety of agents. We have shown that culture of S49 cells, an immature mouse lymphoma cell line, and other thymic lymphoid cells, under hypertonic conditions results in the rapid occurrence of apoptosis (125). In contrast, exposure of nonlymphoid cells such as COS, HeLa, and L-cells to similar conditions resulted in an initial decrease in cell volume, followed by an RVI response without the occurrence of apoptosis, which suggests that inherent volume regulatory responses must be either inhibited or overridden for programmed cell death to occur.

Recent studies have also shown that organic osmolytes such as taurine act in conjunction with ions to maintain a near-normal cell volume during anisotonic stress (126–128). Organic osmolytes also stabilize cellular proteins (129). AntiFas treatment of Jurkat cells resulted in the release of taurine along with other presumed organic osmolytes early after stimulation of the Fas receptor (130). A similar loss of taurine was detected in cerebellar granule neurons undergoing apoptosis in the absence of depolarizing conditions (131). Furthermore, elevated blood glucose levels, similar to those observed in uncontrolled diabetes, can induce accelerated endothelial cell apoptosis and microvascular disease, which can be prevented by taurine (132). The mechanism by which taurine prevents apoptosis in this system is thought to involve ROS inhibition and calcium stabilization. Thus, the role of taurine release in response to apoptosis and the effects of taurine in regulating both cell volume changes and stability of intracellular proteins critical for activation of the apoptotic machinery remain areas of active investigation.

Ionic Repression of Apoptosis

While inherent volume regulatory mechanisms clearly play an important role in protecting cells from changes in their extracellular environment, an increasing number of studies suggest that ions do more than regulate cell size, particularly during apoptosis (133, 134). A universal and defining characteristic during apoptosis is the loss of cell volume, or cell shrinkage. Recently, a number of studies have shown that the characteristic loss of cell volume that occurs during apoptosis results from a dramatic decrease in intracellular ions, specifically intracellular potassium (Figure 3; 135–141). Additionally, the potassium ionophore valinomycin induces apoptosis (142, 143). Thus a link between potassium efflux and cell shrinkage during apoptosis has been established in a number of experimental apoptotic models.

Corresponding to this, inhibition of potassium efflux protects cells from undergoing apoptosis (144, 145). Inhibition of potassium channels with 4-aminopyridine,

sparteine, or quinidine prevents apoptosis in eosinophils (146). The potent potassium channel blockers, tetraethylammonium (TEA), tetrapentylammonium (TPA), and quinine, have a protective effect in neuronal, HL-60, and liver cells, respectively (138, 147–150). Additionally, elevation of extracellular potassium concentrations to prevent potassium efflux during apoptosis resulted in protection from apoptosis in a number of model systems (151–153). Together these studies suggest that ion movement plays a key role in the regulation of apoptosis and specifically that inhibition of intracellular potassium loss can protect cells from cell death. Further support of a role for potassium in apoptotic regulation comes from studies that demonstrate that depolarizing concentrations of extracellular potassium in the culture medium promote the survival of primary neurons in vitro in the absence of specific neurotrophic factors (154). In this model system, however, elevated extracellular potassium is not preventing potassium efflux, but rather is controlling the intracellular ionic environment to support cell survival, which suggests a specific relationship between various intracellular ions and cellular homeostasis to maintain life.

In addition to the role of potassium in controlling the loss of cell volume during apoptosis, the capacity of ions to regulate other aspects of the cell death process has recently become apparent. Over the past several years, the concept of ionic control of apoptosis, and thus the importance of maintaining a normal physiological ionic environment in the cell, has become an area of active research. Induction of apoptosis results from the addition of low concentrations of staphylococcal alpha-toxin to T cells, which created small pores in the plasma membrane that allowed passage of monovalent but not larger ions (155). In addition, normal intracellular concentrations of potassium directly inhibit apoptotic nuclease activity and effector caspase activation (151, 152). Figure 3 shows that in the normal cellular environment where the intracellular ions are balanced by various ionic transporters, apoptotic enzymes such as caspases and nucleases are inactive. However, the loss of cell volume and the associated loss of intracellular ions, especially potassium, during apoptosis results in an intracellular ionic environment conducive for apoptotic nuclease activity and effector caspase activation. Therefore, the normal physiological concentration of intracellular potassium can prevent apoptosis by holding in check both apoptotic nuclease activity and effector caspase activation and suggests that the loss of intracellular ions play an important role in the progression of apoptosis. Furthermore, the role of ions controlling effector caspase activation is supported by an earlier study in human monocytes, which demonstrated that low intracellular potassium levels permit the processing of proIL-1β to its mature form via the interleukin beta converting enzyme (ICE), also known as caspase-1 (156). Likewise, the release of mature IL-1β from LPS-activated monocytes through the activation of ICE occurs in response to hypotonic conditions, where potassium ions are lost from cells owing to the RVD response (157), which again suggests that the ionic strength within a cell plays an important role in maintaining cell survival or promoting cell death.

In summary, the control of the intracellular ionic environment plays an important role in repressing apoptosis. The normal ionic environment of living cells does not favor cell death, and changes in this intracellular environment must occur during apoptosis to permit the programmed cell death process to proceed. This suggests that ions may be the gate keepers for maintaining cell survival or granting cell death.

PROSPECTUS

Cells contain numerous protective mechanisms at various levels of the apoptotic process to guard against errant or inappropriate activation of cell death. At the level of caspases, IAPs, crmA, and p35 prevent the activation of these enzymes, and thus the destructive effects associated with the proteolytic cascade of events that ultimately leads to cell death. At the level of the mitochondria, antiapoptotic Bcl-2 family proteins function to maintain mitochondrial metabolism and prevent the release of proapoptotic factors from this organelle. And at the level of the plasma membrane, regulation of various ions, particularly potassium, plays a critical role in holding the apoptotic machinery in check and in maintaining cellular homeostasis suitable for sustaining life.

In this review, we have described these protective mechanisms individually, each one independently forming a line of defense to inhibit apoptosis. However, it is important to realize that under many circumstances, these protective mechanisms may not act individually, but may rather act in concert with each other. An important question during apoptosis has always been: where in the cell death process does a cell commit itself to die? As our understanding of the various ways in which a cell protects itself against death continues to expand, one proposal is that during apoptosis a cell may have to pass through various check points to permit continual activation of the cell death program.

A first line of defense may simply be the ability of cells to regulate their cell volume in response to an apoptotic stimulus that signals the cells to shrink. To survive, the cells attempt to maintain a normal ionic balance by activating a volume regulatory response to support vital cellular functions, holding apoptotic nuclease activity and effector caspase activation in check in an effort to endure a death signal. Therefore, for a cell to undergo apoptosis, these volume regulatory mechanisms need to be either inhibited or overridden to permit the cell death process to continue. If this initial checkpoint is passed, proteins such as IAPs, crmA, or p35 may function as a second line of defense to inhibit the destructive nature of caspases and further cellular injury associated with the continual activation and progression of apoptosis.

Apoptotic stimuli that act directly on the mitochondria may initially depend on antiapoptotic members of the Bcl-2 family to inhibit the release of proapoptotic factors such as cytochrome c and AIF from this organelle. However, if these inhibitory Bcl-2 proteins are not present or fail to maintain protection, IAPs may in turn limit the amount of destruction by specifically preventing the activation and activity of caspase-9, along with subsequent effector caspase activity. Concurrent with this may be the activation of volume regulatory mechanisms either in

concert with Bcl-2 proteins or independently if these proteins become ineffective in maintaining protection against apoptosis. Additionally, antiapoptotic members of the Bcl-2 family may function as ion channels themselves contributing to the protective nature of the inherent volume regulatory mechanisms.

In conclusion, the commitment point for a cell to undergo apoptosis may occur at various stages of the cell death process and is defined by the presence or absence of a specific protective mechanism inherent for any particular cell type. Consequently, no one stimulus, factor, or event universally condemns a cell to death. A series of defensive checkpoints may exist in cells which, once passed, permit the continual progression of the cell death process in response to a given apoptotic signal and eventually lead to complete, nonevasive destruction of the cell.

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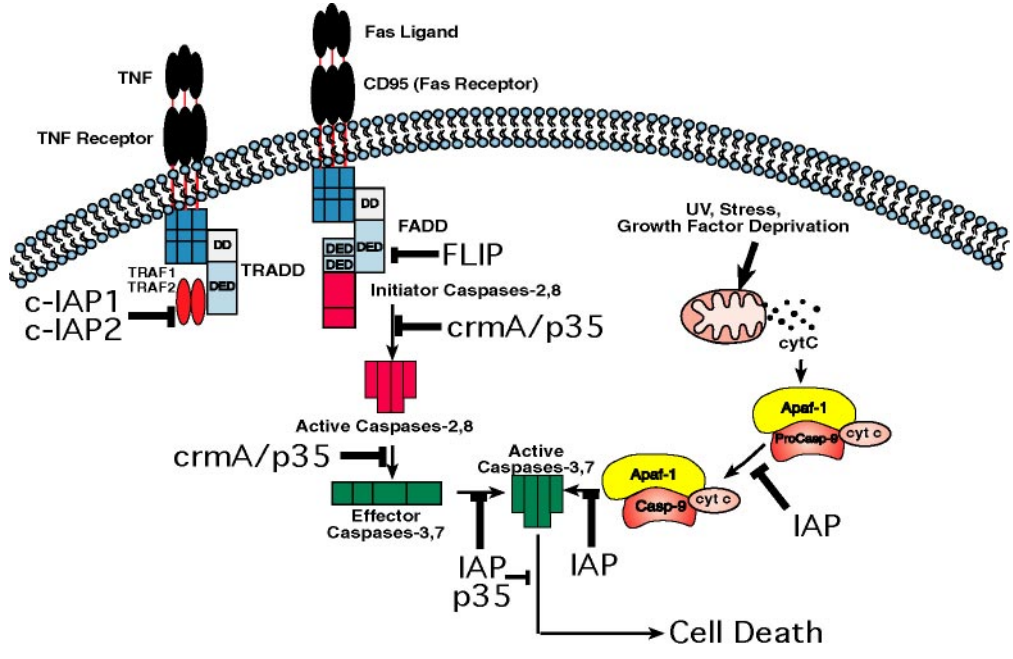

Figure 1 Repression of apoptosis at the level of caspases. Numerous proteins that specifically inhibit caspases during apoptosis have been identified. IAPs have been shown to inhibit both initiator caspases, such as caspase-9, and effector caspases, such as caspase-3 and -7; however, these proteins are ineffective in preventing the activity of caspases associated with the death receptors, such as caspase-2 and -8. CrmA, a caspase inhibitor from cowpox virus, has been shown to be most effective in preventing apoptosis initiated from the death receptors, with limited inhibitory capacity for other downstream proteases. A specific caspase inhibitor for caspase-8 has been identified, known as FLIP, which prevents apoptosis by binding to the DISC. A final caspase inhibitor, p35 isolated from the baculovirus, has a broad-range ability in preventing apoptosis induced from both death receptors, as this protein inhibits both initiator and effector caspases associated with the death receptor pathway. Therefore, the identification of multiple proteins that inhibit caspases through various cell death pathways suggests a critical role of these enzymes in the initiation and execution of apoptosis.

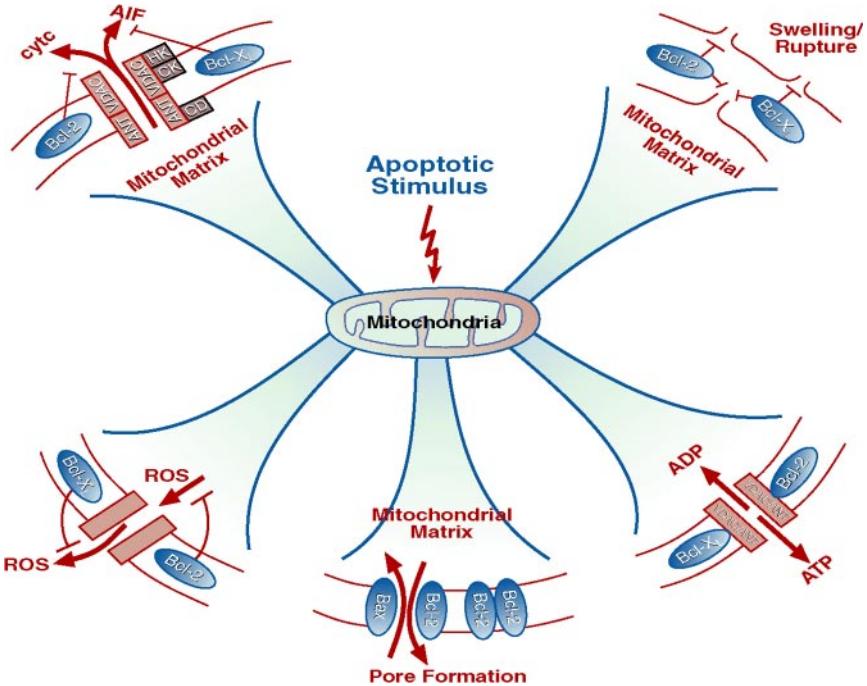

Figure 2 Repression of apoptosis at the level of the mitochondria. Mitochondria have been shown to play a significant role during apoptosis. The antiapoptotic members of the Bcl-2 family inhibit cell death at the level of the mitochondria by maintaining normal physiological function of this organelle and preventing the release of proapoptotic factors, such as cytochrome c and AIF. A variety of mechanisms has been proposed to explain the protective nature afforded by these proteins, including prevention of mitochondrial swelling and rupture, maintaining mitochondrial metabolism through appropriate nucleotide exchange, limiting the effects of reactive oxygen species, and regulating the mitochondrial permeability pore. Direct interactions between pro- and antiapoptotic members of the Bcl-2 family may sequester the protection provided by anti-apoptotic Bcl-2 proteins. Each of these mechanisms may function independently or act in concert to prevent cell death depending on the specific apoptotic signal.

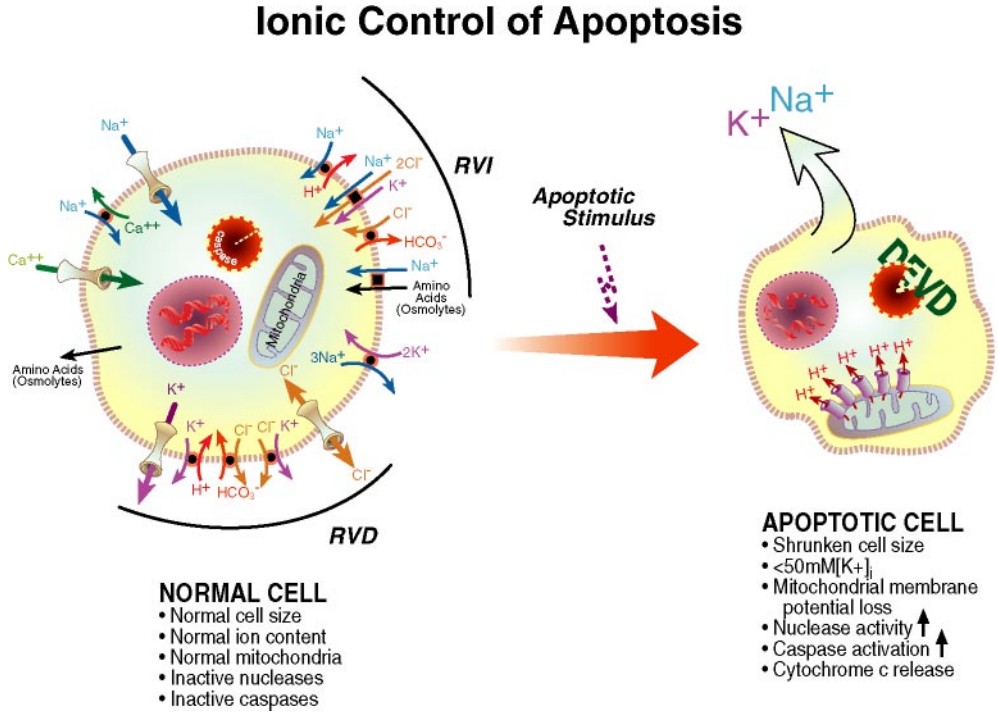

Figure 3 Repression of apoptosis at the level of the plasma membrane. The loss of cell volume or cell shrinkage is a universal characteristic of the apoptotic process, which occurs by the dramatic loss of intracellular ions, especially potassium. Most cells have the inherent ability to regulate changes in their cell size through the specific activation of various ionic transporters and channels, known as a regulatory volume response. An increase in cell volume results in the activation of an RVD response, while a decrease in cell volume results in the activation of an RVI response. Normal physiological concentrations of intracellular potassium have been shown to prevent apoptotic nuclease activity and effector caspase activation, which suggests that apoptosis can be controlled at the level of the plasma membrane through the regulation of the cells' ionic environment.