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Insights into Programmed Cell Death through Structural Biology

Review

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Programmed cell death plays a critical role in controlling the number of cells in development and throughout an organism's life by the removal of cells at the appropriate time. It is an important biological process for the elimination of unwanted cells such as those with potentially harmful genomic mutations, autoreactive lymphocytes, or virally infected cells. Alterations of this normal process can result in the disruption of the delicate balance between cell proliferation and cell death and can lead to a variety of diseases (Thompson, 1995). For example, in many forms of cancer, key proapoptotic proteins are mutated or antiapoptotic proteins are upregulated, leading to the accumulation of cells and the inability to respond to harmful mutations, DNA damage, or chemotherapeutic agents. Since effective chemotherapy depends on the induction of programmed cell death, cancers with defects in the cell death signaling pathways are particularly difficult to treat. Programmed cell death is also important for eliminating autoreactive T cells after an immune response. When this normal process is disrupted through mutations of the proteins that trigger apoptosis (e.g., Fas ligand or the Fas receptor), an autoimmune lymphoproliferative syndrome (ALPS) can result, with complications such as hypersplenism, autoimmune hemolytic anemia, thrombocytopenia, and neutropenia (Strauss et al., 1999). Inappropriate apoptosis also contributes to several neurological disorders. In Alzheimer's, Parkinson's, and Huntington's disease, specific neurons prematurely commit suicide, which can lead to irreversible memory loss, uncontrolled muscular movements, and depression.

In the last few years, much has been learned about the signal transduction pathways of programmed cell death, providing us with insight into how programmed cell death works and how dysregulation of apoptosis contributes to disease. The overall process of programmed cell death occurs in several stages. In the first step, the apoptotic pathway is triggered, which can be accomplished by a wide variety of stimuli, including DNA damage, growth factor withdrawal, toxins, and radiation. Once activated, the signal is transduced by a series of protein–protein interactions that involve a conserved set of signaling modules. In the next stage, cell death is executed by the activation of specific proteases called caspases that cleave multiple substrates, leading to changes characteristic of apoptotic cells such as DNA fragmentation, chromatin condensation, cell shrinkage, and membrane blebbing.

The signaling pathways of programmed cell death can be divided into two components, involving either the mitochondria or death receptors (Figure 1) (Budihardjo et al., 1999). In the death receptor pathway, receptors such as TNFR1, Fas, DR-3, DR-4, or DR-5 interact with their cognate ligands. This binding event allows the recruitment of downstream signaling partners, which

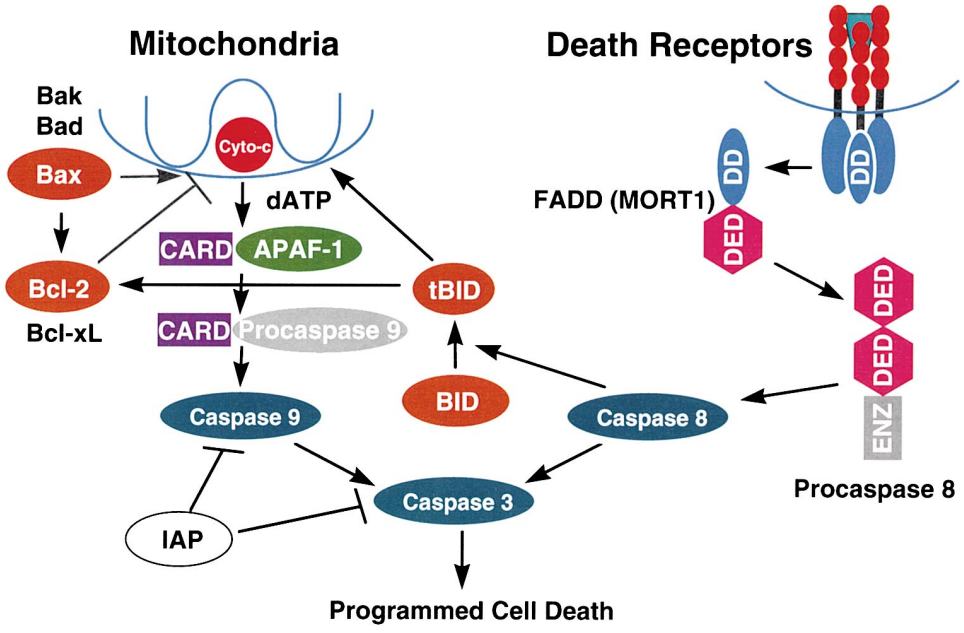

Figure 1. Signaling Pathways of Programmed Cell Death

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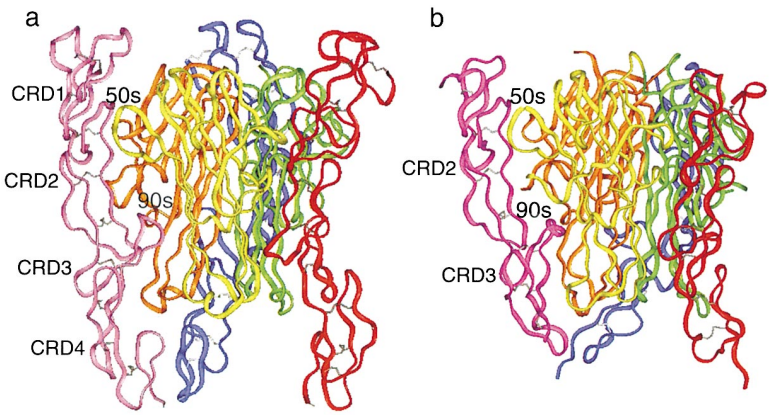

Figure 2. Three-Dimensional Structures of Death Receptor/Ligand Complexes

(A) The structure of the TNFβ trimer (orange, yellow, and green) complexed to three TNF receptor molecules (magenta, blue, and red). The side chains of the cysteines that form disulfides are shown in gray (Banner et al., 1993).

(B) The X-ray structure of TRAIL (orange, yellow, and green) complexed to DR5 (magenta, blue, and red) (Hymowitz et al. 1999).

ultimately results in the activation of caspases and subsequent cell death. For example, in Fas-mediated apoptosis, the Fas ligand interacts with the Fas receptor, which leads to an interaction between the death domain of the cytoplasmic region of the Fas receptor and the death domain of the adaptor protein, FADD. FADD recruits and activates procaspase-8 through interactions between the death effector domains of these two proteins. Once activated, caspase-8 activates downstream caspases such as caspase-3 (for a review, see Nagata, 1997).

In the mitochondrial pathway, cytochrome c is released from the intermembrane space of mitochondria upon activation via a death signal. Although the details of this process at the molecular level are unknown, cytochrome c and ATP or dATP bind to the protein Apaf-1 to form a multimeric complex that recruits and activates procaspase-9. This is followed by the activation of caspase-3 and -7 (Budihardjo et al., 1999).

As one might imagine, apoptosis is highly regulated. One class of regulators is the Bcl-2 family of proteins. Antiapoptotic family members such as Bcl-2 and Bcl-xL inhibit the release of cytochrome c whereas the proapoptotic proteins Bax and tBID promote cytochrome c release from the mitochrondria. Pro- and antiapoptotic members of the Bcl-2 family interact with one another and modulate each others' activities (Yin et al., 1994). Additional regulators of this pathway are the IAPs. One of their major functions is to bind to and inhibit the effectors of apoptosis—the caspase family of enzymes (Deveraux and Reed, 1999).

The three-dimensional structures for a number of proteins and protein domains involved in the signaling pathways of programmed cell death have been determined. These structures have helped us understand apoptosis at the molecular level by defining the interactions that stabilize complex formation, guiding site-directed mutagenesis experiments, elucidating enzyme mechanisms, and characterizing protein function. In this review, a compendium of recently determined three-dimensional structures of proteins involved in the cell death signaling pathways is described along with the information learned from these structures relevant to the chemistry and biology of apoptosis.

Death Receptors—Initiators of Apoptosis

Tumor necrosis factor (TNF) is the prototypic member of a family of cytokines that interact with a large number of receptors. Activation of these receptors by ligand binding leads to many diverse activities such as cell proliferation, differentiation, and apoptosis. The subset of receptors responsible for eliciting programmed cell death all contain an intracellular death domain (Tartaglia et al., 1993).

TNFα and TNFβ were found to crystallize as trimers (Jones et al., 1989; Eck and Sprang, 1989; Eck et al., 1992). The structures of these proteins are similar and adopt the shape of a truncated pyramid. Each monomer consists of a β sandwich with a jellyroll topology. The structure of the complex between TNFβ and the soluble extracellular region of the TNF receptor (TNF-R1) provided the first view of the receptor and the interactions that stabilize complex formation between these two families of proteins (Figure 2A) (Banner et al., 1993). The extracellular portion of TNF-R1 consists of four pseudo repeats, called cysteine-rich domains (CRDs), which each contain three disulfides formed from six conserved cysteines. Three elongated receptor molecules bind to

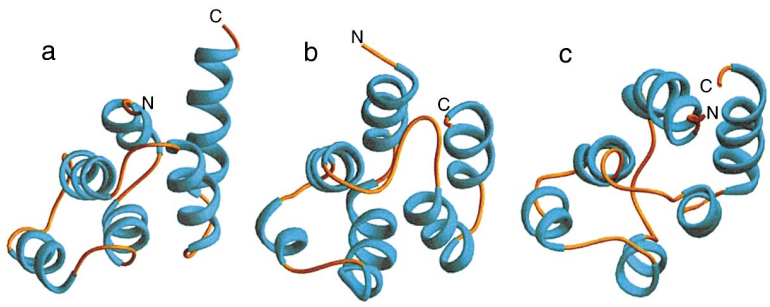

Figure 3. The Structures of Three Modules Involved in the Signal Transduction of Programmed Cell Death

Ribbons (Carson, 1987) depiction of the: (A) Fas death domain (Huang et al., 1996), (B) FADD death effector domain (Eberstadt et al., 1998), and (C) RAIDD CARD (Chou et al., 1998).

one TNF trimer at the interfaces formed between the TNF monomers (Figure 2A). There are two principal regions of contact in the complex that involve loops from the second (50s loop) and third (90s loop) cysteine-rich domains of the TNF receptor and two distinct regions of TNFβ. On the basis of the structure of the TNFβ/TNF-R1 complex, a model was proposed for TNF-mediated signaling in which the TNF trimer induces trimerization of the receptor, which causes the cytoplasmic regions of the receptor to form a cluster. The cytoplasmic region of TNF-R1 and other death receptors contain death domains that oligomerize and recruit death domain–containing adaptor proteins, leading to the downstream signaling of programmed cell death (Figure 1).

Recently, the crystal structure was determined of another death receptor, DR5, complexed to the ligand TRAIL (also called Apo2L) (Figure 2B) (Hymowitz et al., 1999; Mongkolsapaya et al., 1999). The overall structure of the TRAIL/DR5 complex is similar to TNFβ/TNF-R1. However, there are some important differences that define the binding specificities observed within these families of ligands and receptors. Unlike the TNF receptor that contains four extracellular cysteine-rich domains, DR5 contains only two CRDs, which correspond to domains 2 and 3 of TNF-R1. CRD2 of DR5 interacts with TRAIL in a similar fashion as observed for the corresponding residues in the TNF-β/TNF-R1 complex (Figure 2). However, CRD3 of DR5, when bound to TRAIL, adopts a different relative orientation compared to CRD3 of TNF-R1 in the TNFβ/TNF-R1 complex and forms a different set of interactions. The binding specificity observed in the TNF receptor superfamily could be important for developing selective therapeutic agents. Unlike TNF and Fas ligand, which are highly toxic, TRAIL selectively triggers apoptosis in tumor cells versus normal tissues and, when given to mice with human tumors, causes tumors to shrink without any apparent toxicity (Walczak et al., 1999).

Signaling Modules that Transduce the Death Signal

A common theme in the signaling pathways of programmed cell death is the association of proteins that contain similar domains. Four protein modules have been identified that participate in cell death signaling through protein–protein interactions. These include the death domain (DD), death effector domain (DED), caspase recruitment domain (CARD), and the N-terminal domains of DFF45, DFF40, and cell death-inducing DFF45-like effector (CIDE) proteins.

The death domain is found in the intracellular portion of death receptors whereas the death effector and caspase recruitment domains are found in adaptor proteins such as FADD and procaspases. The three-dimensional structure of the Fas death domain was the first structure reported for one of these signaling modules (Huang et al., 1996). The structure contains six antiparallel, amphipathic α helices with an unusual topology (Figure 3A). The surface of the Fas death domain mainly consists of charged residues, suggesting that charge–charge interactions mediate complex formation between the death domains. This was supported by site-directed mutagenesis in which the mutation of charged residues in the second and third α helices of the Fas DD reduced binding to FADD.

The overall fold of other death domains were found to be similar to the Fas death domain with only minor differences in the length and orientation for some of the α helices (Liepinsh et al., 1997; Jeong et al., 1999). However, the manner in which death domains or modules with similiar folds interact with one another was found to be different. This was recently illustrated in the X-ray structure of a complex between the interacting domains of the serine/threonine kinase Pelle and the adaptor protein Tube that recruits Pelle to the plasma membrane during *Drosophila* embryogenesis (Xiao et al., 1999). Although not involved in apoptosis, the interacting domains of Pelle and Tube adopt the same overall fold as death domains but do not appear to interact in the same manner as the Fas and FADD death domains. Unlike the second and third α helices, which have been implicated in the Fas death domain for binding to FADD, residues in the fourth and fifth α helices in Pelle and the sixth α helix in Tube were found to play a critical role in complex formation for these proteins such that the binding interface involves a unique surface that is not even present in other death domains.

The death effector domain of FADD (Figure 3B) adopts a fold that is similar to that of the death domains (Eberstadt et al., 1998). However, mutations in the Fas death domain that inhibit the binding of Fas to FADD have no effect when introduced in the FADD DED. In contrast to the charged surface of the Fas DD, the FADD DED has two hydrophobic patches. One of these patches contains a conserved set of hydrophobic residues that is important for its apoptotic activity and binding to the DEDs of procaspase-8.

The three-dimensional structures of RAIDD CARD (Figure 3C) (Chou et al., 1998) and Apaf-1 CARD (Day et al., 1999; Qin et al., 1999; Vaughn et al., 1999; Zhou et al., 1999) also adopt the same fold as the death domain and the death effector domain. Thus, all three signaling modules (DD, DED, and CARD) that participate in homophilic interactions to transduce the cell death signal adopt very similar structures. This is consistent with the presence of a conserved set of hydrophobic amino acids in all of these domains that compose the hydrophobic core of the proteins (Hofmann et al., 1997). The differences between the domains are the surface residues that stabilize complex formation. In the Apaf-1 CARD/procaspase-9 CARD complex (Qin et al., 1999), the positively charged surface formed by the basic residues Arg13, Arg52, and Arg56 of procaspase-9 CARD interacts with the negatively charged face of Apaf-1 CARD, which is composed of Asp27 and Glu40 (Figure 4).

Another domain that participates in homophilic interactions in the programmed cell death cascade is found in the N terminus of DFF45, DFF40, and CIDE proteins. DFF45 (also called ICAD) forms a complex with DFF40 (also called CAD) through interactions involving their N-terminal domains and inhibits the DNA nuclease activity of DFF40, which is localized in the C-terminal portion of the protein (Liu et al., 1997; Enari et al., 1998). Caspase-3 cleaves DFF45, which causes the release of DFF40 from the DFF45/DFF40 complex and triggers DNA fragmentation and nuclear condensation. The three-dimensional structure of the N-terminal domain of

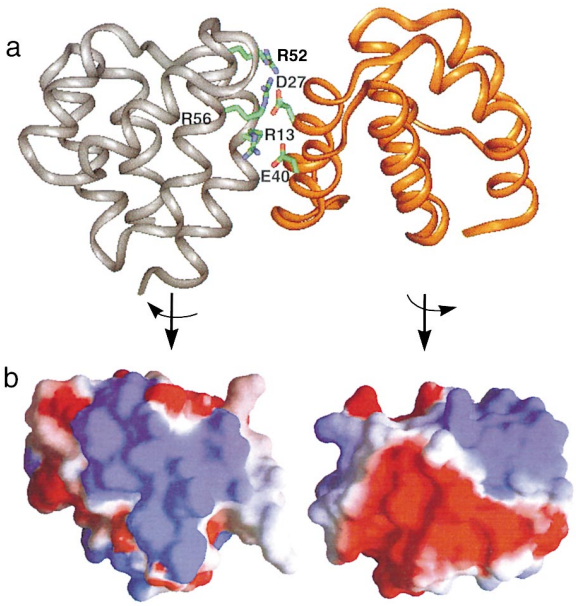

Figure 4. X-Ray Structure of a CARD/CARD Complex

(A) The positively charged Arg13, Arg52, and Arg56 of procaspase-9 CARD (left) interact with the negatively charged residues (Asp27 and Glu40) of Apaf-1 CARD (right) (Qin et al., 1999).
(B) GRASP (Nicholls et al., 1991) depiction of the electrostatic surface of procaspase-9 CARD (left) and Apaf-1 CARD (right).

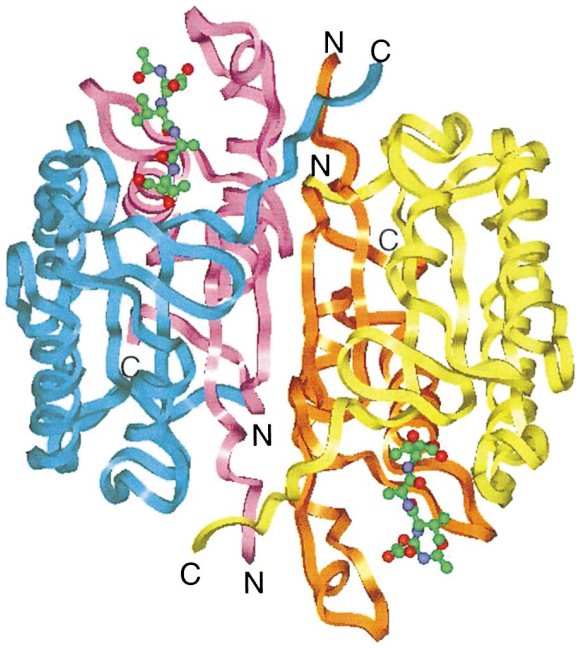

Figure 5. X-Ray Structure of a Caspase-3/Inhibitor Complex

After processing, activated caspases form a tetramer in which the C-terminal ends of the large subunits (blue and yellow) are closer to the N-terminal ends of the small subunits (orange and magenta) in the adjacent heterodimer. The inhibitor (color coded by atom type) interacts in a pocket formed between the two subunits on each side of caspase-3 (Mittl et al., 1997).

the proapoptotic CIDE-B protein was determined (Lugovskoy et al., 1999). The structure consists of a twisted, five-stranded β sheet and two α helices arranged in an α/β roll fold that is very different from the structure of the DD, DED, and CARDs that also participate in homophilic interactions in cell death signaling. On the basis of the structure, a model was proposed for the homophilic interactions involving the N-terminal CIDE domains involving complementary neutralization of their charged surfaces.

Executioners of Programmed Cell Death

The family of cysteine proteases called caspases play a central role in the execution of programmed cell death by cleaving a wide variety of substrates (e.g., DFF45), leading to the characteristic morphological changes associated with apoptosis. Caspases can be divided into two types—those with large prodomains that function upstream as initiators of the death cascade and those with a small prodomain that act downstream as effectors. As described above, the prodomains of the initiator caspases contain either a DED or CARD. These domains interact with other signaling proteins that contain these modules through homophilic interactions. This binding event triggers the activation of the initiator caspases by bringing together the proenzymes, allowing autolytic processing in trans as a result of their induced proximity (Salvesen and Dixit, 1999).

All caspases have a distinct substrate specificity that requires cleavage after an aspartic acid at the P_1 position N-terminal to the cleavage site. The preference of amino acids further to the N terminus of peptide substrates at the P_2–P_4 positions differs among caspase family members and defines their substrate specificity (Thornberry et al., 1997). The initiator caspases such as caspase-8 and caspase-9 prefer (V,L)EXD-containing substrates like those found in the cleavage sites used to process caspase zymogens into active enzymes. In contrast, the effector caspases cleave DEXD-containing substrates such as those found in structural proteins that are cleaved during apoptosis, resulting in the typical morphological changes in the cell.

X-ray crystal structures of caspase-1, (Walker et al., 1994; Wilson et al., 1994) caspase-3 (Rotonda et al., 1996; Mittl et al., 1997), and caspase-8 (Blanchard et al., 1999; Watt et al.,1999) covalently attached to irreversible peptidic inhibitors have been determined. The overall architecture of these three caspases is similar and consists of two heterodimers composed of a large and small subunit that form a tetramer (Figure 5). The mature enzymes are derived by proteolytically removing the N-terminal prodomains and cleaving the proenzymes between the large and small subunits. In the X-ray structures of the processed enzymes, the C terminus of the large subunit (Figure 5, blue) is far away from the N terminus of the small subunit (magenta) in the heterodimer, but is very close to the N terminus of the small subunit (orange) in the adjacent protein. This observation suggests that the large subunit in the heterodimer may have been linked before processing to the small subunit in the adjacent heterodimer. Alternatively, a large conformational change may occur upon autoprocessing.

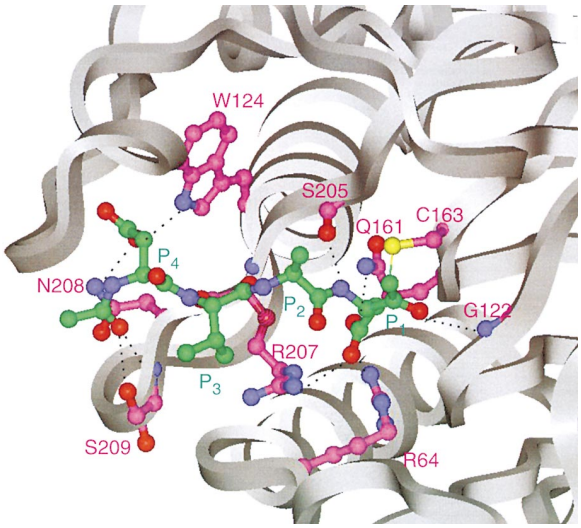

Figure 6. Blow-Up Depicting Caspase-3/Inhibitor Interactions

In addition to providing information on the possible mechanisms of activation, the X-ray structures of caspase/inhibitor complexes revealed the structural basis for the observed substrate specificity. The requirement for an aspartic acid at the P_1 position in all substrates and substrate-based inhibitors could be explained by the favorable interaction of this aspartic acid with two highly conserved arginines and a glutamine. In caspase-3, these residues correspond to Arg64, Arg207, and Gln161 (Figure 6). The differences in substrate specificity within the caspase family can also be explained from the X-ray structures. In caspase-1, the P_4 binding site (S_4 subsite) is a large hydrophobic pocket that can accommodate several residues. In contrast, caspase-3 has a relatively narrow pocket that forms hydrogen bonds with the aspartic acid preferred at the P_4 position (Figure 6). Caspase-8 differs from the other caspases in both the S_3 and S_4 specificity pockets, resulting in the preference for glutamic acid at P_3 and for small hydrophobic amino acids at P_4.

Based on the location of the amino acids in the active site observed in the X-ray structures, an enzymatic mechanism was proposed for the caspases (Figure 7). Like other cysteine proteases, the sulfur of the active site cysteine acts as a nucleophile to form a tetrahedral intermediate (Figure 7B). A nearby histidine increases the nucleophilicity of the sulfur by acting as a base and abstracting a proton (Figure 7A). Some investigators have postulated that a Cys/His dyad is involved in the enzymatic mechanism whereas others have proposed a catalytic triad. The high resolution (1.2 Å) structure of a caspase-8/inhibitor complex supports a catalytic triad in which a clear interaction is observed between the backbone carbonyl of Arg258 to His317 that enhances the basicity of the histidine. Arg258 in caspase-8 is equivalent to Pro177 in caspase-1 and Thr62 in caspase-3. Thus, it appears that the identity of the third residue of the catalytic triad is irrelevant, which is consistent with the observed interaction involving a backbone carbonyl. In addition to the importance of the histidine as a general base in the reaction mechanism, the same

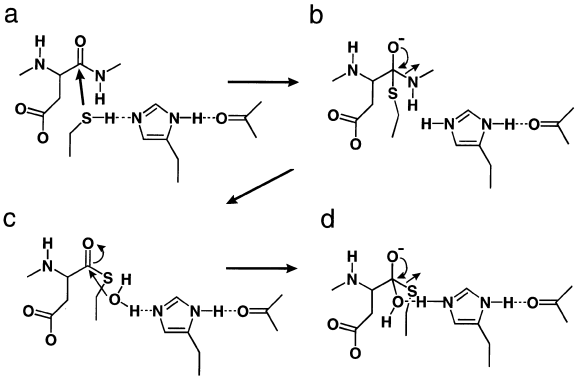

Figure 7. Proposed Enzymatic Mechanism of the Caspases

histidine is critical for protonating the α-amino group of the scissile bond that favors the release of the leaving group and increases the nucleophilicity of a water molecule to aid in the cleavage of the thiol ester (Figures 7C and 7D).

Regulators of Apoptosis

Several proteins have been discovered that regulate apoptosis. In cancer, the levels of these proteins are often altered to allow cancer cells to stay alive even though the cell death pathways have been triggered. Moreover, viruses have engineered mimics of these proteins that allow the host cell to stay alive long enough for the virus to replicate. Thus, these proteins serve as possible targets for the regulation of apoptosis.

IAPs

The inhibitor of apoptosis (IAP) family of proteins represent an important class of regulators of programmed cell death. They were initially discovered in baculoviruses where they were found to inhibit apoptosis in host cells during viral infection (Crook et al., 1993). Subsequently, IAPs have been found in other viruses, yeast, flies, worms, and mammals (Uren et al., 1998).

All IAPs contain one to three baculovirus IAP repeat (BIR) domains which are composed of about 70 amino acids and have a characteristic signature sequence ($CX_2CX_{16}HX_6C$). Some of the IAPs also contain a C-terminal ring finger. Several functions have been attributed to the IAPs. In *Drosophila*, IAPs have been shown to interact with the proapoptotic proteins REAPER, HID, and GRIM (Vucic et al., 1998; Goyal et al., 2000). The mammalian IAPs, cIAP-1 (MIHB) and cIAP-2 (MIHC), bind to TNF-receptor associated factors (TRAFS) 1 and 2, while survivin (Ambrosini et al., 1997), has been implicated in the cell cycle (Li et al., 1998a). Some IAPs have also been shown to potently inhibit caspases. For example, human XIAP inhibits caspases-3 and -7 with Ki's of 0.2–0.7 nM (Deveraux et al., 1997) and also inhibits caspase-9 (Deveraux et al., 1999). The portion of XIAP responsible for inhibiting caspase-3 was found to contain the BIR2 domain (Takahashi et al., 1998) whereas the BIR3 domain of XIAP was shown to inhibit caspase-9 (Deveraux et al., 1999; Sun et al., 2000).

The NMR structure of a BIR2-containing portion of XIAP that inhibits caspase-3 has been recently determined (Sun et al., 1999). The structure of the BIR2 domain of XIAP consists of a three-stranded antiparallel

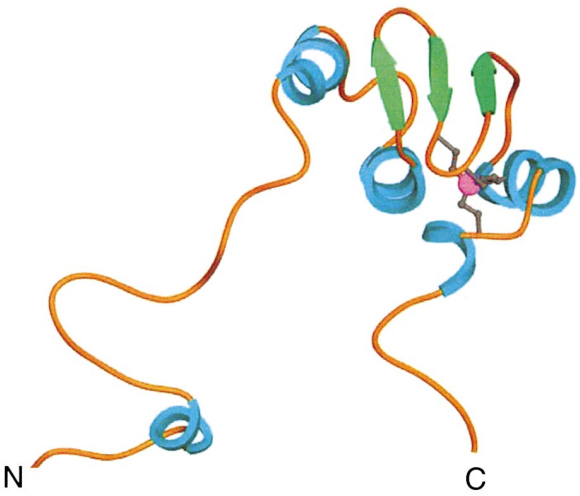

Figure 8. NMR Structure of a region of XIAP containing the BIR2 Domain

The zinc (magenta) chelates to three cysteines and one histidine (Sun et al., 1999).

β sheet and four α helices and resembles a classical zinc finger (Figure 8). The three conserved cysteines and histidine in the signature sequence chelates a zinc. To determine which amino acids of XIAP are responsible for its antiapoptotic activity, site-directed mutants of XIAP were prepared and tested for their ability to inhibit caspase-3. Surprisingly, the residues found to be most important for caspase-3 inhibition are not in the BIR domain but in the linker region between the BIR1 and BIR2 domains. Based on NMR studies on the interaction of XIAP with caspase-3, it was postulated that this linker region binds to the active site and the BIR2 domain interacts with an adjacent site on the enzyme (Sun et al., 1999). This is consistent with the amino acid sequence (DISD) within the linker region that resembles the sequence found in substrates and inhibitors of caspase-3.

The NMR structures of the BIR3 domain of XIAP (Sun et al., 2000) and cIAP-1 (MIHB) (Hinds et al., 1999) have also been determined. The structure of the BIR3 domain of XIAP is very similar to the BIR2 domain of XIAP (Sun et al., 2000). However, the structure of the BIR3 domain of c-IAP-1 is different than the structure of the BIR2 and BIR3 domain of XIAP, which is surprising in light of the high sequence homology among the IAP family of proteins (Uren et al., 1998). The major difference in the BIR3 domain of c-IAP-1 is the lack of the β sheet found in the BIR2 and BIR3 domain of XIAP and classical zinc fingers.

X-ray structures of the IAP, survivin, have also been recently reported (Chantalat et al., 2000; Muchmore et al., 2000; Verdecia et al., 2000). The structure consists of an N-terminal BIR domain that resembles the structures of the BIR2 and BIR3 domains of XIAP and a long, C-terminal α helix. Unlike the BIR domains of XIAP and c-IAP-1, which are monomers, both human and mouse survivin exist as dimers (although the dimeric arrangement differs in the different crystal structures). Since survivin is expressed in many cancers but not in normal adult tissues (Ambrosini et al., 1997), survivin represents a possible target for the development of drugs that selectively eliminate cancer cells. The X-ray crystal structures of survivin reveal basic, acidic, and hydrophobic patches on the surface of the protein that could bind to small molecules capable of inhibiting survivin's function.

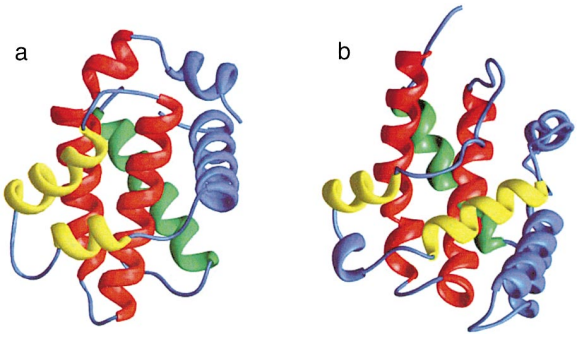

Figure 9. Structural Comparison of Bcl-xL and the Pore Forming Domain of Diphtheria Toxin

Both Bcl-xL (A) and diphtheria toxin (B) contain two central hydrophobic helices (red) surrounded by amphipathic α helices (Muchmore et al., 1996).

Bcl-2 Family of Proteins

Other important regulators of programmed cell death are the Bcl-2 family of proteins. Members of this family, such as Bcl-2 and Bcl-xL, can inhibit apoptosis, whereas others promote cell death (e.g., Bax, Bak, Bad, and Bid). The antiapoptotic proteins interact with the proapoptotic family members to form heterodimers that modulate each other's activity. In fact, the relative levels of these proteins in a cell determine whether a cell will live or die (Yin et al., 1994).

Many of the Bcl-2 proteins contain a hydrophobic C-terminal tail that is responsible for localizing the proteins to the mitochondrial outer membrane, endoplasmic reticulum, and nuclear membrane. The antiapoptotic family members also contain Bcl-2 homology (BH) regions that are important for their antiapoptotic activity (BH1, BH2, BH3, and BH4). Some of the proapoptotic Bcl-2 proteins also contain BH1, BH2, and BH3 regions while others only contain a BH3 region (Kelekar and Thompson, 1998). The BH3 region is responsible for both binding to the antiapoptotic proteins and the ability to promote programmed cell death (Chittenden et al., 1995). Interestingly, the proapoptotic family members that contain BH1 and BH2 regions such as Bax can promote cell death independent from their ability to interact with antiapoptotic proteins through interactions with mitochondrial membranes.

The three-dimensional structure of Bcl-xL was determined by both X-ray crystallography and NMR spectroscopy (Muchmore et al., 1996). The structure consists of two central, predominantly hydrophobic α helices that are flanked on both sides by amphipathic α helices (Figure 9A). A large, flexible loop of about 50 amino acids is located between α1 and α2. To determine whether this loop was functionally important, it was removed. Instead of inhibiting the antiapoptotic activity of Bcl-xL and Bcl-2, loop-deleted mutants of these proteins enhanced their ability to inhibit programmed cell death,

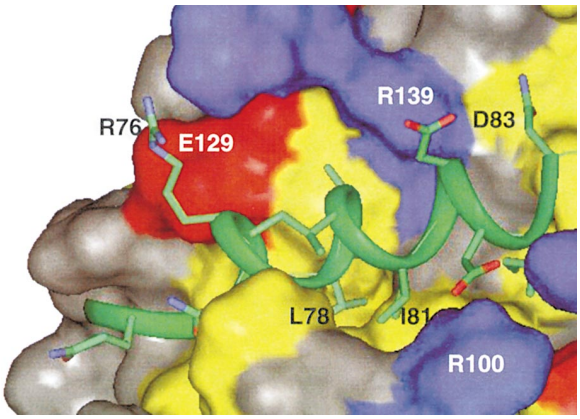

Figure 10. Blow-Up of the Bcl-xL/Bak Peptide Complex

The Bak peptide (green) adopts an amphipathic α helix and binds into a hydrophobic cleft (yellow) in Bcl-xL. The complex is stabilized by hydrophobic interactions involving Leu78 and Ile81 of Bak and electrostatic interactions between R76 and D83 of Bak and E129 and R139 of Bcl-xL. (Sattler, et al., 1997).

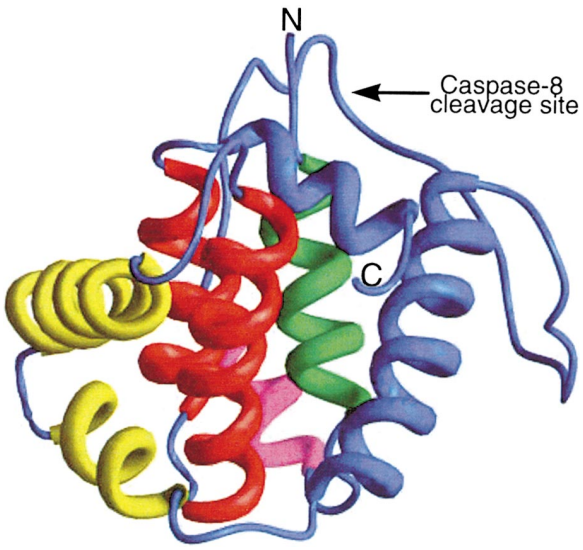

Figure 11. NMR Structure of BID (Chou et al., 1999)

suggesting that the loop region plays a negative regulatory role in the signaling of programmed cell death (Chang et al., 1997).

Another interesting feature of the Bcl-xL structure is its similarity to the pore-forming domains of bacterial toxins (Muchmore et al., 1996). Both Bcl-xL and bacterial toxins such as diphtheria toxin and the colicins contain two central, hydrophobic α helices of about 30 Å in length that are surrounded by amphipathic α helices (Figure 9). Based on this structural similarity, it was postulated that Bcl-2 proteins may form pores in membranes (Muchmore et al., 1996) analogous to the structurally similar bacterial toxins (London, 1992). Indeed, Bcl-xL (Minn et al., 1997) as well as other members of the Bcl-2 family (Antonsson et al., 1997; Schendel et al., 1997; Schlesinger et al., 1997) were shown to insert into synthetic lipid vesicles or planar lipid bilayers and form pH-dependent, ion-conducting channels. Although it is still unclear if these channels are functionally involved in apoptosis, pore formation by Bcl-2 proteins has been hypothesized to be important for regulating the release of cytochrome c and perturbing mitochondrial physiology (Vander Heiden et al., 1997). The structure of the membrane-associated form of Bcl-2 proteins could be very useful for understanding their mode of action. However, the structures of membrane-associated proteins are difficult to obtain. As a first step, the structure of Bcl-xL in detergent micelles was characterized (Losonczi et al., 2000). The structure of Bcl-xL in membrane-like environment was very different from its structure in aqueous solution. In the micelle, Bcl-xL has a loosely packed dynamic structure with helices 1, 6, and 5 partially buried in the hydrophobic interior and the other portions of the protein near or on the outside of the micelle.

The functionally important BH1, BH2, and BH3 regions of Bcl-xL are distant in the primary sequence; however, they are located in the same portion of the tertiary structure and form a hydrophobic cleft that interacts with proapoptotic Bcl-2 family members. The NMR structure of Bcl-xL complexed to the death-promoting BH3 region of the Bak protein indicated that the Bak peptide adopts an amphipathic α helix that interacts with Bcl-xL through specific hydrophobic and electrostatic interactions (Figure 10) (Sattler et al., 1997). The relative importance of these interactions revealed from the structure of the complex and binding measurements of Bak mutant peptides define the binding affinity and specificity observed within the Bcl-2 family of proteins.

The NMR structure of a proapoptotic member of the Bcl-2 family, BID, has also been recently determined (Chou et al., 1999; McDonnell et al., 1999). Despite the lack of sequence homology, the structure of BID (Figure 11) is very similar to that of Bcl-xL (Figure 9A). The only differences are in the length and orientations of some of the helices and the extra α helix (Figure 11, magenta) found in BID. On the basis of the structure, a mechanism was postulated for the increase in BID's proapoptotic activity upon cleavage in the loop region by caspase-8 (Figure 11) (Li et al., 1998b; Luo et al., 1998). This proposed mechanism involves the exposure of the BH3 region, which enhances the binding of BID to Bcl-xL, and the exposure of a hydrophobic surface, leading to the translocation of truncated BID to the mitochondrial membrane. Consistent with the structural analysis, the ion channel activity of BID in model membranes was found to be dependent on the caspase-mediated cleavage of the protein (Schendel et al., 1999).

Conclusions and Future Perspectives

Three-dimensional structures of many proteins that regulate or directly participate in the major signaling pathways of programmed cell death have been determined. These structures have taught us how ligands such as TNF-β and TRAIL recognize death receptors and how small protein modules (death domains, death effector domains, caspase recruitment domains, and the N-terminal domain of CIDE proteins) participate in homophilic interactions important in cell death signaling. From the structures of caspase/inhibitor complexes, we've learned

about their mechanism for protease cleavage and the structural basis for substrate and inhibitor recognition. This information will be important for designing selective inhibitors of this family of enzymes that have potential clinical utility for the treatment of stroke and acute myocardial infarction. Structural and mutagenesis studies of the IAPs have defined the important residues for the interaction of XIAP with caspase-3 and suggest how these two proteins interact with one another. In addition, the structural similarity of Bcl-xL and bacterial toxins, led to the hypothesis that the Bcl-2 family of proteins may function to form pores in membranes, which has subsequently been shown to be true. Finally, the structure of Bcl-xL complexed with the death-promoting region of Bak defines how members of the Bcl-2 family of proteins interact with one another and serves as a starting point for the design of small molecules that might interact with Bcl-xL and induce apoptosis. These compounds may be useful for the treatment of cancers in which the normal mechanisms for cell suicide have been dismantled.

Despite much progress, there are still many unanswered questions in the signal transduction of apoptosis where structural studies could provide answers. The structure of the proform of a caspase should provide insight on the mechanism of activation for this family of enzymes. The structure of an IAP bound to a caspase will reveal the molecular details of the interactions between these proteins and the mechanism of IAP-mediated caspase inhibition. It would also be useful to determine the three-dimensional structure of a Bcl-2 protein in a membrane to structurally characterize channel formation. As new proteins are discovered that participate in the signaling of programmed cell death, the structures of these new proteins could provide clues on their function, mechanisms of action, or how they interact with other proteins. Clearly, the three-dimensional structures of proteins involved in apoptosis will continue to play an important role for understanding cell death signaling at the molecular level.

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