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A structural view of mitochondria-mediated apoptosis

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Mitochondria-mediated apoptosis plays a central role in animal development and tissue homeostasis, and its alteration results in a range of malignant disorders including cancer. Upon apoptotic stimuli, the mitochondrial proteins cytochrome *c* and Smac/DIABLO are released into the cytosol, where they synergistically activate caspases by activating Apaf-1 and relieving the apoptotic inhibition by IAPs. Recent biochemical and structural studies reveal a molecular basis for these important events and identify an evolutionarily conserved mechanism of apoptosis from fruit flies to mammals.

Apoptosis, the dominant form of programmed cell death, refers to the shedding of the leaves from trees in Greek. The distinct morphological changes of cells undergoing apoptosis are sequentially characterized by shrinkage of the cell, hypercondensation of chromatin, cleavage of chromosomes into nucleosomes, violent blebbing of the plasma membrane, and packaging of cellular contents into membrane-enclosed vesicles called 'apoptotic bodies'. These apoptotic bodies are subsequently phagocytosed by surrounding cells. The orderly execution of apoptosis is orchestrated by an evolutionarily conserved paradigm from worms to humans (Fig. 1).

The fundamental significance of apoptosis as an intrinsic and integral component of cell growth and differentiation was only realized in the last decade, with the discovery and characterization of the cell death machinery in *Caenorhabditis elegans* and mammals[1–4] (Fig. 1). In metazoans, homeostasis is maintained through a delicate balance between cell proliferation and programmed cell death. Alterations in apoptotic pathways result in loss of this balance, leading to a number of diseases in humans. For example, abnormal down-regulation of apoptosis is an important contributor to cancer and autoimmune diseases, whereas excessive up-regulation of cell death is implicated in neurodegenerative disorders such as Alzheimer's disease[5,6].

There are two major apoptotic pathways known to date, initiated by either the mitochondria (the 'intrinsic' pathway) or the cell surface receptors (the 'extrinsic' pathway). Mitochondria-mediated apoptosis occurs in response to a wide range of death stimuli, including activation of tumor suppressor proteins (such as p53) and oncogenes (such as c-Myc), DNA damage, chemotherapeutic agents, serum starvation, and ultraviolet radiation. It is generally recognized that this pathway must be inactivated in all cancer cells. Thus elucidation of this pathway has important ramifications for the therapeutic treatment of several malignant diseases.

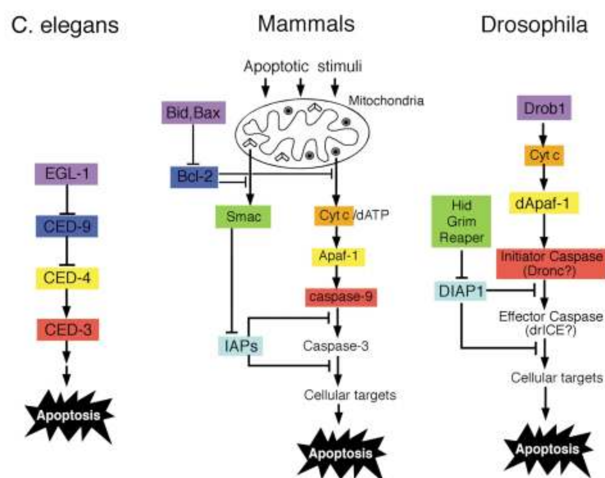

Fig. 1 Conserved apoptotic paradigms in *C. elegans*, *Drosophia*, and mammals. Functional homologs across species are represented by the same color. CED-3 in *C. elegans* may be both the initiator and the effector caspase. The *Drosophila* Apaf-1 are named Dapaf-1 (ref. 34), or HAC-1 (ref. 35), or Dark[36]; the *Drosophila* protein Drob1 (ref. 82) is also named dBorg1 (ref. 83), or Debcl[84], or DBok[85].

Conserved genetic and biochemical paradigms

Apoptosis is executed by the initiator and effector caspases[7,8]. Caspases are a family of cysteine proteases that cleave their substrates after an aspartate residue. Caspases are produced in cells as catalytically inactive zymogens and must be proteolytically processed to become active proteases. The activation of an effector caspase, such as caspase-3, is performed by an initiator caspase, such as caspase-9, through proteolytic cleavage at specific internal Asp residues to separate the two subunits of the mature caspase. Once activated, the effector caspases are responsible for the cleavage of a broad spectrum of cellular targets that ultimately lead to cell death[7]. The activation of the initiator caspases is in turn regulated by upstream protein complexes (Fig. 1).

Genetic studies have identified four genes that act sequentially to control the onset of apoptosis in *C. elegans*[1,9,10] (Fig. 1). Among these four components, CED-3 (CED stands for cell death abnormal) is the apoptotic initiator caspase. The activation of CED-3 is likely to be mediated by an adaptor protein CED-4, involving mutual recognition of their respective caspase recruitment domains (CARDs) and oligomerization of CED-4 (ref. 11). The pro-apoptotic activity of CED-4 is in turn regulated by the anti-apoptotic protein CED-9, again through direct physical interaction. The negative regulation of CED-4 by CED-9 can be removed by EGL-1, a BH3-only member of

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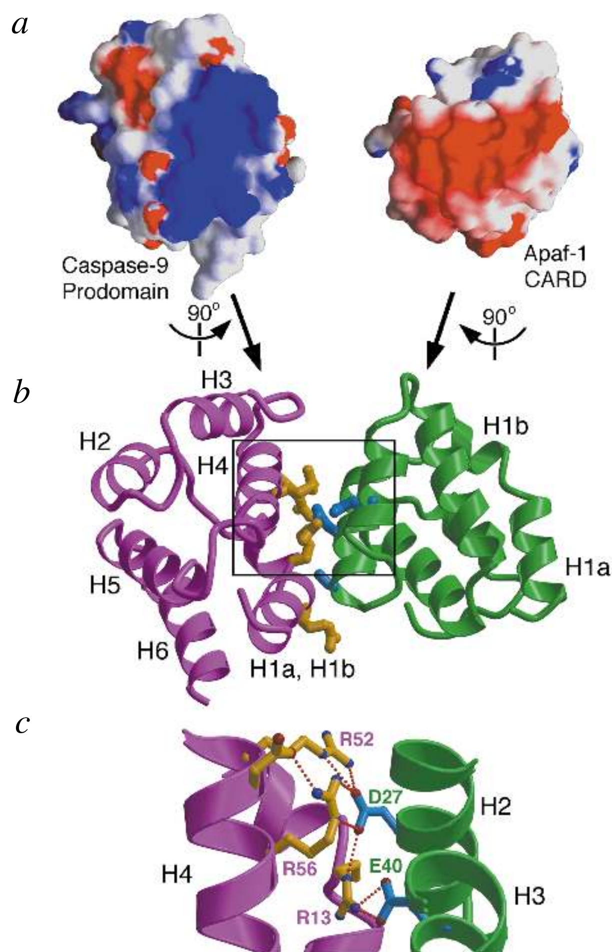

Fig. 2 Recruitment of procaspase-9 by Apaf-1 through CARD–CARD interactions. ***a***, The procaspase-9 prodomain and the Apaf-1 CARD are colored on the basis of electrostatic potential, with blue and red representing positively and negatively charged surface areas, respectively. ***b***, Recognition between procaspase-9 and Apaf-1 involves the positively charged residues from the prodomain and the negatively charged residues from the CARD. The boxed region is shown in detail in (*c*). ***c***, Close-up view of the central interactions involving three Arg residues on helices H4 and H1 of caspase-9 and two acidic residues (Asp 27 and Glu 40) on helices H2 and H3 of Apaf-1. Hydrogen bonds are represented by red dashed lines.

the Bcl-2 superfamily. During *C. elegans* development, the interplay among CED-3, CED-4, CED–9 and EGL-1 results in the death of 131 cells at precise times during development and in precise locations[1].

In mammalian cells, Bid and Bcl-2 exhibit homology in both sequence and function to the *C. elegans* proteins EGL-1 and CED-9, respectively (Fig. 1). Using classic biochemical fractionation and characterization, Xiaodong Wang and coworkers identified caspase-9 and Apaf-1 (apoptotic protease activating factor 1) as the mammalian homologs of *C. elegans* CED-3 and CED-4, respectively[12,13]. Apaf-1 exists in three distinct splice forms, each containing an N-terminal CARD domain, a central CED-4-homology domain, and 13 or 14 repeats of the WD-40 motif at the C-terminal half. The activation of procaspase-9 is similar to the activation of CED-3 except with an added complexity involving cytochrome *c* (Cyt *c*)[13].

Upon receiving an apoptotic stimulus, Cyt *c* is released from the intermembrane space of mitochondria into the cytoplasm, a process regulated by the Bcl-2 family members[14,15] (Fig. 1). Once in the cytosol, Cyt *c* binds tightly to Apaf-1, presumably changing its conformation from an inhibitory to an active form[16,17]. The binary complex of Apaf-1 and Cyt *c* then binds its critical cofactor, dATP or ATP, forming a multimeric complex dubbed the 'apoptosome' (refs 11, 18–21). The only known function of the apoptosome is to recruit and to facilitate activation of procaspase-9[11,18–21]. Once activated, caspase-9 stays with the apoptosome as a holoenzyme to maintain its catalytic activity, as the free caspase-9 is marginally active[17,22,23]. In this respect, the apoptosome serves as an allosteric regulator for the enzymatic activity of caspase-9.

The primary target of the caspase-9 holoenzyme is procaspase-3, one of the most deleterious effector caspases (Fig. 1). The active caspase-3, composed of a heterotetramer of subunits p17 and p12, cleaves a wide range of protein substrates, including caspase-2, caspase-6, nuclear lamin, DNA-dependent protein kinase, and DNA fragmentation factor 45 (DFF45 or ICAD)[7]. In particular, degradation of DFF45 releases its inhibition of DFF40 (or CAD), which functions as a nuclease and degrades chromatin into nucleosomal fragments, a hallmark of apoptosis.

The Inhibitor of Apoptosis (IAP) family of proteins, originally identified in the genome of baculovirus based on their ability to suppress apoptosis in infected host cells, interact with and inhibit the enzymatic activity of mature caspases[24,25] (Fig. 1). Several distinct mammalian IAPs including XIAP, c-IAP1, c-IAP2 and survivin, have been identified, and they all exhibit anti-apoptotic activity in cell culture[24]. The functional unit in each IAP protein is the so-called baculoviral IAP repeat (BIR), which contains ~80 amino acids folded around a zinc atom. Most mammalian IAPs have more than one BIR domain, with the different BIR domains exhibiting distinct functions. For example, in XIAP, the third BIR domain (BIR3) potently inhibits the activity of processed caspase-9 whereas the linker region between BIR1 and BIR2 selectively targets active caspase-3 (refs. 26–29).

In normal surviving cells that have not received an apoptotic stimulus, aberrant activation of caspases can be inhibited by IAPs. In cells signaled to undergo apoptosis, however, this inhibitory effect must be suppressed, a process mediated by a mitochondrial protein named Smac (second mitochondria-derived activator of caspases)[30], also called DIABLO (direct IAP binding protein with low pI)[31]. Smac, synthesized in the cytoplasm, is targeted to the intermembrane space of mitochondria[30]. Upon apoptotic stimuli, Smac is released from mitochondria into the cytosol, together with Cyt *c*. Whereas Cyc *c* directly activates Apaf-1 and caspase-9, Smac interacts with multiple IAPs and relieves their inhibitory effect on both initiator and effector caspases[26,30–32] (Fig. 1).

Homologs of many components in mammalian apoptosis have been identified in the fruitfly *Drosophila Melanogaster*[33] (Fig. 1). The *Drosophila* Apaf-1 (Dapaf-1/HAC-1/Dark) closely resembles its mammalian counterpart and is critically important for the activation of *Drosophila* caspases[34–36]. Two *Drosophila* IAPs, DIAP1 and DIAP2, have been identified, and DIAP1 binds and inactivates several *Drosophila* caspases[37–39]. DIAP1 contains two BIR domains; the second BIR domain (BIR2) is necessary and sufficient to block cell death in many but not all contexts[40]. In *Drosophila* cells, the anti-death func-

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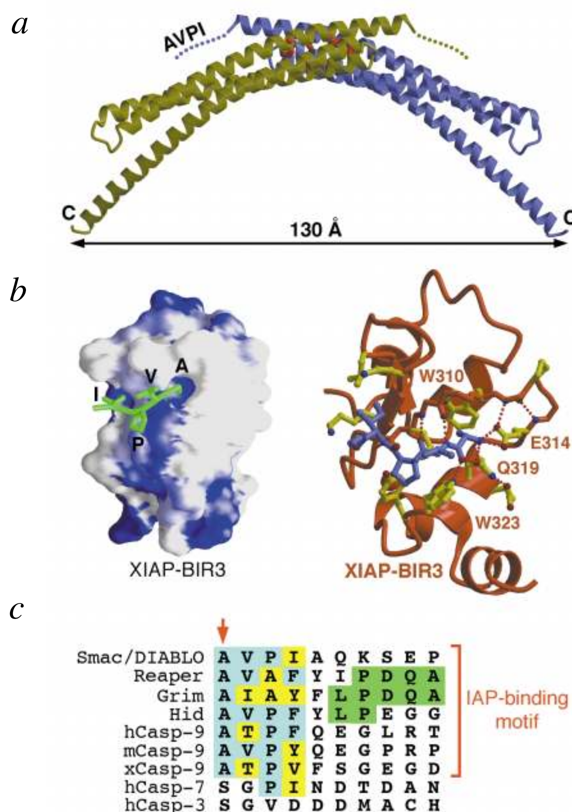

Fig. 3 Smac function and a conserved IAP-binding motif. ***a***, Schematic diagram of the mature dimeric Smac protein. The disordered N-terminal residues are shown as dotted lines. ***b***, Interaction of the Smac N-terminal tetrapeptide (Ala-Val-Pro-Ile) with the BIR3 domain of XIAP. The BIR3 domain is represented as a surface model in the left panel, with the hydrophobic areas colored blue. Detailed atomic interactions are depicted in the right panel. ***c***, A family of IAP-interacting motifs. The tetrapeptide motif has the consensus sequence A-(V/T/I)-(P/A)-(F/Y/I/V). The Drosophila proteins may have an additional binding component (conserved residues 6–8, shaded in green).

tion of DIAP1 is removed by three pro-apoptotic proteins, Hid, Grim and Reaper, which physically interact with the BIR2 domain of DIAP1 and prevent its inhibitory effect on caspases[39,41–43]. Thus Hid, Grim and Reaper represent the functional homologs of the mammalian protein Smac/DIABLO. Although generally regarded as having similar functions in *Drosophila* development, Hid, Grim and Reaper only share homology in the N-terminal 14 residues in their primary sequences. These N-terminal sequences are responsible for interaction with DIAP1, and this function is essential to the induction of cell death in both *Drosophila* and mammalian cells[44].

Recruitment of caspase-9 by Apaf-1

Apaf-1 and caspase-9 play a dominant role in the regulation of programmed cell death in mammalian development and in oncogene- and p53-dependent apoptosis[45–48]. The central importance of this pathway is made clear by the observation that Apaf-1 is frequently inactivated in cancers such as malignant melanoma[49]. The recognition of procaspase-9 by Apaf-1, primarily through a CARD–CARD interaction, is essential to the formation of the apoptosome holoenzyme and subsequent activation of caspases[13]. The 97-residue CARD domain of Apaf-1 shares ~20% sequence identity with the prodomain of procaspase-9, which also belongs to the CARD family of apoptotic signaling motifs[50]. The significance of the CARD-homology domain is supported by its presence in a number of other caspase-activating proteins, such as CED-4 and RAIDD/ CRADD, and other initiator caspases, such as CED-3 and caspase-2/ ICH-1.

The structure of the CARD has been determined for both RAIDD[51] and Apaf-1 (refs 52–55). The CARD adopts a compact globular fold, with six α-helices tightly packed around a central hydrophobic core (Fig. 2). The H1 helix of the CARD domains in both Apaf-1 and procaspase-9 is severely bent (54–65°), resulting in two short α helices H1a and H1b connected by a two-residue intervening linker[52]. Despite close similarity between the CARD domains of RAIDD and procaspase-9, the H1 helix of RAIDD CARD is only slightly bent (15°) and retains its integrity as a single α-helix through maintenance of all intrahelical hydrogen bonds[51].

The surface of the CARD domain contains two oppositely charged surface patches located on two adjacent sides of the molecule[51,52]. These charged surfaces constitute the recognition motifs between Apaf-1 and procaspase-9. The positively charged surface of procaspase-9 CARD formed by the helices H1a/H1b and H4 is recognized by Apaf-1 CARD through a negatively charged surface formed by the helices H2 and H3 (ref. 52). (Fig. 2). Mutation of the important interface residues in procaspase-9 (Arg 13 and Arg 56) or Apaf-1 (Asp 27 and Glu 40) resulted in the loss of mutual interaction and abolishment of procaspase-9 activation in a cell-free system[52]. In addition, wild type but not mutant CARD of procaspase-9 completely inhibited catalytic processing of procaspase-9[52]. Analysis of the *C. elegans* homologs suggests that CED-3 recruitment by CED-4 is mediated by the same set of conserved structural motifs, with a corresponding change in the specificity-determining residues[52]. These results raise the possibility of using small molecules to target the recognition of procaspase-9 by Apaf-1, a concept reinforced by the rich surface features of Apaf-1 and procaspase-9.

The CARD domain is only one representative member in a conserved family of homophilic interaction motifs[50], which also include the death domain (DD)[56–59] and the death effector domain (DED)[60]. These motifs share the same structural topology with different surface features and minor variation in interhelical packing, which give rise to specific homophilic recognition. The recent structure of a death domain complex in *Drosophila* reveals an interesting addition to the diverse recognition mechanisms by these simple motifs[59].

Function of Smac/DIABLO

Inhibition of both caspase-9 and caspase-3 by IAPs is relieved by the mature Smac protein through its interactions with XIAP, c-IAP1, c-IAP2 and survivin[26,30–32]. Smac functions as an elongated arch-shaped dimer, spanning over 130 Å in length[26] (Fig. 3*a*). The wild type Smac protein forms a stable complex with both the BIR2 and the BIR3 domains of XIAP but does not interact with the BIR1 domain[26]. In contrast, the monomeric Smac mutants retain strong interaction with the BIR3 domain but can no longer form a stable complex with the BIR2 domain[26]. Consequently, monomeric Smac mutants cannot relieve XIAP's inhibition of caspase-3 because the linker sequence immediately preceding the BIR2 domain is involved in binding and inhibiting caspase-3. Despite maintenance of interactions with

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the caspase-9-binding BIR3 domain, the monomeric Smac mutants also exhibit significantly compromised activity in terms of relieving XIAP's inhibition of caspase-9[26]. This is likely due to the loss of cooperative binding as caspase-9, Smac and possibly XIAP, all exist as homodimers in solution.

The full-length Smac protein contains 239 amino acids, with the N-terminal 55 residues encoding the mitochondria-targeting sequence that is removed upon import[30]. Thus the mature Smac protein has 184 amino acids, with the N-terminal four residues being Ala-Val-Pro-Ile. These N-terminal residues, although disordered in the Smac crystals, play an indispensable role in Smac function; a seven-residue peptide derived from the N-terminus of Smac can remove the inhibition of caspase-9 by XIAP[26]. Strikingly, a single missense mutation involving the N-terminal residue Ala (Ala 1 to Met) in Smac leads to complete loss of interactions with XIAP and concomitant loss of Smac function[26]. As IAPs are highly amplified in cancer cells[61–63], these findings suggest potential therapeutic applications using these peptides or small molecules as prototypical drugs.

IAP binding by Smac tetrapeptide

The structural explanation for the indispensable role of the Smac N-terminal sequences is provided by both NMR[64] and X-ray[65] analyses. The NMR study focused on the recognition between the BIR3 domain of XIAP and an eight-residue Smac peptide while the crystallographic analysis employed the mature Smac protein[64,65]. Both structures reveal that the Smac N-terminal tetrapeptide (Ala-Val-Pro-Ile) recognizes a surface groove on the BIR3 domain, with the first residue, Ala 1, binding a hydrophobic pocket and making several hydrogen bonds to neighboring XIAP residues (Fig. 3*b*). Stereochemical parameters indicate that replacement of Ala 1 by any other residue except Gly will cause steric hindrance in this pocket, likely weakening binding and abolishing hydrogen bonds by the amino and carbonyl groups of Ala 1. This observation explains the finding that the mutation Ala 1 to Met in Smac completely eliminated interaction with the BIR domains.

The surface groove on XIAP comprises highly conserved residues among the BIR3 domains of c-IAP1 and c-IAP2 and the BIR2 domain of the *Drosophila* protein DIAP1. This conservation and the realization that a mere tetrapeptide suffices IAP-recognition prompted a reexamination of the sequence homology between Smac and the *Drosophila* proteins Hid/Grim/Reaper. Indeed, the N-terminal four amino acids of the *Drosophila* proteins share significant similarity with the mammalian protein Smac[64,65] (Fig. 3*c*). This sequence conservation strongly suggests that the N-terminal sequences of the *Drosophila* proteins Hid/Grim/Reaper may recognize a similarly conserved surface groove on DIAP1. Because the Hid/Grim/Reaper proteins are translated with an initiation Met residue that likely impedes DIAP1-binding, this analysis predicts that the N-terminal Met residue must be removed by an amino peptidase in *Drosophila* cells that are programmed to die[65]. Since removal of the initiation Met may be a regulated process in development and homeostasis, this analysis also implicates the regulation of methionine amino peptidases (MAPs) in diseases such as cancer. Interestingly, small molecule modulators of MAPs, such as TNP-470, have been identified as promising anticancer drugs. Perhaps there are other yet-to-be-identified pro-apoptotic proteins bearing N-terminal homology to the Ala-Val-Pro-Ile sequence[65]. It is also possible that, during apoptosis, a similar motif in the middle of a protein can be released upon caspase cleavage before the Ala residue.

Two residues in XIAP, Trp 310 and Glu 314, which are important for binding and inhibiting caspase-9 (ref. 29), are also involved in mediating critical contacts to the Smac tetrapeptide[64,65]. Hence a mutual exclusion model, in which caspase-9 and Smac compete for binding to the common surface groove in the XIAP-BIR3 domain, was proposed[64,65]. These analyses validate the BIR3 domain of XIAP as the potential target for anti-cancer drug screening and suggest that potential drugs may mimic the Smac tetrapeptide to occupy the surface groove on XIAP.

A conserved IAP-interaction motif

Unlike any other caspase, the unprocessed form of procaspase-9 can activate downstream caspases to similar levels as the wild type caspase-9 (ref. 66). However, XIAP selectively interacts with and inhibits the activity of the processed caspase-9 but exhibits no effect on the unprocessed procaspase-9 (refs 23, 67). Through sequence comparison, a Smac-like tetrapeptide (Ala 316-Thr 317-Pro 318-Phe 319) was discovered in procaspase-9 (ref. 23). Interestingly, the proteolytic cleavage that gives rise to the mature caspase-9 occurs after Asp 315, thus releasing a potential XIAP-binding motif[23]. Indeed, this tetrapeptide motif in the p12 subunit of caspase-9 is primarily responsible for the interactions with the BIR3 domain of XIAP[23]. Although the XIAP-BIR3 domain does not target the wild type caspase-3, it potently inhibits an engineered caspase-3 in which the caspase-9 tetrapeptide motif is inserted behind the caspase-3 processing site[23].

These observations reveal a novel mechanism for the activation and inhibition of caspase-9. Unlike other caspases, proteolytic processing of procaspase-9 serves as a mechanism for inhibition, rather than activation. In the absence of proteolytic processing, XIAP is unable to interact with procaspase-9 or inhibit its enzymatic activity. Upon apoptotic stimuli, procaspase-9 undergoes autocatalytic processing after Asp 315, exposing its internal tetrapeptide motif and resulting in the recruitment of and inhibition by XIAP. The release of the mature Smac from mitochondria titrates the inhibiting XIAP protein, again using the same conserved N-terminal tetrapeptide in Smac. Thus, a conserved XIAP-interaction motif in caspase-9 and Smac mediates opposing effects on caspase activity and apoptosis. During apoptosis, the active caspase-9 can be further cleaved after Asp 330 by downstream caspases such as caspase-3. This positive feedback completely removes XIAP inhibition to caspase-9 and releases a 15-residue peptide that is free to relieve XIAP's inhibition of other caspases. Thus, this peptide represents a pro-apoptotic drug made by the apoptotic cells themselves.

Together with the N-terminal sequences of the *Drosophila* proteins Hid/Grim/Reaper, the tetrapeptides in Smac and caspase-9 define an evolutionarily conserved family of IAP-binding motifs (Fig. 3*c*). Given the serendipitous nature of this discovery, it is certain that this family will further expand to include additional members. For example, there may be mammalian homologs of the *Drosophila* proteins Hid/Grim/Reaper and a *Drosophila* homolog of the mammalian protein Smac.

Inhibition of caspases by IAPs

Because the inhibition of caspase activity could prove important for the therapeutic intervention of many diseases, structural characterization has been performed on several caspases including caspase-1, caspase-3, caspase-7, and caspase-8 (refs 7,68). On the basis of function and substrate specificity, caspases are

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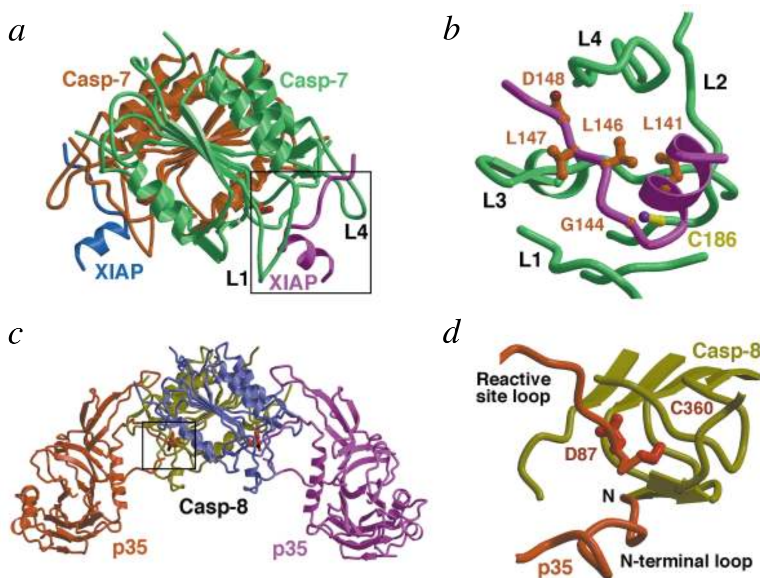

Fig. 4 Inhibition of caspases by IAP and p35. ***a***, Structure of caspase-7 bound to an XIAP fragment. The same XIAP fragment also binds identically to caspase-3 although the BIR2 domain appears to contribute additional interactions and inhibition. ***b***, Close-up view of the XIAP fragment bound to the catalytic cleft of caspase-7. The key residues in XIAP and the catalytic residue in caspase-7 (Cys 186) are labeled. ***c***, Structure of caspase-8 bound to the baculoviral inhibitor p35. ***d***, Close-up view of the covalent inhibition of caspase-8 by p35. The thioester intermediate is shown between Asp 87 of p35 and Cys 360 (active site residue). The N-terminus of p35 restricts solvent access to this intermediate.

divided into three families, the initiator caspases such as caspase-9 and caspase-8 (with long prodomains), the effector caspases such as caspase-3 and caspase-7 (with short prodomains), and those with function closely related to interleukin-1β converting enzyme (ICE) or caspase-1.

IAPs use distinct domains to inhibit the enzymatic activity of both the initiator caspase, caspase-9, and the effector caspase, caspase-3. For caspase-9 inhibition, the primary determinant is the conserved surface groove on the BIR3 domain of XIAP and the conserved tetrapeptide motif in caspase-9 (refs 23,29). The close proximity of the N-terminus of the p12 subunit and the catalytic site of caspase-9 suggests that XIAP may block substrate entry to the active site. Despite the major contribution from the binding of the caspase-9 tetrapeptide to XIAP, other weaker interactions also contribute to the inhibition of caspase-9. For example, mutation of His 343 in XIAP-BIR3 results in complete loss of inhibition to caspase-9, suggesting that His 343 makes important contacts to caspase-9 (ref. 29). Furthermore, IAPs are likely to dimerize in solution[69], which could further block substrate entry.

If the Smac tetrapeptide interacts with the BIR3 domain of XIAP in the same manner as does caspase-9, how can Smac gain an edge in removing the inhibition of capase-9 by XIAP? First, in addition to tetrapeptide binding, Smac uses an additional interface to bind the BIR3 domain of XIAP[65]. Second, Smac also interacts with the BIR2 domain of XIAP[26], which could facilitate the Smac–BIR3 interactions. Third, in apoptotic cells, the amount of Smac released from the mitochondria could overwhelm XIAP, as long as the affinity of the Smac–XIAP interaction is comparable to that of the XIAP-caspase-9 interaction. Finally, caspase-3 activated by small amounts of active caspase-9 can cleave caspase-9 after Asp 330, providing a positive feedback loop. This ensures the termination of inhibition by IAPs. Thus, Smac protein could just serve as the trigger and may not need to overwhelm IAPs in quantity.

XIAP employs a different strategy to inhibit the effector caspase, caspase-3. Mutational analysis reveals that the linker region between the BIR1 and BIR2 domains of XIAP (residues 124–156) play a central role in the inhibition of caspase-3 (ref. 28). In the XIAP-linker-BIR2 fragment (residues 124–240), mutation of Asp 148 or Leu 141 to Ala nearly abolished the inhibition, while mutations of several other residues, including Val 147 and Ile 149, decreased inhibition significantly[28]. Interestingly, although neither BIR1 nor BIR2 exhibits any inhibition to caspase-3, the intervening linker peptide alone failed to inhibit caspase-3 (ref. 28). More surprisingly, an engineered protein with the linker peptide fused either N- or C-terminal to the BIR1 domain was fully able to bind and inhibit caspase-3 (ref. 28).

Recent biochemical and structural analyses provide an explanation for these observations[70–72] (Fig. 4). The linker peptide by itself appears to adopt an 'unproductive' conformation that renders it unable to bind and inhibit caspase-3 or caspase-7 (ref. 70). Tethering at least one terminus of this linker region by either a BIR domain or even GST prevents this unproductive conformation and allows it to bind and inhibit both caspase-3 and caspase-7 (refs 70,71). Although the BIR2 domain by itself does not inhibit caspase-3 or caspase-7, it appears to augment the inhibitory effect of the linker peptide[70–72]. Indeed, the BIR2 domain makes additional contacts to caspase-3 in the crystals[72]. Upon binding to caspase-3 or caspase-7, an 18-residue XIAP peptide from the linker region fills the catalytic groove, making extensive contacts to the residues that are essential for the catalytic activity of caspases (Fig. 4). Four residues from the XIAP linker peptide, Gly 144, Val 146, Val 147, and Asp 148, occupy the corresponding positions for the P1-P2-P3-P4 residues of the substrates, respectively. The P1 position is occupied by the N-terminal Gly 144 of these four residues. Thus this orientation is the reverse of that observed for the tetrapeptide caspase inhibitor, in which the P1 position is occupied by the C-terminal Asp. Interestingly, despite a reversal of relative orientation, a subset of interactions between caspase-7 or caspase-3 and XIAP closely resemble those between caspase-7 or caspase-3 and its tetrapeptide inhibitor DEVD-CHO[68]. Nature has apparently chosen an opposite orientation of caspase inhibitor, which may have important implications for the design of novel caspase inhibitors.

IAPs are not the only natural inhibitors to caspases. In fact, the p35 protein from baculovirus is a pan-caspase inhibitor, as it potently inhibits all three families of caspases[25]. Caspase inhibition by p35 correlates with the cleavage of its reactive site loop after residue Asp 87 (refs 73,74), which leads to the relocation of the N-terminus of p35 into the active site of caspases[75]. The crystal structure of caspase-8 in complex with p35 reveals a surprising mechanism for inhibition: the catalytic residue Cys 360 of caspase-8 is covalently linked to the Asp 87 of p35 through a thioester bond[75]. This fragile thioester bond is stabilized by the N-terminus of p35, which prevents access by water

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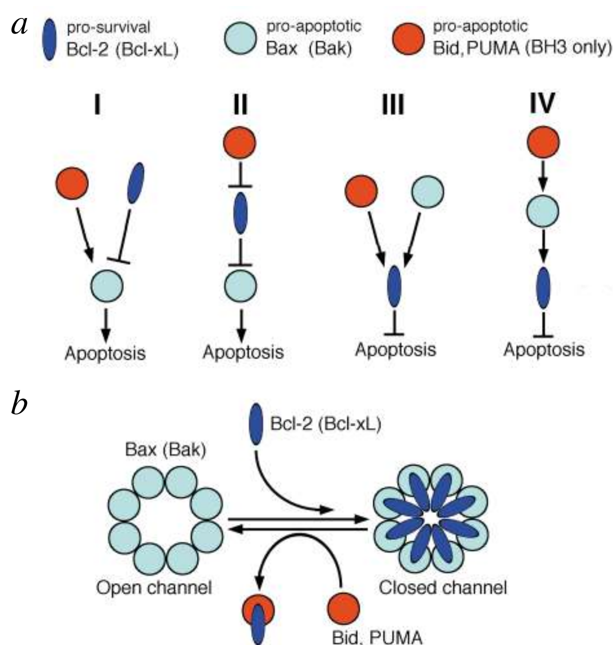

Fig. 5 Regulation of mitochondria permeability by the Bcl-2 family members. ***a***, Bcl-2 and Bcl-xL inhibit apoptosis by preventing release of mitochondrial proteins whereas both the Bax/Bak and the Bid/Bim/PUMA subfamilies promote this process. Four contrasting models are shown. ***b***, A proposed biochemical model. In this model, Bax, either by itself or in association with other factors, is able to form an open channel through which cytochrome *c* and Smac are released. This channel can be negatively modulated by Bcl-2, and this negative modulation can be removed by the BH3-only members.

molecules to this covalent intermediate[75]. Another protein, the serpin CrmA derived from the cowpox virus, can also potently inhibit multiple caspases, likely through covalent modification. This unique mechanism adds to the complexity of caspase inhibition by natural proteins.

Gatekeepers — the Bcl-2 family of proteins

The mitochondria-mediated apoptosis is initiated by the release of Smac and Cyt *c* into the cytosol. This process is controlled by the Bcl-2 family of proteins[15,76,77] (for a comprehensive review on Bcl-2 proteins, see ref. 76). On the basis of function and sequence similarity, the diverse Bcl-2 members can be grouped into three subfamilies. The Bcl-2/Bcl-xL subfamily inhibits programmed cell death by preventing the release of mitochondrial proteins, while the Bax/Bak and Bid/Bim subfamilies induce apoptosis by allowing release. Members in the Bcl-2/Bcl-xL subfamily contain all four conserved Bcl-2 homology domains (BH4, BH3, BH1 and BH2) while the Bax/Bak members lack the BH4 domain and the Bid/Bim subfamily only contain the BH3 domain. Members of the opposing subfamilies as well as between the two pro-apoptotic subfamilies can dimerize, mediated by the amphipathic BH3 helix.

Although Bcl-2 family members are known to control the permeability of the mitochondrial outer membrane, how this is achieved remains highly controversial, with several contrasting models[15,76,77]. Some conflicting observations cannot be explained by any current model. The confusion is in part due to the technical difficulties in studying Bcl-2 family members *in vitro*, as these proteins remain mostly membrane-associated under physiological conditions and undergo significant conformational changes depending upon the surrounding environment. For example, Bax is a monomer in aqueous solution but forms a homo-oligomer in the presence of detergent or lipid.

There are at least four mutually exclusive models that cannot be differentiated by available evidence; in fact, each model is compatible with most experimental observations (Fig. 5*a*). In models I and II, Bax or Bak is responsible for release of mitochondrial proteins and is subject to negative regulation by Bcl-2 or Bcl-xL. Thus, in homeostatic cells, Bax or Bak is quenched by Bcl-2 or Bcl-xL. Recent evidence suggests that model I or II is likely to be correct[78,79]. On the basis of these biochemical observations, a working model is proposed for how Bcl-2 family members regulate the permeability of the mitochondrial outer-membrane (Fig. 5*b*). Bax, the pro-apoptotic representative with three BH domains (BH1, BH2 and BH3), forms a channel either by itself or with the assistance of additional factors. In apoptotic cells, this channel is responsible for the release of all proteins from the intermembrane space of mitochondria to the cytosol. However, binding of Bcl-2 to Bax results in the closure of this channel, as is the case in homeostatic cells. Upon apoptotic stimuli, Bid (BH3-interacting domain death agonist, which mediates the receptor-initiated signal) or PUMA[80] (p53-upregulated modulator of apoptosis, which mediates the p53-initiated signal) is activated and subsequently interacts with the Bcl-2 protein to relieve its inhibition to the Bax-dominated channel. Although this model does not explain all controversies, it is in agreement with a large body of biological and biochemical observations. More importantly, this model differentiates the functions of the two pro-apoptotic subfamilies, the BH3-only (Bid or PUMA) and the BH4-lacking (Bax or Bak) members.

Pressing issues

Cytochrome *c* plays an essential role in the mammalian paradigm of apoptosis, perhaps one of the most important discoveries in recent years. Cells lacking cytochrome *c* exhibit reduced caspase-3 activation and resistance to apoptosis induced by UV irradiation, serum withdrawal, or staurosporine[81]. Evidence hints a similar role for cytochrome *c* in *Drosophila*[34–36].

Recent progress in mitochondria-mediated apoptosis defines a new framework for the final deciphering of this complex process. Many significant questions remain unanswered. From the biological perspective, two central questions are (i) how Bcl-2 family members control the release of pro-apoptotic proteins from the intermembrane space of mitochondria into the cytosol, and (ii) how the various internal apoptotic stimuli, such as DNA damage, are relayed to the mitochondria. Although Bcl-2 family proteins are known to control the permeability of mitochondria, whether they are the primary components of the pores or they merely regulate the open-close status of the pores remain unknown. How the three subfamilies of Bcl-2 proteins work in vivo is also unclear. In *Drosophila*, where powerful genetic manipulations can be employed to reveal mechanisms, mitochondria appear to play a similar role as in mammals. However, only one Bcl-2 family member has been reported in *Drosophila*[82–85], and the identification of additional Bcl-2 homologs is essential for mechanistic studies.

The p53 tumor suppressor protein mediates a range of mitochondria-dependent apoptotic responses including those initiated by DNA damage and hypoxia[86]. Although Bax is

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transcriptionally regulated by p53, it is not necessary to mediate the p53-initiated apoptosis. The recent identification of PUMA may provide the critical missing link[80]. PUMA but not Bax is vigorously activated following p53 expression and induces rapid apoptosis in colorectal cancer cells[80].

Mitochondria appear to play an indispensable role in apoptosis from fruit flies to mammals. Despite the generally conserved apoptotic paradigm (Fig. 1), there is no evidence to support a critical role of mitochondria in the worm *C. elegans*. It remains to be seen whether proteins within mitochondria also contribute to cell death in this lower organism. Although the basic paradigms are clear for the fruit flies and mammals, the intricate inner-workings remain mysterious. Although one Smac splice variant has been reported[32], it is unclear how many Smac homologs exist in mammalian cells and how they function. The *Drosophila* proteins are only homologous at their N-termini; and they display differing abilities to induce cell death in specific contexts. These and other observations argue that these proteins are likely to have more functions than simply to relieve the IAP inhibition of caspases. Their lack of overall homology may also point to distinct mechanisms of regulation. What is the functional difference among the mammalian proteins c-IAP1, c-IAP2, and XIAP? Recent studies suggest that IAPs exhibit E3 ubiquitin ligase activity[87]. How this activity is regulated and what its targets are remain open questions.

Structural and biochemical studies on proteins and protein–protein complexes have begun to reveal valuable insights into the biological functions of the key components in apoptosis. Indeed, the past year saw the pioneering role of structural biology in defining the function of Smac/DIABLO rather than its often confirmatory role. Despite these advances, numerous important issues are yet to be addressed. A structural view of the homo-oligomeric Bax protein may reveal unexpected insights into the functions of the Bcl-2 family members. The structural elucidation of the engine of apoptosis, the 1.4 MDa apoptosome holoenzyme, represents a formidable but tremendously interesting task.

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