PERSPECTIVES: STRUCTURAL BIOLOGY

Controlling the Caspases

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Apoptosis or programmed cell death is an evolutionarily conserved process that removes damaged or unwanted cells (*1*). It is essential for animal development and tissue homeostasis and, when dysregulated, can result in cancer, neurodegenerative disease, or autoimmunity (*2*). There are two major signaling pathways that induce apoptosis. One pathway is initiated by death receptors such as Fas, and the other is activated by molecules released from mitochondria, the energy generators of the cell. Both pathways eventually converge, leading to activation of the central effectors of apoptosis: a group of cysteine proteases, called the caspases, which cleave a number of different substrates (*3, 4*).

Among the most important regulators of the caspases are the IAPs or inhibitor of apoptosis proteins (*5*). Additional regulation is provided by Smac (second mitochondria-derived activator of caspases) or its murine homolog DIABLO (direct IAP-binding protein with low pI), which binds to IAPs and abrogates caspase inhibition (*6, 7*). Structural analyses of IAP and caspase or of IAP and Smac, together with site-directed mutagenesis and biochemical studies, are revealing how apoptosis is regulated.

Members of the IAP family, originally identified in baculoviruses (*8*), contain one or more modules called the baculoviral IAP repeat (BIR) (*5*). Some family members also contain a carboxyl-terminal RING finger through which they are targeted for ubiquitination and subsequent proteolysis (*9, 10*). The BIR domains may be responsible for directly and specifically inhibiting the caspases. For example, a region of X-linked IAP (XIAP) containing the BIR2 domain specifically inhibits caspase-3 and caspase-7 (*11*), whereas the BIR3 domain of XIAP blocks caspase-9 activity (*12*).

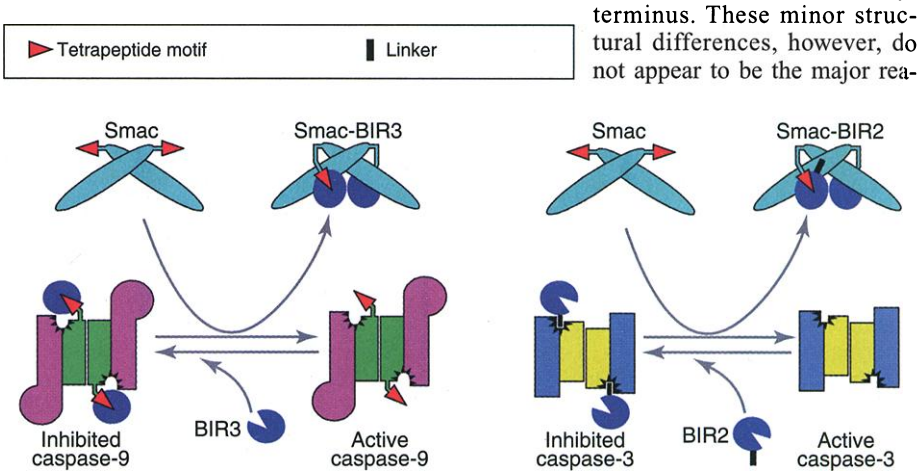
The XIAP BIR2 domain consists of a three-stranded antiparallel β sheet and four α helices, with three highly conserved cysteines and one histidine, which chelates a zinc atom (*13*). Site-directed mutagenesis reveals that the BIR2 domain of XIAP is a minor player in caspase-3 inhibition. Instead, residues in the amino (NH_2) terminal linker sequence of XIAP are critical for caspase-3 inhibition (*13*). These linker amino acids may bind to the active site of caspase-3, with the BIR2 domain interacting with an adjacent site on the enzyme (*13*).

Three x-ray crystal structures reveal how the BIR2-linker region of XIAP interacts with caspase-3 and caspase-7 (*14–16*). The XIAP linker segment completely occupies the active site of caspases—but surprisingly in a reverse orientation compared with peptide-based inhibitors and other substrates—and substrate entry to the caspase catalytic site is completely blocked. An aspartic acid (Asp^{148}) in XIAP is critical for the inhibition of caspase-3. Formation of a complex between caspase-3 or caspase-7 and the XIAP BIR2-linker region appears to be driven by interactions between XIAP's Leu^{141} and Val^{146} and a hydrophobic site present on both caspases. This hydrophobic site is not found in caspase-8 or caspase-9, perhaps explaining the binding specificity of XIAP (*16*).

The BIR2 domain is less important than the linker segment for binding to caspases. In fact, BIR2 was not even observed in the two x-ray structures of caspase-7 complexed with XIAP (*14, 15*). The BIR2 domain can be replaced by either BIR1 (*13*) or glutathione S-transferase (GST) (*14, 15*) without significant loss of caspase inhibition. Although the linker segment, in combination with either of the BIR domains or with GST, is a potent inhibitor of caspase-3 and caspase-7, it cannot bind or inhibit either caspase on its own (*13, 14*). One possibility is that the linker segment by itself adopts a conformation that precludes binding to the caspase active site (*14, 15*). Alternatively, the linker peptide contains a caspase cleavage site (Asp-Ile-Ser-Asp) and thus may have to be cleaved for caspase inhibition to occur.

The structure of the BIR3 domain of XIAP (*17*) is similar to that of BIR2 except for an extra α helix at the carboxyl terminus. These minor structural differences, however, do not appear to be the major reason for the selectivity of caspase inhibition. The BIR2 and BIR3 domains of XIAP inhibit caspase-3 and caspase-9 by very different mechanisms (*17*). For example, the BIR3 domain binds to the NH_2-terminus of the small subunit of caspase-9, which becomes exposed after procaspase-9 is proteolytically cleaved at Asp^{315} (*18*). The peptide sequence of caspase-9 (Ala^{316}-Thr^{317}-Pro^{318}-Phe^{319}) that binds to XIAP resembles the NH_2-terminal residues of Smac that interact with the BIR3 domain of XIAP (*19, 20*) (see the figure). The linker residues NH_2-terminal to the BIR2 domain forge major interactions with caspase-3 and caspase-7, whereas BIR3 itself binds directly to the small subunit of caspase-9.

Additional caspase regulation depends on Smac (*6*) or its murine homolog DIABLO



In the groove. Blocking of caspase-3 and caspase-9 activity by XIAP and abrogation of this inhibition by Smac. (**Left**) The conserved tetrapeptide motif (red arrows) present in the NH_2-terminus of Smac (turquoise) and in the small subunit of caspase-9 (green) binds to a groove on the BIR3 domain of XIAP (dark blue circle). This motif promotes inhibition of caspase-9 (pink and green) by interacting with the BIR3 domain. But by displacing the caspase-9 tetrapeptide motif, the same motif in Smac abrogates inhibition of caspase-9 by XIAP. (**Right**) The linker segment (black rectangle) in XIAP—which is NH_2-terminal to the BIR2 domain (dark blue)—is primarily responsible for inhibiting caspase-3 (blue and yellow). Binding of Smac to the BIR2 domain of XIAP may render the linker segment unavailable for binding to caspase-3.

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(*7*). The proform of Smac contains an NH_2-terminal sequence that targets this protein to the intermembrane space of mitochondria. During apoptosis, Smac is released into the cytosol together with several other mitochondrial proteins, including cytochrome c and AIF (apoptosis-inducing factor). The mature form of Smac (but not the proform) binds to several IAPs and removes the ability of IAPs to block caspase-mediated apoptosis. The x-ray structure of Smac reveals that this protein is a homodimer containing two elongated three-helix bundles (*19*). Smac can interact with the BIR2 and BIR3 domains of XIAP (*19*), abrogating inhibition of caspase-3 and caspase-9, respectively (*19, 20*). Inactivation of dimer formation compromises Smac activity. The NH_2-terminus of Smac is disordered in the crystals, but is critically important for its interactions with XIAP. Small peptides corresponding to this region of Smac bind to XIAP and promote caspase-9 activity. Interestingly, the NH_2-terminal residues of Smac that interact with IAPs are similar to the NH_2-terminal residues of three fruit fly proteins: Reaper, Hid, and Grim (*21*). These proteins promote apoptosis by interacting with an IAP in *Drosophila* (DIAP1), causing the disruption of DIAP1-mediated caspase inhibition (*22, 23*). Thus, Reaper, Hid, and Grim are functional homologs of the mammalian protein Smac. Although the overall amino acid sequences of these proteins are different, their NH_2-termini are similar and bind to IAPs by a similar mechanism (*24*).

The structural basis for IAP recognition by Smac is revealed by crystal structures of the XIAP BIR3 domain together with either the monomeric Smac protein (*25*) or a nine-residue peptide derived from Smac's NH_2-terminus (*26*). Only the first four NH_2-terminal residues of Smac are critical for binding to the BIR3 domain of XIAP (see the figure). The complex is stabilized by several intermolecular hydrogen bonds and by hydrophobic interactions between the positively charged NH_2-terminal alanine of Smac and Glu^{314} of BIR3. Smac's NH_2-terminal alanine is crucial for binding to XIAP, although the next three residues also contribute to the overall affinity (*25*). In addition, the methyl side chain of this alanine is necessary for binding of Smac to a small hydrophobic pocket on the surface of the XIAP BIR3 domain. Larger amino acids at this position (*19*) or substitution of this alanine by a glycine (*25*) result in a loss of binding to XIAP. These results explain why only the mature (proteolytically cleaved) form of Smac binds to IAPs, and why XIAP only interacts with the activated form of caspase-9 in which the sequence containing the NH_2-terminal alanine has been cleaved (*18*).

How does Smac prevent the IAPs from inhibiting the caspases? In the case of XIAP, it is likely that the NH_2-terminus of the Smac protein simply displaces the NH_2-terminus of the small subunit of caspase-9, releasing the activated enzyme (see the figure). This mechanism is consistent with the observation that BIR3 mutants incapable of binding to Smac do not interact with caspase-9 (*26*). Activated caspase-9 (clipped after Asp^{315}) cleaves procaspase-3 and converts it into active caspase-3, which then forms a positive feedback loop to cleave caspase-9 (after Asp^{330}) (*18*). This cleavage does not affect the catalytic activity of caspase-9 but permanently removes a 15-residue peptide (residues 316 to 330) from the caspase-9 small subunit. Because this 15-residue peptide contains the tetrapeptide IAP-binding motif, the resulting caspase-9 can no longer be inhibited by XIAP. In addition, the released peptide forms a complex with XIAP and further attenuates XIAP's ability to block caspase activity.

How Smac opposes the inhibition of caspase-3 by the XIAP BIR2 domain is less clear. The predominant interaction between XIAP and caspase-3 or caspase-7 involves amino acids NH_2-terminal to the BIR2 domain, with BIR2 as a minor player. In the crystal structure of the XIAP BIR2–caspase-3 complex, the NH_2-terminus of the caspase-3 p10 subunit interacts with the surface of BIR2 (*16*) (although this interaction may be an artifact of crystallization). It is noteworthy, however, that when these amino acids in caspase-3 were replaced with a Smac-like sequence, caspase-3 was then able to bind to the BIR3 domain of XIAP (*18*). In any case, the likely Smac binding site on the BIR2 domain is spatially different from the BIR2–caspase-3 interface. A possible explanation for BIR2-mediated caspase-3 inhibition comes from modeling studies, which suggest that steric clashes preclude the XIAP BIR2 domain from simultaneously binding to caspase-3 and Smac (*14*).

The abrogation of caspase-3 inhibition by XIAP is strictly dependent on a Smac homodimer. Although monomeric Smac mutants could bind to the BIR3 domain, hence abrogating blockade of caspase-9 by BIR3, their interactions with the XIAP BIR2 domain appear to be much weaker (*19, 26*) and were unable to abrogate inhibition of caspase-3 by XIAP (*19*). Although short peptides derived from the Smac NH_2-terminus relieve the inhibition of caspase-9 by XIAP, they do so at much higher concentrations than does the mature Smac protein, suggesting a secondary interface between Smac and the BIR3 domain (*19*). Indeed, a Smac mutant with the NH_2-terminal four residues deleted was able to bind to both the BIR2 and BIR3 domains and to relieve XIAP-mediated inhibition of both caspase-3 and caspase-9 (*19*).

An interesting theme emerging from studies of caspase regulation by the IAPs and Smac is how nature can accomplish the same task in different ways using similar scaffolds. Despite the structural similarities between members of the caspase family and the BIR domains of IAPs, caspase inhibition can be accomplished by IAPs using different strategies, and Smac can displace IAPs from caspases by different mechanisms. This appears to be a recurring theme in nature. For example, three families of apoptotic signaling motifs—the caspase recruitment domain (CARD), the death domain (DD), and the death effector domain (DED)—all share similar three-dimensional folds, and all mediate the signaling pathways of programmed cell death (*27*). Yet only members within the same family interact with each other and do so with stringent specificity.

In addition to helping us understand the regulation of caspases at the molecular level, this recent structural information teaches us how one might modulate caspase activity. For example, structural information on the functional groups required for the XIAP-Smac interaction could aid in the design of small molecules that could bind to the IAPs and induce apoptosis. These compounds could be used to treat cancers, such as certain leukemias and lymphomas, in which IAPs are overexpressed.

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