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Requirement of Both the Second and Third BIR Domains for the Relief of X-linked Inhibitor of Apoptosis Protein (XIAP)-mediated Caspase Inhibition by Smac*

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The inhibitor of apoptosis proteins (IAP) are endogenous caspase inhibitors in the metazoan and characterized by the presence of baculoviral IAP repeats (BIR). X-linked IAP (XIAP) contains three BIR domains and directly inhibits effector caspases such as caspase-7 via a linker_BIR2 fragment and initiator caspases such as caspase-9 via the BIR3 domain. A mitochondrial protein Smac/DIABLO, which is released during apoptosis, antagonizes XIAP-mediated caspase inhibition by interacting directly with XIAP. Here, using glutathione *S*-transferase pulldown and caspase activity assay, we show that Smac is ineffective in relieving either caspase-7 or caspase-9 inhibition by XIAP domain fragments. In addition, Smac forms a ternary complex with caspase-7 and linker_BIR2, suggesting that Smac/linker_BIR2 interaction does not sterically exclude linker_BIR2/caspase-7 interaction. However, Smac is effective in removing caspase-7 and caspase-9 inhibition by XIAP fragments containing both the BIR2 and BIR3 domains. Surface plasmon resonance measurements show that Smac interacts with the BIR2 or BIR3 domain in micromolar dissociation constants. On the other hand, Smac interacts with an XIAP construct containing both BIR2 and BIR3 domains in a subnanomolar dissociation constant by the simultaneous interaction of the Smac dimer with the BIR2 and BIR3 domains of a single XIAP molecule. This 2:1 Smac/XIAP interaction not only possesses enhanced affinity but also sterically excludes XIAP/caspase-7 interaction, demonstrating the requirement of both BIR2 and BIR3 domains for Smac to relieve XIAP-mediated caspase inhibition.

Apoptosis, or programmed cell death, is a highly controlled process essential for many aspects of biological functions in multicellular organisms, including fetal development, maintenance of cellular homeostasis, and immune system regulation (1–4). Excessive apoptosis is observed in many forms of degenerative disorders such as Alzheimer's disease, ischemic injury from stroke, and post-menopausal osteoporosis. On the other hand, down-regulation of apoptosis is often associated with cancer, autoimmune disorders, and persistent viral infections. Therefore, like cell survival and proliferation, cell death is a central point of intervention for the treatment of many human diseases.

The major players of apoptotic cell death are caspases, a group of cysteine proteases that cleave after aspartate residues (5–7). Based on their preferential recognition of different substrate motifs, caspases can be divided into three groups. Although group I caspases are mostly involved in inflammation, group II and group III caspases comprise the effector (downstream) and initiator (upstream) caspases, respectively. Caspases are synthesized as single chain zymogens. During apoptosis, initiator caspases are recruited to multi-component and oligomeric signaling complexes and are activated by dimerization (8), followed by auto-processing. Activated initiator caspases in turn produce specific proteolytic cleavages to activate effector caspases. Cleaved mature caspases are dimeric enzymes with two active sites, comprising two large and two small subunits.

Caspases are subject to inhibition by specific viral and cellular caspase inhibitors (9, 10). Three different classes of caspase inhibitors have been identified: the p35 protein from baculoviruses (11, 12), the CrmA protein from the cow pox virus (13), and the family of proteins known as the inhibitor of apoptosis proteins (IAP)[1] (14, 15). IAPs are the only endogenous caspase inhibitors for diverse organisms in the metazoan. They are characterized by the presence of one to three tandem baculoviral IAP repeats (BIR), which exhibit a structural architecture of classical zinc fingers (16). Examples of human IAPs include X-chromosome linked IAP (XIAP), c-IAP1, c-IAP2, neuronal apoptosis inhibitor protein, Survivin, and others.

XIAP is a prototypical member of the IAP family, which has been shown to directly inhibit both effector and initiator caspases (17). XIAP possesses three BIR domains followed by a RING domain (Fig. 1*A*). Structural and biochemical studies revealed that the linker proceeding the BIR2 domain, in the context of a linker_BIR2 construct of XIAP, directly block the active sites of effector caspases such as caspase-3 and caspase-7 (18–21) (Fig. 1*B*). In the linker_BIR2/caspase-7 structure, the BIR2 domain is completely disordered and does not contact the caspase. In the linker_BIR2/caspase-3 structure, the BIR2 domain interacts with a neighboring caspase in the crystal lattice, but the significance of the interaction in solution is unclear. The BIR3 domain is necessary and sufficient for

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[1] The abbreviations used are: IAP, inhibitor of apoptosis proteins; BIR, baculoviral IAP repeats; XIAP, X-linked inhibitor of apoptosis proteins; RING, really interesting new gene; GST, glutathione *S*-transferase; SPR, surface plasmon resonance; P20, Tween-20.

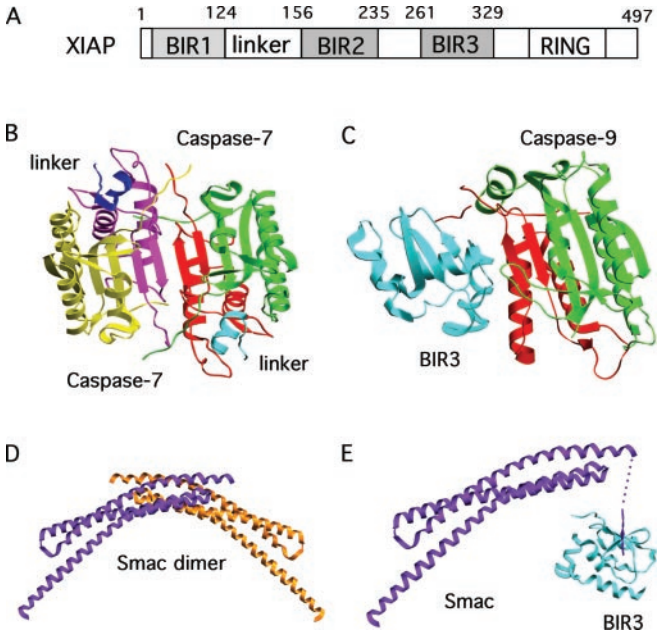

FIG. 1. **Molecular interactions among Smac, XIAP, and caspases.** *A*, domain organization of full-length XIAP. *B*, crystal structure of the linker_BIR2/caspase-7 complex. The linker (*blue* and *cyan*) interacts symmetrically with the active sites of mature dimeric caspase-7 (*yellow* and *green* for large subunits; *magenta* and *red* for small subunits). The BIR2 domain is invisible in the structure. *C*, crystal structure of the BIR3/caspase-9 complex. BIR3 is shown in *cyan* and caspase-9 in *green* and *red*. *D*, crystal structure of dimeric Smac (*purple* and *gold*). *E*, model of Smac/BIR3 interaction, based on the crystal structure of the mutant Smac monomer/BIR3 complex. BIR3 is in *cyan* and Smac monomer is in *purple*. The *dotted purple line* connects the N-terminal BIR-binding peptide of Smac to the remainder structure of Smac.

inhibition of caspase-9, an initiator caspase in the mitochondrial cell death pathway, by sterically hindering caspase-9 dimerization (22) (Fig. 1*C*). The RING domain appears to act as a ubiquitin ligase for ubiquitination and degradation of IAPs and caspases (23, 24). The BIR1 domain of XIAP has so far no ascribed function.

Caspase inhibition by XIAP may be counteracted by Smac/DIABLO, a dimeric mitochondrial protein released into the cytosol during apoptosis (25, 26) (Fig. 1*D*). The biochemical basis of Smac function relies on a direct physical association with a groove on the surface of BIR domains of XIAP, mostly via a four-residue IAP-binding motif (AVPI) at the amino terminus of Smac (27, 28) (Fig. 1*E*). Interestingly, part of the BIR3/caspase-9 interaction is mediated by the binding of the amino terminus of the small subunit of caspase-9 (ATPF) with the same BIR3 surface groove.

Although a wealth of structural and biochemical data are available for XIAP, caspases and Smac, detailed quantitative biochemical and biophysical characterization of the system is lacking. An initial effort to fill this gap surprisingly revealed that linker_BIR2 interacts much more strongly with caspase-7 than with Smac. Furthermore, we found that even when bound with linker_BIR2, Smac does not effectively displace caspase-7, but rather forms a ternary complex with linker_BIR2 and caspase-7. These findings raise the question how and whether Smac could relieve effector caspase inhibition by XIAP.

Here we report a series of experiments using GST pulldown, gel filtration chromatography, caspase activity assay, surface plasmon resonance (SPR), and equilibrium ultracentrifugation. These experiments show that Smac is not only unable to effectively remove effector caspase inhibition by linker_BIR2, it is also inefficient in relieving caspase-9 inhibition by BIR3. On the other hand, Smac can efficiently antagonize inhibition of both effector and initiator caspases by constructs of XIAP containing both BIR2 and BIR3 domains. Although earlier studies have implied that BIR2 interacts only weakly with Smac (27, 28), our affinity characterization shows that Smac interacts similarly with BIR2 and BIR3 domains. In addition, each Smac dimer interacts with the BIR2 and BIR3 domains of an XIAP molecule to form a 2:1 stoichiometric complex. This interaction not only possesses much enhanced affinity because of avidity, but it also provides steric hindrance to disallow the formation of an XIAP, caspase-7, and Smac ternary complex. Therefore, although the individual domains of XIAP are sufficient for caspase inhibition *in vitro*, both BIR2 and BIR3 domains are required for XIAP to interact with Smac and to release caspases from inhibition.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Various human XIAP fragments including BIR2 (residues 156–240), linker_BIR2 (residues 124–240), BIR1 (residues 1–135), BIR3 (residues 252–356), linker_BIR2_3 (residues 124–356), BIR1_2_3 (residues 1–356), BIR1_linker_BIR2 (1–240), and linker (124–158) were expressed as GST-fusion proteins using the pGEX-4T-3 vector (Amersham Biosciences). They were purified by glutathione-Sepharose (Amersham Biosciences) affinity chromatography followed by gel filtration chromatography. To remove the GST tag, the GST fusion proteins were subjected to overnight thrombin cleavage on glutathione-Sepharose beads at room temperature using ~1:200 molar ratios of enzyme to protein. The cleaved proteins were further purified by gel filtration chromatography. Full-length caspase-7, caspase-9, and Smac were expressed as C-terminally His-tagged proteins using the pET-28a vector (Novagen). They were purified by using nickel-nitrilotriacetic acid (Qiagen) affinity chromatography followed by gel filtration.

SPR Biosensor Measurement—All surface plasmon resonance measurements were performed at 20 °C using a BIACORE 2000 SPR-based biosensing system equipped with a research-grade B1 sensor chip. The immobilization buffer was 10 mM HEPES, 150 mM NaCl, and 0.005% P20, pH 7.4; the running buffer was 20 mM Tris, 150 mM NaCl, 2 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 0.005% P20, pH 7.5. The surfaces of four flow cells were activated for 1 min with a 1:1 mixture of 0.1 M N-hydroxysuccinimide and 0.1 M 3-(N,N-dimethylamino)propyl-N-ethylcarbodiimide at a flow rate of 20 μ l/min. Caspase-7 and Smac in 10 mM sodium acetate at pH 5.0 were immobilized at densities of 600–4000 response units on the sensor chip surface. The remaining activated groups were blocked with a 7-min injection of 1 M ethanolamine at pH 8.0. To collect kinetic binding data, concentration series of the XIAP analyte proteins in running buffer were injected over the ligand and reference flow cells at a flow rate of 50 μl/min. During each injection, the ligand/analyte complex was allowed to associate and dissociate for 30 and 60 s. When necessary, the caspase-7 and Smac surfaces were regenerated with a 5-s injection of 5 mM H_3PO_4. All response data were double referenced (30), and data were fit globally to a 1:1 interaction model (A + B = AB) using CLAMP (31).

GST Pulldown—GST-pulldown experiments were performed at room temperature. Purified GST-XIAP proteins and excess caspase-7 or caspase-9 were incubated with glutathione beads for 1 h in phosphate-buffered saline buffer followed by three washes. Increasing amounts of Smac were then added to the beads and the mixture was incubated for another hour. After three washes, the bound proteins were eluted by 10 mM free reduced glutathione in phosphate-buffered saline.

Gel Filtration Chromatography—Gel filtration experiments were performed with Superdex 200 (HR26/60, Amersham Biosciences).

Caspase Activity Assay—Relief of XIAP-mediated caspase inhibition by Smac was monitored by enzyme progress curves (32). The fluorogenic caspase substrates Ac-DEVD-AFC for caspase-7 and Ac-LEHD-AFC for caspase-9 were purchased from Enzyme Systems. Fluorescence detection upon substrate cleavage by caspases was carried out on a PerkinElmer LS50B luminescence spectrometer (plate reader) using an excitation wavelength of 400 nm and an emission wavelength of 505 nm. Caspase-7 (20 nM) or caspase-9 (200 nM), an XIAP construct (0.5 μM), and various concentrations of Smac (0.1 μM, 1 μM, 10 μM, and 100 μM) were mixed in 50-μl reactions in buffer containing 50 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM dithiothreitol, and 10% sucrose, and equilibrated at 37 °C for 10 min. The reactions were initiated by adding pre-warmed caspase-7 or caspase-9 substrate (200 μM) to the mixtures.

TABLE I
SPR Biosensor measurements of caspase-7/XIAP and Smac/XIAP interactions
NB, no binding; ND, not determined. Numbers in parentheses indicate errors in the last digits.

Chip	Solution	k_{on}	k_{off}	K_D
		$M^{-1}\ s^{-1}$	s^{-1}	
Caspase-7				
	BIR1_linker BIR2 (1–240)	$1.592\ (5) \times 10^6$	$1.69\ (4) \times 10^{-3}$	1.06 (3) nM
	linker_BIR2 (124–240)	$2.078\ (4) \times 10^6$	$2.07\ (2) \times 10^{-3}$	0.99 (1) nM
Smac				
	linker_BIR2 (124–240)	$7.415\ (4) \times 10^5$	0.531 (3)	0.720 (4) μM
	BIR1 (1–135)			NB
	BIR2 (156–240)	ND	ND	0.97 (2) μM[a]
	BIR3 (252–356)	$5.5\ (3) \times 10^4$	0.237 (2)	4.4 (2) μM
	linker_BIR2 3 (124–356)	$7.00\ (8) \times 10^6$	$2.21\ (2) \times 10^{-3}$	0.316 (4) nM
	linker (124–158)			NB

[a] Affinity determined from equilibrium analysis.

Substrate hydrolyzes expressed as relative fluorescence units were monitored at 20-s intervals for 45 min.

Equilibrium Ultracentrifugation—Equilibrium sedimentation of the Smac/linker_BIR2_3 complex was performed at 20 °C at 1 mg/ml concentration using an XL-A Optima analytical ultracentrifuge (Beckman Coulter) running at 11,000 rpm. Data were fit to a single species model (33). The apparent molecular weight was calculated by assuming a partial specific volume of 0.74 ml/g and a solvent density of 1.045 g/ml.

RESULTS

linker_BIR2/caspase-7 Interaction Is Much Stronger than linker_BIR2/Smac Interaction—To understand how Smac relieves the inhibition of effector caspases such as caspase-7 by XIAP, we characterized the interactions of XIAP with caspase-7 and Smac using SPR biosensor measurements (Table I). Dimeric caspase-7 and Smac were coupled to the sensor chips to avoid avidity effects during SPR experiments.

Consistent with the crystal structures of linker_BIR2/caspase-7 and linker_BIR2/caspase-3 complexes, the linker_BIR2 region of XIAP interacted with caspase-7 with high affinity. The dissociation constant, as calculated from the kinetics of the interaction, was 1.0 nM. The BIR1_linker_BIR2 protein interacted with caspase-7 with equal affinity. Surprisingly, however, the linker_BIR2/Smac interaction exhibited a dissociation constant of 0.72 μM, which is 3 orders of magnitude weaker than the linker_BIR2/caspase-7 interaction. The weaker linker_BIR2/Smac interaction was mostly the result of a much faster off-rate (257-fold), as shown by comparing the kinetics of the interactions. The on-rates of the two interactions, on the other hand, are fairly similar (3-fold). These observations raise the question whether and how Smac would be able to remove caspase-7 from the grip of linker_BIR2.

Smac Forms a Ternary Complex with Caspase-7 and linker_BIR2 and Is Inefficient in Relieving Caspase-7 Inhibition by linker_BIR2—To determine whether Smac can effectively compete with caspase-7 for linker_BIR2 interaction, we first used GST-linker_BIR2-mediated pulldown experiments. GST-linker_BIR2 and excess of caspase-7 were incubated with reduced glutathione beads and washed with binding buffer to remove excess caspase-7. Smac was then added, followed by incubation, washing, and elution with free reduced glutathione. The resulting SDS-PAGE analyses of the bound proteins showed that even in the presence of excess Smac, most caspase-7 stayed bound with GST-linker_BIR2, suggesting that Smac is very inefficient in relieving caspase-7 from inhibition by linker_BIR2 (Fig. 2*A*).

Interestingly, significant amounts of both Smac and caspase-7 were pulled down by GST-linker_BIR2 (Fig. 2*A*). To determine whether Smac, caspase-7, and linker_BIR2 exist as a ternary complex or as linker_BIR2/Smac and linker_BIR2/caspase-7 binary complexes, we performed a gel filtration experiment on a mixture of the linker_BIR2/caspase-7 complex and excess Smac. The experiment showed that approximately half of the linker_BIR2/caspase-7 complex co-eluted with Smac, demonstrating the formation of a ternary complex of linker_BIR2/caspase-7/Smac (Fig. 2*B*). About equal amounts of the linker_BIR2/caspase-7 complex stayed as the binary complex, and only a tiny fraction of the linker_BIR2 was in a linker_BIR2/Smac binary complex. Therefore, excess Smac not only did not effectively compete with the linker_BIR2/caspase-7 interaction to form linker_BIR2/Smac complex, it did not necessarily disrupt linker_BIR2/caspase-7 interaction, even when bound to linker_BIR2. This result is in contrast to the earlier suggestion that Smac sterically competes with linker_BIR2 for caspase-7 interaction (19, 20) and is consistent with the structural observation that Smac and caspase-7 interact with different regions of the linker_BIR2 construct of XIAP (19, 20), whereas most of the linker_BIR2 is occupied by caspase-7. But, does it form a ternary complex?

To determine whether the failure of Smac to effectively disrupt the linker_BIR2/caspase-7 interaction is translated into ineffective relief of linker_BIR2-mediated caspase-7 inhibition, we used enzyme kinetic analyses to determine the efficacy of Smac in relieving caspase-7 from linker_BIR2 inhibition (Fig. 2*C*). In the absence of Smac, linker_BIR2 inhibited caspase-7 enzymatic activity, as shown by the complete suppression of fluorescent substrate conversion. Increasing concentrations of Smac were ineffective in restoring caspase-7 activity. Even at a 200-fold excess of Smac (100 μM) *versus* linker_BIR2 (0.5 μM), only ~20–25% of the caspase-7 activity was relieved.

Smac Interacts with Both BIR2 and BIR3 Domains of XIAP—Having established that the linker_BIR2 region of XIAP is not sufficient for Smac regulation, we went on to determine whether other regions of XIAP may contribute to Smac interaction and, therefore, increase the sensitivity of XIAP-mediated caspase inhibition to Smac. SPR measurements between Smac and individual domains of XIAP, BIR1, BIR2, and BIR3 showed that both BIR2 and BIR3 domains interacted similarly with Smac (Table I). In contrast to the previous suggestion that BIR2 interacts weakly with Smac (27, 28), these quantitative measurements showed that BIR2 domain interacts with Smac with at least an equal if not somewhat higher affinity.

Smac Is Efficient in Relieving Caspase-7 Inhibition by XIAP Constructs Containing Both BIR2 and BIR3—Because both BIR2 and BIR3 domains interact with Smac, we used XIAP constructs containing both BIR2 and BIR3 domains and the linker_BIR2_3 and BIR1_2_3 proteins to assess the responsiveness to Smac. GST-XIAP-mediated pulldown experiments showed that Smac is much more efficient in liberating caspase-7 from interaction with linker_BIR2_3 and BIR1_2_3. At an approximately equal molar concentration, Smac removed

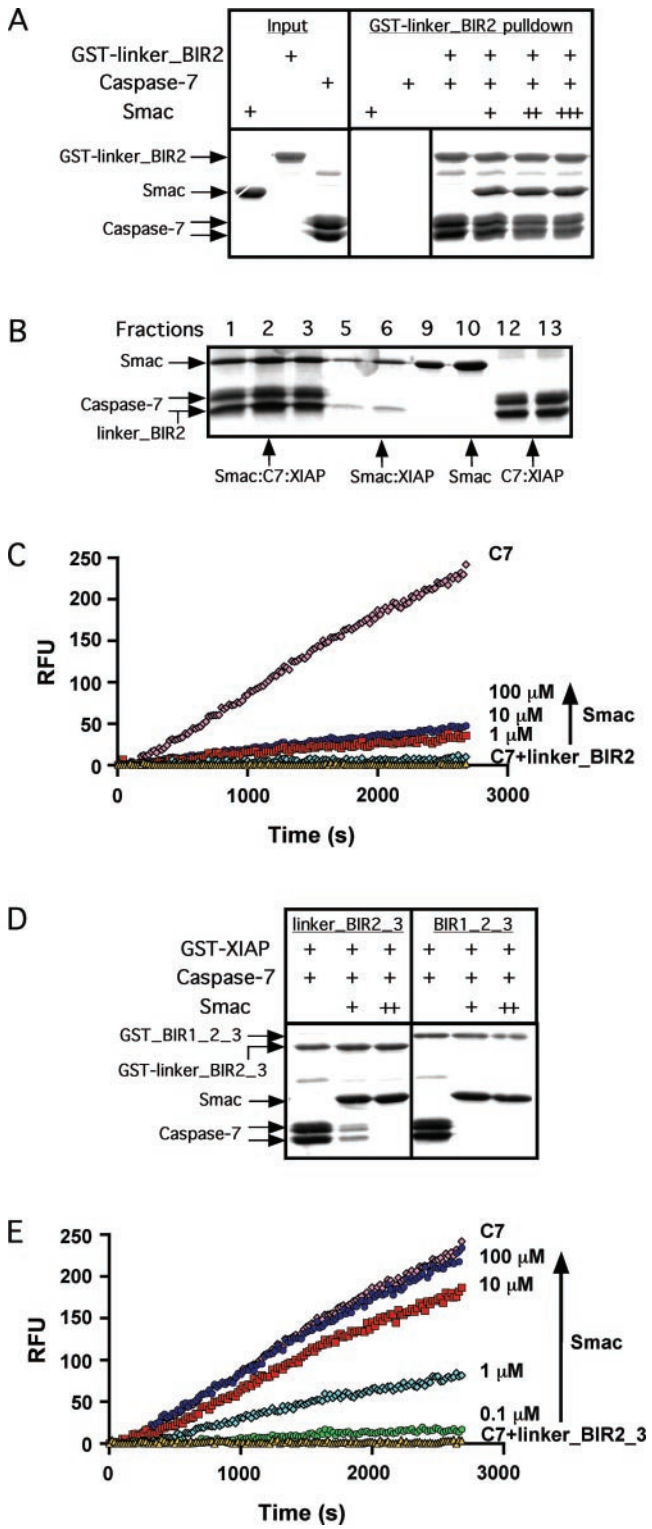

FIG. 2. **Inhibition of XIAP/caspase-7 interaction by Smac.** *A*, inefficient competition by Smac of GST-linker_BIR2 pulldown of caspase-7. *B*, gel filtration profile of a mixture of the linker_BIR2/caspase-7 complex and excess Smac, showing the formation of the ternary complex. Fractions begin at 140 ml and contain 5 ml each. *C*, kinetic analysis for the relief of linker_BIR2-mediated caspase-7 inhibition by Smac. *Pink*, caspase-7 (20 nM) alone; *yellow*, caspase-7 + linker_BIR2 (0.5 μM); *cyan*, *red*, and *blue*, caspase-7 + linker_BIR2 + Smac (1 μM, 10 μM, and 100 μM, respectively). *D*, efficient competition by Smac of pulldown of caspase-7 by GST-linker_BIR2_3 and GST-BIR1_2_3. *E*, kinetic analysis for the relief of linker_BIR2_3-mediated caspase-7 inhibition by Smac. *Pink*, caspase-7 (20 nM) alone; *yellow*, caspase-7 + linker_BIR2_3 (0.5 μM); *green*, *cyan*, *red*, and *blue*, caspase-7 + linker_BIR2_3 + Smac (0.1 μM, 1 μM, 10 μM, and 100 μM, respectively).

almost all caspase-7 from interaction with GST-linker_BIR2_3. Similarly, Smac effectively removed caspase-7 from interaction with GST-BIR1_2_3 (Fig. 2*D*).

Enzymatic kinetic analyses further showed that Smac is effective in relieving caspase-7 inhibition by linker_BIR2_3. Approximately 40% of the caspase-7 activity was restored in the presence of a mere 1 μM Smac, a 2-fold excess of linker_BIR2_3 (0.5 μM). More than 80% restoration of caspase-7 activity was achieved in the presence of 10 μM Smac, and nearly complete restoration was achieved at 100 μM Smac. This result is in contrast to the 20% relief in the presence of 100 μM Smac for linker_BIR2-mediated caspase-7 inhibition (Fig. 2*E*).

Smac Is Inefficient in Relieving BIR3-mediated Caspase-9 Inhibition—The observations that Smac is inefficient in releasing linker_BIR2-mediated caspase-7 inhibition and that both the BIR2 and BIR3 domains are required for efficient function of Smac prompted us to examine the effect of Smac on BIR3-mediated caspase-9 inhibition. Using GST-BIR3 pulldown experiments, we showed that caspase-9 could form a tight complex with BIR3. Because Smac binds to the same pocket on the surface of BIR3 as the N terminus of the small subunit of caspase-9, it is predicted that Smac should directly compete with caspase-9 for BIR3 interaction. Interestingly, the addition of approximate one-, two-, and 3-fold molar excess of Smac did not effectively remove caspase-9 from interaction with BIR3 (Fig. 3*A*). A majority of the caspase-9 stayed bound with BIR3, and significant amounts of both caspase-9 and Smac were pulled down by GST-BIR3 under these conditions. Unlike the situation with the linker_BIR2/caspase-7 complex, however, gel filtration analyses showed that BIR3, caspase-9, and Smac do not form a ternary complex but rather exist as two separate binary BIR3/caspase-9 and BIR3/Smac complexes (Fig. 3*B*). This finding is consistent with the overlapping binding sites for caspase-9 and Smac on the surface of BIR3.

To determine whether the inefficiency in relieving caspase-9 interaction is translated into inefficient relief of caspase-9 inhibition, we used enzymatic analyses to determine the efficacy of Smac in relieving caspase-9 from BIR3 inhibition (Fig. 3*C*). In the absence of Smac, BIR3 completely inhibited caspase-9 enzymatic activity, as shown by the complete suppression of fluorescent substrate conversion. Increasing concentrations of Smac were ineffective in restoring caspase-9 activity. At a 20-fold molar excess of Smac (10 μM) *versus* BIR3 (0.5 μM), only ~20% of caspase-9 activity was restored. Even at 200-fold excess of Smac (100 μM) *versus* BIR3 (0.5 μM), only ~50% of the caspase-9 activity was relieved.

Smac Is Efficient in Relieving Caspase-9 Inhibition by XIAP Constructs Containing Both BIR2 and BIR3—Because both BIR2 and BIR3 domains interact with Smac, we tested the responsiveness to Smac of XIAP constructs containing both the BIR2 and BIR3 domains and the linker_BIR2_3 and BIR1_2_3 proteins (Fig. 3*D*). GST-XIAP-mediated pulldown experiments showed that Smac is much more efficient in liberating caspase-9 from interaction with linker_BIR2_3 and BIR1_2_3. At an approximately equal molar concentration, Smac removed almost all caspase-9 from interaction with GST-linker_BIR2_3. Similarly, Smac effectively removed caspase-9 from interaction with GST-BIR1_2_3.

Further, enzymatic kinetic analyses showed that Smac is effective in relieving caspase-9 inhibition by linker_BIR2_3 (Fig. 3*E*). Approximately 50% of the caspase-9 activity was restored in the presence of a mere 1 μM Smac, a 2-fold excess of linker_BIR2_3. Almost complete restoration of caspase-9 activity was achieved in the presence of 10 μM or more Smac. This result is in contrast to the 50% relief in the presence of 100 μM Smac for BIR3-mediated caspase-9 inhibition.

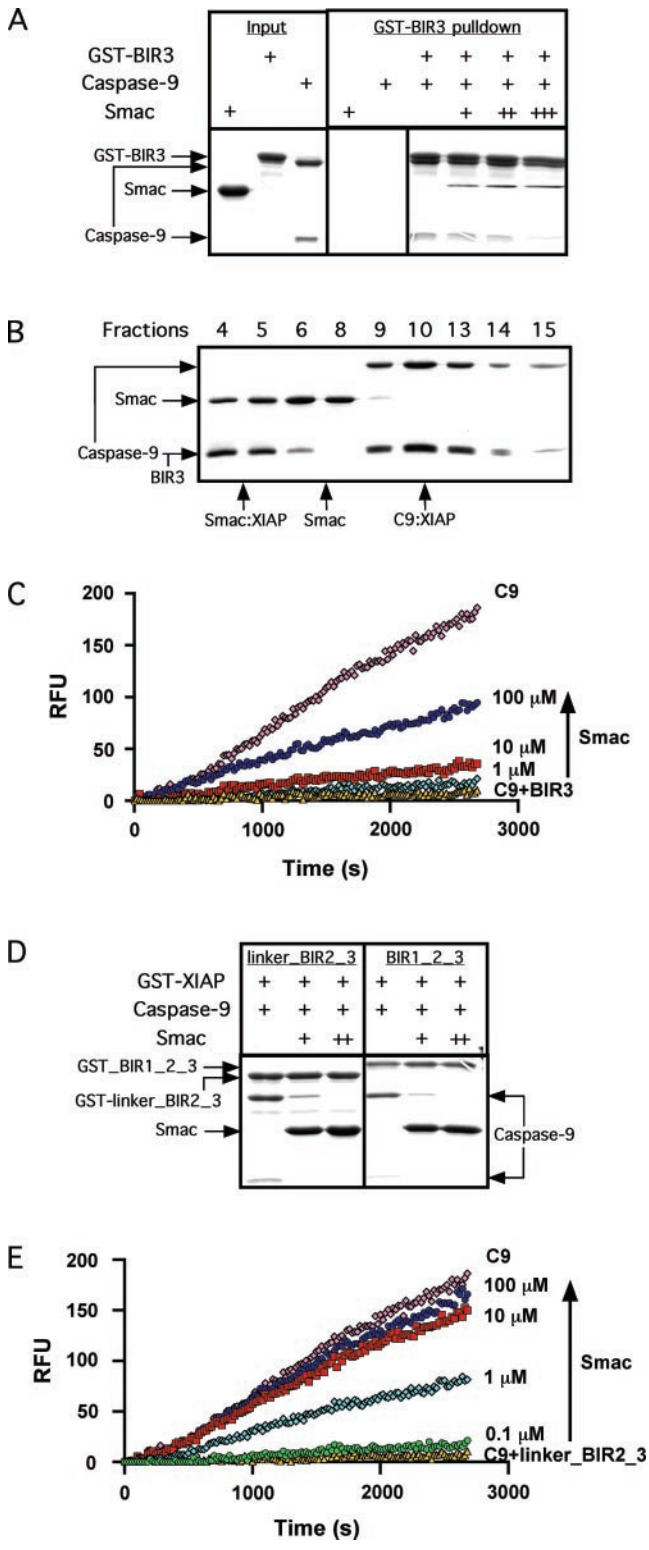

FIG. 3. **Inhibition of XIAP/caspase-9 interaction by Smac.** *A*, inefficient competition by Smac of GST-BIR3 pulldown of caspase-9. *B*, gel filtration profile of a mixture of the BIR3/caspase-9 complex and excess Smac, showing the binary BIR3/Smac and BIR3/caspase-9 complexes. Fractions begin at 140 ml and contain 5 ml each. *C*, kinetic analysis for the relief of BIR3-mediated caspase-9 inhibition by Smac. *Pink*, caspase-9 (200 nM) alone; *yellow*, caspase-9 + BIR3 (0.5 μM); *cyan*, *red*, *blue*, caspase-9 + BIR3 + Smac (1 μM, 10 μM and 100 μM, respectively). *D*, efficient competition by Smac of pulldown of caspase-9 by GST-linker_BIR2_3 and GST-BIR1_2_3. *E*, kinetic analysis for the relief of linker_BIR2_3-mediated caspase-9 inhibition by Smac. *Pink*, caspase-9 (200 nM) alone; *yellow*, caspase-9 + linker_BIR2_3 (0.5 μM); *green*, *cyan*, *red*, *blue*, caspase-9 + linker_BIR2_3 + Smac (0.1 μM, 1 μM, 10 μM, and 100 μM, respectively).

Smac Dimer Forms a 2:1 High Affinity Complex with XIAP by Interacting Simultaneously with the BIR2 and BIR3 Domains of an XIAP Molecule—Because of the affinity of Smac for both BIR2 and BIR3 domains and the dimeric nature of Smac, it may be expected that Smac dimer interacts with the BIR2 and BIR3 domains of XIAP and forms a 2:1 stoichiometric complex. However, previous studies have suggested that XIAP may dimerize (27), which could create many possibilities of interaction stoichiometry. To determine the stoichiometric state of a Smac/XIAP complex, we used the linker_BIR2_3 construct containing BIR2 and BIR3, and isolated a Smac/linker_BIR2_3 complex by gel filtration chromatography. The complex was then subjected to equilibrium ultracentrifugation (Fig. 4*A*), from which a molecular mass of 63 kDa was derived. This finding is consistent with a 2:1 stoichiometry of Smac/linker_BIR2_3 complex with a calculated molecular mass of 71 kDa.

Using the crystal structures of the Smac dimer (27) and the complex between a mutant Smac monomer and BIR3 (28), a dimeric Smac/BIR3 model was constructed. Then, one of the BIR3 molecules in the model was replaced by a crystal structure of the BIR2 domain (21) to generate a Smac/BIR2_3 complex (Fig. 4*B*). In the model, the BIR2 and BIR3 domains come from a single XIAP molecule, because the C-terminal end of the BIR2 domain (residue 237) lies close to the N-terminal end of the BIR3 domain (residue 256).

Strikingly, SPR measurement showed that linker_BIR2_3 interacted with Smac with much higher affinity (Fig. 4*C* and Table I). The derived dissociation constant is 0.3 nM, which is comparable or stronger than the XIAP/caspase-7 interaction and, therefore, would be poised to compete with the XIAP/caspase-7 interaction. Similarly, the increased affinity would enhance the ability of Smac to remove XIAP from inhibiting caspase-9.

DISCUSSION

linker_BIR2_3/Smac Interaction Sterically Hinders linker_BIR2_3/caspase-7 Interaction—The higher affinity of linker_BIR2_3/Smac interaction could readily explain the enhanced ability of Smac to relieve caspase-9 from inhibition by XIAP constructs containing both BIR2 and BIR3 domains. However, the issue for the disruption of the caspase-7/linker_BIR2_3 interaction by Smac is more complex because Smac and caspase-7 interact directly with different regions of XIAP.

The most likely explanation is that the simultaneous interaction of Smac with both BIR2 and BIR3 domains sterically hinders the interaction of the linker with caspase-7 (Fig. 4*B*). On the other hand, in the absence of BIR3, the linker/caspase-7 interaction could occur concurrently with BIR2/Smac interaction to form a ternary linker_BIR2/caspase-7/Smac complex in which one of the Smac monomers in the Smac dimer is not bound with a BIR domain. The role of BIR3 here may be analogous to the role of a second linker_BIR2 molecule bound to a Smac dimer. It has been proposed previously that the binding of a second linker_BIR2 molecule to the Smac dimer may create a steric hindrance for caspase-7 (19). However, because this weak second linker_BIR2/Smac interaction competes with the strong linker_BIR2/caspase-7 interaction, it is a rare event, and this explains the inefficient competition of Smac to remove caspase-7 from linker_BIR2. On the other hand, the strong linker_BIR2_3/Smac interaction efficiently removes caspase-7 from XIAP.

Domains of XIAP Coordinate to Sense Smac Regulation—XIAP is a multi-talented molecule that puts brakes on apoptosis by means of several different mechanisms (34). *In vitro*, each of these anti-apoptotic mechanisms of XIAP seems to be mediated exclusively by a different region of the molecule. For

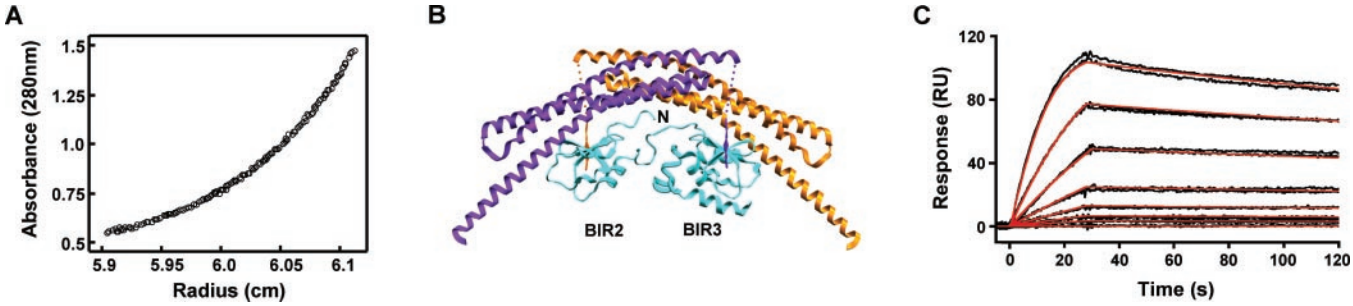

FIG. 4. **Biochemical characterization of the Smac/linker_BIR2_3 complex, showing the formation of a 2:1 stoichiometric and high affinity complex.** *A*, equilibrium ultracentrifugation of the linker_BIR2_3/Smac complex. A molecular mass of 63 kDa was derived from the experiment, consistent with a 2:1 Smac/linker_BIR2_3 complex. *B*, molecular model of the Smac/BIR2_3 complex. Each monomer of the Smac dimer is shown in *purple* and *gold*, respectively, and the BIR2 and BIR3 domains are shown in *cyan*. *Cyan* dotted line connects the C-terminal end of BIR2 to the N-terminal end of BIR3. *Purple* and *gold* dotted lines connect the N-terminal BIR-binding peptides of Smac to the remainder structures of Smac. The location of the N terminus of BIR2, to which the linker for caspase-7 interaction is attached, is labeled. *C*, SPR biosensor measurement of the Smac/linker_BIR2_3 interaction. A dissociation constant of 0.3 nM was derived.

example, linker_BIR2 seems to be both necessary and sufficient for the inhibition of caspase-7 or caspase-3, whereas BIR3 mediates caspase-9 inhibition. However, for Smac regulation, individual domains of BIR2 or BIR3, although having the ability to interact with Smac, are not sufficient for Smac to relieve either caspase-7 or caspase-9 from the grip of linker_BIR2 or BIR3, respectively. In this case, both BIR2 and BIR3 domains are required for XIAP to respond to Smac regulation. Interestingly, in the context of the apoptosome, XIAP seems to associate simultaneously with both caspase-9 and caspase-3, and the ability of XIAP to maintain inhibition of caspase-9 is influenced by its ability to simultaneously inhibit caspase-3 (29). Therefore, *in vivo*, for both caspase inhibition and Smac regulation, the different domains of XIAP cooperate to achieve more effective inhibition and sensitive regulation.

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