

A Dimeric Smac/Diablo Peptide Directly Relieves Caspase-3 Inhibition by XIAP

DYNAMIC AND COOPERATIVE REGULATION OF XIAP BY SMAC/DIABLO*

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Caspase activation, the executing event of apoptosis, is under deliberate regulation. IAP proteins inhibit caspase activity, whereas Smac/Diablo antagonizes IAP. XIAP, a ubiquitous IAP, can inhibit both caspase-9, the initiator caspase of the mitochondrial apoptotic pathway, and the downstream effector caspases, caspase-3 and caspase-7. Smac neutralizes XIAP inhibition of caspase-9 by competing for binding of the BIR3 domain of XIAP with caspase-9, whereas how Smac liberates effector caspases from XIAP inhibition is not clear. It is generally believed that binding of Smac with IAP generates a steric hindrance that prevents XIAP from inhibiting effector caspases, and therefore small molecule mimics of Smac are not able to reverse inhibition of the effector caspases. Surprisingly, we show here that binding of a dimeric Smac N-terminal peptide with the BIR2 domain of XIAP effectively antagonizes inhibition of caspase-3 by XIAP. Further, we defined the dynamic and cooperative interaction of Smac with XIAP: binding of Smac with the BIR3 domain anchors the subsequent binding of Smac with the BIR2 domain, which in turn attenuates the caspase-3 inhibitory function of XIAP. We also show that XIAP homotrimerizes via its C-terminal Ring domain, making its inhibitory activity toward caspase-3 more susceptible to Smac.

Apoptosis, a cellular process that plays crucial roles in multiple physiological and pathological events, is executed by the activity of a family of cysteine proteases known as caspases (1, 2). In mammals, a major caspase activation pathway is the mitochondrial cytochrome *c*-mediated pathway (3). This pathway mediates apoptosis triggered by a plethora of death stimuli including DNA damage, growth factor deprivation, and various other stressful conditions. Mechanistically, these stimuli converge on mitochondria and cause release of cytochrome *c* (4), which subsequently induces the mediator protein Apaf-1 to form an oligomeric protein complex, the apoptosome (5, 6).

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The apoptosome recruits and activates the initiator caspase, caspase-9, and the apoptosome-caspase-9 holoenzyme activates the downstream effector caspases, caspase-3 and caspase-7. This caspase cascade leads to apoptotic cell death associated with a series of hallmark morphological features.

In cells, the mitochondrial apoptotic pathway is regulated at multiple stages. The Bcl-2 family of proteins regulates release of mitochondrial apoptotic proteins such as cytochrome *c* (7–10). The pathway can also be regulated downstream of cytochrome *c* release. For example, it has been found that oncoprotein prothymosin- α and tumor suppressor putative HLA-associated protein proteins modulate the assembly and activity of the apoptosome-caspase-9 holoenzyme, likely via regulating the nucleotide binding/exchanging function of Apaf-1 (11, 12). Further, the enzymatic activity of caspases can be directly regulated by IAP (inhibitor of apoptosis) proteins (13). IAP proteins all contain one or more BIR (baculoviral IAP repeat) domains, which are thought to be responsible for caspase inhibition. For example, XIAP, a ubiquitous member of IAP family, contains three BIR domains (from the N terminus, BIR1, BIR2, and BIR3). During the process of apoptosis, the inhibitory function of IAP can be antagonized by Smac/Diablo (14, 15) and Omi/HtrA2 (16–20), which are also released from mitochondria as cytochrome *c*.

The molecular basis underlying neutralization of IAP by Smac is intriguing and elegant. Smac is a dimeric protein and is post-translationally processed in mitochondria (14, 15, 21). The N terminus of processed Smac, starting with the residues AVPI, has been shown to be necessary and sufficient for removal of inhibition of caspase-9 by XIAP (21). Structural studies demonstrated that the AVPI residues of Smac interact specifically and tightly with a conserved groove within the XIAP BIR3 domain (22, 23). Subsequently, it was revealed that the XIAP BIR3 domain interacts with caspase-9 in a strikingly similar manner. The cleaved, activated caspase-9 exposes a new N terminus similar to that of Smac, which is required for interaction of caspase-9 with the groove region of XIAP BIR3 domain (24, 25). Therefore, Smac neutralizes XIAP inhibition of caspase-9 by competing for the same specific binding site of XIAP with caspase-9.

In addition to the initiator caspase-9, XIAP can inhibit the effector caspases of the mitochondrial pathway as well (26). Such inhibition can also be counteracted by Smac (21). However, the mechanism by which Smac does so is not clear, because Smac can only interact with the BIR2 and BIR3

domains of XIAP (21), whereas XIAP utilizes a linker region between its BIR1 and BIR2 domains, instead of one of the BIR domains, to inhibit the effector caspases. This linker region can strongly interact with the active sites of both caspase-3 and caspase-7, as defined biochemically and structurally (26–30). Currently, it is generally accepted that binding of Smac with the BIR2 and BIR3 domains of XIAP creates a steric hindrance that is essential for preventing binding of XIAP linker region with effector caspases, thus achieving neutralization of XIAP inhibition (27–29, 31).

IAP proteins are frequently overexpressed in human cancers, indicating their oncogenic function and the potential to be therapeutic targets (32–35). Indeed, small molecule compounds and antisense oligonucleotides targeting IAP have been developed as anticancer proto-drugs (36–38). Interestingly, the mechanism by which the short N terminus of Smac antagonizes IAP was also explored for anticancer agent development. A successful example for such mechanism-based design is a synthesized chemical compound mimicking the structure of the dimeric Smac N-terminal sequence, AVPI (33). The nonpeptide nature of this molecule provides desirable cell permeability and stability, and this molecule possesses a strong ability to potentiate apoptosis in cancer cells, thus a promising anticancer drug lead. However, it is predicted by the current model that the effect of such a Smac N-terminal mimic might be limited, because it is supposed to be able to only antagonize inhibition of caspase-9 by IAP but not to antagonize inhibition of effector caspases by IAP, which should require the bulky Smac protein to create steric hindrance (32, 39).

In this study, we investigate the biochemical mechanisms by which Smac antagonizes inhibition of the effector caspase, caspase-3, by XIAP. We show that the dimeric Smac N-terminal peptide alone can effectively restore caspase-3 activity inhibited by XIAP. Our study also indicates that Smac interacts with the BIR3 and then BIR2 domains of XIAP sequentially, and such dynamic interaction cooperatively neutralizes inhibition of caspase-3 by the linker region of XIAP.

EXPERIMENTAL PROCEDURES

Reagents—Caspase-3 substrate, Ac-DEVD-AMC (catalog number 235425), and caspase-9 substrate, Ac-LEHD-AFC (catalog number 218765), were purchased from Calbiochem. Anti-Smac antibody (catalog number 05-681) was obtained from Upstate Biotechnology Inc.; anti-FLAG antibody M2 was from Sigma.

Plasmids—For His-tagged recombinant proteins, full-length human XIAP (residues 10–497) or XIAP- Δ Ring (residues 10–356) was generated by PCR and subcloned into pET21a (Novagen). Site-directed mutagenesis was performed to generate BIR2 (D214S) and BIR3 mutant (E314A) of full-length XIAP. For GST-tagged² recombinant proteins, full-length human XIAP (1–497) or BIR3 domain (238–358) was generated by PCR and subcloned into pGEX2T (GE Healthcare). For

purification of heterodimer Smac, PCR product from pET15b-Smac (AVPI)-His was inserted into pOKD4 to generate pOKD4-Smac (MVPI)-FLAG.

Peptide Synthesis—All of the peptides were prepared using standard Fmoc solid phase chemistry on a peptide synthesizer (Protein Technologies, Inc.). The dimeric peptide (dAVPI) was synthesized using Universal PEG NovaTag resin (catalog number 04-12-3911; EMD Biosciences). The Fmoc protecting group of the Universal PEG NovaTag resin was first removed using 20% piperidine in dimethylformamide. Then individual Fmoc-AA-OH was coupled to the resin. The amino group of N-terminal amino acids was protected by the *t*-butyloxycarbonyl group, rather than Fmoc. After the first peptide was synthesized, the (4-methoxyphenyl)diphenylmethyl protecting group was removed with 1.0 M *N*-hydroxybenzotriazole in trifluoroethanol/dichloromethane. The produced resin was used for the synthesis of second peptides using the same procedure. The final cleavage and deprotection were performed with a mixture (trifluoroacetic acid:water:triisopropylsilane, 95:2.5:2.5, v/v/v). The cleaved peptides were extracted repeatedly with ethyl ether and purified by reverse phase HPLC on a C18 column (ZORBAX 300 SB-C18, Agilent Technologies), using a 0–30% water/acetonitrile gradient in the presence of 0.1% trifluoroacetic acid. Peptide identity was verified by liquid chromatography-tandem mass spectrometry (Agilent Technologies).

Preparation of Cytosolic Fractions from HeLa Cells—HeLa cells are harvested by centrifugation at $1000 \times g$ for 5 min. After washed with phosphate-buffered saline, cell pellets were suspended in $5 \times$ volume of Buffer A (20 mM HEPES, pH 7.5, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM MgCl₂, 1 mM dithiothreitol) supplemented with mixture proteases inhibitors (Roche Applied Science). After incubation on ice for 15 min, the cells were broken by passing through 22-gauge needles 25 times. The cell lysates were then subject to centrifugation at $750 \times g$ for 10 min at 4 °C. The supernatants were further centrifuged at $12,000 \times g$ for 10 min at 4 °C, followed by final centrifugation at $100,000 \times g$ for 2 h at 4 °C to obtain HeLa S-100 fraction (HS100).

Recombinant Proteins—Generally, for His-tagged proteins, plasmids were transformed into BL21 (DE3) and cultured at 37 °C. When optical density reached 0.6, 0.1 mM isopropyl β -D-thiogalactopyranoside was added to induce protein expression at 18 °C for 16 h. The cells were pelleted and subjected to standard Ni²⁺ affinity chromatography. For expression of recombinant SMAC proteins, pET15b-Smac-His and pOKD4-SMAC-FLAG were cotransformed into BL21 (DE3), followed by isopropyl β -D-thiogalactopyranoside induction and Ni²⁺ affinity chromatography. The eluted protein was then subjected to anion exchange Q column purification (1 ml of HiTrapQ; GE Healthcare) eluted with 20 ml of gradient from 100 to 400 mM NaCl in Buffer A, which resulted in two protein peaks. The first one was wild type homodimer Smac (Smac-WT), and the second was mutant heterodimer Smac (Smac-Mut). For GST-tagged protein, a similar procedure was performed to get the expression of proteins in BL21, followed by GST affinity chromatography. All of the recombinant proteins obtained by affinity purification were dialyzed in Buffer A for 2 h at 4 °C. Aliquots were flash frozen with liquid nitrogen and stored at –80 °C.

² The abbreviations used are: GST, glutathione S-transferase; Fmoc, 9-fluorenylmethoxycarbonyl; HPLC, high pressure liquid chromatography; WT, wild type; Mut, mutant; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; WT, wild type; MUT, mutant.

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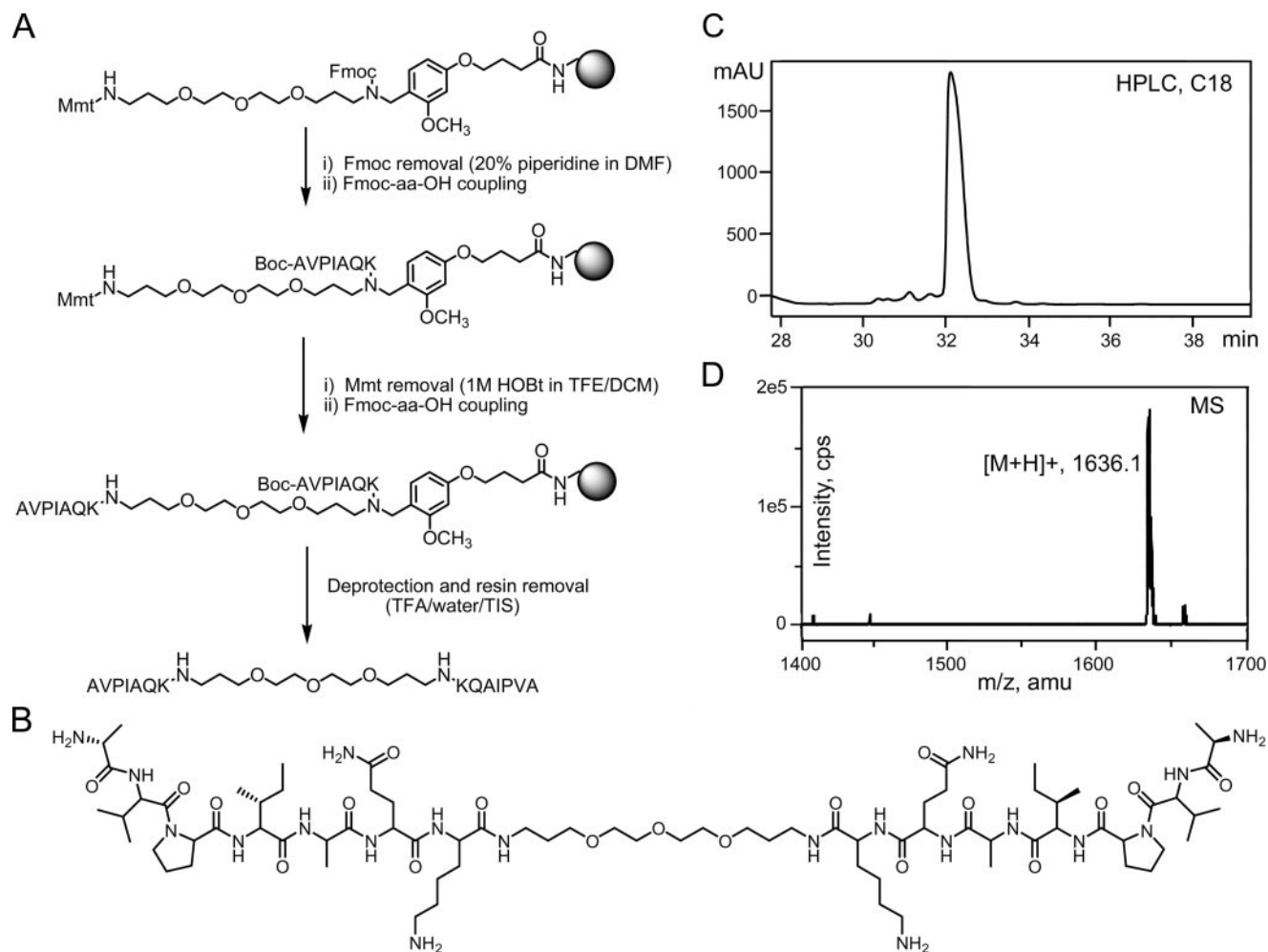


FIGURE 1. Chemical synthesis of the dimeric SMAC N-terminal peptide (dAVPI). *A*, scheme of the synthesis of dAVPI. The detailed methods are described under "Experimental Procedures." *B*, atomic structure of dAVPI. *C*, purification of synthesized dAVPI by reverse phase HPLC. The detailed method is described under "Experimental Procedures." *D*, confirmation of purified peptides by liquid chromatography-tandem mass spectrometry. The detailed method is described under "Experimental Procedures."

Caspase Activity Assay—For caspase-3 or caspase-9 activity assay in a total reconstituted system, each reaction with a final volume of 30 μ l was assembled on ice, including: 3 μ l of 10 \times caspase assay buffer (300 mM HEPES, pH 7.5, 1 M NaCl, 50% sucrose, 1% CHAPS, 200 mM β -mercaptoethanol, 20 mg/ml bovine serum albumin) (40), 3 μ l caspase-3 substrate (Ac-DEVD-AMC, 1 mM), or 3 μ l caspase-9 substrate (Ac-LEHD-AFC, 1 mM), respectively. Wild type or mutant XIAP and Smac or peptides were added as indicated. The mixture was first incubated at 30 $^{\circ}$ C for 10 min and then supplemented with 3 μ l of 10 nM caspase-3 or 3 μ l of 200 nM leucine zipper-linked dimeric caspase-9 (41), respectively, to initiate the enzyme reaction. Generation of fluorescent signal (relative fluorescent units) because of the cleavage of substrates, indicative of caspase-3 or caspase-9 activity, was measured by an automated spectrophotometer (TECAN, Inc.) at wavelengths of 360/465 nm (excitation/emission) or 400/505 nm, respectively.

For caspase-3 activity assay with HeLa cytosolic extract, caspase-3/7 in HS100 was first activated with 1 mM dATP and 5 μ M cytochrome *c* at 30 $^{\circ}$ C for 3.5 h. Then 9 μ l of preactivated HS100 was mixed with 21 μ l of reaction solution prewarmed at 30 $^{\circ}$ C for 10 min, consisting of the indicated amounts of XIAP,

dAVPI, and caspase-3 substrates. Substrate cleavage was measured as above.

GST Pull-down Experiment—For each sample, 5 μ g of purified full-length XIAP (GST-XIAP) or BIR3 domain (GST-BIR3) was coupled to 20 μ l of glutathione beads. Subsequently, in a 200- μ l Buffer A system, the beads were incubated with 1 μ M Smac in the presence of indicated concentration of dAVPI for 1 h followed by three washes. Then beads were boiled in 4 \times SDS loading buffer, and eluted proteins were subject to SDS-PAGE, followed by Western blotting with an anti-Smac antibody.

Molecular Mass Measurement—Molar mass of purified XIAP-WT or XIAP- Δ Ring was determined by static multi-angle light scattering. Protein was injected onto a Superdex 200 HR 10/300 gel filtration column equilibrated in Buffer A. The chromatography system was coupled to a three-angle light scattering detector (mini-DAWN EOS) and refractive index detector (Optilab DSP, Wyatt Technology). The data were collected every 0.5 s at a flow rate of 0.25 ml/min. Data analysis was carried out using the ASTRA program (Wyatt Technology), yielding the molar mass and mass distribution (polydispersity) of the sample.

RESULTS

The Dimeric N-terminal Peptide of Smac Is Sufficient to Antagonize Inhibition of Caspase-3 by XIAP—Small molecules mimicking the Smac N-terminal peptide, AVPI, are potential anticancer agents, because they are able to restore caspase-9 activity inhibited by IAP proteins. On the other hand, according to the current understanding, the effect of such agents is limited to antagonizing IAP inhibition of caspase-9 but not of the downstream effector caspases (32, 39). However, a recently reported dimeric AVPI mimic showed potent activity to potentiate apoptosis in cancer cells, and in cell extracts, this mimic was able to stimulate caspase-3/7 activity as effectively as Smac protein itself (33). This report immediately suggested that AVPI peptide might be able to antagonize inhibition of caspase-3/7 by IAP as well, a possibility clearly conflicting with the current view. On the other hand, several questions should be addressed before such a new mechanism needs to be invoked. First, one might argue that the nonpeptide nature of this Smac mimic might provide the molecule certain functions irrelevant to Smac; second, it is possible that in the cell extracts, the concentrations of effector caspases are much higher than that of endogenous IAP; therefore, once inhibition of caspase-9 is released by the Smac mimic, IAP cannot prevent the downstream activity of the effector caspases any more.

To address these questions and explore whether the Smac N-terminal peptide is sufficient to antagonize XIAP inhibition of caspase-3 activity, we performed chemical synthesis to generate authentic Smac N-terminal peptide rather than its non-peptide mimic. We synthesized the monomer Smac N-terminal peptide AVPIAQQ (mAVPI), as well as its reverse sequence KQAIPVA (mIPVA) as a negative control. Because Smac is a dimeric protein, and the chemical mimic of AVPI dimer possesses greater activity than the monomer mimic (33), we also synthesized the AVPIAQQ dimer (dAVPI) by covalently linking the C-terminal Lys residues of the two AVPIAQQ peptides (Fig. 1, A and B). The synthesized dimer was purified by reverse phase HPLC (Fig. 1C), and its identity was confirmed by mass spectrometry (Fig. 1D). We further confirmed that the synthesized dAVPI behaved as Smac in terms of its binding activity to XIAP; thus, as shown in Fig. 2A, dAVPI competes for GST-tagged full-length XIAP (GST-XIAP) and its BIR3 domain (GST-BIR3) against Smac in a dose-dependent manner.

Subsequently, we examined the effect of the synthesized peptides on XIAP inhibition of caspase-3/7 activity in HeLa cell extracts. To ensure that we were observing caspase-3/7 activity directly, we first activated caspase-3/7 by preincubating the cell extracts with 5 μM cytochrome *c* and 1 mM dATP at 30 °C for 3.5 h. This incubation maximally activated caspase-3/7 in the cell extracts (data not shown). Subsequently, we added recombinant XIAP in the presence of different peptides to test whether the inhibitory function of XIAP can be reversed. Indeed, the added recombinant, full-length XIAP completely inhibited caspase-3/7 activity, and both dAVPI and mAVPI could restore caspase-3/7 activity but not the control peptide mIPVA (Fig. 2B). As expected, dAVPI does so much more efficiently than mAVPI (Fig. 2C). Our particular experimental setup clarified that the Smac N-terminal peptide alone is able to

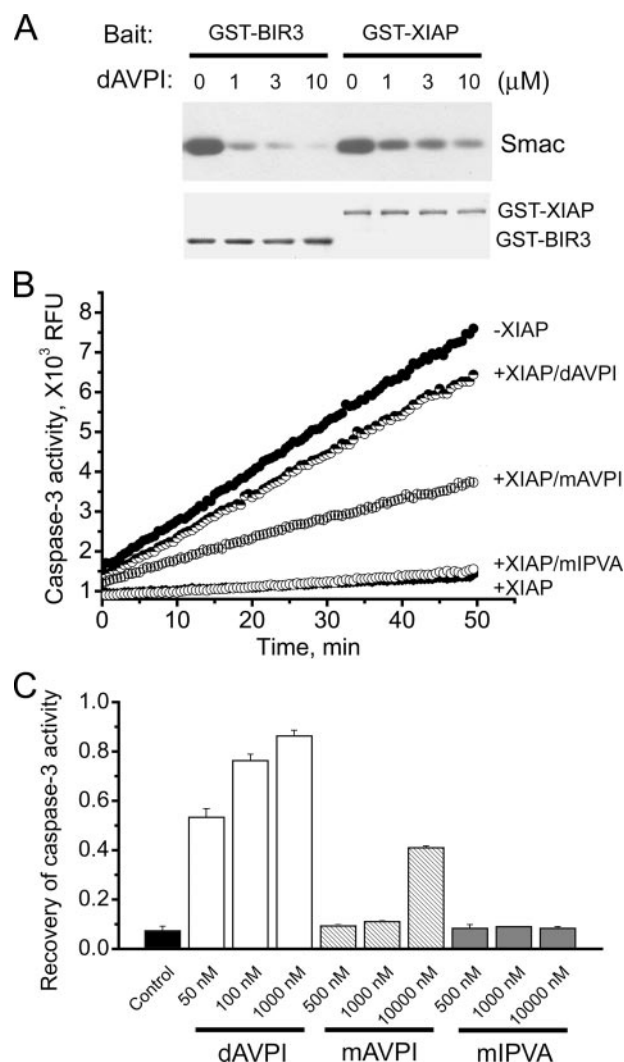


FIGURE 2. dAVPI antagonizes XIAP inhibition of caspase-3 in HeLa cell extracts. A, dAVPI competes with Smac for binding with BIR3 domain (GST-BIR3) or full-length XIAP (GST-XIAP). As described under "Experimental Procedures," GST-BIR3 or GST-XIAP was first conjugated to glutathione beads and then incubated with Smac in the presence of various concentrations of dAVPI as indicated. Bound Smac was resolved by SDS-PAGE followed by Western blotting using an antibody against Smac (upper panel), and the total GST-BIR3 and GST-XIAP were stained by Ponceau S (lower panel). B, HeLa cell cytosolic extracts (HS100) was activated with dATP and cytochrome *c* as described under "Experimental Procedures." An aliquot of activated HS100 was then added into the prewarmed reaction mixture without XIAP (–XIAP), or with 100 nM XIAP in the absence (+XIAP) or presence of dAVPI (+XIAP/dAVPI), mAVPI (+XIAP/mAVPI), or the control mIPVA (+XIAP/mIPVA), as indicated. C, dAVPI is more potent than mAVPI in liberating caspase-3 activity from XIAP inhibition. Assays were performed as in B, with individual synthetic peptides added with indicated concentration. Control indicates reactions in the absence of peptides. All of the data are presented as velocity of Ac-DEVD-AMC cleavage normalized against the maximal velocity (that is, in the absence of XIAP. Same for other figures). The error bars represent standard error calculated from three independent experiments (same for other figures).

antagonize inhibition of caspase-3/7 by XIAP. Thus, the steric hindrance generated by the bulky Smac protein is dispensable for antagonization of XIAP function in cell extracts.

Interaction of Smac N Terminus with the BIR2 Domain Antagonizes XIAP Inhibition of Caspase-3—How does dAVPI antagonize XIAP inhibition of caspase-3, a function of the linker region between the BIR1 and BIR2 domains of XIAP? This linker region cannot interact with AVPI, and dAVPI

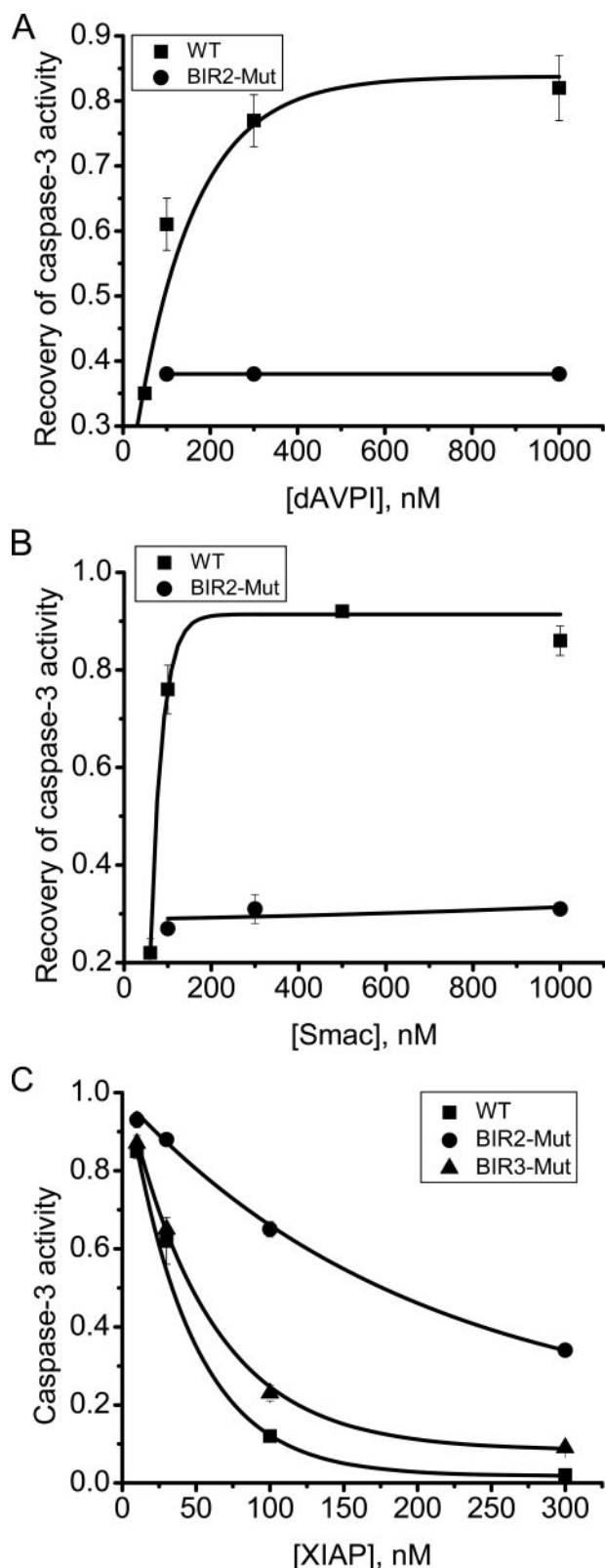


FIGURE 3. A functional BIR2 domain of XIAP is required for dAVPI and Smac to antagonize inhibition of caspase-3 by XIAP. *A*, dAVPI neutralizes inhibition of recombinant caspase-3 by XIAP-WT (WT) but not by XIAP with BIR2-Mut. XIAP-WT (50 nM) or BIR2-Mut (300 nM) was incubated in the presence of indicated concentrations of dAVPI at 30 °C for 10 min. Then 1 nM caspase-3 was added, and the activity was measured. Molar concentrations of all proteins were calculated using their monomer molecular mass in this figure and all other figures. *B*, as with dAVPI, Smac neutralizes inhibition of

apparently is not bulky enough to generate any steric hindrance. Because AVPI can specifically interact with both the BIR2 and BIR3 domains of XIAP, and the BIR2 domain is directly adjacent to the linker region, we propose that interaction of AVPI with the BIR2 domain is required for removing XIAP inhibition of caspase-3. To examine this possibility, we generated a XIAP mutant (BIR2-Mut) in which a conserved residue, aspartate 214, is mutated to serine. Based on the structure of Smac-BIR3 complex, this mutant should lose its ability to interact with AVPI (22, 23). Subsequently, we analyzed the effect of dAVPI and Smac on the inhibitory activity of both XIAP-WT and BIR2-Mut in a total reconstituted system. In this assay, we found that inhibition of recombinant caspase-3 activity by XIAP-WT can be neutralized by both dAVPI (Fig. 3*A*) and recombinant Smac protein (Fig. 3*B*), consistent with our results obtained with HeLa cell extracts (Fig. 2); However, both dAVPI (Fig. 3*A*) and recombinant Smac (Fig. 3*B*) failed to neutralize the inhibition of caspase-3 activity by BIR2-Mut. Collectively, these results indicate that the interaction of the Smac N-terminal peptide with the BIR2 domain of XIAP is both necessary and sufficient for an efficient restoration of caspase-3 activity inhibited by the linker region of XIAP.

AVPI-BIR2 interaction antagonizes the inhibitory activity of the linker region with two possible mechanisms. First, AVPI-BIR2 interaction might result in a conformational change of XIAP and thus make the linker region more buried in the protein and less available for its inhibitory function. Second, as reported previously, the BIR2 domain and the linker region of XIAP might cooperatively interact with effector caspases, although the BIR2 domain itself cannot inhibit caspase activity (29, 40); therefore, by competitively preventing interaction of BIR2 with effector caspases, AVPI can dramatically decrease the inhibitory binding of the linker region with effector caspases. Consistent with this previous report, we also observed the cooperative effect of the BIR2 domain in caspase-3 inhibition; thus, BIR2-Mut inhibited caspase-3 with a much lower efficacy than wild type XIAP, probably because of a weaker interaction of the linker region with caspase-3 caused by mutation of the BIR2 domain (Fig. 3*C*). As a negative control, the XIAP mutant whose BIR3 domain can no longer interact with Smac (BIR3-Mut, mutation of glutamate 314 to alanine) inhibited caspase-3 as effectively as the wild type XIAP (Fig. 3*C*).

Although our result (Fig. 3*C*) supported the second mechanism, this mechanism is not mutually exclusive with the first possibility. A careful dissection of these two possibilities requires crystal structure analysis of both full-length XIAP alone and its complex with dAVPI. In any case, interaction of AVPI with the BIR2 domain of XIAP modulates the inhibitory function of a different region of XIAP, the linker region. Therefore, this is a cooperative regulation.

Dynamic Regulation of XIAP by Smac—Surprisingly, mutation of the BIR3 domain of XIAP, originally intended to be a

recombinant caspase-3 by wild type XIAP but not by BIR2-Mut. Different amount of recombinant Smac was used as indicated, and 100 nM wild type XIAP and 300 nM BIR2-Mut were used in the assay. *C*, BIR2 mutation makes XIAP a weaker inhibitor for caspase-3. Indicated concentrations of XIAP-WT, BIR2-Mut, or BIR3-Mut were mixed with 1 nM caspase-3 to measure caspase-3 activity.

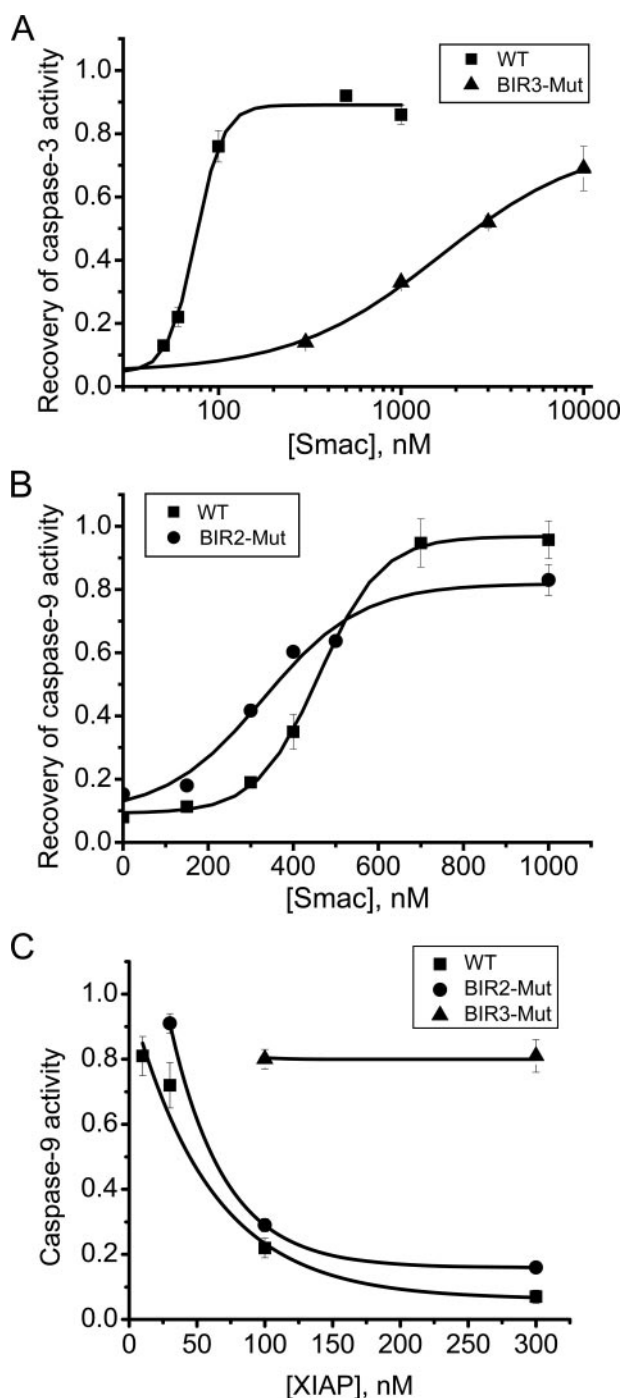


FIGURE 4. A functional BIR3 domain of XIAP is critical for Smac to restore caspase-3 activity inhibited by XIAP. *A*, Smac relieves inhibition of recombinant caspase-3 by XIAP-WT (WT) more efficiently than that by XIAP with BIR3-Mut. 100 nM XIAP-WT or BIR3-Mut were used to inhibit caspase-3 (1 nM), and Smac was added with indicated concentrations. *B*, Smac antagonizes inhibition of caspase-9 by XIAP-WT and BIR2-Mut with same efficiency. The indicated concentrations of Smac, 300 nM of WT-XIAP, or BIR2-Mut and 20 nM of active leucine zipper-linked caspase-9 were used in the analysis. Caspase-9 activity was measured by monitoring cleavage of Ac-LEHD-AFC. *C*, BIR3-Mut loses the ability to inhibit caspase-9, whereas BIR2-Mut remains as active as wild type XIAP. XIAP-WT, BIR2-Mut, or BIR3-Mut was mixed with 20 nM leucine zipper-linked caspase-9 at the indicated concentrations, and caspase-9 activity was measured.

negative control, also made the caspase-3 inhibitory function of XIAP rather difficult to be counteracted by Smac (Fig. 4A). This result is unexpected, because it has been clearly established that

BIR3 is responsible for caspase-9 inhibition and is not involved in caspase-3 inhibition; Further, mutation of BIR3 abolishes one of the two AVPI-binding sites in XIAP, so if neutralization of caspase-3 inhibition, *i.e.* Smac-BIR2 interaction, is independent of Smac-BIR3 interaction, BIR3 mutation should facilitate rather than prevent restoration of caspase-3 activity by Smac or dAVPI.

Thus, a conceivable mechanism is that Smac-BIR2 interaction and Smac-BIR3 interaction are not independent of each other. Based on the previous measurements that BIR3 has a much higher affinity than BIR2 to the N-terminal peptide of Smac (22), we hypothesize a dynamic mode of interaction between XIAP and Smac to explain our result (Fig. 4A). The interaction of Smac with the two BIR domains of XIAP is sequential: first, one N terminus of the Smac dimer binds to the BIR3 domain of XIAP, and this interaction precedes and facilitates the subsequent binding of the other N terminus of Smac dimer with the BIR2 domain, which in turn liberate caspase-3 activity from inhibition by the linker region of XIAP.

If this dynamic mechanism is correct, it immediately predicts several results. First, if BIR3-Smac interaction occurs first and is independent of the BIR2 domain, the prediction should be that mutation of BIR2 would not affect the effect of Smac on restoration of caspase-9 activity inhibited by the BIR3 domain of XIAP, in contrary to the dramatic effect of BIR3 mutation on restoration of caspase-3 activity (Fig. 4A). Indeed, caspase-9 inhibition by the wild type XIAP and BIR2-Mut was antagonized by Smac with similar efficiency (Fig. 4B), which is consistent with our dynamic model. We also confirmed that the wild type XIAP and BIR2-Mut proteins possess similar inhibitory activity toward caspase-9, whereas BIR3-Mut is not able to inhibit caspase-9 (Fig. 4C).

The dynamic mechanism also predicts that a mutant Smac protein that has only one functional N terminus should neutralize XIAP inhibition of caspase-9 with a relatively similar ability compared with wild type Smac, whereas the mutant Smac should neutralize XIAP inhibition of caspase-3 with a weaker efficiency. To generate such a dimeric, mutant Smac recombinant protein (Smac-Mut), we cotransformed into bacteria a wild type Smac plasmid (C-terminal His-tagged) and a mutant Smac plasmid (the critical N-terminal alanine was mutated to methionine, and C-terminal FLAG-tagged) and then expressed and purified a mixture of wild type Smac and Smac-Mut (both dimer) using nickel affinity purification. Subsequently, wild type Smac and Smac-Mut were separated by anion exchange chromatography, taking advantage of the highly negatively charged FLAG tag sequence (Fig. 5, A and B). As expected, wild type Smac and Smac-Mut antagonized XIAP inhibition of caspase-9 with same efficacy, when the concentration of the functional N termini was compared (Fig. 5C); on the contrary, wild type Smac was a stronger antagonist than Smac-Mut for neutralizing XIAP inhibition of caspase-3 (Fig. 5D), which is in agreement with the dynamic mechanism for Smac-XIAP interaction.

XIAP Is a Homotrimeric Protein—Another unexpected observation is that the purified full-length XIAP recombinant protein has an apparent molecular mass of ~150 kDa measured by gel filtration chromatography (Fig. 6A), suggesting potential

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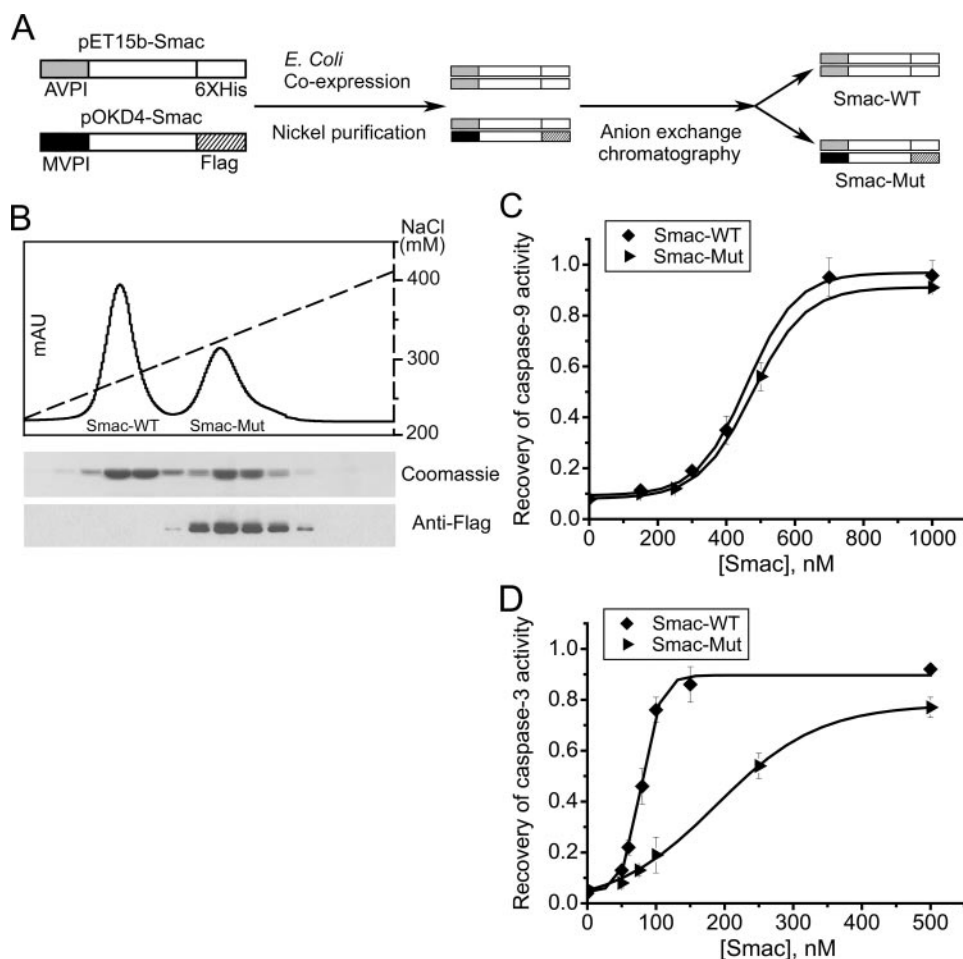


FIGURE 5. Both N-terminal AVPI motifs of Smac dimer are required for efficient restoration of caspase-3 activity inhibited by XIAP. *A*, scheme for expression and purification of heterodimeric Smac-Mut with one of the two AVPI motifs mutated to MVPI. Smac-WT indicates wild type homodimeric Smac. *B*, anion exchange chromatogram for purification of Smac-Mut. The first peak shows Smac-WT, and the second peak shows Smac-Mut, confirmed by both Coomassie Blue staining and Western blotting using an anti-FLAG antibody (bottom panel). *C*, Smac-Mut and Smac-WT have a similar efficiency to remove caspase-9 inhibition by XIAP. Twenty nM of leucine zipper-linked caspase-9 and 300 nM of XIAP were incubated in the presence of indicated concentrations of Smac. The effective concentration of Smac-Mut was treated as 50% of that of Smac-WT because it has only one active AVPI motif per dimer (same for *D*). *D*, compared with Smac-WT, Smac-Mut is less efficient in antagonizing caspase-3 inhibition by XIAP. 1 nM caspase-3 and 100 nM XIAP were incubated in the presence of the indicated concentrations of Smac to measure caspase-3 activity.

oligomerization. Interestingly, it was previously reported that XIAP molecules can interact with each other intermolecularly in cells via their C-terminal Ring domains (42). Thus, we generated XIAP recombinant protein with the Ring domain deleted (XIAP- Δ Ring). When this protein was resolved by gel filtration chromatography, it showed a much lower apparent molecular mass than the full-length XIAP (Fig. 6A). Such a difference cannot be explained by size of the C-terminal deletion alone, which is less than 20 kDa, further suggesting Ring domain-mediated oligomerization of XIAP. To accurately measure the absolute molecular mass of full-length XIAP and XIAP- Δ Ring, we conducted multi-angle light scattering measurement. The measured molecular mass of full-length XIAP is 169.8 ± 3.7 kDa (Fig. 6B), indicating that it is a homotrimer. Intriguingly, the measured molecular mass of XIAP- Δ Ring is 55.5 ± 2.2 kDa, ~ 1.5 -fold of a monomer XIAP- Δ Ring. This value can be explained by the fact that the BIR1 domain of XIAP tends to form a homodimer in a weak yet dynamic manner (43).

inhibition of caspase-3 by a different region of XIAP, the linker region between the BIR1 and BIR2 domains. Further, we found that XIAP is a homotrimeric protein, and its trimerization also facilitates Smac to neutralize inhibition of caspase-3 by XIAP (Fig. 6).

Our findings are novel and unexpected. Currently, it is generally accepted that Smac neutralizes inhibition of caspase-3 by XIAP via steric hindrance, and small molecules that mimic the N-terminal peptide of Smac can only restore caspase-9 activity rather than caspase-3 activity inhibited by XIAP. It is believed so because XIAP inhibits caspase-3 by its linker region rather than one of the BIR domains (27–29, 31, 39). Surprisingly, we clearly show that a synthetic dimeric Smac N-terminal peptide (dAVPI) alone is able to remove XIAP inhibition of caspase-3 as efficiently as Smac protein (Fig. 3). Interestingly, a recent report suggested that interaction of the BIR2 domain of XIAP with caspase-3/7 is critical for the potent inhibitory function of the XIAP linker region (40). If so,

What is the biochemical impact of XIAP homotrimerization? We compared the inhibitory activity of full-length XIAP with XIAP- Δ Ring and found that they possess similar abilities in inhibiting either caspase-9 or caspase-3 (data not shown). However, when Smac was added to restore caspase-3 activity, we found that the full-length XIAP can be more effectively antagonized by Smac than XIAP- Δ Ring (Fig. 6C), whereas the difference of the full-length XIAP versus XIAP- Δ Ring in restoration of caspase-9 activity by Smac is less obvious (data not shown).

DISCUSSION

This study reveals the detailed mechanism by which Smac antagonizes XIAP inhibition of caspase-3 (Fig. 7). Smac first interacts with the BIR3 domain of XIAP utilizing one of its two N-terminal peptides. This interaction not only neutralizes the function of XIAP to inhibit caspase-9 but also primes and facilitates the other N-terminal end of the dimeric Smac to interact with the BIR2 domain of XIAP; this follow-up Smac-BIR2 interaction alone effectively attenuates the function of the adjacent linker region of XIAP to inhibit caspase-3 (Figs. 3–5). Thus, Smac interacts with the two BIR domains of XIAP in a sequential and dynamic manner, and such interaction cooperatively disrupts

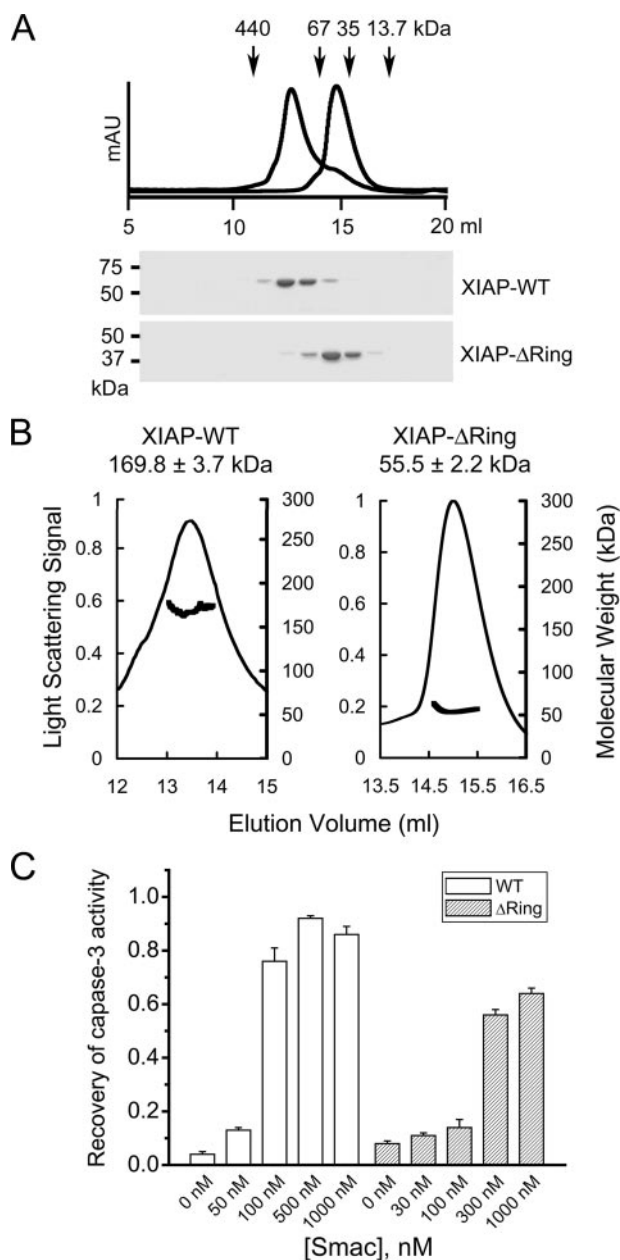


FIGURE 6. Trimerization of XIAP improves the efficiency of Smac to remove its inhibition on caspase-3. *A*, gel filtration profile of full-length XIAP (XIAP-WT) and XIAP with the Ring domain deleted (XIAP- Δ Ring). Coomassie Blue staining shows the staining pattern correlated with the eluted peaks. *B*, molecular mass measurement of XIAP-WT and XIAP- Δ Ring. The trace with the *thin line* corresponds to light scattering signals at 90°. The trace with the *thick line* shows the variation in molecular mass determination as a function of elution volume. *C*, Smac antagonizes inhibition of caspase-3 by XIAP- Δ Ring less effectively than that by full-length XIAP. One nM of caspase-3 and 100 nM of XIAP-WT or XIAP- Δ Ring were incubated in the presence of indicated concentrations of Smac to measure caspase-3 activity.

competitive prevention of BIR2-caspase-3/7 binding by the Smac N-terminal peptide should be able to effectively release inhibition of the effector caspases by XIAP, as shown by our results (Figs. 2 and 3).

However, although this same previous report showed that very high concentrations of Smac N-terminal peptide (monomer) considerably weakened inhibition of caspase-3 by the XIAP linker-BIR2 fragment (40), it was not considered impor-

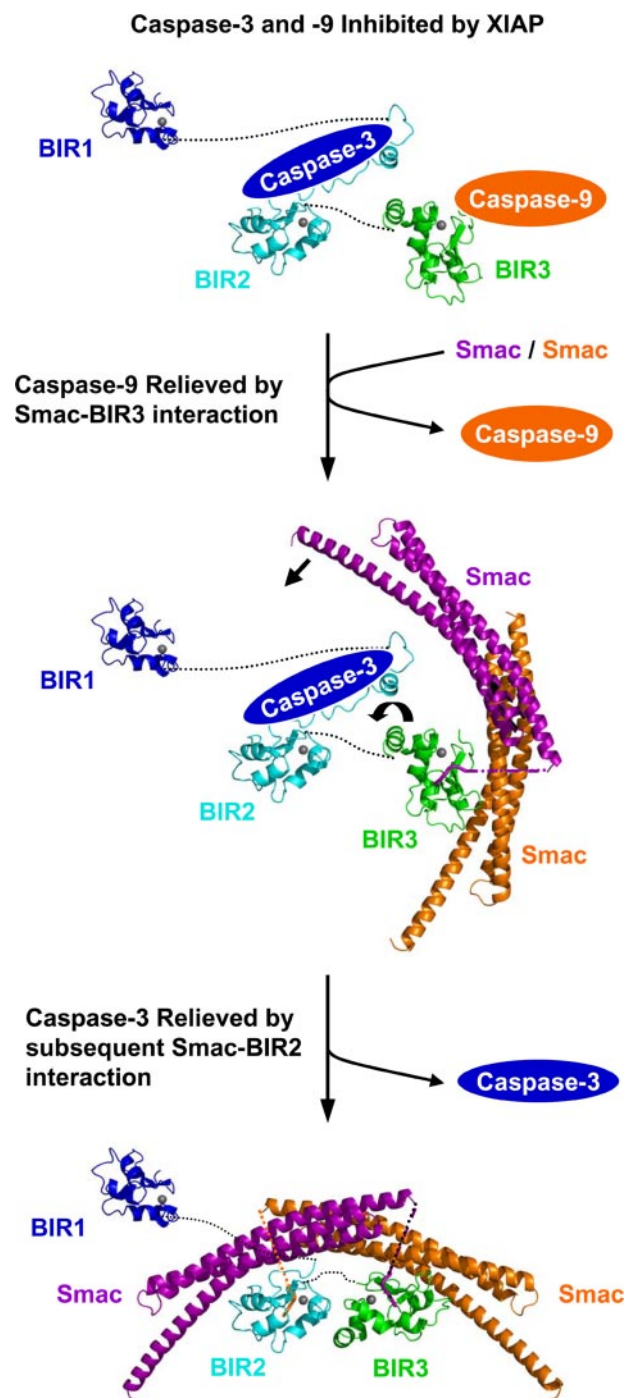


FIGURE 7. A model for dynamic and cooperative regulation of XIAP by Smac. See text for detailed description. XIAP is shown in the model as a monomer for simplicity.

tant because 1) much lower concentration of Smac protein could achieve a better antagonization and 2) much lower concentration of the same peptide could release inhibition of caspase-9 by XIAP. Such comparison strengthened the notion that the steric hindrance generated by the bulky size of Smac holoprotein is required to neutralize XIAP inhibition of effector caspases. If this is the case, then why do dAVPI peptide (Fig. 3) and the reported nonpeptide dimeric Smac small molecule mimic (33) neutralize IAP inhibition of caspase-3 as effectively as Smac protein?

Dynamic and Cooperative Regulation of XIAP by Smac/Diablo

This apparent discrepancy can be explained by the second novel finding of our study: interaction of one of the Smac N termini with the BIR3 domain is critical for the subsequent interaction of the other SMAC N terminus with the BIR2 domain. Also importantly, most previous studies were conducted using individual XIAP BIR domains instead of the full-length protein. As a consequence, the absence of a functional BIR3 domain made the relief of caspase-3 inhibition by the XIAP linker-BIR2 fragment rather inefficient, and under such condition, the Smac protein, which can create steric hindrance, could gain decisive advantage over its N-terminal peptide or chemical mimics.

If the dimeric N-terminal peptide is sufficient to antagonize inhibition of effector caspases by XIAP, why is Smac a bulky protein? Part of the answer is that dimerization of Smac, an essential property for it to antagonize XIAP, requires the bulky protein structure. It is also likely that the Smac protein architecture can interact with other proteins to provide additional regulation. In addition, it has not been ruled out that Smac might have nonapoptotic function inside of mitochondria. Further, because the linker region of XIAP possesses very weak but still detectable inhibitory activity toward effector caspases independent of the BIR2 domain (27, 28, 40), one might argue that the steric hindrance created by the bulky Smac protein is necessary for a more complete restoration of caspase-3 activity inhibited by XIAP, especially when the inhibitor concentration is very high. Although Smac protein indeed antagonizes high concentrations of XIAP more completely (data not shown), the cellular and physiological relevance of such a high IAP concentration is questionable.

Our finding that XIAP is a homotrimeric protein (Fig. 6) is also intriguing. Structurally, the trimerization is mediated by the C-terminal Ring domain of XIAP. However, the Ring domain, which is a putative ubiquitin ligase catalytic domain (44) and was also reported to be able to mediate protein dimerization (45), has not been shown to mediate protein trimerization. Functionally, although trimerization of XIAP has no measurable effect on its caspase inhibitory activity, it facilitates Smac to neutralize its own caspase-3 inhibitory function, which might be physiologically relevant considering that the inhibitory function of IAP should be readily removed to ensure effective apoptosis when needed. It is not clear mechanistically how trimerization makes XIAP more susceptible to Smac, and steric hindrance should not be the reason because Smac protein and dAVPI neutralize full-length XIAP with similar efficacy. On the other hand, trimerization of XIAP appears to have less obvious effect on neutralization of its caspase-9 inhibitory activity by Smac. This scenario might be more complicated in cells, because in cells the active caspase-9 is in a million-dalton protein complex, the apoptosome-caspase-9 holoenzyme (46, 47). Speculatively, binding of a substoichiometric amount of bulky homotrimeric XIAP with the caspase-9 holoenzyme might create a steric barrier to effectively prevent the holoenzyme from acting on the downstream effector caspases; thus, trimerization makes XIAP a more efficient inhibitor; conversely, the substoichiometric amount of XIAP should be more effectively counteracted by Smac when required. Furthermore, Ring domain-mediated XIAP trimerization should be able to stabilize the

dynamic dimerization of the BIR1 domain of XIAP, a property essential for activation of NF- κ B by XIAP (43).

In summary, this study delineated a mechanism by which Smac antagonizes the inhibitory activity of XIAP toward caspase-3, an essential regulatory event in the process of apoptosis and a promising target for cancer treatment.

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