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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Abstract

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 towards caspase-3 more susceptible to Smac.

INTRODUCTION

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). 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 oncprotein prothymosin-α and tumor suppressor PHAP 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 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 dimeric protein, and is posttranslationally 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 XIAP BIR3 domain (22,23). Subsequently, it was revealed that 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 how 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 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 non-peptide nature of this molecule provides desirable cell-permeability and stability, and this molecule possesses 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 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 able 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 (Cat # 235425), and caspase-9 substrate, Ac-LEHD-AFC (Cat # 218765), were purchased from Calbiochem. Anti-Smac antibody (Cat # 05-681) was obtained from Upstate; anti-Flag antibody M2 was from Sigma.

Plasmids
For His-tagged recombinant proteins, full-length human XIAP (residue 10-497) or XIAP-ΔRing (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-tagged 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 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 (Cat # 04-12-3911, EMD Biosciences). The Fmoc protecting group of the Universal PEG NovaTag resin was first removed using 20% piperidine in DMF. Then individual Fmoc-AA-OH was coupled to the resin. The amino group of N-terminal amino acid was protected by Boc group, rather than Fmoc. After the first peptide was synthesized, the Mmt protecting group was removed with 1.0 M HOBt in TFE/DCM. The produced resin was used for the synthesis of second peptides using the same procedure. The final cleavage and deprotection were performed with a cocktail (TFA: water: TIS, 95:2.5:2.5, 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 (TCA). Peptide identity was verified by LC-MS/MS (Agilent Technologies).

Preparation of Cytosolic Fractions from HeLa Cells
HeLa cells are harvested by centrifugation at 1000 x g for 5 min. After washed with PBS, cell pellets were suspended in 5 x 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 DTT) supplemented with mixture proteases inhibitors (Roche Diagnostics). After incubation on ice for 15 min, cells were broken by passing through 22-gauge needles 25 times. Cell lysates were then subject to centrifugation at 750 x g for 10 min at 4°C. Supernatants were further centrifuged at 12,000 x g for 10 min at 4°C, followed by final centrifugation at 100,000 x g for 2 hr 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 OD reached 0.6, 0.1 mM IPTG was added to induce protein expression at 18°C for 16 hours. Cells were pelleted and subject to standard Ni\(^{2+}\) affinity chromatography. For expression of recombinant SMAC proteins, pET15b-Smac-His and pOKD4-SMAC-Flag were co-transformed into BL21 (DE3), followed by IPTG induction and Ni\(^{2+}\) affinity chromatography. The eluted protein was then subject to anion exchange Q column purification (1ml HiTrapQ, GE Healthcare) eluted with 20 ml of gradient from 100 mM 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, similar procedure was performed to get the expression of proteins in BL21, followed by GST affinity chromatography. All recombinant proteins obtained by affinity purification were dialyzed in Buffer A for 2 hours at 4°C. Aliquots were flash frozen with liquid nitrogen, and stored at −80°C.

Caspase Activity Assay

For caspase-3 or caspase-9 activity assay in total reconstituted system, each reaction with a final volume of 30 μl was assembled on ice, including: 3 μl 10 × Caspase Assay Buffer (300 mM HEPES, pH 7.5, 1 M NaCl, 50% sucrose, 1% CHAPS, 200 mM β-mercaptoethanol, 20 mg/ml BSA) (40), 3 μl caspase-3 substrate (Ac-DEVD-AMC, 1 mM) or 3 μl 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°C for 10 min, and then supplemented with 3 μl of 10 nM caspase-3 or 3 μl of 200 nM leucine-zipper-linked dimeric caspase-9 (41), respectively, to initiate the enzyme reaction. Generation of fluorescent signal (relative fluorescent units) due to the cleavage of substrates, indicative of caspase-3 or caspase-9 activity, was measured by an automated spectrophotometer (TECAN) at wavelengths of 360/465 (excitation/emission) nm 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 μM cytochrome c at 30°C for 3.5 hr. Then 9 μl of pre-activated HS100 was mixed with 21 μl of reaction solution pre-warmed at 30°C for 10 min, consisting indicated amount of XIAP, dAVPI, and caspase-3 substrates. Substrate cleavage was measured as above.

GST Pull-Down Experiment

For each sample, 5 μg of purified full-length XIAP (GST-XIAP) or BIR3 domain (GST-BIR3) was coupled to 20 μl glutathione beads. Subsequently, in a 200-μl Buffer Asystem, the beads were incubated with 1 μM Smac in the presence of indicated concentration of dAVPI for 1 hr followed by three washes. Then beads were boiled in 4 × SDS loading buffer, and eluted proteins were subject to SDS-PAGE, followed by Western blotting with an anti-Smac antibody.

Molecular Weight Measurement

Molar mass of purified XIAP-WT or XIAP-ΔRing was determined by static multi-angel light scattering (MALS). 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). Data were collected every 0.5 second 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 non-peptide 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 AVPIAQK (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 AVPIAQK dimer (dAVPI) by covalently linking the C-terminal K residues of the two AVPIAQK peptides (Fig. 1A 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 pre-incubating the cell extracts with 5 μM cytochrome c and 1 mM dATP at 30°C for 3.5 hr. 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 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 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 wild-type (WT) XIAP and BIR2-Mut in a total reconstituted system. In this assay, we found that inhibition of recombinant caspase-3 activity by WT XIAP can be neutralized by both dAVPI (Fig. 3A) and recombinant Smac protein (Fig. 3B), consistent with our results obtained with HeLa cell extracts (Fig. 2); However, both dAVPI (Fig. 3A) and recombinant Smac (Fig. 3B) failed to neutralize the inhibition of caspase-3 activity by BIR2-Mut. Collectively, these results indicate that the interaction of 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 due to a weaker interaction of the linker region with caspase-3 caused by mutation of the BIR2 domain (Fig. 3C). 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. 3C).

Although our result (Fig. 3C) 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 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, ie, 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; 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, while 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 to wild-type Smac, while 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 co-transformed 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. 5A 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 weight (MW) around 150 kDa measured by gel-filtration chromatography (Fig. 6A), suggesting potential oligomerization. Interestingly, it was previously reported that XIAP molecules can interact with each other intermolecularly in cells via its C-terminal Ring domain (42). Thus we generated XIAP recombinant protein with the Ring domain deleted (XIAP-ΔRing). When this protein was resolved by gel-filtration chromatography, it showed a much lower apparent MW than the full-length XIAP (Fig. 6A). Such 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 MW of full-length XIAP and XIAP-ΔRing, we conducted multi-angle light scattering measurement (MALS). The measured MW of full-length XIAP is 169.8 +/- 3.7 kDa (Fig. 6B), indicating it is a homotrimer. Intriguingly, the measured MW of XIAP-ΔRing is 55.5 +/- 2.2 kDa, about 1.5-fold of a monomer XIAP-ΔRing. This value can be explained by that the BIR1 domain of XIAP tends to form a homodimer in a weak yet dynamic manner (43).

What is the biochemical impact of XIAP homotrimerization? We compared the inhibitory activity of full-length XIAP with XIAP-ΔRing, and found they possess similar ability 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-ΔRing (Fig. 6C); while the difference of the full-length XIAP vs XIAP-ΔRing 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 (Fig. 3, 4, 5). Thus Smac interacts with the two BIR domains of XIAP in a sequential and dynamic manner, and such interaction cooperatively disrupts 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, competitive prevention of BIR2-caspase3/7 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 (Fig. 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 important 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 non-peptide dimeric Smac small molecule mimic (33) neutralize IAP inhibition of caspase-3 as effectively as Smac protein?

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 non-apoptotic 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 sub-stoichiometric 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 sub-stoichiometric 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-κB by XIAP (43).

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

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References

34. Verhagen AM, Coulson EJ, Vaux DL. 2001; Genome Biol. 2:REVIEWS3009. [PubMed: 11516343]


FIGURE 1. Chemical Synthesis of the Dimeric SMAC N-Terminal Peptide (dAVPI)

A, Scheme of the synthesis of dAVPI. The detailed methods are described in Experimental Procedures. B, Atomic structure of dAVPI. C, Purification of synthesized dAVPI by reverse phase HPLC. The detailed method is described in Experimental Procedures. D, Confirmation of purified peptides by LC-MS/MS. The detailed method is described in Experimental Procedures.
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 in Experimental Procedures, GST-BIR3 or GST-XIAP was first conjugated to glutathione beads, 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 in Experimental Procedures. An aliquote of activated HS100 was added into the pre-warmed 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.

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from XIAP inhibition. Assays were performed as in panel B, with individual synthetic peptides added with indicated concentration. Control indicates reactions in the absence of peptides. All 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). Error bars represent standard error calculated from 3 independent experiments (same for other figures).
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 wild-type XIAP (WT) but not by XIAP with BIR2 mutation (BIR2-Mut). 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 weight in this figure and all other figures.

B, As dAVPI, Smac neutralizes inhibition of 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 WT XIAP, 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 wild-type XIAP (WT) more efficiently than that by XIAP with BIR3 mutation (BIR3-Mut). 100 nM 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 wild-type XIAP and BIR2-Mut with same efficiency. 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, while BIR2-Mut remains as active as wild-type XIAP. WT, BIR2-Mut, or BIR3-Mut was mixed with 20 nM leucine-zipper-linked caspase-9 at indicated concentrations, and caspase-9 activity was measured.
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 (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 panel D).

D. Compared to Smac-WT, Smac-Mut is less efficient in antagonizing caspase-3 inhibition by XIAP. One nM caspase-3 and 100 nM XIAP were incubated in the presence of indicated concentrations of Smac to measure caspase-3 activity.
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-ΔRing). Coomassie blue staining shows the staining pattern correlated with the eluted peaks. B, Molecular weight measurement of XIAP-WT and XIAP-ΔRing. The trace in thin line corresponds to light-scattering signals at 90°. The trace in thick line shows the variation in molecular mass determination as a function of elution volume. C, Smac antagonizes inhibition of caspase-3 by XIAP-ΔRing less effectively than that by full-length XIAP. One nM of caspase-3 and 100 nM of XIAP-WT or XIAP-ΔRing were incubated in the presence of indicated concentrations of Smac to measure caspase-3 activity.
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.