Smac/DIABLO selectively reduces the levels of c-IAP1 and c-IAP2 but not that of XIAP and Livin in HeLa cells

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Summary

The inhibitors of apoptosis (IAP) bind and inhibit caspases via their baculovirus IAP repeat (BIR) domains. Some of these IAPs are capable of ubiquitylating themselves and their interacting proteins through the ubiquitin-protein ligase (E3) activity of their RING domain. The Drosophila IAP antagonists Reaper, Hid and Grim can accelerate the degradation of Drosophila IAP1 (DIAP1) and some mammalian IAPs by promoting their E3 activity. Smac/DIABLO is a mammalian functional homolog of Reaper/Hid/Grim. Here we show that Smac selectively causes the rapid degradation of c-IAP1 and c-IAP2 but not XIAP and Livin in HeLa cells, although it efficiently promotes the auto-ubiquitylation of them all. Smac binding to c-IAP via its N-terminal IAP-binding motif is the prerequisite for this effect, which is further supported by the findings that Smac N-terminal peptide is sufficient to enhance c-IAP1 ubiquitylation, and Smac no longer promotes the ubiquitylation of mutant c-IAP1 lacking all three BIR domains. In addition, different IAPs require the same ubiquitin conjugating enzymes UbcH5a and UbcH6 for their ubiquitylation. Taken together, Smac may serve as a key molecule in vivo to selectively reduce the protein level of c-IAPs through the ubiquitin/proteasomal pathway.
Introduction

Apoptosis is genetically controlled cell death that is essential for development and homeostasis of multi-cellular organisms. Both excessive and insufficient cell death can lead to anomalies or diseases. The molecular machinery involved in apoptosis is highly conserved across divergent species. The apoptotic death of cells requires proteolytic activation of caspases which are synthesized as latent proenzymes. Once activated, caspases cleave a wide range of molecules that eventually result in the dismantlement of cells. Caspases are therefore tightly controlled within the cells. Active caspases can be specifically inhibited by the inhibitors of apoptosis (IAP), a family of proteins containing one to three copies of characteristic baculovirus IAP repeat (BIR) (1). The BIR domains, in some cases together with the intervening linker regions, directly bind and inhibit caspases. Some of the IAPs also have a C-terminal RING finger domain.

IAP proteins are counteracted by Reaper, Hid, Grim, Sickle and Jafra2 in Drosophila, and by Smac/DIABLO, Omi/HtrA2 and GSPT1/eRF3 in mammals (2-13). Despite the overall sequence differences, these IAP antagonists share a conserved N-terminal IAP-binding motif (IBM) or Reaper/Hid/Grim (RHG) motif. This motif is indispensable and sufficient for counteracting IAP's inhibition of caspases. The small subunit of active caspase-9 also binds to XIAP through the same IBM produced by auto-processing (14). This mechanism allows these IAP antagonists to compete with caspases for IAP-binding and consequently relieves caspases and promotes cell death.

Some IAPs also regulate apoptosis through the ubiquitin-protein ligase activity (E3) of their RING domain (1,15). These IAPs are capable of targeting the poly-ubiquitylation of IAP-binding
proteins such as caspases and IAP antagonists (16-21). On the other hand, the E3 activity of IAP also leads to the ubiquitylation of IAPs themselves and such auto-ubiquitylation can be enhanced by the Reaper, Hid and Grim (22-27). An alternatively spliced form of Smac, Smac3, was recently reported to promote XIAP ubiquitylation and degradation (28). These observations demonstrate that these IAP antagonists accelerate the disposal of IAPs in addition to releasing captive caspases from IAPs. Moreover, the mammalian IAP-binding protein Omi/HtrA2 can directly degrade IAP molecules through its serine protease activity (29-31).

Five of the eight human IAPs have a C-terminal RING domain (32). However, little is known about the characteristics of the E3 activity of various IAPs. Even though the ubiquitylation and proteasomal degradation of XIAP have been reported to be enhanced by Smac3 rather than Smac, it is not known if c-IAP1 and c-IAP2 are also modulated by their cognate antagonists via ubiquitin/proteasomal degradation. In this paper we show that Smac stimulates the rapid degradation of c-IAP1 and c-IAP2 but not XIAP and Livin in HeLa cells, although Smac does promote auto-ubiquitylation of all four of these human IAPs. These human IAPs require the same ubiquitin conjugating enzymes (E2) UbcH5a and UbcH6 for their E3 activity in vitro. We also demonstrate that c-IAP degradation promoted by Smac is dependent on RING E3 activity and direct binding to Smac and the Smac N-terminal peptide is sufficient to accelerate c-IAP1 auto-ubiquitination. These observations, together with the recent report that Smac is recruited by the tumor necrosis factor receptor (TNFR) signaling pathway to disrupt TRAF2-c-IAP1 complex (33), indicate that Smac may selectively down-regulate the levels of c-IAP1 and c-IAP2, thereby facilitating caspase-8 activation initiated by TNFR signaling in vivo.
Experimental procedures

Antibodies and reagents—The polyclonal antibodies that recognize respectively the residues 527-546 of human c-IAP1, residues 507-524 of human c-IAP2 and residues 244-263 of human XIAP, and the monoclonal antibody against human ubiquitin were purchased from R&D Systems. HRP-conjugated anti-GST antibody and anti-FLAG M2 antibody, and anti-c-Myc monoclonal antibody were from Sigma. The monoclonal anti-Livin antibody was from IMGENEX and the monoclonal anti-Actin antibody was from Santa Cruz Biotechnology. The polyclonal antiserum against Smac was generated by immunizing rabbits with recombinant Smac protein as described previously (7). The mammalian ubiquitin, rabbit ubiquitin activating enzyme (E1) and various recombinant human ubiquitin conjugating enzymes (E2) were from Boston Biochem.

Plasmids—The plasmids for the GST fusion proteins of human c-IAP1, c-IAP2 and XIAP (29), and the C-terminal (His)_9-tagged mature form Smac (7) have been described elsewhere. The cDNAs for Livin α and Livin β were individually inserted into the pGEX-4T-2 (Amersham) to generate GST fusion proteins. The p3×FLAG-CMV-7 vector (Sigma) was used for the expression of IAP proteins in mammalian cells. The N-terminal ubiquitin-fused and C-terminal c-Myc-tagged Smac wild type (WT) and the Ala¹ deletion mutant (ΔA) were generated by PCR and subcloned into the pcDNA3.1(−) mammalian expression vector (Invitrogen) according to the reported technique (34,35). The mutation of the conserved His residue to Ala in the RING domain of respective IAPs (c-IAP1 H588A, c-IAP2 H574A, XIAP H467A, Livin α H269A and
Livin β H251A) was made using the QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene). Full length c-IAP1 and XIAP and different c-IAP1 truncation mutants were generated by PCR and subcloned into the pTYB11 (New England Biolabs). All constructs were confirmed by sequencing.

**Protein expression and purification**—All of the recombinant proteins were expressed in E. coli strain BL21 (DE3). The GST-fused IAP proteins were purified with Glutathione Sepharose affinity chromatography, followed by Superdex 200 gel-filtration chromatography if necessary. The C-terminal (His)$_9$-tagged wild type Smac and SmacΔA were purified with Ni-NTA Sepharose affinity chromatography. The non-tagged full length c-IAP1 and XIAP, and the truncated c-IAP1 mutants were purified from the Chitin affinity column after DTT induction according to the manufacture's protocols (New England Biolabs). The protein concentrations were determined by the modified Bradford method (36).

**In vitro ubiquitylation assays**—In vitro ubiquitylation assays were carried out as previously described (29). IAP proteins (200 nM) were incubated with or without Smac for 2 hours at 30°C in a reaction system containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2 mM Mg-ATP, 20 μM mammalian ubiquitin, 100 nM rabbit ubiquitin-activating enzyme (E1) and 400 nM of recombinant human ubiquitin-conjugating enzymes (E2). The reactions were stopped by adding equal volumes of 2×SDS sample loading buffer followed by Western Blot analysis.
Transfection of cultured Cells—HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum. Cells were seeded onto 6-well plates the day before transfection and transfected at ~70% confluence by using Lipofectin combined with Plus reagent (Invitrogen) according to the manufacture's protocols. The IAP expression plasmids (3 μg each for c-IAP1, c-IAP2 and XIAP, and 250 ng each for Livin α and Livin β) together with 2.5 μg of Smac expression plasmids or pcDNA3.1(-) blank plasmid were used for single-well transfection in the absence of antibiotics. The cells were harvested 12 hours after the transfection and lysed with 0.5% CHAPS in 20 mM HEPES (pH 7.4), 10 mM KCl, 1 mM MgCl2 and 1 mM DTT. The lysates were centrifuged and the protein concentrations of the supernatants were quantified by the modified Bradford method (36).

Western Blot analysis—Proteins were resolved by SDS-PAGE and transferred to nitrocellulose filters. The filters were blocked with 5% milk and probed with antibodies as indicated in the text. The signals were visualized with the enhanced chemiluminescence method.
Results

Smac prevents the accumulation of both c-IAP1 and c-IAP2 in HeLa cells

To investigate the effect of Smac on IAP protein levels, we transfected HeLa cells with the expression vectors for Smac and various IAPs. The ubiquitin fusion technique was used here to secure the production of mature Smac protein with the expected N-terminal AVPI motif (6,37). The HeLa cells transiently transfected with c-IAP1 or c-IAP2 expression vector produced not only the expected full length c-IAP1 or c-IAP2, but also the multiple higher molecular weight forms that are characteristic of ubiquitylated products (Lane 2 and 4 of Fig. 1). This was consistent with the previous reports that both c-IAP1 and c-IAP2 are ubiquitin-protein ligases (E3) and are capable of directing auto-ubiquitylation (16,22). In contrast, the transiently expressed XIAP, under the same conditions, showed only one or two bands above the major band of full length XIAP (Lane 6 of Fig. 1). Surprisingly, the protein levels of both c-IAP1 and c-IAP2, but not XIAP, were significantly decreased when the mature form of Smac was co-expressed (Lane 2, 4 and 7 of Fig. 1).

Previous works have revealed that the Drosophila functional homologs of Smac, Reaper, Hid and Grim can promote the degradation of mammalian IAP proteins bearing normal E3 activity (23-26). Two of these Smac homologs, Grim and Reaper, also reduce the levels of IAPs by suppressing global protein synthesis (23,26). It was unlikely that the decrease of c-IAP1 and c-IAP2 was caused by the Smac-mediated inhibition of protein synthesis since XIAP under similar conditions was unaffected. To test whether Smac stimulated the ubiquitylation-mediated degradation of both c-IAP1 and c-IAP2 we mutated His588 in c-
IAP1 and His$^{574}$ in c-IAP2 to Ala. This conserved His residue in the RING finger domain has proven important for the IAP E3 activity (22). The effects of Smac on these IAP E3 activities were examined as described below.

**Smac promotes the degradation of c-IAP1 and c-IAP2 by enhancing their auto-ubiquitylation in HeLa cells**

The H588A mutant c-IAP1 exhibited a much higher expression level than the wild type in HeLa cells (compare Lane 3 and 6 of Fig. 2A). The amount of wild type c-IAP1 was also dramatically increased by the addition of proteasome inhibitor MG132 to the culture medium (Lane 10, Fig. 2A). MG132, however, did not cause a significant increase in the H588A c-IAP1 protein level (Lane 13). These observations were in agreement with the previous report that c-IAP1 is continuously down-regulated by ubiquitylation-dependent degradation and such ubiquitylation is mediated by the E3 activity of c-IAP1 itself (22).

In the presence of MG132, co-expression of wild type Smac led to a reduction in the level of non-ubiquitylated and mono-/di-ubiquitylated wild type c-IAP1 and a concomitant increase in the level of highly ubiquitylated c-IAP1 forms (Lane 11 of Fig. 2A). In the absence of MG132, co-expression of Smac resulted in nearly complete disappearance of c-IAP1 (Lane 4 of Fig. 2A). This data indicated that Smac promoted the poly-ubiquitylation and degradation of c-IAP1.

It is known that the processed Smac and other IAP antagonists have a conserved N-terminal IAP-binding motif (IBM) with an initial Ala residue. Mutation of this Ala or deletion of the IBM abolishes the specific binding of these proteins to IAP (38,39). To
assess whether Smac enhancement of c-IAP1 ubiquitylation and degradation requires the same binding, we co-transfected c-IAP1 with a Smac mutant lacking the initial Ala (Smac ΔA) that was also produced in the ubiquitin fusion expression vector. As expected, Smac ΔA did not enhance the auto-ubiquitylation of c-IAP1 (Compare Lane 5 and 3, and Lane 13 and 10 of Fig. 2A), suggesting that the specific binding of Smac to c-IAP1 via its IBM is required for Smac to enhance the auto-ubiquitylation of c-IAP1. In agreement with the above observations, Smac did not cause a drastic change in the level of E3-negative H588A c-IAP1, either in the absence or in the presence of MG132 (Lane 6 and 7, and Lane 13 and 14 of Fig. 2A, respectively). Smac likewise strongly promoted the ubiquitylation and degradation of c-IAP2 (Fig. 2B). Mutation of His574 to Ala in c-IAP2 also significantly increased the expression level of c-IAP2 (Fig. 2B).

As mentioned above, the level of c-IAP1 was significantly elevated by the addition of MG132. In contrast, c-IAP2 did not show such a significant change when MG132 was added (compare lane 3 and 10 in Fig. 2B). Such a difference made it reasonable to speculate that in the absence of Smac, c-IAP2’s E3 was much less active than c-IAP1 in directing its auto-ubiquitylation.

It should be noted that although His588 in c-IAP1 and His574 in c-IAP2 are critical for their E3 activities, mutation of this residue to Ala greatly reduced but did not completely abolish their E3 activity, as revealed by the background ubiquitylated product ladders (Lane 6, 7, 13 and 14 of Fig. 2). This residual activity was also observed in an in vitro ubiquitylation assay using purified mutant c-IAP proteins (data not shown).
Smac does not promote the degradation of XIAP and Livin in HeLa cells

XIAP has been reported to be ubiquitylated by its own E3 activity when expressed in HEK 293T cells and this auto-ubiquitylation and subsequent degradation is stimulated by Reaper (26). In this current study, XIAP transiently expressed in HeLa cells was ubiquitylated as well; however, mono-ubiquitylation rather than poly-ubiquitylation of XIAP seemed to be the major product (Fig. 3A). Similar to c-IAP1 and c-IAP2, XIAP ubiquitylation also depended on its E3 activity, for the H467A E3-negative mutant gave either no band or a much weaker mono-ubiquitylation band (Lane 3, 5, 8 and 10 of Fig. 3A). Although more poly-ubiquitylated XIAP products could be detected in the presence of MG132, they were much less significant compared to that of c-IAP1 and c-IAP2.

Unlike that of c-IAP1 and c-IAP2, the overall amount of XIAP was not quite affected by the co-transfected Smac, although Smac still promoted XIAP ubiquitylation. In the absence of MG132, co-expression of Smac only had a negligible effect on the level of XIAP (Lane 4 of Fig. 3A), whereas under similar conditions Smac reduced both c-IAP1 and c-IAP2 to a nearly non-detectable level (Fig. 2). This result was consistent with the report by Silke et al. that Smac does not promote XIAP degradation (27).

Unexpectedly, the Smac ΔA mutant, despite its failure to bind to various IAPs (Fig. 4), also stimulated the auto-ubiquitylation of XIAP and Livin (Lane 4 and 5 and 11 and 12 in Fig. 3). The reason for this IBM-independent acceleration on XIAP and Livin ubiquitylation remains unknown.

Smac effectively stimulates auto-ubiquitylation of Livin α, which slightly promoted the degradation of Livin α (Lane 4 of Fig. 3B). Livin β, an alternatively spliced form of Livin,
lacks 18 residues between the BIR and the RING domains compared to Livin \( \alpha \) \((40, 41)\).

The transfection results of Livin \( \beta \) were basically the same as that of Livin \( \alpha \) (data not shown).

In contrast to c-IAP1 and c-IAP2, the mutation of the corresponding His residue in the RING finger domain of XIAP and Livin did not considerably enhance their expression, although the mutation did greatly reduce their E3 activity. Taken together, these observations suggested that the levels of XIAP and Livin were not mainly regulated by auto-ubiquitylation/proteasomal degradation in HeLa cells.

**Different IAP proteins require the same ubiquitin-conjugating enzymes *in vitro* for their E3 activity**

To compare the E3 activities of these human IAPs, we used an *in vitro* ubiquitylation assay system containing purified ubiquitin, ubiquitin-activating enzyme (E1) and ubiquitin-conjugating enzymes (E2) to screen a panel of ubiquitin-conjugating enzymes available from Boston Biochem. All of these IAP proteins exhibited strong E3 activity when UbcH5a and UbcH6 were used as the E2 as manifested by the characteristic poly-ubiquitylation ladders (Lane 4 and 7 of Fig. 5).

**Smac does not promote auto-ubiquitylation of XIAP *in vitro***

The purified XIAP was active in directing poly-ubiquitylation. However, the auto-ubiquitylation of XIAP was not enhanced by Smac. The amount of ubiquitylated forms of
XIAP with a high molecular weight of ~150 kD was even slightly reduced by Smac (Fig. 6).

**Smac promotes auto-ubiquitylation of c-IAP1 in vitro**

The observation that Smac promotes c-IAP1 auto-ubiquitylation in HeLa cells was further confirmed *in vitro* by using UbcH6 as the E2. Smac at equimolar to c-IAP1 enhanced c-IAP1 auto-ubiquitylation to the maximum (Lane 5, Fig. 7A). Consistent with the tranfection result, SmacΔA failed to promote such auto-ubiquitylation (Lane 7 of Fig. 7A). Similar results were also obtained with Smac and c-IAP2 (data not shown).

**Smac promotes the auto-ubiquitylation of c-IAP1 through specific association with the BIR domains**

The Smac mutant lacking the initial Ala residue did not bind IAP proteins, and consequently did not promote the E3 activity of either c-IAP1 or c-IAP2 in HeLa cells or *in vitro*. These results strongly indicated that the interaction between the IBM of Smac and the BIR domains of IAP was necessary and that the BIR domains may regulate the E3 activity of the RING finger. To verify this we made stepwise deletion of the first BIR (ΔBIR1, residues 1-161), the first two BIRs (ΔBIR1-2, residues 1-265), or all the three BIR domains (ΔBIR1-2-3, residues 1-339) from c-IAP1. All these mutants and the full-length wild type c-IAP1 were purified as a non-tagged form by using the pTYB11 intein system (New England Biolabs).
The c-IAP1 mutants with one, two or all three BIR domains deleted were still active in
directing their auto-ubiquitylation (Fig. 7A). The basal activity of ΔBIR1 and ΔBIR1-2
was very low but was significantly enhanced by Smac, whereas the basal activity of
ΔBIR1-2-3 was comparably high and could no longer be enhanced by Smac. This
suggested that the second and the third BIR domains of c-IAP1 strongly inhibit the E3
activity of the C-terminal RING. Smac binding to the BIR domains of c-IAP1 may help
expose the RING domain to generate a higher E3 activity.

Smac N-terminal hexapeptide is sufficient to stimulate the ubiquitin-protein ligase
activity of c-IAP1

Previous work has shown that the N-terminal peptides of Smac can mimic Smac protein in
removing XIAP's inhibition on caspase-3 activation (38,39). Here we examined if binding
to the BIR domains of c-IAP1 was sufficient to promote its ubiquitin-protein ligase
activity by using Smac hexapeptide in vitro. The Smac N-terminal hexapeptide (Smac-6)
stimulated E3 activity of c-IAP1ΔBIR1 as effectively as the Smac protein (Fig. 7B). As
controls, both Smac ΔA and the peptide with an extra Met before the AVPI motif (Smac-
7M) failed to activate c-IAP1.
Discussion

In agreement with previous reports, the human RING-containing IAP proteins tested in this paper exhibited significant E3 activities. The processed Smac promoted the auto-ubiquitylation of c-IAP1 and c-IAP2 both in HeLa cells and in vitro, and such a promotion resulted in a dramatic decrease in the protein levels of these two IAP proteins in HeLa cells. In contrast, the levels of both XIAP and Livin were not dramatically affected by Smac, although their auto-ubiquitylation was enhanced by Smac as well. This may reflect the differences in E3 activity between these two groups of IAPs: c-IAP1 and c-IAP2 are more efficient than XIAP and Livin in catalyzing auto-ubiquitylation. Consistent with the report by Hu et al. that c-IAP1 and c-IAP2 rather than XIAP induce significant Smac ubiquitylation and consequent degradation in 293T cells (20), we also noticed an apparent reduction in the level of Smac by co-transfected c-IAP1 or c-IAP2 but not by XIAP. These observations suggest that Smac may selectively interact with c-IAPs in live cells.

Both c-IAP1 and c-IAP2 are known to be involved in the apoptotic signaling mediated by the TNFR family (33,42-47). Smac was recently found to coexist with c-IAP1, TRAF2 and TRAF3 in an endogenous ligand-receptor complex (48). Deng et al. reported that Smac makes c-IAP1 dissociate from TRAF2, which in turn releases the inhibition of caspase-8 activation by TRAF2-c-IAP1 complex (33). Our observations, together with these reports, imply that Smac, being a potent activator of cIAP E3 in vitro, may accelerate TNFR-mediated caspase activation in live cells by promoting E3 activity and proteasomal degradation of c-IAP1 and c-IAP2.
To our surprise, Smac ΔA, which was unable to bind to IAPs, also promoted the ubiquitylation of XIAP and Livin in HeLa cells, whereas it failed in HEK 293 cells (data not shown). How Smac ΔA exerts such an influence on XIAP and Livin in HeLa cells is not readily explicable. It is, however, unlikely that Smac ΔA is further processed to expose an additional internal IAP-binding motif, for Smac ΔA activated neither c-IAP1 nor c-IAP2. These observations make us speculate that in HeLa cells Smac ΔA might require other unknown factor(s) to indirectly cause XIAP and Livin ubiquitylation, and that such an effect has nothing to do with the IBM in Smac. Despite the fact that Smac ΔA is merely an artificial Smac mutant in vitro, one of the alternatively spliced Smac proteins, Smac β, is naturally lacking IBM but also promotes cell death (49). It is possible that Smac β might enhance cell death by regulating XIAP or Livin ubiquitylation in the same manner as Smac ΔA. We are currently investigating this possibility.

The different fates of various IAPs after Smac stimulation indicated that Smac differentially regulates these IAPs. XIAP is the strongest inhibitor of caspases among the IAP family proteins (32,50,51). Since Smac did not promote ubiquitylation-mediated XIAP degradation, it is reasonable to propose that Smac counteracts XIAP mainly through direct binding. Smac may regulate Livin in a similar manner. On the other hand, it has been reported that XIAP is mono-ubiquitylated in the central nervous system (52). Such a modification may be more related to XIAP's biological function instead of its proteasomal degradation. Our results showed that Smac effectively promoted XIAP mono-ubiquitylation in HeLa cells and therefore may regulate the function of XIAP as well.
It has been recognized that RING domains, along with non-RING sequences, can bind E2s in a specific and catalytically productive manner (53). Given the overall sequence similarity in their RING fingers, it is not surprising that all of the IAPs tested in this paper required the same E2 enzymes UbcH5a and UbcH6 for their maximal activity. Our results were in agreement with the report by Hu et al., that c-IAP1, c-IAP2 and XIAP associate with UbcH5a as determined by an immunoprecipitation assay (20). UbcH5b can also support IAP E3 activity as reported by Yang et al. and MacFarlane et al. (19,22), it, however, produced only negligible activity in our assays. Interestingly, a non-IAP RING finger E3, herpes simplex virus type 1 immediate-early protein ICP0, also requires UbcH5a and UbcH6 in vitro for its E3 activity (54,55). UbcH5a is at least 88% identical to UbcH5b and UbcH5c, whereas it has only 74% similarity to UbcH6 (56,57). UbcH5a may be structurally more similar to UbcH6 than UbcH5b and UbcH5c regardless of their primary amino acid sequences. The structural basis for IAP preference for UbcH5a and UbcH6 and if they are the cognate E2 enzymes for these IAPs remain to be determined.

Our results here demonstrated that Smac stimulated c-IAP1 auto-ubiquitylation through specific binding to the BIR domains. Apparently the BIR domains have an inhibitory effect on the E3 activity of the RING domain. The binding of Smac or Smac peptide to the BIR domains may help unfold the molecule to expose the RING domain, thus making the RING more active in catalyzing ubiquitylation.

It has been reported that Smac N-terminal peptides can strongly enhance the anti-tumor activity of Apo-2L/TRAIL in a xenograft mouse model of intracranial malignant glioma (58). Smac N-terminal peptides also enhance pro-apoptotic activities and long term antiproliferative effects of diverse antineoplastic agents and potentiate apoptosis in a
number of cancer cell lines (58-61). These effects are attributed to the currently known mechanism that Smac binding to IAP releases caspases that are bound to IAPs (38,39). Here we have demonstrated an additional mechanism for Smac to potentiate cell death by enhancing c-IAP auto-ubiquitylation and proteasomal degradation. This mechanism may also contribute to the pro-apoptotic effect of Smac in those cancer models described above. This mechanism therefore offers additional biochemical support for the potential to apply Smac N-terminal peptides in anti-tumor therapy.
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Figure Legends

FIG. 1. **Smac reduces the protein level of c-IAP1 and c-IAP2 in HeLa cells.** HeLa cells were transfected with IAP alone (Lane 2, 4 and 6) or together with Smac (Lane 3, 5 and 7), or Smac alone (Lane 1). Equal amounts of cell lysates (7 µg) were immunoblotted with the HRP-conjugated anti-FLAG antibody (top panel) to detect N-terminal 3×FLAG tagged IAPs, or the anti-c-Myc antibody (bottom panel) to detect C-terminal c-Myc tagged Smac.

FIG. 2. **Smac promotes the rapid degradation of c-IAP1 and c-IAP2 by enhancing their auto-ubiquitylation.** (A). The c-IAP1 WT or H588A mutant plasmids together with Smac WT or Smac ΔA mutant, or with pcDNA3.1(-) blank plasmid, were co-transfected into HeLa cells. (B). The c-IAP2 WT or H574A mutant plasmids together with Smac WT or Smac ΔA mutant, or with pcDNA3.1(-) blank plasmid were co-transfected into HeLa cells. As controls, HeLa cells were also transfected with Smac WT or ΔA plasmids together with the blank p3×FLAG-CMV-7 plasmid. The transfected cells were cultivated either in the absence (Lane 1-7) or in the presence (Lane 8-14) of MG132. Equal amounts of soluble proteins (7 µg) from the transfected cells were immunoblotted with the HRP-conjugated anti-FLAG antibody (top panel) to detect N-terminal 3×FLAG tagged IAPs, or the anti-c-Myc antibody (middle panel) to detect C-terminal c-Myc tagged Smac. The anti-Actin immunoblotting results were to show equal protein loading (bottom panel).
FIG. 3. **Smac promotes auto-ubiquitylation but not degradation of XIAP and Livin.** (A). The XIAP WT or H467A mutant plasmids together with Smac WT or ΔA, or with pcDNA3.1(-) blank plasmid, were co-transfected into HeLa cells. (B). The Livin α WT or H269A mutant plasmids together with Smac WT or Smac ΔA mutant, or with pcDNA3.1(-) blank plasmid, were co-transfected into HeLa cells. As controls, HeLa cells were also transfected with Smac WT or ΔA plasmids together with the blank p3×FLAG-CMV-7 plasmid. The transfected cells were cultivated either in the absence (Lane 1-7) or in the presence (Lane 8-14) of MG132. Equal amounts of soluble proteins (7 μg) from the transfected cells were immunobblotted with the HRP-conjugated anti-FLAG antibody (top panel) to detect N-terminal 3×FLAG tagged IAPs, or the anti-c-Myc antibody (middle panel) to detect Smac. The anti-Actin immunoblotting results were to show equal protein loading (bottom panel).

FIG. 4. **IAP proteins bind Smac WT but not Smac ΔA mutant.** The purified IAP proteins (GST fusion form) were incubated with equimolar concentrations of Smac or Smac ΔA in buffer containing 20 mM phosphate (pH 7.4), 200 mM NaCl and 0.05% Tween 20 for 2 hours at 4°C. The GST-fused IAP proteins were pulled down by Glutathione Sepharose beads. The supernatants after Glutathione Sepharose beads deprivation and the washed beads were subjected to SDS-PAGE followed by silver-staining.
FIG. 5. **Different IAP proteins require the same ubiquitin-conjugating enzymes for their E3 activity in vitro.** The purified IAP proteins (200 nM) were incubated with Smac (400 nM) for 2 hours at 30°C in a reconstituted assay system consisting of 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2 mM Mg-ATP, 20 μM mammalian ubiquitin, 100 nM rabbit ubiquitin-activating enzyme (E1) and 400 nM of different recombinant human ubiquitin-conjugating enzymes (E2). The reactions were stopped by adding equal volumes of 2×SDS sample loading buffer and the products were subjected to SDS-PAGE followed by immunoblotting with different antibodies. (A). The ubiquitylation reaction products for c-IAP1 (GST fusion form) and Smac were immunoblotted with anti-c-IAP1 antibody (top panel) and anti-Smac antibody (middle panel), respectively. The filter for c-IAP1 detection was stripped and re-probed with anti-ubiquitin antibody (bottom panel). (B). The ubiquitylation reaction products for c-IAP2 (GST fusion form), XIAP (natural form, without tag) and Livin α and β (GST fusion form) were immunoblotted with the respective antibodies against each IAP protein.

FIG. 6. **Smac does not promote the auto-ubiquitylation of XIAP in vitro.** The purified XIAP (without tag) was incubated for 2 hours at 30°C with or without different concentrations of Smac or with Smac ΔA in the reconstituted ubiquitylation reaction system (Ub Mix) consisting of 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2 mM Mg-ATP, 20 μM mammalian ubiquitin, 100 nM rabbit ubiquitin-activating enzyme (E1) and 400 nM UbcH6 (E2). The reactions were stopped by adding equal volumes of 2×SDS sample loading buffer and the products were subjected to SDS-PAGE followed by immunoblotting with anti-XIAP antibody.
FIG. 7. **Smac promotes the auto-ubiquitylation of c-IAP1 in vitro.** In the reconstituted ubiquitylation reaction system (Ub Mix) described in FIG. 6 the purified c-IAP1 proteins were incubated for 2 hours at 30°C without or with various concentrations of Smac (A), or c-IAP1 ΔBIR1 was incubated with various concentrations of Smac N-terminal peptide Smac-6 (B). Smac ΔA and the peptide Smac-7M were used as negative controls. The reactions were stopped by adding equal volumes of 2×SDS sample loading buffer and the products were subjected to SDS-PAGE followed by immunoblotting with anti-c-IAP1 antibody. The arrows indicate the unmodified full length or deleted c-IAP1.
**FIG. 1**

|        | IAP | c-IAP1 | c-IAP2 | XIAP |
|--------|-----|--------|--------|------|
| Smac   | +   | –      | +      | –    |
|        | –   | +      | –      | –    |
|        | +   | –      | +      | –    |
|        | –   | +      | –      | +    |
| Lane   | 1   | 2      | 3      | 4    |
|        | 5   | 6      | 7      |      |

**Lane 7**

- Smac
- XIAP
- c-IAP1/c-IAP2

**Lane 6**

- XIAP

**Lane 5**

- Smac

**Lane 4**

- XIAP

**Lane 3**

- Smac

**Lane 2**

- XIAP
FIG. 2A

|            | – MG132 | + MG132 (2 μM) |
|------------|---------|----------------|
| c-IAP1     | –       | –              |
| Smac       | WT      | WT             |
| Lane       | 1       | 2              |

```latex
\begin{tabular}{|c|c|c|c|c|c|c|c|c|c|c|}
\hline
  & –       & –       & WT & WT & WT & H588A & H588A & – & – & WT & WT & WT & H588A & H588A \\
\hline
  c-IAP1     & –       & –       & WT & WT & WT & H588A & H588A & – & – & WT & WT & WT & H588A & H588A \\
  Smac       & WT      & ΔA      & –   & WT & ΔA & –   & WT & ΔA & –   & WT & ΔA & –   & WT & ΔA & –   \\
  Lane       & 1       & 2       & 3   & 4   & 5   & 6   & 7   & 8   & 9   & 10  & 11  & 12  & 13  & 14  & 15  \\
\hline
\end{tabular}
```
FIG. 2B

|           | – MG132 | + MG132 (2 μM) |
|-----------|---------|-----------------|
| c-IAP2    | –       | –              |
| Smac      | WT      | WT              |
|           | ΔA      | ΔA              |
| Lane      | 1       | 2              |
|           | 3       | 4              |
|           | 5       | 6              |
|           | 7       | 8              |
|           | 9       | 10             |
|           | 11      | 12             |
|           | 13      | 14             |

**Figure Description:**

- **c-IAP2 (Ub)n**
- **Smac**
- **Actin**

**Legend:**

- **WT:** Wild Type
- **ΔA:** Delta A
FIG. 3A

|       | – MG132                  | + MG132 (2 μM)                  |
|-------|--------------------------|---------------------------------|
|       | – | – | WT | WT | WT | H467A | H467A | – | – | WT | WT | WT | H467A | H467A |
| XIAP  |   |   |    |    |    |       |       |    |    |    |    |    |       |       |
| Smac  | WT | ΔA | –  | WT | ΔA | –  | WT    | ΔA | –  | WT | ΔA | –  | WT    |
| Lane  | 1  | 2  | 3  | 4  | 5  | 6  | 7     | 8  | 9  | 10 | 11 | 12 | 13 | 14 |

The image displays a gel electrophoresis diagram with bands for XIAP, Smac, and Actin labeled. The table correlates the lane numbers with the protein expressions under different conditions. The gel shows bands at various molecular weights, indicating the presence of XIAP, Smac, and Actin proteins.
### FIG. 3B

|       | – MG132 | + MG132 (2 μM) |
|-------|---------|----------------|
| Livin α | – | – | WT | WT | WT | H269A | H269A | – | – | WT | WT | WT | H269A | H269A |
| Smac  | WT | ΔA | – | WT | ΔA | – | WT | ΔA | – | WT | ΔA | – | WT |       |
| Lane  | 1   | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14   |

Graph showing protein expression levels and bands for Livin α, Smac, and Actin.
FIG. 4
FIG. 5B

| E2 | – | H2 | H3 | H5a | H5b | H5c | H6 | H7 | H10 |
|----|---|----|----|-----|-----|-----|----|----|-----|
| Lane | 1 | 2  | 3  | 4   | 5   | 6   | 7  | 8  | 9   |

- **c-IAP2 (Ub)n**
- **c-IAP2**
- **XIAP (Ub)n**
- **XIAP**
- **Livin α (Ub)n**
- **Livin α**
- **Livin β (Ub)n**
- **Livin β**
FIG. 6

| XIAP (0.2 μM) | - | + | + | + | + | + | - | - | - |
|---------------|---|---|---|---|---|---|---|---|---|
| Smac (μM)     | 0 | 0 | 0 | WT | 0.1 | WT | 0.2 | WT | 0.5 |
| Ub Mix        | + | - | + | + | + | + | + | + | + |
| Lane          | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |

- XIAP (Ub)n
- XIAP
**FIG. 7A**

|                | c-IAP1 (0.2 μM) | Smac (μM) | Ub Mix | Lane |
|----------------|-----------------|-----------|--------|------|
|                | −               | 0         | +      | 1    |
|                | Full length     | .1        | −      | 2    |
|                | ΔA              | .2        | +      | 3    |
|                | WT              | .3        | +      | 4    |
|                | ΔA              | .4        | +      | 5    |
|                | WT              | .5        | +      | 6    |
|                | ΔA              | .6        | −      | 7    |
|                | WT              | .7        | −      | 8    |
|                | ΔA              | .8        | −      | 9    |
|                | WT              | .9        | −      | 10   |
|                | ΔA              | 1.0       | −      | 11   |
|                | WT              | 1.1       | −      | 12   |
|                | ΔA              | 1.2       | −      | 13   |
|                | WT              | 1.3       | −      | 14   |
|                | ΔA              | 1.4       | −      | 15   |
|                | WT              | 1.5       | −      | 16   |
|                | ΔA              | 1.6       | −      | 17   |
|                | WT              | 1.7       | −      | 18   |
|                | ΔA              | 1.8       | −      | 19   |
|                | WT              | 1.9       | −      | 20   |
|                | ΔA              | 2.0       | −      | 21   |
|                | WT              | 2.1       | −      | 22   |
|                | ΔA              | 2.2       | −      | 23   |
|                | WT              | 2.3       | −      | 24   |

The image shows a gel electrophoresis with lanes labeled from 1 to 24. The gel bands indicate the presence of c-IAP1 and its variants under different conditions of Smac and Ub Mix.
| Lane | Ub Mix | Smacpeptide (μM) |
|------|--------|------------------|
| 1    | +      | 0                |
| 2    | -      | 0                |
| 3    | +      | 0.2 WT           |
| 4    | +      | 0.2 ΔA           |
| 5    | +      | 0.1 Smac-6       |
| 6    | +      | 0.2 Smac-6-7M    |
| 7    | +      | 0.6 Smac-6-7M    |
| 8    | +      | 6.0 Smac-6-7M    |
| 9    | +      | 6.0 Smac-6-7M    |
| 10   | +      | 6.0 Smac-6-7M    |
| 11   | +      | 6.0 Smac-6-7M    |

FIG. 7B

Left: c-IAP1 ΔBIR1 (Ub)n

Right: Table of experimental conditions.
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