THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 26, Issue of June 25, pp. 26906–26914, 2004
Printed in U.S.A.

Smac/Diablo Antagonizes Ubiquitin Ligase Activity of Inhibitor of Apoptosis Proteins*

Received for publication, December 18, 2003, and in revised form, March 22, 2004
Published, JBC Papers in Press, April 12, 2004, DOI 10.1074/jbc.M313859200

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Inhibitor of apoptosis proteins (IAPs) can block apoptosis through binding to active caspases and antagonizing their function. IAP function can be neutralized by Smac/Diablo, an IAP-binding protein that is released from mitochondria during apoptosis. In addition to their ability to interact with caspases, certain IAPs also display ubiquitin-protein isopeptide ligase activity because of the presence of a RING domain. However, it is not known whether the ubiquitin-protein isopeptide ligase activities of human IAPs contribute to their apoptosis inhibitory activity or whether this IAP property can be modulated through association with Smac/Diablo. Here we demonstrate that the ubiquitin ligase activities of XIAP, and to a lesser extent c-IAP-1 and c-IAP2, are potently repressed through binding to Smac/Diablo. We also show that mutation of the XIAP RING domain rendered this IAP a less effective inhibitor of apoptosis, suggesting that the ubiquitin ligase activity of XIAP contributes to its anti-apoptotic function. These data suggest that Smac/Diablo potentiates apoptosis by simultaneously antagonizing caspase-IAP interactions and repressing IAP ubiquitin ligase activities.

The caspase family of cysteine proteases plays a pivotal role in apoptosis through coordinating the ultrastructural alterations that take place within the cell during this process (1, 2). Caspase activities can be regulated through association with certain members of the inhibitor of apoptosis protein (IAP)[1] family, most notably XIAP, c-IAP1, and c-IAP2 (3–6). Because of their ability to antagonize caspase function, several IAPs can significantly delay the onset of apoptosis. In common with other proteins that possess the RING motif, XIAP and c-IAP1 have been shown to be capable of acting as ubiquitin ligases in reactions that culminate in the transfer of ubiquitin to the IAPs themselves, or molecules that associate with these proteins (7–9). However, although there is good evidence that XIAP and c-IAP1 can function as ubiquitin ligases, it is not clear whether this activity contributes to the ability of these proteins to regulate apoptosis.

Studies in *Drosophila* have shown that DIAP1 plays a critical role in regulating the onset of apoptosis in this organism (10–12). The *Drosophila* IAP-binding proteins, Reaper (Rpr), Hid, and Grim (collectively called the RHG proteins), are important triggers of programmed cell death in the fly (13). Recent evidence suggests that the *Drosophila* RHG proteins promote apoptosis through their ability to interact with and neutralize DIAP1 function (11, 12). Although Rpr, Hid, and Grim appear to achieve this in subtly distinct ways, one way in which they can neutralize DIAP1 function is through promoting the ubiquitin ligase activities of this IAP (14). In this regard, several groups have shown recently that Rpr can provoke the destruction of DIAP1 through stimulation of DIAP1 auto-ubiquitination (15–19). Polyubiquitination of DIAP1 targets this protein to the proteasome, which rapidly degrades polyubiquitinated proteins into short peptides. These observations raise the interesting possibility that mammalian IAP stability may also be regulated through association with functional homologues of the RHG proteins, Smac/Diablo or Omi/Htra2.

Here we have used *in vivo* and *in vitro* ubiquitination assays to explore whether the ubiquitin ligase activity of XIAP is modulated through association with Smac/Diablo. We show that Smac/Diablo can act as a potent repressor of the ubiquitin ligase activities of the mammalian IAPs; XIAP, c-IAP1, and c-IAP2. Smac/Diablo antagonized both XIAP auto-ubiquitination as well as XIAP-dependent ubiquitination of caspase-7. We also report that XIAP mutants lacking RING domain function were significantly less effective at inhibiting apoptosis than wild type XIAP, suggesting that the ubiquitin ligase activity of this IAP contributes to its anti-apoptotic function. Therefore, we propose that Smac/DIABLO lowers the threshold for apoptosis by binding to IAPs and repressing the intrinsic ubiquitin ligase activities of these proteins.

EXPERIMENTAL PROCEDURES

Plasmid Constructs and Antibodies—Constructs encoding XIAP and c-IAP1 in pEBB and pEBG vectors have been described previously (20). pEBB and pEBG-based c-IAP2 constructs were generated by inserting the c-IAP2 coding sequence into the BamHI and NotI sites of the pEBB and pEBG vectors as described for c-IAP1 and XIAP (20). Myc-pcDNA3.XIAPK322/328R was kindly provided by Dr. Guy S. Salvesen (Burnham Institute, La Jolla, CA). The cysteine residues 450 and 453 of the XIAP RING domain were mutated to serine using QuickChange site-directed mutagenesis using the following primers, followed by ligation into pcDNA3.myc: XIAPC450/453SF, GAGGAGAAGCTTTCCAAAATCTCTATGGATAGAAATATTGC; XIAPC450/453SR, GCAATATTTCTATCCATAGAGATTTTGGAAAGCTTCTCCTC.

* This work was supported in part by European Union Grant QLG1-1999-00739 and Science Foundation Ireland Grant PI1/B038. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by a Health Research Board of Ireland fellowship.

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[1] The abbreviations used are: IAP, inhibitor of apoptosis protein; HEK, human embryonic kidney; Rpr, reaper; Ub, ubiquitin; E3, ubiquitin-protein isopeptide ligase; GST, glutathione *S*-transferase; HA, hemagglutinin; EGFP, enhanced green fluorescent protein; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

Smac and Smacδ isoforms were cloned using standard reverse transcriptase-PCR techniques from total RNA extracted from Jurkat cells. The following primers were used to amplify both Smac isoforms: SmacF, ATGGCGGCTCTGAAGAGTTGG; SmacR, TCAATCCTCACGCAGGTAGGC. PCR products were cloned into pcDNA3 (Invitrogen) by using the above primers incorporating specific restriction enzyme sites and the FLAG epitope tag sequence. Plasmid constructs were verified by using standard automated sequencing methods (MWG Biotec). The His_6-ubiquitin plasmid, pCW7, was a kind gift from Dr. Ron Kopito (Stanford University). The Smacβ plasmid pcDNA6.1Smacβ was a kind gift from Dr. Marion MacFarlane (MRC Toxicology Unit, Leicester, UK).

Anti-XIAP, anti-GST, and anti-caspase-7 antibodies were purchased from BD Transduction Laboratories. Anti-polyubiquitin antibody was purchased from Affiniti Research Products; anti-c-IAP2 polyclonal antibody was from R&D Systems; anti-actin mAb was from ICN. The generation and affinity purification of anti-Smac rabbit polyclonal antibody have been described previously (21) and a similar strategy was used to generate c-IAP1 antisera. Briefly, an N-terminal truncated form of c-IAP1 (amino acids 351–618) containing the CARD and RING domains was cloned into pET15b. Recombinant c-IAP1-(351–618) was expressed in *Escherichia coli* (BL-21/DE3/pLysS strain) and purified over Ni^{2+}-nitrilotriacetic acid-agarose, followed by immunization of rabbits with the purified protein.

Cell Death Assays—Cell death assays were carried out by co-transfection of plasmids along with pCMVβ or pEGFP reporter plasmids (50 ng/well in 6-well plates). After exposure to pro-apoptotic stimuli, cells were fixed and then stained for β-galactosidase activity (where the reporter pCMVβ was used), as described previously (22). Alternatively, where pEGFP reporter plasmid was used, cells were fixed and examined under a UV microscope for EGFP-associated fluorescence. Blue or green (transfected) cells were then scored as either apoptotic or viable according to standard morphological criteria. Apoptotic cells were typically retracted from the substratum, shrunk, and exhibited dramatic membrane blebbing along with the formation of numerous apoptotic bodies. A minimum of 300 transfected cells per treatment was evaluated.

Transient Transfections and Immunoprecipitation Assays—HEK293T cells were seeded at a density of 2×10^6 cells/10-cm plate (or 10^5/well of a 6-well tissue culture plate) 24 h prior to transfection. Cells were transfected with plasmids according to the standard calcium phosphate precipitation method, and DNA complexes were typically allowed to remain on cells for 6 h before replacing with fresh medium. Where indicated, the proteasome inhibitor, MG132 (Affiniti Research Products), was included in the medium to stabilize polyubiquitinated proteins. Following transfection, cells were lysed in 800 μl of triple detergent lysis buffer (50 mM Tris-Cl, pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 0.02% sodium azide) containing 100 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 2 μg/ml aprotinin. Following centrifugation, clarified lysates were subjected to immunoprecipitation using 1 μg of the appropriate antibody and 30 μl of agarose-coupled protein A/G (Santa Cruz Biotechnology). For GST-pulldown assays, lysates were incubated with 40 μl of glutathione-Sepharose beads (Amersham Biosciences).

Samples were rotated at 4 °C for 4 h, and complexes were washed three to four times in lysis buffer. Immunoprecipitates were then analyzed by immunoblotting by using appropriate primary and secondary antibodies. Alternatively, for precipitation of His_6-tagged proteins, cells were lysed in triple detergent lysis buffer supplemented with 20 mM imidazole. Lysates were incubated for 4 h at 4 °C with 40 μl of Ni^{2+}-nitrilotriacetic acid resin (Qiagen), followed by washing three times in lysis buffer supplemented with 20 mM imidazole to reduce nonspecific binding. Captured proteins were subsequently eluted from the resin by boiling in SDS-PAGE sample buffer.

For transfection of HeLa cells, an alternative transfection method was employed. Cells were seeded at 5×10^4/well in 6-well tissue culture plates 24 h prior to transfection. FuGENE 6 (Roche Applied Science) was used at a ratio of ~2 μl/μg input plasmid, and FuGENE-DNA complexes were incubated with cells for 6 h at 37 °C. DNA complexes were then removed, replaced with fresh medium, and incubation continued to 24 h.

Preparation of Cell-free Extracts—Cell-free extracts of Jurkat and HEK293 cells were prepared as described previously (23, 24). Briefly, 5×10^8 cells were packed into a 2-ml Dounce homogenizer, and an equal volume of ice-cold cell extract buffer was added (CEB: 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 100 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 2 μg/ml aprotinin). Cells were allowed to swell in CEB for 20–30 min on ice and were then lysed by homogenization with ~20–30 strokes of a B-type pestle. Crude extracts were then centrifuged for 30 min at 15,000 × *g* to remove nuclei, unbroken cells, and other debris. Extracts were stored in aliquots at −70 °C until required.

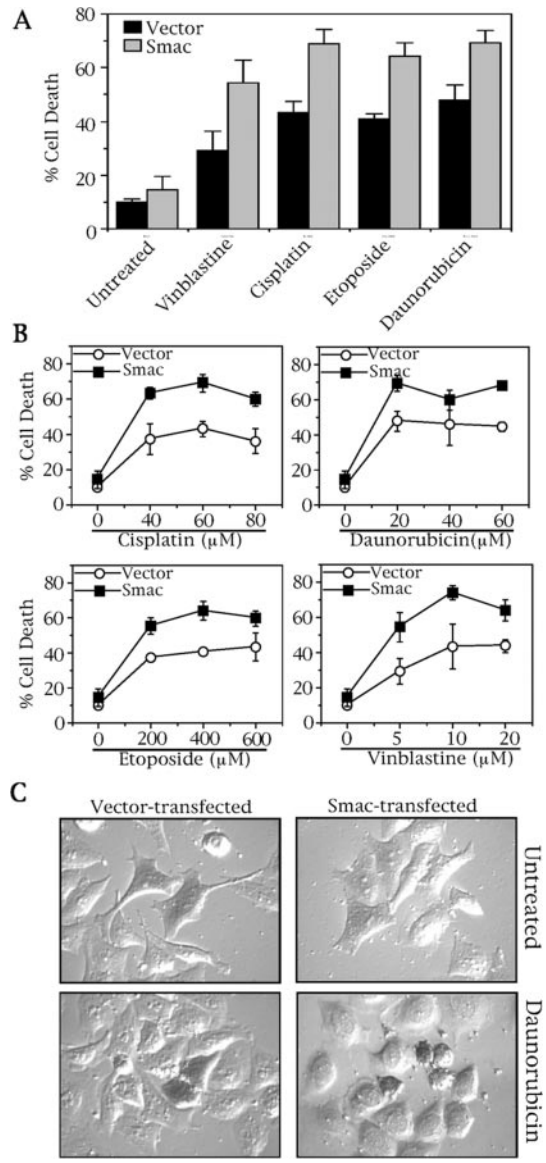

FIG. 1. **Smac potentiates chemotherapeutic drug-induced apoptosis.** HeLa cells were transfected with either empty vector (pcDNA3; 1 μg) or pcDNA3-Smac expression plasmid (1 μg) in combination with a pCMVβ reporter plasmid (50 ng). 24 h post-transfection, cells were treated for a further 6–8 h with a panel of chemotherapeutic drugs. *A*, vector- or Smac-transfected HeLa cells were treated for 8 h with vinblastine (5 μM), cisplatin (60 μM), etoposide (400 μM), or daunorubicin (20 μM) as shown. Treated cells were fixed, stained with X-gal, and scored for features of apoptosis. *Bars* represent mean of triplicate counts ± S.E. *B*, chemotherapeutic drugs were titrated over the indicated concentration ranges, and cells were scored for features of apoptosis as in *A*. *C*, representative fields of X-gal-stained HeLa cells transfected with either empty vector (1 μg) or Smac (1 μg) expression plasmid, followed by incubation in the presence or absence of daunorubicin (20 μM) for 8 h as shown.

In Vitro Ubiquitination Assay Using Cell-free Extracts—*In vitro* ubiquitination assays were typically set up in 100-μl reaction volumes. For 100-μl scale reactions, 25 μl of Jurkat cell-free extract (~30 mg/ml protein) and 25 μl of rabbit reticulocyte lysate (Promega) were supplemented with 10 μg/ml ubiquitin-activating enzyme, 15 μg/ml ubiquitin conjugating enzyme, 250 μg/ml His_6-ubiquitin (Affinity, UK), 10 μM lactacystin, 1 mM ATP and were brought to a final volume of 100 μl in CEB. Caspase activation was initiated by addition of 50 μg/ml bovine heart cytochrome *c* (Sigma, UK) and 1 mM dATP. Samples (10–20 μl) of cell-free reactions were removed at the times indicated in the figure and frozen at −70 °C for subsequent Western blot analysis.

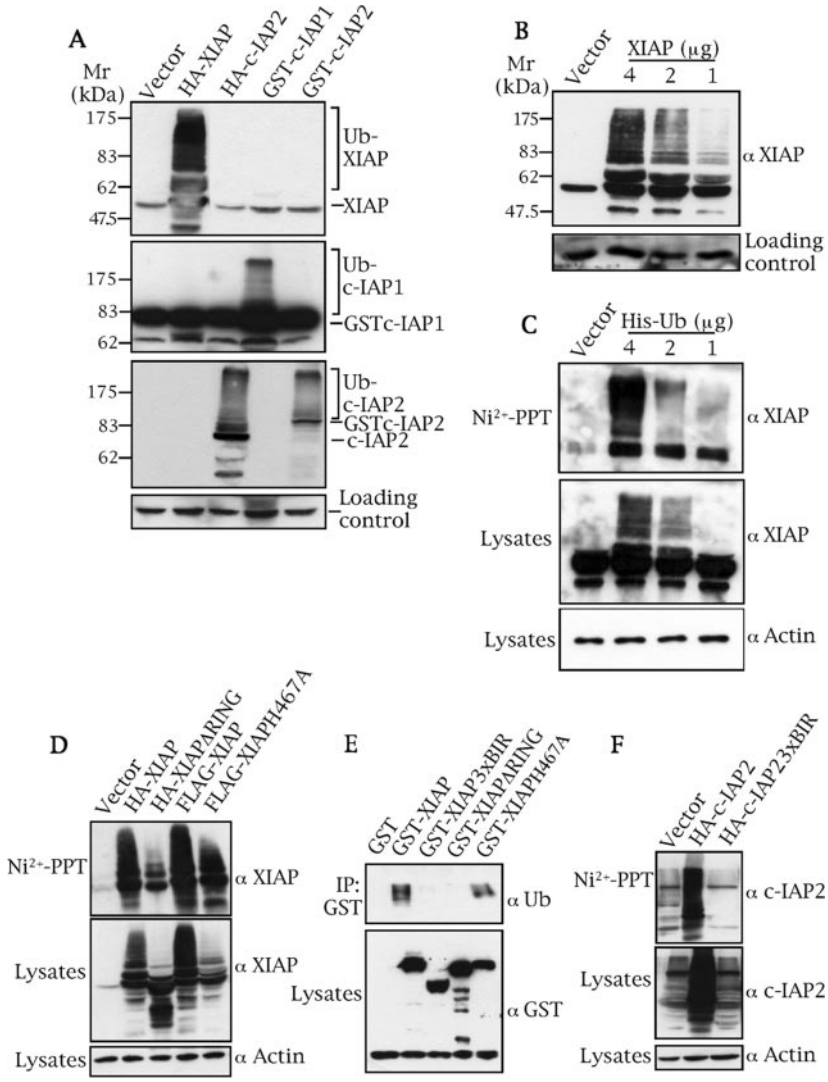

FIG. 2. **XIAP, c-IAP1, and c-IAP2 undergo RING-dependent auto-ubiquitination.** *A*, HEK293T cells were co-transfected with His_6-tagged ubiquitin (2 μg) and the indicated HA- or GST-tagged IAP expression plasmids (4 μg). Cell lysates were prepared 24 h post-transfection, and Western blots were probed for XIAP, c-IAP1, and c-IAP2, as indicated. The loading control is a nonspecific band that resulted from the c-IAP1 probing. *B*, HEK293T cells were transfected with a plasmid encoding His_6-ubiquitin (2 μg) along with the indicated amounts of XIAP expression plasmid or empty vector (4 μg). Cell lysates were prepared 24 h post-transfection, and Western blots were probed for XIAP. Nonspecific bands from the XIAP probing serve as a loading control. *C*, HEK293T cells were transfected with either empty vector (1 μg) or XIAP plasmid (1 μg) together with the indicated amounts of a plasmid encoding His_6-ubiquitin. His_6-ubiquitin-conjugated proteins were subsequently precipitated from cell lysates using nickel immobilized on agarose beads (Ni^{2+}-PPT). Bead-immobilized precipitates were then probed for XIAP (*upper panel*). Cell lysates (5% of input protein) were also probed for XIAP and actin (loading control) as shown. *D*, HEK293T cells were co-transfected with a panel of HA epitope-tagged XIAP constructs (3 μg of each plasmid) along with His_6-ubiquitin (2 μg). Cell lysates were prepared 24 h post-transfection and were subjected to Ni^{2+} affinity precipitation (*Ni^{2+}-PPT*), followed by probing for XIAP. Lysates represent 5% of the input used for the Ni^{2+} affinity precipitations. *E*, HEK293T cells were transfected either with GST vector or the indicated GST-XIAP fusions, along with His_6-ubiquitin expression plasmid (2 μg). Cell lysates were prepared 24 h post-transfection and were incubated with glutathione-conjugated Sepharose beads followed by probing for ubiquitin. Cell lysates (5% of input) were probed for GST, as shown. *IP*, immunoprecipitation. *F*, HEK293T cells were co-transfected with His_6-ubiquitin (3 μg) and HA epitope-tagged vector (4 μg), c-IAP2 (4 μg), or c-IAP2–3xBIR (4 μg). Cell lysates were prepared as in *D*, followed by probing for c-IAP2. Lysates (5% of input protein) were also probed for actin as a loading control.

RESULTS

IAPs Undergo RING-dependent Auto-ubiquitination in Vivo—Smac/Diablo has been reported to interact with several mammalian IAPs and antagonize their caspase-inhibitory functions (25–28). Consistent with this, transient overexpression of Smac/Diablo readily sensitized HeLa cells to several chemotherapeutic drugs over a range of drug concentrations (Fig. 1). However, it remains unclear whether Smac/Diablo functions simply to block interaction between IAPs and caspases or whether this protein can also regulate the Ub ligase activities of the IAPs. To address this question, we used an *in vivo* ubiquitination assay where polyhistidine-tagged ubiquitin (His-Ub) was co-expressed with XIAP, c-IAP1, or c-IAP2 in human embryonic kidney (HEK) 293T cells. Transient overexpression of these RING-containing proteins along with His-Ub resulted in the formation of high molecular weight Ub conjugates of all three IAPs, as demonstrated by a ladder-like pattern of immunoreactive bands upon Western blot analysis (Fig. 2*A*). Titration of either XIAP or His-Ub in this context resulted in a corresponding decrease in XIAP-Ub conjugates, suggesting that XIAP is capable of acting as a Ub ligase toward itself (Fig. 2, *A–C*). We further confirmed this through use of XIAP deletion mutants lacking the RING domain, as well as a XIAP point mutant lacking an important His residue within the RING domain (29, 30) (Fig. 2, *D* and *E*). Both mutants exhibited significantly reduced levels of polyubiquitination

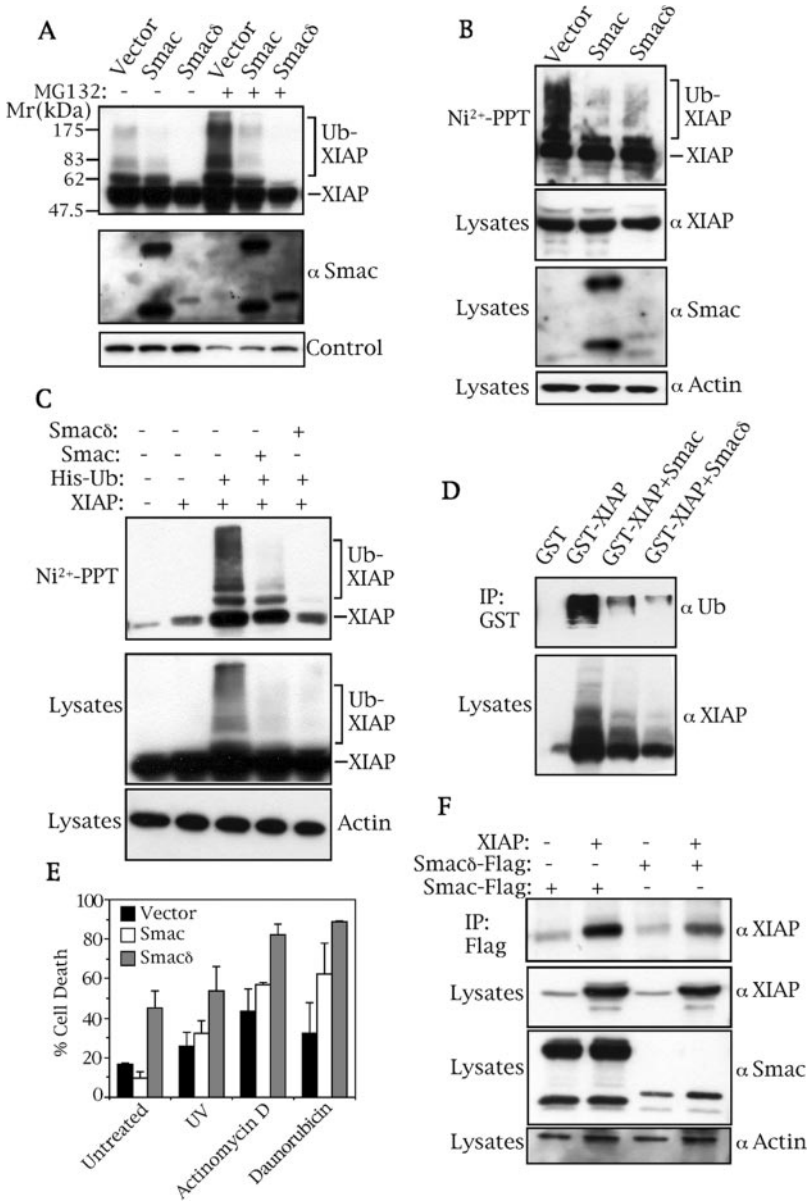

FIG. 3. **Smac inhibits XIAP auto-ubiquitination *in vivo*.** *A*, HEK293T cells were transfected with expression plasmids for His_6-ubiquitin (2 μg) and XIAP (1 μg) in combination with either empty vector (2 μg), Smac (2 μg), or Smacδ (2 μg). Smacδ is a splice variant that lacks the region encoded by exon 4 (amino acids 62–105) but retains the mitochondrial targeting sequence and IAP binding domain (31). Where indicated (+), the proteasome inhibitor, MG132 (1 μM), was included in the medium to stabilize polyubiquitinated proteins. Lysates were prepared 24 h post-transfection and were probed for XIAP, Smac, or actin, as shown. *B*, HeLa cells were transfected with His_6-ubiquitin (3 μg) and XIAP (2 μg) expression plasmids in combination with either empty vector (4 μg), Smac (4 μg), or Smacδ (4 μg). Cell lysates were prepared 24 h post-transfection and were subjected to Ni^{2+} affinity precipitation (*Ni^{2+}-PPT*), followed by probing for XIAP. Lysates were immunoblotted for XIAP, Smac, and actin, as shown. *C*, HEK293T cells, transfected with 2 μg of each of the indicated plasmids, were subjected to Ni^{2+} affinity precipitation, followed by probing for XIAP (*upper panel*). Total lysates (5% of input protein) were also probed for XIAP and actin. *D*, HEK293T cells were transfected with GST or GST-XIAP expression plasmids (3 μg), in combination with plasmids encoding Smac (3 μg) or Smacδ (3 μg). Cell lysates were subjected to glutathione-Sepharose bead immunoprecipitation (*IP*) and were subsequently probed for ubiquitin (*upper panel*). Cell lysates (5% of input protein) were also probed for GST-XIAP expression (*lower panel*). *E*, HeLa cells were transfected with either empty vector (pcDNA3; 0.2 μg), pcDNA3-Smac (0.2 μg), or pcDNA3-Smacδ expression plasmids (0.2 μg) in combination with a pCMVβ reporter plasmid (50 ng). 24 h post-transfection, cells were treated for a further 6–8 h with the indicated pro-apoptotic stimuli (drugs were used at 20 μM final concentration). Treated cells were then fixed, stained with X-gal, and scored for features of apoptosis. *Bars* represent the mean of triplicate counts ± S.E. *F*, Smac and Smacδ interact with XIAP. HEK293T cells were transfected with plasmids encoding XIAP (2 μg) and FLAG epitope-tagged vector (4 μg), Smac (4 μg), or Smacδ (4 μg), as indicated. Cell lysates were prepared 24 h following transfection and immunoprecipitated using anti-FLAG antibodies. The resulting FLAG immunoprecipitates were probed for XIAP. Lysates, representing 5% of the total input protein, were probed for XIAP, Smac, and actin as shown.

when expressed in HEK293T cells (Fig. 2, *D* and *E*). These data demonstrate that XIAP is capable of undergoing auto-ubiquitination and that this activity requires an intact RING domain. Similarly, a c-IAP2 deletion mutant lacking the RING domain also exhibited drastically decreased levels of polyubiquitination when co-expressed with His-Ub in HEK293T cells (Fig. 2*F*).

Smac/Diablo Inhibits XIAP Auto-ubiquitination—To explore whether Smac/Diablo could enhance or repress XIAP auto-ubiquitination, we co-expressed Smac along with XIAP and His-Ub in HEK293T cells (Fig. 3, *A, C* and *D*) or HeLa cells (Fig. 3*B*). However, and in direct contrast to what has been reported in the case of the *Drosophila* RHG proteins, Smac/Diablo potently repressed the formation of XIAP polyubiquitin conjugates, suggesting that this protein can antagonize the E3 ligase activities of XIAP (Fig. 3). We also explored the effects of

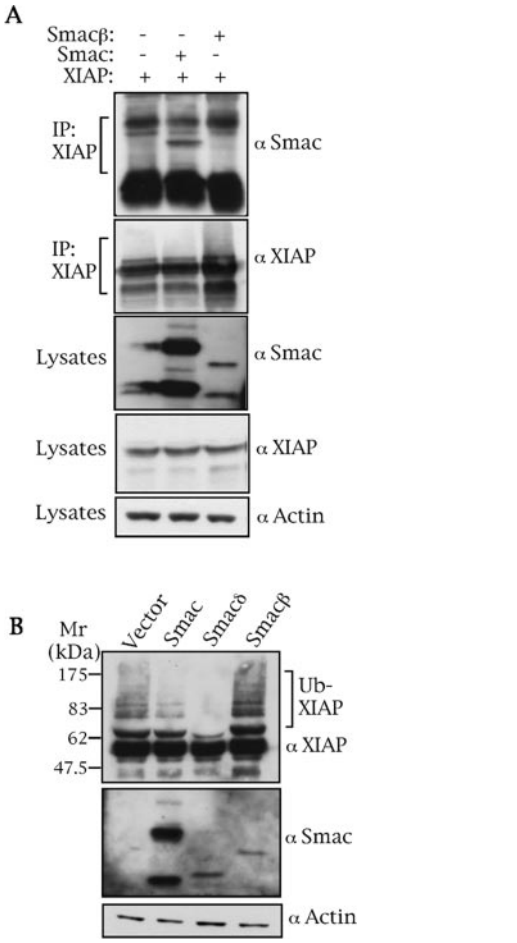

FIG. 4. **Smacβ fails to inhibit XIAP auto-ubiquitination.** *A*, HEK293T cells were co-transfected with XIAP (3 μg) and either vector (6 μg), Smac (6 μg), or Smacβ (6 μg). Smacβ, an alternative splice variant of Smac, lacks the mitochondrial targeting sequence and the tetrapeptide IAP-binding motif AVPI (31). Cell lysates were prepared 24 h post-transfection and were subjected to immunoprecipitation (*IP*) using anti-XIAP antibodies. The resulting immunoprecipitates were probed for Smac and XIAP as shown. Lysates, representing 5% of the total input protein, were probed for Smac, XIAP, and actin as shown. *B*, HEK293T cells were co-transfected with plasmids encoding His_6-ubiquitin (2 μg) and XIAP (1 μg) in the presence or absence of vector (3 μg), Smac (3 μg), Smacδ (3 μg), or Smacβ (3 μg). Cell lysates were prepared 24 h following transfection and immunoblotted for XIAP, Smac, and actin as shown.

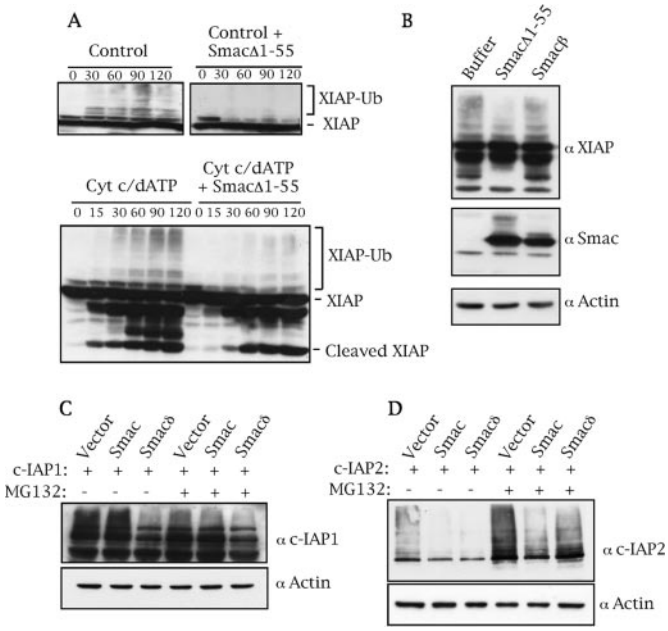

FIG. 5. **Smac-mediated inhibition of XIAP, c-IAP1, and c-IAP2 auto-ubiquitination.** *A*, *in vitro* ubiquitination assay in Jurkat cell-free lysates. Control and cytochrome *c* (*Cyt c*)/dATP-treated cell-free reactions were incubated at 37 °C for the indicated times in the presence or absence of 5 μM recombinant His_6-Smac-(Δ1–55). Time course samples were resolved by SDS-PAGE and immunoblotted for XIAP. *B*, *in vitro* ubiquitination assay in Jurkat cell-free lysates. Cytochrome *c*/dATP-treated cell-free reactions were incubated at 37 °C for 90 min in the presence of either buffer, 5 μM recombinant His_6-Smac-(Δ1–55), or 5 μM recombinant His_6-Smacβ, as indicated. Samples were resolved by SDS-PAGE and immunoblotted for XIAP, Smac, and actin as shown. *C*, HEK293T cells were transfected with plasmids encoding His_6-ubiquitin (2 μg) and c-IAP1 (3 μg) in the presence or absence of Smac (4 μg) or Smacδ (4 μg) expression plasmids as shown. Where indicated (+), the proteasome inhibitor, MG132 (1 μM), was included to stabilize ubiquitin-conjugated proteins. Cell lysates were prepared 16 h post-transfection and were immunoblotted for c-IAP1 and actin as indicated. *D*, HEK293T cells were transfected with plasmids encoding His_6-ubiquitin (2 μg) and c-IAP2 (3 μg), in the presence or absence of Smac (4 μg) or Smacδ (4 μg) expression plasmids. Samples were treated as in *C* and immunoblotted for c-IAP2 and actin as indicated.

a Smac/Diablo splice variant, Smacδ (31, 32),[2] in the same assays (Fig. 3, *A–D*). Smacδ is similar in structure to the wild type Smac isoform except that it lacks amino acids 62–105 that follow the IAP-binding motif (AVPI). Smacδ retains the ability to bind XIAP (Fig. 3*F*) and was even more effective than full-length Smac/Diablo in terms of its ability to block the formation of XIAP-Ub conjugates (Fig. 3, *A–D*). Moreover, Smacδ reproducibly sensitized HeLa cells to a range of pro-apoptotic drugs more effectively than full-length Smac (Fig. 3*E*). This suggests that the ability of Smac to lower the threshold for entry into apoptosis may be correlated with its ability to repress the Ub ligase activity of one or more IAPs.

Significantly, both Smac and Smacδ were found to interact with XIAP under the conditions employed in our co-expression assays, providing a direct means to enable Smac to modulate the ubiquitin ligase activities of XIAP (Fig. 3*F*). However, to confirm that interaction between XIAP and Smac was required for the observed effects on XIAP auto-ubiquitination, we employed the Smacβ isoform that does not interact with XIAP due to the absence of the IAP-binding motif in this splice variant (31). We confirmed that Smacβ failed to interact with XIAP (Fig. 4*A*), as reported previously (31). We also explored whether Smacβ could antagonize XIAP auto-ubiquitination. As Fig. 4*B* illustrates, in sharp contrast to the effects seen with either the wild type Smac or Smacδ isoforms, Smacβ failed to modulate XIAP auto-ubiquitination.

The inhibitory effects of Smac/Diablo on XIAP auto-ubiquitination were also observed by using an *in vitro* ubiquitination assay based upon Jurkat cell-free extracts. As shown in Fig. 5*A*, spontaneous auto-ubiquitination of XIAP was inhibited by the addition of recombinant His-Smac-(Δ1–55) to the extracts. We also used this system to explore whether the rate of XIAP auto-ubiquitination was altered by triggering caspase activation in the extracts. Caspase activation was initiated by addition of cytochrome *c*/dATP (which promotes assembly of the Apaf-1/Caspase-9 apoptosome) to the extracts. It has been reported previously (33) that XIAP undergoes caspase-dependent proteolysis during apoptosis, and this was readily observed in the cell-free system (Fig. 5*A*). XIAP polyubiquitination was also observed under these conditions, and this was once again inhibited through addition of recombinant His-Smac-(Δ1–55) to the extracts (Fig. 5*A*). Addition of Smac also blocked the appearance of one of the XIAP proteolytic fragments, presumably because Smac binding masked this particular caspase cleavage

[2] E. M. Creagh and S. J. Martin, unpublished data.

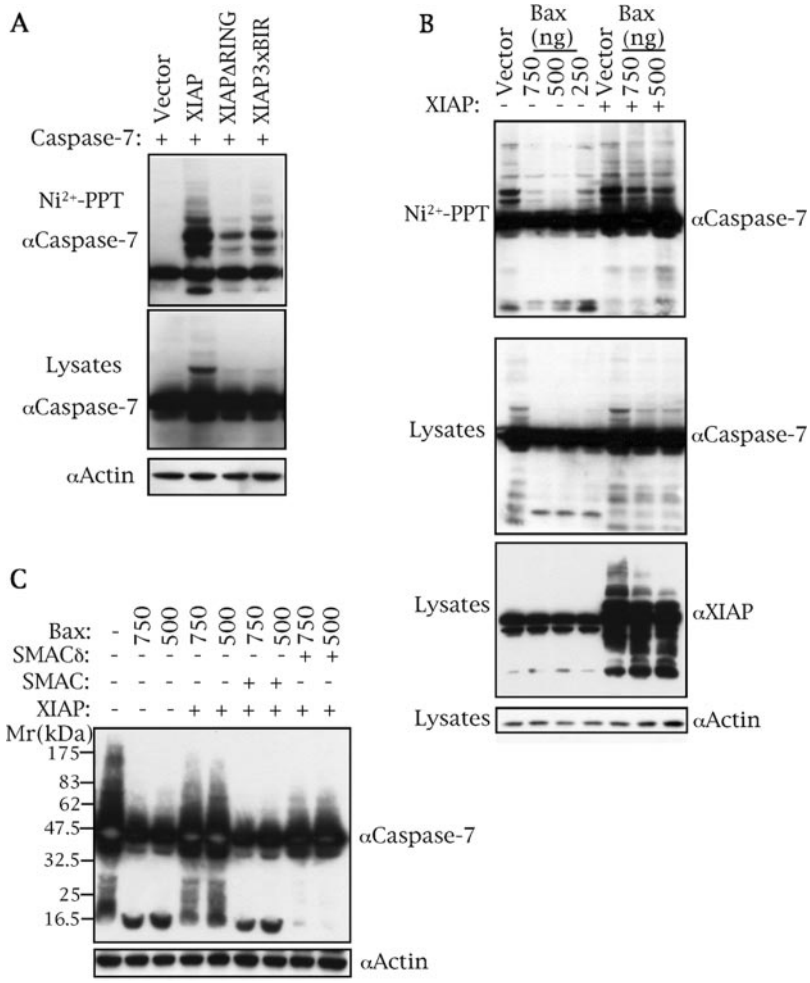

FIG. 6. **Smac/Diablo inhibits XIAP-dependent ubiquitination of caspase-7.** *A*, HEK293T cells were transfected with pcDNA3-caspase-7 (4 μg) and His_6-ubiquitin (3 μg) together with a panel of XIAP constructs (2 μg) as indicated. Lysates were prepared 48 h post-transfection and subjected to Ni^{2+} affinity precipitation to pull down His_6-ubiquitin-tagged proteins. Bead-immobilized precipitates were immunoblotted for caspase-7. Lysates (5% of input protein) were probed for caspase-7 and actin as shown. *B*, HEK293T cells were transfected with plasmids encoding caspase-7 (3 μg) and His_6-ubiquitin (2 μg) in the presence or absence of XIAP (3 μg) and the indicated amounts of Bax plasmid. Cell lysates were prepared 18 h post-transfection and were subjected to Ni^{2+} affinity precipitation. Precipitates were then separated by SDS-PAGE followed by immunoblotting for caspase-7 (*upper panel*). Cell lysates (5% of input protein) were also probed for caspase-7, XIAP, and actin as shown. *C*, Smac inhibits XIAP-mediated ubiquitination of caspase-7. HEK293T cells were transfected with the indicated plasmids (amounts were as in *B*) in the presence or absence of Smac (2 μg) or Smacδ (2 μg) as shown. Cell lysates were prepared 24 h post-transfection and were immunoblotted for caspase-7 and actin as shown.

site in XIAP (Fig. 5*A*). Significantly, the non-IAP binding Smacβ isoform failed to inhibit XIAP auto-ubiquitination in the same context (Fig. 5*B*).

Smac/Diablo Is a General Inhibitor of IAP Ub Ligase Activity—Notably, we also found Smac/Diablo to have inhibitory effects on the ubiquitin ligase activity of the other RING-containing IAPs, c-IAP1 and c-IAP2, *in vivo* (Fig. 5, *C* and *D*). However, the effects of Smac and Smacδ on polyubquitination of c-IAP1 were relatively modest and may reflect a lower affinity of Smac/Diablo for this IAP relative to XIAP. Thus, Smac appears capable of acting as an antagonist of the E3 ligase activities of several IAPs. An alternative interpretation of our observations is that rather than acting as an inhibitor of IAP Ub ligase activities, Smac/Diablo may instead divert these activities away from the IAP and toward IAP-binding proteins, such as Smac/Diablo itself (34). Thus, we also explored whether Smac/Diablo could be ubiquitinated by XIAP, but we found no evidence for this either in the *in vivo* HEK293T cell assay (Fig. 3, *A* and *B*) or in the Jurkat cell-free ubiquitination assay (data not shown). We then asked whether XIAP could act as a Ub ligase toward any of its caspase binding partners.

To address this question, we co-expressed caspase-7 and His-Ub in the presence or absence of XIAP, and we assessed the appearance of high molecular weight forms of caspase-7 under these conditions. Caspase-7 appeared to be weakly polyubiquitinated *in vivo* in an XIAP-dependent manner (Fig. 6*A*). Moreover, this was attenuated when caspase-7 was co-expressed with XIAP mutants lacking the RING domain (Fig. 6*A*). To determine whether Smac/Diablo could enhance or suppress XIAP-mediated poly-ubiquitination of caspase-7, we used two approaches. First, we overexpressed Bax to promote release of cytochrome *c* and Smac/Diablo in cells transfected with caspase-7 in the presence or absence of XIAP. We then assessed the formation of caspase-7 conjugates under these conditions. As Fig. 6*B* illustrates, overexpression of XIAP in this system resulted in an inhibition of caspase-7 activation and enhancement of caspase-7 ubiquitination. Furthermore, we observed a marked decrease in the abundance of caspase-7 conjugates upon Bax overexpression. These observations are consistent with the interpretation that Smac/Diablo also inhibits XIAP-mediated polyubiquitination of caspase-7. In the second approach, we co-transfected caspase-7 and XIAP in the presence or absence of Smac or Smacδ (Fig. 6*C*). Once again, Bax overexpression was used to promote Smac release from mitochondria. We again observed that XIAP-dependent polyubiquitination of caspase-7 was inhibited by overexpression of Smac or Smacδ. Taken together, these observations suggest that Smac/Diablo acts as a general antagonist of the Ub ligase activities of XIAP toward itself or its binding partners.

The XIAP RING Domain Plays a Role in Apoptosis Suppression—The effect of Smac/Diablo on the Ub ligase activities of the IAPs was intriguing and suggested that this IAP function might make an important contribution to the apoptosis inhibitory properties of these proteins. Because the RING domain of XIAP is essential for its E3 ligase activity (Fig. 2, *D* and *E*), we compared XIAP and XIAPΔRING in terms of their ability to block daunorubicin- or etoposide-induced apoptosis in HeLa cells. These experiments revealed that full-length XIAP was a

more potent inhibitor of apoptosis than the XIAPΔRING deletion mutant (Fig. 7). The enhanced protection seen with full-length XIAP does not appear to be due to more efficient expression of this construct as compared with the XIAPΔRING mutant, as both were expressed with equal efficiency in HeLa cells (Fig. 7*B*). It is important to note that the XIAPΔRING mutant was clearly able to block apoptosis when overexpressed at high levels but that its effects titrated more readily than full-length XIAP.

Because deletion of the RING domain of XIAP could have additional effects beyond disruption of the Ub ligase activities of this protein, we also explored the apoptosis inhibitory activity of two additional XIAP mutants (Fig. 8*A*). Thus, we used a XIAP mutant (XIAPK322R/K328R) that has been reported to be defective in auto-ubiquitination because of mutation of two lysine residues within BIR3 that are sites of ubiquitin attachment (35). Significantly, although incapable of auto-ubiquitination, this mutant is predicted to retain Ub ligase activity toward other proteins because of its intact RING domain. We also generated an additional XIAP mutant where two of the conserved cysteine residues within the RING consensus motif were mutated to serine (XIAPC450S/C453S; Fig. 8*A*). As expected, both mutants displayed a marked reduction in auto-ubiquitination when co-transfected with His-Ub in HEK293T cells (Fig. 8*B*). These XIAP mutants were expressed with similar efficiency to wild type XIAP (Fig. 8*C*), enabling their apoptosis inhibitory properties to be directly compared. Consistent with our earlier observations using the XIAPΔRING deletion mutant, the XIAPC/S RING mutant also inhibited apoptosis less effectively than wild type XIAP (Fig. 8, *D* and *E*). In contrast, the auto-ubiquitination defective mutant, XIAPK/R, inhibited apoptosis as effectively as wild type XIAP (Fig. 8*E*). These data suggest that the Ub ligase activity of XIAP toward other proteins (such as caspases), but not toward itself, contributes to its apoptosis inhibitory properties.

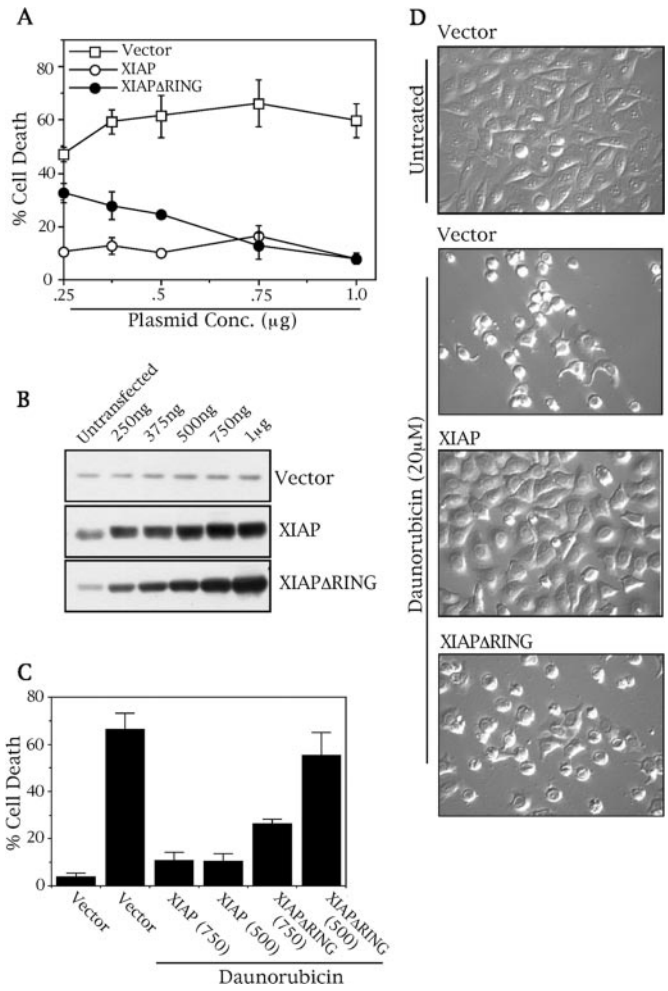

FIG. 7. **The XIAP RING domain contributes to apoptosis suppression.** *A*, HeLa cells were transfected with the indicated amounts of empty vector, XIAP, or XIAPΔRING constructs along with pEGFP reporter plasmid (50 ng). 24 h post-transfection, cells were treated with 400 μM etoposide for 8 h, and EGFP-positive cells were then scored for features of apoptosis. *B*, HeLa cells were transfected with vector, XIAP, or XIAPΔRING plasmids as shown, followed by probing for XIAP. *C*, HeLa cells were transiently transfected with vector (750 ng) or the indicated amounts of XIAP or XIAPΔRING plasmids, along with EGFP reporter plasmid (100 ng). 24 h post-transfection, cells were treated with daunorubicin (20 μM) for 8 h followed by morphological assessment of apoptosis on EGFP-positive cells. *Bars* represent mean ± S.E. *D*, HeLa cells transfected with 500 ng of empty vector, XIAP, or XIAPΔRING plasmids (for 24 h) were then treated with daunorubicin (20 μM) for 8 h. Representative images of cell populations were taken under phase contrast microscopy.

DISCUSSION

Here we have shown that Smac/Diablo can antagonize the Ub ligase activities of several IAPs, suggesting that this property may contribute to the ability of Smac/Diablo to potentiate apoptosis. In line with this, we have also shown that Ub ligase-defective XIAP mutants were less effective inhibitors of apoptosis than their wild type counterparts. The latter observation suggests that IAPs may neutralize caspase function through binding to these proteases and targeting them for ubiquitination. Taken together, these data argue that Smac/Diablo lowers the threshold for apoptosis by simultaneously neutralizing the caspase binding and Ub ligase properties of the IAPs.

The significance of the RING domain for IAP function has been controversial. Some reports have implicated the RING domain as a negative regulator of IAP function, whereas others have reported it to have a role in the anti-apoptotic function of IAPs (7, 36–40). One reason for the disagreement in the literature may be due to the fact that the differences between full-length IAPs and their RING mutants may be difficult to observe at high levels of overexpression. Recently, several reports (15–19, 40) have emerged to suggest that IAP Ub ligase activity plays an important role in regulating the onset of apoptosis in *Drosophila*. In the fly context, the RING domain of DIAP1 was found to be required for efficient suppression of apoptosis possibly through ubiquitination of the caspase-9 orthologue, DRONC (40). However, upon binding of Rpr to DIAP1, the latter was found to undergo auto-ubiquitination and destruction (15–19). Loss of DIAP1 results in the spontaneous assembly of complexes between the Apaf-1 orthologue, DARK, and DRONC, leading to cell death (reviewed in Ref. 14). Thus, Rpr appears to divert the Ub ligase activities of DIAP1 away from DRONC and toward itself, thereby facilitating DRONC activation. DIAP1 appears to play a critical role in regulating the onset of cell death in the fly, as loss of this IAP is sufficient to allow apoptosis to proceed (11, 41). In contrast, targeted disruption of XIAP appears to have few, if any, deleterious effects in the mouse (42). The latter result may be because of functional redundancy between the mammalian IAPs. Alternatively, IAPs may play a less critical role in mammals than in the fly. However, mammalian IAPs do appear to play an important role in setting a threshold for apoptosis because overexpression of Smac/Diablo does indeed lower this threshold (Ref. 43) (Fig. 1).

As outlined above, interaction of *Drosophila* Rpr with DIAP1 is thought to promote apoptosis through triggering auto-ubiquitination of DIAP1. However, in direct contrast to this, we found that Smac/Diablo inhibits rather than promotes auto-

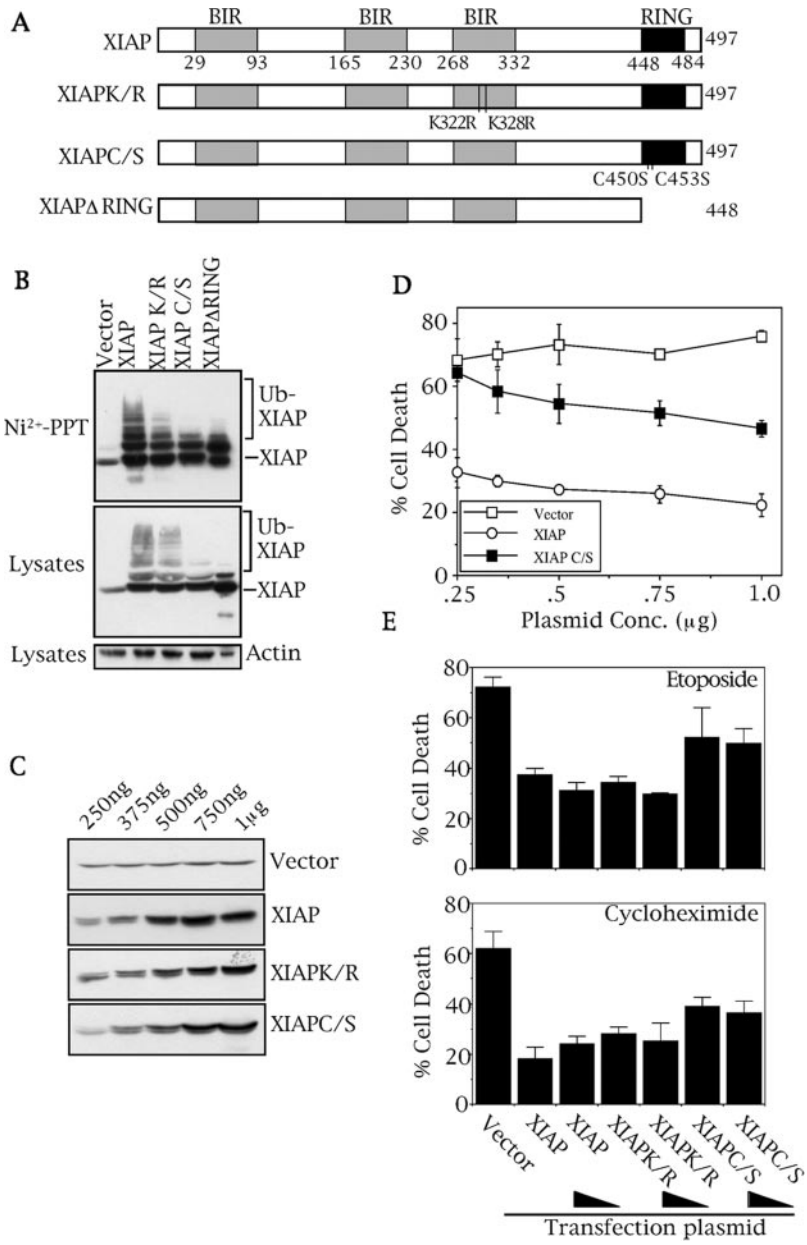

FIG. 8. **The ubiquitin ligase activity of XIAP contributes to apoptosis suppression.** *A*, schematic representation of the following XIAP constructs used: wild type XIAP, XIAP K322R/K328R point mutant, XIAP C450S/C453S point mutant, and XIAPΔRING deletion mutant. *B*, HEK293T cells were transfected with the indicated XIAP plasmids (2 μg) along with a plasmid encoding His_6-ubiquitin (3 μg). Cell lysates were prepared 18 h post-transfection and were subjected to Ni^{2+} affinity precipitation. Precipitates were then separated by SDS-PAGE followed by immunoblotting for XIAP (*upper panel*). Cell lysates (5% of input protein) were also probed for XIAP and actin as shown. *C*, HEK293T cells were transfected with the indicated amounts of either empty vector, XIAP, XIAPK/R, or XIAPC/S plasmids as shown, followed by probing for XIAP. *D*, HeLa cells were transfected with the indicated amounts of empty vector, XIAP, or XIAP C450S/C453S mutant (*XIAPC/S*) along with pEGFP reporter plasmid (50 ng). 24 h post-transfection, cells were treated with 10 μM actinomycin D for 8 h. EGFP-positive cells were then scored for features of apoptosis. *E*, HeLa cells were transiently transfected with empty vector (625 ng) or the indicated amounts (625 or 375 ng) of XIAP, XIAP K322R/K328R, or XIAP C450S/C453S plasmids, along with a pEGFP reporter plasmid (100 ng). 24 h post-transfection, cells were treated with etoposide (400 μM) or cycloheximide (25 μM) for 8 h followed by morphological assessment of apoptosis on EGFP-positive cells. *Bars* represent mean ± S.E.

ubiquitination of mammalian IAPs. This discrepancy is puzzling, but it is clear that Rpr and Smac/Diablo are not orthologues; thus it is not surprising that they exhibit differences in their activities. In support of our observations, a recent study by Vaux and co-workers (44) has also shown that Smac/Diablo can antagonize XIAP Ub ligase activity. Our observation that Smac/Diablo inhibits the Ub ligase activities of the mammalian IAPs suggests that IAP-mediated modification of caspases may contribute to their ability to act as caspase inhibitors. Thus, IAP-bound caspases may still pose a threat to the viability of the cell if they subsequently become displaced from the IAP. However, if caspases become modified (via mono- or polyubiquitination) through even transient IAP interaction, this may render the caspase permanently inactive. This scenario would provide an explanation for the decrease in apoptosis inhibitory activity seen with XIAP upon deletion or mutation of the RING domain.

It remains unclear how Smac/Diablo suppresses the E3 ligase activities of XIAP. The region within XIAP that has been implicated as the site of attachment of polyubiquitin chains has been mapped recently to residues (Lys-322 and Lys-328) within the BIR3 domain (35). Because Smac can interact with XIAP via the same BIR domain, it is possible that Smac antagonizes auto-ubiquitination by masking these lysine residues on XIAP. However, it remains to be determined how Smac antagonizes the E3 ligase activity of XIAP toward other substrates (such as caspases), but this may be simply be due to the displacement of the caspase from this IAP. Alternatively, once Smac is released from mitochondria, it may complex with XIAP and its substrate to form a ternary complex. A complex of this nature has been demonstrated recently between the linker-BIR2 region of XIAP, caspase-7, and Smac (45). Moreover, under these conditions Smac binds XIAP with a lower affinity than caspase-7, failing to displace it. The ability of Smac to inhibit the ubiquitin ligase activity of XIAP may be important in this scenario, rescuing the caspase from a fate of degradation.

In conclusion, we provide evidence to suggest that Smac/Diablo may use a dual mechanism to inhibit IAP-mediated protection

from active caspases. By disrupting IAP-caspase interactions and repressing the ubiquitin ligase activities of IAPs, Smac/Diablo may rescue caspases from the fate of ubiquitination.

Acknowledgments—We thank Drs. Doug Green, Ron Kopito, Marion MacFarlane, Guy Salvesen, and Xiaodong Wang for generous provision of plasmids. We also thank Dr. Stephanie Birkey Reffey for generating some of the constructs used in this study.

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