Inhibitors of apoptosis (IAPs) are crucial regulators of programmed cell death. The mechanism by which IAPs prevent apoptosis has previously been attributed to the direct inhibition of caspases. The function of mammalian IAPs is counteracted by cell death inducer second mitochondria-derived activator of caspases (Smac)/DIABLO during apoptosis. Here we show that cIAP1 and cIAP2 are E3 ubiquitin-protein isopeptide ligases (ubiquitin ligases) for Smac. cIAPs stimulate Smac ubiquitination both in vivo and in vitro, leading to Smac degradation. cIAP1 and cIAP2 associate with overlapping but distinct subsets of E2 (ubiquitin carrier protein) ubiquitin-conjugating enzymes. The substrate-dependent E3 activity of cIAPs is mediated by their RING domains and is dependent on the specific interactions between cIAPs and Smac. Similarly, Drosophila IAP1 also possesses ubiquitin ligase activity that mediates the degradation of the Drosophila apoptosis inducers Grim and HID. These results suggest a novel and conserved mechanism by which IAPs block apoptosis through the degradation of death inducers.

Apoptosis is an evolutionarily conserved process of cell autodestruction that plays a critical role in development, the maintenance of tissue homeostasis, and the protection against diseases (1, 2). The central executioner of apoptosis is a family of aspartic acid-specific cysteine proteases known as caspas (3, 4). A major mammalian apoptosis pathway leading to caspase activation is the intrinsic/mitochondrial pathway, which is induced by diverse intracellular death stimuli such as developmental cues, cellular stresses, severe DNA damages, and oncogenic transformation (5, 6). These stimuli lead to the release of several cell death inducers from the mitochondria, including cytochrome c and Smac (also known as DIABLO). Cytochrome c promotes the formation of a cytosolic protein complex that activates an initiator caspase, caspase-9 (7). Smac, on the other hand, promotes caspase-9 activation by neutralizing the inhibitory effect on caspases of the X-linked inhibitor of apoptosis (XIAP) (8, 9).

The IAPs comprise a group of conserved proteins crucial for the regulation of apoptosis (reviewed in Ref. 10). Initially identified as baculoviral proteins that can functionally substitute the baculovirus-encoded caspase inhibitor p35, the IAP family now includes several mammalian cellular proteins (e.g. XIAP, MIHA/h-ILP, cIAP1/HAIP2/hMIHB, cIAP2/HIAP-1/hMIHC, NAIP, ML-IAP, and survivin) and two Drosophila proteins (DIAP1 and DIAP2/dILP). Structurally, all IAPs contain one to three baculovirus IAP repeat (BIRs). IAPs can directly inhibit caspases through their BIR domains and intervening linker regions. For example, XIAP has been shown to inhibit caspases-3, -7, and -9 through the BIR region (11, 12), whereas the corresponding region of DIAP1 inhibits Drosophila caspases, including DRICE and DCP-1 (13, 14). Smac counteracts the inhibitory effect of XIAP by masking the caspase-9-binding site on XIAP (8, 9, 15, 16). The XIAP binding motif of Smac was subsequently found to be conserved in three Drosophila death inducers, HID, Grim, and Reaper (17), that are required for Drosophila cell death (reviewed in Ref. 18). These three proteins induce apoptosis through the inhibition of DIAP1 (13, 19), likely via a mechanism similar to that of Smac (17). In addition to XIAP, Smac also interacts with cIAP1 and cIAP2 (8, 9). Unlike XIAP, the inhibitory effect of the cIAPs on caspases is weak (100–1,000-fold less than that of XIAP) (20). The mechanism by which cIAP1 and cIAP2 regulate apoptosis remains less understood.

Many IAPs, including XIAP, cIAP1, cIAP2, and DIAP1, also contain a COOH-terminal RING domain. The RING domains on XIAP and cIAP1, like those found in many other proteins (21), have been shown to possess E3 ubiquitin ligase activity (22, 23). Protein polyubiquitination, which typically targets proteins for degradation in the 26 S proteasome, involves the sequential action of three enzymes: the ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (Ubc or E2), and a ubiquitin ligase (E3) (24, 25). The substrate specificity is mainly determined by E3, which binds to both E2 and the substrate and facilitates the assembly of a multiquitin chain on the substrate. The E3 activity of XIAP and cIAP1 can mediate their self-degradation during the apoptosis of thymocytes in response to certain apoptosis stimuli. cIAP1 also regulates the level of TRAF2, an intracellular signaling molecule for the tumor necrosis factor receptor 2. The degradation of TRAF2 by cIAP1 appears to sensitize cells to TNF-mediated apoptosis (26). On the other hand, the RING domain of IAPs has been shown to inhibit apoptosis in a cell type- and/or death stimulus-dependent manner (27, 28). The cIAP1 RING domain can mediate the monoubiquitination of caspases-3 and -7 (23), and the XIAP RING domain promotes caspase-3 degradation through polyubiquitination (29). However, it remains unclear whether the E3 activity of IAPs targets proapoptotic proteins other than caspases.

Here we present evidence that cIAP1 and cIAP2 are E3 ubiquitin ligases for Smac and promote the degradation of this
cIAP-mediated Ubiquitination of Smac/DIABLO

EXPERIMENTAL PROCEDURES

Cell Culture, Antibodies, and Recombinant Proteins—Human embryonic kidney 293T and cervix carcinoma HeLa cells were cultured in complete Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. Rabbit polyclonal antibodies against FLAG and hemagglutinin (HA) tags (Santa Cruz Biotechnology), anti-FLAG monoclonal antibody M2 and M2 conjugated on agarose beads (Sigma), and yeast ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) GST-UbcH5c, and His6-ubiquitin (Boston Biochem) were purchased from the indicated sources.

Expression Plasmids—All mammalian expression constructs were made in pR5K. The cDNAs for cIAP1, cIAP2, XIAP, caspase-9, and all E2s were of human origin. The cDNA fragments for the full-length E2s made in pRK5. The cDNAs for cIAP1, cIAP2, XIAP, caspase-9, and all indicated sources.

Transfection, Immunoprecipitation, and Western Blotting Analysis—Transient transfection was performed using the calcium phosphate precipitation method. Unless indicated otherwise, cells were transfected with 0.1 μg of the indicated plasmids to minimize the difference in the expression levels of various proteins. Sixteen to 22 h after transfection, cell lysates were prepared in 1% Nonidet P-40 lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 2 mM dithiothreitol, 1% Nonidet P-40, 1 mM EDTA) and immunoprecipitated with anti-FLAG monoclonal antibody M2 beads. The immunoprecipitates and cell lysates were resolved by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies.

In Vitro Ubiquitination Assay—Mature Smac protein was obtained by the trimer coupled transcription/translation system (Promega) according to the manufacturer’s instructions. FLAG-tagged cIAP1, XIAP, and cIAP2mut (which lacked the first BIR domain) were expressed in 293T cells and immunoprecipitated from the cell lysates with the anti-FLAG M2 monoclonal antibody conjugated on agarose beads. For the experiments shown in Fig. 5, A and B, the immunoprecipitates were washed twice with washing buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.01% Nonidet P-40, 10% glycerol, 1 mM EDTA) and then added to a ubiquitination reaction mixture containing 120 ng of yeast E1, 2.4 μg of UbcH5c, 4 μg of His6-ubiquitin, and 3 μl of radiolabeled mature Smac in 50 μl total volume. After incubation at 30 °C for 90 min with vigorous shaking, the reaction was terminated by boiling in the Laemmli SDS-loading buffer. Four microliters of the reaction mixture was resolved by SDS-PAGE followed by autoradiography. For the experiment in Fig. 5C, in vitro translated mature Smac was first incubated with the FLAG immunoprecipitating beads at 30 °C for 90 min to allow Smac to bind to the bead-bound E3s. After the ubiquitination reaction, the beads were washed three times with 1% Nonidet P-40 lysis buffer, resolved on SDS-PAGE, and analyzed by autoradiography.

Apoptosis Assay—HeLa cells were transfected with 0.5 μg of Grim-HA together with 0.1 μg of a β-galactosidase reporter plasmid and 1 μg of DIAP1, DIAP mutant, or control vector. Twenty h after transfection, cells were fixed in 0.5% glutaraldehyde and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The percentage of apoptotic cells was determined by the number of membrane blebbed cells divided by the total number of blue cells, as previously described (30).

RESULTS

Expression of cIAP1 and cIAP2 Decreased the Steady-state Level of Smac—The cell death inducer Smac is synthesized as a precursor, which becomes the mature form with the cleavage of its NH2-terminal mitochondrial targeting sequence upon entering the mitochondria (8). During apoptosis, Smac is released into the cytosol, where it becomes associated with IAPs. To examine whether IAPs can target Smac for degradation, we co-expressed in 293T cells the mature Smac protein together with cIAP1, cIAP2, or XIAP. Interestingly, the amount of Smac protein decreased significantly upon co-expression with either cIAP1 or cIAP2 but not with XIAP (Fig. 1A). The down-regulation of the mature Smac protein was unlikely due to the inhibition of apoptosis because XIAP effectively blocked apoptosis (data not shown). In addition, because the expression levels of XIAP were much higher than those of cIAP1 and cIAP2 (Fig. 1A), the decrease in Smac expression was unlikely caused by the squelching effect of the simultaneous overexpression of multiple proteins. To rule out the possibility that overexpression of cIAPs led to the generalized inhibition of protein expression, we examined the effect of cIAPs on endogenous Smac and found that its expression level was not changed by cIAP overexpression (Fig. 1B).

Ubiquitination of Smac by cIAP1 and cIAP2—The cIAP-induced destabilization of Smac and the low expression levels of cIAPs suggest that cIAP1 and cIAP2 might target Smac as well as themselves for degradation via ubiquitination. To compare the self-ubiquitination of the cIAPs with XIAP, we expressed separately FLAG-tagged cIAP1, cIAP2, and XIAP in 293T cells together with HA-tagged ubiquitin. Both cIAP1 and cIAP2 were modified with multiple ubiquitins, as indicated by the appearance of higher molecular weight products (Fig. 2A, top panel). In contrast, the self-ubiquitination of XIAP was substantially weaker, consistent with its relatively high expression levels (Fig. 2A).

To determine whether cIAPs and XIAP can ubiquitinate mature Smac, we expressed cIAPs and XIAP together with mature Smac in 293T cells. The mature Smac protein that bound to cIAP1 and cIAP2 was markedly ubiquitinated, showing a ladder pattern characteristic of mono- and polyubiquitination (Fig. 2B, top panels). The cIAP1-mediated Smac ubiq-
cIAP-mediated Ubiquitination of Smac/DIABLO

The ubiquitination was so strong that the majority of mature Smac proteins were modified even without the addition of exogenous ubiquitin. The cIAP self-ubiquitination and cIAP-mediated Smac ubiquitination were further confirmed by immunoblotting analysis using an antibody against ubiquitin (Fig. 2C). XIAP, however, did not induce significant ubiquitination of mature Smac (Fig. 2B).

The RING domains of cIAPs are required for their self-ubiquitination (22). To test whether these domains are also needed for the cIAP-mediated ubiquitination of Smac, we deleted the RING domains of cIAP1 and cIAP2. As shown in Fig. 2D, these RING deletion mutants failed to ubiquitinate mature Smac. Thus, ubiquitination of Smac by IAPs is RING domain-dependent.

To examine whether other cellular factors are involved in the cIAP-induced Smac ubiquitination, we expressed cIAPs and mature Smac-HA in HeLa cells. As a positive control, we also expressed them in 293T cells. We found that the binding efficiency of mature Smac to cIAPs in these two different cells was comparable. However, ubiquitination of mature Smac was observed in HeLa cells whereas the Smac ubiquitination was readily detected in 293T cells (Fig. 2E, top two panels). This result supports the notion that the cIAP-dependent ubiquitination of mature Smac is regulated by other cellular factors.

Ubiquitination of Smac by cIAPs Requires Their Physical Association—The interactions between IAPs and mature Smac are mediated by the IAP BIR domains and the Smac NH2-terminal region, particularly a 4-amino acid stretch (AVPI). To confirm the specificity of cIAP-dependent ubiquitination of Smac, we generated a mutant cIAP1 construct lacking all the BIR domains and a mutant mature Smac construct with the AVPI motif deleted. The BIR deletion mutant of cIAP1 failed to bind to and degrade Smac (Fig. 3A), even though this region retained E3 activity (22). In addition, the AVPI deletion mutant of Smac only weakly associated with cIAPs. This mutant was not ubiquitinated by cIAP1 and showed higher stability in the presence of cIAP1 compared with the wild-type mature Smac (Fig. 3B). Thus, cIAP-mediated Smac ubiquitination and degradation requires specific interactions between cIAPs and Smac.

cIAP1, cIAP2, and XIAP Associate with Overlapping but Partially Distinct Subsets of E2s—A eukaryotic genome encodes only one, or at most a few, E1. In contrast, it encodes a dozen or so E2s. To determine which E2s can associate with cIAP1, cIAP2, and XIAP, each IAP was expressed in 293T cells with the E2s that have been previously found to associate with various RING domain E3s. The COOH-terminal region of cIAP2 was used in these experiments because it had higher stability.

Co-immunoprecipitation assays revealed that cIAP1 preferentially interacted with UbcH7 and its close homologue UbcH8, as well as with UbcH5C and UbcH5A, whereas cIAP2 associated strongly with all UbcH5 family E2s and weakly with UbcH7, but not with UbcH8 (Fig. 4A and B). XIAP, on the other hand, associated with all E2s tested but with a weak binding affinity to UbcH5C and UbcH8 (Fig. 4C). It is common for an E3 to bind to multiple E2s. For example, Parkin, a....
RING-containing E3 that is associated with Parkinson’s disease, interacts with several E2s (31). E2 recruitment in vivo can also be determined by the presence of other regulatory proteins and the availability of E2s. Because XIAP binds to several E2s, the weak ligase activity of XIAP toward Smac was unlikely caused by a defective E2 binding. In addition, because only the cIAP2 COOH-terminal region that encompasses the RING domain was used for the binding assay (Fig. 4B), the NH$_2$-terminal BIR domains are dispensable for E2 recruitment.

**In Vitro Reconstitution of Ubiquitination of Smac by cIAPs**—Having identified putative E2s for cIAP1, cIAP2, and XIAP, we next examined the ubiquitination of Smac by these IAPs using an in vitro ubiquitination assay. FLAG-tagged full-length cIAP1 and XIAP and a deletion mutant of cIAP2 lacking the first BIR domain were expressed in mammalian cells and affinity purified with an anti-FLAG antibody. The mutant cIAP2 was used because it was expressed at a higher level than the wild-type proteins yet still retained the capacity to bind to Smac (data not shown). When the purified cIAP1 or cIAP2:BIR1 protein was added to a ubiquitination reaction mixture containing recombinant E1 and E2 (UbcH5C) and in vitro translated, metabolically labeled mature Smac, ubiquitination of Smac was evident as shown by the appearance of higher molecular weight materials (Fig. 5, A and B, lane 4 versus 1–3). The ubiquitination was significantly enhanced when recombinant ubiquitin was also included in the reaction (lane 5 versus 4). In comparison, XIAP ubiquitinated Smac very weakly even though the amount of XIAP used was much higher than that used with cIAP1 (Fig. 5C, lane 3 versus 2). These results confirm that both cIAP1 and cIAP2 are strong ubiquitin ligases but suggest that XIAP is a weak ubiquitin ligase for mature Smac.

**DIAPI1-dependent Ubiquitination of Grim and HID**—*Drosophila* IAP1 (DIAPI1) plays a critical role in apoptosis inhibition. Flies defective with DIAPI1 die in the embryonic stage because of excessive cell death (13). Upon activation of apoptosis, the *Drosophila* death inducers Grim, HID, and Reaper bind to and eliminate the inhibitory effect of DIAPI1 on caspases, via an NH$_2$-terminal motif that is shared with each other and with the mammalian functional homologue Smac (13, 17, 19). Several recent studies showed that Hid, Grim, and Reaper promote the autoubiquitination and degradation of DIAPI1 (32–35). To determine whether DIAPI1, like cIAPs, can mediate the ubiquitination of the death inducers, we examined the effect of DIAPI1 on Grim and HID expression levels. Expression of DIAPI1 in 293T cells resulted in marked self-ubiquitination, with the appearance of a ladder of higher molecular weight materials typical of ubiquitination. This DIAPI1 self-ubiquitination depended on an intact RING domain (Fig. 6, A and B, middle panel, lane 2 versus 3). DIAPI1 also ubiquitinated the co-expressed Grim and HID (Fig. 6, A and B, top and middle panels). The expression levels of Grim and HID were increased, rather than decreased, upon the expression of DIAPI1 (Fig. 6, A and B, middle panel), consistent with a previous report that the BIR domains of DIAPI1 could enhance the stability of Grim/HID likely through a direct protein-protein interaction (36). However, the levels of Grim and HID were even higher in the presence of the RING deletion mutant of DIAPI1, which lost the ability to ubiquitinate itself and Grim/HID (Fig. 6, A and B, lane 3 versus 2). To further confirm that the DIAPI1-mediated ubiquitination leads to degradation of Grim, we deleted the only lysine residue in the Grim protein. The resulting Grim mutant could no longer be ubiquitinated by DIAPI1, and con-
currently the level of Grim protein was markedly increased compared with the wild-type protein (data not shown). Taken together, these results show that the E3 activity of DIAP1 can trigger degradation of both Grim and HID.

To address the functional significance of the IAP E3 activity in apoptosis regulation, an attempt was made to compare the apoptosis inhibition ability of the wild-type and the E3-defective cIAP mutants. Paradoxically, overexpression of cIAPs enhanced apoptosis in 293T cells. Although an anti-apoptotic function of cIAPs has been previously demonstrated (37, 38), overexpression of cIAPs in some cells has also been reported to enhance apoptosis, unless the RING domain was deleted (26, 39). This proapoptotic activity of cIAPs could be because of the existence of anti-apoptotic cIAP1 substrates, such as TRAF-2 (26). The E3 activity of cIAP thus plays a complex role in the regulation of apoptosis. Overexpression of DIAP1, on the other hand, was not cytotoxic in either 293T or HeLa cells. Rather, DIAP1 potently inhibited Grim-induced apoptosis in these cells in a transient transfection apoptosis assay (Fig. 6, top panel, and data not shown). In comparison, the E3 defective RING mutant of DIAP1 was substantially less effective in blocking Grim-induced apoptosis (Fig. 6). Therefore, the DIAP1 E3 activity contributes to its apoptosis inhibitory function.

**DISCUSSION**

The IAPs family is a major group of apoptosis regulators. IAPs have been shown to directly block caspase activity. In this study we found that cIAP1 and cIAP2 can also target the mitochondrial cell death inducer Smac for degradation via the COOH-terminal RING domain-mediated ubiquitination. Similarly, *Drosophila* DIAP1 possesses E3 activity that can mediate the ubiquitination and degradation of the *Drosophila* death inducers Grim and HID. These results suggest a novel and
IAPs and XIAP both mediate the ubiquitination of Smac (40). The authors also detected XIAP-mediated ubiquitination in vitro. However, by directly comparing IAPs and XIAP both in vivo and in vitro, we are able to show the marked difference in their E2 activities toward Smac. XIAP may directly bind to and inhibit caspases, cIAP1 and cIAP2 may function as single molecules E3s that interact with both the substrate and E2 (Fig. 7A), and these interactions may be regulated by other cellular factors, as suggested by the lack of XIAP-mediated ubiquitination in HeLa cells. In contrast, in the SCF (Skp1, Cullin, F-box) ubiquitin ligase complex, which plays an important role in cell cycle regulation, the RING-containing E3 (Roc) interacts with the substrate through several intermediary proteins and the substrate specificity is determined mainly by the F-box-containing protein (Fig. 7B) (24, 25). Given that both Smac and Grim/HID interact with the BIR region of IAPs, this region would be interesting to identify additional IAP substrates that associate with this region. The inhibition of IAPs by the death inducer Smac/Grim/HID and the ubiquitination of death inducers by IAPs indicate that there are dynamic interactions between these two groups of anti- and proapoptotic proteins. These interactions, like those between the anti- and proapoptotic members of the Bcl-2 family proteins, may greatly influence the lives and deaths of cells.

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Shimin Hu and Xiaolu Yang

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