J. Biochem. **137**, 125–132 (2005)
DOI: 10.1093/jb/mvi029

X-Linked Inhibitor of Apoptosis Functions as Ubiquitin Ligase toward Mature Caspase-9 and Cytosolic Smac/DIABLO

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Received January 13, 2004; accepted April 5, 2004

Members of the IAP (inhibitor of apoptosis) family function as anti-apoptotic proteins by binding directly to caspase-3, -7, and -9 to inhibit their activities. During apoptosis, the activities of IAPs are relieved by a second mitochondria-derived caspase activator, named Smac/DIABLO. Some IAPs have a C-terminal RING finger domain that has been identified as the essential motif for the activity of ubiquitin ligase (E3). Here we show that X-linked IAP (XIAP) mediates the polyubiquitination of caspase-9 and Smac. The large subunit of mature caspase-9 was polyubiquitinated by XIAP *in vitro*, while procaspase-9 was not. Furthermore, the polyubiquitinated form of caspase-9 accumulated in an XIAP-dependent manner in intact cells. The ubiquitination of caspase-9 was significantly inhibited in the presence of mature Smac, whereas XIAP was also found to promote the polyubiquitination of cytosolic Smac both *in vitro* and in intact cells. These ubiquitination reactions require the RING finger domain of XIAP. These findings suggest that XIAP functions as ubiquitin ligase toward mature caspase-9 and Smac to inhibit apoptosis.

Key words: caspase-9, IAP, RING finger, Smac/DIABLO, ubiquitin, ubiquitin ligase.

Abbreviations: IAP, inhibitor of apoptosis; XIAP, X-linked inhibitor of apoptosis; c-IAP, cellular inhibitor of apoptosis; BIR, baculoviral IAP repeat; casp-9, caspase-9; casp-9LS, large subunit of mature caspase-9; Smac, second mitochondria-derived activator of caspases; Ub, ubiquitin; E1, ubiquitin activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; HA, hemagglutinin; GST, glutathione *S*-transferase.

Apoptosis, programmed cell death regulated by signal transduction mechanisms, plays a critical role in the development and homeostasis of multicellular organisms (*1*, *2*). One of the important steps in the induction of apoptosis is the activation of caspases, a family of cysteine proteases that cleave their substrates at specific aspartate residues (*3*, *4*). Caspases are expressed as inactive zymogens (procaspases) and activated during apoptosis through proteolytic processing that generates large and small subuints to form the mature heterotetramer. Activated caspases amplify their mature molecules by processing other procaspases, and finally induce apoptotic morphological changes by cleaving a variety of cellular proteins.

Caspase-9 is the initiator caspase that is activated first on the mitochondria-triggered caspase activation pathway. In response to various apoptotic stimuli including irradiation and chemotherapeutic drugs, cytochrome *c* is released from the mitochondria into the cytosol and binds to apoptosis promoting factor-1 (Apaf-1) in the presence of dATP/ATP (*5*–*7*). The resulting oligomeric Apaf-1/cytochrome *c* complex recruits procaspase-9, forming apoptosome, which induces the autocatalytic processing and activation of procaspase-9 (*8*, *9*).

Once activated, caspase-9 in turn cleaves and activates effector caspases such as caspase-3 and -7, resulting in a cascade of additional caspase activation and apoptosis.

IAP (inhibitor of apoptosis) family proteins are regulators of the caspase activation cascade (*10*, *11*). IAPs were first identified as baculovirus proteins that inhibit apoptosis in host cells (*12*, *13*), and their homologs are found in many animal species including mammals (*10*, *11*). Several human IAP homologs, such as X-chromosome linked IAP (XIAP), c-IAP1 and c-IAP2, have been shown to bind active caspase-3, caspase-7 and caspase-9 and to inhibit their activities (*14*–*16*). The inhibition of caspases by IAPs can be relieved by the mitochondrial proteins Smac/DIABLO (*17*, *18*). Smac is released from the mitochondria into the cytosol with cytochrome *c* during apoptosis, binds directly to IAPs, and eliminates the interaction of IAPs with caspases.

IAPs are structurally characterized by having one to three copies of the baculoviral IAP repeat (BIR) domain at their N-termini; additionally, some IAPs, including XIAP, c-IAP1 and c-IAP2, contain a C-terminal RING finger domain (*10*, *11*). The BIR domains are thought to be necessary for IAPs to bind to and inhibit caspases. In the case of XIAP, the BIR2 domain, together with its N-terminal linker region is sufficient to bind caspase-3 (*19*, *20*), and the BIR3 domain selectively targets caspase-9 (*21*, *22*).

Several lines of evidence suggest that the RING finger domain is an essential motif for the activity of ubiquitin ligases (*23*). The ubiquitination of proteins is performed

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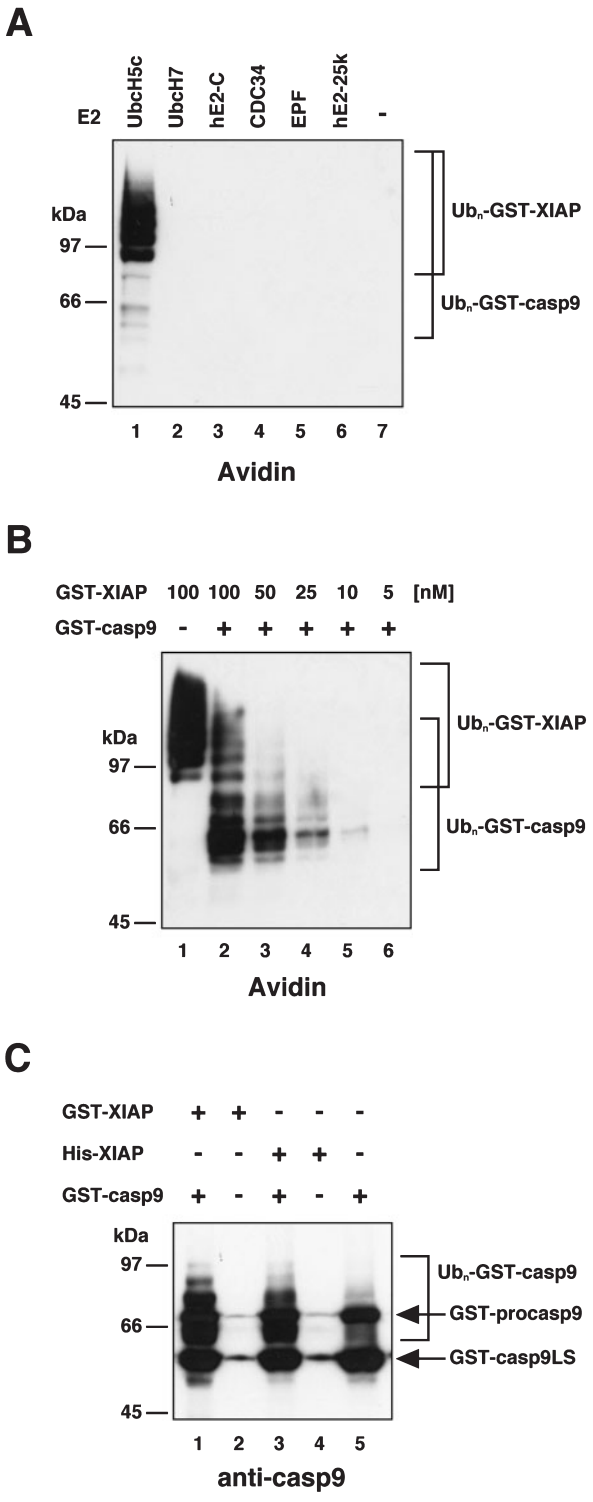
in three enzymatic steps (*24*, *25*). First, the ubiquitin activating enzyme (E1) activates a ubiquitin moiety and forms a thioester bond between the cysteine residue of the E1 and the C-terminus of ubiquitin in an ATP-dependent manner. The activated ubiquitin is then transferred to the ubiquitin-conjugating enzyme (E2) *via* the thioester bond, and, finally, the ubiquitin ligase (E3) recognizes the target protein to be ubiquitinated and transfers the ubiquitin moiety from the E2 to the target. The resulting polyubiquitin chain serves as a recognition signal for the proteasomal degradation. At present two major types of E3s are known: the HECT type and the RING finger type. E6AP (*26*), Smurf1 (*27*) and Nedd4 (*28*) have been identified as HECT type ligases, whereas MDM2 (*29*), c-Cbl (*30*, *31*), Parkin (*32*), Roc1/Rbx1 (SCF and VBC) and APC11 (APC) (*33–35*) are known as RING finger type ligases in mammals. IAPs containing RING finger domains have also been shown to promote auto-ubiquitination and self-degradation in response to apoptotic stimuli (*36*). Moreover, several reports suggest that these IAPs also function as E3s toward other substrates. For example, caspase-7 has been shown to be monoubiquitinated by c-IAP2 *in vitro* (*37*) and caspase-3 has been shown to be polyubiquitinated by XIAP in intact cells (*38*). In addition, it has also been reported that some kinds of IAPs promote the ubiquitination of Smac (*39*, *40*).

Here we identify caspase-9 as another candidate for the target of the E3 activity of XIAP. Furthermore, we show that XIAP can also polyubiquitinate mature cytosolic Smac in intact cells.



EXPERIMENTAL PROCEDURES

Plasmid Constructions and Expression of Recombinant Proteins—Human XIAP, caspase-9, and Smac/DIABLO cDNAs were amplified by the RT-PCR method. The point mutant (C471A) of XIAP was obtained by the PCR mutation method using a specific mutation primer. The ubiquitin-fused mature Smac coding region was generated by overlapping PCR according to Hunter *et al.* (*41*). The resulting cDNAs were subcloned into each expression vector. pFLAG-CMV-2 vector (Sigma) was used to express N-terminally Flag-tagged proteins, and pcDNA3.1/myc-His (–) A vector (Invitrogen) was used to express C-terminally Myc-tagged proteins in 293T cells. Mouse E1, N-terminally GST- or His$_6$-tagged XIAP, and GST-tagged full-length procaspase-9 were expressed in Sf-9 cells using a baculovirus protein expression system (Gibco BRL) according to the manufacturer's protocol. Recombinant mature Smac was expressed in *Escherichia coli* (BL21, *LysS*) as C-terminal intein-chitin binding domain (CBD)

Fig. 1. **XIAP functions as E3 toward caspase-9 *in vitro*.** (A) XIAP and caspase-9 are ubiquitinated in the presence of UbcH5c. GST-caspase-9 (150 nM) and GST-XIAP (400 nM) immobilized on glutathione-Sepharose 4B were applied to the *in vitro* ubiquitination assay together with E1, the indicated E2, and biotinylated ubiquitin. The resins were subjected to SDS-PAGE and the ubiquitinated proteins were detected by Western blotting using peroxidase-conjugated avidin (ExtrAvidin). (B) Ubiquitination of caspase-9 is dependent on the amount of XIAP. The indicated amounts of GST-XIAP were applied to the *in vitro* ubiquitination assay with (lanes 2–6) or without (lane 1) GST-caspase-9 (150 nM), and the ubiquitinated proteins were detected as shown in (A) using purified UbcH5c as E2. (C) Ubiquitination of the large subunit of mature caspase-9 and/or procaspase-9 by XIAP. Resin-immobilized GST-caspase-9 (lanes 1, 3 and 5) or control resin (lanes 2 and 4) was incubated with purified GST-XIAP (lanes 1 and 2) or His-XIAP (lanes 3 and 4) before the ubiquitination assay. The *in vitro* ubiquitination reaction was performed as described in (B), and GST-tagged procaspase-9 and the large subunit of mature caspase-9 (casp-9LS) were detected by Western blotting using an anti-caspase-9 antibody (specific for residues 1–250).

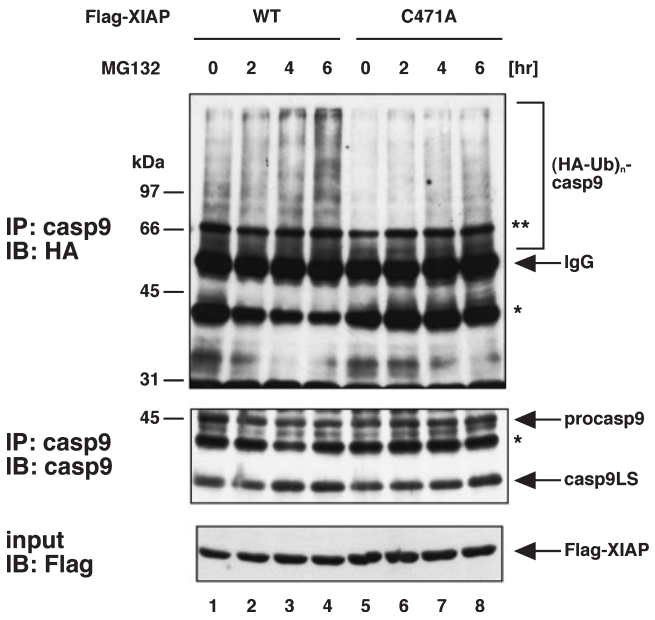


Fig. 2. **A RING finger mutant of XIAP does not enhance the ubiquitination of caspase-9 in intact cells.** 293T cells were co-transfected with expression plasmids encoding untagged full-length procaspase-9, N-terminally Flag-tagged XIAP (wild-type or C471A mutant), and HA-tagged ubiquitin (HA-Ub). Thirty six hours after transfection, the cells were treated with 40 μM MG132 for the indicated times and then harvested. Untagged caspase-9 was immunoprecipitated (IP) from the cell lysates using the anti–caspase-9 antibody (1–250) and protein A/G-agarose as described in "EXPERIMENTAL PROCEDURES." Each immunocomplex was subjected to SDS-PAGE and the ubiquitinated caspase-9 was detected by immunoblotting (IB) using an anti-HA antibody (upper panel). The amounts of immunoprecipitated caspase-9 (middle panel) and the expression level of Flag-XIAP (lower panel) were confirmed using the indicated antibodies. Asterisks (*, **) show the nonspecific antibody-reacting bands derived from the immunocomplexes.

fusion proteins, and purified as untagged proteins using the IMPACT T7 system (New England Biolabs). UbcH5c and other E2s were expressed in *E. coli* (BL21, *LysS*) and purified as described previously (*42*).

In Vitro Ubiquitination Assay—GST-tagged caspase-9 expressed in Sf-9 cells was immobilized on glutathione-Sepharose 4B (Amersham Bioscience) and incubated with purified GST- or His-XIAP at 4°C for 1 h before the ubiquitination reaction. The *in vitro* ubiquitination assay was carried out essentially as described previously (*42*) using biotinylated ubiquitin. Briefly, 30 μl of reaction mixture containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 3 mM ATP, 2 mM DTT, E1, E2, GST- or His-XIAP, GST-caspase-9 and biotinylated ubiquitin was incubated at 25°C for 30 min. After incubation, the resin was washed with buffer [10 mM Tris-HCl (pH 7.4), 0.15 M NaCl, and 3 mM $MgCl_2$] containing 0.01% Brji-35. In the case of ubiquitination of Smac, C-terminally intein-CBD tagged Smac immobilized on chitin beads (New England Biolabs) was used as a substrate. After the ubiquitination reaction, the resin was treated with 50 mM DTT at 4°C overnight in order to remove the intein-CBD tag from Smac and to elute the untagged Smac from the beads. Each sample was subjected to SDS-PAGE and ubiquitinated proteins were detected by Western blotting with peroxidase-conjugated avidin (ExtrAvidin, Sigma) or with the antibody indicated in the figure legends. Primary antibodies used for Western blotting were anti–caspase-9 monoclonal antibody (specific for residues 1–250, MBL), anti–caspase-9 polyclonal antibody (specific for residues 316–330, New England Biolabs) and anti-Smac polyclonal antibody (MBL).

Cell Culture, DNA Transfection and Immunoprecipitation—293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells were transfected with plasmids using the FuGENE 6 reagent (Roche) and replated in 35-mm diameter plates (5×10^5 cells/plate) 24 h after transfection. Thirty six hours after transfection, some cultures were treated with 40 μM proteasome inhibitor MG132 (Peptide Institute) for the number of hours indicated in the figure. Then, the cells were lysed with Tris buffer containing 2% SDS and heated at 100°C for 5 min to inactivate the isopeptidase activity and disrupt protein-protein interactions. For immunoprecipitation, the lysates were diluted 10-fold with Tris buffer containing 0.5% NP-40, protease inhibitor cocktail (Roche) and 1 mM PMSF, sonicated, and clarified by centrifugation. The supernatants were incubated with the indicated antibodies at 4°C for 2 h followed by the addition of protein A–Sepharose CL-4B (Amersham Bioscience) or protein A/G-agarose (Santa Cruz) for overnight. The resulting immunocomplexes were washed three times with Tris buffer containing 0.5% NP-40 and 0.2% SDS, subjected to SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The blots were incubated with the appropriate primary antibodies and then with peroxidase-conjugated anti-mouse secondary antibody (Dako). The detection of proteins was carried out by the ECL method (Amersham Bioscience). Antibodies used for immunoprecipitation and/or immunoblotting in this study were anti–caspase-9 monoclonal antibody (specific for residues 1–250, MBL), anti–HA-tag antibody (clone 12CA5, Roche), anti–Myc-tag antibody (clone PL14, MBL), anti–Flag-tag antibody (clone M2, Sigma) and anti–Flag-tag polyclonal antibody (Sigma).

RESULTS

XIAP Promotes the Ubiquitination of Caspase-9—Human IAPs have been shown to bind specifically to active caspases-3, -7 and -9 (*14–16*). In addition, recent reports demonstrate that IAPs containing a RING finger domain promote the ubiquitination of caspases-3 and -7 (*37*, *38*). These reports raise the possibility that caspase-9 may be also targeted for ubiquitination by these IAPs. Therefore, we examined whether XIAP, the most potent caspase inhibitor among human IAPs, functions as ubiquitin ligase, E3, toward caspase-9.

First, GST-caspase-9 and GST-XIAP were applied to the *in vitro* ubiquitination assay with a variety of E2s in the presence of biotinylated ubiquitin as described in "EXPERIMENTAL PROCEDURES," and then the ubiquitinated proteins were detected with peroxidase-conjugated avidin. When UbcH5c was used as E2, some ubiquitinated proteins were detected below the ladder corresponding to the auto-ubiquitinated GST-XIAP (Fig. 1A, lane 1). When other kinds of E2 were used, neither auto-

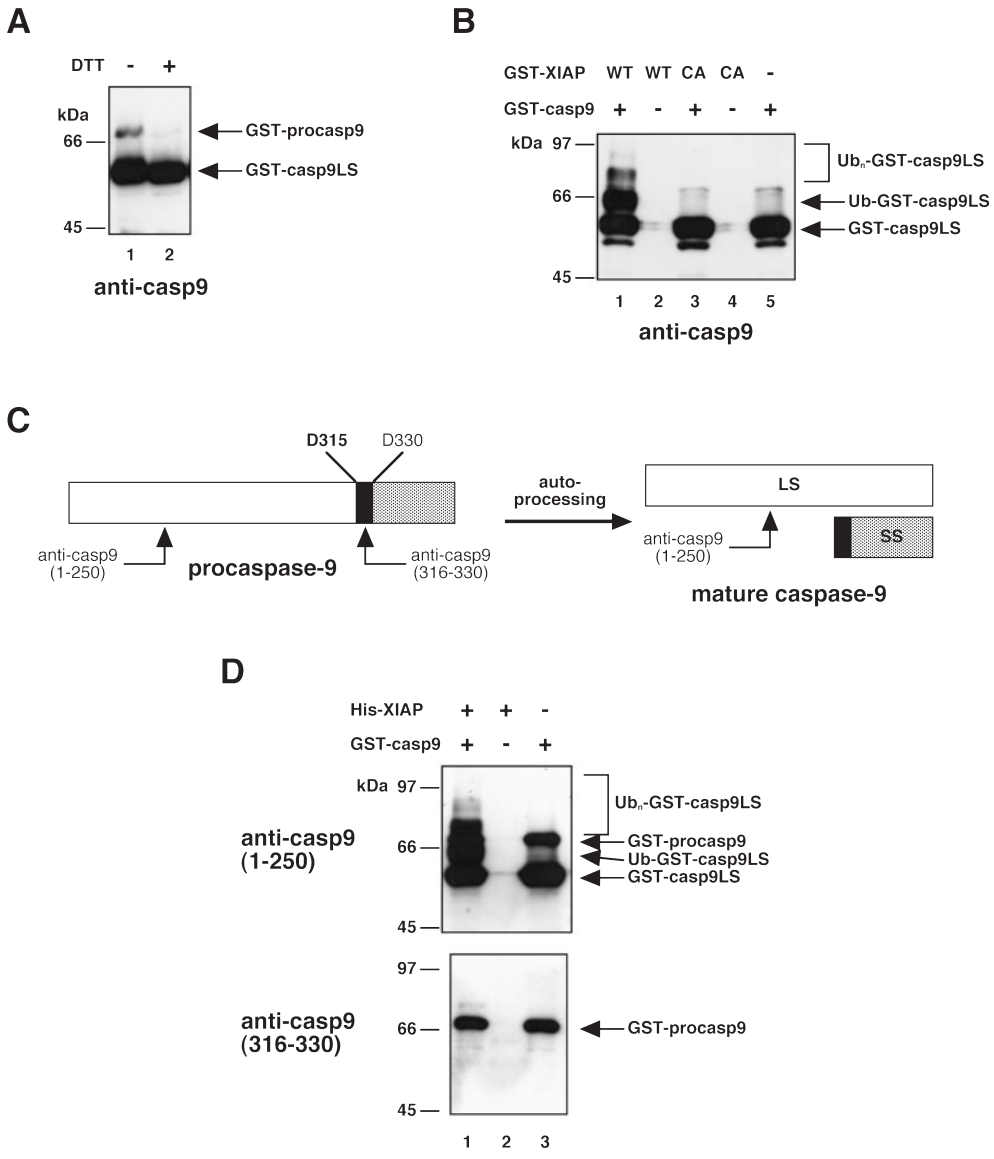
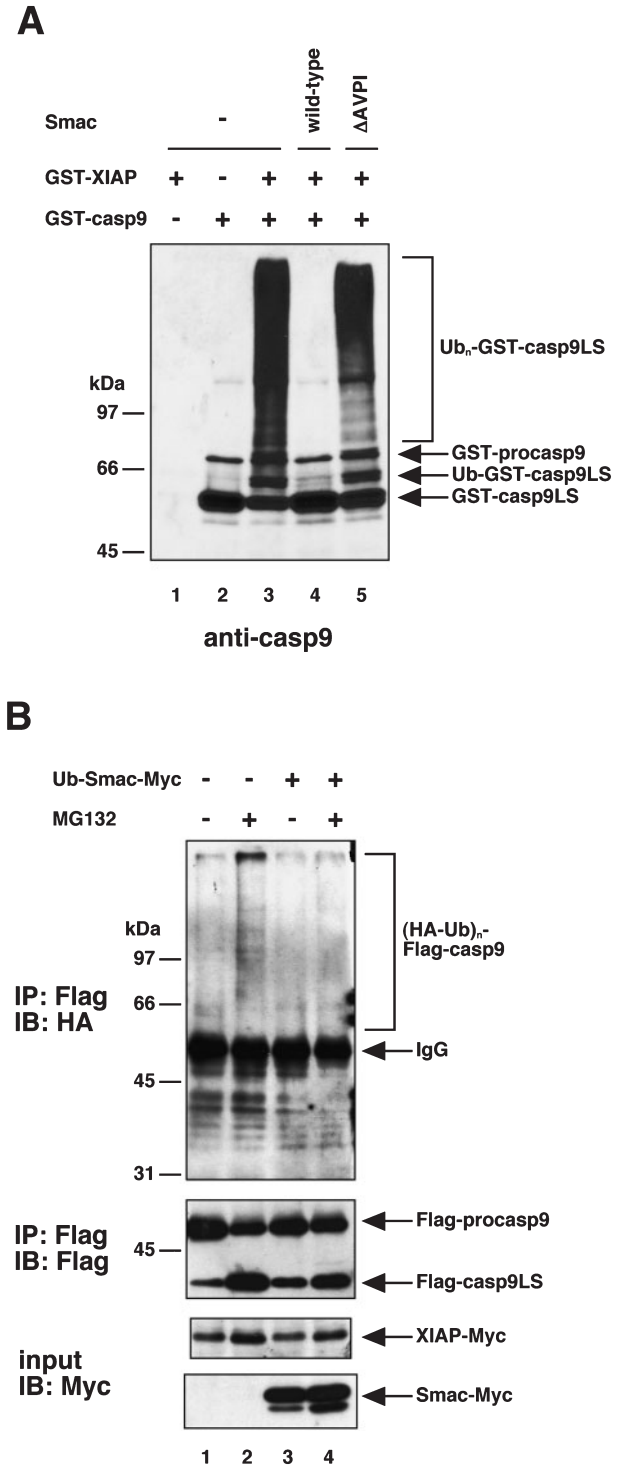


Fig. 3. **The large subunit of mature caspase-9, but not procaspase-9, is polyubiquitinated by XIAP *in vitro*.** (A) GST-caspase-9 is autocatalytically processed *in vitro*. GST-caspase-9 immobilized on glutathione-Sepharose 4B was incubated in buffer [10 mM Tris-HCl (pH 7.4), 0.15 M NaCl, and 3 mM $MgCl_2$] in the presence (lane 2) or absence (lane 1) of 2 mM DTT at 37°C for 30 min. The resins were subjected to SDS-PAGE and the cleavage of GST-procaspase-9 was detected by Western blotting using the anti–caspase-9 antibody. (B) The large subunit of mature caspase-9 is polyubiquitinated by XIAP. Immobilized GST-caspase-9 (lanes 1, 3 and 5) or control resin (lanes 2 and 4) was treated with 2 mM DTT as shown in (A), and then incubated with wild-type GST-XIAP (WT, lanes 1 and 2) or C471A mutant (CA, lanes 3 and 4). The *in vitro* ubiquitination reaction was performed as shown in Fig. 1. and the ubiquitinated GST-casp-9LS was detected by Western blotting using the anti–caspase-9 antibody. (C) The antibody regonition sites of procaspase-9 and autocatalytically processed mature caspase-9 are illustrated. Procaspase-9 and the small subunit (SS) of its mature form, but not the large subunit (LS), can be detected by the anti–caspase-9 antibody (specific for residues 316–330). (D) Procaspase-9 is not ubiquitinated by XIAP. Immobilized GST-caspase-9 (lanes 1 and 3) or control resin (lanes 2) was incubated with (lanes 1 and 2) or without (lane 3) purified His-XIAP and applied to the *in vitro* ubiquitination assay as shown in Fig. 1. GST-procaspase-9 and GST-casp-9LS were detected by Western blotting using the anti–caspase-9 antibody (1–250, upper panel), and GST-procaspase-9 was detected with anti–caspase-9 antibody (316–330, lower panel).

ubiquitinated GST-XIAP nor other ubiquitinated proteins were detected (Fig. 1A, lanes 2–6). The bands around 66 kDa that were detected in the presence of UbcH5c disappeared in the absence of GST-caspase-9 (Fig. 1B, lane 1), suggesting that these bands could be mono- or poly-ubiquitinated GST-procaspase-9 and/or the large subunit of mature caspase-9 (casp-9LS). Moreover, the amount of the ubiquitinated form of GST-caspase-9 was increased by GST-XIAP in a dose-dependent manner (Fig. 1B, lanes 2–6), indicating that the ubiquitination of caspase-9 is induced by XIAP. To confirm that GST-caspase-9 was ubiquitinated, the *in vitro* ubiquitination reaction mixtures were analyzed by Western blotting using an anti–caspase-9 antibody that recognizes the N-terminal regions of procaspase-9 and casp-9LS. A part of the GST-procaspase-9 expressed by the baculovirus expression system was autocatalytically cleaved in Sf-9 cells, and thus both GST-procaspase-9 and the mature caspase-9, produced by the processing of procaspase-9, were present in the reaction mixtures (Fig. 1C, lane 5). When GST- or His-tagged XIAP was added to the reaction mixture, several bands migrating more slowly than the GST-procaspase-9 and/or GST-casp-9LS bands were observed (Fig. 1C, lanes 1 and 3) These bands seemed to be multi-ubiquitinated forms of the caspase. These data show that XIAP can function as E3 toward procaspase-9 and/or casp-9LS *in vitro*.

Next, we tested whether caspase-9 is ubiquitinated by XIAP in intact cells. 293T cells were co-transfected with untagged caspase-9, Flag-XIAP, and HA-ubiquitin, and then treated with a proteasome inhibitor, MG132. The ubiquitination of caspase-9 was observed by immunoprecipitation with anti–caspase-9 antibody. In the presence of wild-type XIAP, a ladder of HA-ubiquitin-conjugated caspase-9 was detected (Fig. 2, lane 1) and the ubiquitinated species accumulated by treatment with MG132 for

4–6 h (Fig. 2, lanes 3 and 4). When the cells were transfected with a RING finger mutant of XIAP (C471A) in place of the wild-type, the ubiquitination of caspase-9 was much weaker, and the accumulation of ubiquitinated species by MG132 was significantly delayed (Fig. 2, lanes 5–8). Therefore, a RING finger of XIAP seems to be essential for this ubiquitination. These results suggest that XIAP promotes the ubiquitination of caspase-9 both *in vitro* and in intact cells.



XIAP Promotes the Polyubiquitination of Mature Caspase-9 but Not of Procaspase-9—From the results of the assay described above, it was not clear which form of caspase-9 was ubiquitinated, because both procaspase-9 and the mature form of caspase-9 co-existed in the reaction mixtures. In order to know which form is ubiquitinated, we tried to detect the ubiquitination of each form of caspase-9 separately.

To detect mature casp-9LS solely, we attempted to remove procaspase-9 from the reaction mixture using an *in vitro* autoprocessing reaction. GST-procaspase-9 immobilized on glutathione-Sepharose 4B was incubated with 2 mM DTT at 37°C. After incubation, the GST-procaspase-9 in the reaction mixture had almost disappeared (Fig. 3A, lane 2). The disappearance of procaspase-9 did not occur in the absence of DTT (Fig. 3A, lane 1). The resulting mature GST-caspase-9 was applied to the *in vitro* ubiquitination assay with GST-XIAP. Although GST-procaspase-9 was hardly detected, a ubiquitinated protein ladder was detected in the presence of wild-type GST-XIAP, indicating that the ladder corresponded to ubiquitinated GST-casp-9LS (Fig. 3B, lane 1). When a Ring-finger mutant of XIAP, C471A, was applied to the reaction, ubiquitinated caspase was not detected (Fig. 3B, lane 3). These data prove that mature casp-9LS is polyubiquitinated by XIAP in a RING finger domain-dependent manner.

Next, we tried to detect the ubiquitination of procaspase-9 by using anti–caspase-9 antibody specifically recognizing residues 316–330 of procaspase-9. As described above, mature GST-caspase-9 is generated by the autocatalytic processing of GST-procaspase-9. It has been shown that the autocatalytic cleavage of procaspase-9 occurs at Asp 315, which results in the generation of the p35 (large subunit) and p12 (small subunit) fragments (*7*, *22*). Therefore, the caspase-9 antibody (316–330) recognizes procaspase-9, but not mature casp-9LS generated by autoprocessing (Fig 3C). In the presence of XIAP, the ubiquitination of GSTcasp-9LS was observed as shown in

Fig. 4. **The ubiquitination of Caspase-9 by XIAP is inhibited by Smac.** (A) Smac decreases the XIAP-mediated ubiquitination of caspase-9 *in vitro*. Purified GST-XIAP (400 nM, lanes 1, 3–5) or control (lane 2) was incubated with purified mature wild-type Smac (50 μM, lane 4), the N-terminal deletion mutant (ΔAVPI, 50 μM, lane 5) or control buffer (lanes 1–3) at 4°C for 1 h, followed by the addition of immobilized GST-caspase-9 (50 nM, lanes 2–5) or control resin (lane 1). The *in vitro* ubiquitination reaction was performed as shown in Fig. 1, and GST-caspase-9 was detected by Western blotting using the anti–caspase-9 antibody (1–250). (B) Cytosolic mature Smac inhibits the ubiquitination of caspase-9 in intact cells. 293T cells were co-transfected with expression plasmids encoding the C-terminally Myc tagged Ubiquitin-Smac fusion protein (Ub-Smac-Myc), N-terminally Flag-tagged procaspase-9, C-terminally Myc-tagged XIAP, and HAUb. Thirty six hours after transfection, the cells were treated with (lanes 2 and 4) or without (lanes 1 and 3) 40 μM MG132 for 4 h, and then harvested. Flag-caspase-9 was immunoprecipitated (IP) from the cell lysates using anti-Flag polyclonal antibody and protein A-Sepharose CL-4B. Each immunocomplex was subjected to SDS-PAGE and the ubiquitinated Flag-caspase-9 was detected by immunoblotting (IB) with an anti-HA antibody (upper panel). The amounts of immunoprecipitated Flag-caspase-9 (middle panel) and the expression level of cytosolic mature Smac-Myc and XIAP-Myc (lower panel) were confirmed using their respective antibodies.

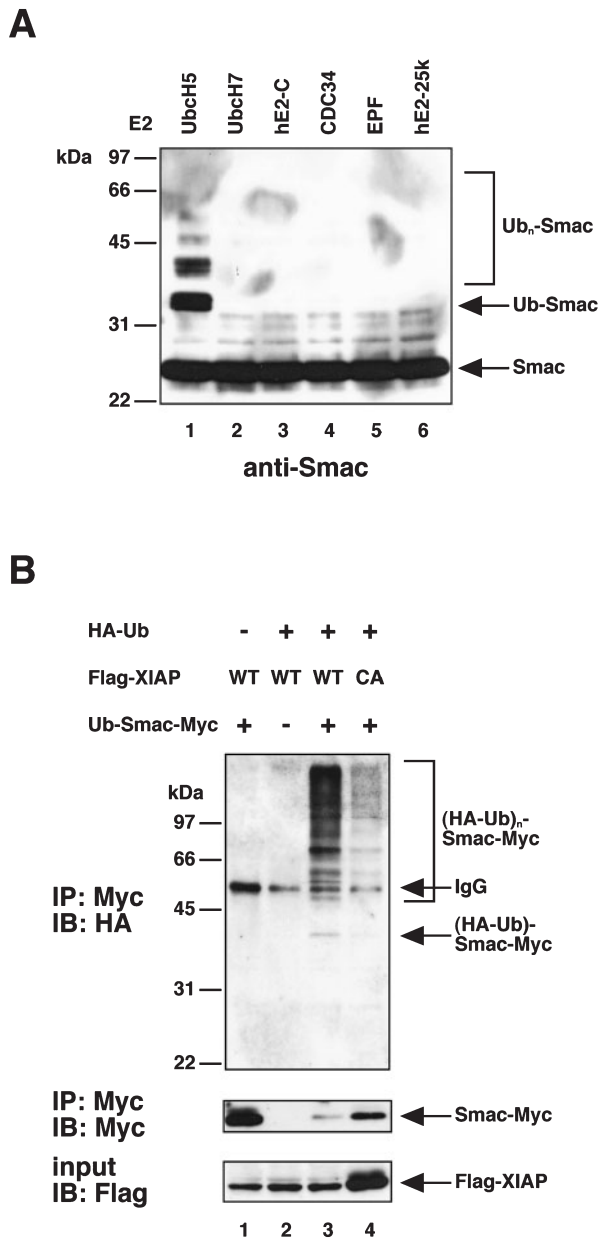


Fig. 5. **Polyubiquitination of Smac by XIAP.** (A) Smac is polyubiquitinated in the presence of UbcH5c and XIAP *in vitro*. C-terminally intein-CBD tagged mature Smac immobilized on chitin beads was applied to the *in vitro* ubiquitination assay, together with E1, the indicated E2, purified GST-XIAP and biotinylated ubiquitin. The intein-CBD tag was then removed from Smac by treatment with 50 mM DTT. The eluted untagged Smac was subjected to SDSPAGE, and detected by Western blotting using the anti-Smac antibody. (B) The RING-finger of XIAP is important for the ubiquitination of cytosolic mature Smac in intact cells. 293T cells were co-transfected with expression plasmids encoding Ub-Smac-Myc, Flag-XIAP and HAUb. Thirty-six hours after transfection, the cells were treated with 40 μM MG132 for 2 h, and then harvested. Cytosolic mature Smac-Myc was immunoprecipitated (IP) from the cell lysates using an anti-Myc polyclonal antibody and protein A–Sepharose CL-4B. Each immunocomplex was subjected to SDS-PAGE, and the ubiquitinated Smac-Myc was detected by immunoblotting (IB) using the anti-HA antibody (upper panel). The amounts of Smac-Myc immunoprecipitated (middle panel) and the expression level of Flag-XIAP (lower panel) were confirmed by reactions using their respective antibodies.

Fig 1C using anti–caspase-9 antibody (1–250), which recognizes both procaspase-9 and casp-9LS (Fig. 3D, lane 1, upper panel); the monoubiquitinated form of procaspase-9 was detected very weakly using anti–caspase-9 antibody (1–259), and polyubiquitinated forms were not detected using anti–caspase-9 antibody (316–330), which recognizes procaspase-9 but not mature casp-9LS (Fig. 3D, lane 1, lower panel). Taken together, these results suggest that mature casp-9LS, but not procaspase-9, is targeted for polyubiquitination by XIAP.

Smac Inhibits the XIAP-Mediated Ubiquitination of Caspase-9—Smac is synthesized as a precursor molecule with a N-terminal mitochondrial targeting sequence that is removed upon import into mitochondria, and released to the cytosol in response to the apoptotic stimuli (*17*). Smac has been shown to interact directly with XIAP and interfere with the binding of XIAP to caspase-9 (*17*, *18*, *22*, *43*). Thus we wondered whether Smac would also prevent the ubiquitination of caspase-9 by XIAP. When a large amount of mature Smac was added to the *in vitro* ubiquitination reaction, ubiquitinated GST-casp9LS disappeared completely (Fig. 4A, lane 4). This inhibition was not observed when a Smac deletion mutant (ΔAVPI) lacking Ala-Val-Pro-Ile in its N-terminus was added in place of wild-type Smac (Fig. 4A, lane 5). This result indicates the inhibition of XIAP-mediated caspase-9 ubiquitination by active Smac. We also investigated the effect of Smac on the ubiquitination of caspase-9 in intact cells. In this experiment, we used the ubiquitin-Smac fusion protein expression system, which allows the expression of active Smac in the cytosol of non-apoptotic cells (*41*). In the case of the culture without Smac, polyubiquitinated Flagcaspase-9 accumulated in a manner dependent on MG132 as shown in Fig. 2 (Fig. 4B, lanes 1 and 2). In the presence of cytosolic mature Smac, however, the accumulation of ubiquitinated species was not observed even after 4 h-treatment with MG132 (Fig. 4B, lanes 3 and 4). These results demonstrate that Smac antagonizes the E3 activity of XIAP toward caspase-9.

Smac Is Polyubiquitinated by XIAP—Smac is known as a proapoptotic protein that relieves the caspase-inhibitory activity of IAPs. On the other hand, recent reports have revealed that IAPs promote the ubiquitination and proteasomal degradation of Smac (*39*, *40*). It has been reported that XIAP promotes the ubiquitination of Smac *in vitro* (*39*), while another report argues that the E3 activity of XIAP toward Smac is very weak compared to that of c-IAP1 or c-IAP2, and that it cannot promote significant ubiquitination of Smac in intact cells (*40*). To confirm the E3 activity of XIAP toward Smac, mature Smac and GST-XIAP were applied to the *in vitro* ubiquitination assay. In the presence of UbcH5c, the ubiquitinated mature Smac was detected as a ladder of bands which is characteristic of polyubiquitinated proteins (Fig. 5A, lane 1). Next, to observe the ubiquitination of cytosolic mature Smac in intact cells, 293T cells were transfected with a C-terminally Myc-tagged Ub-Smac fusion protein, along with Flag-XIAP and HA-Ub. Contrary to the previous report (*40*), both mono- and polyubiquitinated mature Smac-Myc were strongly detected in the presence of wild-type XIAP and HA-Ub (Fig. 5B, lane 3). Furthermore, when cells were transfected with the RING-finger mutant XIAP, the ubiquitination of Smac

was significantly decreased (Fig. 5B, lane 4). The amount of nonubiquitinated Smac in the immunocomplex was larger than when the wild-type XIAP was used (Fig. 5B, middle panel). These results suggest that XIAP is able to polyubiquitinate cytosolic mature Smac as well as c-IAP1 or c-IAP2. When the Smac precursor with a mitochondrial targeting sequence was used in place of Ub-Smac, the ubiquitination of mitochondrial mature Smac was hardly detected (data not shown), indicating that the cytosolic localization of mature Smac is necessary for the XIAP-mediated ubiquitination.

DISCUSSION

IAP family proteins are key regulators of apoptosis. IAPs have been shown to bind specific caspases directly *via* their BIR domains, whereas the RING-finger domain has been shown to function as an active center of E3 and to be responsible for the ubiquitination of substrates. In this study, we demonstrate that caspase-9, the initiator caspase of the mitochondrial apoptotic pathway, is one of the substrates of the E3 activity of XIAP. XIAP can promote the polyubiquitination of caspase-9 both *in vitro* and in intact cells in a RINGfinger domain-dependent manner. Taken together with previous reports indicating that IAPs with a RING finger domain can ubiquitinate caspases-3 and/or -7 (*37*, *38*), these results suggest that these IAPs inhibit caspases not only by suppressing their activities through direct binding, but also by degrading their active species through the ubiquitin–proteasome pathway.

We found that XIAP polyubiquitinates the large subunit of mature caspase-9 but not procaspase-9 *in vitro*. This result is consistent with the previous report that revealed that the processing of caspase-9 is essential for XIAP-mediated inhibition (*22*). In addition, mature Smac prevented the ubiquitination of caspase-9 by XIAP to the same extent as it prevents the binding of XIAP to caspase-9.

The RING-finger type E3s can be separated into two groups: those that do not bind directly to target proteins and can function as E3s by forming an E3 complex with a subunit that recognizes the substrates, such as Roc1/Rbx1 and APC11 (*33–35*), and those that have both the binding and catalytic domains in their own molecules, such as MDM2 and c-Cbl (*29–32*). As shown here, XIAP belongs to the latter group, and the binding of XIAP to mature caspase-9 *via* its BIR3 domain may be required for the subsequent ubiquitination.

Smac has been identified as an inhibitor of the caspase-inhibitory activity of IAPs, and we have demonstrated that Smac also inhibits the XIAP-mediated ubiquitination of caspase-9. We have also shown that cytosolic mature Smac is polyubiquitinated by XIAP *in vitro* and in intact cells. These data indicate that Smac may be a target protein for IAP-mediated ubiquitination, consistent with data reported by other groups (*39*, *40*). Thus, IAPs seem to be able to remove cytosolic mature Smac through the ubiquitin-proteasome pathway. IAPs have been shown to have the auto-ubiquitination activity (*36*). Taken together, these studies suggest that IAPs promote the ubiquitination of caspases and Smac to inhibit apoptosis, or promote auto-ubiquitination to induce apoptosis. Based on this suggestion, we speculate that the selection of the target for IAP-mediated ubiquitination may be involved in the determination of cell death or survival. Recently, Smac3, a splicing variant of Smac, and the *Drosophila* proapoptotic proteins Reaper, Grim and Hid have been shown to enhance the auto-ubiquitination of IAPs (*44–48*). Such factors, which change the target of the E3 activity of IAPs, probably play a critical role in the regulation of apoptosis.

This work was supported in part by the Scientific Grant from the Ministry of Education, Science, Sports and Technology, Japan, and also in part by the Uehara Memorial Foundation o H. Y. We thank Dr. Tomohiko Ohta, St. Marianna University School of Medicine, for kindly providing the HA-ubiquitin expression plasmid. We are grateful to Drs. Hirofumi Tanaka and Tamotsu Nishida, Tokyo University of Pharmacy and Life Science, for helpful advice throughout the course of this work.

REFERENCES

1. Steller, H. (1995) Mechanisms and genes of cellular suicide. *Science* **267**, 1445–1449
2. Thompson, C.B. (1995) Apoptosis in the pathogenesis and treatment of disease. *Science* **267**, 1456–1462
3. Thornberry, N.A. and Lazebnik, Y. (1998) Caspases: Enemies within. *Science* **281**, 1312–1316
4. Cryns, V. and Yuan, J. (1998) Proteases to die for. *Genes Dev.* **12**, 1551–1570
5. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S., and Wang, X. (1997) Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91**, 479–489
6. Zou, H., Henzel, W.J., Liu, X., Lutschg, A., and Wang, X. (1997) Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome *c*-dependent activation of caspase-3. *Cell* **90**, 405–413
7. Srinivasula, S.M., Ahmad, M., Fernandes-Alnemri, T., and Alnemri, E.S. (1998) Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. *Mol. Cell* **1**, 949–957
8. Zou, H., Li, Y., Liu, X., and Wang, X. (1999) An APAF-1-cytochrome *c* multimeric complex is a functional apoptosome that activates procaspase-9. *J. Biol. Chem.* **274**, 11549–11556
9. Saleh, A., Srinivasula, S.M., Acharya, S., Fishel, R., and Alnemri, E.S. (1999) Cytochrome *c* and dATP-mediated oligomerization of Apaf-1 is a prerequisite for procaspase-9 activation. *J. Biol. Chem.* **274**, 17941–17945
10. Deveraux, Q.L. and Reed, J.C. (1999) IAP family of proteins-suppressors of apoptosis. *Genes Dev.* **13**, 239–252
11. Miller, L.K. (1999) An exegesis of IAPs: salvation and surprises from BIR motifs. *Trends Cell Biol.* **9**, 323–328
12. Birnbaum, M.J., Clem, R.J., and Miller, L.K. (1994) An apoptosis-inhibiting gene from a nuclear polyhedrosis virus encoding a polypeptide with Cys/His sequence motifs. *J. Virol.* **68**, 2521–2528
13. Crook, N.E., Clem, R.J., and Miller, L.K. (1993) An apoptosis-inhibiting baculovirus gene with a zinc finger-motif. *J. Virol.* **67**, 2168–2174
14. Deveraux, Q.L., Takahashi, R., Salvesen, G.S., and Reed, J.C. (1997) X-linked IAP is a direct inhibitor of cell-death proteases. *Nature* **388**, 300–304
15. Roy, N., Deveraux, Q.L., Takahashi, R., Salvesen, G.S., and Reed, J.C. (1997) The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. *EMBO J.* **16**, 6914–6925
16. Deveraux, Q.L., Roy, N., Stennicke, H.R., Vanarsdale, T., Zhou, Q., Srinivasula, S.M., Alnemri, E.S., Salvesen, G.S., and Reed, J.C. (1998) IAPs block apoptotic events induced by caspase-8 and cytochrome *c* by direct inhibition of distinct caspases. *EMBO J.* **17**, 2215–2223

17. Du, C., Fang, M., Li, Y., Li, L., and Wang, X. (2000) Smac, a mitochondrial protein that promotes cytochrome *c*-dependent caspase activation by eliminating IAP inhibition. *Cell* **102**, 33–42
18. Verhagen, A.M., Ekert, P.G., Pakusch, M., Silke, J., Connolly, L.M., Reid, G.E., Moritz, 14 R.L., Simpson, R.J., and Vaux, D.L. (2000) Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* **102**, 43–53
19. Takahashi, R., Deveraux, Q., Tamm, I., Welsh, K., Assa-Munt, N., Salvesen, G.S., and Reed, J.C. (1998) A single BIR domain of XIAP sufficient for inhibiting caspases. *J. Biol. Chem.* **273**, 7787–7790
20. Sun, C., Cai, M., Gunasekera, A.H., Meadows, R.P., Wang, H., Chen, J., Zhang, H., Wu, W., Xu, N., Ng, S.C., and Fesik, S.W. (1999) NMR structure and mutagenesis of the inhibitor-of-apoptosis protein XIAP. *Nature* **401**, 818–822
21. Sun, C., Cai, M., Meadows, R.P., Xu, N., Gunasekera, A.H., Herrmann, J., Wu, J.C., and Fesik, S.W. (2000) NMR structure and mutagenesis of the third Bir domain of the inhibitor of apoptosis protein XIAP. *J. Biol. Chem.* **275**, 33777–33781
22. Srinivasula, S.M., Hegde, R., Saleh, A., Datta, P., Shiozaki, E., Chai, J., Lee, R.A., Robbins, P.D., Fernandes-Alnemri, T., Shi, Y., and Alnemri, E.S. (2001) A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. *Nature* **410**, 112–116
23. Joazeiro, C.A. and Weissman, A.M. (2000) RING finger proteins: mediators of ubiquitin ligase activity. *Cell* **102**, 549–552
24. Ciechanover, A. and Schwartz, A.L. (1998) The ubiquitin-proteasome pathway: the complexity and myriad functions of proteins death. *Proc. Natl Acad. Sci. USA* **95**, 2727–2730
25. Hershko, A. and Ciechanover, A. (1998) The ubiquitin system. *Annu. Rev. Biochem.* **67**, 425–429
26. Scheffner, M., Huibregtse, J.M., and Howley, P. (1994) Identification of a human ubiquitin-conjugating enzyme that mediates the E6-AP-dependent ubiquitination of p53. *Proc. Natl Acad. Sci. USA* **91**, 8797–8801
27. Zhu, H., Kavsak, P., Abdollar, S., Wrana, J.L., and Thomsen, G.H. (1999) A SMAD ubiquitin ligase targets the BMP pathway and affects embryonic pattern formation. *Nature* **400**, 687–692
28. Hatakeyama, S., Jensen, J.P., and Weissman, A.M. (1997) Subcellular localization and ubiquitin-conjugating enzyme (E2) interactions of mammalian HECT family ubiquitin protein ligases. *J. Biol. Chem.* **272**, 15085–15092
29. Honda, R., Tanaka, H., and Yasuda, H. (1997) Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett.* **420**, 25–27
30. Joazeiro, C.A., Wing, S.S., Huang, H., Leverson, J.D., Hunter, T., and Liu, Y.C. (1999) The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitinprotein ligase. *Science* **286**, 309–312
31. Yokouchi, M., Kondo, T., Houghton, A., Barkiewicz, M., Home, W.C., Zhang, H., Yoshimura, A., and Baron, R. (1999) Ligand-induced ubiquitination of the epidermal growth factor receptor involves the interaction of the c-Cbl RING finger and UbcH7. *J. Biol. Chem.* **274**, 31707–31712
32. Shimura, H., Hattori, N., Kubo, Si., Mizuno, Y., Asakawa, S., Minoshima, S., Shimizu, N., Iwai, K., Chiba, T., Tanaka, K., and Suzuki, T. (2000) Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. *Nat. Genet.* **25**, 302–305
33. Ohta, T., Michel, J.J., Schottelius, A.J., and Xiong, Y. (1999) ROC1, a homolog of APC11, represents a family of cullin partners with an associated ubiquitin ligase activity. *Mol. Cell* **3**, 535–541
34. Skowyra, D., Koepp, D.M., Kamura, T., Conrad, M.N., Conaway, R.C., Conaway, J.W., Elledge, S.J. and Harper, J.W. (1999) Reconstitution of G_1 cyclin ubiquitination with complexes containing SCF_{Grr1} and Rbx1. *Science* **284**, 662–665
35. Kamura, T., Koepp, D.M., Conrad, M.N., Skowyra, D., Moreland, R.J., Iliopoulos, O., Lane, W.S., Kaelin, W.G.Jr., Elledge, S.J., Conaway, R.C., Harper, J.W., and Conaway, J.W. (1999) Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. *Science* **284**, 657–661
36. Yang, Y., Fang, S., Jensen, J.P., Weissman, A.M., and Ashwell, J.D. (2000) Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. *Science* **288**, 874–877
37. Huang, H., Joazeiro, C.A., Bonfoco, E., Kamada, S., Leverson, J.D., and Hunter, T. (2000) The inhibitor of apoptosis, cIAP2, functions as a ubiquitin-protein ligase and promotes *in vitro* monoubiquitination of caspases 3 and 7. *J. Biol. Chem.* **275**, 26661–26664
38. Suzuki, Y., Nakabayashi, Y., and Takahashi, R. (2001) Ubiquitin-protein ligase activity of X-linked inhibitor of apoptosis protein promotes proteasomal degradation of caspase-3 and enhances its anti-apoptotic effect in Fas-induced cell death. *Proc. Natl Acad. Sci. USA* **98**, 8662–8667
39. MacFarlane, M., Merrison, W., Bratton, S.B., and Cohen, G.M. (2002) Proteasomemediated degradation of Smac during apoptosis: XIAP promotes Smac ubiquitination *in vitro*. *J. Biol. Chem.* **277**, 36611–36616
40. Hu, S. and Yang, X. (2003) Cellular inhibitor of apoptosis 1 and 2 are ubiquitin ligases for the apoptosis inducer Smac/DIABLO. *J. Biol. Chem.* **278**, 10055–10060
41. Hunter, A.M., Kottachchi, D., Lewis, J., Duckett, C.S., Korneluk, R.G., and Liston, P. (2003) A novel ubiquitin fusion system bypasses the mitochondria and generates biologically active Smac/DIABLO. *J. Biol. Chem.* **278**, 7494–7499
42. Honda, R. and Yasuda, H. (1999) Association of $p19^{ARF}$ with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53. *EMBO J.* **18**, 22–27
43. Chai, J., Du, C., Wu, J.W., Kyin, S., Wang, X., and Shi, Y. (2000) Structural and biochemical basis of apoptotic activation by Smac/DIABLO. *Nature* **406**, 855–862
44. Fu, J., Jin, Y., and Arend, L.J. (2003) Smac3, a novel Smac/DIABLO splicing variant, attenuates the stability and apoptosis-inhibiting activity of XIAP. *J. Biol. Chem.* **278**, 52660–52672
45. Yoo, S.J., Huh, J.R., Muro, I., Yu, H., Wang, L., Wang, S.L., Feldman, R.M., Clem, R.J., Muller, H.A., and Hay, B.A. (2002) Hid, Rpr and Grim negatively regulate DIAP1 levels through distinct mechanisms. *Nat. Cell Biol.* **4**, 416–424
46. Hays, R., Wickline, L., and Cagan, R. (2002) Morgue mediates apoptosis in the *Drosophila melanogaster* retina by promoting degradation of DIAP1. *Nat. Cell Biol.* **4**, 425–431
47. Ryoo, H.D., Bergmann, A., Gonen, H., Ciechanover, A., and Steller, H. (2002) Regulation of *Drosophila* IAP1 degradation and apoptosis by reaper and ubcD1. *Nat. Cell Biol.* **4**, 432–438
48. Holley, C.L., Olson, M.R., Colon-Ramos, D.A., and Kornbluth, S. (2002) Reaper eliminates IAP proteins through stimulated IAP degradation and generalized translational inhibition. *Nat. Cell Biol.* **4**, 439–444