Smac3, a Novel Smac/DIABLO Splicing Variant, Attenuates the Stability and Apoptosis-inhibiting Activity of X-linked Inhibitor of Apoptosis Protein*

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X-linked inhibitor of apoptosis protein (XIAP), the most potent member of the inhibitor of apoptosis protein (IAP) family, plays a crucial role in the regulation of apoptosis. XIAP is structurally characterized by three baculovirus IAP repeat (BIR) domains that mediate binding to and inhibition of caspases and a RING domain that confers ubiquitin ligase activity. The caspase inhibitory activity of XIAP can be eliminated by the second mitochondria-derived activator of caspases (Smac)/direct IAP-binding protein with low PI (DIABLO) during apoptosis. Here we report the identification and characterization of a novel isoform of Smac/DIABLO named Smac3, which is generated by alternative splicing of exon 4. Smac3 contains an NH2-terminal mitochondrial targeting sequence required for mitochondrial targeting of Smac3 and an IAP-binding motif essential for Smac3 binding to XIAP. Smac3 is released from mitochondria into the cytosol in response to apoptotic stimuli, where it interacts with the second and third BIR domains of XIAP. Smac3 disrupts processed caspase-9 binding to XIAP, promotes caspase-3 activation, and potentiates apoptosis. Strikingly, Smac3, but not Smac/DIABLO, accelerates XIAP auto-ubiquitination and destruction. Smac3-stimulated XIAP ubiquitination is contingent upon the physical association of XIAP with Smac3 and an intact RING domain of XIAP. Smac3-accelerated XIAP destabilization is, at least in part, attributed to its ability to enhance XIAP ubiquitination. Our study demonstrates that Smac3 is functionally additive to, but independent of, Smac/DIABLO.

Apoptosis, programmed cell death, is an evolutionarily conserved and genetically regulated biological process that plays a fundamental role in the development and tissue homeostasis of metazoans (1–3). Dysregulation of apoptosis has been linked to the pathogenesis of a variety of human diseases (4).

Apoptosis is mainly orchestrated by a family of aspartate-specific cysteine proteases known as caspases. Caspases are synthesized as inactive zymogens that bear three domains: an NH2-terminal prodomain, a large subunit, and a small subunit (5). Caspases involved in apoptosis are generally divided into two categories, the initiator caspases, which include caspase-2, -8, -9, and -10, and effector caspases, such as caspase-3, -6, and -7. A pro-apoptotic signal culminates in activation of an initiator caspase, which, in turn, activates effector caspases (6, 7). There are two well characterized signal pathways leading to the activation of caspases, the death receptor pathway and the mitochondrial pathway. In the mitochondrial pathway, death signals induce the release of cytochrome c from mitochondria into the cytosol and the assembly of an apoptosome consisting of cytochrome c, adapter protein Apaf-1, and procaspase-9, triggering activation of caspase-9, which activates the effector caspases such as caspase-3, resulting in cleavage of a broad spectrum of cellular targets and leading ultimately to apoptosis (8).

Among the most important regulators of caspases are the inhibitor of apoptosis proteins (IAPs) (9, 10). The first IAP was discovered in baculoviruses (11), and many cellular IAP orthologues have since been found in a number of species, ranging from insects to humans (9, 10). Eight IAPs have been identified in mammalian cells to date (9, 10, 12). The anti-apoptotic activity of IAPs, including XIAP, c-IAP1, c-IAP2, and ML-IAP, has been attributed to their ability to bind to and inhibit caspases (13, 14). Of these IAPs, XIAP is the most potent inhibitor of caspases and apoptosis (12, 13). XIAP is structurally characterized by three tandem repeats of the baculovirus IAP repeat (BIR) domain at its NH2 terminus and a COOH-terminal RING finger domain. The inhibitory activity is mediated through the BIR domains. Different BIR domains have been shown to exhibit distinct functions. The second BIR (BIR2) domain together with the immediately proceeding linker region is responsible for binding to and inhibition of active, processed caspase-3 and -7 (15), whereas the third BIR (BIR3) domain is involved in interacting with and suppressing caspase-9 (16, 17). Like many RING domain-containing proteins, several IAPs, including XIAP, c-IAP1, and c-IAP2, serve as ubiquitin ligases toward themselves and other target proteins, which are subsequently degraded by the 26S proteasome (18–21). Protein ubiquitination and degradation are subject to

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1 The abbreviations used are: IAP, inhibitor of apoptosis protein; XIAP, X-linked IAP; BIR, baculovirus IAP repeat; Smac, second mitochondria-derived activator of caspases; DIABLO, direct IAP-binding protein with low PI; MTS, mitochondrial targeting sequence; HA, hemagglutinin; GFP, green fluorescent protein; Ub, ubiquitin; FBS, fetal bovine serum; MEPA, mouse embryonic fibroblasts; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; mAb, monoclonal antibody; GST, glutathione S-transferase; MG132, carboxbenzoxyl-leucyl-leucyl-leucinal; IBM, IAP-binding motif; RT-PCR, reverse transcriptase-PCR; PBS, phosphate-buffered saline; HEK, human embryonic kidney; STA, staurosporine; E3, ubiquitin-protein isopeptide ligase; CDDP, cisplatin.
tight control (22). It remains to be determined how the auto-
ubiquitination and degradation of IAPs are regulated.

The caspase-inhibiting activities of IAPs can be relieved by a
mitochondrial protein named Smac (second mitochondria-de-
river activator of caspases) (23), also known as DIABLO (direct
IAP-binding protein with low pI) (24). Similar to cytochrome c,
Smac/DIABLO is encoded by a nuclear gene and is subse-
quently compartmentalized in mitochondria. Upon receiving pro-
caspase signals, Smac/DIABLO is released from mito-
chondria into the cytosol where it interacts with IAPs (XIAP,
BIR-2 and -3 domains of XIAP (25). A cytosolic isoform
function through mediating the binding of Smac/DIABLO to
the first four residues named IAP-binding motif (29) of mature
mitochondria into the cytosol where it interacts with IAPs (XIAP,
pro-apoptotic signals, Smac/DIABLO is released from mito-
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cies. Smac3 is localized to mitochondria and can be released
into the cytosol during apoptosis. Smac3 is able to interact with
XIA. By binding to XIA, Smac3 disrupts the association
between XIAP and processed caspase-9, facilitates the activa-
tion of caspase-3, and antagonizes the anti-apoptotic function
of XIAP. Most importantly, our results indicate that Smac3,
but not Smac/DIABLO, promotes XIAP ubiquitination and de-
struction. Our study suggests that Smac3 is functionally addi-
tional to, but independent of, Smac/DIABLO and Smac/S-β.
Smac3 is the first molecule identified in mammalian cells to
participate in the regulation of XIAP stability.

EXPERIMENTAL PROCEDURES

Expression Constructs—To create full-length Smac3 with a COOH-
terminal hemagglutinin (HA) tag in pcDNA3 (Invitrogen), PCR ampli-
fication was performed using Pfu polymerase (Stratagene) with the
following primers. The forward primer 5'-AAGAGTCCTCCGACCAT-
GCGGGGTTCCTGGAAGTGTTCGTTCTGCATTGTTTGGC-3' contains a BamHI site (underlined)
and a 27-bp sequence encoding an
and a 27-bp sequence encoding an
expression vector in mature
expression vector in mature

Western Blotting—Cells

Far Western Blotting—Affinity-purified recombinant GST-XIAP
fragments were fractionated by SDS-PAGE and transferred onto a
PVDF membrane. Proteins were denatured by incubating the mem-
brane for 1 h at ambient temperature in denaturation buffer (10 m M
NaOH, pH 7.4), 150 mM NaCl, 5 mM MgCl2, and 1 mM dithiothreitol)
containing 6 m guanidine HCl. Proteins were then renatured by several
washes of the membrane using gradual reduction of guanidine HCl to
0.3 M. The membrane was incubated in the same buffer at 4 °C over-
night. After blocking, the membrane was incubated with whole cell
lysate of HeLa cells (clone TH27C) (kind gift of Dr. G. Nunez) and
immunoprecipitated with anti-Smac polyclonal antibody (Upstate Biotechnology, Inc.).

Immunoprecipitation—Immunoprecipitation was performed as de-
scribed previously (38). The precleared supernatants were incubated with
anti-FLAG M2 (Sigma), anti-HA mAb (clone 12CA5, Roche Applied Science),
anti-HA polyclonal antibody (Santa Cruz Biotechnology), anti-Myc mAb (clone 9E10, Santa
Cruz Biotechnology), anti-beta-actin mAb (Sigma), anti-GFP antibody (Santa Cruz Biotechnology and Clontech), antibody specific for ubiquitin (Zymed Laboratories Inc.), rabbit anti-caspase-9 cleavage site 315/316 antibody (BIOSOURCE International Inc.), anti-caspase-9 polyclonal antibody (Cell Signaling Technology), anti-caspase-3 anti-
body (Upstate Biotechnology, Inc.), anti-XIAP mAb (Transduction Lab-
ories, Inc.), anti-cytochrome c mAb (clone 7H8.2C12, Pharmingen), and
anti-Smac polyclonal antibody (Upstate Biotechnology, Inc.).

Immunostaining—Cells

Immunoprecipitation was performed as de-
scribed previously (38). The precleared supernatants were incubated with
anti-FLAG M2 (Sigma), anti-HA mAb conjugated to agarose beads (Santa Cruz Biotechnology), or anti-HA mAb conjugated
agarose beads (Sigma) (clone TH27C) (kind gift of Dr. G. Nunez).

Immunoprecipitation—Immunoprecipitation was performed as de-
scribed previously (38). The precleared supernatants were incubated with
anti-FLAG M2 (Sigma), anti-HA mAb conjugated
agarose beads (Santa Cruz Biotechnology), or anti-HA mAb conjugated
agarose beads (Sigma) (clone TH27C) (kind gift of Dr. G. Nunez).
Identification and Characterization of Smac3

**Fig. 1.** Cloning and expression of Smac3. A, schematic representation of the Smac/DIABLO and Smac3 genes. Diagram shows the nucleotide position of exon 4 within the Smac/DIABLO gene. B, alignment of the NH$_2$-terminal residues from the mature Smac/DIABLO and Smac3 proteins. C, the amino acid sequence of Smac3 with the putative MTS underlined and the IBM highlighted in boldface type. D, alignment of the NH$_2$-terminal residues from human mature Smac/DIABLO, Smac3, HtrA2/Omi, together with human caspase-9. The IBM is highlighted. E, Western blotting (WB) analysis of ectopic expression of Smac3. Molecular mass marker is indicated at right. F, RT-PCR analysis of expression of Smac3 mRNA in human tissues and cell lines. First-strand cDNAs from various adult human tissues (Clontech) and cell lines were PCR-amplified using primers specific for Smac3 (top) or β-actin (bottom).

4 °C overnight, followed by incubation with fluorescein isothiocyanate-conjugated anti-rabbit IgG (Molecular Probes) for 45 min. Following extensive washing with PBST, cells were probed with an anti-cytochrome c mAb (clone 6H2.B4, Pharmingen) overnight at 4 °C, followed by incubation with Texas Red-conjugated anti-mouse IgG (Santa Cruz Biotechnology) for 45 min at room temperature. Cells were visualized by a laser scanning confocal microscope system (Leica).

**Cycloheximide Experiment**—Two or three μg of XIAP construct was cotransfected into 293T cells grown onto 60-mm plates with equal amounts of Smac3/FLAG, Smac/FLAG, or empty vector. After 36 h, cells from each transfection were equally split into multiple plates and cultured overnight. Cells were then treated with 30 μg/ml cycloheximide (Sigma) for 90 s, and 1 time 72 °C. For analysis of Smac3 expression when cells were harvested. Cellular extracts were normalized for total protein content and subjected to immunoblotting using antisera recognizing Myc for XIAP and FLAG for Smac3 or Smac. The blot was stripped and re-probed with anti-β-actin as the loading control.

**Reverse Transcription-PCR**—For cloning of Smac3, total RNA was extracted from HEK293 cells using TRIzol Reagent (Invitrogen). Five μg of total RNA was utilized to synthesize the first strand cDNA using the primer specific for Smac with Moloney murine leukemia virus reverse transcriptase (Invitrogen). PCR amplification was performed using Pfu polymerase (Stratagene) under the following conditions: 1 time at 94 °C for 45 s, 30 times (94 °C for 45 s, 65 °C for 45 s, and 72 °C for 90 s), and 1 time 72 °C for 10 min. For analysis of Smac3 expression in human cell lines, RT-PCR was carried out essentially as described previously (39).

**Subcellular Fractionation**—Cytosolic and mitochondrial proteins of HeLa cells were prepared essentially as described previously (27, 40). The cytosolic and mitochondrial fractions were subjected to Western blotting analysis.

**Expression and Purification of GST Fusion Proteins**—Overnight cultures of Escherichia coli DH5α (Invitrogen) transformed with parental or recombinant pGEX4T-1 plasmid were diluted in LB medium containing ampicillin and incubated at 37 °C with shaking to an A$_{600}$ of 0.6. Isopropyl-β-thiogalactopyranoside (Amersham Biosciences) was then added to a final concentration of 0.1–0.2 mM. After an additional 3–5 h of growth at 30 °C, cells were pelleted at 6,000 × g for 20 min at 4 °C and resuspended in PBS containing 1 mg/ml lysozyme (Amersham Biosciences). After sonication, Triton X-100 was added to a final concentration of 1%, followed by centrifugation at 12,000 × g for 20 min at 4 °C. The GST fusion proteins were adsorbed to Glutathione-Sepharose 4B beads (Amersham Biosciences) and eluted with 10 mM reduced glutathione (Sigma) in 50 mM Tris-HCl (pH 8.0).
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RESULTS

Cloning of Smac3—To obtain the entire open reading frame of Smac, we designed primers specific for the Smac gene (23) and performed RT-PCR using total RNA prepared from HEK293 cells as template. Two transcripts were amplified (data not shown). Negative control reactions performed with RNA without reverse transcriptase or H2O as template did not yield any PCR product (data not shown). To verify the identity of the PCR products, the bands were excised, subcloned, and subjected to DNA sequencing. Sequence analysis identified one transcript as Smac/DIABLO as expected, and the other as a novel gene product. Comparison of cDNA sequence of each transcript with genomic data revealed that the smaller transcript is a new splicing variant of Smac/DIABLO with exon 4

FIG. 2. Smac3 localizes to mitochondria and translocates into the cytosol during apoptosis. A, immunolocalization of Smac3. HeLa cells were transfected with Smac3/HAs. After 24 h, cells were challenged with the indicated apoptotic agents. Cells were stained with anti-HA antibody plus fluorescein isothiocyanate-conjugated anti-rabbit IgG (green, panels a–f) and then with anti-cytochrome c (panels g–l) plus Texas Red-conjugated anti-mouse IgG (red) to identify mitochondria. Merged Smac3 and cytochrome c signals yielded yellow images (panels m–r). B, subcellular fractionation analysis of Smac3 distribution in untreated and apoptotic cells. HeLa cells were transfected with Smac3/HA. 30 h post-transfection, cells were treated with 1 μM sta or DMSO (as control) for 6 h. Cytosolic (Cyto.) and mitochondrial (Mito.) fractions were prepared and subjected to immunoblotting (IB) with anti-HA and anti-cytochrome c antibodies. C, subcellular fractionation analysis of Smac3 translocation in response to apoptotic agents and Bcl-XL-modulated Smac3 release. Smac3/HA was transfected into HeLa cells with or without (−) or with (+) Bcl-XL. After 30 h, cells were exposed to DMSO, STA (1 μM), VP16 (50 μM), or CDDP (30 μM) for 6 h. Cells were then harvested and processed as described in C.
Fig. 3. Interaction between Smac3 and XIAP. A, far Western blot analysis of interaction between Smac3 and the BIR domains of XIAP (top panel). Coomassie Blue-stained gel of the GST fusion proteins (bottom panel). B, GST pull-down assay of interaction of Smac3 and the XIAP fragments. Purified mature Smac3 with a COOH-terminal HIS tag (Smac3/C-HIS) (top) and an NH2-terminal HIS tag (N-HIS/Smac3) (bottom) were individually incubated with the GST fusion proteins or GST alone immobilized to Glutathione-Sepharose beads. The precipitated proteins were subjected to SDS-PAGE, followed by Coomassie Blue staining. The inputs of Smac3/C-HIS and N-HIS/Smac3 are shown. C, immunoprecipitation
deleted (GenBank™ accession numbers AC048338 and NT_009438) (Fig. 1A). This splicing event results in the deletion of 44 amino acids (Fig. 1B). Given that one Smac/DIABLO isoform has been described (26, 30), we therefore referred to this novel isoform as Smac3, which is composed of 195 amino acids (Fig. 1C). Data base search using the BLAST program revealed that Smac3 matches a previously uncharacterized cDNA in the EST data base (accession number AW247557).

The Smac3 protein contains a putative mitochondrial targeting sequence (MTS) and an IAP-binding motif (IBM) (7, 29), which fits the IAP binding tetrapeptide consensus of A(V/T/I)(A/P)(F/Y/I/V/S) (7, 29) (Fig. 1D).

To detect the expression of the Smac3 protein in mammalian cells, we expressed Smac3 in 293T cells. Western blot analysis showed that Smac3 migrated as a doublet (Fig. 1E). The higher molecular weight band indicates the precursor protein, and the lower molecular mass band represents processed Smac3.

To determine the expression pattern of Smac3 in various adult human tissues and human cell lines, RT-PCR was performed. Fig. 1F shows that Smac3 can be detected in almost all tissues and cell lines examined, although expression levels were different. This result indicates that Smac3 is ubiquitously expressed in human tissues.

Subcellular Localization and Translocation of Smac3—Sequence analysis suggested that the NH₂-terminal 55 residues might serve as a putative MTS. To examine whether Smac3 resides in mitochondria in living cells, double immunostaining of Smac3 and cytochrome c was conducted. As expected, the staining pattern of Smac3 was consistent with that of cytochrome c (Fig. 2A, panels a and g). When superimposed, the fluorescence signals of Smac3 and cytochrome c produce a yellow image (Fig. 2A, panel m), suggesting that the two molecules colocalized in the intermembrane space of mitochondria.

To demonstrate further the mitochondrial targeting of Smac3, biochemical fractionation was carried out. As shown in Fig. 2B, Smac3 and cytochrome c were both observed in the mitochondrial fraction, indicative of cofractionation of two proteins. We then transiently transfected a truncated mutant of Smac3 lacking the MTS into HeLa cells, followed by immunostaining. Expressing this mutant resulted in a diffuse cytoplasmic staining of Smac3 (data not shown). When the MTS is fused to GFP, it directed GFP to mitochondria in 293T cells, whereas GFP alone distributed diffusely throughout the cells (data not shown). Thus, this MTS is necessary and sufficient for the mitochondrial targeting of Smac3. This finding also supports the prediction that Smac3 is produced as a precursor molecule and processed by cleavage of the NH₂-terminal MTS by the mitochondrial processing peptidases upon import into mitochondria, generating mature Smac3 (Fig. 1E).

To investigate further whether Smac3 redistributes during...

FIG. 4. Smac3 negatively regulates the protein level of XIAP. A, 293T cells were transiently transfected using cDNAs encoding XIAP along with various amounts of cDNA expressing Smac3. Total cellular lysates were prepared, normalized for total protein content, and analyzed for the expression of XIAP and Smac3 proteins. The blot was stripped and reprobed with an antibody against β-actin to confirm equivalent loading of each sample. IB, immunoblotting. B, cycloheximide experiment as described under “Experimental Procedures.”
**Identification and Characterization of Smac3**

**Fig. 5. Smac3 promotes XIAP degradation through the ubiquitin-proteasome pathway.** A, FLAG-tagged XIAP was transfected with (+) or without (−) Smac3/HA in the absence (−) or presence (+) of Myc-tagged ubiquitin or its mutant K48R. Cell extracts were immunoblotted (IB) with antibodies specific for FLAG, HA, actin, and GFP (to verify equivalent transfection efficiencies). B, 293T cells were transfected with the indicated plasmids in the absence (−) or presence (+) of proteasome inhibitor MG132 (10 μM final concentration, Calbiochem). Cell extracts were subjected to immunoblotting (IB) using anti-Myc and anti-FLAG. The blot was stripped and reprobed with an anti-actin antibody to confirm the loading of samples. C, Myc-XIAP was transfected with HA-tagged ubiquitin together with wild-type (WT) Smac3, mutant (mut) Smac3, or Smac with a COOH-terminal FLAG tag. Immunoprecipitation (IP) and immunoblotting (IB) were carried out with the appropriate antibodies. Ub(n)-XIAP indicates ubiquitinated XIAP. D, 293T cells were transfected with FLAG-tagged XIAP, XIAP H467A, or XIAP 3xBIR, followed by treatment with (+) or without (−) MG132. Cell lysates were subjected to Western blot analysis. WT, wild-type; mut, mutant. E, 293T cells were transfected with the indicated cDNAs. Cell lysates were immunoprecipitated (IP) with anti-FLAG beads followed by immunoblotting (IB) with an anti-ubiquitin antibody. F, cell extracts prepared from 293T cells transfected with the indicated constructs were subjected to immunoblotting. WT, wild type; mut, mutant. Mutant XIAP is XIAP D214S/W310A/E314S. Mutant Smac3 is Smac3A56M. (Ub)n-XIAP, the ubiquitinated species of XIAP.

| A | B | C |
|---|---|---|
| Flag-XIAP | Myc/Ub | HA-Ub |
| Smac3/HA | Myc-XIAP | Myc-XIAP |
| Myc/Ub K48R | WT Smac3/Flag | mut Smac3/Flag |
| IB: α-Flag | IB: α-HA | IB: α-HA |
| IB: α-actin | IB: α-actin | IB: α-actin |

| D | E | F |
|---|---|---|
| WT/mut XIAP-Flag | Ub | Smac3/HA |
| Wild-type | XIAP | mut |
| H467A | XIAP-Flag | + |
| 3xBIR | MG132 | WT |
| IB: α-Flag | IB: α-Flag | IB: α-Flag |
| IB: α-actin | IB: α-actin | IB: α-actin |

| Ub | XIAP-Flag | XIAP H467A-Flag | XIAP 3xBIR-Flag | Smac3 |
|---|---|---|---|---|
| IB: α-Flag | IB: α-Flag | IB: α-Flag | IB: α-Flag | IB: α-Flag |
| IB: α-Ub | IB: α-Ub | IB: α-Ub | IB: α-Ub | IB: α-Ub |
apoptosis, we treated HeLa cells expressing Smac3/HA with several pro-apoptotic agents. Immunostaining analysis demonstrated that cells challenged with H$_2$O$_2$, anti-Fas antibody, staurosporine (STA), etoposide (VP16), and cisplatin (CDDP), exhibited a diffusely cytoplasmic distribution of Smac3 (Fig. 2A, panels b–f) and cytochrome c (Fig. 2A, panels h–l). Overlapping the Smac3 and cytochrome c signals yielded images (Fig. 2A, panels n–r), implying that Smac3, like cytochrome c, was released from mitochondria into the cytosol during apoptosis. The result of subcellular fractionation assay is consistent with the immunostaining finding. The Smac3 protein could be detected in the cytosolic fraction derived from cells exposed to STA, VP-16, and CDDP (Fig. 2B, C, lanes 2–4, respectively), whereas in untreated cells the Smac3 protein was exclusively found in the mitochondrial fraction (Fig. 2C, lane 1). Importantly, Smac3 efflux is susceptible to inhibition following coexpressing Bcl-X$_L$, molecule, as revealed by the appearance of negligible Smac3 in the cytosol from cells challenged with CDDP (Fig. 2C, lane 5) and STA (Fig. 2C, lane 6).

Taken together, these experiments demonstrate that Smac3 can shift from the mitochondria into the cytosol in response to apoptotic stimuli, and this event can be blocked by Bcl-X$_L$.

Interaction of Smac3 with XIAP—Smac3 bears an IBM that is highly conserved in mammalian Smac/DIABLO, HtrA2/Omi, and caspase-9, as well as Drosophila Reaper, Hid, Grim, Sickle, and Jafra2 (7, 41) (Fig. 1D). In each case, IBM is involved in IAP binding. We therefore sought to characterize the interaction between Smac3 and XIAP by using in vitro and in vivo protein-protein interaction assays. GST fusion proteins of different XIAP fragments (GST-BIR1, -BIR2, -BIR3) were generated to map the domains of XIAP responsible for Smac3 binding using far Western blot analysis. As shown in Fig. 3A, Smac3 bound to the BIR2 and BIR3 domains, but not to the BIR1 domain, of XIAP.

To substantiate further that Smac3 directly binds the BIR2 and BIR3 domains, we performed in vitro GST pull-down assays using purified mature Smac3 and GST fusion proteins produced in E. coli. GST-BIR2 and GST-BIR3 captured mature

Fig. 6. Smac/DIABLO does not affect the steady-state level of XIAP. A, Western blotting analysis of endogenous XIAP, Smac/DIABLO, β-actin in Smac$^{+/+}$, and Smac$^{-/-}$ MEFs. B and C, Smac$^{-/-}$ MEFs were transfected with the appropriate control vector, FLAG-tagged Smac (B), or FLAG-tagged Smac3 (C). Whole cell proteins were subjected to Western blotting using anti-XIAP, anti-actin, and anti-FLAG antibodies. D, 293T cells were transiently transfected using cDNAs encoding XIAP along with various amounts of cDNA expressing Smac. Total cellular lysates were prepared, normalized for total protein content, and analyzed for the expression of XIAP and Smac proteins. The blot was stripped and reprobed with an antibody against β-actin to confirm equivalent loading of each sample. E, cycloheximide experiment as described under “Experimental Procedures.” IB, immunoblotting.
Smac3 with the COOH-terminal His tag (Smac3/C-HIS), whereas GST alone or GST-BIR1 failed to do so (Fig. 3B, top panel). This observation further demonstrated that Smac3 directly and specifically binds both the BIR2 and BIR3 domains in vitro. In sharp contrast, the addition of a hexahistidine tag at the NH$_2$ terminus of Smac3 abrogated its ability to bind XIAP (Fig. 3B, bottom panel). A similar result was also produced in the yeast two-hybrid assay (data not shown). In the case of Smac3/C-HIS, the first amino acid Met is followed by the tetrapeptide Ala-Val-Pro-Ile. The Met residue is removed in bacteria in this context (42). Together, the results presented here highlight that freshly exposed NH$_2$-terminal tetrapeptide of mature Smac3 is necessary for XIAP binding.

To verify that Smac3 and XIAP are associated in vivo, coimmunoprecipitation experiments were carried out. Smac3, like Smac/DIABLO, was present in XIAP immunoprecipitates prepared from whole cell lysates of 293T cells cotransfected with Smac3/FLAG and myc-XIAP using anti-Myc-agarose (Fig. 3C, top left panel). To provide further evidence, we did reciprocal immunoprecipitation experiments. As expected, XIAP can be detected in Smac3/FLAG immunoprecipitates (Fig. 3C, bottom left panel). These experiments revealed that Smac3 is able to interact with XIAP in mammalian cells.

The Ala residue within the IBM is highly conserved (Fig. 1D) and has been shown to be essential for the interaction of XIAP with mature Smac/DIABLO (25, 45, 46). To elucidate whether this Ala residue is instrumental in mediating the interaction between mature Smac3 and XIAP, we generated a Smac3 mutant called Smac3A56M, in which Ala was substituted by Met. Subcellular distribution and expression patterns of mutant Smac3 were indistinguishable from those of the wild-type counterpart (data not shown). We tested this mutant for its ability to bind XIAP using coimmunoprecipitation assays. As shown in Fig. 3C, a very faint band could be observed in the anti-Myc immunocomplex only after long exposure of the blot (top left panel), indicating that this mutant is severely impaired in its ability to interact with XIAP. An identical observation was achieved in the reciprocal immunoprecipitation experiment (Fig. 3C, bottom left panel). Thus, the IBM is essential for Smac3 binding to XIAP.

To characterize further the interaction between mutant Smac3 and XIAP, GST pull-down assay was performed. In agreement with our earlier finding, the BIR2 and BIR3 domains could retrieve wild-type Smac3 (Fig. 3D). Smac3A56M retained the ability to bind the BIR2 domain albeit to a substantially less extent than the wild-type Smac3 (Fig. 3D). However, this mutant was unable to bind GST-BIR3 (Fig. 3D). The combined results from the GST pull-down assay and coimmunoprecipitation analysis prompted the conclusion that different BIR domains exhibit distinct affinity to bind Smac3 with the third BIR domain possessing the greatest ability.

To explore whether the amino acids within XIAP that are essential for Smac/DIABLO binding are also involved in the interaction of XIAP with Smac3, we transiently transfected 293T cells with Smac3/HA and different XIAP mutants (33). As shown in Fig. 3E, XIAP D148A/D214S/W310A/E314S nor XIAP D148A/D214S/H343A could be communoprecipitated nearly as efficiently as wild-type XIAP by Smac3 (lanes 1–3). However, neither XIAP D214S/W310A/E314S nor XIAP D148A/D214S/W310A/E314S were detected in the anti-HA immunoprecipitates (Fig. 3E, lanes 4 and 5). Thus, the residues of XIAP essential for Smac/DIABLO binding are also required for the interaction with Smac3.

**Smac3 Negatively Regulates the Steady-state Level of the XIAP Protein**—To investigate the effect of Smac3 on the XIAP protein level, XIAP was transfected into 293T cells with increasing amounts of Smac3, followed by Western blotting analysis. Fig. 4A shows that Smac3 reduced the XIAP abundance in a dose-dependent manner. To evaluate the effect of Smac3 on the half-life of XIAP, we conducted cycloheximide chase experiments. As shown in Fig. 4B, Smac3-expressing cells exhibited a much lower expression of XIAP than control cells expressing XIAP alone. Moreover, the half-life of XIAP was substantially reduced upon expressing Smac3, whereas no significant change in XIAP half-life was observed in the absence of Smac3 expression (Fig. 4B). Of note, Smac3 could be detected in the cytosol.
when overexpressed in 293T cells (data not shown), consistent with previous observations with Smac/DIABLO and HtrA2/ Omi (28, 33, 43, 44). This result demonstrated that Smac3 decreases the stability of the XIAP protein. Nevertheless, mutant Smac3 failed to destabilize the XIAP protein (see below, Fig. 5C), suggesting that Smac3-mediated reduction in XIAP steady-state levels was dependent on Smac3-XIAP interaction.

**Smac3 Promotes XIAP Degradation through the Ubiquitin-Proteasome Pathway**—Given that previous studies have shown that the RING finger domain of XIAP confers E3 ubiquitin ligase activity (18, 20), we sought to determine whether the ubiquitin-proteasome system might be implicated in the Smac3-accelerated decline of XIAP. Degradation of a protein by the ubiquitin (Ub)-proteasome system involves two successive and distinct steps: (i) covalent attachment of poly-Ub chains to the target protein; and (ii) degradation of the tagged protein by the 26S proteasome (47).

We first cotransfected XIAP with either Smac3 or control vector in the presence of ubiquitin. As shown in Fig. 5A, cotransfection with Smac3 attenuated the XIAP protein level. Interestingly, the substitution of UbK48R mutant, in which Lys was replaced by Arg, for wild-type Ub caused no discernible change in XIAP abundance compared with the empty vector. Interestingly, this dominant negative Ub mutant (K48R) has been shown to inhibit the elongation of polymerized Ub chains and proteasomal degradation of several proteins (34, 48, 49). It has been well established that proteasome inhibitors are able to inhibit proteasome-mediated proteolysis and stabilize proteins destined for degradation in the proteasome (50). As shown in Fig. 5B, treatment with proteasome inhibitor MG132 resulted in the accumulation of the XIAP protein, as expected. MG132 could rescue XIAP from Smac3-stimulated degradation in the proteasome (Fig. 5B). Nevertheless, no significant restoration of XIAP was observed following treatment of Smac3-expressing cells with caspase inhibitor N-benzyloxy carbonyl-Val-Ala-Asp-(OMe)-fluoromethyl ketone or calpain inhibitor calpeptin (data not shown). Thus, observations presented here implied that the proteasome contributes to the Smac3-induced XIAP destruction.

To investigate if Smac3 enhances the ubiquitination of XIAP in vivo, we expressed XIAP and Ub with or without Smac3 in 293T cells, followed by immunoprecipitation/Western blot anal-

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**Fig. 8. Smac3 antagonizes the anti-apoptotic activity of XIAP.** A–E, HeLa cells were transfected with pEGFP and the indicated plasmids. After 36 h, HeLa cells were induced to undergo apoptosis by treatment with 30 μM cisplatin (A), 100 μM etoposide (B), 0.1 μM staurosporine (C), 500 μM H2O2 (D), or 1 μg/ml anti-Fas antibody (E). Apoptosis was assessed by morphological analysis as described under “Experimental Procedures.” The results represent the percentage of apoptotic cells relative to the transfected cells and expressed as the mean ± S.D. of three independent experiments. F. 293T cells were transfected with pEGFP and the plasmids indicated. The results were expressed as the mean ± S.D. of two independent experiments. Casp9, caspase-9.
ysis. When XIAP is expressed in 293T cells, a smear of the high molecular weight XIAP protein characteristic of the ubiquitinated species was observed (Fig. 5C, top panel), in line with previous observation that XIAP catalyzes auto-ubiquitination (18). Coexpression of XIAP and Smac3 resulted in a robust increase in XIAP ubiquitination. This result shows that Smac3 greatly accelerated XIAP ubiquitination. Thus, ubiquitin-dependent proteasome-mediated proteolysis is implicated in the destabilization of XIAP by Smac3.

To examine whether the intact RING finger domain is required for the Smac3-stimulated ubiquitination of XIAP, two XIAP mutants in this region were employed. One mutant, XIAP H467A, in which the metal-coordinating residue His-467 was mutated to Ala, was designed to generate an E3-deficient version of XIAP (18). Another mutant termed XIAP 3xBIR lacks the RING domain. As shown in Fig. 5D, MG132 protected wild-type XIAP against degradation, whereas it had no effect on the protein abundance of XIAP H467A or XIAP 3xBIR, suggesting that both mutants are deficient in E3 activity. If the intact RING domain is essential for Smac3-induced XIAP polyubiquitination, the ubiquitination extent of the two mutants, if any, should remain unchanged in this context. Smac3 failed to promote ubiquitination of XIAP H467A or XIAP 3xBIR (Fig. 5E), although Smac3 is capable of enhancing the poly-ubiquitination of wild-type XIAP (Fig. 5E). Accordingly, our experiments indicated that the intact RING domain is required for the Smac3-stimulated ubiquitination of XIAP. It is noteworthy that both mutants retain the ability to interact with Smac3 (data not shown).

We next asked if stable association of XIAP with Smac3 is essential for the Smac3-promoted XIAP ubiquitination. As shown in Fig. 5F, in the presence of Smac3, wild-type XIAP underwent ubiquitination as shown by the appearance of a typical smear indicative of the high molecular mass, polyubiquitinated species on Western blot. Significantly increased ubiquitination was only seen when coexpressing wild-type Smac3 and XIAP, both of which can strongly interact with each other (Fig. 3C). In contrast, the Smac3 mutant, Smac3A56M, which displayed the largely diminished XIAP binding activity (Fig. 3C), was unable to increase XIAP ubiquitination (Fig. 5, C and F). Similarly, XIAP mutant, XIAP D214S/W310A/E314S, which was defective in interacting with Smac3 (Fig. 3E), was not subject to Smac3-accelerated ubiquitination (Fig. 5F). Thus, the ability of Smac3 to accelerate XIAP ubiquitination requires a physical association between XIAP and Smac3.

**Smac3/DIABLO Fails to Affect Ubiquitination and Abundance of XIAP**—To explore whether Smac/DIABLO, like Smac3, might also function to destabilize XIAP, we evaluated a role for Smac/DIABLO in XIAP protein levels in MEFs and in 293T cells. We first examined the endogenous XIAP protein levels in wild-type (Smac3+/+) and Smac-deficient (Smac3−/−) MEFs. Western blot analysis of cell lysates prepared from Smac3+/+ and Smac3−/− MEFs indicates that elimination of Smac/DIABLO did not affect the endogenous XIAP abundance (Fig. 6A).

We then transiently overexpressed Smac/DIABLO or Smac3 in SV40-transformed Smac3−/− MEFs. As shown in Fig. 6B, forced expression of Smac/DIABLO did not alter the steady-state level of XIAP in Smac3−/− MEFs. As anticipated, overexpression of Smac3 led to a significant decrease in the XIAP protein levels in Smac3−/− MEFs (Fig. 6C), consistent with our findings with 293T cells. Notably, mature Smac/DIABLO and Smac3 could be found in the cytosol of Smac3−/− MEFs largely due to forced overexpression (data not shown). These data provided evidence that Smac/DIABLO, unlike Smac3, is defective in the capacity to down-regulate the XIAP abundance. To confirm this observation further, we transfected 293T cells with XIAP and increasing amounts of Smac/DIABLO. Fig. 6D indicates that the XIAP protein levels were not reduced upon overexpressing Smac/DIABLO. In addition, the cycloheximide chase experiments of Fig. 6E show that no discernible change in the half-life of XIAP was found in Smac-expressing 293T cells compared with that in mock-transfected cells. Notably, in marked contrast to Smac3, no noticeable change in XIAP ubiquitination was observed following Smac/DIABLO expression (Fig. 5C). Together, Smac/DIABLO had no effect on XIAP ubiquitination and abundance.

**Smac3 Interferes with Caspase-9 Binding to XIAP**—To test the possibility that Smac3 can compete with caspase-9 for XIAP binding, we performed transient transfection experiments in 293T cells with Myc-tagged XIAP and FLAG-tagged caspase-9, followed by immunoprecipitation/immunoblotting assay. When overexpressed, caspase-9 underwent autoproteolytic processing (Fig. 7A and data not shown), congruent with previous observations (28, 36, 43). XIAP was seen in the anti-FLAG immunoprecipitates (Fig. 7A, lane 5, top panel). The reciprocal immunoprecipitation experiment revealed that caspase-9 was present in the XIAP immunoprecipitates (Fig. 7A, lane 5, middle and bottom panels). Intriguingly, XIAP could physically interact with the processed active form (both p35 and p12), but not with the proform, of caspase-9 (Fig. 7A, lane 5, middle and bottom panels). Overexpression of Smac3 weakened the interaction of XIAP with processed caspase-9 (Fig. 7A, lane 6, top panel). Reciprocal coimmunoprecipitation experiments showed that Smac3 compromised the capacity of XIAP to interact with both the small and large subunits of caspase-9 (Fig. 7A, lane 6, middle and bottom panels). Furthermore, in vitro caspase-3 activation assay showed that Smac3 stimulated activation of caspase-3 as indicated by increased signals of processed caspase-3 (Fig. 7B, lane 3), whereas XIAP inhibited caspase-3 activation (Fig. 7B, lane 1) which could be partially relieved by Smac3 (Fig. 7B, lane 2). Therefore, Smac3 disrupts XIAP interacting with processed caspase-9 and promotes caspase-3 activation.

**Smac3 Antagonizes the Anti-apoptotic Activity of XIAP**—To
further address the functional importance of the physical association between XIAP and Smac3, we carried out cell death assays. As shown in Fig. 8A, cisplatin treatment of HeLa cells harboring control vector led to 50% of transfected cells undergoing apoptosis. Approximately 75% of Smac3-expressing cells became apoptotic, indicating that Smac3 could potentiate apoptosis. Cisplatin-induced cell death was significantly attenuated upon expressing XIAP, in keeping with previously reported observations (13, 31, 32) that XIAP can inhibit apoptosis evoked by apoptotic agents. HeLa cells coexpressing Smac3 with XIAP exhibited a greater increase in apoptosis than those expressing XIAP alone, strongly suggesting that Smac3 was able to counteract the cell death-inhibitory effect of XIAP. To rule out the possibility that this was unique to cisplatin, parallel experiments were carried out with etoposide, staurosporine, H2O2, and death receptor agonist anti-Fas antibody. As anticipated, comparable results were produced (Fig. 8, B–E).

To extend the observation with HeLa cells, we also evaluated whether Smac3 can potentiate apoptosis in 293T cells. Upon coexpression of Apaf-1XL and caspase-9, more than 40% of the 293T cells displayed morphological signs of apoptosis (Fig. 8F), consistent with previous observations (36). This cell killing activity was markedly suppressed following cotransfection of an XIAP-producing plasmid, which could be effectively neutralized by Smac3 expression (Fig. 8F). Of note, expression of Smac3 alone triggered barely detectable cell death in 293T cells (Fig. 8F) and HeLa cells (data not shown). These observations prompted us to conclude that Smac3 functions to counteract apoptosis inhibition afforded by XIAP.

**DISCUSSION**

Caspases have been recognized as the central executors of apoptosis. Once activated, effector caspases target cellular proteins for proteolysis, leading eventually to cell death (5, 6). Because of the destructive potential of active caspases, the activation and activity of caspase are subject to stringent control to avoid dire consequences as the result of spontaneous or inadvertent activation of caspases. Cells have developed several strategies to modulate the activation and activity of caspases. Of critical importance are IAPs (9, 10, 12). In mammals, XIAP is thought to be the most potent member of the IAP family. In order for cells to commit to apoptotic cell death, IAP-mediated suppression of apoptosis must be overcome. Smac3, Smac/DIABLO (23, 24), and HtrA2/Omi (44, 45, 53–55) can antagonize the caspase inhibitory effect of IAPs.

In the current study, we characterize the nature and biochemical as well as functional relevance of the Smac3-XIAP association. We provide firm evidence that Smac3 is able to interact with XIAP in vitro and in vivo. Smac3 is able to interact with both BIR-2 and -3 domains. The IBM of Smac3 is required for mediating the interaction of Smac3 with XIAP. Importantly, freshly exposed NH₂-terminal IBM of mature Smac3 is essential for XIAP binding, in concert with the results seen with Smac/DIABLO, HtrA2/Omi, and other IBM-containing IAP-binding partners (25, 26, 28, 41, 44, 45). By binding to XIAP, Smac3 accelerates ubiquitination and destruction of XIAP, displaces processed caspase-9 from the XIAP-caspase-9 complex, and antagonizes the apoptosis-inhibitory effect of XIAP. We have clearly demonstrated that Smac3 is functionally additive to, but independent of, Smac/DIABLO (23, 24) or Smac-S/-β (26, 30).

Smac3 exhibits parallels with Smac/DIABLO (23, 24, 27, 43) in its biochemical and biological functions, such as the ability to interact with XIAP as well as c-IAP1 and c-IAP2, to liberate processed caspase-9 by disrupting the XIAP-caspase-9 association and to antagonize the apoptosis inhibiting activity of XIAP. In contrast, Smac-S/-β, an isoform of Smac/DIABLO, resides in the cytosol and does not bind IAPs in cells, but it possesses apoptosis-potentiating function (30).

One of the most important findings in our study is that Smac3 is able to reduce XIAP protein stability. Our experiments suggest that Smac3-triggered XIAP destruction could be attributed, at least in part, to Smac3-accelerated XIAP ubiquitination. The molecular basis by which Smac3 promotes XIAP ubiquitination remains to be elucidated. In addition, it will be intriguing to investigate the possibility that Smac3 might be involved in suppression of XIAP protein synthesis. In stark contrast to Smac3, Smac/DIABLO is incapable of promoting the ubiquitination and turnover of XIAP. The difference in their ability to facilitate the ubiquitination and destruction of XIAP reflects the functional diversity of this family. Our finding is consistent with the notion that alternative splicing of mRNA precursor is a versatile mechanism for regulating gene expression and producing functionally diverse proteins from a single gene (56).

**In vitro** ubiquitination assay indicates that recombinant Smac/DIABLO was poly-ubiquitinated by XIAP in vitro (58). A recent study demonstrates that c-IAP1 and c-IAP2, but not XIAP, can stimulate poly-ubiquitination and destruction of Smac/DIABLO in 293T cells (51). Whether Smac3 is ubiquitinated by the IAP family of E3 ubiquitin ligase is under investigation.

An additional mechanism of action underlying XIAP inhibition of caspases is emerging from a recent finding (20) that XIAP might promote the ubiquitination and decay of caspase-3. In addition, Drosophila IAP1 (DIP1) also possesses E3 activity that mediates ubiquitination of itself and the Drosophila caspase Droc (59). Efficient ubiquitination of caspases is contingent upon the specific association of IAP with caspases (20, 59). Interestingly, our work and the work of others have shown that Smac3 and the Drosophila homologues Reaper, Grim, and Hid act to accelerate the ubiquitination and destruction of IAPs (51, 52, 57, 60, 61). These events likewise require the stable interaction between IBM-containing proteins and IAPs. Our study has shown that binding of XIAP to Smac3 and caspase-9 is mutually exclusive. Importantly, our present study and the work of others (28, 43) have shown that XIAP can only interact with the activated form of caspase-9. By binding to XIAP, Smac3 displaces active caspase-9 or prevents XIAP from binding active caspase-9. Thus, Smac3-stimulated ubiquitination of XIAP is expected to occur only after they physically interact with each other, when activated caspase-9 has been liberated from XIAP. As a result, free caspase-9 will activate caspase-3 and promote apoptosis. Indeed, we found that Smac3 stimulates caspase-3 activation and potentiates apoptosis elicited by diverse apoptotic agents. Taken together, we currently favor a
model in which Smac3-XIAP interaction leads to XIAP destruction and to the liberation of caspase from XIAP-mediated inhibition, degradation, or inactivation, thereby enhancing apoptosis (see Fig. 9).

Recent efforts suggest that elevated XIAP protein levels can be detected in many human cancers and leukemias (62, 63). A link has been established between elevated XIAP levels and poor prognosis for patients with cancer or leukemias (62). Moreover, elevated XIAP protein levels render cancer cells resistant to chemotherapy, making XIAP a possible therapeutic target (63, 64). In the study presented here, we provide novel insight into the regulation of XIAP stability by Smac3. Although there is much more to learn, advances in our understanding of the regulation of XIAP stability by Smac3 may provide new avenues for therapeutic benefit of some diseases including cancer.

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