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Smac, a Mitochondrial Protein that Promotes Cytochrome c–Dependent Caspase Activation by Eliminating IAP Inhibition

Chunying Du, Min Fang, Yucheng Li, Lily Li, and Xiaodong Wang*
Howard Hughes Medical Institute and Department of Biochemistry
University of Texas Southwestern Medical Center at Dallas
Dallas, Texas 75235

Summary

We report here the identification of a novel protein, Smac, which promotes caspase activation in the cytochrome c/Apaf-1/caspase-9 pathway. Smac promotes caspase-9 activation by binding to *i*nhibitor of *a*poptosis *p*roteins, IAPs, and removing their inhibitory activity. Smac is normally a mitochondrial protein but is released into the cytosol when cells undergo apoptosis. Mitochondrial import and cleavage of its signal peptide are required for Smac to gain its apoptotic activity. Overexpression of Smac increases cells' sensitivity to apoptotic stimuli. Smac is the second mitochondrial protein, along with cytochrome c, that promotes apoptosis by activating caspases.

Introduction

One of the key regulatory steps for apoptosis is the activation of caspases, the intracellular cysteine proteases that cleave substrates after aspartic acid residues (reviewed by Thornberry and Lezebnik, 1998). Existing as inactive zymogens in living cells, apoptotic caspases become activated during apoptosis either through autocatalysis or cleavage by other caspases. Active caspases then cleave many important intracellular substrates, leading to the characteristic morphological changes associated with apoptotic cells. These changes include chromatin condensation, DNA fragmentation into nucleosomal fragments, nuclear membrane break down, externalization of phosphatidylserine, and formation of apoptotic bodies that are readily taken up by phagocytosis (reviewed by Thornberry and Lezebnik, 1998).

One major caspase activation cascade is triggered by cytochrome c released from the intermembrane space of mitochondria. Upon receiving apoptotic stimuli, such as serum deprivation, activation of cell surface death receptors, and excessive damage of DNA, the outer membrane of mitochondria becomes permeable to cytochrome c (reviewed by Reed, 1997). Once released to the cytosol, cytochrome c binds to Apaf-1 in a 2:1 ratio forming an oligomeric Apaf-1/cytochrome c complex (apoptosome) in the presence of dATP or ATP (Purring et al., 1999; Zou et al., 1999). This oligomerized Apaf-1/cytochrome c complex then recruits the initiator caspase of this pathway, procaspase-9 and induces its autoactivation (Li et al., 1997b; Zou et al., 1999). Caspase-9 in turn activates downstream caspases including caspase-3, -6, and -7 (Li et al., 1997b; Srinivasula et al., 1998).

Proteins of the Bcl-2 family are major regulators of the mitochondria-initiated caspase activation pathway (reviewed by Adams and Cory, 1998). The anti-apoptotic members of this family, including Bcl-2 and Bcl-X_L, preserve mitochondrial integrity and prevent the release of cytochrome c in the presence of apoptotic stimuli (Kluck et al., 1997; Yang et al., 1997). Conversely, the proapoptotic members of this family such as Bad, Bax, Bid, and Bim move from other cellular compartments to mitochondria in response to apoptotic stimuli and promote cytochrome c release (reviewed by Gross et al., 1999).

*I*nhibitors of *a*poptosis *p*roteins, IAPs, are another family of proteins that regulate the cytochrome c/Apaf-1 caspase activating pathway (reviewed by Deveraux and Reed, 1999). Initially identified in the genome of a baculovirus as suppressors of apoptosis in host cells, endogenous IAPs have been found in a variety of organisms including seven in mammals so far (Crook et al., 1993; Deveraux and Reed, 1999). The antiapoptotic activity of IAPs has been attributed to the conserved *b*aculovirus *I*AP *r*epeat (BIR) domain (Takahashi et al., 1998). Three human IAPs, XIAP, c-IAP-1, and c-IAP-2 have been shown to bind procaspase-9 and prevent its activation (Deveraux et al., 1998). These IAPs can also directly bind and inhibit active caspases (Deveraux et al., 1998). In *Drosophila*, the anti-apoptotic activity of IAPs is countered by Reaper, Hid, and Grim (Vucic et al., 1998; McCarthy and Dixit, 1998; Goyal et al., 2000). Mammalian homologs of Reaper, Hid, and Grim have not been identified.

Despite significant progress in dissecting the cytochrome c–mediated apoptotic pathway, several puzzling experimental observations remain to be explained. First of all, certain types of cells are responsive to microinjected cytochrome c while others are not (Li et al., 1997a). Second, healthy neurons do not respond to microinjected cytochrome c unless they have been subjected to NGF withdrawal for a certain period of time, gaining the status of "competent to die" (Deshmukh and Johnson, 1998). All these data suggest that there must be one or more additional caspase-activating protein factors that are potentially regulated by the Bcl-2 family of proteins.

In the current study, we report the identification, purification, molecular cloning, and characterization of a novel protein that promotes cytochrome c/Apaf-1-dependent caspase activation. This protein acts by opposing the inhibitory activity of IAPs. Like cytochrome c, this new protein is normally located in mitochondria and released into the cytosol when cells undergo apoptosis. We named this protein Smac, for the *s*econd *m*itochondria-derived *a*ctivator of *c*aspase. Smac is likely the functional equivalent of *Drosophila* Reaper, Hid, and Grim in terms of IAP neutralization and is the

*To whom correspondence should be addressed (e-mail: xwang@biochem.swmed.edu).

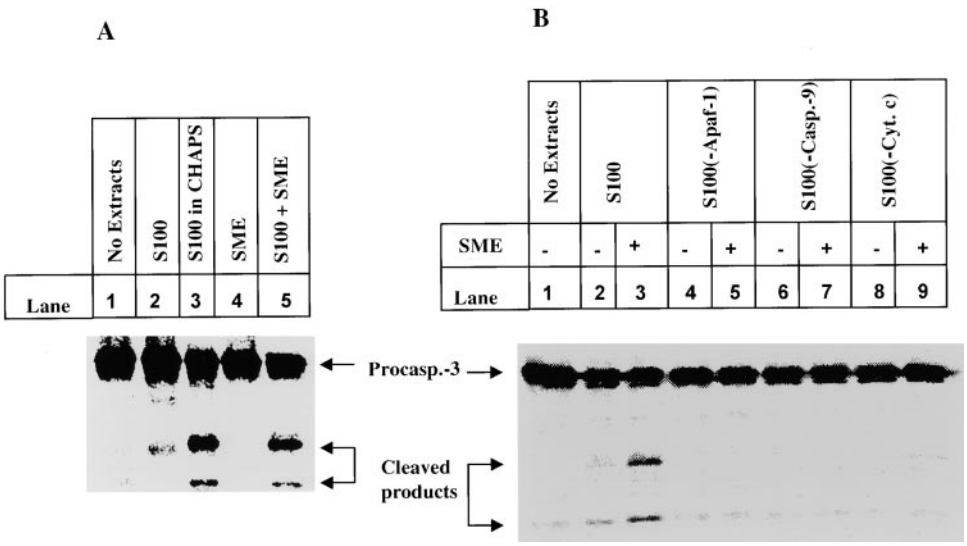

Figure 1. Identification of Smac

HeLa cell extracts (S-100) and solublized membrane extracts (SME) were made as described in Experimental Procedures. S-100 extracts in CHAPS were prepared in the same way as S-100 in buffer A except that 0.5% CHAPS was added to buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM DTT, and 0.1 mM PMSF).

(A) A 12 μl aliquot of buffer A (lane 1), S-100 (30 μg, lane 2), S-100 in CHAPS (30 μg, lane 3), 2 μl aliquot of SME alone (lane 4), or 12 μl aliquot of S-100 plus 2 μl of SME (lane 5) was incubated with 1 μg of purified horse heart cytochrome c, 1 mM dATP, 1 mM additional $MgCl_2$, and 2 μl aliquot of in vitro translated, ^{35}S-labeled procaspase-3 at 30°C for 1 hr in a final volume of 20 μl in buffer A. The samples were then subjected to 15% SDS-PAGE and transferred to a nitrocellulose filter. The filter was exposed to a Phosphorimaging plate for 12 hr at room temperature.

(B) Apaf-1, procaspase-9, and cytochrome c were individually immunodepleted from S-100 extracts as described in Li et al., 1997b. Twelve microliter aliquots of buffer A (lane 1), S-100 (30 μg, lanes 2–3); or S-100 extracts immunodepleted of Apaf-1 (lanes 4–5), procaspase-9 (lanes 6–7), or cytochrome c (lanes 8–9) were incubated alone (lanes 2, 4, 6, and 8), or with 2 μl SME (lanes 3, 5, 7, and 9) in the presence of 1 mM dATP, 1 mM additional $MgCl_2$, and 2 μl aliquot of in vitro translated, ^{35}S-labeled procaspase-3 at 30°C for 1 hr in a final volume of 20 μl in buffer A. The samples were then subjected to 15% SDS-PAGE and transferred to a nitrocellulose filter. The filter was exposed to a phosphorimaging plate for 12 hr at room temperature.

missing link in the mitochondria-initiated caspase activation pathway.

Results

Identification of Smac

In the course of in vitro biochemical experiments on caspase-activating activities, we noticed that cell extracts prepared in a buffer containing detergents had significantly greater ability to activate caspase-3 as compared with extracts prepared without detergent (Figure 1A, lanes 2 and 3). Reasoning that there could be a membrane-bound factor that promotes caspase-3 activation in addition to the known water-soluble factors Apaf-1, cytochrome c, and procaspase-9, we obtained a crude membrane pellet that contained mitochondria as well as other membranes and organelles. We solublized this pellet in a detergent and added it back to the 100,000 × g supernatant of soluble extracts, (S-100). As shown in Figure 1, the detergent solublized "membrane" extract (SME) did not have caspase-3 activating activity by itself (lane 4), but it significantly stimulated caspase-3 activation when added to the S-100 fraction (lanes 2 and 5). Simply adding the same amount of detergent to the S-100 had no effect on caspase-3 activation (data not shown). These experiments indicated a factor in extracts from the membrane fraction that promotes caspase-3 activation in the presence of the water-soluble fraction.

Our previous biochemical fractionation and reconstitution experiments were based solely on S-100 extracts. Fractionating the active factors in the S-100 extracts allowed us to purify three proteins that are necessary and sufficient to reconstitute the caspase-3 activation. These proteins are Apaf-1, a 130 kDa protein that is the mammalian homolog of the CED-4 protein in *C. elegans* (Yuan and Horvitz, 1992; Zou et al., 1997); procaspase-9 (Li et al., 1997b), and cytochrome c, which was released into the S-100 during the homogenization procedure (Liu et al., 1996). To examine whether the detergent-solublized membrane extracts contain proteins that substitute for Apaf-1, or cytochrome c, or procaspase-9, we first immunodepleted these three proteins individually from the S-100 extracts and added the detergent-solublized membrane fraction to these depleted extracts. As shown in Figure 1B, no caspase-3 activation was observed when we used any of these depleted extracts (lanes 4, 6, and 8). This observation suggested that this detergent-soluble factor cannot substitute for Apaf-1, cytochrome c, or procaspase-9. Furthermore, the activity of this factor requires the presence of Apaf-1, cytochrome c, and procaspase-9. We named this activity Smac for the *s*econd *m*itochondria-derived *a*ctivator of *c*aspase, because this protein is normally located in mitochondria (see below).

Purification of Smac

Using the reconstitution experiment described in Figure 1A as an assay, we fractionated the membrane extracts

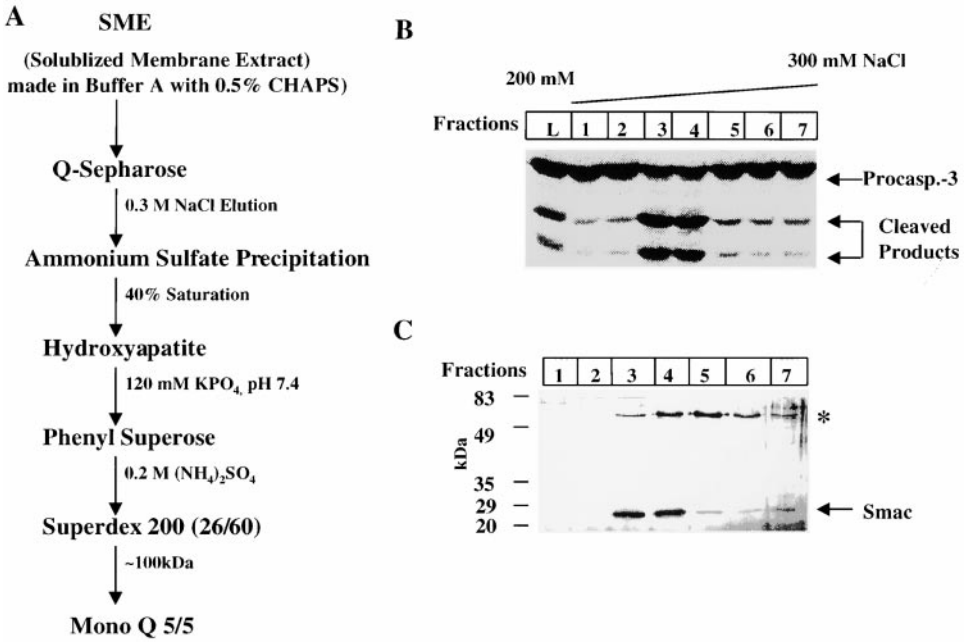

Figure 2. Purification of Smac

(A) Diagram of the purification scheme for Smac. See Experimental Procedures for details.

(B) Aliquots of 2 μl of loading material on Mono Q column (L), or 2 μl of Mono Q column fractions (1–7) were incubated with aliquots of 12 μl (30 μg) of S-100, 1 μl (1 μg) of cytochrome c, 1 mM dATP, 1 mM additional $MgCl_2$, and 3 μl of in vitro translated, ^{35}S-labeled procaspase-3 at 30°C for 1 hr in a final volume of 20 μl in buffer A. After 1 hr incubation, the samples were subjected to 15% SDS-PAGE and transferred to a nitrocellulose filter. The filter was exposed to a phosphorimaging plate for 16 hr at room temperature.

(C) Aliquots of 30 μl of the indicated Mono Q column fractions were subjected to 15% SDS-PAGE and the gel was subsequently stained with silver using a Silver Stain Plus kit from Bio-Rad. The 25-kDa Smac (arrow) and a 50 kDa copurified protein (asterisk) were denoted.

from cultured HeLa cells solublized in 0.5% CHAPS. The activity of Smac was scored for its ability to stimulate caspase-3 activation in the presence of S-100. Although Smac required detergents for its initial release from the membrane/organelle pellet, the protein itself was thereafter soluble in aqueous buffer. The purification was therefore carried out in the absence of detergent. The purification of Smac was achieved through a six-step procedure (Figure 2A). The last step of purification, a Mono Q column chromatographic step, is shown in Figure 2B. The Smac activity was eluted from the Mono Q column as a single peak by ~250 mM NaCl (Fractions 3 and 4). The same protein fractions were subjected to SDS-PAGE followed by silver staining (Figure 2C). A protein band that migrated at 25 kDa correlated with the Smac activity. Native Smac runs ~100 kDa in a gel filtration column, suggesting that Smac is a multimeric protein in its native form (data not shown). A contaminating protein of 50 kDa (asterisk) that was also observed in the active fraction did not correlate with the activity peak (Figure 2C).

Molecular Cloning of Smac

The 25 kDa protein from fractions 3 and 4 eluted from the Mono Q column was subjected to tryptic digestion. Four resulting peptides were purified by reverse-phase HPLC and sequenced in an automated sequencer by the Edman degradation method. Data base searches revealed that these peptide sequences match a previously uncharacterized cDNA in the EST data base (T53449). Using the EST sequence as a probe, a cDNA encoding Smac was cloned from a HeLa cell cDNA library and the deduced amino acid sequence is shown in Figure 3A. An in-frame stop codon was found before the initiating methionine, indicating that the cDNA encodes the full-length coding region of Smac (data not shown). The protein sequence of Smac was used to search against the protein data base (GenBank and Prosite) and no known protein sequence or motif was found to resemble Smac.

To test the tissue distribution of Smac, Northern blot analysis was performed using mRNA blots from multiple human adult tissues (Figure 3B). In all tissues examined, a predominant mRNA of ~1.5 kb was detected, indicating ubiquitous expression of Smac. Expression of Smac mRNA was highest in adult testis and high in heart, liver, kidney, spleen, prostate, and ovary. Smac mRNA expression is low in brain, lung, thymus, and peripheral blood leukocytes.

Smac Stimulates Caspase Activation by Removing the Inhibition of IAPs

Smac could promote caspase activation through two different mechanisms: eliminating an inhibitor(s) in the S-100, or directly acting on Apaf-1, cytochrome c, and procaspase-9 to enhance the activation efficiency of caspases. To differentiate these two possibilities, we first purified recombinant Smac fused with a histidine tag at its COOH terminus from a baculovirus expression system (Figure 4B). This fusion protein is fully functional in promoting caspase-3 activation in S-100 extracts (Figure 4A, lane 3). We then coupled this protein to nickel

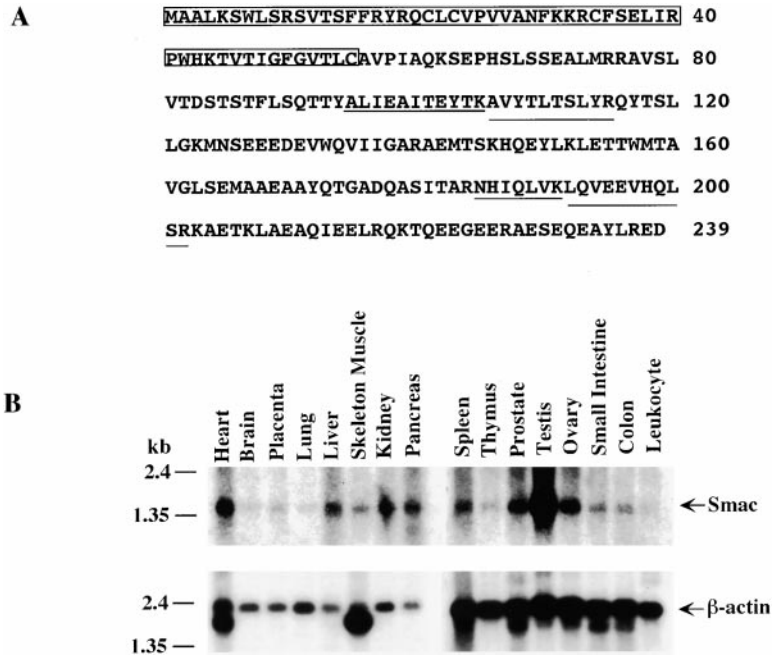

Figure 3. Amino Acid Sequence of Human Smac and Distribution of Smac mRNA in Human Tissues

The cloning of cDNA encoding Smac was described in Experimental Procedures.

(A) Protein sequence of Smac as predicted from the cDNA sequence. Amino acid residues are numbered on the right. Four tryptic peptides that are found in the purified Smac are underlined. The mitochondrial import signal sequence is boxed.

(B) Distribution of Smac mRNA in human tissues. The messenger RNA of Smac was detected in poly(A)$^+$ RNA from the indicated tissues (2 μg/lane) as described in Experimental Procedures. The filters were exposed to an X-ray film with an intensifying screen at −80°C for 5 days (upper panel). The same filters were subsequently stripped and hybridized with human β actin cDNA probe and exposed to film for 2 hr at −80°C with an intensifying screen (lower panel).

beads and incubated the Smac-coupled nickel beads with the S-100 extracts. After incubation, the beads were removed by centrifugation and the supernatants were tested for caspase-3 activation. As shown in Figure 4A, the supernatant from the S-100 treated with Smac-coupled nickel beads showed dramatically increased caspase-3 activation compared to that from the S-100 treated with uncoupled nickel beads (Figure 4A, lanes 1 and 2). These extracts were also subjected to Western blot analysis using an antibody against Smac and no difference in the amount of Smac was detected, indicating that the increased caspase-3 activation was not due to the leakage of Smac from the nickel beads (data not shown). When free Smac protein was again added to these extracts, only the extracts treated with uncoupled nickel beads were still responsive to Smac, whereas the caspase-3 activation was already high in the extracts treated with Smac-coupled nickel beads and the activity could not be stimulated further (Figure 4A, lanes 3 and 4). These results suggested that Smac stimulated caspase-3 activation by removing inhibitors from the S-100 extracts.

Since the mouse Smac ortholog DIABLO has been shown to specifically interact with IAPs (Verhagen et al., 2000 [this issue of *Cell*]), we tested whether the inhibitors that were removed by Smac-coupled nickel beads from the S-100 extracts were IAPs using Western blot analysis. As shown in Figure 4C, c-IAP1, c-IAP2, XIAP, and survivin were readily detected in the Smac-coupled nickel beads after the beads were incubated with S-100. None of these IAPs were detected in the uncoupled nickel beads after the same incubation. Consistently, c-IAP2 and XIAP were depleted from the S-100 after incubation with Smac-coupled nickel beads. We could not unambiguously identify c-IAP1 and survivin in the supernatants due to their lower abundance and/or cross-reactive contaminating bands (data not shown).

To further confirm that Smac promotes caspase-3 activation by removing inhibitors rather than directly acting on Apaf-1, procaspase-9, and cytochrome c, we added Smac to the reconstituted caspase-3 activating system consisting of purified recombinant Apaf-1, recombinant procaspase-9, and cytochrome c. As shown in Figure 5, adding Smac to the reconstituted caspase-3 activating system had minimal effects compared to what was observed in the S-100 (lanes 2 and 3). However, if we added a recombinant human c-IAP1 protein to the reaction, caspase-3 activation was completely inhibited (lane 4), and the inhibition was eliminated in the presence of Smac (lane 5).

Smac Increases Sensitivity of Cells to UV Irradiation In Vivo

To study the role of Smac in vivo, we transiently transfected HeLa cells with a cDNA encoding full-length Smac with a FLAG tag at the C terminus of the protein. We then induced apoptosis by exposing the cells to UV irradiation. As shown in Figure 6, transiently expressed Smac did not induce caspase-3 activation and apoptosis without an apoptotic stimulus. The FLAG-tagged Smac was exclusively localized in mitochondria (data not shown). After 2 s of UV irradiation, ~60% of Smac transfected cells showed signs of apoptosis as measured by the condensation of their chromatin (D). Active caspase-3 was also observed in extracts from these cells by western blot analysis and enzymatic assay as shown in (B) and (C), respectively. In contrast, only ~20% of the vector transfected cells showed signs of apoptosis under the same condition and little cleavage or active caspase-3 was detected. With longer exposure to UV, more apoptosis and caspase-3 activity were observed in the absence of transfected Smac and the difference between Smac and vector transfected cells became smaller.

Smac Is a Mitochondrial Protein That Is Released to Cytosol During Apoptosis

To pinpoint the exact location of Smac in cells, we generated a polyclonal antibody against recombinant Smac

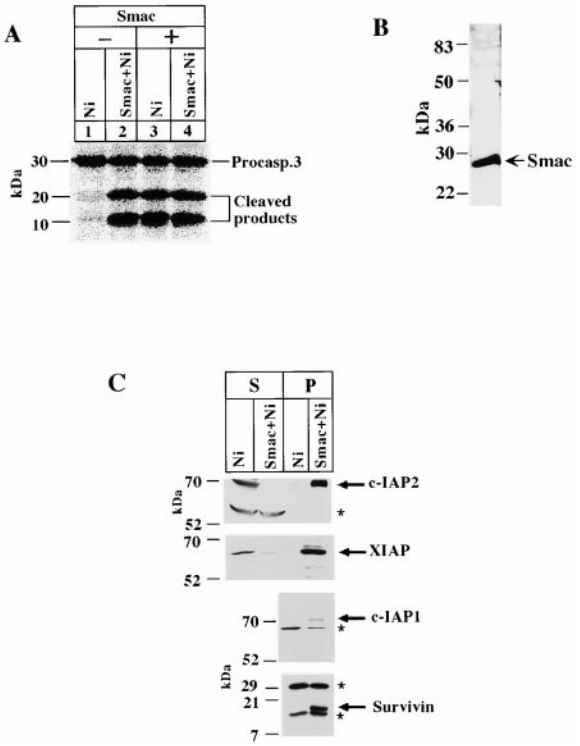

Figure 4. Smac Promotes Caspase Activation by Removing IAPs From S-100

S-100 was prepared from HeLa cells as described in Experimental Procedures. Recombinant Smac was prepared from a baculovirus expression system as described in Experimental Procedures. The purified Smac (10 μg) was coupled to 200 μl of nickel beads by incubating at room temperature for 1 hr in a rotator. The beads were subsequently washed 5 times with buffer A without EDTA and EGTA and used in the following experiments.

Aliquots of 400 μl HeLa S-100 (1.2 mg) were incubated either with 20 μl of uncoupled nickel beads or with 20 μl of Smac-coupled nickel beads for 1 hr at room temperature. The beads were then collected by centrifugation.

(A) Aliquots of 10 μl supernatants from S-100 treated with uncoupled nickel beads (lane 1) or Smac-coupled nickel beads (lane 2) were incubated with 2 μl of in vitro translated, ^{35}S-labeled procaspase-3 in the presence of 1 mM additional $MgCl_2$, 1 μg of horse heart cytochrome c, and 1 mM dATP at 30°C for 1 hr. In lanes 3 and 4, 100 ng of purified Smac was added to these supernatants and the caspase-3 activation reactions were carried out as described above. The samples were then subjected to 15% SDS-PAGE followed by transferring onto a nitrocellulose filter and exposed to a Phosphorimager screen for 12 hr.

(B) The Coomassie blue staining of 1.5 μg of affinity purified recombinant Smac.

(C) The uncoupled nickel and Smac-coupled nickel beads were collected by centrifugation after incubating with the S-100 extracts. After washing 5 times, the protein bound to the beads was eluted in 100 μl of 1× SDS-PAGE loading buffer. Ten microliter aliquots of the eluted materials (P) as well as 30 μl aliquots of the supernatant generated as in (A) (S) were analyzed by 10% (c-IAP1, c-IAP2, and XIAP) or 15% (survivin) SDS-PAGE. The gels were subsequently transferred to nitrocellulose filters and probed with polyclonal antibodies against human c-IAP2, XIAP, c-IAP1, and survivin as indicated. The migration of these proteins on SDS-PAGE was determined by comparing to positive controls provided by the manufacturer (R&D systems, data not shown). For c-IAP1 and survivin, only the results from the pellets were shown because the bands in the supernatants could not be unambiguously identified. Asterisk denotes cross-reactive protein bands.

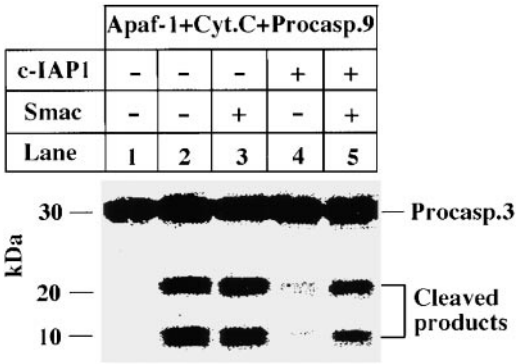

Figure 5. Smac Opposes c-IAP1 Inhibition of Caspase-3 Activation

Recombinant Apaf-1 and procaspase-9 were generated and purified as described in Zou et al., 1999. Recombinant Smac and c-IAP1 were generated as described in Experimental Procedures. Aliquots of 100 ng Apaf-1, 15 ng procaspase-9, and 200 ng cytochrome c were incubated alone (lane 2), or with an aliquot of 300 ng Smac (lane 3), or with 100 ng c-IAP1 (lane 4), or with 300 ng Smac plus 100 ng c-IAP1 (lane 5). The reactions were carried out in the presence of 10 μM dATP, 75 mM KCl, 1 mM additional $MgCl_2$, and 3 μl of in vitro translated, ^{35}S-labeled procaspase-3 at 30°C for 1 hr in a final volume of 20 μl. After 1 hr incubation, the samples were subjected to 15% SDS-PAGE and transferred to a nitrocellulose filter. The filter was exposed to a Phosphorimaging plate for 16 hr at room temperature. Lane 1 is a reaction without incubation.

expressed in bacteria (amino acids 95–239) and used this antibody to locate Smac by immunostaining and biochemical fractionation. As shown in Figure 7A, immunostaining of living cells with the Smac antibody revealed a punctate pattern of mitochondrial localization (middle), colocalizing with cytochrome c (left). The apparent nuclear staining of Smac was due to background staining by this polyclonal antibody since it also showed up when we used preimmune serum (data not shown). However, when cells underwent apoptosis induced by UV irradiation, Smac and cytochrome c staining both changed from a punctate mitochondrial pattern to a more diffuse cytosolic pattern. Both proteins started to show diffuse cytosolic staining 2 hr after UV irradiation and this pattern became more pronounced after 8 hr. At this point, cells showing the most diffuse distribution of cytochrome c and Smac also demonstrated condensed chromatin as measured by DAPI staining (right).

To further confirm the immunostaining results, we isolated mitochondrial and cytosolic fractions from normal or UV irradiated HeLa cells. As shown in Figure 7B, in nonirradiated cells, Smac and cytochrome c were exclusively localized in mitochondrial fractions. Two hours after UV irradiation, cytochrome c and Smac were both observed in the cytosolic fraction. The cytosolic Smac and cytochrome c continued to increase up to 8 hr with a corresponding decrease in the mitochondrial compartment.

Maturation of Smac Requires the Cleavage of Its Signal Peptide

The full-length cDNA of Smac encodes an open reading frame of 239 amino acids, predicting a protein with a molecular weight of 27 kDa. However, Smac purified from HeLa cells is only about 25 kDa (Figure 2B). A helical wheel analysis revealed that the N-terminal region of

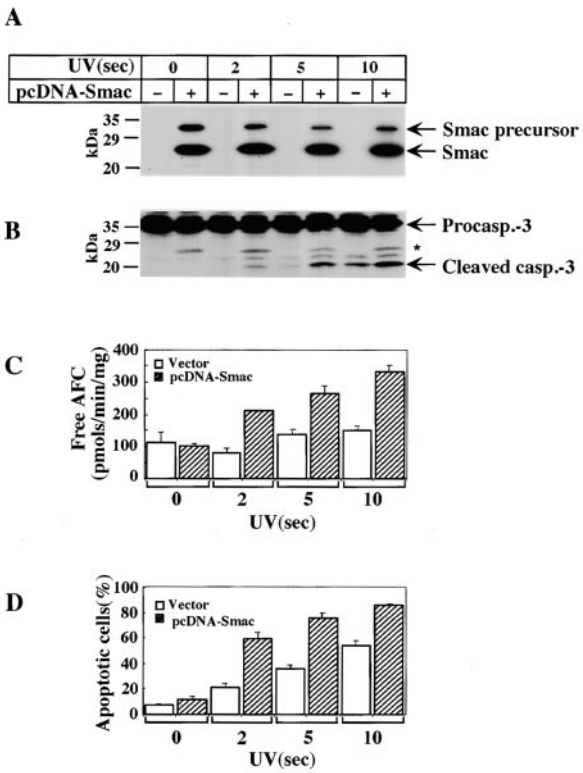

Figure 6. Smac Promotes Caspase Activation and Apoptosis in Vivo

(A) and (B) HeLa cells were transfected with pcDNA-Smac or pcDNA3.1(−) vector as described in Experimental Procedures. 16 hr after transfection, cells were treated with UV irradiation for the indicated length of time. Cells were then harvested 6 hr after UV treatment and extracts were prepared using buffer A containing 0.5% CHAPS. Aliquots of 20 μg protein were subjected to 15% SDS-PAGE followed by transferring to nitrocellulose filters. The first filter was probed with anti-Flag M2 antibody (Sigma, 1:5000 dilution). The second filter was probed with a mouse monoclonal antibody against caspase-3 (Transduction Laboratories, 1:2000 dilution). The antigen/antibody complexes were visualized by an ECL method. Both filters were exposed to X-ray film for 1 min. Asterisk denotes the cross-reactive Smac with the monoclonal anti-caspase-3 antibody.

(C) The spectrofluorometric assay of caspase-3 was carried out as previously described by MacFarlane et al., 1997. Aliquots of 8 μg of S-100 prepared as in (A) were assayed in 150 μl of reaction mixture containing final concentrations of 0.1 mM HEPES (pH 7.4), 2 mM DTT, 0.1% (w/v) CHAPS, and 1% (w/v) sucrose. The reactions were started by adding a caspase-specific fluorogenic substrate benzyloxycarbonyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin-Z-DEVD-AFC (Enzyme System) to the final concentration of 20 μM and continued at 37°C for 30 min. Liberation of AFC from the substrates was monitored continuously using excitation/emission wavelength pairs of 400/505 nm. The caspase-3 activity was calculated and expressed as pmol/min/mg protein.

(D) On day 0, cells were set up the same as in (A) and (B). On day 1, cells were transfected with 1 μg of pEGFP-N plasmid (Clontech) plus either 4 μg of pcDNA-Smac or 4 μg of vector control per dish as in (A). 16 hr after transfection, the cells were treated with UV as in (A). Cells were then harvested and stained with 2 μM Hoechst 33342 in serum-free medium for 10 min at room temperature. After washing three times with serum-free medium, cell suspensions were dropped onto a slide. The green cells that also showed condensed chromatin were counted by changing the excitation from blue light (excited GFP) to UV (excited Hoechst 33342). The results were expressed as the percentage of green cells with condensed chromatin in total green cells.

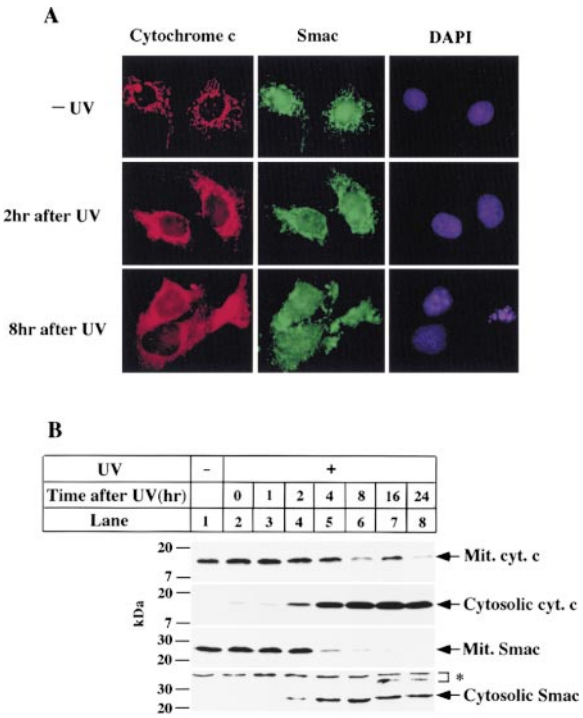

Figure 7. Translocation of Smac During Apoptosis Induced by UV Irradiation

(A) Immunostaining of Smac and cytochrome c was done as described in Experimental Procedures. After UV irradiation, cells were then switched to fresh medium and continued to culture for 2 or 8 hr as indicated. Cells were then fixed and immunostained with a monoclonal antibody against cytochrome c (left panel), a polyclonal antibody against Smac generated as described in Experimental Procedures (middle panel), and 1 μg/ml DNA staining dye DAPI (right panel). The cells were then observed under a Nikon ES 8000 fluorescent microscopy.

(B) On day 0, HeLa cells were set up at 5×10^6 per 150 mm dishes. On day 1, cells were treated with UV as described in (A). After further incubation for the indicated times, cells were harvested and mitochondrial and cytosolic fractions were prepared as described in Experimental Procedures. Aliquots of 10 μg of proteins from the cytosol and 5 μg of protein from the mitochondria were subjected to 15% SDS-PAGE and transferred to a nitrocellulose filter. The filters were probed with antibodies against cytochrome c and Smac. The antigen/antibody complexes were visualized by an ECL method. The filters were exposed to X-ray films for 1 min.

Smac resembles a typical mitochondrial targeting signal sequence: an amphipathic α helix with positively charged amino acid sidechains on one side (Arg-10, Arg-17, Arg-19, Lys-31, Lys-32, Arg-33, and Arg-40) (reviewed by Schatz and Dobberstein, 1996; Figure 3A). To map the exact cleavage site in the mature Smac, we used Edman degradation to determine the N-terminal sequence of mature Smac purified from both HeLa cells and Sf-21 cells. Unfortunately, the N-terminal end of Smac from HeLa cells was blocked (data not shown). However, direct sequencing analysis identified the N terminus of mature Smac purified from Sf-21 cells as amino acid 56 (Figure 3A). Since the 25 kDa recombinant Smac expressed in Sf-21 cells is fully active (see Figure 4), amino acids 1–55 likely represent the mitochondrial targeting signal peptide that is subsequently cleaved after mitochondrial import. Indeed, this polypeptide was

sufficient to target green flourescent protein (GFP) to mitochondria when fused to the N terminus of GFP (data not shown).

Full-length Smac with its signal peptide intact was also observed by Western blot analysis in Sf-21 cells infected with a baculovirus vector encoding Smac. During subsequent purification, the full-length Smac did not show any activity, suggesting that the cleavage of signal peptide inside mitochondria is a required step for Smac to gain its apoptotic activity (data not shown).

Discussion

Smac Promotes Procaspase-9 Activation by Countering IAPs

The data in this paper demonstrate that Smac, a novel mitochondrial protein, is released from mitochondria during apoptosis, whereupon it neutralizes the inhibitory activity of IAPs.

Smac seems to function as a general IAP neutralizer by binding to these proteins (Figure 4 and Verhagen et al., 2000 [this issue of *Cell*]). Multiple IAP molecules, including c-IAP1, c-IAP2, XIAP, and survivin are able to bind Smac. Since these IAPs are the only ones that we checked, it is possible that Smac binds other IAPs as well. The binding of Smac to IAPs could presumably prevent their interactions with caspases and therefore release their inhibition on caspase activation and caspase activities.

c-IAP1 and c-IAP2 have also been found to interact with TRAF molecules, the proteins that mediate signal transduction pathway induced by TNF receptor-like proteins (Rothe et al., 1995; Uren et al., 1996). Future experiments will also resolve whether Smac could regulate TNF signaling by removing IAPs from TRAFs.

Is Smac a Functional Homolog of *Drosophila* Reaper, Grim, and Hid?

The function of Smac is similar to that of *Drosophila* apoptosis activating proteins Reaper, Hid and Grim, which have been proposed to function by countering the activity of IAPs (Vucic et al., 1998; Goyal et al., 1999). Both biochemical and genetic evidences indicate that these three proteins interact directly with *Drosophila* IAPs (D-IAP1 and D-IAP2), and that this direct interaction is important for their function (McCarthy and Dixit, 1998; Goyal et al., 1999). So far, no mammalian homologs of Reaper, Grim, and Hid have been reported. Interestingly, despite functional similarity, the primary sequences of Reaper, Grim, and Hid show very little resemblance to each other except a short stretch of amino acid residues at their N termini (White et al., 1994; Grether et al., 1995; Chen et al., 1996). It is therefore not surprising that the sequence of Smac does not resemble any of these proteins even though it appears to function similarly by countering the activity of IAPs.

Drosophila Hid protein has also been shown to localize on mitochondria. Its mitochondrial location is mediated by the C-terminal part of the protein, similar to Bcl-2 (Haining et al., 1999). The significance of its mitochondrial location remains to be determined. Unlike Smac, Reaper, Grim, and Hid do not seem to have mitochondrial targeting sequences and therefore must be regulated differently. One regulator of Reaper has been recently identified as the *Drosophila* homolog of p53 protein (Brodsky et al., 2000). It is not clear whether p53 also regulates Smac. Nevertheless, if Smac, Reaper, Grim, and Hid are indeed functional homologs, it will present an interesting case of evolutionary convergence. It also raises the possibility that there might be other Smac-like proteins in mammals that bear little sequence homology to Smac.

Regulation of Smac

Smac is a bona fide mitochondrial protein with a typical amphipathic mitochondrial targeting sequence at its N terminus that presumably is cleaved after mitochondrial entry (Figure 3A). Only the mature Smac has caspase activation promoting activity, whereas the precursor with the signal sequence intact does not (data not shown). Smac therefore requires a maturation process inside mitochondria before gaining its apoptotic activity. Since mature Smac is released only during apoptosis, the major regulatory step for Smac should be its release from mitochondria, a process that is likely to be controlled by the Bcl-2 family of proteins. The requirement for mitochondrial processing to activate Smac is analogous to the requirement for mitochondrial processing of cytochrome c, another protein that is known to promote caspase activation after release from the intermembrane space. The requirement for mitochondrial maturation ensures that newly synthesized Smac and cytochrome c will not provoke apoptosis prior to their import into mitochondria.

The discovery of Smac may help to explain the puzzling observations that certain cells such as neurons must be preexposed to apoptotic stimuli such as NGF deprivation to become "competent to die" in response to microinjected cytochrome c (Deshmukh and Johnson, 1998). These observations could potentially be explained by the presence of relatively high amounts of IAPs and the absence of Smac or Smac-like activity in the cytosol of these cells. If this hypothesis is correct, the coinjection of active Smac and cytochrome c should cause apoptosis in neurons even in the presence of NGF, and apoptosis should not be inhibited by Bcl-2.

On gel filtration columns, native Smac behaves as a multimeric protein with an apparent molecular weight of 100 kDa and it is therefore much bigger than cytochrome c. This may explain why the majority of Smac requires detergents for solubilization while cytochrome c can be easily released from mitochondria by hypotonic buffers. However, the kinetics of Smac release from mitochondria in response to UV treatment is very much the same as that of cytochrome c release (Figure 7B). It remains to be determined whether the release of Smac from mitochondria during apoptosis is through the same route used by cytochrome c.

The identification of Smac further highlights the importance of mitochondria in apoptosis. After receiving apoptotic stimuli, mitochondria not only release cytochrome c to induce the formation of caspase-9 activating apoptosome, but also release Smac to counter the inhibitory activity of IAPs.

Experimental Procedures

General Methods and Materials

We obtained nucleotides from Pharmacia; Horse heart cytochrome c from Sigma; Monoclonal antibodies against cytochrome c from PharMingen; Anti-His antibody from Qiagen; Radioactive materials from Amersham; and molecular weight standards for SDS-PAGE and gel filtration chromatography from Bio-Rad. Protein concentrations were determined by the Bradford method; general molecular biology methods were used as described in Sambrook et al., 1989.

Preparation of S-100 Fractions from HeLa Cells

Human HeLa S3 cells were cultured in monolayer at 37°C in an atmosphere of 5% CO_2 in DMEM medium (Dulbecco's modified Eagle's medium containing 100 U/ml of penicillin and 100 μg/ml of streptomycin sulfate supplemented with 10% fetal calf serum). Cells at 70% confluence were washed once with 1× phosphate-buffered saline (PBS) and harvested by centrifugation at 800 × g for 5 min at 40°C. The cell pellets were resuspended in 3 volumes of buffer A (20 mM HEPES-KOH [pH 7.5], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM DTT, and 0.1 mM PMSF), and cell extracts were prepared as described in Liu et al., 1996. When mitochondria were needed to be kept intact during extract preparation, the cell pellet was lysed in buffer A containing 250 mM sucrose as described in Yang et al., 1997.

Preparation of Solublized Membrane Extracts (SME)

Two hundred fifty milliliters of cell pellet from 100 liters of suspension cultured HeLa cells (5×10^6 cells/ml) were resuspended in 1 liter of buffer A. The cells were homogenized, and nuclei were removed as described in Liu et al., 1996. The supernatant was centrifuged at 10,000 × g for 30 min at 4°C to pellet the heavy membrane fraction. The resulting membrane pellet was resuspended in 1 liter of buffer A containing 0.5% (w/v) CHAPS and the solublized mixture was centrifuged at 100,000 × g for 1 hr at 4°C in a Beckman SW 28 rotor. The resulting supernatant (*s*olublized *m*embrane *e*xtracts, SME) was stored at −80°C and used as the starting material for the purification of protein Smac.

Purification of Smac from SME

All purification steps were carried out at 4°C. The chromatographic steps of Q-Sepharose column (Pharmacia) and Hydroxyapatite column (Bio-Rad) were carried out using conventional stepwise chromatography. The chromatographic steps of Phenyl Superose, Superdex 200, and Mono Q were performed on an automatic fast protein liquid chromatography (FPLC) station (Pharmacia).

One liter of solublized membrane fraction in buffer A plus 0.5% CHAPS (5 g total protein) was applied on a Q-Sepharose column (100 ml bed volume) equilibrated with buffer A. The column was washed with 200 ml buffer A followed by 500 ml buffer A containing 100 mM NaCl. The bound materials on the column were eluted by 500 ml buffer A containing 300 mM NaCl. Fractions of 50 ml were collected and assayed for Smac activity. 150 ml of active protein fractions were pooled and precipitated by adding solid ammonium sulfate to 40% saturation and the protein precipitates were collected by centrifugation at 35,000 × g for 20 min. The resulting protein pellet was dissolved in 170 ml buffer A and loaded on a hydroxyapatite column (50 ml bed volume) equilibrated with buffer A. The column was washed with 150 ml buffer A followed by 150 ml buffer A containing 1 M NaCl, and then with 150 ml buffer A again. The bound materials were eluted with 100 ml of 0.12 M KPO_4 (pH 7.5). Fractions of 10 ml were collected and assayed for Smac activity. A total of 40 ml active protein fractions were pooled and 3.1 g of ammonium sulfate was added to make a final concentration of ammonium sulfate at 0.5 M. The mixture was equilibrated by rotating for 1 hr followed by centrifugation at 35,000 × g for 40 min. The resulting supernatant (44 ml) was loaded onto a Phenyl Superose 5/5 column (Pharmacia) equilibrated with buffer A containing 0.5 M ammonium sulfate. The column was washed with 30 ml buffer A containing 0.5 M ammonium sulfate and eluted with a 100 ml linear gradient from buffer A containing 0.5 M ammonium sulfate to buffer A. Fractions of 5 ml were collected and assayed for Smac activity. A total of 15 ml active fractions eluted at 130–180 mM ammonium sulfate were collected and loaded in two separate runs on a Superdex 200 (26/60) gel filtration column equilibrated with buffer A containing 100 mM NaCl. The column was eluted with the same buffer. Fractions of 4 ml were collected starting from 90 ml of elution and assayed for Smac activity. A total of 24 ml active fractions were pooled and loaded on a Mono Q 5/5 column equilibrated with buffer A containing 100 mM NaCl. The column was washed with 10 ml buffer A containing 200 mM NaCl and eluted with a 20 ml linear gradient from 200 mM NaCl to 500 mM NaCl, both in buffer A. Fractions of 1 ml were collected and assayed for Smac activity. Active fractions (2 μg total protein) were eluted at 270–300 mM NaCl in buffer A and were aliquoted with the addition of 20% glycerol and stored at −80°C.

Protein Sequencing of Smac

The 25 kDa band (~8 pmol) from the Mono Q column was excised from a 15% SDS-PAGE gel stained with Coomassie blue. The band was digested by trypsin (Promega) and the resulting peptides were separated by a capillary reverse-phase FPLC column (LC Packing, Inc.). Four individual peptides were sequenced in an Applied Biosystems sequencer.

cDNA Cloning of Smac

Four polypeptide sequences were obtained and used to search for the EST database (tBlastn). One positive EST clone (T53449) was identified and used as the template to design oligo nucleotides to obtain the cDNA clones from HeLa cDNA library by PCR. One microliter (10^8 pfu) aliquot of λExlox HeLa cDNA library (Zou et al., 1997) was heated at 99°C for 15 min to release the DNA, which was then directly amplified with 30 pmol of primer 5′-GTC CGA GCG GAT CCA TGA AAT CTG ACT TCT ACT TCC-3′ and 30 pmol of primer 5′-GCC GAG GCG GCC GCT CAA TCC TCA CGC AGG TAG G-3′ using a PCR reaction with 1 cycle of 94°C for 1 min and 35 cycles of 94°C for 30 s; 55°C for 30 s; and 72°C for 1 min, followed by an extension at 72°C for 10 min. A 559 bp PCR product was obtained and subsequently sequenced after subcloning into the PCR II vector using a TA cloning kit (Invitrogen). The 559 bp PCR product was labeled with α-^{32}P-dCTP using *redi* prime II random prime labeling kit (Amersham) and used as the probe to screen a HeLa λExlox cDNA library by hybridizing duplicate filters at 42°C overnight in Rapid-hyb buffer (Amersham). The filters were washed twice with 1× saline citrate (SSC)/0.1% SDS for 15 min at room temperature and once with 0.5× SSC/0.1% SDS for 10 min at 65°C. Out of 5×10^5 plaques screened, 14 positive clones were identified and sequenced. The 719 bp full-length cDNA was obtained.

Production of Recombinant Smac in Bacteria

The primers 5′-ATG CTC GAG GCG TTG ATT GAA GCT ATT ACT GAA TAT-3′ and 5′-AGC CGG ATC CTC AAA TGG GTA AGA GCA GCT GTA CAG AGT-3′ were designed to PCR amplify a 437 bp plasmid Smac cDNA. The amplified DNA fragment encoding the amino acids 95–239 of Smac was subcloned in-frame into the XhoI/BamHI sites of the bacterial expression vector pET-15b (Novagen). The expression plasmid was transformed into bacteria BL21(DE3) and the recombinant protein was purified as described in Zou et al., 1997.

Production of Recombinant Smac Protein in a Baculovirus Expression System

A 719 bp cDNA encoding the full-length Smac fused with a 9-histidine tag at the carboxyl terminus was subcloned into BamHI/NotI sites of the baculovirus expressing vector pFastBacI (Life Technologies, Inc.). The expression plasmid was transformed into DH10Bac *E. Coli* cells (Life Technologies, Inc). The recombinant viral DNA, bacmid, was purified according to the Bac-To-Bac Baculovirus Expression procedure and confirmed by PCR amplification analysis. The generation of recombinant Smac was as described in Zou et al., 1999.

Production of Recombinant c-IAP1 Fusion Protein

The cDNA encoding the first 359 amino acid residues of c-IAP1 was cloned from a HeLa cDNA library by PCR with primers "CATGGATC CGAATTCAAGGAGATGCACAAAACTGCC" and "GATGAGGATAT

CTTGCGGCCGCCTCATCAACAAACTC". The PCR product was cloned into the EcoRI and NotI sites of pET 25 b(+) vector (Novagen) and transfected into BL21 DE3 pLys cells (Novagen). Recombinant c-IAP1 was prepared as in Liu et al., 1996, except EDTA and EGTA were omitted from all buffers.

Western Blot Analysis

Western blot analysis for Apaf-1, Caspase-9, and cytochrome c was performed as described previously (Li et al., 1997b). Anti-Smac serum was generated by immunizing rabbits with a recombinant Smac fusion protein (see above). Antibodies against IAPs are from R&D Systems and used according to manufacturer's suggestion. Immunoblot analysis was performed with a horseradish peroxidase conjugated goat anti-rabbit immunoglobulin G using enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham).

Northern Blot Analysis

Poly(A)$^+$ RNA blots containing 2 μg of poly(A)$^+$ RNA per lane from multiple human adult tissues were purchased from Clontech. Blots were hybridized with 2×10^6 cpm/ml random primed 559 bp Smac PCR fragment used in cDNA library screening (see above) in a procedure described in Zou et al., 1997.

Immunostaining

Adhesive HeLa cells were seeded at 1×10^4 cells per chamber slide (Nalge Nunc International) in DMEM medium supplemented with 10% (v/v) fetal calf serum, and grown in monolayer at 37°C in an atmosphere of 5% CO_2. Twenty-four hours later, the medium was removed and the HeLa cells were irradiated at 3.5 cm under a UV lamp (5,000 μW/cm^2, Philips, G36T6L) for 15 s. The treated cells were then continued to culture in fresh DMEM medium for several hours as indicated. The cell cultures were terminated by washing three times in PBS followed by fixation in freshly prepared 3% paraformaldehyde in PBS for 10 min. The fixed cells were washed three times in PBS for 15 min each followed by permeabilization in 0.15% Triton X-100 in PBS for 15 min. The cells were then blocked for 60 min in blocking buffer (2% bovine serum albumin in PBS) followed by a 4 hr incubation with either an antiserum against Smac (1:200) or a mouse monoclonal antibody against cytochrome c (1:200). The cells were washed three times at 10 min each in blocking buffer followed by 1 hr incubation with either a fluorescein-conjugated goat anti-rabbit antibody (1:500) (Molecular Probe) for Smac, or Texas-red labeled goat anti-mouse IgG (1:500) (Molecular Probe) for cytochrome c. The cells were then washed three times at 10 min each in PBS followed by staining with 1 μg/ml DAPI (Molecular Probe) and examined under a Nikon Eclipse E800 Fluorescence Microscope.

Transfection of HeLa Cells with Smac cDNA

A 719 bp cDNA containing the entire coding region of Smac and a Flag tag at the carboxyl terminus were subcloned into Xho/EcoRI sites of a pcDNA 3.1(−) vector (Invitrogen) and the plasmid was designated as pcDNA-Smac and prepared using a Qiagen Midi plasmid kit. HeLa cells were transfected using the Fugene 6 transfection reagent (Roche). After 16 hr, the culture medium was removed and the cells were irradiated with UV as described above for different lengths of time. After irradiation, the transfected cells were cultured in fresh DMEM medium for additional 6 hr and harvested for preparation of S-100 extracts in the presence of 0.5% CHAPS in buffer A. A total of 20 μg protein was loaded on a 15% SDS-PAGE and the gel was transferred to a nitrocellulose filter, which was subsequently blotted with a polyclonal antibody against Smac or a monoclonal antibody against human caspase-3 (Transaction Laboratories).

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GenBank Accession Number

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