The Inhibitor of Apoptosis Protein-binding Domain of Smac Is Not Essential for its Proapoptotic Activity

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Abstract. Smac/DIABLO, a recently identified inhibitor of apoptosis protein (IAP)-binding protein, is released from the mitochondria during apoptosis and reportedly potentiates apoptosis by relieving the inhibition of IAPs on caspases. We now describe the molecular characterization of Smac β, an alternatively spliced form of Smac, which lacks the mitochondrial-targeting sequence found in Smac and has a cortical distribution in both human embryonic kidney 293 and breast epithelial tumor MCF-7 cells. Smac β, which binds IAPs in vitro, does not bind IAPs in intact cells due to cellular processing and removal of its NH₂-terminal IAP-binding domain. Despite its inability to interact with IAPs in cells, processed Smac β is proapoptotic, as demonstrated by its ability to potentiate apoptosis induced by both death receptor and chemical stimuli. Furthermore, expression of a NH₂-terminally truncated Smac mutant (Δ75), which lacks the entire IAP-interacting domain, potentiates apoptosis to the same extent as Smac and Smac β. Our data support the hypothesis that the main proapoptotic function of Smac and Smac β is due to a mechanism other than IAP binding.

Key words: TRAIL • XIAP • alternative splicing • MCF-7 cells • mitochondria

Introduction

Apoptosis is an essential mechanism of cell death required for the correct development and homeostasis of multicellular organisms. Two major apoptotic pathways have been identified: (a) triggering of cell surface death receptors of the tumor necrosis factor (TNF) receptor superfamily, including TNFα, CD95 (Fas/Apo-1), and TNF-related apoptosis-inducing ligand (TRAIL), where caspase-8 is activated following its recruitment to a trimerized receptor–ligand complex via adaptor molecules (for reviews see Ashkenazi and Dixit, 1999; Bratton et al., 2000); and (b) stress-induced apoptosis, caused by chemicals and growth factor deprivation, which results in perturbation of mitochondria and the ensuing release of proteins, such as cytochrome c, from the intermitochondrial membrane space (Green and Reed, 1998; Bratton et al., 2000). Released cytochrome c binds to the human CED4 homologue Apaf-1 (Zou et al., 1997), which in the presence of dATP, results in the recruitment and activation of caspase-9 (Li et al., 1997; Cain et al., 1999; Zou et al., 1999). The release of cytochrome c from the mitochondria is also regulated partly by Bcl-2 family members with antiapoptotic and proapoptotic members inhibiting or promoting the release, respectively (Kluck et al., 1997; Yang et al., 1997; Li et al., 1998; Luo et al., 1998). The activated initiator caspases-8 and -9 then activate the effector caspases-3, -6, and -7, which are responsible for the cleavage of important cellular substrates, resulting in the classical biochemical and morphological changes associated with the apoptotic phenotype (Cohen, 1997; Earnshaw et al., 1999).

Caspase activity is also regulated by the inhibitor of apoptosis proteins (IAPs), which are found in a range of organisms and are characterized by one or more baculovirus IAP repeats, which are responsible for their antiapoptotic activity (for reviews see Deveraux et al., 1997; Deveraux and Reed, 1999). One major function of IAPs, particularly c-IAP-1 and -2 and X-linked IAP (XIAP), is their propensity to bind to and inhibit key initiator and effector caspases including caspases-9, -3, and -7 (Deveraux et al., 1997; Deveraux and Reed, 1999).

Recently, a novel protein, Smac, and its murine homologue, DIABLO, were described, which promoted caspase activation by eliminating IAP inhibition of caspases (Du et
In this study, we describe Smac β, an alternatively spliced form of Smac, which lacks the mitochondrial-targeting sequence and has a cortical as opposed to a mitochondrial subcellular distribution. Both Smac β and truncated forms of Smac that lack the IAP interacting domain, markedly potentiate apoptosis induced by diverse apoptotic stimuli. These data demonstrate that the main proapoptotic function of Smac and Smac β is not mediated through an interaction with the IAPs.

Materials and Methods

Materials

Medium and serum were purchased from Life Technologies. c-IAP-1 and -2 polyclonal antibodies were from R&D Systems, and XIAP and herpes simplex virus (HSV) monochonal antibodies were from BD Transduction Labs and Novagen, respectively. The HRP-conjugated secondary antibodies, goat anti-rabbit and goat anti-mouse, were from Dako and Sigma-Labs and Novagen, respectively. The HRP-conjugated secondary antibody (Promega). The HRP-conjugated secondary antibodies, goat anti-rabbit and goat anti-mouse, were from Dako and Sigma-Aldrich, respectively. Anti-mouse Alexa 480 and MitoTracker™ red CMXRos were from Molecular Probes. Carbofurfuryl-lysyl-lysyl-lysyl-leucine (MG132) was obtained from Calbiochem. TRAIL was produced as described previously (MacFarlane et al., 1997a). Unless stated otherwise, all other chemicals were from Sigma-Aldrich.

Generation of Constructs

All Smac and Smac β constructs were generated by PCR amplification and cloned into the expression vector pTriEx-1 (Novagen). Total RNA was isolated using TriReagent and reverse transcribed using Expand™ reverse transcriptase (Roche Diagnostics) before amplification of full-length Smac and Smac β cDNA using Pfu DNA polymerase (Stratagene). Deletion mutants were generated in the same manner, and all constructs were verified by DNA sequencing.

Cell Culture, Transfection, and Induction of Cell Death

Human embryonic kidney 293 cells were obtained from the European Collection of Animal Cell Cultures and grown in high glucose DMEM supplemented with 10% FBS, MCF-7-Fas (MCF-7) human breast epithelial cells (from Dr. M. Jaattela, Danish Cancer Society Research Center, Copenhagen, Denmark) were grown in RPMI 1640 supplemented with 10% FBS and 2 mM Glutamax™. Both cell lines were cultured in an atmosphere of 5% CO₂ in air at 37°C and maintained by routine passage every 3–4 d. Cells were transiently transfected using Fugene 6™ (Roche Biochemicals) and 0.5–4 μg DNA, where indicated in the presence of pRSC lacZ (MacFarlane et al., 1997a). After 24 h, cells were either harvested or exposed to an apoptotic stimulus. Apoptosis was induced by treatment for 9 h with either etoposide, MG132, TNF (in the presence or absence of cycloheximide), or TRAIL. The extent of apoptosis was assessed by counting the percentage of apoptotic blue- (β-galactosidase–expressing) transfected cells.

Immunofluorescence Microscopy

Cells were grown on coverslips and 24 h after transfection, stained with MitoTracker™ (100 nM) for 15 min at 37°C before fixation in 3.8% formaldehyde for 20 min at room temperature. Cells were rinsed three times in PBS, permeabilized with 0.1% Triton X-100 in PBS and blocked overnight in 5% BSA in PBS at 4°C. Cells were incubated with anti–mouse Alexa 488 (1:300) for 45 min at room temperature. The cells were then washed and nuclei were stained with Hoechst 33258 (0.25 μg/ml) for 20 min before mounting onto glass slides using Vectashield® (Vector Laboratories). Optical sections were taken using argon-krypton, UV lasers, and a Leica TCS-4D confocal imaging system.

Production of Recombinant Proteins

Recombinant proteins were produced in Escherichia coli BL21(DE3) as described previously (Zou et al., 1997) and retained on beads. The purified proteins were stored in aliquots at 4°C, and their identity was confirmed by Western blotting using anti-HSV monoclonal antibody.

Production of 35S-labeled Proteins and In Vitro Interaction Assay

35S-labeled proteins were produced using the TNT®-coupled reticulocyte lysate system (Promega). The constructs used were pcDNA3 human MIHB (c-IAP-1), human MIHC (c-IAP-2), and pSp72 XIAP (gifts from Dr. D. Vaux, The Walter and Eliza Hall Institute, Melbourne, Australia). For the in vitro interaction assay, 10 μl of Ni-NTA agarose beads (QIAGEN) or beads bound to recombinant protein were added to 5 μl of TNT mix, and the final volume was adjusted to 100 μl with buffer A (50 mM Tris, 150 mM NaCl, 0.1% IGEPA, pH 7.6), before incubation for 1 h at room temperature. The beads were then pelleted, washed in buffer A, and resuspended in SDS-PAGE sample buffer before resolving on a 13% SDS–polyacrylamide gel. As a marker, 1 μl of the TNT reaction was run alongside the beads. The gel was then dried and visualized by autoradiography.

Immunoprecipitation Studies

Cells were harvested 24 h after transfection, and immunoprecipitation of XIAP was carried out as described previously (Srinivasula et al., 1997). To precipitate Smac and Smac β via the His tag, lysates were prepared and incubated overnight with Ni-NTA agarose beads at 4°C and then washed and prepared for SDS-PAGE.

Gel Electrophoresis and Western Blotting

Equal amounts of lysate were diluted in SDS-PAGE sample buffer and separated on 13% (HSV) or 10% (XIAP and c-IAP-1 and -2) polyacrylamide gels followed by electrophoretic transfer onto polyvinylidene difluoride membrane (Bio-Rad Laboratories). Immunodetection was carried out as described previously (MacFarlane et al., 1997b) using ECL detection (Amersham-Pharmacia Biotech).

Results and Discussion

Identification of Smac β

The protein sequence of Smac was used to search the human ESTs deposited in GenBank using the programme TBLASTN. This search revealed several ESTs, which corresponded to the previously reported Smac and some that contained an alternative prosence (for example, sequence data available from GenBank/EMBL/DDBJ under accession number AA156765) or other sequence differences. A search of the nonredundant database resulted in the identification of Smac β, a form of Smac, with an alternative NH₂ terminus (sequence data available from GenBank/EMBL/DDBJ under accession number AK001399). Translation of this sequence revealed an ORF of 186 amino acids that was identical to the reported Smac molecule in all but the prosence (Fig. 1, A and B) with an in-frame stop codon upstream of the initiator methionine, indicating a full-length reading frame.
shown). Primers were designed to amplify either Smac or Smac β cDNA and were used in reverse transcriptase–PCR using total RNA isolated from various cell lines as a template. The mRNA for both Smac and Smac β was detected in all the cell lines examined and showed similar expression levels (Fig. 1 C). To confirm that the Smac β mRNA was in fact a spliced form of Smac, both the protein and cDNA sequence of Smac were used to search the HTGS section of GenBank. These searches revealed a genomic sequence (sequence data available from GenBank/EMBL/DDBJ under accession number NT_009438) that contained regions of identity to Smac and Smac β sequences, indicative of the exon structure of the gene. The genomic sequence was composed of sequence from the q arm of chromosome 12, mapping the Smac gene close to the APAFI gene (Kim et al., 1999). From the genomic sequence, it was deduced that the Smac gene is composed of seven exons (Fig. 1 A), with the prosequence of Smac encoded by exons 1 and 3, and the prosequence of Smac β encoded by exon 2.

Like many other genes encoding proteins involved in apoptosis, the Smac gene may produce several alternatively spliced forms (Fig. 1 A). As database searching revealed at least three other putative short splice forms of Smac, we have designated these Smac β, γ, and δ. Smac β is identical to the recently described Smac-S (Srinivasula et al., 2000). Smac γ (sequence data available from GenBank/EMBL/DDBJ under accession number BE383154) should have a similar cellular distribution to Smac β, due to the loss of amino acids 18–62 in the mitochondrial-targeting sequence (exon 3) and may bind IAPs only weakly. Smac δ (sequence data available from GenBank/EMBL/DDBJ under accession number AW247557) should be targeted to the mitochondria due to the presence of an intact targeting sequence (amino acids 1–55) and may bind to the baculoviral IAP repeat-5 domain of XIAP as amino acids 56–61 are retained. However, Smac δ lacks amino acids 62–105 due to differential splicing of exon 4 (Fig. 1 B), probably weakening its binding to XIAP (Chai et al., 2000; Srinivasula et al., 2000).

**Smac β Localizes to the Cell Cortex**

Smac and Smac β were overexpressed in 293 and MCF-7 cells, and their subcellular localization was determined by confocal microscopy using an anti-HSV tag antibody (Fig. 2). Smac colocalized with MitoTracker™, indicating a mitochondrial distribution (Fig. 2, A–C). Thus, overexpressed Smac showed the same cellular localization as endogenous Smac (Du et al., 2000). In contrast, Smac β showed a distinct cortical distribution with no mitochondrial localization evident (Fig. 2, E–G and I–K). The subcellular localization of Smac β was confirmed by fractionation of transfected cells (data not shown). To determine the effects of both death receptor and chemically mediated apoptosis on the distribution of Smac and Smac β, cells were exposed to either TRAIL or etoposide, a DNA topoisomerase II inhibitor. TRAIL causes a rapid induction of apoptosis in MCF-7 cells, with caspase-8 as the apical caspase (MacFarlane et al., 2000). Both apoptotic stimuli induced the release of Smac from the mitochondria (Fig. 2 D; data not shown) in agreement with previous reports (Du et al., 2000; Verhagen et al., 2000). In some apoptotic cells, Smac redistributed to the cell periphery and displayed a similar distribution to Smac β. 
changes in the distribution of Smac β were observed in apoptotic cells, but it appeared more punctate and accumulated closer to the cell periphery (Fig. 2 H; data not shown). Thus, in contrast to Smac, Smac β showed a different distribution, and its cellular compartmentalization did not significantly change upon induction of apoptosis.

**Smac β Does Not Interact with IAP Family Members**

To test whether Smac β also interacted with IAPs, lysates were prepared from transfected 293 cells. Immunoprecipitation of endogenous XIAP demonstrated a strong interaction with Smac but little or no interaction with Smac β (Fig. 3 A, top, lanes 5 and 6), although XIAP was completely depleted from the lysates (Fig. 3 A, top, lanes 1–3). Similarly, immunoprecipitation of Smac/Smac β, which completely removed Smac/Smac β (Fig. 3 B, middle, lanes 2 and 3), confirmed that XIAP interacted with Smac but not Smac β (Fig. 3 B, top, lanes 5 and 6). As Smac β was distributed to the cell periphery, it raised the possibility that it bound specifically with c-IAP-1 or -2 rather than with XIAP. Reprobing the blots revealed no interaction of Smac or Smac β with endogenous c-IAP-1 or -2 (Fig. 3 B, bottom, lanes 5 and 6). Taken together, these data strongly suggest that the in vivo interaction of Smac with endogenous XIAP is much stronger than with c-IAP-1 or -2 and that Smac β in cells does not interact with endogenous XIAP or c-IAP-1 or -2.

**NH₂-terminal Processing of Smac β in Cells Prevents Its Interaction with XIAP**

The NH₂-terminal 55-amino acid residues of Smac are removed upon mitochondrial import, yielding mature ~21-
kD Smac. Since Smac β has no mitochondrial targeting sequence, its predicted molecular weight is \( \sim 21.2 \) kD. Although transiently expressed Smac and Smac β should comigrate on SDS-PAGE since they are similarly tagged, Smac β appeared to be processed intracellularly as it consistently migrated below mature Smac (Fig. 4 A, compare lanes 2 and 3). To further examine this possibility, the migration of transiently transfected Smac and Smac β was compared with partially purified recombinant mature Smac (Δ55), Smac β, or Smac Δ75 (Fig. 4 A). Transiently expressed Smac β migrated below both recombinant Smac Δ55 and Smac β but similarly to Smac Δ75. These results further supported the hypothesis that Smac β was processed intracellularly. This processing must have occurred at the amino terminus, since the protein retained the COOH-terminal HSV and His₆ tags, and Smac β appeared to be processed \( \sim 20 \) amino acids from its NH₂ terminus. Initial experiments to identify this cleavage site were unsuccessful because Smac β isolated from cells was NH₂-terminally blocked. Because this NH₂-terminal processing would be predicted to remove the IAP binding domain of Smac β, we assessed the ability of full-length recombinant Smac β and Smac Δ75 to interact with IAPs in an in vitro interaction assay. Recombinant Smac β but not Smac Δ75 interacted with XIAP and c-IAP-1 and -2 (Fig. 4 B, compare lanes 3 and 4). Taken together, these data suggest that the NH₂ terminus of Smac β is removed by cellular processing, therefore explaining its inability to interact with XIAP in intact cells (Fig. 3, A and B, top, lane 6).

**Smac β Potentiates Apoptosis**

Processed Smac β may act as a dominant negative inhibitor of apoptosis, since it did not interact with IAPs in intact cells. To test this hypothesis, cells were cotransfected with constructs for full-length Smac or Smac β together with the reporter construct pRSC lacZ and then treated with different apoptotic stimuli, including death receptor stimuli (TRAIL and TNF) and etoposide and the proteasome inhibitor, MG132. In 293 cells, both Smac and Smac β potentiating apoptosis induced by all five apoptotic stimuli (Fig. 5 A). Similar potentiation was also observed in MCF-7 cells (Fig. 5 B). As Smac β is NH₂-terminally processed to a form, which is unable to interact with IAPs, we propose that the domain(s) for the proapoptotic function of Smac β resides in the remaining COOH-terminal frag-

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**Figure 3.** Smac but not Smac β can bind XIAP. Lysates were prepared from transfected 293 cells. (A) Endogenous XIAP was immunoprecipitated, and the supernatant and pellet were examined either for Smac or Smac β using the HSV antibody (top) or for XIAP using the XIAP antibody (bottom). (B) Lysates were also incubated with Ni-NTA beads to precipitate His₆-tagged Smac and Smac β and the samples analyzed for XIAP (top) or Smac and Smac β (middle). The blots were reprobed for e-IAP-1 or -2 (bottom). (C) Expression levels of Smac and Smac β were measured in the transfected cell lysates. Nonspecific bands detected by the anti-XIAP antibody (?) and the heavy chain of the antibody used for immunoprecipitation (†) are indicated. Occasionally, an alternative start site in the Smac and Smac β constructs gave rise to a smaller product (†), which did not interact with IAPs.

**Figure 4.** Smac β cannot bind XIAP in vivo due to NH₂-terminal processing. (A) 293 cells were transfected with empty vector, Smac, or Smac β and harvested at 24 h (lanes 1–3), and the migration on SDS gels of Smac and Smac β was compared with recombinant Smac Δ55, Smac Δ75, and Smac β (lanes 4–6). (B) Smac β was incubated with beads alone, recombinant Smac β, or Smac Δ75 bound to Ni-NTA beads, washed, and analyzed by autoradiography. (C) Input control showing the beads bound to Smac β and Smac Δ75.
and the deletion mutants was significantly different (from the vector controls as assessed by single factor ANOVA. The experiments, the potentiation of apoptosis by Smac, Smac three independent experiments performed in triplicate. In all of empty vector. The bars represent the mean ± standard error of three independent experiments performed in triplicate. In all of the experiments, the potentiation of apoptosis by Smac, Smac β, and the deletion mutants was significantly different (P < 0.05) from the vector controls as assessed by single factor ANOVA.

Figure 5. NH2-terminal truncations of Smac, lacking the IAP-binding domain, potentiate apoptosis. (A) 293 and (B and C) MCF-7 cells were cotransfected with either empty vector, Smac, Smac β, or NH2-terminal deletions of Smac together with pRSC lacZ. Similar levels of transfected proteins were expressed (data not shown). After 24 h, cells were treated for 6 h with DMSO (vehicle control), etoposide (10 μM), MG132 (0.1 μM), TRAIL (0.25 μg/ml in MCF-7 or 1 μg/ml in 293 cells), or TNF (10 ng/ml) in the presence or absence of cycloheximide (1 μM). Cells were then fixed and stained with X-gal. Apoptosis was assessed by morphological analysis and expressed as a percentage of transfected (blue) cells. The extent of apoptosis represents the fold increase over DMSO-treated cells transfected with empty vector. The bars represent the mean ± standard error of three independent experiments performed in triplicate. In all of the experiments, the potentiation of apoptosis by Smac, Smac β, and the deletion mutants was significantly different (P < 0.05) from the vector controls as assessed by single factor ANOVA.

NH2-terminal Truncations of Smac, Lacking IAP Binding, Potentiate Apoptosis

The IAP-binding domain of Smac resides in the NH2-terminal amino acids (Chai et al., 2000; Srinivasula et al., 2000). This was confirmed in the current study; removal of the NH2-terminal 5 (Δ60) or 20 (Δ75) amino acids from mature Smac resulted in deletion mutants that did not bind to endogenous XIAP in cells or to recombinant XIAP in vitro (Fig. 4 B; data not shown). Both of these deletion mutants exhibited a similar cortical distribution to Smac β (Fig. 2 L; data not shown). Deletion of the NH2-terminal 75 amino acids of Smac resulted in a protein, which migrated similarly to Smac β in cells, suggesting that ~22 amino acids are removed during the maturation of Smac β (Fig. 4 A). Most interestingly, both Smac Δ60 and Δ75 potentiated apoptosis to a similar extent as Smac β in response to different apoptotic stimuli (Fig. 5 C).

Thus, NH2-terminal deletion mutants of Smac, which are unable to bind XIAP or c-IAP-1 or -2, potentiate apoptosis to a similar extent as Smac and Smac β. These data strongly support the hypothesis that the proapoptotic activity of Smac and Smac β is not primarily mediated via relief of the inhibitory action of these specific IAPs. Rather, we propose that Smac binds XIAP as a secondary function and that its primary mechanism for promoting apoptosis is via a domain in the COOH terminus. Because induction of apoptosis by both death receptor and chemical stimuli was enhanced, it suggested that Smac and its deletion mutants may act at a common point where these two apoptotic pathways converge, possibly at the level of postmitochondrial activation of caspases by enhancing the formation or activation of the apoptosome.

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