Molecular Determinants of the Caspase-promoting Activity of Smac/DIABLO and Its Role in the Death Receptor Pathway*

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Smac/DIABLO is a mitochondrial protein that is released along with cytochrome c during apoptosis and promotes cytochrome c-dependent caspase activation by neutralizing inhibitor of apoptosis proteins (IAPs). We provide evidence that Smac/DIABLO functions at the levels of both the Apaf-1-caspase-9 apoptosome and effector caspases. The N terminus of Smac/DIABLO is absolutely required for its ability to interact with the baculovirus IAP repeat (BIR3) of XIAP and to promote cytochrome c-dependent caspase activation. However, it is less critical for its ability to interact with BIR1/BIR2 of XIAP and to promote the activity of the effector caspases. Consistent with the ability of Smac/DIABLO to function at the level of the effector caspases, expression of a cytosolic Smac/DIABLO in Type II cells allowed TRAIL to bypass Bcl-xL inhibition of death receptor-induced apoptosis. Combined, these data suggest that Smac/DIABLO plays a critical role in neutralizing IAP inhibition of the effector caspases in the death receptor pathway of Type II cells.

Apoptosis is a highly conserved cell suicide program essential for development and tissue homeostasis of all metazoan organisms (1–3). Key to the apoptotic program is a family of cysteine proteases termed caspases that cleave their substrates after specific aspartic acid residues (reviewed in Refs. 4 and 5). Caspases reside in cells as inactive zymogens, known as procaspases, and can be activated by proteolytic cleavage after specific aspartate residues present between what will become the large and small subunits of the active caspase (4, 5). During apoptosis, the initiator caspase zymogens are activated by autocatalytic cleavage, which then activate the effector caspases by cleaving their inactive zymogens (6, 7). Active caspases can then induce the characteristic apoptotic changes through their ability to cleave certain key protein substrates in the cell (4, 5).

The initiator caspase zymogens are activated by adaptor proteins such as FADD and Apaf-1, which associate in a stimulus-dependent manner with the prodomains of these zymogens and promote their activation via oligomerization (6, 7). For example, ligation of the cell surface death receptors triggers binding of procaspase-8 to FADD and its subsequent activation and release from the death receptor complex (reviewed in Refs. 6 and 8). Likewise, release of cytochrome c from the mitochondria in response to apoptotic stimuli such as serum starvation, ionization radiation, DNA damaging agents, etc. triggers oligomerization of Apaf-1 in an ATP- or dATP-dependent manner (7, 9–11). The oligomeric Apaf-1 apoptosome then recruits and activates procaspase-9.

Given the potentially irreversible caspase cascade triggered by activation of the upstream initiator caspases, it is crucial that activation of caspases in the cell be tightly regulated. A number of cellular proteins have been shown to modulate caspase activation and activity. One of these, FLAME/FLIP, inhibits death receptor-mediated activation of caspase-8 by binding to FADD (12, 13). Others, such as the antiapoptotic members of the Bcl-2 family, inhibit Apaf-1-mediated activation of caspase-9 by blocking cytochrome c release from the mitochondria (reviewed in Refs. 14 and 15). Heat shock proteins Hsp70 and Hsp90 also interfere with the mitochondrial apoptotic pathway by modulating the formation of a functional Apaf-1 apoptosome (16–18). Finally, members of the IAP family, such as XIAP, c-IAP-1, and c-IAP-2, block both the death receptor and mitochondrial pathways by inhibiting the activity of the effector caspase-3 and -7 and the initiator caspase-9 (reviewed in Ref. 19).

Recently, Smac/DIABLO, a mitochondrial protein that is released together with cytochrome c from the mitochondria in response to apoptotic stimuli, was found to promote caspase activation by binding and neutralizing the IAPs (20, 21). In this report, we investigated in detail the caspase-promoting activity of Smac/DIABLO and its interaction with XIAP. We demonstrate that Smac/DIABLO promotes the caspase activity of the initiator caspase-9 and the effector caspase-3 and -7, by neutralizing the inhibitory effect of IAPs. Like the Drosophila Reaper, Grim, and Hid proteins (19, 22, 23), Smac/DIABLO requires its N terminus to interact with IAPs and promote caspase activation. Moreover, Smac/DIABLO can potentiate death receptor-induced apoptosis in the presence of Bcl-xL, suggesting that it could play an important role in this pathway.

EXPERIMENTAL PROCEDURES

cDNA Cloning and Expression Constructs—The entire open reading frames of human Smac/DIABLO-L and Smac/DIABLO-S cDNAs were

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1 The abbreviations used are: IAP(s), inhibitor of apoptosis protein(s); Smac/DIABLO-S, short Smac/DIABLO isoform; Smac/DIABLO-L, long Smac/DIABLO isoform; RT, reverse transcriptase; PCR, polymerase chain reaction; GST, glutathione S-transferase; GFF, green fluorescent protein; MTS, mitochondrial targeting sequence; DEVD-AMC, Asp-Glu-Val-Asp-7-Amino-Y-methyl coumarin; DEVD-A disappearance. The costs of publication were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF298770.

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FIG. 1. Expression and subcellular localization of the short isoform of Smac/DIABLO. a, a schematic representation of the N termini of the Smac/DIABLO precursor (Smac/DIABLO-L) and the alternatively spliced short isoform of Smac/DIABLO (Smac/DIABLO-S). Smac/DIABLO-L begins with the MKSDFYF sequence, which replaces the MTS and residues 55–60 (AVPIA) of the Smac/DIABLO-L. Smac/DIABLO-L and Smac/DIABLO-S are 100% identical after residue 60. The arrow indicates the cleavage site, which removes the MTS to generate mature Smac/DIABLO-L. b, RT-PCR analysis of the expression of Smac/DIABLO-L (lane 1) and Smac/DIABLO-S (lane 2) in different cell lines (Jurkat, 293, THP1, MCF-7, A431, and 697). The cloned Smac/DIABLO-L or Smac/DIABLO-S cDNAs were used as control templates (cDNA lane). c, MCF-7 cells were transfected with constructs encoding GFP (A) or C-terminal GFP-tagged Smac/DIABLO-S (B) or Smac/DIABLO-L (C). 24 h after transfection, cells were visualized by confocal microscopy and photographed. Note the uniform cytoplasmic fluorescence in panels A and B and the punctate perinuclear (mitochondrial) fluorescence in panel C.

cloned from Jurkat mRNA by RT-PCR using complementary PCR adapter primers spanning the initiation and stop codons of these cDNAs. The PCR primers were designed based on the sequence of mouse DIABLO and human GenBank™ clones AW16150 and AK001399. All deletion mutants were also generated by PCR using modified complementary PCR adapter primers. All Smac/DIABLO constructs were cloned in pET 28(a) in the NcoI/XhoI site with C-terminal His6 tag or GST tag. All XIAP constructs were cloned in the EcoRI/XhoI site of pGEX-4T or MYC-pcDNA3. The MYC-pcDNA3 constructs were used for in vitro translations.

In Vitro Interaction Assay—All in vitro interactions were performed by incubating 10 μl of TNT® (Promega) translation reaction at 4 °C for 2 h with equal amounts of proteins (200 ng) bound to 50 μl of glutathione-Sepharose (Amersham Pharmacia Biotech) or TALON™-agarose (CLONTECH) or GFP-Smac/DIABLO expression construct together with 0.5 μg of empty vector plasmids or plasmids encoding GST-XIAP or GST-tagged Smac/DIABLO or XIAP were purified using TALON™ resin or glutathione-Sepharose by standard affinity-purification procedures as described previously (7).

Caspase-3 Activation and DEVD Cleavage Assays—Cytosolic c-dependent activation of caspase-9 and caspase-3 was done in 293T extracts as described previously (7, 20). Caspase-3 and -7 enzymatic assays were performed in a 20-μl volume in buffer A (20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol) at 37 °C. Pure GST-XIAP was incubated with 5 nM of pure caspase-3 (at 20 nM concentration) or caspase-7 (at 5 nM concentration) with various amounts of Smac/DIABLO and 50 μM Asp-Glu-Val-Asp-7-Amino-Y-methyl coumarin (DEVD-AMC) substrate for 30 min. The release of AMC from the DEVD-AMC substrate was measured using a PerkinElmer Life Sciences luminescence spectrometer. The results were expressed as percent of DEVD cleavage from at least three experiments.

Apoptosis Assay—MCF-7 cells (0.5 × 10⁵ cells/well) in 12-well plates were transfected with 0.5 μg of pEGFP-N1 reporter plasmid (CLONTECH) or GFP-Smac/DIABLO expression construct together with 0.5 μg of empty vector plasmids or plasmids encoding Bcl-xL using the LipofectAMINE™ method. 24 h after transfection cells were treated with TRAIL (0.5 or 2 μg/ml) for 10 h and then the normal (flat and attached) and apoptotic (round and detached) GFP-expressing cells were counted using fluorescence microscopy. The percentage of apoptotic cells in each experiment was expressed as the mean percentage of apoptotic cells as a fraction of the total number of GFP-expressing cells.

RESULTS AND DISCUSSION

Identification of a Cytosolic Isoform of Smac/DIABLO—The Smac/DIABLO precursor contains a 55-residue mitochondrial targeting sequence (MTS) at its N terminus that is cleaved in the mitochondria to generate the mature mitochondrial Smac/DIABLO. Genomic analysis of the Smac/DIABLO gene on chromosome 12q (GenBank™ accession number AC048338) revealed that the MTS is encoded by the first two exons. We found that the exons encoding the MTS are spliced out in two human expressed sequence tag clones (AA156765 and AA305624) and one full-length clone (AK001399) in the data base. The three clones contain an open reading frame that generates Smac/DIABLO-S lacking the MTS (Fig. 1a). The alternatively spliced Smac/DIABLO-S mRNA is expressed in
XIAP (100%) in the absence of XIAP (100%).

The caspase activity in all the samples is plotted as a percentage of the activity of caspase-3 or -7 in the presence of XIAP. Smac/DIABLO was able to promote the enzymatic activity of both caspase-3 and -7 (Fig. 2c). To examine whether Smac/DIABLO promotes caspase-9, Smac/DIABLO-S had ~30% of the activity of wild type Smac/DIABLO with caspase-3 and -7, suggesting that Smac/DIABLO-S may play a role in regulating caspase-3 and -7 activity in vivo. Combined, these data suggest that Smac/DIABLO has a dual role in the caspase cascade, to promote the activities of the initiator caspase-9 and the effector caspases.

The ability of Smac/DIABLO to promote the enzymatic activity of caspases depends on its interaction with IAPs (20, 21). To determine whether the weak activity of Smac/DIABLO-S is due to altered interaction with XIAP, we performed in vitro interaction assays with 35S-labeled full-length XIAP or isolated BIR domains of XIAP (Fig. 2c). Consistent with its weak activity, Smac/DIABLO-S was not able to interact with the BIR3 domain (XIAP-BIR3/RING) of XIAP but was still able to interact with the BIR1/BIR2 domains (XIAP-BIR1/2) of XIAP. Because Smac/DIABLO-S can interact with the isolated BIR1/BIR2 domains (XIAP-BIR1/2) of XIAP, it was not surprising that it was also able to interact with full-length XIAP, although to a lesser extent than the wild type Smac/DIABLO.

The Caspase-promoting Activity of Smac/DIABLO Resides in Its N Terminus—The low activity of Smac/DIABLO-S compared with that of mature Smac/DIABLO suggests that the N terminus of mature Smac/DIABLO is very important for its activity. To understand the molecular basis for this difference, we generated a series of Smac/DIABLO N-terminal deletion mutants (Fig. 3a) and expressed them in bacteria. The recombinant proteins were purified to homogeneity and assayed for their ability to promote cytochrome c-dependent caspase-3 activation in XIAP-containing S100 extracts. Deletion of the first 21 residues (Δ21) further reduced Smac/DIA-
deletion mutants were expressed in bacteria with C-terminal His6 tags. The C-terminal deletion mutants were expressed with C-terminal GST tags.

Mature Smac/DIABLO and the N-terminal and C-terminal deletion mutants of Smac/DIABLO. The reactions were carried out as in Fig. 2c. Those in Fig. 2c were carried out in the presence of DEVD-AMC as a substrate.

BLO activity to undetectable levels. Other larger N-terminal deletions produced insoluble mutant proteins, which could not be used in this assay. However, one N-terminal deletion mutant lacking the first 139 residues (Δ139) was soluble but found to be completely inactive. The N-terminal deletion mutants were then assayed for their ability to enhance caspase-3 and -7 activity in the presence of XIAP (Fig. 3c). Like Smac/DIABLO-S, the N-terminal deletion mutants Δ4 and Δ21 had ~30–40% of the activity of wild type Smac/DIABLO with caspase-3 and -7. However, the N-terminal deletion mutant Δ139 was also completely inactive in this assay.

The results with the N-terminal deletion mutants suggest that the N terminus harbors the caspase-promoting activity of Smac/DIABLO. To test this hypothesis, we generated three C-terminal deletion mutants fused at their C termini to GST. These mutants, which contain the first 7, 30, or 39 N-terminal residues of mature Smac/DIABLO (N7, N30, and N39, respectively) (Fig. 3a), were expressed in bacteria and purified to homogeneity. As shown in Fig. 3b, lower panel, N30 and N39 were able to promote caspase-3 activation in S100 extracts containing XIAP, although at a higher concentration than the wild type Smac/DIABLO. The smallest mutant, N7, was the least effective among the mutants. N30 and N39 were also able to relieve the XIAP inhibition of caspase-7 and caspase-3 (Fig. 3c), although they were slightly more effective with caspase-7 than caspase-3. However, within the range of concentrations used in this experiment N7 had no detectable activity with caspase-3 and -7. Taken together, these results suggest that the caspase-promoting activity of Smac/DIABLO resides within an approximately 30-residue-long domain at its N terminus.

Like Smac/DIABLO-S, the N-terminal deletion mutants Δ4 and Δ21 were not able to interact with the BIR3 domain (XIAP-BIR3/BIRING) of XIAP but were still able to interact with the BIR1/BIR2 domains (XIAP-BIR1/2) of XIAP and to a slightly lesser extent with full-length XIAP (Fig. 3d, left panel). Based on these observations, we suggest that the first 4 residues of mature Smac/DIABLO are essential for its ability to interact with the BIR3 domain of XIAP. Because the BIR3 domain of XIAP is the domain that binds and inhibits caspase-9 (25), this could explain the very weak activity of Smac/DIABLO-S and the N-terminal deletion mutants Δ4 and Δ21 in the caspase-3 activation assay, which measures caspase-9 activity. Thus, deletion or substitution of the first 4 residues impairs the ability of Smac/DIABLO to interact with the BIR3 domain of XIAP and consequently its ability to promote caspase-3 activation by the caspase-9 apoptosome in the presence of XIAP.
less, because Smac/DIABLO-S and the N-terminal deletion mutants Δ4 and Δ21 can still interact with the BIR1/BIR2 domains of XIAP, which is important for caspase-3 and -7 inhibition (25), they had better caspase-promoting activity with caspase-3 and -7 (Figs. 2b and 3c) compared with that with caspase-9 in the presence of XIAP.

Interestingly, all the three C-terminal deletion mutants (N7, N30, and N39) were able to interact with full-length XIAP, as well as the isolated BIR domains of XIAP (Fig. 3d, right panel). However, the interaction of N7 with XIAP and its isolated BIR domains was weaker than that observed with N30 and N39. The weaker activity of N30 and N39 compared with wild type Smac/DIABLO (Fig. 3b and c) suggests that the sequences C-terminal to residue 39 are still required to achieve optimal activity, probably because these sequences are necessary to maintain an overall structure capable of disrupting the interaction of XIAP with the active site of caspases at lower concentrations of Smac/DIABLO. Indeed, according to the crystal structure of Smac/DIABLO (26), the residues that are involved in dimerization of Smac/DIABLO are located within a hydrophobic interface C-terminal to residue 25. Mutations that disrupt Smac/DIABLO dimerization were found to diminish its caspase-promoting activity (26). Thus we believe that the weak activity of the short N-terminal peptides, although they can still interact with XIAP, is perhaps due to removal of the dimerization domain C-terminal to these peptides.

Chemically Synthesized Smac/DIABLO N-terminal Peptides Can Promote Caspase Activation—The finding that short sequences derived from the N terminus of Smac/DIABLO can still interact with XIAP, cIAP-1, and cIAP-2 (Fig. 3d and data not shown) and promote the caspase activity of caspase-9, -3, and -7 (Figs. 2 and 3) suggests that these sequences can be used to make peptides that could enhance the ability of chemotherapeutic agents to induce apoptosis in cancer cells with elevated levels of IAPs. To test this hypothesis, we synthesized four peptides based on the N-terminal sequences of mature Smac/DIABLO and Smac/DIABLO-S and tested their ability to promote cytochrome c-dependent activation of caspase-3 in S100 extracts containing XIAP (Fig. 3e). The activity of caspase-3 in the S100 extracts was measured using the peptide substrate DEVD-AMC. The peptides containing the first 7 or 35 residues (Smac/DIABLO-N7 (pept-1) and Smac/DIABLO-N35 (pept-4), respectively) of mature Smac/DIABLO were very effective in promoting caspase-3 activation. The longer peptide (Smac/DIABLO-N35) was noticeably better than the short peptide (Smac/DIABLO-N7) in promoting caspase-3 activation. The peptide derived from the N terminus of Smac/DIABLO-S (Smac/DIABLO-S-N9 (pept-2)) or derived from an internal Smac/DIABLO sequence (Smac/DIABLO 27–35 (pept-3)) were almost completely inactive in this assay. These results provide a proof of principle that short peptides derived from the N terminus of mature Smac/DIABLO could be used in the future as effective activators of caspases at attainable concentrations to kill cancer cells that overexpress IAPs.

Expression of a Cytosolic Smac/DIABLO Can Convert Type II Cells to Type I Cells—In Type II cells (e.g. breast adenocarcinoma MCF-7 cells) death receptor-induced apoptosis can be blocked at the level of the effector caspases by IAPs such as XIAP (29). In this scenario, the cleavage of BID by caspase-8 might be required to release Smac/DIABLO to neutralize the inhibitory effect of XIAPs on the initiator and effector caspases.
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