Generation and Characterization of Smac/DIABLO-Deficient Mice


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Apoptosis, or programmed cell death (PCD), is a physiological cell suicide program essential for both embryonic development and the maintenance of tissue homeostasis in multicellular organisms (6, 11, 13). The family of cysteine proteases known as caspases are key components of mammalian PCD (1). Caspases are expressed in cells as inactive precursors, which are activated by proteolytic processing (22, 26). Two classes of caspases, initiators and effectors, are involved in mammalian apoptosis (3). Activated initiator caspases, such as caspase 8 and caspase 9, cleave the precursor forms of effector caspases, such as caspases 3, 6, and 7. Activated effector caspases in turn cleave a specific set of cellular substrates resulting in the biochemical and morphological changes associated with the apoptotic phenotype (26).

The activation of initiator caspases is thought to irreversibly trigger the caspase cascade, necessitating that caspase activation be tightly regulated by layered control mechanisms. Among the growing number of cellular proteins that have been shown to regulate caspase activation and activity are the IAPs, including c-IAP1, c-IAP2, XIAP, and survivin. These proteins have been reported to block both death receptor- and mitochondrion-mediated apoptotic pathways by directly inhibiting Smac blocks IAP activity, it has been proposed that Smac is a mitochondrial protein released into the cytosol and promotes caspase activation by binding to IAPs, thereby blocking their function. These observations have suggested that Smac is a new regulator of apoptosis. To better understand the physiological function of Smac in normal cells, we generated Smac-deficient (Smac<sup>−/−</sup>) mice by using homologous recombination in embryonic stem (ES) cells. Smac<sup>−/−</sup> mice were viable, grew, and matured normally and did not show any histological abnormalities. Although the cleavage in vitro of procaspase-3 was inhibited in lysates of Smac<sup>−/−</sup> cells, all types of cultured Smac<sup>−/−</sup> cells tested responded normally to all apoptotic stimuli applied. There were also no detectable differences in Fas-mediated apoptosis in the liver in vivo. Our data strongly suggest the existence of a redundant molecule or molecules capable of compensating for a loss of Smac function.

The mitochondrial proapoptotic protein Smac/DIABLO has recently been shown to potentiate apoptosis by counteracting the antiapoptotic function of the inhibitor of apoptosis proteins (IAPs). In response to apoptotic stimuli, Smac is released into the cytosol and promotes caspase activation by binding to IAPs, thereby blocking their function. These observations have suggested that Smac is a new regulator of apoptosis. To better understand the physiological function of Smac in normal cells, we generated Smac-deficient (Smac<sup>−/−</sup>) mice by using homologous recombination in embryonic stem (ES) cells. Smac<sup>−/−</sup> mice were viable, grew, and matured normally and did not show any histological abnormalities. Although the cleavage in vitro of procaspase-3 was inhibited in lysates of Smac<sup>−/−</sup> cells, all types of cultured Smac<sup>−/−</sup> cells tested responded normally to all apoptotic stimuli applied. There were also no detectable differences in Fas-mediated apoptosis in the liver in vivo. Our data strongly suggest the existence of a redundant molecule or molecules capable of compensating for a loss of Smac function.

Materials and Methods

**ES cell culture.** E14K embryonic stem (ES) cells from 129/Ola mice were maintained on a layer of mitomycin C-treated mouse embryonic fibroblasts (MEFs) in Dulbecco's modified Eagle's medium (DMEM) supplemented with leukemia inhibitory factor, 15% heat-inactivated fetal calf serum (HyClone, Logan, Utah), l-glutamine, and β-mercaptoethanol.

**Generation of Smac-deficient mice.** A 129/Ola mouse phage genomic library was screened with a murine Smac gene containing the IAP binding region (with a cassette in which the neomycin resistance gene is under the control of the PGK promoter (PGK-neo)). The diphtheria toxin A gene (DT-A) driven by the PGK1 promoter was incorporated into the 5' end of the vector to allow for negative selection (35). The targeting vector was linearized with NotI and electroporated into ES cells (Bio-Rad Gene Pulser; 0.34 kV, 250 μF). We obtained 380 G418-resistant ES cell colonies, which were screened for homologous recombination by PCR. Homologous recombination events were confirmed by Southern blot analysis as described previously (19) with
conjugated goat anti-rabbit secondary antibody or goat anti-mouse secondary antibody (Santa Cruz Biotechnology), or anti-Omi antibody (a kind gift from E. Alnemri, Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, Pa.). Immunoblot analysis was performed with horseradish peroxidase-conjugated rabbit polyclonal antibody to Smac (Cell Signaling), an antibody recognizing procaspase 3 (Transduction Laboratories), and anti-cIAP-1 antibody (R&D Systems), anti-cIAP-2 antibody (A145-2C11; BD-Pharmingen) per ml and interleukin-2 (50 U/ml) (Genzyme) for 48 h to induce activation. The activated cells (0.5 × 10⁶) were replated in 24-well plates precoated with 1 μg of anti-CD3ε per ml and harvested 72 h after restimulation. The number of viable cells was determined by trypan blue exclusion.

For T-cell proliferation, purified Smac−/− and Smac+/- T cells were plated into round-bottom 96-well plates (10⁵ cells per well) in freshly prepared RPMI 1640 (10% FCS, 10 μM β-mercaptoethanol). The cells were stimulated with 10 ng of phorbol myristate acetate per ml (Sigma) plus 100 ng of Ca²⁺ ionophore A23187 per ml, 0.1 to 100 μg of soluble anti-CD3ε per ml, 0.02 μg of soluble anti-CD28 per ml (clone 73-51; BD-PharMingen), or 2 μg of the mitogen concanavalin A per ml (Amerham-Pharmacia). For B-cell proliferation, purified B cells (10⁵ cells per well) were stimulated with 20 μg of anti-immunoglobulin M (IgM) (61-6800; Zymed), 10 μg of anti-IgM F(ab’)² fragment per ml (61-5900; Zymed), 1 μg of anti-CD40 per ml (clone HM40; BD-PharMingen), or 2 μg of lipopolysaccharide per ml (Sigma). Cells were stimulated in triplicate for different time periods and pulsed for the last 12 to 18 h with 1 μCi of [,H]thymidine per well (NEN, Boston, Mass.). Labeled cells were harvested with a Filtermate-196 harvester (Canberra Packard, Mississauga, Canada), and thymidine incorporation was determined with a Matrix-996 direct counter (Canberra Packard).

Histological analysis. Tissues were fixed in freshly prepared 4% paraformaldehyde (Sigma) overnight at 4°C. Samples were dehydrated in an ethanol series and embedded in wax. Sections were stained with either hematoxylin and eosin (H&E) or by terminal deoxynucleotide transferase nick-end labeling (TUNEL) staining. TUNEL staining was performed with the Roche In Situ Cell Death Detection kit according to the manufacturer’s instructions.

Induction of apoptosis in the liver. Young adult mice (8 to 10 weeks old) were injected intraperitoneally with anti-Fas antibody (Jo2; BD-PharMingen) at a dose of 10 or 100 μg per animal. Some animals were monitored for survival, while others were killed for injection for histological or immunofluorescent studies. Sections of liver were stained with H&E or TUNEL as described above. Numbers of TUNEL-positive cells were counted in at least five fields per liver.

RESULTS

Targeted disruption of the Smac gene in mice. To determine the physiological role of Smac in vivo, we used homologous recombination in ES cells to disrupt the Smac gene and generate Smac knockout mice (Fig. 1A). The targeting vector was designed to replace most of the Smac coding region, including exons 2 to 4, with the PGK-neo cassette. A polymorphism was discovered in a BamHI restriction site in the Smac locus when DNA from the C57BL/6J and 129/Ola genetic backgrounds was compared. Probe A indicated in Fig. 1A recognizes a 7-kb fragment from the 129/Ola wild-type allele, a 12.0-kb fragment from the C57BL/6J wild-type allele, and a 5.0-kb fragment from the targeted allele. We obtained 19 independent ES cell clones carrying the mutated Smac allele, and 3 (sm-7, sm-24, and sm-33) were injected into C57BL/6J blastocysts to generate chimeric mice. F₁ heterozygotes originating from the sm-24 or sm-33 clones were intercrossed to produce the F₂ progeny

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FIG. 1. Targeting of the murine Smac gene by homologous recombination. (A) Schematic representation of the wild-type mouse Smac locus (top), the targeting construct (middle), and the mutated Smac allele (bottom). The coding exons are shown as clear boxes. Exons 2 to 4 were replaced with PGK-neo, and DT-A was added for negative selection. The 5'-flanking probe A used for Southern blot analysis is shown, as are the predicted sizes of the hybridizing fragments. The primer pairs used for PCR (a and b or a and c) are also indicated. R, EcoRI; B, BamHI; X, XhoI; N, NotI. (B) Southern blot analysis of Smac-deficient ES cells and mice. (Left panel) DNA prepared from C57BL6/J (+/+) and F1 offspring (+/−) of chimeric mice. (Right panel) DNA was from 129/Ola ES cells (−/−), Smac−/− ES cell clones, C57BL6/J (+/+) mice, and F2 offspring (+/+ +/− −/−) of intercrosses of Smac mutant F1 mice. In all cases, tail genomic DNA was digested with BamHI and hybridized to probe A. (C) Western blot analysis of Smac protein expression. Protein samples were prepared from Smac+/+, Smac+/−, and Smac−/− MEFs and incubated with anti-Smac antibody. Actin was used as the loading control. (D) Genotypic analysis of F2 littermates by PCR. PCR was performed on genomic tail DNA templates with primer pair a and b to detect the wild-type allele (W) or primers a and c to detect the mutant allele (M).
Female Smac−/− mice were healthy and fertile, and F3 Smac−/− offspring obtained by homozygous intercrosses were also healthy. Both Smac+/− and Smac−/− mice developed normally and did not exhibit any obvious macroscopic or microscopic abnormalities (data not shown). Aged mice (more than 12 months of age) did not show any sign of anomalies, such as autoimmune disease or tumor formation (data not shown).

Caspase 8 activation in vitro is impaired in Smac−/− cell lysates. The Smac protein is localized in the soluble membrane fraction of cell lysates, which promotes caspase 3 activation in a manner dependent on caspase 9, Apaf-1, and cytochrome c (5). We therefore compared in vitro cleavage of procaspase 3 in cell lysates prepared from Smac−/− or Smac+/+ MEFs. Caspase 3 activation was induced by the addition of dATP and cytochrome c to cell lysates, and the presence of cleaved, endogenous caspase 3 was detected by immunoblotting with antibody specifically recognizing active caspase 3. While procaspase 3 cleavage could be detected within 30 min in Smac+/+ lysates, activated caspase 3 was not observed in Smac−/− lysates even after 60 min (Fig. 2A). To confirm that this phenotype was caused by the loss of Smac, we reconstituted the Smac−/− lysate with recombinant Smac protein (5). Procaspase 3 cleavage was restored in the Smac−/− lysate in a dose-dependent manner (Fig. 2B). To exclude the possibility that the loss of Smac affected the protein levels of the IAPs, we compared the levels of several IAPs in Smac+/+ and Smac−/− lysates by Western blotting. Equivalent amounts of c-IAP1, c-IAP2, XIAP, and survivin were detected in the presence and absence of Smac (data not shown). We also investigated the expression of Omi/Htra2, a recently reported functional homologue of Smac (10, 14, 25, 27). Equivalent amounts of Omi protein were detected in Smac−/− and Smac+/+ lysates (data not shown). From these observations, we conclude that we have introduced a functionally null mutation into the Smac gene and confirm that Smac potentiates procaspase 3 cleavage in vitro as previously reported (5).

Induction of apoptosis is normal in primary and transformed Smac−/− cells. Since procaspase 3 cleavage is a defining event in most instances of apoptosis, we investigated whether the loss of Smac had any effect on apoptosis induced in MEFs subjected to UV irradiation. It has previously been shown that overexpression of Smac itself does not induce apoptosis in cells, but rather sensitizes them to UV-induced cell death (5). We subjected Smac−/− and Smac+/+ primary MEFs to UV irradiation and assessed viability. As shown in Fig. 3A, similar levels of PCD were induced in Smac−/− and Smac+/+ MEFs at 24 h after UV irradiation. It has been reported that Smac starts to be released from the mitochondria into the cytosol within the first few hours after UV irradiation (5). To examine the possibility that Smac might inhibit procaspase 3 cleavage at early time points, we compared the amount of

**Fig. 1B**. Southern blot analyses of DNA from C57BL/6J wild-type mice, C57BL/6J-129/Ola F1 offspring, 129/Ola ES cells, and F2 offspring from F1 intercrosses. To confirm that the knockout allele was a null mutation, MEFs were prepared from Smac+/+, Smac−/−, and Smac−/− littersmates and lysates were subjected to Western blotting to detect Smac expression. As shown in Fig. 1C, Smac−/− MEFs do not express Smac. Genotypic analysis of F2 offspring was confirmed by PCR (Fig. 1D). Of 243 F2 pups, 65 were wild type (26.7%), 117 were heterozygous for the mutation (48.2%), and 61 were homozygous mutants (25.1%), consistent with the ratio expected from Mendelian inheritance. Both male and female Smac−/− mice were healthy and fertile, and F3 Smac−/− offspring obtained by homozygous intercrosses were also healthy. Both Smac+/− and Smac−/− mice developed normally and did not exhibit any obvious macroscopic or microscopic abnormalities (data not shown). Aged mice (more than 12 months of age) did not show any sign of anomalies, such as autoimmune disease or tumor formation (data not shown).

**Fig. 2**. Smac deficiency impairs caspase 3 cleavage in vitro. (A) Impaired in vitro caspase 3 cleavage in Smac−/− MEFs. Lysates of Smac+/+ or Smac−/− MEFs were incubated in vitro with the indicated assay reagents, and the cleavage of procaspase 3 was detected by Western blotting with antibodies recognizing either the cleaved (active) form of caspase 3 or procaspase 3. One of the representative results from three independent samples is shown. cyt C, cytochrome c. (B) Restoration of procaspase 3 cleavage by addition of recombinant Smac protein. Lysates of Smac−/− MEFs were incubated with the indicated reagents plus 1 nM to 1 μM recombinant Smac protein. Detection of activated caspase 3 was as for panel A.

**Fig. 3**. Smac-deficient cells respond normally to apoptotic stimuli. (A) PCD induced by UV irradiation in primary MEFs. Smac+/+ or Smac−/− MEFs were subjected to UV irradiation, and cell viability was determined by annexin-PI staining at 24 h. (B) Procaspase 3 cleavage in UV-treated MEFs. Protein samples were prepared from Smac+/+ or Smac−/− MEFs at 0, 4, 8, 12, or 16 h after UV irradiation. The amount of active caspase 3 was determined by Western blot analysis as for Fig. 2A. (C to F) Effect of Smac deficiency on PCD induced by various other stimuli in MEFs and other cell types. MEFs (C), ES cells (D), thymocytes (E), and E1A- or c-Myc-overexpressing MEFs (F) were treated with the indicated reagents for 24 h, and cell viability was determined by annexin-PI staining. The data shown are means ± standard deviations of triplicate measurements and are representative of three experiments with similar results.
active caspase 3 accumulated in Smac<sup>−/−</sup> and Smac<sup>+/+</sup> MEFs at 0, 4, 8, 12, and 16 h after UV irradiation. No difference in the level of active caspase 3 was detected at any time point (Fig. 3B), indicating that UV-induced apoptosis of primary MEFs is not affected by the loss of Smac.

We next examined a range of chemical agents to determine whether Smac is associated with PCD induced only in certain cell types or only by certain apoptotic stimuli. Staurosporine, adriamycin, anisomycin, etoposide, and TNF/CHX all induced PCD equally in Smac<sup>−/−</sup> and Smac<sup>+/+</sup> MEFs (Fig. 3C) and also in Smac<sup>−/−</sup> and Smac<sup>+/+</sup> ES cells (Fig. 3D [TNF/CHX not tested]). Similarly, Smac<sup>−/−</sup> thymocytes treated with etoposide, dexamethasone, or Fas/CHX underwent normal PCD (Fig. 3E). PCD induced by staurosporine or by UV or gamma irradiation was also indistinguishable in thymocytes from Smac<sup>+/+</sup> and Smac<sup>−/−</sup> mice (data not shown). Thus, despite the involvement of IAPs in apoptosis, loss of Smac has no effect on PCD induced in these cell types under these circumstances.

Oncogenes can sensitize cells to apoptosis mediated by the mitochondrial pathway (7, 8). We therefore infected Smac<sup>−/−</sup> and Smac<sup>+/+</sup> MEFs with retroviruses causing overexpression of the oncogene c-Myc or E1A. These cells were then treated with adriamycin, TRAIL, or paclitaxel to induce PCD. Cells of both genotypes became equally hypersensitive to apoptotic stimuli (Fig. 3F). This result was somewhat unexpected in view of the fact that transformed caspase 3-deficient MEFs are resistant to PCD induced by these stimuli (32). Our data suggest that other molecules must be able to substitute for Smac in the induced PCD of both primary and oncogene-overexpressing cells.

Smac-deficient T and B cells show normal proliferation and survival. In the absence of caspase 3, T cells are less susceptible to AICD (30), and activated B cells hyperproliferate in response to mitogenic stimulation (M. Woo, C. Furlonger, R. Hakem, C. Paige, and T. W. Mak, submitted for publication). Since Smac deficiency decreased the amount of activated caspase 3 in MEFs, we examined AICD of T cells and proliferation of both activated T and B cells. T cells were activated in vitro as described in Materials and Methods, and cell viability at 24, 48, and 72 h was evaluated by trypan blue exclusion. No significant differences in AICD were observed between Smac<sup>+/+</sup> and Smac<sup>−/−</sup> T cells (Fig. 4A). Proliferation of T and B cells activated as described in Materials and Methods was measured by [H]thymidine incorporation at 24, 48, or 72 h after activation for T cells and at 24, 48, or 96 h after activation for B cells. There was no difference between Smac<sup>+/+</sup> and Smac<sup>−/−</sup> cells in [H]thymidine incorporation by either activated T or B cells (Fig. 4B). These results show that Smac does not affect either cell proliferation or survival of activated lymphocytes.

Fas-mediated apoptosis occurs normally in Smac<sup>−/−</sup> hepatocytes. It has been reported that Fas-mediated hepatocyte cell death is delayed in caspase 3-deficient mice (31). Since caspase 3 activation was impaired in vitro in the absence of Smac, we investigated the role of Smac in Fas-mediated PCD in vivo by injecting Smac<sup>−/−</sup> mice with agonistic anti-Fas antibody. Both Smac<sup>+/+</sup> and Smac<sup>−/−</sup> mice died within 3 or 10 h after injection of 100 or 10 μg of anti-Fas antibody, respectively, with similar kinetics (Fig. 5A). Hepatocyte apoptosis was verified by staining liver sections with H&E (Fig. a, c, and e) or TUNEL (Fig. b, d, and f). No statistically significant differences in numbers of TUNEL-positive cells were observed between Smac<sup>+/+</sup> and Smac<sup>−/−</sup> mice livers (data not shown). Similar results were obtained when apoptosis was induced in heart or kidney by injecting adriamycin (12) or tunicamycin (15), respectively (data not shown).

**DISCUSSION**

Our finding in vivo that targeted disruption of the Smac gene has no effect on apoptosis stands in sharp contrast to previous evidence established by biochemical, cell biological and structural analyses that favor a role for Smac/DIABLO in PCD (5, 29). One of the best examples of this apparent contradiction in our report is the discrepancy between the results of the in vitro caspase 3 activation assay and the phenotype of Smac<sup>−/−</sup> cells. We were able to demonstrate differences in procaspase 3 cleavage in Smac<sup>−/−</sup> and Smac<sup>+/+</sup> MEF lysates, but could not detect any differences in induced PCD in MEFs. The simplest explanation for this discrepancy is that a functional homologue of Smac exists in vivo, but was not present or functional in cell lysates. We found equivalent protein expression of Omi/HtrA2, a functional homologue of Smac, in wild-type and Smac-deficient cell lysates, but the possibility remains that Omi may have been inactive or only weakly active under the in vitro assay conditions used. In this context, it is noteworthy that some activated caspase 3 was detected in Smac<sup>−/−</sup> samples after prolonged incubation (data not shown); however, this amount of activated caspase 3 was still significantly less than that in the wild type.

The phenotype of Smac-deficient mice could easily be accounted for if Omi was fully active and sufficient to compensate for the loss of Smac in vivo. It will therefore be useful to generate double mutant mice lacking both Smac and Omi to clarify the precise physiological role of the Smac-mediated pathway. Alternatively, other mitochondrial proapoptotic molecules, such as AIF (24), NOXA (17), and p53AIP (18), may be able to compensate for a loss of Smac function in certain contexts in vivo. Finally, the phenotype of Smac<sup>−/−</sup> mice could result if Smac does not play an essential role in the common apoptotic machinery, but instead participates in regulating PCD only in a specific situation or in tissues yet to be identified.
A role for Smac in apoptosis is not evident from our gene disruption study. However, no obvious abnormalities were found in Smac<sup>−/−</sup> mice, meaning that investigation of alternative functions for the Smac protein will require extensive efforts to find specific conditions under which loss of Smac results in pathology. The generation of combined mutants by crossing Smac-deficient mice with transgenic mice prone to cancer formation or autoimmune disease might help to reveal covert phenotypes. On the other hand, the phenotype of Smac-deficient mice presented in this study strongly suggests the existence of a redundant molecule or molecules. Research to identify such molecules may bring to light new regulatory mechanisms of apoptosis, which could ultimately lead to novel therapeutic targets for diseases caused by the deregulation of apoptosis.

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