SMAC Negatively Regulates the Anti-apoptotic Activity of Melanoma Inhibitor of Apoptosis (ML-IAP)*

Inhibitors of apoptosis (IAPs) physically interact with a variety of pro-apoptotic proteins and inhibit apoptosis induced by diverse stimuli. X-linked IAP (X-IAP) is a prototype IAP family member that inhibits several caspases, the effector proteases of apoptosis. The inhibitory activity of X-IAP is regulated by SMAC, a protein that is processed to its active form upon receipt of a death stimulus. Cleaved SMAC binds X-IAP and antagonizes its anti-apoptotic activity. Here we show that melanoma IAP (ML-IAP), a potent anti-cell death protein and caspase inhibitor, physically interacts with SMAC through its BIR (baculovirus IAP repeat) domain. In addition to binding full-length SMAC, ML-IAP BIR associates with SMAC peptides that are derived from the amino terminus of active, processed SMAC. This high affinity interaction is very specific and can be completely abolished by single amino acid mutations either in the amino terminus of active SMAC or in the BIR domain of ML-IAP. In cells expressing ML-IAP and X-IAP, SMAC coexpression or addition of SMAC peptides abrogates the ability of the IAPs to inhibit cell death. These results demonstrate the feasibility of using SMAC peptides as a way to sensitize IAP-expressing cells to pro-apoptotic stimuli such as chemotherapeutic agents.

Programmed cell death, or apoptosis, is a genetically regulated mechanism that plays an important role in development and homeostasis in metazoans (1). Abnormalities in programmed cell death that lead to early cell death or the absence of normal cell death have been linked to a variety of human diseases, including neurodegenerative disorders and cancer (2). Currently there are two well characterized apoptotic pathways, one initiated through the engagement of cell surface death receptors by their specific ligands (3) and the other triggered by changes in internal cellular integrity (4). Both pathways eventually converge, resulting in activation of caspases, cysteine-dependent aspartate-specific proteases that comprise the effector arm of the apoptotic process (5).

The major regulators of caspases are the IAPs1 or inhibitors of apoptosis (6). Originally identified in baculoviruses by their ability to substitute functionally for P35, a potent anti-apoptotic gene product (7–9), IAPs have been discovered in both invertebrates and vertebrates (10–18). Members of the IAP family are characterized by one to three tandem baculovirus IAP repeat (BIR) motifs, and most of them also possess a carboxyl-terminal RING finger motif (6). IAPs inhibit apoptosis induced by a variety of stimuli and interact with multiple cellular partners (19). The anti-apoptotic activity of several IAPs has been attributed to their ability to inhibit caspases (15, 20, 21). Human X-chromosome-linked IAP (X-IAP), for example, inhibits active caspases-3 and -7 and Apaf-1-cytochrome c-mediated activation of caspase-9 (22, 23). This inhibitory activity is mediated through distinct BIR domains of X-IAP; the BIR2 domain and preceding linker region inhibit caspases-3 and -7, while BIR3 blocks caspase-9 (24–26). Similarly, the anti-apoptotic activity of melanoma IAP, or ML-IAP, is attributed to its lone BIR domain (15). ML-IAP can bind and inhibit caspase-9 through its BIR domain, and mutations in the BIR reduce both inhibition of caspase-9 and general anti-apoptotic activity (15).

IAPs are themselves regulated by proteins that block their anti-apoptotic activity (27). In Drosophila, Reaper (RPR), HID, and GRIM physically interact with and inhibit the anti-cell death activity of D-IAP1 and D-IAP2, fly members of the IAP family (28–30). SMAC/DIABLO performs a similar function to RPR, HID, and GRIM in mammals (31, 32). An amino-terminal signal sequence targets SMAC to mitochondria (31), but during apoptosis, SMAC is processed into the active form and released into the cytosol where it binds IAPs and prevents them from inhibiting caspases (31, 33). Thus, SMAC, by binding to the BIR2 and BIR3 domains of X-IAP, abrogates inhibition of the caspases-3 and -9 (34). Interestingly, the only sequence homology between insect RPR, HID, and GRIM and human SMAC is in the four amino-terminal residues of the active proteins (35). The same region is present in the linker peptide of processed caspase-9 (35) and in HtrA2, a recently identified SMAC-like IAP antagonist (36–39). This short peptide fits into a small hydrophobic pocket on the surface of the X-IAP BIR3 domain and is essential for binding IAPs and blocking their caspase inhibitory activity (34, 40, 41).

In this study we demonstrate that SMAC physically interacts with ML-IAP and abrogates the ability of ML-IAP to inhibit apoptosis. Peptides derived from the amino terminus of active SMAC are also shown to bind ML-IAP and attenuate its anti-apoptotic activity. The specificity of the SMAC–ML-IAP interaction is supported by the finding that single amino acid changes in the amino terminus of active SMAC or the BIR domain of ML-IAP completely abolish their association.

EXPERIMENTAL PROCEDURES

Expression Constructs—Plasmids expressing β-galactosidase, p35, Myc-XIAP, and Myc-ML-IAP, as well as ML-IAP deletions and site-
specific mutants, have been described previously (15, 42, 43). cDNA encoding human SMAC was PCR-amplified from a HeLa cDNA library with SMAC-specific primers. SMAC cDNA was then subcloned into the mammalian expression vector pCFNA.31 with a carboxyl-terminal FLAG tag (Invitrogen). The SMAC55M construct (amino acids 56–239) was also PCR-amplified and similarly subcloned into the pCFNA3.1 vector. Sequences encoding X-IAP BIR3 (amino acid residues 241–356) and ML-IAP BIR (amino acid residues 63–179) were subcloned into pET15b vector (Novagen) for bacterial expression.

Cell Culture, Antibodies, and Immunoprecipitations—Human 293T embryonic kidney cells and MCF7 human breast carcinoma cells were cultured as described previously (43). The primary antibodies used were anti-FLAG M2 (Sigma-Aldrich), anti-Myc (Covance), anti-caspase-9 (PharMingen), and anti-SMAC (Imgenex). Immunoprecipitations were performed as described previously (15, 43).

Protein Purification, Peptide Generation, and Peptide Binding Assays—Escherichia coli strain BL21 (DE3) transformed with pET-15b-FL-SMAC or pET-15b-ML-IAP-BIR was induced with 1 mM isopropyl-β-D-thigalactopyranoside for 4 h at 30 °C, pelleted, and resuspended in 100 ml of Buffer A (50 mM Tris (pH 8.0), 300 mM NaCl, 5 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine) containing 5 mM imidazole. Lysate produced by homogenization and centrifugation was passed through nickel-agarose and Superdex 75 sizing columns, washed, eluted, and dialyzed against buffer containing 50 mM Tris (pH 8.0), 300 mM NaCl, 5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 50 mM zinc acetate, and 1 mM sodium azide.

Antennapedia (RQIKIWFQNRRMKWKK-NH2) fusions were constructed using standard solid phase methods utilizing Wang resin with the condensation reagent. The control peptide biotin-TGWETWVCOOH was made by biotinylating the amino terminus of the peptide. Trifluoroacetic acid cleavage and HPLC purification obtained the labeled peptides.

Fluorescence polarization experiments were performed in 96-well plates on the Analyst HT 96–384 (Molecular Devices Corporation).

Binding experiments were performed using 1.3 serial dilutions of ML-IAP BIR and X-IAP BIR3 domains starting from 300 nM in 50 mM Tris buffer (pH 7.2), 120 mM NaCl, 5 mM dithiothreitol. An approximately 1 mM concentration of each 5-carboxyfluorescein-tagged probe was added to a set of wells containing the protein dilutions. The Kd values of the probe-protein interactions were calculated using Klotz plots and confirmed by Scatchard analysis.

Three-dimensional Modeling and Sequence Analysis—The high resolution crystal structure of SMAC/DIABLO complexed with the BIR3 domain of X-IAP (40) was used to model the NH2-terminal four residues (Ala-Val-Pro-Ile) of SMAC complexed into the binding groove of the previously modeled structure of ML-IAP (15). Both protein structures, X-IAP and ML-IAP, were superimposed by their α carbons (root mean square of Ca – 0.47 Å), and docking of the SMAC peptide was manually performed on the ML-IAP binding site. The ML-IAP-SMAC complex was energy minimized using DISCOVER (Molecular Simulations, Inc.). Amino acid sequence alignments were performed using ClustalW (44).

RESULTS AND DISCUSSION

ML-IAP Physically Interacts with SMAC—SMAC has been shown to physically associate with several IAP family members, most prominently with X-IAP (31). To determine whether SMAC can bind ML-IAP, we coexpressed SMAC with X-IAP, ML-IAP, or vector control. Upon overexpression, SMAC was processed to its active form whereby the first amino-terminal 55 amino acids are cleaved (Fig. 1A). Association of ML-IAP and X-IAP with active SMAC was demonstrated by immunoprecipitation, and the interactions occurred with similar efficiency (Fig. 1A). We investigated the portion of ML-IAP that is responsible for interaction with SMAC using truncation mutants of ML-IAP containing either the BIR or the RING finger domains (Fig. 1B). The BIR domain, like full-length ML-IAP,
immunoprecipitated active SMAC but the RING finger domain did not (Fig. 1B). Thus, ML-IAP physically interacts with SMAC through its BIR domain.

**ML-IAP BIR Binds RPR-like Peptides**—Sequence comparison of the active forms of human SMAC, HtrA2, and caspases-9 and fly RPR, HID, and GRIM revealed a strong similarity in their amino termini (Fig. 2A) (35). Since the four amino-terminal residues (Ala, Val, Pro, Ile) of active SMAC fit into the binding groove on the surface of the X-IAP BIR3 (40), we investigated whether a similar complex might form between ML-IAP BIR and SMAC peptide. A three-dimensional model based on the reported structure of the SMAC/X-IAP BIR3 complex predicted that SMAC peptide should bind ML-IAP BIR much as it does X-IAP BIR3 (Fig. 2B).

To test the validity of our model, we examined whether SMAC, HtrA2, caspase-9, or control peptides could bind the BIR3 domain of X-IAP or the BIR of ML-IAP. SMAC, HtrA2, and caspase-9 peptides efficiently pulled down purified X-IAP BIR3 and ML-IAP BIR, whereas a control peptide did not (Fig. 3A). The binding affinities of these peptides for X-IAP BIR3 and ML-IAP BIR were determined by a fluorescence polarization-based assay. $K_d$ values of 5-FAM-coupled peptides were 1.5 μM (SMAC), 1.8 μM (caspase-9), and 8.0 μM (HtrA2) for X-IAP BIR3 and 0.15 μM (SMAC), 0.15 μM (caspase-9), and 0.50 μM (HtrA2) for ML-IAP BIR.

**SMAC Negatively Regulates ML-IAP**—Processing of SMAC exposes the four amino-terminal residues that mediate binding to X-IAP and are required for SMAC to block caspase inhibition by X-IAP. We investigated whether this region of SMAC is also required for SMAC to bind ML-IAP and abrogate its anti-cell death activity. Active SMAC was mimicked in coimmunoprecipitation experiments using an amino-terminally truncated form of SMAC (amino acids 56–239) (SMAC55M) (Fig. 4A). Full-length SMAC that was processed to its active form was able to bind ML-IAP or X-IAP (Fig. 4B). In contrast, there was no interaction between SMAC55M and ML-IAP or X-IAP (Fig. 4B). Consistent with these results, SMAC but not SMAC55M was able to abrogate ML-IAP-mediated inhibition of adriamycin-induced apoptosis (Fig. 4C). Addition of a single methionine to the amino terminus of active SMAC was therefore sufficient to block its inhibitory effect on ML-IAP.

We also tested whether peptides corresponding to the nine

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**Fig. 3.** A, binding of X-IAP BIR3 and ML-IAP BIR to biotinylated SMAC, HtrA2, caspase-9, or control (irrelevant) peptides. X-IAP BIR3 and ML-IAP BIR were incubated in Nonidet P-40 buffer with the indicated peptides (10 μM) for several hours and the complexes precipitated with streptavidin-agarose. Following acrylamide gel electrophoresis, precipitated proteins were visualized by silver staining. Binding affinities of 5-FAM-labeled peptides to X-IAP BIR3 (B) and ML-IAP BIR (C) as determined by fluorescence polarization-based assay. $K_d$ values of 5-FAM-coupled peptides were 1.5 μM (SMAC), 1.8 μM (caspase-9), and 8.0 μM (HtrA2) for X-IAP BIR3 and 0.15 μM (SMAC), 0.15 μM (caspase-9), and 0.50 μM (HtrA2) for ML-IAP BIR.

**Fig. 4.** SMAC blocks the anti-apoptotic activity of ML-IAP. A, schematic representation of SMAC and SMAC55M constructs. Numbers designate the coding region boundaries expressed in each construct. B, 293T cells were transiently transfected with SMAC or SMAC55M and X-IAP, ML-IAP, or vector. After 40 h, cells were lysed in Nonidet P-40 lysis buffer, and samples were processed as in Fig. 1A. C, MCF7 cells were transiently transfected with the reporter plasmid pCMV-βgal and either vector control alone or ML-IAP plus vector, SMAC, or SMAC55M. Following transfection, cells were exposed to adriamycin, stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, and apoptosis assessed as described previously (42). D, MCF7 cells were transiently transfected with the reporter plasmid pCMV-βgal and either vector control, X-IAP, or ML-IAP. Following transfection, SMAC or M-SMAC peptides (50 μM) were added where indicated and cells exposed to adriamycin. Apoptosis was assessed as described previously (42).
residues at the amino terminus of active SMAC would reverse
ML-IAP-mediated inhibition of apoptosis. SMAC and M-SMAC
peptides, the latter having an additional amino-terminal me-
thionine, were synthesized as fusions with antennapedia pep-
tide. Antennapedia peptides permit chimeric fusions to gain
entry into the cell where they can engage their targets (45).
Expression of ML-IAP or X-IAP efficiently blocked adriamycin-
induced apoptosis, and addition of M-SMAC peptides did not
have a significant inhibitory effect on the protective activity of
IAPs (Fig. 4D). However, addition of SMAC peptides almost
completely negated the ability of ML-IAP and X-IAP to inhibit
apoptosis (Fig. 4D). Thus, coexpression of full-length SMAC or
addition of SMAC-like peptides abrogates the anti-apoptotic
activity of ML-IAP.

SMAC Disrupts Binding of ML-IAP to Processed Caspase-9—To better understand the mechanism by which SMAC an-
tagonizes the anti-apoptotic function of ML-IAP, we investiga-
ted the effect of SMAC on the ability of ML-IAP to bind
caspase-9. When overexpressed, caspase-9 undergoes autocat-
alytic processing, and it is the processed form that physically
interacts with X-IAP (35, 46). Similarly, ML-IAP commun-
precipitated processed caspase-9 but not its zymogen precursor
(Fig. 5A). The interaction between ML-IAP and caspase-9 is
highly specific, because mutation of aspartate 138 to alanine in
the BIR domain of ML-IAP completely abolished the ability of
ML-IAP to bind processed caspase-9 (Fig. 5A).

Coexpression of caspase-9 and ML-IAP with SMAC pre-
vented ML-IAP from interacting with caspase-9 (Fig. 5B).
Instead, SMAC associated with ML-IAP (Fig. 5B), indicating that
SMAC binding to ML-IAP disrupts the interaction of ML-IAP
with caspase-9.

Mutation in the Binding Pocket of ML-IAP Abrogates Inter-
action with SMAC—Previously, we demonstrated that mutat-
ing aspartates 120 and 138 to alanine in ML-IAP abolishes its
anti-apoptotic activity (15). The effect of these mutations on
ML-IAP interaction with SMAC was characterized. As an ad-
ditional control we expressed a double glutamate ML-IAP mu-
tant (E87A,E88A) that possessed equivalent anti-apoptotic
activity to wild type ML-IAP. SMAC immunoprecipitated ML-
IAP and E87A,E88A ML-IAP, but no interaction was observed
between SMAC and the D120A or D138A mutants (Fig. 6A).
The same was true in the inverse experiment where ML-IAP
and E87A,E88A mutant, but not D138A mutant, immunopre-
icipitated SMAC (Fig. 6B).

To examine the interaction of endogenous SMAC with ML-
IAP, we generated stably transfected MCF-7 cell lines expressing
FLAG-tagged ML-IAP or the D138A mutant. Consistent with our earlier results (Fig. 6, A and B), endogenous SMAC
was communoprecipitated from cells expressing ML-IAP, but
not the D138A mutant (Fig. 6C). To determine whether SMAC
peptide can bind IAPs expressed in cells, lysates prepared from

![Image](92x413 to 254x728)

**FIG. 5.** SMAC disrupts binding of ML-IAP to processed
caspase-9. A, 293T cells were transiently transfected with caspase-9
and ML-IAP, ML-IAP mutant, or vector. After 40 h, cells were lysed in
Nonidet P-40 lysis buffer and lysates immunoprecipitated (IP) with
anti-Myc antibody. Samples were then immunoblotted (W) with anti-
caspase-9 and anti-Myc antibodies. B, 293T cells were transiently
transfected with indicated constructs. Vector plasmid was used to keep
the amount of transfected DNA equal. After 40 h, cells were lysed in
Nonidet P-40 lysis buffer and lysates immunoprecipitated (IP) with
anti-Myc antibody. Samples were then immunoblotted (W) with anti-
caspase-9, anti-FLAG, and anti-Myc antibodies. IgG designates mono-
clonal immunoglobulin G antibody.

![Image](308x456 to 555x728)

**FIG. 6.** Mutation in the binding pocket of ML-IAP abrogates
interaction with SMAC. A and B, 293T cells were transiently trans-
fected with SMAC and the indicated ML-IAP constructs. After 40 h,
cells were lysed in Nonidet P-40 lysis buffer and lysates immunoprecipitated (IP) with anti-FLAG antibody (A) or anti-Myc antibody (B). Samples were then immunoblotted (W) with anti-FLAG and anti-Myc antibodies. C, MCF-7 cells stably expressing FLAG-tagged ML-IAP or
ML-IAP D138A mutant were lysed in Nonidet P-40 lysis buffer and
lysates immunoprecipitated (IP) with an anti-FLAG antibody. Samples were then immunoblotted (W) with anti-SMAC antibody. D, 293T cells
were transiently transfected with X-IAP, ML-IAP, or ML-IAP D138A
mutant. After 40 h, cells were lysed in Nonidet P-40 lysis buffer and
lysates incubated with SMAC or control (irrelevant) biotinylated pep-
tides (10 μM) for several hours. Complexes were precipitated with
steptavidin-agarose, washed extensively, and immunoblotted (W) with
anti-Myc antibody.
SMAC Negatively Regulates ML-IAP

293T cells transfected with the X-IAP, ML-IAP, or ML-IAP D138A mutant were incubated with biotinylated SMAC peptide or control peptide. Immuno blotting following peptide precipitation revealed that the SMAC peptide precipitated X-IAP and ML-IAP but not the ML-IAP D138A mutant (Fig. 6D). Therefore, aspartate 138 in the BIR domain of ML-IAP is a critical residue for the binding of SMAC.

Inhibition of caspases by IAPs occurs at the core of the apoptotic machinery, and thus regulation of IAPs by SMAC and SMAC-like proteins represents a key control point in deciding cell fate. We have shown that ML-IAP is regulated by SMAC, since SMAC physically associates with ML-IAP and suppresses the anti-apoptotic activity of ML-IAP. Interaction with SMAC is mediated through the BIR domain of ML-IAP and amino-terminal residues of active SMAC. Three-dimensional modeling together with protein binding studies demonstrated that SMAC binds the BIR of ML-IAP with high affinity and in a manner similar to which it binds the X-IAP BIR3 domain. Further highlighting the similarity of these interactions, mutation of residues in ML-IAP BIR that correspond to functionally important amino acids in X-IAP BIR3 (41) interrupted binding to SMAC. Aspartate 138 of ML-IAP is predicted to be in contact with alanine at the amino terminus of active SMAC peptide and, therefore, is critical for the interaction of ML-IAP with SMAC.

Previous studies have shown the importance of the amino terminus of active SMAC for binding X-IAP (33, 40, 41). We have demonstrated that this short region is important for binding ML-IAP and antagonizing its anti-apoptotic activity. We have also demonstrated that SMAC peptides can specifically bind and inhibit IAPs. SMAC peptide bound purified BIR domains and X-IAP and ML-IAP from cell lysates, and in doing so, nullified IAPs’ ability to inhibit apoptosis. To our knowledge, this is the first report in which the functional potential of SMAC or SMAC-like peptides has been explored in a cellular context. Such peptides have obvious therapeutic potential in the treatment of cancer cells that resist conventional cytotoxic therapies. IAPs do contribute to the resistance of cancers to chemotherapy agents, since they are widely and, in some cases like ML-IAP, specifically expressed in human malignancies (15, 19). Acknowledgments—We thank Karen O’Rourke and Kim Newton for critically reading the manuscript, Grace Mausisa for input in the fluorescence polarization assays, the sequencing laboratory for help with sequencing, and the members of the Dixit laboratory for helpful discussions.