Proteasome-mediated degradation of Smac during apoptosis: XIAP promotes Smac ubiquitination \textit{in vitro}

Marion MacFarlane, Wendy Merrison, Shawn B. Bratton and Gerald M. Cohen

MRC Toxicology Unit, Hodgkin Building, University of Leicester, PO Box 138, Lancaster Road, Leicester, LE1 9HN, UK.

Running Title: Proteasomal degradation of mitochondrial-released Smac

Corresponding author: Dr. G. M. Cohen, MRC Toxicology Unit, Hodgkin Building, University of Leicester, PO Box 138, Lancaster Road, Leicester, LE1 9HN, UK. Tel: 44-116-2525601 Fax: 44-116-2525616. E-mail: gmc2@le.ac.uk

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SUMMARY

During apoptosis, Smac/DIABLO, an IAP (inhibitor-of-apoptosis protein)-binding protein, is released from mitochondria and potentiates apoptosis by relieving IAP inhibition of caspases. We demonstrate that exposure of MCF-7 cells to the death-inducing ligand, TRAIL, results in rapid Smac release from mitochondria, which occurs before or in parallel with loss of cytochrome c. Smac release is inhibited by Bcl-2/Bcl-xL or by a pan-caspase inhibitor demonstrating that this event is caspase-dependent and modulated by Bcl-2 family members. Following release, Smac is rapidly degraded by the proteasome, an effect suppressed by co-treatment with a proteasome inhibitor. As the RING finger domain of XIAP possesses ubiquitin-protein ligase activity and XIAP binds tightly to mature Smac, an *in vitro* ubiquitination assay was performed which revealed that XIAP functions as an E3 ligase in the ubiquitination of Smac. Both the association of XIAP with Smac and the RING finger domain of XIAP are essential for ubiquitination, suggesting that the ubiquitin-protein ligase activity of XIAP may promote the rapid degradation of mitochondrial-released Smac. Thus, in addition to its well-characterised role in inhibiting caspase activity, XIAP may also protect cells from inadvertent mitochondrial damage by targeting pro-apoptotic molecules for proteasomal degradation.
Introduction

Apoptosis is a form of cell death that is essential for the correct development and homeostasis of multicellular organisms. Two major apoptotic pathways have been identified: one activated via death receptor activation and the other by stress-inducing stimuli (1). Triggering of cell surface death receptors of the TNF receptor superfamily, including CD95 (Fas/Apo-1) and TNF-Related Apoptosis-Inducing Ligand (TRAIL), result in a rapid activation of caspase-8 following its recruitment to a trimerized receptor/ligand complex via adaptor molecules (2). Secondly, stress-induced apoptosis caused by chemicals and growth factor deprivation, results in perturbation of mitochondria and the ensuing release of proteins, such as cytochrome c, from the inter-mitochondrial membrane space (3). Once released cytochrome c binds to Apaf-1, which in the presence of dATP results in the recruitment and activation of caspase-9 (4-6). The release of cytochrome c from mitochondria is also regulated, in part, by Bcl-2 family members with anti-apoptotic and pro-apoptotic members inhibiting or promoting the release, respectively (7-10). 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 (11-13).

Caspase activity is also regulated by the Inhibitor of Apoptosis Proteins (IAPs), which are characterized by one or more baculovirus IAP repeats, called BIR domains that are responsible for their anti-apoptotic activity (14, 15) and in some IAPs a RING finger domain near their COOH terminus. One major function of IAPs, particularly XIAP, c-IAP1, and c-IAP2, is their propensity to bind to and inhibit the processed forms of key initiator and effector caspases including caspases-9, -3 and -7.
XIAP has also recently been shown to bind to and inhibit active caspases -9 and -3 within the apoptosome complex (17, 18).

Protein ubiquitination is a post-translational protein modification that involves the sequential action of ubiquitin activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin protein ligase (E3) (19). Recently, the RING finger domain has been implicated in the ubiquitination of proteins with a functional relationship being established between the RING finger and ubiquitin ligase activity (20, 21). Several E3s, including Mdm2, are highly homologous to IAP with respect to their RING finger domains and promote degradation of both themselves and specific substrates, such as p53 (22). Recent reports indicate that XIAP and c-IAP1 promote self-ubiquitination in response to apoptotic stimuli (23), and that both XIAP and c-IAP2 promote in vitro ubiquitination of caspase-3 and -7 (24, 25).

Recently, a novel protein Smac (Second Mitochondrial-derived Activator of Caspases), and its murine homologue DIABLO, were described which promote caspase activation by eliminating IAP inhibition of caspases (26, 27). Smac is synthesized as a 239 amino acid precursor protein, with the N-terminal 55 amino acids serving as a mitochondrial matrix-targeting signal. In response to apoptotic stimuli, mature Smac is released into the cytoplasm and binds to XIAP thereby relieving XIAP inhibition of caspases. Recently this interaction has been mapped to the N-terminal 20 amino acids of mature Smac and removal of this region completely blocks its ability to bind XIAP (28, 29). As Smac acts to prevent IAP activity, it is proposed to be a human equivalent of the Drosophila proteins Reaper, Grim and Hid (26, 27). However, we have shown recently that Smac β, a Smac variant lacking the N-terminal IAP binding domain, still potentiates apoptosis suggesting that Smac may also possess proapoptotic activity independent of its IAP inhibition (30).
In this study, we investigate the kinetics and modulation of Smac release from mitochondria following death receptor-mediated apoptosis. Caspase-8 is the most apical caspase in TRAIL-induced apoptosis in MCF-7 cells (31), and is recruited to the native TRAIL Death-Inducing Signalling Complex (DISC) in various cell types (32-34). In MCF-7 cells, which are caspase-3 null (35), TRAIL induces rapid Bid cleavage resulting in engagement of the mitochondrial amplification loop that is essential for apoptosis in certain cells (31). MCF-7 cells therefore provide a good model in which to study death receptor-mediated, and hence apical caspase-induced, release of pro-apoptotic molecules from mitochondria. We now demonstrate that TRAIL-induces a rapid release of mitochondrial Smac in MCF-7 cells, which is inhibited by Bcl-2. Once released, Smac is rapidly degraded by the proteasome, an effect promoted \emph{in vitro} by the ubiquitin-protein ligase activity of XIAP. This study identifies Smac as a target for ubiquitination and further demonstrates the versatility of XIAP as a potent inhibitor of apoptosis.
Experimental Procedures

**Materials** - Media and serum were purchased from Life Technologies, Inc. Cytochrome c mouse monoclonal antibody was from Pharmingen, His\(_6\) mouse monoclonal antibody from Qiagen, and ubiquitin mouse monoclonal antibody from Zymed. The horseradish peroxidase-conjugated secondary antibodies, goat anti-rabbit and goat anti-mouse, were from Dako and Sigma-Aldrich, respectively. Anti-human cytochrome oxidase subunit II, anti-rabbit Alexa 488 and anti-mouse Alexa 568 were from Molecular Probes Inc. The ubiquitin activating enzyme E1 (rabbit), GST-UbcH5b and His\(_6\)-ubiquitin were from Affiniti Research Products Ltd. The caspase inhibitor, benzoyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethyl ketone (z-VAD.FMK) was purchased from Enzyme Systems Inc., and the proteasome inhibitor carbobenzoxy-leucinyl-leucinyl-leucinal (MG132) from Calbiochem. Recombinant TRAIL was produced as described previously (36). Unless stated otherwise all other chemicals were from Sigma-Aldrich.

**Cell Culture and Induction of Cell Death** - MCF-7-Fas (MCF-7), MCF-7-Bcl-2 and MCF-7-BCL-xL, human breast epithelial cells (from Dr. M. Jaattela)(37), were grown in RPMI 1640, supplemented with 10% FBS and 2 mM Glutamax™, in an atmosphere of 5% CO\(_2\) in air at 37°C and maintained in logarithmic growth phase by routine passage every 3-4 days. Cells were plated in either 6-well dishes, or 100 mm plates, and 24 h later cells were exposed to an apoptotic stimulus. Apoptosis was induced by treatment for up to 3 h with recombinant soluble TRAIL (250 ng/ml), or for 16 h with etoposide (50 μM). Where indicated, cells were pre-treated for 30 min with either MG132 (1 μM) or z-VAD.FMK (20 μM) before exposure to the apoptotic stimulus.
**Immunofluorescence confocal microscopy** - Cells were grown in 8-well chamber slides (Labtek) and 24 h later were either left untreated or were exposed to an apoptotic stimulus as described. Cells were fixed in 3.8% formaldehyde for 20 min at room temperature, then rinsed three times in PBS, permeabilised with 0.1% Triton X-100 in PBS and then blocked for 2 h in 3% BSA in PBS at 4°C. Cells were incubated with cytochrome c antibody (1:250 in 3% BSA) or Smac antibody (1:200 in 3% BSA) for 2 h at room temperature, rinsed three times in PBS, and then incubated with anti-mouse Alexa 568 (1:300) or anti-rabbit 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 prior to mounting onto glass slides using Vectashield® (Vector Laboratories). Optical sections were taken using argon-krypton and UV lasers and a Leica TCS-4D confocal imaging system.

**Generation of recombinant proteins and a polyclonal antibody to Smac/Smac β** - A polyclonal antibody to Smac was raised using recombinant Smac β as the immunogen. Briefly, Smac β pTriEx-1 was expressed in *E. coli* (BL21(DE3)) as described previously (30), and purified on Ni-beads (Qiagen) prior to immunization. The rabbit polyclonal antibody obtained was further purified on an IgG column and then characterised by both ELISA and Western blot analysis, which verified that the antibody recognised both recombinant Smac/Smac β (data not shown) and endogenous Smac. The antibody was also characterised for use in immunofluorescence confocal microscopy, which confirmed that it specifically labelled endogenous Smac in intact cells. Recombinant mature Smac protein was produced in *E. coli* (BL21(DE3)) using SmacΔ55 pET15b, as described previously (26), and purified to homogeneity on Ni-beads. The purified protein was stored in
aliquots at -80°C and its identity confirmed by N-terminal protein sequencing. GST-tagged XIAP, BIR1/2 and BIR3-RING were expressed and purified on GSH-Sepharose beads as described previously (16).

**Isolation of mitochondrial and cellular lysate fractions** - Cells were collected at the indicated times and mitochondrial and cytosolic fractions prepared by the method described previously (38). Samples were then analysed by Western blotting for Smac, cytochrome c, or cytochrome c oxidase subunit II.

**Ubiquitination Assays** - *In vitro* reactions were carried out by adding rabbit ubiquitin activating enzyme E1 (250 nM), GST-UbcH5b (2 µM), His6-ubiquitin (100 µM), ATP (2 mM), recombinant Δ55 Smac-His6 (2 µM) and GST-XIAP (0.5-3 µM) in ubiquitination assay buffer (50 mM Tris-HCl, pH 7.5, 2.5 mM MgCl2, 0.05 % Nonidet P-40 and 0.5 mM dithiothreitol). Reactions were incubated at 25°C for up to 90 min and then stopped by adding an equal volume of 2x SDS-PAGE sample buffer. The samples were then resolved by SDS-PAGE and blotted for ubiquitinated protein products using monoclonal antibodies to either His6 or ubiquitin.

**Gel electrophoresis and Western blotting** - Mitochondrial or cytosolic fractions and *in vitro* assay samples were separated on 15% (cytochrome c or Smac) or 13% (His6 and ubiquitin) polyacrylamide gels followed by electrophoretic transfer onto nitrocellulose membrane (Hybond-C extra; Amersham). Immuno-detection was carried out as described previously (39) using enhanced chemiluminescence detection (Amersham-Pharmacia).
Results

**TRAIL-induced apoptosis results in a rapid release of Smac from mitochondria that is inhibited by Bcl-2/Bcl-xL**

To determine whether TRAIL-induced apoptosis in MCF-7 cells is associated with the release of Smac from mitochondria, MCF-7 cells were treated with TRAIL and the subcellular distribution of endogenous Smac determined by immunofluorescence confocal microscopy. Smac displayed a punctate mitochondrial staining pattern and co-localised with cytochrome c (Fig. 1A-C). Exposure of MCF-7 cells to TRAIL for up to 2 h resulted in a rapid release of Smac from mitochondria (Fig. 1E). This release occurred either earlier, or coincided with the release of cytochrome c (Fig. 1D), as evidenced by the presence of individual cells exhibiting cytochrome c staining within the mitochondria in the absence of any Smac staining (Fig. 1F).

Bcl-2 and Bcl-xL inhibit mitochondrial cytochrome c release and in some cell types can also inhibit TRAIL-induced apoptosis. In MCF-7 cells overexpressing Bcl-2 (Fig. 1G and J) or Bcl-xL (Fig. 1H and K), TRAIL-induced apoptosis and the mitochondrial release of both cytochrome c and Smac was inhibited, confirming that TRAIL-induced apoptosis in MCF-7 cells is, in part, dependent on the release of pro-apoptotic molecules from the mitochondria. We have shown previously (31) that pre-treatment of MCF-7 cells with the pan-caspase inhibitor, z-VAD.FMK, potently inhibits TRAIL-induced apoptosis. Pre-treatment with z-VAD.FMK also inhibited Smac and cytochrome c release from mitochondria (Fig. 1I and L), an effect attributed to the ability of z-VAD.FMK to inhibit caspase-8 activation at the DISC.
Mitochondrial-released Smac is rapidly degraded in MCF-7 cells following exposure to either TRAIL or etoposide

In order to determine whether both Smac and cytochrome c were released with similar kinetics, their subcellular localisation was determined in MCF-7 cells exposed to TRAIL for up to 1 h. Mitochondrial Smac release was first evident 30 min after addition of TRAIL (Fig. 2A-C), with approximately 10% of the cells displaying Smac release from mitochondria. After 45 min of TRAIL treatment, almost 40% of the cells analysed had released Smac from their mitochondria (Fig. 2D-E).

Interestingly, in some cells where Smac release was already evident there was still no evidence of cytochrome c release, whereas in other cells release of both Smac and cytochrome c was clearly evident (compare Figs. 2D and E). After 60 min of TRAIL treatment the number of cells displaying loss of mitochondrial Smac had increased to approximately 60% (data not shown). To determine whether release of Smac from mitochondria was also observed following DNA damage-induced apoptosis, MCF-7 cells were exposed to the DNA topoisomerase II inhibitor etoposide for 16 h, a time-point when significant apoptosis was observed. This was accompanied by the release of both Smac and cytochrome c from the mitochondria of cells undergoing apoptosis together with an overall loss of Smac staining in apoptotic cells (Fig. 2F).

To investigate further the time-dependent release of Smac, MCF-7 cells were exposed to TRAIL for up to 3 h and mitochondrial and cytosolic fractions analysed by Western blot analysis. Significant release of both Smac and cytochrome c occurred between 30 min and 1 h after TRAIL-treatment (Fig. 3A). However, as this method relies on analysis of cell populations rather than single cells, it did not allow us to determine whether Smac release had occurred prior to, or in parallel with, release of cytochrome c.
Both Bcl-2 (Fig. 3A, lanes 7 and 14), and z-VAD.FMK (Fig. 3B, lanes 3 and 6) significantly inhibited TRAIL-induced release of both Smac and cytochrome c.

During our investigation of the kinetics of TRAIL-induced Smac release, a significant loss of cytosolic Smac staining was observed in cells that had released mitochondrial Smac (Fig. 1E and Fig. 2A, D, and G). This effect was unlikely to be the result of a conformational change in the Smac protein following its release from mitochondria, as Western blot analysis of cytosolic fractions under denaturing conditions also revealed that released Smac was rapidly degraded within 2 h (Fig 3A, lane 4). Therefore the loss of Smac staining observed by immunofluorescence microscopy was also evident at the biochemical level. These results indicated that both death receptor and chemical-induced apoptosis in MCF-7 cells resulted in Smac degradation following its release from mitochondria.

**Smac is degraded via the ubiquitin-proteasome pathway in apoptotic MCF-7 cells**

Activation of caspases during apoptosis results in the cleavage of many important cellular proteins (11, 12). As immunoblot analysis provided no direct evidence for the formation of Smac-derived caspase cleavage fragments, we wished to determine whether the ubiquitin-proteasome pathway was involved in Smac degradation. MCF-7 cells were pre-treated with the proteasome inhibitor, MG132, and then exposed to TRAIL for 3 h. Treatment with MG132 inhibited the TRAIL-mediated degradation of Smac, but did not markedly interfere with its release into the cytosol (Fig. 3A, lane 6, and Fig 3C, lane 3).
To extend these studies, confocal analysis of Smac release was performed on cells pre-treated with MG132. In cells exposed for up to 2 h with TRAIL alone, a significant number of cells displayed a complete loss of Smac staining, which coincided with the release of Smac from the mitochondria (Fig. 2G-I). Pre-treatment with MG132, alleviated this loss with significant amounts of Smac now detectable in the cytosol of apoptotic cells (Fig. 2J-K). Taken together, these results suggest that, following its release from mitochondria the degradation of Smac is proteasome-
dependent.

**The ubiquitin-protein ligase activity of XIAP promotes Smac ubiquitination**

Ubiquitination targets proteins for degradation by the proteasome (19). The IAP family of proteins can act as E3 ubiquitin-protein ligases, both for themselves and caspases -3 and -7 (23, 24). As XIAP binds very tightly to mature Smac via an N-terminal AVPIA motif (40, 41), this led us to question whether IAPs may be responsible for targeting Smac for degradation. The ability of XIAP to act as an E3 ligase capable of targeting Smac was therefore assessed using an *in vitro* ubiquitination assay.

Recombinant mature Smac (C-terminally His$_6$-tagged) was incubated in the presence or absence of GST-XIAP, ubiquitin-activating enzyme E1, ubiquitin conjugating enzyme E2 (UbcH5b) and His$_6$-ubiquitin. When probed with an anti-His$_6$ antibody, which recognizes His$_6$-tagged ubiquitin and Smac-His$_6$, a higher molecular weight species of Smac was detected only in the presence of all the components (Fig. 4A, upper panel). Moreover, the higher molecular weight Smac species displayed a time-dependent formation in the presence of XIAP. As His$_6$-ubiquitin is an 8.5 kDa protein,
the ubiquitinated Smac protein appears as ~32 or ~41 kDa bands, with the other higher molecular weight bands corresponding to a mixture of ubiquitinated Smac and XIAP proteins (Fig. 4A, upper panel). When the same blot was probed with an anti-ubiquitin antibody, which detects ubiquitin itself and ubiquitinated products, the time-dependent ubiquitination of Smac was again observed, (Fig. 4A, lower panel). These results demonstrate that XIAP can act as an E3 ligase for the ubiquitination of Smac in vitro.

Previous studies have suggested that the RING finger domain of XIAP is essential for the proteasome-mediated degradation of its target proteins. To examine whether the E3 activity of XIAP for Smac resides in its RING finger domain, the E3 activity of wild-type GST-XIAP was compared to that of GST-BIR1/2 and GST-BIR3-RING. In the presence of full-length XIAP, but not GST-BIR1/2, the higher molecular species of Smac were again detected (Fig. 4B). However, in the presence of GST-BIR3-RING, the higher molecular weight Smac species were even more evident than with full-length XIAP (Fig. 4B, compare lanes 9 and 13). As Smac is known to bind XIAP, primarily via the BIR-3 domain, this may explain the observed increase in E3 activity of the BIR3-RING domain versus that of wild-type XIAP. Taken together, these results demonstrate that the BIR3-RING domain of XIAP is essential for XIAP to exhibit E3 activity for Smac ubiquitination in vitro.
Discussion

Recent studies have shown that Smac can act as a potent pro-apoptotic molecule, an effect primarily attributed to its propensity to bind to IAP family proteins (26, 27), although other mechanisms cannot be excluded (30). However, in order to exert its pro-apoptotic effect Smac must be released from mitochondria and, until now, few studies have investigated the kinetics or modulation of Smac release in intact cells. We now demonstrate that death receptor-mediated apoptosis induces rapid and complete release of mitochondrial Smac, with kinetics similar to that observed for cytochrome c. As studies with Bid deficient mice have confirmed that Bid is a critical substrate for in vivo signalling by death receptor agonists (42), TRAIL-induced release of Smac and cytochrome c in MCF-7 cells is likely to be tBid-dependent. Our observation that z-VAD.FMK prevented the release of both Smac and cytochrome c would support this, however, we cannot exclude the possibility that other apical caspase substrates may also play a role. Importantly, our results demonstrate that mitochondrial release of pro-apoptotic molecules is an apical caspase-dependent event in receptor-mediated apoptosis. This is in contrast to that recently reported for cell stress-associated mitochondrial Smac release which is proposed to be a caspase-catalyzed event occurring downstream of cytochrome c release (43).

We also show that Smac release in MCF-7 cells is sensitive to inhibition by Bcl-2/Bcl-xL, with Bcl-2 exhibiting greater potency than Bcl-xL. The ability of Bcl-2 family members to inhibit the release of mitochondrial intermembrane pro-apoptotic molecules, such as cytochrome c, is now well documented (3). Intriguingly, in some experiments, Bcl-2 appeared more potent in preventing Smac than cytochrome c release. Structural analysis revealed that Smac exists as a homodimer (28), with an
apparent molecular mass of ~100 kDa (26), whereas cytochrome c is known to have a monomeric molecular weight of only ~12 kDa. If Bcl-2 were to act simply by blocking a tBid-induced pore, then one might predict that Smac release would be more readily inhibited than release of cytochrome c. Thus, mitochondrial release of Smac and cytochrome c may occur by different mechanisms, perhaps involving tBid and/or another as yet unidentified pore-forming molecule.

Following its release from mitochondria, Smac was rapidly degraded by the proteasome, an effect we observed in both death receptor-mediated and chemical-induced apoptosis. Recent studies have revealed that XIAP can function as an E3 ligase, and promotes the degradation of both itself and its substrates. Until now, the only XIAP substrates identified in vitro have been caspases-3, -7 and -9 (25). Thus, XIAP exhibits its anti-apoptotic effect by directly binding to and inhibiting active caspases-3, -7, and -9 (14) but in some circumstances may additionally exert its effect through ubiquitination and degradation of active caspases (25). We now demonstrate that XIAP can also act as an E3 ligase for Smac and thus may promote the proteasome-mediated degradation of Smac in intact cells. Importantly, the BIR3-RING domain of XIAP was both necessary and sufficient to ubiquitinate Smac in vitro. Structural analysis has revealed that the primary binding of Smac to XIAP occurs via the N-terminal four residues of Smac and the BIR3 domain of XIAP, with significantly weaker binding observed between Smac and the BIR2 domain of XIAP (41). Smac is proposed to compete with, and thereby antagonize the ‘caspase-inhibitory’ effect of XIAP (28, 29, 40, 41). Importantly, our data suggest that ubiquitination and degradation of Smac may represent another mechanism for modulating apoptosis. Degradation of small amounts of Smac released from
mitochondria would provide a safeguard against inadvertent perturbation of mitochondria and subsequent caspase activation. It may be the case that cells exhibiting high levels of XIAP are protected from apoptosis in several ways: through the ability of XIAP to directly inhibit and degrade caspases, and additionally through its ability to bind and target Smac for degradation. Recently, the serine protease Omi/HtrA2, which contains an N-terminal AVPS motif similar to that found in mature Smac, was shown to be released from mitochondria and inhibit XIAP by direct binding (44-47). Thus Omi, in addition to being a Smac-like inhibitor of IAP activity, may be another pro-apoptotic molecule with the potential to be regulated by proteasomal-degradation. Other E3 ligases, in addition to XIAP, may also target Smac or Omi for proteasomal-mediated degradation. Others have reported that c-IAP2 also possesses E3 ligase activity and can monoubiquitinate caspases-3 and -7 in vitro. However, as mature Smac binds preferentially to XIAP, but not c-IAP1 or 2 in intact cells (30), it is unlikely that c-IAP2 contributes greatly to the proteasomal-mediated degradation of Smac in vivo.

Taken together, our results demonstrate that receptor-mediated Smac release from mitochondria occurs prior to or in parallel with the release of cytochrome c. Once released, Smac is rapidly degraded by the proteasome, an effect we propose from our in vitro data may be mediated in part via the E3 ligase activity of the BIR3-RING domain of XIAP. Based on our results and those of others, apoptosis can now be viewed as a process that has features in common with the control of other cellular processes by ubiquitination.
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Figure Legends

Fig. 1. (A-F) TRAIL induces release of Smac from mitochondria. Confocal analysis of control MCF-7 cells (A-C) or cells after a 2 h exposure to TRAIL (250 ng/ml)(D-F) with cytochrome c (red fluorescence)(A and D) and Smac (green fluorescence)(B and E). Co-localisation is seen as orange/yellow (C and F). Arrows indicate cells that have released Smac from mitochondria yet still retain some cytochrome c.

(G-L) Smac release is inhibited by Bcl-2/Bcl-xL and z-VAD.FMK. MCF-7-Bcl-2 (G and J) cells, MCF-7-Bcl-xL cells (H and K) or MCF-7 cells pre-treated for 30 min with z-VAD.FMK (20 μM)(I and L) were exposed to TRAIL (250 ng/ml) for 2 h and then analysed by confocal microscopy for cytochrome c (G-I) or Smac (J-L). Nuclei are counterstained with Hoechst 33258 (blue fluorescence). Bar, 10 μm.

Fig. 2. (A-E) Smac release occurs either prior to or in parallel with the release of cytochrome c. Confocal analysis of MCF-7 cells for Smac (A and D) and cytochrome c (B and E) following exposure to TRAIL (250 ng/ml) for 30 min (A-C) or 45 min (D-E). Co-localisation is seen as orange/yellow (C). Arrows indicate cells that have released Smac from the mitochondria prior to or in parallel with the release of cytochrome c.

(F) Etoposide induces Smac release from mitochondria. MCF-7 cells were exposed to etoposide (50 μM) for 16 h and then analysed by confocal microscopy for Smac and cytochrome c. Arrows indicate cells that have released both Smac and cytochrome c.

(G-L) Proteasomal degradation of released Smac. MCF-7 cells were exposed to TRAIL (250 ng/ml) for 2 h (G-I), or pre-treated with MG132 (1 μM) for 30 min prior
to exposure to TRAIL (250 ng/ml) for 2 h (J-L), and then analysed by confocal microscopy for Smac (G and J) and cytochrome c (H and K). Co-localization is seen as orange/yellow (I and L).

In all panels, Smac is shown as green fluorescence and cytochrome c as red fluorescence. Nuclei are counterstained with Hoechst 33258 (blue fluorescence) (A-F). Bar, 10 μm.

**Fig. 3. (A) Bcl-2 inhibits TRAIL-induced time-dependent release of Smac and cytochrome c from mitochondria.** MCF-7 or MCF-7-Bcl-2 cells were incubated for up to 3 h in the presence of TRAIL (250 ng/ml) and the time-course for the release of Smac and cytochrome c from mitochondria into the cytosol was assessed by Western blot analysis as described in Materials and Methods. The effect of a 30 min pre-treatment with MG132 (1 μM) on TRAIL-induced Smac release at 3 h is shown in lanes 6 and 13.

**(B) z-VAD.FMK inhibits TRAIL-induced release of Smac and cytochrome c.** MCF-7 cells were incubated either alone (Con), or in the presence of TRAIL (250 ng/ml) for 3 h, or pre-treated with z-VAD.FMK (20 μM) for 30 min prior to exposure to TRAIL (250 ng/ml) for 3 h. The release of Smac and cytochrome c was then assessed as described above. The integrity of the mitochondrial isolation procedure was confirmed by blotting for the mitochondrial matrix protein cytochrome c oxidase subunit II as described in Materials and Methods.

**(C) MG132 inhibits the proteasomal-mediated degradation of Smac following TRAIL-induced apoptosis.** MCF-7 cells were incubated, either alone (Con), or in the presence of MG132 (1 μM) for 30 min and then incubated for a further 3 h in the
presence or absence of TRAIL (250 ng/ml). The release of Smac and cytochrome c into the cytosol was then assessed as described above.

**Fig. 4. (A) XIAP promotes Smac ubiquitination in vitro.** Purified recombinant GST-XIAP (3 μM) was incubated in the presence of all the other assay components, but in the absence of Smac-His₆, His₆-ubiquitin, ATP, or E2, for 90 min (lanes 1-4, respectively). All the assay components were incubated for up to 90 min (lanes 5-8, respectively), as described in Materials and Methods. The effect of incubating all the components in the presence of lower concentrations of XIAP (0.5-2 μM) for either 0 or 90 min is shown in lanes 9-14. His₆-ubiquitin-tagged protein products are shown following probing of the membrane with an anti-His₆ antibody (upper panel), which also detects recombinant Smac-His₆. The presence of His₆-ubiquitin and ubiquitinated protein products is also shown following probing of a matched membrane with an anti-ubiquitin antibody (lower panel).

**B) The BIR3-RING domain of XIAP is sufficient to ubiquitinate Smac in vitro.** Assays were performed in the presence of all components but in the absence of purified recombinant Smac-His₆, E1, E2, GST-XIAP (1 μM) or His₆-ubiquitin for 90 min (lanes 1-5, respectively). All the assay components were then incubated for up to 90 min (lanes 6-9, respectively). The effect of incubating for 0 or 90 min in the presence of the truncated XIAP forms, GST-BIR1/2 and GST-BIR3-RING is shown in lanes 10-13. His₆-ubiquitin-tagged protein products are shown following probing of the membrane with an anti-His₆ antibody (upper panel), which also detects recombinant Smac-His₆.
### A

|       | Smac | Ubiquitin | ATP | E2 | XIAP (µM) |
|-------|------|-----------|-----|----|-----------|
|       | -    | -         | -   | -  | 0.5       |
| Time (min) | 0   | 30        | 60  | 90 | 0         |
|        | 0   | 90        | 0   | 90 | 0         |

**anti-His\textsubscript{6}**

- Smac-His\textsubscript{6}
- (His\textsubscript{6}-Ub\textsubscript{n})
- His\textsubscript{6}-Ub-Smac
- Smac-His\textsubscript{6}

**anti-Ubiquitin**

- (Ub\textsubscript{n})
- Ub-Smac
- His\textsubscript{6}-Ubiquitin

### B

|       | Smac | E1 | E2 | XIAP | - Ubiquitin | XIAP | BIR1/2 | BIR3-R |
|-------|------|----|----|------|-------------|------|--------|--------|
| Time (min) | 0   | 30 | 60 | 90   | 0           | 90   | 0      | 90     |

**anti-His\textsubscript{6}**

- Smac-His\textsubscript{6}
- (His\textsubscript{6}-Ub\textsubscript{n})
- His\textsubscript{6}-Ub-Smac
- Smac-His\textsubscript{6}
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