Uncoupling of the Signaling and Caspase-inhibitory Properties of X-linked Inhibitor of Apoptosis*

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In addition to its well described function as an enzymatic inhibitor of specific caspases, X-linked inhibitor of apoptosis (X-linked IAP or XIAP) can function as a cofactor in Smad, NF-κB, and JNK signaling pathways. However, caspases themselves have been shown to regulate the activity of a number of signaling cascades, raising the possibility that the effect of XIAP in these pathways is indirect. Here we examine this question by introducing point mutations in XIAP predicted to disrupt the ability of the molecule to bind to and inhibit caspases. We show that whereas these mutant variants of XIAP lost caspase-inhibitory activity, they maintained their ability to activate Smad, NF-κB, and JNK signaling pathways. Indeed, the signaling properties of the molecule were mapped to domains not directly involved in caspase binding and inhibition. The activation of NF-κB by XIAP was dependent on the E3 ubiquitin ligase activity of the RING domain. On the other hand, the ability of XIAP to activate Smad-dependent signaling was mapped to the third baculoviral IAP repeat (BIR) and loop regions of the molecule. Thus, the apoptotic and signaling properties of XIAP can be uncoupled.

The *iap (inhibitor of apoptosis) genes were originally described in baculoviruses (1, 2) and have subsequently been identified in a wide range of cellular genomes (3). The antiapoptotic activity of IAPs was first recognized. This property has been largely attributed to their ability to directly inhibit members of the caspase family of cysteine proteases, the central effectors of the apoptotic cascade (4–8). However, IAPs can also participate in various cellular functions unrelated to caspase inhibition; certain IAPs have been found to be cofactors of distinct signal transduction pathways (9–12), and others play an integral part in cell division and cytokinesis (13–16). IAPs contain between one and three imperfect repeats of an ~65-residue motif termed the baculoviral IAP repeat (BIR), which exhibits structural similarity to zinc fingers (17, 18). Many IAPs also contain a carboxyl-terminal RING finger domain that possesses E3 ubiquitin ligase activity (10, 19).

One mammalian member of this family is X-linked IAP (XIAP), a 56-kDa protein composed of three amino-terminal BIRs and a RING domain at the carboxyl terminus (20). XIAP has potent antiapoptotic properties and has been shown to directly suppress the enzymatic activity of caspase-3, -7, and -9 in vitro and in intact cells (4, 5, 21). Recent studies have determined the crystal structure of XIAP interacting with caspase-3, -7, and -9 and revealed great detail of the molecular determinants necessary for these interactions. The linker region between BIR 1 and BIR 2 is important for binding to caspase-3 and -7 (6–8). The amino acids in the linker region upstream of BIR 2 bind to the active site of caspase-3 and -7 in an antiparallel orientation, inhibiting the enzymatic activity of these caspases. The residue Asp148 in XIAP is particularly important for the ability of XIAP to bind to these caspases (7, 17). The interactions between XIAP and caspase-9 depend on a different region of the molecule (22, 23). A hydrophobic pocket in the surface groove of BIR 3 of XIAP mediates this interaction. This pocket is occupied by the first four amino acids of processed caspase-9 (p12 subunit). The residue Trp310 in BIR 3 forms part of this pocket and is critical for binding to caspase-9 (22, 24).

The antiapoptotic activity of XIAP can be suppressed by two nuclear encoded, mitochondrially localized proteins, Smac/DIABLO and Omi/HtrA2 (25–30). These molecules are released from mitochondria into the cytoplasm during apoptosis, where they bind to the same hydrophobic pocket in BIR 3 that is responsible for XIAP interactions with caspase-9 (23, 31, 32). This binding is mediated by tetrapeptide sequences present in the amino-terminal portion of the mature form of these proteins that have significant similarity to amino-terminal sequences present in mature caspase-9 (p12 subunit). The binding of these molecules or caspase-9 to XIAP is mutually exclusive (17, 23, 32). Thus, Smac/DIABLO and Omi/HtrA2 are negative regulators of XIAP that function to release XIAP from caspases; in the case of Omi/HtrA2, proteolytic cleavage of XIAP also participates in this negative regulation (33, 34).

In addition to the ability of XIAP to inhibit caspases, other roles for this molecule have been demonstrated, including its participation in a number of signaling pathways. XIAP has been found to be a cofactor in transforming growth factor-β and bone morphogenetic protein (TGF-β/BMP) signaling pathways.

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¶ The abbreviations used are: IAP, inhibitor of apoptosis; XIAP, X-linked IAP; BIR, baculoviral IAP repeat; E3, ubiquitin-protein isopeptide ligase; TGF, transforming growth factor; BMP, bone morphogenetic protein; JNK, c-Jun N-terminal kinase; GST, glutathione S-transferase; HA, hemagglutinin; CHAPS, 3-[[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PIPES, piperazine- N,N′-bis-2-ethanesulfonic acid; AFC, 7-amino-4-trifluoromethylcoumarin; BisTris, bis[2-hydroxyethyl]limino-tris[hydroxymethyl]methane.

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The TGF-β superfamily is a group of cytokines that function as growth regulators with diverse effects depending on the target tissue (35, 36). XIAP can bind to the cytoplasmic domain of the type I BMP receptor (ALK-3) and can affect BMP-regulated dorsal-ventral polarity in a Xenopus developmental model (11). Similarly, an association between XIAP and the type I TGF-β receptor (ALK-5) results in synergistic activation of TGF-β-dependent transcription by XIAP (12). In addition, ectopic expression of XIAP has been shown to activate stress-responsive signaling pathways, such as the N-terminal c-Jun kinase (JNK) pathway (37, 38) and the transcription factor NF-κB (12, 39, 40). The activation of JNK1 was reported to contribute to the protective effect conferred by XIAP (41) and is thought to result from interactions between XIAP and factors such as TAB1 (11) and ILPIP (41) that activate the MAP3 kinase TAK1. The activation of NF-κB in endothelial cells can result from activation of IκB kinase by TAK1 (40), but in other cell models it is not mediated by this pathway (12).

Whether the caspase-inhibitory properties of XIAP play any role in its signaling properties is not known. Caspases not only play a central role in apoptosis but can regulate other signaling cascades. Caspase-1, -4, and -5 participate in IL-1β and IL-18 processing and maturation; similarly, a role for caspase-8 in signaling events involved in T-cell proliferation has been demonstrated (42). Caspases can participate in NF-κB signaling through the ability of caspase-3 to cleave the amino-terminal portion of IκB-α, an inhibitory factor that prevents nuclear translocation of NF-κB, leading to a stabilization of IκB-α and blockade of NF-κB-mediated transcription (43, 44). Additionally, Relish, a Drosophila homolog of NF-κB, has been found to be cleaved by the Drosophila caspase Dredd, leading to Relish activation (45). Similarly, the mammalian NF-κB subunit c-Rel can also be cleaved by caspase-3, although the functional significance of this event is unclear (46). Therefore, we set out to determine whether the caspase-inhibitory activity of XIAP was required for its signaling properties. Here we report that point mutations in XIAP that abrogate its antiapoptotic activity do not affect its signaling activities, demonstrating that these properties are separate and independent of each other. In addition, the data presented demonstrate that the various signaling properties of XIAP can be mapped to different regions of the molecule.

Materials and Methods

Cell Culture and Transfections—Human embryonic kidney 293 cells and the Smad4-deficient human breast cancer cell line, MDA-MB-468, were obtained from the American Tissue Culture Collection. All cells were cultured at 37 °C with 5% CO₂ in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 2 mM glutamine.

Transfection of 293 cells was performed using the calcium phosphate precipitation procedure as described previously (47). MDA-MB-468 cells were transfected using Fugene 6 reagent following the manufacturer’s instructions (Roche Applied Science) with a 3:1 ratio of Fugene reagent to DNA.

Plasmids—The pE8B expression vector and the 2xB-luc reporter have been described previously (47). Construction of the pE8B XIAP and HA-JNK1 plasmids have been previously reported (12, 39). The SBE-JONK reporter expression vector was kindly provided by B. Vogelstein. The Bax expression vector was kindly provided by S. Korsmeyer. The Fas expression vector was kindly provided by R. Siegel. The Myc-tagged Smad4 expression vector was kindly provided by M. de Caestecker.

The XIAP caspase-binding and RING mutants were generated using the PCR-based QuickChange site-directed mutagenesis kit (Stratagene) following the manufacturer’s instructions as described previously (24). The bacterial GST expression vectors for the XIAP constructs were prepared by subcloning the inserts into the BamHI and NotI sites of pGEX 4T-1 (Amersham Biosciences). The HA-tagged XIAP plasmids were constructed by subcloning the inserts from the respective pEBG constructs previously described (48, 49) using the BamHI and NotI sites in the pE8B-HA mammalian expression vector. In addition, pEBB-HA-BIR 1, pEBB-HA-BIR 2, pEBB-HA-BIR 3, pEBB-HA-BIR 1-2, pEBB-HA-BIR 2-3, and pEBB-HA-BIR 1-2-3 were generated by PCR using pE8B-XIAP as template with the boundaries for each construct as indicated in Fig. 5A.

Luciferase Assays—For all reporter assays, cells were seeded into 6-well plates, and all treatment groups were performed in triplicate. For the NF-κB reporter assays in 293 cells, 50 ng of 2xB-luc reporter along with 2 µg of the indicated expression vectors were transfected into each well. Total DNA amount was equalized with control vector. At 24 h post-transfection, the cells were washed once with PBS and lysed in 0.5 ml of reporter lysis buffer (Promega). Luciferase activity was measured as described previously (12) using the luciferase assay system (Promega) and a TR717 Applied Biosystems microplate luminometer. For the TGF-β-mediated reporter experiments in MDA-MB-468 cells, 200 ng of SBE-JONK reporter, 20 ng of Myc-Smad4, and 1 µg of the indicated expression vectors were transfected into each well. At 24 h post-transfection, the medium was changed to Dulbecco’s modified Eagle’s medium plus 0.2% FCS with or without 5 ng/ml human TGF-β1 (Roche Applied Science). The luciferase assay was performed 24 h later as described above.
Caspase-3 was monitored by measuring cleavage of DEVD-AFC. Data wild type and various point mutants indicated in the graph.

A vector and lysed 24–30 min at 4°C for an additional 3 h. Bacterial pellets were lysed using GST lysis buffer followed by sonication. The clarified lysates were run over a glutathione-Sepharose column (Amersham Biosciences), washed with GST lysis buffer, and eluted in a buffer containing free glutathione (100 mM Tris, pH 8.0, 0.615 g/dl of glutathione). The eluates were then dialyzed overnight at 4°C (25 mM HEPES, pH 7.9, 50 mM NaCl, 5 mM EGTA, 1 mM MgCl₂, 10% glycerol, 0.1% CHAPS) and stored at −80°C until use.

The S-100 extracts were prepared from 293 cells as described previously (49). The caspase assays were set up by mixing 125 ng of the indicated GST fusion protein with 293 S-100 extracts and incubating this mixture for 30 min at 4°C followed by activation of the caspase-3 activity, an indirect measurement of apoptosis and caspase-9 activation. The assay was performed in a caspase reaction buffer (50 mM PIPES, pH 7.0, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol) with the addition of 20 μM DEVD-AFC; cleavage was measured using a fluorescence plate reader (Cytofluor 4000; Perseptive Biosystems). To measure direct inhibition of caspase-3 activity, increasing amounts of recombinant GST fusion proteins were added to 10 ng of purified recombinant caspase-3 (BD Biosciences) in caspase reaction buffer with the addition of DEVD-AFC, and cleavage was monitored as described above.

Western Blot—Human embryonic kidney 293 cells were transfected with 0.5 μg of HA-JNK1 and 2 μg of the indicated plasmids. HA-JNK1 was precipitated with a monoclonal anti-HA antibody (12CA5; Roche Applied Science), and kinase assays were performed as previously described (12). Activation of JNK1 was determined by in vitro phosphorylation of recombinant GST-e-Jun(1–79) and was quantified using a PhosphorImager (Amersham Biosciences). Expression of HA-JNK1 and XIAP was confirmed by immunoblotting of lysates with HA- and XIAP-specific antibodies as described above. The -fold activation was normalized to the total amount of HA-JNK1 detected in each case.

RESULTS AND DISCUSSION

Generation of XIAP Mutants—XIAP is capable of binding to and inhibiting the enzymatic activity of key caspases involved in the activation of cell death pathways, conferring protection from intrinsic and extrinsic apoptotic stimuli (Fig. 1A). In order to evaluate the potential contribution of caspase inhibition to the signaling properties of XIAP, mutations intended to disrupt the ability of XIAP to bind to and inhibit caspases were introduced in the molecule as previously described (24). Using site-directed mutagenesis, the mutations D148A and W310A were introduced to disrupt the binding to caspase-3 and -7 or caspase-9, respectively (Fig. 1B). These mutations were introduced independently (XIAP D148A or XIAP W310A) or simultaneously (XIAP D148A/W310A) to generate three XIAP caspase-binding mutants.

In order to assess the contribution of the RING domain of XIAP and its E3 ubiquitination activity to its ability to activate signal transduction, two additional mutants of XIAP were generated. A mutation creating a stop codon just prior to the RING domain at amino acid 448 (XIAP ARING) was introduced. In addition, a point mutation at histidine 467 (H467A), which results in loss of E3 ubiquitin ligase activity (19), was also generated.

Functional Characterization of XIAP Caspase-binding Mutants—The caspase-inhibitory activity of XIAP proteins, including the wild type form and the point mutations expected to affect caspase binding, was tested in vitro. The ability of recombinant XIAP proteins to directly inhibit purified recombinant caspase-3 was tested first. To determine whether there was a difference in inhibitory activity between the various recombinant XIAP proteins, increasing amounts of recombinant XIAP were incubated with 10 ng of purified recombinant caspase-3. Both the wild type and W310A mutant showed caspase-3-inhibitory activity starting at 10 ng of recombinant XIAP. The mutants that contained the D148A mutation targeting the caspase-3 binding motif in XIAP were unable to inhibit recombinant caspase-3 activity even at the highest concentrations tested.

![Western Blot](image-url)
Next, an in vitro apoptotic system using S-100 extracts made from 293 cells was also used to test the caspase-inhibitory properties of these proteins. The addition of dATP to the lysates followed by incubation at 37 °C allows for the formation of the apoptosome and activation of caspase-9, which in turn activates caspase-3 in the lysate (50, 51). Incubation of recombinant wild type XIAP protein with the S-100 extracts caused suppression of caspase-3 activation as measured by DEVD-AFC cleavage. Proteins containing a single mutation in either the caspase-3 (D148A) or caspase-9 (W310A) binding domains were able to block caspase-3 activation in cell lysates (Fig. 2B). Whereas XIAP D148A cannot inhibit recombinant caspase-3 (Fig. 2A), it can inhibit caspase-3 activation in S-100 extracts after the addition of dATP (Fig. 2B). This is probably the result of the ability of this molecule to inhibit caspase-9, which is a required step for caspase-3 activation in S-100 extracts (Fig. 1A). However, the D148A/W310A double mutant that is incapable of inhibiting either caspase-9 or -3 was unable to suppress caspase-3 activity in both assays.

The antiapoptotic activity of these mutants when expressed in cells has been previously reported (24). Whereas the mutation D148A did not affect the antiapoptotic activity of XIAP against Bax-induced cell death, this mutation did not protect cells from Fas-mediated apoptosis. Therefore, caspase-3 inhibition was dispensable for the protection against stimuli that mediate cell death primarily by destabilizing the mitochondria, probably because the ability of XIAP to inhibit caspase-9 is sufficient to confer protection in this setting. However, if the death stimulus can activate caspase-3 without involving the mitochondria, as in the case of Fas-mediated apoptosis in type 1 cells, loss of caspase-3-inhibitory activity is sufficient to abrogate the protective properties of XIAP (Fig. 1A).

XIAP Caspase-binding Mutants Retain the Ability to Activate NF-κB, Smad, and JNK1—Having generated mutant forms of XIAP that are incapable of suppressing apoptosis and inhibiting caspases, we examined their ability to activate signaling pathways. In order to address the role of caspase inhibition in XIAP-mediated activation of NF-κB, the XIAP caspase-binding mutants were co-transfected into 293 cells along with a κB-responsive promoter driving expression of luciferase. As reported previously (12, 40), wild type XIAP activated transcription of the NF-κB-responsive promoter (Fig. 3A). The mutations of XIAP that affect caspase binding did not affect the ability of this molecule to activate NF-κB-mediated transcription, although comparable expression levels for XIAP wild type
and caspase-binding mutants were found after transient transfection in 293 cells (Fig. 3A).

The ability of XIAP caspase-binding mutants to activate Smad-dependent transcription was evaluated using the Smad-responsive reporter, SBE-JONK, in the Smad 4-null breast cancer cell line MDA-MB-468. These cells were transfected with the various XIAP mutants, the SBE-JONK reporter, and Smad4. Following TGF-β1 stimulation, a robust activation of the SBE-JONK reporter can be observed, which is greatly enhanced by XIAP expression (Fig. 3B). The responses from this reporter are completely dependent on Smad4, since cells transfected in the absence of Smad4 demonstrate minimal luciferase activity under basal conditions or after TGF-β1 treatment (not shown), highlighting that this promoter faithfully represents Smad-dependent transcription. All XIAP caspase-binding mutants were also able to activate transcription, and the D148A/W310A mutant, incapable of binding caspases, maintains its ability to activate Smad-dependent transcription.

The ability of the caspase binding mutations to affect the activation of JNK1 by XIAP was next examined (Fig. 3C). XIAP wild type and the caspase-binding mutants were transfected into 293 cells along with an HA-tagged JNK1 expression vec-

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**Fig. 5.** Mapping the domains of XIAP required for activation of Smad-dependent signaling. A, schematic diagram presenting the different truncation mutants used in these experiments. B, MDA-MB-468 cells were transfected with the TGF-β-responsive reporter SBE-JONK, along with Smad4 and the indicated XIAP expression vectors. At 24 h post-transfection, the cells were stimulated with human TGF-β1 in a low serum medium (0.2% fetal bovine serum). The luciferase assay was performed 24 h after TGF-β1 stimulation. Assays were performed in triplicate, and the mean and S.D. are presented for each group. C, expression of the various truncation mutants of XIAP used in these experiments was evaluated by Western blot using an HA-specific antibody.
tor. Immune complex kinase assays were performed using GST-c-Jun as substrate. The XIAP single mutants activated JNK1 to levels comparable with wild type XIAP. The double mutant, which is no longer able to bind caspases, shows no impairment in its ability to activate JNK1.

These results show that activation of the TGF-β- or NF-κB-responsive promoters or JNK1 kinase activity by XIAP is insensitive to mutations that abrogate caspase binding and inhibition. Taken together, these findings demonstrate that binding to caspases is not important for the signaling properties of XIAP and suggest that distinct domains in the molecule might be involved in these signaling properties. Similarly, whereas XIAP D148A/W310A has preserved signaling properties, it is incapable of protecting cells from Bax- or Fas-induced cell death (24), demonstrating that under these circumstances, XIAP-mediated protection seems to depend more on its ability to inhibit caspases than on its signaling properties.

The effects of XIAP on NF-κB and Smad Signaling Are Mediated by Distinct Domains Not Involved in Caspase Binding—Given that caspase binding and inhibition is not required for the signaling properties of XIAP, other domains in the molecule would be expected to be involved in these signaling effects. Therefore, a series of experiments were performed to delineate the domains required for NF-κB and Smad signaling events.

It has been previously reported that the RING domain of XIAP is required for its ability to activate NF-κB (12). In order to assess whether this effect results from loss of E3 ubiquitin ligase activity, a point mutant (H467A) devoid of E3 activity was tested. Both XIAP ΔRING and H467A failed to activate NF-κB-dependent reporter gene activity, indicating that the E3 ubiquitin ligase function of the RING is required for the activation of NF-κB (Fig. 4). In addition, it has been reported that the RING domain of XIAP is dispensable for its antiapoptotic activity (21, 48). Both the XIAP ΔRING and XIAP H467A mutant were able to protect from Bax-induced death as well as wild type XIAP, indicating that the function of the RING is not required for protection (data not shown). These results further demonstrate that the antiapoptotic properties of XIAP can be uncoupled from the ability of this molecule to activate NF-κB and suggest that XIAP-mediated ubiquitination of a factor(s) is responsible for the ability of this molecule to activate NF-κB.

Next, the domains necessary for XIAP-mediated activation of Smad-dependent signaling were evaluated. Full-length or truncated HA-tagged versions of XIAP (Fig. 5A) were transfected into MDA-MB-468 cells along with Smad4 and the SBE-JONK reporter. Wild type XIAP strongly enhanced Smad-dependent transcription as assessed by the SBE-JONK reporter (Fig. 5B). Carboxyl-terminal deletion mutants of XIAP involving the RING domain (ΔRING) or in conjunction with part of the loop (3×BIR) activated the SBE-JONK reporter to levels comparable with full-length XIAP. However, a deletion involving the RING and the entire loop (BIR 1-2-3) abrogated the effect of XIAP on Smad signaling, suggesting that the amino-terminal portion of the loop domain (amino acids 351–399) is necessary for this signaling property of XIAP. None of the BIR domains (BIR 1, BIR 2, or BIR 3) individually or in various combinations (BIR 1-2 and BIR 2-3) possessed any signaling activity over background when the loop was completely absent. However, an amino-terminal truncation consisting of the entire loop and RING domains (ΔBIR) was also incapable of mediating these effects. The activity was restored in a truncation mutant including BIR 3 and the loop and RING domains (Δ2BIR). These results held true despite the fact that Δ2BIR was expressed at much lower levels than ΔBIR (Fig. 5C). Therefore, whereas the loop region is necessary, it is not sufficient for the effects of XIAP on TGF-β signaling, and the experiments presented here demonstrate that this property can only be mediated when both the BIR 3 and at least the amino-terminal portion of the loop are present.

Our studies demonstrate that the caspase-inhibitory properties of XIAP are dispensable for its signaling activities, which can be mapped to distinct domains of the molecule. The ability to activate NF-κB depends on the E3 ubiquitin ligase activity of the RING, suggesting that this effect occurs as a result of ubiquitination of a regulator of the NF-κB signaling pathway. This is in contrast to XIAP-mediated JNK1 activation, which has been previously shown not to require the RING domain (12, 38). In addition, the fact that both signaling effects do not map to the same domain suggests that in these cells the ability of XIAP to activate NF-κB is not mediated through the same pathway activating JNK1. This is consistent with prior experiments demonstrating that dominant negative TAK1 abrogated XIAP-mediated JNK1 activation but not NF-κB activation (12) and is in contrast with other experiments done with endothelial cells (40).

Additionally, in this study the ability of XIAP to activate Smad-dependent transcription was mapped to BIR 3 and the amino-terminal portion of the loop. This is in contrast to prior reports showing a dependence on the RING for transcriptional activation of the TGF-β-responsive reporter 3TP-Lux (12). However, the RING domain is not required for activation of SBE-JONK, a different TGF-β-responsive reporter used here. The 3TP-Lux reporter but not SBE-JONK has a substantial basal activity and is not stimulated by Smad4 transfection in Smad4-null MDA-MB-468 cells (data not shown). This indicates that the responses of the 3TP-Lux reporter are more complex and are not strictly Smad-dependent but also involve other transcription factors and might explain these differences. Since SBE-JONK is strictly Smad-dependent, the results presented here more faithfully represent the effects of XIAP on Smad-dependent transcription.

The ability of XIAP to inhibit caspases and confer protection from cell death signals is restricted to specific domains in the molecule. Specific mutations of motifs involved in caspase binding and inhibition are sufficient to abrogate the protective properties of XIAP, leaving the signaling activities of XIAP unaffected. We show here that domains dispensable for caspase inhibition are required for these signaling properties. This finding, together with the high conservation of these regions of the molecule, suggest that the ability of XIAP to participate in signaling is indeed an evolutionarily conserved function of this molecule in addition to its ability to inhibit caspases. The role for the integration of these multiple functions into a single molecule remains to be elucidated.

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