Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases

Quinn L. Deveraux, Eugen Leo,
Henning R. Stennicke, Kate Welsh,
Guy S. Salvesen and John C. Reed¹

The Burnham Institute, Program on Apoptosis and Cell Death
Research, La Jolla, CA 92037, USA

¹Corresponding author
e-mail: jreed@burnham-inst.org

Several human inhibitor of apoptosis (IAP) family proteins function by directly inhibiting specific caspases in a mechanism that does not require IAP cleavage. In this study, however, we demonstrate that endogenous XIAP is cleaved into two fragments during apoptosis induced by the tumor necrosis factor family member Fas (CD95). The two fragments produced comprise the baculoviral inhibitory repeat (BIR) 1 and 2 domains (BIR1-2) and the BIR3 and RING (BIR3-Ring) domains of XIAP. Overexpression of the BIR1-2 fragment inhibits Fas-induced apoptosis, albeit at significantly reduced efficiency compared with full-length XIAP. In contrast, overexpression of the BIR3-Ring fragment results in a slight enhancement of Fas-directed apoptosis. Thus, cleavage of XIAP may be one mechanism by which cell death programs circumvent the anti-apoptotic barrier posed by XIAP. Interestingly, ectopic expression of the BIR3-Ring fragment resulted in nearly complete protection from Bax-induced apoptosis. Use of purified recombinant proteins revealed that BIR3-Ring is a specific inhibitor of caspase-9 whereas BIR1-2 is specific for caspases 3 and 7. Therefore XIAP possesses two different caspase inhibitory activities which can be attributed to distinct domains within XIAP. These data may provide an explanation for why IAPs have evolved with multiple BIR domains.

Keywords: apoptosis/caspase/XIAP

Introduction

First identified in insect viruses, inhibitor of apoptosis (IAP) family proteins appear to be evolutionarily conserved with apparent homologs identified thus far in mammals, flies, worms and yeast. IAP family members are characterized by a highly conserved ~70 amino acid domain termed the baculoviral inhibitory repeat (BIR) that can be present as many as three times in some IAPs (reviewed in Clem and Duckett, 1998; LaCasse *et al.*, 1998; Deveraux and Reed, 1999). In addition to BIR domains, some IAPs contain a C-terminal zinc-binding RING motif (Saurin *et al.*, 1996; Borden, 1998). Structure–function studies of IAP family proteins performed to date have uniformly demonstrated a requirement for at least

one BIR domain for suppression of apoptosis (reviewed in Clem and Duckett, 1998; LaCasse *et al.*, 1998; Deveraux and Reed, 1999). Other reports have indicated that at least under some conditions baculoviral IAPs require both N-terminal BIR domains and a C-terminal RING domain for their anti-apoptotic function in insect cells (Clem and Miller, 1994; Harvey *et al.*, 1997). The relevance of the RING domain for IAP-mediated suppression of apoptosis, however, appears to depend upon cellular context. The BIR2 domain alone of human XIAP was found to be sufficient for inhibition of apoptosis induced by Fas (CD95) (Takahashi *et al.*, 1998). Likewise, the BIR2 domain from baculovirus Op-IAP or *Drosophila* D-IAP1 was reported to be sufficient for inhibition of apoptosis induced by the fly apoptosis protein HID in insect cells (Vucic *et al.*, 1998). In a separate study, cell death-suppressing activity mapped to the N-terminal BIR domains in the *Drosophila* IAPs, D-IAP1 or D-IAP2, and removal of the C-terminal RING domain actually enhanced their ability to suppress developmental programmed cell death and cell death induced by ectopic expression of the fly apoptosis gene *reaper* in the developing fly eye (Hay *et al.*, 1995).

Although the structural requirements for IAP function may vary, numerous reports suggest strongly that at least some IAP family members play a highly conserved role in the regulation of cell death (reviewed in Clem and Duckett, 1998; Deveraux and Reed, 1999). Consistent with this idea, we reported previously that the human IAP family members XIAP, cIAP1 and cIAP2 can directly inhibit specific caspases (Deveraux *et al.*, 1997, 1998; Roy *et al.*, 1997), an evolutionarily conserved family of cysteine proteases that play critical roles in the execution of apoptosis (Salvesen and Dixit, 1997; Thornberry and Lazebnik, 1998). XIAP, cIAP1 and cIAP2 each contain three tandem BIR domains and a single C-terminal RING motif. The BIR domains of XIAP, cIAP1 and cIAP2 are sufficient for both suppression of apoptosis and inhibition of caspase-3 and caspase-7 (Deveraux *et al.*, 1997; Roy *et al.*, 1997). Furthermore, the ability of XIAP to inhibit caspases 3 and 7 was localized to the BIR2 domain alone—supporting the concept that a single BIR domain can be sufficient for suppression of caspases and blockade of apoptosis (Takahashi *et al.*, 1998).

Two prototypical pathways for induction of apoptosis in mammalian cells are induced by Fas and Bax. Fas (CD95) is a member of the tumor necrosis factor (TNF) family of apoptosis-inducing receptors that activate procaspase-8 and possibly other initiator caspases (Boldin *et al.*, 1996; Muzio *et al.*, 1996; Wallach *et al.*, 1997). Caspase-8 then cleaves and activates caspase-3 and other downstream caspases that function as the ultimate effectors of apoptosis. Bax is a pro-apoptotic member of the Bcl-2 family that associates with mitochondria and induces

release of cytochrome *c* (reviewed in Green and Reed, 1998). Once activated by cytochrome *c*, together with cofactor nucleotide triphosphates (dATP or ATP), apoptosis promoting factor (Apaf-1) then binds and activates pro-caspase-9, which in turn cleaves and activates caspase-3 and other downstream caspases (Li *et al.*, 1997; Reed, 1997; Zou *et al.*, 1997). IAPs block cell death at distinct steps in apoptotic pathways induced by Fas or Bax. XIAP, cIAP1 and cIAP2 interfere with the cytochrome *c*-mediated activation of caspase-3, which is induced by Bax, by directly inhibiting caspase-9 (Deveraux *et al.*, 1998). Thus, caspase-9 was identified as a new target for the IAPs upstream of caspase-3. In the Fas pathway, which can activate caspase-3 without necessarily involving mitochondria and caspase-9 (Peter and Krammer, 1998; Scaffidi *et al.*, 1998), XIAP and other IAPs suppress the caspase cascade through direct inhibition of active caspase-3 (Deveraux *et al.*, 1998).

In this study we observed that during apoptosis induced by the TNF family member Fas, XIAP is cleaved, separating the BIR1-2 domains from the BIR3-Ring domain. Although cleavage of XIAP yields a BIR1-2 fragment that is capable of inhibiting active caspases 3 and 7, as well as apoptosis induced by Fas, ectopically expressed BIR1-2 has significantly reduced potency compared with full-length XIAP. Moreover, the BIR1-2 fragment appears to be susceptible to further degradation by caspases. Thus, cleavage of XIAP may be one mechanism for lowering the threshold of caspase activity necessary for inducing apoptosis. In contrast to BIR1-2, ectopic expression of the BIR3-Ring fragment slightly enhances Fas-induced apoptosis. Surprisingly, however, expression of BIR3-Ring potentially inhibited cell death due to Bax. Here we elucidate the mechanism by which BIR3-Ring blocks Bax-induced cell death and compare it with the mode of BIR1-2-mediated inhibition of both the Fas and Bax apoptotic pathways. These results demonstrate unique functions of different BIR domains within XIAP and thus provide a possible explanation for the presence of multiple BIR domains in the IAP family of anti-apoptotic proteins.

Results

XIAP is cleaved during Fas-induced apoptosis

Treatment of Jurkat T cells with antibodies specific for Fas (anti-Fas) results in apoptosis which is preceded by proteolytic processing and activation of pro-caspase-3 (Enari *et al.*, 1996; Cohen, 1997; Games *et al.*, 1998). In a time-dependent manner, we observed that endogenous XIAP is cleaved into at least two fragments during treatment of Jurkat T cells with anti-Fas antibody. Anti-Fas treatment results in depletion of much of the full-length 53 kDa protein and concomitant generation of a 30 kDa fragment that reacts with an anti-XIAP antibody specific for an epitope found within the BIR3-Ring region (Figure 1). Fas-induced proteolysis of XIAP occurred with approximately the same kinetics as processing of pro-caspase-3. Addition of 50 μ M zVAD-FMK, a broad spectrum caspase inhibitor, completely prevented anti-Fas-induced cleavage of XIAP, indicating that this is a caspase-dependent event.

Fig. 1. Endogenous XIAP is cleaved during Fas-mediated apoptosis. CH11 anti-Fas antibody (300 ng/ml) was added to Jurkat T cells in culture at 37°C. Cells were removed at the indicated times, normalized for protein content and analyzed by Western blot analysis. Nitrocellulose filters were incubated with antibodies specific for the region between the BIR3-Ring domain of XIAP (left panel) or with caspase-3 antisera (right panel). Arrows denote endogenous XIAP and the BIR3-Ring cleavage product (left panel) or the pro- and processed forms of caspase-3 (right panel). The apparently non-specific anti-XIAP immune-reactive molecule migrating slightly larger than XIAP serves as a fortuitous loading control (*).

Mapping of XIAP cleavage site

Since XIAP cleavage correlates with caspase activation and can be inhibited with caspase inhibitors, ³⁵S-labeled XIAP was translated *in vitro* and incubated with purified active caspases in an effort to reproduce the cleavage of XIAP observed in intact cells. Incubation of XIAP with purified recombinant caspases 3, 6, 7 or 8 produced at least two ~30 kDa cleavage fragments (Figure 2A). No cleavage of the *in vitro*-translated XIAP was detected if caspases were omitted from the reaction. Caspase-3 and caspase-7 were the most efficient enzymes with respect to XIAP cleavage, although all caspases tested produced similar XIAP fragments. However, in the caspase-3-treated [³⁵S]XIAP reaction, the slower migrating fragment (later determined to be the BIR1-2 region) was not visible—possibly due to further cleavage.

To confirm that caspases cleave XIAP directly and to map the cleavage site, purified recombinant GST-XIAP was incubated with purified active caspase-3 or -7. A 20-fold molar excess of caspase relative to XIAP protein was added in these assays, since at equimolar concentration XIAP completely inhibits caspase-3 or -7 activity and therefore XIAP cleavage. The resulting material was separated on an SDS gel and transferred to PVDF membrane and the faster migrating C-terminal fragment was excised and subjected to N-terminal sequence analysis. Both caspase-3 and -7 treatment of XIAP resulted in identical C-terminal fragments beginning with alanine-243 of XIAP. Based upon these results, we prepared constructs in which aspartic acid-242 in XIAP was replaced by a glutamic acid (XIAP_{D242E}). As a control, *in vitro*-translated XIAP_{D214E} was prepared and incubated with purified caspase-3. The XIAP_{D214E} protein underwent cleavage akin to the wild-type protein, whereas XIAP_{D242E} was not cleaved when incubated with active caspase-3 under the same conditions (Figure 2A).

To further address the idea that XIAP aspartic acid-242 is the relevant caspase cleavage site *in vivo*, wild-type XIAP and XIAP_{D242E} constructs were expressed by transient transfection in 293T cells. When co-transfected with Fas, the expressed wild-type XIAP protein was cleaved, generating a 30 kDa product that reacts with antibodies specific for the BIR3-Ring region (Figure 2B). In contrast, the expressed XIAP_{D242E} mutant protein was intact and

Fig. 2. XIAP is cleaved *in vitro* by purified recombinant caspases. (A) cDNAs encoding full-length XIAP or XIAP mutants XIAP_{D242E} or XIAP_{D214E} were translated *in vitro* in reticulocyte lysate containing [³⁵S]methionine. The ³⁵S-radiolabeled XIAP, XIAP_{D242E} or XIAP_{D214E} proteins were incubated with 100 nM of the indicated caspases for 1 h at 37°C. Reactions were then resolved on SDS gels and analyzed by fluorography. (B) 293T cells were transfected with myc control, wild-type myc-XIAP (WT) or mutant myc-XIAP(D242E) with or without co-transfection of Fas. Cell samples were analyzed on Western blots using antibodies specific for the BIR3-Ring region of XIAP. Arrows denote the endogenous XIAP, Myc-XIAP transfection products and the XIAP cleavage fragment. Expression levels of MYC-XIAP proteins were similar in cells not expressing Fas (not shown). (C) The diagram at the bottom shows a schematic of the full-length XIAP, mapped cleavage site, BIR1-2 and BIR3-Ring fragments. In experiments similar to those presented in (A), we observed no cleavage of XIAP by recombinant active caspase-9; however, recombinant caspase-9 is ~10 000-fold less active than the other recombinant caspases tested in these assays.

present at comparable levels in both control and Fas-transfected 293T cells.

Differential effects of BIR1-2 and BIR3-Ring fragments on apoptotic pathways

Since caspase-mediated cleavage can regulate molecules either by disrupting their previous function or by generating a fragment(s) with new activities (reviewed in Salvesen and Dixit, 1997), we wished to examine the effects of each XIAP fragment on apoptotic events induced by ectopic expression of Fas receptor construct (Fas) or the pro-apoptotic protein Bax. To study the potential effects of expression of the BIR1-2 and BIR3-Ring constructs on cell death we chose 293 and 293T cells as a model system, due to their higher transfection efficiency and their apoptotic sensitivity to expression of both Fas and

the Bax protein. As expected, co-expression of full-length XIAP or BIR1-2 suppressed Fas-induced cell death, although BIR1-2 was significantly less efficient (~2- to 3-fold) than full-length XIAP. In contrast, cells expressing the BIR3-Ring fragment showed a slight enhancement (~10%) of cell death induced by Fas (Figure 3), indicating that the BIR3-Ring fragment lacks anti-apoptotic activity against Fas in these cells. Expression of the non-cleavable mutant XIAP_{D242E} was slightly more protective against Fas-mediated apoptosis than wild-type XIAP.

Expression of full-length XIAP or BIR1-2 resulted in suppression of Bax-mediated cell death. These results were not surprising since we had previously localized the caspase-3 and -7 inhibitory activity of XIAP to the BIR2 domain which behaves similarly to the BIR1-2 construct with respect to anti-apoptotic activity (Takahashi *et al.*, 1998). Surprisingly, however, co-expression of Bax and the BIR3-Ring construct resulted in nearly complete inhibition of cell death caused by Bax (Figure 3). Thus, in contrast to Fas, apoptosis induced by Bax was potently inhibited by the BIR3-Ring fragment. Independent transfection of constructs expressing the BIR1, BIR3 or Ring domains alone showed no significant inhibition of apoptosis in these assays, although co-expression of Ring with Fas resulted in a slight enhancement of cell death (not shown). Western blot analysis of lysates prepared from the transiently transfected 293 cells confirmed production of the expected proteins and revealed that the failure of the BIR3-Ring fragment to suppress Fas-induced cell death was not due to lower expression levels. Note that during Fas-induced apoptosis the expressed wild-type XIAP, but not XIAP_{D242E}, is cleaved. Under these conditions, antibodies specific for the BIR3-Ring region of XIAP readily detect the endogenous Fas-induced BIR3-Ring cleavage fragment, whereas incubation of the same blot with antisera specific for the BIR1-2 domain reveals only small amounts of the BIR1-2 fragment, suggesting that it may be unstable. Interestingly, XIAP is not cleaved during apoptosis induced by Bax.

Recombinant BIR3-Ring suppresses caspase activation induced *in vitro* by cytochrome c

To address the biochemical step at which BIR3-Ring might block the Bax apoptotic pathway, we explored whether recombinant BIR3-Ring protein could inhibit caspase activation induced by cytochrome c and dATP (Cyto-c/dATP) in lysates from cultured cells. Addition of recombinant BIR3-Ring concurrently with or before addition of Cyto-c/dATP to cell lysates suppressed the generation of caspase-3-like protease activity as measured by the hydrolysis of DEVD-7-amino-4-trifluoromethyl coumarin (DEVD-AFC) (Figure 4A). Addition of either BIR3 alone or Ring alone was ineffective at blocking Cyto-c/dATP induction of caspase activity, implying that the combination of these domains is required. While BIR3-Ring suppressed caspase activity when added at the time of, or before the introduction of, Cyto-c/dATP, the addition of BIR3-Ring protein 10 min after the addition of Cyto-c/dATP failed to inhibit caspase activity that had already been generated (Figure 4B). In contrast, addition of full-length XIAP, BIR1-2 or BIR2 recombinant protein still suppressed caspase activity regardless of the timing of Cyto-c/dATP addition. Recombinant BIR1, BIR3 or Ring

Fig. 3. Differential effects of BIR3-Ring and BIR1-2 on Fas and Bax-induced apoptosis in 293 cells. (A) Fas or Bax expression plasmids were co-transfected into 293 cells with the indicated XIAP expression plasmids. Apoptosis (dark bars) or caspase activity (light bars) was measured as described in Materials and methods and the data presented here as a percentage relative to cells transfected with Fas or Bax alone (mean $\pm$ SD; $n = 2$). (B) Western blot analysis of transfected cells using antisera specific for BIR2 (left) or BIR3-Ring (right) of XIAP. The upper arrowhead denotes the transfected full-length XIAP or XIAP_{D242E} expression products. The lower arrow denotes the Fas-induced cleavage products and the expressed BIR1-2 and BIR3-Ring fragments. An asterisk is pictured next to the endogenous full-length XIAP to the right of each panel. All the expression products contain an N-terminal *myc* sequence. Similar results were obtained in 293T cells. Note that XIAP is not cleaved in cells co-expressing Bax whereas it is readily cleaved in cells co-expressing Fas.

domain proteins did not affect Cyto-c/dATP-induced caspase activity in these assays, demonstrating the specificity of these results. Consistent with our observation that expression of the BIR3-Ring fragment does not block Fas-induced cell death in intact cells, addition of recombinant BIR3-Ring had no effect upon caspase-8-induced DEVD-AFC cleaving activity in cell lysates, even when added before the addition of active caspase-8 (Figure 4C). In contrast, full-length XIAP, BIR1-2 or BIR2 alone did suppress accumulation of caspase-3-like activity in the caspase-8-treated lysates. Recombinant XIAP_{D242E} or XIAP_{D242A} behaved similarly to recombinant wild-type XIAP in these assays (not shown), further confirming that

cleavage of XIAP is not a necessary component of its caspase inhibitory mechanism.

BIR1-2 and BIR3-Ring differentially inhibit caspases

Based upon dose-response experiments, BIR3-Ring inhibits Cyto-c/dATP-induced activation of caspase-3 ~3-fold more efficiently than BIR1-2 or BIR2 alone (Figure 5A and B, and data not shown). However, addition of recombinant BIR1-2 or BIR3-Ring had very different effects on pro-caspase-3 processing induced by Cyto-c/dATP, as revealed by Western blot analysis of these lysates (Figure 5C). In control lysates to which XIAP protein fragments were not added, Cyto-c/dATP induced proteo-

Fig. 4. Effect of recombinant XIAP and XIAP fragments on Cyto-c/dATP or caspase-8 induction of caspase activity in lysates from 293 cells. Recombinant XIAP or XIAP fragment proteins (2 μM) were added to lysates from 293 cells concurrently with (A) or 10 min after (B) the addition of 10 μM cytochrome *c* and 1 mM dATP. (C) Recombinant XIAP-derived proteins were added to cell lysates followed by the addition of active caspase-8 (100 nM). Lysates were incubated at 30°C and aliquots containing equivalent amounts of total protein were withdrawn after 10 min and assayed for caspase activity by continuously measuring the release of fluorogenic 7-amino-4-trifluoromethyl coumarin (AFC). Release of AFC from DEVD-AFC (y-axis) was measured from the onset of substrate addition (denoted on the x-axis in seconds). Control lysate samples were incubated with (CNTL) or without (Bkg) Cyto-c/dATP alone (A and B). In (C), caspase-8 alone was designated as control (CNTL). Experiments were repeated three times with similar results.

Fig. 5. Comparison of the effect of BIR1-2 and BIR3-Ring on Cyto-c/dATP-induced caspase-3 activation. Various concentrations of recombinant BIR1-2 (A) or BIR3-Ring (B) were added to 293 cell lysates concurrently with 10 μM cytochrome *c* and 1 mM dATP. Lysates normalized for total protein content were incubated at 30°C and aliquots were removed after 30 min and assayed for the ability to hydrolyze DEVD-AFC using continuous measurements over the time indicated (A and B) or for Western blot analysis using an antiserum specific for caspase-3 (C). (D) Schematic of Cyto-c/dATP-induced processing of caspase-3. Experiments were repeated twice with similar results.

lytic cleavage of the ~36 kDa pro-caspase-3 protein, producing 17 (p17) and 20 (p20) kDa fragments which react with an anti-caspase-3 antiserum specific for epitopes in the large subunit of caspase-3 (the small p10 subunit is not detected). The p20 and p17 caspase-3 products are alternatively cleaved forms of the large subunit of active caspase-3, which have previously been attributed to auto-catalytic processing events that sequentially remove the N-terminal pro-domain from the ~24 kDa form (large subunit + the pro-domain) (Martin *et al.*, 1996; Deveraux *et al.*, 1997). BIR1-2 did not substantially suppress the Cyto-c/dATP-induced proteolysis of ~36 kDa pro-caspase-3 whereas BIR3-Ring did preserve the bulk of caspase-3 in its pro-form. The BIR1-2 recombinant protein,

however, did arrest the Cyto-c/dATP-induced processing of caspase-3 at an intermediate step, resulting in an accumulation of an ~24 kDa fragment which we previously determined to be the large subunit with the pro-domain still attached (Deveraux *et al.*, 1997, 1998). The differences in processing patterns seen with BIR1-2 versus the BIR3-Ring fragment were not entirely attributable to potency since even when added at low concentrations (0.2 μM), BIR3-Ring did not cause the accumulation of the p24 caspase-3 processing intermediate, suggesting qualitative differences in the caspase inhibitory properties of BIR1-2 and BIR3-Ring.

As depicted in Figure 5D, the first cleavage of pro-caspase-3 to the p24 subunit in the Cyto-c/dATP pathway

Fig. 6. Association of recombinant GST-XIAP, GST-BIR1-2 and GST-BIR3-Ring with endogenous caspases 3 and 9 in cell lysates containing Cyto-c/dATP. Cytochrome *c* was added to cell lysates with or without the addition of the indicated GST fusion proteins. Following incubation at 30°C for 30 min, samples were removed and analyzed by Western blotting using antisera specific for caspase-3 (left panel) or caspase-9 (right panel). In lanes 1–5, lysates were analyzed directly. In lanes 6–8, GST fusion proteins were recovered on glutathione–Sephadex beads and bound caspase-3 or caspase-9 molecules were determined by Western blot analysis. Arrowheads denote the pro- and processed forms of caspase-3 and caspase-9. Note that neither the caspase-3 nor the caspase-9 antiserum reacts with the small subunit of their respective immunogens.

is likely made by caspase-9, with subsequent processing of p24 to p20 and p17 dependent upon caspase-3 autocatalytic activity (Martin *et al.*, 1996; Li *et al.*, 1997). Thus, in these assays, BIR1-2 appears to inhibit caspase-3 activity, thereby preventing its maturation beyond the p24 stage of processing, whereas BIR3-Ring functions upstream of pro-caspase-3 by possibly inhibiting caspase-9 or its activation.

To further address the mechanism by which BIR3-Ring suppresses Cyto-c/dATP-induced activation of caspase-3, we recovered the recombinant GST fusion proteins following incubation in cell lysates containing Cyto-c/dATP, and analyzed the bound proteins by Western blotting using antisera specific for caspase-3 or caspase-9. BIR1-2 did, indeed, bind to the p24 form of the large subunit of caspase-3, whereas full-length XIAP or BIR3-Ring bound only pro-caspase-9 or active caspase-9 (Figure 6). The inability to detect the pro-form or p24 partially processed form of caspase-3 in GST pull-downs involving full-length XIAP reflects the ability of this protein to suppress caspase-9, thus blocking the effects of Cyto-c/dATP upstream of pro-caspase-3 (Deveraux *et al.*, 1998).

BIR3-Ring directly inhibits caspase-9

To provide direct evidence for the biochemical function of BIR3-Ring, we tested its ability to inhibit purified recombinant active caspases. Recombinant BIR1-2, like BIR2 alone, inhibited caspase-3 and caspase-7 but not caspase-9 or other caspases tested (Figure 7 and data not shown). BIR3-Ring, however, displayed no ability to inhibit caspases 3 and 7 or other caspases tested but did inhibit active recombinant caspase-9. Recombinant BIR1, BIR3 or Ring proteins had no significant effect in these assays, demonstrating the specificity of these results. Thus BIR3-Ring is a specific inhibitor of caspase-9, whereas the BIR1-2 fragment (like BIR2 alone) is specific for caspases 3 and 7.

Discussion

When ectopically overexpressed, members of the IAP family have been shown to effectively inhibit a variety of cell death programs (reviewed in Clem and Duckett, 1998; Deveraux and Reed, 1999). For at least some IAPs, their anti-apoptotic potential can be explained by their potent

inhibition of caspases 3, 7 and 9, which are often essential for the execution of apoptosis. But how do caspases become activated in the presence of endogenous levels of IAPs? Transcriptional or translational regulation may modulate the ratios of IAPs relative to caspases in some scenarios or IAPs might be regulated by localization, post-translational modifications, binding by other proteins or degradation. Here we provide evidence that during Fas-induced apoptosis, caspases themselves may exert positive feedback by cleaving at least one member of the IAP family.

Cleavage of XIAP produces an N-terminal BIR1-2 fragment with reduced ability to inhibit caspases 3 and 7, and therefore, diminished ability to suppress apoptosis. The BIR1-2 fragment may also be susceptible to further caspase-mediated cleavage, since, following treatment of purified recombinant full-length XIAP with recombinant caspase-3, the BIR1-2 fragment is undetectable after 1 h (data not shown). Thus caspase-mediated degradation of the BIR1-2 fragment may further reduce its anti-apoptotic potential (Figure 8). The remaining BIR3-Ring fragment appears to be more stable and accumulates readily in Fas-treated cells. Although a potent inhibitor of caspase-9, BIR3-Ring does not suppress the Fas apoptotic pathway, at least in 293 or 293T cells, where Fas is known to induce apoptosis through Bcl-2-independent (i.e. mitochondria-independent) mechanisms.

We can only speculate about the physiological significance of the BIR3-Ring cleavage product since inhibition of caspase-9 might be expected to have either no effect or a negative effect on Fas-induced apoptosis, depending on whether Fas does or does not require the participation of the mitochondria-dependent Cyto-c/APAF-1 pathway for inducing apoptosis (Scaffidi *et al.*, 1998). In fact, we observed the opposite; namely that expression of BIR3-Ring enhanced Fas-induced apoptosis, at least in 293 cells. It is possible that, during Fas-mediated apoptosis or other physiological events such as differentiation or cellular division, activation of low levels of some caspases, such as caspase-3 and caspase-7, is necessary whereas activation of caspase-9 might be detrimental. Cleavage of XIAP might facilitate this scenario by yielding a BIR1-2 fragment with reduced ability to inhibit caspases 3 and 7, and a BIR3-Ring fragment which potentially suppresses caspase-9 activity.

Fig. 7. Comparison of the inhibitory effects of BIR1-2 and BIR3-Ring on inhibition of caspases. Recombinant active caspases 3 (**A**), 7 (**B**) and 9 (**C**) were incubated with DEVD-AFC (**A** and **B**) or LEHD-AFC (**C**) in the presence or absence of the indicated recombinant XIAP-derived fusion proteins. Reactions were incubated at 37°C for the indicated times with continuous monitoring of AFC release. Caspase-3 (**A**) and caspase-7 (**B**) were employed at 100 and 300 pM, respectively, using a 20-fold molar excess of XIAP fragments. Recombinant BIR3-Ring did not inhibit caspase-3 or caspase-7 even when present at 100-fold molar excess (**A** and **B**). Recombinant Δ CARD-caspase-9 (**C**) was added at 200 nM due to its lower specific activity, and XIAP-derived proteins were added at 800 nM (4-fold molar excess). However, recombinant BIR1, BIR2, BIR1-2, BIR3 and Ring showed no significant inhibition even at 20-fold molar excess in these assays (**C**). None of the XIAP-derived fusion proteins inhibited caspase-8 in similar assays (not shown), further demonstrating the specificity of these results. Active caspases were incubated alone as controls (CNTL). Recombinant Δ CARD-caspase-9 was used in these assays due to its greater activity and stability relative to recombinant caspase-9 containing the CARD domain; however, similar results were obtained using the recombinant caspase-9 containing the CARD domain in these assays. Experiments were repeated a minimum of three times with similar results.

Fig. 8. Model of the differential inhibition of Fas and Bax apoptotic pathways by XIAP fragments. XIAP is cleaved by upstream caspases which it does not inhibit or by XIAP-inhibitable caspases, such as caspase-3 and caspase-7, when their molar concentration exceeds that of XIAP. The resulting BIR1-2 fragment, although capable of inhibiting caspase-3 and caspase-7, is less potent than full-length XIAP and may be susceptible to further degradation. Thus, XIAP cleavage may reduce its ability to inhibit caspases 3 and 7. The BIR3-Ring fragment blocks Bax-mediated activation of caspase-9 by directly inhibiting caspase-9 activity but is ineffective against Fas-induced apoptosis which activates caspase-3 without involving caspase-9.

Regardless of the physiological significance of endogenously produced BIR3-Ring, the data reported here refine the structure–function analysis of the XIAP protein. Previously, we reported that the BIR1 and BIR3 domains of XIAP apparently lack caspase-binding capability, despite their striking amino acid similarity to BIR2 (42% for BIR1; 32% for BIR3) (Takahashi *et al.*, 1998). Assuming that these results cannot be ascribed to trivial explanations, such as misfolding of protein fragments taken out of their

normal context of the intact protein, these observations suggest that not all BIR domains are created equal. In this regard, we demonstrate that XIAP contains two separate caspase inhibitory activities that can be mapped to distinct domains. BIR1-2, like BIR2 alone, is sufficient and specific for inhibition of caspase-3 and caspase-7, while BIR3-Ring is sufficient and specific for caspase-9 inhibition. Analogous situations can be found in other proteins, such as human kininogen and equistatin, a sea anemone protein.

In the former, different cystatin domains embedded within the protein have specificities for distinct papain family cysteine proteases (Salvesen *et al.*, 1986). In the latter, the first of the three embedded thyroglobulin-type 1 domains inhibits papain, whereas inhibitory activity against the aspartic protease cathepsin D maps to the C-terminal domains (Lenarcic and Turk, 1999).

Interestingly, while the BIR3-Ring fragment was a potent caspase-9 inhibitor, experiments involving either BIR3 or Ring domain alone suggested that neither of these is sufficient to suppress caspases by itself. Although the structural basis of this observation is unknown, it could be that the Ring domain is necessary to stabilize the appropriate conformation of BIR3 necessary for caspase inhibition. Further structure–function studies are required to delineate the exact nature of the BIR3-Ring and the BIR2 interaction with their respective caspase targets.

Previously, we presented evidence that IAPs inhibited the Fas and Bax pathways by interacting with and inhibiting distinct caspases (Deveraux *et al.*, 1998). These results strongly suggested that, at least in some cell types, Fas-induced apoptosis does not necessarily require caspase-9 for activation of caspase-3 (Deveraux *et al.*, 1998; Stennicke *et al.*, 1998). Consistent with this idea, gene knockout studies of caspase-9 in mice showed that anti-Fas antibody induced caspase-3-like activity and concomitant apoptosis in both wild-type and caspase-9^{-/-} thymocytes (Hakem *et al.*, 1998; Kuida *et al.*, 1998). Our results presented here are highly consistent with these observations since ectopic expression of the caspase-9 inhibitor BIR3-Ring does not suppress Fas-induced cell death in 293 cells but potentially suppresses Bax-induced apoptosis.

In light of these new data we speculate that multiple BIR domains may have evolved to regulate particular apoptotic programs. For example, BIR3-Ring inhibits a caspase (i.e. caspase-9) which is targeted by a CED-4 homolog (i.e. APAF-1). These CED-4 homolog-dependent caspase activation pathways seem to be highly conserved in diverse organisms ranging from humans to worms (Seshagiri and Miller, 1997; Metzstein *et al.*, 1998). BIR2-like caspase-3 and -7 inhibitory activity may have evolved in response to the mammalian immune system involving T lymphocytes and Fas-mediated apoptosis, which can activate caspase-3 without the involvement of the mitochondria or caspase-9. Thus organisms lacking Fas-like apoptotic programs would not be expected to possess IAPs with BIR2-like caspase inhibitory function. Consistent with this idea, we have observed that an insect IAP and the insect viral CpiAP protein have activities that parallel the XIAP BIR3-Ring but not the BIR2 fragment (Q.Huang, Q.L.Deveraux and J.C.Reed, manuscript in preparation). Moreover, similar to the BIR3-Ring fragment of XIAP, the insect IAP and CpiAP molecules also require the presence of both a BIR and Ring domain for these anti-apoptotic activities (Q.Huang, Q.L.Deveraux and J.C.Reed, manuscript in preparation).

If the function of IAPs is simply to block caspase activity, then it would be more efficient to have evolved as a broad spectrum inhibitor, similar to the baculoviral p35 protein. Rather, the IAPs appear to be highly specific for distinct caspases, suggesting that inhibition of some caspases but not others may be advantageous for regulation of cell death and possibly other cellular events. The data

presented provide evidence for the regulation of XIAP during apoptosis by caspase-mediated cleavage and shed new light on the structure–function relationships of XIAP by identifying the BIR3-Ring region as a specific inhibitor of caspase-9. Thus, XIAP and possibly other IAPs have potential for regulating different caspases through distinct domains.

Materials and methods

Materials

Carbobenzoxymethyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK) was purchased from Enzyme System Products. The Z-VA(OMe)D-FMK derivative was purchased from Signal Transduction Laboratories. Fluorogenic AFC caspase substrates (Ac-DEVD-AFC and Ac-LEHD-AFC) were from Calbiochem. ECL Western blot detection reagents were purchased from Amersham. All other chemicals were from Sigma. Cell culture media were purchased from Irvine Scientific.

Plasmid constructs

Full-length XIAP (residues 1–497) was constructed in pcDNA-myc and pGEX-4T-1 vectors as previously described (Deveraux *et al.*, 1997). Plasmids encoding fragments of the XIAP protein, including BIR1+2+3 (residues 1–336), RING (residues 337–497), BIR1 (residues 1–123), BIR2 (residues 124–260), BIR3 (residues 261–336) and BIR2+3 (residues 124–336) were constructed in pcDNA-myc, and pGEX-4T-1 vectors as previously described (Takahashi *et al.*, 1998). XIAP (D217A/E) and (D214A/E) mutants were constructed in a similar manner (Takahashi *et al.*, 1998) and used as controls for XIAP cleavage reactions. Based upon N-terminal sequence analysis of the XIAP cleavage, the specific XIAP non-cleavable mutants (D242A) and (D242E) were constructed using standard overlap PCR techniques. BIR1-2 (residues 1–242) was created using a T7 forward primer (Invitrogen) together with the 5'-TATTCTCGAGTTAATCAGATTCACCTCGAAT-3' and BIR3-Ring (243–497) was created using the forward primer 5'-TAC-TTGAATTCGCTGTGAGTTCTGATAGG-3' with a Sp6 (Invitrogen) primer. All sequence constructs were created by a one-step PCR method employing plasmids encoding full-length XIAP as the template (Takahashi *et al.*, 1998). PCR products were cloned into *EcoRI*–*XhoI* sites in pcDNA3 or pGEX4T-1 plasmids for expression in mammalian cells or *Escherichia coli*, respectively (Deveraux *et al.*, 1997).

Protein sequencing

For N-terminal sequence analysis, 10 µg recombinant purified GST–XIAP were incubated with a 20-fold molar excess of recombinant purified caspase-3 or caspase-7 at 37°C for 60 min in caspase buffer [20 mM HEPES pH 7.2, 100 mM NaCl, 10% sucrose, 1% CHAPS, 10 mM dithiothreitol (DTT) and 1 mM EDTA]. Reactions were then resolved on SDS gels and transferred to PVDF. Both caspase-3- and caspase-7-treated GST–XIAP resulted in an ~30 kDa fragment which was excised, and the N-terminal sequence of the fragments was determined by Edman degradation using the Applied Biosystems 476A Protein Sequencer according to the manufacturer's instructions and Stennicke *et al.* (1999). The ~30 kDa fragment generated by caspase-3 or caspase-7 had identical N-terminal residues as determined by sequence analysis.

Protein expression and purification

pGEX4T-1-XIAP- and XIAP-derived pGEX4T-1 constructs were introduced into *E.coli* strain BL21(DE3) containing the plasmid pT-Trx (Deveraux *et al.*, 1997; Takahashi *et al.*, 1998), or expressed in XL-1 blue cells. The GST fusion proteins were prepared as previously described (Deveraux *et al.*, 1997; Takahashi *et al.*, 1998) from the soluble fraction upon induction with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 30°C for 3–20 h, then purified as for GST–XIAP glutathione–Sephadex affinity purification methods. BIR1, 2 and 3 XIAP fragments were expressed in BL21(DE3) cells without pT-Trx using 0.05 mM IPTG to induce expression.

Caspases 3, 6 and 7 containing C-terminal His₆-tags and caspase-8 containing an N-terminal His₆-tag were purified as described previously (Stennicke and Salvesen, 1997, 1998). Full-length caspase-9 was subcloned from pcDNA3 into the *NcoI*–*XhoI* sites of pET-23d as a *NcoI*–*XbaI* fragment by blunt-end ligation of the *XhoI* and *XbaI* blunt sites (Stennicke *et al.*, 1999). The resulting vector was introduced into *E.coli* BL21(DE3) and fully processed enzyme was obtained when induced by

0.2 mM IPTG at OD₆₀₀ = 0.6 for 4 h. The zymogen form of caspase-3 was obtained by expression as described previously by reducing the expression time to 30 min (Stennicke *et al.*, 1998). Caspase-9 lacking the CARD domain (Δ CARD-caspase-9) was constructed by truncating the cDNA and introducing an initiator codon substituting Val139. The resulting vector was introduced into *E. coli* BL21(DE3) and fully processed enzyme was obtained when induced by 0.2 mM IPTG at OD₆₀₀ = 0.6 for 4 h. All constructs were expressed in *E. coli* and isolated by virtue of an engineered N- or C-terminal His₆ purification tag as described previously. The concentrations of the purified enzymes were determined from the absorbance at 280 nm based on the molar absorption coefficients; caspase-3 (A_{280} = 26 000/M/cm), caspase-9 (A_{280} = 30 010/M/cm). Caspase activity was assayed by release of AFC from LEHD- or DEVD-containing synthetic peptides using continuous-reading instruments as described (Quan *et al.*, 1995; Deveraux *et al.*, 1997).

Antibodies

A monoclonal antibody specific for the region between the BIR3 and Ring domains of XIAP was purchased from Transduction Laboratories (#H62120). Antisera specific for the BIR2 region of XIAP were produced by proteolytic removal of the GST domain from the purified recombinant GST-BIR2 protein. The resulting BIR2 protein was purified by chromatography and conjugated to KLH by standard methods. The resulting KLH-BIR2 conjugate was used as an immunogen in rabbits. Antisera specific for caspase-9 were made using recombinant active caspase-9 as an immunogen as previously described (Krajewski *et al.*, 1999). An antiserum specific for caspase-3 was generated as previously described (Krajewska *et al.*, 1997).

Cell culture, transfections and apoptosis assays

Jurkat T cells were cultured in RPMI supplemented with 10% fetal bovine serum (FBS) at 37°C prior to and during treatment with 300 ng/ml of monoclonal anti-Fas antibody (Medical and Biological Laboratories, clone CH-11). Human embryonic kidney 293 or 293T cells were maintained in DMEM supplemented with 10% FBS, 1 mM L-glutamine and antibiotics. Subconfluent 293 or 293T cells were transfected in six-well (3-cm-diameter) dishes using Superfect (Qiagen), as described by the manufacturer, with the following modifications and DNA concentrations: 0.1 μ g of the green fluorescent protein (GFP) marker plasmid pEGFP (CLONTECH), 0.5 μ g of pCMV-Fas expression plasmid or 0.25 μ g of pCDNA-Bax plasmid with various pcDNA3-XIAP constructs or the control plasmid pcDNA3 at 1.7–2.0 μ g (Deveraux *et al.*, 1997; Takahashi *et al.*, 1998). Transfected 293 cells were assayed for apoptosis by collecting both floating and adherent cells 24–36 h later. Cells were then fixed in 3.7% formaldehyde in phosphate-buffered saline for 10 min and stained with 4',6-diamidino-2-phenylindole (DAPI) (Deveraux *et al.*, 1997). The percentage of GFP-positive cells that exhibited apoptotic morphology was determined by staining with 100 μ g/ml of DAPI. Transfected 293T cells were assayed for apoptosis by counting at least 200 GFP-positive cells for morphological alterations typical of adherent cells undergoing apoptosis, including becoming rounded, condensed and detached from the dish. Percentage apoptosis represents the mean value from two independent experiments (mean $\pm$ SD). Generally, 30% transfection efficiency was achieved in 293 cells and ~60–70% in 293T cells. Of the transfected GFP-positive cells, ~50–70% apoptosis was observed 24–36 h after Bax or Fas transfection in 293 or 293T cells.

Caspase assays

Caspase activities were assayed as previously described (Deveraux *et al.*, 1997; Stennicke and Salvesen, 1997). Briefly, caspase activity was assayed at 37°C in 100 μ l of caspase buffer (50 mM HEPES pH 7.4, 100 mM NaCl, 10% sucrose, 1 mM EDTA, 0.1% CHAPS and 10 mM DTT) containing 100 μ M of the indicated fluorogenic peptide. Activity was measured continuously over the indicated time by the release of AFC from short peptides such as DEVD-AFC or LEHD-AFC using a Molecular Devices fluorometer in the kinetic mode and the 405–510 filter pair. The amount of AFC release was calculated based upon the fluorescence of a known concentration of purified AFC in similar assays. To assess caspase activity generated in intact cells, 10 μ l of packed cells were lysed by addition of 20 μ l of buffer A (20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA and 1 mM DTT) containing 0.25% Triton X-100. Suspensions were vortexed briefly and 2 μ l (10–20 μ g) were assayed for caspase activity in 100 μ l caspase buffer (containing 100 μ M DEVD-AFC) at 37°C. In parallel, 5 μ l (~35 μ g) of these lysates were generally employed for Western blot analysis as described previously (Deveraux *et al.*, 1997, 1998).

For initiating caspase activation *in vitro*, cytosolic extracts from 293 embryonic kidney cells or Jurkat T cells were prepared essentially as described (Liu *et al.*, 1996), with several modifications (Deveraux *et al.*, 1997). Briefly, cells were washed once with ice-cold buffer A and pelleted by centrifugation. Packed cell pellets were suspended in 1–2 vol. of buffer A, incubated on ice for 20 min and then disrupted by 15–30 passages through a 26-gauge needle. Cell extracts were clarified by centrifugation at 16 000 g for 30 min and the resulting supernatants were stored at –80°C. For initiating caspase activation, either 10 μ M horse heart cytochrome *c* (Sigma) together with 1 mM dATP or 100 nM of purified recombinant caspase-8 was added to cell extracts (~10 mg total protein/ml). Recombinant GST fusion proteins were added and recovered from cell lysates as previously described (Deveraux *et al.*, 1997, 1998). Briefly, 5 μ g of recombinant GST fusion protein were added to 100 μ l of cell lysate and incubated under the conditions described. GST fusion proteins were recovered by addition of 20 μ l of packed glutathione beads which were previously equilibrated in buffer A. Following incubation for 30 min at 4°C on ice, the glutathione beads were collected by centrifugation and washed twice in 500 μ l of ice-cold buffer A. Proteins were eluted from the beads by incubation at 80°C for 10 min in 40 μ l of SDS sample buffer before electrophoresis.

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