c-IAP1 is Cleaved by Caspases to Produce a Pro-apoptotic C-terminal Fragment

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The abbreviations used are: BHK (baby hamster kidney); CARD (caspase recruitment domain); CHO (Chinese hamster ovary); DEVD-CHO, N-acetyl-Asp-Glu-Val-aspartinal; N18 (murine neuroblastoma); HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; TNF (tumor necrosis factor \( \alpha \)) zVAD-fmk, N-benzyloxy-carbonyl-Val-Ala-Asp-fluoromethyl ketone.
Summary

Although human c-IAP1 and c-IAP2 have been reported to possess anti-apoptotic activity against a variety of stimuli in several mammalian cell types, we observed that full-length c-IAP1 and c-IAP2 failed to protect cells from apoptosis induced by Bax overexpression, TNF treatment or Sindbis virus infection. However, deletion of the C-terminal RING domains of c-IAP1 and c-IAP2 restored anti-apoptotic activity, indicating that this region negatively regulates the anti-apoptotic function of the N-terminal BIR domain. This finding is consistent with the observation by others that the RING domain of c-IAP1 functions as an E3 ligase, promoting autoubiquitination and degradation of c-IAP1. In addition, we found that c-IAP1 is cleaved during apoptosis to 52kD and 35kD fragments. Both fragments contain the C-terminal end of c-IAP1 including the RING finger. In vitro cleavage of c-IAP1 with apoptotic cell extracts or with purified recombinant caspase-3 produced similar fragments. Furthermore, transfection of cells with the spacer-RING domain alone suppressed the anti-apoptotic function of the N-terminal BIR domain of c-IAP1 and induced apoptosis. Optimal death-inducing activity of the spacer-RING required both the spacer region and the zinc-binding RING domain of c-IAP1 but did not require the CARD domain located within the spacer region. To the contrary, deletion of the CARD domain increased pro-apoptotic activity perhaps by stabilizing the C-terminal fragment.

Keywords: BIR domain/CARD domain/caspase/inhibitor of apoptosis (IAP)
protein/RING finger
Introduction

The inhibitor of apoptosis (IAP) proteins Op-IAP and Cp-IAP were originally identified in baculoviruses because they could functionally replace the baculovirus-caspase inhibitor P53 (1,2). The closest homologues to the baculovirus IAPs are the Drosophila IAPs, D-IAP1 and D-IAP2, and the human IAPs, c-IAP1 (MIHB), c-IAP2 (MIHC) and XIAP (hILP, MIHA) (3,4). These molecules all share two or three copies of the BIR (baculovirus IAP repeat) motif at their N-termini and a RING finger at their C-termini, and are direct inhibitors of caspases, a family of death-inducing proteases (5-10).

All of these IAP proteins have been shown to inhibit apoptosis in one or more paradigms. D-IAP1 was consistently retrieved in a genetic screen for caspase inhibitors (11), and both D-IAP1 and D-IAP2 overexpression inhibits apoptosis in the Drosophila retina (12). Human c-IAP1 and c-IAP2 are less potent inhibitors of apoptosis compared to XIAP, correlating with the observation that XIAP is a more potent caspase inhibitor (~100-fold) than c-IAP1 or c-IAP2 in vitro (3). Both biochemical and structural data support a model where the individual BIR motifs of XIAP are specific inhibitors of different caspases. That is, the second BIR motif (BIR-2) and adjacent sequences of XIAP interact directly with activated caspase-3 and are sufficient to inhibit caspase-3 (13,14). In addition, BIR-3 of XIAP binds to and inhibits caspase-9. However, NMR structure analysis predicts that the mechanism of caspase inhibition by BIR-3 will be distinct from that of BIR-2 (15), supporting a model where the individual BIR repeats have independent and non-redundant functions (14).
The role of the C-terminal RING finger seems to be cell type and/or death stimulus-dependent. In some situations the RING is required for anti-apoptotic activity but in others the RING inhibits the anti-apoptotic function of IAP proteins. While the RING finger of Op-IAP is required for protection of insect cells from apoptosis induced by actinomycin D treatment or baculovirus infection, this domain is not required for inhibition of Hid-induced apoptosis in the same insect cell line (16-18). Partial inhibition of cell death induced by HID as well as Reaper and Grim is conferred by BIR-2 of Op-IAP (most equivalent to BIR-3 of c-IAP1) plus a critical carboxyl-proximal flanking sequence. However, BIR-2 plus the RING finger is a more potent protector than BIR-2 (with proximal sequences) alone, implying that the RING finger contributes to the anti-apoptotic function of Op-IAP (19).

The RING fingers of a number of proteins including c-IAP1 and XIAP were recently shown to function as E3 ubiquitin ligases in proteosome-dependent protein degradation (20). This activity may predominantly facilitate self-destruction as shown for c-IAP1 and XIAP resulting in cell death. Alternatively, the anti-apoptotic function of IAP proteins may be explained by ubiquitination and degradation of caspases as suggested for the RING finger of c-IAP2 (21). The RING finger domains of XIAP, D-IAP1 and D-IAP2 also were shown to interact with signaling factors for the bone morphogenetic protein (BMP) kinase receptors and may participate in these signal transduction pathways as well (22).

Earlier work in mammalian cells had suggested that the RING finger of full-length c-IAP1 does not interfere with its anti-apoptotic activity in mammalian cells. That is, in a
variety of stably or transiently transfected cells, c-IAP1 and c-IAP2 were reported to inhibit apoptosis induced by different death stimuli including serum withdrawal, menadione, staurosporine, caspase-1, Bak, or K+ depolarization (23-26). In contrast to these reports using mammalian cells, full length human c-IAP1 failed to inhibit Reaper-induced cell death in a Drosophila eye model unless the RING finger was deleted (12). Thus, in insect cells, the RING finger appears to negatively regulate the anti-apoptotic function of human c-IAP1. Consistent with this finding, expression of the spacer-RING region of D-IAP1 in transgenic flies resulted in a small eye phenotype due to excessive cell death in the eye disc. Furthermore, the RING finger regions of baculovirus Cp-IAP and Drosophila D-IAP1 can induce apoptosis in the lepidopteran cell line SF-21 (17,17).

We found that like the Drosophila system, c-IAP1 and c-IAP2 failed to protect mammalian cells unless the RING finger domain was deleted. Furthermore, c-IAP1 was cleaved during apoptosis to release the C-terminal spacer-RING domain that was capable of killing cells in transient transfections.

**Experimental Procedures**

**Plasmid and virus constructs.** The human IAPs were identified in an expressed sequence tag (EST) database (Human Genome Sciences) by searching with a BLAST program for homology to baculovirus IAP. EST clones were used as probes to identify full-length cDNAs from the libraries that contained the EST of interest. These clones were essentially identical to previously published c-IAP1 and c-IAP2. C-terminal truncations were generated by insertion of oligonucleotides containing stop codons in all
three reading frames into restriction sites at the codon positions indicated in Fig. 2. The CARD deletion mutant lacks an internal MunI restriction fragment. The RING mutant was generated by recombinant PCR mutating Cys586, His588 and Cys592 to alanines. ΔBIR-1 initiates at a naturally occurring Met at position 131. IAPs and derivatives were cloned into the BstEII site of the Sindbis virus vector (dsTE12Q) and recombinant viruses were generated as previously reported (27-29). C-terminal c-IAP1 fragments and HA-tagged fragments of c-IAP1 were generated by PCR and expressed from a modified pSG5 vector for expression in transfected cells. All clones were verified by DNA sequencing.

**Cells, infections and transfections.** Mycoplasma-free BHK and CHO cells were plated at 1 x 10^4 or 2.5 x 10^5 cells per well in a 24 or 6-well dish, respectively, and infected the following day with recombinant Sindbis viruses at a multiplicity of 10 plaque forming units per cell. Cell viability was determined at approximately 30 h post infection by trypan blue exclusion which was previously shown to accurately reflect the apoptotic death induced by Sindbis virus (27,30).

Transfected CHO (10 ul Lipofectamine; GIBCO/BRL), BHK (10 ul Lipofectamine), 293 (10 ul GenePorter; Gene Therapy Systems), MCF-7 (10ul Lipofectamine), and Rat-1 (2.5 ul Lipofectamine) cells were fixed and stained with X-GAL 22-24 h later unless indicated otherwise. DNA concentrations were held constant within each experiment. Cell viability was determined by counting 200-600 live/non-apoptotic blue cells per sample and calculated as indicated in the legends. MCF7 Fas cells (provided by Vishva Dixit) were cotransfected with 0.5 µg GFP plasmid and 2µg of the plasmid of interest,
using 2 µl Lipofectin. Media containing 200 units/ml TNF was added 24 h after transfection and cell viability was determined 18 h later by counting GFP-positive cells showing apoptotic morphology relative to the total number of GFP-positive cells.

**Immunoblot analysis.** GST fused to amino acids 87-618 of c-IAP1 was purified from E. coli, cleaved from GST with thrombin and used to immunize rabbits (HRP, Inc.; Denver, PA) to generate anti-c-IAP1 antibody. Cell lysates were prepared with RIPA buffer and a cocktail of protease inhibitors at 8-24 h post infection with recombinant viruses or 16-24 h posttransfection and separated by SDS-PAGE. Immunoblot analysis was performed with anti-c-IAP1 (1:1000 dilution) or anti-HA antibody 12CA5 (1:1000 dilution, Boehringer Mannheim).

**In vitro cleavage assay.** [35S]Met-labeled c-IAP1 protein and derivatives were produced by vitro translation (TnT T7 Quick System, Promega). 1.5 ul of each translation mix was incubated with 10 ul 293 cell extract prepared as previously described (31) and 1mM ATP at 37°C for ~15 h in the absence or presence of 100 uM caspase inhibitor DEVD-CHO or zVAD-fmk. Cleavage with recombinant caspase-3 (Merck) was performed as previously described (32).

**Zinc binding assay.** GST fusion proteins were purified from E. coli using glutathione-bound resin and dialyzed extensively against phosphate buffered saline to remove unbound zinc. Protein samples of known concentration (determined by BCA protein assay) were subjected to atomic absorption spectroscopy and the absorbence at 214 nm (specific for incinerated zinc) was compared to a standard curve of known ZnSO₄ concentrations to determine the molar ratio of zinc to protein in each sample.
RESULTS

To compare the anti-apoptotic activities of IAP family proteins, human c-IAP1 and c-IAP2 were inserted into the Sindbis virus vector and tested for their ability to inhibit Sindbis virus-induced cell death. Sindbis virus triggers classic apoptotic death in many cell types providing a quantitative analysis of the function of a variety of cell death regulators (30). Mammalian XIAP/hILP and baculovirus Op-iap were previously reported to potently protect cells in this assay (33), and both the pro- and anti-apoptotic functions of several Bcl-2 family members have been assessed in this manner (27). BHK and N18 cells were infected with recombinant viruses encoding human c-IAP1, c-IAP2 or XIAP and cell viability was assessed by dye exclusion. While Bcl-xL and XIAP protected cells from virus-induced cell death, both c-IAP1 and c-IAP2 failed to protect cells compared to Sindbis virus vector controls encoding the same cDNAs in reverse orientation (Fig. 1A and B).

It is possible that Sindbis virus triggers a cell death pathway that is impervious to c-IAP1. In an alternate assay, c-IAP1 was tested for the ability to inhibit apoptosis induced by mBax or hBak in transiently transfected MCF-7 cells. While Bcl-xL protected cells from apoptotic death induced by co-transfected Bax or Bak, c-IAP1 failed to protect in contrast to earlier work in the same assays (Fig. 1C).

To determine if the RING finger could interfere with anti-apoptotic activity, stop codons were inserted into c-IAP1, c-IAP2 or XIAP coding sequences within the Sindbis virus vector to generate truncated proteins lacking the C-terminal RING finger or lacking
both the spacer region and RING (Fig. 2A). CHO cells were infected with recombinant viruses expressing wt or truncated IAPs and cell viability was determined by trypan blue exclusion. Removing the spacer-RING or RING domains of XIAP had no effect on anti-apoptotic function. This result with virus-induced apoptosis is consistent with the observation by others that neither the RING nor the spacer region of XIAP is required for inhibition of TNFα-induced cell death in MCF7-Fas cells (34), and that the BIR domain of XIAP is sufficient to inhibit caspases (10). However, the C-terminal truncations of c-IAP1 conferred a gain of anti-apoptotic activity indicating that the BIR domain was sufficient to inhibit cell death in this assay and that the anti-apoptotic function of the BIR domain was suppressed by the RING domain (Fig. 2B). Similar results were observed in BHK cells (data not shown). The RING deletion mutant was also expressed at slightly higher levels, perhaps contributing to its activity (Fig. 2C), but expression of the BIR domain alone was problematic and difficult to detect (see below). Thus, changes in protein expression levels may not fully account for the gain-of-function by C-terminal truncations. A time course experiment in CHO cells further demonstrated that deletion of the C-terminal RING finger of c-IAP1 restores the anti-apoptotic function of the BIR domain against Sindbis virus (Fig. 2D).

In contrast to c-IAP1, deletion of the C-terminal spacer-RING of c-IAP2 failed to confer anti-apoptotic activity on c-IAP2 in the Sindbis virus assay (Fig. 2B). Both full-length and C-terminal truncated c-IAP2 proteins were consistently difficult to detect by immunoblot analysis in Sindbis virus-infected cells and further analyses of c-IAP2 in this model were abandoned. However, c-IAP2 lacking the spacer-RING region was capable
of inhibiting apoptosis induced by TNF treatment of MCF-7 cells while full-length c-IAP2 was inactive in this assay (Fig. 2E). Deletion of the C-terminal spacer-RING domain of c-IAP2 significantly stabilized c-IAP2 in transfected 293 cells, though respectable levels of c-IAP1 still failed to protect (Fig. 2F). Taken together, the RING domains of c-IAP1 and c-IAP2 but not XIAP were capable of suppressing the anti-apoptotic functions of their BIR domains in the assays tested here, which may in part be due to protein stabilization.

To further investigate the fate of c-IAP1 protein during apoptosis, lysates prepared from 293 cells infected with recombinant viruses encoding wild type or mutant c-IAP1 were immunoblotted with a rabbit polyclonal antiserum generated against recombinant c-IAP1. The 68kD c-IAP1 expressed from recombinant Sindbis virus comigrated with endogenous c-IAP1 (compare c-IAP1 lanes with 68kD band in BIR and ΔCARD lanes in Fig. 3A). Overexpression increased c-IAP1 protein levels only 3 to 5-fold over endogenous levels. In addition to full-length protein, an immunoreactive polypeptide of 52kD and a less stable 35kD polypeptide (open circles in Fig. 3A) were detected following infection with virus encoding full-length c-IAP1, suggesting that c-IAP1 may be cleaved during apoptosis. Similar results were obtained in COS-1 cells (data not shown). The BIR domain alone (lanes 5-8) was only detected on longer exposures (not shown). Longer exposures also detected the 52kD fragment of endogenous c-IAP1 in control virus-infected cells but the less stable 35kD fragment was below detection limits (Fig. 3B, lanes 1 and 2).
Deletion mutants of c-IAP1 were analyzed to determine which portion of c-IAP1 is contained in these apparent cleavage fragments. Deletion of most of the CARD motif (amino acids 471-561) within the spacer region produced a ~10kD smaller c-IAP1 protein as expected (Δ in Fig. 3A). This deletion also shifted the 52kD fragment to 41kD (solid circle in Fig. 3A) and shifted the smaller 35kD fragment to 25kD (see inset). In general, the cleavage fragments increased in abundance with time after infection (Fig. 3A-C). Therefore, both fragments appear to be cleavage products of c-IAP1 and both contain the CARD motif. Both fragments also contain the C-terminal RING finger. Deletion of the 6 kD RING reduced the size of the 52kD cleavage fragment (Fig. 3B), while further deletion of BIR-1 had no effect. Deletion of the RING abolished formation of the 35kD fragment perhaps by destabilizing the polypeptide (Fig. 3C). This possibility is consistent with our difficulty in detecting the spacer region when expressed alone (see Fig. 6B below). Taken together, these data indicate that c-IAP1 is cleaved during apoptosis to yield 52 and 35kD fragments that are both derived from the C-terminus. The 35kD fragment is likely to consist of the spacer and RING domains as the predicted size of this region is 31.3 kD and expression of an engineered spacer-RING fragment migrates at ~35kD on SDS gels (see below). The 52kD fragment probably contains BIR-2 through the carboxyl end based on size estimations. The corresponding N-terminal fragments of c-IAP1 could not be identified among the cleavage fragments and may be degraded.

To determine if the 52kD and 35kD fragments of c-IAP1 are generated by caspase cleavage, in vitro translated 35S-labeled c-IAP1 was treated with 293 cell
extracts that contain activated caspases (31,35,36). Cleavage of c-IAP1 by the apoptotic cell extract produced a 35kD fragment approximately the same size as that observed in apoptotic cells. This cleavage was inhibited by a pan caspase inhibitor zVAD and a caspase-3 inhibitor DEVD (Fig. 4A) but was not inhibited by the caspase-1 inhibitor YVAD (data not shown). c-IAP1 was also cleaved to produce a 35kD fragment by purified recombinant caspase-3 that was inhibited by zVAD (Fig. 4A). The larger 52kD cleavage fragment observed in virus-infected cells was not detected in this in vitro assay unless the CARD domain was deleted, perhaps stabilizing the intermediate cleavage product. Consistent with our observations in apoptotic cells, 293 cell extracts cleaved the in vitro translated ΔCARD mutant to the expected 41kD and 25kD fragments (Fig. 4B and data not shown). This result confirms that the in vitro cleavage fragments also contain the C-terminal region of c-IAP1. Consistent with our results in apoptotic cells (Fig. 3A), the ΔCARD mutant of c-IAP1 was more stable in the in vitro translation mix compared to wt full-length c-IAP1 (Fig. 4B). Cleavage of the ΔCARD mutant of c-IAP1 was detectable as early as 1 hour after addition of caspase-3 and densitometry of the uncleaved protein indicated that 72% was cleaved by 4 hours (Fig. 4C).

To map the cleavage site responsible for producing the smaller 35/25kD fragment, several Asp residues near the beginning of the spacer region were mutated individually in the ΔCARD mutant of c-IAP1. Mutation of Asp372 to Ala abolished formation of the smaller cleavage fragment while mutation of Asp346, Asp364 and Asp387 had no effect on generation of this fragment (Fig. 4D and data not shown).
Surprisingly, mutation of Asp372 also impaired generation of the larger 41kD fragment. Mutation of Asp372 may impair cleavage at a second site, but we can not formally exclude the possibility that the larger fragment is a posttranslationally modified form of the smaller fragment.

Because the RING finger squelched the anti-apoptotic activity of full-length c-IAP1 and because a C-terminal fragment of c-IAP1 containing the RING finger is generated by caspases in vitro and in apoptotic cells, we tested the possibility that the C-terminus of c-IAP1 has pro-apoptotic function. Transfection of BHK cells with a construct expressing the spacer-RING region of c-IAP1 (amino acids 342-618) induced cell death in a dose-dependent manner (Fig. 5A). Similar results were obtained with a fragment containing amino acids 373-618 (data not shown).

To determine which portion of the spacer-RING construct was responsible for the induction of apoptosis, several mutants were tested in transfected BHK cells. Deletion of the CARD domain from the spacer-RING (SR-ΔCARD) enhanced pro-apoptotic activity and correlated with higher protein expression levels, again suggesting that the CARD domain contributes to the instability of the space-RING region (Fig. 5B). Similar results were obtained with HA-tagged constructs in CHO and 293 cells (Fig. 5D and F) and Rat-1 (not shown) except that the ΔCARD mutant of HA-spacer-RING was generally a more potent killer in these cell types, again correlating with increased protein expression compared to the CARD-containing construct (Fig. 5E). The spacer or RING regions alone lacked pro-death activity compared to control vector (Fig. 5B, D and F). The RING domain alone was not detected by the polyclonal anti-c-IAP1 antibody (Fig. 5B) but was
likely to be expressed efficiently based on results with an HA-tagged version (Fig. 5E). The spacer region alone was consistently present at lower levels even when detected via an HA tag (Fig. 5B and E). Thus, these experiments do not eliminate the possibility that the spacer region was sufficient for pro-apoptotic function. However, mutation of three conserved Cys/His residues in the RING (RING*) abolished pro-apoptotic activity of spacer-RING, verifying that the RING was required for pro-death activity (Fig. 5C). The pro-death activity of spacer-RING lacking the CARD domain in 293 cells was abolished by co-transfection with the caspase inhibitor P35, suggesting that caspases mediate apoptosis induced by the spacer-RING. Higher molecular weight species of the spacer, RING, and spacer-RING domains were frequently observed suggesting that these proteins are posttranslationally modified in cells (plus signs, Fig. 2F and 5A-C).

To confirm that the spacer-RING domain of c-IAP1 can interfere with the anti-apoptotic activity of the BIR domain, we asked if spacer-RING could impair protection by the BIR domain from Bax-induced cell death. Viability of cells co-transfected with Bax and the BIR domain confirmed that BIR suppressed Bax-induced cell death. Furthermore, co-transfection with spacer-RING abolished the protective activity of BIR in CHO and 293 cells (Fig. 6).

To formally demonstrate that the predicted RING domain of c-IAP1 indeed binds zinc, a GST-RING protein was purified from E. coli and tested for its ability to liberate zinc during combustion. Consistent with the fact that RING fingers of other proteins are known to bind two moles of zinc, GST-RING bound the same molar ratio of zinc as two BIR domains (GST-BIR2-3) that each coordinate one zinc atom (Fig. 7).
each contain a single zinc finger, confirmed in the recently determined structure of several BIR domains (13,15,37). This result also suggests that the lack of cell-killing activity by the RING finger alone (with or without an HA-tag) is probably not due to defects in protein folding.

**DISCUSSION**

In contrast to XIAP, full-length c-IAP1 and c-IAP2 failed to protect cells from apoptosis induced by one or more death stimuli including Bax overexpression, virus infection and TNFα treatment. However, deletion of the C-terminal spacer-RING domains of c-IAP1 and c-IAP2 restored the latent anti-apoptotic function of the N-terminal BIR domains (see Fig. 2). This finding suggests that the C-terminus of c-IAP1 and c-IAP2 can negatively regulate the anti-apoptotic activity of the BIR domains. Interestingly, deletion of the C-terminal spacer-RING of c-IAP2 occurs as the result of a chromosome translocation event commonly found in mucosa-associated lymphoid tissue (MALT) lymphomas (38). Perhaps this translocation produces a constitutively anti-apoptotic c-IAP2 protein. However, the MALT1 gene to which cIAP-2 is fused in the t(11;18) MALT lymphoma translocations was recently found to encode a paracaspase which is also likely to contribute to disease (39). The fusion effectively deletes the prodomain of the paracaspase in many of these translocation events (40), linking it to the BIR domain of c-IAP2.

Our results in mammalian cells are consistent with those obtained in a Drosophila eye model where deletion of the RING finger domain of D-IAP1 significantly
enhanced its anti-apoptotic function against Reaper overexpression and during Drosophila eye development (12). Similarly, deletion of the c-IAP1 RING was required to protect the third instar eye disc from Reaper-induced cell death. However, in some situations the RING finger may be required for protective function. Full-length c-IAP1 was reported to protect human MCF7 cells from Reaper-induced apoptosis (41), and deletion of the RING finger of D-IAP1, D-IAP2 and MIHA (murine XIAP) impaired their ability to inhibit caspase-1-induced apoptosis in mammalian cells (42). Furthermore, the RING of baculovirus Cp-IAP is required for anti-apoptotic activity and to inhibit caspase-9 (5). Taking all of these studies together, the RING finger of IAP proteins may positively or negatively modulate the function of IAP proteins and this modulatory function is presumably dependent in part on cell type-specific factors.

At least two factors appear to contribute to the lack of anti-apoptotic activity by full-length c-IAP1 during virus-induced apoptosis, protein instability and susceptibility to caspase cleavage. Deletion of the RING of c-IAP1 was reported to prevent autoubiquitination and degradation of c-IAP1 (20). Deletion of the RING appeared to stabilize c-IAP1 in virus-infected cells but the observed increase in protein levels may not fully explain the gain of anti-apoptotic function. c-IAP1 was also cleaved to 52kD and 35kD fragments by recombinant caspase-3, by apoptotic cell extracts and in cells undergoing apoptosis. Furthermore, the 35kD spacer-RING fragment was capable of inducing apoptosis and was capable of inhibiting the anti-apoptotic function of the BIR domain when expressed on separate plasmids. No function has been attached to the CARD of c-IAPs and it is not required for direct binding to caspases (7) nor for the pro-
apoptotic function of the spacer-RING fragment. To the contrary, the CARD of c-IAP1 appears to destabilize both the full-length and cleaved fragments of c-IAP1 though the mechanism is not known. Perhaps through dimerization or recruitment to a protein complex the CARD promotes degradation of c-IAP1, or maybe one or more of several Lys residues in the CARD serves as ubiquitination sites. However, both the spacer and RING domains of c-IAP1 appear to be posttranslationally modified to produce ~7-8kD larger immunoreactive bands (marked + in Figs. 5). This size shift is consistent with mono-ubiquitination although other possibilities remain.

Cleavage of c-IAP1 in apoptotic cells appears to be accomplished by caspases as the same size fragments were generated with caspase-3 in vitro, and a caspase inhibitor blocked cleavage of c-IAP1 by apoptotic cell extracts. Deletion analyses indicate that the 35kD caspase cleavage product of c-IAP1 contains the spacer-RING domain. This domain of c-IAP1 induces apoptosis in transfected cells, consistent with the finding that the analogous region of D-IAP1 induces cell death when overexpressed in the Drosophila eye (12). The generation of pro-apoptotic cleavage products of c-IAP1 during cell death is reminiscent of the finding that Bcl-2 and Bcl-xL are also converted from anti-apoptotic to pro-apoptotic factors by caspase cleavage (32,43). However, the in vivo function of the c-IAP1 spacer-RING fragment that is generated during apoptosis is not known. This fragment of c-IAP1 could potentially decrease cell survival via an independent function perhaps involving ubiquitination of cIAP-1, the BIR domain or possibly heterologous targets (presumably anti-apoptotic factors). The RING domain (without spacer) of cIAP-2 was found to be sufficient for in vitro E3 ligase activity that
selectively promoted ubiquitination of caspases 3 and 7 but not caspase 1 (21). This finding seems inconsistent with the pro-apoptotic function of spacer-RING in cells but may imply a role for the spacer region in directing the pro-apoptotic function of spacer-RING inside cells. Alternatively, spacer-RING could function as a dominant negative inhibitor of c-IAP1, perhaps preventing degradation of caspases. Consistent with this idea, deletion of the N-terminal BIR domain of survivin results in a C-terminal fragment that appears to interfere with endogenous survivin by competing for microtubule binding leading to increased caspase activity (44,45).

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Reference List


Legends to Figures

Fig. 1. c-IAP1 and c-IAP2 fail to inhibit apoptosis compared to XIAP and Bcl-xL. A, BHK and B N18 neuroblastoma cells were infected with recombinant Sindbis viruses encoding Bcl-xL, c-IAP1, c-IAP2, XIAP or their reverse orientations as controls and cell viability was determined ~30h post infection by trypan blue exclusion. Results shown are representative of three independent experiments. Induction of apoptosis was confirmed by cell morphology (not shown). C, MCF-7 cells transfected with a βGAL plasmid (0.8 ug) to mark transfected cells and with plasmids encoding murine Bax (0.8 ug), human Bak (0.8 ug), human Bcl-xL (2 ug) and human c-IAP1 (2 ug) separately or in combination were assessed for cell viability by counting viable/non-apoptotic blue cells. Total DNA concentrations were held constant with control vector DNA. Means +/- SEM are shown for at least three independent experiments.
Fig. 2. Deletion of the C-terminus of c-IAP1 and c-IAP2 confers anti-apoptotic function. 
A, Diagram of IAP family members showing relative positions of the BIR, spacer and 
RING motifs. Numbers indicate amino acid positions of the naturally occurring C-termini 
and of the inserted stop codons for the truncated proteins used in panels B and C. B, 
CHO cells were infected with recombinant Sindbis viruses expressing the indicated IAP 
proteins and their mutant derivatives. Cell viability was determined as described for Fig. 
1. C, Immunoblot analysis with anti-c-IAP1 antisera of BHK cell lysates following 
infection with Sindbis virus expressing c-IAP-1, the BIR domain only, or c-IAP lacking 
the RING finger. D, Cell viability was determined over time as described for panel B. E, 
MCF-7F cells transfected with the indicated plasmids and a GFP plasmid were treated 
with TNF (200 U/ml) or left untreated for 19 hours and percent viability was determined 
by counting cells with apoptotic morphology compared to the total number of transfected 
cells. c-IAP2 BIR contains amino acids 1-382. F, Immunoblot analysis with anti-HA 
antibody of transfected 293 cells expressing the indicated HA-tagged proteins.

Fig. 3. c-IAP1 is cleaved during Sindbis virus-induced apoptosis. A, 293 cells were 
infected with recombinant Sindbis viruses expressing wild type or mutant c-IAP1. At the 
indicated times after infection, cells were harvested, separated by SDS-PAGE and 
immunoblotted with anti-c-IAP1 antibody. Open circles mark the fragments derived from 
wt c-IAP1, solid circles mark the cleavage products derived from the CARD deletion 
mutants of c-IAP1 (ΔCARD), Δ marks the position of the ΔCARD mutant. B and C, 
Experiment similar to those shown in panel A was performed in 293 cells with wild type
c-IAP1 and mutants lacking the RING motif (ΔRING) or lacking both BIR-1 and RING (ΔBIR/ΔRING). The left two lanes of panel B showing endogenous c-IAP1 and are a longer exposure of the same gel as lanes 3-6. Symbols are as indicated for panel A.

Fig. 4. c-IAP1 is cleaved by apoptotic cell extracts and caspase-3. A, Radiolabeled, in vitro translated, wild type c-IAP1 was treated overnight with purified recombinant caspase-3 or apoptotic extract prepared from 293 cells (provided by Yuri Lazebnik) with or without the caspase inhibitors zVAD and DEVD-CHO (Enzyme System Products), and analyzed by SDS-PAGE/autoradiography. The activity of the extract was slightly enhanced by adding ATP. B, In vitro translated wild type c-IAP1 and the CARD deletion mutant of c-IAP1 (ΔCARD) were treated with apoptotic 293 cell extracts for 3-4 hours and analyzed by SDS-PAGE/autoradiography. C, 35S-labeled, in vitro translated ΔCARD c-IAP1 was incubated with recombinant caspase-3 with or without zVAD for the indicated times and analyzed as in panel A. A longer exposure of the 25kD fragment is shown below. D, 35S-labeled, in vitro translated mutants of ΔCARD c-IAP1 in which Asp364 or Asp372 was changed to Ala were incubated with or without recombinant caspase-3 for 3-4 hours and analyzed by SDS-PAGE/autoradiography for susceptibility to caspase cleavage.

Fig. 5. The C-terminal spacer-RING domain of c-IAP1 has pro-apoptotic activity. A, BHK cells were transiently transfected with increasing amounts of a plasmid encoding only the spacer (Sp)-Ring region of c-IAP1 (with compensating amounts of control vector).
Transfected cells were marked by co-transfecting 0.6 ug of a βGal plasmid and percent apoptosis was determined as the percentage of the total number of blue cells that were dead/apoptotic. A representative immunoblot of the corresponding transfections with anti-c-IAP1 antibody is shown below. + indicates possible posttranslationally modified form of cIAP-1 fragments. B, BHK cells were transfected with 2 ug of the indicated plasmid constructs and cell viability was determined as described for panel A. An immunoblot with anti-c-IAP1 antibody is shown below. Symbols are as that described for panel A. C, Viability of BHK cells transfected with HA-tagged spacer-RING or an analogous construct with three mutated Cys residues in the RING (SR-RING*) was determined as in panel A. A corresponding immunoblot of transfected cells is shown using anti-HA antibody. D, Viability of CHO cells transiently transfected with HA-tagged spacer-RING, its mutant derivatives or mBax (2 ug each). Because apoptotic CHO (and 293) cells detach from the dish, cell death was determined as the percent reduction in the number of viable blue cells relative to the total (live+dead) number of blue cells in the pSG5 vector control. E, Cell lysates from an experiment shown in panel D were immunoblotted with anti-HA antibody. F, Cell viability of 293 cells transiently transfected with the indicated plasmids (2 ug) was calculated as described for panel D. All viability data are presented as mean +/- SEM for three or more independent experiments.

Fig. 6 CHO or 293 cells were transfected with mBax (0.5 ug), BIR of c-IAP1 (1.0 ug) with or without spacer-RING lacking the CARD domain (SR-ΔCARD, 1.0 ug) with compensating amounts of vector DNA. Cells were harvested at 10-12 h (CHO) or 26 h
(293) posttransfection and viability was determined as mean +/- SEM for three independent experiments as described in Fig. 5A (CHO) or for duplicate samples in a single experiment as described for panel Fig. 5D (293).

Fig. 7. c-IAP1 BIR and RING motifs bind zinc. A, Diagram of the GST-c-IAP1 fusion proteins that were purified from E. coli. B, Coomassie blue stained gel of 1 ug of each of the purified GST fusion protein. C, The indicated purified GST fusion proteins were analyzed for zinc content by atomic absorption spectroscopy.
Fig. 1

A

B

C

Cell viability (%)

0 20 40 60 80 100

Mock Bcl-XL c-IAP1 c-IAP2 XIAP

Mock Bcl-XL c-IAP1 c-IAP2 XIAP

BHK N18

Cell viability (%)

0 20 40 60 80 100

c-IAP1 Bcl-xL Vector

c-IAP1 Bcl-xL Vector

MCF-7

Cell viability (%)

0 20 40 60 80 100

Vector mBax hBak

Vector mBax hBak

Vector mBax hBak

Sindbis virus vector

Sindbis virus vector

Sindbis virus vector
Fig. 3

A

B

C

ΔBIR-1/
ΔRING

ΔBIR-1/
ΔRING

ΔBIR-1/
ΔRING
A B C D

Fig. 4
Fig. 6
Fig. 7