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The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases

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The inhibitor of apoptosis (IAP) family of proteins are highly conserved through evolution. However, the mechanisms by which these proteins interfere with apoptotic cell death have been enigmatic. Recently, we showed that one of the human IAP family proteins, XIAP, can bind to and potently inhibit specific cell death proteases (caspases) that function in the distal portions of the proteolytic cascades involved in apoptosis. In this study, we investigated three of the other known members of the human IAP family, c-IAP-1, c-IAP-2 and NAIP. Similarly to XIAP, *in vitro* binding experiments indicated that c-IAP-1 and c-IAP-2 bound specifically to the terminal effector cell death proteases, caspases-3 and -7, but not to the proximal protease caspase-8, caspases-1 or -6. In contrast, NAIP failed to bind tightly to any of these proteases. Recombinant c-IAP-1 and c-IAP-2 also inhibited the activity of caspases-3 and -7 *in vitro*, with estimated K_i s of $\leq 0.1 \mu\text{M}$, whereas NAIP did not. The BIR domain-containing region of c-IAP-1 and c-IAP-2 was sufficient for inhibition of these caspases, though proteins that retained the RING domain were somewhat more potent. Utilizing a cell-free system in which caspases were activated in cytosolic extracts by addition of cytochrome *c*, c-IAP-1 and c-IAP-2 inhibited both the generation of caspase activities and proteolytic processing of pro-caspase-3. Similar results were obtained in intact cells when c-IAP-1 and c-IAP-2 were overexpressed by gene transfection, and apoptosis was induced by the anticancer drug, etoposide. Cleavage of c-IAP-1 or c-IAP-2 was not observed when interacting with the caspases, implying a different mechanism from the baculovirus p35 protein, the broad spectrum suicide inactivator of caspases. Taken together, these findings suggest that c-IAP-1 and c-IAP-2 function similarly to XIAP by inhibiting the distal cell death proteases, caspases-3 and -7, whereas NAIP presumably inhibits apoptosis via other targets. **Keywords:** apoptosis/caspase/cell death protease/inhibitor of apoptosis proteins

Introduction

It is now firmly established that cysteine proteases, termed caspases, related to the mammalian interleukin-1 β con-

verting enzyme (ICE or caspase-1) and to the nematode CED-3, play a central role as effectors of apoptosis (reviewed in Nicholson, 1996). Diverse stimuli which cause apoptosis result in activation of these cysteine proteases, implying that they play an essential role in the cell death pathway. The natural substrates of the caspases are key regulatory and structural proteins, including protein kinases and proteins involved in DNA repair and cytoskeleton integrity, thereby contributing to the demise of the cell (Casciola-Rosen *et al.*, 1994, 1996; Lazebnik *et al.*, 1994, 1995; Brancolini *et al.*, 1995; Emoto *et al.*, 1995; Martin *et al.*, 1995; Cardone *et al.*, 1997; Rudel and Bokoch, 1997). The caspases are synthesized as inactive precursors (zymogens) that are proteolytically processed to generate active subunits. Processing of the proenzymes and substrates usually occurs by cleavage at specific aspartic acid residues in the P₁ position. An enzymatic cascade is suggested by this observation and, indeed, several active caspases can process their zymogen forms or other members of the family, at least *in vitro* (Srinivasula *et al.*, 1996; Muzio *et al.*, 1997). Peptide inhibitors corresponding to the substrate P₄-P₁ residues of these cysteine proteases are potent inhibitors of apoptosis, substantiating the central role of caspases in mediating cell death (Enari *et al.*, 1995; Los *et al.*, 1995; Milligan *et al.*, 1995).

Ablation of caspase activity is attained by the viral proteins, p35 from baculovirus and CrmA from cowpox, which appear to be suicide inactivators, strongly inhibiting caspase enzymatic activity (Bump *et al.*, 1995; Xue and Horvitz, 1995; Bertin *et al.*, 1996; Zhou *et al.*, 1997). Overexpression of these viral caspase inhibitors in insect, nematode and mammalian cells results in resistance to apoptotic stimuli, confirming that components of the apoptotic pathway are highly conserved throughout evolution, and leading to the speculation that mammalian functional equivalents of these protease inhibitors may exist.

The inhibitor of apoptosis proteins (IAP) are a family of anti-apoptotic proteins that are conserved across several species. The baculovirus IAPs, Cp-IAP and Op-IAP, were the first members of this family to be identified based on their ability functionally to complement the cell death inhibitor, p35, in mutant viruses (Crook *et al.*, 1993; Clem and Miller, 1994). The first human IAP to be identified, the neuronal apoptosis inhibitory protein (NAIP), was isolated based on its contribution to the neurodegenerative disorder, spinal muscular atrophy (SMA) (Roy *et al.*, 1995). Deletions in *naip* are observed in many individuals with SMA, predominantly those with the most severe form of the disease, consistent with the hypothesis that the motor neuron depletion characteristic of this disorder occurs by the failure to inhibit apoptosis. Subsequently, four human (c-IAP-1/HIAP-2/hMIHB, c-IAP-2/HIAP-1/

hMIHC, X-IAP/hILP, survivin) and two *Drosophila* IAP (DIAP-1, DIAP-2/dILP) homologs have been isolated, which have all been demonstrated to counter cell death (Hay *et al.*, 1995; Rothe *et al.*, 1995; Duckett *et al.*, 1996; Liston *et al.*, 1996; Uren *et al.*, 1996; Ambrosini *et al.*, 1997).

The common structural feature of all IAP family members is a motif termed the baculovirus IAP repeat (BIR) that is present in either two or three copies. The baculoviral IAPs and one of the *Drosophila* IAPs contain two BIR domains, while three of the human family members and a second *Drosophila* IAP contain three such domains. Survivin, in contrast, contains only one BIR domain, implying that a single BIR can be sufficient for anti-cell death activity (Ambrosini *et al.*, 1997). With the exceptions of NAIP and survivin, all other known IAP family members also contain a RING domain at their carboxy-terminus whose exact function remains elusive (Saurin *et al.*, 1996). The fact that the BIR motif is shared by all members suggests a central role for this domain in mediating cellular protection; however, this has not been demonstrated conclusively. It has been suggested, at least in the case of the baculoviral IAPs, that the RING finger domain is also required for suppression of apoptosis in insect cells (Clem *et al.*, 1994). However, overexpression of the *Drosophila* IAP BIR motifs in the absence of the RING domain suppresses cell death induced by X-irradiation, *hid* overexpression or naturally occurring developmental cell death in the eye of transgenic flies (Hay *et al.*, 1995). Moreover, transgenic flies overexpressing the RING domain alone exhibited increased cell death in the eye, suggesting that the RING domain may act as a negative regulator of cell death suppression. In addition, the human IAP family proteins NAIP and survivin are capable of inhibiting apoptosis, although they lack a RING finger domain, further suggesting that the BIR domains may be sufficient.

The human c-IAP-1 and c-IAP-2 proteins were identified originally as proteins that are recruited to the cytosolic domain of the p80 tumor necrosis factor receptor II (TNF-RII) via their association with the TNF-associated factor (TRAF) proteins, TRAF-1 and TRAF-2 (Rothe *et al.*, 1995). The c-IAP-1 protein additionally has been shown to be a component of the p60 TNF-RI complex through its association with TRAF-2 (Shu *et al.*, 1996). Although associated with these receptor complexes, the precise function of c-IAP-1 and c-IAP-2 in TNFR family signal transduction cascades is at present unknown. Both c-IAP-1 and c-IAP-2 have been shown to inhibit apoptosis induced by several stimuli when overexpressed in mammalian cells, similar to other IAP family proteins. However, the mechanism by which these proteins suppress apoptosis has heretofore been enigmatic.

Mutant baculoviruses lacking the caspase inhibitor p35 can be genetically complemented by the IAPs, suggesting that the IAP family of proteins may function at the same level within the apoptotic cascade to suppress cell death. Consistent with this hypothesis, we recently have shown that the human family member XIAP can directly inhibit two members of the cell death protease family, caspases-3 and -7 (Deveraux *et al.*, 1997). These particular cell death proteases function in the distal portions of the cell death pathway, downstream of proximal caspases such as

caspase-8 (FLICE/MACH-1/Mch5) which bind to the TNF-RI and Fas/APO-1 receptor complexes (Boldin *et al.*, 1996; Muzio *et al.*, 1996, 1997; Medema *et al.*, 1997). The association of c-IAP-1 and c-IAP-2 with TRAF-1 and TRAF-2, however, suggests that these IAP family members may act at a different level of the apoptotic cascade and/or have alternative functions. Here, we report that, despite their ability to bind TRAF-1 and TRAF-2, the c-IAP-1 and c-IAP-2 proteins are direct inhibitors of the downstream proteases caspase-3 (CPP32/Yama) and caspase-7 (Mch-3/ICE-Lap3) but not the upstream protease caspase-8. Taken together, our data substantiate a model whereby the biochemical mechanism of suppression of apoptosis by these cellular IAP family proteins involves inhibition of caspases that operate in the distal portions of the cell death pathway.

Results

c-IAP-1 and c-IAP-2 but not XIAP and NAIP bind to TRAF family proteins

Previous studies have indicated that c-IAP-1 and c-IAP-2 can bind to the TRAF family proteins, TRAF-1 and TRAF-2 (Rothe *et al.*, 1995). However, it is unknown whether the ability to bind TRAF family proteins is a general characteristic of IAPs. To address this question, c-IAP-1, c-IAP-2, XIAP and the BIR domain-containing region of NAIP were expressed as GST fusion proteins in bacteria and tested for binding to *in vitro* translated [³⁵S]TRAF-1, TRAF-2, TRAF-3, TRAF-4, TRAF-5 and TRAF-6. As shown in Figure 1, TRAF-1 and TRAF-2 bound to GST-c-IAP-1 and GST-c-IAP-2, as anticipated. In contrast, TRAF-1 and TRAF-2 did not bind to GST-XIAP or GST-NAIP. Moreover, the TRAF-3, TRAF-4, TRAF-5 and TRAF-6 proteins failed to bind to all IAPs tested. These *in vitro* translated TRAF family proteins, however, did bind to GST fusion proteins containing the cytosolic domains of either CD40 or the lymphotoxin- β receptor (LT β R), as expected from prior reports (Figure 1 and data not shown). Thus, c-IAP-1 and c-IAP-2 are unique among these four members of the human IAP family in their ability to bind selected TRAF family proteins.

c-IAP-1 and c-IAP-2 inhibit specific caspases *in vitro*

Though we have reported recently that XIAP can bind to and inhibit certain caspases which participate in the distal portions of the proteolytic cascade involved in apoptosis (Deveraux *et al.*, 1997), it was unknown whether other IAP family proteins could also function as caspase inhibitors. Moreover, the finding that c-IAP-1 and c-IAP-2 uniquely interact with the TNF-R family-binding proteins TRAF-1 and TRAF-2 raised the possibility that these proteins, if they were caspase inhibitors, might target more proximal proteases such as caspase-8, which is known to associate with the TNF-RI and Fas/APO-1 receptor complexes (Boldin *et al.*, 1996; Muzio *et al.*, 1996; Medema *et al.*, 1997).

To address this issue, we tested the ability of recombinant IAPs to repress activity of five different purified active recombinant caspases. Some of these caspases are believed to function at proximal portions of cell death pathways

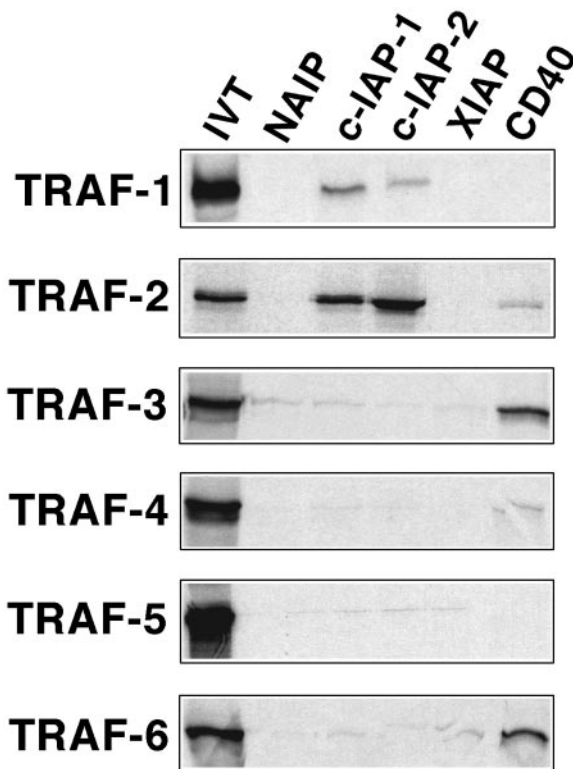


Fig. 1. c-IAP-1 and c-IAP-2 are unique in their ability to bind TRAF family proteins. GST-c-IAP-1, c-IAP-2, XIAP, NAIP (residues 1–368) and CD40 (cytosolic domain) fusion proteins (~3 μ M) were immobilized on glutathione–Sepharose and incubated with 15 μ l of reticulocyte lysates containing *in vitro* translated 35 S-labeled TRAF-1, TRAF-2, TRAF-3, TRAF-4, TRAF-5 or TRAF-6. After extensive washing, bound proteins were analyzed by SDS–PAGE/autoradiography. The first lane (left side) contains 3 μ l of the *in vitro* translation reaction, included as a positive control.

activated by TNF family cytokines (caspases-2 and -8), whereas others appear to act at distal points in cell death pathways (caspases-3, -6 and -7) (Berges *et al.*, 1993; Boldin *et al.*, 1996; Muzio *et al.*, 1996, 1997; Orth *et al.*, 1996; Medema *et al.*, 1997). Moreover, evidence has been presented suggesting that caspase-6 (Mch2) lies at an intermediate position within the apoptotic proteases cascade upstream of the terminal effector caspases-3 and -7 (Liu *et al.*, 1996a; Orth *et al.*, 1996).

For these experiments, c-IAP-1 and c-IAP-2 were expressed as GST fusion proteins in bacteria, affinity purified, and then incubated with active caspase-1, -3, -6, -7 or 8, in the presence of either the fluorogenic tetrapeptide substrate, Z-YVAD-AFC for caspase-1, or Z-DEVD-AFC for the other caspases. Residual enzyme activity was measured by spectrofluorimetry. As shown in Figure 2, c-IAP-1 and c-IAP-2 inhibited caspases-3 and -7 by ~65 and ~75%, respectively, when used at ~0.5 μ M (representing a 100- to 1000-fold molar excess of inhibitor relative to enzyme). In contrast, c-IAP-1 or c-IAP-2 did not alter the activity of caspases-1, -6 or -8, even at this molar excess. Several control proteins including GST, GST–Bcl-2, GST–Bax and GST–CD40 had no effect on the activities of these caspases (data not shown), thus confirming the specificity of the IAPs. Truncated forms of c-IAP-1 (amino acids 1–351) and c-IAP-2 (amino

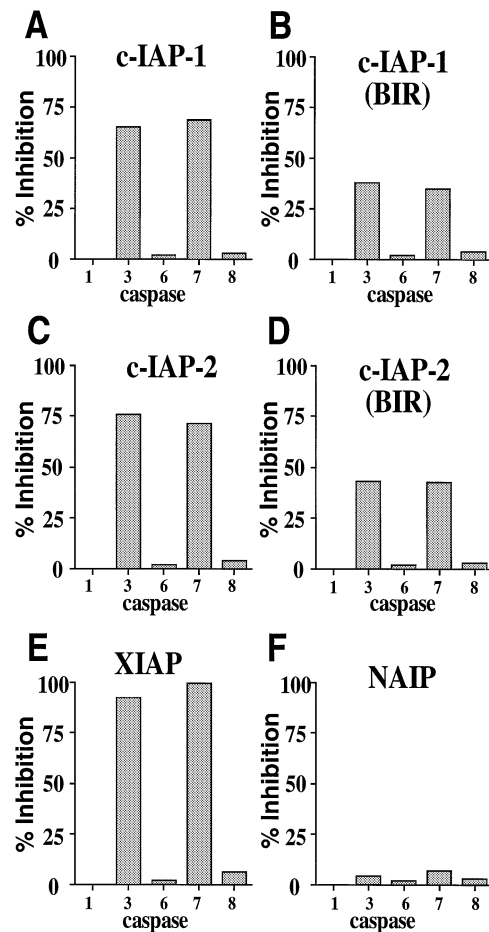


Fig. 2. c-IAP-1 and c-IAP-2 inhibit caspases-3 and -7 but not caspases-1, -6 and -8. Enzyme activity was measured by the release of the AFC fluorophore in the absence or presence of either (A) GST–c-IAP-1, (B) c-IAP-1 (BIR), (C) c-IAP-2, (D) c-IAP-2 (BIR) (E) XIAP or (F) NAIP. The rate of substrate hydrolysis in the presence of inhibitor (V_i) versus the rate in the absence of inhibitor (V_0) was calculated after allowing reactions to reach steady-state and is shown as percentage inhibition. Purified caspases (0.1–1 nM) incubated with a 100- to 5000-fold molar excess of GST–IAP proteins (0.1–0.5 μ M) in the presence of substrate (100 μ M).

acids 1–336) encoding the three BIR motifs but lacking the C-terminal RING domain were also assayed for their ability to inhibit active recombinant caspases *in vitro*. The c-IAP-1 (BIR) and c-IAP-2 (BIR) proteins both decreased caspase-3 and -7 activity by ~40%. Thus, the c-IAP-(BIR) and c-IAP-2-(BIR) proteins retained the ability to inhibit caspases, albeit with somewhat reduced potency compared with full-length c-IAP-1 and c-IAP-2. These data indicate that c-IAP-1 and c-IAP-2 are selective inhibitors of certain caspases, and demonstrate furthermore that their inhibitory function can be ascribed to or within their BIR motifs.

Similarly to c-IAP-1 and c-IAP-2, a GST fusion protein encoding the IAP family member XIAP (~0.1 μ M) potently inhibited caspases-3 and -7 but not caspases-1, -6 and -8, as expected from previous results (Figure 1E) (Deveraux *et al.*, 1997). In contrast, a GST–NAIP fusion protein corresponding to the three BIR motifs did not display any inhibitory effect on any of the caspases tested, including caspases-3 and -7 (Figure 1F). We cannot, however, rule out the possibility that this recombinant protein is inactive.

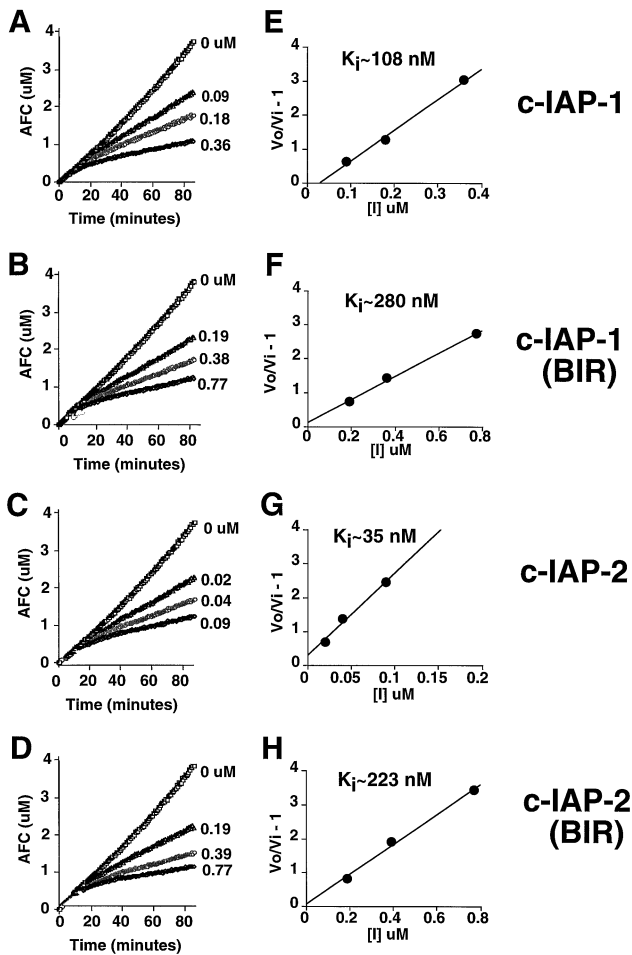


Fig. 3. Concentration-dependent inhibition of caspase-3 by c-IAP-1 and c-IAP-2. Representative progress curves are presented, demonstrating the inhibition of caspase-3 by (A) c-IAP-1, (B) c-IAP-1 (BIR), (C) c-IAP-2 and (D) c-IAP-2 (BIR). Caspase-3 was added to a final concentration of 7 pM with 100 μ M of DEVD-AFC and the indicated concentrations of active IAP protein (μ M). Enzyme activity was determined by the release of the AFC fluorophore (μ M) over time. The inhibition constants (K_i s), for caspase-3 by (E) c-IAP-1, (F) c-IAP-1 (BIR), (G) c-IAP-2 and (H) c-IAP-2 (BIR) were determined by plotting V_o/V_i-1 against inhibitor concentration (I).

Determination of inhibition constants (K_i s) for c-IAP-1, c-IAP-1 (BIR), c-IAP-2 and c-IAP-2 (BIR)

We next sought to determine the kinetics of inhibition of caspases-3 and -7 by c-IAP-1 and c-IAP-2. Representative progress curves for the inhibition of caspases-3 and -7 are shown in Figures 3A–D and 4A–D respectively, using a range of c-IAP-1, c-IAP-1 (BIR), c-IAP-2 and c-IAP-2 (BIR) concentrations. In all cases, the inhibition was concentration dependent. Steady-states of product formation in the absence or presence of inhibitor were attained and utilized to calculate the apparent inhibition constants (K_i) by plotting V_o/V_i-1 (Figures 3E–H and 4E–H), as described (Zhou *et al.*, 1997). The inhibition of caspases-3 and -7 by the IAPs appears to be reversible, given that final steady-states of substrate hydrolysis were attained in the presence of a large excess of the inhibitor. Prior to steady-state rates of substrate hydrolysis, variable relaxation times are observed, indicating binding of the inhibitor to the caspase. This association, however, appears to be quite slow. The c-IAP-1 and c-IAP-2 proteins inhibited

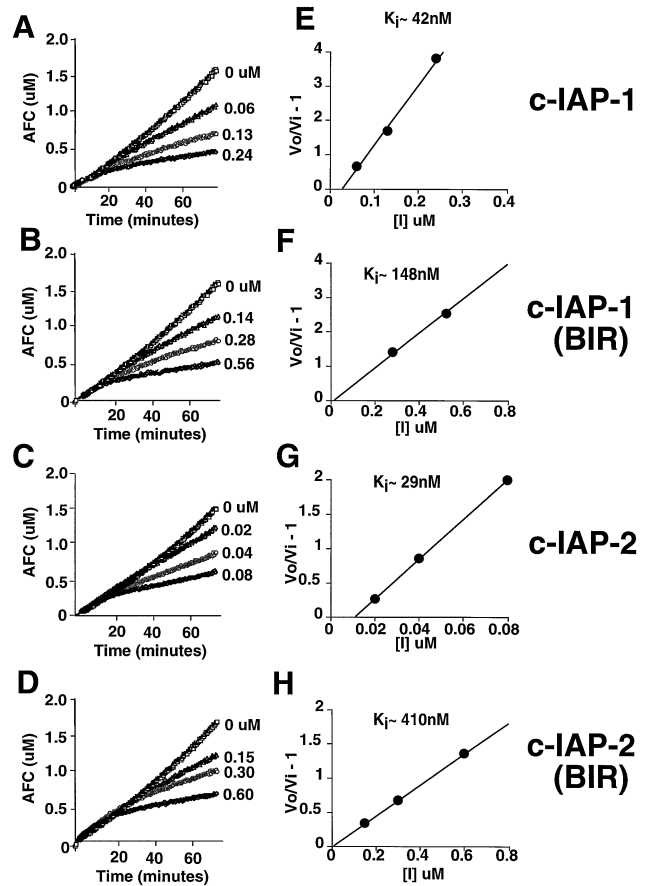


Fig. 4. Concentration-dependent inhibition of caspase-7 by c-IAP-1 and c-IAP-2. Representative progress curves are presented, demonstrating the inhibition of caspase-7 by (A) c-IAP-1, (B) c-IAP-1 (BIR), (C) c-IAP-2 and (D) c-IAP-2 (BIR). Caspase-7 was added to a final concentration of 150 pM with 100 μ M of DEVD-AFC and the indicated concentrations of active IAP protein (μ M). Enzyme activity and inhibition constants (K_i s) were measured as described for Figure 3.

caspase-3 with estimated K_i s of 108 and 35 nM, respectively. The K_i s for caspase-7 were estimated at 42 and 29 nM, respectively. The c-IAP-1 (BIR) and c-IAP-2 (BIR) proteins inhibited caspases-3 and -7, although less potently, with apparent K_i s of 280 and 223 nM, respectively, for caspase-3 and of 148 and 410 nM, respectively, for caspase-7.

c-IAP-1 and c-IAP-2 inhibit caspase-like protease activity and proteolytic processing of caspase-3 in cytochrome c-treated cytosols

Cytochrome *c* recently has been identified as a factor which is released from mitochondria into the cytosol of cells undergoing apoptosis, resulting in the activation of caspase-3 and probably other DEVD-specific caspases by an unknown mechanism (Liu *et al.*, 1996b; Kluck *et al.*, 1997; Yang *et al.*, 1997). We used a cell-free system based on the ability of exogenously added cytochrome *c* to induce activation of the caspases in cytosolic extracts to ask whether recombinant c-IAP-1 and c-IAP-2 could inhibit cellular caspases, in the context of a more physiologic milieu. Cytosols from 293 cells were incubated with 10 μ M of cytochrome *c* and 1 mM dATP for 30 min, and DEVD-AFC hydrolysis was measured. Striking increases

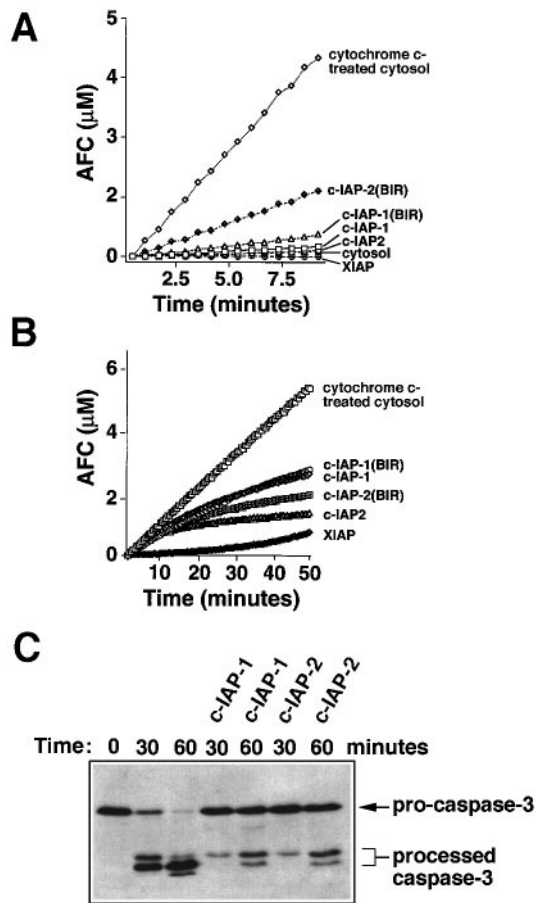


Fig. 5. Inhibition of cytochrome *c*-induced caspase-3 processing and DEVD activity by c-IAP-1 and c-IAP-2. (A) Z-DEVD-AFC hydrolysis was measured in cytosolic extracts from 293 cells into which $\sim 0.3 \mu\text{M}$ GST-c-IAP-1, c-IAP-1 (BIR), c-IAP-2 or c-IAP-2 (BIR) was added prior to activation for 30 min at 37°C by the addition of cytochrome *c* ($10 \mu\text{M}$) and dATP (1 mM). Untreated cytosolic extracts (used as a control) were similarly incubated but without cytochrome *c* and dATP. (B) Cytosolic extracts from 293 cells were pre-activated for 30 min with cytochrome *c* and dATP, and Z-DEVD-AFC hydrolysis was then measured in the presence of the IAPs as indicated. (C) Immunoblot analysis of caspase-3 in cytosolic extracts activated for the indicated times with cytochrome *c* and dATP, either in the presence or absence of c-IAP-1 or c-IAP-2 ($0.3 \mu\text{M}$). The positions of the unprocessed (pro) form and processed (activated) forms of caspase-3 are shown.

in DEVD-specific protease activity were observed in cytochrome *c*-treated extracts, in contrast to control untreated extracts which exhibited negligible amounts of caspase-like activity (Figure 5A). The addition of $0.3 \mu\text{M}$ purified recombinant c-IAP-1 or c-IAP-2 prior to cytochrome *c* resulted in significant suppression of the DEVD-cleaving activity in these extracts (Figure 5A). Similarly, c-IAP-1 (BIR) or c-IAP-2 (BIR) also inhibited cytochrome *c*-induced DEVD-cleaving activity. In contrast, several control proteins, including GST, GST-Bcl-2, GST-Bax and GST-CD40, had no effect. We conclude, therefore, that both c-IAP-1 and c-IAP-2, as well as truncation mutants of these proteins lacking the RING domains, can interfere specifically with the cytochrome *c*-induced generation of caspase-like protease activities in cytosolic extracts.

In addition to experiments where c-IAP-1 and c-IAP-2 were added to cytosolic extracts prior to cytochrome *c*,

we also examined whether the DEVD-cleaving activity in these extracts could be suppressed if the extract was treated with cytochrome *c* for 30 min prior to the addition of c-IAP-1 and -2. As shown in Figure 5B, c-IAP-1, c-IAP-1 (BIR), c-IAP-2 and c-IAP-2 (BIR) all caused reductions in DEVD-cleaving activity in extracts which had been pre-treated with cytochrome *c*.

The ability of c-IAP-1 and c-IAP-2 to inhibit the activities of DEVD-cleaving caspases could be due either to suppression of the active proteins, interference with the processing events that activate their zymogens, or both. To explore this possibility, we performed immunoblot analysis of cytosolic extracts treated with cytochrome *c* and dATP. The zymogen of caspase-3 (32 kDa) is processed initially by cleavage at aspartic acid residue 180 (D175) located between its large and small subunits, followed by autocatalytic removal of the N-terminal prodomain (Nicholson *et al.*, 1995; Han *et al.*, 1997). Whereas the smaller subunit is not recognized by our antibody (Krajewska *et al.*, 1997; Krajewski *et al.*, 1997), the larger subunit is and can exist either as a form that includes the N-terminal prodomain, or as smaller forms produced by removal of part or all of the prodomain (Nicholson *et al.*, 1995; Han *et al.*, 1997). Figure 5C demonstrates that treatment of extracts with cytochrome *c* results in loss of the 32 kDa precursor form of caspase-3 and the appearance of two versions of the large subunit. In contrast, caspases-1 and -2 remained as unprocessed zymogens (data not shown), suggesting that they act proximal to cytochrome *c*-initiated events. This observation also provides evidence that non-specific catalysis of all caspases had not occurred upon addition of cytochrome *c*, implying processing solely of downstream caspases.

Addition of c-IAP-1 or c-IAP-2 to extracts prior to cytochrome *c* and dATP resulted in nearly complete inhibition of pro-caspase-3 processing, with retention of the 32 kDa zymogen at levels comparable with control cytosols (Figure 5C). Thus c-IAP-1 and c-IAP-2 appear to prevent the initial cleavage of pro-caspase-3 at D180. Control GST fusion proteins added at similar concentrations to the extracts did not inhibit processing of pro-caspase-3. GST-NAIP also failed to inhibit cytochrome *c*-induced processing of pro-caspase-3 (not shown). Taken together, these data indicate that c-IAP-1 and c-IAP-2 can inhibit the cytochrome *c*-induced proteolytic processing events which lead to caspase-3 activation and can also inhibit the active caspase-3 enzyme after processing. The inhibition of pro-caspase-3 processing presumably occurs by either inhibiting the actions of an upstream protease that cleaves pro-caspase-3 at the IETDS site (amino acids 172–176) or by interfering with caspase-3 mediated ‘trans-processing’ of pro-caspase-3.

c-IAP-1 and c-IAP-2 inhibit apoptosis and caspase-like DEVD-cleaving activity in etoposide-treated intact cells

The inhibitory effect of c-IAP-1 and c-IAP-2 on cytochrome *c*-induced caspase activity in a cell-free system suggested that these inhibitors could also protect intact cells from apoptosis-inducing stimuli that cause the release of cytochrome *c* from mitochondria. The anticancer drug etoposide inhibits topoisomerase II, resulting in DNA damage and apoptosis (Liu, 1989; Dubrez *et al.*, 1995).

This cell death is accompanied by caspase activation which has DEVD-specific activity (Dubrez *et al.*, 1996; Martins *et al.*, 1997). Additionally, etoposide treatment of cells has been shown to induce release of cytochrome *c* into the cytosol, followed by caspase activation and apoptosis (Yang *et al.*, 1997).

Human kidney epithelial 293T cells were treated with etoposide, and the effect of overexpression of c-IAP-1 and c-IAP-2 on cell survival and caspase activity was examined. Cells were co-transfected with pEGFP as a transfection marker and either pcDNA3-myc-c-IAP-1, pcDNA3-myc-c-IAP-2 or, as a control, pcDNA3-myc. Treatment of 293T cells with 30 μ M etoposide for 2 days resulted in substantial DEVD-specific activity, compared with untreated cells (Figure 6A). Similarly, 293T cells that had been transfected with the control plasmid, pcDNA3-myc, accumulated DEVD-cleaving activity to approximately the same extent as untransfected 293T cells. In contrast, 293T cells transfected with plasmids encoding myc-tagged c-IAP-1 or c-IAP-2 accumulated much less DEVD-cleaving activity after treatment with etoposide. The proteolytic processing of caspase-3 was also inhibited in cells expressing c-IAP-1 or c-IAP-2, as determined by immunoblot analysis of the same cell lysates used for the enzyme assays (Figure 6B). As shown, etoposide induced consumption of the 32 kDa pro-caspase-3 protein, presumably as a result of processing to active protease, in control but not c-IAP-1- or c-IAP-2-transfected cells. Unlike the situation where cytochrome *c* was added to cytosolic extracts, it was difficult to detect the p17 subunit of processed caspase-3 under the conditions of these assays, probably due to the short half-life of the active protease in many types of cells. Finally, the effects of c-IAP-1 and c-IAP-2 overexpression on etoposide-induced apoptosis were determined by examining the morphology of 293T nuclei using the DNA-binding fluorochrome 4',6'-diamidino-2-phenylindole (DAPI). As shown in Figure 6C, etoposide induced apoptosis of ~65% of the control transfected 293T cells under the conditions of these experiments. In contrast, the percentage of cells undergoing apoptosis was reduced substantially in cultures of c-IAP-1- and c-IAP-2-transfected cells following exposure to etoposide. Similar effects of c-IAP-1 and c-IAP-2 on caspase-3 processing and apoptosis were observed in 293 cells when overexpression of Fas (CD 95) was employed as the apoptotic stimulus (data not shown).

c-IAP-1 and c-IAP-2 bind directly to activated caspases-3 and -7

A characteristic property of proteinase inhibitors is their ability to form tight complexes with their target enzymes. We therefore examined the ability of c-IAP-1 and c-IAP-2 to bind caspase-3 and caspase-7, *in vitro*. GST fusion proteins encoding c-IAP-1, c-IAP-1 (BIR), c-IAP-2, c-IAP-2 (BIR), NAIP, XIAP or the cytosolic domain of CD-40 (used as a control) were immobilized on glutathione-Sepharose and incubated with purified recombinant active caspases-3, -6 or -7. The c-IAP-1 and c-IAP-2 fusion proteins, as well as their truncated forms encoding only the three BIR motifs, exhibited specific binding to caspase-3 and caspase-7 but not to caspase-6 (Figure 7A), consistent with our enzyme inhibition data. Though GST-c-IAP-2 (BIR) appeared to bind somewhat less efficiently

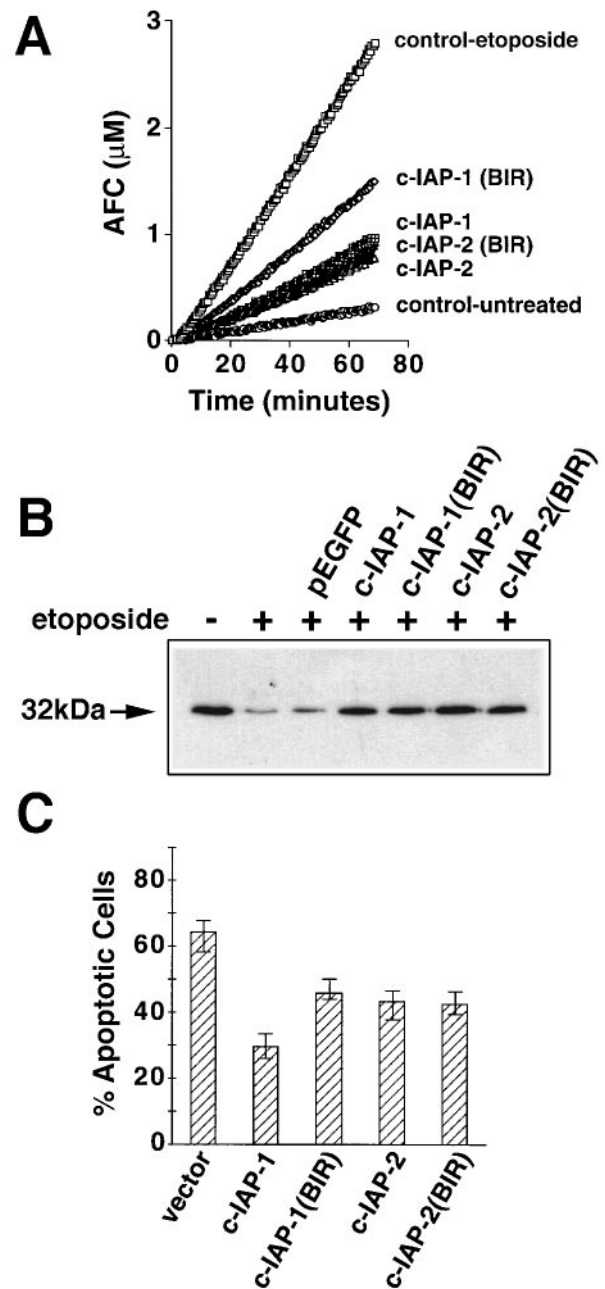


Fig. 6. c-IAP-1 and c-IAP-2 inhibit etoposide-induced apoptosis, processing of caspase-3 and generation of caspase-like protease activity in cells. 293T cells were either used directly or transiently co-transfected with a green fluorescent protein (GFP) marker plasmid pEGFP and either a control plasmid pcDNA3-myc or expression plasmids encoding myc-tagged c-IAP-1, c-IAP-2, c-IAP-1 (BIR) or c-IAP-2 (BIR). After 6 h, etoposide (30 μ M) was added to cultures as indicated (+). Lysates were prepared and apoptosis was assessed at 48 h post-transfection. Immunoblot analysis confirmed production of comparable amounts of the myc-tagged c-IAP-1, c-IAP-2, c-IAP-1 (BIR) and c-IAP-2 (BIR) proteins (not shown). (A) Z-DEVD-AFC hydrolysis was measured using lysates from the transfected 293T cells. Representative progress curves are shown. (B) Immunoblot analysis of pro-caspase-3 levels was performed using lysates from the transfected cells cultured with (+) or without (-) etoposide. (C) The percentage of apoptotic cells was determined by microscopic evaluation of DAPI-stained nuclei in GFP-positive cells. Data represent mean \pm SE ($n = 3$).

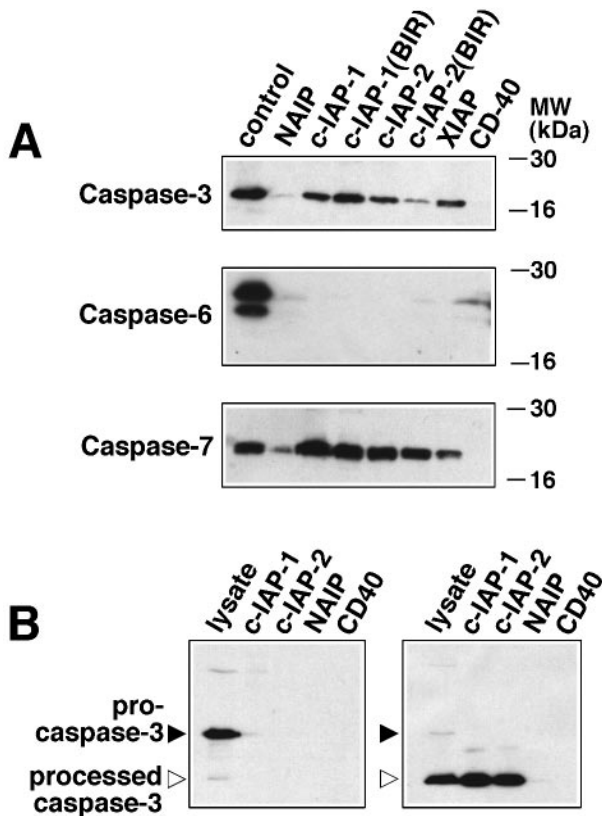


Fig. 7. c-IAP-1 and c-IAP-2 bind caspase-3 and caspase-7 but not caspase-6. (A) GST-c-IAP-1, c-IAP-1 (BIR), c-IAP-2, c-IAP-2 (BIR), NAIP, XIAP or CD40 fusion proteins ($\sim 3 \mu\text{M}$) immobilized on glutathione-Sephadex were assayed for binding *in vitro* to active recombinant caspase-3, caspase-6 and caspase-7 (0.5 μg) in 400 μl of caspase buffer. Caspases were detected by immunoblotting. (B) GST-c-IAP-1, c-IAP-2, NAIP or CD40 immobilized on glutathione-Sephadex were incubated with 293 cytosolic extracts (45 μl) that had been treated with (right panel) or without (left panel) cytochrome *c*/dATP for 30 min. Bound caspase-3 was detected by immunoblotting. An equivalent volume of cell lysate was run directly (first lane), showing the pro-caspase-3 zymogen (dark arrow) and processed large subunit (open arrow).

than full-length c-IAP-2 in the experiment presented in Figure 7A, this was due to loading of less intact GST fusion protein on the glutathione-Sephadex beads. GST-XIAP also bound to caspase-3 and caspase-7, as expected from our previous studies of this member of the IAP family (Deveraux *et al.*, 1997). In contrast to c-IAP-1, c-IAP-2 and XIAP, however, far less binding of caspase-3 and caspase-7 to GST-NAIP was detected (Figure 7A). Although based on a semi-quantitative assay, the less efficient interaction of NAIP with these caspases is in accord with our enzyme inhibition data and suggests that NAIP either has specificity for other caspases not tested here, or that it operates through a different mechanism to inhibit cell death.

We next determined whether c-IAP-1 and c-IAP-2 could bind the inactive zymogens of caspase-3, since we had observed inhibition of their processing. Untreated 293 cytosolic extracts containing unprocessed pro-caspase-3 and cytochrome *c*-treated extracts containing processed caspase-3 were incubated with GST-c-IAP-1 or GST-c-IAP-2 fusion proteins and the bound proteins were analyzed by immunoblotting. As shown in Figure 7B, no

binding of the 32 kDa unprocessed form of caspase-3 to c-IAP-1 or c-IAP-2 was detected. However, the processed large subunits of active caspase-3, produced by the addition of cytochrome *c*/dATP, bound to both c-IAP-1 and c-IAP-2 but not to the control protein, CD40. As before, little or no binding of processed caspase-3 to GST-NAIP was observed (Figure 7B), further confirming the difference in the specificity of this member of the IAP family.

c-IAP-1 and c-IAP-2 act as inhibitors but not as substrates of the caspases

The viral proteins p35 and CrmA are potent inhibitors of several caspases, and appear to act by a suicide mechanism that includes peptide bond hydrolysis at the inhibitory site. We examined if the inhibition by c-IAP-1 and c-IAP-2 occurs by a similar mechanism. c-IAP-1, c-IAP-2 and p35 were *in vitro* translated and transcribed in the presence of [^{35}S]L-methionine and then incubated with either caspase-3, -6, -7 or -8 for 1 h at 37°C. The resulting products were resolved by SDS-PAGE and detected by fluorography. As shown in Figure 8, p35 was cleaved efficiently by all the caspases, with nearly complete loss of the full-length p35 protein and the appearance of a smaller 25 kDa fragment. In contrast, neither c-IAP-1 nor c-IAP-2 underwent any significant cleavage in the presence of caspase-3 or -7, consistent with our kinetic data which demonstrate that these proteins exhibit reversible inhibition. Our data therefore imply that the mechanism of inhibition of caspases by c-IAP-1 and c-IAP-2 differs from that observed for p35. Thus, in contrast to p35 and CrmA, which are apparently suicide inactivators, the IAPs do not seem to require peptide bond hydrolysis as part of their inhibitory mechanism.

Discussion

The IAPs are an evolutionary conserved family of proteins which prevent cell death across species, implying that they act at a central, highly conserved point in the cell death cascade. We report that c-IAP-1 and c-IAP-2 directly interact with and inhibit two members of the caspase protease family, caspases-3 and -7. c-IAP-1 and c-IAP-2, however, did not bind to or inhibit caspases-1, -6 or -8. Thus, c-IAP-1 and c-IAP-2 displayed similar specificities to XIAP for the caspases (Deveraux *et al.*, 1997).

The c-IAP-1 and c-IAP-2 proteins inhibited caspases-3 and -7 with K_i s in the low nanomolar range (~ 30 – 120 nM), significantly lower than CrmA which has been reported to inhibit caspase-3 with a K_i of ~ 500 nM (Zhou *et al.*, 1997). Despite their potent inhibition of caspases-3 and -7, however, XIAP inhibited these same caspases with K_i s of 0.2–0.7 nM, representing 2–3 logs greater potency. This observation suggests that notwithstanding their overall homology, structural differences do exist between XIAP and the c-IAP-1 and c-IAP-2 proteins that affect how well they bind to and inhibit specific caspases. Thus while the K_i s obtained for c-IAP-1 and c-IAP-2 suggest that they are physiologically relevant inhibitors of caspases-3 and -7, presumably it would be necessary for these proteins to be present at higher concentrations than XIAP to achieve the same level of protection against caspases-3 and -7. Moreover, the measured differences in the K_i s for XIAP compared with c-IAP-1 and c-IAP-2 raise the

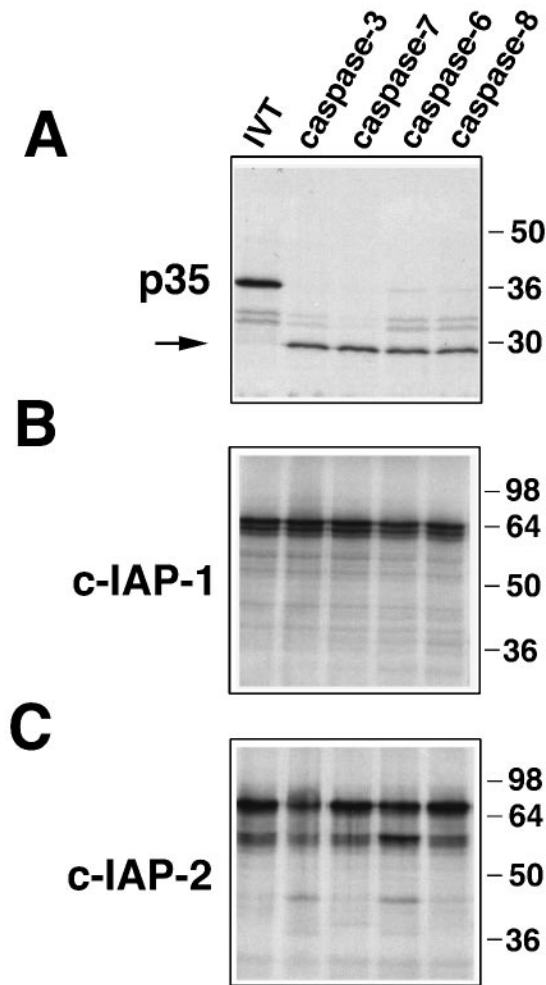


Fig. 8. c-IAP-1 and c-IAP-2 are not cleaved by caspases. *In vitro* translated ^{35}S -labeled p35 (A), c-IAP-1 (B) or c-IAP-2 (C) were incubated in the presence or absence of purified recombinant caspase-3, -6, -7 or -8 for 1 h, resolved by SDS-PAGE and analyzed by autoradiography. The 25 kDa cleavage product of p35 is indicated. The first lane (left side) represents untreated [^{35}S]p35, c-IAP-1 and c-IAP-2. The faint band at ~45 kDa in some c-IAP-2 lanes (C) represents a non-specific cleavage product that was variably present regardless of which caspases were added.

possibility that c-IAP-1 and c-IAP-2 may differ from XIAP in their ability to inhibit other caspases not studied here. Hence, whereas XIAP is a superior inhibitor of caspases-3 and -7, it is conceivable that c-IAP-1 and c-IAP-2 are better inhibitors of other as yet untested caspases. By analogy, NAIP theoretically could be a potent inhibitor of certain caspases, though having little apparent affinity for caspases-3 and -7. Nevertheless, the data reported here confirm that at least three of the currently known human members of the IAP family are direct inhibitors of select caspases and, therefore, establish inhibition of active cell death proteases as at least one mechanism by which this family of evolutionary conserved proteins suppresses apoptosis.

In contrast to the viral protein, p35, which functions as a broad specificity inhibitor of phylogenetically diverse caspases, the c-IAP-1, c-IAP-2 and XIAP proteins are selective inhibitors. The specificity presumably lies in differences in the substrate contact region of the caspases, which is seen when the three-dimensional structures of

caspases-1 and -3 are compared (Wilson *et al.*, 1994; Rotonda *et al.*, 1996). Though of overall similar folds, an additional 10 residues (248–257) are present in caspase-3 that form an extra loop which guards the entrance to the active site of this protease. This loop contributes residues that form part of the S_4 pocket. The size and shape of the S_4 subsite consequently are determined at least in part by these residues. The extra loop region, although conserved in the caspase-3 subfamily, is most similar between caspases-3 and -7. One could speculate, therefore, that this loop is involved in IAP binding. Furthermore, caspases-3 and -7 have the highest overall identity (53%) among the 10 known members of the caspase family, suggesting that additional residues outside the loop may also confer specificity, leading to selective inhibition by c-IAP-1, c-IAP-2 and XIAP.

Suppression of apoptosis induced by gene transfer-mediated overexpression of caspases has been documented for several of the IAPs. The human XIAP and baculovirus Op-IAP, for example, were both shown to inhibit apoptosis induced by overexpression of caspase-1 in mammalian cells (Hawkins *et al.*, 1996; Uren *et al.*, 1996). Op-IAP, however, was unable to protect against caspase-2- or caspase-7-induced apoptosis. Op-IAP may thus inhibit caspases more proximal to caspase-7 but downstream of those, such as caspase-2, which are thought to function at upstream points in protease cascades due to their association with components of plasma membrane receptor complexes (Ahmad *et al.*, 1997; Duan and Dixit, 1997). Moreover, recent evidence suggests that Op-IAP may function upstream of p35 to prevent apoptosis, possibly inhibiting a protease (Manji *et al.*, 1997). This would support a model where the IAPs have specificity for different caspases, a phenomenon that may have evolved over time. If true, it also implies that one of the human homologs may target more upstream caspases. However, we cannot exclude the possibility that Op-IAP does inhibit caspase-7, like c-IAP-1, c-IAP-2 and XIAP, but that within the context of experiments where caspase overexpression is used to induce apoptosis, the amount of caspase-7 produced overwhelmed Op-IAP-mediated protection.

Our data demonstrating that c-IAP-1 and c-IAP-2 target the downstream caspases-3 and -7 are in accordance with their reported ability to inhibit apoptosis induced by the pro-apoptotic Bcl-2 family proteins, Bik and Bak (Orth and Dixit, 1997). Bcl-2 family proteins have been shown to regulate apoptosis at a mitochondria-dependent step, with the anti-apoptotic members such as Bcl-2 preventing release of cytochrome *c* and activation of caspases, and the pro-apoptotic members such as Bax inducing mitochondrial permeability transition and processing of distal caspases including caspases-3, -6 and -7 (Kroemer *et al.*, 1996; Susin *et al.*, 1996; Kluck *et al.*, 1997; Yang *et al.*, 1997). Bcl-2 family proteins, in contrast, do not appear to modulate the processing of upstream proteases such as caspase-8 (Boise and Thompson, 1997). Reports that c-IAP-1 and c-IAP-2 inhibit apoptosis induced by Bik and Bak, and that XIAP inhibits apoptosis triggered by Bax, therefore support the hypothesis that the IAP family proteins function at the level of distal caspases (Deveraux *et al.*, 1997; Orth *et al.*, 1997). These experiments, however, do not exclude the possibility of effects of these IAP family proteins on other caspases not tested here

which operate more proximally in the proteolytic cascades. Moreover, it should be noted that while IAP-mediated inhibition of caspases can prevent apoptosis, this may not necessarily stop cell death from occurring by other caspase-independent pathways.

Cytochrome *c* release from mitochondria into the cytosol represents a recently recognized event associated with caspase activation and apoptosis. Addition of cytochrome *c* to cytosolic extracts, for example, results in the processing of specific caspases, such as caspase-3 (Liu *et al.*, 1996b; Kluck *et al.*, 1997; Yang *et al.*, 1997). In intact cells, the release of cytochrome *c* from mitochondria upon treatment with various apoptotic stimuli, including chemotherapeutic drugs such as etoposide, has been observed and precedes the processing and activation of caspases (Yang *et al.*, 1997). Cytochrome *c* has been reported to induce the generation of caspase-3-like DEVD-specific cleaving activity but not caspase-1-like YVAD-cleaving proteases (Dubrez *et al.*, 1996). Moreover, apoptotic-like destruction of nuclei added to cytosolic extracts derived from etoposide-treated cells can be inhibited by the peptidyl inhibitors containing the sequence DEVD but not YVAD, consistent with the observation that DEVD-specific caspases are activated by cytochrome *c* (Dubrez *et al.*, 1996). Using cytochrome *c*-treated cytosolic extracts, we observed that c-IAP-1 and c-IAP-2 can inhibit DEVD-specific caspases. Furthermore, etoposide-induced DEVD-cleaving activity was also inhibited in intact cells by c-IAP-1 and c-IAP-2. These observations are consistent with a model where c-IAP-1 and -2 can inhibit caspases in more distal portions of the cell death pathway, downstream of cytochrome *c*.

We found that c-IAP-1 and c-IAP-2 proteins can bind to the enzymatically active forms of caspases-3 and -7 but not to their inactive zymogens. Nevertheless, the IAPs can block processing of pro-caspase-3, suggesting that they may inhibit a proximal caspase which is responsible for activating caspase-3, but which lies downstream of cytochrome *c*. Liu *et al.* (1996b) indicated that other cytosolic factors are required for caspase-3 activation by cytochrome *c*. More recently, it has been reported that two enzymatic activities are required for caspase-3 processing (Han *et al.*, 1997). The protease mediating the cleavage between the small and the large subunits is sensitive to a tetrapeptide inhibitor containing the sequence IETD, but not DEVD, whereas the protease that mediates cleavage events removing the prodomain is both ESMD and DEVD sensitive (Han *et al.*, 1997). Maintenance of caspase-3 in its 32 kDa unprocessed form in the presence of c-IAP-1 or c-IAP-2, despite cytochrome *c* treatment, suggests that these proteins may inhibit an IETD-sensitive protease or a protease upstream of it. We cannot exclude the possibility, however, that failure to observe processing of pro-caspase-3 results from a need for a small initial amount of caspase-3 activation through a positive feedback mechanism in which active caspase-3 cleaves pro-caspase-3, not unlike what occurs when pro-caspase-3 is expressed in bacteria where it typically is recovered as fully processed active protease.

At present, the role of c-IAP-1 and -2 recruitment to TNF-RI or TNF-RII upon ligand stimulation is unknown. One possibility is that c-IAP-1 and c-IAP-2 may be sequestered at the receptor and thus unable to reach their

downstream target caspases. Alternatively, the recruitment of IAP family proteins to TNF-R complexes may promote their interaction with caspases that they otherwise would not have opportunities to regulate. Preliminary experiments, however, have failed to reveal an effect of TRAF-1 or TRAF-2 or their combination on c-IAP-1- and c-IAP-2-mediated suppression of cell death induced by either TNF-RI overexpression or etoposide (N.Roy, J.Reed and T.Van Arsdale, unpublished observations). Moreover, the finding that other members of the IAP family, such as XIAP and NAIP, fail to interact with TRAF family proteins suggests that binding to TRAFs is not essential for the caspase-inhibitory function of IAP family proteins.

We have shown that the *in vitro* caspase inhibitory activity of c-IAP-1 and c-IAP-2 resides within their BIR motifs. Consistent with this finding, the death-preventing activity of these IAP family proteins was also retained by overexpression of these BIR motifs. Thus, the preponderance of data available to date support the hypothesis that it is the BIR domains that represent the minimal functional region, at least among the cellular homologs of these proteins. It is of interest, however, that truncation mutants of c-IAP-1 and c-IAP-2 lacking the RING domain were less potent inhibitors of caspases-3 and -7 *in vitro* relative to their full-length forms. Thus, while not essential, the RING domain may make some contributions to c-IAP-1 and c-IAP-2 function, even if indirectly by perhaps stabilizing bioactive conformations of the BIR domains.

Although the mechanism of caspase inhibition by the IAPs remains to be elucidated, it appears to be different from p35. Unlike inhibition by p35 which involves proteolytic cleavage, we did not observe any significant cleavage of c-IAP-1 or c-IAP-2 in the presence of purified caspases. These data suggest that c-IAP-1 and -2 do not require cleavage for their inhibitory activity. Most natural inhibitors are active site-directed, in that the inhibitor directly blocks the active site of the protease. A survey of the known families of natural protease inhibitors reveals a common mechanistic theme. These inhibitors, such as kunitz, kazal and eglin families that inhibit serine proteases, contain a loop of defined structure that is pre-formed to adapt to the substrate groove of the protease (Bode and Huber, 1991). Similarly, the cystatins contain loops that fit the substrate groove of members of the papain family of cysteine proteases (Turk and Bode, 1991). In all cases, inhibition is caused by a lock-and-key type interaction of the pre-formed inhibitory loops with the protease substrate groove, and no peptide bond cleavage takes place. Peptide bond cleavage is rare in protease-protease inhibitor interactions, and is only observed for p35 and serpins (of which CrmA is a member), and the unusual α -macroglobulins that act as protease cages (Barrett and Starkey, 1973). Exactly how a protein can be a substrate yet still an inhibitor is still controversial, but these types of inhibitors are usually called suicide inactivators to indicate that the inhibitor is consumed during the process of inhibition (Patston *et al.*, 1991). Consequently, since the IAPs are not cleaved during inhibition, it is likely that they will operate by a mechanism similar to the conventional lock-and-key inhibitors, and studies are underway in our laboratories to test this hypothesis.

At least two roles can be envisaged for how IAP family

proteins might function physiologically as suppressors of downstream caspases. First, IAPs could be intended to inhibit the small amounts of adventitious caspase activation that surely must occur during the normal functions of healthy cells. In this capacity, IAPs potentially could prevent inappropriate induction of apoptosis which might otherwise be stimulated by small amounts of active proteases amplifying their effects through cascades of proteolytic processing of additional pro-caspases. Second, IAPs conceivably could prevent apoptosis induced by a broad array of cell death stimuli, provided that the IAPs are expressed at sufficiently high concentrations within cells. High levels of IAP protein production might occur normally under some circumstances during cell differentiation, as well as pathologically, such as in tumors. In this regard, striking overexpression of one of the IAP family proteins, survivin, has been reported recently in a wide variety of human tumors (Ambrosini *et al.*, 1997). Regardless of their intended roles, the findings presented here provide further evidence that the major mechanism by which the IAPs promote cell survival is by interfering with specific members of the caspase family of cell death proteases.

Materials and methods

Expression and purification of recombinant IAPs and caspases

c-IAP-1 and c-IAP-2 cDNAs were obtained by RT-PCR of RNA derived from Jurkat T-cells with the following primers for c-IAP-1 (forward, 5'-AGGGAATTCATGCACAAAACCTGCCTCCCA-3'; reverse, 5'-CTCC-TCGAGGATGGCTTCAAGTGTTC AAC-3') and for c-IAP-2 (forward, 5'-AGGGAATTCATGAACATAGTAGAAAACAGCA-3'; reverse, 5'-CTCCTCGAGAGATGATGTTTTGGTTCTTCTT-3'). NAIP constructs encoding the three BIR motifs were generated by PCR from a full-length cDNA in pBluescript (Stratagene) with forward primer 5'-AGGGAATTCATGGCCACCCAGCAGAAA-3' and reverse primer 5'-CTCCTCGAGCAGTAATTGAGAAAGTTCACC-3'. PCR products were digested with *EcoRI* and *XhoI* and ligated into pGEX4T and pcDNA3-myc. c-IAP-1 (BIR) and c-IAP-2 (BIR) constructs were generated by PCR from the full-length constructs with reverse primers 5'-CTCCTCGAGGATCTAACCTTGAATCTCATCAACAAC-3' and 5'-CTCCTCGAGGATCTACTTGAACCTTGACGGATGATGAAC-3' respectively, and the forward primers described above. All IAPs were expressed in *Escherichia coli* strain BL21(DE3) containing the plasmid pT-Trx (Yasukawa *et al.*, 1995). Bacteria were grown at 30°C to an optical density of 0.6, and fusion protein production was induced at 30°C with 0.4 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for 2 h, with the exception of GST-c-IAP-2 which was induced for 1 h at room temperature. Fusion proteins were obtained from the soluble fraction and affinity purified on glutathione-Sepharose by standard methods. Eluted proteins were dialyzed against phosphate-buffered saline (PBS). GST-XIAP was prepared as described (Deveraux *et al.*, 1997).

Caspases-3, -6 and -7 containing C-terminal His₆ tags and caspase-8 containing an N-terminal His₆ tag were purified as previously described (Orth *et al.*, 1996; Quan *et al.*, 1996; Muzio *et al.*, 1997; Zhou *et al.*, 1997).

In vitro protease assays

Caspase activities were assayed at 37°C using a fluorometric plate reader (Perkin-Elmer, LS50B) in the kinetic mode with excitation and emission wavelengths of 400 and 505 nm, respectively. Activity was measured by the release of 7-amino-4-trifluoromethyl-coumarin (AFC) from the synthetic substrate benzyloxycarbonyl-Asp-Glu-Val-Asp (Z-DEVD-AFC). Inhibition rates and equilibria were calculated from progress curves where substrate hydrolysis (100 μ M) by caspase-3 (7 pM), caspase-6 (100 pM), caspase-7 (150 pM) or caspase-8 (125 pM) was measured in the presence of a range of inhibitor concentrations (0.025–1.5 μ M) in caspase buffer [50 mM HEPES, 100 mM NaCl, 1 mM EDTA, 0.1% CHAPS, 10% sucrose and 5 mM dithiothreitol (DTT)]. The observed inhibition constant, K_i , was calculated as described (Zhou

et al., 1997). Given that the mechanism of inhibition is unknown, K_i s were determined without taking into account substrate concentration. Additionally, recombinant c-IAP-1 and c-IAP-2 proteins were titrated against caspase-7 to determine the amount of active protein in our preparations, which ranged from ~25 to 45%, assuming that inhibitor and enzyme form equimolar complexes.

For cell-free experiments, 293 cytosolic extracts were incubated with GST fusion proteins (0.3 μ M) and activated by the addition of cytochrome *c* (10 μ M) and dATP (1 mM) for 30 min at 37°C. For protease assays, 1 μ l of cytosolic extracts was added to 100 μ l of caspase buffer in the presence of 100 μ M substrate, and DEVD hydrolysis was measured. Alternatively, extracts were activated for 30 min with cytochrome *c*/dATP, and DEVD hydrolysis was measured in the absence or presence of GST fusion proteins (0.3 μ M) and substrate (100 μ M).

Preparation of cytosolic extracts

293 cells were harvested by centrifugation at 1800 *g* for 10 min and washed with ice-cold buffer A [20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM DTT and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)]. The cell pellet was resuspended in 1 volume of buffer A and incubated on ice for 20 min. Cells were broken by passing 15 times through a 26 gauge needle, and the extracts were clarified by centrifugation at 16 000 *g* for 30 min. NaCl was then added to a final concentration of 50 mM to the resulting supernatants which were stored at -80°C.

Protein-binding assays

Purified recombinant caspase-3, -6 or -7 (0.5 μ g) was incubated with GST fusion proteins (~3 μ M) immobilized on glutathione-Sepharose beads in 400 μ l of caspase buffer supplemented with bovine serum albumin (BSA) to a final concentration of 1%. The beads were washed three times with 1 ml of wash buffer [50 mM Tris (pH 7.5), 150 mM KCl, 2 mM DTT, 0.1% Triton X-100]. To test binding in cytosolic extracts, GST fusion proteins were incubated for 2 h with 45 μ l of cytosolic extract that had been either unactivated or activated with cytochrome *c*/dATP and then supplemented to 400 μ l with binding buffer (10 mM HEPES, 142 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 0.2% NP-40). TRAF proteins were *in vitro* transcribed and translated in the presence of [³⁵S]methionine and were incubated with IAP-GST fusion proteins (3 μ M) in binding buffer for 3 h. Beads were washed three times in binding buffer; bound proteins were eluted by boiling in Laemmli sample buffer and subjected to SDS-PAGE and immunoblot analysis.

Immunoblot analysis

Proteins were separated by SDS-PAGE using 750 mM Tris-12% polyacrylamide gels and transferred to PVDF membranes. A monoclonal antibody against human caspase-2 was purchased from Transduction laboratories, and polyclonal antibodies against the other caspases were utilized as previously described (Orth *et al.*, 1996; Krajewska *et al.*, 1997; Krajewski *et al.*, 1997). Antibody detection was performed using an enhanced chemiluminescence detection kit (Amersham).

Cell culture

Human embryonic kidney 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Cells were transiently transfected with 12 μ g of pcDNA plasmid DNA and 0.5 μ g of pEGFP (Clontech) by a calcium phosphate precipitation method. Cells were incubated for 6 h with the transfection solution, washed three times with PBS, and then returned to culture with fresh media with or without 30 μ M etoposide (Sigma). Cells were collected by centrifugation at 48 h post-transfection, and washed in ice-cold PBS. For protease-cleaving assays, cells were lysed in binding buffer and incubated on ice for 20 min. Lysates were cleared by centrifugation at 16 000 *g* for 30 min and supernatants stored at -80°C. To determine cell death, cells were fixed with 4% paraformaldehyde, rinsed with PBS and stained with 0.1 μ g/ml DAPI. Nuclear morphology of cells was analyzed by fluorescence microscopy.

Protease inhibitor cleavage assays

Full-length cDNAs encoding c-IAP-1, c-IAP-2 and p35 in pcDNA3 were *in vitro* transcribed and translated in the presence of [³⁵S]methionine using the coupled transcription/translation TNT kit (Promega) according to the manufacturer's instructions. Five μ l of the translation products were incubated with 2 μ l of either caspase-1, -3, -6, -7 or 8 in caspase buffer in a total volume of 12 μ l and incubated for 1 h at 37°C. The reactions were analyzed by SDS-PAGE and autoradiography.

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