The Human Anti-apoptotic Proteins cIAP1 and cIAP2 Bind but Do Not Inhibit Caspases*

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clAs (cellular inhibitor of apoptosis proteins) 1 and 2 are able to regulate apoptosis when ectopically expressed in recipient cells and probably also in vivo. Previous work suggested that this is at least partially due to direct caspase inhibition, mediated by two of the three baculovirus IAP repeat (BIR) domains that are contained in these proteins. In support of this we show that the BIR domains 2 and 3 of the two clAs are able to bind caspases-7 and -9. However, we demonstrate that neither of these BIR domains is able to inhibit caspases because of critical substitutions in the regions that target caspase inhibition in the X-linked IAP, a tight binding caspase inhibitor. The clA BIR domains can be converted to tight binding caspase inhibitors by substituting these critical residues with XIAP residues. Thus, clAs maintain protein scaffolds suitable for direct caspase inhibition but have lost or never acquired specific caspase inhibitory interaction sites. Consequently, although the binding function of the clA BIRs may be important for their physiologic function, caspase inhibition is not.

Apoptosis is normally a highly regulated cellular pathway that ultimately results in the packaging and disposal of unwanted or damaged cells. At the heart of the apoptosis pathway is a family of proteases known as the caspases, the activity of which is responsible for the organized destruction of the cell (reviewed in Ref. 1). Much work has been done to deduce the regulatory mechanisms of caspase activation, and it is now clear that a two-step pathway is required. The pathway consists of an apical (or initiator) phase in which multicomponent protein recruitment complexes provide a platform to activate the zymogens of caspases-8 and -10 (extrinsic pathway) or caspase-9 (intrinsic pathway). Once active, these initiator caspases cleave and activate the zymogens of executioner (or effector) caspases-3 and 7, which in turn are responsible for the majority of proteolytic events that ultimately result in the destruction of the cell.

This proteolytic pathway, like most proteolytic pathways, is regulated at two levels: 1) timing and location of zymogen activation and 2) inhibition of active protease. In mammals, the most well-documented mechanism for influencing caspase activity is driven by direct interaction of members of the IAP family (inhibitor of apoptosis protein) (reviewed in Refs. 2–5). The IAPs, first identified as viral proteins capable of inhibiting active protease, have been shown to be physiologically relevant in mammalian cells (15–19). The IAPs are characterized by the presence of at least one baculoviral IAP repeat (BIR) domain. A characteristic of many BIR domains is the presence of a surface groove that has a preference for binding the extreme N terminus of short peptides of defined sequence (reviewed in Refs. 3 and 5). The mechanism of inhibition of caspases by XIAP has been determined at the atomic and biochemical levels, pinpointing the residues essential for physiologic caspase regulation (7–14). Significantly, in addition to the IBM-interacting exosite, XIAP has an absolute requirement for ancillary interaction surfaces to potently inhibit caspases.

The two closest XIAP paralogs in humans are cellular IAP 1 and 2 (cIAP1 and cIAP2), both of which have been demonstrated to be able to bind caspases because of critical substitutions in the regions that target caspase inhibition (reviewed in Refs. 3 and 5). The BIR domains of cIAP1 and cIAP2 displays residues compatible with inhibition of caspases-9 and -10. Therefore, we hypothesized that cIAP1 and cIAP2 could in fact function as physiologic caspase inhibitors in a similar manner to XIAP.

EXPERIMENTAL PROCEDURES

Recombinant Caspases and IAPs— Full-length caspase-3, full-length caspase-7, and caspase-9 lacking the caspase recruitment domain were expressed in Escherichia coli and purified as previously described (21). A plasmid encoding cIAP1 was the generous gift of John Reed of the Burnham Institute and was used as a template for production of various constructs. Constructs encoded by cIAP2 were directly amplified from a human fetal brain cDNA library. BIR domains and mutants thereof were constructed by cloning into a modified pET15b (Novagen, Madison, WI) that generates N-terminal fusion His6 proteins (22), or pGEX4T-1 (Amersham Bio-

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2 The abbreviations used are: IAP, inhibitor of apoptosis protein; CIAP, cellular inhibitor of apoptosis protein; BIR, baculoviral IAP repeat; IBM, IAP-binding motif; GST, glutathione S-transferase; PIPES, 1,4-piperazinediethanesulfonic acid; Z, benzoyloxycarbonyl; fmk, fluoromethyl ketone; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; GdmCl, guanidinium chloride.

3 The Human Genome Nomenclature Committee recommends the gene and protein designation BIRC1–8 for the human IAP family members. In this system cIAP1 is BIRC3, cIAP2 is BIRC3, and XIAP is BIRC4, although in this paper we use the former (trivial) names.
sciences) to generate N-terminal GST fusion proteins. Full-length cIAP1 was cloned into pET 23b to generate a C-terminal fusion His6 protein. Recombinant proteins were expressed in E. coli strain BL21 DE3. Protein expression was induced with the addition of 0.4 mM isopropyl β-D-thiogalactopyranoside when cells, grown at 37 °C, reached an OD600 of 0.4. The cultures were incubated 4 h at 30 °C following isopropyl β-D-thiogalactopyranoside induction. The full-length cIAP1 construct was transformed in to BL-21 DE3 codon + cells and grown at 37 °C to an OD600 of 0.4. Isopropyl β-D-thiogalactopyranoside (0.2 mM) was added to induce protein expression, and cells were grown at 25 °C for 8 h. His-tagged proteins were purified using chelating Sepharose (Amersham Biosciences) charged with NiSO4 in buffer containing 50 mM HEPES and 100 mM NaCl at pH 7.4 followed by elution using a 0–20 mM linear gradient of imidazole. GST-tagged protein was purified using glutathione-Sepharose 4B (Amersham Biosciences).

Cell-free Lysates—HEK293A cells were grown at 37 °C in 15-cm plates to 70% confluence, harvested by scraping in phosphate-buffered saline, washed, and allowed to swell for 30 min on ice in hypotonic buffer (20 mM PIPES, 10 mM KCl, 2 mM MgCl2, and 4 mM dithiothreitol at a pH of 7.4) as previously described (23). The cells were sheared using a 27-gauge needle and centrifuged at 500 × g for 30 min, and the supernatants were collected and stored at −80 °C. The lysates were activated for cell-free apoptosis by the addition of 10 μM cytochrome c (Sigma) and 0.5 mM dATP (Sigma) for 45 min at 37 °C (24). Following activation the lysates were assayed for caspase activity in the presence of BIR domains as described below or treated with 100 μM Z-VAD-FMK (Enzymes Systems Products, Livermore, CA) for 30 min at room temperature to prevent further caspase activity.

Binding Assays—GST-tagged BIR domains produced in E. coli were bound to glutathione-Sepharose beads for 1 h at room temperature. The beads were washed three times in phosphate-buffered saline-Triton X 100 (1% v/v) and resuspended at 50% (v/v), and 5 μl of beads was incubated with 400 ng (total protein) of cytochrome c-activated HEK293A cell lysate in a total volume of 100 μl overnight at 4 °C. The beads were washed three times in hypotonic buffer, and the proteins were eluted by boiling in SDS sample buffer containing 10 mM dithiothreitol. Equal volumes of input (untreated and cytochrome c-treated) BIR bound, and supernatant (depleted lysates) were loaded on an 8–18% linear gradient acrylamide SDS gel for electrophoresis. The samples were either transferred to polyvinylidene difluoride and immunoblotted or stained with Gel Code Blue Stain reagent (Pierce).

Immunoblotting—Caspase-7 was detected using a rabbit caspase-7 polyclonal antibody (Cell Signaling Technology number 9492). Caspase-9 was detected using a caspase-9 polyclonal antibody (23). Secondary anti-mouse IgG and anti-rabbit IgG conjugated with horseradish peroxidase were purchased from Amersham Biosciences.

Caspase Assays—Recombinant caspases were preactivated with 20 mM 2-mercaptoethanol in buffer containing 50 mM HEPES, 10% sucrose (w/v), and 0.1% CHAPS with 100 mM NaCl (caspases-3 and -7) or without NaCl (caspase-9) at pH 7.4 for 15 min (caspases-3 and -7) or 20 min (caspase-9) at 37 °C. A range of BIR domain concentrations was incubated with the activated caspases for 5 min. Caspase activity was determined by cleavage of Ac-Asp-Glu-Val-Asp-pNA (Bachem, Torrance, CA) on a SpectraMax 340 spectrophotometric plate reader (Molecular Devices, Sunnyvale, CA). Inhibitory constants for each enzyme-inhibitor pair was determined from the uninhibited rate (v0) and inhibited rates (vi), such that a plot of (vi/v0) − 1 versus [I] gives a slope of 1/K(apparent).

Inhibition of endogenous executioner caspase activity by BIR2 was determined using HEK293A cell-free lysates preactivated for 15 min as described above followed by incubation for 5 min with selected recombinant BIR2 domain proteins. Residual activity was determined as for recombinant caspases. Inhibition of endogenous caspase-9 activity by BIR3 was also determined using HEK293A cell-free lysates. In this case cytochrome c/ATP activation was carried out in the presence of recombinant BIR3 proteins, and activity was measured by cleavage of Ac-Asp-Glu-Val-Asp-pNA (Bachem, Torrance, CA) on a SpectraMax 340 spectrophotometric plate reader (Molecular Devices) as a measure of the ability of caspase-9 to activate executioner caspases (23).

Protein Stability Assays—Fluorescence spectra were measured using a PerkinElmer LS50B luminescence spectrometer coupled with the FLWin Lab software using a 1-cm-pathlength cuvette (Helma). Unfolding studies were carried out by incubating protein (dialyzed into 50 mM Tris/HCl, 100 mM NaCl, pH 8.0) in the presence of incremental increases in guanidine chloride (GdmCl, 0–6.35 M) for 1 h at room temperature. Emission spectrum was measured from 300 to 400 nm after excitation at 280 nm. The emission and excitation slit widths were...
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5 and 7 nm, respectively. A shift in the fluorescence emission peak was indicative of protein unfolding. After collecting all of the emission spectra, the area under the curve was calculated by summing the product of wavelength increment and emission intensity for each recorded wavelength-intensity pair. To calculate the average wavelength for each respective GdmCl concentration, the product of emission intensity and corresponding wavelength was calculated for each recorded wavelength-intensity pair and divided by values of the area under the curve. The change in average wavelength from native state (0 M GdmCl) at each GdmCl concentration were normalized to the denatured states (6.35 M GdmCl) and plotted as relative change in the average emission wavelength.

**RESULTS**

The BIR2 and BIR3 Domains of cIAP1 and cIAP2 Bind to Caspases-7 and -9—BIR domains are characterized by their ability to bind proteins possessing conserved sequences at their N terminus. Indeed, this specific binding mode is a necessary step in the inhibition of the apoptotic caspases-3, -7, and -9 by XIAP (7, 8). To test whether the BIR domains of cIAP1 and cIAP2 have this property, we used GST-tagged BIR2 and BIR3 domains (Fig. 2A) and analyzed binding to endogenous caspases-7 and -9 from cell-free lysates programmed to develop apoptotic activity. We performed this by initiating cell-free apoptosis through addition of cytochrome c to hypotonic cytosolic extracts of HEK cells, followed by the addition of recombinant purified GST-BIR domains and adsorption of material on immobilized glutathione. Bound material was eluted by boiling in SDS-PAGE sample buffer followed by immunoblotting with specific antisera raised against caspase-7 or -9. We found that both the BIR2 and BIR3 domains of cIAP1 and cIAP2 associated with caspase-7 (Fig. 2, B and C, top panels). The mode of binding of caspase-7 to IAPs is partly dependent on neoepitopes generated at the large and small subunits during proteolytic activation (Fig. 2D) (8, 25). However, because the caspase-7 antiserum does not recognize the small subunit in immunoblots, we are only able to visualize the associated large subunit, which comes in two differentially processed forms (22–25 kDa; Fig. 2, B and C, top panels). Significantly, BIR2 and BIR3 domains of cIAP1 were able to deplete most of the endogenous caspase-7, whereas the same domains of cIAP2 were less efficient at this task (Fig. 2, B and C, top panels), implying that cIAP1 has a higher affinity for caspase-7 than does cIAP2.

To confirm that the caspase/IAP interaction was dependent on the IBM-interacting exosite maintained within the BIR domain, we mutated key residues that characterize the exosomes in cIAP1 (E239R and H243V in BIR2 and E325R and W329V in BIR3) using the strategy previously shown to attenuate caspase interaction with XIAP (8). These mutations ablated the ability of the BIR domain to efficiently associate with the caspase, demonstrating that this interaction requires the conserved IBM-interacting exosite (Fig. 2B, top panel).

We also tested the ability of the BIR2 and BIR3 domains of the cIAPs to bind caspase-9. The mode of binding of caspase-9 to IAPs is via neoepitopes generated at the small subunit during proteolytic cleavage (Fig. 2, B and C, lower panels) (7, 26, 27). Like the caspase-7 antiserum, the caspase-9 antiserum does not recognize the small subunit in immunoblots, and again we visualize the associated large subunit, which comes in two differentially processed forms (Fig. 2, B and C, lower panels).
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FIGURE 3. Inhibition of caspase-3 and -7. A, recombinant protein expression of the following His-tagged proteins: cIAP1-BIR2 with its flanking N-terminal linker (residues 144–260); the fusion protein (X-cIAP1-BIR2) composed of the XIAP linker (residues 123–162) and the BIR2 domain of cIAP1 (residues 184–260); the exosite mutant (E239R/H234V) of X-cIAP1 BIR2 (Mut); and cIAP2-BIR2 with its flanking N-terminal linker (residues 129–246). The proteins were stained with gel code blue reagent. B and C, inhibition of caspase-3 (100 pM) (B) and caspase-7 (350 pM) (C) by cIAP1-BIR2 (filled circles), the X-cIAP1 BIR2 hybrid protein (open circles) and exosite mutant of X-cIAP1 BIR2 (E239R/H343V) (open squares). The data are presented as relative activity, \( v/v_o \), where \( v_o \) represents the enzyme activity in the presence of the BIR-domain, and \( v \) is the uninhibited enzyme activity.

**A.**

**B.**

**C.**

**D.**

**E.**

panel). The lower panels in Fig. 2 (B and C) reveal that BIRs 2 and 3 of cIAP1 as well as BIR2 of cIAP2 associate with caspase-9. One site of cleavage is the auto-catalytic event in the caspase-9 interdomain linker at Asp\(^{315} \), which is a consequence of apoptosome driven activation (26). The other site of cleavage is at Asp\(^{319} \), and it is controversial whether this latter event uncovers an IBM (26, 27). We found that only the p35 domain of cIAP2 was unable to effectively bind caspase-9 (Fig. 2, C, lower panel). Again this interaction was found to be IBM-binding and exosite-dependent because the mutants were unable to bind caspase-9 (Fig. 2B, bottom panel). Interestingly, the BIR3 domain of cIAP2 was unable to effectively bind caspase-9 (Fig. 2C, bottom panel). This could potentially be attributed to other IAPs within the lysate sequestering the cleaved caspase-9, thereby preventing it from interacting with cIAP2-BIR3. In support of this, we observed incomplete depletion of caspase-9 from the lysates by the cIAPs.

These experiments demonstrate that the BIR2 and BIR3 domains of cIAP1 and cIAP2 are competent to bind caspases in an IBM-dependent manner, with the possible exception of the BIR3 domain of cIAP2. However, binding should not be equated with inhibition, and so we proceeded to test whether the BIR domains are able to inhibit the bound caspases.

**The BIR2 Domains of cIAP1 and cIAP2 Are Poor Inhibitors of the Executioner Caspases-3 and -7**—Tight inhibition of executioner caspases by XIAP requires two surfaces on the BIR2 domain: the IBM-interacting groove (8, 9) and the N-terminal extension between residues 123–163 (11–13). In contrast to the IBM-interacting groove, the N-terminal extension of XIAP exhibits less conservation of important interaction sites when compared with cIAPs (Fig. 1).

To determine whether cIAP1 and cIAP2 are caspase inhibitors, we tested the inhibitory capacity of their BIR2 domains with the respective N-terminally flanking linkers (residues 144–260 of cIAP1, residues 129–246 of cIAP2, equivalent to the caspase-3 and -7 inhibitory unit of XIAP) using purified recombinant caspases-2, -3, -6, -7, -8, -9, and -10 as targets. We observed virtually no inhibition (apparent \( K_i \) values greater than 5 \( \mu \)M) of these caspases by BIR2 from cIAP1 or cIAP2. (Fig. 3 and data not shown). This represents physiologically irrelevant inhibition when compared with the BIR2 domain of XIAP that has low to subnanomolar apparent \( K_i \) values toward caspases-3 and -7. To investigate why cIAP1 and cIAP2 were such poor inhibitors of these caspases, we used structure/sequence-based analysis to predict whether the lack of inhibition stemmed from differences in the putative caspase interacting regions. The IBM-interacting groove of the BIR2 domain of XIAP is highly conserved in many IAPs, including cIAP1 and cIAP2, and so we focused on the N-terminal extension that in XIAP interacts with the Active site region of caspases-3 and -7 (11–13). Although the cIAPs maintain several crucial caspase-interacting residues in the N-terminal linker (starred residues in Fig. 1), this region is slightly more divergent from XIAP, and it is the conformation of this region that we predicted to be the basis for the insignificant inhibition of caspases by cIAPs.

To test this we created a hybrid protein consisting of the linker region from XIAP (residues 123–163) replacing residues 144–183 of cIAP1. As predicted this hybrid protein (X-cIAP1 BIR2) became a tight binding inhibitor of caspases-3 and -7 with low to subnanomolar apparent \( K_i \) values (Fig. 3, B and C, and Table 1). By itself, the linker of XIAP is insufficient for executioner caspase inhibition (8, 9), and this finding tells us two things. First, it reveals that the BIR2 domain of the hybrid protein is sufficient for anchoring caspases through the exosite. Second, it reveals that the N-terminal extension of cIAPs contains residues that disallow the tight interaction seen with the equivalent region of XIAP. To confirm this we disrupted the IBM-interacting exosite in the hybrid protein by the disabling mutations E239R and W243V. With an ablated exosite this protein (X-cIAP1 BIR2-mut) displayed markedly weaker inhibition of both caspases-3 and -7 (Fig. 3, B and C), incidentally confirming the two-site, or exosite, interaction model for the inhibition of caspases by XIAP (8). Together these data suggest that the region we have selected to represent BIR2 in the cIAPs is sufficient to allow binding of caspases-3 and -7 to the exosite but lacks the proper conformation in the linker region to inhibit the proteolytic activity of the enzyme.
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The data also suggest that the BIR2 region in the ciAPs maintains the IAP fold, but there is a formal possibility that the regions are inactive as caspase inhibitors because they lack the correct structure. To test this possibility we performed unfolding studies on ciAP-BIR2, the XIAP ciAP1 hybrid protein, and the exosite mutant of the hybrid protein (Fig. 3D). The conformational stabilities of the three proteins were essentially indistinguishable, ruling out domain stability as a reason for the poor inhibition.

To confirm that the differential inhibitory capacity of the selected BIR2 domains toward recombinant caspases mirrors that of endogenous executioner caspases, we tested BIR2-mediated inhibition of caspase activity in HEK293A cell-free lysates preactivated with cytochrome c and ATP. X-ciAP1-BIR2 displayed robust inhibition of executioner caspases, even slightly better than observed with XIAP-BIR2. Conversely, ciAP1-BIR2 was unable to inhibit caspases, consistent with our studies utilizing recombinant caspases (Fig. 3E).

The BIR3 Domains of ciAP1 and ciAP2 Are Poor Inhibitors of the Initiator Caspase-9—The third BIR domain of XIAP (BIR3) is a specific caspase-9 inhibitor (10, 28), employing an exosite mechanism to anchor caspase-9, similar to the anchoring of caspases-3 and -7 by BIR2, and a second site to ablate activity of caspase-9 (7). We tested the capacity of the third BIR domain (residues 257–356 of ciAP1 and residues 243–333 of ciAP2) of the ciAPs to suppress the proteolytic activity of caspase-9 and observed insignificant inhibition (apparent Ki values greater than 5 μM). The ciAPs are distinct from XIAP at three of the four caspase-9-interacting residues. Mutation of these three residues (R332G, Q349H, and G350L) in ciAP1 to the corresponding residues in XIAP (designated X-ciAP1-BIR3) restored tight binding caspase inhibition (Fig. 4B and Table 2). Similar to the our studies with the BIR2 domain of ciAP1 and ciAP2, it seems that the BIR3 domain of ciAP1 is quite capable of binding to caspase-9 through its IBM-interacting exosite yet lacks the proper residues necessary for inhibition.

The third BIR domain of ciAP1 and the triple mutant thereof were found to be equally stable (Fig. 4C). The equivalent conformational stability of these two proteins, albeit less than that of BIR2, demonstrates that the lack of inhibitory capacity of the BIR3 of ciAP1 cannot be explained by improper protein folding and suggests that the C-terminal helix of BIR3 is not structurally suited for caspase-9 inhibition.

From these studies we predicted that X-ciAP1-BIR3 would be sufficient to inhibit caspase-9-mediated activation of downstream caspases. To test this we incubated cell-free lysates with recombinant ciAP1-BIR3, X-ciAP1-BIR3, or XIAP-BIR3 prior to cytochrome c activation. X-ciAP1-BIR3 potently prevented executioner caspase activation by caspase-9, whereas ciAP1-BIR3 displayed no inhibitory activity. These findings further confirm that minor differences from XIAP in the distal helix of ciAP1-BIR3 are the basis for the loss/lack of caspase-9 inhibitory activity.

Full-length ciAP1 Does Not Function as a Physiological Caspase Inhibitor—We have demonstrated that ciAP1 and ciAP2 are extremely weak inhibitors of caspase-3, -7, and -9 because of distinctions from XIAP in important caspase interacting sites. To properly conclude that
the cIAPs were not caspase inhibitors, this needed to be tested in the context of the full-length protein. Expression of full-length cIAP1 has been problematic, and we were unable to obtain a very pure preparation (Fig. 5A). Nevertheless, at 2 μM of total protein, of which full-length cIAP1 accounts for at least 30%, we observed only 22% inhibition of caspases-3 and -7 and only 12% inhibition of caspase-9 (Fig. 5B). This poor caspase inhibition by at least 600 nM yields an apparent \( K_i \) in excess of 2 μM and suggests physiologically insignificant inhibition.

**DISCUSSION**

The most well understood member of the IAP family is XIAP, a tight binding inhibitor of the executioner caspases-3 and -7 and the initiator caspase-9. On the basis of conservation of function it is usually assumed, and occasionally reported, that other members of this family also function as caspase inhibitors. Based on the established structural requirements for caspase inhibition by XIAP, we predicted that its closest human homologs, cIAP1 and cIAP2, should maintain the capacity to inhibit caspases-3 and 7 and may not be able to potently inhibit caspase-9. Interestingly, we found that the cIAPs are extremely weak inhibitors of both executioner caspases-3 and -7 and the initiator caspase-9. Our findings demonstrate that, although the caspase-binding exosites are retained in cIAPs, the key interacting surfaces that result in protease inhibition are not. Simple mutagenesis of these surfaces to the respective XIAP residues can restore tight binding inhibition in a predictable manner. This finding has two consequences. First it confirms the IAP-mediated mechanism of inhibition by caspases proposed for XIAP on the basis of structural and biochemical studies. Second, it implies that the ancestor of cIAPs lost, or perhaps never acquired, the ability to inhibit caspases.

Importantly, cIAPs are able to bind caspases via IBM-interacting exosites, even though this does not result in inhibition. Unexpectedly, the second BIR domain of each cIAP displayed a higher affinity toward caspase-9 than the third BIR domain, which is counterintuitive because the BIR3 domain of XIAP is the caspase-9 inhibitory domain. This could possibly be due to the enhanced conformational stability of BIR2 compared with BIR3 proteins (Figs. 3D and 4C). But we cannot comment further on this until the individual binding preferences of the BIR domains are more thoroughly mapped.

The IAP literature is somewhat confused with respect to function and mechanism of this important group of proteins. Although it is generally accepted that they have a role in controlling apoptosis (reviewed in Refs. 3–5 and 29), there is a lack of consensus about how they do so. Significantly, several groups have reported that many IAPs are good caspase inhibitors. Although this is now well established for XIAP (reviewed in Refs. 3–5 and 29), controversy exists surrounding the other family members. In a previous publication we postulated that cIAPs 1 and 2 are moderately good direct inhibitors of caspases-3 and -7 (15). However, this was based on the use of GST-tagged BIR domains, which we more recently showed endow them with artificial properties, including caspase inhibition (8). The artifact is related to the propensity of the GST tag to oligomerize, which sets up a scenario where higher order structures probably interact with multiple caspase IBMs and “coat” the proteases in a way that leads to inhibition by steric occlusion in a manner not found naturally. A caveat is that our studies have mainly been performed with isolated BIR domains. The possibility that such higher order structures may be present in full-length cIAPs seems to be ruled out by our finding that full-length cIAP1 also does not inhibit caspases.

There is substantial agreement that cIAP1 and cIAP2 endow cells with protection against apoptotic stimuli (15–19). However, in the absence of a caspase inhibitory function, the means by which the cIAPs obstruct apoptosis is less clear. The simplest hypothesis is that the cIAPs function as protein sinks, binding to proteins that maintain functional IBMs (30). One can envision that binding to IAP antagonists such as SMAC (second mitochondrial activator of caspases) would limit the ability of these pro-apoptotic proteins to disrupt XIAP-mediated caspase inhibition, much as has been suggested for ML-IAP (31, 32). Alternative explanations of the anti-apoptotic activity of the cIAPs propose a regulatory role in the signaling pathways initiated by the tumor necrosis factor receptor because these IAPs were found to directly interact with TRAFs (33, 34) through the BIR1 domain (35) and participate in the activation of NFκB (36). Certain IAPs have been implicated in auto ubiquitination or ubiquitination of targets (reviewed in Ref. 37). Accordingly, cIAPs may maintain an ubiquitin E3 ligase activity conferred by the C-terminal RING domain; thus cIAP2 has been reported to ubiquitinate caspases-3 and 7, at least in vitro (38). The proteosome-mediated destruction of caspases through cIAP dependent ubiquitination would allow the cell to selectively remove caspases after they become activated, which may still allow neutralization the bio-affects of these protease by the cIAPs.

XIAP may be the only member of the mammalian IAP family that has the capacity to inhibit the proteolytic activity of the caspases. Of the eight IAPs in humans, it has previously been demonstrated that ML-IAP and ILP2 are not caspase inhibitors because of a lack of appropriate
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caspase interacting residues or conformational instability (32, 39). The field is divided as to whether the remaining human IAPs, Survivin, NAIP, and Bruce function as physiological caspase inhibitors. Survivin has been reported to directly inhibit caspases-3 and -7, (40, 41), which was later disputed (42) and is now recognized as probably because of stabilization of XIAP rather than direct inhibition of caspases (43). NAIP has been reported to be (44, 45) or not be a caspase inhibitor (15). Bruce has been reported to inhibit caspases-3 and -7 (46). In many cases the observed inhibition by BIR domains may simply be due to aberrant interactions of the GST-tagged constructs, used in several of these studies, with caspases as described above.

On the basis of the experiments with cIAPs 1 and 2 reported here, and structure/function investigations on XIAP, ML-IAP, and ILP2 (7–13, 32, 39), we can now recognize the sequence-specific requirements for caspase inhibition. Specifically, even small deviations from the N-terminal helix of BIR3, which renders inhibition of caspase-9 ineffective. Consequently, it seems that the likelihood of IAPs other than XIAP being physiologic inhibitors of caspases in vivo is diminished and that the primary function of members of this family is not to inhibit caspases. The findings presented herein demonstrate the requirements for IAP-mediated caspase inhibition and based on these restrictions, we predict most IAPs to be incapable of regulating caspase through direct inhibition. Nevertheless, most members of this evolutionarily conserved family clearly maintain an anti-apoptotic activity, and further studies of the IAPs in cellular contexts will provide insights into their apoptotic occluding mechanisms.

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