The inhibitor of apoptosis proteins (IAPs) constitute an evolutionarily conserved family of homologous proteins that suppress apoptosis induced by multiple stimuli. Some IAP family proteins, including XIAP, cIAP-1, and cIAP-2, can bind and directly inhibit selected caspases, a group of intracellular cell death proteases. These caspase-inhibiting IAP family proteins all contain three tandem BIR domains followed by a RING zinc finger domain. To determine the structural basis for caspase inhibition by XIAP, we analyzed the effects of various fragments of this IAP family protein on caspase activity in vitro and on apoptosis suppression in intact cells. The RING domain of XIAP failed to inhibit the activity of recombinant caspases-3 or -7, whereas a fragment of XIAP encompassing the three tandem BIR domains potently inhibited these caspases in vitro and blocked Fas (CD95)-induced apoptosis when expressed in cells. Further dissection of the XIAP protein demonstrated that only the second of the three BIR domains (BIR2) was capable of binding and inhibiting these caspases. The apparent inhibition constants (K_i) for BIR2-mediated inhibition of caspases-3 and -7 were 2–5 nM, indicating that this single BIR domain possesses potent anti-caspase activity. Expression of the BIR2 domain in cells also partially suppressed Fas-induced apoptosis and blocked cytochrome c-induced processing of caspase-9 in cytosolic extracts, whereas BIR1 and BIR3 did not. These findings identify BIR2 as the minimal caspase-inhibitory domain of XIAP and indicate that a single BIR domain can be sufficient for binding and inhibiting caspases.

IAP family proteins have been identified in baculoviruses, Drosophila, and humans, where they function as suppressors of apoptosis (1–8). In all cases examined thus far, apoptosis has been shown to depend upon the activation of a group of intracellular cysteine proteases with specificity for aspartic acid in the P1 position of substrates, called caspases (reviewed in Ref. 20). The human XIAP, cIAP-1, and cIAP-2 proteins can bind to and directly inhibit selected caspases. The caspases identified as targets of these IAP family proteins thus far include caspases-3, -7, and -9 but not caspases-1, 2, 8, or 10 (9, 10, 24). XIAP, cIAP-1, and cIAP-2 all contain three tandem copies of a ~70-amino acid motif termed the BIR (baculovirus IAP repeat) domain, reflecting the historical discovery of IAP family proteins originally in baculoviruses by Miller and colleagues (1, 11). These IAP family proteins also contain a RING zinc finger domain near their C termini. The IAP family protein NAIP, in contrast, contains three tandem BIRs but lacks the RING domain (3). An additional member of the human IAP family, survivin, contains only a single BIR domain but nevertheless can suppress apoptosis in cells (8). The IAP family proteins of Drosophila and the insect-infecting baculoviruses all contain two or three tandem copies of the BIR domain followed by a RING domain (1, 2, 11). Deletional analyses of the Drosophila and baculovirus IAP family proteins have produced conflicting results, with some studies suggesting a critical role for both the BIR and RING domains and others implying that BIRs are directly involved in apoptosis suppression with the RING motif functioning possibly as a negative regulatory domain (2, 12).

The structural similarities and differences among IAP family proteins prompted us to address the question of what constitutes the minimal domain necessary for inhibition of caspases and suppression of apoptosis. Using the human XIAP protein as a model, we determined that only one of the three BIR domains present within this protein was clearly capable of inhibiting caspases-3 and -7, as well as suppressing apoptosis when expressed in cells. These observations demonstrated that a single BIR domain can be sufficient for binding and inhibiting caspases and imply that all BIR domains may not be functionally equivalent, despite their extensive amino acid sequence identity.

MATERIALS AND METHODS

Plasmid Constructions—Plasmids encoding fragments of the XIAP protein, including BIR1+2+3 (residues 1–336), RING (residues 337–497), BIR1 (residues 1–123), BIR2 (residues 124–260), BIR3 (residues 261–336), BIR2+3 (residues 124–336), and BIR1+2 (residues 1–260) were created by a one-step polymerase chain reaction method employing a plasmid encoding a full-length XIAP as the template (10) and using 5′-GGATTCATGACATTTTACAGGTGGAAG-3′ (BIR1+2+3, BIR1 and BIR1+2+2), 5′-GGATTCATGACATTTTACAGGTGGAAG-3′ (RING), 5′-GGATTCATGACATTTTACAGGTGGAAG-3′ (BIR1+2+3, BIR2 and BIR2+3), or 5′-GGATTCATGACATTTTACAGGTGGAAG-3′ (BIR3) as the forward primer and 5′-CTCTGAGATGCTATAGATTGTT-3′ (RING), 5′-CTCTGAGATGCTATAGATTGTT-3′ (BIR1+2+3, BIR3 and BIR3+2), 5′-CTCTGAGATGCTATAGATTGTT-3′ (BIR2 and BIR2+3), or 5′-CTCTGAGATGCTATAGATTGTT-3′ (BIR3) as the reverse primer. The BIR1+3 mutant protein (see Fig. 1) was prepared by ligating the BIR1 and BIR3 polymerase chain reaction fragments directly.

Val-Asp; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; GFP, green fluorescence protein; DAPI, 4′,6-diamidino-2-phenylindole.

* R. Takahashi, Q. Deveraux, I. Tamm, K. Welsh, N. Assa-Munt, G. S. Salvesen, and J. C. Reed, submitted for publication.
gested with EcoRI-NcoI and NcoI-XhoI, respectively. All the fragments were subcloned into pGEXKT-1 (Pharmacia Biotech Inc.) and/or pCDNA3-myc (10) by digestion with EcoRI and XhoI. The proper construction of all the plasmids was confirmed by DNA sequencing.

Production of GST Fusion Proteins—For production of BIR1+2+3 and RING, pGEXKT-1-XIAP mutant plasmids were introduced into Escherichia coli strain BL21(DE3) containing the plasmid pTrX (13). BIR1, BIR2, and BIR3 were produced in XL-1 blue cells (Stratagene, Inc.). The GST fusion proteins were prepared from the soluble fraction upon induction with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside at 30 °C for 12 h, affinity purified using glutathione-Sepharose, and then dialyzed against phosphate-buffered saline. Coomassie staining analysis of GST fusion proteins following SDS-PAGE revealed >90% intact protein in all cases except BIR3 (see text). His6-tagged caspase-3 and -7 recombinant proteins are prepared as described (14, 15).

Enzyme Assays—Caspase-3 and -7 activities were assayed by release of 7-amino-4-trifluoromethyl-coumarin (AFC) from Asp-Glu-Val-Asp (DEVD)-AFC using continuous reading instruments as described (15). Of 7-amino-4-trifluoromethyl-coumarin (AFC) from Asp-Glu-Val-Asp (DEVD)-AFC using continuous reading instruments as described (15).

In Vitro Caspase Binding Assays—Two μg of His6-tagged caspase-3 and -7 were immobilized on 4 μl of Ni2+-Sepharose and incubated with 5 μl of in vitro translated, [35S]-labeled caspase-3 (Fig. 2, A and B), the BIR1+2+3 fragment of XIAP, which proved to be more stable and which contained the BIR domain did not. As shown in Fig. 2 (A and B), the BIR1+2+3 fragment of XIAP inhibited DEVD-AFC substrate cleavage by caspases-3 and -7, whereas the C-terminal fragment of XIAP containing the RING domain did not.

Next, fragments of XIAP containing each of the individual BIR domains (Fig. 1) were expressed in bacteria, purified, and tested for inhibition of recombinant caspases-3 and -7 in vitro. Of the three recombinant BIR domains, only BIR2 prevented DEVD-AFC substrate hydrolysis by caspases-3 and -7 (Fig. 2, C and D). However, because less than half of the purified BIR3 domain appeared to be intact, an additional BIR3-containing fragment of XIAP, which proved to be more stable and which combined BIR1 and BIR3 (Fig. 1), was also produced and tested. This BIR1+3 fragment of XIAP was ineffective at inhibiting caspases-3 or -7 in vitro, whereas BIR1+2 and BIR2+3 fragments that contained BIR2 completely suppressed hydrolysis of DEVD-AFC under the conditions of these in vitro protease assays (not shown).

Determination of the apparent Ki for inhibition of recombinant caspases-3 and -7 by the BIR2 fragment of XIAP (residues 124–260) produced values of 1–5 nM, thus demonstrating potent suppression of these proteases (Table I). Similarly, the BIR1+2 and BIR2+3 fragments of XIAP that contained the BIR2 domain also inhibited caspases-3 and -7 with Ki values of 1–3 nM. Thus, although not as potent as the full-length XIAP molecule that has been reported to inhibit these proteases with Ki values of 0.2–0.7 nM (10), the BIR2 domain nevertheless retains substantial anti-caspase activity. By comparison, the commonly used tetrapeptide inhibitor DEVD-aldehyde has been reported to inhibit caspases-3 and -7 with Ki values of 0.5–3.5 nM (17–19).

In addition to exploring their ability to inhibit the activity of caspases, the binding of recombinant caspases-3 and -7 to BIR2 and other fragments of XIAP was examined using His6-tagged caspases immobilized on Ni2+-Ni2+ resin and 35S-labeled in vitro translated XIAP fragments. All BIR2-containing fragments of XIAP, including BIR2, BIR1+2, and BIR2+3, bound to immobilized caspases-3 and -7 (Fig. 3A). In contrast, fragments of XIAP lacking BIR2, including BIR1 and BIR1+3, failed to bind these caspase mutants. Note that under these in vitro conditions where high concentrations of proteases are involved, caspase-3 cleaved the BIR2-containing fragments of XIAP, causing slightly faster migration of the 35S-labeled XIAP protein fragments in gels, whereas caspase-7 did not cleave these same XIAP fragments (Fig. 3A). Thus, cleavage does not necessarily correlate with enzyme inhibition. Moreover, no evidence has
been obtained to date indicating that XIAP cleavage by caspases occurs under physiological circumstances in intact cells. The specificity of the in vitro binding results was confirmed by experiments in which other His 6-tagged, recombinant proteins, including caspase-6, caspase-8, and TRAF-3 were tested and found not to bind the BIR2-containing fragment of XIAP (Fig. 3B).

Recently, it has been demonstrated that release of cytochrome c from mitochondria occurs during apoptosis and results in caspase activation by inducing formation of a protein complex containing Apaf-1 and pro-caspase-9 (reviewed in Ref. 21). Moreover, we have found that XIAP, cIAP-1, and cIAP-2 can bind and inhibit pro-caspase-9, preventing it from becoming processed when exposed to cytochrome c and Apaf-1 (24). We therefore tested whether BIR2 was sufficient for preventing cytochrome c-induced processing of pro-caspase-9 in cytosolic extracts. As shown in Fig. 4, full-length XIAP and the BIR2-containing fragment of XIAP completely blocked cytochrome c-induced processing of 35S-labeled in vitro translated pro-caspase-9 in cytosolic extracts, whereas BIR1, BIR3, and RING-containing fragments of XIAP were ineffective. Thus, the BIR2 domain of XIAP appears to be sufficient for blocking the release of cytochrome c from mitochondria and caspase activation.

![FIG. 2. In vitro inhibition of recombinant caspases-3 and -7 by BIR2 domain of XIAP.](image)

![FIG. 3. In vitro binding of BIR2 domain of caspases-3 and -7.](image)

![FIG. 4. BIR2 of XIAP blocks cytochrome c-induced processing of pro-caspase-9.](image)

### Table I: Apparent inhibition constants ($K_i$ app (nM)) for GST-XIAP fragments measured against caspases-3 and -7

|       | $K_i$ (nM) |
|-------|------------|
| Caspase-3 | 3.1 ± 0.2  |
| Caspase-7 | 1.3 ± 0.1  |

![FIG. 5. Typical effects of BIR domains on caspase activity.](image)
A Single BIR Domain of XIAP Inhibits Caspases

Fig. 5. BIR2 of XIAP blocks Fas-induced apoptosis. 293 cells were transiently transfected with 0.5 μg of pEGFP (Vector), 1 μg of pcDNA control plasmid, or 1 μg of pcDNA-Fas plasmid together with either 5 μg of pcDNA3 or pcDNA3-myc-XIAP (full-length), myc-XIAP (BIR1+2+3), or myc-XIAP (BIR2). One day later, cells were recovered, and the percentage displaying apoptotic morphology was determined by DAPI staining (mean ± S.E.; n = 3) among GFP positive cells (A), or lysates were prepared for immunoblot analysis of 25 μg of total protein using anti-Myc antibody and an emission chemiluminescence-based detection method (B).

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detection method (BIR2). (a) Using anti-Myc antibody and an emission chemiluminescence-based method (B).

earliest step in the apoptotic pathway triggered by cytochrome c, i.e. processing of procaspase-9.

The effects of the BIR2 portion of XIAP on apoptosis in cells were examined by expressing this or other regions of XIAP in 293 cells, which were induced to undergo apoptosis by overexpression of Fas. Transient transfection of 293 cells typically resulted in over half the cells becoming apoptotic, as determined by DAPI staining and UV microscopic analysis (Fig. 5A). In contrast, co-expressing Fas with plasmids producing Myc-tagged full-length XIAP, BIR1+2+3, or BIR2 alone significantly reduced the percentage of transfected cells undergoing Fas-induced apoptosis. Although the BIR2 fragment was less potent at suppressing Fas-induced apoptosis than full-length XIAP or the BIR1+2+3 fragment of XIAP, it also may have been produced at lower levels in 293 cells (Fig. 5B). In contrast, co-expression of Fas with plasmids producing Myc-tagged fragments of XIAP that lacked BIR2, including myc-BIR1 and myc-BIR1+3, failed to suppress apoptosis (not shown). These BIR2-lacking XIAP mutants, however, were produced at levels comparable with or greater than those of myc-BIR2, based on control transfections experiments using 293 cells that were not induced to undergo apoptosis by Fas (data not presented). Taken together, these findings support the idea that the BIR2 region of XIAP is necessary and sufficient for inhibiting caspases and cellular apoptosis.

Although most IAP family proteins contain two or three copies of the BIR domain, the data provided here suggest that not all BIRs are equivalent in their ability to bind and inhibit caspases. In XIAP, it appears that only the second BIR domain is capable of inhibiting active caspases-3 and -7. Despite their extensive amino acid sequence similarity to BIR2, the BIR1 and BIR3 domains did not inhibit these caspases. Although the issue of protein folding when dealing with mutants is always a potential concern, these findings nevertheless suggest that different BIR domains within IAP family protein may play unique roles in the suppression of cell death. An intriguing idea is that different BIRs may target different subsets of caspases, although no direct evidence for this is yet available. Alternatively, it has been suggested that BIR domains represent a novel type of protein interaction domain. Miller and colleague have noted interactions of the baculovirus IAP family proteins with the Drosophila cell death proteins reaper and doom (22, 23), neither of which are caspases. Efforts to identify the targets of each of the individual BIR domains within IAP family proteins and to understand the structural basis for their differential inhibition of caspases will provide additional insights into the mechanisms by which IAPs suppress apoptosis.
A Single BIR Domain of XIAP Sufficient for Inhibiting Caspases
Ryosuke Takahashi, Quinn Deveraux, Ingo Tamm, Kate Welsh, Nuria Assa-Munt, Guy S.
Salvesen and John C. Reed

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