Sequence Requirements for Hid Binding and Apoptosis Regulation in the Baculovirus Inhibitor of Apoptosis Op-IAP

Hid Binds Op-IAP in a Manner Similar to Smac Binding of XIAP*

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It has been suggested that the Drosophila Hid protein interacts with the baculovirus Op-IAP protein in a manner similar to that of human Smac binding to XIAP, based largely on amino acid sequence homology. However, there is little direct experimental evidence in support of this hypothesis; indeed, evidence exists from previous studies suggesting that the mode of binding is not similar. We have now precisely mapped the interaction between Hid and Op-IAP, and we show clearly for the first time that the biochemical interactions between the amino terminus of Hid and BIR2 of Op-IAP are highly similar to those found between the processed amino terminus of Smac and BIR3 of XIAP. Also similar to Smac, the amino terminus of Hid must be processed to bind Op-IAP. In addition, our data also suggest that a second interaction between Hid and Op-IAP exists that does not involve the amino terminus of Hid, which may explain some of the earlier contradictory results. The evolutionary conservation of this mechanism of binding underscores its importance in apoptotic regulation. Nevertheless, interaction with Hid is not sufficient for Op-IAP to inhibit apoptosis induced by Hid overexpression or by treatment with actinomycin D, indicating that Op-IAP to inhibit apoptosis induced by Hid overexpression or by treatment with actinomycin D, indicating that Op-IAP inhibits caspase-3, whereas the third BIR domain itself binds and inhibits caspase-9 (4–6). Various BIR domains and/or their flanking sequences have been shown to mediate different protein-protein interactions, including self-oligomerization (7), binding, and inhibition of certain caspases (8), and binding to a number of other proteins including the Drosophila pro-apoptotic proteins Hid, Reaper, and Grim (9, 10), as well as the mammalian pro-apoptotic protein Smac/DIABLO (11, 12), among others.

Most cellular IAP proteins, such as DIAP1 from Drosophila and the human proteins XIAP, c-IAP1, and c-IAP2, appear to be able to block apoptosis by binding and inhibiting certain caspases directly, although compelling evidence for additional mechanisms also exists, including E3 ubiquitin-protein ligase activity and interaction in signal transduction pathways (reviewed in Refs. 13 and 14). Expression of the baculovirus Op-IAP protein has been shown to inhibit the processing of a caspase called Sf-caspase-1 in the lepidopteran cell line SF21, a cell line derived from the fall armyworm, Spodoptera frugiperda (15). The processing of Sf-caspase-1 is presumably carried out by an as yet unidentified apical caspase in SF21 cells that has been termed Sf-caspase-X (16). However, even though Op-IAP inhibits the processing of Sf-caspase-1, it has yet to be shown that a direct caspase interaction is responsible for the inhibitory action of Op-IAP. Genetic evidence from Drosophila supports a model wherein Hid, Reaper, and Grim induce apoptosis by binding to the IAP protein DIAP1 and displacing bound caspases, thereby initiating caspase activation (17, 18). Similarly, the mammalian Smac protein appears to induce apoptosis by displacing caspase-9 from XIAP (19).

Recently, the crystal structure of the complex formed by Smac and the BIR3 domain of the human XIAP protein was determined (20, 21). The amino terminus of Smac is processed upon translocation into mitochondria, revealing a sequence element with homology to the amino terminus of Hid, Reaper, and Grim. The first four amino acids of the processed Smac amino terminus bind in a groove within the core BIR3 domain of XIAP, whereas a separate region of Smac is also involved in an additional site of interaction with BIR3. The similarity of the first four amino acids of the processed amino terminus of Smac (Ala-Ala-Pro-Ile) to amino acids 2–5 of Hid (Ala-Ala-Pro-Phe) led to the suggestion that Hid may be post-translationally processed by a methionine aminopeptidase, and the amino terminus of Hid may bind to IAPs by a similar mechanism to

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† The abbreviations used are: IAP, inhibitor of apoptosis; BIR, baculovirus IAP repeat; eGFP, enhanced green fluorescent protein; HA, influenza hemagglutinin epitope tag.
that of Smac. In support of this hypothesis, a peptide lacking the initiating methionine of Hid bound to BIR3 of XIAP, but a peptide containing the initiating methionine did not bind (20). The recent description of the structure of BIR2 of DIAP1 bound to amino-terminal Hid, Reaper, and Grim peptides further indicates a conserved mechanism of binding in insect and mammalian proteins (22). However, a different study indicated that Hid bound to DIAP1 in vitro despite the fact that additional protein sequences, consisting of a glutathione S-transferase tag used for purification, were fused to the amino terminus of Hid, presumably preventing normal amino-terminal processing (18).

Op-IAP has been shown to bind Hid, Reaper, and Grim and inhibit apoptosis stimulated by overexpression of these proteins (9, 10). Previous work by Vuic et al. (23) showed that the region of Op-IAP containing BIR2 and its flanking sequences was necessary and sufficient to co-immunoprecipitate Hid and block Hid-induced apoptosis in Sf21 cells, although the ability to inhibit apoptosis was less efficient than full-length Op-IAP. Furthermore, a sequence required for Hid binding was mapped by deletion analysis to a region encompassing residues 174–190 of Op-IAP, including a portion of the carboxyl-terminal end of BIR2 and some of its downstream flanking sequence. Interestingly, however, this deleted region in BIR2 of Op-IAP does not include the residues that correspond to those in BIR3 of XIAP responsible for interacting with the amino terminus of Smac, as determined by crystallography. In addition, a second deletion mutant lacking residues 157–173 of Op-IAP, which does lack the residues corresponding to those shown in XIAP to bind to Smac, still co-immunoprecipitated with Hid (23). These results, along with those indicating that Hid can bind DIAP1 in vitro without processing at its amino terminus (18), seemed to contradict the hypothesis that Hid binds IAP proteins in a manner similar to that of Smac. Therefore, we decided to investigate the interaction between Hid and Op-IAP more closely.

EXPERIMENTAL PROCEDURES

Cell Line—S. frugiperda IPLS-Sf21 (Sf21) cells were maintained in TC-100 medium (Invitrogen) supplemented with trypton broth and 10% fetal bovine serum (obtained from either BioWhittaker or Invitrogen). Cells were incubated at 27 °C and propagated as described previously (24).

Plasmid Construction—All plasmids used in this study were constructed using a backbone plasmid containing a Drosophila hsp70 promoter that was previously described (25). Plasmids expressing carboxy-terminal FLAG-tagged Hid, loc2, HA-epitope tagged Op-IAP, Op-IAPR21A, Op-IAPG42A, Op-IAPC54A, Op-IAPW64A, Op-IAPR114A, Op-IAPC148A, Op-IAPH168A, Op-IAPG42AW64A, Op-IAPR21AR114A, Op-IAP (Δ106–109), Op-IAP (Δ157–173), Op-IAP (Δ174–190), Op-IAPBIR1, and Op-IAPBIR2, have been described previously (10, 23, 25). The Op-IAP hybrid constructs were created by two-step PCR using primers containing ApaI and EcoRI sites to allow cloning into the heat shock vector. All PCR products were gel-purified with the Geneclean III kit (Bio101), digested with ApaI and EcoRI, and cloned into the pHSeOpIAPV1 vector after the coding sequence of Op-IAP was removed by digesting with ApaI and EcoRI. This allowed for all inserts to be cloned in frame with the HA tag. Point mutations were constructed using the Quikchange kit (Stratagene). The nucleotide sequences of all constructs, including those previously described, were verified before use.

For bacterial expression of OpIAP BIR2, coding sequence encompassing residues 95–190 of Op-IAP, including an amino-terminal HA tag, was cloned into pET-15b (Novagen). This produced a protein containing a His6 and HA tag amino-terminal to BIR2 with its flanking sequences. For expression and Purification of Recombinant BIR2, BL21 (DE3) pLysS cells transformed with pET-15b-BIR2 were used to inoculate 1.5 liters of Luria Broth (LB) containing 100 μg/ml ampicillin. This culture was grown to an OD600 of 0.5 at 37 °C, and protein expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.4 mM for 3 h at 25 °C. The culture was then centrifuged at 10,000 × g for 10 min at 4 °C and the pellet stored overnight at −80 °C to facilitate cell breakage. Once thawed the cells were resuspended in 40 ml of chilled Extraction/Wash Buffer and purified by affinity chromatography using Talon (CLONTECH) metal affinity resin following the batch/gravity-flow protocol provided in the Talon Metal Affinity Resin User Manual (CLONTECH).

Hid Protection Assay—In the Hid protection assay, 6-well plates were seeded at a density of 5 × 10⁶ Sf21 cells/well and transfected with 1 μg of the indicated plasmid by lipid-mediated transfection. A lacZ-expressing plasmid was used as a negative control. 20 h after transfection cells were heat-shocked at 42 °C for 30 min to induce expression. After heat shock, cells were placed back at 27 °C. Four hours after the beginning of heat shock, the media were removed and replaced with fresh media containing actinomycin D to a final concentration of 500 ng/ml. The cells were incubated with actinomycin D for 10 h at 27 °C and then stained with 1 μg/ml Hoechst 33258 dye (Molecular Probes), and all non-apoptotic nuclei were manually scored by fluorescence microscopy. The number of non-apoptotic nuclei were determined and compared with the number of non-apoptotic nuclei of lacZ-transfected controls that were not treated with actinomycin D (set at 100%), and a percent was generated that represented cell viability. This number was then subtracted from 100% and presented as percent apoptosis to be consistent with the remaining figures. Transfection efficiency was monitored in every experiment by transfection of an eGFP-expressing plasmid, and the average transfection efficiency for all experiments was 67%.

In the Hid protection assay, 2 μg of the pHidflagHis plasmid was cotransfected with either 2 μg of wild type Op-IAP plasmid or the full-length Op-IAP point mutant plasmids. Alternatively, 2 μg of the pHidflagHis plasmid was co-transfected with 8 μg of the hybrid mutant Op-IAP plasmids to give a 4:1 concentration ratio of mutant Op-IAP to Hid plasmid. Where necessary, an eGFP-expressing plasmid was used to balance the amount of DNA in each transfection. Twenty hours after transfection cells were heat shocked as described above and then incubated at 27 °C for 1.5 h. At 1.25 h following the beginning of heat shock, cells exhibiting blebbing were scored as apoptotic. For both types of protection assays, 3 randomly chosen high power fields were counted per well, using 2 wells per condition per assay, and each assay was independently repeated at least 3 times.

Hid Peptide Precipitation Assay—To determine whether bacterially expressed BIR2 could bind to Hid, 10 μl of a 1 mg/ml solution of a peptide representing amino acids 2–11 of Hid with a carboxyl-terminal lysine residue and biotin label (AVPFYLFEGGK-biotin) was added to 200 μl of a 10% slurry of streptavidin-conjugated agarose beads (Sigma) in Nonidet P-40 lysis buffer (50 mM Tris·HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride) and gently rocked for 1 h with the beads at 4 °C to allow the peptide to bind to the beads. A similar peptide that included the initiating methionine (MAVPVYLFEGGK-biotin) was used as a control. Peptides were synthesized and purified to ≥95% purity by Sigma. The beads were washed once with 1 ml of Nonidet P-40 lysis buffer to remove any unbound peptide, followed by the addition of 5 μl of biotin. 15 μl of bacterially expressed BIR2 protein was added to each of the three tubes and incubated with gentle rocking overnight at 4 °C. The following day the beads were washed six times with 1 ml of Nonidet P-40 lysis buffer. Protein loading buffer and 1% 2-mercaptoethanol were added, and the mixture was heated to 95 °C to elute the complex from the beads. Proteins were analyzed by immunoblotting with anti-HA.11 mouse monoclonal antibody (Babco) and goat anti-mouse IgG horseradish peroxidase-conjugated antibody. Bound antibody was detected using SuperSignal West Pico chemiluminescent reagent (Pierce).

To analyze binding of proteins expressed in Sf21 cells, 6-well tissue culture plates were seeded with 5 × 10⁶ cells/well, and 2 wells each were transfected with 5 μg of each of the respective plasmids and heat-shocked as above. Hid peptide (1 μl of a 1 mg/ml solution) was added to streptavidin-conjugated beads and incubated for 1 h at 4 °C. The beads and peptide were then washed once with 500 μl of Nonidet P-40 lysis buffer, and Nonidet P-40 lysis buffer (600 μl) was added to the beads and incubated with rocking at 4 °C until use. Three hours following the beginning of heat shock, the cells were harvested in 100 μl/well of Nonidet P-40 lysis buffer. The cells of two wells of similar conditions were combined and lysed for 30 min at 4 °C. After cell lysis, the cells were washed with 100 μl of fresh wash for 10 s and 100 μl of wash buffer for 30 s at 4 °C, to remove cellular debris. Forty μl of the clarified sample was removed from each sample and transferred to a clean tube, followed by addition of protein loading buffer and 1% 2-mercaptoethanol. The samples were stored at −20 °C until analysis by immunoblotting. The remaining 160 μl of clarified supernatant from each tube was added to the beads with the bound Hid peptide and allowed to rock gently at 4 °C overnight. The
Hid Binding and Apoptosis Regulation by Op-IAP

RESULTS

Op-IAP Binds Directly to the Processed Amino Terminus of Hid—It was shown previously that Op-IAP co-immunoprecipitates with overexpressed Hid in SF21 cells and that this interaction involves the BIR2 domain of Op-IAP and the amino terminus of Hid (10, 23). However, direct binding of Op-IAP to Hid has not been demonstrated previously, because co-immunoprecipitation experiments from cell lysates do not exclude the possibility that other proteins are involved in the interaction. Thus, we developed a Hid binding assay that does not rely on proteins expressed in eukaryotic cells. Although full-length Op-IAP proved to be highly insoluble when overexpressed in bacteria, we were able to bacterially express and purify the BIR2 domain of Op-IAP, along with sequences flanking the BIR domain (amino acids 95–199), containing a His6 sequence and an HA epitope tag at the amino terminus. The purified recombinant BIR2 protein was used in binding assays with two carboxyl-terminally biotinylated peptides consisting of either amino acids 1–11 or 2–11 of the Drosophila Hid protein (Fig. 1A). In this assay, recombinant BIR2 protein bound specifically to the peptide lacking the initiating methionine (amino acids 2–11; hereafter called the Hid peptide), but there was no detectable binding to the peptide containing the initiating methionine (amino acids 1–11; hereafter called the Met-Hid peptide). The results of this assay demonstrate a direct and specific interaction between BIR2 of Op-IAP and the processed amino terminus of the Hid protein.

Similar to the bacterially expressed BIR2 protein, full-length Op-IAP protein expressed in SF21 cells also bound to the Hid peptide (Fig. 1B). Interestingly, however, Op-IAP expressed in SF21 cells also appeared to bind the Met-Hid peptide, in contrast to the results obtained with bacterially expressed and purified BIR2 protein. To explain these contradictory results, we hypothesized that the methionine residue on the Met-Hid peptide was being removed by methionine aminopeptidase activity in the SF21 cell lysate during incubation with the Met-Hid peptide. To test this hypothesis we added EDTA to the lysate, which would be expected to inhibit this class of proteases. As predicted, the addition of EDTA to the lysate greatly reduced binding of Op-IAP to the Met-Hid peptide but did not affect binding to the Hid peptide, consistent with the presence of methionine aminopeptidase activity in the lysate (Fig. 1B). Thus Op-IAP expressed in SF21 cells bound specifically to the processed amino terminus of Hid, as did the BIR2 domain with its flanking sequences expressed in SF21 cells (Fig. 1C). As expected based on previous results, the BIR1 and RING domains of Op-IAP, along with the negative control chloramphenicol acetyltransferase protein, did not bind to the Hid peptide (Fig. 1C). The Drosophila DIAP1 protein, which has also been reported to co-immunoprecipitate with Hid (10) and bind Hid in vitro (17, 18), also bound to the Hid peptide (data not shown).

The Conserved Residues in the BIRs of Op-IAP Are Important for Anti-apoptotic Function—To assess the role of the invariant conserved residues of the BIR region in Hid binding and anti-apoptotic function, we determined the effect of mutating a number of these residues to alanine (Fig. 2A). Furthermore, two deletion mutants were constructed by deleting either BIR1 or BIR2 in the context of the entire Op-IAP protein (Fig. 2B). We then assayed whether expression of these mutated proteins could protect SF21 cells against different apoptotic stimuli. Expression of the mutated proteins was verified by immunoblotting (Fig. 3C), whereas transfection efficiency was monitored in each assay using a similar vector expressing eGFP and determining the percentage of fluorescent cells. It should be noted that the level of apoptosis observed upon transfection of wild type Op-IAP was essentially the same as the number of untransfected cells in this assay, suggesting that virtually all of the cells expressing wild type Op-IAP were protected.

SF21 cells were transiently transfected with constructs expressing the respective point mutants, treated with actinomycin D to induce apoptosis, and apoptotic cell death was quantified (Fig. 3A). All of the conserved residues mutated in both BIRs of Op-IAP were important for the anti-apoptotic function of this protein in the context of actinomycin D-induced death, with the notable exception of the C148A mutation, which for unknown reasons retained partial activity. The construct lacking BIR1 (B1D) also retained partial activity in this assay, whereas deletion of BIR2 (B2D) led to a complete loss of pro-

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tection, suggesting that BIR2 is more important than BIR1 for protection against actinomycin D-induced apoptosis. However, whereas BIR2 appears to be crucial, the loss of BIR1 also reduced the ability of Op-IAP to protect against actinomycin D, indicating that BIR1 also contributes to protection against this death stimulus.

To test the ability of the mutated proteins to inhibit another death signal that is less potent than actinomycin D, we co-transfected the mutated versions of Op-IAP with a vector expressing full-length \textit{Drosophila} \textit{Hid} at a ratio of 1:1 (Fig. 3B).

In this assay, a greater difference was observed between point mutations in BIR1 and BIR2 than in the actinomycin D assay. Point mutations in BIR1, such as R21A, G42A, C54A, W64A, and the double mutant G42A/W64A, largely retained the ability to protect against \textit{Hid}-induced death, whereas point mutations in BIR2, such as R114A, C148A, and H168A, showed significantly less protection, including the double mutation R21A/R114A, which behaved like a single mutation in BIR2 (compare R21A/R114A with R21A and R114A). Consistent with these results, deletion of BIR1 (B1D) had no effect on the ability of Op-IAP to protect against \textit{Hid}-induced death, whereas deletion of BIR2 (B2D) resulted in decreased protection. However, deletion of BIR2 (B2D) did not result in total loss of anti-apoptotic activity when compared with the vector control, suggesting that BIR1 and/or the RING have some effect on \textit{Hid}-induced apoptosis. Similar to the actinomycin D results, these results also suggest that BIR2 is more important than BIR1 in the anti-apoptotic activity of Op-IAP and corroborates results published previously (23).

We next correlated the anti-apoptotic activity of the mutated Op-IAP proteins with their ability to bind to the \textit{Hid} peptide (Fig. 3C). Immunoblot analysis of whole cell lysates showed that all of the mutant proteins were expressed at equivalent levels. All of the proteins containing mutations in invariant residues of BIR1 or BIR2 bound the \textit{Hid} peptide, with the exception of R21A/R114A and C175A. In addition, reduced binding was observed for the C54A and H168A constructs. The C175A, C54A, and H168A constructs all contain mutations in zinc-coordinating residues in either BIR1 or BIR2, which might be expected to disrupt the folding of these domains. However, the fact that most of the mutant proteins still bound the \textit{Hid} peptide indicates that they were not completely unfolded or misfolded as a result of the introduced mutations. As expected, B1D, which lacks the entire BIR1 domain, still bound the \textit{Hid} peptide as well as wild type Op-IAP, whereas B2D (lacking BIR2) did not bind the \textit{Hid} peptide (Fig. 3C).
The BIR2 Flanking Regions Do Not Confer Anti-apoptotic Activity to BIR1—Deletions in either of the sequences flanking BIR2 of Op-IAP (deletion of either residues 106–109 or 174–190) were shown previously to eliminate the protective function of BIR2 against Hid (23). The 174–190 deletion also eliminated Hid binding, suggesting that the carboxyl-terminal flanking sequence of BIR2 may be involved in binding Hid (23). Based on these previous results, we wondered whether the sequences flanking BIR2 are what are important for conferring anti-apoptotic function to BIR2, and whether the BIR2 flanking sequences could confer anti-apoptotic activity or Hid binding onto another BIR core sequence. To test this, we created hybrid constructs that contained the core sequence of BIR1 surrounded by either one or both of the flanking sequences of BIR2 (BIR1 N2, BIR1 C2, or BIR1 NC2; Fig. 2B), and we then tested whether expression of these hybrid BIRs could protect Sf21 cells against Hid-induced apoptosis, and whether the BIR2 flanking sequences could confer anti-apoptotic activity or Hid binding onto another BIR core sequence. To test this, we created hybrid constructs that contained the core sequence of BIR1 surrounded by either one or both of the flanking sequences of BIR2 (BIR1 N2, BIR1 C2, or BIR1 NC2; Fig. 2B), and we then tested whether expression of these hybrid BIRs could protect Sf21 cells against Hid-induced apoptosis, and whether the BIR2 flanking sequences could confer anti-apoptotic activity or Hid binding onto another BIR core sequence. 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was not completely disrupted. In addition, the ability of the BIR2 hybrid constructs to bind the Hid peptide indicates that binding to the processed amino terminus of Hid is solely a property of the core BIR2 domain and does not require specific sequences in the flanking regions. From these results we conclude that both the BIR2 core sequence and the sequences flanking BIR2 are required to block Hid-induced apoptosis, but neither are sufficient for inhibitory activity. Instead, both the core and flanking sequences of BIR2 are required in tandem to inhibit Hid-induced apoptosis. We also conclude that the structural elements required to bind the amino terminus of Hid are confined to the core sequence of BIR2, and that simply binding Hid is not sufficient to block Hid-induced death.

Evolutionary Conservation of Hid-Op-IAP Versus Smac-XIAP Interactions—The processed mammalian protein Smac shares a short region of homology with Hid, Reaper, and Grim at their amino-terminal ends, and the first four residues in Smac (Ala-Val-Pro-Ile) have been shown to contact a groove in BIR3 of XIAP, resulting in a stable interaction (20, 21). Five residues lining this groove in BIR3 of XIAP were predicted to be important in creating the chemical interactions with the first four amino acids of processed Smac (21). After comparing BIR2 of Op-IAP to BIR3 of XIAP, we found that these same five residues in BIR3 of XIAP (Gly-306, Leu-307, Trp-310, Glu-314, and Trp-323) were either identical or chemically similar in BIR2 of Op-IAP (Fig. 2A). Furthermore, by comparing the BIR1 and BIR2 sequences of Op-IAP, we discovered that only one of these five residues was not conserved in BIR1. This residue is Gly-306 in BIR3 of XIAP, Gly-154 in BIR2 of Op-IAP, and Glu-60 in BIR1 of Op-IAP. Therefore, we hypothesized that mutating residue Glu-60 in BIR1 of Op-IAP to a glycine might allow Hid binding to BIR1. However, when this mutation was constructed in OpIAP BIR1 (BIR1 E60G), it did not bind the Hid peptide or protect against Hid-induced apoptosis (Fig. 6, A and B). We further hypothesized that in order for this E60G change to have an effect on Hid-induced death, it would have to be made in the BIR1 NC2 construct because the presence of the BIR2 flanking sequences may be required for anti-apoptotic activity based on the results in Figs. 4 and 5. However, the BIR1 NC2 E60G construct also could not bind Hid or protect against Hid-induced apoptosis (Fig. 6, A and B). Thus, simply changing the aspartate residue at position 60 to glycine was not enough to confer Hid binding or anti-apoptotic activity to BIR1. Interestingly, however, when the equivalent glycine residue at position 154 in BIR2 was mutated to a glutamate (BIR2 G154E), there was complete loss of anti-apoptotic activity compared with the BIR2 control, accompanied by loss of binding to the Hid peptide (Fig. 6, A and B). Similar results were observed when the same change was made in BIR2 NC1 (data not shown). These results suggest that the amino terminus of Hid binds Op-IAP in a manner very similar to that of Smac binding to XIAP but that there must be other differences between BIR1 and BIR2 in addition to Glu-60 that preclude Hid binding to BIR1.

Given these results, we decided to take a closer look at the
Sf21 cells transfected with the indicated constructs were lysed, and protein was analyzed for binding to the Hid peptide as described in Fig. 1. Constructs were transfected into Sf21 cells at a 4:1 ratio of Op-IAP:Hid, and the average percentage of apoptotic cells (± S.E.) was determined. As shown in Fig. 6, Sf21 cells transfected with the indicated constructs were lysed, and protein was analyzed for binding to the Hid peptide as described in Fig. 1. Whole cell lysates; Neg. control, no peptide.

The amino terminus of Hid binds to Op-IAP by a mechanism similar to that of Smac binding to XIAP. A, the indicated constructs were transfected into Sf21 cells at a 1:1 ratio of Op-IAP:Hid, and the average percentage of apoptotic cells (± S.E.) was determined. B, Sf21 cells transfected with the indicated constructs were lysed, and protein was analyzed for binding to the Hid peptide as described in Fig. 1. Whole cell lysates; Neg. control, no peptide.

The core sequence of BIR2 is required for binding to the processed amino terminus of Hid, whereas the sequences flanking the core BIR2 domain are not. However, the sequences flanking BIR2 are required for blocking Hid-induced apoptosis. Interestingly, the mechanism of Hid binding to Op-IAP appears to be very similar to that used by the mammalian proteins Smac and XIAP, involving a residue (Gly-154 in Op-IAP and Gly-306 in XIAP) that lines a groove in XIAP BIR3 that contacts the processed amino terminus of Smac. These results further support the hypothesis that Smac is the functional homolog of Hid in mammalian cells (11, 12). This extraordinary conservation of structure and function between insect and mammalian death machinery underscores the importance of this interaction in apoptosis regulation.

We have shown for the first time that the invariant conserved residues in both BIRs of Op-IAP are important for Op-IAP protection against actinomycin D-induced apoptosis, although the level of importance varied somewhat from residue to residue. Mutation of Cys-148 in BIR2 to alanine resulted in only a partial loss of anti-apoptotic function in the actinomycin D assay. This was unexpected because Cys-148 is predicted to be involved in zinc coordination, and we expected that mutation of this residue would have drastic effects on the structural integrity of BIR2. However, the fact that proteins bearing mutations in the zinc-binding residues Cys-148 and His-168 still bound the Hid peptide (Fig. 3C) and still co-immunoprecipitate with full-length Hid (23) leads us to the rather surprising conclusion that mutation of individual zinc-coordinating residues does not necessarily completely disrupt the structure of a BIR. However, mutations in Cys-175 and Gly-154 did eliminate binding to the Hid peptide, indicating that these residues are either important in the interaction between Hid and Op-IAP or that their substitution perturbs the structure of the Hid-binding site in BIR2. Cys-175 is a zinc-coordinating residue, but it is also closer in the predicted structure to the groove predicted to bind the amino terminus of Hid than the other zinc-binding residues in BIR2, and thus mutation of Cys-175 may disturb the folding of the Hid-binding groove more than mutation of the other zinc-coordinating residues in BIR2. Gly-154 is interesting because it is not conserved in BIR1 of Op-IAP, and it is also one of the residues predicted to be a crucial difference between the BIRs of XIAP, because it is not conserved in BIR1 or -2 of XIAP. From their crystallography data, Wu et al. (21) predicted that the residue in XIAP equivalent to Gly-154 (Gly-306) is required to allow tight packing of an isoleucine residue at position 4 of Smac into the groove in BIR3 of XIAP. The equivalent residue in position 4 of Hid is a phenylalanine. Mutation of Gly-154 to...
a glutamate would be predicted to prevent the phenylalanine in position 4 of Hid from fitting into the groove, thus disrupting binding. The fact that the G154E mutation eliminated Hid binding indicates that the contacts between the amino terminus of Hid and BIR2 of Op-IAP are similar to those found between Smac and BIR3 of XIAP.

Another interesting observation that arose from the actinomycin D data was that when the conserved residues in BIR1 were individually mutated, all anti-apoptotic function was lost, but when BIR1 was deleted completely, partial function was retained. This could be contributable to protein misfolding in the point mutants, whereas deletion of the entire BIR1 may allow a more natural fold to occur, and thus partial anti-apoptotic function is retained. However, all of the proteins with point mutations in BIR1 (with the exception of the double mutant R21A/R114A, which also has a mutation in BIR2) still bound the Hid peptide, indicating that the mutated proteins were not completely misfolded or unfolded. Whereas deletion of BIR1 reduced protection partially compared with full-length Op-IAP, deletion of BIR2 led to a complete loss of anti-apoptotic function against actinomycin D, indicating that BIR2 plays a key role in protecting against this death stimulus. However, BIR1 also seems to assist in protection against actinomycin D to some degree.

In the context of Hid-induced apoptosis, we found that expression of BIR2, although sufficient to partially inhibit Hid-induced death, did so at a level lower than that of wild type Op-IAP. This result is consistent with previous reports (25, 26) that the RING domain of Op-IAP is required for optimal anti-death function. The weaker ability of BIR2 to inhibit apoptosis is apparently not due to a problem in protein expression, folding, or stability, because the BIR2 construct expressed high levels of protein that still bound the Hid peptide, indicating that the mutated proteins were not completely misfolded or unfolded. Whereas deletion of BIR1 protected as well as wild type Op-IAP (Fig. 3B) further suggests that the RING plays an important role in protection against Hid-induced apoptosis.

It does not appear that Op-IAP is functioning by simply binding and sequestering Hid in this system, given the existence of several mutants that still bind Hid but do not block Hid-induced death. However, the ability to bind Hid does not appear to be necessary for Op-IAP to block apoptosis induced by overexpression of Hid. This is in contrast to results obtained in Drosophila with DIAP1, in which gain-of-function DIAP1 mutants that have reduced Hid binding are actually better at preventing Hid-induced death than wild type DIAP1 (18). The difference between these two systems may lie in part in the fact that we are overexpressing Hid and Op-IAP. However, it must be remembered that Op-IAP is a viral protein that is not normally present in cells, and so in the context of virus infection, Op-IAP is, by definition, overexpressed. Op-IAP therefore may have a function that is somewhat different from that of cellular IAPs. Both the amino- and carboxy-terminal sequences flanking BIR2 play a critical role in inhibition of Hid-induced apoptosis. The function of these sequences is not known, but it is apparently not simply to stabilize BIR2 or facilitate proper folding, given the hybrid constructs (containing the core BIR2 sequence and flanking sequences from BIR1) that still bind the Hid peptide but do not inhibit Hid-induced apoptosis. The function of the BIR2 flanking sequences may include caspase binding and inhibition, but a caspase that binds Op-IAP has not yet been identified.

Bacterially expressed, purified BIR2 protein bound only to the Hid peptide lacking the initiation methionine and not to the Met-Hid peptide. However, Op-IAP expressed in Sf21 cells bound to both peptides. The observation that Op-IAP did not bind to the Met-Hid peptide when methionine aminopeptidase activity was inhibited by EDTA confirms that Hid must be processed, presumably by a methionine aminopeptidase, before it can bind to Op-IAP. This is perhaps not surprising, because it is common for the initiating methionine residue to be removed from eukaryotic proteins by methionine aminopeptidases, especially when the second position is occupied by a small amino acid (27), such as in the case of Hid where the second amino acid is alanine. Nonetheless, although it was postulated previously that Hid must be processed to bind IAPs (15), this is the first experimental evidence that the amino terminus of Hid is processed in cells.

It was shown previously (23) that deletion of amino acids 174–190 in Op-IAP led to loss of Hid binding, suggesting that the flanking region downstream of BIR2 may be involved in the interaction with Hid. Our results with the C175A mutant indicate that the loss of Cys-175 alone in this deletion is sufficient to eliminate binding to the amino terminus of Hid. This combined with the observation that the BIR2 NC1 domain swapping mutant, which contains the core sequence of BIR2 (amino acids 114–178) but the flanking regions of BIR1, still bound the Hid peptide indicates that the ability to bind the amino terminus of Hid is due to the core BIR2 sequence and does not involve the residues flanking BIR2.

In our assay, deletion of amino acids 157–173 from Op-IAP resulted in a complete loss of binding to the Hid peptide. This is in contrast to the co-immunoprecipitation results of Vucic et al. (23), where this deletion did not affect binding to full-length Hid. The difference in these results could be due to the use of full-length Hid protein in the co-immunoprecipitation experiments versus only amino acids 2–11 in our assay. Thus, it is possible that another domain of the Hid protein in addition to the amino terminus contacts Op-IAP at a second site outside of the 157–173 region and that this second interaction is sufficiently strong to detect by co-immunoprecipitation. In support of this, a second contact site between Smac and BIR3 of XIAP was observed in the co-crystal structure in addition to the amino terminus-binding groove (21). This second contact site involved helices H2 and H3 in Smac and helix α1 in BIR3. The equivalent of helix α1 in Op-IAP lies at the amino-terminal end of BIR2, outside of the 157–173 deletion, and so a similar second interaction may also exist between Op-IAP and Hid.

In conclusion, we have shown for the first time that the interaction between Drosophila Hid and baculovirus Op-IAP is highly similar to the interaction between human Smac and XIAP. This interaction is required for the ability of Op-IAP to inhibit Hid-induced apoptosis, but the interaction alone is not sufficient to block death. A complex set of interactions between Op-IAP, Hid, and presumably other as yet unidentified proteins appears to be involved in the anti-apoptotic function of Op-IAP. These unidentified proteins may include caspases, E2 ubiquitin-conjugating enzyme factors, and/or other proteins.

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