**Amsacta moorei** Entomopoxvirus Inhibitor of Apoptosis Suppresses Cell Death by Binding Grim and Hid

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Inhibitor of apoptosis (**iap** genes) have been identified in the genomes of two independent families of insect viruses, the **Baculoviridae** and the **Entomopoxvirinae**. In this report, we examined the functional attributes of the **Amsacta moorei** entomopoxvirus-encoded IAP protein (**AMV-IAP**). The binding specificity of the individual baculoviral IAP repeat (**BIR**) domains of AMV-IAP was investigated by using a random-peptide, phage display library, and sequences similar to the amino termini of proapoptotic **Drosophila** proteins in the Reaper/Hid/Grims were identified. Furthermore, the BIR domains of AMV-IAP protein were demonstrated to bind the mammalian IAP inhibitor Smac through the AVPI tetrapeptide sequence, suggesting that the peptide binding pocket and groove found in the insect and mammalian IAPs is conserved in this viral protein. Interaction analysis implicated BIR1 as the high-affinity site for Grim, while BIR2 interacted more strongly with Hid. Both Grim and Hid were demonstrated to interact with AMV-IAP in vivo, and Grim- or Hid-induced cell death was suppressed when AMV-IAP was coexpressed.

Apoptosis, or programmed cell death, is used as a strategic response to eliminate infected cells and thus limit the replication of viruses within the host. The coevolution of viruses and their hosts has resulted in the development of viral strategies to circumvent this response (3). Multiple unrelated virus families have independently targeted the extrinsic death receptor pathways and the intrinsic mitochondrial pathways. Both extrinsic and intrinsic apoptotic pathways trigger a cascade of caspases activation, which leads ultimately to apoptosis. The extrinsic pathways can be thwarted through the expression of virally encoded soluble death receptors, inducing the down-regulation of cellular death receptors or the blockade of the signal transduction pathway by viral FLICE-like inhibitory proteins. Intrinsic pathway signaling can also be blocked at several points, most frequently involving the expression of functional inhibitors of *p53* or of viral homologs of the Bcl-2 family (3).

Viruses have also developed strategies to target the caspase cascade constituting the ultimate convergence of the extrinsic and intrinsic apoptotic pathways. Several poxvirus-encoded proteins that regulate apoptosis through inhibition of the caspase cascade have been identified (29). Members of the **Poxviridae** family infect a wide range of vertebrates and invertebrates, and are subdivided into the **Entomopoxvirinae** (insect pathogens) and **Chordopoxvirinae** (mammalian and avian pathogens). Among the **Chordopoxvirinae**, cowpox virus encodes a unique serpin (serine protease inhibitor) called the cytokine response modifier A (**CrmA**, also known as **SPI-2**). **CrmA/SPi-2** is able to inhibit a broad range of initiator caspases, including caspase-1, caspase-8, caspase-4, caspase-5, caspase-9, and caspase-10, in order of decreasing affinity, thus inhibiting apoptosis as well as interfering with the host inflammatory reaction (36).

The only definitive virally encoded inhibitors of the terminal, effector caspases are found in members of the **Baculoviridae**. The baculovirus **Autographa californica** M nucleopolyhedrovirus (**AcMNPV**) expresses a very potent terminal caspase inhibitor termed P35. Like **CrmA**, P35 proteins inhibit the activity of caspases by becoming covalently bound to the protease after initially being recognized as a proteolytic substrate. The P35 protein displays a broad spectrum of caspase inhibition, targeting primarily the effector caspases (caspase-3 and caspase-7) (33). **Spodoptera littoralis** nucleopolyhedrovirus also encodes a P35 homolog, termed P49, which inhibits the activation of initiator caspases, including human caspase-9 (27, 43).

The inhibitor of apoptosis (**IAP** proteins) are a distinct family of caspase inhibitors first identified in the baculoviruses **Orgyia pseudotsugata** nucleopolyhedrovirus (**OpMNPV**) and **Cydia pomonella** granulovirus (**CpGV**) (5, 10). Indeed, most, if not all, baculoviruses encode one or more IAPs (18). Subsequent work has identified cellular IAP homologs in mammals, birds, and invertebrates, such as **Drosophila** DIAP-1 and DIAP-2 and the mammalian members NAIP, XIAP, cIAP1, cIAP2, Livin, Survivin, and Ts-IAP (9, 22). The defining characteristic of an IAP is the presence of one or more baculoviral IAP repeat (**BIR**) domains, with many of the family members also possessing a carboxy-terminal RING zinc finger motif. BIR domains fold into a series of four or five α helices and a three-stranded β sheet with a single zinc ion coordinated by conserved cysteine and histidine residues (23).

Functional characterization of the IAPs has been performed most extensively for the mammalian XIAP and the **Drosophila** DIAP1 proteins. The XIAP protein utilizes the BIR3 domain...
to bind and inhibit caspase-9, the key initiator caspase in the apoptosisome complex (31). The BIR2 domain, together with a short upstream region, binds and inhibits the effector caspase-3 and caspase-7, while the BIR1 domain has no known function (6, 28). DIAP1 and the viral IAPs appear to be organized in a very similar way, but they contain only two BIR domains. The most distal DIAP1 BIR, BIR2, binds and inhibits DRONC (40), the Drosophila initiator caspase most similar to caspase-9 (25). Furthermore, the DIAP1 BIR1 domain inhibits drICE and DCP-1 (16, 20), two effector caspses most analogous to caspase-3 and caspase-7 (13, 32).

The carboxy-terminal RING finger domain present in many of the mammalian, insect, and viral IAPs exhibits E3 ubiquitin ligase activity and can catalyze auto-ubiquitination as well as ubiquitination of IAP-interacting proteins (24, 34, 41). In mammalian systems, the significance of an intact IAP RING finger is controversial (7, 30). However, in Drosophila, the DIAP1 RING finger is absolutely required for in vitro suppression of cell death (11, 40).

Aptotic cell death in Drosophila is controlled to a large extent by the expression of a group of proteins known collectively as Reaper/Hid/Grim or IAP binding motif (IBM) proteins. Members of this family include the Drosophila proapo-

Plasmid constructs. The construction of pGEX-XP-BIR3 (amino acids 241 to 356) has been described previously, as has the construction of Ub-Smac and of Ub-SmacΔAVP1 (19). pGEX-AMV-IAP-BIR1 and pGEX-AMV-IAP-BIR2 were generated by PCR amplification of individual BIR domains with the primers 5′-d-ATAAGGCATCTAGGATGACGACCATCAGATCTCCTGCAGTGTG and 5′-d-ATCGAGTTAGGCGTAATCAGGGACATCGATAGGATATCGCGCCGCA.

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**GST fusion protein purification.** Overnight cultures of pGEX-XP-BIR3, AMV-IAP-BIR1, and AMV-IAP-BIR2 were diluted 1:10 in fresh medium and allowed to grow for 1 h prior to induction. Zinc acetate in a final concentration of 50 μM was then added to the cultures along with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and the cultures were induced at 28°C for 2.5 h. Bacterial pellets were resuspended in STE buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, and 10 mM EDTA) containing protease inhibitors (10 μM aprotinin, 100 μM pepstatin A, 10 μM leupeptin, and 100 μM phenylmethylsulfonyl fluoride). Dithiothreitol was added to a concentration of 1 mM, and the pellet was vortexed for 20 min. Lysis at 20°C for 1 h on a rotating platform. Bead-protein complexes were collected and washed four times in NETN buffer (2 mM Tris-HCl [pH 8.0]), 4 mM NaCl, 0.2% Triton X-100, and 0.5 M EDTA). The bead complexes were then suspended in 200 μM of NETN buffer-50 μM zinc acetate. GST fusion proteins were quantified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with bovine serum albumin as a protein standard prior to pull-down analysis. Coomassie blue staining was used to estimate the amount of full-length GST-BIR fusion protein, which in all cases was ≥90%.

**Cell culture and transfection.** HeLa cells were maintained at 37°C and 5% CO₂ in Dulbecco’s minimal essential medium (Gibco BRL) supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL), penicillin, and streptomycin (Gibco BRL). Transfections were performed by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Plasmid DNAs were pre-

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Phage display library screening. A phage library (Ph.D.-7 Phage Display Peptide Library kit; New England BioLabs) expressing seven random amino acids fused to the amino terminus of the mature PIII phage coat protein was used for screening. For each GST-BIR protein, three sequential rounds of affinity purification and reamplification of the phage were carried out, with ≥20 phage isolates sequenced after each round of selection. Affinity purifications were carried out by using 500 ng of GST-BIR fusion protein and ≥(2 × 10^11) phage particles in Tris-buffered saline containing 0.1% Tween-20. Bound phages were washed 10 times in Tris-buffered saline containing 0.1% Tween-20 and eluted with 1 M Tris (pH 9.1), and titers were determined by using Escherichia coli strain ER2738. Random plaques were chosen for phage minipreparations and sequenced to determine the amino acid sequence encoded by the phage. The eluted phage was reamplified to ≥(2 × 10^11) viral particles according to the manufacturer's direction and subjected to the next round of affinity purification.

GST pull-down analysis. Cell lysates for pull-down analysis were prepared by lysing 100-mm-diameter dishes of untransfected, Ub-Smac-transfected, Ub-Grm-transfected, or Ub-Hid-transfected HeLa cells 24 h posttransfection in lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% Triton X-100, and 10 mM EDTA) and protease inhibitors. Lysates were incubated on ice for 20 min and spun at 17,000 × g for 5 min. For pull-down analysis, 100 μg of total cell lysate and 2.5 μg of GST fusion protein were mixed for 2 h at 4°C, and the protein-bead complexes were washed three times in ice-cold lysis buffer. Beads were syringe dried and resuspended in a small volume of 1× Laemmli sample buffer prior to SDS-PAGE.

Immunoprecipitation analysis. HeLa cells were transfected in 100-mm-diameter dishes. At 24 h posttransfection, cells were rinsed twice in phosphate-buffered saline (PBS), and 500 μl of immunoprecipitation buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% CHAPS, 10% sucrose, and 10 mM dithiothreitol) was immediately added to give a final concentration of 10 mM NaCl, 0.1% CHAPS, 3-(3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate, 1 mM EDTA, 10% sucrose, and 10 mM dithiothreitol (caspase assay buffer) and Dounce homogenized on ice. Protein concentrations were determined by using a Bradford assay (Bio-Rad), and volumes were adjusted with caspase assay buffer to give a final concentration of 10 μg of protein/μl. Caspase activity assays were carried out by using 100 μg of protein in caspase assay buffer with the fluorogenic substrate Ac-DEV-AMC (BioMol) in a Polarstar Galaxy (BMG) plate reader. Readings were taken at 2-min intervals for a total of 120 min.

Clonogenic assay. HeLa cells were transfected as described above. At 12 h posttransfection, cells were trypsinized, counted, and plated in triplicate at 250 cells per 60-mm-diameter dish. All dishes were incubated at 37°C in a humidified atmosphere containing 5% CO2. At 10 days postplating, colonies were fixed with 70% ethanol, stained with Coomassie blue, and counted under a dissection microscope. Only those colonies containing at least 50 cells were considered to be viable. Each experiment was repeated a total of three times, and the data were pooled so that n = 9 for each determination. The number of colonies for each transfection is reported as the average of the number of colonies ± the standard deviation. Results were analyzed by using paired two-tailed Student’s t tests. P values of <0.01 were considered statistically significant.

RESULTS

Identification of peptides that bind to AMV-IAP-BIR1 and AMV-IAP-BIR2. To investigate the binding specificity of AMV-IAP, we screened for peptide interactions with AMV-IAP-BIR1 and AMV-IAP-BIR2 by using a phage display library encoding seven random amino acids at the amino terminus of the PIII phage coat protein. XIAP was selected as the positive control for these experiments, since the binding specificity of the XIAP-BIR3 domain has been established (12). Furthermore, we presupposed that the purification and interaction conditions that allow XIAP to remain active would be compatible with the AMV-IAP protein. After each round of affinity purification, 20 independent isolates were sequenced to determine what peptide was displayed by the phage that bound either XIAP-BIR3, AMV-IAP-BIR1, or AMV-IAP-BIR2. First-round screening revealed that ≥50% of the phage isolates encoded peptides initiating with alanine (data not shown). This percentage increased to virtually 100% by the third round of affinity purification, and isoleucine. No significant patterns were discernible in positions 1, 5, or 8 (Fig. 1B). In the caspase-9 IBM) or one of the three positively charged amino acids (histidine, lysine, and arginine, as found in the GSPTI IBM). The P4 position was occupied predominantly by hydrophobic amino acids, including phenylalanine, leucine, and isoleucine. No significant patterns were discernible in positions 5 through 7. Thus, XIAP-BIR3 bound peptides characteristic of established mammalian IBM-containing proteins, all of which exhibit the AXPX tetrapeptide motif (Fig. 1B). In the parallel screening with AMV-IAP-BIR2, a very similar distribution was observed. All 20 of the isolates encoded either AVP or ATP in positions 1 through 3, and all encoded a hydrophobic residue in P4. In contrast to results with XIAP-BIR3, a clear consensus continued through positions 5, 6, and 7 (Fig. 1A). The P5 position was occupied by phenylalanine or tyrosine, as is found in the Drosophila IEMBS Reaper, Grim, Sickle, and Hid. P6 was occupied predominantly by leucine and isoleucine (present in Reaper, Grim, and Hid), while P7 was...
occupied by serine (not found in established IBMs) or proline (found in Reaper, Grim, and Hid) (Fig. 1B).

Library screening with AMV-IAP-BIR1 also yielded almost exclusively alanine-initiating peptides. In contrast to the BIR2 screen, the BIR1-binding peptides contained either alanine (as found in Reaper and Grim) or proline in the P3 position (Fig. 1A). Isoleucine in P2 was clearly favored, as was phenylalanine in P4 and P5, alanine in P6, and proline in P7. Thus, the library screening suggested that AMV-IAP-BIR2 binds preferentially to the amino acid sequences found in Hid, Sickle, or Jafrac2, while BIR1 appears to prefer interaction with Reaper or Grim-like peptide sequences. These results also suggest that amino acids beyond the tetrapeptide motif contribute to the interaction between Drosophila IBM-containing proteins and the BIR domains of AMV-IAP.

AMV-IAP binds mammalian Smac protein via the AVPI tetrapeptide sequence. To confirm the binding specificity of AMV-IAP-BIR domains with IBM-like peptides observed in the phage library, we investigated the specific binding of the BIR domains of AMV-IAP to wild-type Smac and a mutant version of Smac missing the IBM tetrapeptide (f~AVPI-Smac). XIAP-BIR3, AMV-IAP-BIR1, and AMV-IAP-BIR2 were each expressed as GST fusion proteins and purified from E. coli. Lysates were prepared from HeLa cells transfected with either the Ub-Smac or f~AVPI-Smac plasmid. GST fusions and associated proteins were recovered with glutathione-Sepharose beads. The carboxy-terminal HA epitope tag on Smac or mutant Smac was detected by Western blotting with an anti-HA antibody. Interactions with mature Smac protein and not with recombinant protein lacking the amino-terminal IBM was interpreted as correctly folded protein. The expression of Smac and f~AVPI-Smac in whole-cell lysates was confirmed by Western blotting with anti-HA (lower panel).

Ub-Smac-HA fusion protein was rapidly and completely processed (19). The unprocessed Ub-Smac (31.4 kDa) protein was not detectable by Western blotting with anti-HA, and only the processed, 21.6-kDa mature Smac protein was observed (Fig. 2, lower panel). The ΔAVPI-Smac protein was also prepared from cells transfected with a construct in which the codons encoding the first four amino acids of Smac were deleted by site-directed mutagenesis. A pull-down analysis confirmed that our GST fusion proteins were correctly folded and that the wash conditions allowed Smac-BIR interactions. The ΔAVPI-Smac protein failed to bind to XIAP-BIR3 and the AMV-BIR domains, suggesting that the tetrapeptide motif of this IBM protein was critical. Interestingly, Smac bound equally well to XIAP-BIR3 and both of the AMV-IAP BIR domains. In contrast, previous work has shown that XIAP-BIR2 does not bind Smac with the same affinity as BIR3, and BIR1 has no affinity for Smac (17, 19).
cells transfected with these plasmids and used in GST pull-down analysis with full-length AMV-IAP and individual BIR domains as bait. Our results showed that Hid protein binds equally well to full-length AMV-IAP or the isolated BIR2 domain, but appeared to bind less well to BIR1 (Fig. 3A). In contrast, Grim bound equally well to full-length IAP or BIR1 and less well to BIR2 (Fig. 3B). The interaction of full-length AMV-IAP with Hid and Grim was further confirmed in an in vivo coimmunoprecipitation analysis. HeLa cells were transfected with Myc-tagged AMV-IAP and either Ub-Hid-HA or Ub-Grim-HA. Immunoprecipitation of Myc-tagged AMV-IAP showed that AMV-IAP was readily isolated in a complex with either Hid or Grim (Fig. 4).

AMV-IAPs suppress apoptosis induced by Drosophila IBM proteins. In order to demonstrate the functional significance of AMV-IAP interaction with the Drosophila IBM proteins, we next examined whether expression of this IAP would suppress apoptosis induced by Drosophila IBM proteins as indicated by the appearance of apoptotic markers. HeLa cells were transfected with either AMV-IAP, Ub-Hid-HA, or Ub-Grim-HA, or in combination as indicated in Fig. 5. Cell lysates were prepared and examined for markers of apoptosis, including cytochrome c release from the mitochondria, poly(ADP-ribose)-polymerase (PARP) cleavage, and procaspase-3 activation (Fig. 5A). Expression of either Hid or Grim alone was sufficient to trigger apoptosis in this mammalian system, as has been reported previously (8, 15, 37). The expression of AMV-IAP suppressed the appearance of all of these markers of apoptosis. Furthermore, cytosolic extracts were analyzed for caspase-3 activity by using a fluorogenic substrate (Ac-DEVD-AMC) cleavage assay. Both Hid- and Grim-containing extracts exhibited significantly elevated levels of caspase-3 activity, while concomitant expression of AMV-IAP greatly reduced caspase-3 activity (Fig. 5B and C). These results suggested that AMV-IAP can, at least temporarily, suppress apoptosis induced by the expression of Drosophila IBM proteins in HeLa cells.

AMV-IAP promotes long-term HeLa cell survival. In order to demonstrate that AMV-IAP confers long-term survival rather than a transient delay in the appearance of apoptosis, we performed clonogenic assays. HeLa cells were transfected with either AMV-IAP (plus empty vector) or Ub-Hid (plus empty vector) or both expression plasmids. Shortly after transfection, cells were counted and plated at very low density and allowed to proliferate for 10 days. Colonies containing ≥50 cells were considered viable and scored from triplicate plates. The expression of Hid significantly reduced the number of viable colonies, while coinexpression of AMV-IAP attenuated this effect (Fig. 6A). Parallel experiments with Grim generated similar results (Fig. 6B). These results confirm that AMV-IAP is functional and suppresses apoptosis triggered by Hid or Grim, resulting in long-term cell survival.

DISCUSSION

In this study, we examined the binding specificity and functional attributes of the entomopoxvirus AmEPV IAP homolog. Both AMV-IAP-BIR1 and AMV-IAP-BIR2 are able to bind
IBM-containing proteins but appear to interact preferentially with Grim and Hid, respectively. Precedent for differential BIR domain interactions with IBM proteins has been established for the DIAP1 protein. In vitro binding assays indicate that Grim and Reaper bind both DIAP1-BIR1 and DIAP1-BIR2 equally, while Hid, Sickle, and Jafra2 bind to BIR2 with higher affinity than BIR1 (42). It thus appears that the BIR domains within each IAP, as well as each of the IBM-containing proteins, have unique functional attributes that render them less redundant than was initially suggested.

Interestingly, viral IAPs have now been identified in multiple members of two independent families of insect viruses, the *Baculoviridae* and the *Entomopoxviridae*. Phylogenetic evidence indicates that two, and possibly four, gene capture events have occurred during the evolution of insect viruses (18). In contrast, the only known mammalian virus encoding an IAP-like protein is African swine fever virus (26). If one accepts that viral strategies to subvert the host response are a reflection of the importance of particular pathways, then one must conclude that apoptosis induction is of paramount im-

![Image](http://jvi.asm.org/)

**FIG. 5.** AMV-IAP suppresses Hid- or Grim-induced cell death. (A) Detection of apoptotic markers. HeLa cells were transfected with pCDNA3-6myc-AMV-IAP, Ub-Hid, Ub-Grim, or a combination of plasmids. Expression of AMV-IAP, Hid, and Grim was confirmed by Western blotting with anti-Myc and anti-HA, respectively (top two panels). Subcellular fractionation and Western blotting with anti-cytochrome c was performed to demonstrate that Hid and Grim trigger the endogenous mitochondrial apoptosis pathway. Significantly reduced cytochrome c release occurred in cells cotransfected with AMV-IAP. Additional evidence of apoptosis was observed by using antibodies that detect PARP cleavage (Anti-PARP), accumulation of active caspase-3 (Anti-active-Casp. 3), or the disappearance of pro-caspase-3 (Anti-Pro Casp. 3). Actin Western blot (Anti-Actin) was used as the loading control. Untrans., untransfected. (B) AMV-IAP suppresses Hid-induced caspase-3 activity. Lysates were prepared from untransfected HeLa cells (△) or cells transfected with pCDNA3-6myc-AMV-IAP (●), Ub-Hid (○), or both pCDNA3-6myc-AMV-IAP and Ub-Hid (●). In vitro caspase-3 assays were then performed by using the fluorogenic substrate Ac-DEVD-AMC. (C) AMV-IAP suppresses Grim-induced caspase-3 activity. Cell extracts were prepared from untransfected HeLa cells (△) or cells transfected with pCDNA3-6myc-AMV-IAP (●), Ub-Grim (○), or both pCDNA3-6myc-AMV-IAP and Ub-Grim (●). Samples were analyzed as for panel B. RFU, relative fluorescence units.
importance in the insect response to infection. The development of the vertebrate adaptive immune system as a complement to innate immunity may have reduced the functional significance of apoptosis as an antiviral response. However, many viruses of mammals trigger apoptosis, and numerous viral proteins that block or delay this host response have been identified. IAP overexpression has been demonstrated to efficiently suppress mammalian cell apoptosis in a wide variety of in vitro and in vivo systems (22), and thus it remains unclear why viruses of the vertebrates have not made greater use of a strategy that is clearly effective for insect viruses.

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