Host Insect Inhibitor-of-Apoptosis SfIAP Functionally Replaces Baculovirus IAP but Is Differentially Regulated by Its N-Terminal Leader

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Received 20 June 2010/Accepted 18 August 2010

The inhibitor-of-apoptosis (IAP) proteins encoded by baculoviruses bear a striking resemblance to the cellular IAP homologs of their invertebrate hosts. By virtue of the acquired selective advantage of blocking virus-induced apoptosis, baculoviruses may have captured cellular IAP genes that subsequently evolved for virus-specific objectives. To compare viral and host IAPs, we defined antiapoptotic properties of SfIAP, the principal cellular IAP of the lepidopteran host Spodoptera frugiperda. We report here that SfIAP prevented virus-induced apoptosis as well as viral Op-IAP3 (which is encoded by the Orgyia pseudotsugata nucleopolyhedrovirus) when overexpressed from the baculovirus genome. Like Op-IAP3, SfIAP blocked apoptosis at a step prior to caspase activation. Both of the baculovirus IAP repeats (BIRs) were required for SfIAP function. Moreover, deletion of the C-terminal RING motif generated a loss-of-function SfIAP that interacted and dominantly interfered with wild-type SfIAP. Like Op-IAP3, wild-type SfIAP formed intracellular homodimers, suggesting that oligomerization is a functional requirement for both cellular and viral IAPs. SfIAP possesses a ~100-residue N-terminal leader domain, which is absent among all viral IAPs. Remarkably, deletion of the leader yielded a fully functional SfIAP with dramatically increased protein stability. Thus, the SfIAP leader contains an instability motif that may confer regulatory options for cellular IAPs that baculovirus IAPs have evolved to bypass for maximal stability and antiapoptotic potency. Our findings that SfIAP and viral IAPs have common motifs, share multiple biochemical properties including oligomerization, and act at the same step to block apoptosis support the hypothesis that baculoviral IAPs were derived by acquisition of host insect IAPs.

Apoptosis is a prevalent host cell response to virus infection. Representing an important antivirus defense, apoptotic cell death can limit multiplication and virus dissemination in the host. Thus, the mechanisms by which a host organism detects a viral intruder and initiates the apoptotic response are critical to the outcome of the infection for both the host and virus. The cellular inhibitor-of-apoptosis (IAP) proteins are important candidates for sensing virus infection and determining cell fate by virtue of their central position in the apoptosis pathway (reviewed in references 35, 36, and 44). Affirming their importance in regulation of apoptosis, IAPs are encoded by multiple DNA viruses, including baculoviruses, entomopoxviruses, iridoviruses, and African swine fever virus (reviewed in 3). Nonetheless, the molecular mechanisms by which viral IAPs regulate virus-induced apoptosis and how they biochemically differ from cellular IAPs are poorly understood.

The IAPs were first discovered in baculoviruses because of their capacity to prevent virus-induced apoptosis and thereby facilitate virus multiplication (4, 8). The baculovirus IAPs bear a striking resemblance to the cellular IAPs carried by the host insects that they infect. Cellular IAPs are a highly conserved family of survival factors that regulate developmental and stress-induced apoptosis, as well as inflammation, the cell cycle, and other signaling processes (35, 38, 44). Importantly, misregulation or overexpression of IAPs is associated with neoplasia and tumor chemoresistance (24, 49). The IAPs are defined by the presence of one or more ~80-residue baculovirus IAP repeat (BIR) domains. The BIRs consist of a conserved Zn$^{2+}$-coordinating arrangement of Cys and His residues (CCHC) that interact with diverse proteins, including the cysteinyl aspartate-specific proteases called caspases that execute apoptosis (reviewed in 16 and 37). The antiapoptotic activity of some, but not all, IAPs is derived from their ability to bind and neutralize caspases (reviewed in 35 and 44). The BIRs also interact with proapoptotic factors that contain IAP binding motifs (IBMs). IBM-containing factors have the capacity to bind and dissociate the IAP-caspase complex, thereby liberating active caspases to execute apoptosis (16, 35, 36, 48). Many IAPs, including viral IAPs, also possess a C-terminal RING domain, which is a Zn$^{2+}$-coordinating motif with E3-ubiquitin ligase activity, which can contribute to antiapoptotic activity (48). The best-studied baculovirus IAP is Op-IAP3, which is encoded by Orgyia pseudotsugata nucleopolyhedrovirus. This small IAP (268 residues) contains two BIRs and a C-terminal RING (Fig. 1A). Both BIRs are required for Op-IAP3 antiapoptotic activity (19, 50, 53). Truncation of the Op-IAP3 RING creates a loss-of-function dominant inhibitor (19). Op-IAP3’s capacity to form a complex with this RING-lacking (RINGless) dominant inhibitor and with itself suggests that oligomerization is necessary for IAP function. Upon overex-

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Published ahead of print on 25 August 2010.
expression, Op-IAP3 blocks apoptosis triggered by diverse signals in cells from certain insects and mammals, suggesting that it acts through a conserved mechanism (7, 11, 15, 33, 51, 54). In the baculovirus host moth Spodoptera frugiperda (Lepidoptera: Noctuidae), Op-IAP3 prevents apoptosis by blocking the activation of effector caspases (25, 32, 40). However, in contrast to viral Op-IAP3, SfIAP can bind and inhibit caspases, including Spodoptera frugiperda caspase-1 (Sf-caspase-1) and human caspase-9 (20, 45). Thus, despite their structural similarities, there exist fundamental differences in the biochemical activities of these two IAPs. Importantly, SfIAP fails to prevent baculovirus-induced apoptosis when produced at endogenous levels in permissive Spodoptera cells. Thus, it is expected that SfIAP also possesses regulatory motifs that respond to cellular signals triggered upon virus infection.

SfIAP provides an unprecedented opportunity to investigate the functional and evolutionary relationships between host and viral IAPs and to test the intriguing hypothesis that viral IAPs were acquired by host gene capture (21). We have investigated the biochemical properties of SfIAP as a means to define its molecular mechanisms and to test its relatedness to viral IAPs. We report here that SfIAP shares many biochemical and functional features with viral IAPs. Like Op-IAP3, overexpressed SfIAP prevented virus-induced apoptosis at a step upstream of caspase activation by a mechanism that required BIR1, BIR2, and the RING. SfIAP formed a complex with itself and with a RINGless dominant inhibitor, suggesting that oligomerization is also required for function of cellular IAPs. Unlike viral IAPs, SfIAP possesses an N-terminal leader, which modulates intracellular SfIAP levels and may respond to apoptotic signals to regulate cell survival. Our data are consistent with a model in which baculoviruses acquired a host cell IAP and modified it for virus-specific needs, thereby increasing virus fitness by preventing virus-induced apoptosis.

MATERIALS AND METHODS

Cell lines and viruses. Spodoptera frugiperda IPLB-SF21 cells (47) and Drosophila melanogaster DL-1 cells (39) were maintained at 27°C in TC100 (Invitrogen) and Schneider’s growth medium (Invitrogen) supplemented with 10% and 15% heat-inactivated fetal bovine serum (FBS) (HyClone), respectively. Wild-type L-1 strain Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV) (27) and AcMNPV recombinants Δvnp35 (Δvnp35/p49 Δp35 Δiap), Δvap5/lacZ (Δvap5/lacZ), Δvap9 (Δvap9/p35 Δiap), Δvp5 (Δvp5/p35 Δiap), Δvop (Δvop/p49 Δiap), ΔvIap (ΔvIap/p49 Δiap), ΔvSfIAPΔR (ΔvSfIAPΔR), ΔvSfIAPΔR (ΔvSfIAPΔR) were described previously (18, 26, 56). AcMNPV recombinant mSfIAPΔA (SfIAPΔA ΔvapaΔSfIAPΔR ΔvSfIAPΔR) was generated by gene insertion (32), in which a promoterless plasmid (pBS/KS+) encoding SfIAPΔA (SfIAPΔA ΔvapaΔSfIAPΔR ΔvSfIAPΔR) was inserted into the complete baculovirus genome during infection; SfIAPΔA was tagged at its N terminus with the influenza virus hemagglutinin (HA) epitope (YPYDVPDYA). Recombinant viruses were identified by the lacZ expression and plaque purified. Immunoblot analysis verified expression of SfIAPΔA.

Plasmids. The SfIAP open reading frame (ORF) from the first methionine (M1; residue 1 for SfIAPΔA) or the fourth methionine (M4; residue 97 for SfIAPΔA) to residue 377 (for full-length SfIAP) or to residue 323 (for SfIAPΔA with the RING deleted) was PCR amplified from an SfIAP cDNA (20) (kindly provided by J. Reed). The resulting DNA fragments were substituted for the AscI-SpeI fragment of pIE1IAPHA/PA (32) to create expression vectors pIE1IAPΔA/PA, pIE1IAPΔA/PA, pIE1IAPΔA/PA, pIE1IAPΔA/PA, pIE1IAPΔA/PA, pIE1IAPΔA/PA, pIE1IAPΔA/PA; each plasmid encoded N-terminal HA-tagged versions of SfIAP expressed from the highly active

FIG. 1. SfIAP structure and mutagenesis. (A) Viral and cellular IAPs. Viral Op-IAP3 (268 residues) and SfIAP (377 residues) each contain two BIR motifs (black boxes) and an E3 ligase RING domain (cross-hatched box). Each representing a potential start site, four methionines (M1 to M4) exist in the N-terminal leader of SfIAP. (B) SfIAPΔM mutations. SfIAPΔM (281 residues) begins with the M4 identity localized to its two BIRs and C-terminal RING (20). 42% identical to Op-IAP3, with a higher degree of amino acid identity localized to its two BIRs and C-terminal RING (20). As the principal IAP in Spodoptera, SfIAP suppresses a constitutive push toward apoptosis (34); ablation of SfIAP leads to immediate apoptosis of cultured Spodoptera cells. Upon overexpression, SfIAP also rescues the multiplication of apoptosis-inducing baculoviruses and can prevent apoptosis in certain mammalian cell lines (20, 26). In contrast to viral Op-IAP3, SfIAP can bind and inhibit caspases, including Spodoptera frugiperda caspase-1 (Sf-caspase-1) and human caspase-9 (20, 45). The antiapoptotic activity of wild-type or mutated forms of SfIAP was assayed by virus marker rescue in which replication of p35-deficient vΔp35/lacZ was restored in proportion to the antiapoptotic activity of the mutated Sfiap gene acquired by integration of the Sfiap-encoding plasmid (2). Virus yields were determined by plaque assay using apoptosis-sensitive SF21 cells. Antiapoptotic activity is presented as the ratio of nonapoptotic, lacZ-expressing plaques produced by the indicated Sfiap to those produced by wild-type Sfiap. Values shown are the averages ± standard deviations obtained from triplicate transfections.

SfIAPΔMAR (SfIAPΔMAR) was PCR amplified from an Sfiap cDNA (20) and cloned into the Sfiap wild-type expression and plaque purified. Immunoblot analysis verified expression of SfIAPΔM.

SfIAP/PA, pIE1IAPΔA/PA, pIE1IAPΔA/PA, pIE1IAPΔA/PA; each plasmid encoded N-terminal HA-tagged versions of SfIAP expressed from the highly active

Vol. 84, 2010 COMMON ANCESTRY FOR VIRAL AND CELLULAR IAPs 11449

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AcMNPV ie-1 promoter (prn). These plasmids were used to generate plasmids encoding HA-tagged SfIAP<sub>M4</sub> and SfIAP<sub>RM4</sub> containing BIR and RING subunit substitutions C43A, C67A, H60A, C154S, C67A and C178S, and H251A, as well as SfIAP<sub>M4</sub> with an H347A mutation (H347A-mutated SfIAP<sub>M4</sub>). pIE1<sup>hr5</sup>/hr5/M4-SfIAP<sub>MT</sub>PA, pIE1<sup>hr5</sup>/hr5/M4-SfIAP<sub>MT</sub>PA, and pIE1<sup>hr5</sup>/hr5/M1- SfIAP<sub>MT</sub>PA with and without the indicated BIR or RING mutations were generated by insertion of a T7 epitope tag (MASMTGGQQMG) in place of the HA tag. pIE1<sup>hr5</sup>/hr5/M4-SfIAP<sub>MT</sub>PA/AIEU<sup>lacZ</sup> was generated by inserting the metallothionein promoter-driven luc<sub>Z</sub> cassette from pMl<sub>T</sub>Luc<sub>Z</sub> (Invitrogen) into hr5 enhancer-less pIE1<sup>hr5</sup>/hr5-SfIAP<sub>MT</sub>PA upstream of the ie-1 promoter. pIE1<sup>hr5</sup>/hr5/SfIAP<sub>MT</sub>PA/AIEU<sup>lacZ</sup> was generated by inserting the Drosophila hul ORF (kindly provided by B. Hay) with a C-terminal HA tag under the control of the ie-1 promoter in pIE1<sup>hr5</sup>/hr5/PA-based expression vectors encoding AcMNPV P35 or Spodoptera cettia- torus nucleopolyhedrovirus P49 were described previously (5, 6). All plasmids and mutations thereof were verified by nucleotide sequencing.

Transfections. Expression plasmids (6 μg) were mixed with cationic liposomes (10 μL) consisting of DOTAP-DOPE [N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate]-1-phosphatidylethanolamine, dioleoyl (C<sub>18:1</sub>)/H9262/H11006 and irradiated 24 h later with UV-B (2 min) by using a Blak-Ray lamp (UVP, paschallab.com). Surviving vectors were harvested 20 h later, suspended in 250 μL buffer A (20 mM HEPES at pH 7.5, 10 mM KCl, 1 mM MgCl<sub>2</sub> 1 mM EDTA, 1 mM EGTA, 1 mM DTT) per 1 × 10<sup>6</sup> cells. For immunoprecipitations, 7 × 10<sup>5</sup> SF21 cells in 100-mm plates were transfected with 25 μg plasmid, and 40-μL DOTAP-DOPE. DL-1 monolayers (3 × 10<sup>6</sup> cells per 60-mm plate) were transfected with 2 μg plasmid and 20 μL DOTAP DOPE. All cells were transfected with p35 caused apoptosis (18). Whereas various IAPs, including best-studied Op-IAP<sub>3</sub> (Fig. 1A), inhibit apoptosis by a RING-dependent mechanism. SfIAP blocks apoptosis by a RING-dependent mechanism. Spodoptera frugiperda SfIAP possesses two BIRs and a C-terminal RING motif and thus closely resembles baculovirus IAPs, including best-studied Op-IAP<sub>3</sub> (Fig. 1A). To define biochemical differences between cellular and viral homologs, we first identified the required functional motifs within SfIAP. Site-directed mutations were tested by plasmid transfection of Spodoptera frugiperda SF21 cells, which are highly sensitive to apoptotic stimuli and also permissive for baculovirus AcMNPV. The largest ORF within the Sfiap cDNA (20) is predicted to encode a 377-residue polypeptide (designated SfIAP<sup>M4</sup>) when initiated from the first available methionine (Fig. 1A). However, three other possible start codons are located N terminally to BIR1. Because it most closely resembles the size and structural organization of Op-IAP<sub>3</sub>, we first tested SfIAP<sup>M4</sup>, which initiates from the fourth methionine (Fig. 1B). When expressed from transfected plasmids using the strong AcMNPV ie-1 promoter, HIA epitope-tagged SfIAP<sup>M4</sup> was synthesized as a single 281-residue species that was abundant and fully functional in SF21 cells (see below).

To test the antiapoptotic activity of SfIAP<sup>M4</sup>, we transfected SF21 cells with SfIAP-encoding vectors and monitored cell survival after infection with the AcMNPV mutant vΔ35, which lacks caspase inhibitor P35 and causes widespread apoptosis (18). Whereas vΔ35 caused greater than 75% apoptotic lysis of cells transfected with empty vector (Fig. 2A), overexpressed SfIAP<sup>M4</sup> prevented apoptosis. The survival of SfIAP<sup>M4</sup>-transfected cells was more than 3-fold higher than that of control cells and comparable to that of cells infected with wild-type M. Additionally, the survival of SfIAP<sup>M4</sup>-transfected cells was more than 3-fold higher than that of control cells and comparable to that of cells infected with wild-type P35<sup>+</sup> AcMNPV (Fig. 2B). In contrast, deletion of the C-terminal RING motif caused a loss of antiapoptotic activity (Fig.
this truncation (SfIAPM4ΔR) was readily produced in transfected cells (see below), which indicated that loss of function was not due to loss of protein. SfIAPM4ΔR loss of function was confirmed by marker rescue assays in which transfection with SfIAPM4ΔR-encoding plasmid failed to restore multiplication of the p35-null mutant vΔp35/lacZ (Fig. 1C). In contrast, wild-type SfIAP M4-encoding plasmid increased vΔp35/lacZ multiplication by 500-fold. SfIAP M4, but not SfIAPM4ΔR, also protected cells from UV-induced apoptosis (see below). Thus, overexpressed SfIAP prevented apoptosis triggered by diverse signals in a RING-dependent manner, a finding consistent with earlier studies (20).

SfIAP blocks effector caspase activation. Apoptosis in Spodoptera involves sequential activation of initiator and effector caspases, which includes the principal effector Sf-caspase-1 (1, 25, 31). Activated first in the caspase pathway, initiator caspases proteolytically cleave and thereby activate effector caspases from their inactive proforms (16, 36, 37). Viral Op-IAP3 blocks apoptosis by preventing Sf-caspase-1 activation (25, 40, 56). To define the step at which SfIAP acts, we monitored the proteolytic processing of pro-Sf-caspase-1, which is cleaved to large and small subunits during activation (1, 25). To ensure that SfIAPM4 was overproduced in every cell triggered to undergo apoptosis, we generated an AcMNPV recombinant vSfIAP, which lacks p35 but expresses SfiapM4 from the early ie-1 promoter. Upon infection with vSfIAP, processing of pro-Sf-caspase-1 was blocked (Fig. 2C, lane 1); the abundance of SfIAPM4 (see below) indicated that infection proceeded normally. In contrast, pro-Sf-caspase-1 was processed to active subunits upon infection with p35-recombinant vP35 (Fig. 2C, lane 2); P35 is a substrate inhibitor of effector caspases that blocks virus-induced apoptosis by inhibiting activated Sf-caspase-1 (56). We concluded that SfIAP M4 acts upstream of P35 and thus prior to effector caspase activation.

SfIAPM4 inhibition of pro-Sf-caspase-1 processing was comparable to that by P49 and Op-IAP3, produced by AcMNPV recombinants vP49 and vOpIAP, respectively (Fig. 2C, lanes 4 to 7). Bearing considerable sequence similarity to P35, baculovirus P49 is a substrate inhibitor that is cleaved by and inhibits the initiator caspase that processes Sf-caspase-1 (56). Thus, to determine if SfIAP also inhibits the P49-sensitive initiator caspase, we tested whether SfIAPM4 affects P49 cleavage. When P49 and SfIAPM4 were expressed simultaneously in cells coinfected with recombinants vP49 and vSfIAP, cleavage of P49 was blocked (Fig. 2D). P49 cleavage was detected only in the absence of vSfIAP (Fig. 2D, lane 6), indicating that
Sfiap\textsuperscript{M4} inhibition was dominant to that by P49. We concluded that when overproduced, Sfiap\textsuperscript{M4} inhibits the activation or activity of the Spodoptera initiator caspase responsible for virus-induced apoptosis. Thus, Sfiap can function at a step also inhibited by viral Op-IAP3 (56).

**RINGless Sfiap is a dominant inhibitor of Sfiap.** Viral Op-IAP3 RING truncations are nonfunctional and potently inhibit wild-type Op-IAP3 (19). To determine if Sfiap behaves similarly, we tested the effect of Sfiap\textsuperscript{M4} RING mutations on Sfiap function. To this end, we engineered a p35-null AcMNPV recombinant (rSfiap/lacZ) that produces Sfiap\textsuperscript{M4} at levels minimally sufficient to block apoptosis (see below). rSfiap/lacZ includes a polyhedrin promoter-directed lacZ reporter, which is expressed only when apoptosis is suppressed because the polyhedrin promoter is not activated until very late in infection (18). Thus, \(\beta\)-galactosidase production provided a sensitive measure of apoptotic inhibition, as used previously for studies of Op-IAP3 inhibitors (19).

Sfiap\textsuperscript{M4} produced by rSfiap/lacZ blocked virus-induced apoptosis (Fig. 3A); cell survival 48 h after infection was \(>95\%\). In contrast, Sfiap\textsuperscript{M4}–mediated inhibition of virus-induced apoptosis was reduced by prior transfection with plasmid encoding RINGless Sfiap\textsuperscript{M4\textregistered}; apoptotic lysis affected \(>90\%\) of the cells (Fig. 3A). Sfiap\textsuperscript{M4\textregistered} increased apoptosis of rSfiap/lacZ-infected cells in a dose-dependent manner, as indicated by decreasing \(\beta\)-galactosidase levels with increasing amounts of plasmid (Fig. 3B). Because transfection efficiencies were \(<100\%\), \(\beta\)-galactosidase activity was not reduced to zero. Immunoblots indicated that Sfiap\textsuperscript{M4\textregistered} was more abundant than virus-expressed Sfiap\textsuperscript{M4} in these assays (data not shown). These data suggested that Sfiap\textsuperscript{M4\textregistered} dominantly interferes with Sfiap in heterologous and homologous cells.

To confirm this conclusion, we used heterologous Drosophila DL-1 cells that lack endogenous Sfiap and are sensitive to UV radiation-induced apoptosis. DL-1 cells were transfected with plasmids encoding either Sfiap\textsuperscript{M4} or RINGless Sfiap\textsuperscript{M4\textregistered} and cotransfected with plasmid pMT-lacZ/pIE1-\texttt{Sfiap}\textsuperscript{M4} that encodes \(\beta\)-galactosidase under the control of the Drosophila metallothionein promoter and Sfiap\textsuperscript{M4} under the control of the \texttt{ie-1} promoter (Fig. 3C). After UV irradiation, \(\beta\)-galactosidase production was used to monitor cell viability. Cell survival was highest when only wild-type Sfiap\textsuperscript{M4} was expressed (Fig. 3D), indicating that Sfiap\textsuperscript{M4} blocks UV-induced apoptosis. In contrast, cell survival was lowest upon coexpression of Sfiap\textsuperscript{M4\textregistered} and Sfiap\textsuperscript{M4}; \(\beta\)-galactosidase was reduced by 4- to 5-fold compared to that of cells transfected with vector alone (Fig. 3D). We concluded that Sfiap\textsuperscript{M4\textregistered} dominantly inhibits the antiapoptotic activity of Sfiap in heterologous and homologous cells.

**Sfiap\textsuperscript{M4\textregistered} interacts with Sfiap.** To test whether dominant inhibition involved decreased Sfiap stability, we monitored Sfiap intracellular protein levels. Immunoblot analysis indicated that Sfiap\textsuperscript{M4\textregistered} did not affect Sfiap\textsuperscript{M4} levels either in plasmid-transfected cells (Fig. 4A, lanes 1 to 3) or in rSfiap-infected cells (lanes 5 to 7). Consequently, we determined whether Sfiap\textsuperscript{M4\textregistered} interacts with Sfiap\textsuperscript{M4}. In immunoprecipitation assays, HA-tagged Sfiap\textsuperscript{M4\textregistered} was precipitated with T7-tagged Sfiap\textsuperscript{M4} (Fig. 4B, lane 2). Little, if any, Sfiap\textsuperscript{M4\textregistered} precipitated nonspecifically (Fig. 4B, lane 5). These interactions were confirmed by immunoprecipitations in which the

![FIG. 3. Dominant inhibition by Sfiap\textsuperscript{M4\textregistered}.](http://jvi.asm.org/Downloaded from)

(A) Morphology of infected cells. SF21 cells were transfected with empty vector or plasmid encoding Sfiap\textsuperscript{M4\textregistered}, inoculated 24 h later with Sfiap\textsuperscript{M4\textregistered}-encoding virus rSfiap/lacZ, and photographed (magnification, \(\times 100\)) after 48 h. (B) Virus-based dominant inhibition assay. SF21 cells were transfected with the indicated quantities of empty vector (vect) or Sfiap\textsuperscript{M4\textregistered}–encoding plasmid; the total plasmid level was held constant by including empty vector. After 24 h, the cells were inoculated with rSfiap/lacZ, and intracellular \(\beta\)-galactosidase was measured 48 h later. In this assay, the yield of \(\beta\)-galactosidase is directly proportional to the level of apoptotic protection, which is reported as the average value \(\pm\) standard deviation of activity relative to that of vector-transfected cells (normalized to 100%) obtained from triplicate infections. (C) Expression vectors. The \texttt{ie-1} or metallothionein (MT) promoters (prm) were linked to \texttt{Sfiap}\textsuperscript{M4} or \texttt{lacZ}, respectively, and inserted into the indicated plasmids. An arrow denotes the RNA start sites. (D) Antiapoptotic activity of Sfiap\textsuperscript{M4} in Drosophila cells. DL-1 cells were transfected with empty vector or plasmids encoding the indicated proteins, UV irradiated 24 h later, and immediately overlaid with Cu\textsuperscript{2\textsuperscript{+}}-containing medium to induce \texttt{lacZ} expression. The level of apoptotic protection was directly proportional to the yield of 24-h intracellular \(\beta\)-galactosidase, which is reported as the average value \(\pm\) standard deviation of activity relative to that of cells cotransfected with empty vector and pMT-lacZ/pIE1-\texttt{Sfiap} (normalized to 100%) obtained from triplicate transfections.
epitope tags were reversed (data not shown). Thus, SfIAP\(\Delta R\) forms a stable complex with SfIAP. Moreover, full-length HA-tagged SfIAP\(^{M4}\) coprecipitated with full-length T7-tagged SfIAP\(^{M4}\) (Fig. 4B, lane 1); SfIAP\(^{M4}\) was not precipitated in the absence of SfIAP\(^{T7}\) (Fig. 4B, lane 4). We concluded that SfIAP also interacts with itself and forms homo-oligomeric complexes like viral Op-IAP3 (19).

Intracellular SfIAP is dimeric. The capacity of SfIAP to interact with itself suggested that it forms oligomers. To clarify the nature of homo-oligomerization, we used size exclusion chromatography to characterize the IAP complex. The majority of SfIAP\(^{M4}\) obtained from transfected SF21 cells by freeze-thaw lysis eluted as a complex, with a molecular mass of 60 to 70 kDa (Fig. 5A). Since the monomeric mass of SfIAP\(^{M4}\) (281 residues) is 32 kDa, we concluded that the principal form of SfIAP is a ~64-kDa dimer under these conditions. Minor amounts of SfIAP were reproducibly detected in fractions expected for larger complexes, suggesting the possibility of interaction with other cellular proteins. Collectively, our data argue that a majority of overexpressed SfIAP exists as a homodimer rather than in a complex with other apoptosis-associated molecules. When overexpressed in SF21 cells, 30-kDa Op-IAP3 (268 residues) also eluted with a size expected of a dimeric complex (Fig. 5B), consistent with its capacity to homo-oligomerize (19). Op-IAP3 had a greater tendency to form larger complexes, possibly through multimerization or interaction with cellular components.

RINGless SfIAP triggers caspase activation. Because SF21 cells exhibit a constitutive push toward caspase activation (34), we predicted that SfIAP\(^{M4}\Delta R\)-mediated inhibition of endogenous SfIAP would trigger caspase activity. To test this possibility, we determined the effect of SfIAP\(^{M4}\Delta R\) on caspase activation in SF21 cells. In contrast to transfection of vector or SfIAP\(^{M4}\)-encoding plasmid (Fig. 6A, lanes 1 to 3 and 4 to 6), transfection with SfIAP\(^{M4}\Delta R\)-encoding plasmid triggered the processing of Sf-caspase-1 to active subunits (lanes 7 to 9). SfIAP\(^{M4}\Delta R\)-induced processing was comparable to that induced by overexpression of Drosophila proapoptotic factor HID (Fig. 6A, lanes 19 to 21), which causes widespread apoptosis of SF21 cells (50). The intracellular level of SfIAP\(^{M4}\Delta R\)-induced caspase activity was 3-fold higher than that of vector-transfected cells and comparable to that induced by HID when measured using the effector caspase substrate DEVD-AMC (Fig. 6B). As expected, caspase activity was lowest in SfIAP\(^{M4}\)-expressing cells (Fig. 6B). Immunoblots readily detected transfected SfIAP\(^{M4}\Delta R\), which was more abundant than SfIAP\(^{M4}\) (Fig. 6A, lanes 4 to 6 and 7 to 9). We concluded that SfIAP\(^{M4}\Delta R\) triggers caspase activation and thus mimics RINGless Op-IAP3 (19).

BIR1 of SfIAP\(\Delta R\) is required for dominant inhibition. To define the domain(s) responsible for inhibition by SfIAP\(^{M4}\Delta R\), we first examined the BIRs, which interact with known pro-apoptotic ligands. Substitutions of the Zn-coordinating Cys and His residues (Fig. 1B) were used to disrupt the folding of each BIR. When tested individually by marker rescue assays,
BIR1 substitutions C43A, C67A, and H60A and BIR2 substitutions C154S and C178S caused loss of SfIAP M4 function (Fig. 1C), indicating that both BIRs are required for antiapoptotic activity. These BIR substitutions were then introduced into SfIAP M4/H9004R for tests of dominant inhibition in our rvSfIAP/lacZ/galactosidase assay. Upon transfection, each BIR-mutated SfIAP M4/H9004R was readily detected (see below).

BIR2 substitutions C154S and C178S had no effect on dominant inhibition by SfIAP M4/H9004R, since both proteins reduced antiapoptotic activity of virus-encoded SfIAP M4 just as well as nonmutated SfIAP M4/H9004R (Fig. 7A). Conversely, substitutions C43A, H60A, and C67A within BIR1 caused loss of dominant inhibition by SfIAP M4ΔR, since both proteins reduced antiapoptotic activity of virus-encoded SfIAP M4 just as well as nonmutated SfIAP M4ΔR (Fig. 7A). Conversely, substitutions C43A, H60A, and C67A within BIR1 caused loss of dominant inhibition by SfIAP M4ΔR (Fig. 7B). C67A- and C178S-doubly mutated SfIAP M4/H9004R also failed to inhibit SfIAP M4 (data not shown). Thus, only BIR1 is required for dominant inhibition.

We confirmed these findings by showing that BIR1 is also required for SfIAP M4ΔR-mediated inhibition of endogenous cellular SfIAP. Processing of Sf-caspase-1 to active subunits was blocked or reduced in the presence of BIR1 C43A- or C67A-mutated SfIAP M4ΔR (Fig. 6A, lanes 10 to 15). Conversely, processing in the presence of BIR2 C178S-mutated SfIAP M4ΔR (Fig. 6A, lanes 16 to 18) was comparable to that
FIG. 8. Role of BIRs in SfIAP interactions. (A) BIR2 mutations. NP-40-derived extracts of SF21 cells prepared 24 h after transfection with empty vector (−) or plasmids encoding the indicated HA-tagged BIR2-mutated SfIAPM4ΔR, SfIAPM4ΔR, and T7-tagged wild-type SfIAPM4 (wt) were immunoprecipitated (ppt) by using anti-T7 beads and subjected to immunoblot analysis (blot) by using anti-T7 or anti-HA. HA-tagged proteins prior to immunoprecipitation (total lysate) were included. (B) BIR1 mutations. Immunoprecipitations of NP-40-derived extracts of SF21 cells transfected with empty vector (−) or plasmids encoding the indicated proteins were conducted, as described in the legend to panel A.

of dominant inhibitor SfIAPM4ΔR and HID. Intracellular caspase activity was also reduced in the presence of BIR1 C43A- or C67A-mutated SfIAPM4ΔR but was significantly increased by BIR2 C178S-mutated SfIAPM4ΔR (Fig. 6B), indicating that BIR1, not BIR2, was required for SfIAPM4ΔR-mediated inhibition.

BIR-disrupted SfIAPΔR interacts with SfIAP. To determine whether BIR disruption interfered with SfIAPM4ΔR interaction with functional SfIAP, we used immunoprecipitation assays. Full-length T7-tagged SfIAPM4 formed a complex with BIR2 C178S- and C178S-mutated SfIAPM4ΔR (Fig. 8A, lanes 4 and 6) and with BIR1 C43A-, H60A-, and C67A-mutated SfIAPM4ΔR (Fig. 8B, lanes 4, 6, and 8). These interactions were as strong as or stronger than those with unaltered SfIAPM4ΔR (Fig. 8A and B, lane 2). Full-length T7-tagged SfIAPM4 also interacted with C67A- and C178S-mutated SfIAPM4, in which both BIRs were mutated (data not shown). None of the SfIAPM4 mutations were precipitated in the absence of full-length SfIAPT7 (Fig. 8), and all interactions were confirmed by reciprocal assays (data not shown). We concluded that the proper folding of BIR1 or BIR2 is not required for interaction between SfIAPM4ΔR and SfIAP. These findings suggested that a domain(s) outside or independent of the BIRs mediates SfIAP homophilic interactions.

BIR disruptions within full-length SfIAP generate dominant inhibitors. To further define the role of the BIRs in dominant inhibition, we tested the effect of BIR disruptions within full-length SfIAP. When expressed by plasmid transfection, BIR1 C43A-, H60A-, and C67A-mutated SfIAPM4 and BIR2 C178S- and C178S-mutated SfIAPM4 dominantly interfered with the antiapoptotic activity of SfIAPM4ΔR (Fig. 7A and B); each of these mutated RING-containing proteins reduced the yield of β-galactosidase within rSfIAPlacZ-infected cells, indicating that SfIAP antiapoptotic activity was compromised. Dominant inhibition by these BIR-mutated proteins was comparable to that of RINGless SfIAPM4ΔR. In contrast, C67A- and C178S-mutated SfIAPM4, in which both BIRs were disrupted, failed to alter SfIAPM4 function in this assay (Fig. 7A). This doubly mutated SfIAPM4 was readily produced in transfected cells (data not shown), ruling out the possibility that protein instability caused the loss of dominant inhibition. We concluded that disruption of either BIR1 or BIR2, but not both, can generate a dominant inhibitor of functional SfIAPM4.

As demonstrated by immunoprecipitations, BIR2 C178S- and C178S-mutated SfIAPM4 formed a complex with full-length T7-tagged SfIAPM4ΔR (Fig. 8A, lanes 3 and 5). Likewise, BIR1 C43A-, H60A-, and C67A-mutated SfIAPM4 coprecipitated with full-length SfIAPM4 (Fig. 8B, lanes 3, 5, and 7). Moreover, C67A- and C178S-doubly mutated SfIAPM4 also interacted with full-length SfIAP (data not shown). These findings confirmed our conclusion that although at least one BIR is required for dominant inhibition, properly folded BIRs are not required for SfIAP complex formation. The finding that C67A- and C178S-mutated SfIAPM4 also formed a complex with SfIAP but failed to act as a dominant inhibitor suggests that interaction with a nonfunctional IAP is necessary but not sufficient for dominant inhibition. Additional studies are required to define the mechanisms involved.

The N-terminal SfIAP leader regulates protein abundance. A striking feature of invertebrate (lepidopteran) host IAPs is the possession of a long N-terminal leader sequence that precedes BIR1 (Fig. 1A). The absence of a comparable sequence in viral IAPs (20, 45) suggests that the leader performs a function specific for cellular IAPs. To investigate the potential role of the SfIAP leader, we generated an expression vector for SfIAP M1, which uses the first available methionine (M1) in the SfIap ORF and thus includes the 100 residues prior to BIR1 (Fig. 1A). The absence of a comparable sequence in viral IAPs (20, 45) suggests that the leader performs a function specific for cellular IAPs. To investigate the potential role of the SfIAP leader, we generated an expression vector for SfIAP M1, which uses the first available methionine (M1) in the SfIap ORF and thus includes the 100 residues prior to BIR1 (Fig. 1A). The absence of a comparable sequence in viral IAPs (20, 45) suggests that the leader performs a function specific for cellular IAPs. To investigate the potential role of the SfIAP leader, we generated an expression vector for SfIAP M1, which uses the first available methionine (M1) in the SfIap ORF and thus includes the 100 residues prior to BIR1 (Fig. 1A).
lanes 5 and 7). Nonetheless, leaderless versions of SfIAP M4 (Fig. 9B, lanes 9, 11, 13, and 15) were routinely detected at significantly higher levels than in wild-type or RING-mutated forms of SfIAP M1.

The SfIAP leader confers protein instability. To test whether the higher accumulation of leaderless SfIAP M4 was due to increased stability, we measured the protein half-lives. T7 epitope-tagged SfIAP M1 and SfIAP M4 were overproduced in SF21 cells by using our ie-1 promoter-based expression vector (Fig. 10A). Because both proteins were translated from the same AUG start codon within the same 5’/H11032 noncoding mRNA leader, the transcriptional and translational contexts of each construct were identical. Measurements of protein stability were made after protein synthesis was blocked by cycloheximide. We discovered that leader-containing SfIAP M1 was turned over 5 times faster than SfIAP M4 (Fig. 10B). Whereas the 33-min half-life of SfIAP M1 was comparable to that of Drosophila IAP1 (DIAP1; 1/2 = 30 to 40 min) (52, 55), deletion of the leader increased the half-life of SfIAP M4 to 152 min. Moreover, the longer half-life of SfIAP M4 was comparable to that of I332A-mutated SfIAP M1 (Fig. 10B), which is deficient for E3 ubiquitin ligase activity of the RING (5, 17, 30). The T7 epitope tag at the N terminus had no significant effect on stability of these proteins (data not shown). We concluded that the N-terminal leader of SfIAP decreases protein stability and that both the leader and the RING contribute to SfIAP turnover under steady-state conditions.

Steady-state levels of SfIAP M1 are unaffected by caspases. The SfIAP leader possesses three potential caspase cleavage sites at Asp83, Asp86, and Asp96, immediately N terminal to BIR1 (Fig. 9A). One of these sites, DKTD832N, has been implicated in SfIAP turnover and anti-caspase activity because cleavage here exposes a domain required for N-end rule ubiquitination and degradation (45). To assess the contribution of caspase-mediated cleavage to SfIAP M1 instability, we first determined the effect of caspase inhibitors on SfIAP levels. When effector caspase inhibitor P35 was coexpressed (Fig. 9D), caspase activity as measured by DEVD-AMC cleavage was
SfIAP is the principal regulator of apoptosis in the baculovirus host Spodoptera frugiperda. We report here that on the basis of intracellular activities and biochemical properties, SfIAP closely resembles baculovirus Op-IAP3. Upon overexpression, both IAPs require the same motifs and prevent apoptosis at a step upstream of caspase activation. Nonetheless, endogenous SfIAP is a low-abundance protein that is rapidly depleted upon infection and thus fails to protect cells from virus-induced apoptosis (R. J. Cerio, K. Schultz, R. Vandergaast, and P. D. Friesen, unpublished data). As shown here, this intracellular instability of SfIAP is governed in part by its N-terminal leader sequence, which is absent within viral IAPs. These differences and similarities between cellular and viral IAPs have provided important insight into the mechanisms of IAP function in invertebrates and are consistent with the hypothesis that baculovirus IAPs were derived by capture of host cell IAP (21).

**DISCUSSION**

SfIAP is the principal regulator of apoptosis in the baculovirus host *Spodoptera frugiperda*. We report here that on the basis of intracellular activities and biochemical properties, SfIAP closely resembles baculovirus Op-IAP3. Upon overexpression, both IAPs require the same motifs and prevent apoptosis at a step upstream of caspase activation. Nonetheless, endogenous SfIAP is a low-abundance protein that is rapidly depleted upon infection and thus fails to protect cells from virus-induced apoptosis (R. J. Cerio, K. Schultz, R. Vandergaast, and P. D. Friesen, unpublished data). As shown here, this intracellular instability of SfIAP is governed in part by its N-terminal leader sequence, which is absent within viral IAPs. These differences and similarities between cellular and viral IAPs have provided important insight into the mechanisms of IAP function in invertebrates and are consistent with the hypothesis that baculovirus IAPs were derived by capture of host cell IAP (21).

**SfIAP inhibition of caspase activation and activity.** Site-directed mutagenesis demonstrated that SfIAP requires BIR1, BIR2, and its C-terminal RING motif for antiapoptotic activity when overexpressed in *Spodoptera* cells (Fig. 1 and 2). Given that SfIAP has high (42%) sequence similarity to baculovirus-encoded Op-IAP3 (20), the critical nature of these defining motifs was predictable. For certain cellular IAPs like DIAP1, the BIRs bind and suppress the activity of caspases (36). Indeed, SfIAP can interact with effector caspases through its BIRs (45; R. Vandergaast, D. Tran, and P. D. Friesen, unpublished data). However, when overexpressed, SfIAP functioned upstream of effector caspase activation, as SfIAP blocked the proteolytic cleavage of pro-Sf-caspase-1 in *Spodoptera* cells (Fig. 2D) and pro-DrICE in *Drosophila* cells (26). Furthermore, overexpressed SfIAP blocked the cleavage of baculovirus P49 (Fig. 2C), which is a direct substrate inhibitor of the initiator caspase that activates Sf-caspase-1 (25, 56). Thus, SfIAP can function at or upstream of the activation of the P49-sensitive initiator caspase that mediates baculovirus-induced apoptosis. As such, SfIAP probably inhibits the same step in the apoptosis pathway as baculovirus Op-IAP3 or a step adjacent to that inhibited by Op-IAP3 (56). Nonetheless, whereas SfIAP has the capacity to bind active effector caspases and block apoptosis in lepidopteran and dipteran cells, Op-IAP3 does not (26, 45, 46, 54). Thus, there exist significant differences in the core mechanisms by which SfIAP and viral IAPs block apoptosis.

**Regulation of SfIAP stability.** Endogenous SfIAP is a low-abundance protein that is difficult to detect when directly compared to those produced by our plasmid expression vectors (Fig. 9B). However, by using highly purified antisera and *Sfiap*-specific RNA silencing, we have determined that SfIAPM1 is the principal translation product of *Sfiap* in SF21 cells (R. Vandergaast, R. J. Cerio, and P. D. Friesen, unpublished data). When overproduced at equivalent levels, SfIAPM1 and SfIAPM4 have comparable antiapoptotic activity levels. Nonetheless, we demonstrated here that SfIAPM1 is significantly less stable than SfIAPM4 (Fig. 9 and 10). Full-
length SfIAPM1 exhibited a half-life of ~30 min, which is comparable to that of DIAP1, the principal IAP of *Drosophila*. However, removal of the 99-residue SfIAP leader increased protein stability by a factor of 5 and indicated that the N-terminal leader confers significant instability to SfIAPM1. Thus, although the N-terminal leader does not directly affect antiapoptotic function, it is a principal regulator of intracellular levels of SfIAP.

N-end rule ubiquitination and degradation has been implicated in the regulation of DIAP1 levels (10, 45). Caspase-mediated cleavage of the N-terminal DIAP1 leader at DQVD↓N exposes an N-terminal Asn residue, thereby recruiting N-end rule ubiquitination machinery, which destabilizes DIAP1. Although caspase cleavage sites are located in the N-terminal leader of SfIAP, including DKT↓D83-N (Fig. 9A), our studies here suggested that caspase activity has a minimal effect on SfIAP stability. In particular, the levels of wild-type SfIAPM1 were unaffected when caspase activity was inhibited by coexpression of baculovirus caspase inhibitors P35 and P49 (Fig. 9). Confirming this conclusion, the intracellular level of caspase-resistant D83A-mutated SfIAPM1 was comparable to that of wild-type SfIAPM1 (Fig. 9F). Importantly, our study also demonstrated that leaderless SfIAPM4, which lacks all N-terminal caspase-cleavage sites, exhibited potent antiapoptotic activity in *Spodoptera* and *Drosophila* cell lines (Fig. 2 and 3) (26). These findings suggest that caspase cleavage is not required for SfIAP antiapoptotic function.

Our study also suggests that SfIAP motifs other than that of the N-terminal leader regulate protein stability. For example, disruption of BIR1 also reduced steady-state levels of leaderless SfIAPM4 (Fig. 8B). Inactivation of the C-terminal RING restored protein levels, suggesting that BIR1 and the predicted E3 ubiquitin ligase activity of the RING contribute to SfIAP stability. Loss of its RING also stabilizes DIAP1 (52). The observation that SfIAP possesses multiple determinants that affect its intracellular concentration is consistent with the critical role that this cellular IAP plays in determining cell fate, including that after virus infection.

**Role of oligomerization in IAP function.** Immunoprecipitations and size exclusion chromatography (Fig. 4 and 5) indicated that SfIAPM4 exists primarily as a homodimer. The finding that RINGless SfIAPM4 forms a complex with and inhibits wild-type SfIAP (Fig. 3, 4, and 6) also suggested that oligomerization contributes to antiapoptotic function. As such, SfIAP mimics viral Op-IAP3, which also forms homodimers (Fig. 5) and is potently inhibited by RINGless mutations (19). Homo- and hetero-oligomerization have been described for human IAPs, including XIAP and c-IAP1. The domains required for IAP oligomerization vary (28, 29, 42, 43). In the case of Op-IAP3, the RING is dispensable for oligomerization (19). Likewise, the SfIAP RING and BIRs are not required for homo-oligomeric interactions (Fig. 7). Nonetheless, other experiments have indicated that the isolated SfIAP RING interacts with SfIAP (D. Tran and P. D. Friesen, unpublished data). Thus, it is likely that multiple domains contribute to SfIAP oligomerization.

The biological relevance of IAP oligomerization is unknown. Our findings here that RINGless SfIAP interacts and interferes with wild-type SfIAP suggest that two RING motifs are required for E3 ligase ubiquitination activity. Dimerized RING motifs confer optimal E3 activity from certain RING-contain-
ization domains may be shared (R. J. Cerio and P. D. Friesen, unpublished data). Collectively, these data support a common origin for both proteins.

The most striking feature that distinguishes SfIAP is its N terminus. A 30- to 100-residue leader preceding BIR1 is a prominent attribute of many cellular IAPs of lepidopteran, dipteran, and hymenopteran species. However, most, if not all, viral IAPs either lack the leader or possess just a few residues prior to BIR1. Our studies indicate that the N-terminal SfIAP leader is a principal determinant of protein turnover. We expect that the leader possesses a regulatory motif(s) that provides a critical means by which to govern intracellular concentrations of the host IAP to meet cellular demands for preventing or promoting apoptosis. In the case of virus infection, virus-induced depletion of cellular IAPs would lead to rapid apoptosis, as observed with DIAP1 in RNA virus-infected Drosophila cells (41). In contrast, viral IAPs function over a narrow window of infection to prevent apoptosis and thus would benefit from immunity to the mechanisms downregulating cellular IAP levels. Thus, a virus-encoded IAP that lacks an N-terminal regulatory leader or is deficient in host-responsive destabilizing motifs would have improved protein stability and longer-lasting antiapoptotic activity compared to those of host IAPs. Our study here suggests the simple possibility that viral IAPs evolved from cellular IAPs in a process that included the loss of the N-terminal regulatory leader. Additional studies promise to provide insight into this important aspect of host-virus evolution.

ACKNOWLEDGMENTS

We thank John Reed for the gift of the SfIAP plasmid and Yuri Lazebnik for the anti-P35 serum. We also thank Rebecca Hozak for her preliminary work in generating and characterizing the pIE1 prm-luc construct. We thank Herman-Bachinsky, Y., H. D. Ryoo, A. Ciechanover, and H. Gonen. 2007. Regulation of the Drosophila ubiquitin ligase DIAP1 is mediated via several distinct ubiquitin system pathways. Cell Death Differ. 14:861–871.

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