Caspase Inhibitor P35 and Inhibitor of Apoptosis Op-IAP Block in Vivo Proteolytic Activation of an Effector Caspase at Different Steps*

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Signal-induced activation of caspases, the critical protease effectors of apoptosis, requires proteolytic processing of their inactive proenzymes. Consequently, regulation of procaspase processing is critical to apoptotic execution. We report here that baculovirus pancerase inhibitor P35 and inhibitor of apoptosis Op-IAP prevent caspase activation in vivo, but at different steps. By monitoring proteolytic processing of endogenous Sf-caspase-1, an insect group II effector caspase, we show that Op-IAP blocked the first activation cleavage at TETD↓G between the large and small caspase subunits. In contrast, P35 failed to affect this cleavage, but functioned downstream to block maturation cleavages (DXXD↓(G/A)) of the large subunit. Substitution of P35's reactive site residues with TETDG failed to increase its effectiveness for blocking TETD↓G processing of pro-Sf-caspase-1, despite wild-type function for suppressing apoptosis. These data are consistent with the involvement of a novel initiator caspase that is resistant to P35, but directly or indirectly inhibitable by Op-IAP. The conservation of TETD↓G processing sites among insect effector caspases, including Drosophila drICE and DCP-1, suggests that in vivo activation of these group II caspases involves a P35-insensitive caspase and supports a model wherein apical and effector caspases function through a proteolytic cascade to execute apoptosis in insects.

The caspases are critical protease mediators of apoptosis and thus represent important targets for anti-apoptotic intervention (reviewed in Refs. 1–4). These highly conserved, aspartate-specific proteases are expressed as single-chain zymogens, which upon apoptotic signaling are activated by proteolytic processing, either by autoactivation, transactivation, or cleavage by other caspases (reviewed in Refs. 5 and 6). Once activated, the caspases proteolytically cleave a multitude of cellular substrates, leading to apoptotic death. Thus, caspase activation is a key regulatory point in the commitment to apoptosis.

The molecular mechanisms regulating caspase activation are largely unknown. In mammals, a proteolytic cascade is initiated by group III caspases with long N-terminal prodomains (reviewed in Refs. 2, 3, 6, and 7). Upon activation, initiator caspases proteolytically cleave group II effector caspases at aspartate-containing sites through a regulated sequence of reactions that separate the large and small subunit, and then detach the short prodomain (reviewed in Ref. 4). To date, it is unknown whether analogous cascades are conserved in other organisms, including insects. Nevertheless, diverse apoptotic inhibitors from mammals, insects, and their associated viral pathogens are providing important insight into the regulatory mechanisms of caspase activation (8–14).

The baculoviruses encode two mechanistically distinct apoptotic suppressors, inhibitor of apoptosis (IAP) and P35. Both viral proteins prevent premature insect cell death and thereby promote virus multiplication (reviewed in Ref. 15). The baculovirus IAPs were the first discovered members of the IAP family, which now includes proteins from invertebrates and vertebrates (reviewed in Refs. 16–18). Derived from baculovirus OpMNPV, Op-IAP is a potent inhibitor of apoptosis when overproduced in insect and mammalian cells, suggesting that it functions at a conserved step in the death pathway (19–23). Op-IAP prevents the appearance of caspase activity in vitro (24, 25) and physically interacts with Drosophila pro-apoptotic proteins Reaper, Hid, and Grim (26–28). Although mammalian IAPs can interact with and inhibit select caspases in vitro (29–33), the in vivo target of Op-IAP and other baculoviral IAPs is unknown.

Baculovirus P35 is a substrate inhibitor of group I, II, and III caspases when assayed in vitro (34–37). Caspase inhibition by P35 is correlated with cleavage of the P35 reactive site loop at DMQD↓G and formation of a stable, stoichiometric complex with the target caspase (35, 37–39). Ectopic expression of the p35 gene prevents programmed cell death in phylogenetically diverse organisms and is the basis of P35-mediated anticaspase strategies in transgenic organisms, including nematodes, flies, and mice (40–48). Despite its effectiveness as a universal apoptotic suppressor, the intracellular targets of P35 and its effect on caspase activation are unknown.

To define and contrast the effects of P35 and Op-IAP on caspase activation in vivo, we investigated the proteolytic activation of Sf-caspase-1, a group II-like effector caspase (49) of the insect Spodoptera frugiperda (Lepidoptera: Noctuidae). The established SF21 cell line from S. frugiperda undergoes

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The abbreviations used are: IAP, inhibitor of apoptosis; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; ORF, open reading frame; PCR, polymerase chain reaction; amc, 7-amino-4-methylcoumarin.
widespread apoptosis in response to diverse signals, including UV radiation, baculovirus infection, and ectopic expression of Drosophila death genes reaper, hid, grim, and doom (23, 24, 27, 34, 41, 42, 50–52). Since SF21 apoptosis is fully blocked by P35 and Op-IAP, we subjected these cells to different death stimuli and monitored pro-Sf-caspase-1 processing in the presence and absence of both apoptotic regulators. We report here that pro-Sf-caspase-1 is proteolytically activated in SF21 cells and represents the principal contributor to intracellular caspase activity. Suppression of apoptosis was correlated with inhibition of Sf-caspase-1 activity by Op-IAP and P35, indicating that Sf-caspase-1 activity is a direct or indirect target of these anti-apoptotic proteins. Op-IAP blocked the first in a series of caspase-like cleavages required for pro-Sf-caspase-1 activation.

In contrast, P35 selectively blocked subsequent cleavage events of the large subunit. Thus, P35 prevented in vivo caspase maturation at steps downstream from that affected by Op-IAP. Collectively, our data indicate that signal-induced activation of pro-Sf-caspase-1 involves at least two consecutively acting caspases and suggest that apoptosis in insects involves caspase-mediated proteolytic cascades.

**Experimental Procedures**

**Cells and Viruses—S. frugiperda** IPLB-SF21 (53) cells were propagated at 27°C in TC100 growth medium (Life Technologies, Inc.) supplemented with 2.6 mg/ml tryptose broth and 10% heat-inactivated fetal bovine serum (HyClone Laboratories). Infections with wild-type AcMNPV (L-1 strain) (54) and AcMNPV recombinants Δαaps5 (previously v335K) and vOp-IAP were as described previously (24, 55).

**Transfections—** SF21 cells were transfected with 100 μl of TC100 DNA (2 μg/ml) mixed with an equal volume of TC100 containing 5 μl of N-1(2,3-dioligo(1×10^4)poly(1×10^5))-N,NN-trimethylammonium methyl sulfate liposomes and added to SF21 cells (2 × 10^5 cells/plate). The transfection mixture was replaced 4 h later with supplemented TC100. SF21 cells were UV-irradiated 18 h after transfection and collected with apoptotic bodies as described previously (24).

**Recombinant Plasmids:** Sf-casp1—Total RNA was isolated from SF21 cells by using Trizol reagent (Life Technologies, Inc.). Poly(A)+ RNA was selected by using Oligotex columns (Qiagen) and used to generate a unidirectional λ phage cDNA library with the Lambda Unizap kit (Stratagene). Sf-casp1 was cloned from this library by PCR amplification by using Sf-casp1-specific primers 5′-GGAAATTCCTGTC-3′ and 5′-ACATCTCATATCTCGAGAG-3′. The nucleotide sequence of the SF21-derived Sf-casp1 cDNA was identical to that of Sf-casp1 (49). To generate pET22/Sf casp1, the Sf-casp1 ORF was inserted into the NdeI and Xhol sites of pET22(b) which has been described previously (24). The resultant plasmid pSf-casp1 was prepared by using New Zealand White rabbits (Polyclonal Antibody Service.

**Subcellular Fractionation—** SF21 cells were washed with phosphate-buffered saline (54), and suspended in lysis buffer containing 10 mM HEPES, pH 7.0, 2 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol, and protease inhibitor mixture (PharMingen). Apoptotic vesicles were collected by centrifugation (10,000 × g for 10 min), suspended in lysis buffer, and collected with the intact cells. After a 15 min incubation on ice, cells were removed by centrifugation (10,000 × g for 4°C), and the supernatants were stored at −80°C. The supernatant from 10^7 cells (10 μl) was added to a reaction mix (90 μl) containing 25 mM HEPES, pH 7.5, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 5 mM dithiothreitol, and 10 μM Ac-DEVD-ape (Peptides International) as substrate. Release of the fluorescent product 7-amino-4-methylcoumarin was monitored by using a Molecular Dynamics Biochrom 960 Kinetic Fluorescence/Ab sorption microplate reader with 360-nm and 465-nm filters at 30-s intervals for 20 min. Rates of product formation were obtained from the linear portion of the reaction curves within the first 10% of substrate depletion.

**P35 Inhibition Assays—** Increasing concentrations of recombinant P35-His6, P35(D84A)-His6, or P35(D87A)-His6, were mixed with 200 fmol purified Sf-caspase-1 in 25 mM HEPES, pH 7.5, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, and 2 μM dithiothreitol in a total volume of 100 μl. After 30 min at room temperature, 2 μl of 0.5 mM Ac-DEVD-ape was added to give a final concentration of 10 μM. Product release was monitored as described above. The percent Sf-caspase-1 activity was calculated as the ratio of the rate of product release in the presence and absence of recombinant P35-His6.

**Immunoblot Analysis—** Purified proteins or total cell lysates were subjected to SDS-PAGE and transferred to nitrocellulose membranes. After blocking, the membranes were incubated with 1:2,500 dilutions of anti-Sf-casp1 or a 1:10,000 dilution of anti-T7 (Novagen), followed by alkaline phosphatase-conjugated goat anti-rabbit IgG or alkaline phosphatase-conjugated goat anti-mouse IgG. Details of the blots are described previously (56).
subunits when overproduced in yields an enzymatically active complex of large and small and C-terminal His6 tags are indicated. Subunit joined to the linker. The positions of inserted N-terminal T7 occurs at Asp residues 28, 184, and 195 (49). p19 contains the p18 subunit (p19) and generated subunit p19 coincided with the start of apoptotic blebbing (3 h after infection (Fig. 2C)). Since proc-aspartate-1 is a group II-like caspase with a short prodomain (Fig. 1A), p19 and p18 subunits, and proteins Δpro and Δpro-His are indicated. E. coli-derived Sf-caspase-1His (lane 10) is compared with Sf-casp1T7-transfected proteins (lane 9). Molecular mass markers (sizes in kilodaltons) are indicated at the left.

cloned from cultured S. frugiperda cells (49). Sf-caspase-1 is a group II-like caspase with a short prodomain (Fig. 1A). It yields an enzymatically active complex of large and small subunits when overproduced in E. coli (Fig. 1B). To investigate the in vivo activation of pro-Sf-caspase-1 and to determine its contribution to apoptosis execution, we purified the large subunit (p19) and generated Sf-caspase-1-specific antisera (α-SfCas1). In SF21 cells, pro-Sf-caspase-1 was detected as a prominent 35-kDa protein by immunoblotting using α-SfCas1 (Fig. 1C). Upon transfection with plasmid expressing N-terminal T7-tagged Sf-caspase-1 (SF-caspase-1T7), Sf-caspase-1T7 was also detected (Fig. 1C, lanes 5–8). Induction of apoptosis by UV irradiation caused processing of both SF-caspase-1T7 and endogenous pro-Sf-caspase-1 (Fig. 1C, lanes 1–8). The appearance of the large subunit p19 coincided with the start of apoptotic blebbing after irradiation. As expected, the accumulation of p19 was greater in Sf-caspase-1T7-transfected cells (lanes 5–8). Since processing removed the T7 tag, transfected p19 was indistinguishable from endogenous p19 and E. coli-produced p19 (Fig. 1C, lane 10). Although not recognized by α-SfCas1, the small subunit p12 was detected by immunoblotting using p12-specific anti-

serum (data not shown). A less abundant protein (29 kDa) with a size expected of Sf-caspase-1 lacking its prodomain (Δpro) was also detected (Fig. 1C).

Pro-Sf-caspase-1 Is Activated upon Virus Infection—As a downstream effector caspase, diverse apoptotic signals should promote Sf-caspase-1 activation. We therefore tested whether pro-Sf-caspase-1 is processed during baculovirus infection, a potent apoptotic stimulus for SF21 cells (15). Upon infection of SF21 cells with vΔp35 (−p35/iap), an apoptosis-inducing mutant that lacks functional p35 and iap (55), pro-Sf-caspase-1 was proteolytically processed to its subunits. p19 appeared between 12 and 15 h after infection (Fig. 2A), which coincided with the initiation of apoptotic blebbing. Moreover, the accumulation of p19 paralleled the increase in intracellular caspase activity as measured by DEVD-amc cleavage in extracts of infected cells (Fig. 2B). The observed processing of pro-Sf-caspase-1 by diverse apoptotic signals is consistent with a downstream effector role for this caspase. Interestingly, pro-Sf-caspase-1 levels were only minimally depleted by both apoptotic stimuli (Figs. 1C and 2A), suggesting that only a fraction of the endogenous pro-Sf-caspase-1 requires activation for apoptotic execution.

Sf-caspase-1 Is a Principal Apoptotic Effector in SF21 Cells—The caspase activity present in apoptotic SF21 cells (Fig. 2B) is distinguished by its unique sensitivity to wild-type P35, but not D84A-mutated P35 in which the P1_Asp residue is substituted with Ala (34). By contrast, human caspase-3 is potently inhibited by D84A-mutated P35 (34). To determine if recombinant Sf-caspase-1 exhibits the same P35 inhibition profile, purified Sf-caspase-1His was tested in dose-dependent inhibition assays that used the tetrapeptide DEVD-amc as substrate (Fig. 3). As expected, Sf-caspase-1His was potently inhibited by purified
P35 is an equimolar ratio of wild-type P35-His<sub>6</sub> reduced Sf-caspase-1 activity by 95%. In contrast, even a 12-fold molar excess of D84A-substituted P35-His<sub>6</sub> failed to affect protease activity (Fig. 3). D84A-substituted P35-His<sub>6</sub> was as ineffective as D87A-substituted P35-His<sub>6</sub>, a loss-of-function mutation lacking the requisite cleavage residue Asp<sup>87</sup> (34). Thus, the coincidence of P35 inhibition profiles of purified Sf-caspase-1 and apoptotic extracts of SF21 cells suggested that Sf-caspase-1 is a principal contributor to caspase activity in these cells.

Consistent with this conclusion, overexpression of Sf-casp<sup>1T7</sup>-sensitized cells to apoptosis. SF21 cells transfected with Sf-casp<sup>1T7</sup> underwent apoptosis upon infection with a p35-expressing baculovirus (Fig. 4, lane 3). In this case, the level of apoptotic intracellular DNA fragmentation was comparable to that of cells infected with the p35-deletion mutant vΔp35 (lane 2). Thus, the levels of activated Sf-casp-1<sup>T7</sup> likely exceeded that of P35, which is required in stoichiometric amounts to inhibit caspases (34, 35, 37). The level of virally synthesized P35 was sufficient to block apoptosis in the absence of transfected Sf-casp-1<sup>T7</sup> (Fig. 4, lane 7). Overexpression of Sf-casp<sup>1T7</sup> failed to cause apoptosis in the absence of an apoptotic signal, as indicated by the lack of apoptotic DNA fragmentation (Fig. 4, lane 1) and membrane blebbing (data not shown) in transfected cells. Only low levels of apoptosis were detected in Sf-casp<sup>1T7</sup>-transfected cells that were infected with Op-iap-expressing virus vOp-IAP (Fig. 4, lane 4). This finding was consistent with Op-iap’s function upstream from caspase activation (see below) (24, 25).

Op-IAP Prevents In Vivo Proteolytic Activation of Sf-caspase-1—As an activated effector caspase, Sf-caspase-1 is a likely target for Op-IAP or P35. To determine the in vivo effects of these apoptotic regulators on Sf-caspase-1, we made use of recombinant baculoviruses engineered to encode either Op-IAP or P35. Infection of SF21 cells with these viruses provided an efficient vehicle for uniform delivery of each apoptotic regulator to only those cells stimulated to undergo apoptosis, a situation not possible when transient transfection methods are used. Most importantly, this approach enabled us to assess effects on physiologically relevant levels of caspases in vivo.

Op-IAP efficiently blocks virus-induced apoptosis when synthesized from vOp-IAP, an AcMNPV recombinant that expresses a functional copy of Op-iap, but lacks p35 (24). Immunoblot analyses with anti-Sf-casp1 indicated that pro-Sf-caspase-1 levels remained constant after infection with vOp-IAP (+iap/−p35) (Fig. 5A). At no time were processed forms of Sf-caspase-1 detected, including subunit p19. The AcMNPV capsid protein vp39 cross-reacted with anti-Sf-casp1 (see below), which accounted for the larger protein detected late in infection (lanes 5–8). The inhibition of pro-Sf-caspase-1 processing by Op-IAP is consistent with the absence of detectable caspase activity in SF21 cells constitutively expressing Op-iap and stimulated to undergo apoptosis (24). We concluded that Op-IAP prevents in vivo processing of pro-Sf-caspase-1 and thus its activation.

P35 Blocks Maturation of Sf-caspase-1—To determine the effect of caspase inhibitor P35, we monitored proteolytic processing of pro-Sf-caspase-1 during infection with wild-type baculovirus, which encodes p35 but lacks a functional iap (+p35/−iap). Although P35 synthesized by wild-type virus fully blocked apoptosis (Fig. 4), the levels of intracellular pro-Sf-caspase-1 were dramatically depleted with time (Fig. 5B). However, only low levels of subunit p19 and a smaller fragment (*) of unknown origin were detected by anti-Sf-casp1. Instead, a prominent 25-kDa protein (hereafter designated p25) accumulated (Fig. 5B, lanes 4–8). p25 appeared 12 h after infection when caspase activity is first detected (Fig. 2B). The disappearance of the proenzyme and the concomitant accumulation of p25 suggested that p25 is a processing intermediate of Sf-caspase-1. Thus, these data suggested that, unlike Op-IAP, P35 fails to block the initial activation cleavage of pro-Sf-caspase-1, but inhibits subsequent maturation of the large subunit.

p25 Is a Processing Intermediate of Sf-caspase-1—From its size and early appearance, we predicted that p25 is the promon-18-linker fragment produced by the first cleavage of pro-Sf-caspase-1 at Asp<sup>189</sup> (Fig. 6A). We first verified the presence of the promon in p25 by immunoblot analysis. In SF21 cells transfected with Sf-casp<sup>1T7</sup>, T7-specific antiserum detected both pro-Sf-caspase-1<sup>T7</sup> and p25<sup>T7</sup> (Fig. 6B, lane 4). T7-tagged p25<sup>T7</sup> was only detected in apoptotic-signal (+UV) cells that were cotransfected with p35. No proteins smaller than p25 were detected since proteolytic processing removed the T7-tagged promon. p25<sup>T7</sup> was also detected in cells cotransfected with p35 by using anti-Sf-casp1 (Fig. 6B, lane 12). Electrophoretic comparisons (Fig. 6C) indicated that p25<sup>T7</sup> (lane 13) was larger than endogenous p25 (lane 14) due to the presence of the T7 tag. Moreover, endogenous, untagged p25 was not possible when transient transfection methods are used.
mature p19/p18 subunits were enhanced (compare Sf-casp1 linker) (Fig. 6A). Intermediate p25 consists of the Sf-caspase-1 prodomain, p18, and a protein of unknown origin (*). Molecular mass markers (sizes in kDa) are shown at left. AcMNPV nucleocapsid protein vp39, which cross-reacted with α-Sfcasp1, is indicated. As expected (60), vp39 was synthesized late in infection, localized to the nucleus (see below), and was readily detected in purified preparations of extracellular budded virus (data not shown). The reason for recognition by α-Sfcasp1 is unknown.

**Fig. 5. Effect of Op-IAP and P35 on pro-Sf-caspase-1 processing.** Total SDS lysates of SF21 cells prepared at the indicated times after infection with viruses vOp-IAP (+iap/−p35) (A) or wild-type AcMNPV (+p35/−iap) (B) were subjected to immunoblot analysis by using α-Sfcasp1. Arrows denote pro-Sf-caspase-1 (procasp), p25, p19, and a protein of unknown origin (*). Molecular mass markers (sizes in kDa) are shown at left. AcMNPV nucleocapsid protein vp39, which cross-reacted with α-Sfcasp1, is indicated. As expected (60), vp39 was synthesized late in infection, localized to the nucleus (see below), and was readily detected in purified preparations of extracellular budded virus (data not shown). The reason for recognition by α-Sfcasp1 is unknown.

**Fig. 6. Identification of p25.** A, structure of pro-Sf-caspase-1T7. Intermediate p25 consists of the Sf-caspase-1 prodomain, p18, and linker (cross-hatched). B, immunoblot analysis. SF21 cells were transfected with plasmids encoding Sf-caspase-1T7 or p35 and UV-irradiated (+UV) 18 h later or not (−UV). Total lysates of intact cells and apoptotic bodies prepared 8 h after UV exposure were subjected to immunoblot analysis (1.5 × 10⁵ cell eq) by using α-T7 (lanes 1–4) or α-Sfcasp1 (lanes 5–15). Molecular mass markers (sizes in kDa) are shown. C, electrophoretic comparison of p25 and pro-p18. A lysate of cells transfected with plasmid encoding Sf-caspase-1 pro-p18 (lane 15) and Sf-caspase-1T7 and p35 (lane 13) or infected with p25-producing wild-type AcMNPV (lane 14) was subjected to immunoblot analysis by using α-Sfcasp1.

(lane 14) was larger than a prodomain-p18 (without the linker) fragment (lane 15) synthesized upon transfection with a plasmid encoding the pro-p18 residues of Sf-caspase-1. These analyses confirmed that p25 contains the prodomain, p18, and the linker region of Sf-caspase-1 (Fig. 6A). p25 and p25T7 accumulation was highest in UV-irradiated cells cotransfected with p35 (Fig. 6B, compare lanes 8 and 12). Conversely, p25 and p25T7 levels were lowest in the absence of p35, whereas the mature p19/p18 subunits were enhanced (compare lanes 11 and 12). These findings indicated that P35 blocked the DXXD cleavages at Asp⁹⁰ and Asp⁹⁴ within p25. Thus, in vivo proteolytic activation of pro-Sf-caspase-1 proceeds by an initial cleavage at the p25-p12 junction, followed by additional processing of p25 to generate the final complex of large and small subunits required for activity.

**Fig. 7. Effect of P35 cleavage site substitutions on pro-Sf-caspase-1 processing.** A, cleavage sites. The P₁ to P₉ residues at the pro-Sf-caspase-1 cleavage site (bottom). Cleavage occurs between P₁ and P₉ residues. B, immunoblot analysis. SF21 cells were transfected with plasmids encoding p35 with the wild-type (DQMDG) or the P₁ to P₉-substituted cleavage sites (TETDG, KQVDS, and WEHDG) and inoculated 24 h later with p35 deletion mutant vΔp35. Total lysates of cells 24 h after infection were subjected to immunoblot analysis by using α-Sfcasp1. Lysates of mock- (mit) and wild-type (WT) virus-infected cells (lanes 1 and 2) were included. Molecular mass markers (sizes in kilodaltons) are indicated at the left.

In Vivo Cleavage of Pro-Sf-caspase-1 at TETD ↓ G Is Insensitive to P35 Inhibition—Despite the presence of P35, which blocked apoptosis, pro-Sf-caspase-1 was fully processed to p25 in virus-infected cells (Fig. 5B). P35’s failure to inhibit the first activation cleavage suggested that the protease responsible exhibits a novel resistance to P35 and possibly has a unique substrate specificity. Indeed, initial processing of pro-Sf-caspase-1 occurs at the sequence TETD⁹⁹ ↓ G, which is distinct from the DXXD sites DEGD⁹⁸ ↓ A and DRDL⁹⁴ ↓ G within p25 (Fig. 7A). We therefore hypothesized that the TETD-G-specific protease could be inhibited by modifying the cleavage site residues of P35. To this end, we replaced the P₁ to P₉ residues of P35 (D⁹⁴QMD) with various caspase recognition sites, including TETD. These P35 cleavage site substitutions were introduced into SF21 cells by plasmid transfection, and their effect on pro-Sf-caspase-1 processing was determined after infection with p35 deletion mutant vΔp35, which signaled apoptosis. As indicated by immunoblot analysis (Fig. 7B), the level of pro-Sf-caspase-1 processing was similar in cells transfected with wild-type (DQMDG) P35 and TETDG-substituted P35. Comparable levels of p25 and subunit p19 were detected (Fig. 7B, lanes 4 and 5). Since only ~50% of the cells were trans-
Subcellular distribution of Sf-caspase-1. Normal, non-apoptotic SF21 cell controls (control) or SF21 cells signaled to undergo apoptosis by infection with p35 deletion mutant vΔp35 (−p35) or p35-expressing wild-type virus (+p35) were fractionated by Dounce homogenization and differential centrifugation. Cytosolic (cyto), nuclear (nuc), heavy membrane (HM), and light membrane (LM) fractions were subjected to immunoblot analysis by using α-SfCasp1. The light membrane fraction (lanes 4, 8, and 12) contained 2.5-fold more cell eq (2 × 10^6 cells/lane) relative to the other fractions (8 × 10^6 cells/lane). The positions of Sf-caspase-1 proteins and viral vp39 are shown at the right.

To determine if the primary cleavage of pro-SF-caspase-1 (Fig. 8) is first cleaved at the junction (TETD↓G) of the linker region and p12 by an unidentified caspase (SF-caspase-X) to generate p12 and the pro-p18-link fragment (p25). Subsequent processing of p25 produces p18 and p12 subunits. Op-IAP blocks the first cleavage at TETD↓G within pro-SF-caspase-1. In contrast, P35 has no effect on the first cleavage, but rather blocks subsequent SF-caspase-1 maturation cleavages at DEGD↓A and DRDL↓G. B, comparison of the TETD↓G processing site of Sf-caspase-1 with that of Drosophila caspases drICE, DCP-1, DREDD, and DRONC.

Model for proteolytic activation of Sf-caspase-1. A, pro-SF-caspase-1 is first cleaved at the junction (TETD↓G) of the linker region and p12 by an unidentified caspase (SF-caspase-X) to generate p12 and the pro-p18-link fragment (p25). Subsequent processing of p25 produces p18 and p12 subunits. Op-IAP blocks the first cleavage at TETD↓G within pro-SF-caspase-1. In contrast, P35 has no effect on the first cleavage, but rather blocks subsequent SF-caspase-1 maturation cleavages at DEGD↓A and DRDL↓G. B, comparison of the TETD↓G processing site of Sf-caspase-1 with that of Drosophila caspases drICE, DCP-1, DREDD, and DRONC.

Role of SF-caspase-1 in SF21 Apoptosis—Spodoptera frugiperda IPLB-SF21 cells have provided a highly useful system for the identification and characterization of apoptotic effectors (reviewed in Refs. 15 and 58). Our data, together with that of Ahmad et al. (49), indicate that SF-caspase-1 is a principal effector caspase of SF21 cells. SF-caspase-1 has all the properties of a group II effector caspase on the basis of its short prodomain and sequence similarity to human caspase-3, -6, and -7 (49), its preference for DXXD substrates, and the requirement for apical caspase-mediated cleavage during activation (see below). As expected of a downstream effector, pro-SF-caspase-1 is proteolytically activated by diverse apoptotic signals, including UV radiation (Fig. 1), virus infection (Figs. 3 and 5B), and actinomycin D. Moreover, overexpression of SF-caspase-1 caused apoptosis of SF21 cells in a signal-dependent manner (Fig. 4). Once activated, endogenous SF21 caspase(s) exhibited a pattern of inhibition by wild-type and mutated P35 that was indistinguishable from that of purified recombinant SF-caspase-1 (Fig. 3). Although we cannot rule out the presence of additional Spodoptera caspases that have parallel or redundant functions, our data indicate that SF-caspase-1 is the primary source of intracellular caspase activity and plays a major role in effecting SF21 apoptosis.

Molecular Pathway for SF-caspase-1 Activation: Role of an Apical Caspase—Both P35 and Op-IAP affected SF-caspase-1 activation, but at distinct steps (Fig. 9A). As reported here, signal-dependent activation of pro-SF-caspase-1 involves se-

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* D. J. LaCount, unpublished data.

* G. A. Manji, unpublished data.
quential proteolytic events. The first cleavage separates the small subunit p12 from the prodomain-containing intermediate p25 at the aspartate-containing cleavage site TETD*G. Complete inhibition of in vivo processing of pro-Sf-caspase-1 at TETD*G by caspase-specific synthetic peptides argues that the responsible protease is a caspase. Hereafter, we designate this TETD*G processing activity as Sf-caspase-X. The second activation cleavage for Sf-caspase-1 detaches p25's prodomain at DEGD*G (Fig. 9A), a canonical DXXD site. Less frequent cleavage occurs at Asp*184, but does not appear to be necessary for enzyme activity. The order of pro-Sf-caspase-1 cleavage events is therefore similar to that of human pro-caspase-3, in which cleavage between subunits precedes that of the prodomain (61). Different caspases are thought to mediate processing of pro-caspase-3 (61, 62). The differential inhibition of pro-Sf-caspase-1 processing by Op-IAP and P35 suggest that in vivo activation of pro-Sf-caspase-1 is also mediated by different, but consecutively acting caspases (see below).

Upstream Function of Viral Op-IAP in Sf21 Apoptosis— Upon baculovirus expression, Op-IAP blocked the proteolytic processing of endogenous pro-Sf-caspase-1 (Fig. 5A). This finding definitively places the anti-apoptotic function of Op-IAP upstream from that of P35 with respect to Sf-caspase-1 activation (Fig. 9A). As demonstrated previously (24, 25), Op-IAP prevents the appearance of caspase activity in vivo and can block processing of ectopically expressed pro-Sf-caspase-1 in plasmid-transfected cells. There is no evidence that Op-IAP inhibits active Sf-caspase-1. These data indicate that Op-IAP functions in vivo at or before Sf-caspase-1 activation. Consistent with this conclusion, disruption of Op-IAP function by dominant inhibitors causes caspase activation in SF21 cells (63). Op-IAP may directly inactivate upstream proapoptotic effectors homologous to Reaper, Hid, Grim, or Dromosofila (26–28, 51). However, in light of the capacity of mammalian XIAP to inhibit human apiacal caspase-9 and thereby indirectly prevent activation of effector caspase-3 and -7 (29, 30), Op-IAP may also inhibit the upstream, apical caspase (Sf-caspase-X) responsible for the first activation cleavage of pro-Sf-caspase-1.

In Vivo Targeting of Sf-caspase-1 by P35—Upon apoptotic signaling in the presence of excess caspase inhibitor P35, pro-Sf-caspase-1 was proteolytically processed to the large subunit intermediate p25 (Figs. 5B and 6). However, final processing at DEGD*G to generate the mature p19 subunit was blocked by P35, promoting accumulation of p25 (Fig. 9A). Removal of the Sf-caspase-1 prodomain, which is required for activation of many caspases (reviewed in Refs. 3–5), is therefore sensitive to P35 inhibition. These data provide the first evidence that P35 can block effector caspase processing and maturation in vivo. It is likely that this anti-apoptotic activity is the direct result of P35's capacity to inhibit active caspases. Thus, the P35-inhibitable processing of p25 is mediated by Sf-caspase-1 itself or another unidentified effector caspase in SF21 cells. Sf-caspase-1 may therefore contribute to its own maturation in a feedback pathway.

Evidence That Sf-caspase-X Is a P35-resistant, Apical Caspase—As a consequence of prolonged apoptotic signaling during infection, the intracellular pool of pro-Sf-caspase-1 was fully depleted by the caspase-like cleavage at TETD*G (Fig. 5B). This in vivo processing occurred despite the presence of abundant P35 and thus was surprising, given that P35 is a potent, broad specificity caspase inhibitor (34–37). Biochemical fractionation (Fig. 8) suggested that P35 and pro-Sf-caspase-1 occupy the same cytosolic compartment and are not physically segregated. Thus, the simplest explanation for P35's ineffectiveness in vivo is that the apical protease activity (Sf-caspase-X) responsible for the initial activation cleavage has a novel resistance to P35. We hypothesized that Sf-caspase-X has a unique specificity for the P4 to P1 residues of its substrates. However, substitution of the P4 to P1 cleavage residues comprising P35's reactive site loop with residues TETDG failed to increase P35's capacity to prevent pro-Sf-caspase-1 processing at TETD*G (Fig. 7). Since TETDG-substituted P35 was an effective apoptotic suppressor in SF21 cells, we concluded that the specificity conferred by P4 to P1 residues does not account for the observed resistance to P35 inhibition in vivo. Thus, the molecular mechanism for this novel insensitivity to P35 awaits the biochemical characterization of Sf-caspase-X and identification of specific inhibitors.

Role of Apical Caspases for Activation of Insect Effector Caspases—Sf-caspase-1 is most closely related to Drosophila caspases drICE and DCP-1 (65% and 62% identity, respectively) (49, 64–66). In addition, drICE and DCP-1 possess a DEGD*G— papain-like cleavage site (28). The specificity conferred by P4 to P1 residues does not account for the specificity of these apical caspases to P35 inhibition, which remains to be formally demonstrated. Thus, current evidence argues that pathways for signal-induced caspase activation are conserved in moths and flies (orders Lepidoptera and Diptera, respectively). Furthermore, it is likely that apoptotic execution in insects involves proteolytic cascades mediated by apical and effector caspases analogous to those in mammals.

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