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Baculovirus apoptotic suppressor P49 is a substrate inhibitor of initiator caspases resistant to P35 *in vivo*

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Caspases play a critical role in the execution of metazoan apoptosis and are thus attractive therapeutic targets for apoptosis-associated diseases. Here we report that baculovirus P49, a homolog of pancaspase inhibitor P35, prevents apoptosis in invertebrates by inhibiting an initiator caspase that is P35 insensitive. Consequently P49 blocked proteolytic activation of effector caspases at a unique step upstream from that affected by P35 but downstream from inhibitor of apoptosis Op-IAP. Like P35, P49 was cleaved by and stably associated with its caspase target. Ectopically expressed P49 blocked apoptosis in cultured cells from a phylogenetically distinct organism, *Drosophila melanogaster*. Furthermore, P49 inhibited human caspase-9, demonstrating its capacity to affect a vertebrate initiator caspase. Thus, P49 is a substrate inhibitor with a novel *in vivo* specificity for a P35-insensitive initiator caspase that functions at an evolutionarily conserved step in the caspase cascade. These data indicate that activated initiator caspases provide another effective target for apoptotic intervention by substrate inhibitors.

Keywords: apoptosis/baculovirus P49/caspase inhibitor P35/*Drosophila melanogaster*/IAP

Introduction

Apoptosis is a widely conserved genetic program that deletes unwanted, abnormal or diseased cells in multicellular organisms (Jacobson *et al*., 1997; Vaux and Korsmeyer, 1999). Disruption of normal apoptotic regulation causes adverse effects that are correlated with tumorigenesis, immunodeficiency and degenerative disorders (Thompson, 1995; Yuan and Yankner, 2000). The caspase family of cysteine-dependent aspartate-specific proteases are essential components in the execution of apoptosis (Salvesen and Dixit, 1997; Cryns and Yuan, 1998; Thornberry and Lazebnik, 1998; Earnshaw *et al*., 1999; Chang and Yang, 2000). By selective proteolysis of cellular substrates, the caspases catalyze the biochemical events that lead to cellular disassembly, including DNA fragmentation, chromatin condensation and plasma membrane blebbing. Because of their pivotal role in the commitment to cell death, these proteases are important targets in therapeutic strategies for treating apoptosis-associated diseases (Jacobson, 1998; Nicholson, 2000; Yuan and Yankner, 2000).

Caspases are activated from dormant proenzymes by an ordered series of proteolytic cleavages. Initiator caspases are activated by mechanisms that include signal-induced interactions of specific adaptor proteins with the long N-terminal prodomain, which promotes caspase proteolytic processing (Earnshaw *et al*., 1999; Chang and Yang, 2000). The initiators subsequently activate the short prodomain-containing effector caspases by proteolysis. In the vertebrate mitochondrion/cytochrome *c* pathway, initiator caspase-9 is processed upon recruitment of its CARD-containing prodomain by adaptor Apaf-1 bound to cytochrome *c* released from mitochondria during apoptotic signaling (Li *et al*., 1997; Zou *et al*., 1997; Srinivasula *et al*., 1998). From this apoptosome complex, caspase-9 proteolytically activates effector proteases, including caspase-3 (Adrain and Martin, 2001). In invertebrates, a comparable apoptosome-mediated activation of initiator caspases is likely since initiator caspase candidates DREDD and DRONC of *Drosophila melanogaster* interact with the Apaf-1 homolog designated Ark (White, 2000). The existence of diverse pro- and anti-apoptotic factors that affect initiator caspase activation argues that this process is a key regulatory step in initiation of apoptosis (Chang and Yang, 2000; Adrain and Martin, 2001).

Viruses have evolved novel mechanisms to prevent apoptosis of their host cell and thereby promote virus multiplication (O'Brien, 1998; Roulston *et al*., 1999). To date, two distinct types of apoptotic suppressor, represented by P35 and the inhibitors of apoptosis (IAPs), have been identified in the invertebrate baculoviruses (Clem, 2001). Baculovirus IAPs were the first members of the IAP protein family to be discovered (Deveraux and Reed, 1999; Miller, 1999). The best-studied viral IAP, Op-IAP, prevents apoptosis in insects and mammals by a mechanism that includes interaction with itself and pro-apoptotic proteins like Reaper, HID and GRIM of *Drosophila* (Birnbaum *et al*., 1994; Duckett *et al*., 1996; Hawkins *et al*., 1996; Vucic *et al*., 1997, 1998; Hozak *et al*., 2000). In SF21 cells from *Spodoptera frugiperda* (Order Lepidoptera), a model system for insect apoptosis, Op-IAP blocks proteolytic activation of the principal effector caspase, *Sf*-caspase-1 (Seshagiri and Miller, 1997; LaCount *et al*., 2000). In contrast, caspase inhibitor P35

blocks apoptosis by targeting active *Sf*-caspase-1 (Bertin *et al*., 1996; Ahmad *et al*., 1997; Manji *et al*., 1997; LaCount *et al*., 2000). P35 is a pancaspase inhibitor in which cleavage of its solvent-exposed reactive-site loop (RSL) leads to formation of a stoichiometric complex with the caspase target (Bump *et al*., 1995; Zhou *et al*., 1998; Fisher *et al*., 1999; Xu *et al*., 2001). Despite its broad-spectrum anti-caspase activity, P35 fails to block proteolytic activation of pro-*Sf*-caspase-1, suggesting the existence of a novel P35-insensitive initiator caspase (LaCount *et al*., 2000; Manji and Friesen, 2001). This uncharacterized caspase is designated *Sf*-caspase-X.

Here, we describe a third type of baculovirus apoptotic suppressor, P49, that is distinguished by its capacity to inhibit the *Spodoptera* initiator caspase unaffected by P35. The *p49* gene from baculovirus SlNPV was identified by its capacity to block apoptosis and restore replication of a *p35*-deletion virus (Du *et al*., 1999). P49 is 49% identical to P35 but is unrelated to any known cellular protein. It is characterized by its larger size (446 residues), the presence of a 120 residue insertion absent in P35 (299 residues) and a significantly different sequence (TVTD94↓G) at its predicted cleavage site (Figure 1A). These dissimilarities suggest that if P49 functions as a caspase inhibitor, it may exhibit a unique caspase specificity or target a distinct step in the death pathway. To test these possibilities, we explored the anti-caspase potential of P49 and defined the *in vivo* apoptotic step affected.

We report here that P49 is a caspase substrate inhibitor with a P35-like mechanism. However, unlike P35, P49 functions at an upstream step to inactivate the caspase that proteolytically activates effector caspases of *Spodoptera* cells. Thus, P49 exhibits a distinct *in vivo* target specificity for a novel P35-insensitive initiator caspase. These data indicate that despite comparable structures and mechanisms, caspase inhibitors P49 and P35 have a unique specificity for initiator or effector caspases in the context of the apoptotic cell. Our finding that P49 also has the capacity to block apoptosis in cultured cells of *D.melanogaster* and is a potent inhibitor of human initiator caspase-9 suggests that P49 functions at a highly conserved step in the caspase cascade and should therefore prove useful in delineating cell death pathways in many organisms.

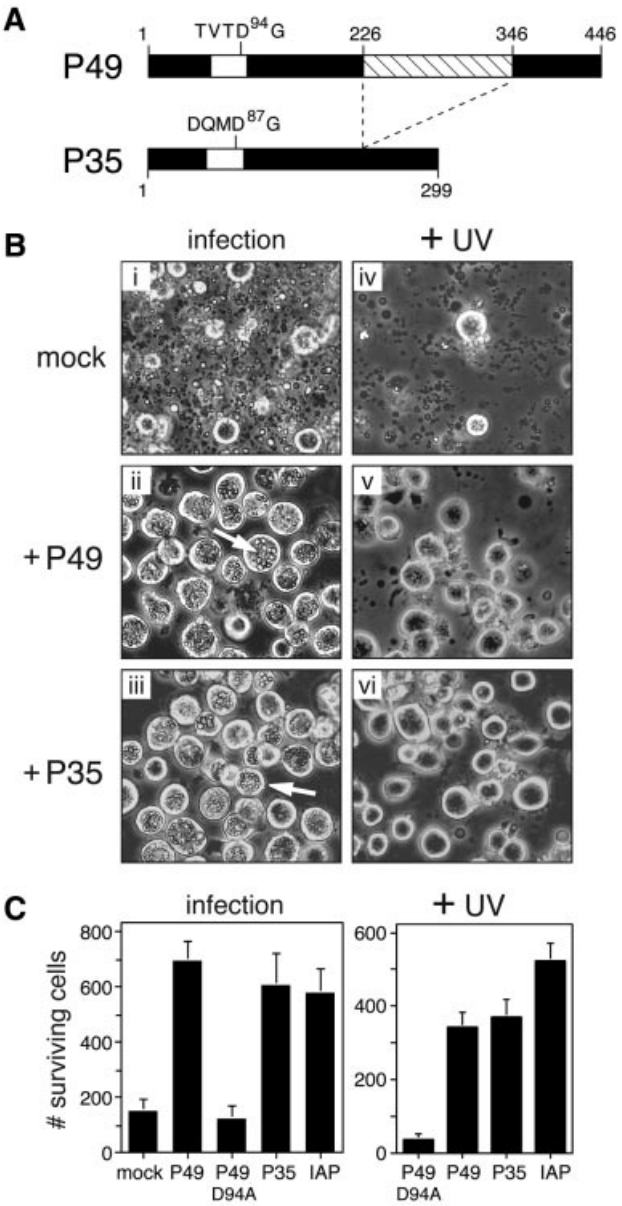

Fig. 1. P49 blocks apoptosis induced by diverse signals. (**A**) P49 and P35 structure. Amino acid similarity between P49 and P35 is colinear with the exception of a 120 residue insert (crosshatched) within P49. The caspase cleavage site within the predicted RSL (open) is indicated. (**B**) Virus- and UV radiation-induced apoptosis. SF21 cells were mock-transfected or transfected with plasmids encoding P49 or P35 and subsequently infected with apoptosis-inducing virus vΔp35 or irradiated with UV-B (125 mJ/cm^2). Photographs (magnification, 100×) were taken 48 or 24 h after infection (i–iii) or UV irradiation (iv–vi), respectively. Arrows depict occluded virus particles in non-apoptotic cells. A representative experiment is shown. (**C**) Cell survival. SF21 cells transfected with plasmids encoding wild-type P49, D94A-mutated P49, P35 or Op-IAP were infected or UV-irradiated as described in (B). Survival was quantified by computer-aided microscopy and is reported as the average number of surviving, non-apoptotic cells ± standard deviation.

Results

Baculovirus P49 blocks apoptosis induced by diverse stimuli

The high sequence similarity with P35 and the presence of a caspase-like cleavage site (Figure 1A) suggested that P49 functions as a caspase inhibitor. Thus, to test the capacity of P49 to block caspase-mediated apoptosis, we expressed *p49* ectopically in cultured SF21 cells that were subsequently induced to undergo apoptosis. Upon plasmid transfection, P49 blocked apoptosis triggered by infection with baculovirus mutant vΔp35, which lacks apoptotic suppressors (Figure 1B). P49 prevented premature cell death and promoted virus replication as indicated by the accumulation of intranuclear virus particles. The level of *p49*-mediated protection was comparable to that conferred by *p35* and baculovirus inhibitor of apoptosis Op-*iap* (Figure 1C). Similarly, P49 was a potent suppressor of UV radiation-induced apoptosis (Figure 1B). Upon transfection, P49 was as effective as P35 in protecting SF21 cells from a dose of UV radiation that caused 95% lethality (Figure 1C). Op-IAP was comparably protective.

To assess the contribution of the P49 predicted cleavage site (TVTD94↓G) to anti-apoptotic activity, Asp94 was

substituted with alanine. Although readily synthesized in transfected cells (see below), D94A-mutated *p49* failed to block apoptosis induced by infection or UV irradiation (Figure 1C). The loss of *p49* function was confirmed by marker rescue assays (Table I) in which the anti-apoptotic activity of a plasmid-borne gene was measured by the extent to which it restored multiplication of a *p35*-deficient baculovirus (Bertin *et al*., 1996; Zoog *et al*., 1999). In contrast to wild-type P49, D94A-mutated P49 failed to rescue virus replication. As expected, wild-type P35 restored replication, but D87A cleavage site-mutated P35 did not (Table I). We concluded that P49 blocks apoptosis induced by distinct death signals through a mechanism requiring an aspartate residue at the predicted caspase cleavage site.

Table I. Marker rescue of vΔp35 mutant replication

Transfected gene	Virus yield[a] ($\times 10^3$ p.f.u./ml)	Anti-apoptotic activity (%)
wt *p49*	125 ± 18	93.3
D94A-*p49*	0.0	0.0
wt *p35*	134 ± 18	100
D87A-*p35*	0.4 ± 0.1	0.3

[a]Virus yields were determined by plaque assay using apoptosis-sensitive SF21 cells. Percentage anti-apoptotic activity is reported as the ratio of non-apoptotic, lacZ-expressing plaques produced by transfection of the gene indicated to that of wild-type *p35*. Values shown are the average ± standard deviation of triplicate transfections.

P49 is a substrate inhibitor of caspases

To determine whether P49 is a direct inhibitor of caspases, we generated C-terminal His_6-tagged P49. When tested in dose-dependent assays that used the tetrapeptide DEVD-AMC as substrate, the principal effector caspase of SF21 cells, *Sf*-caspase-1, was inhibited by purified P49-His_6, but not D94A-mutated P49-His_6 (Figure 2A). Nonetheless, P49-His_6 was ~100 times less effective than P35-His_6, which is a stoichiometric inhibitor of *Sf*-caspase-1 (Figure 2B). In contrast, P49-His_6 was a potent inhibitor of human caspase-3 (Figure 2C). In these assays, P49-His_6

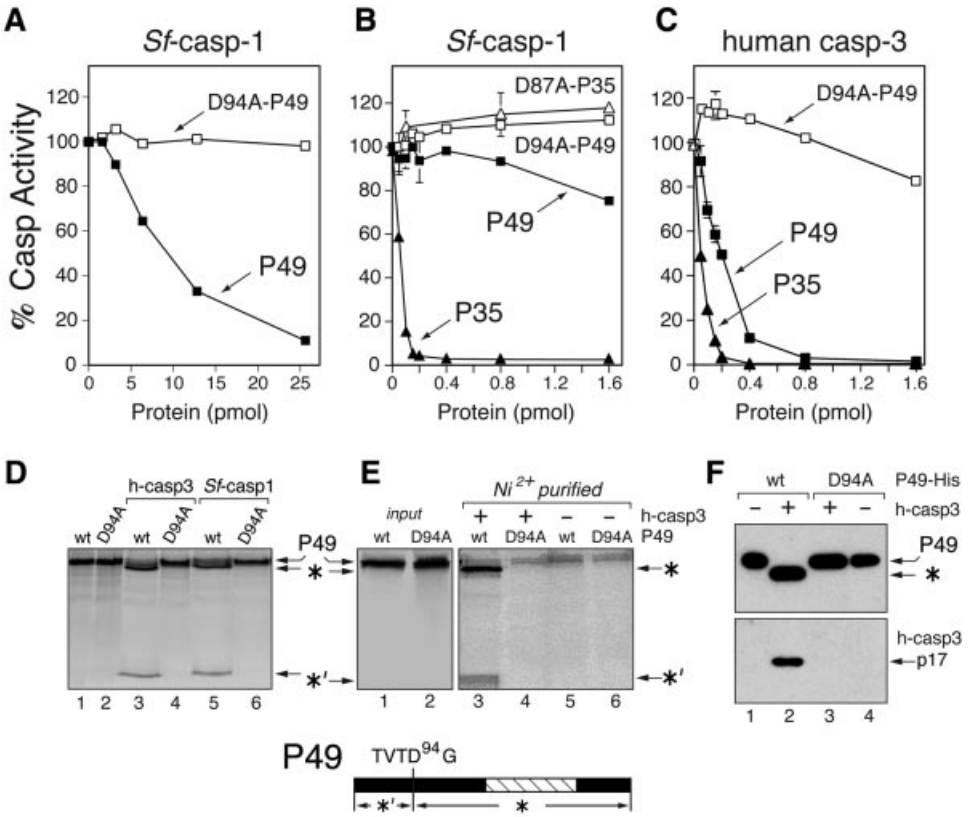

Fig. 2. P49 is a substrate inhibitor. (**A** and **B**) Inhibition of *Sf*-caspase-1. Purified recombinant *Sf*-caspase-1 (0.1 pmol) was incubated with the indicated amounts of purified P49-His_6 (filled square), D94A-mutated P49-His_6 (open square), P35-His_6 (filled triangle) or D87A-mutated P35-His_6 (open triangle). After 30 min, residual caspase activity was measured by using DEVD-AMC as substrate. Plotted values are the average ± standard deviation of triplicate assays and are expressed as a percentage of uninhibited caspase activity. (A) and (B) differ in the range of P49 used. (**C**) Inhibition of human caspase-3. Purified recombinant caspase-3 (0.1 pmol) was incubated with the indicated amounts of purified P49-His_6 (filled square), D94A-mutated P49-His_6 (open square) or P35-His_6 (filled triangle) and assayed as described above. (**D**) P49 cleavage. *In vitro* synthesized, ^{35}S-radiolabeled, wild-type (wt) or D94A-mutated P49 was mixed with buffer (lanes 1 and 2), human caspase-3 (lanes 3 and 4) or *Sf*-caspase-1 (lanes 5 and 6) and analyzed by SDS–PAGE. The 40 kDa (*) and 9 kDa (*′) cleavage fragments are indicated (bottom). (**E**) P49–caspase-His6 complexes. Radiolabeled untagged wild-type or D94A-mutated P49 was mixed with buffer or His_6-tagged human caspase-3 (300 pmol). Complexes recovered by Ni^{2+} affinity chromatography in the presence (lanes 3 and 4) or absence (lanes 5 and 6) of caspase-3 were analyzed by SDS–PAGE. Uncleaved P49 (input) was included (lanes 1 and 2). (**F**) P49-His6–caspase complexes. Purified wild-type (wt) or D94A-mutated P49-His_6 was mixed with buffer or untagged caspase-3. Recovered Ni^{2+}-bound complexes were subjected to immunoblot analysis by using α-P49 (top) or caspase-3 large subunit α-P17 (bottom) antisera.

was nearly as effective as P35-His_6, which is also a stoichiometric inhibitor of caspase-3 (Bump *et al*., 1995; Bertin *et al*., 1996; Zhou *et al*., 1998; Fisher *et al*., 1999). D94A-mutated P49-His_6 had little effect on human caspase-3 over the range of protein concentrations tested (Figure 2C).

Wild-type, but not D94A-mutated P49, was cleaved by human caspase-3 and *Sf*-caspase-1 to produce 40 and 9 kDa fragments (Figure 2D). Amino acid sequence analysis revealed that the N-terminus of the larger 40 kDa fragment was GGGAD (data not shown), indicating that cleavage occurred between P49 residues Asp94 and Gly95 within the predicted cleavage site TVTD94↓GGGAD. To determine whether the P49 cleavage fragments stably associate with the targeted caspase in a manner analogous to that of P35, we used His_6-tagged caspase-3 in Ni^{2+} affinity pull-down assays. Upon recovery of His_6-tagged caspase-3 from cleavage reactions containing untagged P49, both 40 and 9 kDa fragments of wild-type P49 (Figure 2E, lane 3), but not D94A-mutated P49 (lane 4) were detected. Neither wild-type nor mutated P49 was recovered in the absence of His_6-tagged caspase. In reciprocal assays, where the association of untagged caspase-3 with P49-His_6 was tested, caspase-3 interacted stably with cleaved P49-His_6 (Figure 2F, lane 2). In contrast, D94A-mutated P49 was neither cleaved nor associated with caspase-3 (lane 3). Collectively, these data indicate that caspase inhibition *in vitro* includes the formation of a stable P49–caspase complex, which requires cleavage at Asp94.

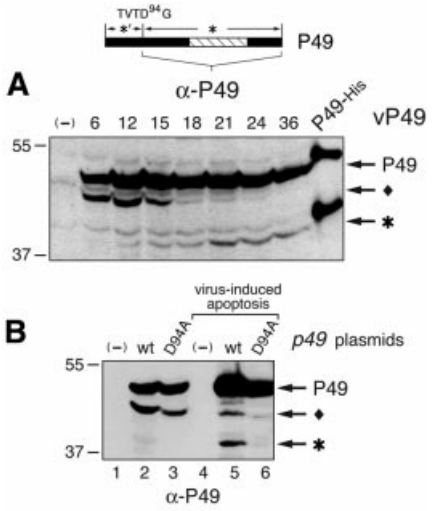

Fig. 3. *In vivo* P49 cleavage. (**A**) Time course. SF21 cell lysates prepared at the indicated hours after infection with vP49 were subjected to immunoblot analysis using α-P49, which recognized full-length P49 and the 40 kDa cleavage fragment (top), but not the N-terminal 9 kDa fragment. Due to the absence of His tags, *in vivo* P49 and its 40 kDa cleavage fragment (*) are smaller than P49-His_6, which was partially cleaved by caspase (right lane). Another P49-related protein (filled diamond) is indicated. (**B**) Asp94-dependent P49 cleavage. SF21 cells were mock-transfected (–) or transfected with plasmid encoding wild-type (wt) or D94A-mutated P49. After mock infection (lanes 1–3) or infection with *p35*⁻ vΔp35 to induce apoptosis (lanes 4–6), cell lysates (5×10^5 cell equiv) were prepared and subjected to α-P49 immunoblot analysis.

P49 functions upstream of P35 but downstream of Op-IAP

Although P49 and P35 function similarly *in vitro*, the structural deviations of these two caspase inhibitors suggested that they act differently *in vivo*, possibly by targeting distinct caspases. This notion was supported by the 100-fold difference in P49 and P35 inhibition of *Sf*-caspase-1 (Figure 2B). To investigate potential differences, we first defined the apoptotic step affected by P49. To this end, we constructed a recombinant baculovirus (vP49) that encodes P49 as its singular apoptotic inhibitor. By inoculating SF21 cultures with this virus, P49 was synthesized in every cell that received an apoptotic stimulus. Importantly, P49 activity could therefore be monitored in the context of physiologically relevant levels of death effectors.

As expected, apoptosis was fully blocked in SF21 cells infected with vP49. Directed by the immediate early *ie-1* promoter, P49 was synthesized early during infection (Figure 3A). Our α-P49 antiserum recognized full-length P49 and its 40 kDa fragment (*) but not the 9 kDa N-terminal fragment as demonstrated by comparing purified P49-His_6 partially cleaved by caspase (Figure 3A). P49 cleavage was first detected between 6 and 12 h and thus coincided with virus-induced caspase activation (LaCount and Friesen, 1997; LaCount *et al*., 2000). To confirm the identity of the 40 kDa cleavage fragment that was present at low levels, we compared cells transfected with wild-type or mutated *p49*. The 40 kDa fragment was detected only in the presence of wild-type P49, not cleavage-impaired D49A-mutated P49 (Figure 3B, lanes 5 and 6). Furthermore, generation of the cleavage fragment required apoptotic signaling, since it was present in virus-infected cells (lane 5) but not mock-infected cells (lane 2). Another P49-related protein (Figure 3B, filled diamond) detected early in vP49 infection and upon transfection with wild-type and D49A-mutated P49 is a likely internal initiation product.

To define the apoptotic step affected by P49, we determined where P49 functions relative to P35 by testing the effect of P49 on *in vivo* cleavage of P35, a sensitive indicator of effector caspase activity (Bertin *et al*., 1996; Manji *et al*., 1997). Upon infection with a *p35*-carrying virus (*p35*⁺), activated caspase(s) readily cleaved P35 to generate the 25 kDa cleavage (*) fragment (Figure 4A, lane 2). However, upon coinfection with vP49, P49 blocked the cleavage of P35 (lane 3). Conversely, P49 was cleaved normally even in the presence of abundant P35 (Figure 4B, lane 3). Taking into account the reduced P49 synthesis due to *p35*⁺ virus coinfection, P49 cleavage was comparable to that in cells infected with vP49 alone (lane 2). We concluded that P49 directly or indirectly inhibits the caspase(s) responsible for P35 cleavage and thus functions upstream of P35.

To determine where P49 functions relative to an IAP, we also assessed the effect of Op-IAP on P49 cleavage. In cells coinfected with vP49 and an Op-*iap*⁺ virus (vOp-IAP), Op-IAP blocked P49 cleavage (Figure 4B, lane 4). To confirm this finding, we infected SF21 cells that constitutively express Op-*iap* with vP49. In contrast to parental (Op-*iap*⁻) cells, P49 cleavage was fully blocked in Op-*iap*⁺ cells (Figure 4B, compare lanes 9 and 10). As expected, Op-IAP also prevented P35 cleavage (Figure 4A,

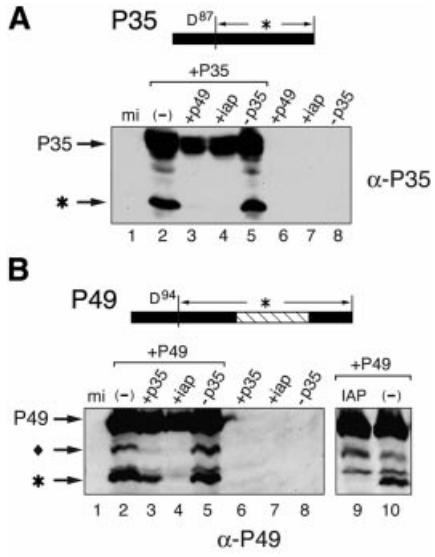

Fig. 4. P49 functions upstream of P35 but downstream of Op-IAP. (**A**) P49 inhibits P35 cleavage. Sf21 cells were mock-infected (mi) or infected with viruses vP49 (+p49), vOp-IAP (+iap) or vΔp35 (–p35) with (lanes 2–5) or without (lanes 6–8) wild-type *p35*$^{+}$ virus (+P35). Lysates (3×10^5 cell equiv) prepared 24 h later were subjected to immunoblot analysis using α-P35. Top: P35 and its C-terminal cleavage product (*) are indicated. (**B**) Op-IAP inhibits P49 cleavage. Lysates (10^6 cell equiv) prepared 24 h after infection with the viruses described in (A) with (lanes 2–5) or without (lanes 6–8) vP49 (+P49) were subjected to immunoblot analysis with α-P49. SF21 cells constitutively expressing Op-*iap* (IAP) were included (lane 9). (Top) P49 and its C-terminal cleavage fragment (*) are indicated. The P49-related protein (filled diamond) is marked.

lane 4) confirming its upstream function relative to P35 (Manji *et al*., 1997). These data indicated that Op-IAP functions at or upstream of the caspase that cleaves P49, which is upstream of the caspase(s) inhibited by P35.

P49, but not P35, prevents in vivo proteolytic processing of effector caspases

During apoptosis of SF21 cells, pro-*Sf*-caspase-1 (Figure 5A) and pro-*Sf*-caspase-2 (Figure 5C) are proteolytically processed (Ahmad *et al*., 1997; LaCount, 1998; LaCount *et al*., 2000). This signal-induced activation is initiated by a P35-insensitive caspase (LaCount *et al*., 2000; Manji and Friesen, 2001). *Sf*-caspase-1 undergoes a second cleavage, which removes the prodomain mediated by a P35-inhibitable protease, probably *Sf*-caspase-1 itself (LaCount *et al*., 2000). To determine whether P49 affects the caspase responsible for processing these effector caspases, we compared *Sf*-caspase activation in the presence of P49 and P35. Upon apoptotic signaling caused by *p35*$^{+}$ virus infection, pro-*Sf*-caspase-1 was processed to its large subunit fragments p25 and p19 (Figure 5B). Thus, cleavage of pro-*Sf*-caspase-1 occurred at TETD↓G despite an abundance of P35. In contrast, P49 blocked the processing of pro-*Sf*-caspase-1 during infection with vP49 (Figure 5B). Levels of pro-*Sf*-caspase-1 were unchanged and cleavage products, including p25, were not detected until very late. In a similar pattern, P49 prevented proteolytic processing of pro-*Sf*-caspase-2, whereas P35 did not (Figure 5D). The large subunit p18 of *Sf*-caspase-2 accumulated in the presence of P35 but not P49. These data confirmed that P49 acts at a distinct step upstream from P35 in the apoptotic pathway of these lepidopteran cells. Furthermore, P49 likely targets the P35-insensitive initiator caspase responsible for activation of pro-*Sf*-caspase-1 and -2.

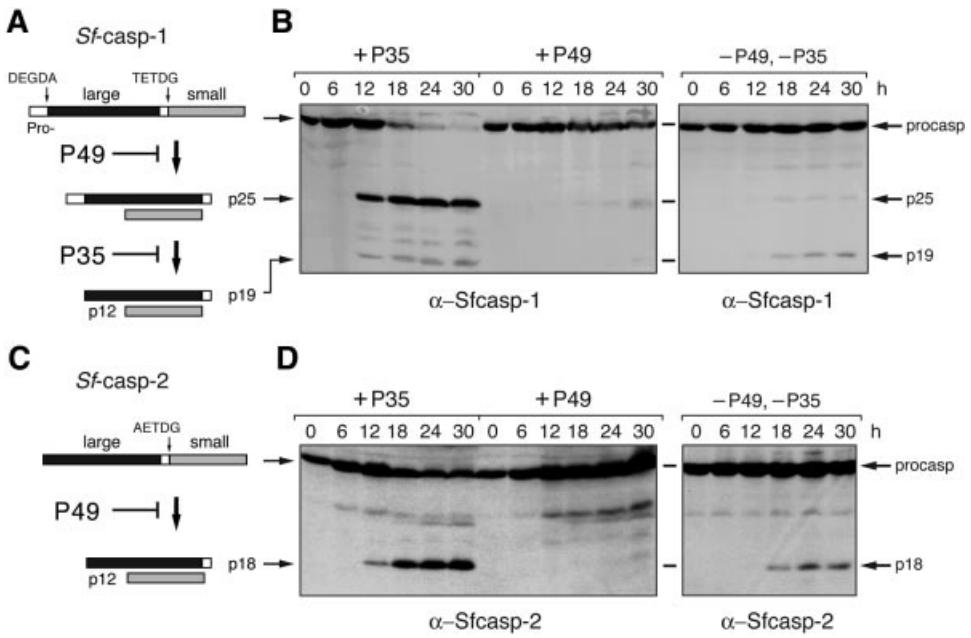

Fig. 5. P49 inhibits effector caspase proteolytic processing. (**A** and **C**) Processing of *Sf*-caspase-1 and -2. Activation of pro-*Sf*-caspase-1 initiates with cleavage at TETD↓G to generate intermediate p25, which is subsequently cleaved at DEGD↓G to generate the mature p19 and p12 subunits. Pro-*Sf*-caspase-2 is cleaved at AETD↓G to generate p18 and p12 subunits. (**B** and **D**) Effect of P49 on *Sf*-caspase-1 and -2 processing. SF21 lysates (3.8×10^5 cell equiv) prepared at the hours (h) indicated after infection with wild-type *p35*$^{+}$ virus (+P35), vP49 (+P49) or vΔp35 (–P49, –P35) were subjected to immunoblot analysis using α-Sfcasp1 (B) or α-Sfcasp2 (D) antiserum.

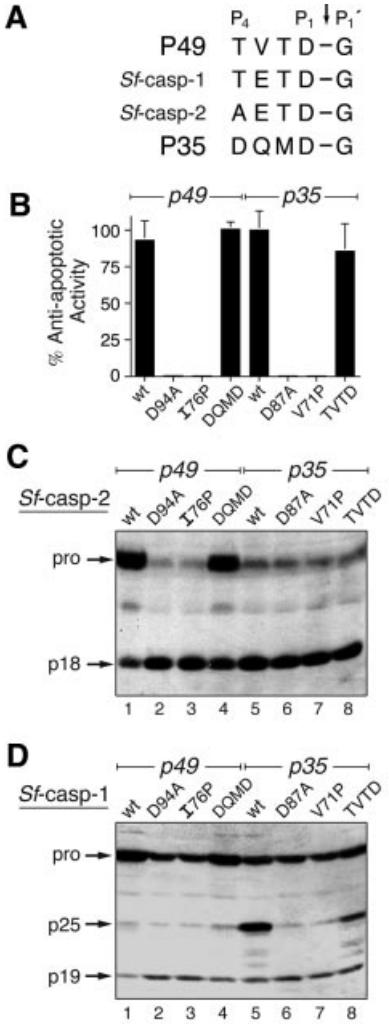

Fig. 6. *In vivo* caspase selectivity by P49 and P35. (**A**) Cleavage sites. The P_4–P_1′ residues of P49 and P35 are compared with the initial processing sites of pro-*Sf*-caspase-1 and -2. (**B**) Anti-apoptotic activity. The capacity of plasmid-borne wild-type (wt) or mutated *p49* and *p35* to block virus-induced apoptosis was determined by marker rescue. The average ± standard deviation of triplicate transfections is reported as a percentage of the capacity of wild-type P35 to restore virus replication. (**C** and **D**) Processing of *Sf*-caspases. SF21 cells were transfected with plasmids encoding wt or mutated P49 (lanes 1–4) or wt or mutated P35 (lanes 5–8), and infected 24 h later to induce apoptosis. Lysates (3.8×10^5 cell equiv) prepared 24 h after infection were subjected to immunoblot analysis with α-Sfcasp2 (C) or α-Sfcasp1 (D).

Does the P49 cleavage site confer caspase specificity?

The similarity between the P49 cleavage site TVTD↓G (Figure 6A) and the initial processing sites TETD↓G and AETD↓G of pro-*Sf*-caspase-1 and -2, respectively, suggested that the apical caspase selectivity of P49 is conferred by its RSL cleavage residues. We hypothesized that the *in vivo* selectivity of P49 and P35 could be switched by exchanging cleavage site residues. Thus, we substituted the P_4–P_1 residues of P49 and P35 and determined the effect of these swaps by monitoring *Sf*-caspase activation in transfected cells.

The exchange of P49 or P35 cleavage sites had no effect on the anti-apoptotic activity of either caspase inhibitor. Marker rescue assays demonstrated that DQMD94G-substituted P49 and TVTD87G-substituted P35 were as effective as the wild-type proteins in blocking virus-induced apoptosis (Figure 6B). In contrast, loss-of-function D94A- or I76P-mutated P49 and D87A- or V71P-mutated P35 failed to block apoptosis. Our previous studies indicated that substitution V71P disrupted the P35 RSL and thus caused loss of function (Fisher *et al*., 1999; Zoog *et al*., 1999). Although fully functional, the cleavage site substitutions failed to alter the effect of P49 or P35 on procaspase processing during virus infection. Both wild-type and DQMD94G-substituted P49 prevented pro-*Sf*-caspase-2 processing (Figure 6C, lanes 1 and 4), whereas loss-of-function D94A- or I76P-mutated P49 did not (lanes 2 and 3). The higher p18 levels (lanes 1 and 4) were attributed to those infected cells that failed to produce P49 because they were not transfected (transfection efficiencies ranged from 75 to 90%). Furthermore, neither wild-type (lane 5) nor TVTD87G-P35 (lane 8) blocked pro-*Sf*-caspase-2 processing. As expected, loss-of-function D87A- and V71P-mutated P35 (lanes 6 and 7) failed to block processing. Likewise, wild-type and DQMD94G-P49 prevented pro-*Sf*-caspase-1 processing (Figure 6D, lanes 1 and 4). TVTD87G-P35 exhibited a slightly increased capacity to prevent processing compared with wild-type P35 (Figure 6D, lanes 5 and 8). Overall, the exchange of cleavage sites had a minimal effect on processing of pro-*Sf*-caspase-1 and -2. We concluded that the P_4–P_1 residues are not sufficient to confer P49 or P35 target specificity *in vivo*. Thus, specificity is conferred by a determinant other than or in addition to the cleavage site residues.

P49 is a substrate inhibitor of human initiator caspase-9

Our data suggested that P49 functions *in vivo* to block activity of an initiator caspase. Therefore, to determine whether P49 is capable of inhibiting a well characterized initiator caspase, we tested the effect of P49 on purified human caspase-9, which functions in the mitochondrion/cytochrome *c* death pathway of mammals (Earnshaw *et al*., 1999; Chang and Yang, 2000; Adrain and Martin, 2001). When tested in dose-dependent assays that used the tetrapeptide LEHD-AFC as substrate, purified P49-His_6 effectively inhibited caspase-9 but D94A-mutated P49-His_6 did not (Figure 7A). Interestingly, wild-type but not D87A-mutated P35-His_6 inhibited caspase-9. Under identical conditions, baculovirus vector purified P49 was ~8-fold less potent than *Escherichia coli*-produced P35. Wild-type but not D94A-mutated P49 was cleaved by caspase-9 into 40 and 9 kDa fragments that were electrophoretically indistinguishable from those generated by human caspase-3 (Figure 7B). Collectively, these data indicate that P49 acts as a substrate inhibitor of caspase-9. To further determine whether P49 blocks the capacity of caspase-9 to proteolytically process and thus activate human effector caspase-3, we tested the effect of increasing concentrations of P49 on cleavage of pro-caspase-3 by active caspase-9 *in vitro*. As expected, caspase-9 cleaved pro-caspase-3 at IETD↓S between its large and small subunits to generate the p19 and p12 subunits (Figure 7C, lanes 2 and 11). However, this cleavage was blocked in a dose-dependent manner by P49-His_6 (lanes 3–6) but not D94A-mutated P49-His_6 (lanes 7–10). P35-His_6 exhibited

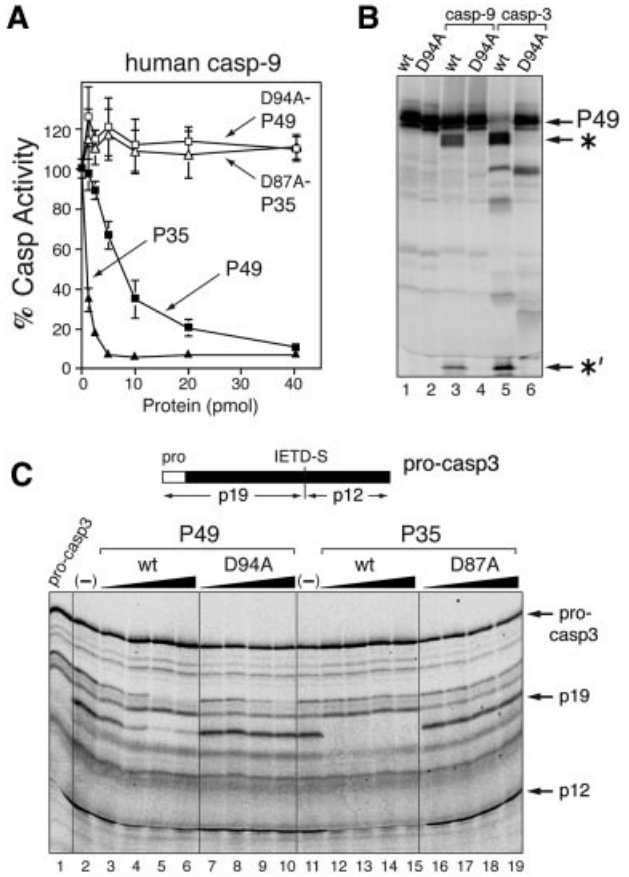

Fig. 7. P49 is a substrate inhibitor of human caspase-9. (**A**) Dose-dependent inhibition. Purified recombinant caspase-9 (40 pmol) was incubated with the indicated amounts (pmol) of purified P49-His_6, D94A-mutated P49-His_6, P35-His_6 or D87A-mutated P49-His_6. After 30 min, residual caspase activity was measured by using the substrate LEHD-AFC as described in the legend to Figure 2. (**B**) P49 cleavage. *In vitro* synthesized, ^{35}S-radiolabeled, wild-type (wt) or D94A-mutated P49 was incubated with buffer alone (lanes 1 and 2), caspase-9 (lanes 3 and 4) or human caspase-3 (lanes 5 and 6) and analyzed by SDS–PAGE. The 40 kDa (*) and 9 kDa (*′) cleavage fragments are indicated. (**C**) Inhibition of procaspase-3 processing. Purified caspase-9 was mixed with buffer alone (–), wild-type (wt) or D94A-mutated P49-His_6 (lanes 3–11), or wild-type or D87A-mutated P49-His_6 (lanes 12–19). After 30 min, *in vitro* synthesized, ^{35}S-labeled, procaspase-3 was added, incubated for 6 h and analyzed by SDS–PAGE. Input procaspase-3 is shown (lane 1). Caspase-3 p19 and p12 subunits are indicated (top).

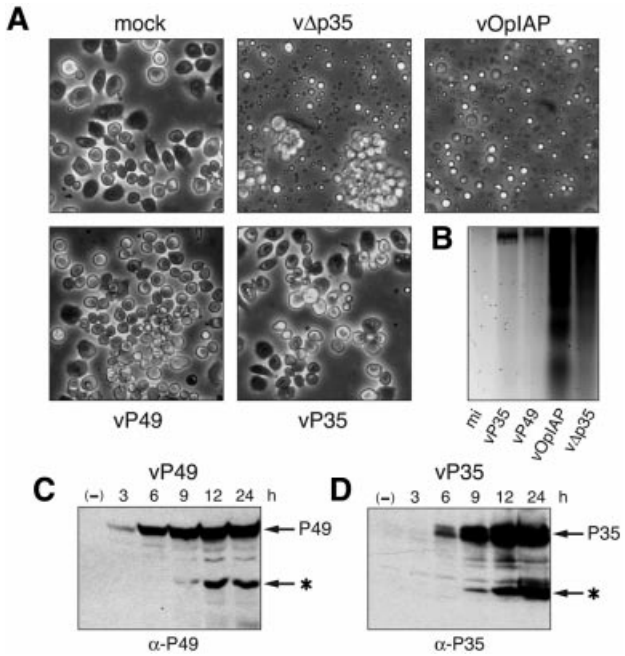

Fig. 8. P49 blocks baculovirus-induced apoptosis in *Drosophila* DL-1 cells. (**A**) Virus-induced apoptosis. *Drosophila* Line-1 (DL-1) cells were photographed 24 h after mock infection (mock) or inoculation with *p35*$^-$ vΔp35, Op-*iap*$^+$ vOp-IAP, *p49*$^+$ vP49 or wild-type *p35*$^+$ virus (vP35). Magnification 100×. (**B**) DNA fragmentation. Low molecular weight DNA was selectively extracted from equivalent numbers of DL-1 cells and associated apoptotic bodies. Only fragmented DNA was visualized by agarose gel electrophoresis since intact cellular DNA was excluded. (**C** and **D**) P49 synthesis and cleavage. DL-1 lysates (3×10^6 cell equiv) prepared at the times indicated after inoculation with vP49 or vP35 were subjected to immunoblot analysis with α-P49 (C) or α-P35 (D). Full-length and C-terminal cleavage (*) proteins are indicated.

a similar pattern of inhibition (Figure 7C, lanes 12–19). We concluded that P49 has the capacity to inhibit the activity of caspase-9 that is required for proteolytic activation of human effector caspase-3.

P49 prevents apoptosis in Drosophila

The capacity of P49 to inhibit vertebrate caspases *in vitro* suggested that P49 would suppress apoptosis in a phylogenetically distinct organism. To explore this possibility, we tested the anti-apoptotic potential of P49 in DL-1 cells of *D.melanogaster* (Order: Diptera). To this end, we used recombinant baculoviruses simultaneously to deliver apoptotic regulatory genes and to induce apoptosis. Although DL-1 cells fail to support productive baculovirus infection (Morris and Miller, 1993), virus entry and gene expression are efficient. Upon inoculation with the *p35*-deletion mutant vΔp35, DL-1 cells underwent widespread apoptosis (Figure 8A). Likewise, vOp-IAP caused apoptosis that affected >95% of the DL-1 culture. Both viruses caused extensive apoptotic body formation (Figure 8A) and intracellular DNA fragmentation (Figure 8B). Consistent with caspase activation, the effector caspase drICE (Fraser *et al.*, 1997) was proteolytically processed and DEVD-AMC cleavage activity was readily detected (E.Lannan, S.Perry, J.Theisen and P.Friesen, unpublished data). Thus, Op-IAP failed to block apoptosis in DL-1 cells despite abundant synthesis (not shown). This finding suggests a fundamental difference between Op-IAP and the other IAPs that regulate apoptosis in *Drosophila* (Miller, 1999).

In contrast, P49 and P35 blocked virus-induced apoptosis of DL-1 cells when expressed from vP49 or *p35*$^+$-virus vP35, respectively. Inoculated cells showed no signs of membrane blebbing (Figure 8A) or DNA fragmentation (Figure 8B). A majority of the inoculated cells detached from the monolayer, but remained intact for several days. Confirming that these cells were infected, P49 (Figure 8C) and P35 (Figure 8D) were synthesized in abundance. Furthermore, P49 was cleaved to generate the 40 kDa fragment (*) indicative of intracellular caspase activity. P49 cleavage was first detected 9–12 h after inoculation (Figure 8C), coinciding with caspase activation in these cells (E.Lannan, S.Perry, J.Theisen and P.Friesen, unpublished data). Previously shown to block

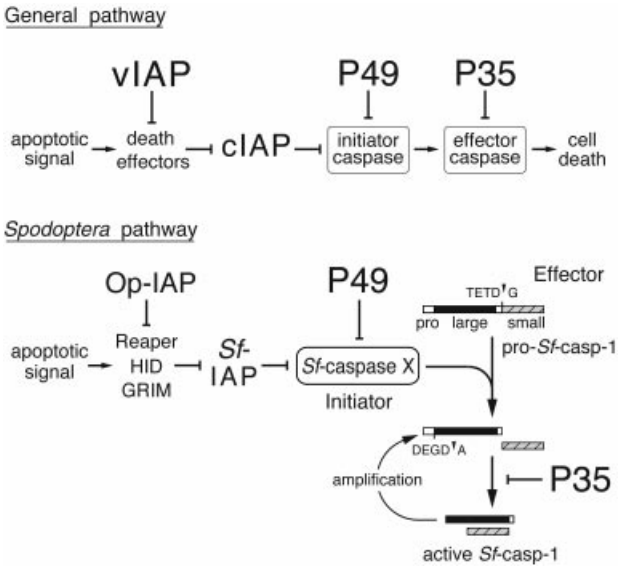

Fig. 9. Cell death regulation by baculovirus apoptotic suppressors. Top: general pathway. Baculovirus IAP (vIAP) and caspase inhibitors P49 and P35 prevent signal-induced apoptosis at distinct steps relative to endogenous cellular IAP (cIAP). A linear pathway is shown but converging paths are possible. Bottom: model for caspase activation in *Spodoptera*. Upon apoptotic signaling, prodeath effectors (Reaper, HID or GRIM homologs) trigger activation of initiator *Sf*-caspase-X by inhibition of *Sf*-IAP. *Sf*-caspase-X proteolytically activates downstream effector caspases, including *Sf*-caspase-1, by cleavage at the large–small subunit junction. Active effector caspases may amplify processing. Distinct steps are inhibited by P49 and P35. Op-IAP functions upstream by association with death effectors, *Sf*-caspase-X or both.

apoptosis in *Drosophila* (Hay *et al*., 1994; White *et al*., 1996; Jiang *et al*., 1997), P35 was cleaved with kinetics similar to those of P49 (Figure 8D). These hallmark caspase-mediated cleavages argue that the anti-apoptotic activity of P49 in *Drosophila*, like that of P35, is due to caspase inactivation by direct substrate inhibition.

Discussion

P49 blocks a central caspase-mediated initiation step in apoptosis

Our study indicates that P49 is a potent apoptotic suppressor that functions as a substrate inhibitor of caspases from invertebrates and vertebrates. Although related to pancaspase inhibitor P35, P49 is distinguished by its unique *in vivo* capacity to inhibit an initiator caspase that is insensitive to P35. As such, P49 is the third distinct apoptotic regulator encoded by the invertebrate baculoviruses (Figure 9). The finding that P49 prevents apoptosis induced by diverse signals in cells from *S.frugiperda* and *D.melanogaster* suggests that this apoptotic regulator acts at a phylogenetically conserved step in the death pathway. In SF21 cells, P49 blocked proteolytic processing of effector caspases *Sf*-caspase-1 and -2 (Figure 5) by preventing cleavage at (T/A)ETD↓G between the large and small subunits. Thus, P49 likely inhibits the P35-insensitive initiator caspase, designated *Sf*-caspase-X, responsible for activation of effector caspases in *Spodoptera* (LaCount *et al*., 2000; Manji and Friesen, 2001). Consistent with the capacity of P49 to block activation of caspases that are targeted by P35, P49 prevented *in vivo* cleavage of P35 by these downstream caspases (Figure 4). Conversely, P35 had no effect on *in vivo* cleavage of P49. Thus, P49 functions at a distinct step upstream from and unaffected by P35 (Figure 9). In support of this conclusion, *in vitro* assays demonstrated that P49 is a poor inhibitor of active *Sf*-caspase-1 when compared with P35, which stoichiometrically inhibits this effector protease (Figure 2).

Collectively, our data support a model for cell death in *Spodoptera* (Figure 9) wherein P49 targets initiator *Sf*-caspase-X, which functions to activate effector caspases, including *Sf*-caspase-1. *Sf*-caspase-X is likely regulated by endogenous *Sf*-IAP, a *Spodoptera* IAP capable of inhibiting human initiator caspase-9 (Huang *et al*., 2000). Consistent with such IAP regulation, Op-IAP blocked *in vivo* caspase-mediated cleavage of P49 (Figure 4B). Thus, this viral IAP acts upstream to inhibit the activation or activity of *Sf*-caspase-X. P35 functions furthest downstream by inhibiting active effector caspases and blocking their maturation. Thus, initiator and effector steps are selectively targeted by these baculovirus caspase inhibitors (Figure 9).

P49 inhibits vertebrate initiator and effector caspases in vitro

Our finding that P49 inhibited invertebrate initiator caspase activity *in vivo* predicted that it would also affect other initiator caspases, including human caspase-9, which is well characterized. Indeed, tetrapeptide cleavage by recombinant caspase-9 was potently inhibited by P49 through a substrate inhibitor mechanism (Figure 7A). Furthermore, P49 blocked proteolytic processing of effector caspase-3 by caspase-9 (Figure 7C). These findings suggest that P49 has the capacity to inhibit *in vivo* activity of caspase-9, which has been reported to be insensitive to P35 (Vier *et al*., 2000). In a purified *in vitro* system, P49 also inhibited human effector caspase-3 (Figure 2). P49 was nearly as potent as P35, a stoichiometric inhibitor with a subnanomolar K_i (Bump *et al*., 1995; Bertin *et al*., 1996; Zhou *et al*., 1998). In addition, P49 inhibited effector *Sf*-caspase-1 but much less effectively than P35. Nonetheless, P49 may have the capacity to target both initiator and effector caspases *in vivo*. Thus, P49's expanded potential to target multiple classes of caspases may confer a selective advantage to its virus during infection of specific tissues or animal hosts. The molecular mechanism determining *in vivo* target selectivity of P49 and P35 is of significant interest (see below).

P49 is a substrate inhibitor with a P35-like mechanism

Cleavage at TVTD94↓G was required for P49's anti-caspase activity *in vitro* (Figures 2 and 7). Like P35, P49's two cleavage fragments remained stably associated with the inhibited caspase (Figure 2E and F). Confirming the mechanistic significance of cleavage, D94A-mutated P49 was not cleaved *in vivo* and failed to prevent apoptosis (Figures 1 and 3). Cleavage-defective P49 also failed to block proteolytic processing of *Sf*-caspase-1 and -2 (Figure 6). The P35 cleavage site DQMD87↓G is located at the apex of a RSL that must be properly anchored to the main protein core for anti-caspase activity (Fisher *et al*., 1999; Zoog *et al*., 1999). The sequence similarity of P49

and P35 (Figure 1A) suggests that P49 has an RSL and a P35-like fold, despite its larger size. Supporting this model, the P49 substitution I76P, which disrupted the predicted α-helix anchoring the RSL, caused loss of function (Figure 6). Substitution of the analogous residue (Val71) in the α-helix of P35 also disrupted function (Fisher *et al*., 1999; Zoog *et al*., 1999). Upon cleavage, the P35 RSL undergoes a conformational rearrangement and forms a stable thioester linkage with the active site of the caspase (de la Cruz *et al*., 2001; Xu *et al*., 2001). Thus, on the basis of structural similarity and the capacity to form a stable inhibitor–caspase complex, it is likely that P49 uses a similar, if not identical, mechanism for suicide inhibition.

P_4–P_1 cleavage residues are insufficient for in vivo caspase selectivity

Although P49 and P35 use similar mechanisms, what accounts for the difference in their *in vivo* specificity? Since cleavage is critical for anti-caspase activity, we tested the contribution of cleavage site residues to caspase targeting. When the P_4–P_1 residues (TVTD94↓G) of P49 were swapped with those of P35 (DQMD87↓G), the exchange had no significant effect on specificity, since inhibition of *Sf*-caspase-1 and -2 processing by DQMD94G-substituted P49 was comparable to that by wild-type P49 (Figure 6). Likewise, the inability of P35 to block processing of both effector caspases was unaffected by substituting its cleavage site with that of P49, even though the TVTD87G-modified P35 was fully functional. In contrast to P49 and P35, alterations of the caspase cleavage residues affected the specificity and potency of CrmA (Xue and Horvitz, 1995; Ekert *et al*., 1999). Although we have not ruled out the possibility that additional RSL residues contribute to selectivity, our data suggest that P49 is not simply a larger version of P35 with a different cleavage site specificity. Rather, the caspase selectivity of P49 is conferred by a determinant absent in P35. An intriguing possibility is that the unique 120 residue insertion of P49 (Figure 1A) is involved.

P49 blocks virus-induced apoptosis in Drosophila

The capacity of P49 to inhibit both invertebrate and vertebrate caspases *in vitro* suggested that P49 should function in phylogenetically diverse organisms. Indeed, P49 blocked virus-induced apoptosis in cultured cells from *Drosophila* (Figure 8). Apoptosis of DL-1 cells was also suppressed by P35, which has been shown to prevent programmed cell death in invertebrates and vertebrates (Hay *et al*., 1994; Sugimoto *et al*., 1994; Grether *et al*., 1995; Izquierdo *et al*., 1999; Hisahara *et al*., 2000; Viswanath *et al*., 2000). Since P49 and P35 were proteolytically cleaved in *Drosophila* cells (Figure 8), a substrate inhibitor mechanism for both apoptotic suppressors is likely. Our preliminary experiments (E.Lannan, S.Perry, J.Theisen and P.Friesen, unpublished data) indicated that P49 and P35 have different targets among the seven *Drosophila* caspases identified to date (Kumar and Doumanis, 2000; Vernooy *et al*., 2000). An obvious *Drosophila* target for P49 is DRONC, a CARD-containing caspase that is resistant to P35 inhibition and likely functions as an initiator in *Drosophila* (Hawkins *et al*., 2000; Meier *et al*., 2000). However, the low *in vitro* activity of recombinant DRONC (Dorstyn *et al*., 1999) has precluded direct tests of P49 inhibition of DRONC (data not shown). Consequently, alternative approaches to identify the *Drosophila* targets of P49 and P35 are underway. We predict that like human initiator caspase-9, DRONC will be inhibited by P49.

Our study also demonstrates for the first time that it is possible to trigger uniform apoptosis in cultured *Drosophila* cells by using baculovirus vectors. Inoculation of DL-1 cells with moderate levels [~10 plaque-forming units (p.f.u.) per cell] of Ac*M*NPV recombinants that lack functional apoptotic suppressors caused complete apoptosis within 24 h. Moreover, the use of recombinant viruses allowed simultaneous expression of functional anti-apoptotic genes to block apoptosis at different steps in the death pathway. The capacity to deliver such apoptotic regulators with near 100% efficiency to cells that are normally refractive to standard transfection methods should facilitate studies on apoptotic pathways in *Drosophila*, a model organism for studies on programmed cell death (Bergmann *et al*., 1998; Abrams, 1999). In particular, P49 promises to be useful in defining the molecular steps of caspase activation in flies and other organisms.

Materials and methods

Cells and viruses

Spodoptera frugiperda IPLB-SF21 (Vaughn *et al*., 1977) and *Drosophila* Schneider DL-1 (Schneider, 1972) cell lines were propagated at 27°C in TC100 and Schneider's growth medium, respectively (Gibco-BRL). For infection, cell monolayers were exposed to ⩾15 p.f.u./cell. Wild-type Ac*M*NPV (*p35*$^+$, *iap*$^-$) and Ac*M*NPV recombinants vΔp35 (*p35*$^-$, *iap*$^-$), vΔp35/lacZ and vOp-IAP (*p35*$^-$, *iap*$^+$) have been described (Hershberger *et al*., 1992; Manji *et al*., 1997). Recombinant vP49 (*p49*$^+$, *p35*$^-$, *iap*$^-$) was generated by allelic replacement (Hershberger *et al*., 1992) whereby the polyhedrin gene of parent vΔp35 was substituted with *Sl*NPV *p49* (Du *et al*., 1999) fused to the Ac*M*NPV *ie-1* promoter and linked to a *lacZ* reporter. To overproduce P49-His_6, an Ac*M*NPV expression vector was constructed with the pFastBac1 donor plasmid (BRL Life Technologies) using standard procedures.

Plasmids

pIE1-P49 was constructed by inserting the *p49* open reading frame from plasmid pES2 (Du *et al*., 1999) downstream from the *ie-1* promoter of pIE1^{hr}/PA at an *Nde*I site introduced at *p49* nucleotide position –1. *p49* and nucleotide substitutions thereof were generated by using overlap extension PCRs and verified by nucleotide sequencing. Plasmids expressing Ac*M*NPV *p35* and Op*M*NPV Op-*iap* using the *ie-1* promoter have been described (Bertin *et al*., 1996; Manji *et al*., 1997; Zoog *et al*., 1999).

Cell survival and marker rescue assays

SF21 monolayers were transfected with plasmid DNAs by using cationic liposomes as described previously (LaCount *et al*., 2000). After infection or UV irradiation, viable and apoptotic cells were distinguished by computer-aided microscopy as described previously (Manji and Friesen, 2001). The mean ± standard deviation was calculated from the number of surviving cells in five evenly distributed fields of view. For marker rescue assays (Bertin *et al*., 1996), SF21 cells were inoculated with vΔp35/lacZ 16 h after transfection with plasmid DNAs. The resulting extracellular virus was quantified by plaque assay using SF21 cells and X-gal to identify rescued (non-apoptotic) virus.

In vitro caspase inhibition assays

P35-His_6 and D87A-mutated P35-His_6 were obtained from *E.coli* (Fisher *et al*., 1999), but similar attempts to produce P49 in *E.coli* yielded only insoluble protein. Thus, wild-type and D94A-mutated P49-His_6 were purified by Ni^{2+} affinity chromatography (Zoog *et al*., 1999) after production in SF21 cells by Ac*M*NPV expression vectors. P49

preparations were judged ⩾95% pure by electrophoretic analysis. Purified His_6-tagged *Sf*-caspase-1 (LaCount *et al*., 2000), His_6-tagged human caspase-3 (Fisher *et al*., 1999) or recombinant human caspase-9 (Calbiochem) was mixed with increasing quantities of P49- or P35-His_6 using reaction conditions described previously (Bertin *et al*., 1996; Fisher *et al*., 1999). After 30 min, residual caspase activity was measured by using substrates Ac-DEVD-AMC or Ac-LEHD-AFC. Reported values are the average of triplicate assays in which the rate of product formation was obtained from the linear portion of the reaction curves within the first 10% of substrate depletion.

P49 cleavage and caspase association

Untagged, [^{35}S]methionine-labeled P49 from coupled transcription–translation reactions (Promega) was mixed with purified His-tagged *Sf*-caspase-1 or human caspase-3 for 30 min in 10 mM HEPES pH 7.5–0.1% CHAPS–10% sucrose. Conversely, purified wild-type or D94A-mutated P49-His_6 (5 μg) was mixed with *E.coli* extract containing untagged human caspase-3. After Ni^{2+} affinity purification (Fisher *et al*., 1999), proteins were subjected to SDS–PAGE and immunoblot analyses. For N-terminal sequence analysis, the large and small fragments of P49-His_6 generated by cleavage with human caspase-3 or *Sf*-caspase-1 were separated electrophoretically and subjected to Edman degradation (Midwest Analytical, Inc.). For cleavage by human caspase-9, reactions containing [^{35}S]methionine-labeled procaspase-3 in 50 mM HEPES pH 7.2, 50 mM NaCl, 10 mM DTT, 10 mM EDTA, 0.1% CHAPS, 5% glycerol were incubated at 37°C for the times indicated. The identity of each caspase was confirmed by use of caspase-specific tetrapeptide inhibitors (data not shown).

Immunoblot analysis and antisera

For P49-specific antiserum (α-P49), insoluble *E.coli*-produced P49-His_6 was used as antigen for immunization of New Zealand white rabbits (University of Wisconsin Medical School Polyclonal Antibody Service). Immunoblot analyses were conducted using the following antisera: α-P49, α-p17 of human caspase-3 (Fisher *et al*., 1999), α-Sfcasp1 (LaCount *et al*., 2000), α-Sfcasp2 (LaCount, 1998) and monoclonal α-P35 (gift from Y.Lazebnik).

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