Baculovirus IAP1 induces caspase-dependent apoptosis in insect cells

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Baculoviruses encode inhibitors of apoptosis (IAPs), which are classified into five groups, IAP1–5, based on their sequence homology. Most of the baculovirus IAPs with anti-apoptotic functions belong to the IAP3 group, with certain exceptions. The functional roles of IAPs from other groups during virus infection have not been well established. We have previously shown that Hyphantria cunea multiple nucleopolyhedrovirus (HycuMNPV) encodes three iap genes, hycu-iap1, hycu-iap2 and hycu-iap3, and that only Hycu-IAP3 has anti-apoptotic activity against actinomycin D-induced apoptosis of Spodoptera frugiperda Sf9 cells. In the present study, we demonstrate that transient expression of Hycu-IAP1 is capable of inducing apoptosis and/or stimulating caspase-3-like protease activity in various lepidopteran and dipteran cell lines. Transient-expression assay analysis also demonstrates that not only Hycu-IAP1 but also IAP1s from Autographa californica MNPV, Bombyx mori NPV and Orgyia pseudotsugata MNPV (OpMNPV) are capable of inducing apoptosis, and that apoptosis induced by Hycu-IAP1 is precluded by the functional anti-apoptotic baculovirus protein Hycu-IAP3. In HycuMNPV-infected Spilosoma imparilis (SpIm) cells and OpMNPV-infected Ld652Y cells, caspase-3-like protease activity is markedly stimulated during the late stages of infection, and the caspase-3-like protease activity stimulated in HycuMNPV-infected SpIm cells is repressed by RNA interference-mediated silencing of hycu-iap1. In addition, initiator caspase Bm-Dronc, the B. mori homologue of Dronc, is cleaved upon transfection of BM-N cells with a plasmid expressing Hycu-IAP1. These results provide the first evidence that baculovirus IAP1s act to induce caspase-dependent apoptosis, possibly by replacing the cellular IAP1 that prevents Dronc activation.

INTRODUCTION

Inhibitor of apoptosis (iap) genes were first identified in baculoviruses, Cydia pomonella granulovirus (CpGV) and Orgyia pseudotsugata multiple nucleopolyhedrovirus (OpMNPV), as genes encoding an anti-apoptotic protein that suppressed virus-induced apoptosis of Spodoptera frugiperda Sf21 cells upon infection with a recombinant Autographa californica MNPV (AcMNPV) having a defective p35 gene (Birnbaum et al., 1994; Crook et al., 1993). Homologues of the baculovirus iap genes were subsequently identified in yeast, nematodes, insects and mammals (Duckett et al., 1996; Fraser et al., 1999; Uren et al., 1998, 1999). However, only a subset of these IAPs function as apoptosis suppressors, and many other IAPs are involved in diverse non-apoptotic roles in cellular survival and differentiation (O’Riordan et al., 2008; Orme & Meier, 2009; Srinivasula & Ashwell, 2008). The IAP proteins are mostly characterized by the presence of one-to-three baculovirus IAP repeat (BIR) motifs in the N-terminal region and most also contain a RING finger domain in the C-terminal region (Mace et al., 2010; O’Riordan et al., 2008; Salvesen & Duckett, 2002; Vaux & Silke, 2005). The BIRs are unique to IAPs and consist of approximately 70 aa with the signature sequence CX2CX16HX6C, which coordinates a zinc ion. The BIRs mediate protein–protein interactions, and individual BIR domains acquire distinct binding properties, choosing different binding partners that participate in apoptosis regulation. The RING domain of IAPs consists of approximately 40 aa, including eight cysteine and histidine residues that form a binding site for two zinc ions. The IAP RING domain functions as an E3 ubiquitin ligase, which promotes the transfer of ubiquitin onto selected protein targets, whereby the IAPs are involved in the proteasome-mediated degradation of target proteins. Consistent with this general feature of IAPs, baculovirus IAPs, including Bombyx mori NPV (BmNPV) IAP2 and OpMNPV IAP3, have E3 ubiquitin ligase activity (Green et al., 2004; Imai et al., 2003).
Baculovirus IAPs, including CpGV IAP3, OpMNPNV IAP3 and Antheraea pernyi NPV (AnpeNPV) IAP1, require both the BIR and RING domains for their anti-apoptotic function (Clem & Miller, 1994; Hozak et al., 2000; Yan et al., 2010). However, certain truncated baculovirus IAP proteins containing separated BIR and RING domains not only lose their anti-apoptotic function but also acquire the capacity to induce apoptosis when these truncated IAP proteins are overexpressed (Harvey et al., 1997). Thus, the roles that individual BIR and RING domains play in anti-apoptotic and pro-apoptotic functions remain obscure.

Individual baculoviruses generally encode multiple IAPs, which are classified into five groups, IAP1–5 based on sequence homology (Luque et al., 2001), and only a limited number of baculovirus IAPs are related to apoptosis (Clem, 2005, 2007). In most of the baculoviruses, IAP3 has an anti-apoptotic function (Birnbaum et al., 1994; Carpes et al., 2005; Crook et al., 1993; Ikeda et al., 2004), although exceptions are reported in Epiphysas postvittanae MNPV (EpPoMNPNV) and AnpeNPV, in which only IAP2 and IAP1, respectively, have apoptosis suppression activity (Maguire et al., 2000; Yan et al., 2010). Functional analyses have also demonstrated that EpPoMNPNV IAP1 and Spodoptera littoralis MNPNV IAP4 are unable to suppress apoptosis but have the ability to delay the onset of apoptosis induced by p35-defective AcMNPNV and other apoptotic stimuli (Liu et al., 2003; Maguire et al., 2000), whereas CpGV IAP5 has a stimulatory effect on the anti-apoptotic function of CpGV IAP3 (Vilaplana & O'Reilly, 2003). In addition, AcMNPNV iap1 and iap2 genes can be disrupted without any loss of viral replication capacity (Griffiths et al., 1999), while AcMNPNV that lacks a functional iap1 gene carries a replication advantage over iap1-containing wild-type AcMNPNV in TN368 cells co-infected with these viruses (McLachlin et al., 2001). The functional roles of baculovirus IAPs, other than IAP3, in virus infection and multiplication are not known.

We have previously shown that Hyphantria cunea MNPNV (HycuMNPNV) encodes three iap genes, hycu-iap1, hycu-iap2 and hycu-iap3, and that only Hycu-IAP3, but not Hycu-IAP1 or Hycu-IAP2, has anti-apoptotic activity against apoptosis induced by actinomycin D treatment in Sf9 cells (Ikeda et al., 2004, 2006). In the present study, we demonstrate that transiently expressed Hycu-IAP1 is capable of inducing caspase-dependent apoptosis, rather than suppressing apoptosis, in various insect cell lines, including Spodoptera frugiperda Sf9, B. mori BM-N, Lymantria dispersa Ld652Y, Spilosoma imparis SpIm and Drosophila melanogaster S2. Transient expression assay analysis has also shown that not only Hycu-IAP1 but also IAP1s from AcMNPNV, BmNPV and OpMNPNV have apoptosis-inducing activities. In addition, we demonstrate in HycuMNPNV-infected SpIm cells that caspase-3-like protease activity is markedly stimulated during the late stages of infection and that this stimulation is suppressed by RNA interference-mediated silencing of hycu-iap1. Furthermore, we show that transfection of BM-N cells with a plasmid expressing Hycu-IAP1 results in the cleavage of Bm-Dronc, the B. mori homologue of the Drosophila initiator caspase Dronc.

**RESULTS**

**Hycu-IAP1 induces apoptosis in Sf9 cells that is suppressed by Hycu-IAP3**

Monolayer cultures of 1×10^6 Sf9 cells in 35 mm tissue-culture dishes were transfected with 2 μg of plE1-2/HAhycu-iap1, plE1-2/HAhycu-iap2 or plE1-2/HAhycu-iap3, which express haemagglutinin (HA)-tagged Hycu-IAP1, Hycu-IAP2 or Hycu-IAP3, respectively, and cultured for 24 h at 28°C in Grace’s medium as previously described (Ikeda et al., 2004). Microscopic examination showed that some of the Sf9 cells transfected with plE1-2/HAhycu-iap1 exhibited morphological changes characteristic of apoptosis, producing apoptotic body-like globules, while transfection with plE1-2/HAhycu-iap2 or plE1-2/HAhycu-iap3, as well as plE1-2/egfp, which expresses EGFP, caused no appreciable apoptotic morphological changes in Sf9 cells (Fig. 1a). The morphological changes that occurred upon transfection with plE1-2/HAhycu-iap1 were precluded by co-transfection with plE1-2/HAhycu-iap3 (Fig. 1a), which expresses functional apoptotic suppressor Hycu-IAP3 (Ikeda et al., 2004). Such plE1-2/HAhycu-iap1-induced morphological changes in Sf9 cells could not be eliminated by plE1-2/egfp co-transfection.

To determine whether the plE1-2/HAhycu-iap1-induced morphological changes in cells were caused by apoptosis, caspase-3-like protease activity was assayed in Sf9 cells at 24 h post-transfection (p.t.) with plE1-2/HAhycu-iap1, plE1-2/HAhycu-iap2 or plE1-2/HAhycu-iap3. Stimulated caspase-3-like protease activity was observed only in plE1-2/HAhycu-iap1-transfected Sf9 cells, while no significant stimulation of caspase-3-like protease activity was detected in Sf9 cells transfected with plE1-2/HAhycu-iap2, plE1-2/HAhycu-iap3 or plE1-2/egfp (Fig. 1b). The stimulated caspase-3-like protease activity in plE1-2/HAhycu-iap1-transfected Sf9 cells was diminished by co-transfection with plE1-2/HAhycu-iap3 (Fig. 1b). These results indicate that Hycu-IAP1 specifically stimulated caspase-3-like protease activity and induced apoptosis in Sf9 cells, both of which were suppressed by the functional anti-apoptotic baculovirus protein Hycu-IAP3.

**Hycu-IAP1 stimulates caspase-3-like protease activity and induces apoptosis in various insect cell lines**

To determine whether the Hycu-IAP1-induced apoptosis that was observed in Sf9 cells also occurred in other insect cell lines, monolayer cultures of one dipteran and four lepidopteran cell lines, including S2, BM-N, Ld652Y, Sf9 and SpIm, were prepared as previously described (Felipe Alves et al., 2009) and transfected with plE1-2/HAhycu-iap1. The cells transfected with plE1-2/HAhycu-iap1...
underwent apoptosis in all cell lines, clearly exhibiting characteristic apoptotic morphological changes in a population of the cells transfected with pIE1-2/HAhycu-iap1 (Fig. 2a). At 24 h p.t., the caspase-3-like protease activity was markedly stimulated in the cell lines undergoing apoptosis (Fig. 2b).

**IAP1s from various NPVs stimulate caspase-3-like protease activity and induce apoptosis in Sf9 cells**

To determine whether IAP1s from other baculoviruses also stimulate caspase-3-like protease activity, expression plasmids pIE1-2/HAac-iap1, pIE1-2/HAbm-iap1, pIE1-2/HAhycu-iap1 and pIE1-2/HAop-iap1, which express HA-tagged IAP1s from AcMNPV (Ac-IAP1), BmNPV (Bm-IAP1), HycuMNPV (Hycu-IAP1) and OpMNPV (Op-IAP1), respectively, were transfected into Sf9 cells. All plasmids successfully expressed their respective IAP1s (Fig. 3a) and stimulated caspase-3-like protease activity in transfected Sf9 cells (Fig. 3b), suggesting that IAP1s from these NPVs had pro-apoptotic activity. Microscopic examination showed that the cell cultures of transfected Sf9 cells with stimulated caspase-3-like protease activity contained a significant population of apoptotic cells (data not shown).

**Changes in caspase-3-like protease activity during NPV infection**

The results in Fig. 3 demonstrated that transiently expressed Ac-IAP1, Bm-IAP1, Hycu-IAP1 and Op-IAP1 stimulated caspase-3-like protease activity and induced apoptosis in Sf9 cells. To gain insight into the possible functional roles of IAP1s during baculovirus infection, SpIm, Ld652Y and BM-N cells were infected with HycuMNPV, OpMNPV and BmNPV, respectively, and caspase-3-like protease activity was determined at different times post-infection (p.i.). Caspase-3-like protease activities were significantly higher in HycuMNPV-infected SpIm cells and OpMNPV-infected Ld652Y cells than in BmNPV-infected BM-N cells (Fig. 4; note differences in the y-axis scales for activity), reflecting the cell line-dependent differences of apoptosis induction. Caspase-3-like protease activity was dramatically stimulated at 72 and 96 h p.i. in HycuMNPV-infected SpIm cells and OpMNPV-infected Ld652Y cells, as compared with that in mock-infected cells (Fig. 4). In the BmNPV-infected BM-N cells, caspase-3-like protease activity was suddenly stimulated to a small extent at 96 h p.i. but decreased to the level of mock-infected cells at 120 h p.i. As in mock-infected SpIm and Ld652Y cells, caspase-3-like protease activity in mock-infected BM-N cells increased slightly during the experiments, probably owing to the increase in cell number.

**RNAi-mediated silencing of hycu-iap1 abrogates stimulation of caspase-3-like protease activity in HycuMNPV-infected SpIm cells**

To confirm that the stimulation of caspase-3-like protease activity in infected cells was caused by virus-encoded IAP1s, RNAi-mediated silencing was performed in HycuMNPV-infected SpIm cells. Monolayer cultures of SpIm cells were transfected with dsRNAs against *hycu-iap1* and EGFP. The transfected SpIm cells were then infected with HycuMNPV at 4 h p.t., and Hycu-IAP1 expression was determined by immunoblot analysis with anti-Hycu-IAP1 polyclonal antibody. Little, if any, Hycu-IAP1 expression was detected in cells transfected with *hycu-iap1* dsRNA, while Hycu-IAP1 expression was clearly observed in cells transfected with EGFP dsRNA from 48 h p.i. onward (Fig. 5a). Immunoblot analysis also demonstrated that there was no detectable difference in the amounts of major capsid protein VP39 between cells transfected with *hycu-iap1* dsRNA and EGFP dsRNA (Fig. 5a), thus indicating that *hycu-iap1* silencing
had little, if any, adverse effects on virus multiplication. Consistent with the data from the immunoblot analysis, stimulation of caspase-3-like protease activity in Hycu MNPV-infected SpIm cells was substantially suppressed by transfection with \textit{hycu-iap1} dsRNA (Fig. 5b). Such ablation of the stimulated caspase-3-like protease activity did not occur upon transfection with \textit{EGFP} dsRNA.

**Hycu-IAP1 stimulates Bm-Dronc cleavage in BM-N cells**

We previously cloned and characterized the \textit{B. mori} homologue of initiator caspase Dronc (Bm-Dronc) (unpublished).

To explore the possibility that Bm-Dronc might be involved in IAP1-induced stimulation of caspase-3-like protease activity late in NPV infection, pIE1-2/HAhycu-iap1 expressing Hycu-IAP1 was transfected into BM-N cells and immunoblot analysis was performed with anti-Bm-Dronc antibody. BM-N cells transfected with pIE1-2/HAhycu-iap1 contained only the full-length Bm-Dronc until 48 h.p.t. However, at 72 h.p.t., a significant, but small, amount of cleaved Bm-Dronc, which probably consisted of the prodomain and the large subunit, was detected in addition to the full-length Bm-Dronc (Fig. 6), thus suggesting that stable activation of Bm-Dronc occurred in BM-N cells expressing Hycu-IAP1. In contrast, BM-N cells transfected

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**Fig. 2.** Apoptosis induction and stimulation of caspase-3-like protease activity in various insect cell lines expressing Hycu-IAP1. BM-N, \textit{Ld652Y}, S2, SF9 and SpIm cells were transfected with 2 \(\mu\)g of pIE1-2/HAhycu-iap1 (Hycu-IAP1) or pIE1-2/egfp (EGFP). At 24 h.p.t., cells were examined for apoptosis (a) and caspase-3-like protease activity (b). (a) Arrowheads indicate the cells undergoing apoptosis. Bar, 100 \(\mu\)m. (b) Caspase-3-like protease activities were normalized to the activity observed in pIE1-2/egfp-transfected cells. Error bars indicate the sd of means from three determinations.
with the plasmid pIE1-2/egfp, expressing EGFP, contained only full-length Bm-Dronc (Fig. 6). These results suggest that lepidopteran insect homologues of the initiator caspase Dronc were involved in the baculovirus IAP1-induced stimulation of caspase-3-like protease activity, which was responsible for the apoptosis occurring in the NPV-infected cells late in infection.

**DISCUSSION**

In the present study, we demonstrate that transiently expressed baculovirus Hycu-IAP1 stimulates caspase-3-like protease activity and induces apoptosis in Sf9 cells. The stimulation of caspase-3-like protease activity by Hycu-IAP1 was not restricted to Sf9 cells but occurred in all cell lines examined, including BM-N, Ld652Y, Splm and S2, which are derived from B. mori, L. dispar, Spilosoma imparilis and...
D. melanogaster, respectively. We also demonstrate that not only Hycu-IAP1 but also IAP1s from AcMNPV, BmNPV and OpMNPV are capable of inducing apoptosis and stimulating caspase-3-like protease activity in Sf9 cells. In addition, we found that caspase-3-like protease activities in HycuMNPV-infected SpIm cells and OpMNPV-infected Ld652Y cells are markedly stimulated during the late stages of infection, and that the caspase-3-like protease activity stimulated in the HycuMNPV-infected SpIm is greatly suppressed by RNAi-mediated silencing of hycu-iap1. Furthermore, our data show that endogenous Bm-Dronc, the B. mori homologue of Drosophila initiator caspase Dronc, is cleaved in BM-N cells upon transfection with a plasmid expressing Hycu-IAP1, indicating that Bm-Dronc is involved in baculovirus IAP1-mediated induction of apoptosis and stimulation of caspase-3-like protease activity. Thus, our results provide the first evidence that baculovirus IAPs induce caspase-dependent apoptosis.

Our results showing that baculovirus IAP1s have a pro-apoptotic function are further supported by circumstantial evidence from studies with truncated Op-IAP3 and Cp-IAP3 (Harvey et al., 1997; Hozak et al., 2000). Both Op-IAP3 and Cp-IAP3 suppress the apoptosis induction and caspase-3-like protease activity stimulation induced by diverse apoptotic stimuli (Birnbaum et al., 1994; Crook et al., 1993; Seshagiri & Miller, 1997). These IAP3s contain two BIR motifs, BIR1 and BIR2, and a RING motif in the N- and C-terminal regions, respectively. For the anti-apoptotic function of Op-IAP3 and Cp-IAP3, both BIR and RING motifs are required (Clem & Miller, 1994). Although the functional roles of individual motifs in the anti-apoptotic function of baculovirus IAPs have not been well-defined, previous studies have shown that both Op-IAP3 and Cp-IAP3 truncations, which contain the BIR1 and BIR2 motifs but lack the RING motif, induce apoptosis or sensitize cells to apoptosis (Harvey et al., 1997; Hozak et al., 2000), rather than prevent apoptosis induced by apoptotic stimuli. These studies have also shown that the Cp-IAP3 truncation containing only the RING motif, but not the corresponding Op-IAP3 truncation, induces apoptosis of Sf21 cells (Harvey et al., 1997).

In addition, our recent studies have demonstrated that the Hycu-IAP3 truncation, which contains both BIR1 and BIR2 motifs but not the RING motif, still retains anti-apoptotic activity, whereas the truncation containing only the RING motif induces apoptosis in Ld652Y cells from L. dispar (unpublished). Furthermore, the Hycu-IAP1 truncation containing BIR2 and RING retains pro-apoptotic activity, while the Hycu-IAP1 truncation lacking a RING domain and containing only BIR1 and BIR2 loses pro-apoptotic activity (unpublished). Collectively, these data indicate that IAPs with BIR and RING motifs may serve as either anti-apoptotic or pro-apoptotic factors, yet these data do not provide a comprehensive understanding of the functional roles of BIR and RING motifs in the anti-apoptotic and pro-apoptotic functions of baculovirus IAPs because of wide variations in the experimental data.

Analyses of HycuMNPV-infected SpIm cells demonstrate that Hycu-IAP1 actually functions in the virus-infected cells to cause apoptosis induction and stimulation of caspase-3-like protease activity at late stages of infection. We have previously shown that hycu-iap1 has a late
promoter and is expressed in HycuMNPV-infected SpIm cells at the late stages of infection, commencing at 12 h p.i., in contrast to the functionally anti-apoptotic hycu-iap3 gene, which has both early and late promoters and is expressed from 4 h p.i. (Ikeda et al., 2004). In the present study, we have demonstrated that caspase-3-like protease activity increases strikingly in HycuMNPV-infected SpIm cells and OpMNPV-infected Ld652Y cells during the late stages of infection, consistent with the previous results in AcMNPV-infected High Five cells from Trichoplusia ni (Hebert et al., 2009). RNAi-mediated silencing analysis further indicates that Hycu-IAP1 is involved in the stimulation of caspase-3-like protease activity, thus showing that the silencing of hycu-iap1 results in suppression of the caspase-3-like protease activity stimulated in the HycuMNPV-infected SpIm cells during the late stages of infection. These results suggest that the induction of apoptosis and the stimulation of caspase-3-like protease activity observed in the baculovirus-infected cells during the late stages of infection are attributable to the baculovirus IAP1s.

It has been demonstrated in D. melanogaster that down-regulation of DIAPI induces Dronc-mediated apoptosis in S2 cells, indicating that DIAPI normally suppresses the activity of Dronc in non-apoptotic cells (Igaki et al., 2002; Muro et al., 2002). Similar cellular IAP1-mediated suppression of Dronc activity in non-apoptotic cells is also observed in Aedes aegypti, in which RNAi silencing of iap1 and dronc in A. aegypti Aag2 cells results in the induction and suppression of apoptosis, respectively (Liu & Clem, 2011). Taking these observations into account, our results suggest that baculovirus IAP1s trigger apoptosis and stimulation of caspase-3-like protease activity by replacing the B. mori cellular IAP1, cmBm-IAP1, which might be physically associated with the initiator caspase Bm-Dronc to suppress its activity. First, our results demonstrate that overexpression of Hycu-IAP1 in BM-N cells causes cleavage of Bm-Dronc, which generally follows the stable activation of Dronc (Dorstyn & Kumar, 2008; Muro et al., 2004; Snipas et al., 2008; Yan et al., 2006). Second, RNAi silencing of cmBm-iap1 in BM-N cells resulted in the cleavage of Bm-Dronc and induction of apoptosis, accompanied by the stimulation of caspase-3-like protease activity (unpublished).

Baculovirus genomic sequence data have shown that baculovirus iap1 genes are found exclusively in group 1 NPVs (Rohrmann, 2011), thus suggesting their important roles in efficient replication and/or dissemination of group 1 NPVs. However, the biological significance of the baculovirus IAP1-mediated apoptosis induction and stimulation of caspase-3-like protease activity is not known. A number of viruses encode various factors that control apoptosis in infected cells for their own benefit (Galluzzi et al., 2008; Hay & Kannourakis, 2002; Roulston et al., 1999; Teodoroi & Branton, 1997). Using these anti- and pro-apoptotic factors, viruses prevent apoptosis induced early in infection to yield sufficient amounts of progeny virions, whereas they actively induce apoptosis late in infection to facilitate the dissemination of progeny virions to neighbouring cells. In the NPV-infected cells, two infectious forms of progeny virions are produced in a temporally regulated fashion (Blissard & Rohrmann, 1990; Williams & Faulkner, 1997). The budded virions (BVs) are produced early in infection by nucleocapsid budding through the plasma membrane of infected cells, whereas the production of the occlusion body-derived virions (ODVs) occurs late in infection within the nucleus, commencing concomitantly with the decrease in BV production (Volkman et al., 1976). Thus, late in infection, when baculovirus IAP1s stimulate caspase-3-like protease activity and induce apoptosis, infected cells exclusively contain virions occluded and pre-occluded in the occlusion bodies, which are more infectious by the per os route of infection than by the haemocoeal route of infection and play a role in insect-to-insect transmission, in contrast to the BVs, which are more potent by the haemocoeal route of infection than by the per os route of infection and play a role in cell-to-cell transmission within the infected insects (Hughes & Wood, 1996; Kawarabata, 1974; Kawarabata & Aratake, 1978; Keddie & Volkman, 1985; Volkman & Summers, 1977). It is therefore tempting to determine whether the ODV and pre-occluded virions liberated from the virus-infected apoptotic cells facilitate viral dissemination in the field and/or within the infected insects.

**Methods**

**Cells, virus and infection.** Sf9 cells from Spodoptera frugiperda were maintained in Grace’s medium (Invitrogen) supplemented with 10 % FBS. BM-N cells from the silkworm, B. mori (Volkman & Goldsmith, 1982) and IPLB-Ld652Y (Ld652Y) cells from L. dispar (Goodwin et al., 1978) were grown in TC100 medium (AppliChem) supplemented with 10 % FBS and 0.26 % tryptose broth (Sigma). S2 cells from D. melanogaster (Schneider, 1972) were cultured in Schneider’s Drosophila Medium (Invitrogen) supplemented with 10 % FBS. FRI-Splm-1229 (Splm) cells from the mulberry tiger moth, Spilosoma imparis (Mitsushashi & Inoue, 1988), were cultured in MM medium (Mitsushashi & Maramorosch, 1964) supplemented with 3 % FBS. These cells were grown at 28 °C.

Viruses used in these experiments were A. californica M nucleopolyhedrovirus E2 (AcMNPV) (Smith & Summers, 1978), B. mori NPV N9 (BmNPV) (Nagamine et al., 1989), O. pseudotsugata MNPV (OpMNPV) (Leisy et al., 1986) and H. cunea MNPV N9 (HycuMNPV) (Kamiya et al., 2003).

Monolayer cultures of 5 × 10^5 cells in 35 mm culture dishes (Falcon 3001) were infected with the respective viruses at an m.o.i. of 5 p.f.u. for 60 min at room temperature as previously described (Ikeda et al., 2004).

**Plasmids.** Expression plasmids pIE1-2/HAbm-iap1, pIE1-2/HAbm-iap2 and pIE1-2/HAcMNPV IAP1 (Hycu-IAP1), Hycu-IAP2 and Hycu-IAP3, respectively, and pIE1-2/egfp, which expresses EGFP, have been described previously (Ikeda et al., 2004). Expression plasmids pIE1-2/HAac-iap1, pIE1-2/ HAbm-iap1 and pIE1-2/HAop-iap1, expressing HA-tagged AcMNPV IAP1 (Ac-IAP1), BmNPV IAP1 (Bm-IAP1) and OpMNPV IAP1 (Op-IAP1), respectively, were constructed according to a previously described method (Ikeda et al., 2004). The BamHI adaptor with HA-tag was constructed by annealing two oligonucleotides: 5’-GATCTTCTACCCTACGATGTCCGATTACGCTC-3’ and 5’-GAGATCCTCTAGGATGCTGGAAGGCGACG-3’.
5′-GATCCAAGGGTAATCCGGAACATGTATGGGTTAA-3′. The BamHI adaptor with the HA tag was inserted into the BamHI site of pIE1-2 (Novagen) to yield pIE1-2/HA. pIE1-2/HAac-iap1 and pIE1-2/HAop-iap1 were generated by inserting the coding regions of ac-iap1, bm-iap1 and op-iap1, respectively, into pIE1-2/HA digested with BamHI and NotI. The coding regions of ac-iap1, bm-iap1 and op-iap1 were amplified by PCR from the genomic DNA of AcMNPV, BmNPV and OpMNPV, respectively, using paired primers with BamHI and NotI restriction sites (underlined): 5′-GGATCCATGAACGAGGACACTCCG-3′ and 5′-GGCGGCCGCTACAGTGACGGACACTCCG-3′ for ac-iap1; 5′-GGATCCATGAACGAGGACACTCCG-3′ and 5′-GGCGGCCGCTACAGTGACGGACACTCCG-3′ for bm-iap1; and 5′-GGATCCATGAACGAGGACACTCCG-3′ and 5′-GGCGGCCGCTACAGTGACGGACACTCCG-3′ for op-iap1.

RNAi procedure. The preparation of dsRNAs against hycu-iap1 and EGFP has been described previously (Ikeda et al., 2004). For production of dsRNA against EGFP, 738 bp of EGFP was PCR-amplified from pIE1-2/egfp by using paired primers that contained a T7 RNA polymerase binding site (underlined): 5′-TAATACGACTCACTATAGGGAGACTTGTACAGCTCGTCCATGC-3′ and 5′-T7-CAATATACGACTCACTATAGGGAGACTTGTACAGCTCGTCCATGC-3′. The PCR products were purified using a MagExtractor kit (Toyobo Life Science), and RNAs were transcribed by using a MEGAscript T7 kit (Ambion). Sense and antisense RNAs were annealed by incubation at 65°C for 30 min, followed by slow cooling at room temperature. dsRNAs were introduced into cells by transfection.

Immunoblot analysis. Polypeptides were resolved on SDS-PAGE gels, transferred onto Immobilon-P transfer membranes (Millipore) and subjected to immunoblot analysis as previously described (Ikeda et al., 2004). Positive signals were visualized by ECL Western blotting detection reagents (Amersham Biosciences). The antibodies against Hycu-IAP1 and major capsid protein VP39 were raised against partial amino acid sequences in mice and rabbits, respectively, as previously described (Ikeda et al., 2004; Katou et al., 2006). The antibody against the B. mori homologue of Dronc (Bm-Dronc) was raised in a rabbit immunized with Bm-Dronc protein expressed in Escherichia coli (unpublished). Anti-HA mAb HA.11 was purchased from Babco, and HRP-conjugated goat anti-mouse IgG and anti-rabbit IgG antibodies (unpublished). Anti-HA mAb HA.11 was purchased from Babco. Antiserum against mouse IgG and anti-rabbit IgG antibodies were obtained from Zymed.

Caspase activity assay. Caspase activity was assayed as previously described (Ishikawa et al., 2003), by using a caspase-3 fluorescent assay kit, ApoProbe-3, containing the synthetic substrate Ac-DEVD-AMC (Biodynamics Laboratory). The infected and transfected cells were collected by a rubber policeman, centrifuged at 800 g for 3 min at 4°C, suspended in the cell lysis buffer included in the kit and incubated on ice for 10 min. The cell lysates thus obtained were centrifuged at 12,000 g for 3 min, and the supernatants were used in the caspase-3-like protease activity assay.

Transfection. Transfection was performed as previously described (Katou et al., 2001, 2006). Monolayer cultures of L6k52Y, S2 or Sp1m cells (5 × 10⁴, 1.5 × 10⁶ and 5 × 10⁶ cells, respectively) in 35 mm dishes were transfected with 2 μg of plasmid or 1 μg of dsRNAs by using 8 μl of Cellfectin (Invitrogen). For the transfection of S9 and BM-N cells (8 × 10⁶ and 5 × 10⁶ cells, respectively), 10 μl of Lipofectin (Invitrogen) and 4 μl of FuGene (Roche Applied Science), respectively, were used.

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