Functional analysis of the putative antiapoptotic genes, p49 and iap4, of Spodoptera litura nucleopolyhedrovirus with RNAi

Qian Yu, Tiehao Lin, Guozhong Feng, Kai Yang and Yi Pang

State Key Laboratory of Biocontrol, Sun Yat-sen University, Guangzhou 510275, PR China

A homology search of a public database revealed that Spodoptera litura nucleopolyhedrovirus (SpltNPV) possesses two putative, antiapoptotic genes, p49 and inhibitor of apoptosis 4 (iap4), but their function has not been investigated in its native host cells. In the present study, we used RNA interference (RNAi) to silence the expression of Splt-iap4 and Splt-p49, independently or together, to determine their roles during the SpltNPV life cycle. RT-PCR analysis and Western blot analysis showed the target gene expression had been knocked out in the SpltNPV-infected SpLi-221 cells after treatment with Splt-p49 or Splt-iap4 double-stranded RNA (dsRNA), respectively, confirming that the two genes were effectively silenced. In SpltNPV-infected cells treated with Splt-p49 dsRNA, apoptosis was observed beginning at 14 h, and almost all cells had undergone apoptosis by 48 h. In contrast, budded virus production and polyhedra formation progressed normally in infected cells treated with Splt-iap4 dsRNA. Cell viability analysis showed that Splt-IAp4 had no synergistic effect on the inhibition of apoptosis of SpLi-221 cells induced by SpltNPV infection. Interestingly, after Splt-iap4 dsRNA treatment, cells did not congregate like those infected with SpltNPV in the early infection phase, implying an unknown role of baculovirus iap4. Our results determine that Splt-p49 is necessary to prevent apoptosis; however, Splt-iap4 has no antiapoptotic function during SpltNPV infection.

INTRODUCTION

Baculoviruses are classified as a group of arthropod-specific viruses with rod-shaped nucleocapsids, and their genomes consist of a circular double-stranded DNA molecule of about 80–180 kbp (Theilmann et al., 2005). The family Baculoviridae comprises the genera Nucleopolyhedrovirus (NPV) and Granulovirus (GV) (Theilmann et al., 2005). Baculoviruses usually have limited host ranges and recent studies suggest that apoptosis plays a role in host range restriction (Zhang et al., 2002; Clarke & Clem, 2003; Feng et al., 2007).

Apoptosis is a type of programmed cell death which is characterized frequently by nuclear condensation and fragmentation, accompanied by vigorous blebbing of the cytoplasm membrane (Kerr et al., 1972; Wyllie et al., 1980). The mechanism of apoptosis is evolutionarily conserved. A wide range of apoptotic stimuli, such as actinomycin D, UV light, virus infection etc., can trigger a family of cysteine protease proteins (caspases) to promote cell death (Roy et al., 1997; Thornberry & Lazebnik, 1998). All caspases are catalytically inactive zymogens and must undergo proteolytic activation during apoptosis. The caspases are generally divided into two classes: the initiator caspases and the effector caspases. Different apoptotic stimuli trigger the activation of the initiator caspases, which then cleave and activate the effector caspases (Riedl & Shi, 2004).

Apoptosis represents an important virus–host interaction process which probably influences viral pathogenesis. As an antiviral response in multicellular organisms, apoptosis can limit viruses in the suicide cells so as to reduce the yield of progeny virus, which results in abortive infection (Hay & Kannourakis, 2002; Roulston et al., 1999). Although many viruses, including baculoviruses, can trigger apoptosis in infected cells, they have the ability to synthesize proteins to prevent apoptosis (Clem et al., 1991; Crook et al., 1993; Birnbaum et al., 1994; Du et al., 1999). The first discovered antiapoptotic baculovirus protein is P35, which can inhibit apoptosis in such diverse animals as nematodes, insects and mammals (Clem et al., 1991; Clem, 2007). P35 is a substrate of caspases and forms a stable caspase–P35 complex to block protease activity (Bump et al., 1995; Xue & Horvitz, 1995). A larger P35 homologue, P49, was found in some baculoviruses (Du et al., 1999; Pang et al., 2001). P49 shows similar three-dimensional structure to and the same mode of action as P35; however, P49 is able to inhibit initiator caspases that P35 is unable to (Du et al., 1999; Pei
et al., 2002; Zoog et al., 2002). The second group of baculovirus antia apoptotic proteins are IAPs (inhibitor of apoptosis). IAPs contain a RING zinc finger at the C terminus and one or more cysteine/histidine-rich motifs, termed baculovirus IAP repeat (BIR), at the N terminus (Crook et al., 1993; Clem & Miller, 1994). The BIR domain can interact with caspases and the RING domain can recruit E2 ubiquitin-conjugating enzymes and catalyse the transfer of ubiquitin onto target proteins (Green et al., 2004; Vaux & Silke, 2005). According to amino acid sequence homology, baculovirus IAPs can be divided into five types from IAP1 to IAP5 (Luque et al., 2001).

An interesting phenomenon is that most baculovirus genomes sequenced so far possess two or more antia apoptotic genes; however, only one gene has usually been determined with antia apoptotic activity in one virus–cell system. For example, there are one \( p35 \) and two \( iap \) genes present in the genome of Autographa californica multiple NPV (AcMNPV), but only \( p35 \) was capable of blocking apoptosis (Clem & Miller, 1994). Hyphantria cunea NPV (HycuNPV) genome possesses three \( iaps \); only one could block apoptosis, while the two others did not show any antia apoptotic functions (Ikeda et al., 2004). The reason that baculoviruses retain the non-functional antia apoptotic genes remains unknown. Studies on the different antia apoptotic genes present in different viral genomes would contribute to our understanding of their diversity and evolution.

Spodoptera litura is an economically important polyphagous pest in China, India and Japan, and causes considerable economic loss to many vegetables and field crops. \( S. \) litura NPV (SpltNPV) is highly specific to \( S. \) litura, an important pest in China, India and Japan, and causes considerable economic loss to many vegetables and field crops.\( S. \) litura, an important pest in China, India and Japan, and causes considerable economic loss to many vegetables and field crops. Spodoptera frugiperda (the fall armyworm) (Yanase et al., 1998) was obtained from Dr Zhihui Su (JT Biohistory Research Hall, Osaka, Japan). The \( S. \) litura cell line is a clonal isolate from IPLB-S21-AE cells which is derived from the fall armyworm (\( S. \) frugiperda) (Vaughn et al., 1977). The cultures were maintained in Grace’s medium (Hink, 1970) (In Vitro) supplemented with 10% fetal bovine serum (In Vitro), glutamine (2 mmol l\(^{-1}\)), penicillin (100 U ml\(^{-1}\)) and streptomycin (100 U ml\(^{-1}\)) at 27 °C.

The SpltNPV genotype strain G2 was isolated from the ZSU strain (Pang et al., 2001). Larvae of \( S. \) litura were reared on an artificial diet (Li et al., 2002) at 27 °C under constant humidity (60%) and photoperiod (14 h light and 10 h dark). SpltNPV polyhedra were propagated by infecting fourth instar \( S. \) litura larval and budded virus (BV) stocks were prepared by extracting haemolymph from infected insects 3 days post-infection (p.i.) as described elsewhere (O’Reilly et al., 1992). BV titre were determined by TCID\(_{50}\) (50% tissue culture infective dose) end-point dilution assay using \( S. \) litura cell line (O’Reilly et al., 1992). In this assay, the cytopathic effects were observed at 6 days p.i.

Generation of dsRNAs and RNAi. In order to get the dsRNA corresponding to \( Splt-iap4 \) and \( Splt-p49 \), the partial regions of these two genes were PCR-amplified and cloned into the LITMUS 28i vector (New England Biolabs). A 318 bp fragment identical to the \( Splt-iap4 \) ORF (SpltNPV nucleotides 58578–589) was PCR-amplified from the SpltNPV genome using the primers SpltNPV-\( iap-1 \) (5′-AGATCTATCTAAAGCACCATACTGAA-3′, BglII site underlined) and SpltNPV-\( iap-2 \) (5′-AGGCCATCTTTTACAAA-CGACCACA-3′, StuI site underlined). With the primers SpltNPV-\( p49-1 \) (5′-AGATCTTGTAGTATGTCGGTG-3′, BglII site underlined) and SpltNPV-\( p49-2 \) (5′-AGGCCCTTCTATTTGTTT-CGTGCGT-3′, StuI site underlined), a 1100 bp fragment identical to the Splt-p49 ORF (SpltNPV nucleotides 50688–51787) was obtained by PCR amplification. The PCR products were digested with StuI and BglII and cloned into LITMUS 28i vector to generate the final plasmids, named pLITMUS-iap4 and pLITMUS-p49, respectively. Then polymerase sites were inserted on both ends of the target genes in the resulting plasmids for production of dsRNAs. A 787 bp fragment identical to chloramphenicol acetyltransferase ORF (cat) (nucleotides 3–789) was PCR-amplified from the vector pCAT 3-Basic (Promega) with the primers CAT-1 (5′-AGATCTATCTGAA-CATTGCCTTTTCTCCTC-3′, StuI site underlined) and CAT-2 (5′-AGGCCCTTCTATTTGTTT-CGTGCGT-3′, StuI site underlined). The cat fragment was then cloned into the LITMUS 28i vector to get the plLITMUS-cat plasmid. pLITMUS-cat was used to obtain the control dsRNA. The plasmids constructed above were linearized in two separate reactions with StuI or BglII, annealed by heating to 65 °C for 15 min, and allowed to cool to room temperature. The AmpliScribe kit (Epitentre Technologies) was then used to transcribe dsRNA strands in vitro from the T7 promoter according to the protocol supplied with the kit. The concentrations of the dsRNA samples were determined with a spectrophotometer.

SpLi-221 cells (5 × 10\(^5\) of each) were seeded in a 6-well plate and adhered for 1 h. The cells were mock-infected with Grace’s medium or infected with SpltNPV at an m.o.i. of 1 plaque forming unit (p.f.u.) per cell. After 1 h absorption period, the virus inoculum was removed and cells were washed twice with 1 ml Grace’s medium without FBS. The cells were mock transfected or transfected with 2 μg Splt-p49 dsRNA or 10 μg Splt-iap4 dsRNA by lipid-mediated transfection using TransMessenger Transfection Reagent (Qiagen) in Grace’s medium without FBS. The reason that five times more Splt-\( iap4 \) than Splt-p49 dsRNA are used in RNAi is that only 10 μg or more Splt-iap4 dsRNA can knock out the transcription of Splt-iap4 efficiently in this study. After 4 h, the lipid–RNA mix was replaced with Grace’s medium containing 10% FBS. The transfected cells were incubated at 27 °C and observed with an inverted microscope (ECLIPSE TE2000 U, Nikon).

METHODS

Cell line and virus. \( S. \) litura cell line TUAT-SpLi-221 (SpLi-221) (Yanase et al., 1998) was obtained from Dr Zhihui Su (JT Biohistory...
Transcription assay (RT-PCR). SpLi-221 cells were infected at an m.o.i. of 1 p.f.u. per cell, followed by dsRNA addition, harvested at indicated time points after dsRNA addition. Total RNA isolation was conducted using RNeasy Protect Mini kit (Qiagen) and was digested with RNase-free DNase Set (Qiagen). RT-PCR was performed using the RNA PCR kit (TaKaRa, Ver.3.0) employing 0.4 µg total RNA as the template per time point. Synthesis of first-strand DNA complementary to mRNA (cDNA) was performed using avian myeloblastosis virus reverse transcriptase and the oligo (dT) primers according to the manufacturer’s instructions. The cDNA mixtures were amplified by PCR using the gene-specific primers. The obtained PCR products were analysed in 1 % agarose gel. Time zero was defined as the time when virus was added.

Antiserum preparation and immunoblot analysis. The whole ORF fragment of the Splitiap4 gene was amplified by PCR using the SplNPV genome as template and primers P1 (5'-GGATCCATTAAAATATACGAAAAAGG-3', BamHI site underlined) and P2 (5'-CTGGAGTTACAAATAAATCTTGAACATT-3', PstI site underlined). The PCR product was inserted into the prokaryotic expression vector pQE30 (Qiagen). Then the resulting recombinant vector was transformed into the expression host Escherichia coli strain M15, and expressed Split-IAP4 protein by IPTG induction. The cells were pelleted and lysed by sonication. The insoluble protein was denatured and purified by metal chelate affinity chromatography. A peptide corresponding to amino acid residues 387–400 of Splt-P49 was synthesized and was conjugated to keyhole limpet haemocyanin (KLH) by AbMART. Afterwards, the 6 x His-IAP fusion or P49-KLH fusion was injected into New Zealand White rabbits to raise the antiserum.

Western blotting was performed with standard procedures described elsewhere (Sambrook & Russell, 2001). Briefly, SpLi-221 cells were infected with virus and then treated with dsRNA as described above. Cells were harvested and boiled in SDS-PAGE sample buffer (62.5 mM Tris/HCl, pH 6.8, 2 % SDS) for 5 min. The protein samples were separated by electrophoresis in 12 % SDS-polyacrylamide gels, then transferred to nitrocellulose membranes (Schleicher & Schuell). The membrane was incubated for 1 h at room temperature with a 1 : 100 dilution of Splt-P49 or for 1 h at room temperature with a 1 : 100 dilution of Splt-P49. Horseradish peroxidase-conjugated secondary antibody (Amersham Bioscience) was used at 1 : 5 000. The signal was visualized by enhanced chemiluminescence (Amersham Bioscience). Time zero was uniform with that for RT-PCR as described above.

DNA laddering. Cells were treated with dsRNA and harvested at 24 h after treatment by centrifugation at 3000 r.p.m. for 10 min. Suicide-Track DNA Ladder Isolation kit (Merk) was then used to extract the total DNA according to the protocol supplied with the kit. DNA was separated by 1.5 % agarose gel electrophoresis and stained with ethidium bromide. The Sf9 cells treated with 0.5 µg actinomycin D ml⁻¹ for 15 h were used as positive control of oligonucleosomal ladder.

Viability assays. SpLi-221 cells were infected alone or infected with virus and then treated with dsRNA as described above. Cell monolayers were stained with 0.04 % trypan blue and blue-excluded cells were scored using an inverted light microscope. For each treatment, three fields of view containing 200–500 cells were scored.

RESULTS
dsRNA can be used to silence Split-p49 and Split-iap4 in SplNPV-infected SpLi-221 cells

Several groups have successfully taken advantage of the RNAi technique to silence baculovirus genes in insect cells (Ikeda et al., 2004; Means et al., 2003; Quadt et al., 2007). Furthermore, RNAi experiments have been performed successfully in S. litura insects (Rajagopal et al., 2002). To find out if we could knock out transcripts specifically, we examined the RNAi silencing efficiencies in SplNPV-infected SpLi-221 cells treated with specific dsRNAs at the beginning of this study. We first used RT-PCR analysis to assess the transcript abundance of the target genes. During SplNPV infection of SpLi-221 cells, there were few transcripts of Split-p49 at 5 h (Fig. 1a) and few transcripts of Split-iap4 at 3–5 h (Fig. 1b), but a highly steady-state

Fig. 1. Effect of Split-p49 or Split-iap4 dsRNA on the transcription of corresponding genes. SpLi-221 cells were infected with SplNPV; 1 h later, the cells were mock-transfected or transfected with control cat, Split-p49 or Split-iap4 dsRNA. Total RNA was isolated at the indicated time points. (a, b) RT-PCR analysis was performed to detect the presence of Split-p49 (a) or Split-iap4 (b) transcripts. (c) Time-courses of Split-p49 or Split-iap4 during SplNPV infection of SpLi-221 cells were determined by immunoblotting with antiserum against the corresponding proteins. (d) Expression of Split-P49 or Split-IAP4 was determined by immunoblotting in SplNPV-infected SpLi-221 cells upon the corresponding RNAi treatments. PC, SplNPV-infected SpLi-221 cell lysates (positive control). Molecular mass is indicated to the right of panel (c) or to the left of panel (d). Time points p.i. are indicated above the lanes.
transcription level of both genes was observed from 7 to 24 h (Fig. 1a, b). By contrast, the transcription of Splt-p49 and Splt-iap4 was significantly downregulated after the corresponding dsRNA treatment. For Splt-p49, few transcripts were detected at 5 h p.i., and there was no detectable signal from 7 to 24 h p.i. For Splt-iap4, few transcripts were detected before 12 h p.i., but much fewer than in cells infected with SpltNPV; there was no detectable signal at 24 h p.i. The transcription pattern of Splt-p49 or Splt-iap4 in SpltNPV-infected cells treated with cat dsRNA was similar to that in SpltNPV-infected cells, suggesting that the RNAi was specific (Fig. 1a, b). These results indicated that the transcription of Splt-p49 or Splt-iap4 could be efficiently and specifically suppressed when the corresponding dsRNAs were transfected into SpltNPV-infected cells.

Further, to detect whether the translation level of Splt-IAP4 or Splt-P49 protein was modified by Splt-iap4 or Splt-p49 RNAi, polyclonal antisera against Splt-P49 or Splt-IAP4 were raised and the two target protein expression patterns were determined by Western blot analysis (Fig. 1c, d). Immunoblot analysis showed that a predicted protein of 51 kDa for Splt-P49 and a 32 kDa protein for Splt-IAP4 could be detected in SpLi-221 cells after infection with SpltNPV. Both proteins were detectable from 12 to 72 h p.i. (Fig. 1c). The 32 kDa protein for Splt-IAP might be the Splt-IAP4 dimers as the molecular mass of Splt-IAP is predicted to be 16 kDa (Pang et al., 2001). A previous study suggested that homo-oligomerization is required for Op-IAP antiapoptotic activity (Hozak et al., 2000). Neither the Splt-IAP4 nor the Splt-P49 protein expressions were detectable from 24 to 48 h after Splt-iap4 or Splt-p49 dsRNA treatment, respectively (Fig. 1d). These results indicated that the Splt-P49 or the Splt-IAP4 protein expression could be suppressed efficiently by the corresponding dsRNAs.

**Splt-p49 dsRNA, not Splt-iap4 dsRNA, treatment can induce apoptosis of SpltNPV-infected SpLi-221 cells**

To explore the roles of the unidentified Splt-iap4 or Splt-p49 in SpltNPV life cycle, we used RNAi to suppress the two genes’ transcription, separately or synchronously, during SpltNPV infection of SpLi-221 cells. Approximately 5 × 10⁵ SpLi-221 cells were infected with SpltNPV at an m.o.i. of 1 p.f.u. per cell, and 1 h later the cells were transfected with dsRNA corresponding to Splt-p49, Splt-iap4, or both. Plasma membrane blebbing (a marker of apoptosis) was first observed on a small proportion of cells treated with Splt-p49 dsRNA or treated simultaneously with Splt-iap4 and Splt-p49 dsRNAs at 14 h post dsRNA addition (h p.r.) (data not shown). The number of cells showing apoptosis increased from then on; by 48 h p.r. almost all cells had undergone apoptosis and the subcellular bodies remained over the next few days. Fig. 2(a) was taken at 72 h p.r. In contrast, the SpltNPV-infected cells transfected with Splt-iap4 dsRNA or the bacterial cat dsRNA showed no signs of apoptosis, but polyhedra were observed within the cells, similar to those in the non-dsRNA-treated SpltNPV-infected cells (Fig. 2a), indicating that the addition of Splt-iap4 dsRNA or the control cat dsRNA could not induce apoptosis and did not abort viral replication. There was no difference among the uninfected cells transfected with dsRNA corresponding to Splt-iap4, Splt-p49, Splt-p49 plus Splt-p49, cat, and the mock-infected cells in global growth and morphology, and apoptosis was not observed in these cells (data not shown).

DNA fragmentation is recognized as a typical character of late apoptosis. To confirm that the observation as described above was actually apoptosis, total DNA of untreated or variously treated cells was extracted at 48 h p.r. and electrophoresed in a 1.5 % agarose gel. As shown in

![Fig. 2. Investigation of the antiapoptotic functions of Splt-p49 and Splt-iap4 during SpltNPV infection of SpLi-221 cells by RNAi.](image-url)
Fig. 2(b), the cells treated with Splt-p49 dsRNA or Splt-iap4/Splt-p49 dsRNA displayed DNA fragments consistent with the positive control of Sf9 apoptosis induced by actinomycin D (Act D). Other treated cells exhibited no DNA fragmentation.

Our results demonstrated that silencing of Splt-p49 could cause apoptosis in SpltNPV-infected SpLi-221 cells, while silencing of Splt-iap4 could not result in apoptosis, indicating that Splt-p49, not Splt-iap4, plays a role in suppressing apoptosis during SpltNPV infection of SpLi-221 cells.

**Splt-IAP4 has no synergistic effect on the inhibition of apoptosis of SpLi-221 cells induced by SpltNPV infection**

To investigate whether Splt-IAP4 has a synergistic effect on inhibition of the apoptosis of SpLi-221 cells induced by SpltNPV infection, we compared the cell viabilities of SpltNPV-infected SpLi-221 treated with Splt-p49 plus Splt-iap4 dsRNA simultaneously with infected cells treated with Splt-p49 dsRNA only. As shown in Fig. 3, the survival percentage for cells treated with Splt-p49 dsRNA and Splt-iap4/Splt-p49 dsRNA declined sharply with increasing time from 0 to 72 h. The cell viabilities declined to a similar extent in both Splt-p49 dsRNA-treated and Splt-iap4/Splt-p49 dsRNA co-treated SpltNPV-infected SpLi-221 cells. These results suggested that Splt-IAP4 neither inhibited the apoptosis of the SpltNPV-infected SpLi-221 cells nor assisted Splt-P49 to block the apoptosis.

An interesting morphological difference between Splt-iap4 dsRNA-treated SpltNPV-infected SpLi-221 cells and non-Splt-iap4 dsRNA-treated infected cells was observed in the present study. The SpltNPV-infected SpLi-221 cells exhibited assembly and formed tiny islands of densely packed cells; however, the Splt-iap4 dsRNA-treated cells did not congregate, but kept separated throughout the infection (Fig. 4a). In order to exclude unspecific effects of the RNAI used, a cat RNAi treatment of the SpltNPV-infected SpLi-221 cells was performed. The SpltNPV-infected SpLi-221 cells treated with cat dsRNA congregated like the SpltNPV-infected SpLi-221 cells (Fig. 4a). These observations implied that iap4 may exert an unexpected role in the baculovirus life cycle. To test whether the cellular morphological change would affect BV production, SpLi-221 cells were infected with SpltNPV for 1 h, and then treated with Splt-iap4 dsRNA or cat dsRNA. At different time points, the supernatants were collected and titred using SpLi-221 cells. As seen in Fig. 4(b), there was no discernible difference in kinetics of progeny viruses among the SpltNPV-infected SpLi-221 cells whether the cells were treated with cat, Splt-iap4 or no dsRNA. The results showed that Splt-iap4 RNAi had little effect on infectious virus production, although the treatment caused a morphological alteration in infected SpLi-221 cells.

**DISCUSSION**

Apoptosis is used by insects as one of the host defence strategies against virus infection (Clem et al., 1991; Zhang et al., 2002; Clarke & Clem, 2003; Feng et al., 2007). Antiapoptotic proteins encoded by viral genomes play a key role in suppressing the apoptotic response (Clem, 2007). Baculovirus genomes have evolved to contain diverse antiapoptotic genes, which can be divided into two families, P35 and IAPs. IAPs can be divided into five types from IAP1 to IAP5 (Luque et al., 2001). A new proposal on classification and nomenclature based on the nucleotide sequences of virus genes and genomes suggested the family Baculoviridae should include four genera: Alphabaculovirus (lepidopteran-specific NPV), Betabaculovirus (lepidopteran-specific GV), Gammabaculovirus (hymenopteran-specific NPV) and Deltabaculovirus (dip+teran-specific NPV) (Jehle et al., 2006). Lepidopteran-specific NPVs show a further division into group I and group II NPVs (Herniou et al., 2003). The antiapoptotic genes present in 43 sequenced baculovirus genomes are summarized in Supplementary Table S1 (available with the online version of this paper). Overall, different groups have their own different features: all lepidopteran-specific group I NPVs contain iap1 and iap2; all lepidopteran-specific group II NPVs except SpltNPV contain iap2 and iap3; iap5 are lepidopteran-specific GV genes; hymenopteran-specific NPVs contain only iap3. The high conservation of different antiapoptotic gene types in different taxonomic groups implies that apoptosis plays an important role in baculovirus phylogenesis. An interesting phenomenon is that nearly all sequenced lepidopteran baculovirus genomes possess two or more antiapoptotic genes (Supplementary Table S1). Several previous studies showed that only one gene had usually been examined for antiapoptotic activity in one virus–cell system (Clem & Miller, 1994; Ikeda et al., 2004). It seems reasonable that it is enough for a virus to choose only one functional antiapoptotic gene to influence a single point of the...
process, even if the apoptotic pathways contain many individual steps. Some of the baculovirus iap genes, which do not have antiapoptotic activity in the context of virus replication, were reported to be capable of delaying apoptosis (Maguire et al., 2000; Liu et al., 2003) or stimulating the ability of the functional IAP (Vilaplana & O’Reilly, 2003). Another study showed that deletion of Ac-iap1 resulted in a competitive advantage for AcMNPV replication in a *Trichoplusia ni* cell line, but not in a *S. frugiperda* cell line (McLachlin et al., 2001). The roles of many other iaps present in baculovirus genomes remain unknown. One possibility is that the non-functional genes are more likely to be needed for different cells, tissues and hosts for the inhibition of different stimuli.

In the present study the putative roles of Splt-P49 and Splt-IAP4 as apoptosis suppressors have been addressed by RNAi. RT-PCR analysis showed that transcription of Splt-p49 and Splt-iap4 was significantly downregulated by the corresponding dsRNA treatment. No Splt-p49 transcript at 7 h p.i. or no Splt-iap4 transcript at 12 h p.i. after treatment was visible upon transfection of the corresponding dsRNAs. In the current study, dsRNA was transacted after viral infection. This is probably why few if any RNA transcripts could be detected at early time points. Immunoblot analysis showed both proteins were undetectable from 24 to 48 h after Splt-p49 or Splt-iap4 dsRNA treatment. These results indicated the efficient silencing of the corresponding genes.

The suppression of Splt-p49 with dsRNA was able to induce extensive apoptosis during SpltNPV infection of SpLi-221 cells (Fig. 2), suggesting that Splt-p49 performed as an antiapoptotic gene. Homology analysis indicates that Splt-P49 displayed 79% amino acid identity with the first P49 identified, Spli-P49. Splt-p49 is present in the *Spodoptera littoralis* NPV genome and encodes a 49 kDa polypeptide with about 48.8% identity to its homologue P35 from AcMNPV (Du et al., 1999). Amino acid similarity between Splt-P49 and P35 is collinear, with the exception of 120 residues within Splt-P49 (Zoog et al., 2002). Although Spli-P49 and P35 have comparable structures and mechanisms, Spli-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; thus, P49 was suggested to be a third type of baculovirus apoptosis suppressor (Zoog et al., 2002). Among the 43 baculovirus genomes sequenced to date there were six baculoviruses containing homologues of P35 (*Rachiplusia* or MNPV, AcMNPV, *Bombyx mori* NPV, *Plutella xylostella* NPV, *Maruca vitrata* NPV and *Culex nigripalpus* NPV) and three containing homologues of P49 (*Choristoneura occidentalis* GV, SpltNPV and *Leucania separata* NPV) (Supplementary Table S1).

The role of Splt-IAP4 in SpltNPV infection of SpLi-221 cells was also assessed in this study. SpLi-221 cells infected with SpltNPV did not undergo apoptosis following transfection with Splt-iap4 dsRNA, and the virus was able to produce polyhedra similarly to wild-type SpltNPV-infected cells in late phase (Fig. 2). We also examined the cell viabilities of co-suppressing Splt-iap4/Splt-p49 and suppressing only Splt-p49 at different time points; no detectable differences were observed, indicating that Splt-IAP4 could not stimulate the antiapoptotic activity of Splt-P49 or delay apoptosis (Fig. 3). Although IAP homologues have been found in all sequenced baculovirus genomes (Supplementary Table S1), no IAPs have been determined to be functional as inhibitors of apoptosis in baculoviruses that contain either p35 or p49.

A previous study showed that Spli-IAP4, which is highly homologous to Splt-IAP4, was able to delay, but not to suppress, apoptosis of S9 cells induced by replication of a recombinant AcMNPV deficient in *p35* in a transient assay (Liu et al., 2003). A recent study reported a p35-null AcMNPV was able to produce progeny virus successfully in S9 cells when transiently expressing Splt-P49 (Yu et al., 2005). We took advantage of the same strategy, i.e. transient expression assay, to determine the antiapoptotic role of Splt-P49 and Splt-IAP4 in S9 cells (data not shown). In our hands, Splt-P49 was able to rescue
replication of a p35-null AcMNPV in Sf9 cells, which is consistent with the findings of Yu et al. (2005). However, we did not observe that Splt-IAP4 inhibited or delayed the apoptosis of Sf9 cells induced by the p35 mutant. SpltNPV is a closely related variant of SpltNPV and also possesses p49 and iap4. Splt-IAP4 and Splt-IAP4 share 73% amino acid sequence identity and were grouped into IAP4 because they have a single BIR motif (Liu et al., 2003). Parallel studies with Splt-IAP4 and Splt-IAP4 would be interesting to determine the difference in their roles in anti-apoptosis.

When infected with baculoviruses, many insect cells, such as AcMNPV-infected Ld652Y cells (Griffiths et al., 1999) or SpltNPV-infected SpLi-221 cells in this study (Fig. 4), show evident cytopathic effects, including cell rounding and clumping, in contrast to normal cells. We found that the Splt-iap4 dsRNA-treated SpLi-221 cells infected with SpltNPV did not assemble and group together throughout infection; in contrast, the SpltNPV-infected SpLi-221 cells treated with the control cat dsRNA conformed like the SpltNPV-infected SpLi-221 cells. Virus titre assay showed that there were no discernible differences in the kinetics of BV production whether the infected cells were treated with Splt-iap dsRNA or not, suggesting the cells’ congregation did not affect BV production. At this point, the reason why and the biological significance of the fact that Splt-iap4 expression can induce the congregation of SpltNPV-infected SpLi-221 cells are still unclear.

In summary, this study has provided evidence for the first time that Splt-P49 is required to suppress apoptosis during SpltNPV replicating in the permissive SpLi-221 cell line; in contrast, Splt-IAP4 could not prevent apoptosis. The study also showed that Splt-iap4 RNAi can induce a morphological alteration, namely abrogating cellular congregation of SpltNPV-infected SpLi-221 cells, and that suppression of Splt-iap4 expression had no effect on virus replication.

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