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Identification and Functional Analysis of LsMNPV Anti-apoptosis Genes

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Three anti-apoptosis genes, Ls-*iap2, iap3* and *p49* were found in *Leucania separata* multiple nuclear polyhedrovirus. Amino acid sequence homology of Ls-IAP2 and Ls-IAP3 with Op-IAP2 and Op-IAP3 from *Orgyia pseddotsugata* MNPV were 20% and 42%, while that of Ls-P49 is 28% with Sl-P49 from *Spodoptera littorolis* MNPV. Ls-IAP2 contains one baculoviral IAP repeat (BIR) domain followed by a RING domain, while Ls-IAP*3* contains two BIRs and a RING. Ls-P49 contains a reactive site loop, predicted cleavage site (KKLD^{74}↓G) that is different from Sl-P49 (TVID^{94}↓G). Expressed Ls-*iap3* or Ls-*p49* under presence of actinomycin D in SF9 cells, DNA ladder assay revealed that Ls- IAP3 or Ls-P49 could block the apoptosis of SF9 cells induced by actinomycin D. Replication of *p35* deficient-mutant *Autographa californica* MNPV in SF9 cells was also rescued when Ls-*iap3* or Ls-p49 was expressed transiently. No anti-apoptotic activity was observed for Ls-IAP*2*. The results showed that both of Ls-IAP*3* and Ls-P49 were functional apoptotic suppressors in SF9 cells.

Keywords: Anti-apoptosis, LsMNPV, Ls-IAP, Ls-p49

Introduction

Apoptosis or programmed cell death is a normal physiological cell suicide program that is highly conserved among vertebrates and invertebrates (Du *et al*., 1999). Apoptosis plays an important role during normal development and tissue homeostasis eliminating unwanted cells, including damaged and virus-infected, from the organism. Apoptosis acts as a host defense mechanism by which virally infected cells are eliminated to limit the propagation of viruses (Pei *et al.*, 2002). To overcome this response, many viruses carry genes whose products inhibit apoptosis (Means *et al.*, 2003).

In baculovirus, two types of anti-apoptotic genes, *p35* and inhibitor of apoptosis (*iap*) have been identified (Clem, 2001). The *p35* gene from *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is required to prevent apoptosis during the virus infection of Sf21 cells, a cell line derived from the *Lepidopteran* insect *Spodoptera frugiperda* (Kamita *et al.*, 1993; Dai *et al.*, 1999; Huang *et al.*, 2001). P35, a 35-kDa protein encoded by the *p35* gene of the AcMNPV, inhibits a broad range of caspases, including human and insect caspases (Pei *et al.*, 2002). The most remarkable feature of P35 structure is the presence of a large loop domain associated with caspase called reactive site loop (RSL), protruding above a central β-sheet core (Pei *et al.*, 2002).

Recently, *p49* was identified from *Spodoptera littorolis* MNPV (Sl-*p49*) as a second member of the *p35* family (Du *et al.*, 1999). Sl-*p49* encodes a predicted 49 kDa protein that showed 48.8% identity to *p35* (Pei *et al.*, 2002). P49 is able to inhibit insect and human effector caspases, require cleavage at its Asp^{94} (^{91}TVTD^{94}) for the anti-apoptotic function.

The first *iap* gene was discovered in *Cydia pomonella* granulovirus (CpGV) and *Orgyia pseddotsugata* MNPV (OpMNPV), during a genetic screen for genes that could rescue the AcMNPV *p35* mutant phenotype (Crood *et al.*, 1997). Cellular homologues of *iap* gene were widely present in baculovirus, yeast, *drosophila*, nematode and mammal cells (Huang *et al.*, 2001). Recently, *iap*s have also been indentified in *lepidopteran* insects that include *Tricoplusia ni*, *Spodoptera frugiperda* and *Bombyx mori*, *Spodoptera litura* (Ikeda *et al.*, 2004).

Baculovirus *IAP*s are currently classified into five members, IAP1-IAP5, based on amino acid sequence homology (Luque *et al.*, 2001). Functional analysis have revealed that only IAP3 is capable of blocking apoptosis in cells infected with *Cydia pomonella* granulovirus (CpGV) or *Orgyia pseudotsugata* MNPV (OpMPV) (Means *et al.*, 2003), although these viruses possess three and four IAPs, respectively. It is IAP1 and IAP2

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in *Epiphyas postvittana* MNPV (EppoMNPV), but not IAP3 and IAP4 that exhibit anti-apoptotic activity (Day *et al.*, 1999).

IAP proteins have distinctive primary structure. They contain one to three copies of baculoviral IAP repeat (BIR) domains and most IAPs also contain a RING domain near their C-termini. The BIR domain contains a highly conserved arrangement of Cys/His residues forming a stable fold that chelates zinc [Maguire *et al.*, 2000]. The BIR region was also found to interact with regulators of IAPs including Grim, Reaper, and Hid from *Drosophila* and may also mediate homo-oligomerization (Seshagiri *et al.*, 1999). The RING finger is a common zinc binding motif that also exists in other cellular proteins. Recent studies showed that several IAPs are inhibitors of caspases and that BIR and RING domains play an important role in apoptosis suppression (Clem and Miller, 1994).

Leucania separata multiple nuclear polyhedrosis virus (LsMNPV) genome was first sequenced by our laboratory in 2002. There are three putative anti-apoptosis genes: Ls-*iap2, iap3* and *p49* in the genome of LsMNPV. Here we report the gene cloning, structural and functional study of Ls-*iap2,* Ls-*iap3* and Ls-*p49*. Our results demonstrate that both Ls-IAP3 and Ls-P49 are functional apoptotic suppressors in SF9 cell, but Ls-IAP2 has no anti-apoptosis function.

Materials and Methods

Cells, viruses and plasmid. *Spodoptera frugiperda* pupal ovarian cell line (SF9) cells were cultured at 27°C in Graces medium (Gibco/BRL), supplemented with 10% fetal bovine serum (Gibco/BRL). *p35* deficient-mutant AcMNPV (AcMNPVΔ*p35*/pol$^+$), pIZ/V5His are gifted from Dr. Lungang Yao.

Expression vector construction. The primers used to amplify Ls-IAP2, Ls-IAP3 and Ls-*p49* genes from LsMNPV genome were used as follows: Ls-IAP2 F (5'-GGAATTCAGATATGGAGACTC GTGCAAA-3'), Ls-IAP2 R (5'-CGGGTCTAGAAGAAAAATTCG AATCAGAG-3'), Ls-IAP3 F (5'-GGAATTCAAACATGGATTCG CTGGAG), Ls-IAP3 R (5'-CGGTCTAGAAGGAGAATAATAGA CACGAATGAT-3'), Ls-p49 F (5'-CGAATTCTCTCAAGCCTCA CCGCA-3'), Ls-p49 R (5'-CGGGTCTAGATGAATATCAATGTAC AGATTGG-3'). PCR fragments were digested with *Eco*RI and *Xba*I and then inserted into the *Eco*RI and *Xba*I sites in pIZ/V5His to produce pIZ-IAP2, pIZ-IAP3 and pIZ-p49, respectively.

Protein expression and Western-blot. SF9 cells were seeded into 24-well plates at about 60% confluence and maintained at 27°C. Transfection was performed with 0.8 μg expression plasmid and reporter plasmid using Cellfectin reagent according to the manufacture protocol (vitrogen). Cells were harvested and lysed at 24 h, 72 h after transfection. The expression protein was subjected to SDS-PAGE and was evaluated by Western- blot using antibody for 6His tag or poyhedrin.

DNA extraction. DNA fragments produced with apoptosis were extracted for 2 h at 37°C from cell samples (SF9 cell) with a 10 mM Tris (pH 8.0), 1 mM EDTA-1% Sodium dodecyl sulfate (SDS) buffer containing 70 μg/ml of protenase K, and 1 M NaCl (final concentration) was added. The extracts were treated with phenol-chloroform and precipitated with ethanol, and resuspended DNA was analyzed by agarose gel electrophoresis.

Flow cytometry. Cell samples were collected by centrifugation at 3,000 rpm for 5 min and then washed three times using PBS buffer (pH 7.0). The cell pellet was resuspended in 1 ml PBS (pH 7.0). RNase A (Gibco) was added in the solution to the final concentration of 50 μg/ml and the cells were incubated at 37°C for 30 min. Then PI (Gibco) was added to the solution to the final concentration of 50 μg/ml and incubated at 37°C for 30 min. After washing twice cells were then fixed in 70% ethanol and flow analysis was performed on Bechman-coulter XL (Beckman coulter).

Microscopy observation. Marker rescue assays were performed as described previously (Clem and Miller, 1994). Routinely, 1 μg VΔ*p35*/pol$^+$ DNA or 50 μg/ml actinomycin D and 1 μg test DNA were cotransfected into 4×10^5 SF9 cells by lipofectin. 3-4 days after transfection, the cells were examined the presence of polyhedra and apoptotic bodies with light microscopy.

Software analysis. Computer- assisted domain structure of Ls-p49 was based on the Predict Protein Server (Rost *et al.*, 2003). Sl-p49 was used model for Ls-p49. Analysis of Ls-IAPs sequences was performed using DNA analysis programs DNAMAN.

Results

Sequence comparison and structural analysis of Ls-p49 and Ls-IAPs. Ls-p49 is 1,335 bp and encodes an about 49 kDa protein which contains one putative caspase cleavage sites KKLD^{74}↓G and shares 28% amino acid sequence identity with Sl-P49 (Fig. 1). Ls-IAP2 is 831 bp and encodes an about 30 KDa protein. Ls-IAP2 contains a BIR domain at N-terminus and a RING finger motif at C-terminus (Fig. 2A). Ls-IAP2 shares 20, 19, 20, 21 and 19% amino acid sequence identity with Op-IAP2, Ep-IAP2, Tn-IAP, Sf-IAP, Bm-IAP, respectively. Ls-IAP3 is 744 bp and encodes an about 28 KDa protein. Similar to Op-IAP3, Ls-IAP3 protein contains two BIR domains followed by a RING domain near its C-terminus (Fig. 2B). Ls-IAP3 shares 42%, 42%, 31%, 30% and 35% amino acid sequence identity with Op-IAP3, Cp-IAP3, Tn-IAP, Sf-IAP and Bm-IAP, respectively. The identity between Ls-IAP2 and Ls-IAP3 was 22%.

Expression of Ls-p49 and Ls-IAPs in SF9 cells. Ls-p49 and Ls-IAPs were amplified using PCR from LsMNPV genome.

PCR fragments were digested with *Eco*Rl and *Xba*l, and then inserted into *Eco*Rl and *Xba*l sites of pIZ/V5His to produce pIZ-IAP*2*, pIZ-IAP*3* and pIZ-p49, respectively. pIZ/V5His plasmid contains the actin promoter of *Drosophila*, which can initiate gene expression in insect cells.

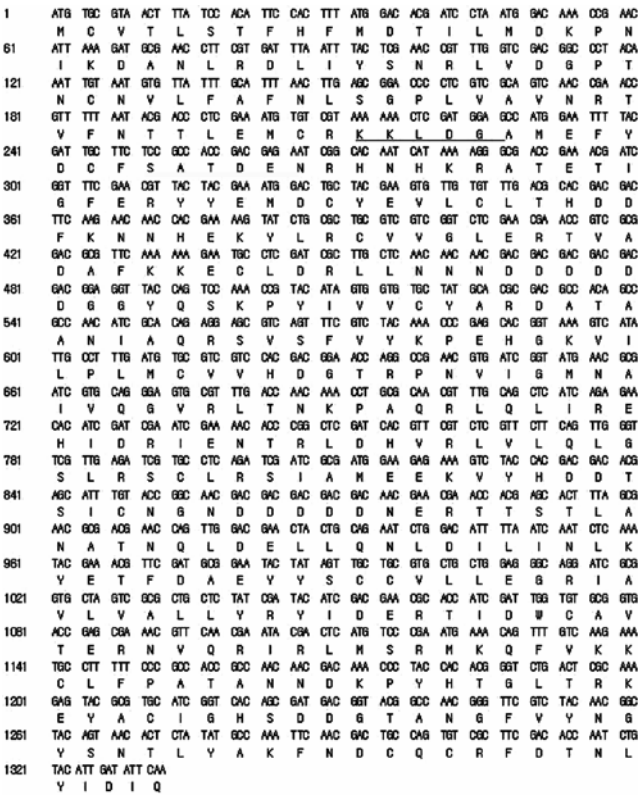

Fig. 1. Nucleotide and predicted amino acid sequence of Ls-*p49*. The putative caspase cleavage sites are underlined.

12 h after transfecting SF9 cells with pIZ-IAP2, pIZ-IAP*3* or pIZ-p49, actinomycinD (ActD) was added and incubated for 24 h. Then the expressed Ls-IAP2, Ls-IAP3 or Ls-P49 proteins was subjected to SDS-PAGE analysis and Western blotting. The results showed that Ls-P49 and Ls-IAPs were expressed under the control of actin promoter of *Drosophila* (Fig. 3A and B).

Ls-P49 and Ls-IAP3 inhibited SF9 cell apoptosis induced by actinomycin D. ActD can block the cell protein synthesis and thus cause the cells to undergo apoptosis. 12 h prior to the addition of ActD, cells were transfected with pIZ-IAP2, pIZ-IAP*3* or pIZ-*p49* and then the transfected cells were treated with ActD for 24 h to induce apoptosis. After treated with ActD for 24 h, the number of SF9 cells that were mock transfected or transfected with pIZ-IAP2 was reduced and obvious characteristic of apoptosis was observed (Fig. 4B and C). While the cells transfected with pIZ-IAP*3* or pIZ-p49 12 h prior to the addition of ActD did not show obvious characteristic of apoptosis after 24 h (Fig. 4D and E).

To examine DNA fragments produced with apoptosis, whole cell DNA was extracted and then subjected to agarose gel electrophoresis. The results showed that the DNA extracted from the cells transfected with pIZ-IAP*3* or pIZ-p49 12 h prior to addition of ActD was intact (Fig. 5 lane 3 and 4), but the DNA in ActD-treated cells that were mock transfected

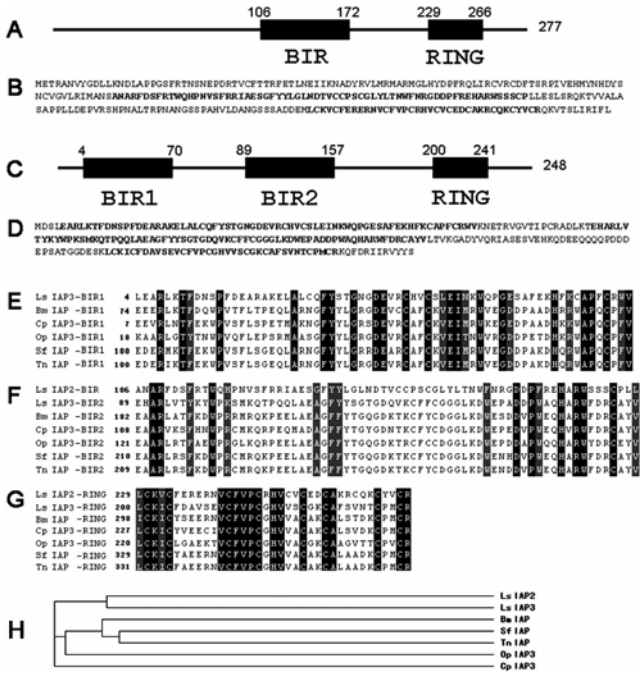

Fig. 2. Comparison of Ls-IAPs amino acid sequences and the predicted domain topology. (A)The BIR and RING domains of Ls-IAP2 are depicted. (B)The predicted amino acid sequence of Ls-IAP2 is presented. (C)The BIR and RING domains of Ls-IAP3 are depicted. (D)The predicted amino acid Sequence of Ls-IAP3 is presented. Sequence alignments of the BIR1 (E), BIR2 (F) and RING (G) domains of Ls-IAP2 and Ls-IAP3 with the corresponding domains of other iap family members are shown. Black and gray indicates identical amino acids. (H) Phylogenetic tree constructed from the alignment displayed in (E, F and G). The Gen Bank accession numbers of sequences used for the alignments are: Ls-IAP2 AAQ11129.1, Ls-IAP3 AAQ11158.1, B. mori IAP (Bm-IAP) AF28107, *Spodoptera frugiperda* IAP (Sf-IAP) AF186378, *Trichoplusia ni* IAP (Tn-IAP) AF195528, *Orgyia pseudotsugata* nucleopolyhedrosis IAP (Op-IAP) P41437 and *Cydia pomonella* granulovirus IAP (Cp-IAP) P41436.

or transfected with pIZ-IAP2 was degraded into fragments, showing typical "DNA ladder" configuration (Fig. 5 lane 1 and lane 2).

Ls-IAP3 and Ls-p49 rescued the replication of AcMNPVΔ*p35*/pol$^+$ in SF9 cells. pIZ-IAP2, pIZ-IAP3 or pIZ-p49 was cotransfected into SF9 cells with AcMNPVΔ*p35*/pol$^+$ DNA. 3-4 days after transfection, the cells were examined the presence of polyhedra with light microscopy.

Polyhedra were observed in the cells cotransfected with AcMNPVΔ*p35*/pol$^+$ DNA and pIZ-IAP3 or AcMNPVΔ*p35*/pol$^+$ DNA and pIZ-p49 (Fig. 6A4 and A5). No polyhedra were observed in the cells transfected with AcMNPVΔ*p35*/pol$^+$ DNA or cotransfected with AcMNPVΔ*p35*/pol$^+$ DNA and pIZ-IAP2 (Fig. 6A2 and A3). To examine polyhedrin in transfected cells, Western-blot was performed using antibody of polyhedrin. Polyhedrin were detected in the cells cotransfected

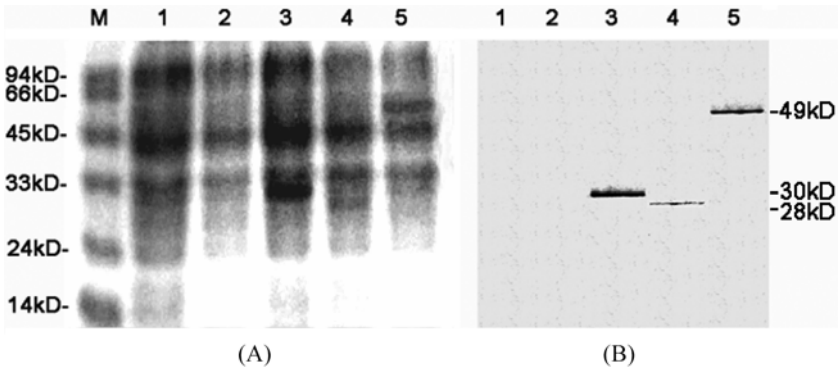

Fig. 3. SDS-PAGE (A) and Western-blot (B) analysis of Ls-IAP2, Ls-IAP3 and Ls-P49 proteins expressed in SF9 cells. (A) Lane M: protein marker. Lane 1: SF9 cells were untransfected with any plasmid for comparison. Lane 2: SF9 cells only were treated with ActD for 24 h after culture 12h. Lane 3- Lane 5: 12 h prior to the addition of ActD, SF9 cells were transfected with pIZ-IAP2 (Lane 3), pIZ-IAP3 (Lane 4) or pIZ-p49 (Lane 5), respectively and then the transfected cells were treated with ActD for 24 h. (B) Western-blot analysis corresponding to (A).

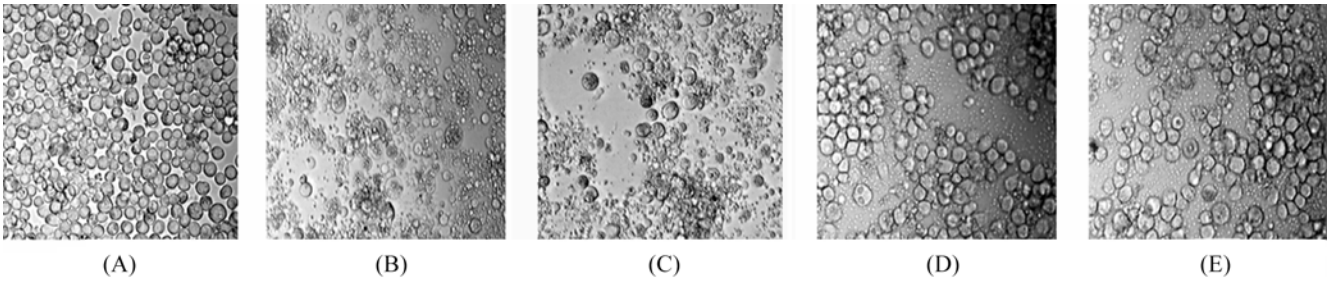

Fig. 4. Transfection of SF9 cells with pIZ-IAP3 or pIZ-p49 prevents apoptosis induced by actinomycinD (morphology observation of SF9 cells). (A) In the normal SF9 cells, no significant characteristic of apoptosis appeared. (B, C) Cells were mock transfected (B) or transfected with pIZ-IAP2(C) 12 h prior to adding ActD and then treated with ActD for 24 h, the number of SF9 cells were decreased and the cells shrank. (D, E) Cells were transfected with pIZ-IAP3 (D) or pIZ-p49 (E) 12h prior to adding ActD and then treated with ActD for 24 h, the morphology of SF9 cells looked like the normal cells (A), i.e. no obvious apoptosis characteristics were observed.

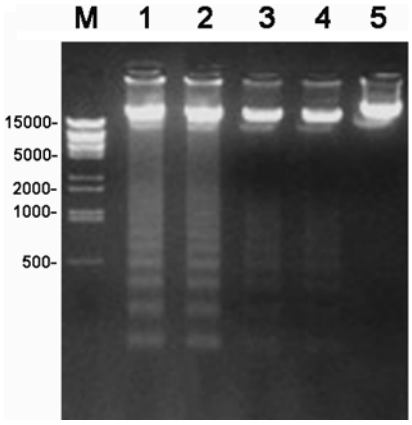

Fig. 5. DNA ladder analysis indicated that Ls-IAP3 and Ls-p49 prevented apoptosis of SF9 cells induced by actinomycin D. Lane 1: Cells were treated with actinomycin D for 24 h. Lane 2-4: Cells were transfected with pIZ-IAP2 (Lane 2), pIZ-IAP3 (Lane 3) or pIZ-p49 (Lane 4) 12 h prior to the addition of ActD and then treated with ActD for 24 h. Lane 5: SF9 cells. Lane M: DNA marker.

with AcMNPVΔ*p35*/pol$^+$ DNA and Ls-*iap3* or AcMNPVΔ*p35*/pol$^+$ DNA and Ls-*p49* (Fig. 6B4 and B5), but were not in the cells transfected with AcMNPVΔ*p35*/pol$^+$ DNA or cotransfected with AcMNPVΔ*p35*/pol$^+$ DNA and pIZ-IAP2 (Fig. 6B2 and B3). Such results demonstrate that the expression of Ls-IAP3 and Ls-P49 could rescue the AcMNPVΔ*p35*/pol$^+$ replication in SF9 cells.

The effect of Ls-IAP3 or Ls-P49 protein that could inhibit apoptosis induced by transfection of AcMNPVΔ*p35*/pol$^+$ was also proved by flow cytometry after the cells stained with PI (Fig. 7). As measured by flow cytometry, the apoptosis in group of normal cells is only 0.42% (Go peak in Fig. 7A), while apoptosis cells (stained by PI) increased to 27.4% (Fig. 7B) when AcMNPVΔ*p35*/pol$^+$ genome DNA was transfected. When equal amount of AcMNPVΔ*p35*/pol$^+$ genome DNA was cotransfected with plasmid pIZ-IAP3 or pIZ-p49, respectively, the apoptosis decreased to 11.3% and 13.9%, respectively (Fig. 7C, D), which indicated that Ls-IAP3 and Ls-P49 could partially inhibit the apoptosis caused by AcMNPVΔ*p35*/pol$^+$ genome in transient experiments.

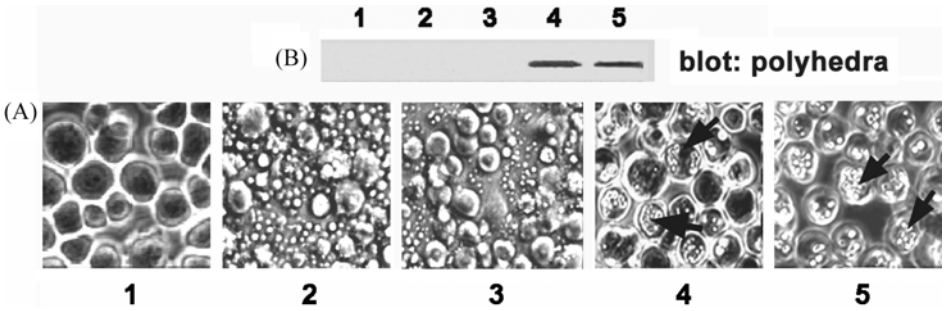

Fig. 6. Polyhedra observation (A) and Western-blot of polyhedrin (B) of AcMNPVΔ*p35*/pol$^+$ rescued by Ls-IAP3 or Ls-P49 in SF9 cells. 1: SF9 cells 72 h after mock transfected. 2: SF9 cells 72 h after transfected with AcMNPVΔ*p35*/pol$^+$ viral DNA. 3-5: SF9 cells 72 h after cotransfected with AcMNPVΔ*p35*/pol$^+$ viral DNA and pIZ-IAP2 (3), AcMNPVΔ*p35*/pol$^+$ viral DNA and pIZ-IAP3 (4) or AcMNPVΔ*p35*/pol$^+$ viral DNA and pIZ-p49 (5). (B) Western blot of polyhedrin corresponding to A.

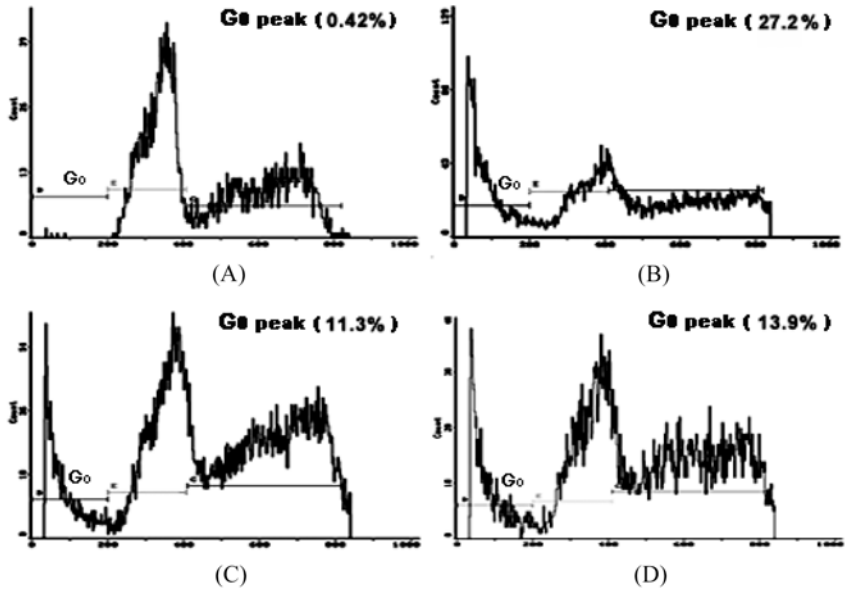

Fig. 7. The apoptosis assay by flow cytometry after transfection with AcMNPVΔ*p35*/pol$^+$. (A) No significant apoptosis peak in normal SF9 cells (0.42%). (B) 24 h after transfected with AcMNPVΔ*p35*/pol$^+$, the apoptosis peak showed that 27.4% of primary cells undergone apoptosis. (C, D) 24 h after cotransfected with AcMNPVΔ*p35*/pol$^+$ DNA and pIZ-IAP3 (C) or AcMNPVΔ*p35*/pol$^+$ DNA and pIZ-p49 (D), the ratio of apoptosis cells was 11.3% (C) or 13.9% (D), respectively.

Discussion

Baculoviruses possess two types of genes with anti-apoptotic activity, *iap* and *p35* that can suppress apoptosis induced by virus infection or by diverse stimuli in vertebrates or invertebrates (Pei *et al.*, 2002). The Baculovirus *p35* gene have been identified in very few baculoviruses (Clen *et al.*, 1994), while the *iap* gene have been identified in all baculoviruses studied to date (Crook *et al.*, 1993).

p49 gene, another member of the famous *p35* family, was first identified in the *Spodoptera littoralis* nucleopolyhedrovirus (SINPV) (Du *et al.*, 1999). *p49* was only possessed by groupII NPV like SINPV and LsMNPV (Du *et al.*, 1999). The P49 from LsMNPV also contained a reactive site loop (RSL), while had different predicted cleavage site (KKLD$^{74}\downarrow$G) from Sl-p49 (TVID$^{94}\downarrow$G).

Two *iap* homologues of LsMNPV were designated as Ls-IAP2 and Ls-IAP3 because of their amino acid sequence homology with other IAP2 and IAP3 that were identified previously in HycuNPV (Ikeda *et al.*, 2004), CpGV (Luque *et al.*, 2001), OpMNPV (Means *et al.*, 2003) and EppoMNPV (Maguire *et al.*, 2000). Hycu-IAP1, -IAP2 and -IAP3 showed the highest homology with corresponding OpMNPV IAPs, exhibiting 81%, 71% and 62% amino acid sequence identities with Op-IAP1, -IAP2 and -IAP3, respectively (Ikeda *et al.*, 2004), but Ls-IAP2, -IAP3 exhibits 44% and 60% amino acid sequence identities with Op-IAP2, -IAP3, respectively. So the identity of IAPs between LsMNPV and OpMNPV was not higher than that of HycuNPV and OpMNPV.

The BIR and RING domains of *B.mori* IAP (Bm-IAP) are required to block apoptosis induced by AcMNPVΔ*p35*/pol$^+$ (Huang *et al.*, 2001). Like other baculovirus IAPs, Ls-IAP2

and IAP3 possess one and two BIR domains in the N-terminal region, respectively, and both IAPs contain a single RING finger motif in the C-terminal region. We tested anti-apoptosis ability of Ls-IAP2, Ls-IAP3 and Ls-p49 using the traditional marker rescue.

Actinomycin D and AcMNPVΔ*p35*/pol$^+$ was used to establish the apoptosis system in SF9 cells. Actinomycin D can suppress cell protein synthesis; induce many kinds of cell to apoptosis (Maguire *et al.*, 2000). SF9 cells infected with AcMNPVΔ*p35*/pol$^+$ could induce apoptosis (Chejanovsky and Gershburg, 1995). Our results from transient expression assays in SF9 cells revealed that both Ls-IAP3 and Ls-p49 could block apoptosis of SF9 cells induced by ActD treatment or p35-deficient AcMNPV transfection, while Ls-IAP2 exhibited no detectable anti-apoptotic activity. The result showed that Ls-*iap3* and Ls-*p49* are two new anti-apoptotic gene homologues that has not been identified before.

Further works should be done to confirm whether the Ls-IAP3 and Ls-P49 proteins are essential for LsMNPV replication or not. We will attempt to isolate various Ls-IAP3 and Ls-p49 deficient LsMNPV mutants to determine their functional domains, and also investigate the interaction of Ls-IAP3 and Ls-p49 with insect and human effector caspase. The findings suggest that more anti-apoptosis genes may exist in virus and probably in the animal kingdom.

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