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Article

Functional analysis of the *Autographa californica* nucleopolyhedrovirus IAP1 and IAP2

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The *Autographa californica* nucleopolyhedrovirus (AcMNPV) contains three apoptosis suppressor genes: *p35*, *iap1* and *iap2*. AcMNPV P35 functions as a pancaspase inhibitor, but the function of IAP1 and IAP2 has not been entirely resolved. In this paper, we analyze the function of IAP1 and IAP2 in detail. AcMNPV with *p35*-deletion inhibited the apoptosis of BTI-Tn-5B1-4 (Tn-Hi5) cells induced by a *Helicoverpa armigera* single nucleocapsid NPV (HearNPV) infection and rescued the replication of HearNPV and BV production in these cells. Transient-expression experiments indicated that both IAP1 and IAP2 suppress apoptosis of Tn-Hi5 cells during HearNPV infection. Recombinant HearNPVs expressing AcMNPV *iap*1, *iap2* and *p35*, respectively, not only prevented apoptosis but also allowed HearNPV to replicate in Tn-Hi5 cells. However, the *iap*1, *iap2* and *p35* genes when expressed in HearNPV were unable to rescue BV production. These results indicate that both AcMNPV *iap1* and *iap2* function independently as apoptosis inhibitors of and are potential host range factors.

***iap*1, *iap*2, AcMNPV, HearNPV**

Apoptosis, a genetically controlled program of celluler suicide, is component of multicellular organisms in physiological and pathological processes, including embryonic development, tissue homeostasis and remodeling, and neurodegenerative, autoimmune and infectious diseases[1,2]. Apoptosis an evolutionally conserved essential process in invertebrates as well as in vertebrates, is initiated by a variety of external and internal stimuli, including virus infections. Host cells often employ apoptosis as a primary antiviral defense strategy in an attempt to inhibit virus multiplications upon viral entry[3].

Many viruses have developed anti-apoptotic mechanisms to delay, suppress or block host cellular suicide and promote virus production during evolution[4]. For example, baculovirus genomes contain two classes of apoptosis suppressors, p35 with its homologue p49, and inhibitor of apoptosis (IAP)[5,6]. The product of the *p35* gene, identified first from the *Autographa californica* nucleopolyhedrovirus (AcMNPV), functions primarily as a substrate inhibitor of effector caspases to block a broad range of downstream apoptotic events in *Spodoptera frugiperda* (Sf21) cells induced by diverse stimuli, including actinomycin D treatment and baculovirus infection[7]. P35 suppresses apoptosis in heterologous systems[8] suggesting that P35 is generally functioning at an evolutionarily conserved point in the apoptosis pathways. The *p*35 family is only reported in baculovirus. Until recently, a P35 homolog was identified from Entomopoxvirus[9]. Various *iap* homologues have been found

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in an array of viruses, including baculoviruses and poxviruses, and in vertebrate and invertebrate cells[7,10].

Members of the IAP protein family structurally contain two classes of signature Zinc coordinating motifs: baculovirus *iap* repeats (BIRs), at least one and usually two to three copies of imperfect 70-amino acid C_2HC motifs at the amino termini, and a RING domain, a specialized C_3HC_4 finger motif at the carboxyl-termini of most IAPs[7]. The requirements of the two motifs involved in protein-protein interaction vary between different IAPs. Generally, IAPs of baculovirus and insect cells, unlike mammalian cellular IAPs[11], require both BIRs and RING finger motifs for functionality[12,13]. IAPs also inhibit apoptosis by physically interacting through the BIR motifs with pro-apoptotic proteins, such as REAPER, HID, GRIM from *Drosophila*, by altering their subcellular localization to punctuate perinuclear structures coinciding with the location of IAPs in Sf21 cells[13,14]. In addition, the anti-apoptotic function of *Orgyia pseudotsugata* MNPV (Op)-IAP3 may be associated with the ability of the RING domain to ubiquitinate pro-apoptotic proteins, such as HID[15], while human XIAP blocks apoptosis by inhibition of caspase-3 and caspase-7[11,16]. Baculoviral and lepidopoteran IAPs, such as Op-IAP3, *Cydia pomonella* granulovirus (Cp)-IAP3, *Spodoptera frugiperda* NPV (Sf)-IAP and BmNPV (Bm)-IAP, also block apoptosis in mammalian cells[13,17]. It is widely held that IAPs may collectively function at an evolutionarily conserved step in the apoptotic pathway at/or upstream of that affected by P35[13].

Nearly all baculoviruses whose genomes have been sequenced contain IAP homologs, which have been classified by sequence homology into five groups, *iap*1, 2, 3, 4 and 5[18]. 14 IAPs from 8 baculoviruses have been analyzed for anti-apoptotic activity. AcMNPV has two *iap*s: *iap*1 and *iap*2, which respectively belong to the *iap*1 and 2 groups[18,19]. The IAP2 of 249 amino acids (aa) is smaller than the IAP1 of 286 aa, with both IAPs having a similar structure to two BIRs and one RING domain[20]. However, the AcMNPV IAPs, unlike P35 or Cp-IAP or Op-IAP, are unable to protect cells against apoptosis in Sf 21[12,21]. It has been hypothesized that the baculovirus IAP which interacts to control apoptosis may occur in a cell-line specific manner, implying that AcMNPV IAPs are functioning in other than Sf-21 cell lines.

It has been reported that *Helicoverpa armigera* single nucleocapsid NPV (HearNPV) induced apoptosis in BTI-Tn-5B1-4 (Tn-Hi5) cells derived from *Trichoplusia ni*[22]. In the current paper we report that the apoptosis of Tn-Hi5 cells induced by HearNPV was blocked by co-infection with the wild strain of AcMNPV. It is notable that AcMNPV with a deletion in the *p35* gene (vP35Z)[23] completely inhibited apoptosis and rescued the replication of HearNPV in Tn-Hi5 cells. Furthermore, we demonstrated that the two putative *iap* genes of AcMNPV suppressed apoptosis of Tn-Hi5 cells. Recombinant HearNPVs respectively over expressing *iap1*, *iap2* and *p35*, rescued the replication of HearNPV in Tn-Hi5 cells and thus extended the host cell range of HearNPV. This is the first evidence that baculoviral apoptosis inhibitors may also function as a host range determinant.

1 Materials and methods

1.1 Cell culture and viruses

T. ni (Tn-Hi5) [24], *H. zea* (BCIRL-HZ-AM1, Hz-AM1)[25]and *S. frugiperda* (Sf21) cells were maintained and propagated in Grace's (GIBCO) medium supplemented with 10% heat-inactivated fetal bovine serum (GIBCO) at 28℃. Wild-strain AcMNPV, the p35-deletion mutant of AcMNPV (vP35Z)[23], HearNPV strain G4[26] and vHaCXW1, a recombinant HearNPV containing a green fluorescent protein gene (*gfp*) cassette and polyhedrin gene[27], were respectively propagated in Sf21, Tn-Hi5 or Hz-AM1 cells. Titration of virus stocks were accomplished by the end point dilution assay method (EPDA).

1.2 Expression of IAP1, IAP2 and P35 and generation of antisera

The complete *iap*1, *iap*2 and *p*35 genes were amplified by PCR from the AcMNPV genome DNA with the primer pairs: p1F and p1aR, p2F and p2aR, p35F and p35aR (Table 1), and cloned into pET32a. The recombinant plasmids pET32a-*iap1*, pET32a-*iap2* and pET32a-*p35* were transformed into the *Escherichia coli* strain *Rosseta* (DE3). To express the proteins, the bacteria were cultured at 37℃ for 4 h, followed by induction with 1 mmol/L IPTG at 20℃ for 16 h. The cell extracts were subjected to SDS-PAGE. The corresponding bands

of the particular fusion protein were excised. The antisera α-AcIAP1, α-AcIAP2 and α-AcP35 were prepared by using New Zealand white rabbits and standard procedures in the Center of Experimental Animals of the Wuhan Institute of Virology, CAS. The antisera were absorbed to an with *E. coli* lysate for one month at 4℃.

1.3 Inhibition of apoptosis

The *iap1*, *iap2* and *p35* genes were amplified by PCR with the primer pairs: p1F and p1bR, p2F and p2bR, p35F and p35bR (Table 1), and cloned into the *Eco*R I-*Xba* I sites of pIZ/V5 (Invitrogen) under the control of the promoter of OpMNPV *ie-2* to construct transient-expressing plasmids designated as pIZ/V5-iap1, pIZ/V5-iap2 and pIZ/V5-p35. Plasmid DNA was prepared for transfection using a plasmid purification kit (OMEGA).

Table 1 Primers for PCR amplification of genes of interest[a)]

Name	Sequence (5′—3′)
p1F	GGGGAATTCATGAACGAGGACACGCC
p1aR	GGGTCTAGACACCACAAATATTTTTATAAAAT
p1bR	GGGAAGCTTTTACACCACAAATATTTTTATAAA
p1cR	GGGCTGCAGTTACACCACAAATATTTTTATAAA
p2F	GGGGAATTCATGAATTTGATGCAA TTT AAT
p2aR	GGGTCTAGACTGAGGTAATGTTTCGATG
p2bR	GGGAAGCTTTTACTGAGGTAATGTTTCGAT
p2cR	GGGCTGCAGTTACTGAGGTAATGTTTCGAT
p35F	GGGGAATTCATGTGTGTAATTTTTCCGG
p35aR	GGGTCT AGATTTAATTGTGTTTAATATTACATT
p35bR	GGGCTGCAGTTATTTAATTGTGTTTAATATTACA

a) The restriction endonuclease sites are underlined.

Tn-Hi5 cells (2×10^5 cells per 35 mm Petri dish) were transfected with 2 μg of plasmid DNA using Lipofectin (Invitrogen, Life Technologies) according to the manufacturers' protocols. HearNPV at a multiplicity of infection (MOI) of 1 $TCID_{50}$ unit/cell was used to induce apoptosis of Tn-Hi5 cells at 24 h post transfection (hpt). The cells were collected at 72 h post infection (hpi) and fixed by 70% ethanol at –20 ℃ overnight. To stain the DNA, fixed cells were resuspended in PBS containing 5 μg/mL propidium iodide (PI) and 10 μg/mL RNase A for 1 h at 37℃. Afterwards, the DNA contents were analyzed by Flow Cytometry (Beckman Coulter XL). The same method was used to analyze apoptosis of recombinant virus-infected cells.

For coinfection assay, 1×10^6 Tn-Hi5 cells were infected with HearNPV at a MOI of 1 $TCID_{50}$ units/cell with or without AcMNPV or vP35Z at the same multiplicity, and harvested at 72 hpi. Then the cells were fixed with 1% paraformaldehyde and stained with Hoechst according to the manufacturer's protocol (Beyotime Biotechnology). The apoptotic cells were counted in the fluorescence microscope from four different fields. The analysis was repeated three times.

1.4 Caspase-like activity assay

Tn-Hi5 cells were harvested at 72 hpi, washed twice with PBS and then subjected to a caspase activity assay. Caspase-like activity were assayed using Caspase-Glo®3/7, 9, 8 assay kit (Promega) according to the technical bulletin, the fluorescence was detected using a $20/20^n$ Luminometer (Turner Biosystems).

1.5 Construction of the recombinant viruses

To generate recombinant HearNPVs expressing AcMNPV *iap* or *p35* genes, donor plasmids were constructed based on pFastBacDual (Invitrogen) for insertion of genes into the HearNPV bacmid HaBacHz8 by Tn7-mediated transposition[28]. Firstly, pFBG was generated by inserting the enhanced *gfp* gene (*egfp*) from pEGFP-N3 (Clontech) into the *Sma*I-*Kpn*I sites of pFastBacDual downstream of the *p*10 promoter. The donor plasmids, pFBG-Aciap1, pFBG-Aciap2 and pFBG-Acp35, were then constructed by insertion of the entire *iap1*, *iap2* and *p35* gene into the *EcoR* I-*Pst* I sites of pFBG under the control of the polyhedrin promoter. To produce recombinant bacmids, transposition was carried out by transforming the donor plasmid into competent cells of *E. coli* DH10B containing a helper plasmid pMON7124 and the HearNPV bacmid HaBacHz8[28]. The authenticity of the recombinant bacmids (inserts) were confirmed by PCR with the primers M13F and M13R (Table 1).

The recombinant viruses were generated by transfecting Hz-AM1 cells with recombinant HearNPV bacmid DNA using lipofectin (Invitrogen). The resulting viruses were named vHa-*Aciap1*, vHa-*Aciap2* and vHa-*Acp35*. Viruses were amplified by propagation in Hz-AM1 cells. Virus titer was determined in Hz-AM1 cells by EPDA.

1.6 Western blotting

Virus-infected or plasmid-transfected cells were washed and harvested in a phosphate saline buffer (pH 7.2). The cell extracts were added to a lysis buffer (100 mmol/L TrisiHCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 15% glycerol, 0.02% bromophenol blue), boiled for 5 min and then subjected to SDS-PAGE. Thereafter the proteins from the gel were transferred onto a nitrocellulose membrane (Millipore). Duplicate blots were prepared and processed for immunoblotting. The membranes were then placed into a blocking buffer (Tris-buffered saline, TBS, supplemented with 2% skimmed milk) and incubated overnight at 4℃. The membranes washed with TBST (TBS with 0.1% Tween-20), were immunoblotted in TBS containing a 1∶2000 dilution of antisera α-AcIAP1, α-AcIAP2 and α-AcP35. Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Sino-American, China) supplied secondary antibodies. The signal was detected using a BCIP/NBT kit (Sino-American, China).

1.7 Electron microscopy

Tn-Hi5 cells (1×10^6) were infected with virus at a MOI of 1 $TCID_{50}$ units/cell. Infected cells were harvested at 96 hpi and washed twice with PBS, then fixed in 2.5% glutaraldehyde and 0.1 mol/L sodium cacodylate (pH 7.3), and embedded in Spurr resin. Ultrathin sections were placed on copper grids, and stained with saturated uranyl acetate. Grids were examined in a HITACHI H7000 transmission electron microscope.

1.8 Growth curves

For one-step growth curves, 1×10^6 Tn-Hi5 cells were infected with vHaCXW1, vHa-*Aciap1*, vHa-*Aciap2* and vHa-*Acp35* at a MOI of 1 $TCID_{50}$ units/cell, or coinfected with vHaCXW1, wild strain AcMNPV or 1 MOI vP35Z at the same multiplicity. Supernatants were collected at the indicated times (0, 48 and 96 hpi). The titers of supernatants were determined by EPDA in Hz-AM1 cells.

2 Results

2.1 *p*35-deletion AcMNPV completely inhibits the apoptosis of Tn-Hi5 cells induced by HearNPV

It has been reported that HearNPV induced apoptosis in Tn-Hi5 cells, which was inhibited by TnMNPV P35[22]. Coinfection of HearNPV and AcMNPV showed that the HearNPV-induced apoptosis of Tn-Hi5 cells was suppressed by wild-strain AcMNPV (Figure 1A(b)). When co-infected with the *p*35-deletion mutant AcMNPV (vP35Z) and HearNPV, Tn-Hi5 cells did not undergo apoptosis and occlusion bodies were normally produced (Figure 1A(c)). Compared to 43% apoptosis of Tn-Hi5 cells infected with HearNPV at a MOI of 1 $TCID_{50}$ units/cell, coinfection of HearNPV with either AcMNPV or vP35Z resulted in very low level apoptosis of less than 3.5% (Figure 1B(b—d)). Apoptotic death of cells could be blocked almost completely by AcMNPV and vP35Z even at 24 h post HearNPV-infection (data not shown). Correspondingly, caspase activity analysis showed that HearNPV infection stimulated intracellular

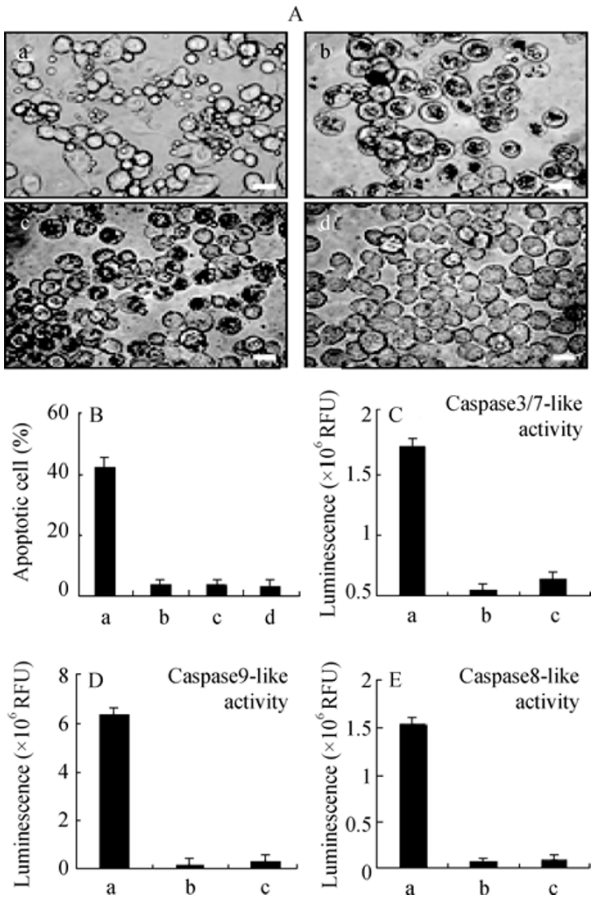

Figure 1 AcMNPV wt and vP35Z inhibits apoptosis of Tn-Hi5 cells induced by HearNPV infection. Tn-Hi5 cells were infected with HearNPV (a), respectively co-infected with HearNPV and AcMNPV (b) or HearNPV and vP35Z (c). At 72 hpi, the cells were observed and photographed in an inverted microscope (A). Mock infection with Grace's medium served as the negative control (d). The cells were harvested, stained with Hoechst and the apoptotic cells were counted utilizing a fluorescence microscope (B). The caspase-3-like (C), caspase-9-like (D), caspase-8-like (E) activities were analyzed.

caspase 3/7, caspase 8 and 9-like activity, while coinfection of vP35Z and HearNPV did not lead to significant activation of caspase-like protease (Figure 1D and E). These results indicated that both AcMNPV and vP35Z completely inhibited Tn-Hi5 apoptosis and significantly inhibited activation of intracellular caspase-like protease induced by HearNPV infection. This implies that HearNPV caused anti-apoptotic genes to block HearNPV-induced apoptosis.

2.2 IAP1 and IAP2 suppressed apoptosis of Tn-Hi5 induced by HearNPV

The *p35 gene*, AcMNPV, contains two *iap* homologs, *iap1* and *iap2*. To test whether or not they are functional as apoptosis inhibitors, Tn-Hi5 cells were transfected with pIZ/V5-*iap1* and pIZ/V5-*iap2*, then infected with wild strain HearNPV. Flow cytometry analysis indicated that both Ac-*iap1* and Ac-*iap2* suppressed apoptosis of Tn-Hi5 induced by HearNPV (Figure 2). Compared to the 44.5% apoptotic cells after transfection with an empty plasmid, the percentage of apoptosis in cells transfected with pIZ/V5-*iap1* and pIZ/V5-*iap2* was 28.4% and 24.3%. This level is similar to a positive control (pIZ/V5-*p35*) giving rise to 20% residual apoptotic cells (Figure 2). These results indicated that both the *iap*1 and *iap*2 of AcMNPV are functional as inhibitors of apoptosis.

2.3 The *p*35-deletion AcMNPV rescues replication of HearNPV in Tn-Hi5 cells

To determine whether or not HearNPV replicated in Tn-Hi5 cells during coinfection, vHaCXW1, a polyhedrin-positive recombinant HearNPV containing an *egfp* gene under control of a heat shock promoter[27], was used to infect Tn-Hi5 cells. Green fluorescence was detected by fluorescence microscopy and flow cytometry. Tn-Hi5 cells infected by vHaCXW1 alone underwent apoptosis and no green fluorescence was detected at 72 hpi (Figure 3A and D), whereas about 60% of the cells expressed eGFP when vHaCXW1 was either coinfected with vP35Z or AcMNPV (Figure 3).

When Tn-Hi5 cells were con-infected with either AcMNPV or vP35Z and the infectious budded virus Hz-AM1 cells, the viral titers of vHaCXW1 were significantly increased during infection and reached 2×10^4 and 1.2×10^4 $TCID_{50}$ units/mL at 96 hpi. No increase in titer was detected when Tn-Hi5 cells were solely infected with vHaCXW1 (Figure 4). These results suggested that

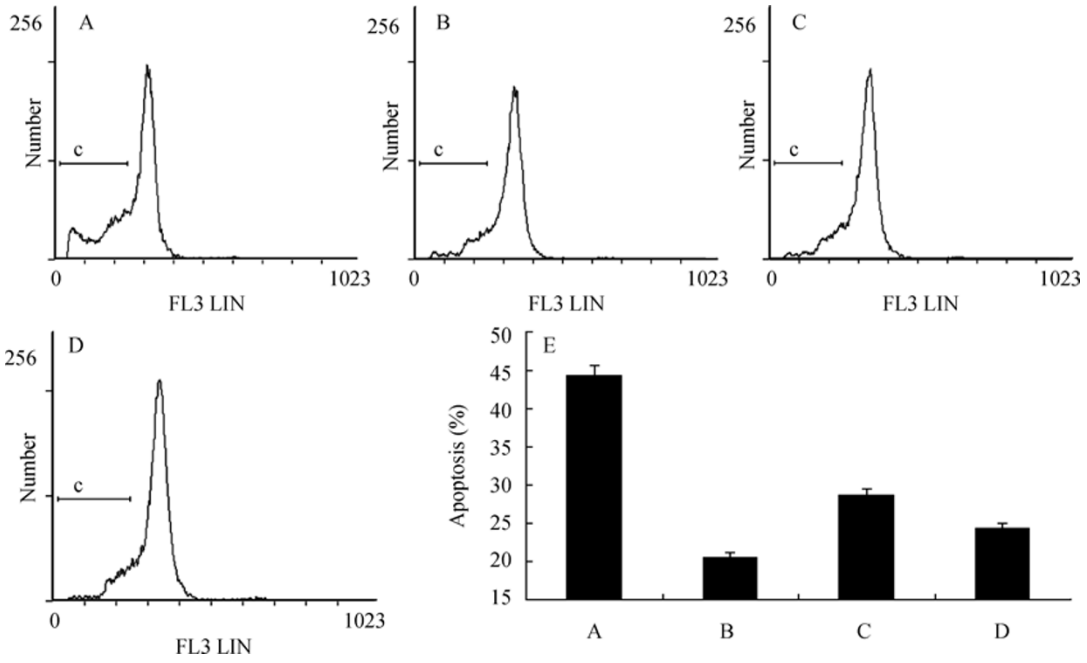

Figure 2 AcMNPV IAP1 and IAP2 inhibited apoptosis of Tn-Hi5 cells. Tn-Hi5 cells were infected by HearNPV G4 to induce apoptosis at 24 h post tranfection with plasmid pIZ/V5 (A), pIZ/V5-*p35* (B), pIZ/V5-*iap1*(C) and pIZ/V5-*iap2* (D). At 72 hpi, the cells were subjected to an apoptosis assay by Flow Cytometry. The percentage of apoptosis is expressed relative to the total number of cells and the data are presented as the mean of triplicate measurements ± SD.

both AcMNPV and vP35Z not only completely inhibited HearNPV-induced apoptosis but also facilitated the production of infectious HearNPV BVs in Tn-Hi5 cells. A similar observation was reported for TnMNPV, which facilitated the replication of HearNPV[22].

2.4 Construction of recombinant HearNPVs

To further investigate whether the apoptosis inhibitors P35, IAP1 and IAP2 could solely rescue HearNPV replication in Tn-Hi5 cells, we constructed three recombinant HearNPVs overexpressing the AcMNPV *iap1*, *iap2* and *p35*. The donor plasmids pFBG-Aciap1, pFBG-Aciap2 and pFBG-Acp35 were first generated, in which *iap1*, *iap2* and *p35* were inserted downstream of polyhedrin promoter. All plasmids also contain a reporter gene, *egfp*, under control of the AcMNPV *p10* promoter. After transposition in *E. coli*, the recombinant HearNPV bacmids (Figure 5A) were selected and identified by PCR.

The recombinant bacmids were transfected into HzAM1 cells to produce the recombinant HearNPVs: vHa-*Aciap1*, vHa-*Aciap2* and vHa-*Acp35*. The GFP expression showed that these recombinant viruses had been produced (Figure 5B). The expression of IAP1, IAP2 and P35 was detected by Western analysis. The antisera α-AcIAP1, α-AcIAP2 and α-AcP35 respectively recognized the corresponding 33, 29 and 35 kD bands in Hz-AM1 cells infected with vHa-*Aciap1*, vHa-*Aciap2* and vHa-*Acp35* (Figure 5C). That indicated that the AcMNPV *iap1*, *iap2* and *p35* genes within HearNPV recombinant viruses were correctly expressed in Hz-AM1 cells. This was further confirmed by RT- PCR (data not shown). These results confirmed that the recombinant HearNPVs have been constructed.

2.5 The AcMNPV *iap1*, *iap2* and *p35* genes rescue HearNPV replication in Tn-Hi5 cells

The infectivity of vHa-*Aciap1*, vHa-*Aciap2*, and vHa-*Acp35* was then analyzed in Tn-Hi5 cells. All the recombinants produced green fluorescence, resulting from *egfp* expression downstream of the HearNPV polyhedrin promoter. Green fluorescence or polyhedra were not seen in Tn-Hi5 cells infected by vHaCXW1 (Figure 6A) or wild-strain HearNPV. Flow cytometry assays showed that the apoptotic rate induced by vHa-*Aciap1*, vHa-*Aciap2* and vHa-*Acp35* was respectively 23.8%, 25.0% and 24.7%, which is significantly lower than the rate by vHaCXW1 (47%) (Figure 6E). However, no infectious BVs were produced or released by Tn-Hi5 cells infected with vHa-*Aciap1*, vHa-*Aciap2* and vHa-*Acp35* (Figure 4). The supernatants of Tn-Hi5 cells infected with these viruses were taken at different time points p.i. to determine the titer of the various viruses in Hz-AM1 cells. For all recombinants the titers remained at background levels (8×10^2 $TCID_{50}$ units/mL) from 0 to 96 hpi (Figure 4). Therefore none of the *p35*, *iap1* and *iap2* genes could rescue the production of budded virion.

2.7 Virions of recombinant HearNPV produced in Hi5 cells

Because no BVs were produced, it was essential ascertain whether or not virions were made in Tn-Hi5 cells. vHa-*Aciap1*, vHa-*Aciap2* and vHa-*Acp35*, produced eGFP, which is the under control of a late promoter, in Tn-Hi5 cells substantially after the infection occured. This suggests that late protein synthesis had occurred.

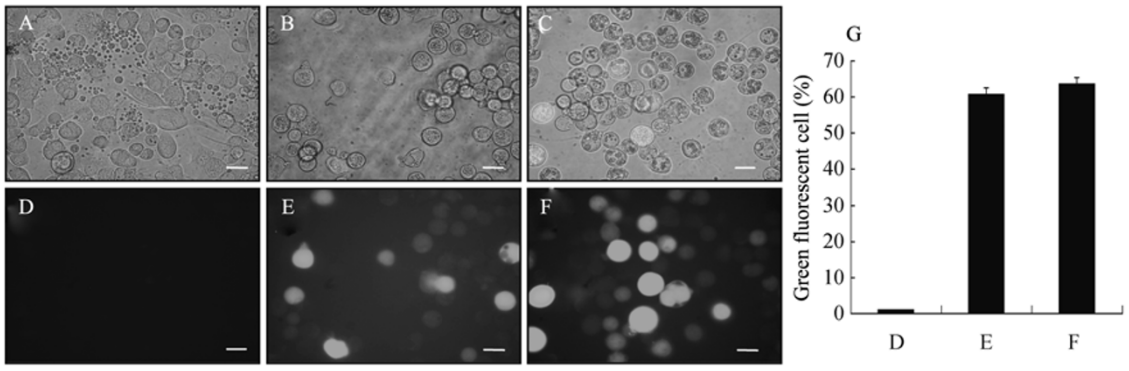

Figure 3 AcMNPV rescued the replication of HearNPV in Tn-Hi5 cells. Tn-Hi5 cells were solely infected by vHaCXW1 (A and D), coinfected with wt AcMNPV (B and E) or vP35Z (C and F). At 72 hpi, Tn-Hi5 cells were observed by fluorescence microscopy and subjected to Flow cytometry to measure the percentage of green fluorescent positive cells (G).

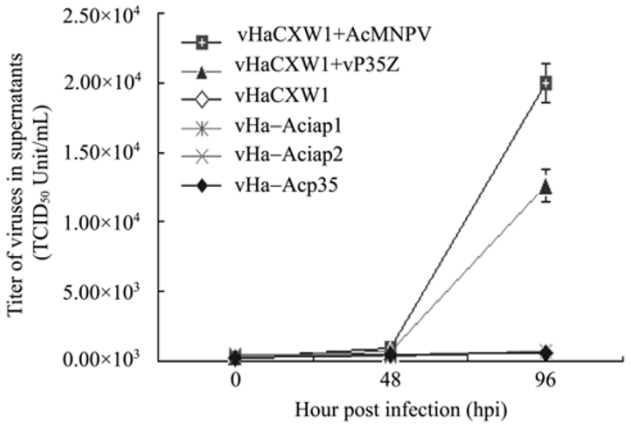

Figure 4 Replication kinetics of HearNPVs in Tn-Hi5 cells. Cells were infected with vHaCXW1, vHa-*Aciap1*, vHa-*Aciap2* and vHa-*Acp35,* or coinfected by vHaCXW1 with AcMNPV or vP35Z. At 0, 48, 96 hpi, the supernatants were sampled and the titers were determined in HzAM1 cells by EPDA.

vHa-*Aciap1*, vHa-*Aciap2* and vHa-*Acp35* -infected Tn-Hi5 cells were then subjected to transmission electron microscopy (Figure 7). Infection by all recombinant viruses resulted in typical cytopathic effects, such as nuclear swelling and abundance of virogenic stromata in the nucleus (Figure 7A—C). Enveloped virions were found interspersed in the nucleus and probably pre-occluded ODVs, which is similar to those produced in Hz-AM1 cells infected with vHaCXW1 (Figure 7D). There were no virions in Tn-Hi5 cells infected with vHaCXW1 (data not shown). The results indicated that recombinant HearNPVs produced progeny virions (ODVs) in Tn-Hi5 cells with the aid of AcMNPV IAPs or P35 as previously reported[22].

3 Discussion

Besides *p35*, two additional putative apoptotic suppressor genes, *Ac-iap1* and *Ac-iap2*, are present in the genome of AcMNPV. The deletion mutant vP35Z, lacking an active *p35* gene, induces apoptosis of Sf21 cells leading to abortive infection[17,29]. However, vP35Z normally replicates in Tn-368 cells, despite the lack of *p35*[21]. When the deletion occurred in *p35* and/or *iap1*, or in both *iap1* and *iap2*, the mutant AcMNPVs nonetheless efficiently produced BV and ODV progeny in *T. ni* (Tn-368), *Mamestra brassicae* and *Panolis flammea* cell lines[30]. This lead to the speculation that host *iaps* might be involved in inhibition of apoptosis in Tn-368 cells and this lead to the discovery of an insect *iap* from *T. ni*[31]. The generation of a triple-mutant AcMNPV lacking *p35*, *iap1* and *iap2* in Tn-Hi5 cells was unsuccessful, suggesting that Ac-*iap1* and/or Ac-*iap2* may need to be functional in the absence of P35[30]. In this report, we showed that both AcMNPV IAP1 and IAP2 are capable of blocking apoptosis of Tn-Hi5 cells (Figures 1—4).

It has been reported that Ac-IAP1 was unable to inhibit apoptosis induced by various stimuli in Sf9 cells and in mammalian cells such as 293 cells[12,13]. It is very likely that different *iap*s may be functional in different specific cells, tissues and hosts. The major functional IAPs fall into the distinct 'IAP3 homology group' by phylogenic analysis based on peptide sequences in CpGV[29], OpMNPV[32,33], and HycuNPV[34]. It has been hypothesized that baculovirus *iap*s were derived from insect host genomes and that *iap3* of NPV was acquired from the insect host through a gene capture event that was different from those involved in gene capture by a possible ancestor of other NPV *iap*s[10,13]. This suggests that NPV IAPs other than IAP3 might have been evolved with differing functions in infected insect cells distinct from that of IAP3. BmNPV IAP2 may function as a ubiquitin ligase[35], while the Eppo-IAP1 and Eppo-IAP2 were identified as functional inhibitors of apoptosis[20]. Comparison of sequences showed that Ac-IAP1 and Ac-IAP2 are most closely related to Eppo-IAP1 and Eppo-IAP2 with respectively 60% and 59% amino acid identity. The innermost sequences of their BIR and RING showed an even higher identity. This is in support of our current finding that each of the Ac-IAPs are functional.

Several lepidopteran cellular IAPs, Tn-IAP[31], Sf-IAP[13] and Bm-IAP[17] have been cloned and identified as functional inhibitors of apoptosis and share striking amino acid sequence similarity with baculovirus IAP[10,13]. Among these insect IAPs, Tn-IAP inhibited cell death induced by Actinomycin D, HID, GRIM and infection of vP35Z in Sf21 cells[31]. The latter two IAPs not only rescued occlusion body formation of vP35Z in Sf21 cells, but also blocked mammalian apoptosis stimulated by the *Bax* gene product. These IAPs also inhibited the activity of mammalian caspase-9, suggesting that insect and baculoviral IAP suppressing apoptosis may involve an insect counterpart of caspase-9[13,17].

This functional Sf-IAP in Sf21 cells was unable to block the widespread apoptosis of Sf21 cells infected by vP35Z. Tn-IAP inhibited apoptosis of Tn-Hi5 cells infected by HearNPV, although Tn-IAP was identified on the basis of its ability to support productive infection of vP35Z in Tn368 cells[31]. One possible explanation might be that different IAPs function in a cell-line-specific fashion as-mentioned above.

The pathway by which HearNPV induces the apoptosis may otherwise be somewhat par- ticular relative to that of vP35Z. This corresponds to the observation that morphologic changes of typical apoptotic cells were observed at a later phase in HearNPV-infected Tn-Hi5 cells than that of vP35Z in Sf21 cells. Additionally, successful coinfection of HearNPV and vP35Z, inhibiting caspase-3/7, 9, 8-like activity, implies that HearNPV

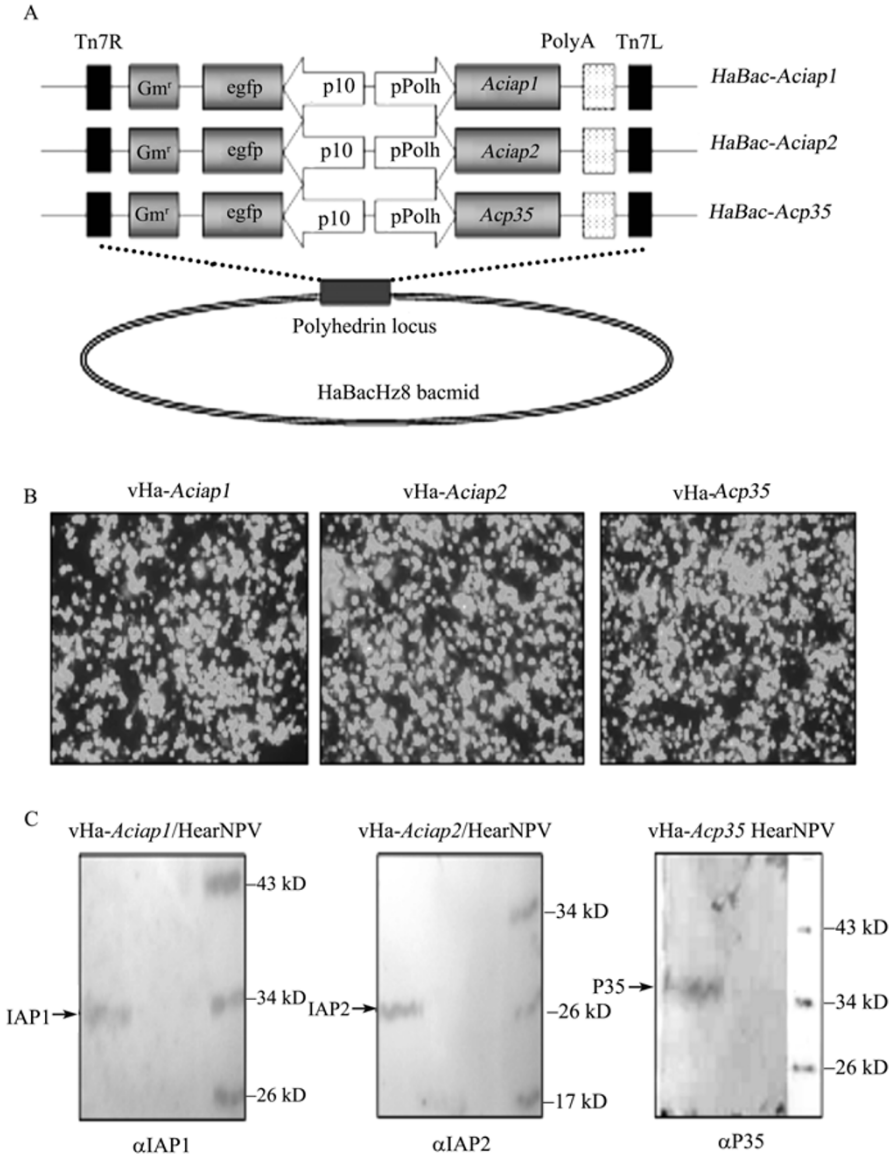

Figure 5 Construction and identification of recombinant HearNPVs (A). Schematic diagram of recombinant bacmid construction showing the *egfp* gene inserted downstream of the *p*10 promoter and the target gene downstream of the HearNPV polyhedrin promoter. The bacmids HaBac-*Aciap1*, HaBac-*Aciap2* and HaBac-*Acp35* were transfected into Hz-AM1 cells and the resulting recombinant viruses were propagated in Hz-AM1 cells (B). Infected Hz-AM1 cells were subjected to Western blotting with specific antisera αIAP1, αIAP2 and αp35 (C). HearNPV infected Hz-AM1 cells served as the control.

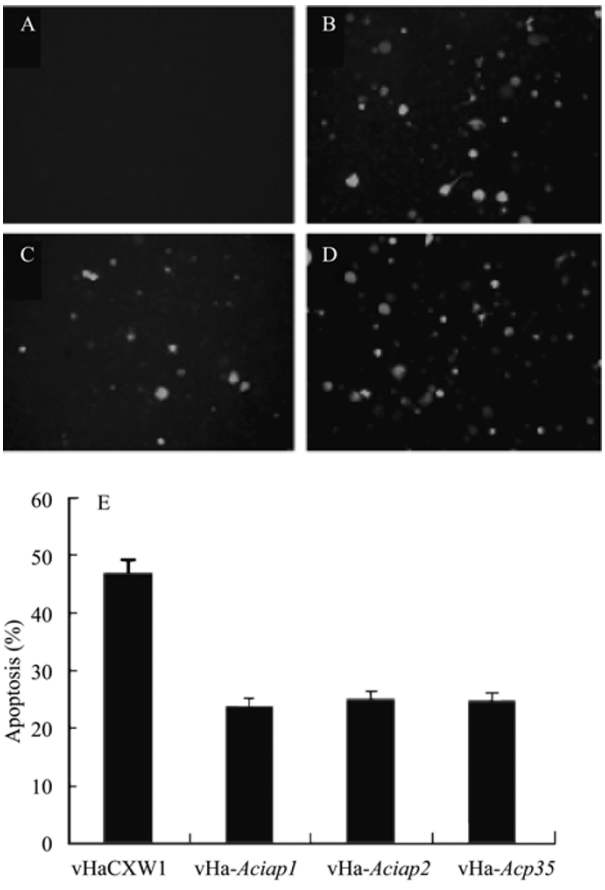

Figure 6 Recombinant HearNPVs infecting Tn-Hi5 cells. Tn-Hi5 cells infected with vHaCXW1 (A), vHa-*Aciap1* (B), vHa-*Aciap2* (C) and vHa-*Acp35* (D), were observed with a fluorescence microscope at 72 hpi, and harvested, stained by 20 μmol/L PI and then subjected to an apoptosis assay by Flow Cytometry (E).

infection could activate the corresponding unknown caspase-like protease to cause apoptosis in Tn-Hi5 cells. Assuming the previously described role of caspase-9 and caspase-8 in the intracellular and extracellular signaling pathway of mammalian cell apoptosis[36,37] HearNPV-induced apoptosis of Tn-Hi5 cells could involve a mitochondrial and death-receptor pathway similar to the situation in mammalian cells.

As an important defense mechanism of host cells, apoptosis reduces both the *in vitro* replication and the *in vivo* infectivity of a baculovirus and results in abortive infection, suggesting that apoptosis is a host range limiting factor for baculovirus infections[23,38]. Hitherto, it was reported that baculovirus genes influencing host range included *p143* (helicase), *hcf-1*, *hrf-1*, *p35*, *iap* and *lef-7*, among which involved late gene expression, DNA replication and apoptotic suppression[39,41]. The AcMNPV P35, IAP1 and IAP2, rescued the replication of HearNPV in Tn-Hi5, supporting the hypothesis that baculovirus IAPs may also be host range determinants. However, the AcMNPV *p35*, *iap1* or *iap2* did not rescue the production of BVs of recombinant HearNPVs, while AcMNPV rescued the complete replication of HearNPV in Tn-Hi5 cells, suggesting that other viral factor(s) are involved in the rescue of BV production. It is also possible that the anti-apoptotic genes in our case are expressed too late in the recombinant HearNPV infected cells.

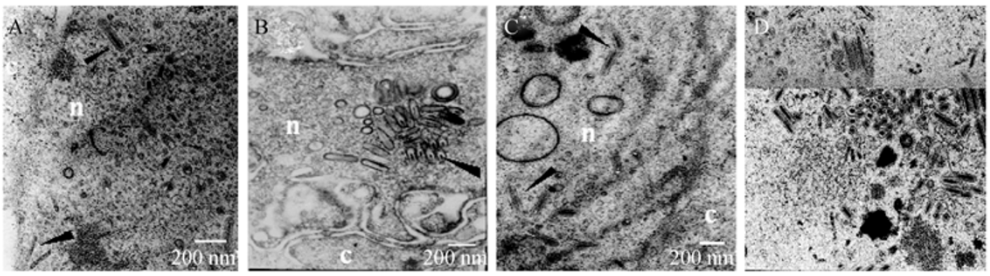

Figure 7 Electron micrographs of Tn-Hi5 cells infected with recombinant HearNPVs. Tn-Hi5 cells were infected with vHa-*Aciap1* (A), vHa-*Aciap2* (B) and vHa-*Acp35* (C), harvested at 96 hpi, subjected to ultrathin sectioning, and examined in a HITACHI H7000 transmission electron microscope. Hz-AM1 cells infected with vHaCXW1 (D) were the positive control. Virions were indicated with black arrows; n, nucleus; c, cytoplasm.

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