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Functional Analysis of the Inhibitor of Apoptosis Genes in *Antheraea pernyi* Nucleopolyhedrovirus

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The inhibitor of apoptosis proteins (IAP) plays an important role in cell apoptosis. We cloned two novel IAP family members, Ap-*iap1* and Ap-*iap2*, from *Antheraea pernyi* nucleopolyhedrovirus (ApNPV) genome. Ap-IAP1 contains two baculoviral IAP repeat (BIR) domains followed by a RING domain, but Ap-IAP2 has only one BIR domain and RING. The result of transient expression in *Spodoptera frugiperda* (Sf21) showed that Ap-*iap*1 blocked cell apoptosis induced by actinomycin D treatment and also rescued the *p35* deficient *Autographa californica* nucleopolyhedrovirus (AcNPV) to replicate in Sf9 cells, while Ap-*iap2* does not have this function. Several Ap-IAP1 truncations were constructed to test the activity of BIRs or RING motif to inhibit cell apoptosis. The results indicated that BIRs or RING of Ap-IAP1 had equally function to inhibit cell apoptosis. Therefore deletion of above both of the above domains could not block apoptosis induced by actinomycin D or rescue the replication of AcMNPV△*p35*. We also screened two phage-display peptides that might interact with Ap-IAP1.

***Keywords*:** apoptosis, ApNPV, *iap*, phage display

Apoptosis is a genetically regulated programmed process of cell suicide that functions not only to eliminate unnecessary cells during normal development and tissue homeostasis, but also serve as a defense strategy against oncogene expression and viral infection (Steller, 1995). In turn, many viruses have evolved ways to inhibit apoptosis of host cells (Uren *et al.*, 1996). *iap* (inhibitor of apoptosis) and *p35* were two types of anti-apoptotic genes in baculovirus (Clem, 2001).

The first members of IAP family, *Cp-iap3* and *Op-iap3*, were identified in the genome of the baculovirus *Cydia pomonella* granulosis virus (CpGV) and *Orgyia pseudotsugata* nucleopolyhedrovirus (OpNPV), respectively (Crook *et al.*, 1993; Birnbaum *et al.*, 1994). *iap* genes have been found in all sequenced lepidoptern baculovirus, and *iap* homologs have also been identified in numerous cellular genomes, from yeast and nematodes to insects and human (Salvesen and Duckett, 2002; Ikeda *et al.*, 2004; Srinivasula and Ashwell, 2008). According to amino acid sequence homology, baculovirus IAPs are classified into five types from IAP1 to IAP5 (Luque *et al.*, 2001) .

The distinguishing feature of the IAP proteins is the presence 1-3 baculovirus IAP repeat (BIR) at the amino terminus and a C3HC4 RING finger at the carboxy terminus (Ince *et al.*, 2008). The BIR motif is unique to IAPs, while RING finger motif have found in many proteins (Borden and Freemont, 1996). The BIRs are regions of about 70 amino acids, which contain a conserved motifs, $CysX_2CysX_{16}HisX_6Cys$ (X, any amino acid) (Srinivasula and Ashwell, 2008). Some IAP proteins can directly bind and inhibit caspases by promoting the degradation of active caspases, or by sequestering the caspases away from its substrates (Tenev *et al.*, 2005), and their interactions are mediated by specific BIR motif or sequences flanking the BIRs (Green *et al.*, 2004). BIR domains also bind to a short IAP-binding motif (IBM) at the processed N-terminus of several pro-apoptotic proteins, such as Reaper, Grim, and Hid (Yoo *et al.*, 2002). Binding of the IBM proteins to the BIR domain prevents caspase inhibition by competing for caspase binding and by promoting IAP degradation (Vucic *et al.*, 1998a). The RING domains of IAPs can act as E3 ubiquitin ligases to mediate the transfer of ubiquitin of associated proteins such as Smac/Diablo, and caspases. However, the importance of the RING domain for the anti-apoptotic activity of the IAPs is unclear (Huang *et al.*, 2000; Srinivasula and Ashwell, 2008).

Not all baculovirus IAPs have been correlated with anti-apoptotic activity. CpGV, OpMNPV, *Epiphyas postvittana* nuclearpolyhedrosis virus (EppoNPV) possess three and four *iap*s respectively, but functional analyses have showed that only IAP3 in CpGV and OpMNPV is capable of blocking apoptosis (Means *et al.*, 2003; Vilaplana and O'Reilly, 2003). As to EppoMNPV, only IAP1 and IAP2 exhibit anti-apoptotic activity (Maguire *et al.*, 2000).

The genome of ApNPV has been sequenced recently. ApNPV does not possess the *p35* gene homologue but encodes two *iap* genes, designated as Ap-*iap*1, and Ap-*iap*2 (Fan *et al.*, 2007). In this study we tested the activity of the two *iap* genes. The results showed that only Ap-*iap*1 is capable of blocking apoptosis induced by actinomycin D (ActD) and *p35*-deficient AcMNPV (AcMNPV△*p35*), but not *iap*2. We reported here that both BIR motifs and RING of Ap-IAP1 were important for anti-apoptotic activity. We also investigated

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the binding specificity of Ap-IAP1 by using phage display library screening.

Materials and Methods

Cells and virus

Spodoptera frugiperda (Sf21) and Sf9 cells were maintained at 27°C in Grace's medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (Hyclone, USA). The virus AcMNPV△*p35*, a mutant AcMNPV defective in *p35*, was gifted by Dr. Lunguang Yao (Nanyang Normal University, China).

Plasmids

ApNPV genomic DNA was extracted from ApNPV polyhedra. Ap-*iap*1 and Ap-*iap*2 were amplified using ApNPV genomic DNA as the template. The primer for Ap-iap1 is G*GAATTC*ACACGCTTGCAAC ATGAAC and CG*GTCTAG*AGGCACTACAAACACTTTTAC, the primer for Ap-iap2 is G*GAATTC*AGTTGCCATGGACAAGATTG and G*GTCTAG*ATGCGGTAGCGTCTCTATTCG. Both PCR products were subcloned into *Eco*RI-*Xba*I site of pIZT/V5-his vector (Invitrogen), named pIZT-Ap*iap*1 and pIZT-Ap*iap*2, respectively. pIZT-Op*iap*3 was constructed by inserting Op-*iap*3 into pIZT/V5-his.

Plasmids encoding fragments of the Ap-IAP1 protein, including BIR1 (residues 1-96), BIR2 (residues 127-200), BIR1+2 (residues 1-200), RING (residues 232-267), were created by a one-step PCR method employing a plasmid encoding a full-length Ap-IAP1 as the template and using GGAATTCACACGCTTGCAACATGAAC (BIR1+2), CGAATTCATGCTGCCAGCGAAGCCAT (RING), GGAATTCAC ACGCTTGCAACATGAAC (BIR1), and GGAATTCCGCCTGGCG CACCCCGATG (BIR2) as the forward primer, and CTCTAGAGCA TTCCATGGCTTCGCTGGC (BIR1+2), GCTCTAGATTACACTA CAAACACT (RING), GCTCTAGAGGTTTACCGGGCGAGACC (BIR1), GCTCTAGATTCCATGGCTTCGCTGG (BIR2) as the reverse primer.

All the fragments were subcloned into pIZT/V5-his vector by digestion with *Eco*RI and *Xba*I. The proper construction of all the plasmids was confirmed by DNA sequencing.

Transfection and actinomycin D treatment

Transfection was performed in cells in 35-mm tissue culture dishes (IWAKI, Japan). Cells were incubated with a mixture containing 10 μl lipofectamine 2000 (Invitrogen) and 2 μg of DNA plasmid in Grace's medium. After 6 h at 27°C, the transfection mixture was replaced with supplement Grace's medium.

At 24 h post-transfection, the media were removed and replaced with medium containing 1 μg actinomycin D (ActD) per ml. After cells were treated with ActD at 27°C for 18 h, cells were observed with an inverted microscope (Eclipse TE2000-U, Nikon, Japan).

DNA fragmentation assay

ActD treated cells were pelleted at 1,000×g and resuspended in a lysis buffer. After 2 h at room temperature, lysates were extracted with phenol/chloroform and precipitated with 2.5 volumes of ethanol. After centrifugation at 12,000×g, DNA pellets were dissolved in Tris-EDTA buffer and subjected to electrophoresis on 1% agarose (Harvey *et al.*, 1997).

Flow cytometry

Cell were harvested and first washed with PBS. Then cells were fixed in cold 70% ethanol for at least 1 h and resuspended in 1 ml of 50 μg/ml propidium iodide/RNase/Triton X-100 for 30 min. Flow cytometry was done on a Coulter Cytomics FC500 (Beckman Coulter, USA). The data were analyzed with Coulter Epocs Expo32 software.

Polyhedron formation

Sf9 cells were first transfected with the following plasmids: pIZT-Ap*iap*1, pIZT-Ap*iap*2, or pIZT-Op*iap*3. At 16 h after transfection, cells were infected with AcMNPV△*p35* (0.5 PFU/cell) and incubated at 28°C for another 2 h, after which the inoculum was removed and replaced with Grace's medium plus 10% FBS. The infected cells were then incubated at 28°C until 72 h post-infection (hpi), and then the cells were examined by inverted microscopy for the presence of polyhedra.

Protein expression and purification

Ap-*iap*1 and Ap-*iap*2 were cloned into *Escherichia coli* strain BL21 (DE3) using the pET28b vector (Novagen, Germany), resulting in expression of protein with carboxyl-terminal His-tagged extension. The samples containing the expressed proteins were used for further purification using HisTrap affinity resin (Amersham Biosciences, USA) according to the instruction manual. The purified poteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Immunoblot analysis

Proteins from transfected cells or purified proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred into an Polyvinylidene fluoride memebrane (Millipore, USA). The blots were probed with antibody against His-tag and visualized by goat anti-mouse IgG antibody conjugated with alkaline phosphatase and BCIP/NBT as substrate.

Phage display library screening

A phage library expressing 15 random amino acids fused to the amino terminus of the mature PIII coat protein was used for screening. Three sequential rounds of affinity purification and reamplification of the phage were carried out, with 20 phage isolates sequenced after each round of selection. Affinity purifications were carried out by using 500 μg of purified protein and (2×10^{11}) phage particles in Tris-buffered saline containing 0.1% Tween 20. Bound phages were washed 10 times in Tris-buffered saline containing 0.1% Tween 20 and eluted with 1 M Tris (pH 9.1), and titers were determined by using Escherichia coli strain K91. Random plaques were chosen for phage minipreparations and sequenced to determine the amino acid sequence encoded by the phage.

Results

Sequence analysis of ApNPV *iap* genes and proteins

Analysis of the genome of ApNPV (GenBank: EF207986) indicated that there were two *iap* genes, namely Ap-*iap*1 and Ap-*iap*2. The Ap-*iap*1 gene encodes a putative 280 amino acid protein with a predicated molecular mass of 31 kDa, while the Ap-*iap*2 gene encodes a putative 189 amino acid protein with a predicated molecular mass of 20 kDa. IAP1 contains two BIRs (BIR1+BIR2) and a RING motif. IAP2 contains only one BIR and a RING motif. Ap-IAP1 and Ap-Iap2 showed significant similarity with BIR and RING sequence of several known baculovirus IAPs (Fig. 1).

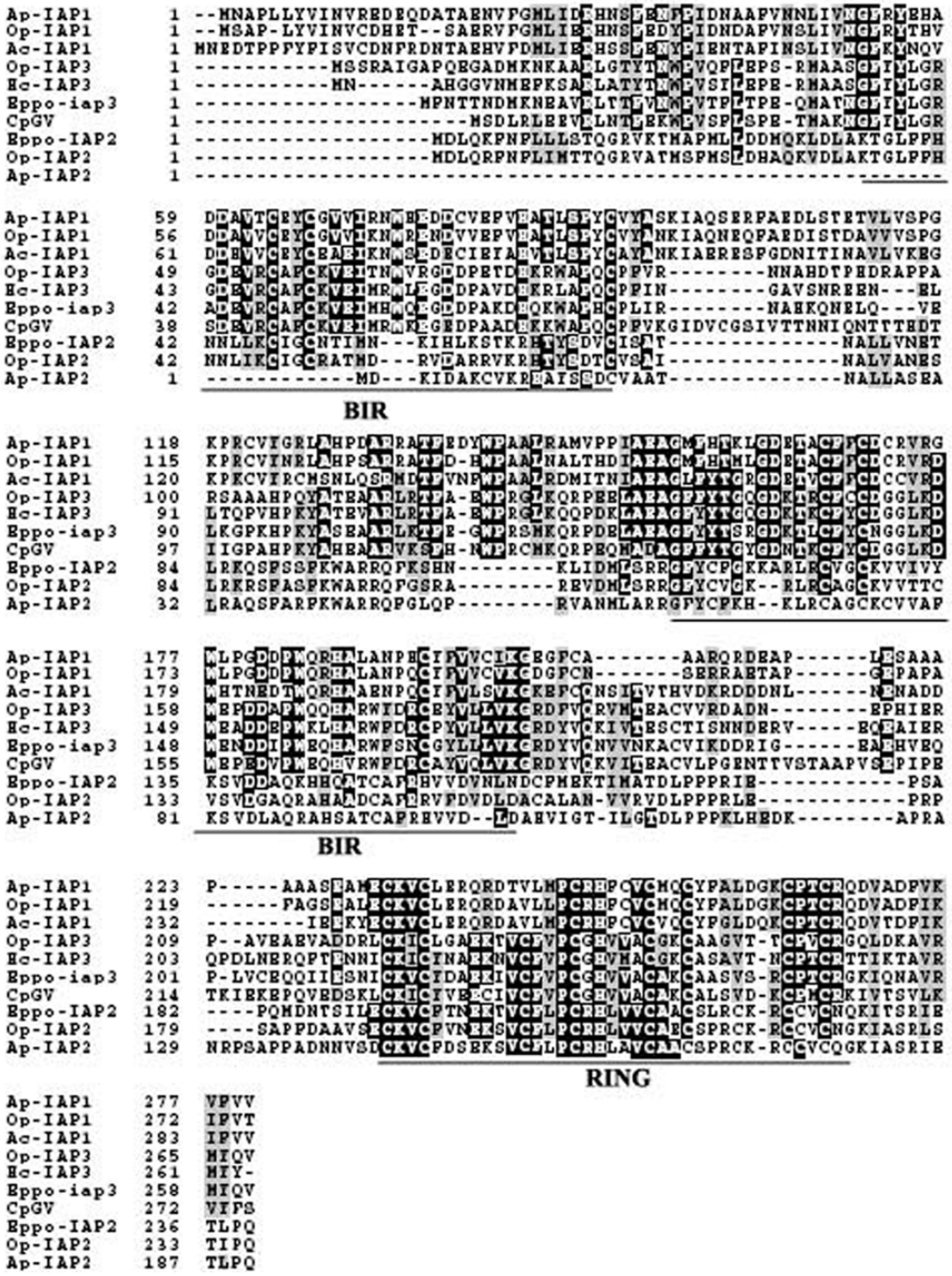

Fig. 1. Multiple alignments of Ap-IAP1 and Ap-IAP2 with amino acid sequences of other IAP family members by CLUSTAL W program. Black and dark grey columns represents amino acids with ≥95% identity, ≥80% similarity, respectively. BIR domains represented by the sequence $GX_{9-11}CX_2CX_{8-10}E/DX_5HX_{3-6}C$ and RING finger domain characterized by the C3HC4 domain are underlined. The GenBank accession no. of used for the alignments are as follow: Ap-IAP1, ABQ12341; Ap-IAP2, ABF50318; Ac-IAP1, NP_054056; Op-IAP1, NP_046197; Eppo-IAP2, AAK85627; Op-IAP2, NP_046230; CpGV-IAP3, AAB39098; Op-IAP3, NP_046191; Hc-IAP3, YP_473308; Eppo-IAP 3, NP_203195.

Ap-*iap*1 functions as an anti-apoptotic gene

Firstly, the expression of Ap-IAP1 and Ap-IAP2 in Sf21 cells were examined by western blot analysis. Sf21 cells were transfected with pIZT-Ap*iap*1, pIZT-Ap*iap*2. At 24 h after the transfection, cells were harvested and were examined by immunoblot analysis using anti-his antibody, because the Ap-*iap*1 and Ap-*iap*2 gene were fused in frame with the polyhistidine tag in pIZT/V5-his vector. Ap-IAP1 and Ap-IAP2 were detected as 31- and 21-kDa, respectively (Fig. 2).

To explore whether the Ap-IAPs have the ability to block

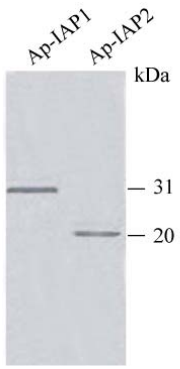

Fig. 2. Western blot analysis of Ap-IAP1 and Ap-IAP2 proteins expression in Sf21 cells. Sf21 cell were transfected with pIZT-Ap*iap*1 and pIZT-Ap*iap*2 and examined by immunoblot analysis with anti-his monoclonal and visualized by goat anti-mouse IgG antibody conjugated with Alkaline Phosphatase and BCIP/NBT as substrate. The approximate molecular mass of two proteins is indicated on the right.

ActD-induced apoptosis, pIZT-Ap*iap*1 and pIZT-Ap*iap*2 were transfected into Sf21 cells, while pIZT-Op*iap*3 as positive control and pIZT/V5-his vector as a negative control. Then cells were incubated with ActD for 18 h to induce apoptosis. The cells transfected with pIZT-Ap*iap*1 and pIZT-Op*iap*3 did not show obvious characteristics of apoptosis, such as cell shrinkage, plasma membrane blebbing and apoptotic bodies. But after treated with ActD, the number of cells transfected with pIZT-Ap*iap*2 and pIZT/V5-his vector reduced and the characteristics of apoptosis could be seen with inverted microscope (Fig. 3A left). Gel electrophoresis result indicated that the DNA of cells transfected with only pIZT-Ap*iap*2 or pIZT/V5-his had been degraded into fragments (Fig. 3A right).

To test the ability of Ap-*iap*s to rescue polyhedron formation in *p35* deficient AcMNPV (AcMNPV△*p35*)-infected Sf9 cells, Sf9 cells were transfected with plamids containing Ap-*iap* genes and then infected with AcMNPV△*p35*. In WT AcMNPV-infected Sf9 cells, polyhedron formation was readily visible.

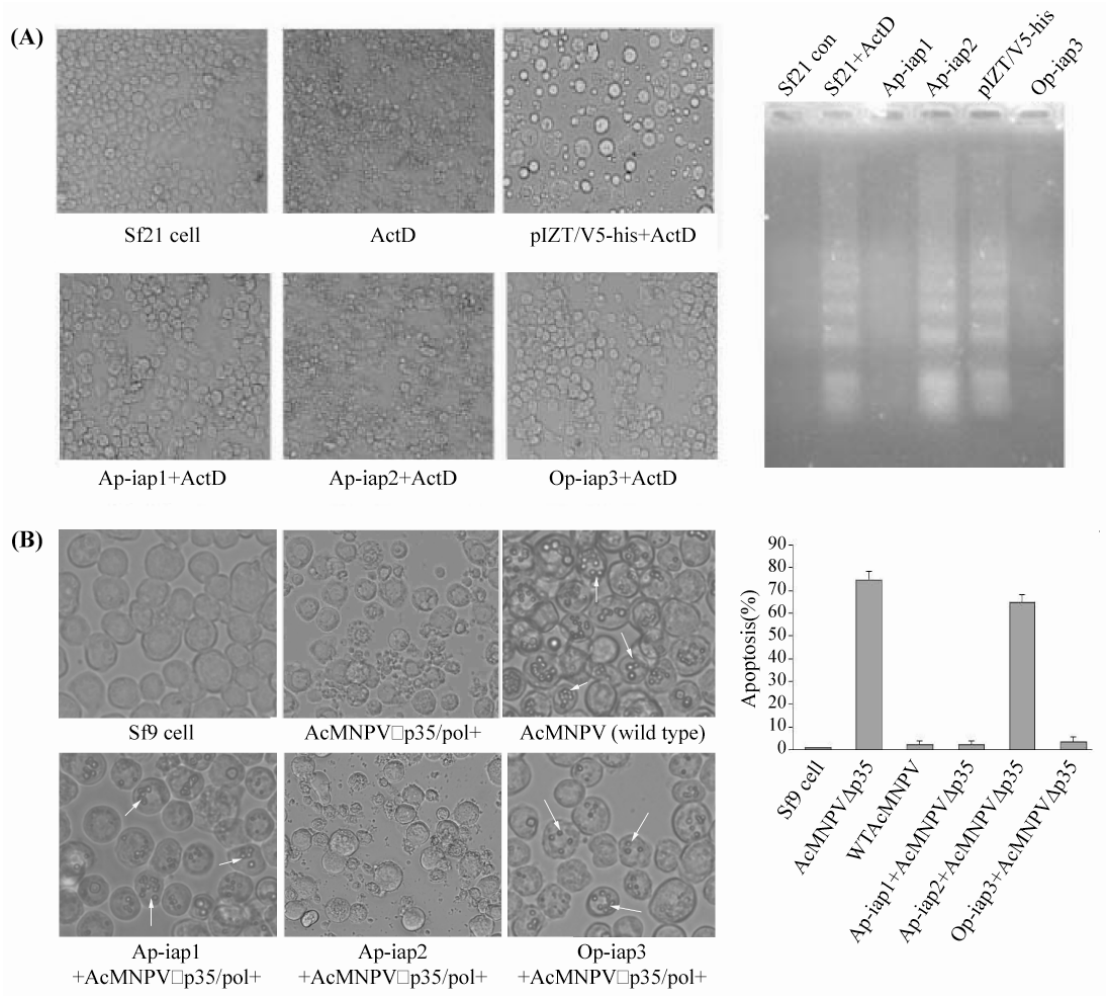

Fig. 3. Ap-*iap*1 functioned as an anti-apoptosis gene. (A) Ap-*iap*1 inhibited Actionmycin D-induced apoptosis in Sf21 cells. Left: Sf21 cells were transfected with plasmid indicated under each photograph and treated with ActD for 18 h. Right: DNA fragmentation was analyzed on 1.0% agarose gel. (B) Ap-*iap*1 rescued polyhedron formation of AcMNPV△*p35*. Left: Sf9 cells were transfected with pIZT-Ap*iap*1, pIZT-Ap*iap*2, and pIZT-Opiap3, then infected with AcMNPV△*p35* virus. Mock-infected Sf9 cells and only infected with AcMNPV△*p35* virus as a control, and infected with WT AcMNPV as a positive control. Polyhedron was indicated by the arrow; Right: Flow cytometry analysis of anti-apoptotic activity of Ap-IAP1 blocking apoptosis induced by AcMNPV△*p35*. Vertical bars represent standard deviations of averages from three indpendent experiments.

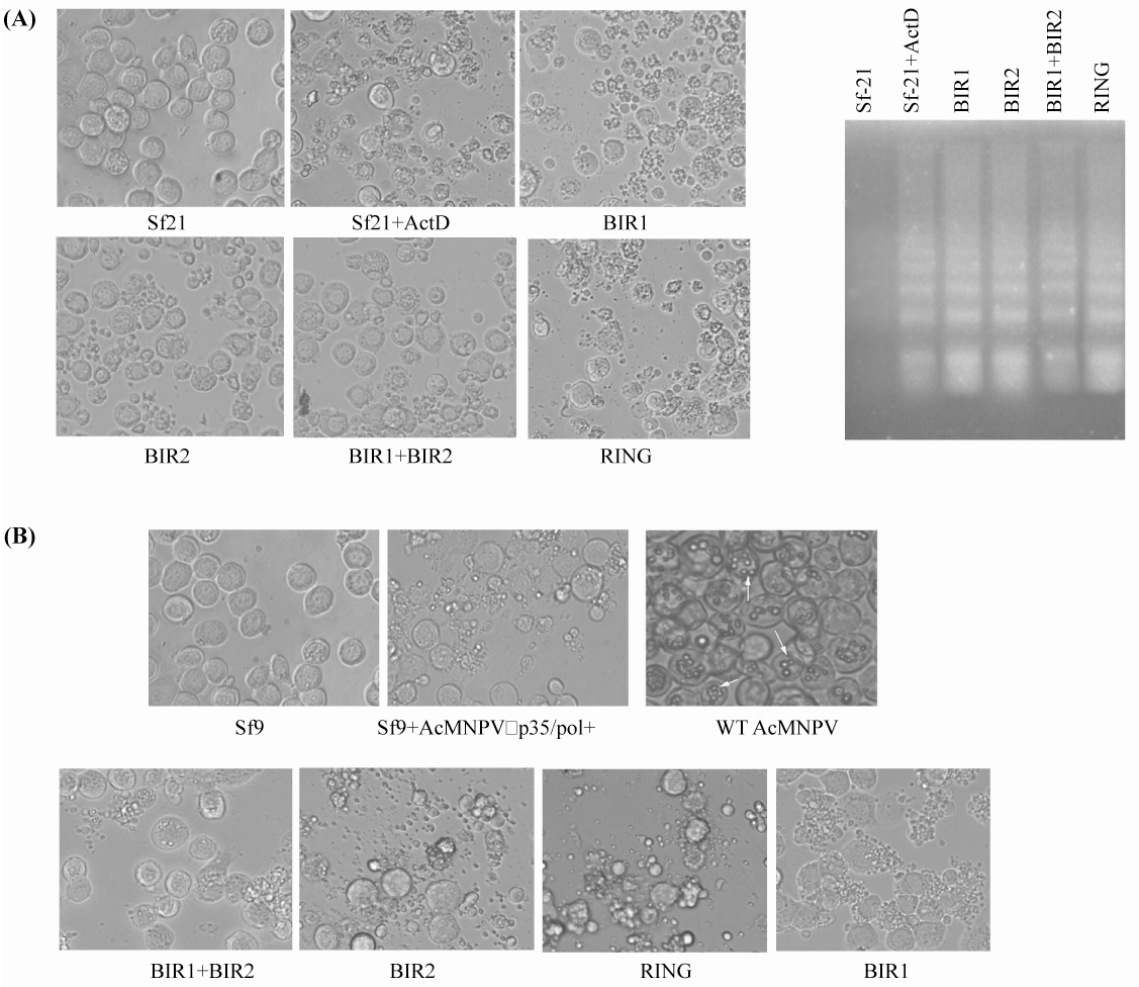

Fig. 4. The anti-apoptotic activity of Ap-IAP1 need BIR and RING motif. (A) Ap-IAP1 truncations did not inhibit apoptosis induced by actinomycin D. Left: Sf21 cells apoptosis were induced by ActD following transfection with plasmids expressing truncations. Right: Cellular DNA was harvested at 8 and 24 h post-heat shock, separated by agarose gel electrophoresis, stained with ethidium bromide. (B) Ap-IAP1 truncations did not block apoptosis induced by infection of AcMNPV△*p35*. Sf9 cells were transfected with Ap-IAP1 truncations, then mock infected or infected with AcMNPV△*p35* or with wild type AcMNPV, ployhedra were observed in cells only infected with wild type AcMNPV and are indicated by the arrows.

But in AcMNPV△*p35*-infected cells previously transfected with empty vector or pIZT-Ap*iap*2, apoptosis was induced and no polyhedron were formed. And in cells transfected with a plasmid expressing pIZT-Ap*iap*1 or pIZT-Op*iap*3, apoptosis was sufficiently delayed to allow polyhedron formation (Fig. 3B left).

The results of flow cytometry assays also showed that Ap-IAP1 and Op-IAP3 could inhibit apoptosis triggered by infection of AcMNPV△*p35* virus (Fig. 3B right). As measured by flow cytometry, the percentage of apoptosis of the mock-infected group was only 1.6%, and the low apoptotic rate was also found in the cells transfected with Ap-*iap*1 and Op-*iap*3. By contrast the apoptotic rate of cells transfected with Ap-*iap*2 or only infected by AcMNPV△*p35* were much higher.

BIRs and RING finger of Ap-IAP1 are required to block cell death

To investigate the individual contributions of BIRs or RING motifs to Ap-IAP1 ant*iap*optotic activity, we constructed Ap-IAP1 truncations Ap-IAP1$^{1\text{-}199}$ and Ap-IAP1$^{229\text{-}280}$, which contain either the BIR motifs (BIR 1 and 2) or the RING finger, respectively. We also constructed truncations containing only BIR1 or BIR2 motif. Each truncation was subcloned into pIZT/V5-his vector for expression in Sf21 cells. The results showed that all truncations failed to protect Sf21 cells from ActD-induced apoptosis. Also the levels of intracellular DNA fragmentation and plasma membrane blebbing were comparable to those of cells transfected with the vector alone. In contrast, the full-length Ap-IAP1 fully protected Sf21 cells from apoptosis (Fig. 4A).

We also tested the ability of Ap-IAP1 truncations to rescue polyhedron formation in AcMNPV△*p35*-infected Sf9 cells. Transfection of pIZT-Ap*iap*1 blocked Sf9 cells death and rescued the formation of polyhedron. However, in AcMNPV △*p35*-infected cells previously transfected with Ap-IAP1 truncations, apoptosis was induced and no polyhedron formed

Table 1. Sequences of the different peptides identified after three rounds of phage selection and amplification. DNA sequencing of the individual clones examined allowed the amino acid sequences to be deduced from the nucleotide sequence. Two of the peptides, B3 and E3, had an identical sequence, GAVAYFIPYQADLDA, and could have arisen from the same clone. Peptide D5, D7, and F2 had the amino acid sequence MAVPYYLPEVGAEDV. Residues conserved in more IBM-proteins are underlined.

Peptide	Nucleotide sequence	Amino acid sequence
B3	GGCGCGGTGGCGTATTTTATTCCGTATCAGGCGGATCTGGATGCG	GAVAYFIPYQADLDA
E3	GGCGCGGTGGCGTATTTTATTCCGTATCAGGCGGATCTGGATGCG	GAVAYFIPYQADLDA
D5	ATGGCGGTGCCGTATTATCTGCCGGAAGTGGGCGCGGAAGATGTG	MAVPYYLPEVGAEDV
D7	ATGGCGGTGCCGTATTATCTGCCGGAAGTGGGCGCGGAAGATGTG	MAVPYYLPEVGAEDV
F2	ATGGCGGTGCCGTATTATCTGCCGGAAGTGGGCGCGGAAGATGTG	MAVPYYLPEVGAEDV

(Fig. 4B).

Identification of Ap-IAP1-binding peptides

The full-length of Ap-*iap*1 and Ap-*iap*2 ORFs were cloned into the pET28b expression vector. Both Ap-IAP1 and Ap-IAP2 proteins were expressed as His-tagged fusion proteins and purified (Figs. 5A and B). To investigate the binding specificity of Ap-IAP1, the Ap-IAP1-binding peptides were identified by selecting phage display 15-mer peptide library. The DNA inserts of 20 randomly chosen phage clones recovered from the third round of bio-panning on Ap-IAP1 were sequenced. The sequencing result showed that two of the peptides, B3 and E3, had an identical sequence, GAVAYFIPYQADLDA, and could have arisen from the same clone. Peptides D5, D7, and F2 had the same amino acid sequence MAVPYYLPEVGAEDV (Table 1). We searched NCBI protein database and found that both the amino acid sequences showed high homology with N-terminal of Drosophila cell death proteins reaper GRIM and HID, respectively.

Discussion

Over expression studies found that IAPs from virus to human can inhibit apoptosis induced by a variety of stimuli, leading to the belief that the primary role of IAPs in to prolong cell survival (Srinivasula and Ashwell, 2008). In this study, we have focused on anti-apoptotic genes of ApNPV. The *iap* genes have been found in all sequenced baculovirus genomes except CuniNPV (Afonso *et al.*, 2001), while *p35*-like genes have only been identified in a few species of baculovirus. However, only some of *iap* gene homologues have shown to have the ability to block apoptosis in some baculovirus containing multiple *iap* genes. The result of genome analysis showed that ApNPV lacks a *p35* homologue, but contains two members from the *iap* family, which were designated as Ap-*iap*1 and Ap-*iap*2 (Fan *et al.*, 2007). Like other baculovirus IAPs, Ap-IAP1 possesses two BIR domains and a single RING finger motif, while Ap-IAP2 contains only one BIR and a RING finger.

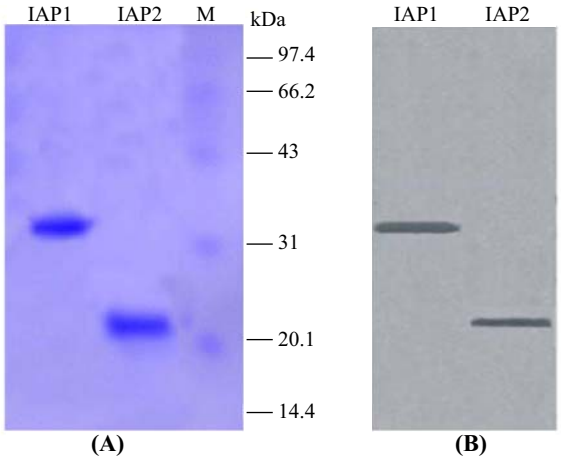

Fig. 5. Expression and purification of Ap-IAP1 and Ap-IAP2 proteins. (A) Two proteins were detected by SDS-PAGE, (B) Western blot analysis with anti-6·His-tag monoclonal antibody.

It has been shown that Op-IAp3 and some other baculovirus IAPs could block ActD-induced apoptosis of Sf21 cells. Terry reported *iap*1 and *iap*2 of EppoMNPV (*Epiphyas postvittana* nucleopolyhedrovirus) can inhibit apoptosis, that is the first report of anti-apoptosis activity for *iap*1 and *iap*2 genes (Maguire *et al.*, 2000). Our results showed that Ap-IAP1 not Ap-IAP2 block apoptosis induced by ActD. Most IAPs with anti-apoptotic activity have two BIR motifs except Survivin (Ambrosini *et al.*, 1997). Ap-IAP1 contains two BIRs, while Ap-IAP2 has only one BIR different with other baculovirus IAP2. In this study we also showed that Ap-IAP1 could block apoptosis induced by infection with AcMNPV△*p35* and rescue AcMNPV△*p35* to replicate in cells, while Ap-IAP2 exhibited none of these activities. All our evidences showed that the Ap-*iap*1 gene is active in inhibiting host cell apoptosis.

The character of IAP family is the presence of BIR motifs and a RING finger (Miller *et al.*, 1998). The number of BIR motifs in different IAPs varies between one and three, but the anti-apoptotic baculovirus IAPs typically contain two BIRs (Seshagiri *et al.*, 1999). In this study, we examined functional attributes of the BIRs and RING motif of Ap-IAP1. Some evidences have shown that the baculovirus IAPs require the BIRs as well as the RING finger for full anti-apoptotic activity (Harvey *et al.*, 1997). Our results added another evidence for this conclusion. We found that either BIRs or RING motif of Ap-IAP1 could not independently inhibit apoptosis induced by actinomycin D or AcMNPV△*p35*. But more remains to be learned about the contribution of BIR and RING motif to Ap-IAP1 function.

We also investigated the binding specificity of Ap-IAP1 protein through phage-display peptide library. We screened two peptides that might interact with Ap-IAP1. We searched in the NCBI protein database and found that their amino acid sequences showed high identity with N-terminal of Drosophila cell death proteins GRIM and HID, respectively (Table 1).

The two proteins are apoptosis-inducing factor in Drosophila, containing an IAP-binding tetra-peptide motif (IBM). There are at least four functional homologues of in Drosophila: reaper (RPR), HID, and GRIM, Sickle (Vucic *et al.*, 1997; Vucic *et al.*, 1998b). These proteins have been shown to be responsible for inducing apoptosis and for binding to either the baculoviral or endogenous cellular IAPs (Vucic *et al.*, 1998b). The baculoviral Op-IAP and Cp-IAP proteins and the Drosophila IAP family proteins DIAP-1 and DIAP-2 have shown to bind the Drosophila cell death proteins reaper (RPR), HID, and GRIM. Further studies are being carried to confirm the interaction between Ap-IAP1 and GRIM, HID.

Acknowledgements

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