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Cloning and Sequence Analysis of *iap*1 Gene of *Bombyx mori* Nuclear Polyhedrovirus ZJ Strain and its Function on Regulating NF-κB Activity in Mammalian Cells

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Abstract The *iap*1 gene of *Bombyx mori* nucleopolyhedrovirus Zhenjiang strain was amplified by Polymerase Chain Reaction and cloned into pGEM-Teasy vector and sequenced. Sequence analysis results showed that *iap*1 of BmNPV-ZJ contains 858 base pairs of nucleotides, with 96% identity in nucleotides, but missing a region which encodes seven continuous aspartic acids, compared to that of BmNPV-T3 strain. DART search result (Domain Architecture Retrieval Tool) indicated that the *iap*1 of BmNPV-ZJ contained two BIR (baculovirus IAP-like repeat) domains, but lacked RING domain. It is not known yet if the iap of BmNPV has any function on apoptosis. Electrophoretic Mobility Shift Assay using NF-κ B as a probe has shown that transfection of BmNPV-ZJ *iap*1 gene into mouse pc12 cells could reverse the effect of TNF-α on NF-κ B binding activity. The action and its pathway of BmNPV-ZJ *iap* 1 on NF-κ B in pc12 cells and other mammalian cells are under way.

Key words Apoptosis *iap*1 Nucleopolyhedrovirus *Bombyx mori* NF-κ B EMSA Sequencing pc12 cell

Apoptosis, also called "programmed cell death", is a physiological process by which cells kill themselves in a controlled manner[1]. Apoptosis is used not only in normal circumstances during development and for homeostatic control of cell numbers, but also used as a defense mechanism to remove unwanted or dangerous cells. It can be used by host cells during viral infection to halt virus spread. Viruses have also acquired anti—apoptotic genes during their revolution to favor their own multiplification. To date, two different families of baculovirus anti—apoptotic genes have been identified, p35 and iap. The first members of the IAP family were identified in search for novel baculoviral anti—apoptotic genes[2]. These proteins have a carboxy terminal RING finger domain, and at their amino termini are two repeats of a motif designated baculovirus IAP-like repeat (BIR). IAPs from *Orgyia pseudotsugata* nuclear polyhedrosis virus and *Cydia pomonella* granulosis virus (OpIAP and CpIAP) could inhibit the apoptosis caused by infection of insect cells with p35 deficient viruses[3].

The mechanism of apoptosis is highly conserved throughout metazoa. A family of a cysteine proteases termed caspases[4] appear to be the key apoptosis effector enzymes in nematodes, insects and mammals. IAPs must interact with conserved components of the apoptotic mechanism, since OpIAP could inhibit death of mammalian cells due to overexpression of caspases, the CD95 signaling molecule FADD, or infection with Sindbis virus[5,6]. Many viral anti—apoptosis proteins have been identified that prevent cell death by either inhibiting caspase activity, or blocking pathways that lead to caspase activation. No paper has been seen which reported the anti—apoptot-

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ic function of *Bombyx mori* nuclear polyhedrosis virus (BmNPV) iap genes. In this study, the *iap*1 gene of BmNPV Zhenjiang strain (BmNPV-ZJ) was cloned and sequenced, its function on regulating mammalian NF-κ B binding activity upon TNF-α stimulation was examined and discussed.

1 Materials and Methods

1.1 Materials

Dulbecco' s Modified Eagle' s Medium (DMEM)-high glucose, FBS (fetal bovine serum), and horse serum, Taq DNA polymerase were purchased from Gibco BRL (Life Technologies, inc.). Poly-D-lysine and TNF-α was obtained from Sigma. PGEM-T easy and pcDNA3.1 (+) plasmids were obtained from Dr. Lim' s lab of University of Missouri-Columbia. (α-p^{32})dCTP was purchased from NEN (New England-Nuclear). Lipofectamine 2000 used for transfection was from Invitrogen Corporation.

1.2 Cells and viruses

BmN cells were maintained at 27 ℃ in TNM-FH medium (Graces medium supplemented with 5% heat-inactivated Fetal Bovine Serum, and 40 μg/mL Gentamicin Sulfate).

The clonal rat pheochromocytoma cell line pc12 was cultured in DMEM-high glucose supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. For all experiments, PC12 cells were plated on poly-D-lysine-coated plastic tissue culture dishes for 24 h before treatments.

1.3 Transient Transfection

PC12 cells were plated onto a 6-well plate ($1-2\times10^6$ cells/well) and grown for 24 h before transfection. Four μg of BmNPV-ZJ *iap*1 or empty vector plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen Life Technologies, Inc.) according to manufacturer' s suggested protocol.

1.4 DNA preparation and PCR amplification

Viral DNA was isolated from BmNPV-ZJ infected BmN cells as described[7]. Primers were designed according to the consensus amino acid sequences between *Bombyx mori* nuclear polyhedrosis virus BmNPV-T3 and BmNPV-ZJ strain: 5′-ATGAACGAGGACACTCCTCCGTTTTAT-TTTATCA-3′ and 5′-TTACACCACAAATATTTTTATAAAA-TCGG-3′. Polymerase Chain Reaction (PCR) was performed for 25 cycles by using 94 ℃ for 50 s, 58 ℃ for 45 s and 72 ℃ for 60 s.

1.5 Plasmid construction

A DNA fragment encompassing the complete open reading frame (ORF) of AcMNPV *iap*1 was PCR amplified and subcloned into pGEM-T easy vector and then sequenced. The correct *iap*1 insert was then subcloned into pcDNA3.1(+) plasmid.

1.6 Nuclear extract preparation

pc12 cells were rinsed with ice-cold phosphate-buffered saline (PBS). The cells were scraped into 1× PBS and centrifuged. The cell pellets were resuspended in hypotonic lysis buffer (10 mmol/L Hepes pH 7.9, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.1 mmol/L EDTA/EGTA, 1 mmol/L DTT) with proteinase inhibitor cocktail (Sigma). The cell suspension was then centrifuged at 4 ℃ for 20 min at 3 000 r/min (Hermle, Z360K). The nuclei pellets were resuspended in hypertonic buffer (420 mmol/L NaCl, 20 mmol/L Hepes, pH 7.9, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, 25% glycerol) supplemented with proteinase inhibitor cocktail (Sigma). The resuspended nuclei were then extracted by rocking in a cold room for 1 hour, then centrifuging for 20 min at 10 000 r/min. The supernatant containing nuclear proteins was aliquoted, frozen immediately in liquid nitrogen and stored at −80 ℃. Protein concentration of the extract was determined using the Bio-Rad protein assay, according to manufacturer' s protocol using bovine serum album as the concentration standard.

1.7 Electrophoretic Mobility Shift Assay (EMSA)

EMSAs were performed with double-stranded synthetic NF-κ B oligonucleotides (5′-AGCGTGATCCAAGG-GGTATTTCCAG-3′ and 5′-CGGCTGGAAATACCCCTTG-GA TCA-3′) radioactively labeled by 3′fill-in with Klenow enzyme (Promega) using standard methods (30). Two to three μg of nuclear extract proteins were incubated with NF-κ B probe (0.05~0.3 ng) for 20 min at 25 ℃ with 2 μg of salmon sperm DNA in a buffer containing 125

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mmol/L HEPES pH 7.9, 5 mmol/L EDTA, 5 mmol/L DTT, 250 mmol/L NaCl and 50% glycerol. The reaction mixture was then immediately loaded on a 5% non-denaturing polyacrylamide gel (29∶1 acrylamide-bis-acrylamide) and electrophoresed in 0.5×TBE (50 mmol/L Tris-borate and 1 mmol/L EDTA) at 8～10 V/cm. The gels were fixed in 10% methanol, 10% glacial acetic acid, dried and exposed to X-ray films.

2 Result

2.1 PCR Cloning of BmNPV-ZJ *iap*1 and construction of expression vector

A pair of primers was designed to amplify the BmNPV-ZJ *iap*1 as described in Material and Method. Fig.1 showed that a 900 bp gene fragment was obtained by PCR from viral DNA extracted from BmNPV-ZJ infected BmN cells. The product was sub-cloned into pGEM-T easy plasmin (Promega). The fragment was sequenced. After digestion by *Eco*RⅠ, the inserted *iap*1 fragment was subcloned into the *Eco*RⅠ site of the expression vector pcDNA3.1(＋) (Fig.2). The orientation of the BmNPV-ZJ iap1 was confirmed by *Sal*Ⅰ digestion of the expression plasmid (Fig. 3).

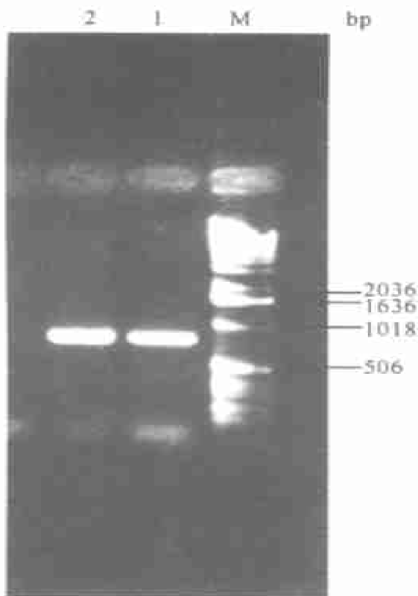

Fig.1 Amplified product of *iap*1 gene from BmNPV-ZJ strain

图 1　BmNPV 镇江株 *iap*1 基因 PCR 扩增结果

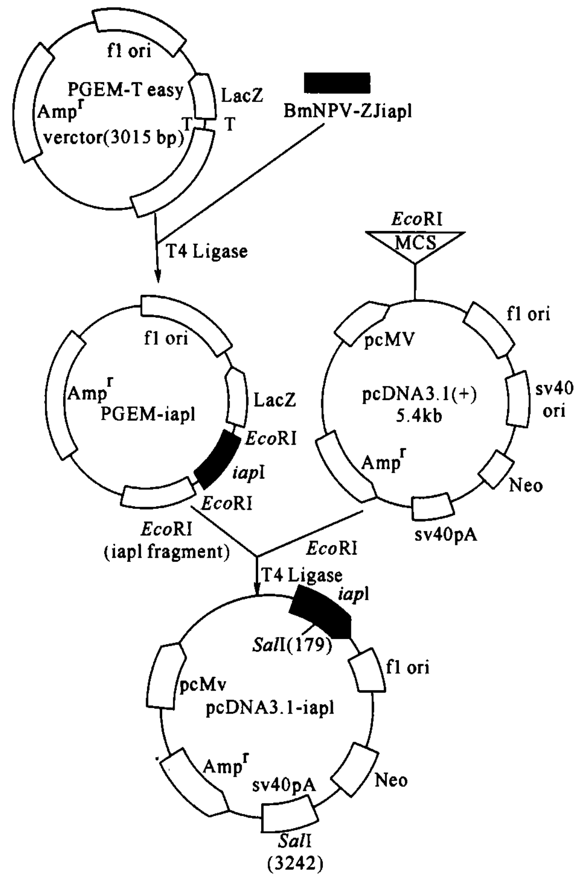

Fig. 2 Schematic diagram of construction of the expression plasmid pcDNA3.1-*iap*1

图 2　表达质粒 pcDNA3.1-*iap*1 构建概图

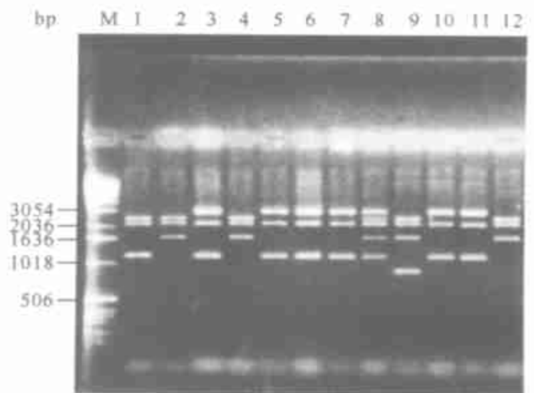

Fig. 3 Identification of recombinant expression plasmid pcDNA3.1-*iap*1 by restriction enzyme digestion

图 3　重组表达质粒 pcDNA3.1-*iap*1 的酶切鉴定

M. DNA marker　Lane 1—12. pcDNA3.1-*iap*1/*Sal*Ⅰ

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2.2 Sequence analysis of BmNPV-ZJ *iap*1 gene

BmNPV-ZJ *iap*1 gene was comprised of 858 bps (GenBank accession number AF481998) which contains an ORF encoding a protein of 285 amino acids. It exhibited high homology with that of the BmNPV-T3 iap1 gene (96% identity of nucleotide sequence as well as amino acid sequence). According to NCBI's DART (Domain Architecture Retrieval Tool) search result, the predicted BmNPV-ZJ *iap*1 protein contains two Cys/His motifs (baculovirus iap repeat, BIR) at its N-terminus, but lacks a C3HC4 or RING finger motif near its C-terminus found in a number of transcriptional regulatory proteins.

A region encoding seven continuous aspartic acids was lost in BmNPV-ZJ, compared to that of BmNPV-T3. The region was flanked by seven continuous aspartic acids at its 5' end and three aspartic acids at its 3' end. Although lost region was not located within any of the BIR domains of the iap, it is still interesting to see if it has any effect on the iap's activity.

The amino acid sequences of the BIR1 and BIR2 domains of *iap*1 of BmNPV-ZJ, BmNPV-T3 and AcMNPV have higher homology than those of OpNPV, CfNPV and BmIAP, as shown in Fig.4. It showed that the BmNPV-ZJ is closer to AcNPV in evolution than with OpNPV. That may be one of reasons why the OpNPV and BmIAP exhibited different anti-apoptotic function compared to AcNPV *iap*[6,8].

Fig 4 Amino acid sequence alignment of BIR1 and BIR2 of BmNPV-ZJ and other baculoviral iap1 or cellular iap (BmIAP). Identical amino acids were shadowed

图 4 BmNPV-ZJ 与其它几种杆状病毒 *iap*1 基因以及宿主细胞 IAP(BmIAP)的 BIR1 和 BIR2 的氨基酸序列同源性比较

2.3 TNF-α induces NF-κB activation in pc12 cells

Tumor Necrosis Factor-α (TNF-α) is a pleiotropic cytokine that may induce proliferation, apoptosis, or inflammatory reactions in target cells. These different cellular reactions toward TNF-α result from the activation of distinct cellular signaling pathways that interact with each other in a complex signaling network and allow a wide range of cellular responses[9]. Although TNF-α is a known activator of the apoptotic signaling cascade, TNF-α commonly does not induce apoptosis in target cells due to the parallel activation of protective signaling pathways that interfere with the onset of apoptosis. The nuclear factor-κ

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B (NF-κ B) pathway is strongly activated by TNF-α and prevents apoptosis. As shown in Fig. 5 (lane 4 and 6), the NF-κ B binding activity was strongly stimulated in TNF α-treated pc12 cells.

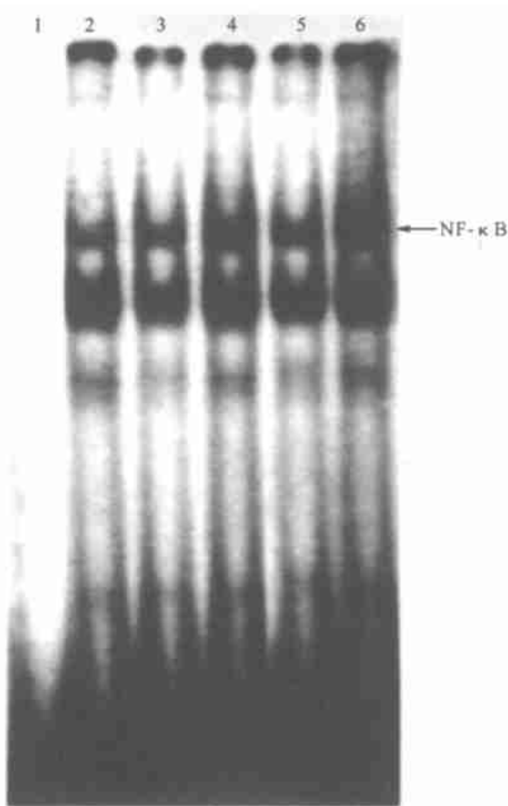

Fig. 5 NF-κ B gel mobility shift assay

图5 核因子κB的凝胶阻滞分析

Lane 1. NF-κB free probe Lane 2. Empty vector transfection control

Lane 3 and 5 BmNPV-*iap*1 transfection plus TNF-α treatment

Lane 4 and 6 Empty vectos pcDNA3 1 transfection plus TNF-α treatment

1. NF-κB 游离探针 2. 空载体 pcDNA3.1 转染对照

3, 5. BmNPV-iap1 转染+TNF-α 处理

4, 6. 空载体 pcDNA3.1 转染+TNF-α 处理

2.4 BmNPV-ZJ *iap*1 can reverse the effect of TNF-α on NF-κ B activity

The nuclear transcription factor NF-κ B was reported to have an anti-apoptotic function in mammalian cells. To investigate if the BmNPV-ZJ *iap*1 has any function on NF-κ B binding activity in TNF-α-treated pc12 cells, BmNPV-ZJ *iap*1 or empty control vector pcDNA3.1 was transfected into pc12 cells using Lipofectamine™ 2000 as described by the manufacturer. After 24 hrs' incubation, the cells were then treated with TNF-α (10 ng/mL) for 6 hrs. The nuclear extracts were harvested and incubated with NF-κ B probe as described in Materials and Methods. As shown in Fig. 5, the NF-κ B binding activity induced by the treatment of TNF-α (lane 3 and 5) was reduced in pc12 cells transfected with BmNPV-ZJ *iap*1, compared to those transfected with empty vector control (lane 4 and 6) (pcDNA3.1).

3 Discussion

The *iap*1 gene of BmNPV-ZJ was cloned and the sequence analysis result has shown that the BIR domains of BmNPV-ZJ *iap*1 had higher homology to BmNPV-T3 or AcMNPV than OpNPV *iap*1 or BmIAP. OpNPV and BmIAP exhibited different anti-apoptotic function compared to AcNPV IAP[6,8]. Whether IAP of BmNPV has any function in their insect host cell or mammalian cell is still unclear. NF-κ B is an important nuclear regulator which responses to diverse environmental stimuli. The function of IAP1 of BmNPV-ZJ on regulating NF-κ B activity upon TNF-α stimulation was an important indication that BmNPV-ZJ IAP1 was also functional in mammalian cells.

To date, at least 10 different baculoviruses are known to contain one or more *iap* homologous genes, although so far only Cp-*iap* and Op-*iap* (from *Orgyia pseudotsugata* NPV) have been shown to have anti-apoptotic function. Both of Cp-iap and Op-iap contain BIR and RING domains. It was believed that both the BIR and RING domains are necessary for anti-apoptotic function in baculoviruses. Recently, Rebecca RH et al[10] reported a Op-IAP$^{1-216}$, a loss-of-function truncation, which contains two BIRs but lacks the C-terminal RING motif potently interfered with OpIAP's capability to block apoptosis induced by diverse stimuli. In this study, our DART search result showed that BmNPV-ZJ *iap*1 contained two BIR domains but lacked the RING domain. It would be very interesting to see if BmNPV-ZJ IAP1 plays a similar role as Op-IAP$^{1-216}$.

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BmNPV-ZJ *iap* 1 基因的克隆、序列分析及其对哺乳动物细胞 NF-κ B 的调节作用研究

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摘 要 利用 PCR 法克隆得到家蚕核型多角体病毒镇江株(BmNPV-ZJ)的 *iap* 1 基因。序列同源分析表明: BmNPV-ZJ 的 *iap* 1 全长为 858 bp, 与 BmNPV-T3 株的碱基同源性为 96%, 与 BmNPV-T3 株相比少了一段编码 7 个氨基酸的区域, 该缺失区域的两侧有着独特的结构: 缺失区的 5′ 侧为连续编码 7 个天冬氨酸的序列; 缺失区的 3′ 侧为连续编码 3 个天冬氨酸的序列。NCBI 的 DART(Domain Architecture Retrieval Tool) 功能域搜索表明: BmNPV-ZJ 的 *iap*1 含有 2 个杆状病毒 BIR 功能域, 但不含 RING 区域。BmNPV 的 *iap* 是否具有抗凋亡作用迄今尚未见报道。利用以 NF-κ B 为探针的凝胶阻滞分析表明, BmNPV-ZJ 的 *iap*1 转染鼠的 pc12 细胞, 可逆转肿瘤坏死因子 TNFα 处理 pc12 细胞引起的核因子-κ B(NF-κ B) 的激活。BmNPV-ZJ 的 *iap*1 对 NF-κ B 的作用途径及作用方式, 以及其对哺乳动物细胞的细胞凋亡相关作用的研究正在进行之中。

关键词 凋亡 *iap*1 核型多角体病毒 家蚕 核因子κ B 凝胶阻滞分析测序 pc12 细胞

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