Acta virologica **65**: 264 – 272, 2021 doi: 10.4149/av_2021_303

Acetylation of nucleopolyhedrovirus P35 is crucial for its anti-apoptotic activity in silkworm, *Bombyx mori*

Enoch Obeng[1,2], Jihai Lei[1,2], Jonas Ngowo[1,2], Fuxiang Mao[1,2], Huihui Yan[1,2], Yajie Zhu[1,2], Wei Yu[1,2*]

[1]College of Life Sciences & Medicine, Zhejiang Sci-Tech University, Zhejiang Province, Hangzhou 310018, P. R. China;
[2]Zhejiang Provincial Key Laboratory of Silkworm Bioreactor and Biomedicine, Zhejiang Province, Hangzhou 310018, P. R. China

Received July 20, 2020; revised January 18, 2021; accepted June 17, 2021

Summary. – Apoptosis is a characteristic feature of a nucleopolyhedrovirus infected insect cells. This defensive strategy of the insect cells also affects the viral infectivity. On the contrary, the P35 baculovirus apoptosis inhibitor impedes the insect cell apoptosis induced by viral infection. Our previous investigation of the *Bombyx mori* nucleopolyhedrovirus (BmNPV) acetylome showed that 3 lysine (K) (70, 127 and 256) sites of P35 were acetylated during infection. How these modifications affect the interaction between the insect cells and BmNPV is still unknown. In order to explore the underlying mechanism of P35 lysine acetylation, mutants with glutamine or arginine substitution were constructed to mimic the acetylated (Q) and deacetylated (R) state. ELISA and DNA fragmentation assay were used to ascertain the acetylation effects on apoptosis. Subsequently the results showed that acetylation of K70 upregulated the anti-apoptotic activity, thereby preventing apoptosis induced by insect cells. Caspase 1 activity assay further confirmed that, acetylated K70 exhibited a strong anti-apoptotic activity in cell lines infected with BmNPV. Intriguingly, an examination with the yeast 2 hybrid (Y2H) assay revealed an interaction with the silkworm caspase 1. Taken together, we demonstrated that acetylation of P35 is crucial for an interaction with caspase 1 and the upregulation of anti-apoptotic activity.

Keywords: Bombyx mori; BmNPV; P35; acetylation; anti-apoptotic; caspase 1

Introduction

Viral infections of insects are in most cases caused by the baculoviruses, which specifically target insects as their hosts. The interaction between the baculovirus and its host, provides an essential model for infectivity and pathogenicity (Kukan, 1999; Fuxa *et al*., 2002). Apoptosis is the most important mechanisms to combat any changes within the host, affecting the viral infectivity in terms of replication and dissemination (Ikuno *et al*., 2004; Khurad *et al*., 2004; Gavrieli *et al*., 1992). Apoptosis is a form of programmed cell death which is very crucial for cell development and homeostasis. The process of apoptosis is achieved through a cysteine proteases known as caspases (cysteine dependent aspartate-specific protease), which are responsible for the cleavage of aspartic acid residue. Among these caspases are the initiators and effectors, which act upstream and downstream, respectively (Koonin and Aravind, 2002; Shaham, 1998). Baculovirus P35 acts antagonistically toward the processes of apoptosis, acting as a downstream anti-apoptotic agent (Bertin *et al*., 1996). The P35 prevents the death of host cells and enables the virus multiplication, undertaking the inhibition process through the formation of a stoichiometric complex with caspase, following cleavage at its aspartate 87 (Asp^{87}) (Zoog *et al*., 1999). Also, the P35 has been reported to inhibit group 1, 2 and 3 of caspases *in vitro* (Bertin *et al*., 1996). The P35 as an apoptosis suppressor possesses about

*Corresponding author. E-mail: mikkyu@163.com; phone: +86-571-86843338.

Abbreviations: AcMNPV = *Autographa californica* multicapsid nucleopolyhedrovirus; BmNPV = *Bombyx mori* nucleopolyhedrovirus; DDO = double drop out; QDO/X = quadruple drop out; RSL = reactive site loop; Y2H = yeast two hybrid

299 amino acids and consists of a main core and regions responsible for its cleavage, an eight stranded β-sheet and a reactive site loop (RSL) (Zoog *et al*., 1999). The RSL serves as an extension above the β-sheet and is attached to the β-sheet through a single amphipathic α-helix (α1), possessing a caspase recognition and cleavage site (Asp^{87}) at its end (Zoog *et al*., 1999). In addition, studies show that the ectopic expression of P35, prevents programmed cell death in phylogenetically diverse organisms (Davidson and Steller, 1998; Martinou *et al*., 1995; Beidler *et al*., 1995).

Studies have demonstrated that, the substitution of lysine to leucine (L) 67 or valine (V) 71 provides a better P35 substrate for cleavage but not anti-apoptotic activity making the α1/ β-sheet association crucial (Zoog *et al*., 1999). Based on our previous studies reporting a number of acetylated sites located within some viral proteins, it was found that acetylation of BmNPV lysine (K) 70 in P35 caused a change in its function following infection (Hu *et al*., 2018). Acetylation may cause neutralization of a lysine residue through the reduction of the positive charge. Lysine possesses a charge of ϵ-amino group as result of the protonation at its physiological pH (Scaglia *et al*., 2002). This process inactivates lysine involvement in catalytic reactions (Scaglia *et al*., 2002). However, it's still capable of coordinating a number of enzyme processes (Wellen *et al*., 2009). Based on our previous work, in this study we report the potency and outcome of acetylation of BmNPV P35. Moreover, our observation provides an insight into conditions that regulate the anti-apoptotic activity and also improve the P35 structural domain.

Materials and Methods

Cells. BmN cell line, derived from *Bombyx mori* ovary, was isolated in our laboratory. BmN cell line was propagated at 27°C in an SF-900 medium (Thermo Fisher Scientific, USA) containing 10% fetal bovine serum (Corning, America).

Mutagenesis. The wildtype *p35* gene (*p35*-wt) was obtained from DH10Bac *E. coli*/BmNPV which was isolated in our laboratory using primers *Bam*HI-P35-FOR and *Hin*dIII-P35-RE. All site directed mutagenesis of *p35* K (70,127 and 256) was accomplished using 70-Q-FOR/RE, 70-R-FOR/RE, 127-Q-FOR/RE, 127-R-FOR/RE, 256-Q-FOR/RE and 256-R-FOR/RE primers which were designed according to the sequence of BmNPV from NCBI (Acc. No. NP_047533, Shanghai Sangon Company, China). The construction of the parallel PCR fragments was done using the forward primer of the *p35*-wt and the reverse primer of each mutant to generate the 1st parallel PCR fragments in a first PCR. The forward primer of the mutants and the reverse primer of the *p35*-wt was used to generate the 2nd parallel PCR fragments in a second PCR. The 1st and 2nd parallel PCR fragments were then overlapped in a fusion PCR (third PCR) using both forward and reverse primers of *p35*-wt. The overlapped fragments generated with the third PCR were the acetylation-mimicking P35 K70Q, K127Q and K256Q mutations and deacetylation-mimicking P35

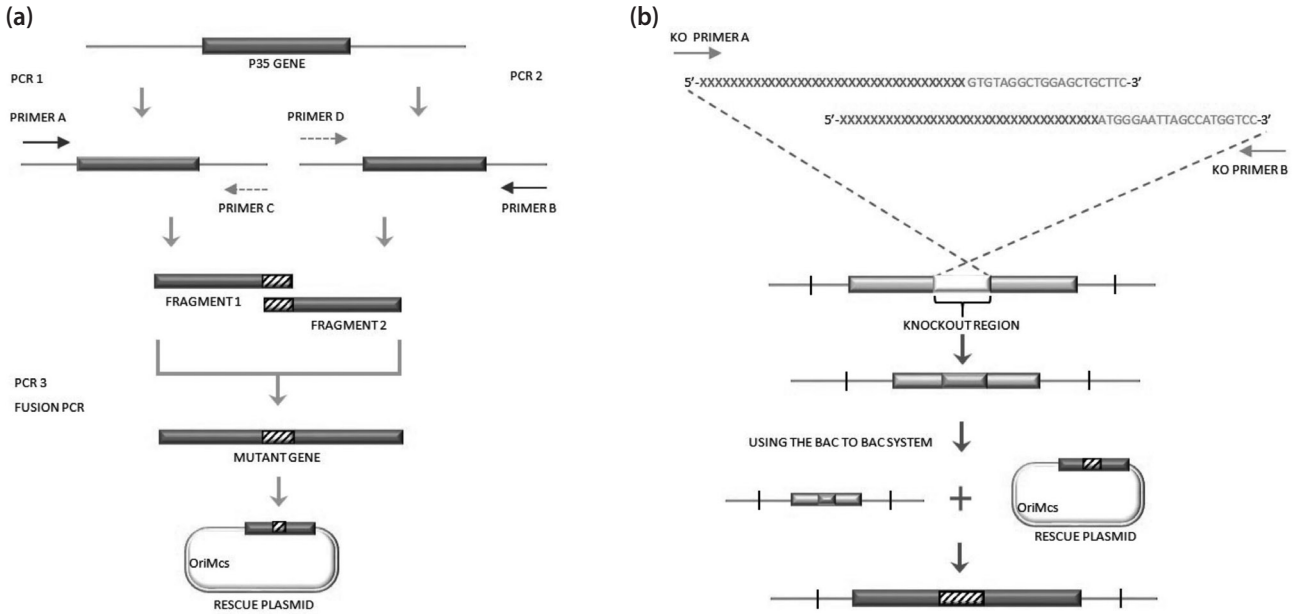

Fig. 1

Confirmation of *p35*-ko/re/-bacmid and other repaired variants
Diagrammatic representation of the gene knockout mechanism **(a)** and the process of repairing **(b).**

Table 1. Primers used in this study

Primer name	Primer sequence
*Bam*HI-P35-FOR	5'-CGCGGATCCTCAATTTGTTGATAATAAATTTG-3'
*Hin*dIII-P35-RE	5'-CCCAAGCTTTTAGTGATGATGATGATGATGT TTAATCATGTCTAATATTA-3'
256-Q-FOR	5'-CGAGCTGGGTCCAATCCAACAAG-3'
256-Q-RE	5'-CTTGTTGGATTGGACCCAGCTCG-3'
256-R-FOR	5'-CGAGCTGGGTCAGATCCAACAAG-3'
256-R-RE	5'-CTTGTTGGATCTGACCCAGCTCG-3'
127-Q-FOR	5'-GATTATACCGATCAAAAGTCTAT-3'
127-Q-RE	5'-ATAGACTTTTGATCGGTATAATC-3'
127-R-FOR	5-GATTATACCGATAGAAAGTCTAT-3'
127-R-RE	5'-ATAGACTTTCTATCGGTATAATC-3'
70-Q-FOR	5'-AAATCACAAGTCGATGAACAATT-3'
70-Q-RE	5'-AATTGTTCATCGACTTGTGATTT-3'
70-R-FOR	5'-AAATCAAGAGTCGATGAACAATT-3'
70-R-RE	5'-AATTGTTCATCGACTCTTGATTT-3'
cat-F	5'-CAGCACCTTGTCGCCTTGCGTAT-3'
cat-R	5'-ATTCAGGTTCATCATGCCGTTTGTG-3'
P35-ko-F	5'-ATGCGTGTTGAAGCCGGGATTTGAGAACGGCAGCAACCA AGTGCTATCTTGTGTAGGCTGGAGCTGCTTC-3'
P35-ko-R	5'-TGTCGTTAATTTCGTGAGCAAACGGCACAATAACTTTGTT ACCAATCGGGATGGGAATTAGCCATGGTCC-3'
EcoRI-P35-FOR	5'-CCGGAATTCATGTGTGTAATTTTTCCGGTAGA-3'
*Bam*HI-P35-RE	5'-CGCGGATCCTTTAATCATGTCTAATATTACATT-3'
EcoRI-CAS 1-FOR	5'-CCGGAATTCATGGCTGATGAAGAAAAGAAAACC-3'
*Bam*HI-CAS 1-RE	5'-CGCGGATCCTTTTTTTCCAAACAAGAGAAGGCG-3'

All site directed mutagenesis were verified by PCR, double digestion and DNA sequencing (Sangon sequencing Company, Shanghai). The bold underlined label represents the base pairs for the site directed mutation and the underlined label represents the upstream and downstream arms for the knocked-out site of the gene.

K70R, K127R and K256R mutations which were further inserted into the pFastBac-Htb plasmid (Fig. 1a). The *p35*-knockout (*p35*-ko) was constructed using P35-ko-F, P35-ko-R primers (Table 1).

Transformation. Competent DH10Bac cells (strain comprising of the wildtype bacmid, helper plasmid pMON7124 and antibiotic resistance gene) containing PKD46 were transformed with purified *p35*-PKD3-cat PCR product. The cells were plated on LB plate containing kanamycin, tetracycline and chloramphenicol antibiotics. Single clones were picked and confirmation was done by PCR.

P35-repaired rescue. The construct *p35*-repaired (*p35*-re) was obtained by rescuing of the knockout through transformation of the DH10Bac *p35*-ko competent cells with the pFastBac-Htb-*p35*-wt. These were plated on LB medium plates containing IPTG, kanamycin, tetracycline, gentamycin and chloramphenicol. Single white colonies were picked and verification was accomplished by PCR.

Mutant transformation. All the mutants (K70Q, K70R, K127Q, K127R, K256Q and K256R) were obtained by the same process as DH10Bac *p35*-ko, by transformation with pFastBac-Htb-P35 K70Q, K127Q, K127R, K256Q and K256R and confirmed by PCR using M13-F (-40) and M13-R primers (Fig. 1b).

Transfection. Superfectin^{TM}II Invitro DNA transfection reagent (Shanghai Pufei Biotech, China) was used according to the manufacturer's instructions with ratio of mass /reagent (volume) = 1:3. BmN cells with concentration of 1×10^6 per well were seeded and left to grow overnight. Two hundred microliters of a mixture of 50 mM NaCl, 2 μg of bacmid and 6 μl of transfection Superfectin reagent were added into each well. The transfected cells were cultivated for 96 h.

DNA fragmentation. Cell death detection ELISA ^{PLUS} (Roche, Switzerland) was used according to the manufacturer's instruction. BmN cell lines were transfected with *p35*- wt, *p35*-ko, *p35*-re, K70Q, K70R, K127Q, K127R, K256Q and K256R bacmids and

harvested after 96 h when cytopathic effect could be observed. Cells were washed with PBS, harvested into a 1.5 ml Eppendorf tubes and centrifuged at 200 rcf for 5 min. Supernatants were discarded and 200 µl of lysis buffer were added to each Eppendorf tube. Cells were gently resuspended and allowed to be lysed at room temperature for 30 min. Again, the samples were centrifuged at 200 rcf for 10 min and 20 µl of lysate supernatants were carefully transferred into the streptavidin-coated micro-plates for subsequent analysis. The normal BmN cells (Nm) were used as a positive control. A mixture of 288 µl of incubation buffer, 16 µl of anti-histone-biotin and 16 µl of detection DNA antibody anti-DNA-POD was prepared and 80 µl added to each well of the streptavidin-coated microplate. Microplates were then sealed with foil and incubated on a rotary shaker at 300 rpm at 25°C for 2h. Supernatants were discarded and pellets were carefully washed 3 times with 250 µl of incubation buffer. One hundred µl of ABTS solution was then added to each well and further incubated at room temperature (25°C), on a rotary shaker at 300 rpm for 30 min. The reaction was stopped by adding 100 µl of ABTS stop solution. The absorbance of each well was measured at 405 and 490 nm with a microplate reader (Molecular Devices SpectraMax ® Absorbance Reader CMax Plus, UK).

Yeast two-hybrid assay (Y2H). Using primers EcoRI-P35-FOR and *Bam*HI-P35-RE for *p35*-wt and its mutants (K70Q, K70R, K127Q, K127R, K256Q and K256R) and primers for silkworm caspase 1 (EcoRI-CAS 1-FOR, *Bam*HI-CAS 1-RE) the sequences were generated and inserted into the various vectors of the yeast two hybrid system, pGBKT7 DNA-BD and pGADT7-AD. The Y2H Gold chemically competent cells (2nd Lab™, Shanghai) were separately transformed with 2 µg of pGBKT7-*p35*-wt, and pGADT7-caspase 1 plasmids, then plated on selective plate lacking tryptophan and leucine amino acid, respectively. This was first used in the determination of the autotoxicity and autoactivation of both P35 and caspase 1. Each of the pGBKT7-(*p35*-wt, K70Q, K70R, K127Q, K127R, K256Q and K256R) constructs were then co-transformed with the pGADT7-caspase 1 using a double drop out plates (DDO) lacking leucine and tryptophan. Clones from all the plates were further picked and re-cultured on a single quadruple drop out (QDO/X) plate, lacking adenine, histidine, leucine and tryptophan with the presence of X-α-Gal, using a dilution factor of 1/100, 1/1000, 1/10000. All samples were plated on a single plate for easy monitoring. Plates were then incubated under 30°C and growth was monitored.

Anti-apoptotic activity. Using the caspase 3 activity detection kit (Beyotime, Shanghai China), caspase 1 activity was evaluated. Cells were washed three times using PBS, followed by an incubation with 200 µl of lysis buffer for 15 min in an ice bath. Centrifugation was carried out at 12,000 rcf at 4°C for 5 min. Supernatants were then transferred into an Eppendorf tube and kept on ice. Fifty µl of each sample were added to the reaction mixture of 40 µl of detection buffer and 10 µl of 2 mM Ac-DEVD-pNA. Mixtures were loaded into a micro plate and incubated at 37°C for 120 h. The absorbance was measured at a wavelength of 405 nm.

Statistical analysis. Statistical analysis was performed using the GraphPad Prism 8 software. One-way analysis of variance (ANOVA) and paired *t*-test were used to compare the various acetylated groups together with the wild type and control groups. *p*-values of the various groups were determined.

Results

Confirmation of p35-knockout and p35-repaired bacmid

To confirm the successful preparation of *p35*-ko and the *p35*-re, λ Red recombinase system for the knockout processes and the Bac-to-Bac system for rescuing the knockout were employed, respectively. λ Red Recombinase systems involve a cassette consisting of an origin of replication, DNA segment with *lacZ* having a site of attachment for transposition of the bacteria Tn7 and antibiotic resistance marker gene. *P35*-ko was determined using CAT primers (cat-F + cat-R) and *p35*-wt primers *Bam*HI-P35-FOR and *Hin*dIII-P35-RE in a PCR, resulting in a band size of 1033 bp and 2223 bp, indicating an internal overlap with the resistance marker PKD3 and the external overlap of the target gene, proving the replacement of the expected ORF by a CAT cassette. *P35*-re, K70Q, K70R, K127Q, K127R, K256Q and K256R were equally confirmed by using the M13 forward and reverse primers together with the primers for the *p35*-wt (M13-F(-40) + M13-R, M13-F(-40) + *Hin*dIII-P35-RE and *Bam*HI-P35-FOR + M13-R). PCR results confirmed band sizes of 3523 bp, 2986 bp and 1832 bp which indicated a successful insertion of rescue cassette with transposition occurring due to *lacZ* complementation, possessing an M13(-40) and M13-R sites flanking the mini-attTn7.

Baculovirus P35 plays crucial role in inhibiting cell apoptosis

The baculoviral P35 is a well-known anti-apoptotic protein. Many reports have shown that *Spodoptera frugiperda* infected with p35 deficient *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) resulted in apoptosis but allowed a normal replication in the *Trichoplusia ni* (Kelly *et al*., 2006). This difference in replication among the hosts might be a result of the host specificity of the virus and also variation in the host strains. However, other studies have also reported that, infection of BmN cells with BmNPV *p35* resulted in some level of apoptosis and that BmNPV defective in *p35 gene* did not in any way enable the production of baculoviruses in BmN cells (Kelly *et al*., 2006; Nakanishi *et al*., 2010). In

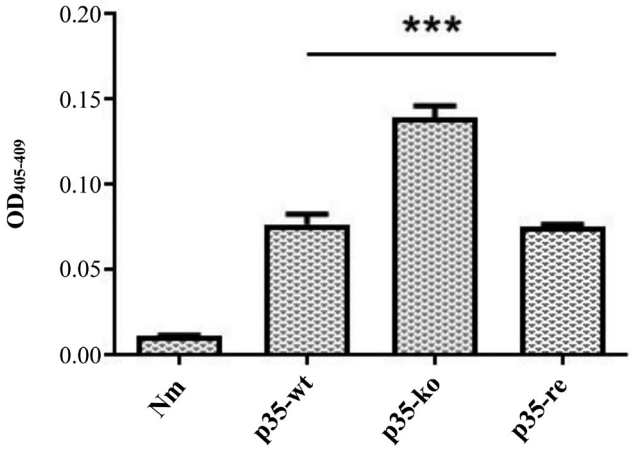

Fig. 2

Baculovirus P35 plays crucial roles in inhibiting cell apoptosis
BmN cells were transfected with *p35*-wt, *p35*-ko and *p35*-re. Following the transfection, DNA fragmentation assay was carried out. Experiment was repeated three times, statistical comparison was done with one-way ANOVA with the error bars indicating the mean ± standard deviation, ***p-value <0.01 was considered as significant difference.

considering this, BmN cell lines were transfected with *p35*-ko and *p35*-re, to observe their effects. According to the DNA fragmentation assay, following 96 h post transfection, results from the analysis showed a high level of DNA fragmentation in the *p35*-ko (Fig. 2). Also, the level of DNA fragmentation in the *p35*-re was significantly lower and almost equivalent to the *p35*-wt indicating a successful rescue process. The results suggested that the *p35* gene is crucial for apoptosis inhibition.

Lysine acetylation is crucial for P35 ability to suppress apoptosis

Modification of some lysine sites within the RSL contributed to a weak interaction between P35 and caspase. As it was reported by Zoog *et al.* (1999), which implicated that the Asp could reduce some nearby positive charges, to further examine whether the acetylation of these various sites would entirely affect the P35 function, we transfected BmN cells with the various mutants, harvested cells after 96 h, and analyzed with ELISA. Results from the DNA fragmentation analysis, indicated that K70Q mutation significantly decreased the level of DNA fragmentation compared with the K70R (Fig. 3a). K127Q and K127R mutants demonstrated significantly lower levels of anti-apoptotic activity, causing an increased level of DNA fragmentation suggesting, that mutation of K127 affected the α3 (Fig. 3b). Also, K256R showed a significant decrease in the level of DNA fragmentation (Fig. 3c). The

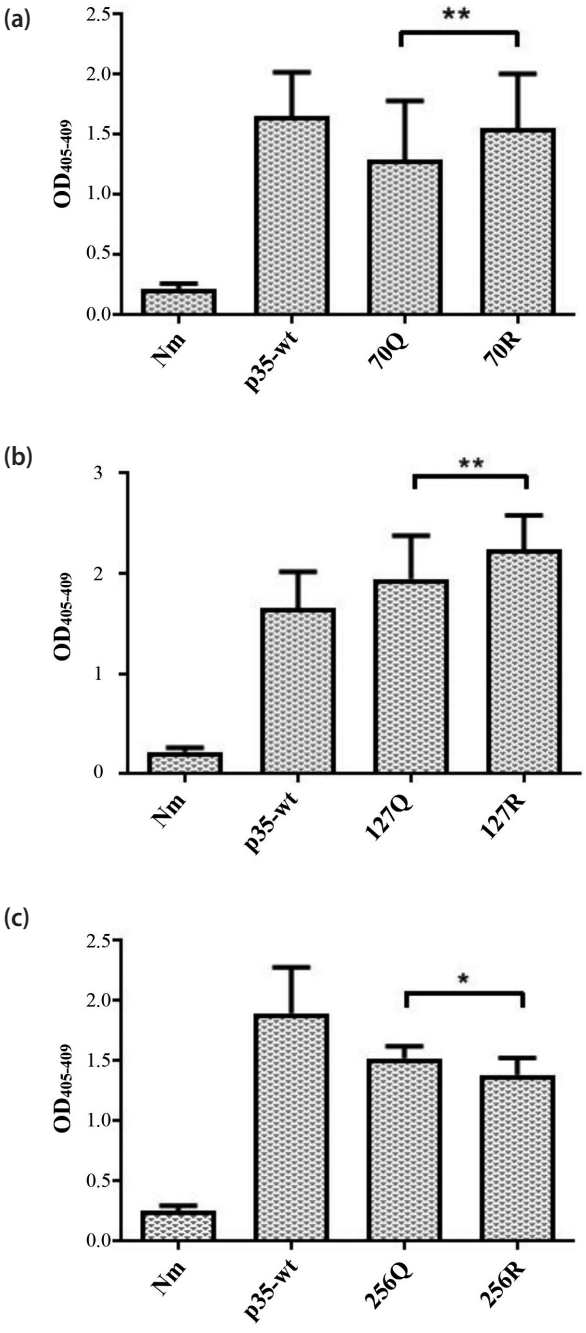

Fig. 3

Lysine acetylation is crucial for P35 ability to suppress apoptosis
(a) The anti-apoptotic activity exhibited by the acetylated sites following 96 h after transfection. DNA fragmentation assay was carried out for the various acetylated sites with K70 mutant. Acetylated P35 moderately inhibited apoptosis. **(b)** DNA fragmentation assay showing modification of α3-helix(α3) depicts the importance of the α3 domain in anti-caspase activity. **(c)** DNA fragmentation assay showing positive charge compensation outside the α1/β-sheet region led to an increase in apoptotic activity. The experiment was carried out four times, statistical comparison was done with the paired t- test with error bar representing the mean ± standard deviation, **p-value <0.05 was considered as significant difference.

(a)

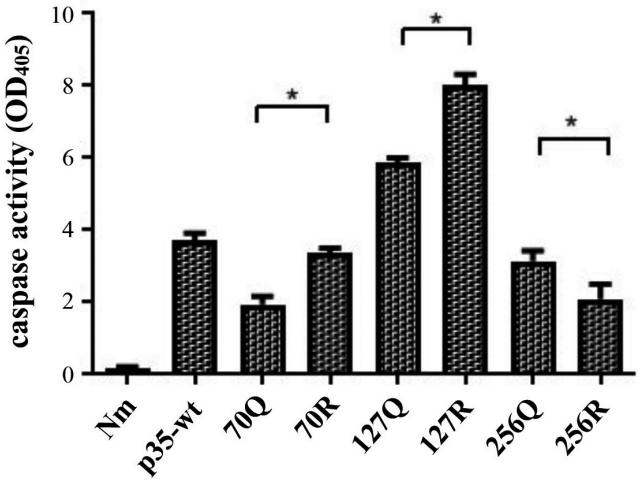

Fig. 4

Acetylated P35 modulates cellular apoptosis associated with the function of caspase 1

(a) BmN cells were transfected with *p35*-wt together with the mutants (K70Q, K70R, K127Q, K127R, K256Q and K256R), and 96 h after transfection, caspase activity assay was carried out for further analysis. The experiment was carried out four times, statistical comparison was done with paired *t*-test with error bars representing the mean ± standard deviation, **p*-value <0.01 was considered as significant difference. **(b)** Y2H assay showing the positive and negative control together with the test of auto-activation of both *p35*-wt and caspase 1. 1-Positive control; 2-Negative control; 3-*p35*-wt with empty pGADT7; 4-caspase 1 with empty pGBKT7 **(c)** Y2H assay showing the interaction between *p35*-wt, various mutants and caspase 1. 1-Represents *p35*-wt, 2-K70Q; 3-K70R; 4-K127Q; 5-K127R; 6-K256Q and 7-K256R. The blue colonies denote interaction with caspase 1 and those without colonies denote loss of interaction. All samples were plated with the same dilution factor 1/100, 1/1000, 1/10000.

(b)

SAMPLE	PLASMID	INTERACTION
1	pGBKT7- 53/pGADT7-T (Positive Control)	YES
2	pGBK7-Lam/pGADT7-T (Negative Control)	NO
3	pGBKT7-*p35*-wt/pGADT7	NO
4	pGADT7-caspase-1/pGBKT7	NO

(c)

SAMPLE	PLASMID	INTERACTION
1	pGBKT7-*p35*-wt/pGADT7-caspase1	YES
2	pGBKT7-70Q/pGADT7-caspase1	YES
3	pGBKT7-70R/pGADT7-caspase1	NO
4	pGBKT7-127Q/pGADT7-caspase1	NO
5	pGBKT7-127R/pGADT7-caspase1	NO
6	pGBKT7-256Q/pGADT7-caspase1	NO
7	pGBKT7-256R/pGADT7-caspase1	YES

results from K256R mutation suggested that, retaining of the positive charge promoted an increase in anti-apoptotic activity. In all, lysine acetylation enhanced the ability of the P35 to suppress apoptosis.

Acetylated P35 modulates cellular apoptosis associated with the function of caspase 1

To further understand the inhibition role of acetylated P35 upon its interaction with caspase 1, caspase activity assay was performed. Since the proper regulation of caspase activity is very crucial for its apoptotic activity, once again BmN cells were transfected with all the P35 mutants, harvested after 96 h, and followed by a caspase activity assay which showed significantly lower levels of caspase activation comparing K70Q and K256R mutants (Fig. 4a). The P35 apoptosis efficiency is known to be linked with the stoichiometric inhibition of the cellular caspase to form a complex after cleavage (Zoog *et al.*,1999). *p35*-wt together with mutants (K70Q, K70R, K127Q, K127R, K256Q and K256R) and caspase 1 were inserted into pGBKT7 and pGADT7-Y2H plasmids respectively. A test for both the *p35*-wt and caspase 1 demonstrated that there was no autoactivation (Fig. 4b). P35 interaction with the silkworm caspase 1 was confirmed on DDO/X plates and reconfirmed on QDO/X plates. The blue colonies indicated the interaction of K70Q and K256R with caspase 1 (Fig. 4c). Taken together, the results demonstrated that for strong anti-apoptotic activity acetylation at K70 would be required while at K256 site within the β-strand loops, positive charge ensures an increase in the anti-apoptotic activity.

Discussion

The ability of the P35 to form a stoichiometric complex with its target caspase is very crucial for the effective functioning of the RSL loop, in contributing to abrogation of the cell death execution (Fisher *et al.*, 1999). It is evident that, the substitution of the Asp^{87} affected the cleavage of P35 and subsequently, the loss of its anti-apoptotic activity (Bertin *et al.*, 1996). However, the mutation of some sites within the RSL (V71) apart from the Asp^{87} also had the ability to impede the caspase inhibitory activity but did not affect the cleavage process of P35, suggesting their active role in the pre and post cleavage process of caspase inhibition and the combine effect from the interaction of the RSL and the β-sheet to accomplish an anti-apoptotic effect (Zoog *et al.*, 1999). Hence, P35 cleavage can occur without a proper interaction but the inhibitory effect requires the interaction between the RSL and β-sheet, making the α1/β sheet association very crucial for cleavage and stabilization of the RSL conformation. Acetylation may reduce a lysine residue by abolishing its positive charge resulting in inactivation of lysine in its involvement in a catalytic reaction (Scaglia *et al.*, 2002; Thao *et al.*, 2010). Besides, it is known to antagonize allosteric activation, causing conformational changes, regulating the accessibility of substrate and regulation of the subcellular localization of enzymes (Spange *et al.*, 2009). Normally, the acetylation of lysine impactfully affects the conformation, size or function and may improve or affect the normal functions of the protein (Terret *et al.*, 2009).

In this current study, acetylation of some lysine residues within the RSL and the β-sheet proved a new finding of P35 involvement in the inhibition of apoptosis, since BmN cells infected with p35 deficient BmNPV did not show any anti-apoptotic activity. In terms of the apoptosis inhibition, the BmNPV P35 operates effectively. Also, K70 which is a residue in the α1 showed an increase in its activity in the K70Q mutant, in terms of its interaction with caspase 1 and its anti-apoptotic activity. However, in the K70R (mutant mimicking deacetylation) only low level of anti-apoptotic activity was evident. These data support the idea that abolishing positive charge in the α1 contributes to its orientation with the β-sheet for an effective interaction and cleavage by caspase. Zoog *et al.* (1999) reported that a charge compensation in the β-sheet was required for strong interaction, it can therefore be said that reduction of the positive charge residue within the α1 could as well enhance its interaction with the β-sheet giving a proper orientation for cleavage by caspase. In addition, other mutated sites such as the K127Q and K127R resulted in the upregulation of the caspase activity but with some significant differences. These data demonstrated that the α3 structure is important for the P35 anti-apoptotic activity. Future studies could result in more other functions. K256Q mutant showed that, the normal positive charge is required by the loops of the β-strand outside the α1/β-sheet region of association, while K256R which mimics the normal state of the lysine proved to keep up with its activity in-terms of caspase interaction and anti-apoptotic activity. Hence, positivity of the β-strand loops is still required for the effective function of the P35. Also, caspase 1 activity assay revealed the downregulation of silkworm caspase 1, among the P35 acetylated residues. Guy *et al.* (2008) reported that P35 has been used as a stoichiometric inhibitor in determination of caspase activity due to the effective inhibition of its targeted caspase. Therefore, it could be said that the silkworm caspase 1 acts as an active effector. Other studies concerning caspase 1 have reported that, the P35 complex with the effector caspase, *Spodoptera frugiperda* caspase 1 (*sf*-caspase 1) inhibited apoptosis (Ahmad *et al.*, 1997). Furthermore, Y2H assay corresponded with previous results

obtained in this work, revealing the interaction between caspase 1 and K70Q or K256R. These data confirmed that acetylation of K70 was required for the effective interaction with caspase 1 and that, K256 still required the positive charge for effective functioning, while acetylation of K127 affected interaction with caspase 1 in Y2H, showing the crucial presence of this lysine within the P35 domain. Taken together, we report that P35 plays a crucial role in apoptosis inhibition by the BmNPV. Lysine acetylation is very important for the suppression of apoptosis by the BmNPV P35 as well. Also, acetylated P35 modulates cellular apoptosis which is associated with the function of caspase 1. In conclusion, results from the DNA fragmentation, caspase activity assay and Y2H regarding the various P35 residues revealed that acetylation of the P35 is crucial for the interaction and inhibition of caspase 1.

Acknowledgment. This work was findect by grants from the National Natural Science Foundation of China (No. 31972623) and Science Foundation of Zheji and Province (No. LY17C170006).

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