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Putative Phosphorylation Sites On WCA Domain of HA2 Is Essential For *Helicoverpa armigera* Single Nucleopolyhedrovirus Replication*

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Abstract: Protein phosphorylation is one of the most common post-translational modification processes that play an essential role in regulating protein functionality. The *Helicoverpa armigera* single nucleopolyhedrovirus (HearNPV) *orf2*-encoded nucleocapsid protein HA2 participates in orchestration of virus-induced actin polymerization through its WCA domain, in which phosphorylation status are supposed to be critical in respect to actin polymerization. In the present study, two putative phosphorylation sites (^{232}Thr and ^{250}Ser) and a highly conserved Serine (^{245}Ser) on the WCA domain of HA2 were mutated, and their phenotypes were characterized by reintroducing the mutated HA2 into the HearNPV genome. Viral infectivity assays demonstrated that only the recombinant HearNPV bearing HA2 mutation at ^{245}Ser can produce infectious virions, both ^{232}Thr and ^{250}Ser mutations were lethal to the virus. However, actin polymerization assay demonstrated that all the three viruses bearing HA2 mutations were still capable of initiating actin polymerization in the host nucleus, which indicated the putative phosphorylation sites on HA2 may contribute to HearNPV replication through another unidentified pathway.

Key words: *Helicoverpa armigera* single nucleopolyhedrovirus (HearNPV); Actin polymerization; Protein phosphorylation; N-WASP

Baculoviruses are rod-shaped, enveloped viruses with circular double-stranded DNA genomes ranging in size from 80 kb to 180 kb. During their life cycle, baculoviruses produce two distinct phenotypes: the budded virus (BV), which is responsible for the spread of virus from cell to cell within and the occlusion-derived virus (ODV), which is responsible for the horizontal transmission of infection between insects [4].

The infection of baculovirus is accompanied with polymerization of granular actin (G-actin) to filamentous actin (F-actin) in the host nucleus, where viral nucleocapsids are assembled and F-actin is supposed

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to facilitate this process [3, 10]. The actin polymerization process is mediated by the interaction between the WCA domain of N-WASP and the actin-related protein 2/3 (Arp2/3) complex[6, 7]. Additionally, the phosphorylation of N-WASP at ^{256}Tyr by Abl kinases has been shown to be critical for the actin comet tail formation [1].

Previously, we had demonstrated that HearNPV HA2, a viral nucleocapsid protein bearing a WCA domain is essential for baculovirus-induced host nuclear actin polymerization [8, 12]. Also, knockout of *ha2* from the viral genome is lethal to HearNPV, as manifested by lack of nuclear F-actin formation and aberrant viral nucleocapsid formation in the nucleus of virus transfected cells [13].

Since the phosphorylation status of N-WASP is correlated with its capability in initiating actin polymerization, and HA2 counterpart P78/83 of *Ac*MNPV is a phosphorylated protein [9], the function of putative phosphorylation sites of HA2 was investigated with respect to viral replication and actin polymerization.

MATERIALS AND METHODS

Cell culture and virus

Insect cell line HzAM1 was maintained in Grace's medium (Invitrogen) with a supplement of 10% fetal bovine serum (FBS) (Invitrogen) at 27°C. All the HearNPV recombinant bacmid constructs were derived from HabacHZ8 and propagated in *Escherichia coli* strain DH10B [11].

Bioinformatical assay

HA2 sequence was submitted to either Prosite (http://www.expasy.org/tools/scanprosite/) to identify potential phosphorylation sites, or NCBI Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to make sequence alignments and for motif recognition.

Plasmid constructs

The bacmid with *ha2* knockout (HaΔha2) was previously described [13]. The site-directed mutagenesis of *ha2* was performed using a two-step polymerase chain reaction (PCR), following the protocols described previously [14]. Briefly, *ha*2 was cloned to pFbdg using primer set Ha2-upper/lower (Table 1) [14]. To introduce mutations to *ha2*, primer sets 232-upper/lower, 245-upper/lower, and 250-upper/lower (Table 1) were used to generate donor plasmids bearing T232A, S245G, and S250A, respectively. The resulted plasmids were transposed to HaΔha2 by using the Bac-to-Bac method [5, 11, 13], and generated vHa$^{\text{ha2-T232A}}$, vHa$^{\text{ha2-T245G}}$, and vHa$^{\text{ha2-S250A}}$ (Fig. 1A). The resulted bacmid constructs were confirmed by PCR with primer set M13+/M13-, according to the Bac-to-Bac protocol (Invitrogen).

Virus infectivity assay

These bacmid constructs were transfected to HzAM1 cells using lipofectin (Invitrogen). At 144 hours post transfection (hpt), the supernatants were collected and filtered through 0.45 μm-diameter syringe filters (Sartorius) to remove cell debris before

Table 1. Primers used in this research

Name	Sequence
Ha2 upper	CG*GAATTC* (*Eco*RI)ATGGTTCAACTGCAAAGTGTT
Ha2 lower	CC*TCTAGA* (*Xho*I)TTAAACTTGCGATTCAGTTGA
232 upper	GCCGCCAGTCCTGCTGCAAAGATTTTA
232 lower	TAAAATCTTTGCAGCAGGACTGGCGGC
245 upper	GCCGTTCAACCGGGTCCTGTAGTTTCG
245 lower	CGAAACTACAGGACCCGGTTGAACGGC
250 upper	CCTGTAGTTTCGGCAGAATCGGAAAAC
250 lower	GTTTTCCGATTCTGCCGAAACTACAGG

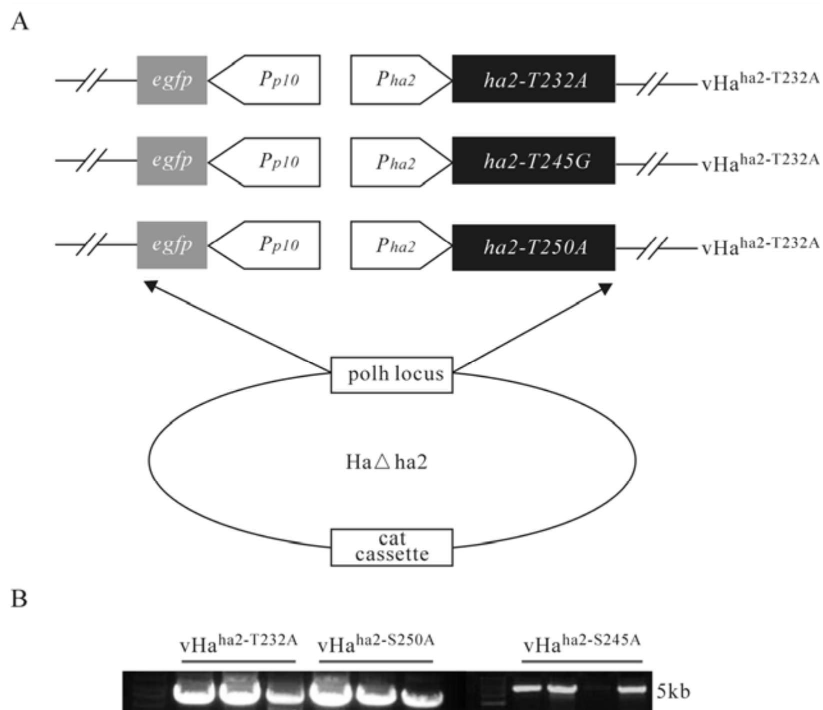

Fig. 1. Construction of the recombinant bacmids. A: Diagram of bacmid constructs. Coding sequences of EGFP and HA2 mutants were respectively cloned into donor plasmid pFastBac-dual. The resulted plasmids were transposed to HaΔha2 to generate recombinant bacmid constructs $vHa^{ha2\text{-}T232A}$, $vHa^{ha2\text{-}S245G}$, and $vHa^{ha2\text{-}S250A}$. B: Confirmation of the recombinant bacmids by PCR with M13± primers.

being added to fresh HzAM1 cells to initiate secondary infection. After 60 min of incubation, the supernatant was discarded and replenished with fresh Grace medium plus 10% FBS. At 144 hours post infection (hpi), the cell images were captured with an Olympus-IX51 microscope.

Nuclear actin polymerization

Cell transfection was achieved using a virus replication assay. At 59 hpt or 96 hpt, the transfected cells were rinsed twice with 1× phosphate buffered saline (PBS) (Beyotime), fixed in 4% paraformaldehyde (Sigma), and penetrated in 0.2% Triton-X (Sigma). The cells were then stained with rhodamine- conjugated phalloidin (Invitrogen) and Hoechst 33258 (Beyotime) for labeling of F-actin and nuclear DNA, respectively. The images were captured using a Leica SP2 confocal laser scanning microscope.

RESULTS

Sequence characterization

N-WASP phosphorylation status plays an important role in regulating actin polymerization [1], and AcMNPV P78/83 is a phosphorylated protein[9]. ScanProsite recognized 18 putative phosphorylated sites of HA2. Of these, ^{232}Thr and ^{250}Ser were located within the WCA domain (Fig. 2). Besides these two putative phosphorylated sites identified by ScanProsite prediction, ^{245}Ser is a highly conserved residue among various baculoviruses and may possibly work as a kinase target.

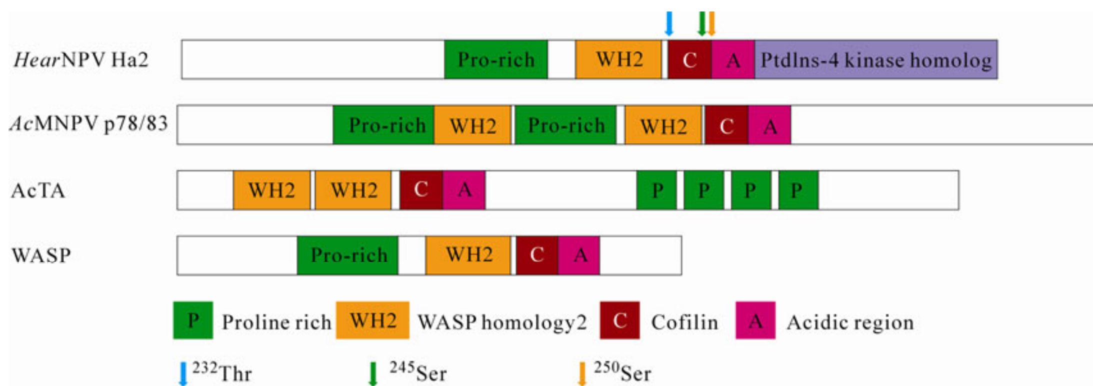

Fig. 2. Diagram of motifs on N-WASP homologues. Important motifs for actin polymerization were labeled with colors, and the arrows indicate the relative locations of the putative phosphorylation sites on WCA motif of HA2 predicted by ScanProsite.

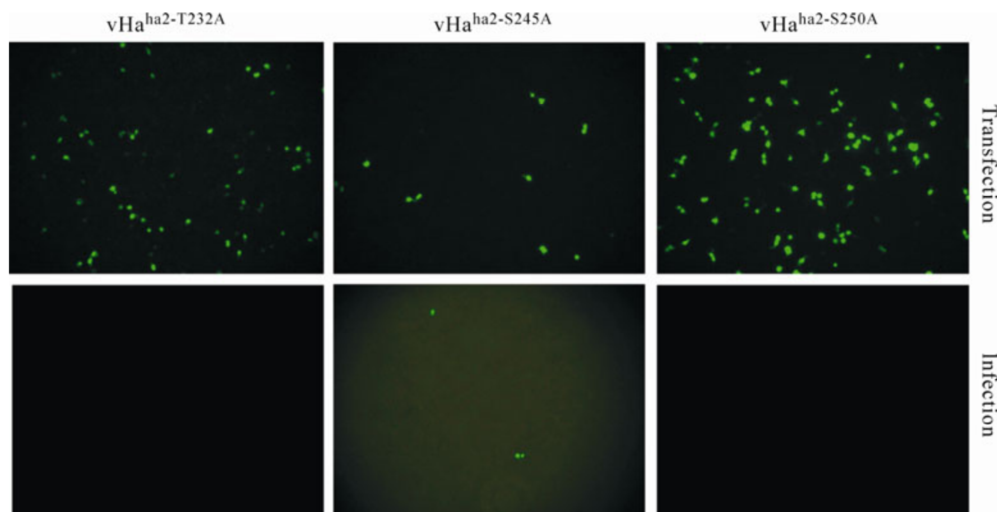

Fig. 3. Infectivity assay of the recombinant bacmid constructs. vHa$^{ha2\text{-}T232A}$, vHa$^{ha2\text{-}S245G}$, and vHa$^{ha2\text{-}S250A}$ were transfected to HzAM1 cells. At 144 htp, the supernatants were collected and added to fresh HzAM1 cells for secondary infection. At 144 hpi, cell images were captured using Olympus IX-51 microscope.

Construction of the recombinant bacmids

All the donor plasmids were sequenced before being submitted to bacmid transposition. After being transposed to the *ha2* knockout bacmid HaΔha2, three *ha*2 repair recombinant bacmids vHa$^{ha2\text{-}T232A}$, vHa$^{ha2\text{-}T245G}$, and vHa $^{ha2\text{-}S250A}$ were generated. All the recombinant bacmids contained *egfp* under the *p*10 promoter and the *ha2* or truncated *ha2* controlled by the native *ha*2 promoter in the polyhedrin (*polh*) locus The recombinant bacmid constructs were confirmed by PCR with M13± primers (Fig. 1B), and all the three constructs generated 5 kb fragments, which was in accordance with the predicted length of the recombinant viruses.

^{232}Thr and ^{250}Ser is essential for HearNPV infectivity

To explore the importance of ^{232}Thr, ^{245}Ser and ^{250}Ser in virus replication, a virus infectivity assay was performed. Supernatants from either vHa$^{ha2\text{-}T232A}$ or vHa $^{ha2\text{-}S250A}$ transfected cells failed to initiate secondary infection, whereas supernatant from vHa $^{ha2\text{-}S245G}$ transfected cells successfully infected the fresh cells as manifested by EGFP fluorescence (Fig. 3). These phenotypes isndicated that ^{232}Thr and ^{250}Ser were critical in defining HA2 function, and subsequently influence the virus infectivity

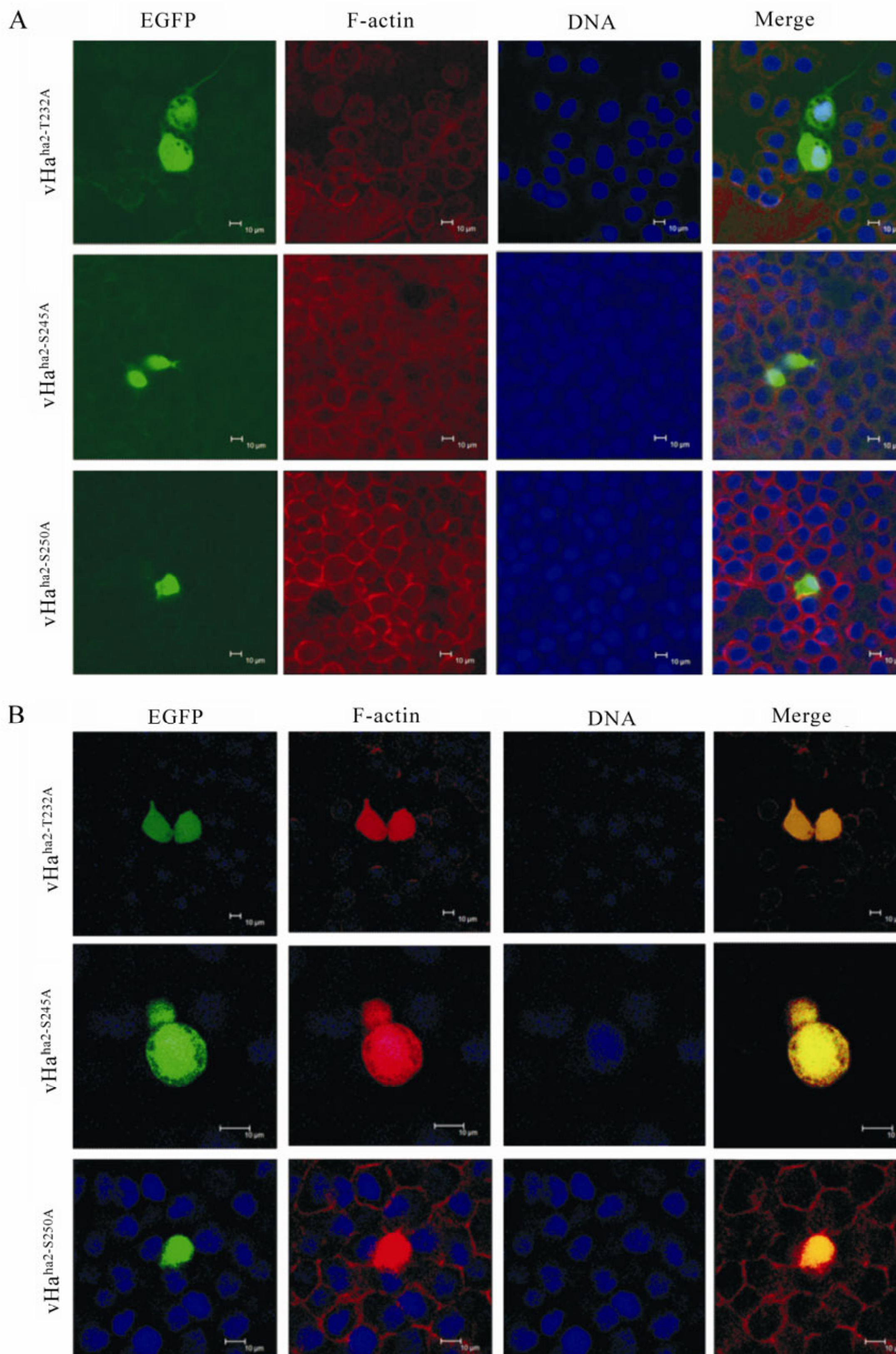

Fig. 4. Nuclear actin polymerization assay of the recombinant bacmids. A: vHa$^{ha2\text{-}T232A}$, vHa$^{ha2\text{-}S245G}$ and vHa$^{ha2\text{-}S250A}$ were transfected into HzAM1 cells. At 59 hpt, cells were fixed and stained with rhodamine-conjugated phalloidin and Hoechst 33258. B: The same treatment as in A, except that cells were fixed and stained at 96 hpt.

Viruses bearing mutated HA2 showed an aberrant phenotype of nuclear actin polymerization

Since ^{232}Thr and ^{250}Ser were located within WCA domain and proved to be critical for virus infectivity, further experiments were performed to investigate their role in virus-induced actin polymerization.

In either vHa$^{ha2\text{-}T232A}$, vHa$^{ha2\text{-}S250A}$ or vHa$^{ha2\text{-}S245G}$ transfected cells, the formation of nuclear F-actin stained by phalloidin-rhodamine was still invisible at 59 hpt (Fig. 4A), and became dispersed throughout the whole cell as late as 96 hpt (Fig. 4B). This aberrant phenotype provides a significant contrast to the wild-type HearNPV, in which infected cells nuclear F-actin forms as early as 24 hpi and disappears as late as 60 hpi [13].

DISCUSSION

Protein phosphorylation as a common post-translational modification process that exists in a wide range of cell types from insect to mammalian, and offers a variety of functions from catalytic activators to protein-protein interaction mediators. A unique feature of baculovirus infection of insect cells is the virus-induced nuclear actin polymerization, which is supposed to function as a scaffold for viral nucleocapsid assembly[2]. Previous research has demonstrated the N-WASP homologous viral proteins P78/83 of *Ac*MNPV or HA2 of HearNPV are essential for baculovirus-induced actin polymerization through their WCA domain [2, 8, 12]. However, unlike the N-WASP protein where phosphorylation status defines its actin polymerization capability [1], the present research indicated HA2-mediated actin polymerization appeared to be marginally affected by its putative phosphorylation sites, as manifested by the delayed actin polymerization. The diffused distribution pattern of F-actin may possibly due to the collapsed nuclear envelope at a very late stage of virus-infection that allows nuclear F-actin to leak outside to the cytoplasm from the nucleus.

The replication cycle of baculovirus contains various steps, including virus entry, viral genome nuclear transportation, replication, and expression, nucleocapsid assembly, and viral particle budding. Any mistake in these steps can result in baculovirus replication failure. Besides its well-known function in mediating actin polymerization, HA2 is at least, but not limited to, one of the major participants in nucleocapsid assembly as a structural protein[9]. The putative phosphorylation sites mutation of HA2 in this research only marginally influence virus-induced actin polymerization, which suggested the phosphorylation of HA2 was possibly involved in other processes, such as viral nucleocapsid assembly.

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