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The *ha72* Core Gene of Baculovirus Is Essential for Budded Virus Production and Occlusion-Derived Virus Embedding, and Amino Acid K22 Plays an Important Role in Its Function

Huachao Huang,[a] Manli Wang,[a] Fei Deng,[a] Dianhai Hou,[a] Basil M. Arif,[b] Hualin Wang,[a] Zhihong Hu[a]

State Key Laboratory of Virology and Joint Laboratory of Invertebrate Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, People's Republic of China[a]; Laboratory for Molecular Virology, Great Lakes Forestry Centre, Sault Ste. Marie, Ontario, Canada[b]

***ha72* of *Helicoverpa armigera* nucleopolyhedrovirus (a homologue of *ac78*) was identified as a conserved late baculovirus gene and characterized. HA72 localizes in the intranuclear ring zone. By generating mutants, we showed that HA72 is essential for budded virus (BD) production and occlusion-derived virus (ODV) embedding. HA72 also interacted with P33, a baculoviral sulfhydryl oxidase. A point mutation of amino acid 22 from lysine to glutamic acid curtailed BV production and precluded ODV occlusion as well as interaction with P33.**

Baculoviruses are pathogens that infect insects from the orders *Lepidoptera*, *Diptera*, and *Hymenoptera* (1). To date, 60 baculovirus genomes have been fully sequenced, and all of them contain the 37 core genes (2) that play critical roles in baculovirus infection. *ac78* is a newly identified baculovirus core gene (2). A previous study showed that deletion of the homologue of *ac78* (*bm64*) in *Bombyx mori* nucleopolyhedrovirus (BmNPV) prevented the production of infectious budded virus (BV) (3). Its homologue in *Helicoverpa armigera* NPV (HA72) was shown to be a component of the occlusion-derived virus (ODV) envelope (4). However, its role in the viral life cycle is still unclear, and the present study was undertaken to provide initial biochemical, microscopic, and genetic characterizations of HA72.

HA72 contains an IPLKL motif and a putative fumarate reductase flavoprotein C-terminal motif. Bioinformatics analysis showed that HA72 has two recognized motifs (Fig. 1A): an IPLKL motif at the N terminus, required for a peroxisome-targeting signal in plants (5), and a putative fumarate reductase flavoprotein (FRF) C-terminal motif that has been implicated in electron transfer in the oxidation-reduction process (6, 7). The IPLKL motif is highly conserved in all baculoviral HA72 homologues, while the FRF-C is conserved at a low amino acid identity in all of the baculoviruses (Fig. 1A and B).

Time course analyses of transcription and expression suggest that *ha72* is a late gene. Reverse transcription-PCR (RT-PCR) and Western blotting were performed to assess the temporal transcription and expression of *ha72*. The data summarized in Fig. 1C show that transcription was first detected at 18 hours postinfection (hpi). A rabbit polyconal antibody against the full length of HA72 was used for Western blotting, and a specific immunoreactive band was initially detected at 36 hpi, indicating that *ha72* is a late gene. The gene-specific primer *ha72*up-R (Table 1) was used for 5′-random amplification of cDNA ends (5′-RACE) and, consistent with the above data, sequencing of the 5′-RACE products showed that expression of the *ha72* transcript was initiated from the first A of a late gene transcriptional start site motif, TTAAG at 87 nucleotides upstream of the predicted start codon. For the 3′-RACE analysis, an oligo(dT)$_{15}$ three-site adaptor primer was used for generating cDNA, and subsequent PCR amplification was performed by using a three-site adaptor primer and an *ha72*-specific forward primer (Table 1). The 3′-RACE analysis showed that *ha72* uses a polyadenylation signal motif, ATTAAA at 480 nucleotides downstream of its stop codon (Fig. 1D). In *Autographica californica* multiple NPV (AcMNPV), *ac78* expression is initiated at 12 hpi from the late transcriptional motif TTAAG, and its mRNA is polyadenylated (8).

HA72 is essential for BV production, and lysine at position 22 plays an important role in its function. To generate an *ha72*-inactivated recombinant bacmid (bHaBacΔ*72*) as a tool to study the function of HA72, a portion (bp 33 to 45) of the coding sequence was replaced with *egfp* under the *hsp70* promoter and also a Cm^r cassette by utilizing homologous recombination. Briefly, an upstream fragment was amplified using the primer pair *ha72*up-F and *ha72*up-R. A downstream fragment was amplified with primers *ha72*down-F and *ha72*down-R. The amplicons were cloned in the pKS-*egfp*-Cm^r vector flanking an *egfp*-Cm^r cassette. The linear fragment, containing *egfp*, Cm^r, and the *ha72* flanking sequences, was amplified with *ha72*up-F and *ha72*down-R and subsequently electroporated into *Escherichia coli* BW25113 harboring bHaBacHZ8 and pKD46. *Polyhedrin* was reintroduced into the original *ph* locus (bHaBacΔ*72-ph*) to monitor morphogenesis of the occlusion bodies (OBs). A rescue bacmid, bHaBacΔ*72-72*R-*ph*, was also generated by using the Bac-to-Bac system to contain the *ha72* open reading frame (ORF) and *ph* under the control of their native promoters. The *ha72* coding sequence was amplified with *ha72*R-F and *ha72*R-R. In order to verify that the IPLKL motif is important for HA72 function, mutants with replacement of its lysine (K, at position 22) with positively charged arginine (R) or negatively charged glutamic acid (E) were constructed and designated bHaBacΔ*72-72*^{K22E}*-ph* and bHaBacΔ*72-72*^{K22R}*-ph*, respectively (Fig. 2A). An *ha72* site-directed mutant was generated by overlap extension PCR. For the K22E mutant, the first round of

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Address correspondence to Zhihong Hu, huzh@wh.iov.cn.

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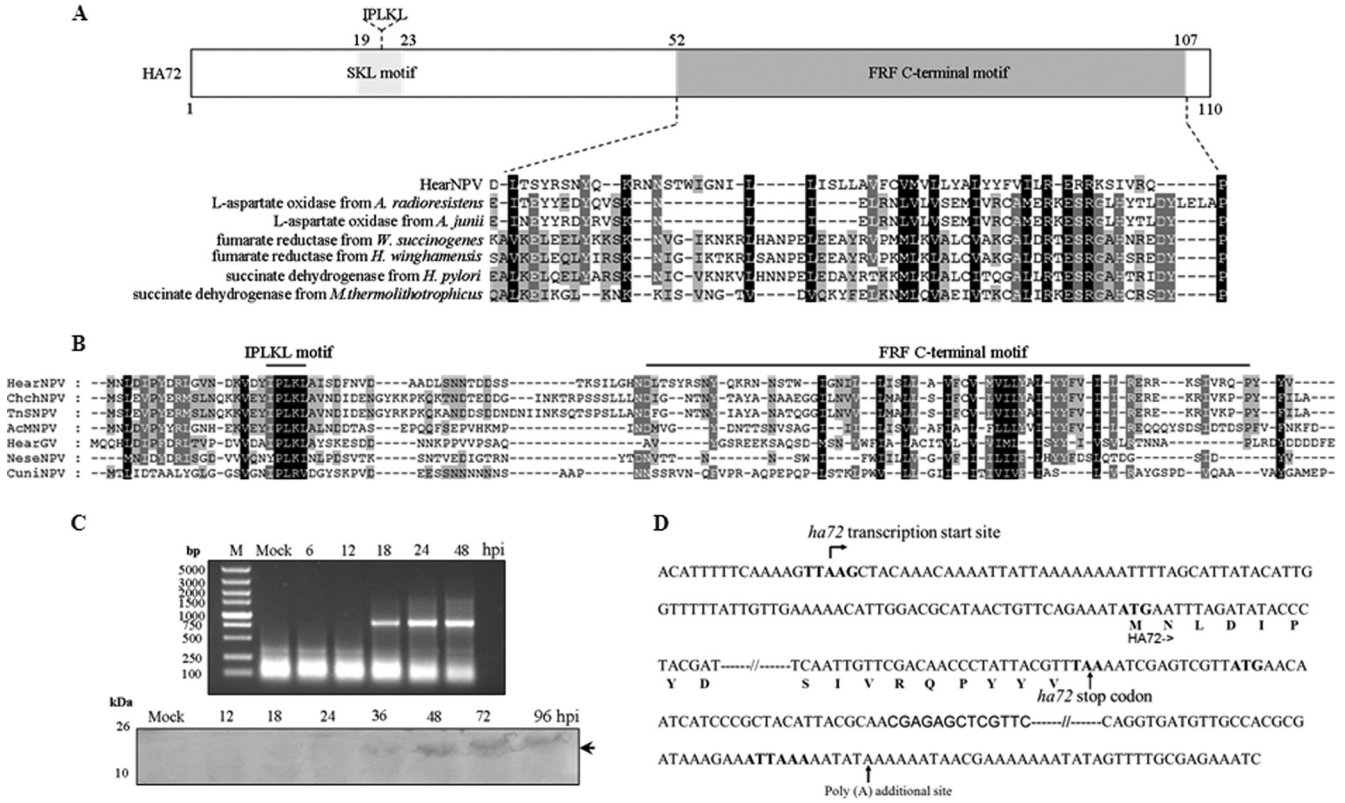

FIG 1 Characterization of HA72. (A) Map of HA72, showing the location of the IPLKL motif and a putative FRF C-terminal motif. An alignment of a portion of HA72 with representative sequences containing FRF C-terminal motifs is shown. GenBank protein accession numbers: WP_005405373.1, L-aspartate oxidase from *Acinetobacter radioresistens* SK82; WP_004953071.1, L-aspartate oxidase from *Acinetobacter junii* NIPH 182; NP_907042.1, fumarate reductase from *Wolinella succinogenes* DSM 1740; WP_006801824.1, fumarate reductase from *Helicobacter winghamensis* ATCC BAA-430; WP_001941531.1, succinate dehydrogenase from *Helicobacter pylori* GAM231Ai; WP_018153189.1, succinate dehydrogenase from *Methanothermococcus thermolithotrophicus*. (B) Multiple sequence alignments of HA72 with selected baculoviral homologues. GenBank protein accession numbers: NP_075141.1, HearNPV; YP_249681.1, *Chrysodeixis chalcites* NPV (ChchNPV); YP_308961.1, *Trichoplusia ni* single NPV (TnSNPV); NP_054108.1, AcMNPV; YP_001649107.1, HearGV; YP_025153.1, *Neodiprion sertifer* NPV (NeseNPV); NP_203338.1, *Culex nigripalpus* NPV (CuniNPV). Alignments were constructed using the Lasergene program MegAlign and displayed with the GeneDoc software. (C) Temporal analysis of *ha72* transcription and expression. HzAM1 cells were infected with vHaBac-*egfp-ph*, harvested at the indicated times, and subjected to RT-PCR (upper panel) or Western blot analysis (lower panel). A polyclonal antibody against full-length HA72 that had been raised in rabbits was used in the Western blotting. (D) Illustration of the 5′- and 3′-RACE results for *ha72*. The sequence of *ha72* with its flanking regions is shown. The late promoter (TTAAG), the start and stop codons, and the poly(A) signals are shown in boldface. The arrow indicates the location of the transcription initiation site for *ha72* mRNA or the additional poly(A) site.

PCR entailed two parallel PCRs with the following primer pairs: *ha72*R-F/*ha72*^{K22E}E-R and *ha72*^{K22E}-F/*ha72*R-R. The second round of amplification was performed with *ha72*R-F and *ha72*R-R primers, using the annealed PCR products from the first round as the template. Similarly, the K22R mutant was generated with primers *ha72*^{K22R}-F and *ha72*^{K22R}-R. All the primers used in this studied are outlined in Table 1. Schematic diagrams for the generated constructs are shown in Fig. 2A.

Cells transfected with bHaBacΔ*72-ph* showed only single fluorescent cells, while neighboring green fluorescent cells were observed for cells transfected with the control bacmids (bHaBac-*egfp-ph* and bHaBacΔ*72-72*R-*ph*) (Fig. 2B). When the supernatants from the transfection mixtures were used to infect a second batch of cells, no infection was observed with HaBacΔ*72-ph*, while control bacmids showed successful infection, suggesting that disruption of *ha72* leads to a defect in the production of infectious BVs (Fig. 2B).

One-step growth curve assays were conducted to detect further effects of the recombinants on the production of progeny virus. Comparable viral growth kinetics were observed with vHaBacΔ*72-72*R-*ph*, vHaBac-*egfp-ph*, and vHaBacΔ*72-72*^{K22R}-*ph*, but statistical analysis showed that vHaBacΔ*72-72*^{K22E}-*ph* had significantly decreased BV titers from 24 hpi to 96 hpi compared to the rescue virus, vHaBacΔ*72-72*R-*ph* (Fig. 2C). The results indicated that K22 is needed for optimal production of infectious BV.

Inactivation of HA72 does not affect viral DNA replication. Quantitative PCR was used to analyze the effect of *ha72* on viral DNA replication by using the paired primers *ha39*-F and *ha39*-R. *Heliothis zea* AM1 (HzAM1) cells were transfected with bHaBacΔ*72-ph*, bHaBacΔ*72-72*R-*ph*, or the control bacmid, bHaBacΔ*133*, which does not contain the membrane fusion protein F (9). At 0 h posttransfection (hpt) and 48 hpt, intracellular viral DNA was extracted and analyzed. The data outlined in Fig. 2D show comparable levels of DNA synthesis for all constructs, suggesting that *ha72* is not essential for DNA replication.

HA72 localizes in the intranuclear ring zone region during infection. Confocal microscopy was carried out on HzAM1 cells

TABLE 1 Primers used in the study

Primers	Sequence[a]	Restriction site(s)
Oligo(dT)$_{15}$ three-site adaptor primer	5′-CTGATCTAGAGGTACCGGATCCTTTTTTTTTTTTTTT-3′	
Three-site adaptor primer	5′-CTGATCTAGAGGTACCGGATCC-3′	
ha72-specific forward primer	5′- ATGAATTTAGATATACCCTACGA-3′	
*ha72*up-F	5′-CCCGGTACCCGATAGAATTAAATTCTTTGTTCA-3′	KpnI site
*ha72*up-R	5′-CCCCTCGAGGTGGACTATATTCCGCTAAAAT-3′	XhoI site
*ha72*down-F	5′-GGGTCTAGACCGAGACGATCGTAGGGTA-3′	XbaI site
*ha72*down-R	5′-GGGTCTAGAGATATCTGTGTCCAGTCGCAAATACC-3′	XbaI and EcoRI sites
*ha72*R-F	5′-CGCCTCGAGAGCGACGTTCCTCAACGCT-3′	XhoI site
*ha72*R-R	5′-CGCGCTAGCGTCCGTGGTCAATTCGAAAAT-3′	NheI site
ha72^{K22E}-R	5′-CTACGTTAAAATCGGATATAGCCAATTCTAGCGGAATATAG-3′	
ha72^{K22E}-F	5′-CTATATTCCGCTAGAATTGGCTATATCCGATTTTAACGTAG-3′	
ha72^{K22R}-F	5′-CTATATTCCGCTAAGATTGGCTATATCCGATTTTAACGTAG-3′	
ha72^{K22R}-R	5′-CTACGTTAAAATCGGATATAGCCAATCTTAGCGGAATATAG-3′	
ha39-F	5′-GAAATGCGAATCAGACAGATTACTCG-3′	
ha39-R	5′-CGCAACCTAACATTTGAGAACACAC-3′	

[a] Sequences of restriction sites are underlined.

infected with vHaBac-*egfp-ph* or vHaBacΔ*72-72-eyfp-ph* (Fig. 3A and B). Fluorescence was captured at 48 hpi by using a laser scanning confocal microscope (PerkinElmer UltraView VOX) with a laser wavelength of 488 nm for DNA (blue) stained with Hoechst 33258 (Beyotime), 561 nm for enhanced green fluorescent protein (EGFP), 605 nm for rhodamine-conjugated secondary antibody (red; Chemicon), and 527 nm for enhanced yellow fluorescent protein (EYFP; yellow). HA72 was mainly localized to the ring zone region (Fig. 3A and B). A small amount of fluorescence was also observed in the center of the nucleus (Fig. 3A and B). By

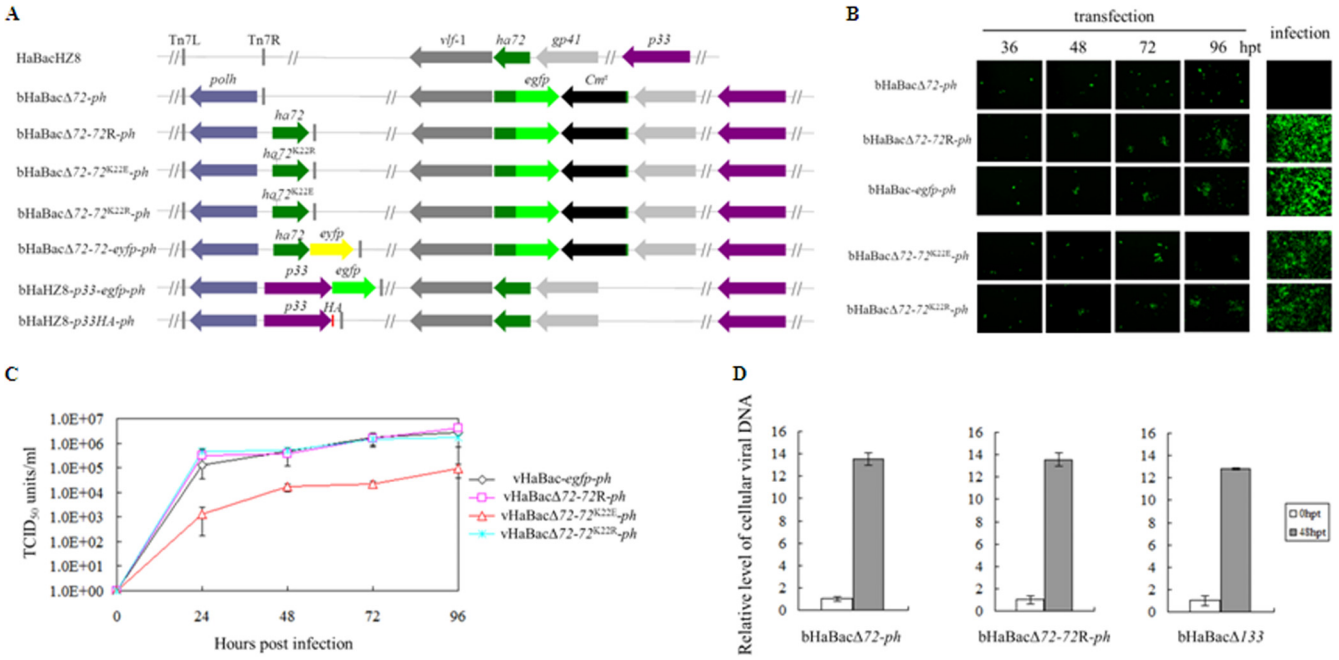

FIG 2 HA72 is essential for BV production but not DNA replication, and the K22E mutation curtails BV production. (A) Schematic diagrams of recombinant bacmids. The *ha72* ORF was partially replaced with an *egfp* and Cm resistance gene cassette from pKS-*egfp*-Cm^r, which contains an *egfp* gene driven by an *hsp70* promoter and a Cm^r gene under the control of the T7 promoter, to generate *ha72*-inactivated bacmid bHaBacΔ*72*. The recombinant bacmids bHaBacΔ*72-ph*, bHaBacΔ*72-72*R-*ph*, bHaBacΔ*72-72*^{K22E}-*ph*, bHaBacΔ*72-72*^{K22R}-*ph*, and bHaBacΔ*72-72-eyfp-ph* were generated by Tn7-based transposition of the indicated genes into the *ph* locus of bHaBacΔ*72*. bHaBacΔ*72-p33-egfp-ph* and *bHaBacHZ8-p33HA-ph* were generated by Tn7-based transposition of the indicated genes into bHaBacHZ8. (B) Analysis of virus replication and BV production in HzAM1 cells. Fluorescence microscopy images were taken at various time posttransfection or at 120 hpi. (C) One-step growth curve analysis of recombinant viruses. HzAM1 cells were infected with vHaBac-*egfp-ph*, vHaBacΔ*72-72*R-*ph*, vHaBacΔ*72-72*^{K22E}-*ph*, or vHaBacΔ*72-72*^{K22R}-*ph* at a multiplicity of infection of 5. Supernatants were harvested at the designated time points postinfection and quantified for production of infectious BV by the endpoint dilution method. Data points represent average titers from triplicate infections. Error bars represent standard errors. (D) Real-time PCR analysis of viral DNA replication. HzAM1 cells were transfected with 3 μg bHaBacΔ*72-ph*, bHaBacΔ*72-72*R-*ph*, or control bacmid, bHaBacΔ*133* (null for the *f* protein gene) through three experiments with three replicates per experiment. At the indicated time points postinfection, cells were collected to extract total DNA. Prior to quantitative PCR, DNA was digested with DpnI to avoid interference from *E. coli*-derived viral DNA. Quantitative PCRs were carried out in triplicates, utilizing 1 μl of DpnI-treated DNA. Error bars represent the standard errors.

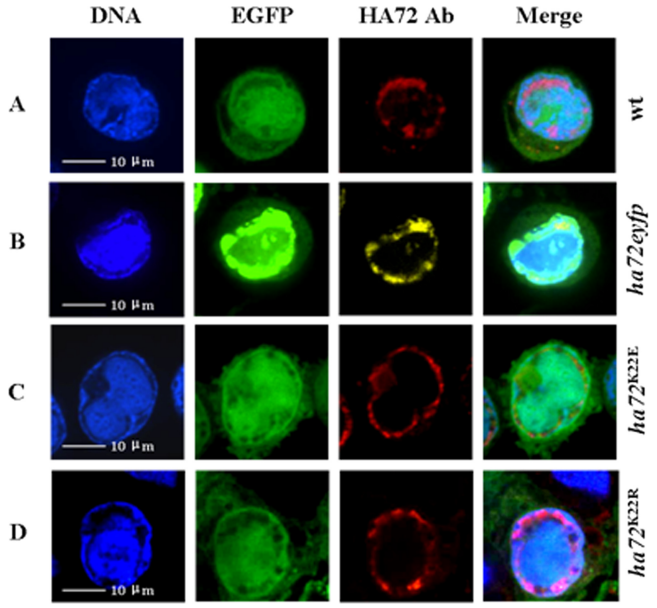

FIG 3 Subcellular localization of HA72 in HzAM1 cells. Cells were infected with vHaBac-*egfp-ph*, vHaBacΔ*72-72-eyfp-ph*, vHaBacΔ*72-72*^{K22E}*-ph*, or vHaBacΔ*72-72*^{K22R}*-ph* at a multiplicity of infection of 5 and prepared for confocal imaging at 48 hpi. For immunofluoresence assays, polyclonal antiserum against HA72 was used as the primary antibody and stained with anti-rabbit IgG (A, B, and D) or observed via autofluorescence of HA72-EYFP chimera (B). DNA was stained with Hoechst stain. The panels on the right are merged images. Bars, 10 μm.

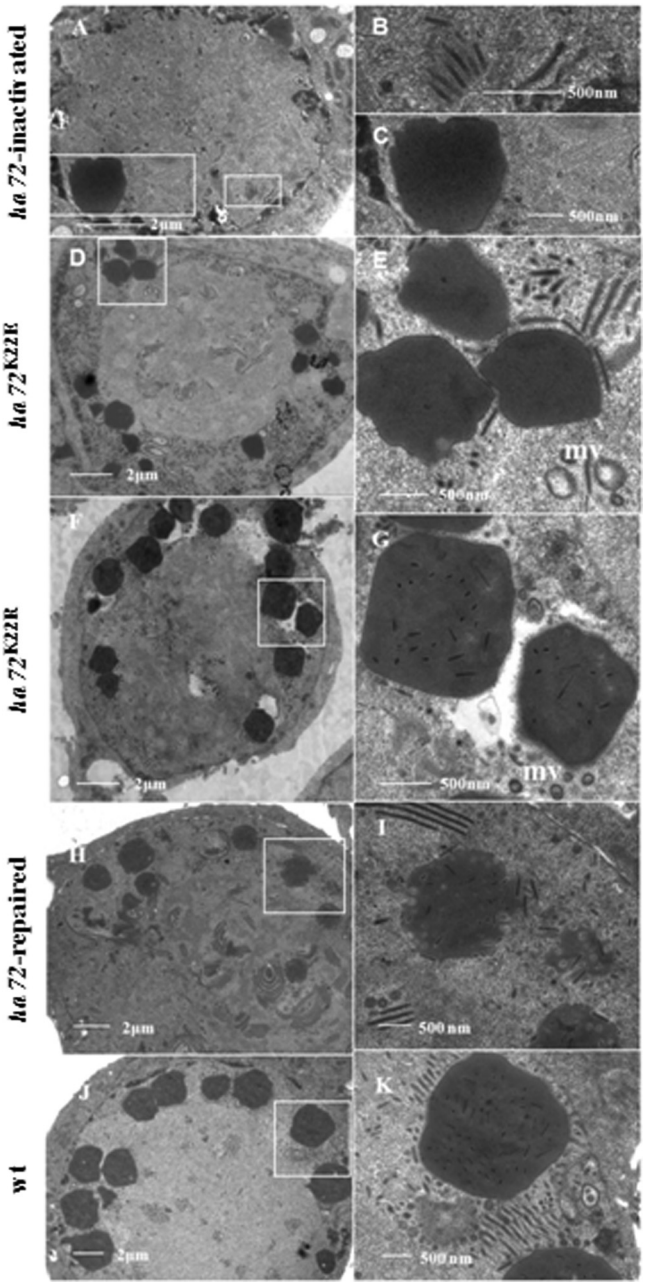

FIG 4 TEM analysis. HzAM1 cells were transfected with bHaBacΔ*72-ph* (A to C) or infected with vHaBacΔ*72-72*^{K22E}*-ph* (D and E), vHaBacΔ*72-72*^{K22R}*-ph* (F and G), vHaBacΔ*72-72*R-*ph* (H and I), or vHaBac-*egfp-ph* (J and K). Samples were collected at 96 hpt or 96 hpi for TEM.

mutating K to R or E, we did not observe changes in the intranuclear localization of HA72 (Fig. 3C and D).

Deletion of *ha72* prevents ODV occlusion. Cells were transfected with bHaBacΔ*72-ph*, harvested at 96 hpt, and prepared for electron microscopy to observe the effects of *ha72* disruption on any morphological alterations. Typical symptoms of infection, such as nuclear hypertrophy and the presence of an electron-dense virogenic stroma (VS), were observed. Nucleocapsids with a normal appearance were also observed; however, they were not embedded into OBs (Fig. 4A, B, and C). Therefore, inactivation of HA72 prevented ODV occlusion.

The K22 mutation interferes with ODV occlusion. Transmission electron microscopy (TEM) was also used to monitor the morphology of mutants generated from mutations in the IPLKL motif. In the K22E mutant, ODVs with a normal appearance were observed, but occlusion within OBs did not seem to take place (Fig. 4D and E). In contrast, in the K22R mutant, OBs with normal ODV occlusion were observed (Fig. 4F and G), similar to the control viruses vHaBacΔ*72-72*R-*ph* (Fig. 4H and I) and vHaBac-*egfp-ph* (Fig. 4J and K).

HA72 interacts with P33 *in vitro* and colocalizes with P33 in infected cells. In order to investigate how HA72 participates in the process of ODV occlusion, a yeast two-hybrid (Y2H) assay was employed to screen interactions of HA72 with *Helicoverpa armigera* NPV (HearNPV) ODV structural proteins as described previously (10). We decided to test interactions with viral structural proteins, because it had been shown already that HA72 is part of the virion (4). The results showed that HA72 interacted with P33 (HA80) when it was expressed in the vectors containing either a binding domain or an activation domain (Fig. 5A). The K22E mutation abolished this interaction, while K22R did not affect the interaction (data not shown). During Y2H experiments, the interactions of HA72 with 38K or P49 were also detected but resulted in weak yeast growth (data not shown).

Recombinant bacmids bHaBacHZ8-*p33-egfp-ph* and HaBacHZ8-*p33HA-ph* were constructed to study the colocalization of HA72 and P33 (Fig. 2A). Confocal microscopy of cells infected with vHaBacHZ8-*p33-egfp-ph* or vHaHZ8-*p33HA-ph* showed that P33 is located at the rim of nuclei at 48 hpi (Fig. 5B). For the hemagglutinin (HA)-tagged P33, the chimera was primar-

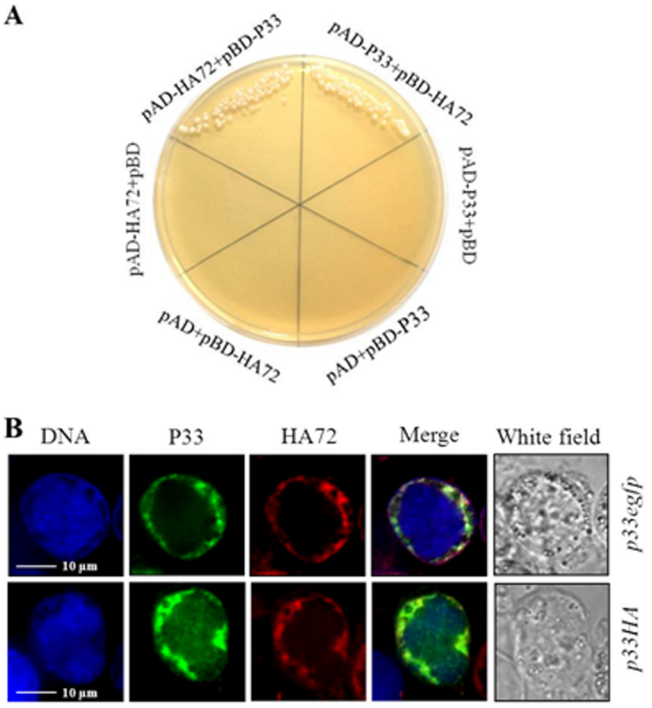

FIG 5 Interaction and colocalization of HA72 and P33. (A) Results from the yeast two-hybrid assay revealed an interaction of HA72 and P33. The AD fusion plasmid and the BD fusion plasmid were used to cotransform AH109 yeast competent cells. The names of the cotransforming plasmids are indicated. (B) Immunofluorescent staining or autofluorescence images, showing colocalization of HA72 and P33. HzAM1 cells were infected with vHaBacHZ8-*p33-egfp-ph* or vHaBacHZ8-*p33HA-ph*, and at 48 hpi HA72 was stained with rhodamine-conjugated antibody. Bars, 10 μm.

ily probed with HA-tagged (6E2) mouse monoclonal antibody (Cell Signaling) and subsequently signaled with fluorescein isothiocyanate-conjugated Affinipure goat anti-mouse IgG (H+L; Proteintech). When anti-HA72 antibody was used as the probe, the colocalization of HA72 and P33 to the ring zone region was observed (Fig. 5B). The colocalization result supported the possibility of an interaction between HA72 and P33.

In summary, this study characterized the functions of a baculovirus core gene, *ha72*. We revealed that K22 is important for the function of HA72, as the K22E mutation curtailed BV production and precluded ODV occlusion. As K22 is located at the conserved IPLKL motif, it will be interesting to see if the motif is important for protein function. Furthermore, protein-protein interactions showed that HA72 interacted with P33, a baculoviral sulfhydryl oxidase that plays an important role in BV production and multiple-enveloped ODV formation (11, 12). As HA72 contains a putative FRF C-terminal motif, we suggest that HA72 and P33 work together in a redox process during baculovirus infection. At this stage, we are expending much effort to obtain enough soluble expressed HA72 to study its enzymatic activity, and we hope to demonstrate its possible role in the proposed redox process in the future. During the process of our manuscript submission, a similar result was published by Tao et al. regarding AC78 (13). Our data corroborate their data indicating that HA72/AC78 is a core baculoviral gene essential for the production of infectious BV and ODV occlusion but is not involved in viral DNA replication.

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