Identification of a Hydrophobic Domain of HA2 Essential to
Morphogenesis of Helicoverpa armigera Nucleopolyhedrovirus†

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The HA2 protein of the Helicoverpa armigera single-nucleocapsid nucleopolyhedrovirus (HearNPV) is a WASP homology protein capable of nucleating branched actin filaments in the presence of the Arp2/3 complex in vitro. To determine the role of ha2 in the HearNPV life cycle, ha2 knockout and ha2 repair bacmids were constructed. Transfection and infection analysis demonstrated that the ha2 null bacmid was unable to produce infectious budded virus (BV), while the repair bacmid rescued the defect. In vitro analysis demonstrated that the WCA domain of HA2 accelerates Arp2/3-mediated actin assembly and is indispensable to the function of HA2. However, analysis of the repaired recombinant with a series of truncated ha2 mutants demonstrated that the WCA domain was essential but not enough to yield infectious virions, and a hydrophobic domain (H domain) consisting of amino acids (aa) 167 to 193 played a pivotal role in the production of BV. Subcellular localization analysis with enhanced green fluorescent protein fusions showed that the H domain functioned as a nuclear localization signal. In addition, deletion of the C terminus of the ha2 product, a phosphatidylinositol 4-kinase homolog, dramatically decreased the viral titer, while deletion of 128 aa from the N terminus did not affect HA2 function.

Rearrangement of the actin cytoskeleton in response to viral infection has been observed for many viruses (8). Since Volkman et al. first demonstrated that microfilaments are involved in the production of budded virions (BV) of Autographa californica nucleopolyhedrovirus (AcMNPV) (26), all evidence to date indicates that nuclear F-actin is essential for nucleocapsid morphogenesis of nucleopolyhedroviruses (20, 25).

AcMNPV can induce sequential rearrangement and nuclear assembly of actin in infected cells (3). It has been reported that monomeric G-actin accumulated within the nuclei of AcMNPV-infected cells during the early stages of infection and that expression of six AcMNPV early genes (ie-1, pe38, he65, Ac004, Ac102, and Ac152) was sufficient for this to occur (19). The dynamic nuclear actin was polymerized by the Arp2/3 complex and the Wiskott-Aldrich syndrome protein (WASP) homolog, such as P78/83 of AcMNPV (9, 17) and HA2 of Helicoverpa armigera NPV (HearNPV) (18, 28), during baculovirus infection. Nuclear F-actin assembly by P78/83 and the Arp2/3 complex was essential for viral progeny production (9).

The overall arrangement of baculoviral WASP exhibits the same proline-rich, WH2 (verprolin homolog: W), coflin homology (C), and acidic (A) motifs as its counterparts, the WASP family proteins (4, 17, 18). The WCA domain of AcMNPV P78/83 stimulates actin polymerization into Y branches with the Arp2/3 complex in vitro and also plays an essential role in AcMNPV replication (9). Although all WASPs and WASP family proteins verprolin-homologous proteins (WAVEs) have a WCA region, which associates with actin and the Arp2/3 complex and is critical for Arp2/3 complex-induced actin polymerization, there still exist differences in the potencies of WCA regions from WASPs and WAVEs in Arp2/3 complex activation (23, 29). On the other hand, the ha2 product shares very low homology with other WASPs (17). Besides the conserved motifs, the N and C termini of HA2 are highly diverse. Sequence analysis indicated that the N-terminal 128 amino acids (aa) of HA2 have no homolog in GenBank, while the C-terminal 152 aa contain a phosphatidylinositol 4-kinase (PtdIns 4-kinase)-homologous domain. It has been reported that PtdIns 4-kinase is involved in normal actin cytoskeleton organization (1). Thus, it is of great benefit to analyze the functions of the different domains of HA2.

In this study, we generated an ha2 knockout bacmid via homologous recombination to determine the role of HA2 in viral replication. Our results showed that ha2 was an essential gene necessary for virus propagation. By the repair of a series of mutants of ha2, we demonstrated that those truncated ha2 mutants which covered the WCA domain could yield infectious virions. Further investigation described the activity of the HA2 WCA region, which could activate the Arp2/3 complex in vitro. Moreover, by combining the results of the recombinant viruses and the subcellular localization of the different fragments, a new hydrophobic domain (aa 167 to 193) was characterized.

MATERIALS AND METHODS

Cells, viruses, bacterial strains, and plasmids. Helicoverpa zea (BCIRL-Hz-AM1 and Hz-AM1) cells were maintained at 28°C in Grace’s medium supplemented with 10% fetal bovine serum (Gibco BRL). HearNPV G4, whose genome has been sequenced entirely (4), and recombinant vHA-eGFP (18) and HaBacHZ8 (27) were propagated in Hz-AM1 cells before use. BW25113(pKD46)(HaBacHZ8) was constructed and preserved in our lab (11); this strain contained a HearNPV bacterial artificial chromosome (Hearbacmid) and could express λ phage red recombinase. Plasmid pKD3 contained the chloramphenicol resistance gene (cat) (kindly provided by B. L. Wanner of Purdue University) (6).

Generation of ha2 knockout HearNPV bacmid. Using the λ red recombinant system, an ha2 knockout HearNPV was generated through homologous recom-
TABLE 1. Oligonucleotides used for this study

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<tr>
<td>CATD</td>
<td>ATA TGA ATA TCC TCC T TAG</td>
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<tr>
<td>PR1</td>
<td>CCG GTA TAC (Bst1107I) ACG TCA</td>
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<td>PR2</td>
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* Restriction enzyme sites are indicated in italics.

bination in *Escherichia coli* as previously described (6, 11). First, a linear fragment for generating ha2-replaced recombinant HearNPV was obtained by the PCR method. Primers used for PCR were HP1 and HP2. Both primers were made up of two parts. The first 40 bp was homologous to the 3’ region of ha2 (HearNPV nucleotides [nt] 738 to 1402), and the other 19 bp was homologous to flanking region, a stream flanking regions. One recombinant bacmid with both correct PCR confirmations was selected and named HaΔha2.

### Construction of donor plasmids

To generate an ha2 repair bacmid, transfer plasmids were constructed for transposition into the polh locus of the ha2 knockout bacmid according to the Bac-to-Bac protocol (Invitrogen). First, the fastBac Dual transfer plasmid (Invitrogen Life Technologies), containing two multicloning regions, was digested with Bst1107I and EcoRI to remove the polh promoter and replaced with the native ha2 promoter (nt 192 to 208), which was amplified from the HearNPV G4 genomic template by using primers PR1 and PR2. A copy of the enhanced green fluorescent protein gene (egfp), which was PCR amplified from pEGFP-N1 using primers egfp1 forward and egfp1 reverse, was inserted into the p10 multicloning region of the altered pFastBac Dual plasmid, and the resulting construct was named pFBGpha2. Finally, ha2 and a series of truncated ha2 fragments were inserted between EcoRI and Xhol sites under the control of the ha2 promoter. All of these fragments were obtained by the PCR method with the primer pairs listed in Tables 1 and 2.

### Construction of knockout and repair HearNPV bacmids containing ha2 promoter and egfp

HearNPV bacmids containing the ha2 promoter and the egfp cassette were generated by using the Bac-to-Bac protocol as described previously (15). To prepare bacmids for transposition, the helper plasmid pMON7124, which confers resistance to tetracycline and encodes a transposase, was transformed into DH10B cells harboring an ha2 knockout bacmid, HaΔha2. These cells were then transformed with donor plasmid pFBGpha2-X ("X" represents empty plasmid or wild-type [WT] or truncated ha2 fragments), incubated at 37°C for 4 h in 1 ml of SOC medium, and placed onto agar medium containing 50 μg/ml of kanamycin, 7 μg/ml of gentamicin, 10 μg/ml of tetracycline, 100 μg/ml of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and 40 μg/ml of IPTG (isopropyl-β-D-thiogalactopyranoside). Primers gp1 forward and M13 forward were used to confirm the phenotypes of recombinant bacmids by PCR.

The correct bacmid constructs were then electroporated back into *E. coli* DH10B cells and screened for tetracycline sensitivity to ensure that the isolated bacmids were free of helper plasmid artifacts. Equimolar amounts of purified bacmid DNA were transfected into Hz-AM1 cells (5 × 10^5) by use of a cationic liposome method. Transfection events were confirmed by egfp expression in purified bacmid DNA-transfected Hz-AM1 cells.

### DNA replication assay

To assess viral DNA replication, a quantitative real-time PCR assay was performed as previously described (24). To prepare total DNA for analysis, 5 × 10^6 Hz-AM1 cells were transfected with 2.0 μg of bacmid DNA. At selected time points, cells were harvested in 1 ml phosphate-buffered saline (PBS), lysed in 500 μl cell lysis buffer (10 mM Tris, pH 8.0, 100 mM EDTA, 20 μg/ml RNase A, 0.5% sodium dodecyl sulfate), incubated for 30 min at 37°C before the addition of 100 μg/ml of proteinase K, and incubated overnight at 56°C. Total DNA was phenol extracted, ethanol precipitated, and suspended in 300 μl of water. Prior to PCR, 10 μl of total DNA from each time point was digested with 10 units of DpnI restriction enzyme (Fermentas) for 24 h in a 50-μl total reaction volume. Quantitative PCR was performed with 1 μl of digested DNA added to SYBR green real-time PCR master mix (Toyobo) according to the manufacturer’s instructions and was analyzed on an Opticon sequence detection system under the following conditions: 50°C for 2 min, 95°C for 2 min, and 45 cycles of 95°C for 30 s and 60°C for 30 s, with a 500 μM concentration of each primer (477F and 643R). Cycle threshold (Ct) values were determined by automated threshold analysis using the Opticon software. Disso- ciation curves were recorded after each run.

### Transcriptional activity assays

Total RNA was extracted from HaΔha2-eGFP- and HaΔha2-ha2-transfected Hz-AM1 cells by using RNAzol according...
FIG. 1. Generation of recombinant bacmids. (A) Schematic diagram of construction of an ha2 knockout bacmid containing a deletion of the HearNPV ha2 gene by recombination in E. coli. A 663-bp portion of the ha2 open reading frame was deleted and replaced with the chloramphenicol resistance gene (cat). (B) Positions of primer pairs used in analysis of the WT locus and ha2 knockout locus to confirm the deletion of the ha2 open reading frame and the correct insertion of the cat cassette. Primers are indicated by arrows. (C) Confirmation by PCR analysis of the presence or absence of sequence modifications in HaΔha2 and Ha-WT. The virus analyzed is shown above each lane, and the primer pairs used.
to the supplier's protocol (Invitrogen). For detection of HearNPV gene expression, the RNA was pretreated for 30 min with RNase-free DNase (Promega) at 0.1 U/μg RNA. Reverse transcription (RT) reactions were carried out using 1 to 2 μg of DNase-treated RNA as the template, a random primer (Promega) as the primer, and Moloney murine leukemia virus reverse transcriptase (Promega). The RT reaction mix was incubated sequentially at 42°C for 60 min and 70°C for 15 min. The reaction was stopped by heating to 95°C for 5 min. 0.5 μl of RNase H (Promega) was added, and the reaction mix was incubated at 57°C for an additional 30 min. Afterward, the cDNA was stored at ~80°C.

PCRs using KOD Plus DNA polymerase (Toyobo) were carried out using cDNAs as templates. To detect specific transcripts, the following forward and reverse primers were used in the PCRs: p10F and p10R for the p10 gene (264-bp product) and vp39F and vp39R for the vp39 gene (882-bp product). Amplifications were carried out for 40 cycles at 94°C for 1 min, 54°C for 1 min, and 72°C for 45 s. For PCRs carried out directly with DNase-treated RNA preparations (controls for the absence of baculovirus genomic DNA contamination), aliquots equivalent to those present in the cDNA preparations used for PCR amplification were used as templates.

**Titration of BV and electron microscopy.** Hz-AM1 cells were infected with the indicated viruses at a multiplicity of infection (MOI) of 0.1. Supernatants were collected at 87 h postinfection (hpi), and cells were dislodged and pelleted at 5,000 × g for 5 min. The titers of supernatants were determined by end-point dilution assay with Hz-AM1 cells. Cells collected at 7 days postinfection were fixed, dehydrated, embedded, sectioned, and stained as described previously (21). Samples were viewed with a JEM-1230 transmission electron microscope at an accelerating voltage of 80 kV.

**Fluorescence microscopy analysis.** Hz-AM1 (1 × 10⁶) cells were grown on glass coverslips in petri dishes and infected with the indicated viruses. For the localization of cellular actin, infected cells were fixed at 60 hpi for 10 min on ice in 2% formaldehyde and 0.2% glutaraldehyde in PBS, washed once with PBS, solubilized with 0.15% Triton X-100, stained with rhodamine-phalloidin (Sigma), and then examined under a Leica S2 laser confocal scanning microscope for fluorescence. vHa2-eGFP was used as a control.

**Subcellular localization.** To detect the localization of HA2 and truncated HA2 fragments in insect cells, pIZ/V5-eGFP-X plasmids ("X" represents empty plasmid or WT or truncated fragments) in insect cells, pIZ/V5-eGFP-X plasmids were constructed. The N-terminal EGFP fusion protein in Hz-AM1 cells, were constructed. The egfp gene cassette and a cat cassette by homologous recombination in Hz-AM1 cells, were constructed. The egfp fragment, which was PCR amplified with primers egfp2 forward and egfp2 reverse, with pEGFP-N1 as the template, was inserted into the multicloning region of pIZ/V5 to produce pIZ/V5-eGFP. All of the ha2 fragments, which were digested from donor plasmids by EcoRI and XhoI, were subcloned into pIZ/V5-eGFP and fused with egfp. Hz-AM1 cells (1 × 10⁶) were transfected with these plasmids. By 48 h posttransfection, cells were fixed, stained with Hoechst (Beyotime), and then examined under a Leica S2 laser confocal scanning microscope for fluorescence. For coinfection experiments, transfected cells were infected with HearNPV G4 at an MOI of 5 at 6 hpt. At 72 hpi, cells were fixed, stained with Hoechst, and then observed under a laser confocal scanning microscope.

**RESULTS**

**Generation of recombinant bacmids.** To investigate the function of ha2 during the viral infection cycle, we constructed an ha2 knockout bacmid of HearNPV in which the major ha2 coding sequences were replaced with a cat gene cassette and a 579-bp segment of the 5' end of the ha2 coding sequence was retained to preserve the havPK promoter (Fig. 1A). Knockout of ha2 was confirmed by PCR (Fig. 1B). PCR products derived with primer pairs CATU/B1 and CATU/CATD from HaΔha2 were 1,039 bp long (Fig. 1C), confirming replacement of ha2 by the cat cassette. In contrast, no signal was detected with the WT control HaBacHZ8. The primer combinations A4/B3 and A5/B1 generated PCR products of 201 bp and 462 bp from HaBacHZ8, but they were unable to amplify PCR products from HaΔha2, indicating the loss of the 663 bp within the ha2 coding region. Furthermore, PCR analysis of HaΔha2 resulted in products of 1,702 bp and 1,039 bp with primer sets A1/CATD and A4/B1, respectively, while only a 663-bp product was obtained from HaBacHZ8 with primer set A4/B1 and no product was obtained with primer pair A1/CATD. Thus, these data indicated that the ha2 gene was deleted and replaced with the cat cassette by homologous recombination in HaΔha2.

To generate an ha2 repair bacmid, a transfer plasmid was constructed for transposition into the polyhedrin (polh) locus of the ha2 knockout bacmid HaΔha2. The transfer plasmid pFastBac Dual was first modified by replacing the polh promoter with a copy of the ha2 promoter (nt 1992 to 2082 of HearNPV) (4) and inserting a copy of egfp downstream of the p10 promoter. The ha2 and truncated ha2 fragments were then inserted downstream from the ha2 promoter. The truncated fragments were C129, C170, C194, C261, N193, N260, PWCA, HWCA, and WCA (Fig. 1D). After transposition, the recombinants were confirmed by PCR with the primers M13 forward and gfp1 forward (Table 1) (data not shown). The resulting repair bacmids were named HaΔha2-ha2, HaΔha2-C129, HaΔha2-C170, HaΔha2-C194, HaΔha2-C261, HaΔha2-N193, HaΔha2-N260, HaΔha2-PWCA, HaΔha2-HWCA, and HaΔha2-WCA (Fig. 1D). All of the recombinant bacmids contained egfp under the control of the p10 promoter and ha2 or truncated ha2 controlled by the native ha2 promoter. HaΔha2-eGFP and Ha-eGFP (5, 18) were the negative and positive controls, respectively.

**ha2 is essential for BV production.** The ha2 null (HaΔha2-eGFP), ha2 repair (HaΔha2-ha2), and WT control (Ha-eGFP) constructs were used in turn to transfect Hz-AM1 cells. Green fluorescence was detected at 72 hpt, and there was no difference among the three viruses, indicating comparable transfection efficiencies (Fig. 2A). However, fluorescence accumulated in both Ha-eGFP- and HaΔha2-ha2-transfected cells, while HaΔha2-transfected cells showed almost no increase in the number of infected cells (Fig. 2A), indicating that there was no spread of the virus beyond the cells initially transfected with the ha2 knockout bacmid DNA. When the supernatant was used to infect Hz-AM1 cells, HaΔha2-ha2 could set up infection, while HaΔha2-eGFP did not produce any EGFP at any time points analyzed for up to 168 h (Fig. 2A). These results indicated that ha2 is essential for infectious BV production. However, HaΔha2-eGFP could also produce EGFP under
the control of the very late gene promoter after transfection, implying that the HaΔha2-eGFP genome could still replicate. Quantitative real-time PCR was performed with DNAs from cells transfected with bacmids, and the results indicated that the HaΔ2 knockout bacmid was still able to synthesize nascent DNA at 72 hpt, like the HaΔ2 repair bacmid, suggesting that Ha2 is not involved in viral DNA replication (Fig. 2B).

We next analyzed the RNA content at 96 hpt of Hz-AM1 cells transfected with HaΔHa2-eGFP and HaΔHa2-ha2, using RT-PCR. As shown in Fig. 2C, the presence of transcripts originating from p10 and vp39 in both HaΔHa2-eGFP- and HaΔHa2-ha2-transfected cells demonstrated that transcription of the late and very late viral genes was not blocked by the lack of ha2.

The WCA domain of HA2 accelerates Arp2/3-mediated actin assembly in vitro. Transfection and infection analysis demonstrated that not only the entire ha2 gene rescued the infectious BV production defect, but some retrieved truncated ha2 fragments, including C129, C170, N260, PWCA, and HWCA (Fig. 3A), all of which contained the WCA domain (Fig. 1D), also did so. The other recombinants, vHaΔHa2-C194/vHaΔHa2-C261 and vHaΔHa2-N193, did not yield progeny upon infection (Fig. 3A). These results demonstrate that the WCA domain is essential for viral replication. The WCA domain of HA2 is a
conservative motif of the WASP family, which includes the G-actin-binding (WH2) domain, a cofilin homology sequence (C), and an acidic segment (A). These sequences have been shown to be necessary and sufficient for activating the Arp2/3 complex (9, 16). For in vitro analysis, we expressed and purified His fusion proteins containing the WCA, WC, or CA domain and first tested their ability to interact with actin in a pull-down assay. Actin bound to His-WC and His-WCA but not to His-CA (data not shown), indicating that WH2 is an actin-binding domain in HA2 (22). We then tested the ability to activate actin polymerization and found that the His-WCA fusion protein together with the Arp2/3 complex accelerated assembly, increased the elongation rate, and shortened but did not eliminate the lag phase, while the WC and CA fusions had almost no activity (data not shown) (9). Compared to full-length HA2, the His-WCA fragment was just as effective in stimulating actin polymerization.

A hydrophobic domain plays a pivotal role in localization/transportation of HA2. To our surprise, vHaΔha2-WCA, which contained the minimal WCA domain, could not produce infectious particles. Comparison of vHaΔha2-HWCA with vHaΔha2-WCA revealed that the insertion of vHaΔha2-HWCA had an extra 27 aa (aa 167 to 193) over the WCA domain, suggesting that this sequence played an important role in rescuing the defect of the knockout of ha2 associated with the WCA domain. Sequence analysis indicated that aa 167 to 193 were highly hydrophobic, and we therefore named the region the H domain.

It has been demonstrated that hydrophobic domains of baculoviral structural proteins, such as AcMNPV ODV-E66 and PIF-3, function as sorting motifs to transport fusion proteins to the nucleus and virions (10, 13). The results shown in Fig. 3B indicate that the distribution of EGFP-HA2 transiently expressed by plasmid was within the cytoplasm (panel c), while the EGFP control was diffused throughout the cells (panel a). The truncated ha2 fragments, including C129, C170, C194, C261, N193, N260, PWCA, HWCA, and WCA, were tagged with EGFP at the carboxyl terminus, and these recombinant plasmids were transfected into Hz-AM1 cells. Consistent with the full-length EGFP-HA2 fusion protein, C129 (Fig. 3B, panel e), C170 (Fig. 3B, panel g), N193 (Fig. 3B, panel i), N260 (Fig. 3B, panel k), PWCA (Fig. 3B, panel m), and HWCA (Fig. 3B, panel o) were preferentially localized in the cytoplasm, and no obvious nuclear accumulation was observed in over 90% of cells transfected. However, the remaining mutants, C194 (Fig. 3B, panel q), C261 (Fig. 3B, panel s), and WCA (Fig. 3B, panel u), spread throughout the whole cell, showing a similar distribution pattern to that observed in cells transfected with pIZ/
V5-eGFP; this was probably due to passive diffusion. After superinfection of HearNPV, the EGFP-HA2 fusion protein was transported from the cytoplasm into the nucleus (Fig. 3B, panel d), while EGFP alone was present throughout the whole cells all the time (Fig. 3B, panel b), as previously reported (5, 18). The truncated fragments C129 (Fig. 3B, panel f), C170 (Fig. 3B, panel h), N193 (Fig. 3B, panel j), N260 (Fig. 3B, panel l), PWCA (Fig. 3B, panel n), and HWCA (Fig. 3B, panel p), all of which were localized in the cytoplasm of the transfected cells, exhibited similar distribution patterns to that of full-length HA2 after infection with virus and were also transported into the nucleus. However, mutants C194 (Fig. 3B, panel r), C261 (Fig. 3B, panel t), and WCA (Fig. 3B, panel v) appeared to display the same distribution as EGFP alone and remained present throughout the whole cell after infection with virus. These results indicated that HA2 itself localizes in the cytoplasm and is recruited to the nucleus with the help of a viral factor(s) and that the H motif (aa 167 to s193) is essential for the localization and translocation of HA2.

**Infectivity of recombinant repair HearNPVs.** To further assess the infectivity and BV production of the recombinant HearNPVs, Hz-AM1 cells were synchronously infected with BV of the recombinant viruses and the titers were determined by an end-point dilution assay at 87 hpi. The titers of vHaΔha2-ha2 and vHa-eGFP were about 1.8 × 10^6 and 4 × 10^6 50% tissue culture infective doses (TCID_{50})/ml, respectively, which were not significantly different (Fig. 4). The mutants succeeding in rescuing the defect of infectious BV production showed delayed viral production and reduced titers. vHaΔha2-C129 and vHaΔha2-C170 produced only 3.2 × 10^5 and 1.1 × 10^5 TCID_{50}/ml, indicating that deleting the N terminus, including the proline-rich region, decreased the infectivity of the recombinant. Moreover, the deletion of C-terminal region PtdIns 4-kinase homology dramatically decreased the production of BV, as the titers of HaΔha2N260, HaΔha2-PWCA, and HaΔha2-HWCA were only 4.6 × 10^3, 2.2 × 10^3, and 0.6 × 10^3 TCID_{50}/ml, respectively, which were about 1,000-fold lower than those of vHaΔha2-ha2 and vHa-eGFP (Fig. 4). Thus, the C terminus of HA2 is significant for high-efficiency BV yields.

**HA2 is involved in virion assembly.** The titer results described above indicated that there could be some defect in BV production of the repaired viruses with truncated ha2 fragments. To further analyze whether the different domains affect nucleocapsid structure, electron microscopy was performed with virus-infected cells at 87 hpi (Fig. 5). Both WT and ha2 repair viruses produced characteristic normal occluded virions.
containing single nucleocapsids and surrounded by tight membranous envelopes (Fig. 5A and B). Compared to the WT, \( v\text{Ha}_{\text{H9004}} \text{ha2-C129} \) could also form many obvious typical nucleocapsids, but there still existed some abnormal structures. Some electron-lucent tubular structures were dispersed in the nucleus, indicating that they lack an electron-dense core, which would be indicative of nucleoprotein (Fig. 5C). \( v\text{Ha}_{\text{H9004}} \text{ha2-C170} \) had the same result (Fig. 5D). We also observed apparent defects in \( v\text{Ha}_{\text{H9004}} \text{ha2-N260} \)-infected cells, and there were abundant membranes without associated nucleocapsids and the same electron-lucent tubular structures (Fig. 5E). Besides a few normal virions, \( v\text{Ha}_{\text{H9004}} \text{ha2-PWCA} \) also formed a mass of free membranes without nucleocapsids and the same electron-lucent tubular structures (Fig. 5F). In \( v\text{Ha}_{\text{H9004}} \text{ha2-HWCA} \)-infected cells, normal occluded virions were very hard to find, and there was an abundance of electron-lucent tubular structures and some hollow tubular structure which probably lacked both envelopes and electron-dense core (Fig. 5G and H).

**Actin kinetics in** \( \text{Ha}_{\text{H9004}} \text{ha2-X}-\text{infected cells}. **Nuclear F-actin is essential for baculovirus infection** (20, 25), and the baculoviral WASP-like protein activates dynamic actin polymerization in the nucleus to enable viral replication and nucleocapsid morphogenesis (18, 28). Changes of the actin cytoskeleton were analyzed during the infection of \( \text{Hz-AM1} \) cells by different repair viruses (Fig. 6A). In \( v\text{Ha}_{\text{H9004}} \text{ha2-ha2} \)- and \( v\text{Ha}_{\text{H9004}} \text{eGFP} \)-infected cells, F-actin aggregated at the plasma membrane during the early stage of infection and then appeared within the nucleus (data not shown), and finally, microfilaments were transported to the cytoplasm and plasma membrane, again at a very late stage of infection (Fig. 6A, panels a to c and d to f), which agreed with the result that F-actin is transported from the cytoplasm into the nucleus and then out of it in the very late phase of baculovirus infection (7, 14, 19). Cells infected with \( v\text{Ha}_{\text{H9004}} \text{ha2-C129} \), \( v\text{Ha}_{\text{H9004}} \text{ha2-C170} \), and \( v\text{Ha}_{\text{H9004}} \text{ha2-N260} \) had a dramatically different configuration of microfilaments, which were located in the nucleus until 60 hpi. F-actin appeared within the nucleus and, more prominently, within the surrounding “ring zone” after \( \text{Hz-AM1} \) cells were infected with \( v\text{Ha}_{\text{H9004}} \text{ha2-C129} \) by 60 hpi (Fig. 6A, panels g to i). Phalloidin labeling of microfilaments in \( v\text{Ha}_{\text{H9004}} \text{ha2-N260} \)-infected cells showed a fine homogeneous network within the central virogenic stroma of the nucleus or the whole nucleus (Fig. 6A, panels m to o). \( v\text{Ha}_{\text{H9004}} \text{ha2-PWCA} \)- and \( v\text{Ha}_{\text{H9004}} \text{ha2-HWCA} \)-infected cells showed microfilaments with small amounts of F-actin aggregated in the plasma membrane (Fig. 6A, panels p to r and s to u). The different subcellular localizations of F-actin in the cells infected with different recombinants implied that they were at different replication stages. Moreover, \( \text{Hz-AM1} \) cells which were transfected with viruses \( \text{Ha}_{\text{H9004}} \text{ha2-eGFP} \) (Fig. 6B, panels a to c), \( v\text{Ha}_{\text{H9004}} \text{ha2-C194} \), and \( v\text{Ha}_{\text{H9004}} \text{ha2-C261} \).
(Fig. 6B, panels d to f), HaΔha2-C261 (Fig. 6B, panels g to i), HaΔha2-WCA (Fig. 6B, panels j to l), and HaΔha2-N193 (Fig. 6B, panels m to o), which could not produce infectious BV, exhibited microfilaments with little F-actin localized in either the plasma or nucleus.

**DISCUSSION**

Baculovirus WASP proteins, such as AcMNPV P78/83 and HearNPV HA2, are activators that direct actin polymerization-based motility of virions and further demonstrate that F-actin-dependent progeny morphogenesis appears to be a characteristic common to viruses that have lepidopteran hosts (12). A ha2 knockout bacmid revealed a phenotype that was unable to produce BV. This defect could be rescued by the repair of ha2, and the repaired virus was equivalent to the WT with respect to replication in host cells, indicating that ha2 was required for production of infectious BV. Quantitative real-time PCR results determined that the deletion of ha2 did not affect viral DNA replication and the transcription of the viral late and very late genes, suggesting that the defect of virus morphogenesis might be related to the assembly of the virus. In AcMNPV, the deletion of P78/83, an HA2 homologue, also leads to the defect in yielding progeny (9).

Electron microscopy performed on ha2 and truncated fragment repair viruses demonstrated that the lower efficiency of production of BV might be due to a defect in nucleocapsid assembly. The full-length repair virus produced a typical occluded virus, like the WT virus did. However, we observed virus assembly defects in the truncated ha2 fragment repair viruses. Together with the results of infectivity assays, the lower the titration was, the greater was the defect in virion organization. It has been reported that long tubular structures largely devoid of viral DNA are located primarily in the nucleus and are associated with the inner nuclear membrane of AcMNPV-infected cells in the presence of cytochalasin D (25). Excessively long tubular structures were located primarily in AcMNPV-infected cells in the presence of cytochalasin D (25). After actin cables are detected within the cytoplasm, the formation of F-actin aggregates at the plasma membrane occurs at the early stage of infection (7). F-actin then appears within the nucleus, and finally, microfilaments are transported to the cytoplasm and plasma membrane once again (19). The distribution of F-actin in vHaΔha2-ha2- and vHa-eGFP-infected cells showed that they were in the very late stage of infection at 87 hpi. However, the presence of F-actin suggested that vHaΔha2-C129, vHaΔha2-C170, and vHaΔha2-N260 might be in the late stage of infection at 87 hpi. Compared to the WT, these three viruses showed a delay of propagation which might be due to the retarding of actin assembly and further confirmed the lower efficiency of the BV titer. The quite different localization of F-actin in vHaΔha2-PWCA- and vHaΔha2-HWCA-infected cells pointed to a hypothesis that in these cases, it was difficult to recruit and polymerize F-actin in the nucleus, which would further affect the assembly of the virus. Those viruses which could not yield viable progeny might be the result of defects in nuclear actin assembly (9).

Sequence alignments indicate that HA2 contains motifs, such as the proline-rich region and the WCA domain, which are common to WASP or WASP-like proteins. The WCA domain of the HA2 fragment is able to interact with actin directly in vitro, and the WH2 domain is a G-actin-binding site, which is consistent with observations for Scar1/WAVE and WASP (16). In addition, the WCA domain is the minimal functional unit of HA2 necessary to activate the Arp2/3 complex and has a similar effect on actin polymerization kinetics (data not shown). Based on these findings, and combined with those reported from the observation of AcMNPV P78/83 (9), we conclude that WCA of the baculoviral WASP also appears to stimulate actin polymerization via the Arp2/3 complex.

However, the WCA region is essential but not enough to rescue the defective phenotype of the ha2 knockout. Comparing the truncated fragments in infectious viruses, we found that the repaired fragment in vHaΔha2-HWCA is the shortest one that contains the WCA domain and a 27-aa sequence (aa 167 to 193) which predominantly comprises hydrophobic amino acids such as leucine, isoleucine, and valine. Subcellular localization analysis of different truncated fragments indicated that this motif might be a nuclear localization signal or sorting motif (Fig. 5) similar to that found in baculoviral structural proteins, such as AcMNPV ODV-E66, and the PIF-3 hydrophobic domain responsible for trafficking of fusion protein to the nucleus and virion (10, 13). However, unlike the sorting motif domain of ODV-E66, which is sufficient to traffic fusion proteins to the inner nuclear membrane and the ODV envelope with an efficiency similar to that of the WT protein (2), HA2 requires another viral factor(s) for efficient transport from the cytoplasm to the nucleus, as reported for PIF-3 (13). Sequence BLAST results showed that there existed no homologous motif in the other ha2 homologues of lepidopteran baculoviruses. However, the sequence between the proline-rich and WCA domains is usually rich in hydrophobic amino acids, which might endow it with a conserved function.

PtdIns 4-kinase functions in the Pck1-mediated mitogen-activated protein (MAP) kinase cascade, and a distinct pool of PIP4 generated by PtdIns 4-kinase is critical for normal actin cytoskeleton organization (1). In the C terminus of HA2, there is an exclusive domain homologous to PtdIns 4-kinase which has not been reported for any WASP family protein to date. Interestingly, the deletion of the C terminus resulted in a dramatic decrease of virus infectivity. Compared to the WT, the BV titers of vHaΔha2-N260, vHaΔha2-PWCA, and vHaΔha2-HWCA were reduced almost 1,000 times. Although the C terminus of HA2 was not required for activating the
Arp2/3 complex or inducing actin polymerization, it is significant for a high efficiency of BV production. Further experiments will be performed to clarify its function.

In summary, we have demonstrated that the HA2 WCA region is required for actin polymerization by the Arp2/3 complex in vitro and for infectious virus assembly and production. At the C terminus, a PtdIns 4-kinase homology region is important for increasing the efficiency of BV progeny. The H motif of aa 167 to 193 is essential for the distribution and transport of HA2. Although the exact roles for some motifs in BV generation are unclear, our results give us a hint about the subcellular localization and elementary function of the HA2 domain in viral replication. Experiments are currently under way to determine how the HA2 domains work in virus morphogenesis.

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