Helicoverpa armigera nucleopolyhedrovirus occlusion-derived virus-associated protein, HA100, affects oral infectivity *in vivo* but not virus replication *in vitro*

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ORF100 (*ha100*) of *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV) has been reported as one of the unique genes of group II alphabaculoviruses encoding a protein located in the occlusion-derived virus (ODV) envelope and nucleocapsid. The protein consists of 510 aa with a predicted mass of 58.1 kDa and is a homologue of poly(ADP–ribose) glycohydrolase in eukaryotes. Western blot analysis detected a 60 kDa band in HearNPV-infected HzAM1 cells starting at 18 h post-infection. Transient expression of GFP-fused HA100 in HzAM1 cells resulted in cytoplasmic localization of the protein, but after superinfection with HearNPV, GFP-fused HA100 was localized in the nucleus. To study the function of HA100 further, an *ha100*-null virus was constructed using bacmid technology. Viral one-step growth curve analyses showed that the *ha100*-null virus had similar budded virus production kinetics to that of the parental virus. Electron microscopy revealed that deletion of HA100 did not alter the morphology of ODVs or occlusion bodies (OBs). However, bioassays in larvae showed that the 50 % lethal concentration (LC_{50}) value of HA100-null OBs was significantly higher than that of parental OBs; the median lethal time (LT_{50}) of *ha100*-null OBs was about 24 h later than control virus. These results indicate that HA100 is not essential for virus replication *in vitro*. However, it significantly affects the oral infectivity of OBs in host insects, suggesting that the association HA100 with the ODV contributes to the infectivity of OBs *in vivo*.

INTRODUCTION

Baculoviruses are large, dsDNA viruses pathogenic to a variety of insect species and many have been developed as alternative insecticides in agriculture and forestry. *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV; also called HaSNPV) was isolated from diseased larvae in the province of Hubei, China, and has been developed as a pesticide against the cotton bollworm in China (Sun *et al.*, 1998). As an initial step to genetically engineer HearNPV to improve its insecticidal activity, the genome of an *in vivo*-cloned strain, HaSNPV-G4 (Chen *et al.*, 2001), was sequenced. Many HearNPV genes have been investigated and their functions annotated (Dong *et al.*, 2005; Fang *et al.*, 2006; Pan *et al.*, 2007; Peng *et al.*, 2010; Song *et al.*, 2008; Tan *et al.*, 2008; Wu *et al.*, 2005). As such, HearNPV has become one of the best-characterized members of group II of the genus *Alphabaculoviruses*.

Recently, HA100, encoded by ORF100, was identified as an occlusion-derived virus (ODV)-associated protein using proteomics technology. Homologues of *ha100* are conserved among group II alphabaculoviruses, suggesting an important role for HA100 in the biology of this group of baculoviruses. HA100 has homology with poly(ADP–ribose) glycohydrolase (PARG), a ubiquitously expressed exo- and endoglycohydrolase in eukaryotic cells (Deng *et al.*, 2007). This enzyme, which is present in various isoforms in eukaryotes, is involved in the cleavage of ADP–ribose polymers. The enzyme may play a physiological role in modulating chromosome structure, transcription, DNA repair and apoptosis (Bonicalzi *et al.*, 2005). As most studies on PARG have focused on mammals and insects, it is of interest to determine the function of HA100 in the baculovirus multiplication cycle and why it is associated with ODVs.

In this paper, the function of HA100 was studied with particular reference to its expression and subcellular localization. We also constructed an *ha100*-null recombinant virus to investigate its role in virus biology, infectivity and replication. These studies provide further insights into the function of this unique protein in group II alphabaculoviruses.

RESULTS

Expression analysis

The *ha100* gene encodes a protein of approximately 58 kDa. A late transcription initiation motif, ATAAG, was found 37 nt upstream of the ATG translation start codon of *ha100* (Chen *et al.*, 2001) suggesting that *ha100* is a late gene. HA100 was detected from 18 to 96 h post-infection (p.i.) as a protein band of about 60 kDa by Western blot analysis (Fig. 1), which indicated that *ha100* is indeed a late gene and is expressed at a time when ODV proteins are made and assembled into ODVs.

Localization of HA100 protein in insect cells

To study the localization of HA100 in insect cells, pIZ/V5-*egfp/ha100* was constructed, in which the *egfp* gene is fused in frame to the N terminus of *ha100*, and used to transfect HzAM1 cells. The intracellular localization of HA100 was examined by confocal laser-scanning microscopy. The GFP-fused HA100 protein by itself was localized exclusively in the cytoplasm (Fig. 2c). When HzAM1 cells were superinfected with HearNPV, most GFP-fused HA100 was found in the nucleus (Fig. 2d). As a negative control, EGFP downstream of the same promoter showed diffuse fluorescence in the cytoplasm and nucleus in uninfected (Fig. 2a) or superinfected (Fig. 2b) HzAM1 cells.

Construction and infection of recombinant HearNPV viruses

Three recombinant bacmids, an *ha100* knockout bacmid (bHaBac*ha100*-KO-*ph*) where the polyhedrin gene (*ph*) was reinserted into the *ph* locus, a repair bacmid (bHaBac*ha100*-REP-*ph*) where *ha100* under the control of its own promoter was inserted at the *ph* locus and a wild-type HearNPV bacmid containing *egfp* and *ph* (bHaBac-*egfp-ph*) as a positive control were constructed as described in Methods (Fig. 3). All recombinants, carrying *egfp* driven by the *hsp70* promoter at the empty *ha100* locus, were authenticated by PCR and restriction enzyme analyses. Supernatant fluid from cells transfected with the *ha100* knockout bacmid, bHaBac*ha100*-KO-*ph*, was infectious to healthy HzAM1 cells [Fig. 4a, panels (i) and (iv)], indicating that HA100 is not essential for virus replication. Transfection and infection of the repair bacmid, bHaBac*ha100*-REP-*ph* [Fig. 4a, panels (ii) and (v)], and positive-control bacmid, bHaBac-*egfp-ph* [Fig. 4a, panels (iii) and (vi)], resulted in a similar infection process to that for bHaBac*ha100*-KO-*ph*.

Western blot analysis showed that HA100 was detectable at 72 h p.i. in cells infected with the virus vHaBac*ha100*-REP-*ph*, vHaBac-*egfp-ph* or wild-type HearNPV, with a band of ~58 kDa (Fig. 4b). The knockout virus vHaBac*ha100*-KO-*ph* did not synthesize the HA100 protein at a detectable level. Likewise HA100 was also not found in uninfected cells. Equal numbers of cells were used for blotting. These results confirmed that the recombinants were constructed correctly.

One-step growth curves of recombinant viruses

HzAM1 cells were infected with vHaBac*ha100*-KO-*ph*, vHaBac*ha100*-REP-*ph* and vHaBac-*egfp-ph* at the same m.o.i. One-step growth curves of budded viruses (BVs) are shown in Fig. 4(c). The results showed that vHaBac*ha100*-KO-*ph*, vHaBac*ha100*-REP-*ph* and vHaBac-*egfp-ph* had similar kinetics of BV production and that the titres of vHaBac*ha100*-KO-*ph* were similar to that of the parental virus at similar times after infection. Statistical analysis of each time point (12, 24, 48, 72 and 96 h p.i.) for these three recombinants viruses revealed that there was no significant difference between HA100-null virus and HA100-repaired virus or the parental virus ($P>0.05$).

Electron microscopic observations

To analyse further whether the deletion of *ha100* had any effect on virus morphogenesis, electron microscopic analysis was performed on cells infected with the different viruses and analysed at 72 h p.i. (Fig. 5). Cells infected with vHaBac*ha100*-KO-*ph* and vHaBac*ha100*-REP-*ph* exhibited cytopathological changes and viral morphogenesis typical of infection by a single nucleopolyhedrovirus and the two

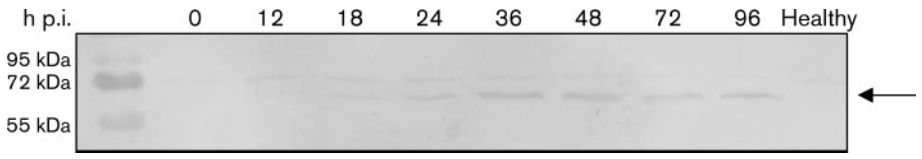

Fig. 1. Expression analysis of HearNPV *ha100*. Cellular proteins were harvested from HearNPV-infected cells at different time points p.i. and separated by SDS-PAGE (10% acrylamide gel). A polyclonal antibody against HA100 was used for Western blot analysis. HA100 is indicated by an arrow. Size markers (kDa) are shown in lane 1.

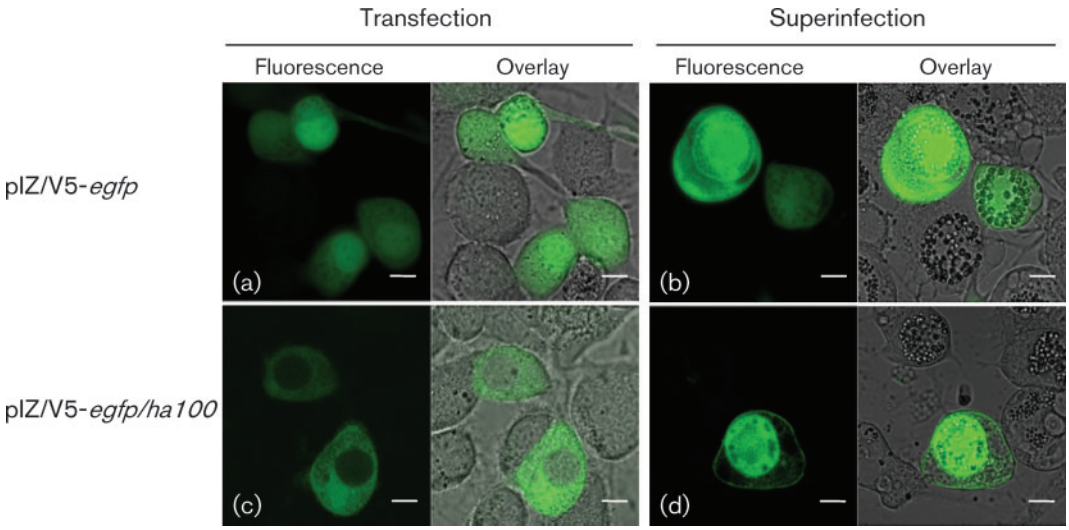

Fig. 2. Subcellular localization of EGFP-fused HA100. Confocal fluorescence analysis was employed to study the localization of the protein in infected cells. HzAM1 cells were transfected with pIZ/V5-*egfp* (a, b) or pIZ/V5-*egfp*/*ha100* (c, d) followed by superinfection (b, d) with HearNPV. Fluorescent microscopy was performed at 72 h p.i. Left panels are images under fluorescent microscopy and right panels show the overlay results under fluorescent and visible microscopy. Bars, 10 μm.

viruses were morphologically indistinguishable (Fig. 5a, b). The morphology of purified occlusion bodies (OBs) (vHaBac*ha100*-KO-*ph*; Fig. 5c) was similar to that of control-virus OBs (Fig. 5d). These studies appeared to demonstrate that HA100 does not play a major role in ODV morphogenesis and occlusion into OBs.

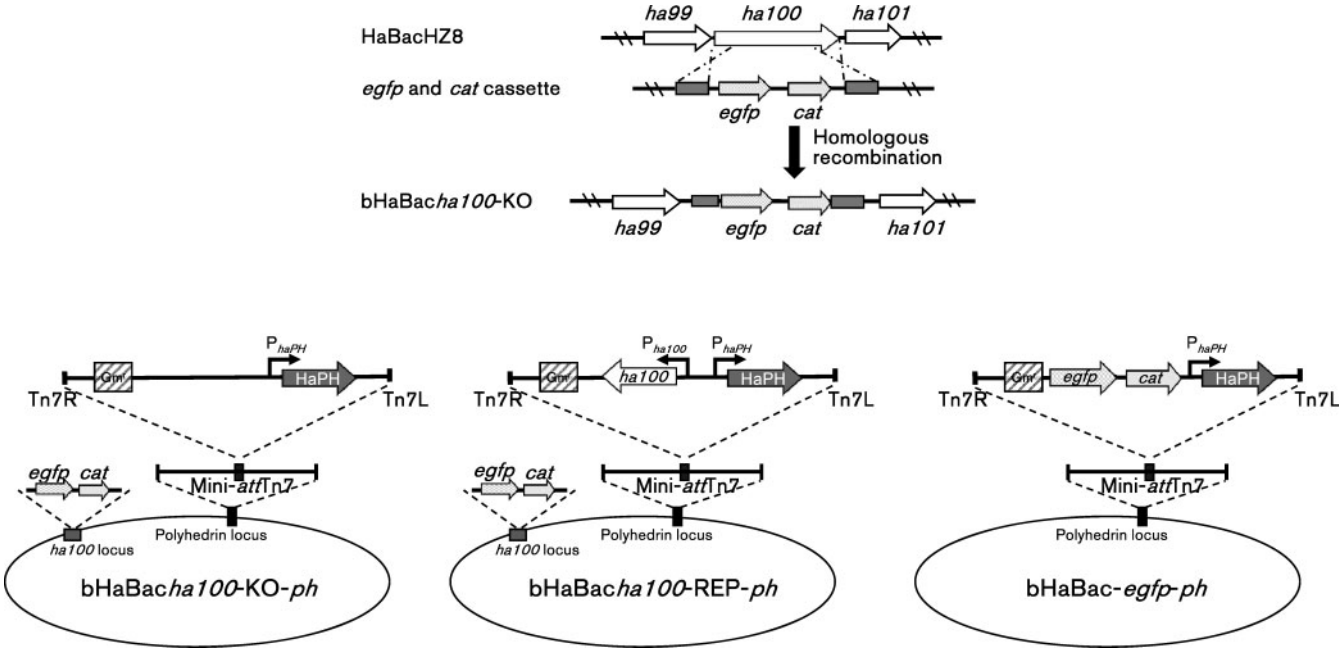

Fig. 3. Construction of recombinant bacmids. The chloramphenicol acetyltransferase (*cat*) and *egfp* genes flanked by *ha100* sequences (57 bp homologous arm) were used for homologous recombination to generate bHaBac*ha100*-KO. The *ph* gene was reintroduced into bHaBac*ha100*-KO to generate bHaBac*ha100*-KO-*ph*. The *ha100* and *ph* genes under their native promoters were inserted into the *ph* locus of HaBacHZ8 by Tn7-mediated transposition to generate bHaBac*ha100*-REP-*ph*. bHaBac-*egfp*-*ph* was a control virus containing EGFP as the marker. Gm^r, Gentamicin resistance gene.

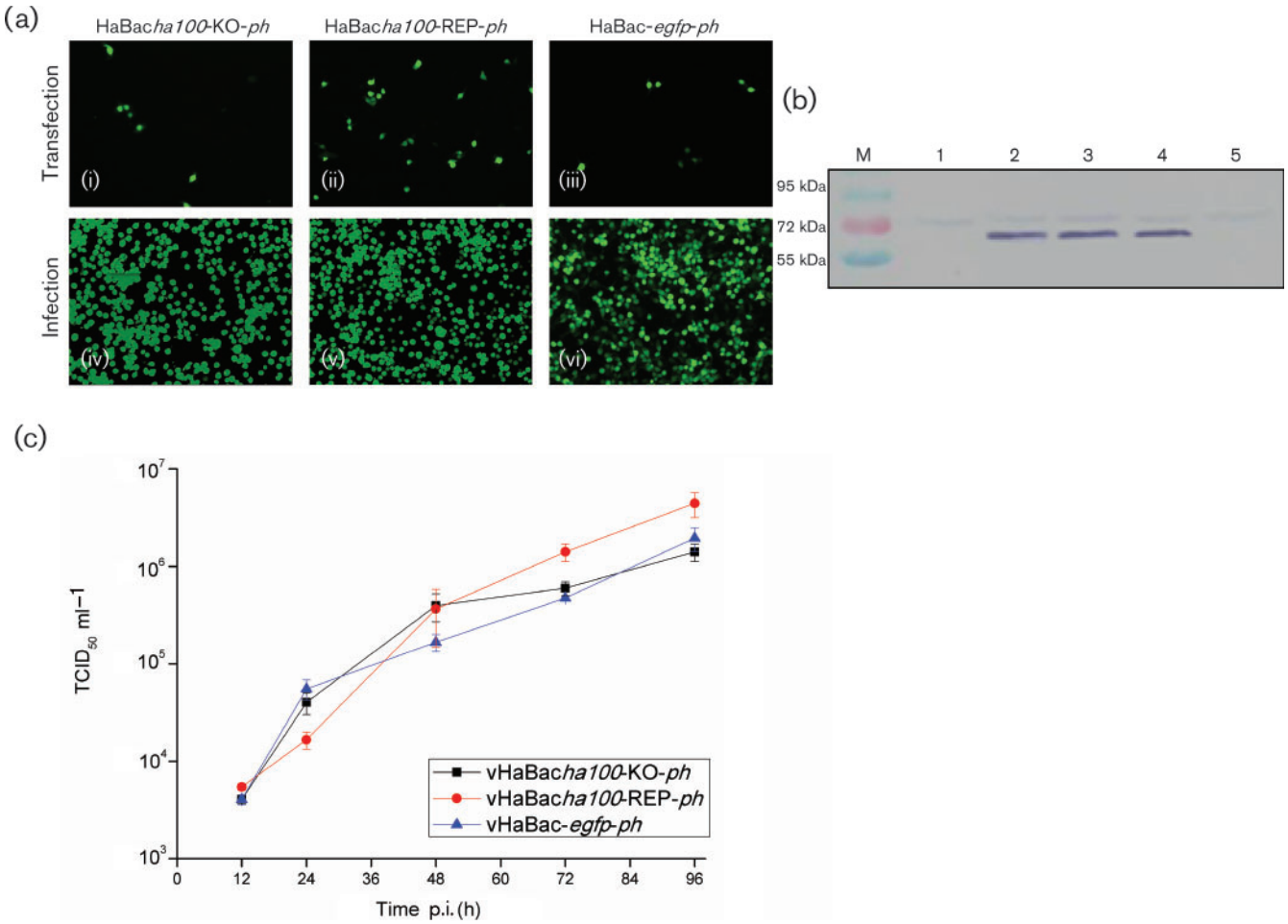

Fig. 4. (a) Transfection and infection analysis results. (i–iii) Fluorescence images of transfection with recombinant bacmid bHaBac*ha100*-KO-*ph*, bHaBac*ha100*-REP-*ph* or bHaBac-*egfp*-*ph* in HzAM1 cells, respectively. (iv–vi) Fluorescence images of infection with vHaBac*ha100*-KO-*ph*, vHaBac*ha100*-REP-*ph* or vHaBac-*egfp*-*ph* at 96 h p.i., respectively. (b) Detection of HA100 expression of the recombinant viruses. Cells were infected at an m.o.i. of 5 $TCID_{50}$ per cell with vHaBac*ha100*-KO-*ph* (lane 1), vHaBac*ha100*-REP-*ph* (lane 2), vHaBac-*egfp*-*ph* (lane 3) or HearNPV (lane 4) or were mock infected (lane 5). Cellular proteins were harvested at 72 h p.i. and separated by 10 % SDS-PAGE. An anti-HA100 polyclonal antibody was used for Western blot analysis. Molecular mass markers (kDa) are shown in lane M. (c) BV one-step growth curves of recombinants viruses. HzAM1 cells were infected with vHaBac*ha100*-KO-*ph*, vHaBac*ha100*-REP-*ph* or vHaBac-*egfp*-*ph*. BV titres were determined at the appropriate times p.i. by an end-point dilution method. Growth curves were generated by arithmetic mean data from three infections.

Biological activity of vHaBac*ha100*-KO-*ph*

To determine the 50 % lethal concentration (LC_{50}) values of vHaBac*ha100*-KO-*ph* and vHaBac*ha100*-REP-*ph*, third-instar *H. armigera* larvae were infected orally with selected doses of OBs and monitored for mortality. The LC_{50} value of vHaBac*ha100*-REP-*ph* was 1.2×10^4 ml^{-1}. In contrast, the LC_{50} of vHaBac*ha100*-KO-*ph* was 6.8×10^5 ml^{-1}, which was significantly higher than HA100-repaired OBs. Statistical analysis also indicated that the LC_{50} value of vHaBac*ha100*-KO-*ph* was significantly higher than that of vHaBac*ha100*-REP-*ph* (ratio potency=57.5; Table 1). The LC_{50} bioassay was repeated once with similar results (ratio potency=79.4). The median lethal time (LT_{50}) of vHaBac*ha100*-KO-*ph* was 114 ± 2.2 h p.i., whilst that of vHaBac*ha100*-REP-*ph* was 92 ± 2.4 h p.i. (Table 2). Survivor analysis indicated that there was a significant difference between the two viruses ($\chi^2=36.4$, $P=1.62 \times 10^{-9}$). Similar results were seen in parallel experiments; mortality resulting from infection with a *ha100*-null virus was delayed by 24 h ($\chi^2=29.7$, $P=5.09 \times 10^{-8}$). Therefore, the deletion of *ha100* from HearNPV impacts the efficacy of the virus in killing the host insect.

DISCUSSION

ha100 is one of the uncharacterized unique genes of group II alphabaculoviruses. This gene encodes a homologue of PARG in eukaryotes. In this paper, we studied the function

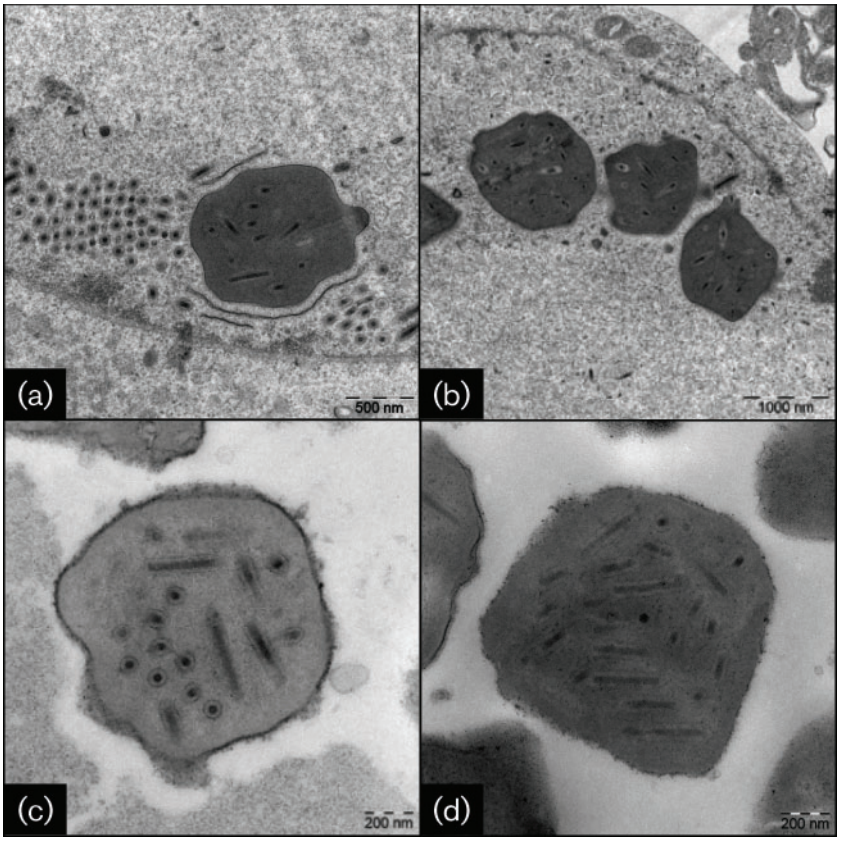

Fig. 5. Electron microscopic analyses. HzAM1 cells were infected with vHaBac*ha100*-KO-*ph* (a) or vHaBac*ha100*-REP-*ph* (b) at an m.o.i. of 5 $TCID_{50}$ per cell. Cells were fixed at 72 h p.i. and OB formation was observed. Purified OBs of HaBac*ha100*-KO-*ph* (c) and HaBac*ha100*-REP-*ph* (d) were also observed. Bars, 500 nm (a); 1000 nm (b); 200 nm (c, d).

of HA100 and its role in virus infectivity. Our analyses showed that the absence of *ha100* did not affect BV propagation in HzAM1 cells (Fig. 4c). Transient expression assays suggested that HA100 by itself localized in the cytoplasm and shuttled into the nucleus after super-infection. In addition, deletion of *ha100* did not appear to affect ODV or OB structure and morphogenesis. However, the *ha100*-null OBs resulted in higher LC_{50} values and longer LT_{50} times in third-instar *H. armigera* larvae, suggesting that the association of HA100 with ODVs could contribute to the virulence of HearNPV OBs. The results of this study showed that HA100 affects the oral infectivity of HearNPV *in vivo* but not replication of the virus *in vitro*.

Previous studies have shown that HA100 is associated with ODV nucleocapsid and envelope components (Deng *et al.*, 2007), which implies that the protein would localize to the ODV in the nucleus at the late stages of infection. Transient expression of GFP-fused HA100 in HzAM1 cells resulted in cytoplasmic localization of the protein, but following HearNPV infection the localization of GFP-fused HA100 changed from the cytoplasm to the nucleus, suggesting that HearNPV provides other factor(s) to chaperone HA100 to the nucleus. In a recent study, an interaction of HA100 was identified with VP80 (HA092; Chen *et al.*, 2001) by yeast two-hybrid and co-immunoprecipitation assays (Peng *et al.*, 2010). The gene for VP80 is highly conserved among baculoviruses and is one of the 'core genes' (Herniou & Jehle, 2007). VP80 is expressed late after infection and is found to be located in infected cell nuclei associated with nucleocapsids (Müller *et al.*, 1990; Wu *et al.*, 2008). Using VP80 knockout viruses, it was shown to be essential for BV

Table 1. Concentration causing mortality of vHaBac*ha100*-KO-*ph* and vHaBac*ha*100-REP-*ph* in third-instar *H. armigera* larvae

Test	Virus	LC_{50} (OBs ml^{-1})	95 % Confidence limit (OBs ml^{-1})		Ratio potency (95 % confidence limit)
			Lower	Upper	
1	HaBac*ha100*-KO-*ph*	6.8×10^5	4.4×10^5	11.8×10^5	57.5 (18.8–299.3)
	HaBac*ha100*-REP-*ph*	1.2×10^4	6.0×10^3	1.9×10^4	
2	HaBac*ha100*-KO-*ph*	6.0×10^5	4.0×10^5	9.8×10^5	79.4 (23.4–496.5)
	HaBac*ha100*-REP-*ph*	7.0×10^3	4.0×10^3	1.2×10^4	

Table 2. Time of mortality of vHaBac*ha100*-KO-*ph* and vHaBac*ha100*-REP-*ph* in third-instar *H. armigera* larvae

Test	Virus	LT_{50} with 95 % confidence interval (h p.i.)
1	HaBac*ha100*-KO-*ph*	114.0 (111.8–116.2)
	HaBac*ha100*-REP-*ph*	92.0 (89.6–94.4)
2	HaBac*ha100*-KO-*ph*	114.0 (112.0–116.0)
	HaBac*ha100*-REP-*ph*	90.0 (87.2–92.8)

and ODV assembly (Marek *et al.*, 2010). VP80 also localizes in the nucleus of *Bombyx mori* nucleopolyhedrosis virus-infected cells (Tang *et al.*, 2008). Further experiments will be performed to elucidate whether VP80 indeed plays a role in the transportation of HA100 into the nucleus and the contribution of HA100 in the assembly of ODVs.

Although HA100 has been identified as a structural protein of both the nucleocapsid and the envelope of ODVs (Deng *et al.*, 2007), our results showed that *ha100* is a non-essential gene for HearNPV and possibly all group II alphabaculoviruses. Moreover, growth-curve kinetics indicated that *ha100* had no effect on BV infectivity (Fig. 4c). Electron microscopy showed that the ODV morphology of the knockout virus was similar to that of control viruses, suggesting that HA100 is not a major protein of ODVs, as its absence did not affect the overall structure of ODV particles and the assembly process.

Bioassay experiments demonstrated that loss of HA100 impacted on HearNPV *per os* infectivity. Deletion of *ha100* resulted in a reduction in oral infectivity of OBs (LC_{50}) by six- to eightfold (Table 1) but had no effect on BV production and titres (Fig. 4c). In addition, survivor analysis results indicated that *ha100* affects the final mortality by accelerating mortality in infected larvae by about a day. Two types of viral processes, primary midgut infection and rapid BV transmission, contribute to the virulence by *per os* infection. So far, several baculovirus genes have been shown to be non-essential for *in vivo* infection but play an important role in accelerating the mortality of the host, i.e. *ac23*, *pe38*, *ac150* and *ac145*, or *sf29*. Deletion of *ac23* results in infectious virus with an extended median survival time (Lung *et al.*, 2003). The protein encoded by *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) *pe38* promotes rapid transmission of BV to tracheolar cells and thereby enhances the virulence of infection initiated *per os* (Milks *et al.*, 2003). In the case of virus infection of *Heliothis virescens* by AcMNPV lacking Ac150 and Ac145, the higher LC_{50} was due to poor establishment of primary infection (Lapointe *et al.*, 2004). In the case of *Spodoptera frugiperda* multiple nucleopolyhedrovirus, deletion of *sf29* reduced *per os* infectivity, which could be explained by an eightfold lower number of ODVs per OB (Simón *et al.*, 2008). In our study, the results confirmed that *ha100* plays a role in *in vivo* infectivity, but the mechanism by which *ha100*-null virus affects oral infection is unknown.

Previously, most investigations on PARG were carried out in higher eukaryotes. It has been reported that PARG is critical for the maintenance of steady-state poly(ADP–ribose) levels and plays important roles in modulating chromatin structure, transcription, DNA repair and apoptosis (Bonicalzi *et al.*, 2005). However, the presence of PARG in a virus has not been reported previously. As shown in Figs 1 and 4(b), an additional band could always be detected, even in mock-infected cells. It is possible that anti-HA100 antibody might recognize the homologue of an insect PARG. The results presented here suggest that, even though HA100 appears to be a homologue of PARG, its function in insect cells in conjunction with baculovirus nucleocapsids appears to be different from that in higher eukaryotes. Further investigations on HA100 may reveal more functions of this viral PARG-like protein in addition to what is already known from research on PARG in higher eukaryotes.

METHODS

Insect cells, virus and infection. HzAM1 cells (McIntosh & Ignoffo, 1983) were maintained in Grace's insect medium supplemented with 10 % FBS (Gibco-BRL) at 28 °C and used for HA100 expression analysis. An *in vivo*-cloned strain of HearNPV (HaSNPV-G4; Sun *et al.*, 1998) was used as the wild-type virus and propagated in fourth-instar *H. armigera*. An infectious bacmid, HaBacHZ8, of HearNPV has been described previously (Wang *et al.*, 2003) and was used as a bacmid source. Viral titres were determined by an end-point dilution assay (Vlak, 1979).

Expression analysis of HearNPV HA100. HzAM1 cells were infected with HearNPV at an m.o.i. of 5 $TCID_{50}$ per cell. At appropriate time points p.i., cells were collected and washed with PBS. Protein samples were separated by SDS-PAGE (10 % acrylamide gel) and transferred onto nitrocellulose membrane using a semi-dry transfer cell (Bio-Rad) as recommended by the manufacturer. The blot was probed with rabbit polyclonal anti-HA100 antibody (Deng *et al.*, 2007) followed by goat anti-rabbit IgG conjugated with alkaline phosphatase (Jackson ImmunoResearch). The signal was detected with a BCIP/nitro blue tetrazolium kit (SABC).

Localization of HA100 in HzAM1 cells. A plasmid containing the *egfp*-fused *ha100* gene was constructed to study the localization of HA100 in HzAM1 cells. The *egfp* gene was digested from pEGFP-C1 (Clontech) with *Nhe*I and *Bam*HI, and subcloned into pIZ/V5-His (Invitrogen) to generate pIZ/V5-*egfp*. The *ha100* sequence was amplified by PCR from HearNPV genomic DNA using primers 100CfusionF (5′-GCGAGATCTATGGCCGTTGAACAATTAAAACA-3′; *Bgl*II site underlined) and 100CfusionR (5′-GCGGATCCGC-CGCATGAATGTAGCGCTT-3′; *Bam*HI site underlined) and cloned into pIZ/V5-*egfp* to generate pIZ/V5-*egfp/ha100*. Plasmid DNA (5 μg) of pIZ/V5-*egfp/ha100* was transfected into HzAM1 cells with Lipofectin (Invitrogen) according to the manufacturer's protocol.

Plasmid pIZ/V5-*egfp* was used as a control. Superinfection of HzAM1 cells was conducted with HearNPV (m.o.i. of 5 $TCID_{50}$ per cell) at 12 h post-transfection. At 72 h p.i., the cells were fixed (4 % paraformaldehyde, 10 min) and observed in a confocal laser-scanning microscope (Leica).

Construction of the *ha100*-null HearNPV bacmid. The *ha100* gene in the HearNPV bacmid HaBacHZ8 (Wang *et al.*, 2003) was replaced by a gene cassette containing a chloramphenicol resistance (Cm^r) gene and an *egfp* gene controlled by the *hsp70* promoter. A 2.3 kb fragment containing the Cm^r and *egfp* genes with 57 bp upstream and downstream flanking sequences of *ha100* was PCR amplified from pKS-*egfp*-cm^r (kindly provided by Dr M. M. van Oers, Wageningen University, The Netherlands) with primers 100delF: 5′-ATGGCCGTTGAACAATTAAAACAGCTCAATGACATTCAAA-AATATTTGCTCGAGGCACTCGAGAAATTTCTCTGGCCG-3′ and 100delR: 5′-TTAATTTAAATTGATACTAGTTTGTTTTCTGTTTCG-TTTACTTGCAGATTTGGATTGAAGCTTTTTAAGGGCACCAATA-A-3′ (nucleotides identical to the *ha100* region are underlined) and transformed into *Escherichia coli* BW25113 containing HaBacHZ8 DNA. The *ha100*-null bacmid was generated by homologous recombination in *E. coli*, which were screened for kanamycin and chloramphenicol resistance as described previously (Fig. 3) (Hou *et al.*, 2002). Recombinant bacmids were identified by PCR, and the correct bacmid was designated bHaBac*ha100*-KO. As the original *ph* gene was disrupted during construction of the bacmid HaBacHZ8 (Wang *et al.*, 2003), *ph* was reintroduced into HearNPV *ha100*-deletion bacmids in the *ph* locus to produce bHaBac*ha100*-KO-*ph* (Fig. 3).

Construction of the *ha100*-rescued bacmid. A fragment containing *ha100* under the control of its own promoter was PCR-amplified with primers REP100F (5′-GGGGGATCCCAGTATTATGTGGATC-AGATG-3′) and REP100R (5′-ATAGGTACCCACAACGACAAATA-GCCGCATGAA-3′), cloned into the transfer vector pFB-DUAL-*ph* (Song *et al.*, 2008) and named pFB100-*ph*. The expression cassette of pFB100-*ph* was transposed into the Tn7 attachment site of bHaBac*ha100*-KO (Wang *et al.*, 2003). The resulting bacmid was named bHaBac*ha100*-REP-*ph* (Fig. 3). bHaBac-*egfp*-*ph* containing the *egfp*–Cm^r cassette and *ph* was used as a control (Fig. 3) (Song *et al.*, 2008).

Transfection and infection assays. Bacmid DNAs of bHaBac*ha100*-KO-*ph*, bHaBac*h*a100-REP-*ph* and bHaBac-*egfp*-*ph* were purified from 5 ml cultures. DNA (1 μg) was used to transfect 5×10^5 HzAM1 cells with the aid of Lipofectin (Invitrogen). After 120 h, the cells were centrifuged at 4000 r.p.m. in a microfuge for 10 min. The supernatants were used to infect 6×10^5 HzAM1 cells for 96 h. All the transfected and infected cells were viewed for fluorescence. Western blot analysis with infected cells was performed to detect HA100 expression. The HzAM1 cells were infected with vHaBac*ha100*-KO-*ph*, vHaBac*ha100*-REP-*ph*, vHaBac-*egfp*-*ph* and HearNPV at an m.o.i. of 5 $TCID_{50}$ per cell. Cells were collected at 72 h p.i., and cellular proteins were separated by 10 % SDS-PAGE, blotted onto nitrocellulose membranes and HA100 was detected as described above.

One-step growth curves of recombinant viruses. HzAM1 cells (2×10^5) were infected with vHaBac*ha100*-KO-*ph*, vHaBac*ha100*-REP-*ph* or vHaBac-*egfp*-*ph* at an m.o.i. of 5 $TCID_{50}$ per cell. Supernatants were collected at various time points (12, 24, 48, 72 and 96 h p.i.) and titres were measured by an end-point dilution assay using green fluorescence as an indicator of infection. The experiment was performed in triplicate for each virus and growth curves were generated by arithmetic mean data of three infections. The titres of the three viruses were compared with one-way analysis of variance (SPSS Inc.).

Electron microscopy. HzAM1 cells (1×10^6) were infected with vHaBac*ha100*-KO-*ph* or vHaBac*ha100*-REP-*ph* at an m.o.i. of 5 $TCID_{50}$ per cell. Infected cells were harvested at 72 h p.i and washed twice with PBS. The OBs of the recombinant viruses (vHaBac*ha100*-KO-*ph* or vHaBac*ha100*-REP-*ph*) were obtained by intrahaemocoelic injection of BVs into late third-instar *H. armigera* larvae and purified from diseased larvae as described previously (Sun *et al.*, 1998). All samples were processed for electron microscopy as described elsewhere (van Lent *et al.*, 1990).

Bioassays. In order to determine whether the *ha100* deletion had any effect on the infectivity of HearNPV, bioassays were performed as described previously (Wang *et al.*, 2003). The OBs of recombinant viruses (vHaBac*ha100*-KO-*ph* or vHaBac*ha100*-REP-*ph*) were purified from separately infected *H. armigera* larvae. The LC_{50} of the viruses was determined by droplet bioassay by exposing third-instar larvae to five concentrations of OBs: 1×10^4, 3×10^4, 1×10^5, 3×10^5 and 1×10^6 OBs ml^{-1} for 10 min and then transferred to a fresh diet. Forty-eight insects were tested per dose and the mortality was checked daily. The experiment was performed twice. The LC_{50} values were determined by probit analysis and compared by standard lethal dose ratio comparison (SPSS Inc.).

The LT_{50} of the viruses was determined with third-instar larvae using a food-contamination method: 10 μl OBs (1×10^6 OBs ml^{-1}) of the viruses was applied to a small amount (3 mm^3) of the diet of early third-instar *H. armigera* larvae. The intake was sufficient to kill 100 % of the larvae. Time 0 was defined as the point at which larvae were transferred to the fresh diet. Forty-eight insects were used for each virus and the mortality was checked every 6 h. The LT_{50} values of the two viruses were calculated using the Kaplan–Meier estimator and further compared using a log-rank test (Mantel–Cox).

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REFERENCES

Bonicalzi, M.-E., Haince, J.-F., Droit, A. & Poirier, G. G. (2005). Regulation of poly(ADP–ribose) metabolism by poly(ADP–ribose) glycohydrolase: where and when? *Cell Mol Life Sci* **62**, 739–750.

Chen, X. W., IJkel, W. F. J., Tarchini, R., Sun, X., Sandbrink, H., Wang, H., Peters, S., Zuidema, D., Lankhorst, R. K. & other authors (2001). The sequence of the *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus genome. *J Gen Virol* **82**, 241–257.

Deng, F., Wang, R., Fang, M., Jiang, Y., Xu, X., Wang, H., Chen, X., Arif, B. M., Guo, L. & other authors (2007). Proteomics analysis of *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus identified two new occlusion-derived virus-associated proteins, HA44 and HA100. *J Virol* **81**, 9377–9385.

Dong, C., Li, D., Long, G., Deng, F., Wang, H. & Hu, Z. (2005). Identification of functional domains required for HearNPV P10 filament formation. *Virology* **338**, 112–120.

Fang, M., Nie, Y., Wang, Q., Deng, F., Wang, R., Wang, H., Wang, H., Vlak, J. M., Chen, X. & Hu, Z. (2006). Open reading frame 132 of *Helicoverpa armigera* nucleopolyhedrovirus encodes a functional *per os* infectivity factor (PIF-2). *J Gen Virol* **87**, 2563–2569.

Herniou, E. A. & Jehle, J. A. (2007). Baculovirus phylogeny and evolution. *Curr Drug Targets* **8**, 1043–1050.

Hou, S., Chen, X., Wang, H., Tao, M. & Hu, Z. (2002). Efficient method to generate homologous recombinant baculovirus genomes in *E. coli*. *Biotechniques* **32**, 783–784, 786, 788.

Lapointe, R., Popham, H. J. R., Straschil, U., Goulding, D., O'Reilly, D. R. & Olszewski, J. A. (2004). Characterization of two *Autographa californica* nucleopolyhedrovirus proteins, Ac145 and Ac150, which affect oral infectivity in a host-dependent manner. *J Virol* **78**, 6439–6448.

Lung, O. Y., Cruz-Alvarez, M. & Blissard, G. W. (2003). Ac23, an envelope fusion protein homolog in the baculovirus *Autographa californica* multicapsid nucleopolyhedrovirus, is a viral pathogenicity factor. *J Virol* **77**, 328–339.

Marek, M., van Oers, M. M., Deveray, F. F., Vlak, J. M. & Merten, O.-W. (2010). Engineering of baculovirus vectors for the manufacture of virion-free biopharmaceuticals. *Biotechnol Bioeng* n/a. (Epub ahead of print).

McIntosh, A. H. & Ignoffo, C. M. (1983). Characterization of five cell lines established from species of *Heliothis*. *Appl Entomol Zool (Jpn)* **18**, 262–269.

Milks, M. L., Washburn, J. O., Willis, L. G., Volkman, L. E. & Theilmann, D. A. (2003). Deletion of *pe38* attenuates AcMNPV genome replication, budded virus production, and virulence in *Heliothis virescens*. *Virology* **310**, 224–234.

Müller, R., Pearson, M. N., Russell, R. L. & Rohrmann, G. F. (1990). A capsid-associated protein of the multicapsid nuclear polyhedrosis virus of *Orgyia pseudotsugata*: genetic location, sequence, transcriptional mapping, and immunocytochemical characterization. *Virology* **176**, 133–144.

Pan, X., Long, G., Wang, R., Hou, S., Wang, H., Zheng, Y., Sun, X., Westenberg, M., Deng, F. & other authors (2007). Deletion of a *Helicoverpa armigera* nucleopolyhedrovirus gene encoding a virion structural protein (ORF107) increases the budded virion titre and reduces *in vivo* infectivity. *J Gen Virol* **88**, 3307–3316.

Peng, K., Wu, M., Deng, F., Song, J., Dong, C., Wang, H. & Hu, Z. (2010). Identification of protein–protein interactions of the occlusion-derived virus-associated proteins of *Helicoverpa armigera* nucleopolyhedrovirus. *J Gen Virol* **91**, 659–670.

Simón, O., Williams, T., Asensio, A. C., Ros, S., Gaya, A., Caballero, P. & Possee, R. D. (2008). *Sf29* gene of *Spodoptera frugiperda* multiple nucleopolyhedrovirus is a viral factor that determines the number of virions in occlusion bodies. *J Virol* **82**, 7897–7904.

Song, J., Wang, R., Deng, F., Wang, H. & Hu, Z. (2008). Functional studies of *per os* infectivity factors of *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus. *J Gen Virol* **89**, 2331–2338.

Sun, X., Zhang, G.,, Zhang, Z., Hu, Z., Vlak, J. M. & Arif, B. M. (1998). *In vivo* cloning of *Helicoverpa armigera* single nucleocapsid nuclear polyhedrosis virus genotypes. *Virol Sin* **13**, 83–88.

Tan, Y., Jiang, L., Wang, M., Yin, F., Deng, F., Liu, M., Hu, Z. & Wang, H. (2008). Mutagenesis and nuclear magnetic resonance analyses of the fusion peptide of *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus F protein. *J Virol* **82**, 8138–8148.

Tang, X.-D., Xu, Y.-P., Yu, L.-L., Lang, G.-J., Tian, C.-H., Zhao, J.-F. & Zhang, C.-X. (2008). Characterization of a *Bombyx mori* nucleopolyhedrovirus with *Bmvp80* disruption. *Virus Res* **138**, 81–88.

van Lent, J. W., Groenen, J. T., Klinge-Roode, E. C., Rohrmann, G. F., Zuidema, D. & Vlak, J. M. (1990). Localization of the 34 kDa polyhedron envelope protein in *Spodoptera frugiperda* cells infected with *Autographa californica* nuclear polyhedrosis virus. *Arch Virol* **111**, 103–114.

Vlak, J. M. (1979). The proteins of nonoccluded *Autographa californica* nuclear polyhedrosis virus produced in an established cell line of *Spodoptera frugiperda*. *J Invertebr Pathol* **34**, 110–118.

Wang, H., Deng, F., Pijlman, G. P., Chen, X., Sun, X., Vlak, J. M. & Hu, Z. (2003). Cloning of biologically active genomes from a *Helicoverpa armigera* single-nucleocapsid nucleopolyhedrovirus isolate by using a bacterial artificial chromosome. *Virus Res* **97**, 57–63.

Wu, D., Deng, F., Sun, X., Wang, H., Yuan, L., Vlak, J. M. & Hu, Z. (2005). Functional analysis of FP25K of *Helicoverpa armigera* single nucleocapsid nucleopolyhedrovirus. *J Gen Virol* **86**, 2439–2444.

Wu, W., Liang, H., Kan, J., Liu, C., Yuan, M., Liang, C., Yang, K. & Pang, Y. (2008). *Autographa californica* multiple nucleopolyhedrovirus 38K is a novel nucleocapsid protein that interacts with VP1054, VP39, VP80, and itself. *J Virol* **82**, 12356–12364.