**Bombyx mori** nucleopolyhedrovirus *BmP95* plays an essential role in budded virus production and nucleocapsid assembly

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**INTRODUCTION**

The family *Baculoviridae* comprises a diverse group of insect-specific DNA viruses, and is characterized by a circular dsDNA genome that varies in size from approximately 80 to 180 kb, packaged into a rod-shaped capsid and enclosed by a lipid envelope (Herniou et al., 2003; Jakubowska et al., 2006). Two structurally and functionally distinct virion phenotypes, budded virus (BV) and occlusion-derived virus (ODV), are produced in the biphasic life cycle (Theilmann et al., 2003). ODVs are responsible for the primary infection of insects, whereas BVs are involved in systemic infection from cell to cell within an infected host.

Although the two virion forms are genetically identical, their envelope source and composition differ and they are produced at different times (Funk et al., 1997). Upon baculovirus infection, viral DNA replication and late gene transcription occur within electron-dense structures within the nuclei called virogenic stroma (VS). The viral genomes appear to be condensed into capsid structures within the nuclei to form nucleocapsids (Lu et al., 1997). Mature nucleocapsids initially migrate from the nucleus and move to the modified plasma membrane, from which they bud to form BVs (Monsma et al., 1996). During the very late phases of infection, nucleocapsids remain within the nucleus, where they align with intranuclear vesicle membranes and acquire an envelope to form ODVs. The different compositions of the envelopes of BVs and ODVs reflect their distinct functions in the viral life cycle.

The viral nucleocapsids are tubular in shape with a flat disk at the basal end, a cylindrical capsid sheath and a nipple cap at the apical end (Fraser, 1986). It has been proposed that P6.9 (Wang et al., 2010a), VP39 (Thiem & Miller, 1989), VP1054 (Olszewski & Miller, 1997), P78/83 (Russell et al., 1997), VLF-1 (Vanarsdall et al., 2006), FP25 (Braunagel et al., 1999, 2001), BV/ODV-C42 (Braunagel et al., 2001), ODV-EC27 (Vanarsdall et al., 2007), vP80 (Marek et al., 2011), P24 (Wolgamot et al., 1993), AC141 (EXON0; Fang et al., 2007), AC142 (McCarthy et al., 2008) and AC98 (38K; Wu et al., 2008) are associated with BV and ODV nucleocapsids. P95, a homologue of Ac83 of *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV), is a virion capsid protein of both BV and ODV and was originally characterized as VP91 in *Oryzias pseudotsugata* MNPV (OpMNPV; Russell & Rohrmann, 1997). The viral nucleocapsids are tubular in shape with a flat disk at the basal end, a cylindrical capsid sheath and a nipple cap at the apical end (Fraser, 1986). It has been proposed that P6.9 (Wang et al., 2010a), VP39 (Thiem & Miller, 1989), VP1054 (Olszewski & Miller, 1997), P78/83 (Russell et al., 1997), VLF-1 (Vanarsdall et al., 2006), FP25 (Braunagel et al., 1999, 2001), BV/ODV-C42 (Braunagel et al., 2001), ODV-EC27 (Vanarsdall et al., 2007), vP80 (Marek et al., 2011), P24 (Wolgamot et al., 1993), AC141 (EXON0; Fang et al., 2007), AC142 (McCarthy et al., 2008) and AC98 (38K; Wu et al., 2008) are associated with BV and ODV nucleocapsids. P95, a homologue of Ac83 of *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV), is a virion capsid protein of both BV and ODV and was originally characterized as VP91 in *Oryzias pseudotsugata* MNPV (OpMNPV; Russell & Rohrmann, 1997). Proteomic analysis identified the homologues of *BmNPV* P95 as a component of ODV of AcMNPV (Braunagel et al., 2003), *Culex nigripalpus* NPV (CuniNPV; Perera et al., 2007), *Pieris rapae* granulovirus (PrGV; Wang et al., 2011) and *Helicoverpa armigera* NPV (HeaNPV; Hou et al., 2013), but a recent study of BV structural proteins of AcMNPV and HeaNPV did not determine the homologues of BmP95 (Braunagel et al., 2003; Hou et al., 2013; Wang et al., 2010b). The role of BmP95 in the *Bombyx mori* NPV (BmNPV) life cycle is still unknown, except for the observation that BmP95 can stimulate gene expression driven by its own promoter and...
the promoter of the cytoplasmic actin gene of *B. mori* (Lu et al., 1998). Characterization of the components of the AcMNPV *per os* infectivity factor (PIF) complex found that P95 is a component of the PIF complex, suggesting that P95 is involved in *per os* infectivity (Peng et al., 2012). The failure to construct a deletion mutant of *BmP95* in BmNPV (*Bm69*) suggested that it is probably an essential gene for *in vitro* infection (Rohrmann, 2011). Whether *BmP95* is involved in the nucleocapsid assembly process is still unclear, so further work with a *BmP95* deletion mutant is necessary to study its functionality in baculovirus infection.

In this study, we took advantage of a BmNPV bacmid to construct a *BmP95* deletion mutant virus via homologous recombination in *Escherichia coli*. The results indicated that *BmP95* is essential for BV production. However, its deletion did not affect viral DNA replication. Electron microscopy analysis demonstrated that deletion of *BmP95* had an obvious effect on nucleocapsid morphology, suggesting that *BmP95* is required for normal nucleocapsid assembly. Normal nucleocapsids were produced when the *BmP95* deletion bacmid was repaired with full-length *BmP95* under the control of its own promoter. We also generated a repair bacmid containing the N-terminal fragment of *BmP95* driven by its own promoter, which enabled us to determine whether the C-terminal region is required for its function. In this way, we showed that the C-terminal region of *BmP95* is also required for normal nucleocapsid formation.

**RESULTS**

**Sequence analysis**

To date, 57 baculovirus genomes have been completely sequenced and analysed: 53 (genera *Alphabaculovirus* and *Betabaculovirus*) are pathogenic to lepidopteran insects, and three (genus *Gammabaculovirus*) are able to infect hymenopteran insects, whereas only CuniNPV has a mosquito (Diptera) host. The *BmP95* gene (ORF69) of BmNPV is a core gene and encodes a putative protein of 839 aa, and its homologues are found in all baculoviruses. Fig. 1(a) shows a phylogenetic tree of a selection of *BmP95* homologues from each baculovirus genus. Homologues of *BmP95* from the genera *Alphabaculovirus*, *Gammabaculovirus* and *Deltabaculovirus* clustered together, whilst those from the genus *Betabaculovirus* appeared to be more diverse than the other baculoviruses. Fig. 1(a) shows a phylogenetic tree of a selection of *BmP95* homologues from each baculovirus genus. Homologues of *BmP95* from the genera *Alphabaculovirus*, *Gammabaculovirus* and *Deltabaculovirus* clustered together, whilst those from the genus *Betabaculovirus* appeared to be more diverse than the other baculoviruses. When an amino acid sequence alignment was performed, a conserved, highly hydrophobic transmembrane domain was found, located in the N-terminal region of the P95 protein (Fig. 1b). Fig. 1(c) shows the predicted functional domain of the selection of *BmP95* homologues,
showing that homologues of BmP95 from members of the Alphabaculovirus and Gammabaculovirus genera had two functional domains: Pfam:Baculo_VP91_N and ChtBD2; however, those from members if the genera Betabaculovirus and Deltabaculovirus only had the Pfam:Baculo_VP91_N domain, which coincided with the results of the phylogenetic tree and suggested that P95 may have different functions in different genera.

**Construction of BmP95 knockout and repair bacmids**

To investigate the function of BmP95 in the BmNPV infection cycle, a BmP95-deleted bacmid was constructed by use of the Red homologous recombination system in *E. coli*, as described previously (Wu et al., 2006). A 1686 bp fragment of the BmP95 locus region was replaced by the chloramphenical resistance gene (Cm<sup>R</sup>) (Fig. 2a). The replacement of BmP95 with the Cm<sup>R</sup> gene was verified by PCR analysis using two specific primer pairs, BmP95-F/BmP95-R and BmP95De-F/BmP95De-R. As expected, PCR with primer pairs BmP95-F/BmP95-R produced a single fragment of 2520 bp from the WT BmNPV bacmid, but a 1934 bp fragment from vBmP95-De. Similarly, a 1200 bp fragment was produced from BmBacP95-D using the primer pair BmP95De-F/BmP95De-R, but did not generate a fragment from the WT BmNPV bacmid (Fig. 2b). Therefore, these results indicated that BmP95 was successfully deleted from its locus in the BmNPV bacmid DNA.

To examine whether deletion of BmP95 had any effect on the occlusion body morphogenesis and to facilitate observation of virus infection, the polh and gfp genes were transposed into the polh locus of the WT BmNPV bacmid and the BmBacP95-D bacmid by Tn7-mediated transposition to ChitBD2, chitin-binding peritrophin A domain.

**Fig. 1.** Evolutionary conservation of BmP95. (a) Phylogenetic analysis (neighbour joining) with bootstrap analysis was conducted using MEGA v4.0 software. Ten representative sequences were selected and the NCBI Protein database accession numbers of the protein sequences used were as follows: NP_047486.1 for BmNPV ORF69, NP_054113.1 for AcMNPV ORF83, NP_046242.1 for OpMNPV ORF86, YP_001036371.1 for Spodoptera frugiperda MNPV B (SimNPv-B) ORF79 and NP_258347.1 for Spodoptera littoralis NPV (SpliNPV) ORF79 (Alphabaculovirus); NP_068303.1 for Plutella xylostella GV (PlxGV) ORF84, NP_148888.1 for Cydia pomonella GV (CpGV) ORF101 and YP_003429409.1 for PrGV ORF85 (Betabaculovirus); YP_025191.1 for Neodiprion sertifer NPV (NeseNPV) ORF84 (Gammabaculovirus); and NP_203339.1 for CuniNPV ORF35 (Deltabaculovirus). (b) Amino acid sequence alignment of the N terminus of the BmP95 homologues. The alignment was performed using CLUSTAL_X v2.11 and edited with GeneDoc software. The transmembrane domain was predicted using the TMPred program (http://www.ch.embnet.org/software/TMPRED_form.html) and is indicated by a box. The different shadings of the amino acids represent the following: black background, 100 % conservation; dark grey, > 80 %; light grey, > 60 %. (c) Analysis of the function domain of the BmP95 homologues. The function domain predictions were made using the SMART program (http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1). ChitBD2, chitin-binding peritrophin A domain.
generate the WT control and vBmP95-De bacmid (Fig. 2a).
To rescue and confirm that the characteristics of the BmP95 deletion were due to removal of the BmP95 gene, a repair bacmid, vBmP95-Re, was constructed by inserting BmP95 under the control of its original promoter as well as polh and gfp into the polh locus by transposition. To determine further whether the functional domain of BmP95 was enough for its function, another repair bacmid, vBmP95-N-Re, was constructed, which contained the N-terminal 500 aa residues of BmP95 driven by its native promoter, in addition to the polh and gfp genes (Fig. 2a and Fig. S1 available in JGV online). Transposition events were confirmed by PCR analysis with primer pair M13F/M13R (Fig. 2c).

Analysis of viral replication in BmN cells
To examine the effect of the BmP95 deletion on virus replication, BmN cells were transfected with the WT, vBmP95-De, vBmP95-Re and vBmP95-N-Re bacmids. Expression of GFP in transfected BmN cells was observed by fluorescence microscopy. At 24 h post-transfection (p.t.), no significant difference was observed among the four viruses, indicating relatively equal levels and efficiencies of transfection (Fig. 3a). By 96 h p.t., GFP fluorescence was observed in almost all WT- and vBmP95-Re transfected cells, suggesting that infectious BV was generated from the initially transfected cells (Fig. 3a). However, vBmP95-De-transfected cells showed no increase in number (Fig. 3a), indicating that the BmP95 knockout bacmid was unable to produce infectious BVs to initiate secondary infection. Similarly, there was also no spread of infection for the vBmP95-N-Re bacmid, indicating that the N-terminal region of BmP95 could not rescue the defective phenotype of the BmP95 deletion bacmid (Fig. 3a).

At 96 h p.t., light microscopy analysis revealed that polyhedra were formed in BmN cells transfected with all
the constructs (Fig. 3b). However, the number of vBmP95-De-transfected cells containing polyhedra corresponded only to individual cells (Fig. 3b). Similar results for vBmP95-N-Re were observed, which was consistent with the results of fluorescence microscopy (Fig. 3b). In contrast, most of the WT and vBmP95-Re transfected cells contained polyhedra, indicating production of BV and spread of the infection (Fig. 3b). These results indicated that P95 deletion led to a defect in infectious BV production but did not affect the formation of polyhedra.

To better assess the effect of deleting BmP95 on virus replication and determine the replication kinetics of the virus constructs, virus growth curve analysis was performed. BmN cells were transfected with each bacmid DNA and at selected time points the BV titres were determined using a TCID₅₀ end-point dilution assay. As expected, BmN cells transfected with WT and vBmP95-Re revealed a steady increase in BV production that reached equivalent titres, and their growth kinetics were similar (Fig. 3c). In contrast, the titre was undetectable at any time point up to 96 h p.t. for vBmP95-De- and vBmP95-N-Re-transfected cells, indicating that no infectious BV was produced (Fig. 3c). These results confirmed that the defect could be rescued by insertion of intact BmP95 into the polh locus of the BmP95 deletion bacmid but not the N-terminal region of BmP95, indicating that full-length BmP95 is required for infectious BV production in BmN cells.

Because the TCID₅₀ assay determines the production of the infectious BV, the titres were also determined by quantitative PCR (qPCR) analysis, which detects the
number of viral genomes regardless of infectivity. BmN cells were transfected with each bacmid, and at various times p.t., BV production was analysed by qPCR (Fig. 3d). Because of the bacmid transfection, there was a background level of viral genomes detected at all time points for all viruses. As expected, in WT- and vBmP95-Re-transfected cells, there was a pronounced increase in BV production from 24 to 96 h p.t. (Fig. 3d). However, there was no increase in BV production detected above the background in vBmP95-De- and vBmP95-N-Re-transfected cell supernatants at any time point (Fig. 3d). Therefore, the qPCR results were in agreement with the TCID50 results. In combination, these data demonstrated that full-length BmP95 is required for BV production in the cultured cells.

**BmP95 does not affect viral DNA replication**

Observation of occlusion body-like structures in vBmP95-De-transfected cells suggested that DNA replication was not severely affected by deletion of BmP95 (Fig. 2c). To quantitatively determine whether deletion of BmP95 impacted on viral DNA replication, the levels of viral DNA replication in vBmP95-De- and vBmP95-Re-transfected BmN cells were detected by qPCR analyses. At the designated time points, total intracellular DNA was extracted from equal amounts of bacmid-transfected cells and treated with DpnI prior to PCR amplification. From 6 to 24 h p.t., similar amounts of viral DNA were obtained from BmN cells transfected with vBmP95-De and vBmP95-Re, as shown by detection of the gp41 gene (Fig. 4). The DNA replication levels generated by vBmP95-Re-transfected cells exhibited a steady increase from 24 to 96 h p.t., with DNA synthesis reaching a plateau at 72 h p.t. (Fig. 4). However, the DNA synthesis levels of vBmP95-De-transfected cells showed no distinct increase (Fig. 4). Thus, the infectious BVs initiated secondary infection, leading to a continuous increase in DNA synthesis levels for vBmP95-Re. For vBmP95-De, which does not produce BV, DNA replication was restricted primarily to the initially transfected cells. These data indicated that the onset of viral DNA replication in BmN cells was unaffected by the deletion of BmP95.

**Electron microscopy analysis of bacmid-transfected BmN cells**

The TCID50 and qPCR results indicated that BmP95 is essential for BV production. To analyse further the cause of the defect in BV production and to investigate whether BmP95 affected the assembly of nucleocapsids and the occlusion of ODV to form functional polyhedra, electron microscopy analysis was performed. BmN cells transfected with vBmP95-Re exhibited the typical characteristics of baculovirus infection, such as enlargement of the nucleus (Fig. 5a), the presence of electron-dense VS with an extensive number of rod-shaped nucleocapsids (Fig. 5b), numerous virus-induced intranuclear microvesicles emerging and pre-occluded virions forming in the ring zone (Fig. 5c), and mature virions (ODVs) embedded in the polyhedra (Fig. 5d). However, in contrast to cells transfected with vBmP95-Re, although a well-defined VS was observed in vBmP95-De transfected cells (data not shown), the nucleocapsid structures were morphologically distinguishable from those observed in vBmP95-Re-transfected cells. Clusters of aberrant nucleocapsid structures were observed to localize to the electron-dense edges of the VS and inner nuclear membrane in vBmP95-De-transfected cells (Fig. 5e–g, arrowheads). The aberrant nucleocapsid structures could be divided into three forms: electron-lucent and elongated tubular structures (Fig. 5e, arrowhead), tubular structures with incomplete viral DNA genomes (Fig. 5f, arrowhead) and electron-dense tubular structures that were longer than normal (Fig. 5g, arrowheads). The electron-lucent tubular structures seemed to be empty capsid sheaths, indicating that the viral DNA genomes failed to be condensed and packaged into these tubular structures. The tubular structures in Fig. 5(f) and (g) appeared to contain the viral DNA genomes; however, the genomes were unable to be packaged into the capsid sheaths accurately, and these electron-dense tubular structures were larger and longer than normal nucleocapsids. Similar phenomena were also seen in cells transfected with vBmP95-N-Re, implying that this defect could not be rescued by supplying only the selected N-terminal domain of BmP95 (Fig. 5i–k, arrowheads). These results indicated that full-length BmP95 is crucial for proper capsid assembly.

In vBmP95-De-transfected cells, bundles of nucleocapsids aligning with intranuclear vesicle membranes, acquiring envelopes and forming mature ODV could be observed clearly (Fig. 5c). However, for vBmP95-De and vBmP95-N-Re, the bundles of nucleocapsids never aligned with the intranuclear microvesicle membranes, and no enveloped
virions formed ODVs (Fig. 5f, j). Enveloped virions were occluded into the polyhedra within the ring zone of vBmP95-Re-transfected cells (Fig. 5d). The shape and size of the polyhedra in the vBmP95-De- and vBmP95-N-Re-transfected cells were comparable to those in vBmP95-Re-transfected cells; however, no virions were observed to be embedded in the polyhedra (Fig. 5h, l). These observations suggested that deletion of BmP95 did not affect the morphogenesis of polyhedra but disrupted the maturation of ODVs.

Taken together, these data indicated that BmP95 is not required for the formation of VS, intranuclear microvesicles and polyhedra but does affect the proper assembly of nucleocapsids and subsequent BV formation, ODV envelopment and embedding of ODVs into polyhedra.

**DISCUSSION**

BmNPV BmP95 and its homologues are conserved and encoded by all baculoviruses and are also found in nudiviruses (Wang *et al.*, 2007) and probably in several insect genomes, such as that of *Anopheles gambiae* (Rohrmann, 2011). However, the function of BmP95 in the baculovirus life cycle remains largely unknown. In this report, we showed that the virus with a BmP95 deletion was unable to propagate and that infection was restricted to the

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**Fig. 5.** Transmission electron microscopy analysis of BmN cells transfected with vBmP95-Re (a–d), vBmP95-De (e–h) and vBmP95-N-Re bacmid (i–l) at 60 h p.t. (a) Image of a whole cell displaying the VS, enlarged nucleus (Nu), nuclear membrane (Nm) and polyhedra (P). Cyt, cytoplasm. (b) Electron-dense nucleocapsids (white arrow) in the VS. (c) Virus-induced intranuclear microvesicles (black arrow), nucleocapsids aligning with the intranuclear vesicles membranes and pre-occluded enveloped virions in the ring zone (white arrow). (d) Normal virions (white arrow) embedded in the polyhedra (P). (e, i) Masses of electron-lucent tubular structures (arrowheads) observed at the VS and inner nuclear membrane. (f, j) Tubular structures with incomplete viral DNA genomes (arrowheads) appeared in the nucleus. In (f) and (i), virus-induced intranuclear microvesicles are indicated by a black arrow. (g, k) Electron-dense and aberrant tubular structures (arrowheads) were seen in the VS. (h, l) Polyhedra (P) devoid of embedded virions.
initially transfected cells, TCID₅₀ end-point dilution assays and qPCR analysis confirmed that no infectious BVs were produced (Fig. 3). To analyse whether or not the defect in BV production was due to inhibition of viral DNA replication, a qPCR assay was performed to compare the initiation and levels of viral DNA replication in bacmid-transfected cells (Fig. 4). The results indicated that the onset and levels of DNA replication in individually transfected cells were unaffected by deletion of BmP95. Our results indicated that BmP95 is required for nucleocapsid assembly and subsequently for BV production, the formation of ODVs and functional polyhedra.

Electron microscopy demonstrated that the failure of production of BVs was due to a defect in the assembly of nucleocapsids. During the early stages of infection, a virus-induced specific nuclear region appears, the VS, which is thought to be the active site for viral DNA replication, condensation and packaging into capsids (Fraser, 1986). The newly formed nucleocapsids are transported from the VS to the ring zone periphery of the inner nuclear membrane and then egress from the nucleus to form BVs in the early stage of infection, or acquire envelopes from intranuclear microvesicles to form ODVs in the late stage of infection (Marek et al., 2011). In the present study, masses of aberrant capsid structures were observed in vBmP95-De-transfected cells. The electron-lucent tubular structures represented incomplete capsid particles containing no viral DNA genomes (Fig. 5e, i). Similar phenomena were also observed in the nuclei of S9 cells transfected with vlf-1, 38K, ac53, alkaline nuclease and ac79 knockouts of the AcMNPV bacmid (Liu et al., 2008; Okano et al., 2007; Vanarsdall et al., 2006; Wu & Passarelli, 2012; Wu et al., 2006). Masses of electron-lucent tubular structures were observed to remain apart from the VS, localizing instead to the inner nuclear membrane of cells transfected with the vlf-1-knockout bacmid. It was proposed that VLF-1 is required for normal capsid assembly and serves an essential function during the final stages of the DNA packaging process (Vanarsdall et al., 2006). In 38K-knockout bacmid-transfected cells, it was found that aberrant capsid structures of normal length appeared at the electron-dense edges of the VS, indicating that 38K is important for facilitating DNA packaging into viral capsids (Wu et al., 2006). In ac53-knockout bacmid-transfected cells, many lucent tubular shells devoid of viral DNA genomes were present in the VS and ring zone, implying that Ac53 might be involved in the efficient condensation of viral DNA or packaging of viral DNA into these tubular structures (Liu et al., 2008). Clusters of elongated tubular structures in the ring zone were monitored in cells transfected with the ac79-knockout bacmid (Wu & Passarelli, 2012). However, in our present study, masses of electron-lucent tubular structures of elongated length were present in the inner nuclear membrane of cells transfected with the BmP95-deletion bacmid, suggesting that transportation of these aberrant structures from the VS to the ring zone had occurred and that BmP95 has a role in nucleocapsid assembly. However, some electron-dense tubular structures with aberrant size and shape were monitored, seeming to contain viral DNA genomes. On occasion, aberrant capsid structures of normal length appeared in the inner nuclear membrane but were not completely filled with viral DNA genomes. These observations indicated that BmP95 is essential for the precise condensation of viral DNA or packaging of the newly replicated viral genomes into these tubular structures, and that it might be involved in the processing of the long tubular structures to generate legitimate precursors (Fig. 5). The aberrant tubular structures were certainly unable to egress from the nucleus and bud through the cytoplasm, leading to the loss of BV production. In previous studies, Ac66, Ac93 and Bm61 were shown to be involved in nucleocapsid egress from the nucleus to the cytoplasm (Ke et al., 2008; Shen & Chen, 2012; Yuan et al., 2011). Later during infections, normal nucleocapsids aligned with abundant virus-induced intranuclear microvesicles to form enveloped ODVs and subsequently embedded in the polyhedra. However, the polyhedra observed in vBmP95-De-transfected cells were completely devoid of ODVs. Although some vesicle-like structures were present within the ring zone, these aberrant tubular structures never aligned with them and no enveloped virions could be observed (Fig. 5e-g, i-k). These results suggested that the normal nucleocapsid is a prerequisite for ODV morphogenesis and that formation of polyhedra does not require mature ODVs. The phenotype resulting from the BmP95-knockout bacmid could be rescued by reinsertion of full-length BmP95 into the polh locus of the deletion bacmid, but not the N-terminal (functional domain) of BmP95, demonstrating that the C-terminal region is also required for the function of BmP95.

Recently, P95 has been reported to be a component of the PIF complex, and it was demonstrated that PIF1, PIF2, PIF3 and PIF4 form a stable core complex, whilst P95 and P74 are more loosely associated with this complex (Peng et al., 2012). The initiation of baculovirus infection in the midgut is mediated by PIFs. So far, seven PIF proteins have been identified, P74 (Faulkner et al., 1997), PIF1 (Kikhno et al., 2002), PIF2 (Pijlman et al., 2003), PIF3 (Ohkawa et al., 2005), PIF4 (Fang et al., 2009), PIF5 (ODV-E56) (Sparks et al., 2011; Xiang et al., 2011a) and PIF6 (Ac68) (Nie et al., 2012). After ODVs are released from the polyhedra, the peritrophic matrix (PM) is the first barrier for ODV infection of the midgut epithelial cells. The PM is composed of chitin, mucopolysaccharides and other proteins, and lines the insect midgut and protects the midgut epithelial cells from mechanical damage and micro-organisms (Hegedus et al., 2009). Conserved domain analysis of the homologues of BmP95 has shown that P95 from members of the genera Alphabaculovirus and Gammanabaculovirus has a ChtBD2 domain, indicating that P95 in these two genera might have chitin-binding ability. However, no ChtBD2 existed in members of the genera Betabaculovirus and Deltabaculovirus (Fig. 1). Coincidently, some baculoviruses encode metalloproteases, known as
enhancins, which are concentrated in the polyhedra and facilitate infection by degrading mucus, a PM component (Wang & Granados, 1997). Nevertheless, only a limited number of baculoviruses encode these enhancins, and it remains unknown how viruses that lack this enzyme pass through the PM. In a previous study, Ac145 and Ac150 were shown to be involved in per os infection and were predicted to encode chitin-binding domains (Lapointe et al., 2004). Therefore, the potential chitin-binding ability of BmP95 might be associated with mediating interactions between ODVs and the PM, or between ODVs and epithelial cells.

In conclusion, full-length BmP95 is required for the production of infectious BVs and is involved in viral nucleocapsid assembly. As P95 is a component of the PIF complex and might be associated with per os infection, it therefore appears to be a bifunctional protein. Further work is necessary to clarify its potential functionality in virus infection in vivo.

METHODS

Cells and viruses. The B. mori cell line BmN was cultured at 27 °C in TC-100 insect medium supplemented with 10 % (v/v) FBS (Gibco). The E. coli strains BW25113 containing plasmid pKD46 and BW25141 harbouring plasmid pKD3 (encoding the CmR gene) were kindly provided by Mary Berlyn (Yale University, CT, USA). The E. coli strain BmDH10Bac containing the BmNPV genome and a helper plasmid pMON7124 was constructed previously in our laboratory.

Construction of the BmP95 knockout bacmid. A recombinant bacmid containing a deletion of BmP95 was generated by homologous recombination in E. coli as described previously (Wu et al., 2006). A CmR cassette with BmP95 (BmNPV ORF69; GenBank accession no. NC_001962) flanking regions was amplified from the pKD3 plasmid using primers BmP95De-F (5'-GGCGATGCTGAGAATGTCGCCAGACAA-TTACACGTGTTCGACAAAAACCGGCTGATGTTAGTGGCTGAG-GCTGCT-3') and BmP95De-R (5'-GTCGACACCAAGATGTTAAT-TGACGGCCGTTGATGTTAGTGGCTGTCTCATATATGATATC-CTCCTT-3') (underlined sequences are homologous to the coding regions of BmP95), and 657 bp of the 5' end and 174 bp of the 3' end were retained so that the deletion would not affect transcription of the adjacent genes (Bm68 and Bm70). The E. coli strain BW25113/pKD46/BmBac competent cells were electrotransformed with the PCR fragment of the CmR cassette. The electrotransformed cells were then mixed with 800 μl pre-heated SOC medium (2 % Bacto tryptone, 0.5 % Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) and incubated at 37 °C for 2 h. The cells were collected and plated onto Luria-Bertani plates containing 50 μg kanamycin cm⁻² and 7 μg chloramphenicol ml⁻¹ for 48 h. Finally, colonies resistant to chloramphenicol and kanamycin were selected and verified by PCR with the primers BmP95F (5’-GAATTCTAGATGTCGCCATGATGTTAGTGGCTGAG-GCTGCT-3’) and BmP95R (5’-CTCGAGTCAGATGTCGCCATGATGTTAGTGGCTGAG-GCTGCT-3’), and BmP95De-F and BmP95De-R.

The identified BmNPV bacmid was extracted and electrophoresed into E. coli DH10β, named DH10β/BmBac/BmP95-De. Subsequently, the helper plasmid pMON7124 was chemically transformed into DH10β/BmBac/BmP95-De to generate DH10β cells containing both the BmP95-deleted bacmid and the helper plasmid, designated DH10β/BmBac/BmP95-De/helper.

Construction of BmP95-deleted, repair and positive-control bacmids containing polh and egfp. To facilitate observation of virus infection, the polh and egfp genes were inserted into the polh locus by Tn7-mediated transposition in the Bac-to-Bac system (Ge et al., 2008; Wu et al., 2006). The polh promoter from pFastBac1 (Invitrogen) was removed, and the intact polh gene (coding sequence plus native promoter and poly(A) signal region) was amplified from the BmNPV genome by PCR using the primer pair PH-F (5’-GAATTCTAGATGTCGCCATGATGTTAGTGGCTGAG-GCTGCT-3’) and PH-R (5’-CTCGAGTCAGATGTCGCCATGATGTTAGTGGCTGAG-GCTGCT-3’) (EcoRI and SmaBI sites are underlined, respectively). The polh and egfp genes driven by the AcMNPV ie1 promoter were inserted to generate the donor plasmid pFB1-PH-GFP. The donor plasmid was then transformed into the DH10β/BmBac/BmP95-De/helper- and DH10β/BmBacmid/helper-competent cells to generate the vBmP95-De bacmid and WT bacmid, respectively.

To generate the BmP95 repair bacmid tagged with the influenza haemagglutinin (HA) epitope (GYPDYDPDYASL) at the C terminus, a 2950 bp repaired fragment was amplified from the BmNPV bacmid using the primer pair BmP95Re-F (5’-CGGAATTCATGTCGCCAGGACAA-TTACACGTGTTCGACAAAAACCGGCTGATGTTAGTGGCTGAG-GCTGCT-3’) and BmP95Re-R (5’-TAATCTTCCACCGTCGCCAGGACAA-TTACACGTGTTCGACAAAAACCGGCTGATGTTAGTGGCTGAG-GCTGCT-3’), and subcloned into pFB1-PH-GFP to generate the repaired transfer plasmid pFB1-BmP95-De-PH-GFP. The DH10β/BmBac/BmP95-De-PH-GFP helper-competent cells were chemically transformed with the repaired transfer plasmid to generate the BmP95 repair bacmid vBmP95-De-Re.

To determine whether the C-terminal region of BmP95 is required for BmP95 function, another repair bacmid containing the native promoter and the N-terminal 500 aa of BmP95 was constructed. The primer pair BmP95Re-F and BmP95-Re-RR (5’-TACGAATGTTCGACCGGTGATGTTAGTGGCTGAG-GCTGCT-3’, Spel site underlined) were used to amplify the native promoter and the N-terminal region of BmP95. The construction of the vBmP95-N-Re bacmid was as described above. The primer pair M13F (5’-GTTTTCCAGTCGACGAC-3’) and M13R (5’-CAGAAAACAGCTATGAC-3’) were used to verify the successful transposition.

Time-course analysis of BV production. BmN cells (1.0 × 10⁶ cells per 35 mm diameter plate) were transfected with 2.0 μg DNA extracted from each bacmid (WT, vBmP95-De, vBmP95-Re and vBmP95-N-Re) using Cellfectin liposome reagent (Invitrogen Life Technologies) (Campbell, 1995). The supernatant containing BV was harvested at various times post-transfection (p.t.) and BV titres at 6 and 72 h p.t. were titrated by qPCR assay. The time-course analysis of BV production was performed as described previously (Vanarsdall et al., 2006). The DNA replication in infected BmN cells, a qPCR assay was performed using an OMEGA Viral DNA kit. An aliquot of each purified DNA sample (6 μl) was mixed with 12.5 μl SYBR Premix Ex Taq (TaKaRa) and the qPCR primers in a 25 μl reaction volume. The primers were designed to amplify a 100 bp genomic fragment of the chitinase gene. The samples were analysed on an ABI 7300 Real-Time PCR System using the following conditions: one cycle of 95 °C for 30 s, and 40 cycles of 95 °C for 5 s and 60 °C for 30 s. BV titres at 6 and 72 h p.t. were also titrated by a TCID₅₀ end-point dilution assay.

Quantitative analysis of viral DNA replication. To analyse viral DNA replication in infected BmN cells, a qPCR assay was performed as described previously (Vanarsdall et al., 2005). BmN cells (1.0 × 10⁶ cells per 35 mm diameter plate) were transfected with 2.0 μg vBmP95-De and vBmP95-Re bacmid DNA and at the indicated time points the cells were collected. Total DNA from each sample was extracted using a Classic Genomic DNA Isolation kit (Bio Basic)
according to the manufacturer’s protocol. Prior to PCR, 5 µl total DNA from each time point was digested with 2 U DpnI overnight in a 20 µl reaction volume to remove the input bacmid. Digested DNA (10 µl) was combined with 12.5 µl SYBR Premix Ex Taq and the qPCR primers (amplifying a 100 bp region of the gp41 gene) in a 25 µl reaction volume, and the PCR was performed as described above.

**Electron microscopy.** BmN cells (2.0 × 10^6 cells per 35 mm diameter plate) were transfected with 2.0 µg vBmP95-De, vBmP95-Re and vBmP95-N-Ne bacmid DNA. At 60 h p.t., the cells were harvested and washed once with PBS (pH 7.2). The cells were then fixed, dehydrated, embedded, sectioned and stained as described previously (Xiang et al., 2011b). Samples were viewed with a JEM-1230 transmission electron microscope at an accelerating voltage of 80 kV.

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