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AcMNPV PKIP is associated with nucleocapsid of budded virions and involved in nucleocapsid assembly

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ABSTRACT

Autographa californica multiple nucleopolyhedrovirus (AcMNPV) *orf24* (*pkip*) is a unique *Alphabaculovirus* gene. A previous study showed that a temperature-sensitive mutant of AcMNPV with a mutation in *pkip* displayed severe defects in progeny budded virion (BV) production and very late gene transcription, however, the underlying mechanism has not been determined. To investigate the function of *pkip* in the baculovirus replication cycle, we constructed a *pkip*-knockout AcMNPV bacmid in this study. Our results showed that deletion of *pkip* led to significant reduction of BV production, while the synthesis of viral DNA and the transcription of early and late genes were not affected. Further examination by transmission electron microscopy analysis showed that deletion of *pkip* resulted in the formation of massive electron-lucent tubular structures in the nucleus of the infected cells, along with some normal electron-dense nucleocapsids. The *pkip*-encoded protein PKIP could be detected at late phase during infection and was distributed in both the cytoplasm and nuclei of viruses-infected cells, with a ring pattern near the inner nuclear membrane and punctate distribution in the virogenic stroma area. Biochemical fractionation of virions into nucleocapsid and envelop components showed that PKIP was associated with the nucleocapsid fraction of BV. Taken together, our results indicated that PKIP is associated with nucleocapsids of BV and involved in nucleocapsid assembly, which contributes to the optimal production of BV.

1. Introduction

Baculoviridae is a family of large, insect-specific viruses with circular double-stranded DNA genomes ranging from 80 to 180 kb that are packaged within rod-shaped nucleocapsids enclosed by lipid envelopes (Rohrmann, 2013). Members of this family exclusively infect larvae of the insect orders Lepidoptera, Hymenoptera and Diptera. The family *Baculoviridae* is divided into four genera: the lepidopteran-specific *Alphabaculovirus* and *Betabaculovirus*, the hymenopteran-specific *Gammabaculovirus*, and the dipteran-specific *Deltabaculovirus*. These subdivisions likely reflect differences in gene content, genetic evolution, their permissive host species, and variations in the manner in which virions are occluded or embedded to form occlusion bodies (OBs) (Herniou and Jehle, 2007; Hu et al., 1998; Jehle et al., 2006). The *Alphabaculovirus* genus falls into two phylogenetic clades representing the group I and group II NPVs based on phylogenetic analysis of *polyhedrin* (*polh*) (Zanotto et al., 1993), and the major difference is which envelope fusion protein is used, GP64 or F protein, respectively.

A striking feature of the baculovirus infection cycle is the production of two phenotypically different but genetically identical types of virions: budded virion (BV) and occlusion-derived virion (ODV) (Blissard and Rohrmann, 1990; Volkman et al., 1976). It was previously thought that the nucleocapsids of these two virions were identical; however, the source and composition of the viral envelope were quite distinct (Braunagel and Summers, 1994; Hou et al., 2013; Slack and Arif, 2007). BVs are formed when synthesized nucleocapsids are transported out of the nucleus and eventually bud from the plasma membrane, while ODVs are formed when nucleocapsids retained in the nucleus are enveloped by intranuclear microvesicles. ODV infects midgut epithelial cells to initiate primary infection in insect larvae, while BV is responsible for cell-to-cell spread of infection within the larval body, which is referred to as systemic infection (Summers, 1971; Volkman and Summers, 1977). In addition, BV production of betabaculoviruses in cultured cells is very low; in contrast, the BV yields of alphabaculoviruses can be very high in cultured cells (Winstanley and Crook, 1993).

The gene expression of baculoviruses is mainly regulated by the transcription cascade as characterized into three phases: immediate-early/early, late, and very late. Immediate-early/early genes are transcribed by host RNA polymerase II after viral DNA is released in the nuclei of the infected cells, and the products regulate viral DNA replication and/or late gene expression; while late and very late genes are

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transcribed by a virus-encoded RNA polymerase and their expressions are dependent of viral DNA replication (Passarelli and Guarino, 2007). When late gene products are produced, nucleocapsid assembly is triggered, BVs and ODVs are formed subsequently. During very late state of infection, the very late viral genes *polh* and *p10* are expressed and the products are to form OBs that embedded with ODVs. The incorporation of virions into the OBs provides stability for the virions and allows them to remain viable for long periods in the environment (Rohrmann, 2013).

Autographa californica multiple nucleopolyhedrovirus (AcMNPV), which is one of the members of *Alphabaculovirus*, is the first baculovirus to have its genome completely sequenced and is currently the most extensively studied baculovirus. *ac24*, a unique gene in the genus *Alphabaculovirus* (Miele et al., 2011; Rohrmann, 2013), encodes a 19.2-kDa protein kinase-interacting protein (PKIP) that stimulates the activity of PK1, a virus-encoded serine/threonine kinase, *in vitro* (Fan et al., 1998). A previous study showed that an AcMNPV temperature-sensitive (ts) mutant with a mutation in *pkip* displayed several properties at nonpermissive temperature, including a significant decrease in progeny BV production and a defect in very late gene transcription (McLachlin et al., 1998), however, the underlying mechanism has not been determined.

In the present study, a *pkip*-knockout AcMNPV bacmid was constructed and the effects of *pkip* deletion on infectious BV production and virion morphogenesis were monitored. The results showed that *pkip* was not an essential gene for virus replication, but deletion of this gene significantly decreased infectious BV production, which are consistent with the study of the *pkip*-ts mutant (McLachlin et al., 1998). Viral DNA replication and transcription of early and late viral genes were not affected by deletion of *pkip*, but transmission electron microscopy analysis showed that the nucleocapsid assembly within the virus-infected cells was impaired by producing massive electron-lucent tubular structures along with normal nucleocapsids upon *pkip* deletion. Further experiments revealed that PKIP was a late viral protein and localized in both the nucleus and the cytoplasm of the viruses-infected cells, with a ring pattern near the inner nuclear membrane and punctate distribution in the virogenic stroma (VS) area, and is associated with the nucleocapsid fraction of BV in a fractionation experiment.

2. Materials and methods

2.1. Cells and viruses

Sf9 (*Spodoptera frugiperda* IPLB-Sf21-AE clonal isolate 9) insect cells were cultured at 27 °C in Grace's medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 30 μg/ml streptomycin. The AcMNPV-resembling virus vAc^{PH-GFP} (defined as vAcWT in the present study) was constructed in our previous study (Wu et al., 2006) and used as a control virus. The *ie1*-knockout virus vIE1KO (Liu, 2010) was used as a negative control for viral DNA replication.

2.2. Construction of recombinant viruses

The bacmid bMON14272, which contains the genome of AcMNPV, was used to generate a *pkip*-null bacmid via homologous recombination in *Escherichia coli* (*E. coli*) as previously described (Wu et al., 2006). To avoid any impact on the transcription of the flanking genes (*ac23* and *ac25*), 27-nucleotide (nt) of the 5′-terminal (nt 1–27) and 41-nt of the 3′-terminal (nt 470–510) regions of the *pkip* open reading frame were preserved according to a transcriptomic study of AcMNPV (Chen et al., 2013). Briefly, a linear fragment containing the 1,028-bp *chloramphenicol resistance* (*CmR*) gene cassette and *pkip* flanking regions was amplified with PCR from plasmid pKOV-KanF (Lalioti and Heath, 2001) using primers PKIP-US-Cm and PKIP-DS-Cm (the primers used in this study are listed in Table S1 of supplemental material). Then, the linear fragment was electrotransformed into *E. coli* DH10Bac cells (Thermo Fisher Scientific), resulting in the replacement of the 442-bp segment of *pkip* (AcMNPV nt 20,675 to 21,116) with the *CmR* cassette to generate a *pkip*-null recombinant bacmid, bPKIPKO.

The construct vPKIPKO was generated by the insertion of AcMNPV *polh* and *enhanced green fluoresence protein* (*egfp*) genes into the *polh* locus of bPKIPKO via a site-specific transposition using the donor plasmid pFB1-PH-GFP as previously described (Wu et al., 2006). To generate the repair virus v2HAPKIP, two copies of influenza virus hemagglutinin (HA)-tagged *pkip* as well as *polh* and *egfp* genes were inserted into bPKIPKO. The native promoter (AcMNPV nt 20,675 to 21,116) of *pkip*, which includes the transcriptional start sites of *pkip* according to the transcriptomic study (Chen et al., 2013), was amplified with PCR from bMON14272 with primers PKIP-Pro-U and PKIP-Pro-D and digested with EcoRI and SacI and ligated to pUC18-SV40 (Cai et al., 2012), which has been digested with the same restriction enzymes to generate pUC18-Pro-SV40. The *pkip* gene fragment fused with two HA-coding sequences at its 5′ end was amplified with PCR from bMON14272 with primers 2HAPKIP-U and 2HAPKIP-D and was digested with SacI and BamHI and ligated to pUC18-Pro-SV40, which has been digested with the same restriction enzymes to generate pUC18-Pro-2HAPKIP-SV40. Then, the Pro-2HAPKIP-SV40 fragment was released from pUC18-Pro-2HAPKIP-SV40 by EcoRI and XbaI digestion and subcloned into pFB1-PH-GFP to generate the donor plasmid pFB1-2HAPKIP-PH-GFP. The donor plasmid was then transformed into the electrocompetent *E. coli* DH10B cells containing the pMON7124 helper plasmid and the bPKIPKO bacmid to generate the repair bacmid v2HAPKIP.

All constructs were confirmed by PCR analysis and DNA sequencing. Bacmid DNA of recombinant viruses was electroporated into DH10B cells and screened for tetracycline sensitivity to remove the helper plasmid. Extrapure bacmid DNA was extracted and purified with the Large-Construct Kit (Qiagen) and quantified by optical density measurement.

2.3. Viral growth curve analysis and plaque assay

For viral growth curves, Sf9 cells (1.0×10^6/35-mm-diameter dish) were transfected in triplicate with 1.0 μg bacmid DNA of recombinant viruses using Lipofectin (Thermo Fisher Scientific). The viral inocula were allowed to be adsorbed by the cells for 5 h upon transfection at 27 °C and were then replaced with fresh medium. Time zero was defined as the time when fresh medium was added. At the indicated time points, the supernatants of transfected cells containing the BVs were harvested, and cell debris was removed by centrifugation at 3000 × *g* for 10 min. The BV titers were determined by a 50% tissue culture infective dose ($TCID_{50}$) endpoint dilution assay in Sf9 cells O'Reilly et al. (1992).

Plaque assays were performed as previously described (Wu et al., 2006). In brief, a total of 2.0×10^6 cells in a monolayer were infected with vAcWT, vPKIPKO, or v2HAPKIP at a multiplicity of infection (MOI) of 0.01 $TCID_{50}$/cell. At 96 h post infection (p.i.), viral plaques were photographed, and the size of plaques was measured.

2.4. Detection of BV production in the supernatant of transfected cells

Sf9 cells of 1.0×10^6 in a monolayer were transfected with 2.0 μg bacmid DNA of vPKIPKO or v2HAPKIP. At 24 h post transfection (p.t.), the transfected cells were collected by centrifugation of 3000 × *g* for 10 min at 4 °C. The supernatants containing BV from the transfected cells were harvested and purified as previously described O'Reilly et al. (1992). The cell pellets and the BV purified from the supernatants were subjected to immunoblot analysis as described below.

2.5. Analysis of viral DNA synthesis by real-time quantitative PCR (qPCR)

qPCR was performed to assess viral DNA synthesis as previously described (Vanarsdall et al., 2005), with some modifications. Briefly, 1.0×10^6 cells were transfected in triplicate with 1.0 μg bacmid DNA of vIE1KO, vPKIPKO, or v2HAPKIP, and transfected cells were collected at the indicated time points. Total DNA from each sample was extracted using the Universal Genomic DNA Extraction Kit (TaKaRa) and re-suspended in 50 μl of sterile water. To eliminate the input bacmid DNA retained from the transfection, 1.0 μg of DNA was digested with 20 units of *DpnI* restriction enzyme (NEB) overnight in a 40-μl reaction volume. qPCR was performed with a 5-μl aliquot of the digested DNA using the SYBR Premix Ex Taq II (TaKaRa) and primers targeting a 100-bp region of the AcMNPV *gp41* gene as described previously (Vanarsdall et al., 2005).

2.6. Analysis of viral gene transcription by reverse transcription qPCR (RT-qPCR)

A monolayer of Sf9 cells (1.0×10^6) were transfected with 1.0 μg bacmid DNA of vPKIPKO or v2HAPKIP, and the transfected cells were harvested at 24 h p.t. Total cellular RNA was isolated using a MiniBEST Universal RNA Extraction Kit (TaKaRa) according to the manufacturer's protocol. cDNA was synthesized using a PrimerScript™RT Reagent Kit (TaKaRa) according to the manufacturer's instruction. Four viral genes, the immediate-early gene *ie1*, the delayed-early gene *gp64*, and the late genes *vp39* and *p6.9*, were selected to represent early and late viral genes and to evaluate the effect of *pkip* deletion on viral gene transcription. qPCR was carried out with the synthesized cDNA as the template to examine gene transcripts using the corresponding primers as shown in Table S1. Host 18S rRNA was used as the endogenous reference gene and the relative transcription levels of the selected viral genes were determined as described previously (Peng et al., 2012).

2.7. Transmission electron microscopy (TEM) analysis

Sf9 cells (1.0×10^6) were transfected with 5.0 μg bacmid DNA of recombinant viruses. At 72 h p.t., the transfected cells were harvested and pelleted by centrifugation at 2000 × *g* for 10 min. The cells were fixed and processed for TEM analysis as previously described (Wu et al., 2006). Ultrathin sections were observed with a JEM-100CX/II transmission electron microscope (JEOL, Japan) at an accelerating voltage of 80 kV.

2.8. Time course analysis of PKIP expression

Sf9 cells (1.0×10^6) were infected with v2HAPKIP at an MOI of 10 $TCID_{50}$/cell. At the indicated time points, the infected cells were collected by centrifugation of 3000 × *g* for 10 min at 4 °C and then subjected to immunoblot analysis.

2.9. Immunofluorescence microscopy

Sf9 cells (0.8×10^6) were seeded onto 35-mm glass-bottom culture dishes (MatTek) and then infected with v2HAPKIP at an MOI of 5 $TCID_{50}$/cell. At the indicated time points, the infected cells were processed for immunofluorescence microscopy as previously described (Yuan et al., 2011). The mouse monoclonal anti-HA antibody (1:200; Abmart) was used as the primary antibody. An Alexa Fluor 555-conjugated donkey anti-mouse antibody (1:200; Thermo Fisher Scientific) was used as the secondary antibody. Prior to observation, the cells were stained with Hoechst 33258 (1:1000; Thermo Fisher Scientific) to identify the nucleus and DNA-rich region. All pictures were collected using a Zeiss LSM 780 confocal laser scanning microscope.

2.10. Fractionation of cytoplasm and nucleus

Sf9 cells (1.0×10^6) were infected with v2HAPKIP at an MOI of 5 $TCID_{50}$/cell. At the indicated time points, the infected cells were collected by centrifugation of 3000 × *g* for 10 min at 4 °C and then fractionated into cytoplasmic and nuclear fractions using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific) according to the manufacturer's protocol.

2.11. Purification and fractionation of BVs and ODVs

BVs were purified from supernatants of v2HAPKIP-infected Sf9 cells, which were then fractionated into envelope and nucleocapsid fractions as previously described (Wu et al., 2008). ODVs were purified from v2HAPKIP-infected third-instar *Spodoptera exigua* larvae as previously described (Wu et al., 2008).

2.12. Immunoblotting

Immunoblot analysis was performed to analyze the expression and localization of PKIP in infected cells or virions. Protein samples from the nuclear/cytoplasmic fractions, purified virions, or cell lysates were separated with SDS-15% PAGE, transferred onto a PVDF membrane (MILLIPORE) and then probed with one of the following primary antibodies: (i) mouse monoclonal anti-HA antibody (1:1000; BioLegend), to detect 2 × HA-tagged PKIP; (ii) mouse monoclonal anti-IE1 antibody (1:1000; a gift from Prof. L. A. Guarino, Texas A&M University), to detect the transactivator IE1; (iii) mouse monoclonal anti-GP64 AcV5 antibody (1:2000; eBioscience), to detect the viral fusion envelope protein GP64; (iv) rabbit polyclonal anti-VP39 antibodies (1:2000), to detect the major capsid protein VP39; (v) or mouse monoclonal anti-actin antibody (1:2000; Proteintech), to detect the cellular actin as a loading control, followed by incubation with goat anti-mouse (1:5000; Thermo Fisher Scientific) or donkey anti-rabbit (1:10,000; Sigma) horse radish peroxidase-conjugated secondary antibodies, as needed.

3. Results

3.1. Construction of the recombinant AcMNPV bacmids

To investigate the role *pkip* plays in the AcMNPV infection cycle, a *pkip*-knockout AcMNPV bacmid (bPKIPKO) was constructed based on bacmid bMON14272 via homologous recombination. Briefly, the 442-bp fragment of *pkip* (nt 20,675-21,116 of AcMNPV genome) was replaced with a *CmR* cassette in bPKIPKO (Fig. 1). For observation of OB morphogenesis and the progression of viral infection, an AcMNPV *polh* gene driven by its native promoter and an *egfp* gene under the AcMNPV *ie1* promoter were inserted into the *polh* locus of bPKIPKO to generate the vPKIPKO; meanwhile, to generate a *pkip* repair virus (v2HAPKIP), a *pkip* cassette under its own promoter and 2 copies of HA-tag at its N terminus, together with the *polh* and *egfp* genes, were inserted into the *polh* locus of bPKIPKO (Fig. 1). vAcWT was constructed as a control to resemble the wild-type AcMNPV by inserting the *polh* and *egfp* genes into the *polh* locus of bMON14272 (Fig. 1).

3.2. pkip is required for optimal BV production in Sf9 cells

Sf9 cells were transfected with 1.0 μg bacmid DNA of vAcWT, vPKIPKO, or v2HAPKIP and were monitored by fluorescence microscopy. At 24 h p.t., no obvious differences in the number of fluorescent cells were observed, indicating comparable transfection efficiencies among all the viruses (Fig. 2A). By 96 h p.t., almost all of the cells transfected with vAcWT or v2HAPKIP showed fluorescence, while approximately only half of the cells transfected with vPKIPKO displayed fluorescence (Fig. 2A), indicating that *pkip* was required for optimal virus propagation. In addition, OBs were observed in most of the cells

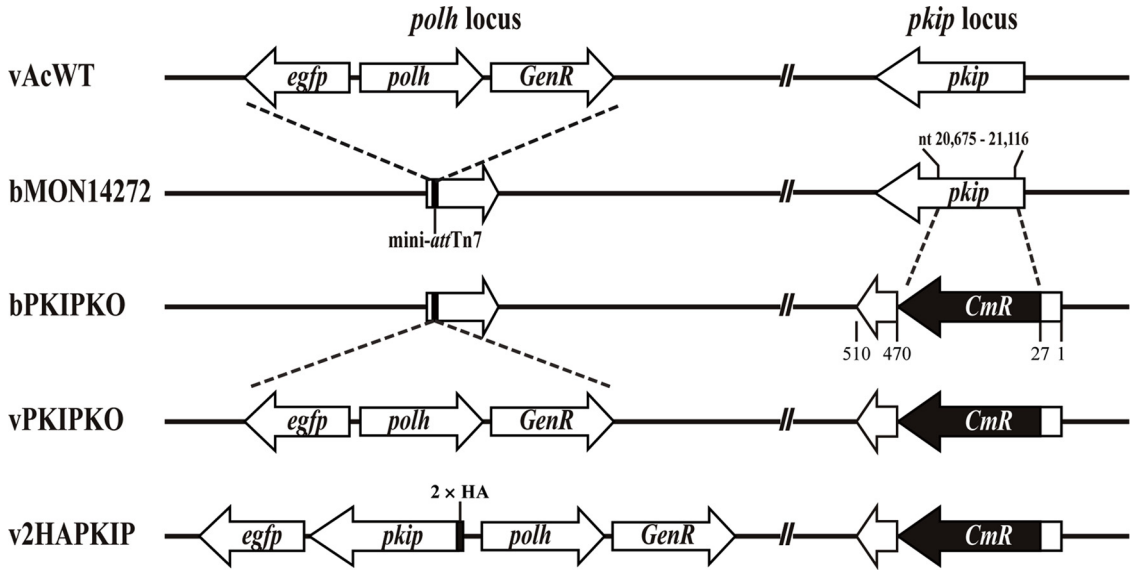

Fig. 1. Schematic diagram of recombinant virus construction. bPKIPKO was constructed by replacing a 442-bp fragment of *pkip* in bMNON14272 with a *CmR* cassette via homologous recombination in *E. coli*. vPKIPKO was generated by transposing the *polh* and *egfp* genes into the mini-*att*Tn7 locus of bPKIPKO. A repaired virus, v2HAPKIP, was constructed by transposing the 2 × HA-tagged *pkip* under its native promoter as well as *polh* and *egfp* genes into the mini-*att*Tn7 locus of bPKIPKO. vAcWT was generated by transposing the *polh* and *egfp* genes into the mini-*att*Tn7 locus of bMNON14272 as a control to resemble the wild-type AcMNPV.

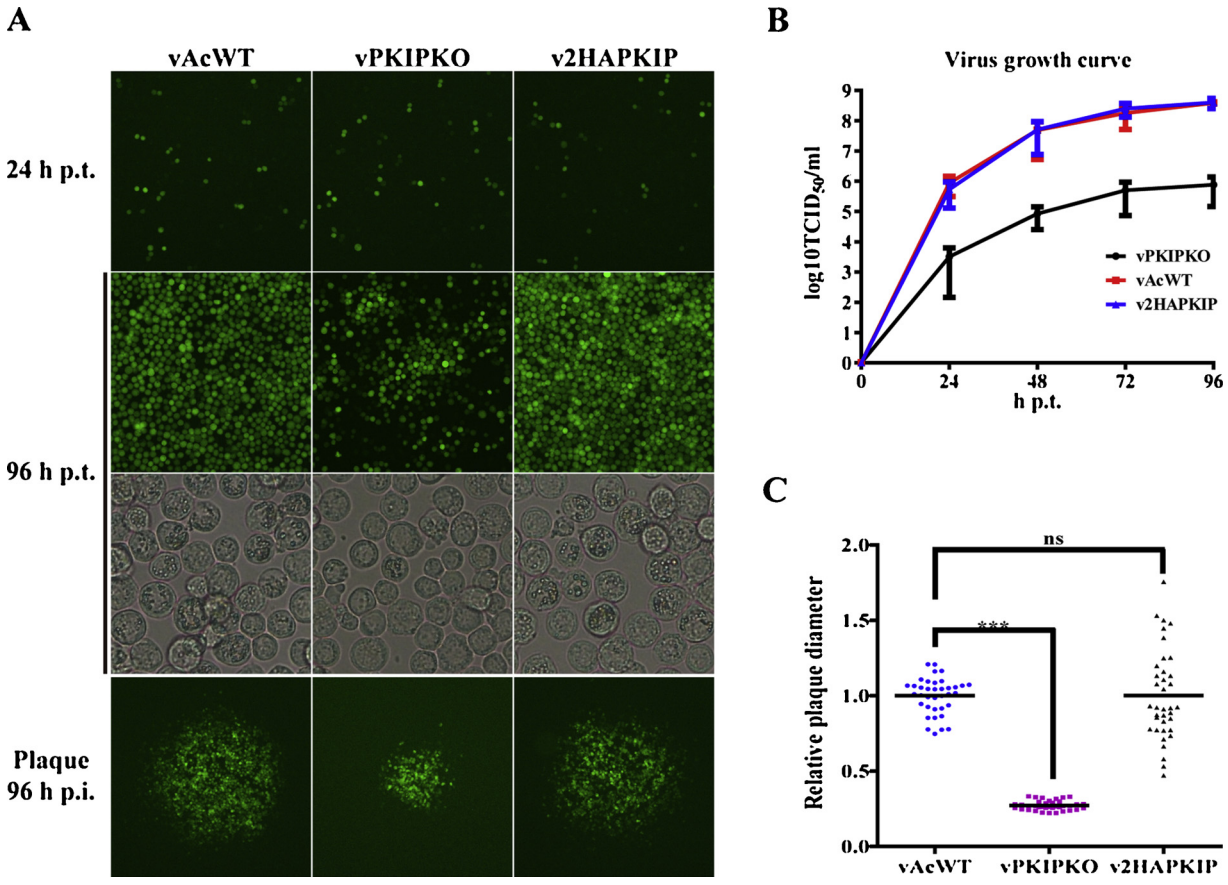

Fig. 2. Analysis of virus replication in Sf9 cells. (A) Sf9 cells were transfected with 1.0 μg bacmid DNA of the indicated recombinant viruses and monitored for infection by fluorescence microscopy or for OB formation by light microscopy. The bottom panels show viral plaques visualized by fluorescence microscopy at 96 h p.i. (B) Viral growth curves generated from transfections. Sf9 cells were transfected with 1.0 μg bacmid DNA of vPKIPKO, vAcWT, or v2HAPKIP. At the indicated time points, supernatants of transfected cells were collected to determine the titers of infectious BV by $TCID_{50}$ endpoint dilution assay. Each data point represents the average titer from three independent infections. The error bars represent the standard deviations from three independent experiments. (C) Plaque diameters in vAcWT-, vPKIPKO- or v2HAPKIP-infected Sf9 cells. A total of 37 plaques for each virus were measured in vAcWT-, vPKIPKO- or v2HAPKIP-infected cells. Plaque size differences were analyzed by Student's *t* test with GraphPad Prism software. Bars indicate the medians of samples. ns, no significance; ***, $P < 0.001$.

transfected with vAcWT or v2HAPKIP when using light microscopy, whereas only a few of the cells transfected with vPKIPKO contained OBs at 96 h p.t. (Fig. 2A, bright field), suggesting that the deletion of *pkip* might affect OB production.

To better evaluate the effect of deletion of *pkip* on virus replication, viral growth curve analyses were performed by determining the BV titers in the supernatants of the transfected cells at different time points p.t. using a $TICD_{50}$ endpoint dilution assay. As shown in Fig. 2B, the virus growth curves of vAcWT and v2HAPKIP were comparable, indicating that v2HAPKIP replicated as efficiently as vAcWT and that the 2 × HA-tag at the N terminus did not affect the function of PKIP. Although infectious BVs were also detected in cells transfected with vPKIPKO, the titers were lower than those of the vAcWT and v2HAPKIP at all selected time points p.t. By 96 h p.t., the total number of infectious BVs generated by vPKIPKO was approximately 500-fold lower than that generated by vAcWT ($P < 0.01$) (Fig. 2B).

Plaque assays were performed to further compare the spreading of virus to neighboring cells. Sf9 cells infected with vAcWT, vPKIPKO, or v2HAPKIP at an MOI of 0.01 $TCID_{50}$/cell were observed at 96 h p.i. and plaques were photographed (Fig. 2A, bottom panel), and the diameter of the plaques was measured. The sizes of the plaques produced by vAcWT and v2HAPKIP were comparable, while the plaques produced by vPKIPKO were significantly smaller than those from vAcWT-infected cells ($P < 0.0001$) (Fig. 2C).

The total BV production regardless of infectivity in the supernatants of the vPKIPKO-transfected cells was examined. Compared to that of the v2HAPKIP-transfected cells, an obvious reduction of BV production was detected in the vPKIPKO-transfected cells, while the protein expression levels of GP64 and VP39 within the transfected cells were comparable (Fig. 3).

3.3. The synthesis of viral DNA is not affected by the deletion of pkip

To investigate whether the deletion of *pkip* affects viral DNA synthesis, the levels of viral DNA synthesis in vPKIPKO-transfected cells were measured by qPCR analysis. Since the baculovirus transactivator IE1 has been shown to be essential for viral DNA replication (Kool et al., 1994; Stewart et al., 2005), an *ie1*-null recombinant virus (vIE1KO) (Liu, 2010) was used as a negative control. As shown in Fig. 4, the viral DNA replication levels between vPKIPKO-transfected cells and v2HAPKIP-transfected cells were comparable and gradually increased from 0 to 24 h p.t. In the negative control, as expected, viral DNA replication was not triggered in vIE1KO-transfected cells over time.

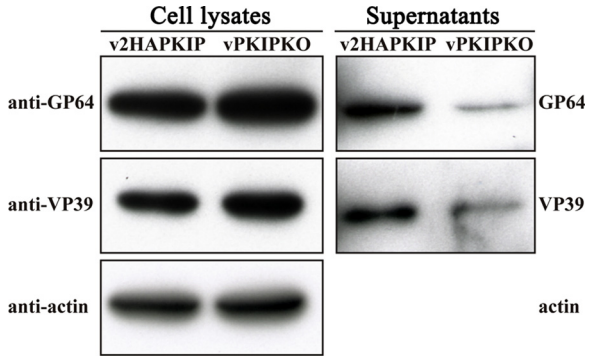

Fig. 3. Detection of total BV production in supernatant of transfected Sf9 cells. Sf9 cells were transfected with 2.0 μg bacmid DNA of vPKIPKO or v2HAPKIP. The supernatants and cell pellets of transfected cells were harvested separately at 24 h p.t. Total proteins of cell pellets (Cell lysates) and BVs purified from supernatants (Supernatants) were subjected to immunoblot analysis. A monoclonal anti-GP64 antibody or polyclonal anti-VP39 antibodies was used to detect the BV envelop-specific protein GP64 or the major capsid protein VP39, respectively. A monoclonal anti-actin antibody was used to detect the expression of actin as the loading control.

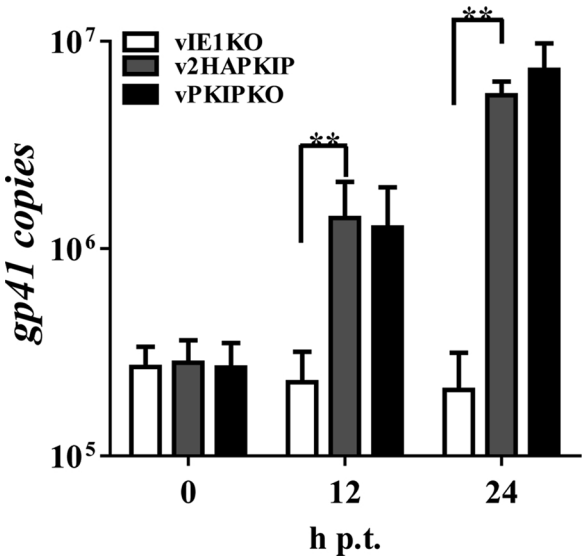

Fig. 4. Analysis of viral DNA replication. Sf9 cells were transfected with 0.5 μg bacmid DNA of v2HAPKIP, vIE1KO or vPKIPKO. At the indicated time points, the total DNA of transfected cells was isolated, further digested with the restriction enzyme DpnI to eliminate input DNA during transfection, and analyzed by qPCR. The results are averages of three independent assays. Data were analyzed by Student's *t* test. Error bars represent standard deviations. **, $P < 0.01$.

Therefore, this result indicated that the deletion of *pkip* did not affect the synthesis of viral DNA.

3.4. The transcription of early and late viral genes was not affected by the deletion of pkip

Since *pkip* is not required for viral DNA replication, we subsequently investigated whether it is required for early or late gene transcription and consequently the deletion of it affects BV production. Sf9 cells transfected with vPKIPKO or v2HAPKIP were collected at 24 h p.t. and total cellular RNA was extracted. The transcription levels of four representative genes, the immediate-early gene *ie1*, delayed-early gene *gp64*, and late genes *vp39* and *p6.9*, were determined by RT-qPCR using corresponding primers (Table S1). As shown in Fig. 5, no significant differences in transcription levels were observed for all of the representative genes between vPKIPKO and v2HAPKIP, suggesting that the deletion of *pkip* did not affect the transcription of both early and late viral genes.

3.5. The deletion of pkip impairs nucleocapsid assembly

To find out why *pkip* is required for optimal BV production, we further investigated the effect of deletion of *pkip* on virion morphogenesis. Ultrathin sections of Sf9 cells transfected with vAcWT, v2HAPKIP, or vPKIPKO were analyzed via TEM. The formations of a net-shaped VS interspersed with rod-shaped electron-dense nucleocapsids (Fig. 6A) and ODV-containing polyhedra within the intranuclear peripheral area, called the ring zone (RZ) (Fig. 6B), were observed in vAcWT-transfected cells. In v2HAPKIP-transfected cells, characteristics of rod-shaped electron-dense nucleocapsids in the VS (Fig. 6C), ODVs and ODV-containing polyhedra within the RZ (Fig. 6D) similar to those of the vAcWT-transfected cells were observed. In the cells transfected with vPKIPKO, although several electron-dense nucleocapsids (white arrowhead) were observed in the VS, masses of electron-lucent tubular structures (black arrow) and electron-dense bodies (white arrow) were present at the intrastromal space of the VS (Fig. 6E) and the RZ (Fig. 6F). In addition, ODVs could be observed, and very few polyhedra with or without ODVs were formed in the RZ of the

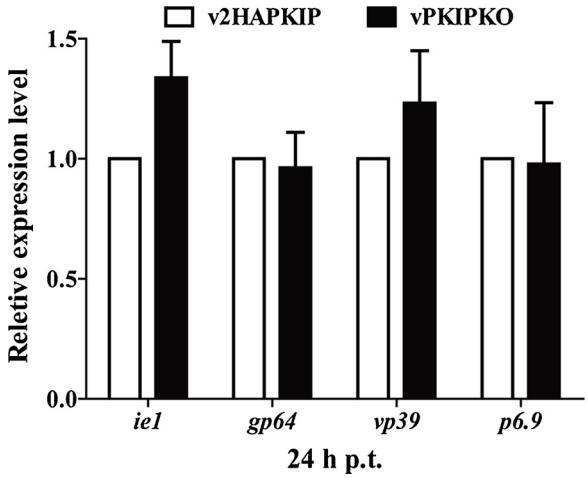

Fig. 5. Analysis of viral gene transcription. Sf9 cells transfected with 1.0 μg bacmid DNA of vPKIPKO or 2HAPKIP were harvested at 24 h p.t. and total RNA was isolated to measure the transcription levels of the indicated viral genes by RT-qPCR. The transcription level of each gene was normalized to that of the host 18S rRNA and was shown as the percentage of the corresponding gene in the v2HAPKIP-transfected cells. Data were analyzed by Student's *t* test. Error bars indicate standard deviations from three indepent experiments.

vPKIPKO-transfected cells (Fig. 6F). These results indicated that *pkip* participated in nucleocapsid assembly and that its deletion might affect the normal production of polyhedra.

3.6. PKIP is a protein product of a late viral gene

To determine the temporal expression of PKIP during the infection cycle, Sf9 cells were infected with v2HAPKIP at an MOI of 10 $TCID_{50}$ and were collected at the indicated time points p.t. The infected cells were subjected to immunoblotting with a monoclonal anti-HA antibody to detect the expression of PKIP. An immunoreactive band of approximately 22 kDa corresponding to the mass of the full-length 2 × HA-tagged PKIP was first detected at 18 h p.i. and persisted up to 96 h p.i. (Fig. 7A). At 24 h p.i., a second band of lower molecular mass, which might be a degradation product of PKIP, was detected and remained up to 96 h p.i. (Fig. 7A). The temporal expression of the early-late gene product GP64 and the late gene product VP39 were probed as controls to verify the infection progress, and cellular actin was detected as a loading control (Fig. 7A).

To further investigate whether the expression of PKIP relies on the synthesis of viral DNA, the DNA synthesis inhibitor aphidicolin was added to the cells infected with v2HAPKIP. At 24 h p.i., the infected cells were collected and subjected to immunoblot analysis. As expected, the synthesis of IE1, an early viral protein that does not require DNA synthesis, could be detected in aphidicolin-treated cells; however, the synthesis of PKIP was abolished in the presence of aphidicolin (Fig. 7B). Similarly, the synthesis of late viral proteins VP39 and P6.9 were abolished in the presence of aphidicolin as negative controls. The requirement for DNA synthesis indicates that PKIP is produced at later times, which is consistent with the result of a previous transcriptomic study that determined *pkip* is a late gene (Chen et al., 2013).

3.7. PKIP is distributed in both the cytoplasm and nuclei of virus-infected cells

To further understand the role PKIP plays in the baculovirus life cycle, Sf9 cells were infected with v2HAPKIP and analyzed by immunoblotting (Fig. 8A) or immunofluorescence (Fig. 8B) to detect PKIP distribution in virus-infected cells. Sf9 cells infected with v2HAPKIP were biochemically fractionated into cytoplasmic and nuclear fractions and were subjected to immunoblot analysis. As shown in Fig. 8A, PKIP was detected in both the cytoplasmic and nuclear fractions of v2HAPKIP-infected cells from 18 to 72 h p.i. It was noted that the molecular mass of PKIP in nuclear fractions appeared to be greater than that detected in cytoplasmic fractions. The same immunoblot membrane was reprobed with a GP64 or IE1 antibody to detect a mainly cytoplasmic or nuclear protein, respectively, to assess the fractionation efficiency (Fig. 8A). In addition, Sf9 cells infected with v2HAPKIP were analyzed by immunofluorescence using a monoclonal anti-HA antibody to examine the subcellular localization of PKIP using a confocal microscope. Although PKIP could be detected throughout the cells from 18 to 48 h p.t., it predominantly localized to the inner nuclear membrane of the infected cells at 18 h p.i. and the signal of the ring pattern accumulated from 24 to 48 h p.i., while discrete foci with the presence of PKIP were also observed in what appears to be the VS area (Fig. 8B).

3.8. PKIP is associated with the nucleocapsid of BV

Since the deletion of *pkip* impairs nucleocapsid assembly, it is intrigued to know whether PKIP is a component of the virions. To this end, BV or ODV was purified from the supernatant of v2HAPKIP-infected cells or OBs isolated from whole larvae, respectively. The purified virions were subjected to immunoblotting using an anti-HA antibody to detect PKIP. Since PKIP was not detected in ODV but was associated with BV (Fig. 9A), BV was further biochemically fractionated into envelope and nucleocapsid fractions and analyzed by immunoblotting. As shown in Fig. 9B, PKIP was present in the nucleocapsid fraction but not in the envelope fraction of BV. As controls to confirm the fractionation efficiency, the major nucleocapsid protein VP39 and the BV envelope protein GP64 were detected in the expected fractions (Fig. 9B).

4. Discussion

As baculovirus genes that are conserved in only some members of the family will provide novelty to the virus and will influence individual phenotypic traits such as host or tissue tropism, virulence, and morphology, studies of these genes will probably reveal how baculovirus is currently changing and acquiring new distinctive properties (Possee, 1997). For example, *ac34* and *ac51* are conserved only in alphabaculoviruses but are both required for efficient BV production, although they function in different ways (Cai et al., 2012; Qiu et al., 2018). Likewise, AcMNPV with a ts mutation in *pkip*, a gene also only conserved in alphabaculoviruses, showed defects in BV production and very late gene transcription at nonpermissive temperature (McLachlin et al., 1998); however, the underlying mechanism is unclear and the knowledge on the precise function of PKIP is very limited. In this study, we generated a *pkip*-knockout AcMNPV bacmid and investigated the role *pkip* plays in the AcMNPV life cycle.

PKIP is a protein product of the late viral gene. Using an anti-HA antibody, we detected two immunoreactive bands in v2HAPKIP-infected cells (Fig. 7A). The predominant band of approximately 22 kDa corresponding to the mass of the full-length 2 × HA-tagged PKIP was first detected at 18 h p.i. and persisted up to 96 h p.i., which was similar to the temporal expression of the late viral gene product VP39. At 24 h p.i., a band of lower molecular mass, which might be a degradation product of PKIP that has the same N terminus as the full-length PKIP, appeared and remained up to 96 h p.i. Further analysis showed that *pkip* requires viral DNA replication for expression (Fig. 7B), indicating that *pkip* is a late viral gene since the expression of late baculovirus genes requires viral DNA replication. Our results are consistent with the conclusions of previous studies that indicate *pkip* is a late gene: by northern blot analysis, *pkip* transcript was first detected at 6 h p.i., the time when viral DNA starts to replicate (Fan et al., 1998), and by a transcriptomic study, where two canonical late gene promoters TAAG were identified as transcription start sites of *pkip* (Chen et al., 2013).

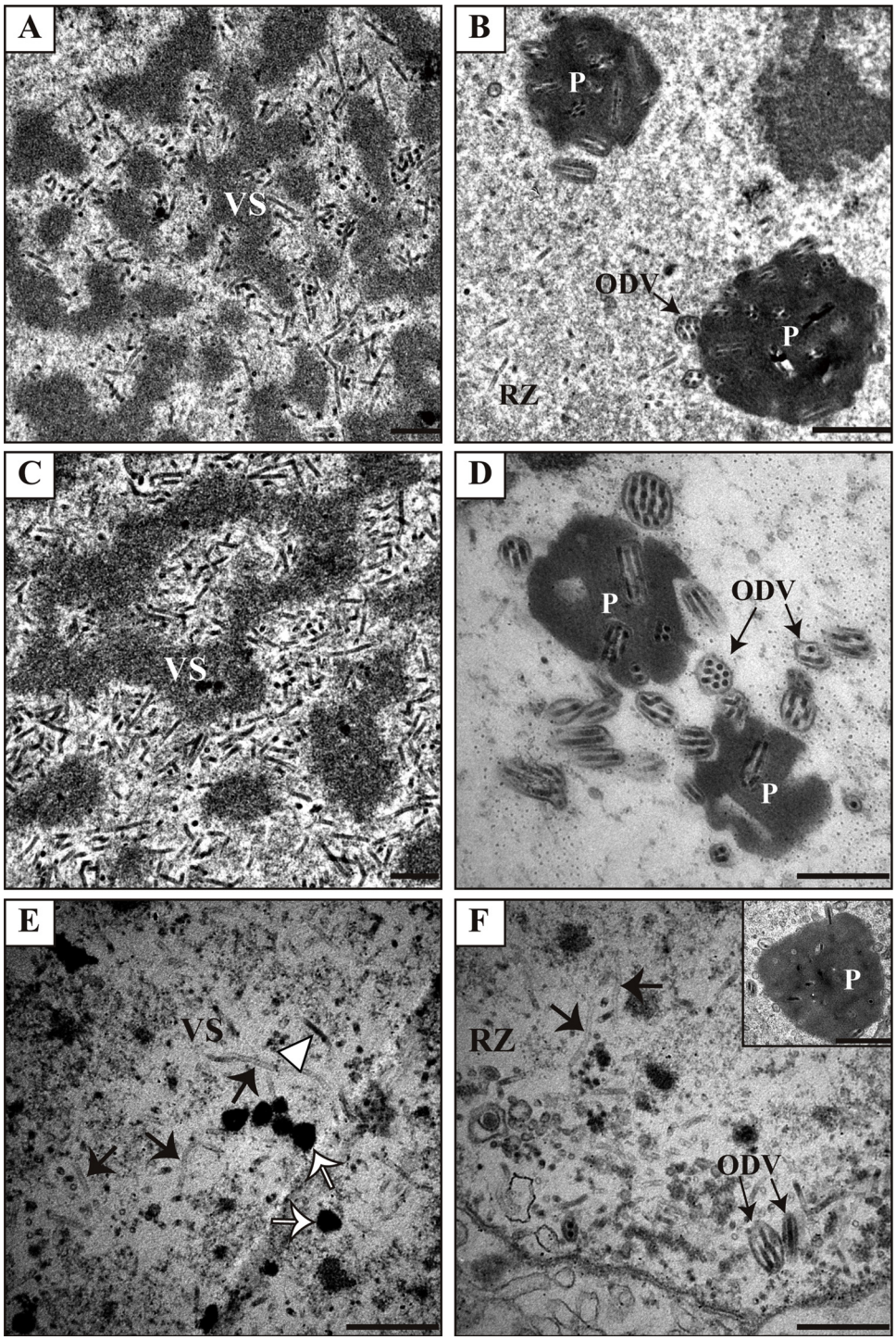

Fig. 6. The deletion of *pkip* impairs nucleocapsid assembly. Sf9 cells were transfected with bacmid DNA of vAcWT (A and B), v2HAPKIP (C and D), or vPKIPKO (E and F). At 72 h p.t., the transfected cells were collected and prepared for TEM. (A and C) Rod-shaped electron-dense nucleocapsids are observed in the VS. (B and D) ODVs and polyhedra (P) embedded with ODVs are found within the RZ. (E) Electron-dense nucleocapsids (indicated by the white arrowhead), electron-lucent tubules (indicated by black arrows) and a mass of electron-dense bodies (indicated with white arrows) can be found in the VS. (F) Electron-lucent tubules (indicated by black arrows), ODVs and polyhedra (P) embedded with ODVs (inset) are observed within the RZ. The bars represent 500 nm.

PKIP is associated with the nucleocapsid of BV. Most baculovirus late genes encode viral structure proteins. Our study demonstrated that PKIP was not detected in ODV but was associated with BV and is a nucleocapsid component of BV (Fig. 9). Comparison of protein components of the two virions by proteomic analyses, many viral proteins are specific to BV or ODV (Braunagel et al., 2003; Hou et al., 2013; Wang et al., 2010). Nevertheless, it does not rule out that the N-terminal shear protein of PKIP was associated with ODV, which cannot be detected in this study. In addition, by proteomic analysis, PKIP was not identified as the BV and ODV structure protein of diverse viruses, including AcMNPV (Braconi et al., 2014; Braunagel et al., 2003; Hou et al., 2016; Liu et al., 2008; Wang et al., 2010), except for the Helicoverpa armigera NPV (HearNPV), in which PKIP was identified in BV (Hou et al., 2013). It is possible that PKIP may be associated with BV at low abundance and may not be easily detected or that it is not amenable to the proteomic techniques used in these studies. There have been viral proteins that were not identified as BV or ODV structure proteins of AcMNPV by proteomic analysis, such as PK1 and Ac75, but were detected to be associated with BV and ODV by immunoblotting (Li et al., 2015; Shi et al., 2018).

PKIP is required for efficient nucleocapsid assembly. *pkip* is a non-essential gene *in vitro*. Deletion of *pkip* resulted in a significant reduction of BV production (Fig. 2 and 3), whereas the synthesis of viral DNA and the transcriptions of early/late viral genes appeared to be

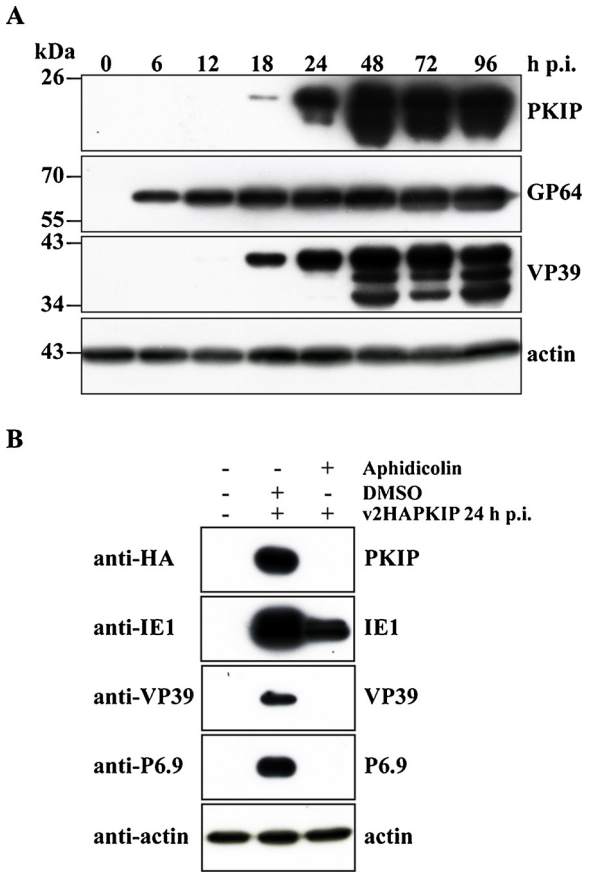

Fig. 7. PKIP is a protein product of the late viral gene. (A) Expression time course of PKIP. Sf9 cells were infected with v2HAPKIP at an MOI of 10 $TCID_{50}$/cell. At the indicated time points, the infected cells were collected, and total proteins were subjected to immunoblotting. (B) PKIP expression depends on viral DNA replication. Sf9 cells were infected with v2HAPKIP at an MOI of 5 $TCID_{50}$/cell in the presence (+) or absence (-) of the DNA synthesis inhibitor aphidicolin. At 24 h p.i., the cells were collected, and total proteins were subjected to immunoblotting. PKIP was detected with an anti-HA antibody; GP64 was detected with an anti-GP64 antibody; VP39 was detected with the anti-VP39 polyclonal antibodies; IE1 was detected with an anti-IE1 antibody; P6.9 was detected with the anti-P6.9 polyclonal antibodies; actin was detected with an anti-actin antibody as the loading control.

unaffected (Fig. 4 and 5). We have been aware of the emerging inconsistent results of the studies of *pkip* orthologs: similar to our result, the deletion of the *pkip* ortholog in Bombyx mori NPV resulted in the markedly slow spread of virus infection (Ono et al., 2012), while deletion of *pkip* orthologs in HearNPV(Pan, 2007) or Spodoptera exigua MNPV did not affect viral replication (Pijlman et al., 2003). AcMNPV and Bombyx mori NPV are pertained to members of group I of the genus *Alphabaculovirus*, while HearNPV and Spodoptera exigua MNPV belong to group II. This suggests that PKIP may have a distinct function in group I. An alanine at 46 of AcMNPV PKIP, which is conserved in orthologs of group I but not group II, was mutated to threonine, leading to the severe defects in BV production at nonpermissive temperature (McLachlin et al., 1998), which may support the hypothesis mentioned above.

Previous studies have shown that some AcMNPV genes are required for optimal infectious BV production but not for viral DNA replication, such as *ac17*, *ac34*, *pp31* (*ac36*), *ac51*, *ac66*, *ac79*, *gp64* (*ac128*), *me53* (*ac139*), and *exon0* (*ac141*). Although deletion of these genes shares a similar phenotype in infectious BV production, the specific functions of each gene in the process of BV production are varied. *ac51*, *ac66*, and *exon0* are required for efficient nuclear egress of nucleocapsids (Biswas et al., 2016; Ke et al., 2008; Qiu et al., 2018); *ac17*, *ac34* and *pp31* are shown to promote late gene expression (Cai et al., 2012; Nie and Theilmann, 2010; Yamagishi et al., 2007); *gp64* encodes a BV envelope protein that is required for efficient budding of nucleocapsid from infected cells (Oomens and Blissard, 1999), while ME53 may provide a scaffold that bridges the viral envelope and nucleocapsid (de Jong et al., 2011); the mechanism of *ac79* for efficient infectious BV production is unclear (Wu and Passarelli, 2012). In this study, to further analyze how *pkip* affects infectious BV production, ultrathin sections of vPKIPKO-transfected cells were observed by TEM, and the results showed that deletion of *pkip* impaired the nucleocapsid assembly by forming massive electron-lucent tubular structures in VS (Fig. 6E), which may be the empty capsid sheaths devoid of viral DNA that could account for the reduction of infectious BV production. However, we cannot rule out the possibility that the deletion of *pkip* may also affect the nuclear egress of the nucleocapsids. Since the formation of electron-dense nucleocapsids is severely affected, we were unable to compare the nucleocapsid egress of the *pkip*-deleted virus with the control viruses at this time. On the other hand, the results that PKIP was detected to be associated with nucleocapsid of BV but not ODV in this study may imply that PKIP promotes the budding of nucleocapsid to form BV but not retains the nucleocapsid in the nucleus to form ODV. In accordance with the results of the previous study on the *pkip*-ts mutant at nonpermissive temperature (McLachlin et al., 1998), electron-dense bodies associated with the VS were also observed by TEM analysis in *pkip*-deleted virus-transfected cells (Fig. 6E, indicated with white arrows). Similar features were observed upon the deletion of some genes that are involved in nucleocapsid assembly, such as *ac54* (Guan et al., 2016) and *ac83* (Zhu et al., 2013). It was shown that viral DNA and P6.9 were both detected to be associated with the electron-dense bodies in *ac54*-deleted virus-transfected cells by immunoelectron microscopy (Guan et al., 2016), indicating an accumulation of viral DNA-P6.9 complex or a defect in incorporation of viral DNA-P6.9 complex to form nucleocapsids. The transcription level of *p6.9* was not affected by the deletion of *pkip*, as shown in Fig. 5, which is consistent with the study on the *pkip*-ts mutant (McLachlin et al., 1998). However, elevated production of P6.9 in *pkip*-ts mutant-infected cells was observed compare to that of the wild-type virus-infected cells at nonpermissive temperature, which may due to a difference in the rate of the accumulation of this protein (McLachlin et al., 1998). These results indicated that *pkip* has a distinct function in the process of BV production, which differs from the other optimal BV production-related genes mentioned above. The BV production promoted by *pkip*, *ac34*, and *ac51*, which are all exclusively conserved in alphabaculoviruses, in contrast with the low BV yields of betabaculoviruses in cultured cells (Winstanley and Crook, 1993), may imply the evolutionary direction of alphabaculoviruses.

The cellular distribution of PKIP was examined, and the results showed that PKIP was predominantly localized to the inner nuclear membrane, with discrete foci in the VS area (Fig. 8B), which was similar to that of PK1 (Li et al., 2015). Deletion of *pk1* (Li et al., 2015) or a mutation in PK1 with disruption of the kinase activity (Liang et al., 2017) resulted in interruption of the nucleocapsid assembly with formation of electron-lucent tubular structures, indicating that the kinase activity of PK1 is required for nucleocapsid assembly. A previous study showed that PKIP interacts with and strongly stimulates kinase activity of PK1 *in vitro* (Fan et al., 1998). Thus, deletion of *pkip* may result in a dramatic reduction of kinase activity of PK1 and lead to inefficient nucleocapsid assembly.

The effect of *pkip* deletion on very late viral gene expression was not examined in the present study. As the deletion of *pkip* led to a dramatic reduction in BV production, it was difficult to use *pkip*-deleted virus vPKIPKO for a synchronous infection to investigate the very late viral gene transcription or expression when compare with those of the control viruses. However, the present study showed that only a few of the cells transfected with the bacmid DNA of vPKIPKO contained OBs at 96 h p.t. (Fig. 2A, bright field), and very few polyhedra could be

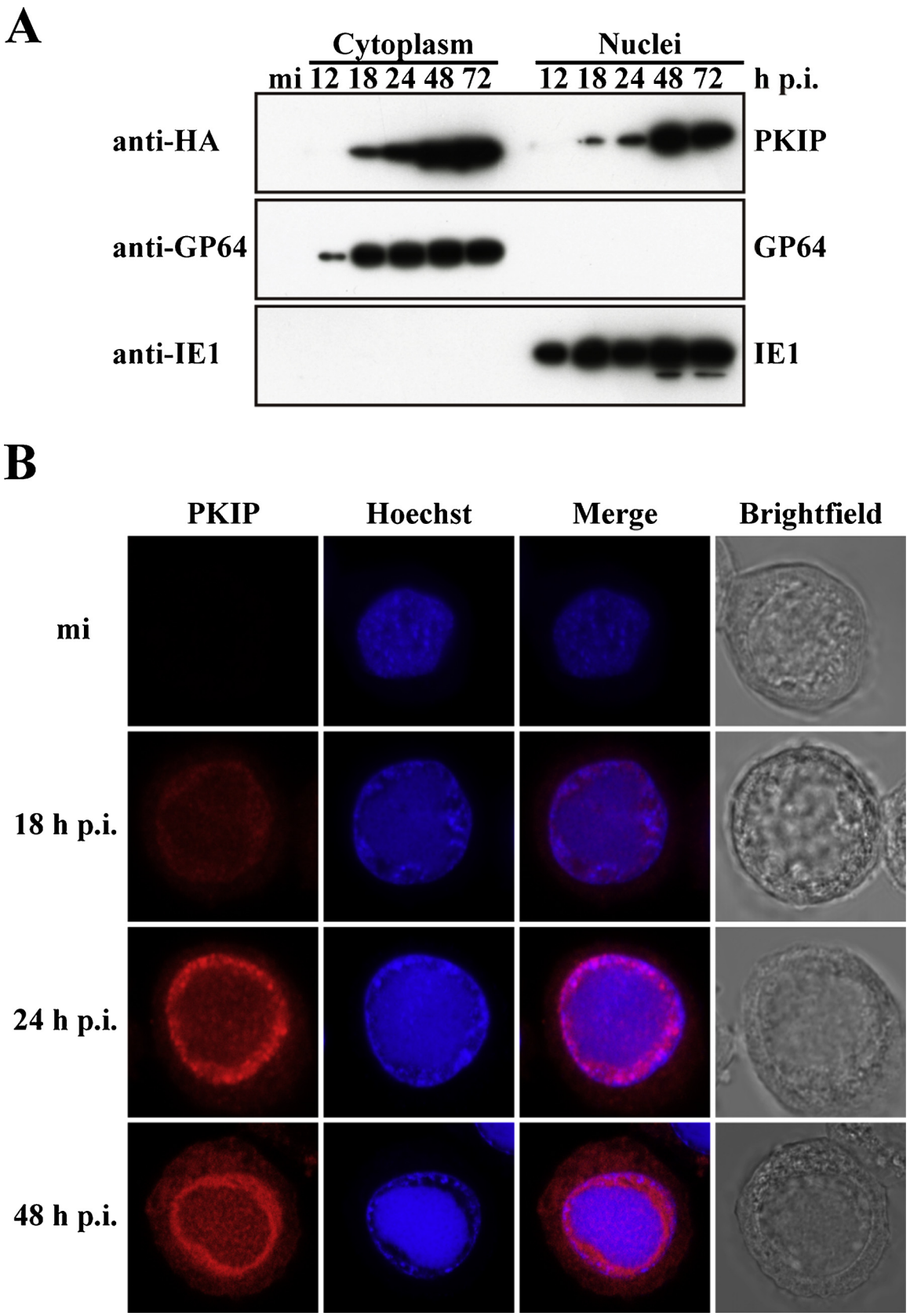

Fig. 8. Localization of PKIP in virus-infected cells. (A) Immunoblotting of the subcellular localization of PKIP in virus-infected cells. Sf9 cells were infected with v2HAPKIP at an MOI of 5 $TCID_{50}$/cell. At the indicated time points, the infected cells were collected and fractionated into cytoplasmic and nuclear fractions. The fractions were subjected to immunoblotting with anti-HA, anti-IE1, or anti-GP64 antibodies to detect PKIP, IE1, or GP64, respectively. Mock-infected (mi) cells were used as a negative control for immunoblotting. (B) Immunofluorescence analysis of the subcellular localization of PKIP. Sf9 cells were mock-infected or infected with v2HAPKIP at an MOI of 5 $TCID_{50}$/cell. At the indicated time points, cells were fixed, probed with an anti-HA antibody, and then visualized using an Alexa Fluor 647-conjugated goat anti-mouse antibody (red). Cell nuclei were stained with Hoechst 33258 (blue).

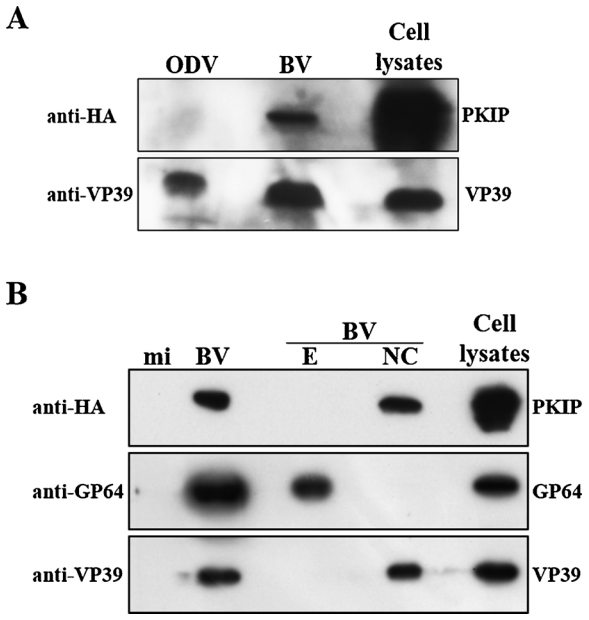

Fig. 9. PKIP is associated with nucleocapsid component of BV. (A) The BV or ODV was purified from v2HAPKIP-infected Sf9 cells or *Spodoptera exigua* larvae, respectively. (B) The purified BV were fractionated into envelope (lane E) and nucleocapsid (lane NC) fractions. Immunoblotting was performed with an anti-HA antibody to detect PKIP, an anti-GP64 antibody to detect the BV envelope glycoprotein GP64, and the anti-VP39 polyclonal antibodies to detect the major capsid protein VP39. Cell lysates were from v2HAPKIP-infected cells at 72 h p.i.

detected in the RZ of the vPKIPKO-transfected cells (Fig. 6F). These observations indicated that *pkip* deletion might affect the normal production of polyhedrin, which is encoded by the very late viral gene *polh*, and were in consistent with the previous study on the ts mutant of AcMNPV with defect in *pkip* (McLachlin et al., 1998).The kinase activity of PK1 was also required for polyhedrin hyperexpression (Liang et al., 2017). It was reported that PK1 interacted with and involved in the hyperphosphorylation of P6.9, which is a precondition for the maximal hyperexpression of baculovirus very late genes (Li et al., 2015). Thus, PKIP might involve in hyperphosphorylation of P6.9 to regulate very late gene expression by interacting with and stimulating the kinase activity of PK1. Since the defect of *pkip*-ts mutant at nonpermissive temperature was resulted from the mutation of an alanine at 46 of AcMNPV PKIP to threonine, this alanine might be required for the interaction between PKIP and PK1, which conferred the function of PKIP in BV production and very late gene expression. While the alanine at 46 of AcMNPV PKIP is only conserved in group I of the genus *Alphabaculovirus*, it might offer a specific feature to PKIP in BV production and very late genes expression during alphabaculovirus evolution.

The present study enriches the knowledge of the alphabaculovirus unique genes, and contributes to the understanding of the evolution of the baculovirus in the long run. Nevertheless, further studies are required to dissect the mechanism on how PKIP involves in nucleocapsid assembly and how it affects very late gene expression.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.05.014.

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