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Autographa californica multiple nucleopolyhedrovirus PK-1 is essential for nucleocapsid assembly

Changyong Liang [a,*], Min Li [a,1], Xuejuan Dai [a,1], Shuling Zhao [a], Yanling Hou [a], Yongli Zhang [a], Dandan Lan [a], Yun Wang [b], Xinwen Chen [b]

[a] College of Bioscience and Biotechnology, Yangzhou University, Yangzhou 225009, People's Republic of China
[b] State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, People's Republic of China

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ABSTRACT

PK-1 (Ac10) is a baculovirus-encoded serine/threonine kinase and its function is unclear. Our results showed that a *pk-1* knockout AcMNPV failed to produce infectious progeny, while the *pk-1* repair virus could rescue this defect. qPCR analysis demonstrated that *pk-1* deletion did not affect viral DNA replication. Analysis of the repaired recombinants with truncated *pk-1* mutants demonstrated that the catalytic domain of protein kinases of PK-1 was essential to viral infectivity. Moreover, those PK-1 mutants that could rescue the infectious BV production defect exhibited kinase activity *in vitro*. Therefore, it is suggested that the kinase activity of PK-1 is essential in regulating viral propagation. Electron microscopy revealed that *pk-1* deletion affected the formation of normal nucleocapsids. Masses of electron-lucent tubular structures were present in cell transfected with *pk-1* knockout bacmid. Therefore, PK-1 appears to phosphorylate some viral or cellular proteins that are essential for DNA packaging to regulate nucleocapsid assembly.

Introduction

The family *Baculoviridae* consists of a diverse group of invertebrate-specific DNA viruses. Members of this family are characterized by having enveloped, rod-shaped virions with a circular double-stranded DNA genome (Miller, 1993). During the baculovirus infection cycle, progeny nucleocapsids have two fates: to be matured as budded virus (BV) or as occlusion-derived virus (ODV). BVs are produced during the early stage of baculovirus infection and obtain their envelopes as they bud off the plasma membranes of the infected cells. BVs mediate the systematic infection within the host (Keddie et al., 1989). ODVs are produced during the very late stage of baculovirus infection and embedded within a crystalline structure made up of polyhedrin proteins that form occlusion bodies (OBs). ODVs are responsible for horizontal transmission between insect hosts (Keddie et al., 1989). BV and ODV share dozens of the same proteins, in which some proteins are essential for nucleocapsid formation including P6.9 (Wang et al., 2010a), VP39 (Thiem and Miller, 1989), C42, AC142, EC27 (Vanarsdall et al., 2007) and 38 K (Wu et al., 2006). In *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), very late factor 1 (VLF-1) (Vanarsdall et al., 2006), VP1054 (Olszewski and Miller, 1997) and AC109 (EC43) (Lin et al., 2009) were also found to be the components of BV and ODV and were essential for nucleocapsid assembly. Nucleocapsid assembly takes place in the virogenic stroma of nucleus, indicating that viral genome is pre-packaged with the basic DNA binding protein P6.9 (Wang et al., 2010a), and the nucleoprotein complex is then inserted into a pre-formed tube-like capsid sheaths composed of VP39.

Protein phosphorylation, which is mediated by protein kinases (PKs), is one of the most important mechanisms for regulating protein activity and enabling the cell to respond to external signals. PKs catalyze the transfer of a phosphoryl group to a hydroxyl group on either Ser/Thr or Tyr residue (Hanks and Hunter, 1995). PKs regulate a wide range of eukaryotic cellular functions, including transcription, translation, cell division and differentiation (Graves and Krebs, 1999). Evidence has now accumulated to suggest that PKs are important in the virus life cycles, including steps in viral infection, uncoating, transcription and replication (Hui, 2002). PK-1 is the baculoviral encoded serine/threonine kinase (Possee et al., 1991; Reilly and Guarino, 1994). In AcMNPV, PK-1 is expressed in the late and very late phases of infection (Possee et al., 1991; Reilly and Guarino, 1994). In contrast, in *Choristoneura fumiferana* granulosis virus (CfGV) and *Lymantria dispar* multiple nucleopolyhedrovirus (LdMNPV), PK-1 is expressed throughout the infection, suggesting it may be categorized as both an early and late baculovirus gene (Bischoff and Slavicek, 1994;

* Correspondence to: No. 12 Wenhui road east, Yangzhou, 225009, P.R.China. Fax: +86 514 87991747.
E-mail address: cyliang@yzu.edu.cn (C. Liang).
[1] These authors contributed equally to the work.

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Giannopoulos et al., 2005). PK-1 of AcMNPV has been shown to regulate very late *polh* promoter expression (Fan et al., 1996; Mishra et al., 2008a). Further studies indicated that PK-1 was a component of the very late gene transcription initiation complex of AcMNPV. And PK-1-mediated phosphorylation was a part of the regulatory mechanism to initiate transcription from the *polh* promoter (Mishra et al., 2008b). Fan et al. (1998) have shown using a yeast two-hybrid system that PK-1 interacts with an AcMNPV-encoded protein, PKIP, which stimulates the activity of PK-1. A temperature-sensitive mutation in the *pkip* gene caused AcMNPV to lose the ability to form plaques and occlusion bodies at the nonpermissive temperature (McLachlin et al., 1998). In *Bombyx mori* nucleopolyhedrovirus (BmNPV), a *pk-1* gene knockout virus failed to spread infection, but showed ability to express *egfp* in the transfection experiments (Ono et al., 2012). So, it is not very clear how PK-1 functions in early and late phases of infection.

In this study, we used an AcMNPV, one of the typical species of the *Baculoviridae*, to generate a *pk-1* knockout mutant by homologous recombination to determine the role of PK-1 in AcMNPV viral replication. Our data indicated that the PK-1 was essential for virion production, and its kinase activity seemed essential in regulating virion assembly.

Results

Generation of recombinant AcMNPV bacmids

To investigate the function of *pk-1* during the viral infection cycle, a bacmid containing a knockout in the *pk-1* gene was generated by the λ- red recombination system in *Escherichia coli*. In all the knockout candidates, nt 7043 to 7739 of the AcMNPV genome were successfully replaced by the *Cm* cassette (Fig. 1A), as all the PCR amplicons from catF/1020 were about 2.5 kb (expected size was 2452 bp) and amplicons from catR/1629 were nearly 2.7 kb (expected size was 2667 bp) (Fig. 1C).

To examine whether the *pk-1* deletion had any effect on virion morphogenesis and to facilitate observation of viral infection, the *polh* gene of AcMNPV and the *gfp* gene were inserted into the *polh* locus of vAc$^{pk\text{-}1\text{-}ko}$ via transposition. The resulting bacmid was named vAc$^{pk\text{-}1\text{-}ko\text{-}PH\text{-}GFP}$ (Fig. 1B). In order to assure the phenotype resulting from the deletion of *pk-1*, a repair bacmid, vAc$^{pk\text{-}1\text{-}rep\text{-}PH\text{-}GFP}$, was generated in which the *pk-1* gene with the *pk-1* promoter and poly (A) signal, as well as *polh* and *gfp*, were inserted into vAc$^{pk\text{-}1\text{-}ko}$ *polh* locus by transposition (Fig. 1B). Additionally, a series of truncated *pk-1* fragments with the *pk-1* promoter and the OpIE2 poly (A) tail, as well as *polh* and *gfp*, were inserted into vAc$^{pk\text{-}1\text{-}ko}$ *polh* locus by transposition. The truncated fragments were Z1, Z2, Z3, Z4, Z5, Z6, Z7 and Z8 (Fig. 1B). The recombinants were named vAc$^{pk1\text{-}Z1}$, vAc$^{pk1\text{-}Z2}$, vAc$^{pk1\text{-}Z3}$, vAc$^{pk1\text{-}Z4}$, vAc$^{pk1\text{-}Z5}$, vAc$^{pk1\text{-}Z6}$, vAc$^{pk1\text{-}Z7}$ and vAc$^{pk1\text{-}Z8}$, respectively. As a positive control, vAc$^{PH\text{-}GFP}$ was generated by transposing the *polh* and *gfp* into the *polh* locus of the bMON14272 bacmid.

All constructs were confirmed by PCR analysis. An amplicon of 819 bp was amplified from vAc$^{pk\text{-}1\text{-}rep\text{-}PH\text{-}GFP}$ or vAc$^{PH\text{-}GFP}$ by primers pk-1F/pk-1R, whereas no amplicon was produced from vAc$^{pk\text{-}1\text{-}ko\text{-}PH\text{-}GFP}$ (Fig. 1C), indicating that the *pk-1* gene was deleted in vAc$^{pk\text{-}1\text{-}ko\text{-}PH\text{-}GFP}$. No amplicon was amplified from vAc$^{PH\text{-}GFP}$ by primers catF/catR, but an amplicon of 1025 bp was produced in vAc$^{pk\text{-}1\text{-}ko\text{-}PH\text{-}GFP}$ or vAc$^{pk\text{-}1\text{-}rep\text{-}PH\text{-}GFP}$ (Fig. 1C), indicating that the *Cm* gene cassette was inserted in vAc$^{pk\text{-}1\text{-}ko\text{-}PH\text{-}GFP}$ or vAc$^{pk\text{-}1\text{-}rep\text{-}PH\text{-}GFP}$. No amplicon was produced by primers catF/1020 or catR/1629 in vAc$^{PH\text{-}GFP}$, whereas an amplicon of 2452 bp was produced using primers catF/1020 and that of 2667 bp was produced using primers catR/1629 in vAc$^{pk\text{-}1\text{-}ko\text{-}PH\text{-}GFP}$ or vAc$^{pk\text{-}1\text{-}rep\text{-}PH\text{-}GFP}$ (Fig. 1C), indicating the recombination junctions of *Cm* gene cassette with flanking regions of *pk-1* gene were generated in vAc$^{pk\text{-}1\text{-}ko\text{-}PH\text{-}GFP}$ and vAc$^{pk\text{-}1\text{-}rep\text{-}PH\text{-}GFP}$.

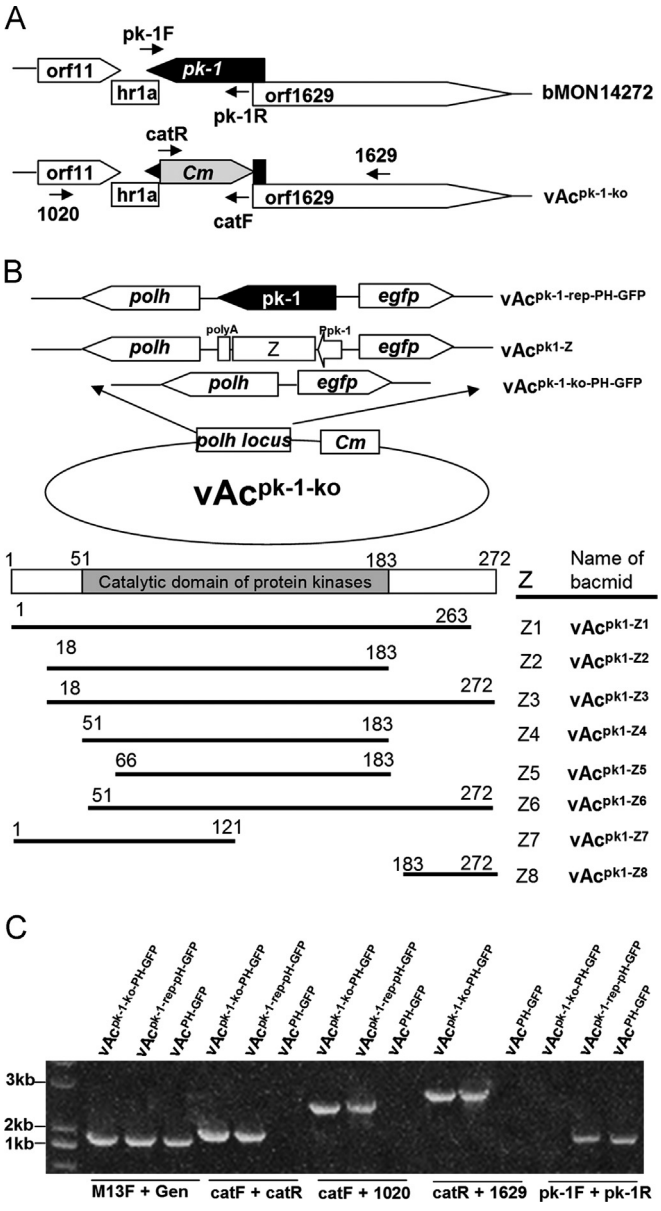

Fig. 1. Generation of recombinant AcMNPV bacmids. (A) Diagram of *pk-1* knockout. The C-terminal coding region (127 to 819 bp) of *pk-1* on AcMNPV bacmid bMON14272 was replaced with a *Cm* expression cassette. The knockout bacmid was named vAc$^{pk\text{-}1\text{-}ko}$. Four sets of primers, pk-1F/pk-1R, catR/catF, 1020/catE and 1629/catR, are labeled with arrows at their corresponding loci, which were used for PCR confirmation in *pk-1* knockout. (B) The schematic diagram of the recombinant bacmids derived from vAc$^{pk\text{-}1\text{-}ko}$. All of transposed bacmids possess a *gfp* gene and a *polh* gene. vAc$^{pk\text{-}1\text{-}rep\text{-}PH\text{-}GFP}$ was inserted a wild type *pk-1* expression cassette driven by *pk-1*'s native promoter and polyA tail. vAc$^{pk1\text{-}Z}$ was inserted Z, which is represented by the truncated *pk-1* mutants, driven by *pk-1*'s native promoter and OpIE2 polyA tail. The designations for *pk-1* mutants are indicated under the "Z", and the names of the corresponding recombinant repair bacmids are shown on the right. The delete sketch map at the top represents the PKc domain (51 to 183 aa). (C) PCR analysis of the presence or absence of sequence modifications in vAc$^{pk\text{-}1\text{-}ko\text{-}PH\text{-}GFP}$, vAc$^{pk\text{-}1\text{-}rep\text{-}PH\text{-}GFP}$, and vAc$^{PH\text{-}GFP}$. The virus templates are shown above each lane, and the primer pairs used are shown below.

PK-1 is essential for viral propagation

To examine the effect of *pk-1* deletion on the viral propagation, *Sf*9 cells were transfected with vAc$^{pk\text{-}1\text{-}ko\text{-}PH\text{-}GFP}$, vAc$^{pk\text{-}1\text{-}rep\text{-}PH\text{-}GFP}$ and vAc$^{PH\text{-}GFP}$, respectively. The GFP fluorescence cells were

observed in all transfection samples and gradually became stronger as the incubation time increased. Unlike the ubiquitous GFP signal in vAc$^{PH\text{-}GFP}$- or vAc$^{pk\text{-}1\text{-}rep\text{-}PH\text{-}GFP}$-transfected cells, only isolated fluorescence cells were observed in the cells transfected with vAc$^{pk\text{-}1\text{-}ko\text{-}PH\text{-}GFP}$ even at 96 h post-transfection (hpt) (Fig. 2A). These GFP expression phenotypes strongly indicated that vAc$^{pk\text{-}1\text{-}ko\text{-}PH\text{-}GFP}$ failed to yield infectious virions when transfected into *Sf*9 cells. Secondary virus infection tests further indicated that PK-1 was essential to BV propagation, as no GFP fluorescence was observed 96 h after the supernatants from vAc$^{pk\text{-}1\text{-}ko\text{-}PH\text{-}GFP}$-transfected cells were added to uninfected cells to initiate a secondary infection (Fig. 2A). As expected, the fluorescence was observed in almost all cells infected with vAc$^{PH\text{-}GFP}$ or vAc$^{pk\text{-}1\text{-}rep\text{-}PH\text{-}GFP}$ at 96 h post-infection (hpi) (Fig. 2A). Light microscopy showed that most of the cells infected with vAc$^{PH\text{-}GFP}$ or vAc$^{pk\text{-}1\text{-}rep\text{-}PH\text{-}GFP}$ contained OBs, whereas no OB was observed in the cells transfected or infected with vAc$^{pk\text{-}1\text{-}ko\text{-}PH\text{-}GFP}$ (Fig. 2B). These results suggested that the deletion of *pk-1* leads to a defect in infectious BV production and polyhedra formation.

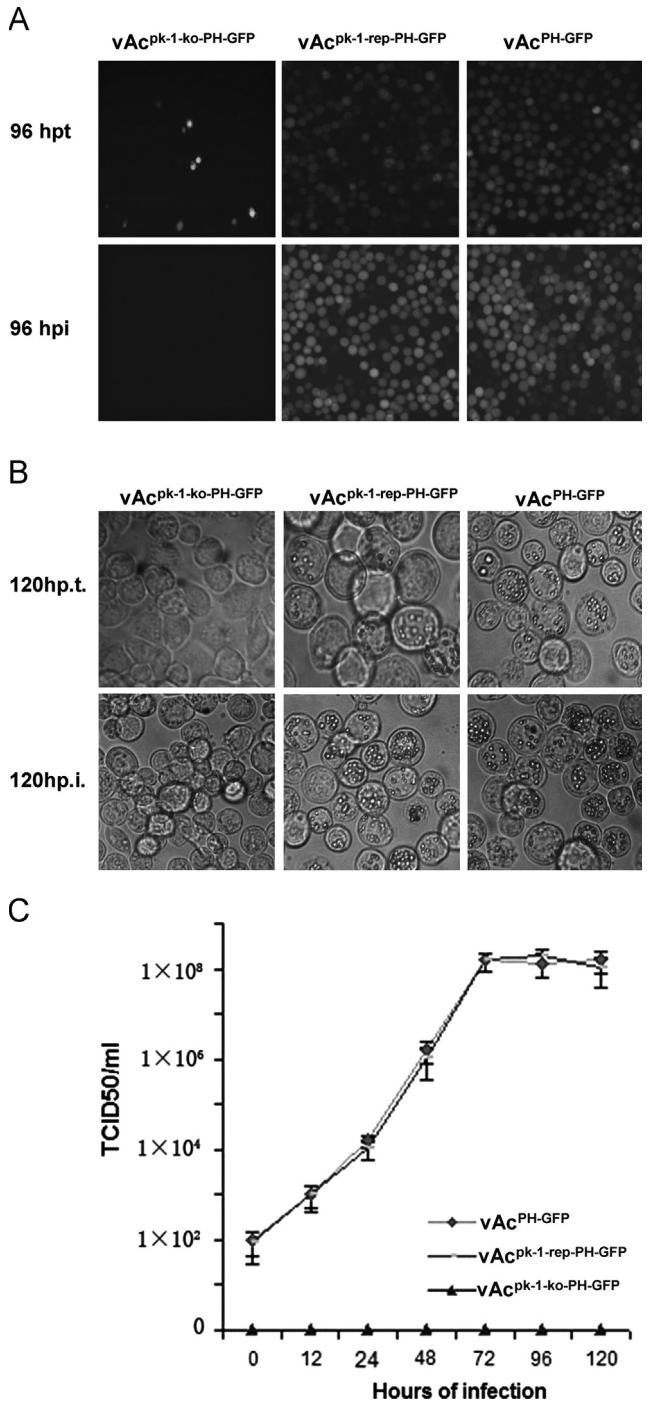

Fig. 2. viral propagation assays. (A) Analysis of viral propagation capability in *Sf*9 cells by transfection and secondary infection. Three bacmids were individually transfected into *Sf*9 cells, which were detected for GFP expression in 96 hpt using fluorescent microscope (top panel). At 120 hpt, the supernatants were collected to initiate secondary infections in *Sf*9 cells, which were detected for GFP expression in 96 hpi using fluorescent microscope. (B) Polyhedra were detected using light microscopy in *Sf*9 cells, which were transfected (top panel) and secondary infected (bottom panel) with vAc$^{pk\text{-}1\text{-}ko\text{-}PH\text{-}GFP}$, vAc$^{pk\text{-}1\text{-}rep\text{-}PH\text{-}GFP}$, or vAc$^{PH\text{-}GFP}$ at 120 hpt or 120 hpi, respectively. (C) Virus growth curve of knockout, rescued, and wild-type bacmids. *Sf*9 cells were infected with BV (vAc$^{pk\text{-}1\text{-}rep\text{-}PH\text{-}GFP}$ and vAc$^{PH\text{-}GFP}$) at an MOI of 5, or viral supernatants (vAc$^{pk\text{-}1\text{-}ko\text{-}PH\text{-}GFP}$) at 120 hpt. Cells culture supernatants were harvested and assayed for the production of infectious virus by TCID50 assay. Each datum point represents the average from three independent transfections or infections. Error bars represent standard errors.

To further evaluate the effect of deletion of *pk-1* on BV production and determine the replication kinetics, a virus growth curve analysis was performed. *Sf*9 cells were infected with either BVs of vAc$^{PH\text{-}GFP}$ and vAc$^{pk\text{-}1\text{-}rep\text{-}PH\text{-}GFP}$ at an MOI of 5 or viral supernatants of vAc$^{pk\text{-}1\text{-}ko\text{-}PH\text{-}GFP}$ from virus-infected cells at 120 hpt. At selected time points, the BV titers were determined by a $TCID_{50}$ endpoint dilution assay. In accordance with the preliminary results obtained from transfection-infection assays, vAc$^{pk\text{-}1\text{-}rep\text{-}PH\text{-}GFP}$ and vAc$^{PH\text{-}GFP}$ shared similar kinetics in virus production (Fig. 2C). In contrast, the titers were not detected by $TCID_{50}$ at any time point up to 120 hpi from vAc$^{pk\text{-}1\text{-}ko\text{-}PH\text{-}GFP}$-infected cells (Fig. 2C). These results indicated that the defective phenotype could be rescued by inserting *pk-1* into the *polh* locus of AcMNPV.

The pk-1 deletion has no effect on viral DNA replication

To determine whether PK-1 affects the viral DNA replication within transfected cells, the level of viral DNA synthesis in vAc$^{pk\text{-}1\text{-}ko}$ transfected cells was analyzed by qPCR assay. The *gp64*-knockout bacmid (*gp64*-KO) was served as the control virus (Vanarsdall et al., 2006). Since the fusion protein GP64 is required for virion budding from infected cells (Oomens and Blissard, 1999), the *gp64*-KO bacmid lacks the ability to initiate cell-to-cell infection, but viral DNA replication is unaffected. The results of this analysis indicated that both DNA synthesis of vAc$^{pk\text{-}1\text{-}ko}$ and *gp64*-KO kept increasing up to 48 hpt at similar levels during a 48 hpt period (Fig. 3). These data confirmed that the deletion of *pk-1* did not result in a defect in the level of viral DNA replication.

The catalytic domain of protein kinases (PKc) in PK-1 is important for viral propagation

To explore whether the PKc domain of PK-1 plays an important role in BV production, a series of recombinant AcMNPV bacmids containing retrieved truncated *pk-1* fragments were constructed (Fig. 1B). Transfection and infection analysis showed that those fragments containing PKc domain (Z1, Z2, Z3, and Z4) could rescue the infectious BV production defect (Fig. 4A), while other fragments without PKc domain (Z5, Z7 and Z8) could not rescue the infectious BV defect (Fig. 4A). The only exception was Z6, which contained PKc domain but failed to rescue the infectious BV defect.

Infectivity of recombinant AcMNPVs repaired with truncated pk-1 mutants

To further assess the infectivity and BV production of the recombinant AcMNPVs repaired with truncated *pk-1* mutants, *Sf*9 cells were synchronously infected with BV of the recombinant viruses and the titers were determined by an end-point dilution

assay. Those mutants that succeeded in rescuing the defect of infectious BV production showed similar replication kinetics with $vAc^{PH\text{-}GFP}$ and $vAc^{pk\text{-}1\text{-}rep\text{-}PH\text{-}GFP}$ (Figs. 2C and 4B). At 96 hpi, $vAc^{pk1\text{-}Z1}$, $vAc^{pk1\text{-}Z2}$, $vAc^{pk1\text{-}Z3}$ and $vAc^{pk1\text{-}Z4}$ produced about 1.6×10^8, 3.0×10^8, 7.4×10^7 and 1.3×10^8 $TCID_{50}$/ml, respectively (Fig. 4B), which were not significantly different with $vAc^{PH\text{-}GFP}$ and $vAc^{pk\text{-}1\text{-}rep\text{-}PH\text{-}GFP}$ whose titers were about 1.9×10^8, and 1.3×10^8 $TCID_{50}$/ml (Fig. 2C). These results indicated that the PKc domain of PK-1 was essential for BV yields.

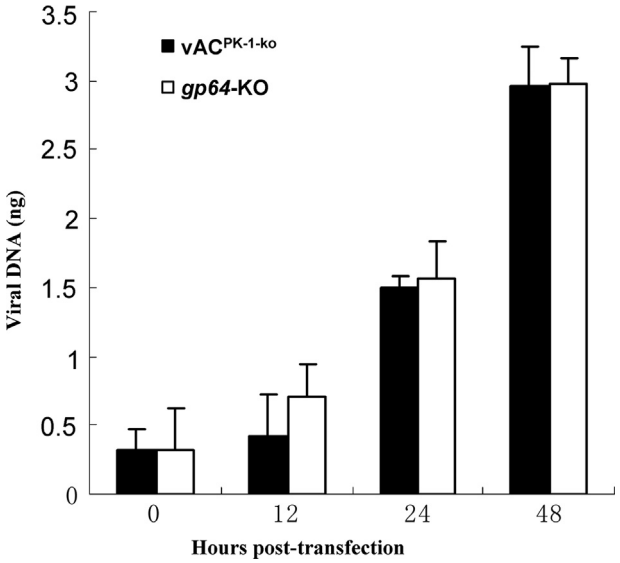

Fig. 3. Real-time PCR analysis of viral DNA replication in *Sf*9 cells. Total DNA was extracted from *Sf*9 cells transfected with $vAc^{pk\text{-}1\text{-}ko}$ or *gp64*-KO at selected time points, digested with DpnI and assayed by real-time PCR. Values represent the means from three independent transfections. Error bars indicate standard deviations.

The PKc domain expressed in E. coli exhibits kinase activity in vitro

Z1∼8 and wt PK-1 were expressed in *E. coli* and purified. Coomassie blue staining of the gels showed that the purity of each protein was greater than 95% (data not shown). wt and PK-1 mutants including Z1∼8 were evaluated for kinase activity using the protamine sulphate as substrate, which has been used as substrate for PK-1 (Miller et al., 1983). The results showed that mutants Z1, Z2, Z3, and Z4 exhibited about 40–80% of the kinase activity compared to wt PK-1 (Fig. 5), whereas mutants Z5, Z6, Z7, and Z8 exhibited kinase activity similar with the control of MBP, which was less than 5% kinase activity of wt PK-1(Fig. 5). All proteins were checked and had no valuable measurement about their autophosphorylation (data not shown). These results showed that mutants containing Z1∼4 of the PKc domain possessed kinase activity, whereas Z5∼8 possessed no detectable kinase activity.

PK-1 is involved in nucleocapsid assembly and polyhedra formation

In order to evaluate the effect of *pk-1* deletion on virion morphogenesis, electron microscopy analysis was performed with thin sections generated from $vAc^{PH\text{-}GFP}$ and $vAc^{pk\text{-}1\text{-}ko\text{-}PH\text{-}GFP}$ transfected cells, respectively. In cells transfected with $vAc^{PH\text{-}GFP}$, a well-defined virogenic stroma containing electron-dense rod-shaped nucleocapsids was observed (Fig. 6B and D). However, only masses of electron-lucent tubule-like structures were present in nucleus of cells transfected with $vAc^{pk\text{-}1\text{-}ko\text{-}PH\text{-}GFP}$ (Fig. 6A and C). These electron-lucent tubular structures seemed to be the empty capsid sheaths which were devoid of a viral DNA-P6.9 core, indicating that the viral DNA genomes failed to be condensed or packaged into the tubular structures. The result indicated that PK-1 was required for proper nucleocapsid assembly.

Additionally, *Sf*9 cells transfected with $vAc^{pk\text{-}1\text{-}rep\text{-}PH\text{-}GFP}$ or $vAc^{PH\text{-}GFP}$ showed obvious polyhedra (Fig. 2B). In contrast, no polyhedra formation was observed in *Sf*9 cells transfected with

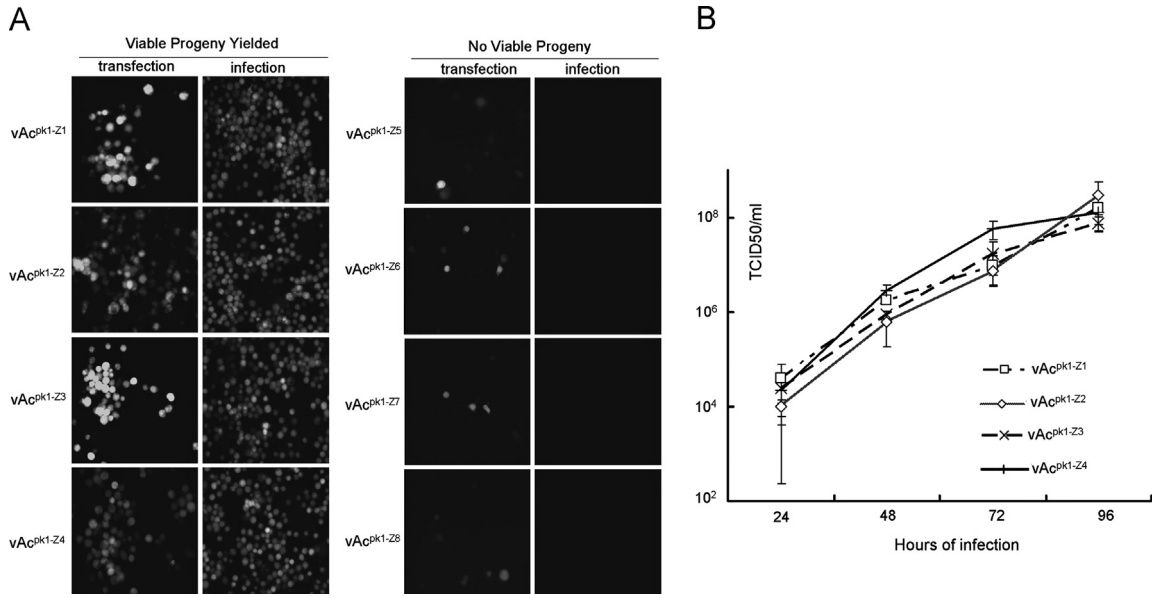

Fig. 4. The PKc domain (aa 51 to 183) plays a pivotal role in BV production. (A) Fluorescence microscopy images of the left panel of two columns show *Sf*9 cells transfected with 2.0 μg of DNA of $vAc^{pk1\text{-}Z1\sim8}$ at 96 hpt, respectively. The right panels of two columns show new monolayer cells which were infected with the cell culture supernatants of transfected cells. The left column shows that the recombinant viruses yield viable progeny by rescue with truncated PK-1 mutants Z1∼4, while the right column shows that the recombinant viruses do not yield viable progeny by rescue with Z5∼8. (B) Virus growth curve of the recombinant viruses by rescue with Z1∼4, which produce infectious progeny. *Sf*9 cells were infected with BV at an MOI of 5. Cells culture supernatants were harvested and assayed for the production of infectious virus by TCID50 assay. Each datum point represents the average from three independent infections. Error bars represent standard errors.

vAc$^{pk\text{-}1\text{-}ko\text{-}PH\text{-}GFP}$ by fluorescence and light microscope (Fig. 2A and B). Electron microscopy analysis also showed that no polyhedra formation was detected in *Sf*9 cell transfected with vAc$^{pk\text{-}1\text{-}ko\text{-}PH\text{-}GFP}$ (Fig. 6A), whereas several polyhedra were observed in *Sf*9 cell transfected with vAc$^{PH\text{-}GFP}$ (Fig. 6B). These results indicated that PK-1 was involved in polyhedra formation.

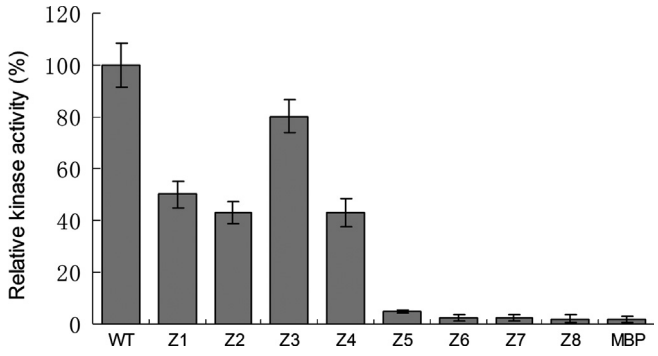

Fig. 5. Comparative Analysis for kinase activity of wt PK-1 and its mutants. MBP, WT or PK-1 mutants (200 nM each) were incubated with 0.1 mM ATP containing 5 μCi of [γ-^{32}P] ATP, 0.8 mg/ml protamine sulphate for kinase assay. The results are graphed relative to wild type PK-1, which was set at 100%. The data shows Z1∼4 exhibit more than 40% kinase activity compare with wt PK-1, while Z5∼8 exhibited less than 5% kinase activity compare with wt PK-1 and similar with the control of MBP. The data represent the average of triplicate determinations from three representative experiments with error bars indicating S.D.

Discussion

Viral serine/threonine kinases appear to be encoded exclusively by large and evolutionarily old DNA viruses, such as herpesviruses, poxviruses, and baculoviruses (Jacob et al., 2011). Previous data indicated that baculoviral *pk-1* encoded a serine/threonine protein kinase which was able to phosphorylate histone H1 in rabbit reticulocyte lysates (Kim and Weaver, 1993; McLachlin et al., 1998) and protamine sulphate (Miller et al., 1983). Sequence comparison of sequenced baculoviruses showed that *pk-1* was present in lepidopteran baculoviruses, but absent in *Culex nigripalpus* NPV (Diptera), *Neodiprion lecontei* NPV (Hymenoptera) and *Neodiprion sertifer* NPV (Hymenoptera). Many baculovirus-encoded proteins of various temporal classes have been reported to be phosphorylated, including GP64 (Maruniak and Summers, 1981), PP31 (Guarino et al., 1992), PP34 (Whitt and Manning, 1988), capsid protein (vp39), ORF1629 (Vialard and Richardson, 1993) and P6.9 (Kelly and Lescott, 1984). Recently, analysis of protein phosphorylation in *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV) BV and ODV was reported (Hou et al., 2013). A total of 23 baculoviral proteins in both BV and ODV of HearNPV were identified as phosphoproteins (Hou et al., 2013). Among all the

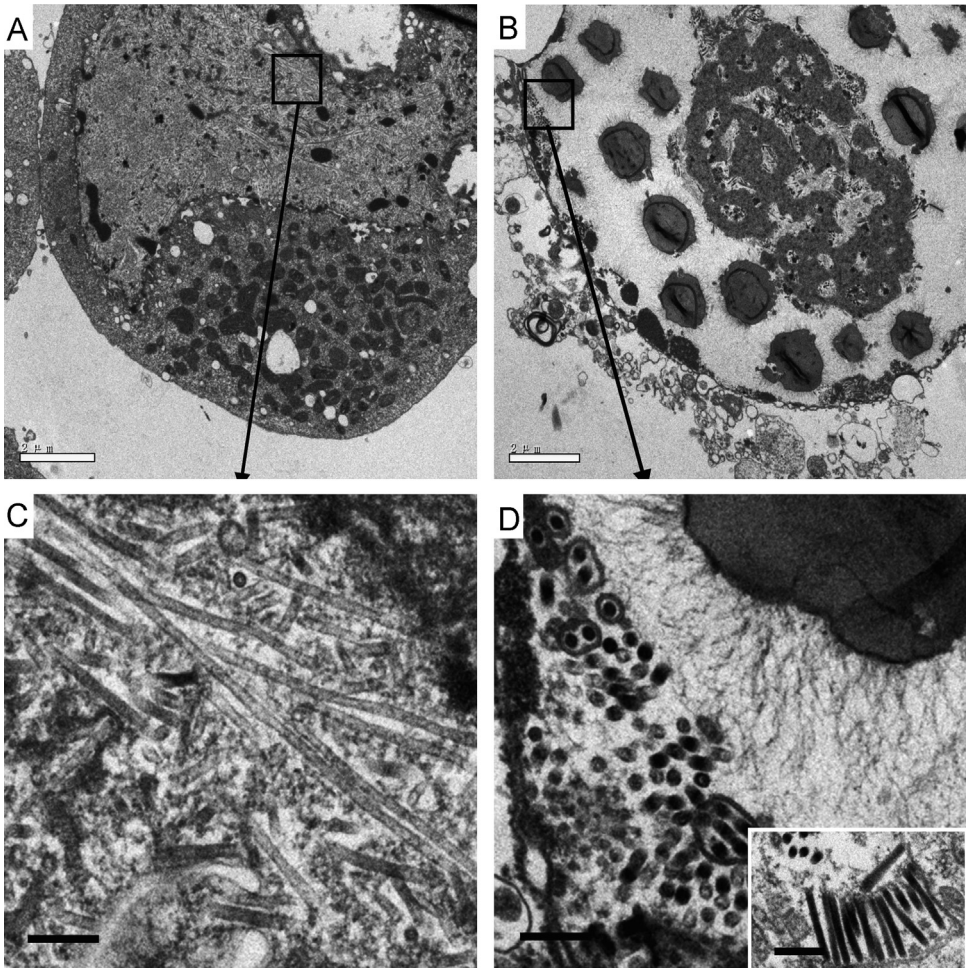

Fig. 6. Electron microscopy analysis of transfected Sf-9 cells to characterize PK-1 mutant. (A) Whole of a *Sf*9 cell transfected with vAc$^{pk\text{-}1\text{-}ko\text{-}PH\text{-}GFP}$. No polyhedra formation was observed in the cell. (B) Sf-9 cell was transfected with vAc$^{PH\text{-}GFP}$. There are several polyhedra within the nucleus. (C) Portion enlarged form the cell showed in figure A. Masses of electron-lucent tubular structures were showed in the nucleus. (D) Portion enlarged from the cell showed in figure B. Normal nucleocapsids are present in the edges of nucleus. The inset shows a vertical section of several nucleocapsids. White bars, 2 μm; black bars, 200 nm.

phosphorylation sites, 38 sites were in ODV-associated proteins, 4 sites were in BV-associated proteins, only 1 site was identified in both BV and ODV-associated protein (Hou et al., 2013). So, phosphorylation plays important roles in the infection process of baculovirus and the differentiation of virion. To date, it is unclear which phosphorylation sites are regulated by PK-1. The function of PK-1 in the infection process of baculovirus is less known.

We investigated the role of AcMNPV PK-1 in viral propagation by using a *pk-1* knockout bacmid. The *pk-1* knockout virus failed to propagate after transfection into *Sf*9 cells. This defection in replication could be rescued by reinsertion of the *pk-1* gene into the *polyhedrin* locus of the same bacmid. And the repaired virus was equivalent to the wt virus with respect to replication in host cells. The results confirmed that this phenotype resulted from the *pk-1* knockout, not from a second site mutation or from disruption of regulatory elements located at the *pk-1* locus. Therefore, our studies indicated that PK-1 is essential for AcMNPV propagation in *Sf*9 cells.

It was possible that the failure of viral propagation was an indirect effect of a defect in viral DNA replication. To eliminate this possibility, a quantitative real-time PCR assay for viral DNA replication was performed. The result demonstrated that PK-1 was not essential for viral DNA replication, as evidenced by the fact that the *pk-1*-null AcMNPV bacmid had replication efficiencies similar to that of the control *gp64*-null AcMNPV bacmids. The result was also in accordance with that reported previously (Fan et al., 1996).

It is important for us to know whether PK-1 regulating viral propagation depends on its kinase activity. Sequence alignments indicate that PK-1 contains a PKc domain, which are common to protein kinases. The PKc is the essential domain of protein kinase for catalysation in the transfer of the gamma-phosphate of ATP to a serine, threonine or tyrosine residue in protein substrates (Johnson, 2009). Those truncated fragments of PK-1 containing PKc except Z6, which could rescue the infectious BV production defect, possessed kinase activity, whereas the other truncated fragments, which could not rescue PK-1 deletion, have no detectable kinase activity. Therefore, it is suggested that the kinase activity of PK-1 is essential in regulating viral propagation. So, PK-1 may regulate viral replication through phosphorylating one or more protein(s), which mediate essential steps in viral infection. Although these truncated PK-1 mutants exhibited about 40–80% of the kinase activity compared to wt PK-1 *in vitro*, all of them appear to possess intact capability to aid the *pk-1-null* AcMNPV to produce high virion yield similar to wt virus. So, the level of kinase activity of PK-1, to some extent, did not influence virion yield. The possible reason is that PK-1 may trigger a switch controlling virion assembly through its kinase activity.

Interestingly, the smaller truncation Z4 (51–183 aa) had kinase activity and could rescue the infectious BV production defect. On the contrary, the larger truncation Z6 (51–272 aa) had no detected kinase activity and failed to rescue the infectious BV production defect. One possible explanation for this is that the C terminal region of PK-1 interferes with the kinase activity of PKc domain. However, the kinase activity of Z4 was not inhibited by Z8 (183–272 aa) when Z4 and Z8 were co-incubated *in vitro* (data not shown). So, we speculated that the C-terminus of PK-1 covered the active site of kinase due to improper three dimensional structure of protein. The similarities of PK-1 in other baculoviruses were analyzed and found that the PKc domain was highly conserved, while N-terminus and C-terminus of PK-1 have low sequence homology (data not shown), which suggested that PKc was essential for the propagation of baculoviruses.

Electron microscopy showed that the failure of a *pk-1* knockout bacmid to produce budded viruses was due to a defect in nucleocapsid assembly. Abundant elongated electron-lucent tube structures were present in cells transfected with the *pk-1* knockout bacmid. These tuber structures were longer than the normal nucleocapsids, and no electron-dense inner nucleoprotein core was present in them, suggesting that they are empty capsid shells. Similar aberrant long nucleocapsid structures were also found in non-BV-producing *orf101*-null (Vanarsdall et al., 2007), *vlf-1*-null (Vanarsdall et al., 2006), *38k*-null (Wu et al., 2006) and *p6.9*-null AcMNPV (Wang et al., 2010a). Interestingly, P6.9, 38K, ORF101 and VLF-1 had been demonstrated to be the essential components of AcMNPV nucleocapsid structure (Wang et al., 2010b), while PK-1 was not detected as a structural component in both BV and ODV (Braunagel et al., 2003; Wang et al., 2010b). P6.9 is a heterogeneous arginine- and serine-rich protein, ranging from 49 to 109 aa, which is believed to bind to and condense viral DNA during nucleocapsid assembly and virion packaging (Tweeten et al., 1980; Wilson et al., 1987; Wilson and Price, 1988). Moreover, 38K may facilitate the P6.9-DNA complex packaging into viral nucleocapsids (Wu et al., 2006). Previous reports indicated that the phosphorylation or dephosphorylation of P6.9 played an essential role in promoting DNA binding and enabling condensation of the viral genome and packaging into the viral nucleocapsid (Funk and Consigli, 1993; Kelly and Lescott, 1984; Maeda et al., 1991; Wilson and Consigli, 1985). PK-1 was detected to catalyze phosphorylation of the VP12, a P6.9 homologue, of granulosis virus (Wilson and Consigli, 1985). Additionally, ORF101, and VLF-1 were detected as phosphoproteins in HearNPV ODV (Hou et al., 2013). Therefore, PK-1 may catalyze phosphorylation of cellular proteins and/or viral proteins that include P6.9 or other baculoviral capsid protein(s) such as VLF, ORF101, or 8K to regulate nucleocapsid assembly. The mechanism needs to be explored in future studies.

Our results showed that deletion of PK-1 from AcMNPV disrupted polyhedra formation. It is possible that PK-1 regulates transcription or translation of very late *polyhedrin* gene, or phosphorylates polyhedrin to influence polyhedra formation. Based on temperature-sensitive AcMNPV mutants assay, PK-1 has been shown to regulate transcription from very late promoters, such as the *polyhedrin* and *p10* promoters, to disrupt polyhedra formation (Fan et al., 1996). PK-1 also played a role in the phosphorylation of LEF8 protein, which is a component of the very late gene transcription initiation complex (Mishra et al., 2008b). So, it is suggested that PK-1 regulates polyhedra formation by phosphorylating some factors involving in very late transcription. Exploring the regulating mechanism of polyhedra formation is of interest to us in future studies.

Materials and methods

Viruses, cells and primers

The AcMNPV bacmid bMON14272 (Invitrogen) was propagated and maintained in *E. coli* DH10B cells as described previously (Luckow et al., 1993). *Spodoptera frugiperda* clone 9 (*Sf*9) cells were maintained at 27 °C in Grace's medium (Invitrogen) supplemented with 10% fetal bovine serum (Gibco). All primers listed in Table 1 were synthesized in Invitrogen Corporation.

Generation of the pk-1-knockout AcMNPV bacmid

Using the bacmid bMON14272, *pk-1* knockout AcMNPV bacmid was generated via homologous recombination in *E. coli* as previously described (Bideshi and Federici, 2000; Vanarsdall et al., 2004). A transfer vector in which the *pk-1* locus region was replaced with a chloramphenicol resistance gene (Cm) for antibiotic selection in *E. coli* was constructed as follows. A 902 bp fragment homologous to the 5′-flanking region (nt 6141 to 7042 in

the AcMNPV genome (GenBank accession number NC_001623.1)) of the *pk-1* gene was amplified from the AcMNPV bacmid using primers pk-upF and pk-upR. The PCR product was digested with XbaI and EcoRI and then ligated into the vector pBlueScriptII SK(+) (Stratagene) to generate the recombinant plasmid pSK-US. With the primers catF and catR, the *Cm* gene cassette was amplified from the plasmid pKD3 (Invitrogen). The PCR product was digested with EcoRI and XhoI and ligated into pSK-US to generate the recombinant plasmid pSK-US-Cm. Using the primers pk-dnF and pk-dnR, a 600 bp fragment homologous to the 3′-flanking region (nt 7740 to 8339) of the *pk-1* gene was amplified from the AcMNPV bacmid. The resulting product was digested with XhoI and KpnI and then ligated into pSK-US-Cm to generate the final *pk-1* knockout transfer plasmid pSK-US-Cm-DS. This transfer vector was digested with StuI, and the resulting linear 2527-bp fragment containing the *Cm* gene cassette and the *pk-1* flanking region was gel purified.

The above gel-purified product was digested by StuI overnight to completely remove any remaining intact plasmids before it was transformed into arabinose-preinduced DH10B competent *E. coli* cells harboring bMON14272 and λ-Red recombinase encoding plasmid pKD46. The resulting transformed DH10B cells were incubated at 37 °C for 4 h in SOC medium (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) with gentle shaking before being spread onto LB agar plate containing kanamycin and chloramphenicol. The colonies resistant to kanamycin and chloramphenicol were selected after 48 h of incubation at 37 °C, and the bacmid constructs were extracted and verified by PCR analysis. The resulting *pk-1* knockout bacmid was named $vAc^{pk\text{-}1\text{-}ko}$ (Fig. 1A).

Four pairs of specific primer were used to confirm the replacement of *pk-1* by the *Cm* gene in the *pk-1* locus of bMON14272. Primers catF and catR were used to detect the correct insertion of the *Cm* gene cassette (Fig. 1A). Primers pk-1F and pk-1R were used to confirm the deletion of *pk-1* gene (Fig. 1A). Primers catF and 1020 or primers catR and 1629 were used to examine the recombination junctions of the upstream and downstream flanking regions (Fig. 1A).

Table 1
Primers used for this study.

Name	Sequence(5′ to 3′)
A1	CTGCAGTCAAGCTCGGATCCCGCAC PstI
A2	TCTAGAGGACCTGTTGTGTGTTATGG XbaI
catF	GAATTCTGTAGGCTGGAGCTGCTTCG EcoRI
catR	CTCGAGCATATGAATATCCTCCTTAG XhoI
Gen	ATCATTCGCACATGTAGGC
M13F	GTTTTCCCAGTCACGAC
P-F	CTGCAGTGTTGTCAAAAACGTCGTTG PstI
P-R	TCTAGAGACGAATCGTAGATATGAATC XbaI
pA-F	GTCGACCATTGAGTTTATCTGACTAAATC SalI
pA-R	GAATTCGCCTTTGAGTGAGCATCG EcoRI
PE38F	AATGGAACAGCAGCGA
PE38R	CGCACGTAGTCGGAATC
pk-1F	ATGGCCACCACAAATGCT
pk-1R	TTACGACAAAAACTCATGTTT
pk-dnF	CTCGAGGCCACTTGTTTTACGAGTAG XhoI
pk-dnR	GGTACCAGGCCTCGTTTGTCGGAAGCG KpnI and StuI
pk-upF	TCTAGAAGGCCTCAACTATTGTACTGG XbaI and StuI
pk-upR	GAATTCGGGCTTGTGCGATAAAATAG EcoRI
1F	TCTAGAATGGCCACCACAAATGCT XbaI
18F	TCTAGAATGTGCAAAAACGTCAAAACTCG XbaI
51F	TCTAGAATGACAATTTCGGCGCACAATTT XbaI
66F	TCTAGAATGCAGTTAATGAGCGACCAC XbaI
121F	TCTAGAATGCTGTGTGAAGCGCTCAAC XbaI
183F	TCTAGAATGCACACA ACTATGCACGTTTC XbaI
121R	GTCGACTTACAGCTGTCTAATAATATTGC SalI
183R	GTCGACTTAGTGTCGAATTTTTTCCGGAC SalI
263R	GTCGACTTATTTGTAATTTGTGAGTCTACAA SalI
272R	GTCGACTTACGACAAAAACTCATGTTTT SalI
1020	ACCAAATTGCAGTAATCAG
1629	TTCAGCGTCTTTATATCTG

Construction of donor plasmids and transposition of bacmid constructs

A series of donor vectors were constructed based on plasmid pFBI-PH-GFP (donated by K.Yang, Sun Yat-sen University), which was constructed by inserting *polyhedrin* and *gfp* into the pFastBacI (Invitrogen) under the control of the *polyhedrin* promoter and the AcMNPV *ie1* promoter, respectively (Wu et al., 2006). A 1375-bp fragment of *pk-1* cassette (nt 6618 to 7992) containing the wild-type (wt) *pk-1* gene with its own promoter and poly (A) tail was PCR amplified from AcMNPV genome using primers A1 and A2. Then, the fragment was cloned into pFBI-PH-GFP plasmid to generate pFBI-pk-1-PH-GFP. Meanwhile, a series of donor plasmids, containing the native *pk-1* promoter, OpIE2 poly (A) tail and different truncated *pk-1* gene sequences were constructed as follows. A 200-bp fragment of *pk-1* native promoter was amplified by PCR from AcMNPV bacmid using primer P-F and primer P-R. Then, the PCR product was cloned into the plasmid pFBI-PH-GFP to generate pFBI-P. With the primers pA-F and pA-R, the OpIE2 poly (A) tail was amplified from the plasmid pIZ/V5-His (Invitrogen), and then, cloned into pFBI-P to generate pFBI-P-pA. Finally, a series of truncated fragments of *pk-1* gene were inserted between XbaI and SalI sites under the control of the *pk-1* promoter to generate pFBI-Z1～Z8, respectively (Table 2). All of these truncated *pk-1* fragments were obtained by PCR with the primer pairs listed in Tables 1 and 2 and sequenced to confirm their fidelity. Competent DH10B cells containing the helper plasmid pMON7124 and bacmid $vAc^{pk\text{-}1\text{-}KO}$ were transformed with donor plasmids pFBI-PH-GFP, pFBI-pk-1-PH-GFP and pFBI-Z1～Z8 to generate the *pk-1*-null bacmid $vAc^{pk\text{-}1\text{-}ko\text{-}PH\text{-}GFP}$, the *pk-1* repair bacmid $vAc^{pk\text{-}1\text{-}rep\text{-}PH\text{-}GFP}$, and a series of *pk-1* mutants repair bacmids $vAc^{pk1\text{-}Z1\sim Z8}$ using the Bac-to-Bac system (Luckow et al., 1993), respectively (Fig. 1B). DH10B cells containing helper plasmid pMON7124 and AcMNPV bacmid bMON14272 were transformed with pFBI-PH-GFP to generate a wt control bacmid named $vAc^{PH\text{-}GFP}$. Transformed cells were placed onto agar medium containing kanamycin (50 μg/ml), gentamycin (7 μg/ml), tetracycline (10 μg/ml), X-Gal (100 μg/ml) and IPTG (40 μg/ml). White colonies were selected and restreaked onto fresh plates to verify the phenotype, which was then confirmed by PCR. Primers M13F and Gen were used to confirm the transposition.

Viral propagation assay

DNA (approximately 1 μg) of each recombinant bacmid was transfected into 1.5×10^6 *Sf*9 cells by 10 μl Cellfectin (Invitrogen) according to the manufacturer's protocol. After 96 hpt, cells were examined for GFP expression and polyhedra formation by fluorescence microscopy. At 120 hpt, the supernatants were harvested

Table 2
Donor plasmids and primers used for this study.

Donor plasmid	Fragment (aa position)	Primers
pFBI-Z1	1–263	1F/263R
pFBI-Z2	18–183	18F/183R
pFBI-Z3	18–272	18F/272R
pFBI-Z4	51–183	51F/183R
pFBI-Z5	66–183	66F/183R
pFBI-Z6	51–272	51F/272R
pFBI-Z7	1–121	1F/121R
pFBI-Z8	183–272	183F/272R

and filtered with 0.45-μm-diameter syringe filters (Sartorius) to remove cell debris before being added to uninfected *Sf*9 cells to initiate secondary infection. After 2 h of incubation, the supernatants were removed and the cells were added with fresh medium. The infected cells were again inspected for GFP signal at 96 hpi, and polyhedra formation at 120 hpi. To evaluate the growth kinetics of viruses derived from these recombinant bacmids, *Sf*9 cells plated at 2.5×10^5 per well on a 24-well plate were infected with BV stocks ($vAc^{pk\text{-}1\text{-}rep\text{-}PH\text{-}GFP}$, $vAc^{PH\text{-}GFP}$, and $vAc^{pk1\text{-}Z1\sim Z4}$) at a multiplicity of infection of 5 or 1,000 μl supernatants from bacmid-transfected cells ($vAc^{pk\text{-}1\text{-}ko\text{-}PH\text{-}GFP}$) at 120 hpt in triplicate. The cell monolayers were incubated for 2 h after infection, washed with Grace's medium, and replenished with fresh Grace's medium supplemented with 10% fetal bovine serum. Virus supernatants were collected at selected time points, and the titers were determined by 50% tissue culture infective dose ($TCID_{50}$) endpoint dilution assay on *Sf*9 cells with GFP expression as an indicator of positive infection.

qPCR analysis of viral DNA replication

To assess viral DNA replication, a quantitative real-time PCR (qPCR) assay was performed as previously described (Vanarsdall et al., 2005). *Sf*9 cells were transfected with 5 μg bacmid DNA of $vAc^{pk\text{-}1\text{-}ko}$ or *gp64*-KO (a kind gift to X. Chen from G. F. Rohrmann, Oregon State University), which is a fusion protein gene *gp64*-knockout bacmid using as a non-infection control virus due to its lack of cell-to-cell infectivity (Oomens and Blissard, 1999; Vanarsdall et al., 2006). At selected time points, cells were harvested and the total DNA was extracted with a Universal Genomic DNA Extraction Kit (TaKaRa) according to the manufacturer's instructions. The total DNA was resuspended in 100 μl of sterile water. Prior to PCR, 10 μl of total DNA from each time point was digested with 10 units of DpnI restriction enzyme (New England BioLabs) for 24 h in a 50-μl total reaction volume to eliminate input bacmid DNA. Quantitative PCR (qPCR) was performed with 1 μl of digested DNA added to SYBR green real-time PCR master mix (Toyobo) according to the manufacturer's instructions and was analyzed using the primers PE38F and PE38R, which target at a 137 bp region of *PE38* gene.

Production and purification of maltose-binding protein (MBP) fusion protein expressed in E. coli

The wt or truncated *pk-1* gene fragments (Z1∼8) (Fig. 2C) were amplified by PCR from AcMNPV bacmid as mentioned above. All PCR fragments were then cloned into pMal-c2x (New England BioLabs) to generate pMal-PK1 and pMal-Z1∼Z8, respectively. The resulting constructs were transformed into *E. coli* Rosetta-gami (DE3) competent cells for expressing wt PK-1 or truncated PK-1 mutants (Z1∼Z8) fused at the C terminus of MBP. After affinity purification by amylose resin (New England BioLabs) and purification by Sephadex G-100 chromatograph, those fusion proteins and MBP were used to analyze kinase activity, respectively.

In vitro kinase assays

Purified MBP fusion proteins captured on amylase beads were rinsed twice with washing buffer (50 mM Tris–HCl [pH 8.0] and 1 mM dithiothreitol). Purified proteins were further purified by Sephadex G-100 chromatograph and quantified for their concentrations with Bradford assay, and were subjected to *in vitro* kinase assays. The assays were performed to determine whether certain MBP fusion proteins could phosphorylate substrate protamine. Protein kinase assays were carried out in 100 μl reaction mixtures containing 20 mM Tris (pH 8.0), 10 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM ATP containing 5 μCi of [γ-^{32}P] ATP, and protamine sulphate (0.8 mg/ml) per reaction. Then, each purified MBP fusion protein was added and the final concentration of each fusion protein was 200 nM. Reactions were incubated 30 min at 32 °C. At the end of the incubation 100 μl samples were spotted onto Whatman 3 MM paper discs which were washed for 15 min periods twice in 20% TCA, four times in 10% TCA, and then rinsed in absolute ethanol, dried, and their radioactivity was measured by scintillation spectrometry.

Electron microscopy

*Sf*9 cells were transfected with 3μg $vAc^{pk\text{-}1\text{-}ko\text{-}PH\text{-}GFP}$ or $vAc^{PH\text{-}GFP}$. At 96 hpt, cells were dislodged and pelleted at 2000 × g for 10 min. Then the cells were fixed, dehydrated, embedded, sectioned, and stained as described previously (Liang et al., 2004). Samples were examined using a Tecnai 12 transmission electron microscope.

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