The FP25K Acts as a Negative Factor for the Infectivity of AcMNPV Budded Virus

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Abstract

Baculoviruses generally produce two progeny phenotypes—the budded virus (BV) and the occlusion-derived virus (ODV)—and the intricate mechanisms that regulate the temporal synthesis of the two phenotypes are critical for the virus replication cycle, which are far from being clearly understood. FP25K was reported to be responsible for the regulation of BV/ODV, and the mutations within result in a decrease of normal ODVs formation and an increase of BVs production. In this study, we demonstrated that the increase of BV titer in an fp25k knockout recombinant (fp25k-negative) was a result of higher infectivity of BVs rather than an increased production of BVs. The constitution of the major structural proteins and genome of parental and fp25k-negative BVs were analyzed. The results showed that the integrity of the majority of DNA packaged into the fp25k-negative BVs was intact; i.e., the genomic DNA of fp25k-negative BV had better transformation and transfection efficiency than that of the parental virus, indicating more intact genomes in the virions. Although the analysis of proteins associated with BVs revealed that more envelope protein GP64 were incorporated into the fp25k-negative BVs, subsequent experiments suggested that overexpression of GP64 did not improve the titer of BVs. Thus, we conclude that the main reason for higher infectivity of BVs is due to better genome integrity, which benefits from the deletion of fp25k resulting in increased stability of the genome and produce a higher proportion of infectious BVs. FP25K acts as a negative factor for the infectivity of BV.

Introduction

Baculoviruses are a diverse group of large double strain DNA viruses targeting insects, which contain four genera: Alphabaculovirus, Betabaculovirus, Gammabaculovirus, and Deltabaculovirus. Except for members of the genus Gammabaculovirus, two morphologically distinct virion phenotypes are produced in the biphasic life cycle of baculoviruses: the budded virus (BV) and the occlusion-derived virus (ODV) [1, 2]. ODV enters the epithelial cells of the insect midgut through direct membrane fusion and initiates primary infection, while BV is transmitted from cell to cell, and is responsible for secondary and systemic infection [3, 4].
Baculoviruses have been successfully developed as bioinsecticides or eukaryotic expression vectors/gene therapy vectors. Genetic modification was applied to improve baculovirus as a more efficient expression vector [5].

During the life cycle of baculoviruses, progeny nucleocapsids of the Autographa californica multiple nucleopolyhedrovirus (AcMNPV) begin to egress from the nucleus after assembly in the intra nuclear virogenic stroma at about 18 h post-infection [6]. Then nucleocapsids bud through the plasma membrane, obtaining a lipid-containing envelope derived from the membrane with glycoproteins, and finally become progeny BVs [7]. In the very late phase of the life cycle, nucleocapsids are retained in the ring zone of the nucleus to become enveloped by intra nuclear microvesicles to form ODVs, and finally to be occluded in a crystalline matrix called the polyhedra [3]. To date, the ODV components of AcMNPV [8] and three other baculoviruses, Helicoverpa armigera single nucleocapsid polyhedrovirus (HearNPV) [9], Culex nigripalpus nucleopolyhedrovirus (CuniNPV) [10], and Bombyx mori ucleopolyhedrovirus (BmNPV) [11], have been analyzed by mass spectrometry-based techniques. A comprehensive proteomics analysis of AcMNPV BV-associated proteins was reported [12]. Comparative proteomics have been recently used to reveal differences in protein compositions between the two phenotypes of HearNPV [13].

Serial passage of nucleopolyhedrovirus (NPVs) in cultured cell lines could result in few polyhedra (FP) phenotype that was first observed in infected Trichoplusiani cells [14]. FP phenotypes usually lose part of the viral genome or acquire a few of host genome fragments through transposon site. Mutations within the fp25k gene were identified to be responsible for the FP phenomenon of AcMNPV [15]. Cells infected with FP mutants produced BVs with higher titer and smaller numbers of occlusion bodies [16]. Braunagel et al. observed that mutations within the fp25k gene resulted in a remarkable change in the accumulation of several baculovirus structural proteins, including GP64, ODV-E26 and ODV-E66 [17]. The expression level of ODV-E66 decreased in the cells infected with FP mutants, whereas production of GP64 and ODV-E26 increased. In addition, FP25K was shown to interact with ODV-E26, ODV-E66 and GP64, and form a complex with ODV-E25, ODV-E66 and VP39. FP25K and the protein complexes associated with it may participate in the intracellular transport of viral proteins and contribute to ODV formation [17]. Deletion of FP25K decreased the accumulation of E66 protein and blocked the transport of E66 to inner nuclear membrane [18]. Further investigations indicated that transport of ODV-E66 to the inner nuclear membrane is mediated via a sorting motif, facilitated by FP25K and other viral proteins [19].

Like FP phenomenon, the defective interfering particle (DIP) mutants, which are missing part of the genome and thus are replication-defective, accumulate in cell culture during virus passage [20]. It has been reported that transposon insertion could be a crucial step in DIP generation during serial passage [20, 21]. A recent report found that the production of baculovirus DIPs during serial passage could be delayed when the target sites for transposon insertion were deleted from the fp25k gene [22]. These results suggest a potential relation between fp25k mutant and genome stability.

Previous studies have indicated that the fp25k gene might be involved in the regulation of BV and ODV ratio and, ultimately, the yield of the two virion phenotypes [23]; however, the precise molecular mechanism behind this remains unclear. In this study, in order to investigate the specific role of FP25K in the formation of BV and ODV and to further improve the baculovirus as an expression vector, the fp25k gene was knocked out from the genome of vAcΔcc, which was deficient in chitinase and v-cathepsin gene and proved to be a expression vector had positive influence on the integrity and production of intracellular or secreted proteins [24, 25]. We found that the deletion of fp25k gene caused a higher BV titer and a decreased ODV formation. Further investigation indicated that the increased BV titer was due to higher infectivity.
The constitution analyses of the major structural proteins and viral genomes of both parental and \(fp25k\)-negative BVs suggested that more envelope proteins and higher proportion of genomes with intact integrity were incorporated into \(fp25k\)-negative BVs. Since overexpression of GP64 could not result in an improvement in the titer of BVs, we speculate that higher proportion of intact virus genomes incorporation is likely to be the main reason for the higher infectivity, and FP25K acts as a negative factor in this process.

**Materials and Methods**

**Cell lines and viruses**
The *Spodoptera frugiperda* (Sf9) cell line [26] (as gift from Prof. Just M. Vlak, Wageningen University, The Netherlands) was cultured in Grace’s insect medium (pH 6.0; Gibco-BRL), supplemented with 10% fetal bovine serum (FBS; Gibco-BRL) at 28°C. The AcBacΔcc bacmid which was deficient in both *chitinase* and *cathepsin* genes [24] was generously provided by Prof. Just M. Vlak (Wageningen University, The Netherlands), and was propagated in *Escherichia coli* strain DH10B. Viruses were harvested from culture supernatants followed by purification (5,000×g for 5 min) to eliminate cell debris. Titers of recombinant AcMNPVs were determined by endpoint dilution assays (EPDA) with Sf9 cells [27].

**Construction of \(fp25k\)-knockout, repair and parental bacmids containing egfp**
The \(fp25k\) gene of AcBacΔcc bacmid was knocked out by homologous recombination in *E. coli* BW25113 containing AcBacΔcc bacmid, in accordance with the method of Hou et al. [28], replacing the \(fp25k\) gene by the zeocin-resistance gene (\(zeo^r\)). Briefly, a 444 bp sequence upstream of the \(fp25k\) gene was amplified by PCR with the forward primer (\(5' - AAGCTTTGTCGCTGTTGGTCT-3'\); *HindIII* site underlined) and the reverse primer (\(5' - GAATTCGGCGCTTGAGCAAGACACGTTAATC-3'\); *EcoRI* and *NarI* sites underlined). A 230 bp sequence downstream of the \(fp25k\) gene was obtained with the forward primer (\(5' - GGCGGCTGCTGTTGGTCT-3'\); *NarI* site underlined) and the reverse primer (\(5' - CTGGAACAGAGACACGTTAATC-3'\); *NheI* site underlined), using AcMNPV genome DNA as template. The PCR products were cloned into a pFastBac-Dual vector (Invitrogen, USA). The \(zeo^r\) gene was further cloned into the pFastBac-Dual vector using the *NarI* site, generating the transfer vector pFastBac-Dual-\(fp25k\). This transfer vector was digested by *HindIII* and *NheI*, and the linear fragment containing \(zeo^r\) and the flanking sequences of the \(fp25k\) gene was used to transform BW25113 competent cells containing AcBacΔcc bacmid with the helper plasmid pKD46. Positive clones were selected through both zeocin and kanamycin resistance. The construction strategy was illustrated in Fig 1A. The correct bacmid clone was verified by PCR using primers flanking the \(fp25k\) locus.

In order to observe the transfection and infection directly, an egfp gene under the control of the p10 promoter was inserted through transposition into the polyhedrin gene locus of AcBacΔcc and AcBacΔcc\(Δfp25k\) bacmids. A fragment of \(fp25k\) gene with its own promoter and egfp gene driven by the p10 promoter was inserted into the polyhedrin gene locus of AcBacΔcc\(Δfp25k\) bacmid to generate the \(fp25k\) repair bacmid containing egfp (Fig 1B).

**Transfection and infection assays**
Sf9 cells (2×10^6) were cultured in 35 mm diameter tissue culture dishes, and transfected with each recombinant bacmid DNA (approximately 10 μg) using 10 μl of Lipofectin (Invitrogen, USA) according to the manufacturer’s specification. At 48 h post transfection (p.t.), cells
were examined for green fluorescent protein (GFP) expression by fluorescence microscopy. For the infection assay, at 5 days p.t., supernatants from the transfections were harvested and centrifuged at 5000 rpm for 5 min to remove cell debris, and then 200 μl of the supernatant were used to infect fresh Sf9 cells. Cells were monitored by fluorescence microscopy at 72 h post infection (p.i.).

One-step virus growth curve

Sf9 cells (1×10⁶ per well; six-well plates) were infected with each recombinant virus at a multiplicity of infection (MOI) of 5. At corresponding time post infection, 15 μl of the supernatants from infected cells were collected, and the titers of each time points were determined by EPDA in Sf9 cells [27]. GFP was the marker of infection used during the assay. All infection experiments and EPDA were performed three times, and the growth curves were generated by the arithmetic mean data of three independent infections.
Quantitative PCR analysis of genomic DNA copies in BVs and infected cells

At 0, 18, 24, 48, 72 and 96 h p.i., 50 μl of the infected cell culture supernatants were collected to isolate BV DNA as previously described [29]. For quantitative PCR (qPCR) analyses, 5 μl of BV DNA were used as a template to determine BV genomic DNA copies as previously described method [30].

For identification of total virus genomic DNA copies in infected cells, 1×10⁶ cells were infected with each recombinant virus (5 MOI) and total cellular DNA was isolated at 0, 72 and 96 h p.i. using a commercial kit (Genomic DNA Rapid Isolation Kit; BioDev, China). 5 μl isolated total cellular DNA was used as template in qPCR analyses to determine viral copy numbers in infected cells with primers of viral gene \( vp80 \):

- \( vp80\)-For: 5'-gacgatgtcgttaatcgtgc-3'
- \( vp80\)-Rev: 5'-atcagcatcgctattcagataa-3'

The measured virus genomic DNA copies in both recombinants infected cells of each time points were compared.

Electron microscopy

Sf9 cells (2×10⁶) were infected with vAcΔcc or vAcΔccΔfp25k (5 MOI). Cells were harvested at 48, 64, 72 and 96 h p.i., and washed three times with phosphate-buffered saline (PBS). All samples were fixed with 2.5% (w/v) glutaraldehyde in 0.1 M sodium phosphate, and processed for transmission electron microscopy (TEM) as described previously [29]. ODV formation in infected cell was observed by TEM (FEI Tecnai G2 microscope; 200 kV).

Western blot analysis

Sf9 cells (2×10⁶) infected with recombinant vAcΔcc/ vAcΔccΔfp25k at 5 MOI were collected at 48 h p.i. and rinsed with PBS. The protein samples were separated through 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Millipore Corporation, USA) by semi-dry electrophoresis. The Western blot analyses were performed with primary polyclonal antibodies generated from rabbit which against AcMNPV structural proteins: BV envelope protein GP64 and Ac23 [30], nucleocapsid protein AC109 (generated in our lab, unpublished data) and 38K [31].

The structural proteins incorporated into BV were identified by Western blot analyses. Genomic DNA was isolated from 100 μl of BV supernatant for each recombinant virus, and quantified by qPCR as described above. BVs containing equal copies of genome were centrifuged at 13,000 rpm for 30 min at 4°C. Samples were disrupted under reducing condition (4×SDS-PAGE sample buffer, 100°C) and separated by SDS-PAGE (12% separation gel). The polyclonal antibodies against AcMNPV structural proteins: GP64, Ac23 and VP39 [12] were used as primary antibodies for Western blot analyses. The experiment was performed as described previously [30].

Quantitative reverse transcription PCR analysis

Sf9 cells (2×10⁶) in 35-mm diameter tissue culture dishes were infected with vAcΔcc or vAcΔccΔfp25k (5 MOI). At 48 h p.i., total RNA was isolated with TRIzol (Invitrogen, USA) and subsequently treated with RQ1 RNase-Free DNase (Promega, USA) to digest the residual DNA. A two-step quantitative reverse transcription (qRT)-PCR method was performed using 0.5 μg of DNA-free RNA as template. The first step of the cDNA synthesis was performed using M-MLV Reverse Transcriptase (Promega, USA) and oligo (dT) primers (5’ - CTGATC TAGAGGTACCGGATCCTTTTTTTTTTTTTTTTTTTTTTTTTTTT -3’ ). The second step of cDNA qPCR using 1 μl template cDNA was performed as previously described [30]. The qRT-PCR primer pairs
were derived from the gp64, 38k, Ac109 and polyhedrin genes. 28S rRNA was used as an internal control (Table 1). Relative viral gene RNA levels were calculated as the quantity of the specific gene RNA normalized to 28S rRNA levels. Each experiment was performed three times.

**BV Genomic DNA transformation and transfection assay**

BV genomic DNA isolated from 100 μl of BVs harvested at 36 and 48 h p.i. was quantified by qPCR. Equal copies (5×10⁸) of fp25k-negative and control BV genomic DNA were used to transform competent E. coli DH10β cells by electroporation, and the number of colony forming units (CFUs) formed under kanamycin selection was calculated. The experiment was carried out for three times.

Genomic DNA isolated from 2 ml of BVs was dissolved in 50 μl of ddH₂O, and then quantified by qPCR. 1×10¹⁰ copies of fp25k-deleted and control BV genome DNA were transfected into Sf9 cells. At 36 h p.t., cells were photographed under fluorescence microscopy. For each analysis, five fields were chosen randomly, and the number of cells expressing EGFP was calculated. The experiment was performed three times.

**Overexpression of GP64**

For generation of vAcΔcc-gp64, the extra gp64 gene driven by gp64 promoter and an egfp gene under the control of the p10 promoter were inserted through transposition into the polyhedrin gene locus of AcBacAcc. The bacmid of AcBacΔcc-gp64 with an egfp gene was transected into Sf9 cells as described above, and supernatant from the transfection was harvested to infect a new batch of Sf9 cells to generate vAcΔcc-gp64. The gp64 expression in infected cells and GP64 incorporation in budded virions were detected as described above. One-step virus growth curves of vAcΔcc and vAcΔcc-gp64 were conducted as described previously.

**Results**

**Generation of recombinant viruses**

The fp25k gene of AcBacAcc bacmid was successfully deleted and the recombinant named AcBacAccΔfp25k was verified by PCR detection (data not shown). An egfp gene under the control of the p10 promoter were inserted through transposition into the polyhedrin gene locus of AcBacAcc. The bacmid of AcBacΔcc-gp64 with an egfp gene was transected into Sf9 cells as described above, and supernatant from the transfection was harvested to infect a new batch of Sf9 cells to generate vAcΔcc-gp64. The gp64 expression in infected cells and GP64 incorporation in budded virions were detected as described above. One-step virus growth curves of vAcΔcc and vAcΔcc-gp64 were conducted as described previously.

In order to confirm the deletion of fp25k result in an increased yield of budded virus [16], Sf9 cells were infected with vAcΔcc, vAcΔccΔfp25k and vAcΔccΔfp25k-rfp25k. The one-step growth curve of each virus is shown in Fig 1D. At 24 h p.i., the fp25k-negative virus had a higher BV titer than parental virus (vAcΔcc), although the difference was not significant (P>0.05, analyzed by a two-tailed Student’s t-test). At 48 h p.i., the fp25k-negative virus had a higher BV titer than parental virus (vAcΔcc), although the difference was not significant (P>0.05, analyzed by a two-tailed Student’s t-test). At 48 h p.i., the fp25k-negative virus had a higher BV titer than parental virus (vAcΔcc), although the difference was not significant (P<0.05, analyzed by a two-tailed Student’s t-test). The fp25k repair virus showed a similar kinetic with the parental virus (Fig 1D). These results showed that the increased titer of fp25k-negative virus was due to the deletion of fp25k.

The fp25k-negative virus produced more infectious progeny BVs

Both previous studies and our result indicated that fp25k mutant virus generated more BV during infection [16, 32], especially around the time point 48h p.i. (Fig 1D). In order to further
investigate whether the deletion of fp25k facilitate virus production or infectivity, the virus genomic DNA copies were determined by qPCR in the same samples for One-step growth curve analysis (Fig 2A), we found that the similar genomic DNA copies were detected in both recombinants at each time points (Fig 2B). The result indicated that the titer of vAcΔccΔfp25k was higher than vAcΔcc whereas the copy number of genomic DNA was the same for each virus (Fig 2). At 48 h p.i., the average BV titer of vAcΔccΔfp25k was about 3 times to that of vAcΔcc. And at the same time point (48 h p.i.), the genomic DNA copies of the BVs in the supernatant were 8.1×10¹⁰ copies/ml for vAcΔcc and similarly for vAcΔccΔfp25k (8.0×10¹⁰ copies/ml). Thus, the viral infectivity unit (copies/TCID50) of vAcΔccΔfp25k was calculated as 4.37×10³, and for parental virus vAcΔcc, it was 1.14×10⁴. The result implied that fp25k gene deletion would lead to producing more infectious progeny BV particles.

Non-enveloped nucleocapsids retained in the nucleus

It has been reported that the envelopment of nucleocapsids within the nucleus of cells infected with FP mutant was incomplete [16]. We observed that the nucleocapsids envelopment and ODV formation of fp25k-negative virus also appeared to be significantly altered compared with control virus. Electron microscopy revealed a large number of completely enveloped nucleocapsids at the ring zone of Sf9 cells infected with control virus (48, 64, 72 and 96 h p.i.), while in cells infected with fp25k-negative virus, envelopment of nucleocapsids was impeded significantly (Fig 3A). It is important to note that nucleocapsids were not enveloped within

Table 1. Primers used for quantitative reverse transcription PCR.

| Primer     | Sequence                      |
|------------|-------------------------------|
| 28sfor     | 5’TGGTTGCTTGAAGTGCAGCC3’       |
| 28rev      | 5’TCCATTCGATTTGCGGAGT3’        |
| 38sfor     | 5’TCCGAGATGGCGCGGCAC3’         |
| 38rev      | 5’TCCGAGATGGCGCGGCAC3’         |
| gp64for    | 5’TGGCGATCCGCTGCAGTCG3’        |
| gp64rev    | 5’TGGCGATCCGCTGCAGTCG3’        |
| ac109for   | 5’ATGAGTGCGCGCGTTCAGATT3’      |
| ac109rev   | 5’TGGCGATCCGCTGCAGTCG3’        |
| polyhedrinfor | 5’GTACCTACGTGTACGACAAACAA3’   |
| polyhedrinrev | 5’GTACCTACGTGTACGACAAACAA3’   |

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Fig 2. Virus titer determination and qPCR analysis of BV genomic DNA copy number in the supernatant. Sf9 cells were infected with vAcΔcc or vAcΔccΔfp25k at an MOI of 5. The supernatants from infected cells were collected at 0, 18, 24, 48, 72 and 96 h. p.i. Virus titers were determined by EPDA (A), genomic DNA was detected by qPCR (B), and the results were transformed logarithmically. Each point represents the average titer from three independent infections. Error bars represent standard deviations.

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ODVs did not participate in BV formation either, as they were still retained in the nucleus at corresponding time points during infection.

In addition, qPCR was carried out to determine the number of viral particles maintained in infected cells at 72 and 96 h p.i. We found that the number of viral genome copies in cells infected with fp25k-negative and control virus showed no significant difference \((P > 0.05, \text{analyzed by a two-tailed Student's } t\text{-test})\) (Fig 3B). The results of the assessment of BV titer, BV genome copy number in supernatant and infected cells as well as the EM observation indicated that vAcΔccΔfp25k was more infectious than vAcΔcc, rather than having a higher absolute production compared to the control.

**The expression of gp64, ac109 and polyhedrin genes were regulated by FP25K at transcriptional level**

It has been reported that mutation within AcMNPV fp25k increased the accumulation of GP64 and decreased production of ODV-E66 [17]. Sf9 cells infected with vAcΔcc/vAcΔccΔfp25k
were harvested at 48 h p.i. to investigate whether the synthesis of other proteins was affected by \( fp25k \) deletion. Western blots were performed to detect the accumulation of structural proteins GP64, AC109, AC23 and 38K. Expression levels of AC109 decreased significantly in cells infected with \( fp25k \)-negative virus, whereas synthesis of GP64 increased. The deletion had no effect on the expression of \( ac23 \) and 38K (Fig 4A). VP39 was used as an internal control to normalize the expression level (data not shown).

Furthermore, qRT-PCR analysis was performed. Total RNA in cells infected with \( vAc\Delta cc \) or \( vAc\Delta cc\Delta fp25k \) was isolated and reverse transcribed into cDNA for qPCR detection. The result showed that the transcription levels of \( ac109 \) and polyhedrin genes were down regulated significantly \((P<0.05, \text{analyzed by a two-tailed Student’s } t\text{-test})\), while the \( gp64 \) gene was up regulated \((P<0.05, \text{analyzed by a two-tailed Student’s } t\text{-test})\) (Fig 4B). It suggested that the expression of \( gp64 \), \( ac109 \) and polyhedrin were regulated by FP25K at transcriptional level.

Higher level of GP64 was incorporated into \( fp25k \)-negative BV particles

Since the infectivity of \( fp25k \)-negative BV was higher than that of the control virus, we questioned whether the deletion confers alterations in the BV structure resulting in higher

Fig 4. Expression analyses of viral structural proteins in infected cells. (A) SF9 cells were infected with \( vAc\Delta cc \) or \( vAc\Delta cc\Delta fp25k \) at an MOI of 5. Infected cells were collected at 48 h p.i. and analyzed by western blotting using the corresponding antibodies, the results were repeated twice. (B) SF9 cells infected with \( vAc\Delta cc \) or \( vAc\Delta cc\Delta fp25k \) (MOI = 5) were collected at 48 h p.i. and analyzed by qRT-PCR. The transcriptional levels of viral genes were normalized to the internal control 28S rRNA, and the transcription difference between \( vAc\Delta cc \)- and \( vAc\Delta cc\Delta fp25k \)-infected cells was analyzed by the \( 2^{-\Delta\Delta Ct} \) method. The results of corresponding genes in \( vAc\Delta cc \)-infected cells were set as 100%. The data are from three independent experiments. Error bars represent standard deviation. Data were analyzed by two-tailed Student’s \( t\)-test. * \( P<0.05 \). doi:10.1371/journal.pone.0128471.g004
infectivity. Western blot analysis showed that the synthesis of BV envelope proteins GP64 increased in the fp25k-negative virus, we decided to investigate if higher amounts of the protein become incorporated into the BVs. Real-time qPCR were performed to determine the level of virus particles in supernatants. At 48 h p.i., supernatants containing equal copies (5×10¹⁰) of vAcΔcc and vAcΔccΔfp25k virions were collected and used in western blot analyses. As shown in Fig. 5A, significantly higher levels of GP64 was detected in the vAcΔccΔfp25k BVs compared with the vAcΔcc BVs, while the incorporations of envelope protein Ac23 and nucleocapsid protein VP39 were unaltered.

The overall genomic integrity of the fp25k-negative BVs was better than that of parental virus

The genomic stability of AcMNPV was shown to increase when the transposon insertion sites in fp25k gene was modified [22]. Since in our study the entire fp25k gene including the transposon insertion site was knocked out, we desired to find out whether the higher infectivity of fp25k-negative virus was due, at least part to better genomic stability. Genomic DNA was isolated from 100 μl of BV supernatant of fp25k-negative virus and control virus and quantified by qPCR. Genomic DNA of each virus in an equal copy number (5×10⁸) was transformed into E. coli DH10B cells, and the number of CFUs was calculated. Data were analyzed by two-tailed Student’s t-test. *P<0.05.

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Fig 5. BV structural proteins incorporation and genome integrity analysis. (A) Western blot analysis of structural proteins incorporated into recombinant BVs. Equal copy numbers of vAcΔcc and vAcΔccΔfp25k virions were purified from the supernatants of infected Sf9 cells at 48 h p.i. and subjected to SDS-PAGE, then blotted with antibodies against GP64, Ac23, and VP39. (B) Transformation assay. Genomic DNA isolated from vAcΔcc or vAcΔccΔfp25k BVs harvested at 36 h and 48 h p.i. was quantified by qPCR. Equal copy numbers (5×10⁸) of genomic DNA was used to transform competent E. coli DH10B cells, and the number of CFUs was calculated. (C) Transfection assay. Equal copy numbers of genomic DNA isolated from vAcΔcc and vAcΔccΔfp25k BVs were transfected into Sf9 cell. Transfection efficiency was calculated as the number of cells expressing EGFP. The mean number of cells transfected by BV genomic DNA of control virus was set as 100%. Data are representative of three independent experiments. Error bars represent standard deviation. Data were analyzed by two-tailed Student’s t-test. *P<0.05.
competent E. coli DH10β cells, and the number of CFUs was calculated (Fig 5B). Genomic DNA formed colonies under antibiotic selection was considered to be complete circular DNA according to the fundamental principle of molecular cloning, since only complete circular DNA containing an antibiotic resistance gene can replicate in E. coli cells and confer on bacteria the ability to survive and proliferate in the selective growth medium with corresponding antibiotic [33]. The result indicated that more number of CFUs was formed when E. coli DH10β was transformed with the fp25k-negative genomic DNA (P<0.05, analyzed by two-tailed Student’s t-test), suggesting more intact virus genomes were incorporated into the fp25k-negative virus particles.

The result of genomic integrity was further confirmed by transfection assay using the host cell line. Equal copy numbers (1×10^10 copies) of fp25k-negative and control BV genomic DNA were used to transfect Sf9 cells. The transfection efficiency was calculated as the number of cells expressing GFP at 36 h p.t. The mean number of cells transfected by BV genomic DNA of control virus was set as 100% (Fig 5C). The transfection efficiency of fp25k-deleted genomic DNA was significantly higher than that of control DNA (P<0.05, analyzed by two-tailed Student’s t-test). Therefore, both results of transformation and transfection assays indicated that a higher proportion of intact viral genome was incorporated into fp25k-negative virus.

**Overexpression of GP64 could not enhance the BV titer**

To investigate whether the increased infectivity of fp25k-negative virus benefited from a higher level of GP64 incorporation, GP64 was overexpressed under its native promoter in vAcAcc-egfp. Western blot analysis of infected cells verified that GP64 was successfully overexpressed (Fig 6B), and that overexpressed GP64 was incorporated into BVs (Fig 6B). However, the results of the one-step growth curve assay showed that the recombinant virus titer was not increased by overexpression of GP64 (Fig 6C), suggesting that a higher level of GP64 incorporation to BVs might not be the major reason for the enhancement of infectivity of BV.

**Discussion**

FP mutants of baculoviruses often result from acquisition of host cell DNA fragments or loss of a portion of the viral genome [34]. The common characteristics of the FP phenomenon are a decrease in the number of OBs, an increase in the production of BVs, and reduced numbers of completely enveloped ODVs [16, 35]. In our investigations, an fp25k-negative mutant virus was constructed. In comparison with the control virus, the mutant produced BVs with higher infectious titer than the parental virus, corroborating previous data [16, 32]. There are at least two hypotheses to explain this increased BV production: (1) that the nucleocapsids destined to form ODVs actually participate in BV formation, or (2) fp25k-negative virus produced BVs with higher infectivity [16]. We found that the number of virus genome copies in supernatant did not increase, when deletion of fp25k caused an increase in BV titer (Fig 2). EM observation of infected cells at different time points revealed that only few normal ODV formed in the fp25k-negative virus-infected cells, as most nucleocapsids were not enveloped (Fig 3A). The nucleocapsids that were not completely enveloped in ODV remained in the nucleus, rather than participated in BV formation, suggesting that the increase of BV production was not due to the nucleocapsids escaping from ODV formation.

Further investigation indicated that FP25K was related to the regulation of expression of the structural proteins, such as the major envelope protein GP64 (Fig 4A). This regulation of viral protein expression occurred at the transcriptional level (Fig 4B). We showed the up-regulated GP64 protein was incorporated into the fp25k-negative virions (Fig 5A). GP64 has been identified as the envelope fusion protein of group I Alphabaculovirus [36], which is essential for
receptor recognition, cell entry, and the budding process [7, 37]. In addition, GP64 is also involved in inducing low pH-dependent membrane fusion, which is indispensable for virus entry into host cells through the endocytic pathway [38]. It was recently reported that incorporation of GP64 into the group II Alphabaculovirus HearNPV resulted in higher fusogenic activity and ultimately in a greater number of infectious HearNPV BVs [39], indicating that extra GP64 may benefit the infectivity of baculovirus. In our study, higher level of GP64 was detected in fp25k-negative BVs (Fig 5A). Other results indicate that the increase in fp25k-negative BV production is a consequence of higher BV infectivity, which might be the result of more GP64 being incorporated into BVs. However, we confirmed that BV infectivity was not increased when GP64 was over expressed and incorporated to BVs (Fig 6). These results suggested that the increased incorporation of BV envelope protein might not be the major cause of the enhancement of BV infectivity.

The DIP mutants lacked some genetic information, including the polyhedrin and DNA polymerase genes, and these mutations accumulated during passage in cell culture. Transposon insertion (like the FP mutants) is a crucial step in DIP mutant generation during serial passage [20, 21]. This is evidenced by a delayed production of DIP mutants during baculovirus serial passage when the transposon target sites (TTAA) were modified. These reports substantiate the idea that modification of the insertion sites contributed to the genomic stability of AcMNPV [22]. In our study, the entire sequence of the fp25k gene was deleted from the genome, including the TTAA sites needed for transposon insertion. The results of genomic integrity assays implied that fewer defective genomes were packaged into the fp25k-negative virus.
than the control virus (Fig 5B and 5C), suggesting that the high proportion of intact genome DNA in the \textit{fp25k}-negative virions is likely to have led to the increase in infectivity.

Our results suggest that the \textit{fp25k}-negative BVs are more infectious than the parental virus, which might benefit from a higher proportion of infectious virions with better genomic integrity. Thus, we propose a model of parental and \textit{fp25k}-negative virus infection (Fig 7). FP25K is a multifunctional protein in the life cycle of AcMNPV. FP25K participates in the protein synthesis (Fig 7A\(1\)) \cite{17} and the transport of several structural proteins from cytoplasm to inner nuclear membrane then associated with ODV formation (Fig 7A\(2\)) \cite{18, 19}, besides being a component of the nucleocapsid (Fig 7A\(3\)) \cite{8} and contributing the polyhedra formation (Fig 7A\(4\)) \cite{40}. In addition, we found out that FP25K acts as a negative factor of genome stability (Fig 7A\(5\)), when \textit{fp25k} was deleted a higher proportion of the newly synthesized genome DNA was intact. In AcMNPV infected cells, normal ODVs can be observed in the nucleus, and the ratio of infectious and non-infectious BVs that bud through the plasma membrane was low (Fig 7A). As for wild-type AcMNPV, the viral infectivity (copies/TCID\textsubscript{50}U) is about 1×10\(^4\), which means that 10\(^4\) copies of viral genome DNA result in one TCID\textsubscript{50} unit \cite{30}. In contrast,
in cells infected with fp25k-negative virus, the expression of gp64 is up regulated while ac109, and polyhedrin were down regulated on transcriptional level, the envelopment of nucleocapsids is incomplete. However, nucleocapsids, which are not completely occluded within ODVs, are retained in the nucleus. The deletion of fp25k gene results in an increase in genome stability, producing a higher proportion of infectious BVs (Fig 7B).

From an evolutionary perspective, FP mutants of baculoviruses accumulate in cell culture caused by fp25k mutations result in higher infectivity of BV, facilitating the transmission from cell to cell. However, the propagation of virus in insect larvae could eliminate the FP mutants, producing more OBs to benefit the spread of virus from insect to insect. A balance of BV/ODV formation will finally be achieved between FP mutants and wild type. This hypothesis could provide guidance in the application of baculovirus as different applications. FP25 mutant with higher infectivity and genome stability could be acquired through deletion of fp25k, which has potential to be applied as a more efficient expression vector.

In summary, our data revealed that the deletion of fp25k gene resulted in an increase in BV infectivity and a decrease in ODV formation. Expression of several structural proteins was regulated by FP25K at the transcriptional level. Furthermore, we demonstrated that fp25k-negative BVs formed with additional GP64 and greater proportion of intact genome, and that the latter one might be the major reason for the higher infectivity of fp25k-negative virus. These results suggest that FP25K acts as a negative factor for the infectivity of AcMNPV BVs, which give us a new insight into the FP25K-mediated regulation mechanism of BV/ODV formation, and might guide the genetic modification of baculovirus BV to be utilized as expression, surface display and gene therapy vector.

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Author Contributions

Conceived and designed the experiments: FD HW ZH. Performed the experiments: SL MW SS. Analyzed the data: SL HW FD. Contributed reagents/materials/analysis tools: SL MW SS. Wrote the paper: SL HW FD.

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