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ORIGINAL ARTICLE

Autographa californica multiple nucleopolyhedrovirus *odv-e25* (Ac94) is required for budded virus infectivity and occlusion-derived virus formation

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Abstract *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) *odv-e25* is a core gene found in all lepidopteran baculoviruses, but its function is unknown. In this study, we generated an *odv-e25*-knockout AcMNPV and investigated the roles of ODV-E25 in the baculovirus life cycle. The *odv-e25* knockout was subsequently rescued by reinserting the *odv-e25* gene into the same virus genome. Fluorescence microscopy showed that transfection with the *odv-e25*-null bacmid vAcBac^{KO} was insufficient for propagation in cell culture, whereas the 'repair' virus vAcBac^{RE} was able to function in a manner similar to that of the control vAcBac. We found that *odv-e25* was not essential for the release of budded viruses (BVs) into culture medium, although the absence of *odv-e25* resulted in a 100-fold lower viral titer at 24 h post-transfection (p.t.). Analysis of viral DNA replication in the absence of *odv-e25* showed that viral DNA replication was unaffected in the first 24 h p.t. Furthermore, electron microscopy revealed that polyhedra were found in the nucleus, while mature occlusion-derived viruses (ODVs) were not found in the nucleus or polyhedra in *odv-e25* null transfected cells, which indicated that ODV-E25 was required for the formation of ODV.

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Introduction

Autographa californica multiple nucleopolyhedrosis virus (AcMNPV), a member of the genus *Alphabaculovirus*, family *Baculoviridae*, is the best-characterized baculovirus. Baculoviruses are arthropod-specific DNA viruses, and more than 600 viruses have been identified to date in a variety of insect orders [1, 13, 19, 22, 24, 33]. Baculovirus genomes are covalently closed circles of double-stranded DNA ranging from 80 to 180 k bp in size. Two viral forms, BV (budded virus) and ODV (occlusion-derived virus), are produced during the infection cycle. Even though the genomes of BV and ODV are genetically identical, the phenotypes of the two virions are structurally and functionally distinct [34]. The fact that ODV envelope proteins are more diverse in composition than those of BV envelope proteins determines the different roles of ODV and BV in viral infection [32]. BV is responsible for horizontal transmission of viral infection from cell to cell within a host, while ODV plays a critical role in vertical transmission of the virus from insect to insect.

Odv-e25 is conserved in all lepidopteran baculoviruses and is not found in other baculoviruses, such as dipteran NPVs and hymenopteran NPVs [34]. ODV-E25 is N-terminally anchored in the envelope, and the C-terminus interacts with the C-terminal region of ODV-E66 [5, 15, 30]. The AcMNPV *odv-e25* (ORF94) is 687 bp long and predicted to code for a 228-aa (25.5 kDa) protein. A late transcriptional initiation motif (ATAAG) is found upstream of this gene. Some ODV envelope proteins contain a hydrophobic domain, such as ODV-E18, ODV-E56, and ODV-E66 [3, 4, 14], and the N-terminal 23 aa of ODV-E25 is conserved in all orthologs and directs the transport of ODV-E25 from the cytoplasm to the nucleus [15, 30].

Many ODV envelope proteins have been identified, such as *odv-e26*, *odv-e66*, *p74*, *pif-1*, *pif-2*, *pif-3*, and *pif-4*, and the functions of several of them have been investigated by deletion of the gene [7, 10, 18, 25, 26, 28]. ODV-E25, which has been identified as a structural protein of ODV that is also found on the BV envelope, is poorly understood because no gene deletion mutants have been generated [15, 29, 30, 38]. In this report, we generated an AcMNPV bacmid in which *odv-e25* was disrupted by homologous recombination and investigated the role of *odv-e25* in AcMNPV infection of *Sf*-9 cells using the *odv-e25* null virus. The infectivity of the *odv-e25*-deletion virus was significantly reduced, and mature ODVs were not found in these virus-transfected cells.

Materials and methods

Cells

Sf-9 cells were maintained in TC-100 insect medium (Gibco) supplemented with 10% fetal bovine serum.

Construction of AcBac^{KO}

Odv-e25 was deleted from an AcMNPV bacmid (AcBac), which was constructed according to the method reported by Xiang *et al*. [39] and maintained in DH10*β* using the *λ* Red recombination system (kindly provided by Dr Mary Berlyn, Yale University, USA) as described by Datsenko and Wanner [8]. In this bacmid, the polyhedrin gene is expressed under the *p10* promoter. The chloramphenicol acetyl transferase (*cat*) gene (also provided by Dr. Mary Berlyn) with the arabinose promoter was amplified using primers 5′-tgaaattgtaaatta aaacaaatcatgtggggaatcgtgttacttttaagtgtaggctggagctgct-3′ and 5′-acaccaaagtgcaattagatttgcgcaattcgcgcacctctcgttcggcatatgaa tatcctcctt-3′ (the nucleotides in the black box represent an artificially incorporated termination codon to terminate translation of the original product). Electrocompetent *E. coli* DH10*β* cells harbouring AcMNPV bacmid and pKD46 (*λ* Red recombinase genes) were transformed with 300 ng of the purified *cat* amplicon using an AxyPrep DNA Gel Extraction kit (Axygen). Cells were supplemented with 900 µl of fresh SOC medium and incubated for 3 h at 37°C with gentle shaking. Then, the cells were pelleted for 5 min at 5,000 rpm, resuspended in 150 µl of SOC broth, and plated on LB plates supplemented with kanamycin (50 µg/ml) and chloramphenicol (20 µg/ml). Colonies were subjected to PCR screening to confirm the deletion of *odv-e25* and proper genomic insertion of *cat*. The colonies were identified by PCR (M13 F, 5′-tgtaaaacgacggccagt-3′ and M13 R, 5′-gaaacagctatgaccatgat-3′, and *odv-e25* primers, odv-e25F, 5′-agtactatgtggggaatcgtg-3′ and odv-e25R, 5′-aggatccttaattcatttctctgta-3′).

Construction of vAcBac, vAcBac^{KO}, and vAcBac^{RE}

AcBac^{KO} DNA and the helper plasmid pMON7124 were electroporated into *E. coli* DH10*β* cells, and subsequently, DH10AcBac^{KO} competent cells were prepared. The enhanced green fluorescent protein gene (*egfp*) was amplified (primers: 5′-aatggatcctatggtgagcaag-3′ and 5′-cactagtgactagtcttgtac-3′) and cloned into pFastBacHTa, creating pFastBacHTa-egfp. Competent DH10AcBac cells (with helper plasmid pMON7124 and AcMNPV bacmid) and competent DH10AcBac^{KO} cells were transformed with the recombinant plasmid, and the *egfp* gene was inserted into the *polyhedrin* locus by site-specific transposition. Thus, the constructed bacmids were subsequently identified and named vAcBac and vAcBac^{KO}, respectively.

Similar to the SpltMNPV *odv-e25* sequence [20], the AcMNPV *odv-e25* sequence displayed a baculovirus consensus late transcriptional initiation motif (TTAAG) [2] at nucleotide residues −71 bp to −67 bp and one host factor binding site (GATA) [17] at nucleotide residues −77 bp to −74 bp with respect to the translational start codon. The whole *odv-e25* gene (containing 327 bp upstream and 237 bp downstream of *odv-e25*) was amplified using primers (e25wh F: 5′-caagcttgtgaaagccttatttatg-3′; e25wh B: 5′-tttcgaacactcgtttgttcagtac-3′) and cloned into the pMD19-T vector (TaKaRa). The positive clone was identified and digested with *Hin*d III, and e25wh was then cloned into the *Hin*d III site of pFastBacHTa-egfp and pFastBscHTa, generating pFastBacHTa-egfp-e25wh and pFastBacHTa-e25wh. All of the recombinant plasmids used in this study were sequenced to confirm the correct form.

vAcBac^{RE} was created by transforming competent DH10AcBac^{KO} cells with pFastBacHTa-egfp-e25wh.

Transfection

Bacmid DNA was purified from 1.5 ml of LB cultures as described before [35] and detected using a NanoDrop ND-2000C. Cells (about 2×10^6/ml) were transfected with 2 µg of bacmid DNA using Cellfectin (Invitrogen) according to the manufacturer's protocol. After 5 hours of incubation, the transfection mixture was removed, and the cells were gently washed twice with fresh TC-100 insect medium, and 2 ml of medium was added to cells.

Analysis of the viral growth curve

Virus supernatants were collected after cells were transfected with the bacmid at the given times (6, 12, 24, 48, 72, 96, and 120 h), and cell debris and cells were removed by centrifugation (5000 rpm for 5 min). An aliquot of each of these supernatants (250 µl) was processed using a viral DNA Kit (OMEGA). 2 µl of the viral DNA was used in triplicate to detect BV production by Q-PCR assay [21] with SYBR Premix Ex Taq^{TM} (TaKaRa). A stock of control vAcBac (2.0×10^8 pfu/ml), previously titered by endpoint dilution and Q-PCR, was used as a standard and diluted tenfold. Q-PCR was performed by using a 7300 Real-Time PCR system (ABI) under the following conditions: 95°C for 30 s; 45 cycles of 95°C for 5 s and 60°C for 31 s, with a 500 µM concentration of each primer. A 50% tissue culture infective dose ($TCID_{50}$) endpoint dilution assay was used to determine BV titers using *Sf*-9 cells in the 96-well microtiter plates.

Quantitative real-time PCR (Q-PCR) DNA replication assay

To detect viral DNA replication, a Q-PCR assay was performed as described previously [36, 37]. The primers gp41 F (5′-CGTAGTGGTAGTAATCGCCGC-3′) and gp41 R (5′-AGTCGAGTCGCGTCGCTTT-3′) were used to amplify a 101-bp region containing four *Dpn* I restriction sites within the gp41 ORF of AcMNPV. *Sf*-9 cells were transfected with vAcBac, vAcBac^{KO}, and vAcBac^{RE} and harvested at different time points. Total DNA was extracted using a Classic Genomic DNA Isolation Kit (Sangon) and digested with *Dpn* I (TaKaRa). Q-PCR was performed by using the 7300 Real-Time PCR system (ABI) under the following conditions: 95°C for 30 s; 45 cycles of 95°C for 5 s and 60°C for 31 s, with a 500 µM concentration of each primer.

Transmission electron microscopy

Sf-9 cells (2×10^6) were transfected with 6.0 µg of vAcBac, vAcBac^{KO}, or vAcBac^{RE}, and the cells were collected at 96 h post-transfection (p.t.) for transmission electron microscopy. The cells were first fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.0) for more than 12 hours, washed three times with phosphate buffer, and then once more for 15 min, postfixed with 1% OsO_4 in phosphate buffer (pH 7.0) for 1 hour, and again washed three times with phosphate buffer. Then, the treated cells were first dehydrated using a graded series of ethanol (50%, 70%, 80%, 90%, 95%, and 100%) for about 15 to 20 minutes at each step, transferred to a mixture of alcohol and isoamyl acetate (V:V = 1:1) for about 30 minutes, and then transferred to pure isoamyl acetate for about one hour. Finally, the specimen was dehydrated in a Hitachi Model HCP-2 critical point dryer with liquid CO_2. The dehydrated specimens were coated with gold-palladium and observed in a Philips Model XL30 ESEM.

Results

Construction and characterization of a recombinant bacmid

To determine the effect of *odv-e25* knockout on virus replication, we generated an *odv-e25*-knockout AcMNPV bacmid via the λ Red homologous recombination system in *E. coli*. A sequence of 525 bp within the *odv-e25* locus was replaced with a *cat* gene cassette (Fig. 1a). The precise deletion was confirmed by PCR; as expected, using primers odv-e25F/R, a PCR product of 1,212 bp was amplified from *odv-e25*-knockout bacmid vAcBac^{KO}, a 696-bp fragment was amplified from vAcBac, and 696- and 1,212-bp fragments were confirmed from vAcBac^{RE} (Fig. 1b). Using primers M13F/R, the PCR products of vAcBac, vAcBac^{KO}, and vAcBac^{RE} were 3,108, 3,108, and 4,359 bp, respectively (Fig. 1b). These results demonstrated that the recombinant bacmids used in this reports had been successfully constructed.

Viral replication in *Sf*-9 cells

Sf-9 cells were transfected with vAcBac, vAcBac^{KO}, or vAcBac^{RE} and monitored by fluorescence microscopy. At 36 h p.t., no significant difference was observed among the cell lines transfected with vAcBac, vAcBac^{KO}, or vAcBac^{RE}, indicating comparable transfection efficiencies (Fig. 2a). The number of cells exhibiting fluorescence increased significantly after transfection with vAcBac and vAcBac^{RE}, but in vAcBac^{KO}-transfected cells, the number of infected cells scarcely changed. At 96 h p.t., the number of fluorescent cells transfected with vAcBac was equivalent to that of vAcBac^{RE}, whereas there was no significant change in vAcBac^{KO} -transfected cells (Fig. 2a). Supernatants from *Sf*-9 cells transfected with the three bacmids (120 h p.t.) were collected and used to infect 2×10^6 *Sf*-9 cells. At 36 h postinfection (p.i.), few cells infected with vAcBac^{KO} were observed to emit fluorescence – far fewer than those infected with vAcBac/vAcBac^{RE}. Fluorescence excitation was detected in nearly all cells infected by vAcBac/vAcBac^{RE} at 96 h p.i., whereas there was no significant change in the number of vAcBac^{KO}-infected cells (Fig. 2b).

(1) BV production and $TCID_{50}$ assays A virus growth-curve analysis was performed to determine the effect of

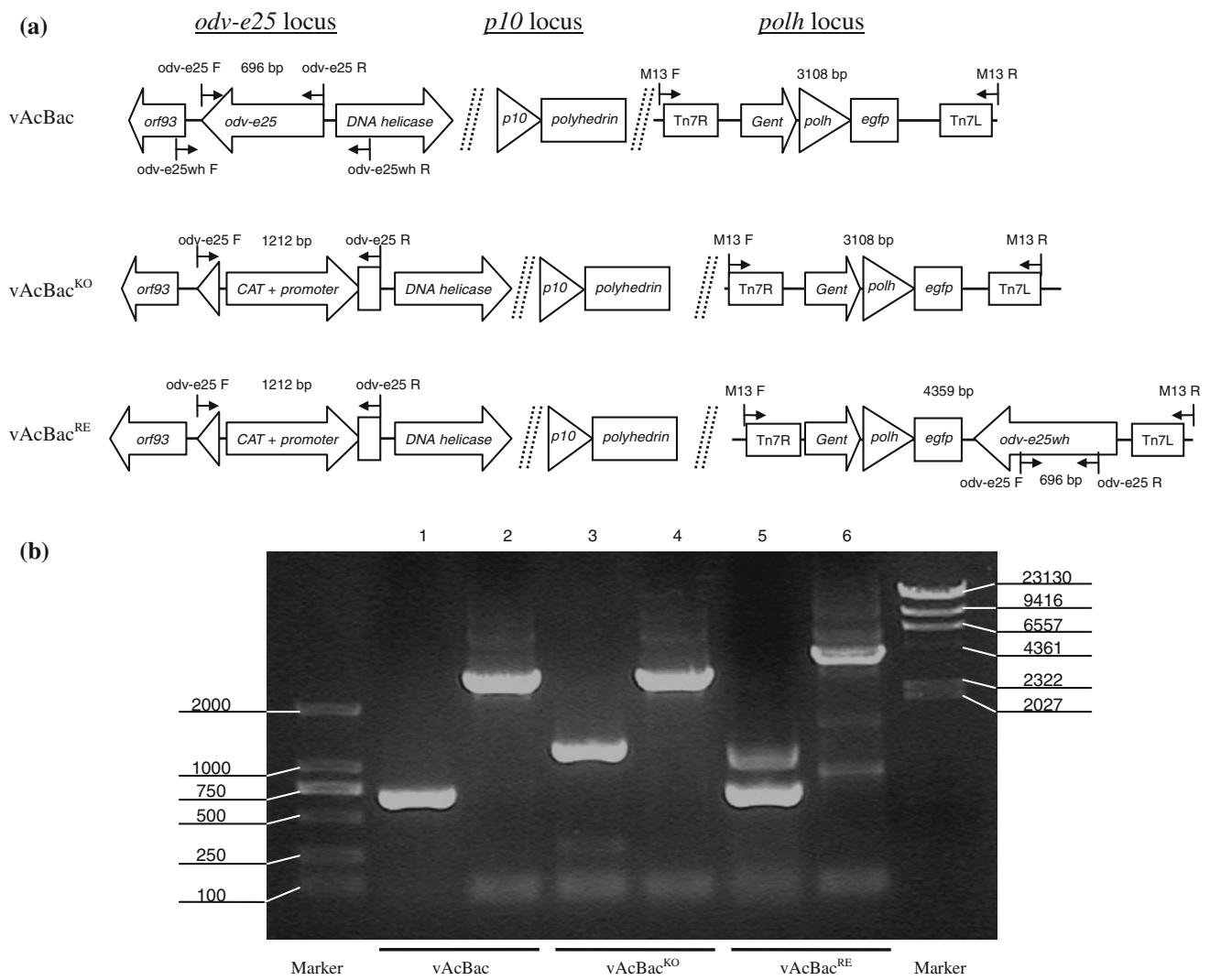

Fig. 1 Schematic diagram and analysis of the bacmids used in this study. (**a**) Schematic diagram of the virus used in this study. Primers and replacement site are indicated by small black arrows. The open triangle and rectangle adjacent to *cat* are residues of *odv-e25* that remained after recombination. (**b**) Identification of vAcBac, vAcBac^{KO}, and vAcBac^{RE} by PCR. The virus templates are indicated below each lane. In lanes 1, 3, and 5, the primer pair odv-e25F/R was used; in lanes 2, 4, and 6, the primer pair M13F/R was used. Marker sizes (bp) are indicated

odv-e25 on virus production. BVs were detected by Q-PCR to measure the extracellular viral DNA produced by cells transfected with each of the three bacmids. BV production was almost equivalent among the three bacmid-transfected cells from 6 to 24 h p.t. This suggested that the release of BVs from transfected cells was unaffected by the deletion of ODV-E25 (Fig. 2d). vAcBac- and vAcBac^{RE}-transfected cells exhibited a steady increase in BV production, while that from cells transfected with vAcBac^{KO} was almost constant starting at 24 h p.t. From 24 to 120 h p.t., the BV production of vAcBac^{KO} increased about threefold, while that of vAcBac and vAcBac^{RE} increased 1,000-fold. The production of BVs of vAcBac and vAcBac^{RE} increased sharply from 24 to 72 h p.t. and reached a plateau at 72 h p.t.

A $TCID_{50}$ endpoint dilution assay was used to detect infectious BVs. As expected, no infectious BVs were detected from cells transfected with the three bacmids transfected at 6 h p.t. The $TCID_{50}$ of vAcBac and vAcBac^{RE} was 2.37×10^4 and 1.78×10^4, respectively, at 24 h p.t., while that of the vAcBac^{KO} was only 1.78×10^2. The viral titers of vAcBac and vAcBac^{RE} were nearly 100 times higher than that of vAcBac^{KO} (Fig. 2e). Considering that BV production was similar at 24 h p.t. with the three bacmids, the number of infectious viruses produced by the *odv-e25* deletion virus was lower (Fig. 2f).

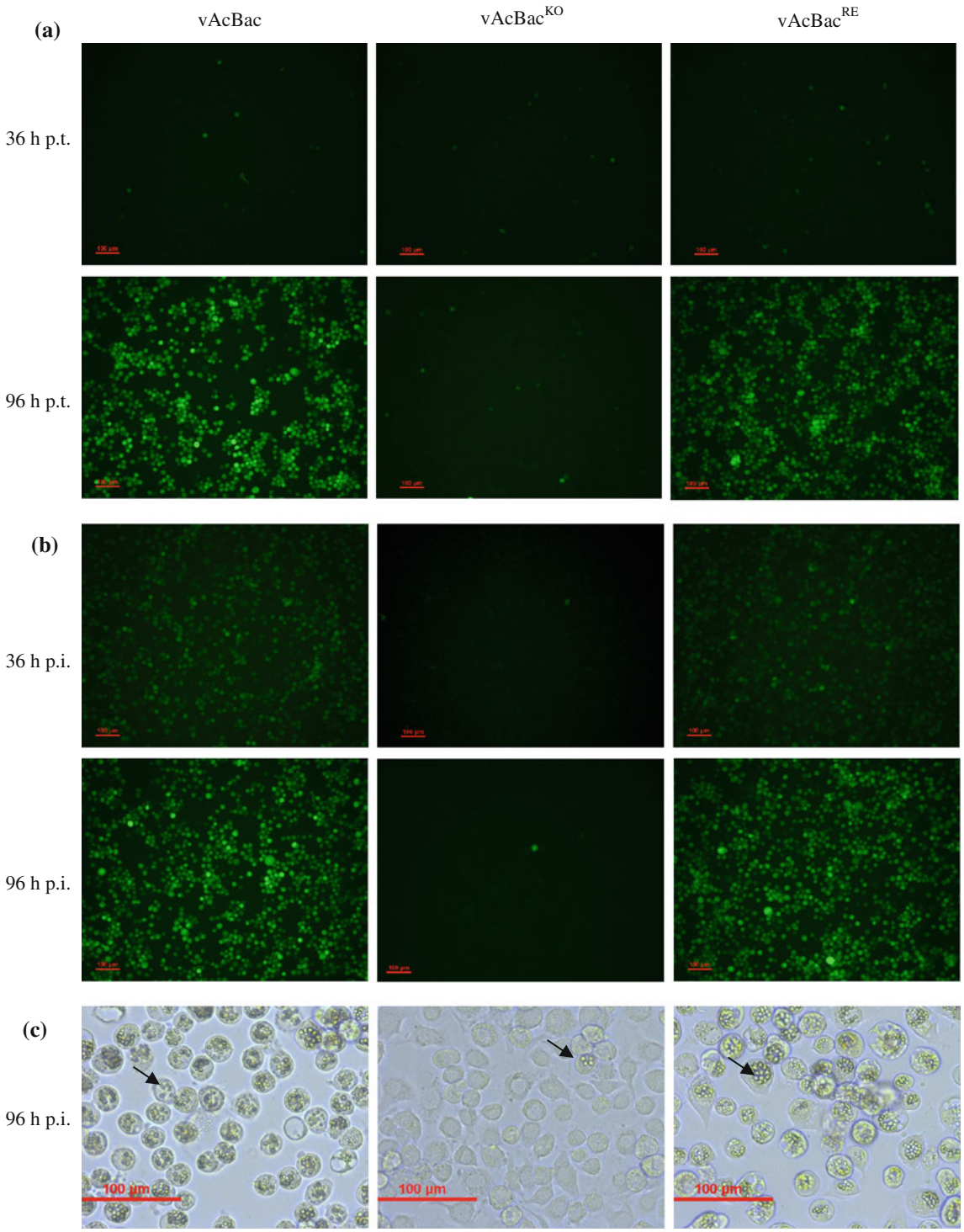

Fig. 2 Analysis of virus replication in *Sf*-9 cells. (**a**) *Sf*-9 cells were transfected with vAcBac, vAcBac^{KO} or vAcBac^{RE} and observed under a fluorescence microscope at 36 and 96 h p.t. (**b**) An infection assay was performed to detect replication and viral infectivity of vAcBac, vAcBac^{KO}, and vAcBac^{RE} in *Sf*-9 cells. (**c**) Brightfield images of vAcBac, vAcBac^{KO}, and vAcBac^{RE} infected cells at 96 h p.i. The arrows indicate cells containing polyhedra. (**d**) BV production curves of vAcBac, vAcBac^{KO}, and vAcBac^{RE} generated in a transfection time course experiment. (**e**) Examination of viral titre by $TCID_{50}$ assay. (**f**) Quantitative analysis of replication of viral DNA. For (**d**), (**e**) and (**f**), error bars represent one standard deviation

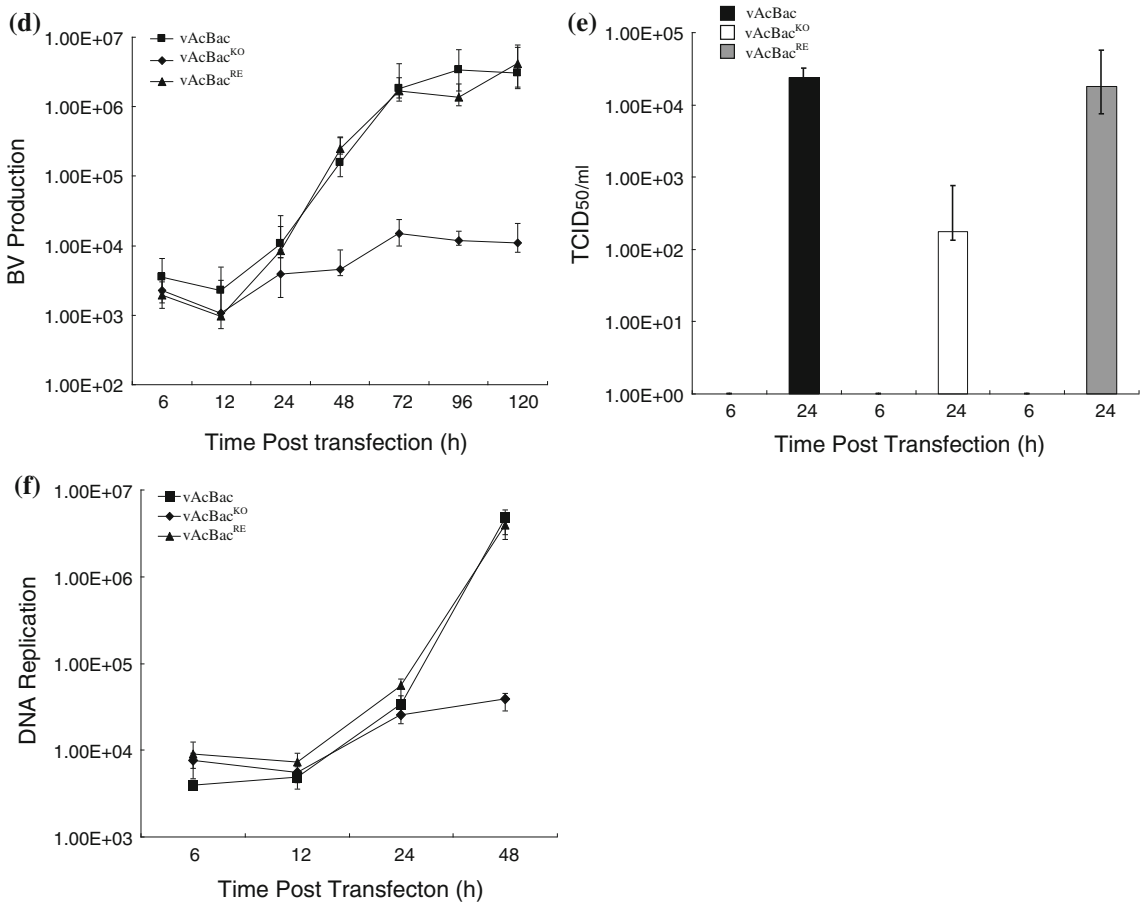

Fig. 2 continued

(2) DNA replication in *Sf*-9 cells To detect viral DNA replication in *Sf*-9 cells, total DNA was obtained from identical numbers of vAcBac-, vAcBac^{KO}- and vAcBac^{RE}-transfected *Sf*-9 cells and used for *gp41*-specific Q-PCR after digestion with *Dpn* I. From 6 to 24 h p.t., similar amounts of viral DNA were obtained from cells transfected with vAcBac, vAcBac^{KO} and vAcBac^{RE}, indicating that viral DNA synthesis was unaffected by the deletion of *odv-e25* (Fig. 2f) during the first 24 h p.t. From 24 to 48 h p.t., the DNA accumulation of vAcBac and vAcBac^{RE} increased 100-fold. In contrast, that of vAcBac^{KO} was almost unchanged.

At 12 h p.i., BVs are produced by wt viruses, and until approximately 20 h p.i., production increases exponentially [16]. It is the infectious BVs that initiate secondary infection and lead to a continuous increase of DNA replication and BV production after 24 h p.i. Viral DNA replication has been detected before 12 h p.i. [31]. As reported before [30], the transcriptional and translational products of *odv-e25* can be detected at 24 h p.i. (supplemental data). As expected, from 6 to 24 h p.t., the accumulation of vAcBac, vAcBac^{KO}, and vAcBac^{RE} DNA was similar. This indicated that all the three bacmids replicated in the transfected cells and that the deletion of ODV-E25 did not affect viral DNA replication during the first 24 h p.t. (Fig. 2f).

Transmission electron microscopic analysis of transfected cells

Electron microscopic analysis was performed to determine whether the deletion of *odv-e25* had any effect on virus morphogenesis. Cells transfected with bacmid were harvested at 96 h p.t., and typical sections were chosen and analyzed. The results showed that typical ODVs were found in the nuclei and polyhedra of vAcBac- and vAcBac^{RE}-transfected cells (Fig. 3a, c). However, there were no virions in the polyhedra or mature ODVs in the nuclei of vAcBac^{KO}-transfected cells (Fig. 3b). For vAcBac^{KO}, 15 cells were found to contain polyhedra in six typical sections, and 35 polyhedra were analyzed. In addition, nucleocapsids were found in all three bacmid-transfected cells, indicating that ODV-E25 was not required for the formation of the nucleocapsid (Fig. 3 and supplemental data).

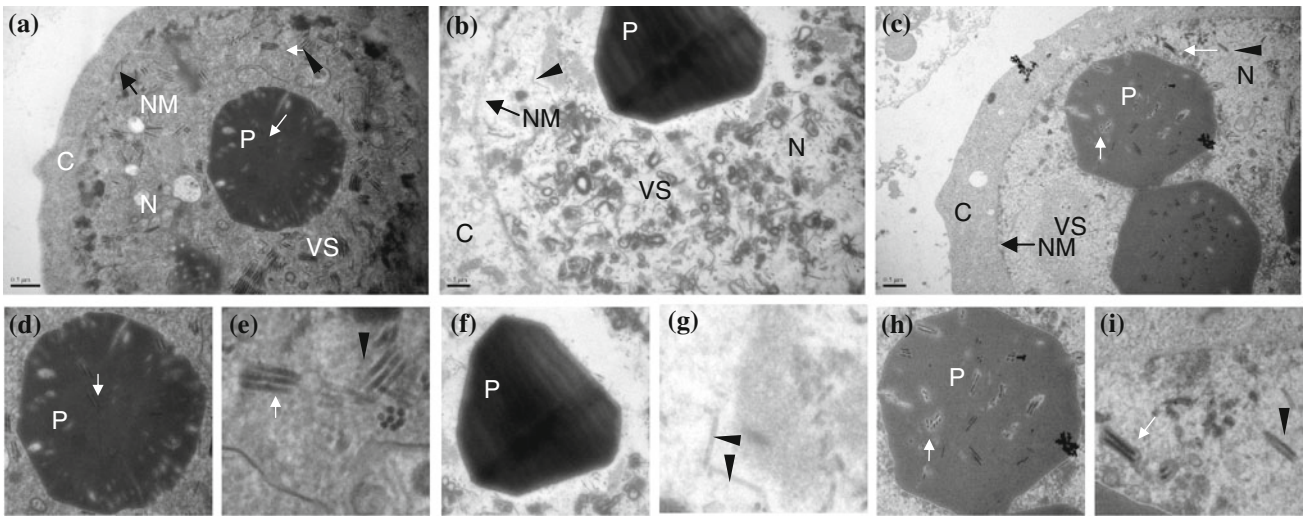

Fig. 3 Electron microscopic analysis of vAcBac (**a**, **d**, **e**), vAcBac^{KO} (**b**, **f**, **g**), and vAcBac^{RE} (**c**, **h**, **i**) bacmid-transfected *Sf*-9 cells at 96 h p.t. Panels **a**-**c** show micrographs of transfected cells. Panels **d**, **f** and **h** show higher-magnification micrographs of polyhedra. The presence of ODVs (white arrows) was found in vAcBac (**e**) and vAcBac^{RE} (**i**) but not in vAcBac^{KO} (**g**). The black triangles indicate nucleocapsids. P, polyhedron; C, cytoplasm; N, nucleus; NM, nuclear membrane; VS, virogenic stroma

Discussion

AcMNPV *odv-e25* and its homologs are conserved in all lepidopteran NPVs and not found in dipteran and hymenopteran NPVs. The N-terminal portion of ODV-E25 is a transmembrane motif that is conserved in all homologs. Although found in BV, ODV-E25 is comparatively more abundant in ODV and is considered a structural protein of ODV [15]. However, the biological function of ODV-E25 remains mainly uncharacterized due to the inability to construct an *odv-e25* knockout virus. In this study, we constructed an *odv-e25* knockout bacmid using the λ Red recombination system. Our results show that ODV-E25 is required for the infectivity of BVs and the formation of ODVs.

The function of *odv-e25* in the context of an AcMNPV infection in *Sf*-9 cells was analyzed with the *odv-e25*-null bacmid. The mutant virus could not be propagated after transfection (Fig. 2a, b), suggesting that BV production or viral infectivity was affected. To test this, BV production and $TCID_{50}$ endpoint dilution assays were performed. The analysis showed that the deletion of *odv-e25* affected infectious BV production. Comparison of the levels of viral DNA accumulation via Q-PCR in vAcBac-, vAcBac^{KO}-, and vAcBac^{RE}-transfected cells showed that viral DNA synthesis was unaffected by the deletion of *odv-e25* in the first 24 h p.t. The difference between vAcBac^{KO} and vAcBac/ vAcBac^{RE} at 48 h p.t. is presumed to be caused by the different infectivity of BVs produced by the three bacmids. Polyhedra were found in *odv-e25*-null-infected cells (Figs. 2c, 3b), indicating that the expression of the polyhedrin gene and viral replication were unaffected [11]. On the other hand, it is concluded from our data that the defects caused by the deletion of *odv-e25* were fully rescued by the 'repair type', and the replacement of *odv-e25* by *cat* did not disturb the transcription of the DNA helicase gene, which is located downstream of *odv-e25*. The ODV-E25 protein appeared as a doublet with about 1 kDa separating the two bands (supplemental data), and a previous study showed that this protein does not appear to be glycosylated [30]. This suggested that another form of processing occurs, and further studies should be performed to see if these modifications affect the functions of ODV-E25.

Electron microscopy showed that nucleocapsids with a normal appearance were found in all three bacmid-transfected cells, indicating that nucleocapsid assembly was not affected by the deletion of *odv-e25*. The normal nucleocapsids also indicated that new viral DNAs were incorporated into capsids. This is consistent with the Q-PCR analysis, which showed that *odv-e25* is not involved in viral DNA synthesis. Mature ODVs were found in the nuclei and polyhedra of vAcBac- and vAcBac^{RE}-transfected cells (Fig. 3a, c) but were not found in vAcBac^{KO}-transfected cells (Fig. 3b). This suggests that ODV-E25 is required for the formation of the mature ODV. Furthermore, ODV-E25 is considered to be part of the FP-25K/ODV-E66 complex [5, 7] and might play important roles in the functions of the complex and the procedure of ODV occlusion.

ODV envelope proteins play biological roles in ODV occlusion and interact with the midgut [32]. Current data indicate that up to 47 proteins are reported to be structural components of ODV [6, 9, 27]. Many of them are related to oral infection, such as PIF factors (PIF-1, PIF-2, PIF-3, and

PIF-4) and do not affect BV infection [10, 12, 26, 28]. Some of ODV proteins have also been found in BVs, such as ODV-E18, which is essential for mediating BV production [23, 38]. AC109 is the first reported structural protein of the envelope and nucleocapsid from BV and ODV. In addition to being a structural protein of ODVs [30], ODV-E25 is suggested to be a structural component of BVs because it is required for producing infectious BVs. It has been demonstrated that the ODV-E25 N-terminal hydrophobic motif was sufficient to direct the protein through the nuclear membrane [15]. ODV-E25 is believed to be synthesized in the cytoplasm and transported into the nucleus to assemble the mature ODVs. However, it is not clear how ODV-E25 attaches to BVs. Additional studies should be performed to uncover the mechanism of action of ODV-E25 on BV infection and ODV formation.

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