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Disruption of the baculovirus core gene *ac78* results in decreased production of multiple nucleocapsid-enveloped occlusion-derived virions and the failure of primary infection *in vivo*

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ABSTRACT

The *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) *ac78* gene is one of the baculovirus core genes. Recent studies showed that *ac78* is essential for budded virion (BV) production and the embedding of occlusion-derived virion (ODV) into occlusion body during the AcMNPV life cycle. Here, we report that an *ac78*-knockout AcMNPV (vAc78KO) constructed in this study had different phenotypes than those described in the previous studies. A few infectious BVs were detected using titer assays, immunoblot analyses and plaque assays, indicating that *ac78* is not essential for BV formation. Electron microscopy confirmed that the *ac78* deletion did not affect nucleocapsid assembly and ODV formation. However, the numbers of multiple nucleocapsid-enveloped ODVs and ODV-embedded occlusion bodies were significantly decreased. Subsequently, the highly conserved amino acid residues 2–25 and 64–88 of Ac78, which are homologous to an oxidoreductase and cytochrome c oxidase, respectively, were demonstrated to play a crucial role in the morphogenesis of multiple nucleocapsid-enveloped ODV. Immunoblot analysis found that Ac78 was an ODV envelope-associated protein. Consistently, amino acid residues 56–93 of Ac78 were identified as an inner nuclear membrane sorting motif, which may direct the localization of Ac78 to the ODV envelope. *In vivo* infectivity assays showed that the occlusion bodies of vAc78KO were unable to establish primary infection in the midgut of *Trichoplusia ni* larvae. Taken together, our results suggest that *ac78* plays an important role in BV production and proper multiple nucleocapsid-enveloped ODV formation, as well as AcMNPV primary infection *in vivo*.

1. Introduction

Baculoviruses are a very diverse group of insect-specific viruses with double-stranded, circular, supercoiled DNA genomes that vary in size from approximately 80 to 180 kb (Rohrmann, 2011). During the baculovirus infection cycle, two functionally distinct virion phenotypes are produced: the budded virions (BVs) and the occlusion-derived virions (ODVs). BVs and ODVs are similar in nucleocapsid structure and genetic material, but they differ in the origin and composition of the envelopes (Braunagel and Summers, 1994; Hou et al., 2013; Slack and Arif, 2007). Early in infection, newly assembled nucleocapsids egress from the nucleus and bud from the plasma membrane to form BVs. BVs are highly infectious to most insect tissues and are responsible for systemic infection (Federici, 1997; Williams and Faulkner, 1997). Late in infection, the nucleocapsids are retained within the nucleus, and they acquire their envelopes from virus-induced microvesicles to form ODVs (Hong et al., 1994; Hu et al., 2010; Yuan et al., 2011). ODVs are subsequently embedded within a proteinaceous crystalline matrix to form occlusion bodies (OBs). ODVs initiate the primary infection in the midgut of a susceptible host and are required for the horizontal spread of the infection among the insect hosts (Federici, 1997; Rohrmann, 2011; Williams and Faulkner, 1997).

Baculoviruses have been reported worldwide in more than 600 host species that predominantly belong to the orders Lepidoptera, Hymenoptera and Diptera (Martignoni and Iwai, 1986; Rohrmann, 2011). Historically, nucleopolyhedroviruses (NPVs) have been designated as either single NPVs (SNPVs) or multiple NPVs (MNPVs),

Abbreviations: AcMNPV, *Autographa californica multiple nucleopolyhedrovirus*; BV, budded virion; ODV, occlusion-derived virion; OB, occlusion body; SNPV, single NPV; MNPV, multiple NPV; HaNPV, *Helicoverpa armigera* NPV; M-ODV, multiple nucleocapsid-enveloped ODV; INM-SM, inner nuclear membrane sorting motif; MOI, multiplicity of infection; SV40, simian vacuolating virus 40; *Cm*, chloramphenicol resistance gene; *polh*, *polyhedrin* gene; *egfp*, *enhanced green fluorescence protein* gene; VS, virogenic stroma; TM, transmembrane domain.

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referring to whether the ODVs contain single nucleocapsid or multiple nucleocapsids within the envelope, respectively (Williams and Faulkner, 1997). MNPVs have been isolated only from Lepidoptera, while SNPVs have been isolated from Lepidoptera, Hymenoptera and Diptera (Rohrmann, 1986). It has been documented that MNPVs are more efficient at establishing systemic infections than SNPVs, although their abilities to establish primary infections may be similar (Washburn et al., 1999, 2003).

To date, more than 80 baculovirus genomes have been completely sequenced, including *Autographa californica multiple* NPV (AcMNPV) (Ayres et al., 1994), which is the archetype of the *Baculoviridae* family. Additionally, 37 genes have been found to be conserved in all sequenced baculovirus genomes and are considered to be the baculovirus core genes (Garavaglia et al., 2012; Herniou et al., 2003; Yuan et al., 2011). Most baculovirus core genes play important roles in the baculovirus life cycle. The *ac78* gene is a baculovirus core gene that is located between AcMNPV nucleotides (nt) 64,958 and 65,287 and encodes a putative protein of 109 amino acids (aa) with a predicted molecular weight of ~12.5 kDa (Ayres et al., 1994; Garavaglia et al., 2012). Proteomic analyses showed that the Ac78 homologs in *Chrysodeixis chalcites* NPV, *Culex nigripalpus* NPV and *Helicoverpa armigera* NPV (HaNPV) were ODV-associated structural proteins (Hou et al., 2013; Perera et al., 2007; Xu et al., 2011). Recently, Ac78 was shown to be associated with the envelopes of both BVs and ODVs (Tao et al., 2013a). Ac78 and its homolog in HaNPV, Ha72, have been found to be essential for infectious BV production and the embedding of ODVs into the OBs (Huang et al., 2014; Tao et al., 2013a).

In this study, an *ac78* knockout AcMNPV (vAc78KO) was constructed to further investigate the role of *ac78* in the AcMNPV life cycle. The phenotypes of vAc78KO were different from those described in the previous studies. Essentially, we demonstrated that *ac78* is not essential for BV production and ODV formation. However, the deletion of *ac78* drastically reduced BV production and multiple nucleocapsid-enveloped ODV (M-ODV) formation. Ac78 was shown to be an ODV envelope-associated protein. Consistently, we revealed that Ac78 contains an inner nuclear membrane sorting motif (INM-SM) which may direct the localization of Ac78 to the ODV envelope. Bioinformatics showed that Ac78 regions 2–25 and 64–88 are homologous to an oxidoreductase and cytochrome c oxidase, respectively. Deletions of either of these regions resulted in reduced virus yield, but not as much as the original vAc78KO. OBs from vAc78KO were non infectious to the host larvae and no signs of infection were observed in the midgut of larvae. Taken together, our results confirmed that Ac78 plays a pivotal role in BV production and revealed the importance of Ac78 in ODV and OB morphogenesis/maturation.

2. Materials and methods

2.1. Bioinformatics analysis

The conserved domains of Ac78 were predicted using NCBI's Conserved Domain Database (Marchler-Bauer et al., 2007), SMART (Schultz et al., 1998), HHpred analysis (Soding et al., 2005) and Phrye (Kelley and Sternberg, 2009). The homologs of Ac78 were searched against the nonredundant protein sequences in the NCBI database using the Position-Specific Iterated BLAST algorithm. Multiple sequence alignments were performed and edited using ClustalX (Larkin et al., 2007) and GeneDoc (Nicholas and Deerfield, 1997), respectively.

2.2. Cell lines and viruses

The bMON14272 bacmid (Invitrogen), which contains the AcMNPV genome, was propagated in DH10B cells as previously described (Luckow et al., 1993). Sf9 (*Spodoptera frugiperda* IPLB-Sf21-AE clonal isolate 9) insect cells were cultured at 27 °C in TNM-FH medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin and 30 μg/ml streptomycin. The BV titers were determined using a 50% tissue culture infective dose ($TCID_{50}$) endpoint dilution assay in Sf9 cells, as previously described (O'Reilly et al., 1992). Time zero was defined as the time when the viral inoculum was added during infection or replaced with fresh medium during transfection.

2.3. Generation of an ac78-knockout AcMNPV bacmid

An *ac78*-knockout AcMNPV bacmid was generated *via* ET homologous recombination in *Escherichia coli*, as previously described (Yuan et al., 2008). Briefly, a 304-bp fragment that was homologous to the 5′ region of the *ac78* ORF (AcMNPV nt 65,272–65,575) was PCR amplified from bMON14272 using the primers ac78PF1/ac78PR1. The PCR products were ligated into the pUC18 vector (Promega) to generate pUC18-US. A simian vacuolating virus 40 (SV40) polyadenylation signal was PCR amplified from the pFastBac1 vector (Invitrogen) using the primer pair SV40PF/SV40PR. For antibiotic selection in *E. coli*, a chloramphenicol resistance gene (*Cm*) was PCR amplified from the pUC18-Cm vector (Wu et al., 2006) using the primer pair CmPF/CmPR. Using the mixture of the PCR products as templates and SV40PF/CmPR as the outmost primers, an overlap PCR was performed as previously described (Daimon et al., 2005). The resulting PCR fragment, SV40-Cm, was inserted into pUC18-US to construct the pUC18-US-SV40-Cm plasmid, in which the SV40 polyadenylation signal was followed by the *Cm* cassette. A 304-bp fragment that was homologous to the 3′ region of the *ac78* ORF (AcMNPV nt 64,958 to 65,261) was obtained using the primer pair ac78PF2/ac78PR2, and the PCR products were ligated into pUC18-US-SV40-Cm to generate pUC18-US-SV40-Cm-DS. pUC18-US-SV40-Cm-DS was digested with SacI and XbaI to obtain a linear 1888-bp fragment, US-SV40-Cm-DS, in which the SV40 polyadenylation signal and the *Cm* cassette were flanked by the *ac78* 5′- and 3′-regions. The replacement of the target deletion region of *ac78* (AcMNPV nt 65,262–65,271) with the SV40 polyadenylation signal and the *Cm* cassette was performed *via* ET homologous recombination and was confirmed by PCR analysis. The resulting *ac78*-knockout AcMNPV bacmid was designated as bAc78KO. All PCR primers are listed in Table 1.

2.4. Construction of an ac78-knockout virus

To determine if the deletion of *ac78* had any effect on OB morphogenesis and to facilitate the detection of viral infection, the donor plasmid pFB1-PH-GFP, which contains the AcMNPV *polyhedrin* (*polh*) gene and the *enhanced green fluorescence protein* (*egfp*; referred to as *gfp* in the present study) gene whose expression was driven by its own promoter and the AcMNPV immediate-early gene *ie1* promoter, respectively, was transformed into electrocompetent DH10B cells harboring the helper plasmid pMON7124 and the bAc78KO bacmid as previously described (Wu et al., 2006), to generate an *ac78*-knockout virus (vAc78KO). Similarly, a wild-type control virus, vAcWT, was constructed by insertion of the *polh* and *gfp* genes into the *polh* locus of bMON14272. The *ac78*-repaired virus was generated using the following steps. A 645-bp fragment containing the *ac78* native promoter and the *ac78* ORF tagged with an HA epitope prior to the stop codon was PCR amplified from bMON14272 using the primers ac78PF1/ac78PR3. The PCR products were cloned into pUC18-SV40 (Cai et al., 2012) to construct the pUC18-Ac78:HA plasmid. pUC18-Ac78:HA was subsequently digested with SacI and XbaI, and the resulting Ac78:HA fragment was cloned into pFB1-PH-GFP to generate the donor plasmid pFB1-Ac78:HA-PH-GFP. The resulting donor plasmid was