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Microscopic investigation of AcMNPV infection in the *Trichoplusia ni* midgut

Muhammad Afzal Javed [a], Stephanie Harris [a], Leslie G. Willis [b], David A. Theilmann [b], B. Cameron Donly [c], Martin A. Erlandson [a,*], Dwayne D. Hegedus [a,d,*]

[a] Saskatoon Research and Development Centre, Agriculture and Agri-Food Canada, Saskatoon, SK S7N 0X2, Canada
[b] Summerland Research and Development Centre, Agriculture and Agri-Food Canada, Summerland, BC V0H 1Z0, Canada
[c] London Research and Development Centre, Agriculture and Agri-Food Canada, London, ON N5V 4T3, Canada
[d] Dept. of Food & Bio-Product Sciences, University of Saskatchewan, Saskatoon, SK S7N 4A8, Canada

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ABSTRACT

Autographa californica multiple nucleopolyhedrovirus (AcMNPV) is the type species for the genus *Alphabaculovirus* in the family *Baculoviridae*. In nature, AcMNPV infection begins with ingestion of viral occlusion bodies (OBs) from which occlusion-derived viruses (ODV) are released to infect midgut cells. This study explored the early stages of *Trichoplusia ni* midgut infection using recombinant viruses expressing green fluorescent protein (GFP) and/or a VP39-mCherry fusion protein under the control of early and late promoters, respectively. Using a recombinant *ie1:GFP* virus, the anterior midgut region was identified as the predominant site for primary infection. Infection of midguts using the GFP-VP39mCherry-dual labelled recombinant virus revealed that active viral replication and cell-to-cell spread was required for the formation of infection foci and the subsequent spread to uninfected midgut cells and tracheoblasts. The spread of the infection from primary infected cells to secondary cells within the midgut was shown to be dependent upon the membrane fusion protein GP64.

1. Introduction

Baculoviruses are enveloped double-stranded DNA viruses that infect invertebrate hosts. *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) is the most well-studied baculovirus species and belongs to the genus *Alphabaculovirus* of the *Baculoviridae* (Jehle et al., 2006). AcMNPV has a wide host range compared to most other baculoviruses and has been developed as a biocontrol agent for some lepidopteran crop pests (Erlandson, 2008; Hasse et al., 2015; http://www.andermattbiocontrol.com/sites/products/bio-insecticides/baculovirus/loopex.html). The AcMNPV life cycle involves the production of two distinct viral phenotypes; occlusion derived virus (ODV) and budded virus (BV). Both have similar nucleocapsid structure, but BV nucleocapsids are enveloped singly while ODV often contain many nucleocapsids within a common envelope. ODV and BV envelopes are acquired within the nucleus or from the plasma membrane, respectively, and the protein composition of the viral envelope differs for each virion type (Braunagel and Summers, 1994). ODVs are released from ingested AcMNPV occlusion bodies (OBs) when exposed to the alkaline environment in the larval midgut. ODVs enter midgut epithelial cells and establish a primary infection that leads to the production of BVs which are responsible for systemic infection of the host. AcMNPV infection culminates with liquefaction of the larva and release of OBs which may be ingested by another host to initiate a new infection.

Several studies have reported details of AcMNPV infection of the insect midgut. After release from the OB, ODVs must first cross the peritrophic matrix to infect midgut epithelial cells (Granados and Lawler, 1981) and both columnar epithelial and regenerative cells become infected in *T. ni* and *Spodoptera exigua* larvae (Flipsen et al., 1995; Keddie et al., 1989; Knebel-Morsdorf et al., 1996). ODV enter host midgut cells through a process that involves a number of ODV envelope proteins referred to as *per os* infectivity factors (PIFs) that have been shown to be essential for oral infectivity and initial midgut infection. The PIF proteins form a complex associated with the ODV envelope in which PIF1 (*ac119*), PIF2

* Corresponding authors at: Saskatoon Research and Development Centre, Agriculture and Agri-Food Canada, Saskatoon, SK S7N 0X2, Canada (M.A. Erlandson, D.D. Hegedus).

E-mail addresses: afzal.javed@agr.gc.ca (M.A. Javed), stephanie.harris@agr.gc.ca (S. Harris), les.willis@agr.gc.ca (L.G. Willis), david.theilmann@agr.gc.ca (D.A. Theilmann), cam.donly@agr.gc.ca (B.C. Donly), martin.erlandson@agr.gc.ca (M.A. Erlandson), dwayne.hegedus@agr.gc.ca (D.D. Hegedus).

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(*ac22*), PIF3 (*ac115*), PIF4 (*ac96*) and P95 (*ac83*) form the core of the complex along with associated proteins PIF0 (*ac138*, P74), PIF5 (*ac148*, ODV-E56) and PIF6 (*ac68*) (Fang et al., 2009; Faulkner et al., 1997; Harrison et al., 2010; Kikhno et al., 2002; Nie et al., 2012; Ohkawa et al., 2005; Peng et al., 2012; Pijlman et al., 2003; Zhu et al., 2013). The PIF complex plays a central role in the binding of ODV to as yet unknown host midgut cell brush border membrane ligands and the entry of ODV into midgut cells (Granados and Lawler, 1981; Haas-Stapleton et al., 2004; Horton and Burand, 1993; Mu et al., 2014; Ohkawa et al., 2005; Peng et al., 2012). Viral nucleocapsids are transported to the nucleus by a mechanism most probably involving F-actin polymerization as was shown for BV nucleocapsids (Ohkawa et al., 2010). Viral DNA replicates in an intranuclear region called the virogenic stroma where new nucleocapsids assemble. BV are then released from the infected cells in a process requiring the membrane fusion protein GP64 (Monsma et al., 1996). Midgut and tracheal epithelial cells are basally bounded by a uniform non-cellular layer, the basal lamina (BL), which precludes most particles larger than 15 nm in diameter, including AcMNPV BV, from entering the hemocoel to initiate systemic infection (Passarelli, 2011). Expression of a virus-encoded fibroblast growth factor activates a cascade of matrix-metalloproteases which mediate remodelling of the BL allowing viral infection of tracheal epithelial cells (Means and Passarelli, 2010). The tracheal system provides a route for viral particles to spread from the midgut to the hemocoel leading to systemic infection (Engelhard et al., 1994).

The goal of this study was to examine the very early stages of midgut infection to delineate the primary sites for infection initiation and to investigate the spread of the virus between midgut epithelial cells. To this end, a recombinant AcMNPV expressing the green fluorescent protein (GFP) under the control of the *ie1* promoter was used as a marker for early stage viral infection. Another recombinant AcMNPV which, in addition to *ie1*-expressed GFP, expresses the major capsid protein VP39 fused to the mCherry fluorescent protein under the control of the *vp39* native promoter, was used to identify cells in the later stages of infection that were actively undergoing viral replication. The role of GP64 in midgut infection was also examined using ODV derived from a *gp64*-knockout virus expressing GFP under the control of the *ie1* promoter. Our results identified the predominant regions within the midgut for initial virus infection and provided insights into the mechanism for establishment of infection foci and for cell-to-cell transmission of virus within midgut tissues.

2. Materials and methods

2.1. AcMNPV wild type and recombinant viruses

For this study, the AcMNPV bacmid bMON14272 backbone repaired with *polyhedrin* and *gfp* under the control of the OpMNPV *ie1* promoter was used as a wild-type (WT-GFP) control virus (Fang et al., 2009). The virus WT-GFP-3xmCh was constructed by repairing bMON14272 with a construct containing GFP under the early promoter *ie1* and VP39 fused with 3x mCherry under the *vp39* native promoter which has been previously described (Biswas et al., 2016; Ohkawa et al., 2010). A GFP expressing Ac*gp64* knockout virus (Ac*gp64*KO-GFP) in which the viral *gp64* gene has been deleted was constructed as previously described (Dickison et al., 2012).

2.2. Production of occlusion bodies

AcMNPV (WT-GFP and WT-GFP-3xmCh) OBs were produced by injecting 100 $TCID_{50}$ (tissue culture infectious units) of BV into the hemocoel of 4th instar *T. ni* larvae which were then reared on artificial diet at 27 °C as described previously (Erlandson et al., 2007). AcMNPV OBs were purified from the cadavers using a standard protocol. Briefly, cadavers were homogenized in OB purification buffer (0.001M EDTA, 0.01M Tris, 0.5% SDS, 0.1% Triton X-100, pH 7.5) and mixed using a vortex until a uniform suspension was obtained. The suspension was incubated at 37 °C for 2 h after which the suspension was mixed using a vortex and then filtered through 4 layers of sterile cheesecloth. The suspension was centrifuged at 3000*g* for 20 min, the pellet washed 3 times with sterile ddH_2O and the purified OBs were resuspended in sterile ddH_2O.

Ac*gp64*KO-GFP lacks GP64 and is unable to cause a systemic infection in larvae, therefore, OBs were produced in the *Spodoptera frugiperda* cell line Sf9 cultured in Grace's supplemented insect medium (Invitrogen) containing 10% fetal bovine serum (FBS) and gentamycin (12.5 μg/ml). Four T75 flasks seeded with Sf9 cells (3×10^6 cells/flask) were transfected with Ac*gp64*KO-GFP DNA following the Sf9 cell *in vitro* transfection protocol using lipofectin (Campbell, 1995). To purify OBs, cells were harvested at 6 days post-transfection by centrifugation at 1000*g* for 10 min and the cell pellet resuspended in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH7.5) supplemented with 0.5% Triton X-100. The suspension was mixed gently using a vortex set to low speed and incubated at 20 °C for 30 min. The cells were lysed by sonication using a Misonix S-4000 sonicator (QSonica, Newtown, CT) set at amplitude 50 and a pulse time of 15 s and then chilled on ice for 5 min. OBs were pelleted by centrifugation at 3500*g* for 5 min, washed twice in sterile ddH_2O and then resuspended in 100 μl of sterile ddH_2O.

2.3. Infection of T. ni larval midgut

Fourth instar *T. ni* larvae were starved for 6 h and then fed a 5 μl droplet containing 1×10^3, 10^4 or 10^5 OBs in a sucrose solution (5 mg/ml) with blue food coloring. The larvae were transferred onto artificial diet and incubated at 27 °C. Midguts were removed at various time points post-infection using a longitudinal excision technique described by Braun and Keddie (1997) with minor modifications. Briefly, the basal lamina was removed by treating the midgut with dispase (2IU in sterile artificial hemolymph solution [SAHS] [STEMCELL Technologies, Vancouver, Canada]) for 30 min. After removal of the basal lamina, the midgut lumen was opened and the peritrophic matrix removed without damaging the midgut epithelial cell layer. To reveal nuclei, the midgut epithelial cell layer was stained with Hoechst 33342 (Invitrogen) and rinsed 3 times in SAHS. The preparations were observed with Carl Zeiss fluorescence stereomicroscope Lumar V12 using a Zeiss Neolumar S 0.8× FWD 80 mm lens or 1.5× FWD 30 mm lens (Carl Zeiss, Canada). Higher magnification microscopy of midgut cell infections was done with a Carl Zeiss optical fluorescence microscope Imager Z1 (Carl Zeiss, Canada). Images were captured using an Axiocam HRm camera and processed with AxioVision software (Carl Zeiss, Canada).

2.4. Viral infection of hemocytes

Viral infection of hemocytes was determined by examining GFP expression by fluorescence microscopy. *T. ni* larvae (4th instar) were fed with 10^4 OBs of WT-GFP as described above and hemolymph was collected by incision of larval proleg at 24 and 48 hpi. All hemolymph (6–8 μl) collected from each larva was immediately examined by fluorescence microscopy and GFP expression was used as marker of viral infection. Hemolymph was observed from two independent experiments and three larvae were bled at each time point.