Intrahaemocoelic infection of *Trichoplusia ni* with the baculovirus *Autographa californica* M nucleopolyhedrovirus does not induce tracheal cell basal lamina remodelling

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Infection of the lepidopteran insect *Trichoplusia ni* with the baculovirus *Autographa californica* M nucleopolyhedrovirus (AcMNPV) by the oral route stimulates activation of host matrix metalloproteases (MMP) and effector caspases, a process dependent on expression of the viral fibroblast growth factor (vFGF). This pathway leads to tracheal cell basal lamina remodelling, enabling virus escape from the primary site of infection, the midgut epithelium, and establishment of efficient systemic infection. In this study, we asked whether the MMP–caspase pathway was also activated following infection by intrahaemocoelic injection. We found that intrahaemocoelic infection did not lead to any observable tracheal cell or midgut epithelium basal lamina remodelling. MMP and caspase activities were not significantly stimulated. We conclude that the main role of the AcMNPV vFGF is in facilitating virus midgut escape.

Fibroblast growth factors (FGFs) are a family of growth factors involved in several biological signalling processes, including cell survival, differentiation and proliferation (Ornitz & Itoh, 2001). Imbalances in FGF levels or mutations affecting FGF receptors have been associated with disease, including cancer (Grose & Dickson, 2005). FGFs are encoded by metazoan organisms ranging from nematodes to humans and are expressed during embryogenesis and in mature organisms (Ornitz & Itoh, 2001). In addition, viruses belonging to the family *Baculoviridae* encode FGFs (Ayres et al., 1994), called viral FGFs (vFGFs).

Baculoviruses are large enveloped DNA viruses. All but four of the sequenced baculovirus genomes carry at least one copy of *vfgf*, and the viruses carrying *vfgf* establish productive systemic infections in their lepidopteran hosts. Among the four viruses lacking *vfgf*, three infect insects in orders other than Lepidoptera, namely, Hymenoptera and Diptera, and do not spread beyond the primary infection site, which is the midgut epithelium. The remaining virus that does not encode *vfgf*, *Maruca vitrata* nucleopolyhedrovirus, infects lepidopteran insects (Chen et al., 2008), but its infection tropism is not well characterized.

The baculovirus *Autographa californica* M nucleopolyhedrovirus (AcMNPV) infects susceptible larvae that ingest an occluded and environmentally stable form of the virus. The released virions, occlusion-derived virions, infect midgut epithelial cells. Midgut cells produce nucleocapsids that reach the basal side and bud through the plasma membrane and are referred to as budded virions (BVs). BVs infect susceptible tissues throughout the host (reviewed by Rohrmann 2011).

In recent years, caspases, cysteine proteinases involved in cell death, have been shown to be involved in processes other than cell death, including cellular differentiation, development, proliferation and fusion (Nhan et al., 2006). We have shown that caspases can also affect components of the extracellular matrix of tracheal cells (i.e. laminin and collagen IV) in a process that leads to baculovirus midgut escape and efficient systemic spread (Means & Passarelli, 2010). This process involves matrix metalloproteases (MMPs) and is stimulated by AcMNPV *vfgf*. Inhibiting either caspases or MMPs abolished AcMNPV midgut escape (Means & Passarelli, 2010). Two other studies also support that the AcMNPV vFGF may facilitate virus spread beyond the midgut epithelium to establish efficient systemic infections. First, AcBAC, a bacmid-based AcMNPV vector carrying *vfgf*, accelerates *Trichoplusia ni* larvae mortality compared with AcBAC-*vfgfKO*, which lacks *vfgf* (Detvisitsakun et al., 2007), when the virus is delivered by the oral route. Second, larvae infected by the oral route with AcBAC show infection of tracheal cells, fat body and haemocytes at earlier times compared with AcBAC-*vfgfKO*-infected larval tissues, indicating that the rate of infection is faster in the presence of *vfgf* (Means & Passarelli, 2010). Together, these studies indicate that the AcMNPV vFGF stimulates the activation of host caspases and MMPs, leading to tracheal cell basal lamina remodelling and virus escape from the primary site of infection.

In this study, we asked whether vFGF had a role once the virus escaped the midgut. Specifically, we sought to determine whether vFGF affected laminin in the extracellular matrix of tissues if the virus was administered intrahaemocoelically and bypassed the midgut. Early fifth
instar T. ni larvae (Benzon Research) were injected between the first pair of prolegs at the third abdominal segment with 10 p.f.u. of AcBAC or AcBAC-vfgfKO, midguts and associated tracheal cells were dissected at 12 h post-infection (p.i.) (Fig. 1a, upper row, right panels), and laminin was detected using anti-laminin polyclonal sera (Sigma) at 1:100 dilution as previously described (Means & Passarelli, 2010). Laminin was detected in midgut-associated tracheae from mock-infected larvae (Fig. 1a, upper row, mock-infected), while control larvae that were infected orally with AcBAC showed a decrease in tracheal cell laminin as previously reported [Fig. 1a, AcBAC-fed panel at left and Means & Passarelli (2010)]. However, when viruses were delivered intrahaemocoelically, no differences in tracheal cell anti-laminin cross-reactivity were observed regardless of the virus used (Fig. 1a, upper right panels), suggesting that the integrity of the basal lamina was not compromised. Since basal lamina proteins are continuously secreted to the extracellular matrix, we considered that when we detected laminin at 12 h p.i., additional laminin could have been synthesized as part of a newly secreted basal lamina and detected in this experiment. Alternatively, it is possible that the basal lamina was being remodelled during virus infection later than 12 h p.i. Thus, we examined tracheal cell basal lamina integrity at earlier (6 h p.i.) and later (24 and 72 h p.i.) times, but still no differences were observed (Fig. 1b, c). Expression of EGFP expressed by AcBAC and AcBAC-vfgfKO was visible at 72 h p.i., verifying infection of the tissues. Thus, intrahaemocoelic virus infection and the presence of vfgf did not drastically affect tracheal cell laminin composition.

Other insect organs besides trachea are also lined by basal lamina, making their vulnerability to viruses more challenging. However, the tracheal branching network has access to these organs and can be used as a gateway to infect them (Engelhard et al., 1994). We thus examined whether we could detect laminin differences in the basal lamina secreted by cells lining a major organ, the midgut. Staining of laminin associated with midgut epithelial cells infected with AcBAC or AcBAC-vfgfKO resembled that of mock-infected midgut epithelial cells at 6 (data not shown) and 12 h p.i. (Fig. 2a), indicating that the basal lamina lining the midgut epithelium was not drastically affected by intrahaemocoelic virus infection. It is not clear if the basal laminae lining midgut are readily accessed by viruses from the haemocoel, since the midgut basal laminae are in close association with connective tissue.

To evaluate the infection process over time in the current experiments, we counted the number of EGFP-positive haemocytes in larvae intrahaemocoelically infected with the EGFP-expressing viruses AcBAC and AcBAC-vfgfKO. In previous work, we did not observe any significant differences in numbers of EGFP-positive haemocytes following
intra-haemocoelic infections with AcBAC or AcBAC-vfgfKO (Means & Passarelli, 2010). Here, we did observe a slight decrease in EGFP-positive haemocytes at 24 h p.i between AcBAC and AcBAC-vfgfKO or AcBAC and infections with caspase or MMP inhibitors, but these differences were not evident at later times (Fig. 2b). Given our previous results, we think that this small and transient difference is not likely to be biologically significant.

We previously reported that T. ni larvae intra-haemo-coelically infected with AcBAC or AcBAC-vfgfKO showed no apparent differences in the timing of infection of several cell types (epithelial tracheal cells, fat body cells and haemocytes), in contrast to infection by feeding where lack of vfgf resulted in a slowed infection process (Means & Passarelli, 2010). Since vFGF stimulates caspases and MMPs following oral infections, we questioned whether vFGF stimulated these enzymes when it was expressed in the haemocoel. To address this, we measured MMP and caspase activation as previously described (Means & Passarelli, 2010), following intra-haemo-coelic infection (10 p.f.u.) of early fifth instar T. ni larvae with or without 100 μM of the caspase inhibitor zVAD-fmk (MP Biomedicals) or the MMP inhibitor GM6001 (Calbiochem). We found that inhibition of either caspases or MMPs did not lead to any observable changes in tracheal cell basal lamina composition (Fig. 1a, panels in lower row).

To more directly evaluate the activation of caspases, we examined caspase activation in T. ni midgut-associated tracheal cells, following T. ni intra-haemocoelic infection with AcBAC or AcBAC-vfgfKO. Caspase activation was assessed using the anti-CM1 antibody (Cell Signalling), an antibody that detects the activated form of effector caspases (Florentin & Arama, 2012). We observed no detectable anti-CM1 binding, suggesting effector caspases were not significantly activated by intra-haemocoelic infection (Fig. 3a). This is in contrast to the strong anti-CM1 staining observed during oral infection [Fig. 3a and Means & Passarelli (2010)]. Since we observed no significant detectable caspase activity using the anti-CM1 antibody, we decided to examine caspase activity in vitro using lysates prepared from infected midgut and the substrate DEVD-afc. The activity in AcBAC or AcBAC-vfgfKO-infected midgut lysate compared to mock-infected midgut or compared between viruses was not significantly different using the unpaired t-test (Fig. 3b).

In addition, the MMP activities from midgut lysates of AcBAC- and AcBAC-vfgfKO-infected midguts or between mock- and virus-infected samples were not significantly different following unpaired t-tests (Fig. 3c). The levels of caspase and MMP activities from AcBAC-infected midgut were significantly lower than those observed during per os
infection (Fig. 3b, c, columns labelled ‘fed’). We also examined effector caspase and MMP activities at 6 h p.i. and observed no differences compared to mock-infected midguts (data not shown). Thus, vFGF did not stimulate significant levels of either caspase or MMP activity in midguts following intrahaemocoelic infection.

MMPs and caspases are activated during oral infection and dependent on vfgf expression. After intrahaemocoelic infections, we did not observe any significant increase in MMP and caspase activities compared to midgut samples from mock-infected larvae and the presence of vfgf did not affect enzymatic activities or laminin detection. Bombyx mori larvae intrahaemocoelically infected with a recombinant lacking vfgf had a reduced number of infected haemocytes than recombinants with vfgf (Katsuma et al., 2008). The differences between AcMNPV-based viruses carrying or lacking vfgf are less pronounced than those observed with the B. mori NPV (BmNPV) vfgf following in vitro and in vivo experiments. It has been suggested that the potential higher expression of the BmNPV vfgf compared to that of the AcMNPV may enhance the phenotype (Passarelli, 2011). Together, data presented here and in previously published studies conclude that the AcMNPV vFGF does not significantly affect infection rates of several tissues, the timing of host mortality or activation of MMP and effector caspases following intrahaemocoelic infections. It is possible that the AcMNPV vfgf may have more obvious effects in other hosts, aiding the virus to establish more efficient systemic infections. It is also possible that some viruses that do not encode fgf stimulate cellular FGF-mediated pathways for efficient replication.

**Acknowledgements**

This work was partially supported by the National Institutes of Health grant NIH R21AI63089 and the National Institutes of Food and Agriculture grant NRI 2008-35302-18849. This is contribution number 14-074 J from the Kansas Agricultural Experiment Station.

**References**


Role of baculovirus vFGF in haemocoelic infections