Review

Barriers to success: How baculoviruses establish efficient systemic infections

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The mechanisms used by baculoviruses to exit the midgut and cause systemic infection of their insect hosts have been debated for decades. After being ingested, baculoviruses reach the midgut, where several host barriers need to be overcome in order to establish successful infection. One of these barriers is the basal lamina, a presumably virus-impermeable extracellular layer secreted by the epithelial cells lining the midgut and trachea. This review discusses new evidence that demonstrates how these viruses breach the basal lamina and establish efficient systemic infections. The biochemical mechanisms involved in dismantling basal lamina during baculovirus infection may also provide new insights into the process of basal lamina remodeling in invertebrate and vertebrate animals.

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Introduction

Viruses encounter cellular and non-cellular physical barriers as they attempt to infect a host. These may include the maternal–fetal barrier, blood–brain barrier, epidermal layers, or basal laminae that...
line epithelial surfaces. Like all viruses, insect viruses must be able to defeat physical barriers in order to successfully establish infection.

The first physical barrier that a virus may encounter in an insect is the outermost exoskeleton or cuticle. However, many viruses gain entry into the insect by the oral route, as they are consumed during feeding, and reach insect intestinal epithelial cells where they establish a primary infection. In the intestine, insect viruses are faced with chemical and physical barriers, which must be overcome to establish productive infections. In this review, I will focus on the mechanisms used by baculoviruses to overcome the basal lamina separating the initial site of infection from other insect tissues. Most mechanistic examples will be derived from studies using the baculovirus Autographa californica M nucleopolyhedrovirus (AcMNPV) in the permissive cabbage looper host, Trichoplusia ni. In the process, I will compare parallels that have been reported in arboviruses vectored by mosquitoes. Since the biochemical mechanisms that baculoviruses use to dismantle basal lamina are reminiscent of observations made during basal lamina breakdown during mammalian developmental and pathological processes, I will also cite selected parallels described in some of these fields.

**Basal laminae as barriers to virus infiltration**

Basal laminae are thin (up to 120 nm thick), flexible, and noncellular sheets that are secreted on the basal side of epithelial cell layers. These extracellular layers are composed of proteins including type IV collagen, laminin, nidogen, and the heparin sulfate proteoglycan perlecán. Basal laminae have diverse functions, including filtering molecules, serving as a support of cellular layers, directing the migration of cells, and preventing cell movement into underlying cells (Sasaki et al., 2004). In insects, all organs secrete basal laminae except the blood cells, called hemocytes, which are free in the hemolymph that bathes all organs.

The insect midgut epithelium is a polarized tissue with different functions and cellular structures at the basal and apical sides. On the apical surface are abundant and long cell projections, microvilli, which form the brush border in the lumen side of the midgut. At the basal side, bordering the hemocoel, midgut epithelial cells secrete a uniform basal lamina. The midgut-lining basal lamina provides a scaffold for regeneration of epithelial cells after mature cells slough off during development and after intestinal damage or insults, and also provides a clear separation between the midgut and the hemocoelic tissues. In addition, the basal lamina secreted by midgut epithelial cells has a protective function, namely preventing the passage of pathogens that enter the midgut from infecting hemocoelic tissues.

Many viruses that infect insects, including mosquito-vectored viruses, are ingested by insects as contaminants of their food, and thus primary infection is established in midgut epithelial cells. Although some viruses are midgut restricted, others escape the midgut and infect other tissues. To access organs beyond the midgut, viruses have to cross into the hemocoel. The basal lamina bordering the midgut has been estimated to have apertures between its protein fibril layers of only 15 nm in diameter (Reddy and Locke, 1990), and this pore size would preclude virus passage. Nevertheless, viruses must cross the midgut basal lamina in order to establish infections in other organs. This problem has prompted numerous studies over the past few decades examining whether insect and insect-borne viruses directly cross the basal lamina, or whether they utilize alternative routes to escape from the midgut.

**Possible mechanisms of virus midgut escape**

Studies asking how viruses escape from the midgut and infect hemocoelic organs have been mainly restricted to descriptive approaches utilizing electron microscopy or histochemistry. In order to catch a virus as it transits across the midgut epithelial cell/basal lamina barrier, timing is crucial. If the number of virus particles necessary for midgut escape and lethality is small, it may also be difficult to detect. Consequently, determining the secondarily infected target cell may be challenging and limitations in technology may have left gaps in the route pathogens follow from primary to secondary infection sites. Two main routes of virus midgut escape have been proposed: directly crossing the midgut basal lamina and using tracheal cells as a conduit between the midgut and the main insect cavity (Fig. 1).

**Escape via midgut cell basal laminae**

Directly passing through the midgut cell basal lamina would seem to be the most direct route to access hemocoelic organs and offers the virus a large target to attempt midgut escape. Although the small size of the pores in basal laminae would be expected to prevent virus passage, basal laminae are dynamic structures which are formed, degraded, and reform and this may create gaps that allow virus escape. The importance of midgut infection and escape barriers is highlighted by findings describing the midgut as the principal organ determining mosquito vector competence (Black et al., 2002).

There are several reports supporting that viruses cross the midgut basal lamina. Changes in midgut basal lamina integrity have been observed during infection with Eastern equine encephalomyelitis virus (Weaver et al., 1988). During Venezuelan equine encephalitis and Rift Valley fever virus infections, a modified basal lamina associated with muscle tissue has been noted (Romoser et al., 2004). Also, Dengue virus appears to enter the hemocoel regardless of basal laminae thickness (Thompson et al., 1993); arguing against a preference for midgut or tracheal cell basal lamina, if there is any difference. It is thought that glycoproteins in luteoviruses regulate access through the basal laminae of salivary glands (Gildow and Gray, 1993); it is possible that a specific virus–host interaction may be a determinant in midgut escape. Finally, AcMNPV has been observed pushing against the resilient basal lamina from the basal side of midgut epithelial cells and within 30 min post infection (p.i.), virus was observed in the hemocoel of T. ni larvae (Granados and Lawler, 1981).

**Escape via the insect respiratory system**

An alternative route for virus egress from the midgut using the insect tracheal or respiratory system has also been proposed. Insects oxygenate their tissues by delivering oxygen via an intricately branched organ, the trachea. Tracheae provide gas exchange for all tissues through a network of chitinous tubes. Surrounding these tubes is a sheath of tracheal epithelial cells that are susceptible to virus infection. Like all epithelial cells, the outermost, basal side of these cells is lined with a basal lamina. The branching pattern of tracheae is genetically predetermined by chemotactic tracheal cell migrations stimulated by a fibroblast growth factor (FGF) (Sutherland et al., 1996). As these cells branch, fine single cell protrusions radiate into insect tissues. The single cells found at the ends of these branches are called tracheoblasts and in Drosophila, they migrate towards oxygen-depleted cells, which secrete FGF (Sutherland et al., 1996).

Rift Valley Fever, Sindbis, LaCrosse, and Dengue viruses and insect pathogens such as the baculoviruses AcMNPV and Bombyx mori nucleopolyhedrovirus (BmNPV) have been observed infecting tracheal epithelial cells following midgut cell infections or infecting other organs via tracheae (Bowers et al., 1995; Chandler et al., 1998; Engelhard et al., 1994; Kirkpatrick et al., 1994; Rahman and Gopinathan, 2004; Romoser et al., 2005). It has been hypothesized that tracheoblasts, which pierce the midgut basal lamina in order to oxygenate midgut epithelial cells, are the secondary sites of infection, thereby establishing a connection between midgut and hemocoelic tissues (Engelhard et al., 1994). Tracheoblasts reaching into the midgut basal lamina have been observed by transmission electron microscopy (Romoser et al., 2005).
In support of the hypothesis that the tracheal system is used for virus dissemination, it has been observed that AcMNPV infects hemocoelic organs where tracheoblasts terminate (Engelhard et al., 1994). Also, expression of basal lamina-degrading enzymes from baculoviruses did not accelerate systemic infection of the virus, suggesting the virus uses an alternate route of escape (Li et al., 2007).

On the other hand, the fact that tracheal branches are themselves wrapped by basal lamina raises the question of how viruses can penetrate the basal lamina covering tracheoblasts, but not that lining the midgut. It is thought that if secondary infection does not take place rapidly enough, midgut cells are sloughed into the gut lumen and virus replication and dissemination will not take place (Engelhard and Volkman, 1994; Washburn et al., 1995). Therefore, viruses need to have an efficient method to cross either the midgut basal lamina or a tracheal element basal lamina and escape into the hemocoel. In the case of some baculoviruses, a viral gene is expressed which optimizes this event by stimulating a cellular signaling pathway that eventually triggers basal lamina turnover and virus escape.

**Baculovirus pathogenesis**

In this section, I will discuss AcMNPV pathogenesis in the susceptible host, the cabbage looper *T. ni*, since the process of establishing systemic infection is well characterized in this system. However, it is important to note that there are differences in pathogenesis such as organ tropism and rate of infections between different baculovirus–host combinations.

One form of AcMNPV, the occluded form, is encased in crystalline proteinaceous occlusion bodies, called polyhedra that confer stability and environmental protection to the embedded virions. AcMNPV occlusion bodies contain groups of co-enveloped nucleocapsids that are orally consumed by larvae and reach the midgut of the insect. The alkaline environment of the midgut dissolves the occlusion body, releasing co-enveloped nucleocapsids (more than one nucleocapsid per envelope) known as occlusion-derived virus. The occlusion-derived virus crosses the peritrophic membrane, a layer separating the midgut epithelium from the food bolus in the midgut lumen, and the viral envelope is thought to fuse with the cell membrane on the apical side of midgut epithelial cells. The virus replicates in the nucleus of these cells and viral genes are expressed, including expression of the viral fusion protein (GP64) that is shuttled to the cell membrane. As virions bud from the basal side of midgut epithelial cells, they wrap the GP64-studded cell membrane around single nucleocapsids to form their envelope. This singly enveloped budded form of the virus serves to spread infection between cells in that host. It is thought that some nucleocapsids do not enter the nucleus but instead, shortly after entry, directly bud through the basolateral midgut epithelial side to ensure a productive infection prior to midgut cell sloughing (Washburn et al., 1999). The production of occluded virus is not efficient in midgut epithelial cells (Granados and Lawler, 1981).

An AcMNPV recombinant expressing the *Escherichia coli lacZ* gene was used to follow virus infection of orally infected 4th instar *T. ni* larvae (Engelhard et al., 1994). Infected midgut epithelial cells were first observed at 4 h p.i. and multiple foci of infection were detected in the midgut by 12 h p.i. (Fig. 2). Between 12 and 24 h p.i., the next cells to become infected after midgut epithelial cells were tracheoblasts and tracheal epithelial cells associated with the midgut. The observation that tracheoblasts, rather than hemocytes, were first infected after midgut cells led to the hypothesis that tracheal cells were the conduits to disseminate infection systemically (Engelhard et al., 1994). By 36 h p.i., hemocytes and tracheoblasts associated with other organs were infected. Infection of additional hemocytes and fat body tissue was observed by 48 h p.i. At this time, infection of midgut epithelial cells was reduced as infected cells were sloughed off and new cells regenerated. Finally, by 70 h p.i., other tissues were infected, including muscle fibers, salivary glands, malpighian tubules, gonads, cuticular epidermis, and midgut epithelia (Fig. 2). In contrast, when
budded virions were directly injected into the hemocoel of T. ni larvae, the first cells to be infected were tracheoblasts and hemocytes (Engelhard et al., 1994).

The role of FGFs in trachea development

Since tracheal cells are the first cell type to be infected following the midgut epithelium, learning about tracheal cell branching morphogenesis is important to understand baculovirus pathogenesis. The process of trachea formation throughout development has been detailed in the fruit fly Drosophila melanogaster, which has served as a model system to study the morphological, genetic, and molecular aspects of this process and branching morphogenesis in other organs.

Drosophila carries an fgf homolog, branchless, which is required for development of the tracheal system, aiding in the formation of primary and secondary branches (Sato and Kornberg, 2002; Sutherland et al., 1996). The receptor for Branchless, called Breathless, is expressed on the surface of tracheal epithelial cells. In the embryo, tracheal sacs consisting of 80 cells each form by invaginations which later become the spiracles. Remarkably, through cell migration and shape changes, these 80 cells later constitute each individual tracheal tree in the larva (i.e., no further cell divisions are observed). Later in larval development, Branchless also plays an essential role in terminal tracheal branching. Terminal tracheal branching is not strictly developmentally programmed, but instead occurs in response to low oxygen. Tracheoblasts are highly motile single-cell tracheal projections that respond quickly to Branchless signaling originating from neighboring oxygen-deficient cells by sending fine processes towards the source of Branchless. Tracheoblasts contain a narrow intracellular lumen (less than 1 μm in diameter) within each extension that is lined with chitin and is continuous with the main tracheal branch (Sutherland et al., 1996).

There are several characteristics of the branching response of tracheoblasts to Branchless that have important implications during baculovirus secondary infection of tracheoblasts: 1) The response to Branchless is very rapid (occurring within minutes); 2) the response does not require cell division (Ghabrial et al., 2003; Sutherland et al., 1996), and can occur over relatively long distances (through at least two cell layers) (Sato and Kornberg, 2002); and 3) based on what is known for other types of epithelia (Bernfield et al., 1984), in order for terminal branching to occur, the tracheoblast would need to locally degrade its basal lamina by secreting degrading enzymes. Following extension of the tracheoblast, the basal lamina would then be resynthesized.

FGF signaling

FGFs are a family of structurally related paracrine signaling molecules that modulate a myriad of developmental processes in different tissues of multicellular organisms. During secretion, FGF binds heparan sulfate proteoglycans on the cell surface prior to this complex being released. The complex of glycosaminoglycan heparan sulfate and FGF activates FGF receptors (FGFRs), stimulating receptor dimerization and tyrosine autophosphorylation. Receptor activation triggers a cascade of activation events leading to the many functions of FGFs in cell differentiation, cell motility, cell proliferation, and disease (Ornitz and Itoh, 2001).

Viral FGFs

Baculoviruses are the only viruses that encode FGFs. The viral fgf, vfgf, is conserved in most alphabaculoviruses (a genus of nucleopolyhedroviruses or NPVs that infect Lepidoptera) and betabaculoviruses (a genus of granuloviruses, which also infect Lepidoptera), but

**Fig. 2.** Pathogenesis of Trichoplusia ni larvae orally infected with AcMNPV carrying lacZ. Schematic illustrating virus infection followed by lacZ expression as described by Engelhard et al. (1994). (1) At 4 h post infection (h.p.i.), 11 to 12% of insects have midgut epithelial cell infections. (2) At 12 h p.i., 80% of insects show multifoci of infection in midgut epithelial cells. (3) At 12 to 24 h p.i., tracheoblasts (t; smallest cell) and tracheal epithelial cells (T; blue cells with black nuclei; chitinous tube is yellow) associated with the midgut are infected. (4) At 36 to 48 h p.i., hemocytes (H) and tracheoblasts are infected and midgut epithelial cell infection is cleared. There is widespread infection in 75% of larvae and 50% of fat body (FB) infections originate at tracheoblasts that contact the tissue. (5) At 48–70 h p.i., infection is widespread in tissues. For simplicity, only part of one tracheal element is shown. See legend to Fig. 1 for definition of structures.
vfgf is lacking in gammabaculoviruses (viruses that infect Hymenoptera) and deltabaculoviruses (viruses that infect Diptera) (Jehee et al., 2006). Interestingly, the lack of vfgf homologs in the characterized gammabaculoviruses and deltabaculoviruses correlates with these viruses being midgut-restricted (Becnel et al., 2001; Federici, 1997; Moser et al., 2001). Furthermore, the sequences of all baculoviruses that infect Lepidoptera larvae, except for Maruca vitrata MNPV (Chen et al., 2008), carry vfgf and cause systemic infection. The infection pattern of *M. vitrata* MNPV has not been reported. The betabaculovirus, *Harrisina brillians* granulovirus, is specific for midgut epithelia, but it is not known whether this virus carries vfgf. There are protein sequence differences between alphabaculoviruses and betabaculovirus vFGFs, and it has been suggested that vFGFs may have independently evolved in the insect hosts (Katsuma et al., 2008b).

In some granuloviruses, several copies of vfgf are carried in the genome. The function of several copies is not clear; however, granuloviruses have varying infection patterns, from midgut-restricted to systemic infections (Federici, 1997). It is possible that different granulovirus vFGFs have tissue-specific roles or expression patterns, providing more cell-specific and temporal functions. This would be reminiscent of mammalian FGF family members having different expression and developmental (timing) patterns (Ornitz and Itoh, 2001). Therefore, there is an excellent correlation among baculoviruses between the presence of vfgf and the ability to cause systemic infections, suggesting that vFGF facilitates systemic infections (Detvisitsakun et al., 2005). Even if the correlation between vfgf and systemic infection is not universal, since vfgf accelerates, but is not essential for, midgut escape (see below), it may only be conserved in viruses which need to exit the midgut rapidly.

### vfgf gene expression

The *AcMNPV* and *BmNPV* *fgf* homologs (herein, *Acfgf* and *Bmfgf*, respectively) are the best characterized vfgfs. vfgf is an early gene; *vfgf* transcripts in *AcMNPV*- or *BmNPV*-infected cells are observed as early as 3 or 6 h p.i., respectively, and expression is independent of prior protein synthesis and viral DNA replication (Detvisitsakun et al., 2005; Katsuma et al., 2004). Accumulation of *Acfgf* is not observed until 24 h p.i. in TN-368 cells (Lehiy et al., 2009), but this is presumably due to the low levels of synthesis characteristic of signaling proteins (Detvisitsakun et al., 2005).

#### FGFRs

There are four mammalian FGFRs, which are activated upon FGF-heparan sulfate binding. Activation leads to receptor dimerization and tyrosine kinase activity, setting up a signaling cascade (Ornitz, 2000). The host FGFRs of *B. mori* (*BmFGFR*) and *Spodoptera frugiperda* have been cloned and shown to be responsive to vFGF stimulation. The BmFGFR was abundantly expressed in trachea and midgut and less abundantly in hemocytes (Katsuma et al., 2006b).

#### Cell motility

vFGFs stimulate cell motility in vitro (Detvisitsakun et al., 2005; Katsuma et al., 2006b; Li et al., 2008). *AcFGF* induces chemotaxis of cells from two insect cell lines (SF-21 and TN-368) and hemocytes obtained from *T. ni* larvae (Detvisitsakun et al., 2005), while BmFGF induced migration of *Sf-9* insect cells and motility was dependent on the BmFGFR (Katsuma et al., 2006b). The vFGF of *Helicoverpa armigera* NPV (*HearNPV*) was able to stimulate motility of *H-AM1* cells, cells permissive for *HearNPV*, but not of *Spodoptera* (*SF-9* or *Se-URC*),- or *mammalian-293* or HepG2)-derived cells, implying that there is specificity between vFGFs and host receptors (Li et al., 2008). This specificity may provide an additional layer of host range determination in baculoviruses.

### vFGF secretion

vfgfs contain predicted N-terminal hydrophobic signal sequences, and they are secreted products (Detvisitsakun et al., 2005; Katsuma et al., 2004; Lehiy et al., 2009; Li et al., 2008). Prediction of N-glycosylation sites differs; for example, *Bmfgf* contains two predicted N-glycosylation sites, *Acfgf* contains none, and the *Spodoptera litura* NPV has four (Detvisitsakun et al., 2005; Katsuma et al., 2006a, 2004). N-glycosylation of AcFGF and BmFGF improves its secretion (Katsuma et al., 2006a). Since vFGF is a chemotactic signaling molecule, it would be interesting to know if there is a relationship between secretion and enhanced chemotaxis, which may have effects on the efficiency of virus replication or dissemination. It has been suggested that these N-glycosylation differences in vFGFs may have evolved into different vFGF functions (Katsuma et al., 2006a).

#### Binding heparan sulfate proteoglycans

vFGFs have biochemical and structural properties similar to those of cellular FGFs. As with cellular FGFs, vFGFs bind heparin in vitro (Detvisitsakun et al., 2005). Heparan sulfate proteoglycans are present on the cell surface and their interaction with FGFs tethers them to the surface of the cell membrane. Antibodies detected either vFGF or epitope-tagged vFGF anchored to the surface of the cell and the budded virus envelope (Lehiy et al., 2009; Li et al., 2008). It was hypothesized that when the budded virus acquires its envelope from the vFGF-dotted cell membranes, it incorporates vFGF on its envelope (Lehiy et al., 2009). It is not clear whether vFGF has a function on the budded virus surface or if this association is simply a consequence of its secretion to the cell surface. Nevertheless, vFGF on the budded virus has activity; vFGF-carrying budded virions are able to stimulate cell motility better than virions without vFGF (Lehiy et al., 2009; Li et al., 2008). One can envision advantageous scenarios for this, including the ability of vFGF-laden viruses or vFGF cleaved from virions to attract hemocytes or tracheal cells, or a function at late times during budded virus production when the synthesis of vFGF may have declined.

#### Role of vfgf in baculovirus replication in vitro

vfgfs carried by *AcMNPV* and *BmNPV* have been deleted from their respective virus genome and the phenotypes of the mutants have been examined in cell culture. No obvious differences were observed in the overall protein or DNA synthesis profiles of cells infected with an *AcMNPV* recombinant lacking vfgf compared to one carrying vfgf (Detvisitsakun et al., 2006). In addition, budded virus production in two cell lines was not affected by deletion of *Acfgf*. To rule out subtle differences, a growth competition experiment between two viruses either lacking or expressing *Acfgf* was conducted by coinfesting with the two viruses and asking if there was a selective advantage to encoding AcFGF. There was no indication that carrying *Acfgf* was advantageous to virus replication over several passages (Detvisitsakun et al., 2006).

In contrast, deletion of *Bmfgf* had some effects on virus replication in vitro. Budded virus production was slightly reduced in the mutant compared to viruses carrying *Bmfgf* (Katsuma et al., 2006c). Nevertheless, budded virus titers were similar after the last sampling, indicating that there may only be a delay in virus production. However, virus entry kinetics were the same for viruses with or without *Bmfgf* (Katsuma et al., 2006c). Upon closer examination, the accumulation of the very late structural protein of occlusion bodies, polyhedrin, and RNA levels of early and late genes was reduced in the *Bmfgf* mutant virus. As in the growth curve experiment, expression of early and late genes in the *Bmfgf* deletion virus caught up with that of the *Bmfgf* carrying viruses at late times. In addition, viral DNA synthesis was also reduced in the mutant virus, although the onset of viral DNA synthesis was not delayed (Katsuma et al., 2006c). Overall,
it appears that there may only be a delay rather than a defect in the in vitro replication of BmNPV lacking vfgf, which was not apparent in the AcMNPV vfgf mutant. Furthermore, it is possible that higher expression and/or secretion levels of the BmFGF compared to AcFGF assist in the manifestation of a phenotype. Overexpression of vFGF in vitro and in vivo magnifies the phenotype (Detvisitsakun et al., 2007).

The different phenotypes of two baculoviruses lacking vfgf evident in cell culture indicate that the dependency for vfgf may be different depending on the virus and/or cell culture line. However, it is clear that vfgf is not an essential gene; deletion of vfgf still allows virus replication. It appears that vfgf has been conserved in baculoviruses to enhance infection of certain cell types or host species. To discern if subtle effects of vfgf could be better appreciated in the insect host, AcMNPV and BmNPV encoding or lacking vFGF have been compared in vivo.

Role of vfgf during baculovirus replication in vivo

Deletion of AcFGF or BmFGF had no effects on the dosages of virus required to establish infection, whether the virus was delivered by injection of budded virus intrahemocoelically or by feeding of occluded virus (Detvisitsakun et al., 2007; Katsuma et al., 2006c). There were slight differences, yet statistically insignificant, infectivities in S. frugiperda and T. ni larvae infected orally with occlusion bodies of AcMNPV carrying a deletion of vfgf, suggesting more dependence for AcFGF in less susceptible species (Detvisitsakun et al., 2007). However, vfgf did have a significant effect on the time required to kill insects. Virus mutants lacking BmFGF or AcFGF killed their hosts more slowly when the viruses were delivered by feeding (Detvisitsakun et al., 2007; Katsuma et al., 2006c). When the viruses were delivered intrahemocoelically, lethal time differences were only observed in the absence of the BmFGF (Katsuma et al., 2006c), mirroring the cell culture phenotype. Time–mortality response differences indicate that vFGF is necessary for virus dissemination and this effect is observed predominantly when the virus enters via the midgut.

vFGF accelerates baculovirus dissemination

The presence of vFGF reduces the time it takes the virus to kill its host. One scenario is that the presence of vFGF allows the virus to disperse within an insect more rapidly, resulting in more rapid host death. If so, one would expect an accelerated infection in the presence of vFGF. Following oral infection of larvae with occluded virus expressing the enhanced green fluorescent protein (eGFP) and AcFGF, eGFP expression was observed in tracheal cells as early as 12 h p.i. and increased through 96 h p.i., when insects were dying from virus infection. In contrast, tracheal cells in larvae infected with the Acfgf deletion virus did not become eGFP-positive until 48 h p.i.; however, at 96 h p.i., the number of eGFP-positive tracheal cells was similar to those in larvae infected with the virus carrying Acfgf. Similar delayed patterns were observed in the infection of fat body cells and hemocytes (Means and Passarelli, 2010). A delay in virus spread is consistent with AcFGF playing a role in accelerating host mortality after oral infection (Detvisitsakun et al., 2007).

A lethal time defect was not observed when the midgut was bypassed by injecting budded virus into the hemocoel (Detvisitsakun et al., 2007). Similarly, when the timing and infection of tissues was analyzed following intrahemocoelic injections, no obvious differences were observed in the timing of infection of tracheal epithelial cells, fat body cells, or hemocytes between viruses carrying or lacking Acfgf (Means and Passarelli, 2010). A statistically insignificant but slight and reproducible increase in the number of tracheal epithelial cell infection foci, but not in the timing of their appearance, was observed in tissues infected with a virus carrying vfgf. It is possible that vfgf has a function optimizing virus spread in the hemocoel, but this may only be more apparent in other, less susceptible hosts. These results support intrahemocoelic lethal time experiments reported previously (Detvisitsakun et al., 2007), and stress that vFGF has a role at the primary or secondary site of infection but is not required for virus spread after the midgut barrier has been breached.

Unlike experiments with AcMNPV, Bmfgf helped establish efficient hemocyte and tracheal cell infection following injection of B. mori larvae with BmNPV budded virions. However, no differences were observed in the timing of fat body infection between BmNPV- and the Bmfgf-deficient viruses (Katsuma et al., 2008a). In orally infected insects, only hemocytes showed reduced infection in the absence of BmFGF. It was concluded that the chemotactic activity of BmFGF stimulated hemocyte motility, resulting in more efficient infection. The differences in the requirement for AcFGF or BmFGF, depending on the route of infection, suggest that vFGF may act on different cellular targets to optimize systemic infections.

Cell death, matrix metalloproteases, and basal lamina remodeling

FGF is crucial during mammalian organogenesis, including branching morphogenesis of neurites, kidney, mammary gland, lungs, and blood vessels, and the patterning of embryos (Klint and Claesson-Welsh, 1999). During cell motility and organogenesis, basal laminae are degraded or remodeled to accommodate cell growth or fusions of cell layers. After these morphological changes are complete, cells secrete new basal laminae, laying a supportive outline for cell layers. It is well known that matrix metalloproteases (MMPs), zinc-dependent proteases found in the extracellular matrix of cells, are involved in the turnover of basal laminae.

In some studies, caspas, the cysteine proteases that execute cell death, have been associated with processes that involve basal lamina degradation. For example, cell death has been reported to be necessary for shelf fusion during palate formation. In this system, inhibition of MMPs does not affect cell death but blocks basal lamina degradation. More importantly, the study found that cell death activation alone led to basal lamina degradation, and it concluded that the activation of basal lamina degradation led to cell death-mediated degeneration of the seam formed by the two cell shelves in palatogenesis (Cuervo and Covarrubias, 2003). Although the exact pathway mediating these enzymatic events was not defined, it is clear that in this craniofacial developmental process, MMPs, caspas, and basal lamina remodeling are all interrelated.

Vascular smooth muscle cells secrete elastin during blood vessel formation, a protein that confers elasticity to tissues, into the extracellular matrix. When these smooth muscle cells are placed on floating collagen gels, they undergo apoptosis and have elastolytic activity (Cowen et al., 2005). Higher elastolytic activity is inhibited by caspase inhibitors but not by MMP inhibitors. Thus, caspas bound to the cell surface of apoptotic cells lead to degradation of matrix proteins (Cowen et al., 2005).

A relationship between MMPs and FGFs has also been established in a number of systems, including myoblast migration, angiogenesis, and cartilage (Allen et al., 2003; Im, 2006; Kowalewski et al., 2009; Partridge et al., 2000). For example, during varicose vein pathogenesis, the extracellular matrix of the vein wall is remodeled, including a reduction in elastin and changes in other proteins. Elevated levels of FGF in the walls of varicose veins affected the expression of extracellular matrix enzymes (Kowalewski et al., 2009).

Remodeling of tracheal basal laminae

Establishment of systemic infection by AcMNPV requires a coordinated series of activation steps that are reminiscent of findings during palatogenesis and aorta myogenesis described above; namely, cross talking between signaling pathways, involving FGFs, MMPs and caspas, and culminating in basal lamina remodeling. One can hypothesize that baculoviruses captured the cellular ffgf and use it to
stimulate similar cellular pathways that ultimately lead to the productive invasion of larval tissues. If this is the case, since the basic factors involved in cellular developmental processes have been conserved in invertebrates, studies on baculovirus pathogenesis can help define protein functions in poorly understood human developmental programs and pathologies. The biochemical pathway downstream of and dependant on AcFGF signaling that allows for basal lamina remodeling and efficient midgut escape was recently described (Means and Passarelli, 2010).

First, since baculoviruses may escape the midgut by crossing the midgut basal lamina, it was determined whether the basal laminae lining the midgut showed any ultrastructural changes in orally infected T. ni larvae, and if AcFGF affected any of the observed changes. The integrity of midgut cell basal laminae infected with AcMNPV encoding vFGF did not appear to have defects in the distribution or overall amounts of laminin, a major protein component of basal laminae (Means and Passarelli, 2010). It is possible that subtle fissures or rearrangements in the midgut basal lamina may allow budded virions to access the hemocoel, but these were not obvious by laser confocal microscopy.

Second, baculoviruses may exit the midgut using tracheal elements; thus, basal lamina surrounding midgut-associated tracheae in orally infected T. ni with viruses expressing AcFGF was examined by transmission electron microscopy. In the presence of AcFGF, the basal lamina of tracheal cells associated with the midgut was disorganized; instead of a thin uniform sheath surrounding the cells, it appeared fragmented (Means and Passarelli, 2010) and reminiscent of that observed when larvae were infected with viruses expressing basal laminae degrading enzymes (Tang et al., 2007). Analysis with a virus lacking Acfgf showed some rearrangement of tracheal cell basal lamina but not as drastically as with the virus carrying Acfgf. This may explain why vfgf is not required for secondary infections but in its absence it takes longer to infect beyond the primary site. In addition, a reduction in laminin was also observed following immunostaining (Fig. 3). Finally, immunoblots of laminin or collagen type IV using lysates from infected midguts and associated trachea indicated that these proteins were being proteolytically cleaved (Means and Passarelli, 2010). Cleavage was drastically reduced in T. ni infected with an Acfgf mutant, suggesting that laminin cleavage and basal lamina degradation was stimulated by AcFGF.

It has been reported that tracheal elements reach into the midgut cell basal lamina of insects (Romoser et al., 2005). It is not clear if the terminal tracheoblast actually protrudes past the midgut cell basal lamina where it would secrete its own basal lamina and come in closer contact with midgut epithelial cell membranes. Cells are known to be able to break through basement membranes or apposed basal laminae (Sherwood et al., 2005). Alternatively, the tracheoblast may not cross the midgut basal lamina and instead may terminate within the basal lamina of the midgut epithelium, oxygenating the midgut epithelium by diffusion. Tracheoblasts protected by their basal lamina or buried within the midgut cell basal lamina may be targets for vFGF-mediated cell motility. Upon stimulation of cell motility, the tracheoblast would shed its basal lamina and become more accessible to the virus as it extends toward the signal. In this scenario, infection would need

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**Fig. 3.** Delamination of trachea infected with AcMNPV. T. ni larvae were mock-infected or orally infected with a virus carrying vfgf (AcBAC) or lacking vfgf (AcBAC-vfgfKO). Midguts and associated trachea were dissected at 6 h p.i., incubated with anti-laminin antibody, and visualized by confocal microscopy. Immunoreactivity is indicated by green staining.
to take place prior to the secretion of new basal lamina post-cell motility.

Further investigation revealed that at least one caspase was directly responsible for cleaving both laminin and collagen IV in tracheal cell basal lamina (Fig. 4; Means and Passarelli, 2010). Caspases are cysteine proteases that are best known for their roles in apoptosis or cell death. This is consistent with the implication of cell death events during organogenesis and disease mentioned above. However, caspases also play roles in other cellular processes and cell death would not necessarily occur upon caspase activation. The caspase(s) involved appear to be effector caspases or the final protease executioners based on their substrate preference. Inclusion of caspase inhibitors during virus feeding blocks both basal lamina degradation and caspase activation in vivo and in vitro (Means and Passarelli, 2010). The purified *Drosophila* effector caspase Drice was able to cleave mammalian laminin in vitro, and this activity was specifically inhibited by the effector caspase inhibitor zVAD-fmk but not by two initiator caspase inhibitors (Means and Passarelli, 2010). This implies that the common caspase activation pathway, where an initiator caspase activates an effector caspase, is being circumvented during this process.

Caspases are normally thought of as intracellular enzymes in charge of dismantling the cell. Given that this process is occurring extracellularly, the effector caspase may be secreted and then activated by other proteases and this extracellular compartmentalization could result in cleavage of different substrates, e.g., basal lamina protein components. The peptide sequences of laminin and collagen type IV contain predicted caspase cleavage sites, supporting the possibility of their degradation during virus infection. In addition, effector caspase activity was detected in the hemolymph of infected insects (Means and Passarelli, 2010). It is not clear yet whether the caspases are shuttled extracellularly or if apoptotic cells release them, or whether the caspase(s) responsible for cleavage of basal laminae are not normally involved in apoptosis. Nevertheless, the extent of apoptosis in caspase activated tissues did not correlate with infection (Means and Passarelli, 2010).

The presence of caspase activity in the extracellular matrix prompts the question of how effector caspases are activated extracellularly. Candidate proteins on the cell surface that have a role in basal lamina turnover are MMPs, which have been associated with cell death and/or basal lamina remodeling in other systems (Cuervo and Covarrubias, 2003; Kim et al., 2007; You et al., 2003). In vitro, purified human MMP-9, a basal lamina remodeling protein, was able to activate human pro-caspase-3. Caspase activation was enhanced in infected midgut lysates in the presence of MMP activity and vFGF expression, and blocking MMP activity also blocked caspase activation. Mock-infected midgut lysates could trigger caspase activity only if purified MMP-9 was added exogenously to an in vitro reaction. Together, this suggests that MMPs were activated during virus infection and active MMPs stimulated the activation of an effector caspase. Also, caspase activation and vFGF expression coincided with tyrosine phosphorylation (Fig. 5), indicative of vFGF receptor engagement (Means and Passarelli, 2010). Finally, including either MMP or caspase inhibitors in the virus inoculums blocked both tracheal cell basal lamina remodeling and virus escape from the midgut, tying together the roles of vFGF, MMPs and caspases in basal lamina remodeling and virus dissemination (Means and Passarelli, 2010). Although these enzymatic events leading to basal lamina remodeling have been identified, it remains possible that there are additional intermediary factors in this pathway that have not yet been identified.

**Concluding remarks**

Insect pathogens are usually ingested orally and thus establish primary infection in the midgut epithelium. In order to escape from the midgut, viruses need to penetrate the host structural barriers (e.g., basal laminae) that protect cells from further infections. In addition, there is a time pressure to exit infected midgut epithelial cells before these are shed into the midgut lumen. Baculoviruses have evolved to overcome these two obstacles. An FGF encoded by baculoviruses improves the timely infection of secondary sites, tracheoblasts, by causing tracheoblast delamination and making them vulnerable to infection. How the virus crosses the midgut in the absence of vFGF is not clear, but the process appears to take longer. Depending on the virus and host, this time delay could be the difference between survival of the host or replication success of the virus. The midgut-restricted and vfgf-deficient gammabaculoviruses and deltabaculoviruses are thought to be more ancient viruses than the midgut-unrestricted and vfgf-expressing alphabaculoviruses and betabaculoviruses. It is possible that acquisition of *vfgf* in part, allowed for host range expansion by the alphabaculoviruses and betabaculoviruses. Conservation of *vfgf* among all but one lepidopteran-infesting baculovirus implies that the role of vFGF in virus pathogenesis is important for virus replication. Although vFGF is not required for virus dissemination, activation of both MMPs and effector caspases is required, even in the absence of vFGF. Thus, it is possible that virus infection, even in the absence of vFGF, stimulates this pathway via a different factor or via the host FGF. Alternatively, in the absence of vFGF, it is possible that normal turnover of basal lamina gives viruses a window of opportunity to infect secondary targets.

![Fig. 4. Caspase activation in AcMNPV-infected tracheal cells. *T. ni* larvae were orally infected with a virus carrying (AcBAC) or lacking (AcBAC-vfgfKO) *vfgf*. Midguts and associated trachea were dissected at 12 h p.i., incubated with an antibody against the active *Drosophila* effector caspase Drice (green), and visualized by confocal microscopy.](image)
Since tracheal basal laminae are partially degraded and show slightly elevated levels of MMP and effector caspase activity even in the absence of vFGF in baculovirus-infected insects, it is plausible that insect-vectored viruses also utilize this pathway to escape midgut barriers. In many cases, arboviruses have been observed infecting tracheal cells. Since factors involved in this signaling pathway are conserved among insects across insect families, arboviruses may be using the same mechanism to escape midgut barriers and reach the insect salivary glands.

It has been reported that the insect FGF, branchless, is upregulated by hypoxia (Sutherland et al., 1996). The promoter element of baculovirus \( \text{fgf} \) contains putative hypoxia response elements, although testing responsiveness to hypoxia using a reporter gene fused to the 5′ \( \text{Acfgf} \) elements did not result in obvious upregulation at the protein or mRNA levels after stimulation under several hypoxic conditions (Berretta and Passarelli, unpublished results).

So far, tracheal cell remodeling has only been explored in \( T. \ ni \) using AcMNPV. Although apoptosis was not observed in this system, the relationship between activation of effector caspases via vFGF and stimulation of apoptosis needs to be tested in a host that more readily undergoes apoptosis (e.g., \( S. \ frugiperda \)). It is possible that alternative factors may be required in a different host. For example, granuloviruses encode MMPs that stimulate melanization of the infected host (Ko et al., 2000); these enzymes may also be involved in basal lamina remodeling.

In summary, many baculoviruses have devised a sophisticated mechanism to escape midgut barriers by capturing a host gene, \( \text{fgf} \), and then pirating the use of a host pathway to establish efficient systemic infections. Clearly, \( \text{vfgf} \) only accelerates the process and is not required. This opens the possibility that arbovirus infection also stimulates basal lamina remodeling, even though arboviruses do not encode \( \text{fgf} \) homologs, perhaps by stimulating the cellular \( \text{fgf} \) signaling pathway or intercepting the pathway at another point. Given that the genes encoding the pathway enzymes are conserved in mammals and insects, it is feasible that other insect pathogens and insect-vectored pathogens use the same pathway to cause disseminated infection.

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