Impact of a basement membrane-degrading protease on dissemination and secondary infection of Autographa californica multiple nucleopolyhedrovirus in Heliothis virescens (Fabricus)

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ScathL is a cathepsin L-like cysteine protease from the flesh fly, Sarcophaga peregrina, that digests components of the basement membrane (BM) during insect metamorphosis. A recombinant baculovirus that expresses ScathL (AcMLF9.ScathL) kills larvae of the tobacco budworm, Heliothis virescens, significantly faster than the wild-type virus and triggers melanization and tissue fragmentation in infected larvae shortly before death. As BMs are a potential barrier to the spread of baculovirus secondary infection to other tissues in the host, this study tested the hypothesis that the rapid death of insects infected with AcMLF9.ScathL was caused by accelerated secondary infection resulting from the degradation of host BMs by ScathL. Viruses expressing catalytically active or inactive ScathL were used to examine the effects of ScathL activity on budded virus release into the haemocoel during infection, the production of polyhedra in infected larvae and the rate of infection of the gut, trachea, haemocytes, fat body and Malpighian tubules. It was concluded that the enhanced insecticidal efficacy of the recombinant baculovirus that expresses ScathL does not result from altered tissue tropism or accelerated systemic infection. Implications for the role of the BM as a barrier to baculovirus dissemination within the host insect are discussed.

INTRODUCTION

Baculoviruses are arthropod-specific pathogens that infect a number of agriculturally important insect pests within the Lepidoptera (butterflies and moths). Baculoviruses have double-stranded, circular DNA genomes contained within enveloped, rod-shaped virions that have two distinct phenotypes, occlusion-derived virus (ODV) and budded virus (BV). Following ingestion by the host insect, ODVs are released from the polyhedra (occlusion bodies) in the alkaline environment of the midgut and initiate infection of the midgut epithelial cells (Bonning, 2005). BVs are produced and released from the infected cells and establish secondary infection of other tissues within the host. At the very late phase of infection, polyhedra are generated in massive quantities. Following death of the host, the cadaver lyses, releasing polyhedra into the environment. Because of their pathogenicity and environmental safety, baculoviruses have been studied as potential biological control agents and used successfully for the management of several insect pests (Moscardi, 1999). Baculovirus insecticides have not been adopted widely, however, in part because of their relatively slow action. Insect pests infected with wild-type baculoviruses may continue to feed and cause crop damage for days and thus often fail to compete with conventional chemical insecticides. Recombinant baculoviruses expressing genes that encode a variety of insect-specific toxins or development-disrupting enzymes and hormones kill insects more rapidly and reduce feeding damage compared with larvae infected with wild-type baculoviruses (Kamita et al., 2005).

Host basement membranes (BMs) have been identified as a potential target for improving baculovirus insecticidal efficacy (Keddie et al., 1989). The BM is an extracellular protein sheet surrounding all tissues of animals, composed primarily of laminin, collagen IV and proteoglycans. The BM functions in cell adhesion, cell signalling and maintenance of tissue structure (Yurchenco & O’Rear, 1993). There is high homology between the BM of invertebrates and vertebrates in composition, structure and function (Fessler & Fessler, 1989). As described for other viruses (Romoser et al., 2005), BMs appear to act as a barrier to dissemination of baculoviruses within infected insects. BVs are too large to diffuse freely through the pores in the BM that surround tissues of the host insect (Reddy &
Locke, 1990). Co-injection of BVs and clostridial collagenase, a protease known to degrade BM, resulted in enhanced infection of host tissues (Smith-Johannsen et al., 1986). Ultrastructural study of infection by the baculovirus *Cydia pomonella* granulovirus revealed a substantial accumulation of BVs in the extracellular spaces between BMs and the plasma membranes of midgut and fat body cells (Hess & Falcon, 1987). Collectively, these observations suggest that insect BM inhibits the movement of BVs.

To see whether disruption of the BM could augment dissemination of BV within an infected host, a recombinant baculovirus, AcMLF9.ScathL, was constructed to express a BM-degrading cathepsin L (EC3.4.22.15) from the flesh fly, *Sarcophaga peregrina* Robineau-Desvoidy (Harrison & Bonning, 2001). In the flesh fly, this cathepsin L (ScathL) degrades two components of the BM (Homma & Natori, 1996). The recombinant virus AcMLF9.ScathL killed *Heliothis virescens* larvae approximately 30 % faster than a virus expressing a scorpion venom-derived neurotoxin and over 50 % faster than the wild-type virus. Larvae infected with AcMLF9.ScathL consumed fivefold less lettuce than wild-type virus-infected larvae. Interestingly, AcMLF9.ScathL caused fragmentation of internal tissues and melanization after death. Larvae infected with a virus expressing a scorpion venom-derived neurotoxin killed *Heliothis virescens* larvae prior to death. Wild-type baculovirus-infected larvae typically melanize after death.

We have tested a number of hypotheses to understand the mechanisms underlying the significantly enhanced insecticidal efficacy of the recombinant baculovirus AcMLF9.ScathL. Here, we describe experiments to test the hypothesis that ScathL damages the BM barrier to virus dissemination, allowing more rapid spread or altered tissue tropism of the virus.

**METHODS**

**Insect cells, insects and viruses.** *Spodoptera frugiperda* (Sf)21 cells (Vaughn et al., 1977) were maintained in TC-100 medium (Sigma) supplemented with 10 % fetal bovine serum (FBS; Intergen) and antibiotics (1 U penicillin ml⁻¹, 1 μg streptomycin ml⁻¹; Sigma). *Trichoplusia ni* BTI-TN-5B1-4 (High Five) cells (Wickham et al., 1992) were maintained in Ex-Cell 405 medium (JRH Biosciences) supplemented with antibiotics only. Both cell lines were maintained at 28 °C. Larvae of *H. virescens* were reared individually from eggs (BioServ, Frenchtown, NJ, USA) on an artificial diet in 1 oz plastic cups (BioServ) at 28 °C with a 14 : 10 h light : dark cycle.

The wild-type C6 strain of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and the recombinant viruses AcMLF9.ScathL (Harrison & Bonning, 2001) and AcMLF9.ScathL.C146A were used for this study. AcMLF9.ScathL expresses a functional fly cathepsin L protease (ScathL), whilst AcMLF9.ScathL.C146A, constructed in the present study, expresses a catalytic site mutant of ScathL. The expression of both proteins was directed by the AcMNPV p6.9 promoter (Harrison & Bonning, 2000; Hill-Perkins & Possee, 1990). Additional recombinant viruses were constructed to express ScathL or ScathL.C146A along with either β-galactosidase or chloramphenicol acetyltransferase (CAT) (see below). Budded virus stocks were produced in Sf21 cells and polyhedra were generated and purified as described previously (Harrison & Bonning, 2001), resuspended in glycerin and water (3 : 2, v/v), quantified using a haemocytometer and stored at 4 °C.

**Construction of recombinant viruses.** To construct the control virus AcMLF9.ScathL.C146A, site-directed mutagenesis of the ScathL sequence was cloned out to substitute the catalytic cysteine residue at position 146 with alanine. The mutated ScathL gene (designated ScathL.C146A) was cloned back into the *Bgl*II site of the pAcMLF9 transfer vector (Harrison & Bonning, 2000). This transfer vector allows production of polyhedrin-positive viruses with protein expression driven by the AcMNPV p6.9 promoter (Hill-Perkins & Possee, 1990).

To construct recombinant baculoviruses carrying reporter genes, the *hsps/lacZ expression cassette was amplified from a recombinant *Rachiplusia ou* MNV (RofEP.hsps/lacZ) in which *hsps/lacZ* had been inserted into the polyhedral envelope protein gene (Jin, 2002). The primers used were 5′-CTCCCTATTGAGACCTC-3′, which hybridized upstream of an *Xba*I site next to the *hsps/lacZ* cassette, and 3′-CATCTCAGAGAGTCTGACAGC-AGTATAAGATACATTG-3′, which hybridized to the 3′ end of the *hsps/lacZ* simian virus 40 (SV40) poly(A)⁺ signal and contained an *Xba*I site (underlined).

A CAT expression cassette was PCR amplified from the recombinant virus *sSynXVI* ml+CAT, which expresses CAT from a very strong tandem baculovirus late/very late promoter array (Wang et al., 1991). The primers used were 5′-GATTCACTAGTAAATTGGCCAG-3′, which hybridizes upstream of an *Xba*I site flanking the CAT cassette, and 3′-CATCTGAGAGAGTCTGACAGC-AGTATAAGATACATTG-3′, which contains an *Xba*I site (underlined). After PCR amplification, the *hsps/lacZ* and CAT PCR products were digested with *Xba*I and ligated into the *Xba*I sites of pAcMLF9.ScathL and pAcMLF9.ScathLC146A between the *polh* gene and p6.9/ScathL or p6.9/ScathLC146A cassette (Fig. 1). Recombinant viruses were obtained through homologous recombination by co-transfection of Sf21 cells with *Bsu36I*-linearized AcRP23.lacZ viral DNA (Kitts et al., 1990) using calcium phosphate precipitation (O’Reilly et al., 1992). Recombinant viruses were isolated by four rounds of plaque assay (Summers & Smith, 1987). Purified recombinant virus clones were checked for correct insertion of the foreign sequence by restriction enzyme analysis, PCR amplification and sequencing of the region where the gene was inserted. Expression of ScathL or ScathLC146A and the reporter genes (lacZ and CAT) by the recombinant viruses were confirmed by Western blotting and activity assays (Harrison & Bonning, 2001).

**Bioassays of recombinant viruses expressing reporter enzymes.** Lethal concentration and survival time bioassays were conducted using the droplet feeding method (Hughes & Wood, 1981). For lethal concentration bioassays, all viruses were assayed against neonate *H. virescens* larvae as described previously with three replicates of 30 larvae per virus concentration (Harrison & Bonning, 2001). Larval mortality was scored after mock-infected larvae had pupated. Median lethal concentration (LC₅₀) values (polyhedra ml⁻¹) were calculated by POLO probit analysis and compared by standard lethal dose ratio comparison (Robertson & Preisler, 1992).

In time–mortality bioassays, the 99 % lethal concentration (LC₉₉) (polyhedra ml⁻¹) for each virus was calculated from the dose–mortality bioassay data and used as an inoculation dose in droplet feeding bioassays of neonate *H. virescens*. Mortality was recorded every 2–8 h until the mock-infected larvae and any larvae that survived the virus treatments had pupated. Median survival times (ST₅₀) values were calculated using the Kaplan–Meier estimator and compared by log-rank test (Kalbfleisch & Prentice, 1980). Three replicates were conducted for each virus with 30 neonates per replicate.
Quantification of BV and polyhedra production. To measure production of progeny BV and polyhedra in larvae, newly moulted fifth-instar larvae within 1 h of ecdisys of the fourth-instar cuticle were inoculated using a microapplicator (Burkard Scientific) for direct delivery of a polyhedral suspension (1 µl) into the midgut, as described previously (Washburn et al., 1995). For BV titre assays, infected larvae at 24, 48 and 72 h post-inoculation (p.i.) were anaesthetized on ice and surface sterilized in 70% ethanol. Haemolymph was collected from an incision in a proleg directly onto a piece of Parafilm on ice. Ten microlitres of haemolymph per larva was transferred into a 1.5 ml sterile tube containing 90 µl TC-100 medium supplemented with 10% FBS, antibiotics (1 U penicillin ml⁻¹, 1 µg streptomycin ml⁻¹; Sigma) and 0.003 % 1-phenyl-2-thiourea (PTU) to prevent melanization. The samples were mixed well and placed on ice. The diluted haemolymph samples were centrifuged at 500 g for 5 min and the cell-free plasma used to determine the BV titre by end-point dilution (Summers & Smith, 1987). Sixteen larvae were tested at each time point for each virus and the experiment was repeated twice. Means of TCID₅₀ values ml⁻¹ in log scale were analysed for statistical significance among the three viruses in a one-way analysis of variance (ANOVA) using SAS (SAS Institute, 1990).

To determine any potential adverse effect of ScathL expression on the virus itself, progeny BV titres from infections of Sf21 cells with AcMLF9.ScathL.hsp70/lacZ, AcMLF9.ScathL.C146A.hsp70/lacZ and wild-type AcMNPV C6 were also compared. Sf21 cells in 35 mm culture dishes (1 × 10⁶ cells per dish) were infected with BV at an m.o.i. of 0.1. Infected cell culture medium was harvested at 48, 96 and 144 h p.i. and titrated by end-point dilution.

For quantification of polyhedra, larvae at 72 h p.i. or cadavers were weighed individually and groups of three larvae or cadavers were selected randomly for isolation of polyhedra, as described previously (Harrison & Bonning, 2001). Six groups of larvae or cadavers were processed for each treatment. Polyhedra were counted using a haemocytometer and counts were analysed by one-way ANOVA.

Visualization of the course of infection by expression of β-galactosidase. Newly moulted fifth-instar larvae of *H. virescens* were inoculated orally with 5.0 × 10⁵ polyhedra per larva of AcMLF9.ScathL.hsp70/lacZ or AcMLF9.ScathL.C146A.hsp70/lacZ. Every 6 h, haemolymph from infected larvae was collected as described above. Ten to fifteen microlitres of haemolymph per larva was transferred into a 1.5 ml sterile tube containing 50 µl TC-100 medium and mixed, and then 10 µl of the mixture was transferred into a well of a 96-well culture plate containing 70 µl TC-100 medium with 10% FBS. After attachment, haemocytes were processed for lacZ expression as described previously (Trudeau et al., 2001). Plates were maintained in the dark overnight and examined for blue coloration under an inverted microscope. The percentage of LacZ-positive haemocytes was determined by first examining 1000–5000 haemocytes for each well of samples harvested at 12, 18 and 24 h p.i. When the percentage of LacZ-positive haemocytes in a well exceeded 1%, 200 haemocytes were selected randomly and the numbers of positive and negative cells were recorded.

After haemolymph collection, larvae were dissected and whole mounts were made (Washburn et al., 2000). Larval tissues were observed under a dissection microscope to assess the presence and distribution of blue coloration indicative of virus infection. Data were...
recorded for the gut, trachea, fat body and Malphigian tubules. The mean percentage of larvae with virus infection of each tissue at each time point was calculated from observations of three replicates, each with eight larvae for each virus. One-way ANOVA was performed to test for statistical significance of infection rate between the two viruses.

Quantification of the extent of virus infection by CAT activity assay.

To quantify the extent of virus infection, CAT activity assays were conducted on tissues from H. virescens larvae infected with AcMLF9.ScathL.CAT or AcMLF9.ScathL.C146A.CAT. Newly moulted fifth-instar larvae were inoculated orally with 5.0 × 10^5 polyhedra per larva. At 6 h p.i., infected larvae were bled and dissected every 6 h for assay of CAT activity in the haemolymph and excised tissues. Haemolymph samples from three larvae were prepared in a 1.5 ml tube containing 100 μl 250 mM Tris/HCl (pH 7.8) on dry ice. Gut or fat body tissues excised from three larvae were pooled in a 1.5 ml tube containing 100 μl 250 mM Tris/HCl (pH 7.8) on dry ice. The samples were stored at −80 °C until further processing. Gut and fat body samples were homogenized for 1 min with a plastic pestle and then refrozen at −80 °C for 10 min. The refrozen samples were thawed in a 37 °C water bath for 1 min, followed by vortexing for 1 min. Haemolymph samples were subjected to three freeze–thaw cycles. All samples were heated at 65 °C for 15 min to inactivate deacetylases. After centrifugation at 10 000 g for 10 min, the supernatants were stored at 4 °C for 10 min and stored at −20 °C until Western blot analysis. The remaining plasma was transferred into clean tubes containing 100 μl 0.1 M sodium acetate buffer (pH 5.0), the haemocyte pellets were resuspended in 50 μl of the same buffer and these samples were stored at −80 °C prior to analysis.

The gut (minus the food bolus) and fat body were excised from the three bled larvae and placed separately into 1.5 ml tubes containing 100 μl 0.1 M sodium acetate buffer on dry ice for ScathL activity assays. Excised tissues were homogenized with a plastic pestle for 1 min. Prior to homogenization, 5 μl 100 mM PMSF was added to each gut sample. Samples were centrifuged at 10 000 g for 5 min at 4 °C and the supernatants were transferred to clean tubes. Western blot analysis was conducted using 15 μg total protein for each sample to examine the expression and molecular size of ScathL in the haemocytes, plasma, fat body and gut tissues. Anti-ScathL antisera were produced in two New Zealand White rabbits against purified yeast-expressed ScathL by the Iowa State University Hybridoma Facility using standard procedures (Harlow & Lane, 1988). For specific activity assays, 5 μl of each sample was incubated with 95 μl 0.1 M sodium acetate buffer containing 5 mg azo dye-impregnated collagen (Azocoll; Sigma-Aldrich) ml⁻¹ and 0.003 % PTU at 37 °C for 3 h. Undigested Azocoll was pelleted by centrifugation at 2000 g for 10 min. Absorbance of the supernatant was measured at 520 nm using a VMax Kinetic Microplate Reader (Molecular Devices). Specific activity data were analysed by one-way ANOVA for different virus treatments within a tissue type.

### RESULTS

Pathogenicity of recombinant viruses expressing reporter enzymes

Dose–mortality bioassays with neonates of H. virescens demonstrated that most of the LC₅₀ values ranged from 94 000 to 235 000 polyhedra ml⁻¹, with no significant differences among the tested viruses (Table 1). This suggested that expression of either catalytically active or inactive ScathL together with either β-galactosidase or CAT did not change the dose–mortality response.

<table>
<thead>
<tr>
<th>Virus</th>
<th>LC₅₀ (95% CL) (×10⁵)⁺</th>
<th>LC₉₀ (95% CL) (×10⁴)⁺</th>
<th>Slope ± SEM (χ²/n)</th>
<th>Heterogeneity</th>
<th>g value†</th>
<th>Potency ratio‡ (95% CL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type C6</td>
<td>1.27 (0.45–2.29)</td>
<td>10.0 (5.52–30.7)</td>
<td>1.428 ± 0.150</td>
<td>3.72</td>
<td>0.192</td>
<td>–</td>
</tr>
<tr>
<td>ScathL</td>
<td>1.95 (0.53–3.46)</td>
<td>10.4 (6.02–31.1)</td>
<td>1.764 ± 0.200</td>
<td>4.59</td>
<td>0.276</td>
<td>0.760 (0.480–1.203)</td>
</tr>
<tr>
<td>ScathLhsp70/lacZ</td>
<td>2.35 (1.45–3.34)</td>
<td>11.1 (7.47–20.5)</td>
<td>1.901 ± 0.174</td>
<td>2.59</td>
<td>0.102</td>
<td>0.637 (0.403–1.002)</td>
</tr>
<tr>
<td>ScathL.CAT</td>
<td>1.07 (0.75–1.41)</td>
<td>6.67 (4.95–9.90)</td>
<td>1.609 ± 0.154</td>
<td>1.08</td>
<td>0.046</td>
<td>1.317 (0.827–2.099)</td>
</tr>
<tr>
<td>ScathL.C146A</td>
<td>1.24 (0.67–1.81)</td>
<td>9.78 (6.44–18.4)</td>
<td>1.426 ± 0.159</td>
<td>1.55</td>
<td>0.090</td>
<td>1.028 (0.640–1.651)</td>
</tr>
<tr>
<td>ScathL.C146A.hsp70/lacZ</td>
<td>0.94 (0.64–1.27)</td>
<td>8.87 (6.49–13.5)</td>
<td>1.316 ± 0.137</td>
<td>0.79</td>
<td>0.042</td>
<td>1.242 (0.783–1.971)</td>
</tr>
<tr>
<td>ScathL.C146A.CAT</td>
<td>1.48 (0.83–2.21)</td>
<td>7.74 (4.77–19.1)</td>
<td>1.780 ± 0.185</td>
<td>2.83</td>
<td>0.151</td>
<td>0.980 (0.610–1.563)</td>
</tr>
</tbody>
</table>

*LC₅₀ and LC₉₀ values (polyhedra ml⁻¹) were obtained by running a POLO probit analysis program and reported with 95% confidence limits (CL). For each treatment, LC₅₀ values with the same letter are not significantly different. Significant difference was based on whether the 95% CL of the potency ratio included the value 1.0 (Robertson & Preisler, 1992).

†If g<0.5, the data fit the probit model. Otherwise, the data do not fit the probit model and the analysis is not valid (Finney, 1971).

‡Potency ratio=LC₅₀ of the wild-type virus divided by the LC₅₀ of the recombinant virus (Robertson & Preisler, 1992).
In time–mortality bioassays with inoculation of neonate larvae at the corresponding LC99 dose, the ST50 values were significantly different among the tested viruses (Table 2). The ST50 for AcMLF9.ScathL of 47 h p.i. was significantly lower than that of 92 h p.i. for the wild-type C6. However, when the catalytic site mutant of the ScathL protease was expressed (AcMLF9.ScathL.C146A), the ST50 increased to 92 h p.i., similar to that of wild-type C6. This demonstrated that the cysteine protease activity of ScathL plays a key role in the rapid death of infected larvae. Addition of sequences encoding either β-galactosidase or CAT resulted in some alteration in the ST50 values of the corresponding viruses with significant differences in some cases following analysis by log-rank comparison (Table 2). Significant differences in ST50 values were seen as expected between viruses expressing active ScathL or inactive ScathL (Table 2). In addition, the ST50 of AcMLF9.ScathL was significantly higher than that of AcMLF9.ScathL.CAT. The ST50 of AcMLF9.ScathL.C146A was also significantly higher than that of AcMLF9.ScathL.C146A.CAT, suggesting that expression of CAT slightly increased the speed of kill by the virus.

Table 2. Time–mortality response of neonates of H. virescens infected with recombinant AcMNPV expressing either ScathL or ScathLC146A protease and the reporter enzyme β-galactosidase or CAT

<table>
<thead>
<tr>
<th>Virus</th>
<th>Inoculating dose (LC99, polyhedra ml⁻¹)</th>
<th>n</th>
<th>ST50 (h p.i.)*</th>
<th>95% CL (h p.i.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type C6</td>
<td>4.7 x 10⁶</td>
<td>78</td>
<td>92d</td>
<td>92–92</td>
</tr>
<tr>
<td>ScathL</td>
<td>4.2 x 10⁶</td>
<td>81</td>
<td>47h</td>
<td>45–49</td>
</tr>
<tr>
<td>ScathL.hsp70/lacZ</td>
<td>4.0 x 10⁶</td>
<td>85</td>
<td>45b</td>
<td>42–47</td>
</tr>
<tr>
<td>ScathL.CAT</td>
<td>3.9 x 10⁶</td>
<td>88</td>
<td>42</td>
<td>42–45</td>
</tr>
<tr>
<td>ScathLC146A</td>
<td>5.3 x 10⁶</td>
<td>79</td>
<td>92d</td>
<td>82–92</td>
</tr>
<tr>
<td>ScathLC146A.hsp70/lacZ</td>
<td>5.5 x 10⁶</td>
<td>83</td>
<td>92d</td>
<td>84–92</td>
</tr>
<tr>
<td>ScathLC146A.CAT</td>
<td>3.0 x 10⁶</td>
<td>87</td>
<td>84</td>
<td>84–92</td>
</tr>
</tbody>
</table>

*ST50 values of insects were determined by the Kaplan–Meier estimator and reported with 95% confident limits (CL). For each treatment, ST50 values with different letters are significantly different at P=0.05 as tested by log-rank comparison.

Fig. 2. BV titres (TCID50 ml⁻¹) in haemolymph plasma of fifth-instar H. virescens larvae that were infected with either wild-type AcMNPV C6 or the recombinant viruses AcMLF9.ScathL or AcMLF9.ScathLC146A (a) and in culture medium of Sf21 cells infected with AcMNPV C6, AcMLF9.ScathLhsp70/lacZ or AcMLF9.ScathLC146A.hsp70/lacZ (b), at the indicated times p.i. The BV titre at 72 h p.i. in melanized and non-melanized larvae inoculated with AcMLF9.ScathL is shown in (c). Means of TCID50 ml⁻¹ at the same time point with the same letter are not significantly different (P=0.05; ANOVA and least significant difference analysis (LSD)). Error bars represent standard errors.
**Effect of expression of ScathL on production of BV and polyhedra**

If disruption of BM by ScathL removes the barrier to movement of BV, the titres of BV and polyhedra would be expected to increase more rapidly than for the control viral treatments. To test this hypothesis, larvae were infected with wild-type and recombinant viruses, and progeny BV and polyhedra production was assessed. BV titres for AcMLF9.ScathL in the haemolymph of infected *H. virescens* larvae were significantly reduced at 48 and 72 h.p.i. compared with titres in larvae infected by either wild-type C6 or AcMLF9.ScathL.C146A (Fig. 2a). The reduced BV titre could result from degradation of BV by ScathL or from some other effect of ScathL on BV assembly and secretion. To evaluate these possibilities, Sf21 cells were infected with wild-type and recombinant viruses and progeny BV titres were measured at different time points. The BV titres of AcMLF9.ScathL within the culture medium of Sf21 cells were not significantly lower than those of the control virus AcMLF9.ScathL.C146A at an m.o.i. of 0.1, but both viruses produced titres that were approximately 1 log lower than that of wild-type virus (*P* = 0.001) (Fig. 2b). At 72 h.p.i., approximately 50% of larvae infected with AcMLF9.ScathL exhibited extensive integumental melanization. To evaluate the impact of this melanization on progeny virus titres, BV from melanized and non-melanized larvae were quantified. Haemolymph from melanized larvae contained a significantly higher BV titre in the haemolymph than non-melanized larvae (Fig. 2c).

The number of polyhedra isolated from infected larvae was compared at 72 h.p.i. and after insect death for AcMLF9.ScathL, AcMLF9.ScathL.C146A and wild-type C6. At 72 h.p.i., the number of polyhedra produced by AcMNPV C6 was significantly higher than that produced by AcMLF9.ScathL. However, there was no significant difference in polyhedra production between AcMLF9.ScathL and AcMLF9.ScathL.C146A. Interestingly, the number of polyhedra present after insect death for AcMLF9.ScathL was significantly reduced relative to both AcMLF9.

**Effect of ScathL on the course of infection as visualized by detection of β-galactosidase**

The time course and tropism of infection of fifth-instar larvae of *H. virescens* for viruses expressing ScathL or ScathL.C146A and β-galactosidase were studied by monitoring expression of β-galactosidase in different tissues (Fig. 4). LacZ was detected in the gut at 6 h.p.i. in approximately 30% of larvae, and at 12 h.p.i. all larvae infected with either AcMLF9.ScathL.hsp70/lacZ or AcMLF9.ScathL.C146A.hsp70/lacZ had blue foci of infection in the gut. Although the tracheae are closely associated with the gut, LacZ activity was not observed in this tissue until 18 h.p.i., when foci were detected on the major branches of the trachea. However, because of the difficulty in distinguishing between blue staining of the gut and blue staining of the fine branches of the trachea, some tracheal signals may have been counted as infection of the gut. At 36 h.p.i., LacZ signals were seen on the trachea of all larvae examined. LacZ staining was first seen on the fat body and Malpighian tubules at 18 h.p.i. in larvae infected with both viruses (Fig. 4b). Staining of these tissues in all larvae was not observed until 48 h.p.i. There were no significant differences in the proportion of larvae expressing LacZ in any tissue at any time point.

We did not observe LacZ staining in haemocytes at 6 h.p.i., but all tested larvae showed staining of haemocytes at 12 h.p.i. for both viruses at a low rate (<0.1%). The infection rate of haemocytes remained at a low level (<1.1%) up to 24 h.p.i. By 30 h.p.i., however, the infection rate had increased to 51% for AcMLF9.ScathL.hsp70/lacZ and 38% for AcMLF9.ScathL.C146A.hsp70/lacZ. By 48 h.p.i., over 90% of haemocytes were infected. No significant difference was observed in the infection rate of haemocytes between the two viruses (Fig. 4c).
Comparison of the extent of infection by CAT assay

Quantification of the extent of viral infection of different tissues was characterized with CAT-expressing viruses. Significant CAT activity was detected after 24 h p.i. in the gut and 30 h p.i. in the fat body and haemolymph of larvae infected with AcMLF9.ScathL.CAT or AcMLF9.ScathL.C146A.CAT compared with the mock-infected larvae (Fig. 5). After 30 h p.i., specific CAT activity increased rapidly in all tissues of larvae infected with either virus. The CAT activity in gut, haemolymph and the fat body of larvae infected with AcMLF9.ScathL.CAT was significantly higher than that infected with AcMLF9.ScathLC146A.CAT at 42 h p.i. (Fig. 5c), but not at other time points. At 48 h p.i., the activity in the fat body of larvae infected with AcMLF9.ScathL.C146A.CAT was higher than that infected with the ScathL-expressing virus (Fig. 5c). Overall, there were no significant differences in CAT activity in any tissue between the two viruses (P=0.2149 for gut, P=0.9425 for haemolymph and P=0.4876 for fat body) across all time points. Melanized larvae infected with AcMLF9.ScathL.CAT exhibited higher CAT activity than non-melanized larvae in all tissues tested (Fig. 5d).

Time course of ScathL expression

Expression of ScathL and the mutant ScathL.C146A in larval tissues was examined at 12, 24 and 48 h p.i. by an activity assay and Western blot analysis. At 12 h p.i., there were no significant differences in cysteine protease activity for different tissues among AcMLF9.ScathL-, AcMLF9.ScathL.C146A-, AcMNPV C6- and mock-infected H. virescens fifth-instar larvae infected with the recombinant virus AcMLF9.ScathL.hsp70/lacZ or AcMLF9.ScathL.C146A.hsp70/lacZ. The percentages of larvae exhibiting LacZ-positive tissues and of LacZ-positive haemocytes are plotted against hours p.i. Error bars represent standard errors.
gut may include such endogenous proteases (Fig. 6c). The processed form (35 kDa) of both ScathL and ScathL.C146A was detected in all tested tissues, whereas the pro-enzyme (45 kDa) was only consistently detected in the plasma. The presence of the pro-enzyme in the plasma results from secretion of the pro-enzyme from cells with the active form (produced either by autocatalytic cleavage or by endogenous proteases) remaining within the cell. More ScathL was detected by Western blotting for all tissues in melanized than non-melanized larvae, and higher ScathL activity was detected by enzyme assay in melanized compared with non-melanized larvae (Fig. 6d).

**DISCUSSION**

In this study, we failed to find evidence for accelerated baculovirus dissemination or altered tissue tropism in *H. virescens* larvae mediated by expression of the ScathL protease.

If ScathL facilitated a more rapid release of BV into the haemocoel from the infected gut and other tissues, a higher titre of BV and larger numbers of polyhedra would be produced in larvae infected with AcMLF9.ScathL at early time points. However, the BV titre of AcMLF9.ScathL in the haemolymph of infected larvae was significantly lower than that of the control virus-infected larvae. Moreover, the overall yield of polyhedra was significantly reduced compared with the control viruses on death of the host insect. These data do not support the hypothesis that AcMLF9.ScathL kills larvae more quickly than the control viruses as a result of more rapid virus dissemination.

It is likely that the significant reduction in polyhedra production by AcMLF9.ScathL resulted in part from the more rapid death of larvae infected with this virus compared with larvae infected with either wild-type or control virus. A similar reduction has been reported previously for fast-killing recombinant AcMNPV clones that express scorpion toxins (Kunimi *et al.*, 1996). Also, the preponderance of data on BV production in larvae and cell culture (Fig. 2) suggests that ScathL expression is accompanied by a moderate reduction in BV titre, although the reason for this reduction is unclear.

The use of β-galactosidase expression for a visual comparison of the course of infection of viruses expressing ScathL or ScathL.C146A did not reveal any significant differences in the timing or pathway of infection by the two...
viruses. In addition, the use of CAT expression to monitor infection did not show a consistent quantitative difference in the extent of infection by ScathL- and ScathL.C146A-expressing viruses.

The results of bioassays with viruses expressing ScathL and an inactivated ScathL mutant demonstrated that the cysteine protease activity of ScathL plays a key role in the accelerated death of AcMLF9.ScathL-infected larvae. ScathL activity was consistently associated with widespread melanization and tissue damage, including ruptured guts and a fragmented fat body. Melanization and tissue damage was not observed in larvae infected with AcMLF9.ScathL.C146A. This association was underscored by a comparison of infected melanized and non-melanized larvae at 48 h p.i. ScathL activity was significantly higher in the tissues of melanized larvae than in those of non-melanized larvae. BV, polyhedra and reporter gene (CAT) activity levels were also higher in melanized larvae, indicating that a higher level of viral gene expression overall had occurred in the melanized larvae compared with the non-melanized larvae. Although larval melanization was associated with ScathL activity, the contribution of melanization to reduced larval survival time remains unclear. The formation of melanin is accompanied by the production of quinones and other cytotoxic reactive species (Carton & Nappi, 1997; Lavine & Strand, 2002). The production of these toxic materials during uncontrolled and widespread melanization may contribute to the pathogenesis of AcMLF9.ScathL infection. We are currently using polydnavirus-derived immunosuppressive genes to separate the effects of melanization and the associated production of toxic free radicals from the potentially lethal impact of BM damage alone.

One possible explanation for why significantly enhanced systemic infection was not observed in AcMLF9.ScathL-infected larvae of *H. virescens* is that the BM may not be a significant barrier to the dissemination of BV within fifth-instar *H. virescens*. In *Trichoplusia ni*, AcMNPV BV

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**Fig. 6.** (a, b) Expression of ScathL and its catalytic-site mutant ScathL.C146A within different tissues of fifth-instar larvae of *H. virescens* infected with AcMLF9.ScathL or AcMLF9.ScathL.C146A at the indicated times p.i. Means with the same letter within a tissue are not significantly different at *P*<0.05 by ANOVA and least significant difference analysis. (c) Western blot analyses of different tissues. Samples from two different groups of insects are shown for melanized and non-melanized larvae infected with AcMLF9.ScathL and for AcMLF9.ScathL.C146A-infected larvae (a C6 haemocyte sample was not available). The 45 and 35 kDa bands represent the pro-enzyme and mature enzyme. (d) Protease activity in tissues harvested from melanized and non-melanized larvae infected with AcMLF9.ScathL at 48 h p.i. Error bars represent standard errors.
appeared to pass through the midgut epithelium and BM directly and establish infection of haemocytes by 4 h p.i. (Granados & Lawler, 1981). However, Keddie et al. (1989) did not find evidence for direct passage of virus into the haemocoel of H. virescens. Engelhard et al. (1994) proposed that baculoviruses bypass the BM by utilizing the tracheae (which penetrate the BM) as a conduit for moving to other tissues (Engelhard et al., 1994). The mechanism of penetration of the BM remains to be determined and there is debate over whether one route predominates over the other (Federici, 1997; Volkman, 1997). Our study showed that a widespread infection of haemocytes in H. virescens larvae did not occur until 30 h p.i., suggesting that the BM surrounding the midgut sheath did serve as a barrier to the passage of virus into the haemocoel.

It is also possible that ScathL was not expressed at a time and at a level that would have perforated the BM to an extent necessary to see an effect on systemic infection. The AcMLF9.ScathL virus utilizes the late p6.9 promoter to drive ScathL expression. A virus that expresses ScathL from the early ie-1 promoter does not kill H. virescens larvae faster than wild-type AcMNPV (Harrison & Bonning, 2001). This virus (AcIE1TV3.ScathL) produced far less ScathL activity than AcMLF9.ScathL, indicating that the level of expression may be more important than the timing of expression for survival time reduction.

In summary, expression of a BM-degrading protease did not hasten secondary infection of H. virescens larvae under the conditions used in this study. The significantly enhanced insecticidal efficacy of AcMLF9.ScathL did not appear to be due to accelerated systemic infection by the virus. We confirmed that the cysteine protease activity of ScathL was necessary for the pathology and reduced host survival time observed for AcMLF9.ScathL. Damage to the BM may also trigger the immune response and the role of this response in the death of the insect remains to be addressed.

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