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Short Communication

Impact of viral *enhancin* genes on potency of *Lymantria dispar* multiple nucleopolyhedrovirus in *L. dispar* following disruption of the peritrophic matrix

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ABSTRACT

Enhancins are metalloproteases found in many betabaculoviruses and several alphabaculoviruses, which enhance alphabaculovirus potency by degrading a protein component of the peritrophic matrix (PM), facilitating passage of virions through this structure. Earlier studies on betabaculovirus enhancins within heterologous systems suggested that enhancins facilitate virion binding to midgut cells. We compared the potency of wild-type *Lymantria dispar* multiple nucleopolyhedrovirus (LdMNPV) with that of single and double *enhancin* deletion viruses in *L. dispar* in the presence and absence of an intact PM. Compared to wild-type virus, the double *enhancin* deletion virus was 6-fold and 14-fold less potent, respectively, indicating that within this homologous system the LdMNPV enhancin genes have a function beyond PM degradation.

1. Introduction

The gypsy moth (*Lymantria dispar*) is a major defoliator of deciduous trees in the US and Canada. One of the gypsy moth's natural enemies is the *Lymantria dispar* multiple nucleopolyhedrovirus (LdMNPV; Genus *Alphabaculovirus*). NPVs consist of multiple virions packaged as single or multiple nucleocapsids that are embedded in occlusion bodies (OB) composed primarily of polyhedrin. Susceptible larval hosts become infected by ingestion of OBs, which dissolve in the midgut releasing occlusion-derived virus (ODV) into the midgut lumen. In order for the virus to infect the insect, ODVs must first traverse the peritrophic matrix (PM) that protects the epithelial cells lining the gut from a variety of pathogens (Lehane, 1997; Terra, 2001).

Early studies with granuloviruses (GV; Genus *Betabaculovirus*) identified a factor in the *Pseudaletia unipuncta* (Psun) GV that increased the infectivity of PsunNPV in NPV/GV mixed infections (Tanada, 1959). The enhancing factor (termed the synergistic factor (SF)) is a protein component of the GV capsule (Hara et al., 1976; Tanada et al., 1973). A synonymous factor, (viral enhancing factor, VEF) was found in *Trichoplusia ni* GV (TnGV) granules that enhanced the infectivity of *Autographa californica* NPV (AcNPV), and the gene for this factor was identified and sequenced (Hashimoto et al., 1991). Subsequent studies identified enhancins in many GVs as well as several NPVs (Jakubowska et al., 2006). Initial studies on enhancin function suggested that the site of action is the cellular membrane of midgut cell microvilli (Tanada et al., 1975; Tanada et al., 1980), and these cells contain specific binding sites for enhancins (Uchima et al., 1988). Subsequent studies demonstrated that the enhancin from TnGV degraded major glycoproteins of the PM (Derksen and Granados, 1988), was a metalloprotease (Lepore et al., 1996), increased the permeability of the PM (Peng et al., 1999), and degraded insect intestinal mucin, a protein associated with the PM (Wang and Granados, 1997). Degradation of intestinal mucin increased access of virions to the midgut epithelial cells, increasing susceptibility of the host insect to viral infection (Wang and Granados, 2000). This is likely the basis for the early observations of enhancement of NPV infections *in vivo* when co-administered with GV granules and purified enhancin.

The LdMNPV contains two genes, *E1* and *E2* (Bischoff and Slavicek, 1997; Popham et al., 2001). These authors produced three LdMNPV *enhancin*-deletion constructs that exhibited decreased potency compared to wild-type virus. The potency of constructs E1cat and E2del were 2.3- and 1.8-fold lower, respectively, in gypsy moths inoculated 24 h post-molt to the second instar. A double deletion construct (E1delE2del) showed a 12-fold potency drop, indicating that the two genes confer a non-additive compensatory enhancement of viral efficacy (Popham et al., 2001). Using immunoelectron microscopy, Slavicek and Popham (2005) demonstrated that the two gene products are distributed in the ODV envelopes and are associated with the nucleocapsids. Bioinformatics analysis for trans-membrane domains indicated that the proteins traverse the ODV envelope with the N-terminus of the

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proteins extending outside of the membrane, placing them in a position to interact with the PM and host midgut epithelial cell membranes (Slavicek and Popham, 2005).

In contrast to all other studies on the function of baculovirus enhancins, only the studies with the LdMNPV were performed within a homologous system. Studies using heterologous systems showed that GV enhancins increased the infectivity of NPVs *per os*, and that GV enhancins degrade the mucin component of the PM thereby compromising the integrity of this structure. Based on these findings, and the finding the enhancins constitute 5% of the protein in GV granules, there is substantial evidence supporting the hypothesis that GV enhancins facilitate GV virus movement through the PM. However, extension of this hypothesis to the function of NPV enhancins within homologous systems may not be valid. Earlier studies found evidence that *P. unipuncta* larval midgut cells contain specific binding sites for GV enhancins that enhance the infection process of NPVs (Uchima et al., 1988), and that GV enhancins increased infection of NPVs in cell culture systems (reviewed in Hukuhara and Zhu, 1989; Kozuma and Hukuhara, 1994; Tanada, 1985).

In our earlier work (Slavicek and Popham, 2005) we hypothesized that the function of the LdMNPV enhancin is to degrade the PM, albeit through a distinct mechanism since the proteins were found to be components of ODV envelopes as opposed to the polyhedron matrix as is the case with GVs (the granule). However, based on the results of early studies and the location of LdMNPV enhancins within the ODV envelopes it is possible that enhancins could facilitate viral entry into midgut cells. To address this hypothesis the PM in gypsy moth larvae was disrupted and the impact on viral potency of single and double *enhancin* gene deletion viruses was compared with the wild-type virus in this study. If the sole function of LdMNPV enhancins is to enable the virus to penetrate the PM, then the potencies of the *enhancin*-deletion recombinant viruses should be similar to wild-type in larvae lacking a PM.

2. Methods

L. dispar eggs from USDA (Otis ANGB, MA) were reared through the third instar on artificial diet using the protocol of Hoover et al. (2002). Three recombinant strains of the A21 variant of LdMNPV, deficient in the production of one or both *enhancin* transcripts, were used in these experiments. The E1cat viral construct has the *E1* gene interrupted by insertion of the *cat* gene. The E2del construct lacks most of the *E2* gene and the E1delE2del construct lacks portions of both *enhancin* genes (Bischoff and Slavicek, 1997; Popham et al. (2001). Each virus was amplified in *L. dispar* larvae and purified as described previously (Hoover et al., 2002). Virus dilutions were made in sterile 60% glycerol and OB concentrations determined using an improved Neubauer hemocytometer. Newly molted fourth-instars were fed on artificial diet with or without surface contamination of 0.5% fluorescent brightener 28 (Sigma) dissolved in sterile milliQ water for 24 h. Preliminary experiments showed that 0.5% brightener was equally effective at removing the PM as 1%, and was less likely to inhibit feeding or produce control mortality (data not shown). After 24 h, each larva was inoculated *per os* with 1 μl of OBs as described previously (Hoover et al., 2002). After inoculation, larvae were placed on artificial diet without brightener and maintained at 25 °C under a photoperiod of 14:10 h (L:D) until they died or pupated. Controls were inoculated with 60% glycerol plus brightener only. A range of at least five viral doses, bracketing the expected LD_{50}, was tested for each construct using 35–40 larvae per dose; doses were replicated 2–4 times. Intact larval midguts were removed from a subset of 10 larvae fed diet with or without brightener at the time larvae were to be inoculated to confirm the presence/absence of the PM using scanning electron microscopy (SEM) as described in Plymale et al. (2008). Dose–response data were analyzed using probit analysis (POLO-PLUS v.2, LeOre Software) to calculate LD_{50}s and 95% confidence intervals. Data from experiments with and without brightener were analyzed separately.

3. Results and discussion

In the absence of brightener, viral potency was significantly lower (6-fold) in larvae treated with the double *enhancin* gene deletion construct (E1delE2del) than for larvae treated with the wild-type virus (A21), with a lethal dose ratio of 0.17 (LD_{50} for A21/LD_{50} for E1delE2del) (Fig. 1a). The LD_{50} of the single deletion viruses were not significantly different from wild-type but were significantly lower than the double deletion virus.

To test the null hypothesis that the *enhancin* genes of LdMNPV have no other function but to increase penetration of virus through the PM, larvae were dosed with virus after feeding for 24 h on diet contaminated with brightener. The brightener markedly reduced the LD_{50} for all viruses, from 139 to 340-fold for E1delE2del and A21, respectively (Fig. 1b). Despite removal of the PM, the E1delE2-

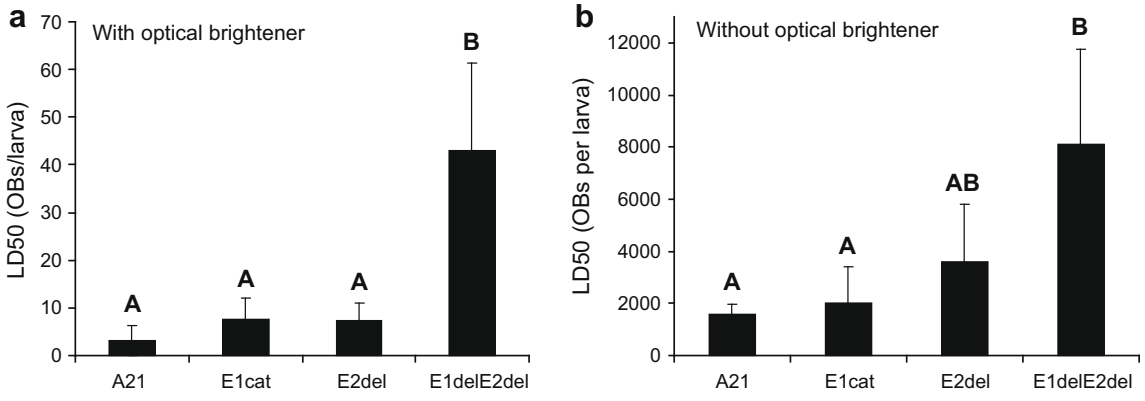

Fig. 1. Mean lethal dose (LD_{50}) of three recombinants of LdMNPV with one or more deletions of *enhancin* genes in comparison with wild-type virus (A21 strain). *L. dispar* larvae were fed on artificial diet for the first 24 h after molting to the fourth instar in the (a) presence or (b) absence of 0.5% fluorescent brightener, inoculated with virus *per os*, and then transferred to fresh artificial diet without brightener. All virus constructs originated with an A21 parent. The *E1* gene of E1cat is disrupted by the *chloramphenicol acetyl transferase* gene, E2del has a partial *E2* deletion, and E1delE2del is missing portions of both *E1* and *E2*. Error bars represent the 95% confidence interval. LD_{50}'s with the same letter over the bar are not significantly different at the $p < 0.05$ level. Tests of equal intercepts and slopes: (a) with brightener intercepts differed: $\chi^2 = 43$, df = 6, $p < 0.001$ but slopes were equal $\chi^2 = 4.3$, df = 3, $p = 0.231$. (b) Without brightener both intercepts and slopes differed: $\chi^2 = 27.6$, df = 3, $p < 0.001$ and $\chi^2 = 62.4$, df = 6, $p < 0.001$, respectively. Fit of the data to probit models verified for each virus by heterogeneities <1.0.

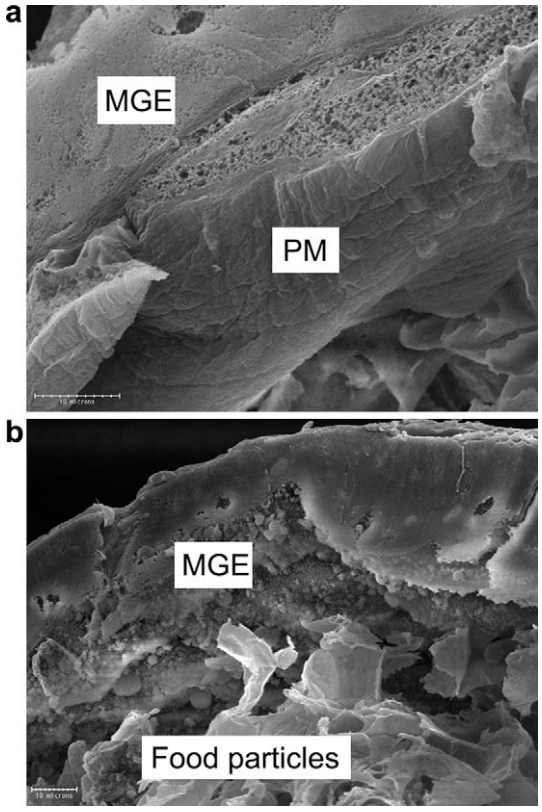

Fig. 2. Scanning electronic micrographs of midguts removed from gypsy moth larvae after feeding on artificial diet for the first 24 h post-molt to the fourth instar (a) without or (b) with addition of 0.5% fluorescent brightener. Timing of dissection coincided with timing of viral challenge. (a) Intact PM; magnification = 2000×; MGE = midgut epithelial cells; PM = peritrophic matrix. To the right of PM label is gut lumen. (b) Magnification = 1000×. Note complete absence of the PM. Blebs on the surface of the midgut are likely discarded contents of disrupted goblet cells due to absence of the PM.

del was 14-fold less potent than the wild-type virus, with an LD_{50} of 43.1 compared with 3.1, respectively (Fig. 1b). There was no significant difference among the LD_{50}s of the single deletion viruses compared with the wild-type virus, but all LD_{50}s were significantly lower than for the double deletion virus, except for virus with E2 deleted.

SEMs of representative midgut samples removed at the time of virus inoculation revealed a robust, multi-layered PM in larvae fed artificial diet only (Fig. 2a). After feeding for 24 h on brightener, the PM was either absent or badly damaged (Fig. 2b).

Our results do not support the hypothesis that the *enhancin* genes of LdMNPV function only to enable the virus to penetrate the peritrophic matrix. If the enhancins only role is to increase penetration of virions through the PM, then complete removal of the PM, as was verified by SEM, should have produced equivalent potencies for the wild-type virus (A21) and the double *enhancin* deletion virus (E1delE2del).

Early studies reported that GV enhancins bind to larval midgut cells at specific sites and it was hypothesized that the enhancin protein serves as a binding protein for some NPVs (Tanada, 1985); significantly more virus particles were attached to or already in midgut microvilli in insects treated with enhancin plus PsunNPV compared to insects without enhancin even though polyhedra and ODV were found in abundance next to microvilli in the control insects. Also, enhancin was found associated with midgut cell microvilli when purified protein was administered *per os* (Tanada et al., 1980). The location of LdMNPV enhancins within ODV envelopes (Slavicek and Popham, 2005) is consistent with the hypothesis of a role in binding to midgut cells. A general PM degradation function of GV enhancin is consistent with their location and amount of protein within the GV granule. However, this does not preclude a dual function of binding to and facilitating entry into midgut cells. Such a dual role for GV enhancins has never been tested within a homologous context. Given that the two enhancin proteins can partially compensate for the lack of the other (Popham et al., 2001), their position in the ODV envelope (Slavicek and Popham, 2005) and the results reported here, we suspect that one or both of the enhancins assist virus-host cell fusion in addition to disruption of the PM. Further studies are needed to address this possibility.

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