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Bacterial, but not baculoviral infections stimulate *Hemolin* expression in noctuid moths

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ABSTRACT

Lepidopteran larvae are regularly infected by baculoviruses during feeding on infected plants. The differences in sensitivity to these infections can be substantial, even among closely related species. For example, the noctuids Cotton bollworm (*Helicoverpa zea*) and Tobacco budworm (*Heliothis virescens*), have a 1000-fold difference in sensitivity to *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) infection.

Recent data were interpreted to indicate that the lepidopteran immunoglobulin protein, *Hemolin*, is synthesized upon viral injection and therefore to participate in anti-viral responses. To investigate whether *Hemolin* synthesis is affected by a natural viral infection, specific transcription in fat bodies and hemocytes of *H. zea* and *H. virescens* larvae was monitored following *per os* infection with the baculovirus HzSNPV (*H. zea* single nucleopolyhedrovirus). Both moths showed the same expression pattern as seen in uninfected animals and coincided with ecdysone responses, previously known to induce *Hemolin* expression. In contrast, injection of lyophilized *Micrococcus luteus* resulted in increased *Hemolin* expression supporting a role for *Hemolin* as an immuno-responsive protein in these species.

The combined data are consistent with the suggestion that while *Hemolin* seems to participate in the response to virus infection in the superfamily Bombycoidea, this is not true in the Noctuoidea.

1. Introduction

Insect immune responses involved in virus defense have lately received increased focus. While the pathways and effector molecules active in defense against bacteria and fungi are well elaborated [1,2], we are only in the beginning of understanding how the innate immune system protects insects from viral infection [3]. Knowledge about the insect immune responses to bacteria and fungi has led to important advances in vertebrate innate immunity; therefore, we expect research on insect viral immunity to have the same effect.

The baculoviruses (Baculoviridae) are a family of viruses that infect arthropods, mainly insects and in particular Lepidoptera [4]. Although the use of baculoviruses in pest control has led to significant knowledge of viral pathobiology and ecology in Lepidoptera [4], little is known about the host immune responses towards viral infections. The anti-viral mechanism of host-cell transcription down-regulation, leading to global translational arrest and apoptosis, is well studied in mammals [5]. Likewise; in Lepidoptera, one of the major responses against virus infection is the apoptosis of infected cells [6]. This is apparent in experiments where baculovirus-encoded anti-apoptotic genes have been deleted genetically or their products silenced by RNA interference (RNAi). For example, the yields of baculovirus decreased 100-fold and the lethal dose required for killing larvae increased more than 1000-fold after mutation of the anti-apoptotic gene p35 in *Spodoptera frugiperda* [7]. However, in addition to apoptosis, hitherto unknown factors also playing a role in the response have been suggested [6,8].

The lepidopteran-specific immunoglobulin protein *Hemolin* was found as the most abundant bacteria-induced protein in *Hyalophora cecropia* and *Manduca sexta* [9–11]. Bacteria-induced *Hemolin* expression has since been documented in several moth species and recently *Hemolin* involvement in anti-viral defense was suggested [12]. Baculovirus infection up-regulated the expression of *Hemolin* in the Chinese oak silkmoth *Antheraea pernyi* and was confirmed by RNAi of *Hemolin* that resulted in accelerated death of virus-infected *A. pernyi* pupae [13]. *Hemolin* involvement in viral infection also was seen for polydnavirus, a virus attached to the injected eggs of parasitic wasps to interfere with the host immune responses. A polydnavirus protein from the

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parasitic wasp *Cotesia congregata* binds to *Hemolin* from *M. sexta* and inhibits the normal *Hemolin* functions of binding LPS and agglutination of bacteria [14].

A robust antibacterial response has been documented in *Heliothis virescens* [15,16], but far less is known about the larval response to baculoviral infection; although evidence exists that dietary selenium may be required to resist infection [17,18] and that the plasma enzyme prophenoloxidase may possess virucidal activity against baculoviruses [19]. Thus, *per os* HzSNPV infection of 5th instar *H. virescens* larvae did not alter expression of prophenoloxidase subunits in fat bodies or in hemocytes; however the expression of prophenoloxidase-1 was mildly suppressed when 4th instar larvae were infected though the expression of prophenoloxidase-2 was unchanged [18]. In contrast, bacterial elicitation of 5th instar *H. virescens* larvae significantly elevated hemocyte and fat body transcript levels of prophenoloxidase-1, but not of prophenoloxidase-2 [18]. While prophenoloxidase subunits are expressed constitutively at high levels in larval *H. virescens* hemocytes and fat bodies, the prophenoloxidase-1 subunit was thus differentially regulated by bacterial and baculoviral infection.

The *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is the most extensively studied virus and infects primarily noctuids [20,21]. In a comparative study of AcMNPV-pathogenicity, baculovirus-infected cells were encapsulated by hemocytes and cleared from the hemocoel in *Helicoverpa zea*, but not in *H. virescens*, resulting in a 1000-fold increased resistance to mortal infection for the former [22].

To determine whether *Hemolin* plays a role in anti-viral immunity of noctuid moths and perhaps be a key factor in the differences in resistance observed, we cloned the *Hemolin* cDNAs from *H. zea* and *H. virescens*, and analyzed their expression in response to baculoviral and bacterial infection.

2. Material and methods

2.1. Insects, infections and tissue sample collections

H. virescens and *H. zea* eggs were received from the North Carolina State Univ. Dept. of Entomology Insectary (Raleigh, NC) and reared individually on an artificial wheat-germ-based diet (BioServe, Frenchtown, NJ) under standard conditions of 14 h:10 h (L:D) photoperiod, 55% RH, 28 °C [23]. For baculoviral response, newly moulted larvae were infected *per os* with an LC_{95} (concentration killing 95% of larvae) at either 5×10^8 (4th instar) or 1×10^9 (5th instar) polyhedra/ml of HzSNPV or mock-infected according to Popham et al. [23]. Larvae were infected with virus mixed with food coloring visible through the integument. Additional trays of larvae were infected to confirm that the virus killed at the expected rate. By 60 h post-infection at the latest, larvae were showing clear signs of infection such as moribund behavior and an absence of burrowing. To activate the antibacterial immune response, newly moulted 5th instar larvae were punctured with a tungsten needle dipped into a suspension of PBS and heat-killed *Escherichia coli* and *Micrococcus luteus* [24]. *Micrococcus luteus* was purchased from Sigma Chemicals as *Micrococcus lysodeikticus* ATCC No. 4698, but is a synonym of *Micrococcus luteus*. Mock-infected controls received a sterile puncture. Tissues were dissected in cold PBS, put in RNAlater (Ambion) and frozen at −84 °C for later isolation of RNA using TriZol® (Invitrogen).

2.2. Cloning of Hemolin

H. virescens DNA (45 ng) was used to clone a fragment corresponding to exon 4 of *Hemolin* by using PCR supermix (InVitrogen). Forward primer Hem Ex4 HvF: 5′-GGT ACA AAA ATG GCC AAC C-3′ (nucleotide positions 227–245 in Fig. 1) and reverse primer Hem Ex4 HvR: 5′-GCT TTA GCC ATC ATA TCG TTG C-3′ (nt pos 761–782) were designed based on the partial *Hemolin* cDNA sequence EF537866 from *H. virescens* (M. Geber, I. Faye, O. Terenius, direct submission). Thermal cycle was 94 °C for 30 s, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, and final extension of 72 °C for 25 min. PCR products were cloned into a TOPO-T/A cloning vector (Invitrogen) and several independent clones were subjected to sequencing at Laguna Scientific, CA, USA.

To obtain full-length cDNA, 5′- and 3′RACE were conducted with a SMART cDNA RACE kit (Clontech) using nested PCR. The first primer set was 5′RACE 1: 5′-CTA TGT TAC CAG CCT TCT CCC A-3′ (nt pos 254–274) and 3′RACE 1: 5′-GTC AGG TGA AGA TGT GGT GTT GG-3′ (nt pos 667–688) and the second primer set was 5′RACE 2: 5′-GGT TGG CCA TTT TTG TAC C-3′ (nt pos 227–245) and 3′RACE 2: 5′-GCA ACG ATA TGA TGG CTA AAG C-3′ (nt pos 761–782). PCR products were cloned into a TOPO-T/A cloning vector and several independent clones were subjected to sequence analysis. Using the sequence data obtained by RACE, new primers were designed outside the coding region and the full-length coding region of *Hemolin* was cloned and re-sequenced. The primers used to clone the entire *H. virescens Hemolin* sequence was also used to amplify *Hemolin* from *H. zea*.

2.3. qRT-PCR

2.3.1. RNA isolation and reverse transcription

Total RNA was extracted from fat bodies and hemocytes using the RNeasy™ kit (Qiagen). RNA was reverse transcribed using the Qiagen Omniscript® Reverse Transcription kit. The reactions were carried out in a total volume of 20 μl using the cDNA synthase buffer, dNTP mix, oligo-dT, RT enhancer, Verso Enzyme mix and 5 μl of template RNA. The reaction was incubated at 37 °C for 60 min and inactivated at 95 °C for 5 min. The samples were either used immediately for qPCR or frozen at −20 °C until use. The expression of *Hemolin* transcripts relative to a house-keeping ribosomal protein transcript was determined using quantitative real-time PCR (qPCR). Reactions were performed in the Eppendorf Master Cycle Rep Realplex 4S and analyzed with the Realplex software. The qPCR reactions were performed using the Qiagen QuantiFast™ SYBR Green® qRT-PCR Kit and the Eppendorf twin.tec® 96 well PCR plates. The sequences of the primers used in the reactions were for *H. virescens Hemolin* transcript measurements: *Hv Hemolin* forward (TGG AGT CGG CAA GAA ACA GAC TCA), *Hv Hemolin* reverse (AGG CCA GTA ACT TGG CAA GGG ATA), *Hv*RPL4 forward (AGA TGC TGA ACG TGG ACA AGC TGA) and *Hv*RPL4 reverse (TTC AGC GGG TTG AGT TTC CTG GTA). The same primers were used for the determination of *H. zea Hemolin* transcript level, except that a different internal standard was used, *Hz*RPL21 forward (CGT CCG CAT TGA ACA CAT CAA GCA) and *Hz*RPL21 reverse (TGG GAG CCA ATA GCA CTG GTT TCT). The specificity of each primer pair was confirmed using agarose gel electrophoresis and by performing melting curve analysis on the completed qPCR reactions. Negative controls consisting of no-template reactions were performed for each primer pair. The PCR reaction was performed at 95 °C for 15 min followed by 50 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. All reactions were performed in duplicate and the *Hv*RPL4 normalized expression ratio was calculated using the $\Delta\Delta C_T$ method [25]. Infection time-courses and dissections were repeated three separate times.

2.3.2. Statistical comparisons

Triplicate real-time-PCR data were analyzed as a randomized complete block design ANOVA. All data was log transformed and