**Drosophila melanogaster** Mounts a Unique Immune Response to the Rhabdovirus Sigma virus†

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Rhabdoviruses are important pathogens of humans, livestock, and plants that are often vectored by insects. Rhabdovirus particles have a characteristic bullet shape with a lipid envelope and surface-exposed transmembrane glycoproteins. *Sigma virus* (SIGMAV) is a member of the *Rhabdoviridae* and is a naturally occurring disease agent of *Drosophila melanogaster*. The infection is maintained in *Drosophila* populations through vertical transmission via germ cells. We report here the nature of the *Drosophila* innate immune response to SIGMAV infection as revealed by quantitative reverse transcription-PCR analysis of differentially expressed genes identified by microarray analysis. We have also compared and contrasted the immune response of the host with respect to two nonenveloped viruses, *Drosophila* C virus (DCV) and *Drosophila* X virus (DXV). We determined that SIGMAV infection upregulates expression of the peptidoglycan receptor protein genes PGRP-SB1 and PGRP-SD and the antimicrobial peptide (AMP) genes *Dipterican-A, Attacin-A, Attacin-B, Cecropin-A1*, and *Drosocin*. SIGMAV infection did not induce PGRP-SA and the AMP genes *Drosomycin-B, Metchnikowin*, and *Defensin* that are upregulated in DCV and/or DXV infections. Expression levels of the Toll and Imd signaling cascade genes are not significantly altered by SIGMAV infection. These results highlight shared and unique aspects of the *Drosophila* immune response to the three viruses and may shed light on the nature of the interaction with the host and the evolution of these associations.

**Sigma virus** (SIGMAV; family *Rhabdoviridae*) occurs naturally in *Drosophila melanogaster* and is maintained in fly populations through vertical transmission via germ cells (31). Other viruses in this family are known pathogens of humans, livestock, fish, and plants (33). Insects commonly serve as vectors and replication hosts for many livestock and all well-characterized plant rhabdoviruses. Black flies, sand flies, and mosquitoes, for example, transmit vertebrate-infecting rhabdoviruses, e.g., *Vesicular stomatitis virus* and *Bovine ephemeral fever virus* (12, 27), whereas aphids, leafhoppers, and planthoppers vector plant rhabdoviruses (17, 19).

While rhabdoviruses can infect a variety of tissues in their invertebrate hosts, they appear to predominantly invade the central nervous system. In humans and other vertebrates, *Rabies virus* spreads throughout the body, including the central nervous system, and most importantly for transmission, the salivary glands (12). SIGMAV and some plant rhabdoviruses have been shown to replicate in neural and other tissues of *Drosophila* and their insect vectors (1, 2, 17, 31). SIGMAV does not appear to adversely affect *Drosophila* in their natural environment; however, SIGMAV-infected flies remain irreversibly paralyzed and die after CO₂ anesthetization (7, 31).

Vesiculoviruses also confer similar CO₂ sensitivity to their black fly hosts (7).

*Drosophila* immune responses to various bacterial and fungal pathogens are well characterized at the molecular level. The elucidation of *Drosophila* antiviral immune responses began only recently and has focused on two other naturally occurring viruses, *Drosophila* C virus (DCV; family *Dicistroviridae*) (13, 28) and *Drosophila* X virus (DXV; family *Birnaviridae*) (35). SIGMAV differs from these two viruses in its mode of transmission, morphology, tissue tropism, and virulence (8, 16, 21, 31, 32, 35) (Table 1). Given SIGMAV’s unique biology, we predicted that the *Drosophila* immune response might also differ with respect to this virus. Using quantitative reverse transcription-PCR (qRT-PCR) approaches, we have examined the expression of a number of innate immune genes in SIGMAV-infected *Drosophila* insects relative to uninfected flies. We have compared these patterns of transcription to those in response to DCV and DXV with the aim of shedding some light on how *Drosophila* responds to diverse viral infections.

**MATERIALS AND METHODS**

**Drosophila** stocks. The *D. melanogaster* Fe strain (SIGMAV infected) and Canton-S strain (SIGMAV free) were used as starting stocks. All *Drosophila* stocks were maintained at 25°C in 70% humidity with a 12-h light-dark cycle on standard cornmeal-yeast medium. To minimize genetic background effects, Canton-S females were crossed with Fe males, and then the progeny females of each generation were backcrossed against Fe males for four generations to create a BC4 strain with 97% Fe background. A small portion of BC4 flies remained infected with SIGMAV because paternal transmission is possible although it is less efficient than maternal transmission (31). SIGMAV-infected BC4 flies

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were treated with CO₂ gas and kept on ice for 10 min. The SIGMAV-infected bad, CA) for 1 h; samples were immersed first in the nuclear stain propidium iodide (Invitrogen Corp.) for 5 min and then in the actin stain phalloidin for 1 h before being examined by CLSM (Leica TCS SP). A subset of samples exhibiting substantial levels of infection (Fig. 1A) was then selected for downstream analysis. The status of SIGMAV-negative samples (Fig. 1B) was further confirmed with qRT-PCR (see below) using primers designed to amplify a fragment spanning the SIGMAV N and P genes (Table 2). Relative SIGMAV abundance per sample was compared following normalization against the host gene, Actin 88F (Table 2).

Sample preparation and qRT-PCR. A total of six samples were prepared for analysis for each SIGMAV-infected and uninfected Drosophila line. Each sample was comprised of paired male and female flies. Total RNA was extracted using Trizol (Invitrogen Corp.) according to the manufacturer’s instructions. Initial homogenization was carried out using a Mini BeadBeater (BioSpec Products, Inc. Bartlesville, OK). The integrity and concentration of the RNA were determined spectrophotometrically using a NanoDrop and associated software. Gene-specific primers were subsequently utilized for qRT-PCRs in a Rotor-Gene 3000 thermal cycler (Corbett). Two assay replicates and five to six biological replicates were compiled and averaged for each treatment.

RESULTS

Relative SIGMAV abundance. The mean relative values of SIGMAV infection, as revealed by qRT-PCR across the six samples that were found positive in the iCLSM study (Fig. 1A), was $2.3 \pm 0.76$ (mean $\pm$ standard error of the mean) with a range of 0.8 to 6.0 (Fig. 2). Of the six putative SIGMAV-negative samples based on the iCLSM study (Fig. 1B), one sample was apparently infected with SIGMAV, as revealed by qRT-PCR using SIGMAV primers (data not shown), and therefore was excluded for further analyses. Thus, iCLSM detected only relatively high levels of SIGMAV infection, which is reflected in the narrow range of qRT-PCR numbers for the six samples found positive in iCLSM (Fig. 2).

In pilot experiments prior to employing selection of SIGMAV-positive samples by iCLSM, extremely variable results were obtained with respect to the transcriptional profiles of various immune genes. This variation can be explained by a polymer-
phism for both infection status and viral titer in laboratory stocks. The CO₂ sensitivity assays are also not 100% accurate in identifying SIGMAV-free flies. Hence, we decided to focus on comparing the transcription profiles of highly infected flies and SIGMAV-negative flies as determined by iCLSM and qRT-PCR.

Expression of innate immunity-associated genes. We tested the transcription levels of 15 immunity-related genes relative to the internal control gene Actin 88F by qRT-PCR. This indicated that six of the immune genes showed a consistent and statistically significant upregulation in the six SIGMAV-infected samples versus the five samples of uninfected flies. For the upstream genes involved in receptor activity and signaling, the peptidoglycan recognition protein (PGRP) genes PGRP-SB1 and PGRP-SD showed clear upregulation in infected flies (Fig. 3A and Table 3), whereas PGRP-LC, PGRP-SC1, and PGRP-SA were not upregulated (Fig. 3A and Table 3). Expression levels of PGRP-SB1 were particularly high (23.3-fold uninfected) whereas the expression level of PGRP-SD was only slightly higher (3.5-fold uninfected) (Table 3). Expression of Toll, Relish, and vir-1 showed increases in expression in SIGMAV-infected flies, but these increases were not statistically significant (Fig. 3B). For the genes encoding antimicrobial peptides (AMPs), significant upregulation was found for Attacin-A, Attacin-B, Cecropin-A1, Diptericin-A, and Drosocin in SIGMAV-infected flies but not for Defensin, Drosomycin-B, and Metchnikowin (Fig. 3C). PGRP-SB1, PGRP-SD, and Diptericin-A are primarily regulated by Relish of the Imd pathway, whereas Attacin-A, Attacin-B, Cecropin-A1, and Drosocin are regulated by Relish of the Imd pathway and Spaetzle of the Toll pathway (11). However, we find no evidence that SIGMAV infection induces expression of Toll and Relish (Table 3). SIGMAV infection also does not activate vir-1 of the Jak-STAT pathway (Table 3).

Comparison of Drosophila immune responses toward SIGMAV, DCV, and DXV. Signaling pathways controlling the

![FIG. 2. Relative abundance of SIGMAV (SiV) per sample based on the expression of the SIGMAV N and P genes normalized against host Actin 88F expression. Error bars represent the range from assay replicates.](image-url)
expression of the *Attacin-A, Attacin-B,* and *Cecropin A* genes. SIGMAV- and DXV-infected flies (35) share upregulated expression of *Drosocin, Diptericin-A, Attacin-A,* and *Attacin-B.*

**DISCUSSION**

The pattern of induced PGRP gene expression by SIGMAV is distinct from that of other viruses, where only *PGRP-SA* shows induced transcription by DCV. SIGMAV induces both *PGRP-SD* and, more notably in terms of the magnitude of expression, *PGRP-SB1.* These two members of the short class of PGRP genes share a number of characteristics. Both genes exhibit low-level constitutive expression in adult *Drosophila* insects, are highly inducible in response to bacterial infection, are expressed mainly in the fat body, and encode proteins that are likely exported from the cell (34). *PGRP-SB1* has also been shown to have amidase activity and bactericidal properties (25). Unlike DCV and DXV that have proteinaceous capsids, SIGMAV particles are surrounded by a lipid bilayer with glycoprotein spikes. PGRPs are the first receptors that recognize, bind, or catalytically cleave specific surface components of bacterial cell membranes (22, 29). Thus, the differential induction of the PGRPs among the viruses may be an indication of the different virus surface properties.

**TABLE 3. Mean ratios of expression for virus-infected relative to virus-free *Drosophila***

<table>
<thead>
<tr>
<th>Gene function and name</th>
<th>GeneID</th>
<th>DCV</th>
<th>SIGMAV&lt;sup&gt;a&lt;/sup&gt; Oral infection route&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Intra-thoracic infection route&lt;sup&gt;c&lt;/sup&gt;</th>
<th>DXV&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
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<td><strong>Upstream genes and receptors</strong></td>
<td></td>
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<tr>
<td><em>PGRP-SA</em></td>
<td>CG11709</td>
<td>x</td>
<td>–</td>
<td>3.2</td>
<td>ND</td>
</tr>
<tr>
<td><em>PGRP-SBI</em></td>
<td>CG9681</td>
<td>23.5</td>
<td>–</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td><em>PGRP-SC1a</em></td>
<td>CG14746</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>PGRP-SC1b</em></td>
<td>CG8577</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>PGRP-SD</em></td>
<td>CG7496</td>
<td>3.5</td>
<td>–</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td><em>PGRP-LC</em></td>
<td>CG4432</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Toll</em></td>
<td>CG5490</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Spätzle</em></td>
<td>CG6134</td>
<td>ND</td>
<td>–</td>
<td>3.0</td>
<td>ND</td>
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<tr>
<td><strong>Signaling cascade</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td><em>Relish</em></td>
<td>CG11992</td>
<td>–</td>
<td>–</td>
<td>3.5</td>
<td>ND</td>
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<tr>
<td><em>vir-1</em></td>
<td>CG31784</td>
<td>–</td>
<td>–</td>
<td>6.4</td>
<td>ND</td>
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<tr>
<td><strong>Antimicrobial peptides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><em>Drosomycin-B</em></td>
<td>CG10810</td>
<td>–</td>
<td>3.0</td>
<td>2.1</td>
<td>70</td>
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<tr>
<td><em>Defensin</em></td>
<td>CG1385</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>4.8</td>
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<tr>
<td><em>Metchnikowin</em></td>
<td>CG8175</td>
<td>–</td>
<td>–</td>
<td>3.0</td>
<td>60</td>
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<tr>
<td><em>Drosocin</em></td>
<td>CG10816</td>
<td>10.3</td>
<td>–</td>
<td>–</td>
<td>3.2</td>
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<tr>
<td><em>Diptericin-A</em></td>
<td>CG12763</td>
<td>5.6</td>
<td>–</td>
<td>–</td>
<td>3.2</td>
</tr>
<tr>
<td><em>Diptericin-B</em></td>
<td>CG10794</td>
<td>ND</td>
<td>–</td>
<td>5.0</td>
<td>ND</td>
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<tr>
<td><em>Attacin-A</em></td>
<td>CG10146</td>
<td>7.2</td>
<td>6.3</td>
<td>8.7</td>
<td>2.5</td>
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<tr>
<td><em>Attacin-B</em></td>
<td>CG18372</td>
<td>ND</td>
<td>–</td>
<td>4.7</td>
<td>ND</td>
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<tr>
<td><em>Attacin-C</em></td>
<td>CG14740</td>
<td>ND</td>
<td>–</td>
<td>2.7</td>
<td>ND</td>
</tr>
<tr>
<td><em>Attacin-D</em></td>
<td>CG7629</td>
<td>ND</td>
<td>–</td>
<td>+</td>
<td>ND</td>
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<tr>
<td><em>Cecropin-A1</em></td>
<td>CG1385</td>
<td>8.8</td>
<td>2.6</td>
<td>–</td>
<td>1.8</td>
</tr>
<tr>
<td><em>Cecropin-A2</em></td>
<td>CG1387</td>
<td>ND</td>
<td>–</td>
<td>3.3</td>
<td>ND</td>
</tr>
<tr>
<td><em>Cecropin-B</em></td>
<td>CG1878</td>
<td>ND</td>
<td>–</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td><em>Cecropin-C</em></td>
<td>CG1373</td>
<td>ND</td>
<td>–</td>
<td>+</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> – no difference in expression levels; <sup>b</sup> – no detectable expression; <sup>c</sup> – determined; <sup>d</sup> – induced expression relatively to virus-free flies.

<sup>a</sup> qRT-PCR data from this study; data reported for *P* values <0.05.

<sup>b</sup> Microarray data from reference 13.

<sup>c</sup> Microarray data from reference 28.

<sup>d</sup> Microarray data from reference 13.
Unlike the case with DCV (13), we found little evidence of increased transcription in the signaling cascade genes of the Imd, Toll, and Jak-STAT pathways. However, one would expect a chance in expression of these signaling genes, because PGRP-SB1 and PGRP-SD expressions are primarily regulated by Relish of the Imd pathway (11), and PGRP-SD function is required for activation of the Toll pathway (4, 30). Also, Diptericin-A is primarily regulated by Relish of the Imd pathway, whereas Attacin-A, Attacin-B, Cecropin-A, and Drosocin are regulated by Relish of the Imd pathway and Spaetzle of the Toll pathway (11). On the other hand, we did not find upregulation of Metchnikovin, which is also induced by both the Toll and Imd pathways (23). The type of infection dictates how the Toll and Imd pathways contribute to the expression of each AMP gene (11). For the AMP gene expression levels, the SIGMAV infection appears to be most similar to that of the gram-negative bacteria that also induce Dipterocin, Attacin, Cecropin, and Drosocin but not Drosomycin and Metchnikovin (18). The outcomes of the Drosophila immune response to SIGMAV and gram-negative bacteria may be similar because both microbes have outer lipid bilayers and glucose.

Since SIGMAV is a vertically transmitted parasite, there would be substantial selection pressure for reduced virulence and for evasion of the host immune response in the virus (14). Like SIGMAV, DCV has an old and established relationship with Drosophila. Laboratory experiments comparing responses of flies following infection by intrathoracic injection with the more natural route of feeding indicate a weaker Toll response in the latter case (9, 13, 28). This may be due entirely to differences in the mode of immune system activation via the gut but could also reveal a history of adaptation. The constitutive upregulation of immunity genes in SIGMAV-infected flies nonetheless indicates evidence of host recognition and energetic investment in fighting the SIGMAV infection. Extreme overactivation of the Imd pathway has been associated with developmental defects and larval death (5), and a number of published works reveal tradeoffs between immune function and fitness in insects (3, 10, 24). Not surprisingly, SIGMAV has been shown to cause mild reductions in host egg viability; however, the 10 to 20% SIGMAV infection frequency in natural Drosophila populations (31) suggests that infected flies can compete in terms of fitness to some degree with virus-free flies.

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