REGULATION OF LIPID METABOLISM GENES, LIPID CARRIER PROTEIN LIPOPHORIN AND ITS RECEPTOR, DURING IMMUNE CHALLENGE IN THE MOSQUITO Aedes Aegypti

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Running Title: Aedes Lp/LpR in Mosquito Systemic Immune Response

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In the mosquito Aedes aegypti, the expression of two fat body genes involved in lipid metabolism—a lipid carrier protein lipophorin (Lp) and its lipophorin receptor (LpRfb)—was significantly increased after infections with Gram (+) bacteria and fungi, but not with Gram (-) bacteria. The expression of these genes was enhanced after the infection with Plasmodium gallinaceum. RNA interference (RNAi) knockdown of Lp strongly restricted the development of Plasmodium oocysts, reducing their number by 90%. In Vg-ΔREL1-A transgenic mosquitoes, with gain-of-function phenotype of Toll/REL1 immune pathway activated after blood feeding, both the Lp and LpRfb genes were over-expressed independently of septic injury. The same phenotype was observed in the mosquitoes with RNAi knockdown of Cactus, an IκB inhibitor in the Toll/REL1 pathway. These results showed that, in the mosquito fat body, both Lp and LpRfb gene expression were regulated by the Toll/REL1 pathway during immune induction by pathogen and parasite infections. Indeed, the proximal region of the LpRfb promoter contained closely linked binding motifs for GATA and NF-κB transcription factors. Transfection and in vivo RNAi knockdown experiments showed that the bindings of both GATA and NF-κB transcription factors to the corresponding motif were required for the induction of the LpRfb gene. These findings suggest that lipid metabolism is involved in the mosquito systemic immune responses to pathogens and parasites.

Lipophorin (Lp) is the major lipid carrier protein in insects. It delivers lipids to various organs via the hemolymph as a reusable shuttle, with no concomitant degradation of the protein matrix of the Lp particle. The fat body, which is an insect analog of vertebrate liver and adipose tissue combined, plays a key role in lipid metabolism by being the site of lipid storage and mobilization. The loading and unloading of lipids into and from fat body cells is accomplished by a shuttle mechanism involving Lp and a multi-protein complex called a lipid transfer particle. The lipophorin particle consists of three apolipoproteins: apolipoprotein I, apolipoprotein II, and apolipoprotein III (1, 2, 3). ApoLpI and apoLpII are encoded by a single gene (4, 5, 6). Likewise, mosquito Aedes aegypti Lp consists of apoLpI and apoLpII, the mRNAs of which originate from a single large precursor RNA, indicating that they are encoded by the same gene. Aedes Lp protein level increases upon ingestion of a blood meal, when the mosquito needs an increased rate of lipid transport to developing oocytes. Lipophorin in the whole bodies reaches its maximal levels by 40-48 h post-blood meal (PBM) when major events of egg yolk and lipid deposition have been completed. However, Lp mRNA and the rate of Lp synthesis in the fat body reach their maximal levels at 18-20 h PBM (7, 8, 9, 10).

The intracellular uptake of Lp is mediated by its cognate lipophorin receptor (LpR), which belongs to the superfamily of low-density lipoprotein receptors (LDLRs) (11, 12, 13, 14, 15). LpRs have been cloned from several insects, including Locusta migratoria (11), Ae. aegypti (14, 15), and Galleria mellonella (16). Previously, we have identified two splice variants of Ae. aegypti LpR gene products specific to the fat body (AaLpRfb) and ovary (AaLpRow) (14, 15). The expression of the fat body-specific AaLpRfb transcript is restricted to the post-vitellogenic
period, during which production of yolk protein precursors is terminated and the fat body is transformed into a storage depot of lipid, carbohydrate, and protein (15). In the mosquito fat body, the regulation of expression of most genes involved in vitellogenesis is governed through a blood meal-driven hormonal cascade, with the terminal signal being a steroid, 20-hydroxyecdysone (20E) (17). Transcription of the LDLR gene in the animal cells is regulated by intracellular cholesterol concentration, hormones, and growth factors (18). In the mosquito, a rising Lp titer and/or a falling titer of 20E could serve as signals activating transcription of the AaLpRfb gene (10, 14, 15).

In addition to its role in providing energy for various physiological processes, lipid metabolism has been implicated in infectious and parasitic diseases (19). Lipid carrier proteins have been reported to reduce the toxicity of lipopolysaccharide in both invertebrates (20, 21) and mammals (22, 23). Lipophorins have been reported to be components of the wound-response clot Periplaneta (24) and Galleria (25). Several studies have suggested that another component of the insect lipid transport system, the exchangeable apoLpIII, plays a role in immunological responses (26, 27, 28, 29, 30, 31). Interestingly, the human exchangeable apolipoprotein E, a constituent of LDL, has also been implicated in immunomodulatory effects; apo-E-deficient knockout mice possess altered immune responses (32) and are more susceptible to bacterial infections than wild-type mice (33). Recent evidence shows that a retinoid and fatty-acid binding protein (RFABG) is up-regulated in the midgut of Anopheles gambiae during infection by Plasmodium and is important for the parasite development (34).

In this study, we systematically investigated the responses of two genes associated with lipid metabolism, Lp and LpR, during pathogen and parasite infections in the mosquito Ae. aegypti. We showed that, in the fat body—the tissue of insect systemic immunity and metabolism—Lp and LpRfb gene expression were up-regulated as a result of infection and regulated by the Toll/REL1 pathway. The results of this study have shed further light on the link between the lipid metabolism and immunity and infection in these vectors of major human diseases.

**EXPERIMENTAL PROCEDURES**

**Insects.** The mosquitoes, Ae. aegypti, were maintained in laboratory culture as described previously (35). Adults were provided with water and a 10% sucrose solution. Vitellogenesis was initiated by feeding females, 3–5 days after eclosion, with a blood meal from rats. All dissections were performed in Aedes physiological saline (36).

**RNA Extraction, Reverse Transcription, and Real-Time PCR.** Dissected fat bodies from abdomens of 10-15 individual mosquitoes were homogenized using a motor-driven pellet pestle mixer (Kontes, Vineland, New Jersey, USA) and lysed by Trizol reagent (Invitrogen). RNA was isolated following the manufacturer’s protocol. Contaminating genomic DNA was removed by treatment with RNase-free DNase I (Invitrogen). Reverse transcription was carried out using an Omniscript reverse transcriptase kit (Qiagen) in a 20-µl reaction mixture containing oligo-dT primers and 2 µg total RNA at 37°C for 1 h. Real-time PCR was performed using the iCycler iQ system (Bio-Rad, Hercules), and reactions were performed in 96-well plates with a QuantiTect SYBR PCR kit (Qiagen). In order to quantify relative gene expression, standard curves were generated using 10-fold serial dilution of cDNA pools containing high concentrations of the gene of interest. The protocol for amplifying the cDNA product was 40 cycles of 95°C for 30 s, then 59°C for 45 s, followed by melting curve analysis to detect specific product amplification. Each sample was analyzed in triplicate and normalized to the internal control, β-actin mRNA. Real-time data were collected by the iCycler iQ Real-Time Detection System software V. 3.0 for Windows. Raw data were exported to Excel (Microsoft, Seattle, WA) for analysis. Real-time PCR primers are as follows:

- **LpR** forward primer: CGAAAGTCAGTGCAAGTTCATCAG; LpR reverse primer: CTGGCTTCGGTCCCTTCTGAG; Lp forward primer: CAGCCAGAACAATGTGGGTAAGCTC; Lp reverse primer: GACCTTACGTGCAGCAACTTGTTC; Actin...
Synthesis and Micro-injection of dsRNA. Synthesis of dsRNA was accomplished by simultaneous transcription of both strands of template DNA with a HiScribe RNAi Transcription Kit (NEB). The plasmid LITMUS 28iMal containing a nonfunctional portion of the E. coli male gene that encodes maltose-binding protein was used to generate control dsRNA. After RNA synthesis, the samples were treated by phenol/chloroform extraction and ethanol precipitation. The dsRNA was then suspended in diethyl pyrocarbonate-treated distilled water with a final concentration of 5 µg/µl. The formation of dsRNA was confirmed by running 0.2 µl of these reactions in a 1.0% agarose gel in TBE (90 mM Tris-borate/2 mM EDTA, pH 8.0). A Picospritzer II (General Valve, Fairfield, NJ) was used to introduce 200 nl of this dsRNA into the thorax of CO2-anesthetized mosquito females at 2–3 days post-eclosion.

Septic Injury and Infection Experiments. Septic injuries were performed by pricking mosquitoes in the rear part of the abdomen with an acupuncture needle dipped into either bacterial culture or a fungal spore suspension. Plasmodium gallinaceum is routinely maintained in the laboratory by natural sporozoite transmission between the Ae. aegypti RED strain and chicks. One-week-old birds are infected by exposure to infected mosquitoes. The parasitemia was monitored daily on thin Giemsa-stained blood smears from 1 week after the infection until a gametocytemia range of 1–3% was reached. For the infections, 4-day-old female mosquitoes were fed on anaesthetized chicken that had been infected with P. gallinaceum 9 days previously. To determine parasite oocyst development, midguts were dissected 7 days post-infection and then stained with 1% mercurochrome. Parasite oocyst numbers were determined by means of light microscopy (Nikon E400, Japan).

Western blotting analysis. SDS-PAGE and Western blot analysis were conducted as described previously (35). Proteins were resolved on 4-12% SDS-PAGE, followed by electroblotting, to polyvinylidene difluoride membranes (Invitrogen). The membranes were probed with AaLp and β-actin (Sigma) antibodies. Immune complexes were visualized by the addition of SuperSignal WestDura Extended-Duration Substrate (Pierce).

Isolation of the 5’ Upstream Region of the Fat Body-Specific LpR Gene. To clone the 5’ upstream region of the LpRfb gene, a Vectorette library was constructed in accordance with the instructions provided by the manufacturer (Vectorette II; Sigma Genosys Ltd.). Genomic DNA was digested by restriction enzyme Cla I and then ligated with the corresponding Vectorette units to the digested end of the DNA. A PCR was performed using universal vectorette and specific primers from the Vectorette library. The Vectorette amplicons were then subcloned and sequenced.

Electrophoretic Gel Mobility Shift Assay (EMSA). Each protein was synthesized by a coupled in vitro transcription-translation (TNT) system (Promega). The corresponding cDNA clones were subcloned into pcDNA3.1/Zeo (+) (Invitrogen). The in vitro transcription-translation reactions programmed by the circular plasmid DNA utilized the T7 promoter. To confirm the synthesis of proteins with expected size, the control TNT reactions of each protein were performed in the presence of [35S] methionine, and the resulting reactions were analyzed by means of SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography. The annealed deoxyoligonucleotide of NF-κB and GATA motifs were purified from 15% TBE Criterion Precast Gel (Bio-Rad), and labeling of double-stranded oligonucleotides and EMSA was performed with a gel shift assay system (Promega). The protein-DNA complex was separated on 5% TBA Criterion Precast Gel (Bio-Rad) and visualized by means of autoradiography.

Cell Culture and Transient Transfection Assay in the Aag-2 Cell Line. Cell line Aag-2 from Ae. aegypti (37) was maintained in Schneider medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone). The coding region sequences of the AaREL1-A, AaREL1-B, AaREL2, and AaGATAa were inserted into the pAc5.1/V5/HisA (Invitrogen) vector. The LpR2.4 kb promoter was

Forward primer: GACTACCTGATGAAGATCCTGAC; Actin reverse primer: GCACAGCTTCTCCTTAATGTCAC.
inserted into pGL3/firefly luciferase vector (Promega) to form the reporter construct pLpR2.4-luc. Cells were incubated at 26°C until they reached at least 70% confluency (approximately 24 h). Transfection was conducted using Effectin (QIAGEN) with an optimal DNA-lipid ratio of 1:25 (w/v), following the manufacturer’s instructions. Typically, 150 ng each of pLpR2.4-luc, AaREL1, AaGATAA, and pRLCMV/renilla luciferase (Promega) were mixed in a 24-well plate with a total volume of 250 µl growth medium and then incubated at room temperature for 20 min. The expression vector pAc5.1/V5/HisA was used as carrier DNA so that each well received an equal amount of total DNA. Renilla luciferase served as an internal control for transfection efficiency. The cells transfected with empty expression vectors pAV5.1/V5/HisA were used as a negative control. The transfection cocktail was added to Aag2 cells for 6 h at 27°C. Transfection mixtures were then removed and replaced with fresh growth media. After 48 h of incubation, the medium was aspirated and the cells were lysed in 100 µl passive lysis buffer (Promega). Dual luciferase activities were measured using Lumimark (Bio-Rad). The relative luciferase activity was obtained by normalization of the firefly luciferase activity against renilla luciferase activity.

RESULTS

The effect of bacterial and fungal infection on Lp and LpR gene expression. To investigate whether Aedes aegypti Lp gene expression was affected as a result of infection, we measured its transcript levels after infections with Gram (-) bacterium Escherichia cloacae, Gram (+) bacterium Micrococcus luteus, and spores of the entomopathogenic fungus Beauveria bassiana. Mosquitoes were injected at 20 h, and the expression of the Lp gene was monitored at 5, 12 and 24 h post-infection. In infected vitellogenic female mosquitoes, the Lp transcript expression level was significantly increased by either M. luteus or B. bassiana infections but not by E. cloacae (Fig. 1C). The elevation of this gene in response to infection exhibited a maximal elevated response at expression 12 h post-infection (Fig. 1C). When infections were performed in blood-fed female mosquitoes, the LpRfb transcript responded similarly to that of pre-vitellogenic mosquitoes, elevating its level 12 h post-infection in response to Gram (+) bacteria and fungi (Fig. 1D). In the control blood-fed female mosquito, LpRfb transcripts in the fat body reached their maximal levels as reported previously (14, 15).

Therefore, we observed a significant effect of infections on the expression of two essential genes of lipid metabolism, Lp and LpRfb. Importantly, expression of both these genes was elevated in the Aedes fat body in response to Gram (+) bacteria and fungal infections, but not to Gram (-) bacteria. This type of immune regulation suggested involvement of the Toll/REL1 pathway in controlling expression of these two genes (38, 39, 40).

The effect of Plasmodium gallinaceum infection on Lp and LpR gene expression. To investigate a possible effect of Plasmodium infection on the expression of Lp and LpR transcripts in Ae. aegypti, wild-type mosquitoes
were fed on chicks infected with *P. gallinaceum*, and RNA samples were collected from fat bodies of infected mosquitoes throughout several stages of *Plasmodium* development. The level of *Lp* transcripts was significantly elevated in the fat body at 24 h after feeding on an infective blood meal, the stage when ookinetes invade the midgut (Fig. 2A). The expression levels of the *LpRfb* gene were also elevated in parasite-infected mosquitoes compared with those of uninfected mosquitoes, but at a later stage (32 h PBM) (Fig. 2B). These experiments clearly showed that malaria infection caused the elevated expression of both *Lp* and *LpRfb* genes in the *Aedes* fat body.

To examine the phenotypic effects on parasite development of silencing *Lp* and *LpR* genes, we prepared knockout mosquitoes by injecting the females at 1-2 days post-eclosion with respective dsRNA. Mosquitoes with knockdown phenotype for *Lp* and *LpR* were infected with *P. gallinaceum* at 4 days after dsRNA injection. Infection levels were scored by the number of oocysts per midgut at 7 days post-infection (Fig. 3A). Controls showed that treatments with either anti-*Lp* or anti-*LpR* dsRNAi resulted in successful knockdown of both target genes (Fig. 3B and 3C). Table 1 summarizes statistically evaluated results from three independent experiments. The *Lp* knockdown showed a marked and statistically significant effect (U-test, *P*<0.05, two-tailed) (Fig. 4A). Likewise, the *LpRfb* mRNA expression level was greatly increased in the *REL1* gain-of-function transgenic mosquitoes from 32 h PBM, lasting until 56 h PBM. The expression of *LpR* gene at 32 h PBM between the REL1 gain-of-function and control mosquitoes was significantly different (U-test, *P*<0.01, two-tailed) (Fig. 4B). Thus, this elevation of *Lp* and *LpRfb* gene expression suggested their regulation by the Toll/REL1 pathway.

Additionally, we utilized the dsRNA knockdown of Cactus, an IκB inhibitor of REL1 (39, 40). Double-stranded RNA of *Aedes* Cactus was injected into the thorax of 1- to 2-day-old female mosquitoes, and, after 2-4 days of recovery, the treated mosquitoes were challenged by *B. bassiana* spores. Both the *Lp* and *LpRfb* transcripts were elevated by the fungal challenge in the wild-type UGAL and control iMal dsRNA-treated mosquitoes (Fig. 5A and 5B). However, in the Cactus knockdown mosquitoes without any infection, the *Lp* transcript was increased to a level comparable with that of the activation by *B. bassiana* (Fig. 5A). Likewise, the *LpRfb* gene expression was fully activated without any infection in the Cactus dsRNA knockdown mosquitoes (Fig. 5B). The control experiment shown in Figure 5C shows that Cactus mRNA was dramatically reduced by its dsRNA. Taken together, these reverse-genetics experiments strongly suggest the REL1-mediated Toll pathway regulation of *LpRfb* and *Lp* genes.
Cloning and analysis of the regulatory region of the fat body-specific LpR gene transcription unit (LpRfb). The Ae. aegypti LpR gene, consisting of two transcription units—ovarian-specific LpRov and fat body-specific LpRfb—has been cloned (14, 15). Having this information for the LpR gene, we focused on the cloning of the 5′ upstream region of the LpRfb transcription unit in order to examine whether or not it was directly controlled by the REL1 factor. PCR was performed using a Clal Vectorette library as a template. The 2.4-kb Vectorette ampiclon was subcloned into the pCRII-TOPO vector and sequenced. This fragment contained the 5′ upstream region of the LpRfb gene as well as a partial sequence encoding an N-terminal portion of the LpR protein (Fig. 6A). We analyzed this DNA fragment for transcription factor binding sites and determined that the 2.4-kb fragment of the upstream region from -108 bp to -682 bp of the 5′ upstream LpRfb gene contained the 5′ upstream region of the LpR gene (Fig. 6A). This 5′-GGGAAAACCC-3′ motif (κB-like motif, 5′-GGGAAAACCC-3′), had high affinity to both in vitro-translated AaREL1-A and AaREL2 binding. The addition of 50-fold excess of the unlabeled specific oligonucleotides (S(N)) effectively competed with the binding to the labeled probe. The addition of a nonspecific competitor (NS), SP1, had no effect suggesting that there is no difference in transactivation activity resulting from any combination of REL proteins in vitro (Fig. 7A).

Next, we analyzed the binding specificity of five putative GATA binding motifs (G1–G5). In Ae. aegypti, six GATA factors have been identified (42, and Park et al., unpublished data). The best characterized among them were the fat body-specific AaGATAa, with two isoforms (42, and Park et al., unpublished data) and a high homology to DmGATAb (serpent), the GATA factor required for the regulation of a number of fat body-specific promoters (43, 44). We utilized in vitro-translated AaGATAa for this analysis.
transfection of pLpR2.4-luc with either Ae. aegypti REL factor (Fig. 7A). This result clearly demonstrated that the kB-like (N) motif located in the regulatory region of the LpRfb gene was required for its transcriptional activation in vitro by the Ae. aegypti REL factors.

Next, we investigated whether all three GATA binding sites that exhibited specific binding to AaGATAa serpent homologue were required for the transcriptional activation of the LpRfb gene in vitro. When AaGATAa was co-transfected with the pLpR2.4-luc in Aag-2 cells, AaGATAa marginally enhanced the basal luciferase activity (Fig. 7B). However, when we mutated these three GATA motifs individually by means of site-directed mutagenesis and tested transactivation of these mutated constructs of the pLpR2.4-luc (ΔG1, ΔG2, and ΔG3) by co-transfecting into cells with the AaGATAa expression plasmid, the mutation of G2 and G1 sites resulted in a 90% reduction of luciferase activity, whereas no significant difference was observed in the mutation of the G3 sites (Fig. 7B). The binding specificity of AaGATAa for these mutated GATA binding sites was not detected by the EMSA controls (Fig. 7B inset). Therefore, although AaGATAa showed no effect on transactivation activity of the pLpR2.4-luc in vitro, the site-directed mutagenesis experiments suggested that the G1 and G2 GATA binding sites were required for the activation of the LpRfb gene. Additionally, when AaGATAa was co-transfected together with either Ae. aegypti REL1 or REL2 factors, the transactivation of the reporter luciferase was higher than the action of either REL1 or REL2 factors alone. This result suggested that GATA and REL factors could result in cooperate transactivation of the pLpR2.4-luc reporter gene (Fig. 7C).

Finally, to confirm the involvement of REL and GATA factors in the regulation of the LpRfb gene expression in vivo, we carried out knockdown experiments using dsRNAs of REL1, REL2, and GATAa. When REL1, REL2, and GATAa dsRNAs were introduced into the mosquitoes, the mRNA level of LpRfb transcripts decreased by 90% when compared with those in the wild type UGAL and iMal-injected control mosquitoes (Fig. 7D). Thus, the in vivo reverse-genetics experiments supported in vitro data suggesting that both the REL and GATAa factors were involved in the regulation of Ae. aegypti LpRfb gene expression.

DISCUSSION

We have investigated the effect of infection on activation of genes encoding two essential proteins involved in lipid trafficking—lipid carrier protein lipophorin (Lp) and its receptor (LpR)—in adult, female Ae. aegypti mosquitoes. The activation of the Lp transcript was rapid, rising by 5 h post-infection, while the LpRfb transcript levels became elevated at 12 h (Fig. 1). Such a lag in expression of the LpRfb after that of the Lp gene is reminiscent of what occurs during the egg development cycle, when the Lp gene expression peaks at 20 h PBM, coinciding with maximal Lp synthesis and secretion, and the LpRfb transcript level peaks at 36 h PBM, during postvitellogenic internalization of Lp (10, 14, 15). This sequential pattern in the activation of Lp and LpRfb transcripts during infection suggests that the elevation of the Lp titer in the hemolymph is the primary response followed by the LpRfb production for the fat body uptake of the hemolymph Lp.

Our study has shown that the activation of Lp and LpRfb genes by the mosquito fat body is one of the responses to Plasmodium infection. As a result of Plasmodium infection, the fat body activation of Lp and LpRfb transcripts had a sequential pattern similar to that observed with Gram (+) bacteria or fungal infections (Figs. 1 and 2). Introduction of Gram (+) bacteria or fungal spores into the mosquito hemolymph generates a rapid immune systemic response by the fat body, the mechanism of which has been studied in great detail in Drosophila (39, 40). However, in the case of Plasmodium infection, the response by the fat body with respect to elevated transcription of the Lp gene coincides with the timing of ookinete penetration of the midgut wall (24 h PBM). Thus, it appears that the mosquito midgut by the Plasmodium ookinete likely generates a direct or indirect signal activating a systemic lipid metabolic response by the fat body.

A recent study has shown that the lipophorin precursor gene (RFABG) is up-regulated in the midgut of An. gambiae during infection by Plasmodium and is important for the parasite development (34). Therefore, it appears that
elevation of lipid metabolism represents an essential response to Plasmodium infections. In An. gambiae, silencing the RFABG gene has led to significant and consistent 4-fold reduction in oocyst numbers (34). Our reverse-genetics data have demonstrated that, as a result of silencing of the Lp gene, oocyst numbers were reduced by 90%. Silencing of either RFABG (34) or Lp (this study, Fig. 3) also resulted in inhibition of egg development. Interestingly, we have not observed an inhibiting effect on the specific development after silencing of the fat body-Toll/REL1 pathway in the regulation of both genes involved in immune reactions via a nested REL factor (41) has provided a particularly important tool for demonstrating the regulation of Lp and LpRfb genes in the fat body, the tissue of the systemic immunity and the central metabolism. Although injection of the Cactus dsRNA has technically a systemic nature, affecting many tissues in the mosquito, controlling its response in the fat body has provided strong supportive evidence that the Toll/REL1 pathway is involved in regulating the Lp and LpRfb gene expression in this tissue (Fig. 5).

For additional confirmation of immune regulation of genes involved in lipid metabolism, the 5’-regulatory region of the AaLpRfb gene has been cloned and functionally characterized. The proximal region of LpRfb promoter contains a GATA motif in close proximity to a NF-κB site, indicating the possibility that LpR expression is regulated by the immune-related regulation via a nested GATA/NF-κB site. Computer-assisted analysis has revealed apparent important regulators of a close association between GATA and NF-κB-like sites located in many insect immune genes (49). The GATA sequence is present in proximity to a known regulatory region of antimicrobial genes in Drosophila and in several other insects as well (49). The GATA sequence is present in proximity to a specific NF-κB site in the same orientation in all of the known regulatory regions of antimicrobial genes in Drosophila and in several other insects as well (49). Importantly, our in vitro experiments utilizing the pLpR2.4-luc have shown that two GATA motifs located in the same orientation with the NF-κB site are important for the transactivation activity of the LpRfb expression.

Shin et al. (37) have reported that AaREL1 and AaREL2 selectively bind to different κB motifs than insect immune gene promoters. The NF-κB motif present in the LpRfb regulatory region matches well to the κB motif consensus, GGG(W)nCCM, of Drosophila zen Ventral Repression Element (52). This type of κB motif was present in the promoter regions of Drosophila immune genes, the expression of which was activated exclusively by the Toll pathway (49). Recently, SELEX (systematic evolution of ligands by exponential enrichment) assays identified a more specific NF-κB binding sequence in the regulatory region of Drosophila immunity genes (49, 50). Although both AaREL1 and AaREL2 bound to the NF-κB motif in LpRfb promoter, the
NF-κB motif identified in this study closely matches the SELEX data for Drosophila Dorsal, a direct target of the Toll pathway.

In *Ae. aegypti*, AaGATAa, expressed in the fat body and ovary, has a high homology with DmGATAb (serpent) having one zinc finger (Park, J.H., Attardo, G. M., Hansen I.A. Raikhel, A.S., unpublished data). Serpent is required for the regulation of a number of fat body-specific promoters; it mediates fat body-specific transcriptional activation of the alcohol dehydrogenase-1 (Adh-1) gene, and it has also been shown to be required for fat body- and blood cell-specific activation of immune genes in response to immune challenge (50; 51; 52). In *Drosophila*, κB and GATA sites are both required for the induction of Cecropin A1 gene expression (52). Although, AaGATAa has a weak in vitro trans-activating effect on the fat body-specific *LpR* gene reporter, in vitro mutational analysis of its binding sites and in vivo GATA dsRNA silencing strongly suggest its requirement for regulation of this gene. Our in vitro results further suggest that AaGATA works in cooperation with the REL factor on the promoter of the *LpRfb* gene. These findings imply that the NF-κB and GATA subset on the *LpRfb* promoter are required for immune-mediated activation of this gene.

This study has demonstrated that the interactions of the mosquito host with microorganisms and *Plasmodium* require an increase in expression of genes essential for lipid metabolism. Our results show that in female *Ae. aegypti* mosquitoes, in addition to blood feeding, *AaLp* and *AaLpRfb* gene expression are activated by infection with Gram (+) bacteria, fungal spores, and *Plasmodium*. Reverse genetics has confirmed involvement of the Toll/REL1 pathway in immunoregulation of these genes. We have further shown that the *LpR* gene regulation depends on the NF-κB/GATA cassette in its promoter, which is a signature of immune regulation. In our analysis for activation of the *AaLpRfb* gene expression, REL2 has exhibited activating ability of this gene, raising the possibility of more complex interaction of immune regulation of the lipid metabolism. This question will require further study.

REFERENCES


FOOTNOTES

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1 The abbreviations used are: Dif, Dorsal-related immune factor; LDLR, low-density lipoprotein receptor; Lp, Lipophorin; LpR, Lipophorin receptor; PBM, post-blood meal; RFABG retinoid and fatty-acid binding gene; RNAi, RNA interference

FIGURE LEGENDS

Fig. 1. Effect of immune septic infections with different types of pathogens (Gram (-), Gram (+), and fungi) on AaLp and AaLpRfb gene expression in Aedes aegypti mosquitoes. (A) Activation of the AaLp gene expression by Gram (+) and fungal infection in vitellogenic mosquitoes; (B) Effect of immune activation on the fat body AaLp protein level; note continuous elevated levels of AaLp protein after Gram (+) and fungal infection at 44 h PBM (corresponding to 24 h post-infection). Western blot analysis with antibodies against AaLp and actin in the mosquito fat bodies infected with bacteria and fungi. (C) The AaLpRfb gene was induced by Gram (+) and fungal infection in previtellogenic mosquitoes 12 h after septic injury. (D) The increased expression of AaLpRfb in the vitellogenic mosquitoes after Gram (+) and fungal infection. For the experiments in vitellogenic mosquitoes, septic injury was introduced into female adults 20 h PBM. For experiments in A, C, and D, total RNA was collected from fat bodies, and cDNA was synthesized from equal amounts of DNase I-treated total RNA. Real-time PCRs were performed in triplicate. Data were normalized by means of real-time PCR analysis of actin levels in the cDNA samples. Data represent means ± SE of triplicate samples. Standard errors are shown. ASI, after septic injury; PBM, hours after blood meal.

Fig. 2. The expression profiles of AaLp and AaLpRfb gene after Plasmodium gallinaceum infection. AaLp (A) and AaLpRfb (B) genes exhibited increased levels of expression after feeding in Aedes aegypti mosquitoes on the Plasmodium-infected blood. Real-time PCR was performed as described in Fig. 1. Data represent means ± SE of triplicate samples. Standard errors are shown.

Fig. 3. Effect of RNAi-mediated knockdown of AaLp and AaLpRfb on Plasmodium gallinaceum development in the mosquito midgut. Aedes aegypti female mosquitoes were injected with AaLp dsRNA (iLp), AaLpR dsRNA (iLpR), or with a control dsRNA derived from the noncoding region of a bacterial gene (iMal). Four days later, mosquitoes were infected with P. gallinaceum, and midguts were collected 7 days post-infection. (A) AaLp knockdown (iLp) shows 12-fold reduction of oocyst numbers compared with control mosquitoes, whereas AaLpR knockdown (iLpR) shows no significant effect on parasite development. The data represent the pooled data set of three independent experiments. (B) and (C) Controls showing effect of AaLp dsRNA (iLp), AaLpR dsRNA (iLpR), and (iMal) on the levels of
Fig. 4. **The expression profiles of AaLp and AaLpRfb genes in the transgenic Vg-ΔREL1-A mosquitoes with the fat body-specific gain-of-function REL1 phenotype.** The expression of AaLp (A) and AaLpRfb (B) genes was over-expressed after blood feeding in gain-of-function REL1-A transgenic mosquitoes. AaLp and AaLpRfb mRNA levels were determined by real-time PCR as described above in Fig. 1. Data represent means ± SE of triplicate samples. Asterisk indicates statistical significance between gain-of-function REL1-A transgenic and control mosquitoes at 24 h for Lp mRNA (A) and 40 h PBM for LpR mRNA, respectively.

Fig. 5. **The effect of Cactus dsRNA (iCactus) on expression of the AaLp and AaLpRfb genes.** RNAi-mediated knockdown of AaCactus increased AaLp (A) and AaLpRfb (B) mRNA expression without fungal challenge. Female mosquitoes were injected with either dsAaCactus (iCactus) or a control dsRNA (iMal). AaLp and AaLpRfb mRNA levels were determined by means of real-time PCR, as described above. Data represent means ± SE of triplicate samples. (C) Control showing the effect of iCactus dsRNA (iLp) and (iMal) on the level of AaCactus mRNA.

Fig. 6. **Analysis of GATA and NF-κB motifs on the 5’ upstream region of the LpRfb gene.** (A) Schematic illustration of five GATA (G1–5) motifs and one NF-κB (N) motif within the -682 bp upstream portion of the fat body-specific LpR gene in Aedes aegypti mosquitoes. Numbers refer to nucleotide positions relative to the transcription start site. (B) Electrophoretic mobility shift assay (EMSA) for the binding of AaREL1-A and AaREL2 to NF-κB motifs. DNA binding was carried out with in vitro translated AaREL1-A and AaREL2 (33) and a 32P-labeled NF-κB probe. The binding of AaREL1-A and AaREL2 to the NF-κB motif from the Aedes LpRfb gene promoter was abolished by the addition of 50-fold unlabeled specific competitor (S(N)) but not by the non-specific probe SP1 (NS). (C) EMSA with in vitro-translated AaGATAa and 32P-labeled GATA probe and box A probe from the D. mulleri alcohol dehydrogenase (Adh) gene (36). A 50-fold molar excess of unlabeled GATA probe was added as a specific competitor (S). The binding of AaGATAa to the GATA motif was competed with 50-fold unlabeled boxA probe (bA). Arrows indicate the protein/DNA-specific binding complexes. The asterisks indicate the GATA motifs that have high affinity with AaGATAa.

Fig. 7. **Activation of LpRfb gene expression in vitro.** (A, B) The 2.4-kb promoter region was placed upstream of a luciferase reporter and co-transfected with AaREL1-A, AaREL1-B, AaREL2 (A), and AaGATAa (B) into the Aag2 cell line. EMSA assays were conducted on radio-labeled oligos bearing normal or mutant binding site sequences. Insets show that successive mutation of NF-κB (ANF-κB) and GATA (ΔG1-3) motifs resulted in no promoter activity except mutation of the G3 site, indicating a crucial role for the NF-κB and GATA sites. (C) The cooperative effect of AaREL and AaGATAa on the LpR promoter activity in mosquito Aag2 cells. (D) The decreased expression of AaLpRfb gene in AaREL and AaGATAa dsRNA-treated mosquitoes. AaLpRfb mRNA level was analyzed by means of real-time PCR, as described above. iMal, iREL1, iREL2, and iGATAa indicate MalE-, AaREL1-, AaREL2-, and AaGATAa dsRNA-treated mosquitoes, respectively. Data represent means ± SE of triplicate samples.
Table 1. Lipophorin influences vectorial capacity for *Plasmodium gallinaceum*

<table>
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<tr>
<th>dsRNA</th>
<th>Number of experiments</th>
<th>Total number of midguts</th>
<th>Parasite load</th>
<th>Range of oocyst numbers</th>
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</tbody>
</table>
Figure 2

A

Relative Lp mRNA expression level

PV 24h 32h 40h 48h 56h 64h 72h PBM

Naive  Plasmodium

B

Relative Lph mRNA expression level

PV 24h 32h 40h 48h 56h 64h 72h PBM

Naive  Plasmodium
Figure 3

A

The population of midguts with oocysts (%)

- 0-20 oocysts
- 21-50 oocysts
- >51 oocysts

iMal  iLpR  iLp

B

Relative Lp mRNA expression level

UGAL  iMal  iLp

C

Relative LpBD mRNA expression level

UGAL  iMal  iLpR
Figure 7

A

B

C

D

Relative luciferase activity (phosphorimetric units)

Relative luciferase activity (phosphorimetric units)

Relative luciferase activity (phosphorimetric units)

Relative LpR mRNA content
Regulation of lipid metabolism genes, lipid carrier protein lipophorin and its receptor, during immune challenge in the mosquito aedes aegypti
Huang-Mi Cheon, Sang Woon Shin, Guowu Bian, Jong-Hwa Park and Alexander S. Raikhel

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