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RNAi knockdowns support a role for the mucin-like (*AeIMUC1*) gene and short-chain dehydrogenase/reductase (*SDR*) gene in *Aedes aegypti* susceptibility to *Plasmodium gallinaceum*

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Abstract

The mosquito midgut represents the first barrier that the *Plasmodium* parasite encounters after blood ingestion from an infected vertebrate. Previous studies identified the *Aedes aegypti* (L.) mucin-like (*AeIMUC1*) and short-chain dehydrogenase/reductase (*SDR*) genes as midgut-expressed candidate genes influencing susceptibility to infection by *Plasmodium gallinaceum* (Brumpt). Here we used RNA interference (RNAi) by double-stranded RNA injections (dsRNA) to examine ookinete survival to the oocyst stage following individual gene knockdowns. dsRNA gene knockdowns were performed three days prior to *P. gallinaceum* infections and oocyst development was evaluated at seven days post infection. Mean numbers of parasites developing to the oocyst stage were significantly reduced by 52.3% among *dsAeIMUC1* injected females and by 36.5% among *dsSDR* infected females compared to females injected with a *dsβ-gal* control. Prevalence of infection was significantly reduced among *dsAeIMUC1* and *dsSDR* injected females compared to females injected with a *dsβ-gal* control; this represented a two and three fold increase in the number of uninfected individuals, respectively. Overall, these results suggest that both *AeIMUC1* and *SDR* play a role in *Ae. aegypti* vector competence to *P. gallinaceum*.

Keywords

Aedes aegypti; *Plasmodium gallinaceum*; vector competence; innate immunity; malaria; mosquito midgut

Malaria remains one of the most devastating infectious diseases with a global burden for 2002 estimated at 300 to 600 million episodes of clinical *Plasmodium falciparum* (Welch) malaria (Snow *et al.*, 2005). In previous studies we have investigated molecular interactions between the avian malaria parasite *Plasmodium gallinaceum* (Brumpt) and the mosquito *Aedes aegypti* (L.). Our investigations and others have demonstrated that the mucin-like protein (*AeIMUC1*) is a component of the PM, and is expressed in the mosquito midgut (Morlais & Severson, 2001; Rayms-Keller *et al.*, 2000). The *AeIMUC1* gene showed differential allelic polymorphism between a *P. gallinaceum* susceptible and refractory strain of *Ae. aegypti*, and it has been suggested that it might be involved in the interaction of ookinetes with the PM and epithelium surface (Morlais & Severson, 2001). Moreover, this gene is upregulated in response to heavy metal exposure in *Ae. aegypti* (Rayms-Keller *et al.*, 2000) and has heme-binding activity (Devenport *et al.*, 2006). Another gene differentially expressed in the female mosquito midgut is a short-chain dehydrogenase/reductase enzyme (*SDR*). We have shown that *SDR* expression is up-regulated within 12–24 hrs post feeding in

response to an uninfected or infected blood meal in a *P. gallinaceum* susceptible strain compared with a refractory strain (Morlais *et al.*, 2003). SDR is a member of a family of enzymes involved in enzymatic metabolism and oxidative/reductive reactions (Persson *et al.*, 2003). Both the *AeIMUC1* and *SDR* genes map to chromosome 2 in *Ae. aegypti* and are located within previously identified quantitative trait loci (QTL) for *P. gallinaceum* susceptibility (Morlais & Severson, 2001; Morlais *et al.*, 2003). In this study, we used RNAi techniques to knock down the expression of these genes and investigated the effect on the number of oocysts as a measure of infection prevalence and intensity.

The *Aedes aegypti* RED strain, which is highly susceptible to *P. gallinaceum* (Thathy *et al.*, 1994), was reared and maintained in an environmental chamber following our standard procedures (Clemons *et al.*, 2010). Infection with *P. gallinaceum* was performed generally as previously described (Morlais & Severson, 2001). For each independent replicate, both control and test mosquitoes were allowed to feed on the same chicken within a less than one hour interval. Only completely engorged females were selected and maintained for further studies. Midgut dissections were performed seven days after blood feeding to assess prevalence (frequency of infection) and intensity (numbers of developing oocysts) of infection, respectively.

Fragments of the *AeIMUC1* and *SDR* genes for double-stranded RNA (dsRNA) production were selected based on conserved sequences represented in GenBank. Sequence alignments and consensus sequences were determined using the BioEdit Sequence Alignment Editor 4.7.3 (North Carolina State University, 1999). Individual fragments were amplified from the previously cloned *Ae. aegypti* RED strain cDNAs (GenBank accession nos. AF308864, AY033626) of each gene (Morlais & Severson, 2001; Morlais *et al.*, 2003). A 478 bp fragment (gene positions 406 to 884) was selected for *AeIMUC1* and a 605 bp fragment (gene positions 262 to 867) for *SDR*; as a control, a 357 bp fragment of the β -galactosidase gene (β -gal) was amplified from pBluescriptIIKS (Info S1). Sense and antisense RNAs were synthesized using the MegaScript kit and purified according to the manufacturer's instructions. CO₂-anesthetized females (2–7 days old) were injected in the thorax with 0.1–0.2 μ l of dsRNA solution (~1.5 μ g/ μ l) using a Narishige IM300 Microinjector.

Quantitative real-time PCR (qRT-PCR) was performed using an ABI 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Total RNA was extracted from 5–10 mosquito midguts per replicate with Trizol Reagent (Invitrogen) according to the manufacturer's instructions. cDNA was prepared by reverse transcription in a 20 μ l reaction with Superscript II (Invitrogen) following the manufacturer's instructions. All qRT-PCR reactions were performed in triplicate according to the manufacturer's instructions with 0.4 μ l of cDNA in a total volume of 25 μ l containing 12.5 μ l of SYBR Green PCR Master Mix, 300 nmol of each primer and 0.5 μ l of Reverse Transcriptase reaction. Standard curves were prepared for each gene using 10-fold serial dilutions of *Ae. aegypti* RED genomic DNA and the amplification efficiency for each primer pair was calculated (Info S2). Expression levels were normalized to the ribosomal gene *RpS17* (GenBank accession no. AF164153) as an endogenous control (Morlais *et al.*, 2003).

We used qRT-PCR to assess gene expression following *dsAeIMUC1* or *dsSDR* induced knockdown. We injected adult RED strain females ~3 days prior to a *P. gallinaceum* infected blood meal. Our aim was to reduce target gene expression after the first 24 hours following an infected blood meal, when *Plasmodium* ookinetes are moving from the gut lumen through the midgut epithelium. RNAi mediated reductions in transcript levels of *AeIMUC1* and *SDR*, compared with transcript levels of the control β -gal, at four days post-injection with dsRNA are shown in Fig. 1A. For *dsAeIMUC1*, mean transcription levels were reduced 82% compared to control levels. For *dsSDR*, mean transcriptional levels were

reduced 91% compared to control levels. Additionally, we analyzed the transcription levels for *AeIMUC* at several earlier time points, in light of the evidence provided by Devenport *et al.* (2006) that mRNAs encoding *AeIMUC1* protein are stored in the midgut cells previous to blood feeding. Our results confirm reductions in the transcript levels of *AeIMUC1* compared to the control β -gal at four hours before blood feeding (76%) as well as four hours (88%) and 20 hours (69%) after blood feeding (Fig. 1B).

We observed that *dsAeIMUC1* induced knockdown had significant effects on *Plasmodium* development in *Ae. aegypti* females (Figure 2A, Table 1). The number of parasites developing to the oocyst stage (intensity of infection) was significantly reduced among *dsAeIMUC1* injected females compared to females injected with *ds β -gal* ($P=0.0032$, U-test), wherein mean parasite load was reduced by 52.3% among the *dsAeIMUC1* injected females. In addition, we observed a significant reduction in prevalence of infection among *dsAeIMUC1* injected females compared to females injected with *ds β -gal* ($P=0.0261$, Fisher's exact test), which represented an ~2-fold increase in the number of uninfected individuals among the *dsAeIMUC1* injected females.

We observed that *dsSDR* induced knockdown had significant effects on *Plasmodium* development in *Ae. aegypti* females (Figure 2B, Table 1). The number of parasites developing to the oocyst stage was significantly reduced among *dsSDR* injected females compared to females injected with *ds β -gal* ($P=0.0086$, U-test), wherein mean parasite load was reduced by 36.5% among the *dsSDR* injected females. In addition, we observed a significant reduction in prevalence of infection among *dsSDR* injected females compared to females injected with *ds β -gal* ($P=0.0383$, Fisher's exact test), which represented an ~3-fold increase in the number of uninfected individuals among the *dsSDR* injected females.

AeIMUC1 protein has been shown *in vitro* to bind to chitin, the major component of the PM and also has been associated with microvilli of the midgut epithelium (Devenport *et al.*, 2006). In addition, the protein contains multiple potential *O*-glycosylation sites and the *AeIMUC1* glycoprotein has been suggested as a candidate for a PM recognition molecule by the parasite (Morlais & Severson, 2001). It is well-documented that ookinetes recognize and bind to carbohydrate ligands of glycoproteins on the midgut epithelium (Dinglasan *et al.*, 2005; Ramasamy *et al.*, 1997; Rudin & Hecker, 1989; Zieler *et al.*, 1999, 2000). Further, a potential transmission blocking monoclonal antibody (MG96) appears to recognize a mannose α 1–6 substitution on an *O*-linked oligosaccharide (Dinglasan *et al.*, 2005).

SDRs are a complex family of enzymes that includes greater than 3000 primary protein structures (Kallberg *et al.*, 2002; Oppermann *et al.*, 2003; Persson *et al.*, 2003). These display considerable substrate specificity and intracellular localization, and play important roles in steroid metabolism and carbonyl reduction of potentially toxic xenobiotic compounds including aldehydes, ketones and quinones. It has been suggested that SDRs and other metabolic detoxification enzymes may play important roles in longevity assurance in *Caenorhabditis elegans* (Maupas), yet because these reactions are energetically costly their relative enzyme activities and subsequent longevity would reflect balancing selection that maximized overall fitness (Gems & McElwee, 2005). The *Ae. aegypti* Moyo-R strain which shows limited up-regulation of *SDR* following a blood meal (Morlais & Severson, 2001), was selected for high refractoriness to *P. gallinaceum* and shows reduced fitness relative to several life history traits including longevity when compared to another closely related strain, Moyo-S which was selected for high susceptibility to *P. gallinaceum* (Yan *et al.*, 1997). In the present study we observed no differences in survival of the *dsSDR* and control *ds β -gal* groups, indicating that *dsSDR* induced knockdowns did not influence survival for at least ten days post-injection with dsRNA (data not shown).

In summary, in this study we investigated the roles of the *AeIMUC1* and *SDR* genes in determining *Ae. aegypti* vector competence to *P. gallinaceum*. Based on results from RNAi induced gene knockdowns, we conclude that both *AeIMUC1* and *SDR* likely play a role during *Plasmodium* infection in the mosquito midgut. Although neither gene appears to be a major effector in the immune response against the parasite, their individual actions were observed to significantly impact parasite invasion of the midgut epithelium and subsequent development to the oocyst stage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Relative abundance in transcript levels of *AeIMUC1* and *SDR* genes in female midguts of *Aedes aegypti* injected with the corresponding dsRNAs compared with control mosquitoes injected with *dsβ-gal* RNA. Transcript data for control and test genes were normalized using *RpS17*. (A) Results represent means $\pm$ SD for real-time PCR analysis of four replicates in each assay. Quantifications were performed at 24 hours after blood feeding. (B) Results represent means $\pm$ SD for *AeIMUC1* gene real-time PCR analysis of three replicates. Quantifications were performed at 4 hours before and 4 hours and 20 hours after blood feeding.

Figure 2.

Number of oocysts on midguts of female *Aedes aegypti* at seven days following a *Plasmodium gallinaceum* infected blood meal among test gene knockdowns compared to a control (*dsβ-gal*). Mean numbers of oocysts per midgut are indicated for each dsRNA injected group. (A) *dsβ-gal* vs. *dsAeIMUC1* injected females. (B) *dsβ-gal* vs. *dsSDR* injected females. The nonparametric Mann-Whitney U-test was used to compare numbers of oocysts per midgut (intensity of infection) and Fisher's exact test to compare frequencies of infected vs. uninfected (prevalence of infection) individuals between control and test mosquitoes.

Table 1

Oocyst development following *dsAeIMUC1* and *dsSDR* induced knockdowns.

dsRNA	Replicates	Total ^a	Oocysts per midgut ^b	P-value ^c	Prevalence (%) ^b	P-value ^d
<i>β-gal</i>	4	74	13.2±18.2		84.0	
<i>AeIMUC1</i>		74	6.3±11.6	0.0032	69.0	0.0261
<i>β-gal</i>	3	48	17.8±20.6		93.75	
<i>SDR</i>		55	11.3±20.3	0.0086	80.0	0.0383

^aTotal number of midguts examined across all replicates.

^bMean ± SD for all replicates combined.

^cMann-Whitney U-test.

^dFisher's exact test, one-tailed.