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The immunogenic properties of protozoan glycosylphosphatidylinositols in the mosquito *Anopheles gambiae*

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ABSTRACT

In contrast to humans, mosquitoes do not have an adaptive immune response to deal with pathogens, and therefore must rely on their innate immune system to deal with invaders. This facilitates the recognition of different microbes on the basis of surface components or antigens. Such antigens have been identified in various types of microbe such as bacteria and fungi, yet none has been identified in the genus protozoa, which includes pathogens such as the malaria parasite, *Plasmodium falciparum* and *Toxoplasma gondii*. This study allowed us to test the antigenic properties of protozoan glycosylphosphatidylinositol (GPI) on the mosquito immune system. We found that both *P. falciparum* GPI and *T. gondii* GPI induce the strong expression of several antimicrobial peptides following ingestion, and that as a result of the immune response against the GPIs, the number of eggs produced by the mosquito is reduced dramatically. Such effects have been associated with malaria infected mosquitoes, but never associated with a *Plasmodium* specific antigen. This study demonstrates that protozoan GPIs can be considered as protozoan specific immune elicitors in mosquitoes, and that *P. falciparum* GPI plays a critical role in the malaria parasite manipulation of the mosquito vector to facilitate its transmission.

1. Introduction

Many pathogen-associated molecular patterns (PAMPs) and pattern recognition receptors (PRRs) of both the model insect *Drosophila*, and the major vector for the malaria parasite, *Anopheles gambiae*, have been well characterized [1–4].

Specific PAMPs have yet to be identified for the malaria parasite, *Plasmodium falciparum*, but one type of candidates are GPIs. Found in all eukaryotes, and demonstrating variability in sugar and lipid composition, their primary function is to anchor surface proteins to the cell membranes [5,6]. GPIs are believed to constitute the major carbohydrate modification in all stages of *Plasmodium* development [7,8]. It has been demonstrated that *P. falciparum* GPI (PfGPI) along with hemozoin hyper activate the human innate immune response, contributing to the pathogenesis of the parasite [9–12]. Within the infected mosquito, two major GPI anchored surface proteins, P25 and P28, are shed during ookinete maturation and play important roles in midgut invasion [13–15]. It has also been shown that the GPI anchor of the circumsporozoite protein plays a crucial role in sporogenesis [16].

A plethora of vector genes are induced following ingestion of a *Plasmodium* infected bloodmeal [17], and studies looking at nitric oxide synthase activation implies that PfGPI and hemozoin may play a direct role in the *Anopheles stephensi* immune response [18,19]. In addition to changes in the mosquito transcriptome, the fecundity of the mosquito is also affected by the malaria infection [20–22]. This may be a result of parasite manipulation to increase nutrient availability, or from the hosts perspective, the re-allocation of resources to the immune response, although it has yet to be determined whether the mere presence of the parasite, specific stage of sporogony, or parasite protein(s) are primarily responsible for this fecundity effect.

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Abbreviations: AMP, antimicrobial peptide; GPI, glycosylphosphatidylinositol; LAM, laminarin; LPS, lipopolysaccharide; PAMP, pathogen associated molecular pattern; PfGPI, *Plasmodium falciparum* glycosylphosphatidylinositol; PRR, pattern recognition receptor; TF, transcription factor; TgGPI, *Toxoplasma gondii* glycosylphosphatidylinositol; TLR, toll like receptor.

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In our study, the immunogenic properties of purified PfGPI or *Toxoplasma gondii* GPI (TgGPI) in *A. gambiae* are investigated for the first time, and compared to the response evoked by non-protozoan microbial antigens. In addition, we monitored mosquito fecundity after feeding with PfGPI and TgGPI. We show that protozoan GPIs strongly induce the expression of three antimicrobial peptides, and more specifically, that the activation of the mosquito immune response by PfGPI or TgGPI leads to a significant reduction in fecundity. With regards to malaria sporogonic development, this implies that PfGPI may be the primary cause of the observed reduction in mosquito fecundity due to the parasite infection, and has strong implications in the design of future control strategies directed towards the human and mosquito stages of parasite development.

2. Experimental procedures

2.1. GPI purification and antigen membrane feeding

GPIs from *P. falciparum* strain FCBR and *T. gondii* RH strain were extracted as described previously [23,24]. The activity of *T. gondii* and *P. falciparum* GPI purified from 10^8 parasites was checked by induction of TNF-α secretion measured in macrophage culture supernatants by using a specific sandwich ELISA (BD Biosciences, San Jose, CA).

Groups of 50–100 female mosquitoes (*A. gambiae*, Ifakara strain, 4–6 days old) were fed with either *P. falciparum* GPI (PfGPI, isolated from 5×10^9 trophozoites); *T. gondii* GPI (TgGPI, isolated from 7×10^8 tachyzoites); *Escherichia coli* 026:B6 lipopolysaccharide (LPS 10 μg/ml; Sigma); or *Laminaria digitata* laminarin polysaccharide (LAM 10 μg/ml; Sigma). Each antigen, as well as a PBS control, was mixed into a 1 ml blood meal and then transferred to a separate feeding reservoir covered with a collagen membrane, held at 37 °C (*Hemotek* 5W1 membrane feeding system, Discovery Workshops, UK). Following overnight starvation, the mosquitoes were membrane-fed for 15 min. Only fully engorged females were used for subsequent analyses.

2.2. Quantitative PCR

Five engorged mosquitoes were removed from each cohort treatment at 1 h, 3 h, 6 h, and 24 h post-bloodfeeding, and frozen at −80 °C until RNA extraction. Total RNA was extracted from each group of mosquitoes using TRIzol reagent (Invitrogen), and genomic DNA was removed using the TURBO DNA-free kit (Ambion). First strand cDNA synthesis was carried out using Ready-To-Go RT-PCR beads (Amersham Biosciences). We selected seven immune genes from *A. gambiae*, to monitor transcriptional profiling following ingestion of antigen supplemented bloodmeal; one PRR, Toll9 [25], two transcription factors (TFs), STAT1 and Rel2 [26,27], and four effector molecules, Defensin 1 (Def1 [28]), Cecropin A (CecA [29]), Gambicin (Gamb [30]), and Lysozyme c-7 (Lys c-7 [31]). These proteins are known to be activated or expressed in the mosquito midgut upon immune stimulation. Also, *Rel2* and *Toll9* were both recently identified within a genomic *Plasmodium*-resistance island in *Anopheles* mosquitoes [32]. Gene specific primers (listed in Table 1) were obtained through the TaqMan® assay-by-design facility (Applied Biosystems). The ribosomal S7 gene was used as the reference. RT-qPCR reactions were carried out using an ABI prism 7000 thermocycler according to the Applied Biosystems recommended protocol: 95 °C, 10 min; then 40 cycles of 95 °C, 15 s and 60 °C, 1 min. The data were analyzed using Sequence Detection Software v.1.2.3 (Applied Biosystems) and fold changes in expression were calculated using the comparative ΔC_T method against the bloodfed control. The experiment was repeated twice.

Table 1
Primers used in this study

Primer name	Primer sequence (5′–3′)
CecAF	GGCTGAAGAAGCTGGGAAAGA
CecAR	TTCTCTGCTGCCTTGAACACT
CecA probe	CCGGCTCCCTCAATTT
GambF	CCGGAAGGGCGTTTCGT
GambR	GCTTGCAGTCCTCACAGCTATT
Gamb probe	ACGGACAGACGACCATC
Def1F	AGCGGTGGTGCCAATCT
Def1R	CGGCATGGTGCGTTTCC
Def1 probe	TTCGTCCAGAAGGGTATTG
STAT1F	CCCGTGTTAATACGACAGCATCT
STAT1R	GGCGCGTTGTGCAAACTAT
STAT1 probe	CTTTGATCCAATTTCCC
Toll9F	GCGGTCAGCTTCCTTATCCTTAC
Toll9R	AAGCTAAGTACGGCCGAGTTTT
Toll9 probe	CTGGTTCCACATAAAGTACTTC
Lysc-7F	GGCGGCAAATGTAACATGAAGT
Lysc-7R	ATGATTTTGGAACACTTAATTGCATTGGT
Lysc-7 probe	CCGTCACAAGATCTTCA
Rel2F	TCGACGCTGCTGAATTTGGA
Rel2R	CATCGACAGGACGGTGTACTG
Rel2 probe	TTGTTGCTCGTACAGCTC
S7F	GGTGCACCTGGATAAGAACCA
S7R	CTTGTACACCGACGCAAAAGTG
S7 probe	CCATCGAACACAAAGTTGA

Each assay mix consists of unlabelled primer pairs and TaqMan® MGB probes (FAM™ dye-labelled).

2.3. Fecundity measurement

Groups of 50–100 mosquitoes (*A. gambiae*, G3 strain, MR4, ATCC®, Manasses) 4–6 days old, were offered a bloodmeal with PfGPI, TgGPI, or PBS (as described earlier in Section 2). Immediately after the feed, only fully engorged mosquitoes were transferred to individual tubes, and provided with a 10% sugar solution. After 3 days, the surviving mosquitoes were moved to new tubes containing water to allow oviposition. During this time, the mosquitoes had excreted haematin following bloodmeal digestion, and bloodmeal size was indirectly estimated according to the amount of haematin excreted [33]. Fecundity was measured as the number of eggs laid over the following 2 days, as well as the number of mature, stage V eggs counted after dissection [34]. Wing length was measured microscopically as an index of body size. Each block of experiments was repeated three times.

2.4. Statistical analysis

In the qPCR study, statistical analysis was carried out using Student's paired *t*-test (two tailed) to determine whether the expression of the target gene differed significantly compared to the control ($P \leq 0.05$). In the fecundity experiments, statistics were carried out using MINITAB® V.15. All data on bloodmeal size, wing length, and fecundity were tested for normality by the Anderson Darling test, and then the effects between PfGPI, TgGPI, and the control feed were tested using a General Linear Model ANOVA, using Tukey's pairwise comparisons. Linear regression analysis was used to determine any association between bloodmeal size and fecundity.

3. Results

In order to investigate the immunogenic properties of protozoan GPIs in *A. gambiae*, GPIs were purified from *T. gondii* tachyzoites and *P. falciparum* trophozoites by sequential extraction, phase partition, and thin-layer chromatography as described in Section 2. The *T. gondii* GPI has the following structure (Fig. 1A):