Differential roles of an Anopheline midgut GPI-anchored protein in mediating Plasmodium falciparum and Plasmodium vivax ookinet invasion

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ABSTRACT

Novel strategies to directly thwart malaria transmission are needed to maintain the gains achieved by current control measures. Transmission-blocking interventions (TBIs), namely vaccines and drugs targeting parasite or mosquito molecules required for vector-stage parasite development, have been recognized as promising approaches for preventing malaria transmission. However, the number of TBI targets is limited and their degree of conservation among the major vector-parasite systems causing human disease is unclear. Therefore, discovery and characterization of novel proteins involved in vector-stage parasite development of Plasmodium falciparum and Plasmodium vivax is paramount. We mined the recent Anophelles gambiae midgut lipid raft proteome for putative mosquito-derived TBI targets and characterized a secreted glycoprotein of unknown function, AgSGU. We analyzed molecular variation in this protein among a range of anopheline mosquitoes, determined its transcriptomic and proteomic profiles, and conducted both standard and direct membrane feeding assays with P. falciparum (lab/field) and P. vivax (field) in An. gambiae and Anophelles dirus. We observed that α-AgSGU antibodies significantly reduced midgut infection intensity for both lab and field isolates of P. falciparum in An. gambiae and An. dirus. However, no transmission-reducing effects were noted when comparable concentrations of antibodies were included in P. vivax-infected blood meals. Although antibodies against AgSGU exhibit transmission-reducing activity, the high antibody titer required for achieving 80% reduction in oocyst intensity precludes its consideration as a malaria mosquito-based TBI candidate. However, our results suggest that P. falciparum and P. vivax ookinetes use a different repertoire of midgut surface glycoproteins for invasion and that α-AgSGU antibodies, as well as antibodies to other mosquito-midgut microvillar surface proteins, may prove useful as tools for interrogating Plasmodium-mosquito interactions.

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1. Introduction

Over the last decade, more than 200 million cases of malaria have been reported annually, with an estimated 473,000–789,000 people succumbing to the disease in 2012 (WHO, 2013). These statistics, though eye opening, fail to capture the tremendous social and economic burdens that the disease imposes on the developing world. Although recent financial and political commitments to malaria control and elimination have led to significant declines in disease transmission, progress isimenently threatened by mosquito resistance to insecticides and delayed parasite clearance times following treatment with Artemisinin (Alonso et al., 2011; Dondorp et al., 2009; Enayati and Hemingway, 2010; Phyo et al., 2012). Novel strategies to curb transmission are critical to ensuring continual progress towards malaria elimination and eradication. The etiologic agents of human malaria are Apicomplexan parasites in the genus Plasmodium. For mosquito-to-human transmission to occur, a mosquito must first take a blood meal from an infected individual with gametocytes in peripheral circulation.
Once inside the lumen of the mosquito midgut, gametocytes egress from parasitized red blood cells and develop into male or female gametes. These stages undergo sexual reproduction to form zygotes that transform into motile ookinetes, which attach to and invade the mosquito midgut epithelium. Upon successful invasion, ookinetes traverse the cell to reach the basal lamina of the basement membrane, where they develop into oocysts. Oocysts mature over a 10–14 day period during which sporogony occurs, resulting in the production of hundreds to thousands of sporozoites.

Among blood meals, salivary-gland sporozoites are injected into the capillary bed, thereby transmitting parasites to a new host. Midgut invasion by ookinetes represents a critical bottleneck in the life cycle of Plasmodium and offers a unique opportunity to interrupt the parasite’s life cycle (Dinglasan and Jacobs-Lorena, 2008).

One promising approach to combating malaria is the use of transmission-blocking interventions (TBIs), namely vaccines or drugs that target parasite stages in the blood meal and therefore prevent developmental steps in the mosquito vector required for subsequent transmission to human hosts. The rationale is that if a susceptible population is adequately treated with a TBI, the average blood meal ingested by a mosquito will contain antibodies (vaccine) or small molecules (drug) that target parasite sexual stages ingested in the blood (gametocytes) and/or those that develop in the midgut after feeding (e.g., macrogametocytes, microgametocytes, oocytes, ookinetes). TBIs may either kill the parasites or interfere with molecular interactions necessary for specific developmental steps to occur (e.g., fertilization, ookinete invasion of the midgut epithelium) (Dinglasan and Jacobs-Lorena, 2008; Lavazec and Bourgoin, 2008; Mathias et al., 2013). Strategies aiming to prevent Plasmodium invasion of the mosquito midgut may target surface antigens on either the ookinete or the midgut epithelium, and several such TBIs have shown encouraging results (Armistead et al., 2014; Mathias et al., 2012, 2013; Shimp et al., 2013; Miyata et al., 2010). However, the various mechanisms through which an ookinete invades a midgut epithelial cell remain poorly understood. Recent invasion studies have suggested that Plasmodium ookinete sequences multiple ligands on the apical midgut plasma membrane during the invasion process, which may be the result of multiple pathways of midgut invasion (Angrisano et al., 2012; Parish et al., 2011; Vega-Rodríguez et al., 2014). As such, an integral understanding of the parasite’s entire invasion process will contribute greatly to improving current strategies to interrupt parasite transmission with TBIs.

Lipid-raft microdomains play a fundamental role in the invasion pathways of a diverse array of pathogens (Riethmuller et al., 2006). Lipid rafts are dynamic, ordered structures of proteins and lipids, rich in cholesterol and sphingolipids, in the plasma membrane of eukaryotes. These microdomains can fuse together to form platforms that facilitate key cellular functions including cell signal transduction, membrane trafficking and pathogen invasion (reviewed by Simons and Gerl, 2010). Recent evidence suggests that lipid rafts may be an important component of ookinete invasion of the midgut epithelium. Six out of the seven known ookinete-interacting proteins, including the recently reported enolase binding protein (EBP) (Vega-Rodríguez et al., 2014) and the two mosquito-banded TBI antigens Anophelines alyalin aminopeptidase N (AnAPN1) (Dinglasan et al., 2007) and Anophelines gambiae carboxy-peptidase B (CPB1) (Lavazec et al., 2007), were found associated with apical midgut-microvillus detergent resistant membranes (DRM), which are enriched in lipid rafts (Parish et al., 2011). We argue that mining the midgut DRM proteome will likely result in the identification of novel TBI candidates and thus, provide insight into invasion models proposed in the literature, a stance validated by the work on EBP.

The An. gambiae protein AGAP000570, a secreted glycoconjugate of unknown function, which we refer to as AgSGU, was consistently identified in replicate DRM or lipid raft preparations from An. gambiae midguts (Parish et al., 2011) and was among the most highly abundant proteins in one of the replicates. Interestingly, AgSGU was also the second most abundant protein in the An. gambiae peritrophic matrix (PM) proteome (Dinglasan et al., 2009). The presence of AgSGU in the DRM fraction suggests it is partitioned into the same raft structures as the ookinete-interacting proteins mentioned above. Given the presence of AgSGU in midgut DRMs, we hypothesized that this protein plays a role in ookinete invasion of the mosquito midgut. Because these rafts may act as platforms for ookinete invasion, AgSGU may interact either directly with Plasmodium ookinetes (trans-acting) or with other important ligands within midgut lipid-raft structures (cis-acting) during the invasion process. As such, characterization of this protein may be relevant to the molecular biology underlying Plasmodium transmission and therefore yield new insights into the midgut invasion pathway(s) of Plasmodium species.

2. Methods

2.1. Homology searches and evolutionary analyses of AgSGU and putative orthologs

To investigate homology and potential functions of AgSGU, its amino acid sequence was searched against the Conserved Domain Database (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) using RPS (Reverse Position Specific)-BLAST. To identify putative orthologs among anophelines, the AgSGU amino acid sequence was searched against the assembled genome sequences from all anophelines in VectorBase (Release VB-2014–02) using BLASTp (peptide vs. peptide). Where putative orthologs were not found or gene sets were not yet available, all additional datasets were searched using tBLASTn (peptide vs. translated nucleotide), including genome scaffolds, contigs, assembled trascriptomes, and sets of EST sequences. To identify homologs outside of Anophelesinae, the amino acid sequence of AgSGU was searched against the NCBI non-redundant database using BLASTp.

Protein sequences for 15 anophelines were selected for further analyses based on confidence of orthology. The signal peptide for each was identified using the SignalP 4.1 server (Petersen et al., 2000; http://www.cbs.dtu.dk/services/SignalP/) and then removed manually. All sequences were then aligned with TCOFFEE (Notredame et al., 2000; http://tcoffee.crg.cat/) and that alignment was used to align the corresponding DNA sequences while taking the reading frame into account with the program TranslatoR (Abasal et al., 2010; http://translatorx.co.uk/). The DNA alignment was then uploaded to Datamonkey (Delport et al., 2010; Pond and Frost, 2005a; www.datamonkey.org/), the web-based server of the HyPhy package (Pond et al., 2005), and used to construct a phylogenetic tree (neighbor joining method) and select an appropriate model of nucleotide substitution. The latter process identified model 012302 (LRT = 9.435, AIC = 8579.799, p = 0.00893), a time-reversible model of nucleotide substitution that accounts for biases in substitution rates inherent in the sequences analyzed (e.g., transversion/transversion biases). In subsequent analyses of selection, this model was used to estimate \( \omega \), or dN/dS, the ratio of the number of non-synonymous substitutions per non-synonymous site to the number of synonymous substitutions per synonymous site. Estimates of \( \omega \) were generated across AgSGU using three separate methods in HyPhy, single-likelihood ancestor counting (SLAC), random effects likelihood (REL), and fixed effects likelihood (FEL).

Predictions of secondary structure and putative protein-binding residues were made using the PredictProtein server (Rost et al., 2003).
2004; www.predictprotein.org) with the amino acid sequence from An. gambiae minus the signal peptide.

2.2. Expression, purification, and analysis of AgSGU

A 510 bp fragment of AgSGU, representing the complete exon, minus the predicted signal peptide and transmembrane domain, was amplified from An. gambiae midgut cDNA with the gene-specific primers AgSGU F1 5'-GACCCGTACTGCAGGTTTC-3' and AgSGU R1 5'-GAACCGTAGATGGCAGC-3', cloned into the pBAD/Thio-TOPO vector (Life Technologies, Carlsbad, CA) with a C-terminal 6xHis tag, and expressed in Erichesica coli BL21 (DE3) cells. Cells were grown at 37 °C to an optical density (O.D.) of ~0.4 and induced with 0.02% arabinose per liter for 5–6 h. Soluble protein was extracted from the final cell pellet with BugBuster™ Protein Extraction Reagent (Novagen, Madison, WI), loaded onto a Ni-NTA agarose column (Qiagen, Venlo, Netherlands) and eluted with 300 mM imidazole. Elutions were concentrated using Amicon Ultra 3 KD centrifugal filters (Millipore, Billerica, MA), and purity was assessed by SDS–PAGE using 12% Tris–glycine gels and SimplyBlue™ SafeStain (Life Technologies).

2.3. Antibody purification and Fab preparation

Rabbit polyclonal antibody (Pab) generation was outsourced and produced against the purified recombinant protein (Washington Biotechnology, Baltimore, MD). AgSGU-specific antibody was purified from serum by affinity chromatography using AminoLink Plus Coupling Resin (Thermo Fisher, Rockford, IL) to which recombinant AgSGU was immobilized. To generate Fab fragments AgSGU-specific antibody, normal rabbit IgG (Life Technologies) and normal rabbit IgM (Southern Biotech, Birmingham, AL) were digested with papain (Thermo Fisher) overnight at 37 °C.

2.4. SDS–PAGE and Immunoblot analysis

Mosquito (An. gambiae, Anopheles dirus, Anopheles stephensi, or Aedes aegypti) midgut, salivary gland, abdomen (minus midguts), or thorax (minus salivary glands) lysates were loaded (~5 μg per well) into 4–20% Tris–glycine gels. Proteins were separated under reducing conditions and transferred to a nitrocellulose membrane. The membranes were blocked in a 1:1 solution of Odyssey blocking buffer (Li-COR, Lincoln, NE) and PBS plus tween-20 (0.1%) (PBST), to equilibrate to room temperature for 15 min and hydrated 3 times with PBS for 5 min. Cryosections were blocked for 1 h with PBS-BSA (3%) and probed with rabbit α-AgSGU Pabs (1:500) or normal rabbit IgG (controls) for 2 h at 37 °C in a humidified chamber. Slides were washed 3 times with PBS for 5 min and probed with Alexa Fluor 488-conjugated α-rabbit IgG secondary antibodies (Invitrogen, Carlsbad, CA) in 0.02% Evans blue in blocking buffer. Slides were washed as above and imaged under oil immersion at 1000x total magnification using a Nikon Eclipse E800 epifluorescence microscope and SPOT imaging software (version 4.9).

2.6. Immunofluorescence microscopy

Paraformaldehyde (4%)-fixed, sucrose (20%)-protected An. gambiae KEELE midguts (sugar-fed and blood-fed) were embedded in Tissue Tek OCT compound (Sakura Finetek USA, Torrence, CA) and flash frozen in a butanol bath on ethanol-dry ice. Prior to immunohistochemistry, slides of 7 μm cryosections were allowed to equilibrate to room temperature for 15 min and hydrated 3 times with PBS for 5 min. Cryosections were blocked for 1 h with PBS-BSA (3%) and probed with rabbit α-AgSGU Pabs (1:500) or normal rabbit IgG (controls) for 2 h at 37 °C in a humidified chamber. Slides were washed 3 times with PBS for 5 min and probed with Alexa Fluor 488-conjugated α-rabbit IgG secondary antibodies (Invitrogen, Carlsbad, CA) in 0.02% Evans blue in blocking buffer. Slides were washed as above and imaged under oil immersion at 1000x total magnification using a Nikon Eclipse E800 epifluorescence microscope and SPOT imaging software (version 4.9).

2.7. Transmission-blocking assays

For standard membrane feeding assays (SMFAs), approximately 50 female An. gambiae (Keele strain) mosquitoes per treatment group (5–6 days old) were allocated to pint-sized cup cages and starved for ~16 h prior to each assay. Plasmodium falciparum (NF54) gametocyte cultures (15–19 days post-initiation) were pelleted and diluted to 0.3% gametocytemia with human blood that had been washed with RPMI and brought up to 40–50% hematocrit with normal AB serum (Interstate Blood Bank, Inc., Memphis, TN). Gametocytemic blood was mixed with pre-immune rabbit IgG (control), rabbit α-AgSGU whole antibody (0.1, 0.2, 0.4, or 0.6 mg/ml) or Fab fragments (0.6 or 0.9 mg/ml) and then delivered to water-jacketed membrane feeders at 37 °C. Fifty mosquitoes per treatment (starved overnight prior to experiment) were allowed to feed for 30–60 min. Midguts were dissected 8 days post-blood feeding, stained with 0.2% mercuricchrome for 15 min, and scored for oocysts with a compound microscope at 200× total magnification. Note that in one SMFA experiment (reported in Fig. 4B), two pre-immune replicates were included and the oocyst counts for each were pooled prior to making comparisons with α-AgSGU treatments.

Direct membrane feeding assays (DMFAs) were performed using blood from gametocyteic volunteers infected with P. falciparum or Plasmodium vivax collected from patients in the Bongti, Thasao, and Lumsum malaria clinics in Kanchanaburi Province, Thailand. The protocol for the use of parasite-infected blood from volunteers (TEMEC 11-033) was approved by the Ethics Committee of the Faculty of Tropical Medicine of Mahidol University, Thailand (Approval # MUTM 2011-040-01) and by the Johns Hopkins Bloomberg School of Public Health Institutional Review Board (protocol #IRB00001629). Infected blood-cell pellets were washed with RPMI and resuspended with human AB serum alone or mixed with rabbit α-AgSGU antibodies (0.3, 0.4, or 0.6 mg/ml) at 40% hematocrit, and fed to ~100 An. dirus mosquitoes per treatment (starved using random hexamers (Fermentas, Waltham, MA). Real-time quantitative PCR (qRT-PCR) was performed using SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA) and the StepOnePlus Real-Time PCR System (Applied Biosystems). AgSGU was amplified with gene-specific primers AgSGU F2 5'-GGATCC-GGTAGCTGCTGT-3' and AgSGU R2 5'-GATAGTGGACGAGCTGATGC-3'. An. gambiae ribosomal protein L32 (AgRpL32; AGAP002122) was amplified with the primers AgRpL32 F 5'-GCGGAAGATTGTAAGAACG-3' and AgRpL32 R 5'-GACGTGTTGGACGAGGAAC-3' to serve as an internal control for normalization of cDNA templates. All qRT-PCR reactions were performed in triplicate.

overnight prior to experiment) for 30 min using water-jacketed membrane feeders at 37 °C. Mosquito midguts were dissected 7 (P. vivax) or 9 (P. falciparum) days after blood feeding, stained with 0.2% mercuricchrome for 15 min, and scored for oocytes with a compound microscope at 200× total magnification. Due to low numbers of malaria cases at the field clinics, only one isolate of P. falciparum and four isolates of P. vivax from infected individuals were used in DMFA experiments. Two of the P. vivax experiments failed to generate infections in mosquitoes, so data from only two of those experiments are reported in the Section 3. Differences in oocyst intensity between control and α-AgSGU groups for both SMFA and DMFA experiments were analyzed by nonparametric statistical analysis using the Mann–Whitney U test (z = 0.05, one tailed) using GraphPad Prism (v5) software.

2.8. Enzyme-linked immunosorbent assay (ELISA)

The presence of IgG and IgM was detected in rabbit α-AgSGU whole antibody and Fab fragments by enzyme-linked immunosorbent assay (ELISA). Maxisorp 96-well ELISA plates (Nunc, Fisher Scientific) were incubated overnight at 4 °C with recombinant AgSGU at 1 μg/ml in 100 mM Na2CO3/100 mM NaHCO3 buffer and washed three times with PBS. Plates were blocked for 1 h at room temperature with 1:1 solution of 5% milk and 1% gelatin and probed with rabbit α-AgSGU antibody (10 μg/ml) or Fab fragments (10 μg/ml) diluted in blocking buffer for 1 h at room temperature. Plates were washed five times with PBS plus Tween-20 (0.05%) (PBST20), followed by incubation with 100 μl per well of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (KPL, Gaithersburg, MD) at 1:1000 in blocking buffer, or mouse anti-rabbit IgM (BD Biosciences, San Jose, CA) at 1:3000 followed by HRP-conjugated goat anti-mouse IgG (KPL) at 1:5000. Plates were washed five times with PBST20 and developed by adding 100 μl of TMB (3,3′,5,5′-tetramethylbenzidine) peroxidase substrate (KPL) to each well. Development was stopped after 5 min by the addition of 100 μl of TMB Stop solution (KPL), and plates were read at 450 nm using a SpectraMax 384 Plus plate reader (Molecular Devices, Sunnyvale, CA) and analyzed by SOFTmax Pro 5.3 software. Anti-AgSGU absorbance readings were normalized against that of normal rabbit IgG, IgM, or Fab isotype controls.

3. Results and discussion

3.1. In silico analyses suggest conservation of AgSGU among mosquitoes and dipterans with notable exceptions

AgSGU is a relatively short, intronless gene (507 bases) on the X chromosome of An. gambiae coding for an 18.1 kDa protein highly expressed in the midgut epithelium (Parish et al., 2011) and found in the peritrophic matrix (Dinglasan et al., 2009). The molecular function of the protein is unknown and a search of its amino acid sequence against the Conserved Domain Database using RPS-BLAST failed to identify a single domain with known function. The protein has identifiable homologs in other mosquitoes and insects in the order Diptera (Table 1, Table S1), a diverse group of insects that includes fruit flies and taxa of medical and veterinary importance such as mosquitoes, sand flies, biting midges, and black flies. Mosquitoes belong to the family Culicidae, which is divided into two monophyletic sub-families, Culicinae and Anophelinae. Only the latter includes vectors of human malaria, all of which are found in the genus Anopheles. Currently, Anopheles is divided into seven sub-genera, although the major malaria vectors are restricted to just four, Cellia (Old World), Anopheles (Cosmopolitan), Nyssorhynchus (Neotropical), and Kerteszia (Neotropical) (Harbach, 2013). An gambiens and its sibling species belong to the subgenus Cellia, and BLAST searches of AgSGU against the genomic and/or transcriptomic sequences from other anopheline mosquitoes included in VectorBase returned single putative orthologs for all species with the exception of those in the subgenus Nyssorhynchus, represented only by two species Anopheles darlingi and Anopheles albimanus. Percent identity at the amino acid level among putative orthologs ranged from 59–98% with the greatest similarity in the An. gambiae species complex as expected (Table 1). For the two Nyssorhynchus species, An. darlingi and An. albimanus, the top BLAST hits were only 37.1% and 26.7% identical at the amino acid level, with low alignment scores (<79) and poor E values (E > 0.06) in both cases. Reciprocal BLAST searches of these proteins against the An. gambiae genome returned different genes with greater percent identity than AgSGU indicating that orthology is unlikely. Moreover, neither a transcriptomic (Martinez-Barnette et al., 2012) nor a proteomic study (Ubdai Mohien et al., 2012) of the An. albimanus midgut identified putative orthologs of this protein, suggesting that the AgSGU ortholog in the Nyssorhynchus lineage was either lost or diversified beyond recognition following divergence from the Anopheles-Cellia lineage, which according to mtDNA data occurred approximately 79 million years ago (Moreno et al., 2010). However, caveats that must be considered are that these analyses were based only on two species of the Nyssorhynchus subgenus and that transcriptomic and proteomic data were from colonies that may not fully reflect genomes occurring in nature.

Outside of Anophelinea, homologs were identified in the culicine mosquitoes Ae. aegypti and Culex quinquefasciatus, as well as in the biting midge Culicoides sonorensis, the sand flies Phlebotomus dubosci, Phlebotomus papatasii, and Lutzomyia longipalpis, and in numerous fruit flies (Table S1). Taken together, the analysis of homology suggests that AgSGU has descended from an ancient protein conserved across the order Diptera, including families of the suborder Nematocera, such as Culicidae (mosquitoes), Ceratopogonidae (biting midges), and Psychodidae (sand flies), as well as at least one lineage of the suborder Brachycera, the family Drosophilidae (fruit flies).

3.2. Evolutionary analyses of AgSGU and putative orthologs indicate a prominent role for purifying selection during anopheline diversification

Compared to other mosquito-midgut proteins implicated in Plasmodium ookinete invasion in the literature, the degree of divergence among anophelines is greater than expected for AgSGU (Table 1). Thus, to further analyze the evolution of AgSGU and its putative orthologs among anophelines, we used Datamonkey, the web-based server (www.datamonkey.org) of the HyPhy package (Delport et al., 2010; Pond et al., 2005; Pond and Frost, 2005a), to investigate signatures of selection over the protein’s evolutionary history by estimating ω, or dN/dS, the ratio of the number of non-synonymous substitutions per non-synonymous site to the number of synonymous substitutions per synonymous site. When estimated on a site-by-site basis, this ratio provides insight into the selection pressure experienced by each codon in a gene of interest over its evolutionary history (Pond and Frost, 2005b; Yang and Bielawski, 2000). Ratios equal to 1 indicate neutral evolution, those greater than 1 indicate positive selection, and those less than 1 indicate negative, or purifying, selection (Yang and Bielawski, 2000). Estimates of ω were generated across AgSGU using three separate methods, single-likelihood ancestor counting (SLAC), random effects likelihood (REL), and fixed effects likelihood (FEL). Gene-wide estimates of ω ranged from 0.216835–0.244641, suggesting that negative selection has played a prominent role in AgSGU evolution. On a finer scale, REL failed to identify specific codons under negative selection, while SLAC and FEL identified
Among anophelines, of which 18 correspond to codons with significantly less than 1 identified by both SLAC and FEL (Table 2).

Eighteen additional amino acids are conserved among more than 80% (>12/15) of the anophelines in the alignment, and 3 of these aminos are also under negative selection (Table 2). Considering that structural details and the molecular function of the AgSGU remain unknown, the nature of the highly conserved negatively selected amino acids may provide important clues about the molecular function of this gene. Table 1 includes a list of putative orthologs of AgSGU.

Fig. 1 suggests that 52 out of 152 amino acids (34.2%) are invariant among anophelines, of which 18 correspond to codons with significantly less than 1 identified by both SLAC and FEL (Table 2). Eighteen additional amino acids are conserved among more than 80% (>12/15) of the anophelines in the alignment, and 3 of these are also under negative selection (Table 2).

about AgSGU structure and function. In the secreted version of the protein (i.e., no signal peptide), hydrophobic amino acids are the most common residues, collectively comprising ~53% with valine being the most frequent (23/144, ~16%). Polar amino acids make up one third of the protein (47/144, ~33%), while the remaining residues are charged (20/144, ~14%) and likely to be on the protein surface. Of these amino acid categories, negative selection appears to be operating on 13 hydrophobic, 7 polar, and 5 charged residues (Table 2). Predictions of secondary structure and protein-binding residues using the PredictProtein server (Rost et al., 2004) suggest that AgSGU consists of 8 beta sheets and a single alpha helix (Fig. 1). Twenty-two amino acids within the peptide sequence were predicted to take part in protein–protein interactions, of which 20 were invariant among the anophelines investigated (Kato et al., 2006), suggesting a role for AgSGU in the salivary glands removed (Neira Oviedo et al., 2009). These data, as well as evidence for negative selection (Fig. 1, Table 2), are noteworthy that both threonines are flanked by lines investigated. Although not predicted to be glycosylated (see Section 3.3), it is noteworthy that both threonines are flanked by at least one proline in the majority of species in the alignment, which increases the likelihood of O-linked, or mucin-type, glycosylation in Drosophila (Gerken et al., 2008). Thus, as an alternative to protein binding, conservation at these two residues may instead be due to important sites of post-translational modification. Interestingly, of the remaining amino acids predicted to take part in protein binding, 14 of 20 were relatively less conserved among the anophelines investigated (Fig. 1) and were mostly polar or charged. These data, along with evidence for negative selection predominantly occurring on hydrophobic residues, suggest that the protein’s interactive motifs have been more evolutionarily flexible while purifying selection has maintained the protein’s general structure.

Table 2

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<th>Codon Position</th>
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⁴ For each codon position, all amino acids present in the alignment are provided with the frequency indicated by the subscript.

³ Amino acids were categorized into three broad groups based on physicochemical properties of side chains (Wolfenden et al., 1981): hydrophobic - A, F, G, I, L, P, V, W; polar – C, H, M, N, Q, S, T, Y; charged – D, E, K, R.

3.3. AgSGU expression data suggest conserved roles in physiology of blood feeding

Published microarray data indicated that AgSGU transcription in An. gambiae adult females was down-regulated 29.9-fold at 3 h post-blood feeding relative to the non-blood fed condition (p < 0.001) (Marinotti et al., 2006). However, at 24 h post-blood feeding, transcription partially rebounded and was up-regulated 9.8-fold (p < 0.001) relative to the previous time point. At 48 h the upward trend continued with a 2.2-fold increase (p = 0.0019), but transcription plateaued thereafter with no significant change at 72 or 96 h post-blood feeding, and a small but significant increase of 1.7-fold 15 days after taking a blood meal (p = 0.016) (Marinotti et al., 2006). Moreover, in a comprehensive analysis of the adult tissues in An. gambiae, AgSGU was most highly expressed in the female midgut (Baker et al., 2011), and was also the second most abundant protein detected in the peritrophic matrix (PM) proteome (Dinglasan et al., 2009), which forms after the mosquito has taken a blood meal. This indicates that despite a reduction in transcript, this protein is highly abundant in the midgut following the ingestion of blood. Together, these data suggest that the transcript is translated immediately in response to blood feeding, which is in line with the association of AgSGU with the PM. Transcription is then likely initiated within 24 h after blood feeding to replenish mRNA transcript in preparation for subsequent blood meals. An increase in transcription levels of AgSGU was also found in larval salivary glands relative to the whole larvae with the glands removed (Neira Oviedo et al., 2009). These data, as well as homology with salivary-gland proteins empirically identified in sand flies (Kato et al., 2006), suggests a role for AgSGU in the salivary glands or saliva, in addition to the midgut.

Although AgSGU lacks a transmembrane domain, it is predicted to have a GPI-anchor according to both algorithm-derived (GPI-SOM) and empirical data, as it was identified in two preparations of the GPI-anchored An. gambiae midgut proteome (<1% False Discovery Rate, 2 unique peptides) (Parish et al., 2011). A GPI-anchor on AgSGU provides further support for its presence in lipid rafts, since GPI-anchored proteins are enriched in lipid rafts (Simons and Ikonen, 1997). Finally, AgSGU is predicted to lack either N- or O-linked glycans as determined by the glycosylation prediction programs NetOGlyc (Steenoft et al., 2013; Gerken et al., 2008). Thus, as an alternative to protein binding, conservation at these two residues may instead be due to important sites of post-translational modification. Interestingly, the remaining amino acids predicted to take part in protein binding, 14 of 20 were relatively less conserved among the anophelines investigated (Fig. 1) and were mostly polar or charged. These data, along with evidence for negative selection predominantly occurring on hydrophobic residues, suggest that the protein’s interactive motifs have been more evolutionarily flexible while purifying selection has maintained the protein’s general structure.

Given the above, AgSGU cannot be called a glycoprotein since the GPI anchor appears to be the only empirically supported glycosylation on this protein. Following convention, it is therefore referred to as a glycoconjugate.

3.4. Empirical evidence for AgSGU expression in the An. gambiae midgut

To confirm the presence of AgSGU in the midgut and begin investigating its function, midgut proteins were resolved by SDS–PAGE and antibodies against full-length recombinant AgSGU were used for immunoblotting. A distinct protein doublet with a more prominent lower band was observed at an apparent Mr of ~14–16 kDa under reducing conditions (Fig. 2B and C), corresponding to the Mr of the predicted size of AgSGU without a signal sequence (15.5 kDa). These data provide additional support for the presence of a GPI-anchor, since the signal peptide is cleaved upon GPI attachment (Englund, 1993). Some GPI-anchored proteins undergo shifts in mobility causing either an apparent increase or, in some cases, a decrease in Mr compared to what is predicted due to the lipid anchor altering protein conformation or binding to SDS (Eugster et al., 2007; Li et al., 2007; Luders et al., 2003). Since AgSGU is aglycosylated except for the anchor, we suspect that the ~16 kDa band in the SDS–PAGE is AgSGU with a GPI-anchor, while the lower band is without anchor. This could be due to a simple loss of the GPI anchor or its targeted removal for the secondary release of the secreted glycoconjugate from the midgut surface.

Contrary to what we observed in An. gambiae midgut lysates, only a faint single band at ~35 kDa was observed in the salivary gland lysate (Fig. 2B). Because similarly faint bands were seen in both abdomen (minus midguts) and thorax (minus salivary glands) lysates, the salivary-gland band is likely due to cross reactivity. Thus, we find no definitive evidence for protein expression of AgSGU in adult female salivary glands, results congruent with a published proteome for this tissue in An. gambiae (Kalume et al., 2005) despite its transcription in the salivary glands of larvae (Neira Oviedo et al., 2009).

A comparative immunoblot analysis of midgut lysates from An. gambiae, Anopheles stephensi, An. dirus and Ae. aegypti support homology as predicted by in silico analyses. AgSGU appears as a
protein doublet in both *An. gambiae* and *An. stephensi* at ~14–16 kDa with the lower band being much more prominent (Fig. 2C). For *An. dirus* sample, a ~15 kDa band is present but very faint. However, across all samples, faint bands are consistently observed from ~35–55 kDa, which may indicate AgSGU SDS-resis-tant complexes or simply the presence of cross-reactive proteins. It is noteworthy that the signal for *An. dirus* was not as strong as it was for *An. stephensi* despite similar percent amino acid identities with *An. gambiae*, suggesting differences in protein abundance between species or variation in the sequence of the epitope recog-nized by the antibodies. As described above no ortholog to AgSGU was found in the midgut transcriptome (Martinez-Barnetche et al., 2012) or proteome (Ubaida Mohien et al., 2012) of the neotropical mosquito, *An. albimanus* (subgenus Nyssorhynchus). Nevertheless, the confirmed presence of AgSGU in the midguts of Old World malaria vectors from the subgenus *Cellia* (Fig. 2C), as well as predicted conservation among other vectors from subgenera *Cellia* and *Anophelles*, suggests an important role for the protein in mosqui-to physiology and perhaps in vector-parasite interactions. AgSGU shows a considerable degree of conservation with four *Ae. aegypti* proteins: AAEL009985 and AAEL013885 (Table S1). The full-length homologs of *Ae. aegypti*, AAEL009985, AAEL013885, AAEL004809, and AAEL013885, are 17.12 kDa, 17.15 kDa, 16.44 kDa, and 16.55 kDa, respectively (Table S1). In the immuno-blot we observed a faint low Mr, band in *Ae. aegypti* close to the pre-dicted M, of all four homologs, as well as more prominent bands at 35 and 45 kDa (Fig. 2C). Although the higher molecular weight bands may also indicate multi-protein complexes in *Aedes*, we again cannot rule the fact that the antibody is simply cross reacting with other proteins. Further characterization of these homologs in the biology of *Aedes* mosquitoes may help elucidate the function of AgSGU in *An. gambiae*.

Secretion to the membrane surface is one function of the GPI post-translational modification (Englund, 1993), but it is unclear if AgSGU is further secreted following the cleavage of the GPI-anchor or if it remains associated with the extracellular matrix through interactions with surface glycoproteins. A *bona fide* GPI anchor would indicate localization of AgSGU to the midgut micro-villi (MV) on the luminal surface. Indeed, AgSGU was found to be present in the *An. gambiae* midgut luminal brush border microvilli (BBMV) proteome (Ubaida Mohien et al., 2012). Immunofluores-cence microscopy of sugar-fed *An. gambiae* midgut cryosections indicates the presence of AgSGU on the MV and surprisingly, also on the midgut basal surface (Fig. 2D). As expected from our previous work, normal rabbit IgG does not stain midgut sections (Fig. S1). Therefore, several orthogonal lines of evidence supports the presence of AgSGU on the apical membrane and its association with lipid rafts, which primarily localize to the apical plasma membrane of polarized cells. Additionally, the presence of AgSGU on the basal side of the midgut suggests a different, albeit unknown function on the basal lamina extracellular matrix.

3.5. AgSGU expression throughout mosquito development

To further characterize AgSGU expression, both immunoblot and quantitative RT-PCR (qRT-PCR) analyses of anopheline midgut lysates at various life stages, and adult ages were performed (Fig. 3). AgSGU transcript expression in sugar-fed mosquitoes appears to peak at ~10 days post-eclosion, and then decreases incrementally (Fig. 3A). Protein expression, however, is highest at the beginning of the female mosquito’s adult life and decreases until 16 days post-emergence, when it appears to stay relatively stable for its remaining life span (Fig. 3B). This discordance in transcript and protein expression in *An. gambiae* suggests that transcription occurs either in the pupal stage or within the first two days following eclosion. The protein is then expressed in the

emerged adult, perhaps in preparation for the first blood meal. In the absence of blood feeding, transcripts likely accumulate in the midgut, while protein levels slowly decrease over time. In contrast, protein expression in *An. stephensi* midguts is relatively stable with little variation over the adult life span (Fig. 3D), although transcript expression peaks later at greater relative levels than *An. gambiae*, near day 17 (Fig. 3C). Interestingly, relative protein expression is similar at day 3 for each species but quite distinct thereafter. These differences in relative transcript and protein expression between *An. gambiae* and *An. stephensi* may indicate differences in AgSGU function between the two species, or may reflect variation in expression of this protein during senescence.

Quantitative RT-PCR (Fig. 3E) and quantitative immunoblots (Fig. 3F) of AgSGU pre- and post-blood feeding (8 h, 24 h and 48 h) show that levels of both RNA and protein are comparatively higher prior to blood feeding, diminish immediately after the blood meal, and then slowly rise back to pre-blood fed levels as digestion progresses. These data support the previously reported transcriptomic data discussed above (Marinotti et al., 2006), and in combination with data from the peritrophic matrix (PM) proteome, suggest that AgSGU has a role in midgut physiology related to blood feeding. In the PM study, AgSGU was identified as the second most abundant protein present in all fractions of the PM (Dinglasan et al., 2009), suggesting the presence of AgSGU during blood feeding, and a possible direct or indirect role in PM structure. This is not unexpected, since many midgut-surface proteins associated with the PM in blood-fed females are apparently sheared off the midgut microvillar surface and then regenerated after blood feeding, indicating that the majority of AgSGU may be purged with the digested blood meal (Klowden, 2007; Parish et al., 2011). Importantly, data from quantitative immunoblots in Fig. 3F are from blood fed midguts with the blood meal and PM removed, and thus represents protein that is present only in the midgut tissues themselves. Following a blood meal, it is therefore unlikely to find abundant levels of AgSGU in the midgut tissue until its epithelial surface is regenerated.

To confirm this hypothesis the localization of AgSGU in blood fed midguts was examined by immunofluorescence microscopy on midgut cryosections from mosquitoes dissected 24 h post-blood feeding (Fig. 3G). AgSGU is present on the apical MVsurface, on the midgut basal lamina and at the interface of the blood meal and the apical midgut epithelium, which roughly corresponds to the PM interface. As with cryosections from sugar-fed midguts, normal rabbit IgG does not stain sections from blood-fed mosquitoes (Fig. S1). These staining patterns are consistent with those observed for the localization of AgAPN1 (Dinglasan et al., 2007) and AgMUC1 (Devenport et al., 2005), both of which are *bona fide* apical microvillar surface proteins, as well as Ag-Aper-14 (Devenport et al., 2005), which was independently confirmed to be a PM protein (Dinglasan et al., 2009).

These data demonstrate that AgSGU is present and accessible during ookinete invasion, and is consistently expressed throughout the mosquito life span, although it is unclear whether it is bound to the PM or not. The PM is thickest at 24 h (Fig. 3G) and while there is previous proteomic evidence for its presence, AgSGU may simply be intercalated into the PM and not necessarily attached to it.

3.6. Inhibition of *P. falciparum* oocyst formation by \(\alpha\)-AgSGU polyclonal antibodies (PAbS)

Due to the presence of AgSGU in lipid rafts on the mosquito midgut, and its putative role in blood feeding, we hypothesized that if AgSGU has a role in ookinete attachment to the midgut, then \(\alpha\)-AgSGU PAbS may prevent *Plasmodium* ookinete invasion. Concentrations of 100, 200, 400 and 600 \(\mu\)g/mL of antigen-specific PAbS significantly reduced *P. falciparum* oocyst formation in *An.
gambiae mosquitoes, resulting in 40–100% inhibition of median oocyst intensity ($P < 0.05$) with reductions in mosquito infection prevalence of ~20–42% across groups (Fig. 4A–C) and no apparent reductions in vector survivorship at the time of dissection (8 days post-blood feeding). Inhibition by PAbs implies either a direct or indirect role for AgSGU in Plasmodium ookinete invasion. High concentrations necessary for inhibition may be indicative of high levels of AgSGU present in the midgut that neutralize or sequester PAbs prior to reaching lipid rafts, or an abundance of the AgSGU on the midgut surface during ookinete invasion.

Inhibition of ookinete invasion by $\alpha$-AgSGU PAbs could occur through various mechanisms. For example, AgSGU may interact with a portion of ookinete-interacting proteins on lipid rafts, such as AgAPN1 (Mathias et al., 2012; Ubaida Mohien et al., 2012), and prevent ookinete inhibition by steric hindrance of the ookinete-interacting protein; or the PAbs may efficiently diffuse through the blood meal and bind to AgSGU on lipid rafts and prevent lipid raft clustering.

Immunizing mice with mosquito-midgut lysate elicits a repertoire of antibodies that have transmission-blocking or reducing activity against murine and human malaria parasites (Almeida and Billingsley, 2002; Lal et al., 2001). Midgut proteins that are recognized by mouse antisera (Almeida and Billingsley, 2002) or the monoclonal antibody MG25E (Lal et al., 2001) span a large
spectrum of molecular masses from ~15 to 200 kDa, but to date the identities of these proteins remain unknown. It should be noted that MG25E exhibited a multi-banding profile by immunoblot, suggesting that like α-AgSGU and α-AgAPN1 (Dinglasan et al., 2007), MG25E may also see a multimer of the same protein. Only seven bona fide ookinete-interacting, apical midgut-surface proteins have been identified to date (Parish et al., 2011; Vega-Rodriguez et al., 2014) and of these, only two proteins from An. gambiae have thus far been shown to be potent transmission-blocking vaccine antigens: carboxypeptidase B, CPBAg1 (Lavazec et al., 2007) and alanyl aminopeptidase N, AgAPN1 (Dinglasan et al., 2007; Mathias et al., 2012; Armistead et al., 2014).

Fig. 4. α-AgSGU antibodies inhibit parasite invasion in laboratory and field Plasmodium falciparum isolates but not Plasmodium vivax. (A-C) α-AgSGU PAbs inhibit Plasmodium falciparum laboratory isolates in three independent standard membrane feeding assays (SMFAs). Each panel shows P. falciparum oocyst counts in Anopheles gambiae mosquitoes fed on gametocytic blood along with CTRL (pre-immune IgG) or α-AgSGU antigen-specific antibodies. (A) α-AgSGU inhibits parasite invasion at 100 and 400 µg/mL. (B) α-AgSGU inhibits parasite invasion at 200 and 400 µg/mL. (C) α-AgSGU inhibits parasite invasion at 200 and 600 µg/mL. (D) α-AgSGU inhibits parasite invasion in P. falciparum field isolates. SMFA showing P. falciparum oocyst counts in An. dirus mosquitoes fed on gametocytic blood with CTRL (human AB serum) or α-AgSGU antibodies (300 and 600 µg/mL). (E, F) α-AgSGU does not block parasite invasion in P. vivax field isolates. SMFA showing P. vivax oocyst counts in An. dirus mosquitoes fed gametocytic blood with CTRL (human AB Serum) or α-AgSGU antibodies (400 and 600 µg/mL). N: numbers of mosquitoes dissected per group (Sample Size); Range: range of oocyst numbers per midgut observed for each group; Prevalence: percent of blood fed mosquitoes in which at least one oocyst developed; Inhibition: reduction in median oocyst intensity in the treatment group relative to the control reported as the % inhibition, calculated as follows: 100 x [(median oocystcontrol – median oocysttreatment)/ median oocystcontrol]. P-values were determined by the non-parametric Mann Whitney U Test (α = 0.05).

Since *P. falciparum* NF54 parasites maintained in the laboratory do not represent naturally circulating *Plasmodium* populations worldwide, we conducted a limited study to determine whether α-AgSGU PAbs would block field isolates of *P. falciparum* equivalently to our laboratory observations. Although the numbers of cases were low in the field site (Kanchanaburi, Thailand) we were able to test one human isolate of *P. falciparum* and two isolates of *P. vivax* from infected individuals in *An. dirus* mosquitoes, a primary *Plasmodium* vector in Southeast Asia. PAbs significantly inhibited oocyst development of local Thai *P. falciparum* at concentrations of 300 and 600 μg/ml (45–74% inhibition, *P* < 0.05 and *P* < 0.0001 respectively) (Fig. 4D), corroborating our laboratory data. Interestingly, α-AgSGU PAbs did not inhibit *P. vivax* oocyst development in *An. dirus* (Figs. 4E and F). These data suggest different invasion strategies by *P. falciparum* and *P. vivax* in the anopheline midgut although our sample size was limited.

One potential explanation for this difference is the speed of oocyst invasion following blood meals. *P. vivax* ookinetes invade within 18–24 h, while *P. falciparum* invasion begins around 24 h post-blood feeding (Baton and Ranford-Cartwright, 2004; Zollner et al., 2006). In *An. gambiae*, AgSGU levels appear lowest in midgut tissue at 8 h post-blood feeding (Figs. 3E and F). Therefore, discordant AgSGU expression during that same time frame may eliminate its potential as a transmission-blocking vaccine candidate for *P. vivax*. At 24 h, AgSGU is clearly present on the midgut surface in addition to the lumen (Figs. 3E–G), and can thus affect *P. falciparum* invasion (Figs. 4A–D). Moreover, if our hypothesis above is correct, it implicates the role of AgSGU in oocyst invasion as a bystander through its lipid raft association with other ookinete-interacting proteins, rather than as a critical component to oocyst invasion. The presence of α-AgSGU IgM in our antisera (Fig. S2) and its potential to cross-link proteins on the midgut surface or influence oocyst invasion by non-specific steric hindrance provides some support to the possible bystander inhibition phenomenon. However, we generated Fab fragments from the AgSGU IgG and IgM and observed a significant reduction in *P. falciparum* oocyst numbers in mosquitoes, albeit only at the 900 μg/ml concentration of Fab (Fig. S2).

### 4. Conclusions

We have shown that AgSGU is a GPI-anchored midgut protein conserved among many of the major vectors of malaria with the likely exception of some in the neotropics (i.e., subgenus *Nyssororhynchus*). The evolutionary history of AgSGU and its putative orthologs has been governed predominantly by purifying selection within 18–24 h, while *P. falciparum* invasion begins around 24 h post-blood feeding (Baton and Ranford-Cartwright, 2004; Zollner et al., 2006). In *An. gambiae*, AgSGU levels appear lowest in midgut tissue at 8 h post-blood feeding (Figs. 3E and F). Therefore, discordant AgSGU expression during that same time frame may eliminate its potential as a transmission-blocking vaccine candidate for *P. vivax*. At 24 h, AgSGU is clearly present on the midgut surface in addition to the lumen (Figs. 3E–G), and can thus affect *P. falciparum* invasion (Figs. 4A–D). Moreover, if our hypothesis above is correct, it implicates the role of AgSGU in oocyst invasion as a bystander through its lipid raft association with other ookinete-interacting proteins, rather than as a critical component to oocyst invasion. The presence of α-AgSGU IgM in our antisera (Fig. S2) and its potential to cross-link proteins on the midgut surface or influence oocyst invasion by non-specific steric hindrance provides some support to the possible bystander inhibition phenomenon. However, we generated Fab fragments from the AgSGU IgG and IgM and observed a significant reduction in *P. falciparum* oocyst numbers in mosquitoes, albeit only at the 900 μg/ml concentration of Fab (Fig. S2).

**Authors’ contributions**

DKM, JGJ, LAP, and HVT carried out the molecular biology studies and analysis of data. DKM performed the analyses of molecular evolution and generated the sequence alignment. JGJ, DKM, LAP, JSA, HVT and CK performed standard and direct membrane feeding assays. JSA performed immunoassays, the preparation and provision of critical reagents and analysis of data. JGJ, RRD, and DKM performed the microscopy. JGJ, LAP and RRD participated in the design of the study and performed the statistical analysis. JGJ and JS participated in the design, coordination and execution of field studies in Thailand. RRD conceived of the study, and participated in its design and coordination. All authors drafted the manuscript with final edits by DKM and RRD.

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2014.05.025.

**References**


