The mosquito midgut ookinete stage of the malaria parasite, Plasmodium, processes microneme secretory organelles that mediate locomotion and midgut egress to establish sporogonic stages and subsequent transmission. The purpose of this study was 2-fold: 1) to determine whether there exists a single micronemal population with respect to soluble and membrane-associated secreted proteins; and 2) to evaluate the ookinete micronemal proteins chitinase (PgCHT1), circumsporozoite and TRAP-related protein (CTRP), and von Willebrand factor A domain-related protein (WARP) as immunological targets eliciting sera-blocking malaria parasite infectivity to mosquitoes. Indirect immunofluorescence localization studies in Plasmodium gallinaceum using specific antisera showed that all three proteins are distributed intracellularly with a similar granular cytoplasmic appearance and with focal concentration of PgCHT1 and PgCTRP, but not PgWARP, at the ookinete apical end. Immunogold double-labeling electron microscopy, using antisera against the membrane-associated protein CTRP and the soluble WARP, showed that these two proteins co-localized to the same micronemal population. Within the microneme CTRP was associated peripherally at the microneme membrane, whereas PgCHT1 and WARP were diffuse within the micronemal lumen. Sera produced against Plasmodium falciparum WARP significantly reduced the infectivity of P. gallinaceum to Aedes aegypti and P. falciparum to Anopheles mosquitoes. Antiseras against PgCTRP and PgCHT1 also significantly reduced the infectivity of P. gallinaceum for A. aegypti. These results support the concept that ookinete micronemal proteins may constitute a general class of malaria transmission-blocking vaccine candidates.

Transmission of malaria occurs after a female mosquito ingests infected blood, thereby initiating subsequent parasite sexual and sporogonic development. Within minutes in response to midgut environmental cues, gametocytes fertilize to form zygotes that transform into motile ookinetes over the following 15–25 h. Mature midgut lumen ookinetes penetrate and traverse the peritrophic matrix and midgut epithelium and then develop into oocysts on the luminal side of the epithelial basement membrane. Strategies to block the transmission of parasites from vertebrate host to mosquitoes seek to interrupt parasite development at some point in the continuum from gametocytes to ookinete penetration of the mosquito midgut epithelium.

The Plasmodium ookinete contains a single type of specialized secretory organelle, the microneme, which is thought to be involved in host-cell recognition, binding, and motility via secretion of soluble and cell surface molecules involved in interaction with different compartments within the mosquito midgut. Ookinete differ from other Plasmodium invasive stages, such as sporozoites and merozoites, in that they lack rhoptry and dense granule organelles. Plasmodium ookinete appear to secrete micronemal contents constitutively, and it has not been determined whether ookinete apical secretion is regulated. The question remains, however, whether the numerous micronemes visible within the apical end of Plasmodium ookinete are functionally equivalent or whether classes of proteins with different destinations are associated with distinct micronemal populations.

Previous studies suggest that soluble ookinete-secreted/released proteins (1) as well as cell surface molecules (2, 3) are transmission-blocking targets. This study focused on the following three micronemal proteins of Plasmodium gallinaceum ookinete: the soluble, secreted chitinase PgCHT1 (4), the membrane-associated protein CTRP (5–9), and the recently described von Willebrand adhesive (vWA) domain-related protein (WARP) (10). In this study we test the hypothesis that micronemal secreted proteins are candidate targets of blocking parasite infectivity for mosquitoes. After verifying that the Escherichia coli-produced recombinant...
proteins had appropriate biological activity and elicited specific antisera, the antibodies were used to determine subcellular localization and transmission blocking activity in mosquito membrane feeding assays. Transmission blocking assays determined the ability of both single sera and combinations to inhibit \textit{P. gallinaceum} ookinetic infectivity for the \textit{Aedes aegypti} mosquito midgut. The results have implications for developing a mechanistic understanding of \textit{Plasmodium} cell biology and parasite-mosquito interactions and suggest that ookinete micronemal proteins may be a general class of malaria transmission-blocking targets.

**EXPERIMENTAL PROCEDURES**

**Parasites—**The \textit{P. gallinaceum} TA strain was maintained by mechanical blood passage and transmission through mosquitoes. Ookinetes were cultured in serum-free, chemically defined M199 medium and were obtained as described (11).

**Membrane Feeding Assays—**Female \textit{A. aegypti} mosquitoes between 5 and 7 days post-emergence from pupae were starved for 24 h and then fed on membrane feeders containing 200 \(\mu\)l of \textit{P. gallinaceum}-infected blood (10–100% parasitemia) plus 70 \(\mu\)l of antisera for 20 min. After feeding, nonengorged mosquitoes were removed, and engorged mosquitoes were maintained with 10% glucose at 26°C and 80% relative humidity. Midguts were dissected without digestion 7 days after feeding. Statistical analysis was done using the Mann Whitney \(U\) test to compare differences in geometric mean oocyst density and the proportion of mosquitoes infected between groups, as described previously (1, 12–15).

**Construct Preparation, Production of Recombinant Protein, and Antibody Preparation—**Enzymatically active recombinant PgCTP1 was produced in \textit{E. coli} as described previously (4). The full-length PgCTP gene was isolated, and the DNA sequence was determined following amplification from \textit{P. gallinaceum} genomic DNA using degenerate PCR primers designed based on conserved regions identified following alignment of \textit{PyC}TP and \textit{PvC}TP. The first vWA domain of PgCTRP—first vWA domain of \textit{PgCTRP} was expressed in \textit{E. coli} and was amplified from \textit{P. gallinaceum} genomic DNA template using gene-specific primers designed based on the full-length \textit{P. gallinaceum} CTRP gene. The primers used are as follows: 5'-GGCCGATGCTGAACTGAA-CAAAAGTAGAATC-3' and 5'-GCGCTGAGCTTTAGCTTGT-GTG-3' (Ncol and Xhol restriction sites are underlined, respectively). The PCR product was cloned into the \textit{E. coli} expression vector, PET22b, and expressed in \textit{E. coli} strain AD494 (DE3) (Novagen). Protein was induced at \(A_{600}\) of 0.6 with 0.1 mm isopropyl-1-thio-\(\beta\)-galactopyranoside at 15°C in a shaking incubator for 16 h. Bacterial pellets were lysed by sonication in a buffer of 20 mm Tris, 50 mm NaCl, 10 mm imidazole, pH 8.0, in the presence of a protease inhibitor mixture (BD Protease Inhibitor cocktail, Roche Applied Science), sodium deoxycholate, and sucrose by sonication. Insoluble debris, and resolved in 4–20% Tris-glycine gradient gels, electroblotted to nitrocellulose membranes, and blocked in PBS containing 5% nonfat milk, 0.01% Tween 20, and incubated with primary antibodies diluted in PBS/milk/Tween 20. Grids were incubated with 5 or 15 nm gold particle-labeled anti-mouse or anti-rabbit IgG conjugated to 105 g/ml collagen type I, collagen type IV, fibrinogen, or 105 g/ml for use as a negative control. For the rWARP binding assays, recombinant (r) GST was expressed and purified by glutathione affinity chromatography as above. For the rPfWARP binding assays, recombinant (r) GST was expressed and purified for use in the ELISA.

**Immunofluorescence Microscopy—**Ookinetes were dried onto 10-well Teflon-coated slides. The samples were blocked and permeabilized by incubation overnight in PBS containing 20% human serum and 3% Triton X-100. The samples were incubated with primary antisera recognizing the first vWA domain of PgCTP and PgWARP or PfCTP and PfWARP for 1 h at room temperature. After washing five times with PBS, the samples were incubated with Alexa Fluor 546 goat anti-rabbit IgG conjugation and Alexa Fluor 488 goat anti-mouse IgG conjugation for 30 min at room temperature and washed five times with PBS and one time with water. Cellular immunolocalization was examined by using an Olympus BX51 fluorescence microscope. As negative controls, preimmune mouse and rabbit sera were used as primary antibody.

**Immunoelectron Microscopy—**Cultured ookinetes were fixated in 1% paraformaldehyde, 0.1% glutaraldehyde in PBS (pH 7.4) and embedded in LR White (Ladd Research Industries, Burlington, VT). Sections were double-stained with 100 g/ml collagen type I, collagen type IV, fibrinogen, or 100 g/ml for use in the ELISA. Binding of recombinant protein was assessed by using alkaline phosphatase-conjugated rabbit anti-GST (Amersham Biosciences), with para-nitrophenyl phosphate (Sigma) as substrate, and \(A_{405}\) measured in a HT37000 microplate reader (Hewlett-Packard).

**Western Immunoblot—**For Western immunoblot (nonreducing, denaturing), \textit{P. gallinaceum} ookinetes were heated (5 min, 90°C) in sample buffer (25 mm Tris, pH 6.8, 2.2% (w/v) SDS, 15% (v/v) glycerol, 0.01% (w/v) bromophenol blue), centrifuged (10,000 \(\times\) g, 5 min) to remove intact membranes and resolved in 4–20% SDS-polyacrylamide gels in 0.1% (w/v) Triton X-100 containing 5% (w/v) nonfat milk (NDM). Separated proteins were electroblotted to nitrocellulose membranes by using the NOVEX Xcell Blot II module. After blocking with Buffer A (0.15 mm NaCl, 0.1 mm Tris, pH 7.5) containing 5% (w/v) nonfat milk (NDM), blots were incubated (22°C, 1 h) with primary antibodies in Buffer B (Buffer A containing 0.1% (w/v) SDS, 0.1% (v/v) Triton X-100) containing 5% (w/v) NDM. Specificity of antibodies was confirmed using preimmune sera as negative controls. After washes (5 min each) with Buffer B containing NDM, blots were incubated (22°C, 1 h) with alkaline phosphatase-conjugated goat anti-mouse IgG/M/A (Kirkegaard & Perry) 1/2000 in Buffer B containing NDM. Blots were washed three times with Buffer B containing NDM, three times with water, and finally incubated in 100 mm Tris, pH 9.0, containing 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium alkaline phosphatase substrate (Kirkegaard & Perry).

**Purification and Identification of \textit{P. gallinaceum} WARPS—**Pooling culture supernatants and soluble \textit{P. gallinaceum} ookinetes extracts were subjected to high pressure liquid chromatography under the conditions described (4). Fractions were size-fractionated by SDS-PAGE in 4–20% gel (NOVELX gels, Invitrogen), which was stained with Coomassie Blue R-250 (Bio-Rad) and destained for 4 h with Gel-Stain Destain Solution (NOVELX) with three changes of destaining solution. Stain bands were excised from the gel, rinsed twice with 50% acetonitrile in HPLC-grade water, and frozen on dry ice. Subsequent protein sequences were determined at the Howard Hughes Medical Institute Protein Sequencing Laboratory (Cambridge, MA). The band was subjected to in-gel reduction, S-carboxy-amidomethylation and tryptic digestion (Promega), and a 10% aliquot of the resultant mixture was analyzed. Sequence information was determined by capillary (180 \(\mu\)m \(\times\) 15 cm column, LC.
Novel Ookinet Microneme Transmission-blocking Antigens

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Identification of WARP in the Ookinete Stage—As part of a previous study of *P. gallinaceum* ookinete-secreted chitinases (4), an unidentified 220-kDa Coomassie Blue-stained protein band was nearly co-purified through multiple chromatographic steps from *P. gallinaceum* ookinete lysates combined with spent culture supernatant by SDS-PAGE (Fig. 1). This band was excised and subjected to in-gel tryptic digestion followed by HPLC separation. Amino acid sequences of two peptides were determined by *de novo* MS/MS sequencing as follows: one with sequence (L)D(L)E(L)V(G)QG(L)GKNK, and the other with sequence V(L)n(F/M)YGDDYGANK. BLAST search of the *Plasmodium* genome data bases demonstrated that these peptide sequences belonged to a new gene encoding a protein containing a predicted secretory signal sequence (Fig. 2, underlined amino sequences) followed by a single vWA domain. This family of *Plasmodium* proteins was recently identified by computational genome analysis and a subtractive hybridization approach (10, 21), demonstrated to be present within *P. berghei* micronemes, and named WARP (10). WARP is highly conserved between *Plasmodium* species (Fig. 2). WARP is the third vWA domain-containing protein, after TRAP and CTRP (Fig. 3), to be described as a *Plasmodium* mosquito stage protein (Fig. 2). In contrast to TRAP and CTRP, WARP notably lacks a transmembrane domain and is either secreted or remains associated with the surface membrane via interaction with membrane-associated proteins.

A *P. falciparum* WARP cDNA was amplified by reverse transcriptase-PCR from total RNA of *in vitro* cultivated *P. falciparum* gametocytes. Comparison with *P. falciparum* genomic sequence information showed no nucleotide sequence differences and indicated that the gene is encoded on a single exon. Alignment of the predicted amino acid sequences of the WARP proteins from *P. falciparum*, *P. gallinaceum*, *Plasmodium berghei*, *Plasmodium yoelii*, *Plasmodium knowlesi*, and *Plasmodium vivax* shows significant cross-species conservation of sequence (PyWARP versus PfWARP 62% identical; PfWARP versus PyWARP 58% identical; and PyWARP versus PvWARP 67% identical), including rigorous conservation of seven cysteines (indicated by an asterisk above the cysteines in Fig. 2). The four most carboxyl-terminal cysteines delineate the vWA domain and are conserved with the vWA domain of the *Plasmodium* TRAP gene. The amino-terminal regions (in PyWARP, amino acids 25–71) of all WARP proteins share significant sequence similarity, but similar proteins were not identified in GenBank™ (nonredundant) via reiterative PSI-BLAST queries. This region contains an odd number of conserved cysteines, one of which lies within a nonconserved proline-rich low complexity region (in PyWARP, amino acids 69–90) that separates the conserved amino-terminal region and the vWA domain.

**RESULTS**

Identification of WARP in the Ookinete Stage—As part of a previous study of *P. gallinaceum* ookinete-secreted chitinases (4), an unidentified 220-kDa Coomassie Blue-stained protein band was nearly co-purified through multiple chromatographic steps from *P. gallinaceum* ookinete lysates combined with spent culture supernatant by SDS-PAGE (Fig. 1). This band was excised and subjected to in-gel tryptic digestion followed by HPLC separation. Amino acid sequences of two peptides were determined by *de novo* MS/MS sequencing as follows: one with sequence (L)D(L)E(L)V(G)QG(L)GKNK, and the other with sequence V(L)n(F/M)YGDDYGANK. BLAST search of the *Plasmodium* genome data bases demonstrated that these peptide sequences belonged to a new gene encoding a protein containing a predicted secretory signal sequence (Fig. 2, underlined amino sequences) followed by a single vWA domain. This family of *Plasmodium* proteins was recently identified by computational genome analysis and a subtractive hybridization approach (10, 21), demonstrated to be present within *P. berghei* micronemes, and named WARP (10). WARP is highly conserved between *Plasmodium* species (Fig. 2). WARP is the third vWA domain-containing protein, after TRAP and CTRP (Fig. 3), to be described as a *Plasmodium* mosquito stage protein (Fig. 2). In contrast to TRAP and CTRP, WARP notably lacks a transmembrane domain and is either secreted or remains associated with the surface membrane via interaction with membrane-associated proteins.

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**Biological Activity of Recombinant PgCHT1, PfWARP, and the First PgCTRP A Domain—Recombinant PgCHT1, Pf-WARP, and the PgCTRP vWA domains were assayed for binding activity, in part to assess if protein was properly folded after expression in *E. coli*. Recombinant PgCHT1 was able to hydrolyze 4-methylumbelliferone chitotrioside, as reported previously (4). In a solid phase assay, *E. coli*-expressed recombinant PfWARP (Fig. 4) and the first vWA domain of PgCTRP (Fig. 5) (expressed according to the schematic in Fig. 3), were tested for their ability to bind to a variety of components found in intercellular matrices. Recombinant *P. falciparum* TRAP was used as a positive control in these assays; either recombinant GST or an irrelevant His₆-tagged protein (PfCHT1) were used as negative controls. Recombinant PfWARP (Fig. 4) and the PgCTRP first vWA domain (Fig. 5) did not demonstrably bind to collagen, fibrinogen, or, as a negative control, bovine serum albumin. Both proteins had significant binding to heparin conjugated to bovine serum albumin, suggesting specific binding to a highly negatively charged carbohydrate-type ligand. Although these data do not demonstrate the *in vivo* ligand recognized by these proteins, they suggest that the recombinant proteins are properly folded and bind to an appropriate matrix substrate that might be predicted to be present on target cells/basement membrane encountered during ookinete transit through the midgut wall.

**In Vivo Expression of PgCHT1, PgCTRP, and PgWARP in *P. gallinaceum* Ookinetes—Western immunoblotting assays were performed to determine whether hyperimmunized mouse polyclonal antisera recognized *P. gallinaceum* ookinete-produced, native PgCHT1, PgCTRP, and PgWARP. An appropriately sized band was detected for all three proteins (Fig. 6). As predicted, the proteins were not detected in 3–4-h-old zygotes (Fig. 6); the parasite stage formed immediately after gamete fertilization but appeared in ookinetes, which begin to differentiate 10–15 post-fertilization. PgWARP was detected as a high molecular mass protein (∼250 kDa) under nonreducing conditions; under reducing conditions, the protein appeared as ∼45 kDa. This observation strongly suggested that the anti-WARP antiserum did not cross-react with the high molecular weight, monomeric protein PgCTRP, despite the presence of...
multiple vWA domains in this high molecular protein. Furthermore, this observation suggests that \(P_{gWARP}\) exists in the high molecular weight complex as a disulfide-bonded homo- or heteromultimer.

Two-color immunofluorescence microscopy was performed to determine \(P_{gCHT1}\), \(P_{gCTRP}\), and \(P_{gWARP}\) localization within mature \(P. gallinaceum\) ookinetes and to provide insight into the overall intracellular distribution and spatial relationships of these three proteins (Fig. 7). \(P_{gCHT1}\) and \(P_{gCTRP}\) were present in a granular appearance throughout the ookinete cytoplasm, with notable concentration at the apical end of the parasite. When visualized simultaneously with \(P_{gCHT1}\), \(P_{gWARP}\) was also present in a granular pattern throughout the cytoplasm, but with less focal concentration at the apical end of the parasite in comparison with the appearance of \(P_{gCHT1}\) and \(P_{gCTRP}\). These findings were reproducible in more than 40 morphologically mature ookinetes examined. As a negative control, polyclonal antisera raised against \(E. coli\)-produced recombinant His6-tagged \(P. vivax\) chitinase \(PvCHT1\) (from inclusion bodies; an irrelevant antigen because there was no reactivity to the protein on Western immunoblot (data not shown)) produced no signal by immunofluorescence microscopy.

**FIG. 2.** Alignment of \(WARP\) proteins from \(P. falciparum, P. gallinaceum, P. yoelii, P. berghei, P. vivax,\) and \(P. knowlesi\). Identically conserved amino acid residues are indicated by an asterisk; highly conserved amino acid residue by a colon, and weakly conserved by a period. An 85% consensus sequence is shown; identical residues are shown in capital letters. Below the protein sequences are consensus positions identified as follows: s, amino acids with small side chains (Ala, Gly, Ser, Pro, Asp, Asn, and Val); a, amino acids with aromatic side chains (Phe, Tyr, and Trp); p, amino acids with polar side chains (Lys, Arg, His, Glu, Asp, Gln, and Asn); h, amino acids with hydrophobic side chains (Leu, Ile, Val, Met, Tyr, Phe, Trp, and Ala); l, amino acids with aliphatic side chains (Ala, Leu, Ile, and Val); c, amino acids with charged side chains (Glu, Asp, Lys, His, and Arg); t, amino acids with basic side chains (Lys, His, and Arg); and u, amino acids with tiny side chains (Gly, Ala, and Ser). Predicted signal peptide sequences are indicated in **boldface** at the amino terminus. Experimentally determined peptides initially identifying the protein as \(P_{gWARP}\) are underlined.
staining immunoelectron microscopy and morphological appearance of the micronemes. To investigate whether another soluble protein, \( \text{PgCHT1} \), was present in a similar or distinct population of micronemes compared with CTRP, dual immunogold electron microscopy was performed on \( P. \text{gallinaceum} \) ookinetes (Fig. 8). CTRP, a protein with a predicted single transmembrane \(/H9251\)-helix, was found in a circumferential “rim” distribution within micronemes concentrated at the ookinete apical end (large (15 nm) gold dots). In contrast, \( \text{PgCHT1} \), a protein that is not cell surface-associated and lacks predicted transmembrane domains, was found soluble within the central lumen of the same micronemes as those containing CTRP (small (5 nm) gold dots). Similarly, \( \text{PgCHT1} \) and \( \text{PgWARP} \) are both secreted in close proximity through the electron-dense area of the apical complex and appear within the same micronemes (\( \text{PgCHT1} \) labeled with large (15 nm) gold dots; \( \text{PgWARP} \) labeled with small (5 nm) gold dots) (Fig. 9, inset). \( \text{PgWARP} \), despite the absence of identifiable transmembrane domains or glycosylphosphatidylinositol-anchoring motifs, appears to be present on the ookinete cell surface, including at the posterior end of the parasite. These observations suggest that there is likely a single pathway for extracellular secretion of proteins through the micronemal pathway and that the same set of micronemes transports soluble, membrane-associated, and complexed proteins for secretion through the electron-dense region of the ookinete apical complex.

Effect of Antibodies against Micronemal Proteins on the Infectivity of \( P. \text{gallinaceum} \) for \( A. \text{aegypti} \) Mosquitoes—Membrane feeding experiments were performed to test the hypothesis that the micronemal proteins chitinase, WARP, and CTRP would be immunological targets of blocking malaria transmission. Antisera to all three \( P. \text{gallinaceum} \) proteins significantly inhibited the formation of oocysts in \( A. \text{aegypti} \). Polyclonal anti-\( \text{PgCHT1} \) antisera reduced the number of oocysts by 67–96% and the proportion of infected mosquitoes by 37–76% (Table I), statistically strongly significant compared with both infectious blood meal sham controls and with negative control antisera obtained from mice vaccinated with two different irrelevant proteins (administered with Freund’s adjuvant to control for the effects of the vaccination procedure). Polyclonal anti-\( \text{PgCTRP} \) antisera reduced the number of oocysts by 78–88% and the proportion of mosquitoes infected by 38–68% (Table II). Polyclonal \( \text{PgWARP} \) antisera reduced oocyst counts 70–82% and the proportion of mosquitoes infected by 34–51% (Table II). To determine the effect antiserum combinations on \( P. \text{gallinaceum} \) infectivity to \( A. \text{aegypti} \), mosquitoes were fed with either a combination of anti-\( \text{PgCTRP} \) + anti-\( \text{PgWARP} \) or all three antisera mixed together in the membrane feeder (Table III). The combination of polyclonal antisera against \( \text{PgCTRP} \) and \( \text{PgWARP} \) further reduced the number of oocysts in a reproducible and statistically significant fashion. The presence of all three antisera in the blood meal did not additionally reduce transmission, compared with combinations of two anti-
sera in terms of either reduction in oocyst counts or proportion of mosquitoes infected.

Effect of Antibodies against Micronemal Proteins on Infectivity of P. falciparum for Anopheles Mosquitoes—Membrane feeding assays using cultured P. falciparum strain 3D7 gametocytes were performed to determine whether antibodies to the P. falciparum chitinase PfCHT1 and the P. falciparum WARP orthologue inhibited parasite infectivity to Anopheles mosquitoes similar to what was observed with homologous proteins in the P. gallinaceum/A. aegypti system.

Monoclonal antibody 1C3, raised against enzymatically active recombinant PfCHT1 (13), when added to infectious blood meals fed to colonized Anopheles gambiae strain G3 and Anopheles stephensi mosquitoes, significantly inhibited oocyst formation and reduced the proportion of mosquitoes infected in a dose-dependent manner (Table IV). In A. gambiae, oocyst counts were reduced by 85–94% with a concomitant reduction in the proportion of mosquitoes infected by 73–86%. In A. stephensi mosquitoes, the effect of mAb 1C3 was even more profound, with 92–100% reduction in oocyst counts and 84–100% reduction in proportion of mosquitoes infected.

Mouse and rabbit polyclonal antisera raised to biologically active recombinant PfWARP had nearly complete transmission blocking activity in membrane feeding assays of P. falciparum to An. gambiae mosquitoes (Table V).

**DISCUSSION**

Most strategies for identifying malaria transmission-blocking vaccine targets have focused on early sexual stage parasite surface proteins, i.e. present on gametocytes, gametes, or zygotes (23, 24). In this study, we demonstrate that antibodies directed against three late mosquito midgut stage proteins have significant transmission blocking activity, namely the ookinete-secreted proteins PfCHT1 chitinase, WARP, and CTRP. The transmission blocking activity of antisera persisted despite the presence of high levels of proteolytic activity in blood meal digestion, secreted by epithelial cells into the midgut. The antisera likely act on ookinete stages of the parasite because the proteins first appear in ookinetes (Fig. 6). Immunolocalization studies demonstrate that all three ookinete-secreted proteins are present in micronemes (7, 10, 25). Furthermore, evidence presented here suggests that PfCHT1, PfWARP, and PfCTRP are present within the same microneme population and are secreted through a common pathway through the electron-dense apical end of the ookinete. Finally, transmission-blocking results in the avian model of malaria transmission were confirmed by parallel studies of the lethal human malaria parasite, P. falciparum. Taken together, these observations suggest that as a class, ookinete-secreted micronemal proteins are candidate target antigens blocking malaria transmission. One could speculate that interruption of microneme formation or trafficking of secreted/chaperone proteins into micronemes may be useful as a novel approach to interventions against malaria, including transmission-blocking approaches.

The experiments presented here used the following E. coli-produced recombinant proteins: PfCHT1, PfWARP, and PfCTRP (the first vWA domain), and PfCTRP. Assays of adhesive activity performed in this study indicate that the proteins were
properly folded and had appropriate biological activities. Chitinase activity for rPfCHT1 and rPgCHT1 has been reported previously (4, 17); additionally in the presence of 100 mM di-thiothreitol, chitinase activity was maintained indicating that the enzymatic activity of these proteins for small oligosaccharide substrates is not disulfide-dependent (data not shown). We demonstrate here that the first vWA domain of PgCTRP and recombinant PfWARP bound to highly negatively charged glycosaminoglycans (heparin) but not to other proteins found in basement membrane such as fibrinogen and collagen type I or IV or to highly negatively charged albumin. These findings do not confirm that heparin is the natural ligand of these ookinete proteins. Rather, in the limited present study we interpret these results simply to indicate that these disulfide-dependent proteins, as produced in E. coli, likely induce conformationally dependent, neutralizing protective antibodies. Experiments are in progress to use recombinant PfWARP to identify whether it has a natural mosquito midgut ligand, which would

![Image of dual immunoelectron microscopy](image)

**TABLE I**

| Exp. | Group | Geometric mean no. of oocysts in midgut | No. infected/total engorged (mosquitoes) |
|------|-------|-----------------------------------------|-----------------------------------------|
| 1    | Control (blood alone, nothing added) | 4.16 (0–62) | 24/36 (66.7%) |
|      | Control (KLH peptide with CFA) | 3.40 (0–48) | 21/34 (61.8%) |
|      | Control (recombinant His6-tagged protein) | 4.36 (0–66) | 28/38 (73.7%) |
|      | Anti-PgCHT1 | 1.37 (0–24) | 14/38 (36.8%) |
| 2    | Control (blood alone, nothing added) | 6.20 (0–28) | 30/35 (85.7%) |
|      | Control (KLH peptide with CFA) | 6.39 (0–30) | 31/35 (88.6%) |
|      | Control (recombinant His6-tagged protein) | 4.67 (0–33) | 33/40 (82.5%) |
|      | Anti-PgCHT1 | 1.61 (0–14) | 20/37 (54.1%) |
| 3    | Control (blood alone, nothing added) | 2.42 (0–56) | 21/40 (52.5%) |
|      | Control (KLH peptide with CFA) | 2.46 (0–60) | 20/39 (51.3%) |
|      | Control (recombinant His6-tagged protein) | 1.92 (0–35) | 19/37 (51.4%) |
|      | Anti-PgCHT1 | 0.10 (0–2) | 5/39 (12.8%) |

a Statistical significance was determined by Mann-Whitney U test, controls versus PgCHT1, p ≤ 0.01.

b KLH, keyhole limpet hemocyanin; CFA, complete Freund’s adjuvant.
be of substantial interest as a potential ookinete invasion receptor.

Fundamental studies of *Plasmodium* ookinete cell biology are likely to yield novel, practical, and pre-clinical development of malaria transmission-blocking strategies and identification of potential vaccine targets. We demonstrate that three micronemal proteins of the ookinete are secreted via a common mechanism by which mAb 1C3 reduced transmission-blocking activity (13). Regardless of the mechanism by which mAb 1C3 reduced transmission-blocking activity, it is clear that mAb 1C3 had strong transmission blocking activity in both *P. falciparum* and *P. berghei* ookinetes. Hence, in blocking parasite transmission to mosquitoes, the data presented here are consistent with the possibility that mAb 1C3 may be a potential target of blocking parasite transmission to mosquitoes.

To further investigate the potential importance of ookinete-secreted chitinase as a transmission-blocking target, in the experiments presented here, mAb 1C3 had strong transmission blocking activity in both *P. falciparum* and *P. berghei* ookinetes. Hence, in blocking parasite transmission to mosquitoes, the data presented here are consistent with the possibility that mAb 1C3 may be a potential target of blocking parasite transmission to mosquitoes.

**TABLE II**
Effect of antisera raised to recombinant *PgCCTR* vWA domain and *PgWARP* on *P. gallinaceum* infectivity to *A. aegypti* mosquitoes

| Exp. | Group | Geometric mean no. of oocysts in midgut | No. infected/total engorged (mosquitoes) |
|------|-------|----------------------------------------|----------------------------------------|
| 1    | Control (KLH* peptide with CFA) | 4.76 (0–54) | 31/40 (77.5%) |
|      | Anti-*PgCCTR* | 0.55 (0–19) | 10/40 (25.0%) |
|      | Anti-*PgWARP* | 0.85 (0–22) | 16/40 (40.0%) |
| 2    | Control (KLH peptide with CFA) | 4.47 (0–50) | 23/35 (65.7%) |
|      | Anti-*PgCCTR* | 1.00 (0–48) | 12/40 (30.0%) |
|      | Anti-*PgWARP* | 1.18 (0–58) | 13/40 (32.5%) |
| 3    | Control (KLH peptide with CFA) | 6.57 (0–54) | 32/40 (80.0%) |
|      | Anti-*PgCCTR* | 1.36 (0–14) | 20/40 (50.0%) |
|      | Anti-*PgWARP* | 1.98 (0–28) | 21/40 (52.5%) |

*KLH, keyhole limpet hemocyanin; CFA, complete Freund’s adjuvant.

**TABLE III**
Effect of combining antisera raised to micronemal proteins on *P. gallinaceum* infectivity to *A. aegypti* mosquitoes

| Exp. | Group | Geometric mean no. of oocysts in midgut | No. infected/total engorged (mosquitoes) |
|------|-------|----------------------------------------|----------------------------------------|
| 1    | Control (recombinant His*γ*-tagged protein) | 4.28 (0–28) | 29/39 (71.8%) |
|      | Anti-*PgCCTR* + control | 0.73 (0–26) | 11/40 (27.6%) |
|      | Anti-*PgWARP* + control (recombinant His*γ*-tagged protein) | 0.56 (0–11) | 10/38 (26.3%) |
|      | Anti-*PgCHT1* + control (recombinant His*γ*-tagged protein) | 0.50 (0–10) | 12/40 (30.0%) |
|      | Anti-*PgCCTR* + anti-*PgWARP* + control (recombinant His*γ*-tagged protein) | 0.26 (0–7) | 8/40 (20.0%) |
|      | Anti-*PgCHT1* + anti-*PgWARP* + anti-*PgCCTR* | 0.31 (0–5) | 9/40 (22.5%) |
| 2    | Control (recombinant His*γ*-tagged protein) | 41.39 (3–129) | 40/40 (100%) |
|      | Anti-*PgCCTR* + control (recombinant His*γ*-tagged protein) | 13.58 (0–69) | 34/40 (85.0%) |
|      | Anti-*PgWARP* + control (recombinant His*γ*-tagged protein) | 7.22 (0–63) | 33/40 (82.5%) |
|      | Anti-*PgCHT1* + control (recombinant His*γ*-tagged protein) | 17.47 (0–120) | 18/40 (45.0%) |
|      | Anti-*PgCCTR* + anti-*PgWARP* + control (recombinant His*γ*-tagged protein) | 7.15 (0–89) | 29/40 (72.5%) |
|      | Anti-*PgCHT1* + anti-*PgWARP* + anti-*PgCCTR* | 5.98 (0–67) | 28/40 (70.0%) |

*Statistical significance determined by Mann-Whitney U test, controls versus treatment group, p ≤ 0.01.

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the membrane-feeding experiments using anti-PfWARP sera in both the *P. gallinaceum* and *P. falciparum* systems. We showed that anti-PfWARP sera reacted specifically with a single, reduction-sensitive, high molecular weight protein present in *P. gallinaceum* ookinetes but not in 3–4-h-old zygotes (Fig. 6), consistent with previous reports (10). Furthermore, numerous negative control sera had no transmission blocking activity in the experiments where anti-PfWARP sera were added to membrane feeds. These results confirm that the anti-PfWARP sera were specific for PfWARP and reduce the potential concern whether such antibodies might cross-react with CTRP (30).

Similar to the experience of others in using membrane feeding assays to determine the effect of various antisera on *Plasmodium* infectivity to mosquitoes, we observed experiment-to-experiment variability in the numbers of oocysts obtained, with relatively low geometric means oocyst counts observed in both *P. gallinaceum* and *P. falciparum* experiments. Such variability has been observed by others with *P. gallinaceum*, *P. berghei*, *P. vivax*, and *P. falciparum* (15, 22, 31, 32). Each of the membrane feeding experiments presented here, both of *P. gallinaceum* and *P. falciparum*, was analyzed independently with internal negative controls included in each assay. Each membrane feeding assay was performed at least three times for each treatment group, and similar magnitudes of statistically significant oocyst reduction were observed, despite varying numbers of oocysts seen in the negative control groups. Therefore, we conclude that despite the variability of oocyst counts in the experiments presented, statistically robust conclusions about the effect of the anti-micronemal protein antisera can be made.

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**Table IV**

Effect of monoclonal antibody 1C3 raised to recombinant PfCHT1 on *P. falciparum* infectivity to *A. gambiae* and *A. stephensi* mosquitoes.

| Exp. | Antibody | Geometric mean no. of oocysts in midgut | No. infected/total engorged (mosquitoes) |
|------|----------|----------------------------------------|----------------------------------------|
| 1    | Control (blood alone, nothing added) | 0.61 (0–6) | 18/40 (45.0%) |
|      | Control (100 µg/ml isotype) | 0.63 (0–5) | 15/40 (37.5%) |
|      | Control (200 µg/ml isotype) | 0.52 (0–4) | 15/40 (37.5%) |
|      | 1C3 (100 µg/ml) | 0.33 (0–5) | 11/40 (27.5%) |
|      | 1C3 (200 µg/ml) | 0.09 (0–2) | 4/40 (10.0%) |
|      | Control (100 µg/ml isotype) | 0.68 (0–6) | 16/40 (40.0%) |
|      | Control (200 µg/ml isotype) | 0.66 (0–5) | 17/40 (42.5%) |
|      | 1C3 (100 µg/ml) | 0.33 (0–4) | 11/40 (27.5%) |
|      | 1C3 (200 µg/ml) | 0.08 (0–2) | 4/40 (10.0%) |
| 2    | Control (blood alone, nothing added) | 0.69 (0–7) | 18/40 (45.0%) |
|      | Control (100 µg/ml isotype) | 0.08 (0–6) | 16/40 (40.0%) |
|      | Control (200 µg/ml isotype) | 0.77 (0–8) | 14/38 (36.8%) |
|      | 1C3 (100 µg/ml) | 0.22 (0–5) | 6/38 (15.8%) |
|      | 1C3 (200 µg/ml) | 0.05 (0–2) | 2/39 (5.1%) |
| 3    | Control (blood alone, nothing added) | 0.79 (0–10) | 14/39 (35.9%) |
|      | Control (100 µg/ml isotype) | 0.68 (0–8) | 13/37 (35.1%) |
|      | Control (200 µg/ml isotype) | 0.77 (0–8) | 14/38 (36.8%) |
|      | 1C3 (100 µg/ml) | 0.22 (0–5) | 6/38 (15.8%) |
|      | 1C3 (200 µg/ml) | 0.05 (0–2) | 2/39 (5.1%) |
| 4    | Control (blood alone, nothing added) | 1.33 (0–24) | 21/39 (53.8%) |
|      | Control (100 µg/ml isotype) | 1.30 (0–18) | 20/38 (52.6%) |
|      | Control (200 µg/ml isotype) | 1.31 (0–16) | 19/36 (52.8%) |
|      | 1C3 (100 µg/ml) | 0.76 (0–8) | 16/37 (43.2%) |
|      | 1C3 (200 µg/ml) | 0.10 (0–3) | 3/25 (12.0%) |
| 5    | Control (blood alone, nothing added) | 0.83 (0–9) | 14/38 (36.8%) |
|      | Control (100 µg/ml isotype) | 0.91 (0–8) | 15/39 (38.5%) |
|      | Control (200 µg/ml isotype) | 0.75 (0–8) | 15/38 (39.5%) |
|      | 1C3 (100 µg/ml) | 0.10 (0–3) | 4/40 (10.0%) |
|      | 1C3 (200 µg/ml) | 0.02 (0–2) | 1/38 (2.6%) |
| 6    | Control (blood alone, nothing added) | 1.12 (0–15) | 16/39 (41.0%) |
|      | Control (100 µg/ml isotype) | 1.04 (0–14) | 15/37 (40.5%) |
|      | Control (200 µg/ml isotype) | 1.05 (0–11) | 15/36 (41.7%) |
|      | 1C3 (100 µg/ml) | 0.31 (0–6) | 7/38 (18.4%) |
|      | 1C3 (200 µg/ml) | 0.00 (0–0) | 0/40 (0.0%) |

a Statistical significance determined by Mann-Whitney U test, controls versus 1C3, p ≤ 0.01.

**Table V**

Effect of antisera raised to recombinant PfWARP on *P. falciparum* infectivity to *A. gambiae* mosquitoes.

| Exp. | Antibody | Geometric mean no. of oocysts in midgut | No. infected/total engorged (mosquitoes) |
|------|----------|----------------------------------------|----------------------------------------|
| 1    | Control (PBS) | 1.00 (0–9) | 20/37 (54.1%) |
|      | Control (NMS) | 0.98 (0–7) | 18/36 (50.0%) |
|      | Control (NRS) | 0.92 (0–7) | 18/36 (50.0%) |
|      | Control (anti-GST) | 1.07 (0–8) | 18/33 (54.5%) |
|      | MS anti-PfWARP | 0.00 (0–0) | 0/37 (0.0%) |
|      | RS anti-PfWARP | 0.02 (0–1) | 1/38 (2.6%) |
| 2    | Control (PBS) | 0.77 (0–9) | 15/38 (39.5%) |
|      | Control (NMS) | 0.70 (0–8) | 14/40 (35.0%) |
|      | Control (NRS) | 0.70 (0–10) | 16/39 (41.0%) |
|      | MS anti-PfWARP | 0.02 (0–1) | 1/40 (2.5%) |
|      | RS anti-PfWARP | 0.00 (0–0) | 0/40 (0.0%) |
| 3    | Control (PBS) | 0.56 (0–8) | 16/40 (40.0%) |
|      | Control (NMS) | 0.57 (0–9) | 14/40 (35.0%) |
|      | Control (NRS) | 0.49 (0–7) | 13/40 (32.5%) |
|      | MS anti-PfWARP | 0.04 (0–1) | 2/40 (5.0%) |
|      | RS anti-PfWARP | 0.02 (0–1) | 1/40 (2.5%) |

a Statistical significance determined by Mann-Whitney U test, controls versus anti-PfWARP, p ≤ 0.01.

b NMS, normal mouse serum; NRS, normal rabbit serum; MS, mouse antiserum; RS, rabbit antiserum.
In summary, we present evidence that validates, as proof-of-principle, three *Plasmodium* ookinete-secreted proteins (chitinase, WARP, and CTRP) as malaria transmission-blocking targets in both an avian model system and with the human pathogen *P. falciparum*. Despite the contrasting final destinations of these proteins, they are secreted by a common microneural pathway through the electron-dense region of the apical complex of the ookinete. With ongoing genome-scale efforts to delineate the proteome and gene expression repertoire of *Plasmodium* sexual stage genes, novel insights into the fundamental biology of malaria parasites will be gained, with the anticipation of applying such results to the amelioration and control of human malaria.

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