SURFACE PROTEINS OF \textit{PLASMODIUM FALCIPARUM} MEROZOITES BINDING TO THE ERYTHROCYTE RECEPTOR, GLYCOPHORIN

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For the major part of its life cycle in the vertebrate host, the malarial parasite is intracellular. Only the sporozoite, the form that is introduced by the mosquito vector, and the merozoite are extracellular and both only for a brief period. The sporozoite invades the liver cell and the merozoite the erythrocyte. The intracellular localization of the parasite affords it protection from the host's immune system. Efforts to achieve immune protection against \textit{Plasmodium falciparum}, one of four species that infect humans, have concentrated on the extracellular forms, the sporozoite and the merozoite (1, 2).

The attachment of the merozoite to the erythrocyte is receptor-mediated (3). For \textit{P. falciparum} the erythrocyte receptors appear to be the sialoglycoproteins, glycophorin A and B (4–7). We have recently shown that [$^{125}$I]glycophorins added to the isolated merozoite bind to saturable sites on the merozoite surface (7). This raised the possibility that specific proteins on the merozoite surface, possibly with high affinity for the glycophorins, mediate the attachment to the erythrocyte surface. Immunization with merozoite surface molecules could prove to be protective against \textit{P. falciparum} malaria. Using glycophorin coupled to an acrylamide matrix, we have identified two parasite proteins of molecular weight 155,000 and 130,000 that specifically bind to glycophorin. Some of the results described here have been published in preliminary notes (8, 9).

Materials and Methods

\textit{Culture and Biosynthetic Labeling of P. falciparum Merozoites.} \textit{P. falciparum} (FCR-3, Gambia strain) was maintained in vitro (10) and synchronized as described previously (4). Schizont-infected erythrocytes were concentrated to $>$80% parasitemia with 2% gelatin treatment (11), 50–56 h after the median invasion time. 5 h before the beginning of merozoite release, $5 \times 10^9$ schizont-infected erythrocytes were labeled with [$^3$H]proline (100 $\mu$Ci/ml), [$^3$H]glycine (100 $\mu$Ci/ml), or [$^{35}$S]methionine (100 $\mu$Ci/ml) for 1 h in 5 ml RPMI plus 10% human serum. Subsequent analysis was performed on (a) the intraerythrocytic or schizont stage collected immediately after labeling; (b) the merozoites collected from the same population of schizonts (3–4 h after the labeling period) by centrifugation of the culture supernatant at 12,000 g for 3 min; (c) the culture supernatant (4 ml) after removal of the merozoites. The number of merozoites collected during a 1-h period varied from 2 to $10 \times 10^9$.

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**Preparation of Glycophorin-Acrylamide Matrix.** Glycophorins were prepared from human erythrocyte ghosts (12). The preparation contained glycophorins A, B, and possibly small amounts of C and D (13). After chloroform/methanol extraction the aqueous phase containing sialoglycoproteins was concentrated to 10 ml by vacuum and dialyzed against three changes of 0.1 M NaHCO₃, pH 8.0. Precipitated material was removed by centrifugation at 10,000 g for 20 min and the solution containing the sialoglycoproteins was diluted to 2.0 mg protein/ml and adjusted to pH 4.5 with 1 N HCl. The glycophorin fraction was coupled to amino-ethyl polyacrylamide beads (Bio-gel, P 150), swollen in water, as follows. Ethyl dimethyl aminopropyl carbodiimide (5 mg) was added to 2 ml of the glycophorin fraction (2 mg/ml), adjusted to pH 4.5 with 0.1 N HCl and added to 2 ml (packed volume) of swollen acrylamide beads. The suspension was mixed gently at room temperature for 5 h and then centrifuged at 600 g for 2 min. The supernatant was removed and its protein content determined by measuring Abs₂₈₀. The beads were washed three times in phosphate-buffered saline (PBS) and the protein concentration of the combined supernatants determined. By difference it was estimated that the amount of glycophorin coupled to the beads was 0.66 mg/ml packed beads, or 33% of that added. As a control, another sialoglycoprotein, fetuin (10 mg) was coupled to 2 ml of beads in the same manner. Fetuin concentration was estimated to be 3.20 mg/ml of packed beads. The biological activity of glycophorin coupled to acrylamide was tested, and found to be almost as effective as soluble glycophorin in inhibiting merozoite invasion.

**Glycophorin Affinity Chromatography.** Schizont-infected erythrocytes (10⁹) were lysed in 10 ml of cold 10 mM Tris-HCl, pH 8.0 and centrifuged at 12,000 g for 5 min. The pellet was washed twice in the same buffer and then solubilized with 1 ml of extraction buffer (1% Nonidet P-40(NP-40)/0.1% deoxycholate/5 mM iodoacetamide/1 mM ZnCl₂ in PBS, pH 7.4). The merozoites (5 × 10⁹) were not washed in hypotonic buffer, but were solubilized immediately in 1 ml of extraction buffer. Insoluble material was removed by centrifugation at 12,000 g for 5 min. The culture supernatant fraction was assayed without further treatment.

Extracts of [³H]glycine-labeled schizonts (0.4 ml), merozoites (0.4 ml), and [³H]proline-labeled culture supernatant fraction (0.4 ml) were added to 0.6 ml of glycophorin-acrylamide beads or fetuin-acrylamide beads. The suspensions were mixed gently for 30 min at room temperature then centrifuged at 600 g for 2 min. The supernatant (flow-through fraction), was removed and saved for analysis. The beads were washed in 0.3 ml of 2× PBS, centrifuged at 600 g for 2 min and the supernatant removed. In all subsequent steps the elution solutions were added to the beads, shaken for 1 min and the suspension centrifuged at 600 g for 2 min. The beads were treated sequentially with 10 ml PBS, 0.3 ml 0.5 M NaCl in PBS, 10 ml PBS, 0.3 ml 2% sodium dodecyl sulfate (SDS) in PBS, and finally, boiled in 0.3 ml of 4% SDS in PBS. Equal volumes of each supernatant fraction were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE). In other experiments, either soluble glycophorin (1 mg/0.6 ml beads) or immune serum (0.2 ml) was included in the suspension of parasite extracts 30 min before addition to glycophorin-acrylamide beads.

**Preparation of Rabbit Immune Sera.** Rabbit antibodies to the parasite proteins released into the culture supernatant were prepared as follows. When merozoite release from schizont-infected erythrocytes (10⁹) began, the medium was replaced with 8 ml of RPMI and 5% preimmune serum from the same rabbit to be immunized. The cultures were agitated for 3 h, and the schizonts and merozoites removed by centrifugation at 3,000 g for 5 min. The parasite proteins of interest were heat stable and this property was used to remove all other parasite proteins from the culture supernatant as follows. The culture supernatant was heated to 100°C for 10 min and centrifuged at 10,000 g for 20 min. The supernatant was concentrated to 1 ml in an Amicon ultrafiltration unit (XM 50 membrane), and then centrifuged at 30,000 g for 1 h. The supernatant that contained

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1 *Abbreviations used in this paper:* BSA, bovine serum albumin; kD, kilodalton; NP-40, Nonidet P-40; PBS, phosphate-buffered saline; pH 7.4; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.
only heat-stable proteins was mixed with an equal volume of Freund's incomplete adjuvant
and used to immunize a New Zealand white rabbit by three injections of 1 ml every 2 wk. 

**Immunoprecipitation.** Extracts prepared as for affinity chromatography were immu-
noprecipitated with rabbit immune sera as follows. Each fraction (0.4 ml) was incubated
with 0.4 ml preimmune or immune serum for 1 h at 37°C. Formaldehyde-fixed *Staphy-
lococcus aureus* (Cowan I strain) (0.1 ml) was washed three times in 0.1% SDS/0.1% bovine
serum albumin (BSA)/0.5% NP-40 in PBS and was added to each sample. After 1 h at
room temperature and 3 h at 4°C the *S. aureus* was pelleted by centrifugation at 12,000
g for 2 min and washed three times with the above buffer. The pellet was boiled in
electrophoresis sample buffer, (2% SDS/10% glycerol/0.1 M Tris-HCl, pH 6.8/0.01%
bromophenol blue) to solubilize the antibody-antigen complex. *S. aureus* was removed by
centrifugation at 12,000 g for 2 min and the supernatant analyzed by SDS-PAGE.

**Merozoite Invasion Assay.** The effect of antibodies on merozoite invasion was tested by
adding immune serum to merozoites before the addition to erythrocytes. The procedure
for isolation of merozoites was described previously (4).

**Immunoelectron Microscopy.** Merozolites (10⁶) collected from the culture supernatant
were washed five times in 0.5% glutaraldehyde, 0.25 M sucrose in 0.1 M cacodylate buffer,
pH 7.4 for 15 min at 0°C, centrifuged at 3,000 g for 10 min, and washed in 1% BSA,
0.1 M glycine in PBS. The pellet was resuspended in 0.4 ml preimmune or immune rabbit
serum and mixed intermittently for 2 h at room temperature. The cells were washed
three times in PBS and resuspended in 0.1 ml of 20 nm gold particles bound to protein
A (14). The suspension was allowed to stand, with intermittent mixing, for 45 min at
room temperature and then centrifuged at 3,000 g for 10 min. The merozoite pellet was
washed once in PBS, fixed in 1.0% glutaraldehyde/0.25 M sucrose in 0.1 M cacodylate
buffer, pH 7.4 and then processed for transmission electron microscopy (15).

**Results**

**Glycophorin Affinity Chromatography.** [³H]Glycine-labeled merozoites (Fig. 1)
were added to glycophorin-acrylamide or fetuin-acrylamide. Several of the
numerous labeled merozoite proteins present in the starting material (Fig. 1b)
were absent or reduced in the flow through (Fig. 1a). PBS eluted most of the
loosely bound proteins, including a 200-kilodalton (kD) protein (Fig. 1d). The
NaCl wash eluted small amounts of several proteins (Fig. 1e). The only proteins
that remained bound to glycophorin after NaCl elution were a major labeled
protein of 155 kD and a minor protein of 130 kD. Both of these were removed
by 2% SDS (Fig. 1f). None of the 20 or so labeled merozoite proteins bound to
fetuin-acrylamide (Fig. 1, g-j).

Proteins of 155 kD and 130 kD were released into the culture supernatant
and the ability of these proteins to bind to glycophorin was also tested. All of the
155-kD protein was bound and most of the 130-kD protein bound (Fig. 2b).
This suggests their identity with the merozoite proteins of identical size that
bound to glycophorin. Preincubation of merozoites with soluble glycophorin
reduced the binding (Fig. 2a). NaCl did not elute either protein (Fig. 2d); 2%
SDS eluted approximately half of the proteins (Fig. 2e) and boiling in 4% SDS
was required to remove the remainder (Fig. 2f). None of the labeled proteins in
the culture supernatant bound to fetuin (Fig. 2, g-i). Results shown in Fig. 2, a–
h and Fig. 2, g–i were from two separate experiments, and demonstrate the
variability in the distribution of label between the 155-kD and 130-kD proteins.
The inverse relationship in the amount of label between the two supernatant
proteins suggests that the 130-kD protein may be a breakdown product of the
155-kD protein.
FIGURE 1. Glycophorin binding of merozoite proteins. [3H]Glycine-labeled merozoites were incubated for 30 min with glycophorin-coupled acrylamide and eluted with different solutions. 

a, [3H]Glycine merozoite proteins not binding to beads, (flow through); b, [3H]glycine merozoites added to beads (starting material) and c, kept at 4°C for 30 min; d, 2× PBS eluate; e, 0.5 M NaCl/PBS eluate; f, 2% SDS/PBS eluate; g, 4% SDS, 100°C eluate; h, starting material (identical to b); i, proteins not binding to fetuin-acrylamide beads. Eluate from fetuin beads with (j) 2× PBS; (k) 0.5 M NaCl/PBS; (l), 2% SDS/PBS. A 5–15% polyacrylamide gradient gel was used in all experiments shown in Fig. 1–5. Molecular weight standards were myosin, 200 kD, β-galactosidase, 116 kD, phosphorylase 94 kD, albumin 68 kD.

Stage Specificity of Glycophorin-binding Proteins. Schizont-infected erythrocytes (42 h post invasion) were labeled for 1 h with [3H]proline (Fig. 3a). The major protein labeled was one of 200 kD shown to be located on the merozoite surface coat.2 In merozoites collected from this population, a 155-kD protein was labeled prominently (Fig. 3b). In merozoites washed in PBS (Fig. 3c), the 155-kD protein was reduced and appeared in the PBS supernatant (Fig. 3d). The only proline-labeled proteins released into the supernatant were those of 155 kD and 130 kD (Fig. 3e). After heat treatment and removal of denatured proteins, the 155-kD and 130-kD proteins remained in the supernatant (Fig. 3f), suggesting their heat stability. Almost identical results were observed when the parasites were labeled with [3H]glycine (data not shown). All the serum proteins detected in the culture supernatant by Coomassie Blue staining were precipitated by the heat treatment (data not shown). Many [35S]methionine-labeled proteins were released into the culture supernatant (Fig. 3g), but only the 155 kD was heat stable (Fig. 3h). The

2 Pirson, P. J., and M. E. Perkins. 1984. Characterization with monoclonal antibodies of a surface antigen of Plasmodium falciparum merozoites. Submitted for publication.
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FIGURE 2. Glycophorin binding of proteins in culture supernatant. Proteins released into the culture supernatant from \(^{3}H\)proline-labeled \textit{P. falciparum} cultures were added to glycophorin-matrix. 
\begin{enumerate}
\item[a] Proteins not binding to glycophorin-matrix in presence of soluble glycophorin (1.0 mg/ml);
\item[b] proteins not binding to glycophorin-matrix;
\item[c] parasite proteins in culture supernatant added to glycophorin-matrix (starting material); 
\item[d] 0.5 M NaCl/PBS eluate;
\item[e] 2% SDS/PBS eluate;
\item[f] 4% SDS/PBS eluate;
\item[g] culture supernatant added to fetuin-acrylamide (different experiment from c);
\item[h] proteins not binding to fetuin beads;
\item[i] NaCl/PBS eluate;
\item[j] SDS/PBS eluate from fetuin-acrylamide.
\end{enumerate}

FIGURE 3. Stage-specificity of glycophorin-binding proteins. 
\begin{enumerate}
\item[a] Schizont-infected erythrocytes (1 h \(^{3}H\)proline label, 42 h post invasion);
\item[b] merozoites collected from \(^{3}H\)proline-labeled schizonts, 3-4 h after labeling;
\item[c] merozoites shown in b resuspended in PBS and centrifuged;
\item[d] PBS supernatant from c;
\item[e] culture supernatant collected with merozoites in b;
\item[f] culture supernatant heated to 100°C for 10 min;
\item[g] Culture supernatant collected from \(^{35}S\)methionine-labeled cultures;
\item[h] supernatant in g heated to 100°C for 10 min.
\end{enumerate}
FIGURE 4. Characterization of rabbit immune serum against glycophorin-binding proteins. (a–i) Immunoprecipitation with rabbit serum. a, e: [³H]proline-labeled culture supernatant proteins; b, supernatant after precipitation with preimmune serum; c, precipitate with preimmune serum; d, [³H]proline-labeled schizont; f, supernatant after precipitation with immune serum; g, precipitate with immune serum; h, [³H]glycine-labeled schizonts; i, precipitate of schizont with immune serum; j–p, inhibition of binding to glycophorin-matrix with immune serum. [³H]Glycine-labeled schizonts were incubated with immune serum (0.2 ml) for 30 min at room temperature and then added to glycophorin-matrix for 30 min. j, [³H]Glycine-labeled schizonts; k, [³H]glycine-labeled merozoites added to glycophorin-beads; l, flow-through fraction from merozoites in absence of antiserum (same as Fig. 2a); m, flow through fraction in presence of immune serum; n, 2× PBS eluate; o, NaCl/PBS eluate; p, 2% SDS/PBS eluate.

Relative amount of [³⁵S]methionine incorporated into the 155-kD protein was very low, estimated to be <0.1% of the total incorporated into schizonts.

Characterization of Antibodies Directed Against Glycophorin-binding Proteins. Serum from a rabbit immunized with the culture supernatant proteins immunoprecipitated a 130-kD protein from [³H]glycine-labeled schizonts (Fig. 4, a and b) and both the 155 kD and 130 kD proteins from the culture supernatant (Fig. 4, g–i). Preimmune serum did not precipitate either of the proteins (Fig. 4,
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TABLE I
Inhibition of Merozoite Invasion by Immune Serum Against Glycophorin-binding Proteins

| Additions          | Amount* | Invasion² |
|--------------------|---------|-----------|
| None               | —       | 100       |
| Preimmune serum    | 0.25    | 92        |
|                   | 0.50    | 79        |
| Immune serum       | 0.25    | 16        |
|                   | 0.50    | 13        |

* Amount refers to the IgG content of serum added.

² Serum added to merozoites (10⁶/ml) before addition to erythrocytes (10⁵) and ring stages counted 4 h later. Control invasion rate was 10.3, 15.4, and 12.6 rings per 100 erythrocytes and were standardized to 100%. Results represent the average of three separate experiments.

FIGURE 5. Immunocytochemistry of glycophorin-binding proteins. a, Merozoites treated with immune rabbit serum and protein A-gold; b, merozoites treated with preimmune rabbit serum and protein A-gold. × 45,000. Bar, 500 nm.

c–e). The serum, when preincubated with the merozoite extract, partially inhibited binding of the 155 kD and 130 kD to the glycophorin-matrix (Fig. 4, j–p). The inability to immunoprecipitate the 155-kD protein from schizonts may be due to its low solubility at this stage in 1% NP-40.

Immune serum (0.5 mg/ml IgG) directed against the 155-kD and 130-kD proteins inhibited merozoite invasion by 87% (Table I). Inhibition of schizont development to rings by the immune serum was less marked, but still significant (data not shown).

Immunoelectron Microscopy. Electron micrographs of merozoites treated with
immune serum and protein A–gold (Fig. 5a) showed that gold particles were present only on the surface coat of the extracellular merozoites. Although the number of gold grains was few, they were completely absent from contaminating infected erythrocyte membrane and in control samples (Fig. 5b). There were also no gold particles on the cytoplasm of broken cells.

Discussion

The *P. falciparum* merozoite, the invasive form of the erythrocytic stage, recognizes and binds to the sialoglycoproteins glycophorin A and B on the surface of human erythrocytes during entry. The binding specificity seems to be located in both the N-terminal tryptic fragment, T1, and the hydrophobic transmembrane fragment, T6 of glycophorin A (7, 16, 17). Electron microscopic studies show that the merozoite enters the erythrocyte by a process that resembles endocytosis (18), although a molecular mechanism that could account for these observations is not known. Since the erythrocyte is not indiscriminant in its endocytosis of microorganisms, it has been postulated that components of the merozoite surface and possibly the rhoptries initiate the invasive process. Merozoite surface components that recognize the erythrocyte receptor may trigger the subsequent events of invasion. In the present work we identified a merozoite surface protein of 155 kD and a less prominent protein of 130 kD that bound with high affinity and specificity to glycophorin immobilized on an acrylamide matrix. Immunoelectron microscopy demonstrated that these proteins were localized on the merozoite surface coat. Antibodies directed against the proteins significantly inhibited both merozoite invasion into erythrocytes and binding to glycophorin-acrylamide, suggesting that the 155-kD and 130-kD proteins play a role in merozoite attachment to the erythrocyte surface.

The heat stability of the glycophorin-binding proteins suggests that they could belong to the class of heat-stable, strain-variant soluble antigens of *P. falciparum* known as S-antigens, first described by Wilson and colleagues (19, 20). S-antigens of several strains of *P. falciparum* have been identified recently and in all cases they are rich in glycine and poor in methionine (21, 22), as are the glycophorin-binding proteins identified in this study. In addition, we find the latter to be rich in proline, often identified as an abundant amino-acid in heat-stable proteins. Identification of glycophorin-binding proteins in other strains will be necessary before their identity with strain-variant S-antigens can be verified.

Immunoprecipitation experiments show that the glycophorin-binding proteins are not related to the glycoprotein (190–200 kD) identified previously to be on the surface of *P. falciparum* merozoites (23, 24). This antigen is processed to fragments of lower molecular weight on the merozoite surface (23, 24). The polyclonal rabbit antibodies against the 155-kD and 130-kD proteins do not immunoprecipitate a 200-kD protein or any of its breakdown products (Fig. 5h). Furthermore, polyclonal rabbit antibodies against the 200-kD protein do not cross-react with the heat-stable proteins (P. Pirson and M. Perkins, unpublished data). The 155-kD and 130-kD proteins are also unrelated to a 140-kD protein that binds to N-acetylglucosamine (25). The 140-kD protein is a major protein in schizonts labeled with $^{35}$S)methionine. Further experiments will be required to decide whether the 130-kD glycophorin-binding protein is a breakdown
product of the 155-kD protein, as suggested by their common properties. The
155-kD protein appeared to be a major [3H]glycine-labeled or [3H]proline-labeled
protein in the merozoite, but only a minor protein in the schizont, suggesting
that the merozoite protein could have been derived from a protein of higher
molecular weight synthesized by the schizont. However, it should be kept in
mind that the experiment was not an authentic pulse-chase procedure. The
merozoites collected for 1 h, shown in Fig. 3b, were derived from the most
mature cells representing at most 20% of the schizont population shown in Fig.
3a.

An interesting characteristic of the glycophorin-binding proteins is that they
are readily released from the merozoite surface, either spontaneously or by
washing with buffer (Fig. 1), indicating that they are loosely attached to the
merozoite surface. Release occurs only from extracellular merozoites, since the
proteins were absent from supernatants of all intracellular stages (data not
shown). The loose attachment of these proteins may be necessary for the rapid
shedding of the merozoite surface coat, which occurs during invasion into
erthrocyte (18). It is not known whether an antibody against a merozoite
receptor protein would be effective in blocking invasion into erythrocytes in
vivo. The glycophorin-binding proteins may offer an advantage over other
merozoite surface proteins in that the site that binds to glycophorin, which is
essential for parasite survival, would be more likely to be conserved.

Summary
Invasion of erythrocytes by the malarial parasite is a receptor-mediated process.
*P. falciparum* merozoites recognize and bind to erythrocyte surface sialoglyco-
proteins, glycophorins A and B, and the glycophorins bind to saturable sites on
the merozoite surface. The purpose of the present work was to identify a receptor
or ligand molecule on the merozoite surface that mediates binding to the
erythrocyte. A fraction containing the sialoglycoproteins was coupled to an
acrylamide matrix and incubated with metabolically labeled merozoites. A mer-
ozoite protein of 155 kD that labeled prominently with [3H]glycine bound to
glycophorin. A minor protein of 130 kD also bound. Both proteins are rich in
proline and glycine, poor in methionine, and may be related. The proteins are
also stable to heating to 100°C for 10 min. Immunoelectron microscopy dem-
onstrated that the 155 kD and 130 kD proteins are located on the merozoite
surface coat. The antibodies significantly inhibited merozoite invasion into
erthrocytes and also binding of the proteins to the glycophorin-matrix. The
specific binding of the 155-kD and 130-kD proteins to the erythrocyte receptor
and the demonstration that they are located on the merozoite surface suggest
they could be receptor proteins that mediate binding of the merozoite to the
erythrocyte surface.

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