Distinct Protein Classes Including Novel Merozoite Surface Antigens in Raft-like Membranes of *Plasmodium falciparum* **

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Glycosylphosphatidylinositol (GPI)-anchored proteins coat the surface of extracellular *Plasmodium falciparum* merozoites, of which several are highly validated candidates for inclusion in a blood-stage malaria vaccine. Here we determined the proteome of gradient-purified detergent-resistant membranes of mature blood-stage parasites and found that these membranes are greatly enriched in GPI-anchored proteins and their putative interacting partners. The GPI-anchored proteins were identified, and a number of novel proteins that are predicted to localize to the merozoite surface and/or apical organelles were detected. Three of the putative surface proteins possessed six-cysteine (Cys6) motifs, a distinct fold found in adhesive surface proteins expressed in other life stages. All three Cys6 proteins, termed Pf12, Pf38, and Pf41, were validated as merozoite surface antigens recognized strongly by antibodies present in naturally infected individuals. In addition to the merozoite surface, Pf38 was particularly prominent in the secretory apical organelles. A different cysteine-rich putative GPI-anchored protein, Pf92, was also localized to the merozoite surface. This insight into merozoite surfaces provides new opportunities for understanding both erythrocyte invasion and anti-parasite immunity.

Developing a vaccine to control human malaria is a global health priority. The recent availability of the genome sequence of the protozoan parasite *Plasmodium falciparum*, the major cause of malaria, allows the use of genomic technologies such as microarray and proteomics to identify novel vaccine and drug targets (1–5). Most membrane proteins that coat the surface of the erythrocyte-invasive merozoite form of the parasite are attached to the plasma membrane via a C-terminal glycosylphosphatidylinositol (GPI) anchor. To date, four GPI-anchored merozoite surface proteins (MSP-1, -2, -4, and -5) have been identified, and two others (MSP-10 and rhoptry-associated membrane antigen (RAMA)) appear to reside at least in part in organelles at the apical end of the parasite (6–10). Another protein originally localized to the merozoite surface, MSP-8, appears to instead reside in the ring stage (11). Most other merozoite surface proteins (e.g. MSP-3/6 family members, MSP-7, and acidic basic repeat antigen (ABRA)) are not directly membrane-associated but are indirectly linked to the surface, probably in most cases via interactions with GPI-anchored proteins (12–15). In contrast to the apical and peripheral classes of blood-stage antigens, the GPI-anchored proteins appear to be essential to blood-stage growth with repeated attempts to genetically disrupt six GPI-anchored merozoite proteins resulting in only one “knock-out,” that of the *msp-5* gene (16). This, together with considerable data highlighting their potential as targets of protective antibodies, places the merozoite GPI-anchored proteins among the most highly validated blood-stage vaccine targets.

GPI-anchored plasma membrane proteins of many eukaryotes, including *P. falciparum* (17), appear to be enriched in detergent-resistant membrane (DRM) domains, sometimes referred to as lipid rafts. To further characterize the surface coat of the blood-stage form of *P. falciparum* parasites, we determined the proteome of DRMs in this life stage and found that these membranes are greatly enriched in GPI-anchored proteins and their interacting partners. Also prominent in DRMs were apical organelle (rhoptry), multimembrane-spanning, and a novel set of proteins that are destined to be exported from the intracellular form parasite into the host erythrocyte. A number of new surface proteins were identified, and three of these were confirmed as merozoite surface antigens that elicit antibody responses in naturally infected individuals. This study provides a more comprehensive picture of the nature of the *P. falciparum* merozoite surface and generates a range of new possibilities for malaria vaccine development.

**EXPERIMENTAL PROCEDURES**

Preparation of Detergent-resistant Membranes—Sorbitol-synchronized parasite cultures at 8–10% parasitemia were pelleted (1500 rpm Beckman GS-6 centrifuge), and late stage schizonts (~44 h after invasion) were purified using Miltenyi Biotec Vario MACS CS magnetic separation columns. Parasites were washed twice in culture medium prior to saponin lysis (0.15% saponin, 10 min, on ice). Samples were pelleted at 2800 rpm for 10 min (Beckman GS-6 centrifuge) and washed three times in MES-buffered saline (25 mM MES (Sigma) pH 6.5, 150 fluorescent protein; MBP, mannose-binding protein; MES, 4-morpholinethanesulfonic acid.

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mM NaCl). Parasites were resuspended to a volume of 1.5 ml in MES containing a Roche Applied Science Complete protease inhibitor mixture tablet and chilled to 0 °C on an ice water slurry. Parasite samples were cooled to 0 °C, and an equal volume of chilled (0 °C) 1% Triton X-100 (SigmaUltra) in MES at 0 °C was added to give a final concentration of 0.5% Triton X-100. Samples maintained at 0 °C for 30 min (3 ml total) were resuspended every 10 min and then centrifuged at 30,000 rpm (2 °C for 30 min) in a pre-chilled TLA 100.3 rotor in a Beckman Optima MAX-E ultracentrifuge. The supernatant was discarded, and the pellet was washed with an additional 3 ml human tonicity-PBS (0 °C) and recentrifuged as above. Supernatant was discarded, and the pellets were snap-frozen on dry ice for proteomic analysis.

Protein Digestion and Multidimensional Protein Identification Technology Analysis—Each membrane/protein pellet was resuspended in 20 μl of 90% formic acid containing 500 mg/ml cyanogen bromide and incubated overnight at room temperature protected from light. 2 volumes (40 μl) of 30% NH4OH were added drop by drop followed by the addition of 3 volumes (180 μl) of saturated NH4HCO3 drop by drop. After verifying that the pH of each solution was ~8.5, solid urea was added to a final concentration of 8 M. Disulfide bonds were reduced by adding tris(2-carboxyethyl)phosphine to 5 mM and incubated at room temperature for 30 min. Cysteines were alkylated by adding iodoacetamide to 20 mM and incubating at room temperature for 30 min protected from light. Sequencing grade endoproteinase Lys-C (Roche Applied Science) was then added at an estimated ratio of 1:100 (enzyme:substrate, w/w) and incubated at 37 °C for 16 h. The solution was then diluted 2-fold by adding an equal volume (345 μl) of 100 mM Tris, pH 8.5, and adding CaCl2 to 2 mM. Sequencing grade modified trypsin (Protext) was then added at an estimated ratio of 1:100 and incubated at 37 °C for ~16 h. 90% formic acid was then added to a final concentration of 4%.

For proteomic analysis, the 15 sucrose gradient fractions were pooled according to the distribution pattern of MSP-1200 (determined by Western blotting), such that Fractions 1–4 formed Pool 1, Fractions 5–9 formed Pool 2, Fractions 10–13 formed Pool 3, and Fractions 14–15 formed Pool 4 (pellet-associated material). Pooled fractions were diluted to 3 ml with human tonicity-PBS at 0 °C and centrifuged at 30,000 rpm for 1 h (2 °C) in a TLA 100.3 rotor in a Beckman Optima MAX-E ultracentrifuge. The supernatant was discarded, and the pellet was washed with an additional 3 ml human tonicity-PBS (0 °C) and recentrifuged as above. Supernatant was discarded, and the pellets were snap-frozen on dry ice for proteomic analysis.
tandem mass spectrometry spectra were collected as described previously (19). Tandem mass spectrometry spectra were searched against a P. falciparum protein data base (10/03/02 release, with the manually added sequence of RAMA) combined with human, mouse, and rat data-bases using the search algorithm SEQUEST. Peptide identifications were filtered using default settings (except where noted) using the program DTASelect, and samples were compared using the program Contrast (20).

Fluorescent Imaging—GFP fusion proteins for localization studies were encoded in transfection constructs under the regulation of the tetracycline-inducible expression system (21). Primer sets used for the amplification of the following genes were:

- **pf12**: 5'-H11032-GATCAGCGTGTTGATTTTCGAACAGAA-3' and 5'-H11032-GATCAGCTAGTTTAAAAGTTAAGAA-3';
- **pf38**: 5'-H11032-GATCAGCGTGTTGATTTTCGAACAGAA-3' and 5'-H11032-GATCAGCTAGTTTAAAAGTTAAGAA-3';
- **pf92**: 5'-H11032-TAACAAGGTCAGTTGTTTGGGCGGGA-3' and 5'-H11032-TAACAAGGTCAGTTGTTTGGGCGGGA-3'.

PCR products were cut with MluI and SpeI and were cloned in-frame C-terminal to secreted GFP. Anhydrotetracycline was removed from parasite cultures 72 h prior to live imaging (in the presence of 2.5 nM WR99210) to allow expression of the GFP fusion. Prior to microscopy, parasites were incubated in culture medium containing 100 ng/ml 4',6-diamidino-2-phenylindole.

Antigen Discovery in Malaria Merozoites

**FIGURE 2.** Proteomic analysis of P. falciparum DRMs. The proteomes of DRM fractions floated on a sucrose gradient prepared from P. falciparum (3D7) schizont-stage parasites with and without saponin treatment. The relative intensity of the red boxes is proportional to the degree of peptide coverage for each protein. The first 28 proteins on the saponin-treated list are expanded (top right). The N-terminal signal sequence (SS), number of predicted transmembrane domain(s) (TM), and predicted GPI-addition signal (asterisks) are indicated. By way of comparison with other whole schizont proteome data, we show the percentile rank ordered position by peptide coverage (Prot. %) of each protein in the previously determined whole schizont proteome (32) (i.e., the values 1–100% represent the most to least peptide coverage; absent means no peptides were detected for this protein). The timing of peak transcription (mRNA) in the life cycle is also shown (LR, late rings; ET, early trophozoites; ES, early schizonts; LS, late schizonts; Sp, sporozoites; Gm, gametocytes).

### Gene model (Name) | Features | SS | TM | Prot. % | mRNA
--- | --- | --- | --- | --- | ---
**PF13_0338 (Pf92)** | GPI-anchored/predicted | 1 | 1 | 23.0 | ES
**PF00250c (Pf96)** | GPI-binding/predicted; 6-cys | 1 | 0 | 27.0 | ES, LS
**PF00395c (Pf98)** | GPI-anchored/predicted; 6-cys | 1 | 0 | absent | LS, Sp, Gm
**PF13_0197 (MSP-7)** | GPI-binding/surface | 1 | 0 | 7.5 | LS
**PF02010c (MSP-3)** | GPI-anchored/surface | 1 | 0 | absent | LS
**PF13_0198 (MSP-7-like)** | GPI-binding/predicted | 1 | 0 | 50.3 | LS (low)
**PFI12.0126** | multimembrane span | 0 | 5 | absent | ES, Sp, Gm
**PFI1.0437** | ribosomal contaminant | 0 | 0 | absent | LR/ET
**PFI14.0453** | multimembrane span | 0 | 4 | 65.5 | weak
**PFB0015w (PCDK1)** | membrane-associated kinase | 0 | 0 | 17.4 | LS
**PF14.0056** | membrane-associated; MORN repeats | 0 | 0 | absent | LS
**PF14.0071 (Pf113)** | GPI-anchored/predicted | 1 | 1 | 39.6 | broad/Sp
**PF13.0009c (MSP-2)** | GPI-anchored/surface | 1 | 1 | absent | LS
**PF00615c (Pf12)** | GPI-anchored/predicted; 6-cys | 1 | 1 | 20.0 | LS, Sp, Gm
**MALP1.29** | parastrophophorous vacuole mem. | 0 | 0 | absent | ET (low)
**MALP1.25** | Possible surface/hotpy | 1 | 1 | 5.1 | broad
**PF02010c** | monosac. transporter | 0 | 12 | absent | broad
**PF00250c** | monosac. transporter | 0 | 12 | absent | broad (low)
**PFI1.0024 (Exp-1)** | monosac. transporter | 0 | 0 | absent | ET
**PFI14.0031 (MSP-1)** | GPI-anchored/surface | 1 | 1 | 1.8 | LS
**PF14.0012 (MSP-25)** | mitochondrion membrane? | 0 | 0 | 37.0 | broad (low)
**PF14.0030 (MSP-25)** | apicoplast | 0 | 1 | absent | LR/ET
**MALP1.130** | multimeric membrane | 1 | 1 | 40.2 | LS
**MALP1.144** | Possible surface/hotpy | 1 | 1 | 87.3 | ManSp
**MALP1.72** | high mobility group | 0 | 0 | 3.2 | broad/Gm
**chrBLOB_004173.gen_1 (RAMA)** | GPI-anchored/hotpy | 1 | 1 | ND | ND*
Antigen Discovery in Malaria Merozoites

FIGURE 3. Localization of Cys$_6$ proteins to the merozoite surface and apical organelles. A, localization of Pf38 and Pf12 fused to a GFP reporter and expressed in an inducible system that allows strong surface expression (21). MS, mid-schizont; LS, late schizont; M, merozoite. B, antisera raised to an MBP-Pf41 fusion protein (Pf41 amino acids 115–229) recognize a protein of ~40 kDa in P. falciparum (3D7) parasites that were either saponin-lysed (S) or untreated (NS) prior to solubilization in non-reducing sample buffer. The reactivity of mouse anti-Pf41 antibodies with schizonts and free merozoites in a double labeling immunofluorescence assay with rabbit anti-MSP1 antibodies is shown. The arrows indicate the apical end of the parasite (dense structures).

RESULTS AND DISCUSSION

The DRM Proteome of P. falciparum Schizonts Is Dominated by Surface, Rhoptry, Multimembrane-spanning, and Exported Proteins—Sucrose gradient flotation was used to purify DRMs from schizonts, an intraerythrocytic stage that consists of maturing merozoites enclosed in a parasitophorous vacuole. The effectiveness of this approach was examined by western blotting (Fig. 1). Each of the GPI-anchored proteins examined was recovered in the buoyant fractions. These proteins were generally well separated from the single-pass type 1 integral membrane proteins, which remained in the bottom fractions. It was evident that peripheral proteins located in the parasitophorous vacuole or rhoptry organelles also tended to associate with DRMs (Fig. 1). These proteins each possess a signal sequence but do not have an obvious membrane anchor element, suggesting that they interact with a membrane-associated protein. Unlike all other proteins examined by western blotting, SERA5 and SERA6 were only observed in DRM extracts from schizont-infected erythrocytes not treated with saponin (a process that removes much of the surrounding erythrocyte proteins). This was expected as it is well known that the peripheral SERA proteins do not remain with the parasite pellet upon saponin lysis (29, 30). Thus, the

nm anti-rabbit IgG and Alexa Fluor 488 nm anti-mouse IgG (Molecular Probes) diluted 1:1000. Parasites were mounted in Vectashield containing 4',6-diamidino-2-phenylindole (Vector Laboratories).

Immunodetection of Detergent-resistant Membrane Proteins—DRM fractions from the sucrose gradient were resuspended in an equal volume of 2 × non-reducing sample buffer and placed at 70 °C for 10 min. Samples were run under non-reducing conditions because a number of the antibodies used in the subsequent western blotting (e.g. those recognizing MSP-1, -4, and -5) recognize reduction-sensitive epitopes. Samples (20 μl) were loaded onto a 4–20% gradient Gradipore long-life Tris-HEPES-SDS precast polyacrylamide mini gel, spiking the first fraction with 6 μl of Bio-Rad prestained Precision Plus protein standards. Protein samples were electrophoresed under non-reducing conditions and transferred to Immobilon-P transfer membranes (Millipore) for Western blotting as described (24).

For immunoprecipitation of GFP fusion proteins expressed in P. falciparum, anhydrotetracycline was removed from parasite cultures 72 h prior to solubilization to induce expression of the transgene. Schizonts were solubilized in 1% Triton X-100, PBS, pH 7.6, at room temperature for 30 min in the presence of Roche Applied Science Complete protease inhibitor mixture. Samples were preabsorbed with control (from individuals not exposed to malaria) IgG-coated Sepharose (100 μl of a 50% suspension, 2 h, 4 °C) and then incubated with Pool M human IgG-coated Sepharose (from individuals not exposed to malaria) (Melbourne) beads (100 μl of a 50% suspension) and incubated at 4 °C for 4 h. Pool M (as with Pool P; see below) was prepared from pooled sera obtained from highly exposed (P. falciparum immune) Papua New Guinean adults (24). The unbound fraction (~1 ml) was retained, and beads were washed 3 × 10 min in immunoprecipitation buffer (PBS, pH 7.6, containing 1% Triton X-100 and protease inhibitors). Bound material was eluted with the addition of an equal bed volume of non-reducing SDS sample buffer (on ice, 5 min) followed by the subsequent addition of an equal bed volume of non-reducing sample buffer at 70 °C for 10 min.

P. falciparum Culture and Transfection—P. falciparum 3D7 strain parasites were cultured and synchronized using standard procedures (25, 26). Ring-stage parasites (~1% parasitemia) were transfected with 100 μg of purified plasmid DNA (Plasmid Maxi kit, Qiagen) as described previously (27), using modified electroporation conditions (28).

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SERAs are potentially membrane-associated by virtue of adherence to protein(s) that are themselves solubilized by saponin. Alternatively, membrane-bound SERA proteases may be activated and degraded by autolysis following exposure to the extracellular environment; recombinant forms of both SERA5 (31) and SERA6 7 are capable of autolysis.

To analyze the proteome of schizont-stage DRMs, extracts from both untreated and saponin-treated parasites were combined into four pools (Fig. 1), and the proteome of washed membranes present in each pool was determined. Pools 1–3 represent buoyant membrane fractions that the western blotting experiments in Fig. 1 suggested would comprise genuine DRM-associated proteins. Pool 4 represents material that remains at the bottom of the sucrose gradient and is predicted to contain mostly non-DRM parasite proteins that remain with a pellet of whole parasites and/or parasite organelles that are not effectively solubilized by the cold Triton X-100 extraction. However, we did expect some genuine DRM proteins to be present in Pool 4. 94 and 70 parasite proteins were detected in the saponin and non-saponin DRM proteomes, respectively (Fig. 2 and supplemental Tables S1 and S2). There was strong overlap between proteomes with 60% of the non-saponin DRM proteins also found in the saponin-treated DRM proteome; a total of 122 DRM proteins were detected. Only high confidence proteins were included in these lists, criteria that included the detection of at least two different peptides for each protein. Proteins were ordered according to the degree of buoyancy in their respective sucrose gradients (Fig. 2 and supplemental Tables S1 and S2).

Overall, both DRM proteomes (incorporating all four pools) were substantially enriched for proteins predicted to be membrane-associated by means of an encoded signal sequence and/or a transmembrane domain or known acylation site, from 24% in the whole schizont proteome (32) to 65–73% in the DRM proteomes. As expected, the enrichment for membrane proteins was greater (75–76%) in Pools 1–3 with the bottom fractions (Pool 4) containing a higher number of apparently contaminating proteins presumably associated with poorly solubilized parasite pellet material. Among the DRM proteins without a signal sequence or transmembrane domain were a number of proteins known to be associated with membrane proteins (e.g. the mitochondrial Tom40 homologue). Thus, the vast majority of proteins in the buoyant fractions, especially from the saponin-lysed material, appeared to be genuine DRM proteins. It was anticipated that the DRM proteome from saponin-lysed parasites would include fewer contaminants because this preparation is devoid of contaminating material from the cytosol of uninfected and infected erythrocytes.

Four classes of protein were particularly prominent in buoyant fractions (Pools 1–3): GPI-associated proteins (defined below), rhoptry proteins, multimembrane-spanning proteins, and proteins predicted to be exported from the parasite into the host erythrocyte cytosol by virtue of a PEXEL motif (33, 34). With respect to this latter group, the presence of a number of ring-infected erythrocyte surface antigen (RESA) homologues suggests that these virulence-associated exported proteins may largely constitute a subset of PEXEL motif proteins that are initially stored in merozoite dense granule organelles prior to their export after erythrocyte invasion (35).

Analogous to proteomic analyses of mammalian lipid rafts (36), the fractionation procedure appears to have enriched for lipid-anchored membrane proteins, with a strong peptide coverage of GPI-associated proteins in the most buoyant fractions (Fig. 2). Good peptide coverage was obtained for known, seemingly abundant GPI-anchored proteins such as MSP-2 and MSP-4, peptides of which were surprisingly poorly represented, or absent altogether (Fig. 2, absent) in previously described whole schizont P. falciparum proteomes (2, 3, 32). Thus, our procedures used to isolate, extract, and digest DRMs have facilitated the identification of a subset of relatively abundant proteins that were seemingly...
not effectively solubilized in the whole cell extracts examined in previous studies.

Thirteen GPI-associated proteins were detected, which can be categorized as follows: (i) known GPI-anchored proteins, (ii) predicted GPI-anchored proteins (i.e. possessing an N-terminal signal sequence and a characteristic C-terminal hydrophobic domain), (iii) proteins that bind to GPI-anchored proteins (MSP-7 (13)), and (iv) proteins that are predicted to bind to GPI-anchored proteins (i.e. protein homologues of groups 2 or 3 and the SERAs). Most GPI-associated proteins detected in the non-saponin extract were also present in the saponin DRM proteome; only SERA5 and SERA6 were unique to the non-saponin list consistent with the western blot data described above. Genes encoding all of the 13 GPI-associated proteins are transcribed in blood stages and are mostly co-regulated with maximal levels of expression late in the blood-stage cycle (Fig. 2 and supplemental Fig. S1) (4, 5).

Four of the six known GPI-anchored merozoite proteins (MSP-1, -2, -4, and RAMA) were in the DRM proteomes, and each was prominent in buoyant fractions. The absence of MSP-10 from the list was expected as this protein does not appear to incorporate into DRMs (17). It is less clear why MSP-5 was not detected, although it is perhaps only a minor component of the merozoite surface. Consistent with this, gene knock-out experiments have shown that MSP-5 is the only GPI-anchored merozoite protein not required for normal blood-stage growth.6

Four novel putative GPI-anchored proteins were detected in DRMs, and each was prominent in both saponin and non-saponin proteomes. Two of these, encoded by PF0395c and PFF0615c, comprise dual six-cysteine (Cys6) domains, a fold found in a family of Plasmodium proteins, some of which localize to the surface of gametocyte stage parasites and have potential as transmission blocking vaccines (37) (supplemental Fig. S2). Gene knock-out experiments have confirmed a key role for the best-characterized member of this family, Pfs48/45, in gamete fertilization, seemingly as an adhesive surface protein (38). Genes encoding PF0395c or PFF0615c have been identified previously, and the respective predicted proteins were termed Pf38 (renamed Pf38 here) and Pf12 (39–41). The two other putative GPI-anchored proteins detected, PF13_0338 and PF14_0201 (termed Pf92 and Pf113), are large novel proteins that do not possess obvious homology to other proteins, although both possess cysteine-rich domains.

PFD0240c (Pf41) represents another dual Cys6 protein that segregates into the DRM fraction. As with some other members of this family (e.g. Pf230), Pf41 does not encode a C-terminal GPI-attachment sequence (supplemental Fig. S2), and thus, is presumably peripherally associated with the surface. As with its cousin, MSP7-like protein (PF13_0196) is a peripheral protein that presumably associates with DRMs by virtue of an interaction with MSP-1 (13). Several other probable peripheral proteins (i.e. those with a signal sequence in the absence of other known organelle-targeting elements) were detected, some of which potentially represent proteins that associate with GPI-anchored species (Fig. 2, orange dots). Notably absent from the DRM proteome are peripheral proteins of the chromosome 10 MSP-3/6 family (15). Although some of these proteins appear to interact to a degree at least with DRM surface proteins (Fig. 1) (14), our analysis suggests that none are major structural components of the merozoite surface.
With respect to rhoptry proteins, the GPI-anchored rhoptry protein RAMA (10) was detected in the DRM proteome (Fig. 2). In addition, all members of the high (RhopH) and low (Rhoptry-associated protein (RAP)) protein complexes were identified (Fig. 2). It is likely that one or both of these complexes interact with a rhoptry resident GPI-anchored protein. Indeed, fluorescence resonance energy transfer analysis has suggested that both could be interacting with RAMA (10). Given that the majority of known *P. falciparum* rhoptry proteins were present in the proteome, it is likely that a number of the uncharacterized DRM proteins described in this report will reside in these organelles.

The resident parasitophorous vacuole membrane proteins EXP-1 and -2 were prominent in the DRM proteomes, especially in untreated schizonts, consistent with the previously described presence of EXP-1 in lipid rafts (42). Also of note is PfGAP50, the homologue of which in *Toxoplasma gondii* is associated with the inner membrane complex and is considered a key component of the myosin motor system that drives gliding motility and invasion (43). As expected, several membrane-spanning proteins were also detected in the DRM proteome including some transporters. Perhaps the most interesting in terms of a likely surface or apical organelle location are the hypothetical proteins encoded by PFL1825w and MAL13P1.130 (Fig. 2). Both genes are transcribed most strongly late in blood-stage development. Finally, in other cells, acylated proteins are also commonly found in DRMs, and indeed, one such protein, calcium-dependent protein kinase 1 (CDPK1; PFB0815w) (44), was among the prominent buoyant proteins. This membrane-associated kinase is particularly interesting because it is transcribed late in the blood-stage cycle, suggesting a possible role in erythrocyte invasion (Fig. 2).

**All Three Cys$_6$ Proteins and Pf92 Localize to the Merozoite Surface**—As mentioned previously, some Cys$_6$ family members appear to be adhesins and are prominent targets of transmission blocking vaccines. Because of this, we sought to investigate the localization and antigenicity of the three blood-stage Cys$_6$ proteins detected in the DRM proteomes. A GFP-targeting approach using an inducible expression system that directs strong, schizont-stage expression of transgenes (21) was employed to localize Pf12 and Pf38. The temporal expression of transgenes in this system mimics that of MSP-2 (21), which has an identical transcriptional profile to Pf12 and Pf38 (supplemental Fig. S1). Both Pf12- and Pf38-GFP fusion proteins were localized to the merozoite surface using this approach (Fig. 3A). Interestingly, Pf38-GFP was mostly concentrated in secretory organelles at the apical end of the parasite, exclusively at an early stage of schizont development, suggesting that Pf38 probably predominantly resides in these apical organelles. As two distinct dots are clearly evident in some Pf38-GFP merozoites, it is likely that this protein resides in the rhoptry organelles.

To investigate the localization of Pf41, we raised antibodies to the unusually large interdomain region of this protein, which we reasoned was likely to contain epitopes that are less dependent on correct conformation than the flanking cysteine-rich regions (supplemental Fig. S2). These antibodies recognized an ~40-kDa saponin-resistant protein that is spread over the parasite surface in whole schizonts but is concentrated toward the apical end in free merozoites (Fig. 3B). This implies that Pf41, which has no direct means of membrane attachment, interacts with another GPI-anchored surface protein and is potentially involved in erythrocyte invasion.

Localization on the merozoite surface suggested that the three merozoite Cys$_6$ proteins are likely to elicit an antibody response in infected individuals. Consistent with this, Pf12 and Pf38 fusion proteins expressed in *P. falciparum* were strongly recognized by antibodies present in individuals naturally exposed to *P. falciparum* (Fig. 4A). Although not previously defined as a blood-stage protein, reactivity with human antibodies was described previously for a recombinant form of Pf12 expressed on the surface of mammalian cells (40). Furthermore, an *E. coli*-expressed version of the central region of Pf41 also bound to antibodies present in human immune sera (Fig. 4B). Together, these data highlighted the antigenic potential of all three merozoite surface-localized Cys$_6$ proteins.

Using the inducible GFP expression system, we also show that another predicted GPI-anchored protein, Pf92, also localizes to the merozoite surface (Fig. 5). Transcription of the gene encoding Pf92 is also co-regulated with MSP-2 (supplemental Fig. S1). This large protein includes 14 cysteine residues that are relatively evenly spaced throughout the entire molecule. Thus, as with the Cys$_6$ proteins, it is likely that this protein is extensively disulfide-bonded. This conformational constraint probably contributed to the previous inability to detect these proteins using the prokaryotic expression methods commonly adopted in past studies to identify surface antigens.

In summary, we have identified proteins that comprise the DRM subcompartment of mature *P. falciparum* blood stages. Given that most, if not all, of the major known integral membrane surface proteins were prominent in DRMs, we propose that the DRM proteome includes the majority of the core structural components of the merozoite surface (Fig. 6). Although this already includes a number of novel proteins, it is likely that upon further investigation other previously uncharacterized proteins in the DRM proteome will also be found to localize to the surface and/or the apical end of the parasite (Fig. 6, inset). New proteins identified include three surface Cys$_6$ proteins, Pf12, Pf38, and Pf41, and two other previously uncharacterized putative GPI-anchored proteins, Pf92 and Pf113. Pf92 appeared to be spread evenly over the parasite surface, whereas the location of Pf113 remained unclear. In a complementary study, we verified the presence of GPI anchors on Pf12, Pf38, and Pf92 using $[^3]$H]glucosamine labeling and revealed that each of these proteins is relatively abundant in mature blood-stage parasites being present at copy numbers similar to well characterized MSPs.

Of particular note with respect to vaccine development, the three Cys$_6$ proteins were well recognized by antibodies from naturally infected individuals. Moreover, both Pf12 and Pf38 display remarkably broad expression across the different life stages, most notably Pf38, which is expressed strongly in schizont, gametocyte, and sporozoite stages (supplemental Fig. S1). Not only does this raise interesting questions about the potential broad nature of the biological function of such surface proteins, but it also suggests that a recombinant form of a single protein may have potential as a blood-stage, transmission blocking, and pre-erythrocytic vaccine.

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Antigen Discovery in Malaria Merozoites

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