Functional Characterization of *Plasmodium falciparum* Surface-Related Antigen as a Potential Blood-Stage Vaccine Target

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*Plasmodium falciparum* erythrocyte invasion is a multistep process that involves a spectrum of interactions that are not well characterized. We have characterized a 113-kDa immunogenic protein, PF3D7_1431400 (PF14_0293), that possesses coiled-coil structures. The protein is localized on the surfaces of both merozoites and gametocytes, hence the name *Plasmodium falciparum* surface-related antigen (PfSRA). The processed 32-kDa fragment of PfSRA binds normal human erythrocytes with different sensitivities to enzyme treatments. Temporal imaging from initial attachment to internalization of viable merozoites revealed that a fragment of PfSRA, along with PfMSP119, is internalized after invasion. Moreover, parasite growth inhibition assays showed that PfSRA P1 antibodies potently inhibited erythrocyte invasion of both sialic acid–dependent and –independent parasite strains. Also, immunoepidemiological studies show that malaria-infected populations have naturally acquired antibodies against PfSRA. Overall, the results demonstrate that PfSRA has the structural and functional characteristics of a very promising target for vaccine development.

**Keywords.** *Plasmodium falciparum*; malaria vaccine, erythrocyte invasion, novel antigens, naturally acquired immunity.

Malaria is a deadly infectious disease that affects inhabitants of the tropics and subtropical regions of the world and accounts for approximately 212 million cases and 429 000 deaths annually [1]. The clinical manifestation of the disease begins from the blood stage of the infection, during which the parasite invades human erythrocytes [2]. *Plasmodium falciparum* erythrocyte invasion is a complicated process that involves an array of receptor–ligand interactions [3] and/or protein–protein interactions [4–8] that occur at the parasite–host cell interface and facilitate the recruitment of the parasite’s invasion machinery. Thus, recent identification of *P. falciparum* surface proteins that are accessible to both humoral and cellular immune systems is a major advancement toward vaccine development against malaria [9–12]. This has given great impetus to the idea of a multiantigen vaccine as an intervention strategy against blood-stage malaria. Therefore, the selection and prioritization of candidate antigens are critical aspects of this strategy. Although several blood-stage antigens have been extensively studied, few have demonstrated the desired qualities for a vaccine candidate. One of the remarkable observations from the *P. falciparum* genome sequencing project was that nearly 60% of the parasite’s genes lacked sequence similarity to genes from other known organisms, and thus these genes have remained hypothetical with no defined functional roles [13]. Subsequently, the availability of more comprehensive genomic, proteomic, and transcriptomic datasets from both humans and *Plasmodium* has paved the way for further characterization of these hypothetical proteins using informatics-based approaches. This is required for successful identification of a repertoire of novel *P. falciparum* merozoite antigens that could be explored as targets for a rational vaccine design [14].

A detailed understanding of the functional roles of *P. falciparum* novel merozoite antigens, their localization, and their fate during invasion is critical to the identification of targets of host immunity and prioritization of merozoite antigens for inclusion in blood-stage malaria vaccines. Herein, we have identified a novel *P. falciparum* protein (PlasmoDB ID: PF3D7_1431400/PF14_0293) that we have named *P. falciparum* surface-related antigen (PfSRA), based on its dual subcellular localization on both merozoites and gametocytes. Native PfSRA is proteolytically processed into multiple fragments in parasite culture supernatant, and the 32-kDa fragment of PfSRA exhibits erythrocyte-binding activity. More important, antibodies against PfSRA potently inhibited merozoite invasion of erythrocytes by both sialic acid–dependent and sialic acid–independent parasite strains. The data also demonstrated that PfSRA is a target for naturally acquired immune responses in humans.
**METHODS**

**Screening for New Blood-Stage Vaccine Candidates**

To identify new blood-stage proteins as potential vaccine candidates, a systematic screening procedure was implemented. This included analysis of temporal gene expression relative to other well-characterized invasion-type genes and in silico interrogation of protein structural features. Once all of these selection criteria were ascertained, sequence alignment analysis was done to evaluate the conservation level of the target gene across the different *Plasmodium* species orthologs. Finally, we scanned the entire protein sequence using a current state-of-the-art online threading program to identify coiled-coil regions [15].

**Peptide Synthesis and Immunogenicity Studies**

Three synthetic peptides corresponding to the immunogenic epitopes were synthesized by GeneScript on the basis that they harbor coiled-coil signatures corresponding to the conserved regions in *Pf*SRA orthologs.

**Plasma Samples and Immunoreactivity**

Ethical approval was obtained from the ethics committees as documented previously [16, 17]. Immunoreactivity screenings of plasma samples were performed by enzyme-linked immunosorbent assay (ELISA) [12] and immuno-dot blotting [18] using the synthetic peptides *Pf*SRA P1 (NNKDNHNKDKTNENC); *Pf*SRA P2 (CENDNDEYGNKNNK); and *Pf*SRA P3 (CSNNKKKKKNDKKKK). R1 peptide (LFSKFGRSMHLKC) was used as control (R1 peptide blocks AMA 1–RON 4 interaction), and naive plasma was used as negative control.

**Affinity Purification of *Plasmodium falciparum* Surface-Related Antigen C-Terminal Human Antibodies**

The C-terminal α-*Pf*SRA human antibodies (α-*Pf*SRA P3) were affinity-purified from plasma samples of malaria-exposed children as described previously [19].

**Parasite Culture Supernatant, Ring-Stage Invasion Supernatant, and Erythrocyte-Binding Assay**

*Plasmodium falciparum* strains 3D7 and W2mef were maintained in culture as described previously [16]. Schizonts were purified using Percoll-alanine gradient centrifugation [20], followed by saponin lysis, and the recovered parasite pellets were further lysed in sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer. Parasite culture supernatant or ring-stage invasion supernatant were used as the source for native *Pf*SRA, which was detected by immunoblotting using primary α-rabbit antibodies to the 3 peptides (α-*Pf*SRA P1, α-*Pf*SRA P2, and α-*Pf*SRA P3). The immunoblots were developed using goat α-rabbit horseradish peroxidase–conjugated, secondary antibody, and enhanced chemiluminescence reagents (Thermo Scientific). Erythrocyte-binding assay (EBA) was performed using parasite culture supernatant as described previously [21].

**Permeabilized and Nonpermeabilized Immunofluorescence Assays**

Immunofluorescence microscopy was carried out using *P. falciparum* (3D7)–infected erythrocytes smeared onto glass slides and fixed in prechilled methanol for 30 minutes. Fixed erythrocytes were permeabilized using 0.1 % Triton X-100 formulated in phosphate-buffered saline. Nonpermeabilized, liquid immunofluorescence assay (IFA) was carried out as described previously [22]. After the washing step for both IFA conditions, the slides were blocked for 1 hour in PBS containing 3 % bovine serum albumin. Slides were probed with primary and secondary antibodies for the respective antigens and mounted in vectashield (Vector Laboratories Inc) supplemented with 4’,6-diamidino-2-phenylindole for staining the nucleus. Fluorescence microscopy was performed on an Olympus fluorescence microscope (BX41). Images captured were processed using the open access Fiji-Image J software (National Institutes of Health).

**Antibody Internalization Assay**

To test for the internalization of α-*Pf*SRA P3 antibodies, α-*Pf*SRA P3 and α-*Pf*MSP-1α polyclonal antibodies were incubated with tightly synchronized segmenting schizonts that were allowed to rupture and release merozoites. The viability of the released merozoites was assessed at the different stages (early, mid, and late) of an invading merozoite. For each stage, smears were prepared, fixed, and examined by immunofluorescence microscopy as described above.

**Growth Inhibition Assays**

Growth inhibition assays (GIAs) were performed as described previously [23], and parasitemia levels were determined using flow cytometry on a BD FORTESSA X-20 with Flo J software. Erythrocyte invasion inhibitory effects of the α-*Pf*SRA antibodies were estimated by comparison of percentage of invasion of controls with test assay.

**RESULTS**

**Identification of *Plasmodium falciparum* Surface-Related Antigen as a Potential Malaria Vaccine Candidate**

In a systematic screen of uncharacterized *P. falciparum* proteins for potential blood-stage vaccine candidates, we performed data-mining analysis for genes with peak mRNA expression levels in late schizogony using data from *P. falciparum* transcriptome studies [24, 25] and another study on the prediction of PISUB-1 protease specificity [26]. The details of all analyses have been described in the Supplementary Material (Supplementary Figure 1A and 1B). Overall, *Pf*SRA emerged as the top hit with both signal peptide and a predicted glycosylphosphatidylinositol attachment site.

Furthermore, we generated sequence alignments with other orthologs of *Pf*SRA and showed that the C-terminus of *Pf*SRA had 5 positionally conserved cysteine residues across
the different \emph{Plasmodium} species orthologs (Supplementary Figure 2). Additional predictive analysis from PSI-Pred revealed that the PfSRA protein sequence harbored coiled-coil signatures (Supplementary Figure 3). This signature forms stable structures that elicit functional antibodies and was considered as a basis for the design of 3 PfSRA chemically synthesized peptides used for antibody generation.

**Induction of \emph{Plasmodium falciparum} Surface Related Antigen-Specific Antibodies Using Synthetic Peptides**

Despite several optimization procedures for expression in \emph{Escherichia coli}, attempts to express recombinant PfSRA protein were unsuccessful. However, we designed 3 peptides for synthesis that corresponded to the conserved regions of PfSRA in other \emph{Plasmodium} species orthologs (Figure 1A). These PfSRA-derived peptides (PfSRA P1, PfSRA P2, and PfSRA P3) include coiled-coil signatures (Supplementary Figure 3). Immunization of rabbits with the 3 synthetic peptides (PfSRA P1, PfSRA P2, and PfSRA P3) by Genescript resulted in high titers of PfSRA peptide-specific antibodies, which were used in subsequent experiments.

**Proteolytic Processing and Erythrocyte Binding Activity of \emph{Plasmodium falciparum} Surface-Related Antigen**

Preimmune sera were used for all immunoblotting as a negative control that did not detect native PfSRA. As expected, α-EBA-175 (R217) antibodies did not detect native EBA-175 under reduced condition (Figure 1B, I). The antibodies (α-PfSRA P1, α-PfSRA P2, and α-PfSRA P3) consistently detected multiple processed fragments (17, 32, and 58 kDa) of the native PfSRA in ring-stage invasion supernatants (Figure 1B, II–V) or parasite culture supernatants (Figure 1C, I–IV) and schizont lysates (Figure 1D, I–IV).

To identify the fragment(s) of PfSRA that possess erythrocyte-binding activity, we performed erythrocyte binding assays. The 32-kDa fragment of PfSRA was clearly detected in the eluate, suggesting that it bound erythrocytes. Under higher exposure, the 113-kDa full-length PfSRA and the 58-kDa processed fragment were also detectable (Supplementary Figure 3), suggesting a weaker binding relative to the 32-kDa fragment. Of interest, the binding was sensitive to neuraminidase and trypsin treatments but resistant to chymotrypsin treatment (Figure 1F). As a control, recombinant EBA-175 (R217) bound erythrocytes with the same sensitivity to enzyme treatments (Figure 1F).

**Subcellular Localization of Native \emph{Plasmodium falciparum} Surface-Related Antigen in Asexual Stage**

Stage-specific expression analysis in \emph{P. falciparum} asexual stages by IFAs showed that all α-PfSRA peptide antibodies labeled the surface membranes of merozoites in intact schizonts and released merozoites, respectively (Figure 2A–C). However, α-PfSRA antibodies did not label developing ring-stage parasites, which served as a useful internal control for subsequent experiments in asexual stages.

Colabeling of segmenting or rupturing schizonts and released merozoites with α-PfSRA P1 antibodies and the micronemal marker α-PfAMA-1 showed no colocalization (Figure 2D), even though reports exist about the circumferential staining pattern of PfAMA-1 [27]. However, α-PfSRA P1 antibodies and α-PfMSP119 antibodies colocalized at the parasitophorous vacuole in late trophozoites, segmenting schizonts and released merozoites (Figure 2E). In segmenting schizonts and released merozoites, both proteins colocalized on the merozoite surface (Figures 2E and 3A). Liquid IFA shows that both α-PfSRA P1 antibodies and α-PfAMA-1 antibodies labeled the surface of nonpermeabilized merozoites (Figure 3B). A control panel shows α-PfSRA P1 labeling of an invading merozoite under permeabilized condition, but no labeling was observed for α-Pfs48/45 antibodies (Figure 3B).

**Fate and Shedding Patterns of Native \emph{Plasmodium falciparum} Surface-Related Antigen During Erythrocyte Invasion**

To determine the fate of native PfSRA during invasion, invading merozoites at different time-points (early, mid, late, and postinvassion) were colabeled with α-PfSRA P3 and α-PfMSP119 antibodies. We observed labeling of both α-PfSRA P3 and α-PfMSP119 antibodies in all of the time points of invasion (Figure 3C and 3D) suggesting that a fragment or the unprocessed forms of PfSRA are carried into erythrocytes during invasion. As a control, late-stage parasites and internalized merozoites were colabeled with α-PfSRA P3 and the gamocyte surface marker α-Pfs48/45 antibodies. Whereas α-PfSRA P3 antibody labeled internalized merozoite, no labeling was observed with α-Pfs48/45 antibody (Figure 3B).

**Recognition of \emph{Plasmodium falciparum} Surface-Related Antigen Peptides by Human Plasma**

A panel of plasma samples from malaria-infected children resident in 3 malaria-endemic sites in Ghana (Accra, Navrongo, and Kintampo) was evaluated for reactivity to PfSRA synthetic peptides by ELISA. Plasma antibodies from children in Accra and Navrongo showed no reactivity to the R1 peptide beyond background (normal human serum [NHS]) levels, whereas those from Kintampo were only slightly above background; however, this difference was statistically significant (P = .04) (Figure 4A). Plasma samples from all 3 sites appeared to recognize all 3 PfSRA peptides, and all groups showed reactivity above background levels (Figure 4B–D). Statistically, these differences in reactivity against PfSRA P1 were significant compared with NHS for plasma from Navrongo (P < .0001) and Accra (P < .0001) but not Kintampo (Figure 4B). Reactivity for PfSRA P2 was significant compared with NHS for plasma from Kintampo (P < .0001) and Accra (P = .04) but not Navrongo (Figure 4C). However, the reactivity against PfSRA P3 was significant for all 3 sites (Kintampo, P < .0001; Navrongo, P = .02; and Accra, P < .0001) (Figure 4D).
Figure 1. Domain organization and proteolytic processing of native Plasmodium falciparum surface-related antigen (PfSRA). A, PfSRA possesses a signal peptide and a predicted glycosylphosphatidylinositol (GPI) anchor. Cut-1 and -2 represent PfSUB-1 cleavage sites analyzed by the Prediction of Protease Specificity tool. Cut-3 represents the GPI–transamidase cleavage site for the predicted GPI attachment signal. PfSRA P1, PfSRA P2, and PfSRA P3 designate conserved regions of PfSRA in different orthologs that possesses coiled-coil signatures for which chemically synthesized peptides were designed. C designates the carboxyl terminus, and N designates the amino terminus.

B, α-PfSRA antibodies detect multiple processed fragments of native PfSRA during immunoblotting of 3D7 ring-stage invasion supernatants (ISs) that were probed with mouse α-EBA-175 (R217) antibody (I), rabbit α-PfSRA P1 antibody (lot no.: A417040387) (II), α-PfSRA P2 antibody (lot no.: A417040332) (III), and α-PfSRA P3 antibody (lot no.: A417040334) (IV). The colored bars at the far right represent the predicted processed fragments (70, 58, 32, and 17 kDa) (V).

C, Anti-PfSRA antibodies detect multiple processed fragments of native PfSRA during immunoblotting of 3D7 parasite culture supernatants (CSs) that were probed with preimmune sera (I), α-PfSRA P1 antibody (II), α-PfSRA P2 antibody (III), and α-PfSRA P3 antibody (IV).

D, Anti-PfSRA antibodies detect multiple processed fragments of native PfSRA during immunoblotting of 3D7 schizont lysates (SLs) that were probed with preimmune sera (I), α-PfSRA P1 antibody (II), α-PfSRA P2 antibody (III), and α-PfSRA P3 antibody (IV). E, The colored bars at the far right represent the predicted processed fragments (70, 58, 32, and 17 kDa) (V).

F, Recombinant EBA-175 was used as a control, and it bound erythrocytes in a neuraminidase-sensitive, trypsin-sensitive, and chymotrypsin-resistant manner. Similarly, the 32-kDa processed fragment of native PfSRA bound erythrocytes in a neuraminidase-sensitive, trypsin-sensitive, and chymotrypsin-resistant manner. Abbreviations: CT, chymotrypsin; GH, normal human erythrocyte ghost; NT, neuraminidase; TT, trypsin; UT, untreated control.
Figure 2. Subcellular localization of native *Plasmodium falciparum* surface-related antigen (PSRA) in asexual stages. Methanol-fixed 3D7 ring, trophozoite, schizont, and released merozoites were stained with α-PSRA antibodies. A, Anti-PSRA P1 antibody (green; 1:100). B, Anti-PSRA P2 antibody (green; 1:100). C, Anti-PSRA P3 antibody (green; 1:100). D, PSRA does not colocalize with *PfAMA-1* on the merozoite surface. Colabeling of rabbit α-PSRA-1 (red) with mouse α-*PfAMA-1* (1:100; green) antibodies in intact schizonts show that PSRA is localized on the merozoite surface. Secondary antibodies used are Alexa 488–conjugated goat α-mouse immunoglobulin G (IgG) Alexa 568-conjugated goat α-rabbit IgG (1:200; Life Technologies). Exposure times were identical for all images of the same channel. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). E, PSRA colocalizes with *PfMSP1* at the parasitophorous vacuolar membrane in late trophozoites and on the merozoite surface. Colabeling of rabbit α-PSRA-1 (1:100; red) with mouse α-*PfMSP1* (1:100; green) antibodies in late trophozoites and intact schizonts show that PSRA is localized at the PV and on the merozoite surface. Colocalization coefficient (R) is displayed at the far right. Secondary antibodies used are Alexa 488–conjugated goat α-mouse IgG and Alexa 568–conjugated goat α-rabbit IgG (1:200; Life Technologies). Exposure times are identical for all images of the same channel. Nuclei were stained with DAPI (blue). Abbreviations: BP, brightfield; DAPI, 4′,6-diamidino-2-phenylindole; PSRA, *Plasmodium falciparum* surface-related antigen.
Recognition of Plasmodium falciparum Surface-Related Antigen by Naturally-Acquired Antibodies

Consistent with the ELISA data, we also established by immunodot blotting that plasma samples from malaria-infected children recognized PfSRA P1 (Figure 5A). Thus, α-PfSRA P3-specific human antibodies against the C-terminus of the protein were purified from a pooled sample of plasma from Kintampo children that showed immunoreactivity (Figure 4D). Consistent with the data from rabbit α-PfSRA antibodies (Figures 2E and 3A), human α-PfSRA P3 immuno-affinity purified antibodies stained schizonts and released merozoites that colocalized with α-PfMSP1_19 antibodies on the merozoite surface (Figure 5B).

Subcellular Localization of Native Plasmodium falciparum Surface-Related Antigen in Gametocytes

We performed stage-specific expression analysis in gametocytes (stage III–V) by microscopy and observed that α-PfSRA P3 antibodies specifically labeled the membranes of gametocytes, whereas the control antibody (PfMSP1_19) did not label gametocytes (Figure 6A). Similarly, we colabeled gametocytes...
(stage II–V) with the respective α-PfSRA peptide antibodies and the gametocyte surface marker α-Pfs48/45 antibody and showed close colocalization that appeared to be stage-dependent (Figure 6B–D). Furthermore, PfSRA expression in gametocytes appeared not to be sex-specific, with α-PfSRA P1 antibodies labeling both male and female gametocytes and α-tubulin antibodies labeling only male gametocyte (Supplementary Figure 5).

Growth Inhibitory Activity of Plasmodium falciparum Surface-Related Antigen Peptide-Induced Antibodies

We evaluated the invasion inhibitory activity of α-PfSRA antibodies against P. falciparum 3D7 and W2mef and showed that all PfSRA peptide antibodies exhibited 70%–80% inhibition at 750 μg/mL, whereas the preimmune control did not show any inhibition (Supplementary Figure 6A and 6B). Because all 3 PfSRA peptide antibodies were inhibitory at higher concentrations (100–750 μg/mL) (Supplementary Figure 6A and 6B), we have shown that, whereas the preimmune sera and a control immunoglobulin G (IgG) did not inhibit parasite invasion at lower concentrations (25–75 μg/mL), α-PfSRA antibodies exhibited a concentration-dependent inhibition of parasite invasion (Figure 7A). Of all 3 of the PfSRA peptide antibodies tested, only α-PfSRA P1 antibodies showed 60% inhibition of parasite invasion at 75 μg/mL. As a control, anti-Basigin antibodies showed 75% inhibition of parasite invasion at 10 μg/mL (Figure 7A).
To exclude the possibility that serum contaminants might be interfering with parasite invasion, we generated antibodies against a shorter construct of R1-peptide using the same purification procedures for α-\(\text{PfSRA}\) peptide-specific antibodies, and no inhibition of parasite invasion was observed (Figure 7A). Because all 3 α-\(\text{PfSRA}\) antibodies were inhibitory at 100–750 μg/mL, we tested combinations of α-\(\text{PfSRA}\) peptide antibodies against 3D7 and observed that this strategy did not greatly impact on parasite invasion inhibition (Figure 7B). Furthermore, we performed invasion inhibition assays with 2 Ghanaian clinical isolates (MISA010 and MISA011) and observed similar patterns of parasite invasion inhibition for α-\(\text{PfSRA}\) peptide antibodies (Figure 7C and 7D).

**DISCUSSION**

The focus of this work was the identification and functional characterization of a potential blood-stage malaria vaccine candidate (\(\text{PfSRA}\)). Using Bioinformatics and data-mining analysis of published \(\text{P. falciparum}\) transcriptomes and proteomes [24, 25], we identified \(\text{PfSRA}\) to be reminiscent of a surface protein, defined by the presence of a signal peptide and a predicted glycosylphosphatidylinositol attachment signal [28, 29]. Indeed, \(\text{PfSRA}\) has been shown to have \(\text{PfSUB-1}\) cleavage sites that were assigned using the Prediction of Protease Specificity analysis platform [25]. The presence of these proteolytic cleavage sites in \(\text{PfSRA}\) may allude to processing events occurring prior to invasion of erythrocytes. Besides, proteolytic processing in the malaria parasite has been shown to be relevant for cascades of interaction occurring at the parasite–host cell interface [21, 30]. Furthermore, because orthologs are good candidates for multispecies vaccines, we generated sequence alignments with other \(\text{PfSRA}\) orthologs, which showed that the C-terminus of \(\text{PfSRA}\) has 5 positionally conserved cysteine residues across the different species orthologs. Our attempt to express the recombinant \(\text{PfSRA}\) that possesses 67% unstructured regions was unsuccessful. Similarly, others have attempted the expression of the mature recombinant \(\text{PfSRA}\) in HEK293E cells using a codon-optimized gene (Geneart) and have also been unsuccessful [28]. Considering that \(\text{PfSRA}\) is an asparagine-rich protein with numerous potential N-linked glycosylation sites, we did not attempt expression in insect cells because the system produces proteins with more complex N-linked glycosylation [31] that may not present the relevant sugar epitopes of native glycosylation [32]. Therefore, the challenges associated with the expression of recombinant \(\text{PfSRA}\) necessitated the design of chemically synthetized peptides for an epitope-based vaccine strategy.

\(\text{Plasmodium falciparum}\) surface-related antigen harbors coiled-coil domains that are known to be less polymorphic [33, 34], and they form stable structures that elicit functional antibodies that block relevant domains in many organisms [35–37]. Interestingly, these domains have been evaluated as potential targets for peptide-based vaccines [38–40]. The 3 \(\text{PfSRA}\) peptide antibodies from rabbits specifically detected breakdown products of native \(\text{PfSRA}\) in ring-stage invasion supernatant or parasite culture lysates. This indicated...
that \textit{Pf}SRA synthetic peptides are antigenic mimics of the native parasite protein. Our data showed that \textit{Pf}SRA is postsynthetically processed by cleavage into parasite culture supernatant, and this is consistent with the \textit{Pf}SUB-1 cleavage sites it harbors. The merozoite surface is remodeled by a series of proteolytic processing events; the physiological relevance of these events in the malaria parasite remains poorly described. However, there are reports suggesting that proteolytic processing may result in activation, structural rearrangement, or acquisition of other new functional properties of native parasite proteins [41].

We have described the fate and shedding pattern of native \textit{Pf}SRA by temporal immunofluorescence imaging using \( \alpha\text{-Pf}SRA \) and \( \alpha\text{-PfMSP1}_{19} \) antibodies that bound the merozoite surface and were internalized during erythrocyte invasion. Consistent with this observation is a previous report that \( \alpha\text{-PfMSP1}_{19} \) antibodies were carried into invaded erythrocytes,
disrupted intra-erythrocytic development, and inhibited erythrocyte invasion [42–44]. Although the molecular mechanism underlying the internalization of antibodies remains debatable, it was suggested that the tight junction between the merozoite and the erythrocyte might consist of transient interactions that allow the passage of antibodies or surface proteins [19].
Because all rabbit α-PfSRA peptide antibodies recognized different PfSRA polypeptide fragments in ring-stage invasion supernatant or parasite culture supernatant and schizont lysates at varying thresholds, it was expedient to investigate whether processing could influence differential subcellular localization of PfSRA in the parasite. Interestingly, all 3 rabbit α-PfSRA peptide antibodies showed circumferential association on the merozoite surface at the timing of schizont rupture and merozoite release. Similarly, we performed colabeling in IFAs with gametocytes (stage II–V) using all 3 rabbit α-PfSRA peptide antibodies with the gametocyte surface marker PfS48/45. A clear, punctate rim-fluorescence pattern was observed for all 3 rabbit α-PfSRA peptide antibodies that appeared to colocalize with PfS48/45 in a stage-dependent manner based on the colocalization coefficient. Therefore, the consistency in the staining patterns of all 3 rabbit α-PfSRA peptide antibodies in both asexual and gametocyte stages suggested that proteolytic processing of PfSRA does not cause changes in the subcellular localization of the protein. Although the distribution of proteins in male or female gametocytes could be linked to functional divergence between the sexes [45], we observed the expression of PfSRA in both male and female gametocytes. Consistent with this observation is an existing report on Plasmodium berghei gametocyte egress and sporozoite traversal protein (PbGEST) expression in both sexes [46].

Also, it was imperative to determine whether PfSRA in released merozoites was accessible to humoral immune surveillance during the short period of erythrocyte invasion. Our serological screens, buttressed by immuno-dot blot assays, with plasma samples from malaria-infected children residing at different endemic sites showed differences in total IgG recognition frequencies for PfSRA peptides. This could be linked to varying transmission intensity rates as reported for samples collected from different endemic sites in previous studies [33, 47]. In most cases, the reactivity of all 3 PfSRA synthetic peptides was low, and the likely explanation for this could be the hindered accessibility of PfSRA in the native context. However, our data from IFAs showed that the immuno-affinity purified, human α-PfSRA peptide antibodies labeled native PfSRA, which suggests that malaria-infected populations have naturally acquired antibodies against PfSRA.

Generally, several receptors have been characterized based on their sensitivities to different enzyme treatments. Notably, neuraminidase removes sialic acids from glycoporphins, trypsin cleaves peptide backbones of several receptors (glycoporphin A, glycoporphin C, and complement receptor 1), and chymotrypsin cleaves glycoporphin B and complement receptor 1, among others [48]. We have shown that the 32-kDa–processed fragment of native PfSRA binds normal human erythrocytes, but the molecular identity of the receptor remains unknown. However, we have classified the putative receptor for PfSRA as sialic acid–dependent on the basis of its binding specificity, which is sensitive to treatments with both neuraminidase and trypsin but resistant to chymotrypsin, a binding phenotype that fits the description of the receptor glycoporphin C. The enzyme sensitivity profile of PfSRA binding to erythrocytes is similar to that observed for PfEBA-140 (region II), the parasite ligand for glycoporphin C [49]. Additional investigations are required to determine whether PfSRA also interacts with glycoporphin C, possibly via a different binding site.

Our data revealed that PfSRA peptides induce functional antibodies that inhibited P. falciparum erythrocyte invasion of both laboratory strains and clinical isolates. The observed invasion inhibitory activity of rabbit α-PfSRA peptide antibodies could be attributed to indirect effects of antibody binding to the merozoite surface or direct inhibition of proteolytic processing events. The demonstration that PfSRA synthetic peptides induced erythrocyte invasion inhibitory antibodies and the successful purification of a limited amount of PfSRA-specific human antibodies from patient plasma suggested that the synthetic peptides possessed structural integrity or conformation that mimics the native PfSRA.

In summary, this study has provided relevant new information regarding the proteolytic processing of PfSRA that supports the idea of targeting these cleavage events for development of antimalarial therapies. The expression of PfSRA in late stages of gametocytes is an unprecedented opportunity that should be explored for potential transmission-blocking vaccines. Also, PfSRA-specific immune responses triggered in natural infections may inform the inclusion of PfSRA as a candidate for epitope-based, blood-stage malaria vaccine development.

Supplementary Data
Supplementary materials are available at The Journal of Infectious Diseases online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes
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25. Le Roch KG, Zhou Y, Blair PL, et al. Discovery of gene function by expression profiling of the malaria parasite life cycle. Science 2003; 301:1503–8.
26. Gilson PR, Nebl T, Vukcevic D, et al. Identification and stoichiometry of glycosylphosphatidylinositol-anchored membrane proteins of the human malaria parasite Plasmodium falciparum. Mol Cell Proteomics 2006; 5:1286–99.
27. Douglas AD, Williams AR, Knuepfer E, et al. Neutralization of Plasmodium falciparum merozoites by antibodies against PIRH5. J Immunol 2014; 192:245–58.
28. Crosnier C, Wanaguru M, McDade B, et al. A library of functional recombinant cell-surface and secreted P falciparum merozoite proteins. Mol Cell Proteomics 2013; 12:3976–86.
29. Silmon de Monerri NC, Flynn HR, Campos MG, et al. Global identification of multiple substrates for Plasmodium falciparum SUB1, an essential malarial processing protease. Infect Immun 2011; 79:1086–97.
30. Das S, Hertrich N, Perrin AJ, et al. Processing of Plasmodium falciparum merozoite surface protein MSP1 activates a spectrin-binding function enabling parasite egress from RBCs. Cell Host Microbe 2015; 18:433–44.
31. Patel SD, Ahouidi AD, Bei AK, et al. Plasmodium falciparum merozoite surface antigen, PIRH5, elicits detectable levels of invasion-inhibiting antibodies in humans. J Infect Dis 2013; 208:1679–87.
32. Bushkin GG, Ratner DM, Cui J, et al. Suggestive evidence for Darwinian selection against asparagine-linked glycans of Plasmodium falciparum and Toxoplasma gondii. Eukaryot Cell 2010; 9:228–41.
33. Villard V, Agak GW, Frank G, et al. Rapid identification of malaria vaccine candidates based on alpha-helical coiled coil protein motif. PLoS One 2007; 2:e645.
34. Kulantha C, Kajava AV, Corradin G, Felger I. Sequence conservation in Plasmodium falciparum alpha-helical coiled coil domains proposed for vaccine development. PLoS One 2009; 4:e5419.
35. Gustchina E, Li M, Ghirlando R, et al. Complexes of neutralizing and non-neutralizing affinity matured Fabs with a mimetic of the internal trimeric coiled-coil of HIV-1 gp41. PLoS One 2013; 8:e78187.
36. Jiang Z, Gera L, Mant CT, et al. Platform technology to generate broadly cross-reactive antibodies to α-helical epitopes in hemagglutinin proteins from influenza A viruses. Peptide Science 2016; 106:144–59.
37. Tripet B, Kao DJ, Jeffers SA, Holmes KV, Hodges RS. Template-based coiled-coil antigens elicit neutralizing antibodies to the SARS-coronavirus. J Struct Biol 2006; 155:176–94.
38. Adda CG, Tilley L, Anders RF, Foley M. Isolation of peptides that mimic epitopes on a malarial antigen from random peptide libraries displayed on phage. Infect Immun 1999; 67:4679–88.
39. Demangel C, Rouyre S, Alzari PM, et al. Phage-displayed mimotopes elicit monoclonal antibodies specific for a malaria vaccine candidate. Biol Chem 1998; 379:65–70.
40. Stoute JA, Ballou WR, Kolodny N, Deal CD, Wirtz RA, Lindler LE. Induction of humoral immune response against Plasmodium falciparum sporozoites by immunization with a synthetic peptide mimotope whose sequence was derived from screening a filamentous phage library. Infect Immun 1995; 63:934–9.
41. Pachebat JA, Kadekoppala M, Grainger M, et al. Extensive proteolytic processing of the malaria parasite merozoite surface protein 7 during biosynthesis and parasite release from erythrocytes. Mol Biochem Parasitol 2007; 151:59–69.
42. Dluzewski AR, Ling IT, Hopkins JM, et al. Formation of the food vacuole in Plasmodium falciparum: a potential role for the 19 kDa fragment of merozoite surface protein 1 (MSP1(19)). PLoS One 2008; 3:e3085.
43. Blackman MJ, Scott-Finnigan TJ, Shai S, Holder AA. Antibodies inhibit the protease-mediated processing of a malaria merozoite surface protein. J Exp Med 1994; 180:389–93.
44. Woehlbier U, Epp C, Kauth CW, et al. Analysis of antibodies directed against merozoite surface protein 1 of the human malaria parasite Plasmodium falciparum. Infect Immun 2006; 74:1313–22.
45. Khan SM, Franke-Fayard B, Mair GR, et al. Proteome analysis of separated male and female gametocytes reveals novel sex-specific Plasmodium biology. Cell 2005; 121:675–87.
46. Talman AM, Lacroix C, Marques SR, et al. PbGEST mediates malaria transmission to both mosquito and vertebrate host. Mol Microbiol 2011; 82:462–74.
47. Céspedes N, Habel C, Lopez-Perez M, et al. Plasmodium falciparum sporozoites by immunization with PfRH5. J Immunol 2009; 180:389–93.
48. Woehlbier U, Epp C, Kauth CW, et al. Analysis of antibodies directed against merozoite surface protein 1 of the human malaria parasite Plasmodium falciparum. Infect Immun 2006; 74:1313–22.
49. Céspedes N, Habel C, Lopez-Perez M, et al. Plasmodium vivax antigen discovery based on alpha-helical coiled coil protein motif. PLoS One 2014; 9:e100440.
50. Bowyer PW, Stewart LB, Aspeling-Jones H, et al. Variation in Plasmodium falciparum erythrocyte invasion phenotypes and merozoite ligand gene expression across different populations in areas of malaria endemicity. Infect Immun 2015; 83:2575–82.
51. Mayer DG, Kaneko O, Hudson-Taylor DE, Reid ME, Miller LH. Characterization of a Plasmodium falciparum erythrocyte-binding protein paralogous to EBA-175. Proc Natl Acad Sci U S A 2001; 98:5222–7.