SURFIN is a polymorphic antigen expressed on Plasmodium falciparum merozoites and infected erythrocytes

Gerhard Winter, Satoru Kawai, Malin Haeggström, Osamu Kaneko, Anne von Euler, Shin-ichiro Kawazu, Daniel Palm, Victor Fernandez, and Mats Wahlgren

1Microbiology and Tumorbiology Center, Karolinska Institutet, SE-17177 Stockholm, Sweden
2Department of Tropical Medicine and Parasitology, Dokkyo University School of Medicine, Tochigi 321-0293, Japan
3Department of Molecular Parasitology, Ehime University School of Medicine, Ehime 791-0295, Japan
4Research Institute, International Medical Center of Japan, Tokyo 162-8655, Japan
5The Swedish Institute for Infectious Disease Control, SE-17182 Stockholm, Sweden

The surfaces of the infected erythrocyte (IE) and the merozoite, two developmental stages of malaria parasites, expose antigenic determinants to the host immune system. We report on surface-associated interspersed genes (surf genes), which encode a novel polymorphic protein family, SURFINs, present on both IEs and merozoites. A SURFIN expressed in 3D7 parasites, SURFIN4.2, was identified by mass spectrometric analysis of peptides cleaved off the surface of live IEs with trypsin. SURFINs are encoded by a family of 10 surf genes, including three predicted pseudogenes, located within or close to the subtelomeres of five of the chromosomes. SURFINs show structural and sequence similarities with exported surface-exposed proteins (PvSTP1, PKSICavar, PvVIR, Pf332, and PfEMP1) of several Plasmodium species. SURFIN4.2 of a parasite other than 3D7 (FCR3S1.2) showed polymorphisms in the extracellular domain, suggesting sequence variability between genotypes. SURFIN4.2 not only was found cotransported with PfEMP1 and RIFIN to the IE surface, but also accumulated in the parasitophorous vacuole. In released merozoites, SURFIN4.2 was present in an amorphous cap at the parasite apex, where it may be involved in the invasion of erythrocytes. By exposing shared polymorphic antigens on IEs and merozoites, the parasite may coordinate the antigenic composition of these attachment surfaces during growth in the bloodstream.

As Plasmodium falciparum parasites mature in the infected erythrocyte (IE) into pigmented trophozoites, parasite-derived molecules emerge on the cell’s surface. These confer unique adhesive properties to the IE and thereby determine the clinical manifestations of the disease. The most intensively studied IE surface protein, P. falciparum erythrocyte membrane protein–1 (PfEMP1), is a 200–400-kD variant antigen expressed in the mature IE. The clonal ex-

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one clade with a putative \textit{P. vivax} transmembrane protein, PvSTP1. The structure of the SURFIN–PvSTP1 clade incorporates features of several exported and surface-expressed proteins from human, rodent, and monkey malaria parasites. The predicted ectodomains of SURFIN–PvSTP1 show structural similarity to the external cysteine-rich domains (CRDs) of VIR proteins in \textit{P. vivax} (5). In contrast, the predicted endodomains of SURFIN–PvSTP1 are related in sequence to the internal domains of PfEMP1, the exported erythrocyte giant membrane-associated antigen 332 (6), and the \textit{P. knowlesi} variant surface antigen SICA\textsubscript{var} (7). We show that a SURFIN expressed in the laboratory parasite 3D7S8 is cotransported with RIFIN and PfEMP1 to Maurer’s clefts and presented on the IE surface. Moreover, SURFIN is part of an amorphous layer attached to the apex of released merozoites, which establishes for the first time an antigenic link between the merozoite and IE surfaces. We therefore conclude that the IE and the merozoite surfaces share molecular features not previously recognized.

\section*{RESULTS}
\subsection*{Identification of SURFIN, a high molecular mass exported antigen}

To identify proteins exposed at the IE surface, we developed a proteomic approach in which we directly targeted protein moieties displayed on the surface of live IEs. For this purpose, we used the recently established placenta-binding parasite 3D7S8 (8). Parasite cultures grown in fresh red blood cells were tightly synchronized by consecutive sorbitol treatments and harvested at 24 h after invasion (a.i.). Live trophozoite-stage IEs were treated with mild trypsin in order to release, or “shave off,” peptides derived from surface-exposed proteins. The conditions for the trypsination were based on previous results in which PfEMP1 was removed quantitatively from the IEs, even though no or only limited cleavage was observed in major human erythrocyte proteins (Band III) and glycoporphins (9). Thus, the obtained peptide-containing supernatant was analyzed by matrix-assisted laser desorption/ionization (MALDI)–time of flight mass spectrometry, resulting in 54 significant mass peaks. A Mascot peptide search against a human database identified only hemoglobin with significant probability (five peptides; \( P < 0.05 \)), which constitutes >95% of the total cellular erythrocyte proteins (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20041392/DC1). This may have been because of very limited cell breakage, although no other internal erythrocyte protein was identified with significant probability.

After human protein–derived mass peaks were subtracted, a total of 49 peptide masses were matched against a \textit{P. falciparum} database. We performed a mass fingerprint analysis using the PeptideSearch and Mascot search engines and retrieved a list of candidate proteins from which a gene, PFD1160w, was selected for further analysis based on criteria detailed in Fig. S1. The gene structure of PFD1160w consists of two exons of 2,292 and 4,891 bp, respectively, separated by a relatively small intron of 159 bp (Fig. S1). The 286-kD predicted protein encoded by PFD1160w contains a putative transmembrane (TM) region located at the 3’ end of exon 1. Mass fingerprint analysis initially identified six tryptic peptides that matched the PFD1160w gene, four of which were located in the smaller exon 1– and two in the larger exon 2–encoded domains. An increasingly stringent analysis, however, reduced the number of matched peptides to four, all of which were derived from the smaller exon 1–encoded domain. This was in agreement with a surface-restricted proteolytic cleavage of this domain.

\textbf{The SURFIN\textsubscript{4,2}–encoding PFD1160w gene is a member of the multigene surf family}

PFD1160w is one of a small family of 10 \textit{surf} genes whose translation products, here referred to as SURFINs, exhibit structural features related to surface proteins of human, monkey, and rodent malaria parasites (Fig. 1 and Figs. S2–S4, available at http://www.jem.org/cgi/content/full/jem.20041392/DC1). Structurally, the \textit{surf} family is closely related to \textit{PvSTP1} (5). The NH\textsubscript{2}–terminal end of the SURFIN–PvSTP1 clade exhibits a moderately conserved, cysteine-rich putative globular domain that shares positionally conserved cysteine residues with almost the entire external CRD of the \textit{P. vivax} VIR protein family (Fig. 1 and Fig. S2). The CRD of SURFIN–PvSTP1 precedes a variable segment not found in VIR, followed by a putative transmembrane domain. No apparent similarity was found between the predicted internal domains of SURFIN–PvSTP1 and VIR. The COOH-terminal regions of SURFINs feature conserved tryptophan-rich domains (WRDs 1–4) intersected by stretches of higher variability. PfSTP1 exhibits one WRD of \( \sim 145 \) amino acids (aa), whereas SURFINs contain three to four of these segments. Notably, tryptophan residues are highly conserved between the WRDs, implicating functional restraints. We performed a BLAST search analysis with the SURFIN–WRD1 domain (145 aa) against the nonredundant gene bank database (all organisms) and consistently retrieved PfEMP1, SICA\textsubscript{var}, and Pf332, together with the members of the SURFIN–PvSTP1 clade. The exported protein Pf332 contains a COOH-terminal WRD domain (\( \sim 250 \) aa). The complex sequence relationship between the WRD of Pf332 and SURFIN is explained in detail in Fig. S3. SURFIN and Pf332 WRDs are characterized by two sequence segments, S1 (30–40 aa) and S2 (60 aa) that define an ancestral relationship of the WRD with PkSICA\textsubscript{var} and PfEMP1. SICA\textsubscript{var} contains S1, including a highly conserved sequence motif recently identified as a \textit{P. falciparum} export element (Pexel) at the NH\textsubscript{2}–terminal end of exported proteins (10). S1 is highly conserved between PkSICA\textsubscript{var} members, which apparently lack S2. In contrast, PSI-BLAST analysis with the SURFIN\textsubscript{4,2} WRD–1 retrieved a sequence in the internal PfEMP1 acidic terminal sequence (ATS) comprising the entire S2 segment. The highest homology was found in segment S2* (Figs. S3 and S4), which was also found as the most conserved region between Pf332 and SURFIN S2 segments.
Figure 1. SURFINs share structural features with exported proteins of several Plasmodium species. (A) Common structure of SURFIN proteins and their relation to other Plasmodiidae surface proteins. SURFINs and a putative transmembrane protein of P. vivax, PvSTP1, form a clade with a common structure (gray box). A predicted TM separates a variable NH2-terminal region from a semiconserved COOH-terminal region. The NH2-terminal domain features a moderately conserved CRD with six highly conserved cysteine residues and a modified Pexel, which precedes a region of higher sequence variability. The COOH-terminal domain is characterized by one (PvSTP1) or three to four (SURFIN) relatively conserved WRDs. SURFIN–PvSTP1 is related to the surface/exported proteins Pf332, PiEMP1, PvVIR, and PiSICAvar. PSI-BLAST and ClustalW alignment analysis revealed conserved cysteine residues shared between the CRD of SURFIN–PvSTP1 and the variable extracellular NH2-terminal domain of VIR. In contrast, the COOH-terminal WRDs feature similarities with the exported protein Pf332 and the surface-exposed proteins PiEMP1 and PiSICAvar. Two sequence segments can be distinguished in the SURFIN WRDs: S1 (30 aa) and S2 (60 aa) which both occur with high homology in a WRD found at the COOH-terminal end of Pf332, here intersected by a stretch of \( \sim 130 \) aa containing a partial duplication of S2 (S2*, Fig. S3). A modified S1 segment, characterized by a highly conserved Pexel-like motif (Fig. S3), is also found in the COOH-terminal domain of PiSICAvar, which lacks an S2 segment. S2 is present in the ATS-domain of PiEMP1, which lacks a S1 segment, although a strictly conserved Pexel-like motif related to S1 is \( \sim 150 \) aa upstream of S2. (B) General structural features shared by related surface proteins in P. vivax and P. falciparum include a variable external domain containing conserved cysteine residues and a conserved internal domain.

PSI-BLAST analysis with S2 retrieved only the SURFIN–PvSTP1 clade, PiEMP1, and Pf332. In contrast, PSI-BLAST analysis with S1 alone retrieved only Pf332, SICAvar, and the SURFIN–PvSTP1 clade (two to five iterations), suggesting that PiEMP1 does not contain this segment. However, alignments of 49 PiEMP1 ATS regions revealed a conserved Pexel-like motif \( \sim 150 \) aa upstream of the PiEMP1 S2 element, which is almost identical the motif in SURFIN S1. In summary, the WRDs of PiSURFIN–PvSTP1 provide a link to the internal domains of Pf332, PkSICAvar, and PiEMP1. In contrast, the CRD of PiSURFIN is related to the external domain of PkVIR, which is consistent with the predicted topology of these domains.

SURFINs lack a predicted NH2-terminal ER-type signal sequence. At approximately amino acid position 125, a conserved pentameric motif (K/R)xLxD is found, which is congruent to a Pexel motif (KLLAD) in two SURFINs (MAL8P1.162 and PFA0725w), but is modified in other SURFINs, mainly in position 4 (predominant E; Fig. S2).

The surf genes are present close to or within the subtelomere of five P. falciparum chromosomes (Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20041392/DC1). 6 of the 13 annotated open reading frames (ORFs) presumably have been misannotated as individual genes, but actually represent three truncated genes comprising six ORFs: the ORF pairs PFD0105c/PFD0100c and PFA0650w/PFD0655 are truncated as a result of an internal frameshift and the ORF pair PF13_0074/PF13_0075 shows an internal stop codon (compare with Fig. S6, available at http://www.jem.org/cgi/content/full/jem.20041392/DC1). This interpretation of the gene family was confirmed by PCR, RT-PCR, and sequence analysis (unpublished data). Of the seven full-length surf genes, we have dissected two groups that differ in their gene architecture (group A has two exons and group B has three exons). ClustalW alignment of the exons in both groups revealed that the COOH-terminal domain is relatively conserved overall, whereas the NH2-terminal domain is comparably variable, as is to be predicted for an exterior domain (Fig. S5). The seven full-length surf genes also exhibit an elevated codon volatility (between 0 and 0.11744), suggesting a diversifying pressure as also observed for other falciparum surface proteins (i.e., PiEMP1; reference 11).

In line with a terminology applied to var genes by Mok et al. (unpublished data), we propose a similar terminology for the surf genes based on their position in the 3D7 genome. The PFD1160w gene will accordingly be referred to as 3D7surf1.2 gene (note that here we interpret the ORF pair PFD0105c/PFD0100c as one predicted pseudo-gene, 3D7surf4.2).

The 3D7surf1.2 gene (PFD1160w) is transcribed in 3D7S8 and a homologous gene transcript is present in parasite FCR3S1.2.

The presence of the 3D7surf1.2 transcript was verified by RT-PCR with a primer set (P1/P2; see Materials and methods) that matched gene sequences on either side of the intron sequence. In time course experiments with a synchro-
of reverse transcriptase. (B) Northern blot analysis with total RNA prepared from an asynchronous parasite culture. Total RNA separated by agarose gel electrophoresis and stained with ethidium bromide before transfer to a membrane (lane 1) is shown. Northern blot analysis with a probe specific for the 3D7surf4.2 identified a full-length transcript of ~9 kb, which was in the expected size range for SURFIN-encoding mRNA (lane 2).

nized culture, a transcript of predicted size was amplified throughout parasite development, notably also in the late schizont stage (Fig. 2 A).

Furthermore, RT-PCR with primer sets P1/P5 and P6/P7 (see Materials and methods) amplified SURFIN transcript sequences of 1,776 bp in exon 1/2 and 447 bp located at the 3’ end of exon 2, respectively (not depicted). In addition, Northern blot analysis with a gene-specific probe confirmed the presence of a 3D7surf4.2 full-length transcript (Fig. 2 B).

To analyze the transcription of surf genes in a parasite genotypically distinct from the 3D7 strain, we conducted RT-PCR with RNA derived from the parasite clone FCR3S1.2 (not depicted). First, we used a primer set (P3/P8) targeting sequences in the variable exon 1 region. Next, we used a primer set (P6/P7) targeting the relatively conserved exon 2 of the 3D7surf4.2 gene. After sequencing, the obtained amplification products revealed a high degree of homology with the 3D7surf4.2 gene. Divergence in sequence was restricted to the exon 1–derived amplification product (88% identical amino acids). In contrast, the exon 2–derived sequence was completely conserved between 3D7 and FCR3S1.2.

Expression of 3D7surf4.2 in parasite stages
In a time course experiment, samples of a synchronized culture were harvested at various stages of development and one part was extracted with Triton X–100. Insoluble pro-

Analysis of 3D7surf4.2 protein expression
We raised antibodies against synthetic peptides S1.3 and S1.4 and rSURFIN4.2, which were derived from the COOH- and NH2-terminal domain of 3D7 SURFIN4.2, as shown in Fig. 3 A. A Western blot analysis was performed with lysates of 3D7S8 parasites in the trophozoite stage (25–30 h), sequentially extracted first by 1% Triton X-100 (Triton soluble), then by 2% SDS (Triton insoluble), and finally by boiling in reducing SDS sample buffer (SDS insoluble). Anti-S1.3 serum reacted specifically with SURFIN4.2 at an apparent molecular mass of 280–300 kD (286 kD predicted), which partitioned into the Triton-insoluble fraction and the SDS-insoluble fraction (Fig. 3 B). Interestingly, a protein band stained by Ponceau S comigrated with SURFIN4.2 and was also found predominantly in the Triton X-100– and SDS-insoluble fractions and, thus, was likely to be identical to SURFIN4.2.

The specificity of the anti-S1.3 reaction was confirmed with anti-rSURFIN4.2 serum directed against the NH2-terminal SURFIN4.2 domain, which specifically recognized a protein of the same size in the Triton X-100 insoluble fraction (Fig. 3 C). Neither antibody reacted with any other 3D7S8 protein in Western blots with 5 or 12% gels and no reaction was found with the preimmune sera (not depicted).

Recently, we have established surface biotinylation of the IE and identified a 280–300-kD Triton-insoluble protein (8). Here, we find that this major surface-biotinylated band comigrated with SURFIN4.2 and was specifically immunoprecipitated by anti-S1.3 serum from lysates of surface-biotinylated 3D7S8 trophozoites (Fig. 3 D). In contrast, a PfEMP1 variant expressed in the 3D7S8 parasite identified in Western blots with anti-PfEMP1 antibodies was only faintly labeled by biotin and of significantly lower apparent molecular mass compared with the biotinylated SURFIN4.2 (Fig. 3 E).

To further investigate the surface composition of the 3D7S8 clone, we performed surface iodination of trophozoite-stage IEs and analyzed a Triton X-100 insoluble protein fraction by SDS-PAGE and phosphoimaging. A protein comigrating with SURFIN4.2 appeared only very faintly labeled (Fig. 3 F), whereas labeling of a putatively expressed var variant was obtained as was suggested by a comparative Western blot stain with anti-PfEMP1 and anti-S1.3 antibodies (not depicted). The apparent differences in the labeling of SURFIN and PfEMP1 are puzzling, but may reflect differences in the chemical properties of the labeling reagents.

Figure 2. surf4.2 gene expression profile in 3D7. (A) RT-PCR with RNA from synchronized 3D7S8 cultures. Samples were taken at the trophozoite stage (<6 h), the mid trophozoite stage (20–24 h), and the matured schizont stage (>40 h). Forward primer P1 and reverse primer P2 were used to amplify a segment of 440 bp of the PFD1160w transcript across the intron boundary. To further control for a genomic DNA-derived transcript, the RT step was performed in the presence (+) or absence (−) of reverse transcriptase. (B) Northern blot analysis with total RNA prepared from an asynchronous parasite culture. Total RNA separated by agarose gel electrophoresis and stained with ethidium bromide before transfer to a membrane (lane 1) is shown. Northern blot analysis with a probe specific for the 3D7surf4.2 identified a full-length transcript of ~9 kb, which was in the expected size range for SURFIN-encoding mRNA (lane 2).
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proteins were used for immunoblot analysis with anti-S1.3 serum. Alternatively, cells were first surface biotinylated, and then processed for analysis in blots with a streptavidin reagent. SURFIN4.2 was detectable with anti-S1.3 serum at 24 h a.i. and was similarly detected in late schizonts (45 h a.i.; Fig. 4 A, *). A low amount of SURFIN 4.2 was also faintly detected in early ring stages (4 h a.i.). A similar result was obtained with surface-biotinylated 3D7S8 parasites with anti-S1.3 serum and anti-S1.3 antibodies. (D) Immunoprecipitation of surface-biotinylated 3D7S8 parasites with anti-SURFIN4.2 serum S1.3. Total lysate and antibody-precipitated materials were analyzed in Western blots with streptavidin reagent. A major biotinylated band in total lysates was specifically precipitated (lanes shown were from the same gel). A control with preimmune serum did not precipitate any biotinylated protein (not depicted). (E) Western blot analysis of lysates of surface-biotinylated 3D7S8 parasites probed with streptavidin reagent or anti-PFEMP1 revealed SURFIN4.2 (*) as of considerably higher molecular mass to an expressed PFEMP1 (O). Lanes shown were run in the same gel and the position of spectrin was deduced in a transient stain with Ponceau S. (F) 3D7S8 parasites were surface iodinated. Triton-insoluble fractions were analyzed by 5% SDS-PAGE and exposed by phosphoimaging. The position of SURFIN4.2 and PFEMP1 was deduced by Western blot with 3D7S8 lysate incubated with anti-SURFIN4.2 or anti-PFEMP1 antibodies (not depicted). Note that a band comigrating with SURFIN4.2 is barely visible in the exposed surface-iodinated lysate, whereas a strongly labeled protein comigrated with anti-PFEMP1 reactive antibodies.

Figure 3. Anti-3D7 surf4.2 (PFD1160w) antibodies identify SURFIN4.2 as a Triton X-100 insoluble protein strongly labeled in surface biotinylation experiments. (A) Relative position of S1.3 and S1.4 peptides, and rSURFIN4.2 used to raise anti-SURFIN4.2 serum. (B) Western blot analysis with anti-S1.3 serum on protein fractions of 3D7S8 sequentially extracted with 1% Triton X-100 (Triton soluble), 2% SDS (Triton insoluble), and boiling sample buffer (SDS insoluble). Also, a transient total protein stain with Ponceau S is shown. Anti-S1.3 serum reacted specifically with a 280–300-kD band, as is predicted for SURFIN4.2 (286 kD), which separated predominantly into the Triton-insoluble and SDS-insoluble fraction (*, SURFIN4.2). A copartitioning band of the same size was also visualized with Ponceau S. (C) Anti-rSURFIN4.2 and anti-S1.3 react with the same antigen in Western blots with Triton-insoluble fractions. Lanes were stained with Ponceau S, cut in half, and then probed with either S1.3 or rSURFIN4.2 antibodies. (D) Immunoprecipitation of surface-biotinylated 3D7S8 parasites with anti-SURFIN4.2 serum S1.3. Total lysate and antibody-precipitated materials were analyzed in Western blots with streptavidin reagent. A major biotinylated band in total lysates was specifically precipitated (lanes shown were from the same gel). A control with preimmune serum did not precipitate any biotinylated protein (not depicted). (E) Western blot analysis of lysates of surface-biotinylated 3D7S8 parasites probed with streptavidin reagent or anti-PFEMP1 revealed SURFIN4.2 (*) as of considerably higher molecular mass to an expressed PFEMP1 (O). Lanes shown were run in the same gel and the position of spectrin was deduced in a transient stain with Ponceau S. (F) 3D7S8 parasites were surface iodinated. Triton-insoluble fractions were analyzed by 5% SDS-PAGE and exposed by phosphoimaging. The position of SURFIN4.2 and PFEMP1 was deduced by Western blot with 3D7S8 lysate incubated with anti-SURFIN4.2 or anti-PFEMP1 antibodies (not depicted). Note that a band comigrating with SURFIN4.2 is barely visible in the exposed surface-iodinated lysate, whereas a strongly labeled protein comigrated with anti-PFEMP1 reactive antibodies.

3D7 surf4.2-encoded SURFIN is colocalized with proteins (PFEMP1 and RIFIN) exported to the erythrocyte cytosol and PM

To examine the transport pathway of SURFIN4.2, we performed indirect immunofluorescence experiments with air-dried monolayers of 3D7S8 IEs. With anti-S1.3 serum, a parasite-restricted fluorescence was detected at ~4 h a.i., which increased strongly with the development of mature trophozoites (Fig. 5 A). At ~24 h a.i., SURFIN4.2 was detected in the erythrocyte cytosol of a subpopulation (~25%) of cultured cells. A comparable reactivity was observed with anti-rSURFIN serum that confirmed the specificity of the anti-S1.3 reaction (Fig. 5 A). Double staining of 3D7S8 parasites with PFEMP1-directed antibodies and anti-S1.3 serum revealed that, in ~25% of trophozoites, SURFIN4.2 and
PfEMP1 colocalized in single small vesicles (SSVs), which were previously implicated in PfEMP1/RIFIN transport (reference 12; Fig. 5 B). In midstage trophozoites (16–24 h a.i.), FCR3 parasites and parasites freshly obtained from patients generally show aggregation of SSVs to large multimeric vesicles (LMVs; reference 12). A similar structural arrangement was not observed in 3D7-derived parasites, possibly because of an impaired transport machinery in the 3D7 lineage. The finding that FCR3S1.2 parasites express a surf gene highly homologous to 3D7 surf4.2 led us to examine the SURFIN transport in this parasite. In contrast to 3D7S8, we found colocalization of PfEMP1 and SURFIN within the same subcellular compartments, LMVs, in almost all (>90%) mature stage FCR3S1.2 parasites, indicating a common export route to the PM (Fig. 5 B). We further examined the SURFIN location by immunoelectron microscopy and conducted these experiments on both 3D7S8 and the FCR3S1.2 parasite. With embedded cuts of mature-stage IEs, the antibody anti-S1.3 not only reacted strongly with structures inside the parasite and the parasitophorous vacuole (PV), but also with Maurer’s clefts and the IE PM of both 3D7S8 and FCR3S1.2 parasites (Fig. 5 C). Notably, in 3D7S8 the SURFIN4.2 was also present in protruding knob structures, suggesting colocalization of PfEMP1 and SURFIN4.2 at the parasite surface. A reaction comparable to anti-S1.3 serum was obtained with the peptide antibody anti-S1.4, which was used as an Ig fraction affinity purified on rSURFIN4.2 (Fig. 5 C). The orientation of the S1.3 and S1.4 epitopes was, however, difficult to assess because of the relatively poor preservation of membranes in paraformaldehyde fixed cell preparations. No specific reaction was detected with membranes of noninfected IEs or with preimmune serum (not depicted) even though background staining with parasite nuclei was observed. Attempts to verify SURFIN4.2 surface expression in immunofluorescence experiments on live IEs did not produce conclusive results. Similar poor reactivity in live immunofluorescence experiments with Escherichia coli–produced recombinant protein or peptide antisera has been reported by others for PfEMP1 and RIFIN, and may reflect the predominant exposure of conformational epitopes (13, 14).

**A SURFIN-containing structure adheres to free merozoites**

Merged fluorescence patterns obtained with propidium iodide (PI) and anti-S1.3 on late schizont stage 3D7S8 parasites suggested accumulation of 3D7 surf4.2–derived SURFIN in the PV, which was closely associated with the developing merozoite (Fig. 6 A). Double staining with PI and anti-S1.3 serum revealed a cap–like concentrated zone of SURFIN4.2 at the merozoite apex of released merozoites (Fig. 6 B), which was also observed, albeit with decreased intensity, after several washes (not depicted). The localization of SURFIN4.2 in merozoite-associated material (MAM) in late schizonts and free merozoites was confirmed in immunofluorescence experiments with anti-rSURFIN4.2 serum and immunoelectron microscopy with affinity-purified anti-S1.4 immunoglobulin (Fig. 6, C and D). Interestingly, SURFIN4.2 was also detected in the PM of merozoites (Fig. 6 D), although this may reflect a tight association of MAM with the merozoite. In double-labeling experiments, SURFIN4.2 did not colocalize with the microneme apical merozoite antigen (AMA)-1 (15, 16), but was present in a crescent-shaped cap attached to the merozoite apical pole (Fig. 6 E). The extracellular location of SURFIN4.2 containing MAM was further suggested by experiments with paraformaldehyde–fixed cells under conditions that preserved the integrity of the cells. No difference in fluorescence intensity was observed (not depicted). In conclusion, the immunofluorescence and immunoelectron microscopy data confirm that SURFIN4.2 antigen is exported in the trophozoite stage IE to the erythrocyte cytosol and is associated with the PM.
Furthermore, in the rupturing schizont, SURFIN4.2 is found accumulated in the parasite PV and is associated with developed merozoites in the form of an amorphous layer in juxtaposition to the apical prominence.

Invasion inhibition assays, summarized in Fig. S7 (available at http://www.jem.org/cgi/content/full/jem.20041392/DC1), reduced infectivity was observed in the presence of SURFIN4.2. The assay was conducted using a combinatorial approach to evaluate the potential role of SURFIN4.2 in the invasion process.

Figure 5. SURFINs expressed in 3D7S8 and FCR3S1.2 are cotransported with PfEMP1 to the host PM.

(A) Indirect immunofluorescence assay (IFA) on air-dried monolayers of 3D7S8 IEs at various stages of maturation. Top: Conventional IFA with SURFIN4.2-directed anti-S1.3 antibodies at 4, 24, 36, and 44 h a.i.; middle and bottom: merged double-stained confocal IFA of PI-stained parasite nuclei (red) with Alexa 488–labeled SURFIN4.2 (green). SURFIN was detected with either anti-S1.3 (middle) or anti-rSURFIN4.2 antibodies (bottom); yellow, label overlay. Both antibodies detected SURFIN4.2 in ~25% of examined parasites at ~24 h a.i. in dot-like transport vesicles outside the parasitophorous vacuole. (B) Dual localization of TRITC-labeled SURFIN4.2 (red) and Alexa 488–labeled PfEMP1 (green) showed that SURFIN4.2 and PfEMP1 are colocalized in cytosolic structures of ~25% 3D7S8 IEs (compare with the merged fluorescence pattern). An expressed SURFIN was detected in >90% of trophozoite-stage FCR3S1.2 IEs (>24 h a.i.) and colocalized with PfEMP1 in LMVs. Dashed circles indicate the area of the IE. (C) Immunoelectron microscopy studies on 3D7S8 and FCR3S1.2 IEs. SURFIN detected with either anti-S1.3 serum or affinity-purified anti-S1.4 immunoglobulins is present in the PM, as well as in the erythrocyte cytosol associated with Maurer's clefts (MC) as indicated by arrows. Bars: (A and B), 2 μm; (C), 0.5 μm.

Figure 6. SURFIN accumulates within the parasitophorous vacuole in late schizonts and is associated with released merozoites.

(A) Alexa 488–labeled SURFIN4.2 (green) detected with anti-S1.3 antibodies in parasites developing from late trophozoite stage to schizonts. PI staining (red) was used to visualize nuclei. (B–D) SURFIN4.2 was found localized in MAM and the PM of late and bursting schizonts (>48 h), and associated with the released merozoite surface. Dashed circles indicate the area of the IE. (B) IFA with anti-S1.3 and PI on bursting schizonts. IFA (anti-rSURFIN4.2) and immunoelectron microscopy (anti-S1.4 Ig) on late schizonts (C) and released merozoites (D). SURFIN associated with PM or MAM is indicated by arrows. (E) SURFIN4.2 did not colocalize with the microneme AMA-1. A detailed analysis of SURFIN4.2 (anti-S1.3 antibodies) and AMA-1 in relation to the nuclei and to each other in merozoites is shown. (B and E) A schematic representation of the antigen localization is shown on the right. Bars: IFA, 2 μm; EM, 0.5.

Furthermore, in the rupturing schizont, SURFIN4.2 is found accumulated in the parasitophorous vacuole and is associated with developing merozoites in the form of an amorphous layer in juxtaposition to the apical prominence.

SURFIN4.2 may be involved in merozoite invasion

Invasion inhibition assays, summarized in Fig. S7 (available at http://www.jem.org/cgi/content/full/jem.20041392/DC1), reduced infectivity was observed in the presence of
2–5 mg of purified rSURFIN4.2 IgG in which parasite invasion was reduced to ~80% compared with control IgG (anti-PfEMP1 IgG). Because of the high amounts of antibody required, these data cautiously indicate that SURFIN4.2 has a functional relevance at the merozoite apex.

**DISCUSSION**

*P. falciparum* has created a formidable machinery to export families of variant proteins to the erythrocyte surface and, similarly, the structures needed in the merozoite to attach and invade its host cell are complex. We describe for the first time the presence of a novel antigen, SURFIN, at both the IE and the merozoite surfaces.

3D7surf4.2-encoded SURFIN4.2 was identified by a novel proteome approach in which we specifically targeted surface proteins by treating the live trophozoite IEs with trypsin. Mass spectrometry of the released peptides generated a peptide profile of relatively low complexity in accordance with a surface-restricted proteolysis. Surprisingly, mass fingerprint analysis with the PeptideSearch or Mascot search engines did not identify any erythrocyte surface proteins, possibly because the mild conditions of the proteolysis, which previously had been shown to leave major erythrocyte proteins largely unaffected (9). In addition, the cleavage of erythrocyte surface proteins may have generated glycosylated tryptic peptides, which would not have matched the respective predicted masses.

An initial peptide search analysis assigned six peptides to the 3D7surf4.2 gene located upstream and downstream from the TM. This was in conflict with structural predictions in which the larger COOH-terminal domain was presumed internal and, therefore, inaccessible to proteolysis. However, more stringent window settings in the peptide search exclusively removed those two peptides assigned to the predicted internal domain and were, therefore, most likely spurious matches, whereas the four peptides derived from the smaller, variable, and presumably exterior NH2-terminal domain remained (Fig. S1). This was in agreement with a larger internal region shielded from the trypsin reagent. The correct assignment of the surface-derived tryptic peptides to the 3D7surf4.2 gene was confirmed by a series of experiments. First, RT-PCR and Northern blot analysis identified 3D7surf4.2 mRNA throughout the blood stage development. Second, antibody probes generated against sequences in either the COOH-terminal or the NH2-terminal domain of 3D7surf4.2 reacted in Western blots with a protein of the predicted size in the Triton-insoluble fraction of mature stage 3D7S8 parasites. Third, 3D7surf4.2-encoded SURFIN, but not PfEMP1 variant expressed in 3D7S8, was a prominently labeled protein in surface biotinylation experiments. This was shown by Western blot analysis and immunoprecipitation of biotinylated proteins with anti-SURFIN4.2 peptide antibody and comparison with PfEMP1-directed antibodies. Anionic biotin reagent may gain limited access to the cytosol (17), but is unlikely to label proteins within the parasitophorous vacuole, which is consistent with the export of SURFIN4.2 into the host cytosol. Furthermore, to limit a cellular uptake of the reagent, we used a noncharged NHS-PEO-biotin derivative with which we did not find labeling of major cytosolic proteins such as hemoglobin or spectrin (compare Fig. 3 with Fig. 4). Fourth, immunofluorescence experiments and immunoelectron microscopy studies with two anti-SURFIN4.2 peptide sera and serum raised against recombinant SURFIN4.2 coherently suggested the export of SURFIN4.2 into the erythrocyte cytosol and its display on the IE surface.

In indirect immunofluorescence experiments, we found that SURFIN4.2 colocalized with PfEMP1 in SSVs of ~25% of 3D7S8 parasites, which linked SURFIN to other previously characterized surface proteins (12). Parasites of the 3D7 lineage do not appear to sustain a completely developed export machinery, which is indicated by the absence of LMVs (12). In contrast, FCR3S1.2 parasites contain LMVs and, thus, more closely resemble parasites freshly obtained from patient samples (12). In >90% of FCR3S1.2 trophozoite stage parasites, we found that PfEMP1 and an expressed surf gene product colocalized in SSVs, and also prominently in LMVs, from where surface proteins appear to be delivered to the exterior. Furthermore, in immunoelectron microscopy with the SURFIN4.2-directed peptide serum anti-S1.3, we could confirm that FCR3S1.2 SURFIN is associated with the PM, as well as Maurer’s clefts.

The structural analysis of the surf gene family provided important support for a surface exposure of SURFIN on the IE. SURFIN and the phylogenetically closely related PvSTP1 feature sequence similarities with PfEMP1, PvVIR, PkSICAvar (compare Fig. 1 with Figs. S2–S4). In addition, the cellular loci of the homologous sequence motifs are in complete agreement with the predicted location of the SURFIN domains. The NH2-terminal domains of SURFINs are related to the surface-exposed domains of PfEMP1, whereas the predicted internal WRD of SURFIN–PvSTP1 feature sequence similarities with PfEMP1, PvVIR, whereas the predicted internal WRD of SURFIN–PvSTP1 provides a structural link between the internal domains of exported (Pf332) and surface-expressed molecules (PfEMP1 and PkSICAvar) from human, rodent, and monkey malaria parasites.

It was surprising to find SURFIN4.2 in a crescent-shaped amorphous structure attached to the apex of merozoites. SURFIN4.2-containing material was present even after repeated washes of released merozoite clusters and, thus, is unlikely to be only transiently adhering residual material. However, SURFIN-containing MAM may be lost by applying harsher conditions. Interestingly, *P. falciparum* merozoites can be isolated structurally intact but are, in contrast to *P. knowlesi* merozoites, noninvasive (18). We speculate that MAM may be removed during the isolation procedure, yet is required for the successful invasion process.

The localization pattern of SURFIN establishes for the first time a link between key molecules at the merozoite and the IE surface (a model summarizing the SURFIN expres-
sion in the blood stages is shown in Fig. S8, available at http://www.jem.org/cgi/content/full/jem.20041392/DC1). This discovery is relevant because it suggests that the parasite expresses polymorphic antigens on the surface of merozoites and IEs. SURFIN$_{4.2}$ was present in 3D7-derived clones irrespective of their binding phenotype (Fig. S9, available at http://www.jem.org/cgi/content/full/jem.20041392/DC1) and therefore cannot, at the present time, be associated with a particular adhesion phenotype. However, this does not preclude a differential expression (switch) of SURFINs under host immune pressure. A Western blot analysis of genotypically distinct parasite strains with an anti-SURFIN$_{4.2}$ antibody found size polymorphism in the expressed SURFIN protein (Fig. S9 A). Sequencing data of a surf$_{4.2}$ homologue in an FVO strain (available from GenBank/EMBL/DDBJ under accession no. AB207820) identified an important sequence polymorphism in the ectodomain (Fig. S9 B) that may contribute to immune evasion.

The finding that several parasite stages express the same antigens located in distinct virulence- and therefore expressed at various immunodominant epitopes designed for each purpose, the parasite may have adapted key molecules to various distinct tasks. In support of this concept, a rhoptry-derived antigen (ring surface protein–2) was implicated in merozoite invasion, as well as the sequestration of ring stage–infected IEs (19), presumably as the antigen is passively transferred onto the IE surface during the invasion. STEVOR was also recently proposed as a multifunctional protein on the basis of expression in sporozoites and gametocytes (20). In addition, STEVOR, RIFIN, and PIIEMP1, as well as SURFIN-derived peptides (PFD1160w [one peptide], PFA0725w [two peptides], and MAL8P1.162 [one peptide]), were found in sporozoites, suggesting that key parasite molecules are commonly present during different developmental stages of the parasite (21).

In summary, 3D7 parasites contain a family of 10 closely related surf genes. In 3D7S8 parasites, we found the 3D7surf$_{4.2}$ expressed as a novel protein, SURFIN$_{4.2}$, which is exposed at the surface of the IE and the released merozoites. The association with the surfaces of stages throughout blood stage development suggests that SURFINs are crucial for parasite survival. We provide initial evidence that SURFIN$_{4.2}$ may be involved in the invasion of merozoites into erythrocytes, though a detailed assignment of the function for SURFINs in immune evasion/receptor binding and/or erythrocyte invasion needs further clarification.

**MATERIALS AND METHODS**

**Parasites and cultures.** Parasite clones 3D7S8 and FCR3S1.2(K) were maintained in continuous culture according to standard procedures (22, 23). NF54 parasites passed over CSA were provided by L. Hvid (University of Copenhagen, Copenhagen, Denmark).

**Trypsin treatment of the IE surface and mass spectrometry analysis.** 3D7S8 IEs were enriched by Percoll gradient centrifugation as previously described (23). Cells floating between the 40 to 60% Percoll layers (97–99% erythrocytes containing parasites ≥18 h) were recovered and gently washed with ice-cold PBS. 300 µl of washed IEs were resuspended in ice-cold PBS to a final volume of 1 ml and digested with 1 µg/ml of porcine-modified trypsin (Promega) for 30 min at 37°C. The reaction was terminated by the addition of 10 mg/ml of soybean trypsin inhibitor (Sigma-Aldrich). The cell suspension was centrifuged at 725 g for 30 s and the supernatant again was centrifuged at 18,000 g for 3 min at 4°C. Supernatants of four identical digestions were pooled and concentrated in a Speedvac to 100 µl, desalted, and further concentrated using reversed-phase C$_{8}$ ZipTip microcolumns (Millipore).

MALDI mass spectra were recorded using a Voyager DE-Pro (PE Biosystems) instrument and a Reflex 3 (Bruker) using α-cyan-4-hydroxycinnamic acid as the matrix. The peptide mass maps were searched against simulated tryptic digests of predicted proteins either in the whole *P. falciparum* genome (PlasmoDB) or in GenBank/EMBL/DDBJ using the PeptideSearch software (EMBL Analytical Research Group), as well as the MS-FIT (Protein Prospector) and Mascot (Matrix Science) peptide mass fingerprint tools.

**SURFIN transcript analysis.** Total RNA samples were prepared from highly synchronized cultures of 3D7S8 and FCR3S1.2 parasites that were harvested at various stages of development, extracted for RNA using TRIzol reagent (Invitrogen), and reverse transcribed with the SuperScript First-Strand Synthesis System (Invitrogen) according to the manufacturer’s instructions. RNA priming was conducted with random hexamer primers or with oligo dT primers with comparable results. Each RT experiment included a sample in which the reverse transcriptase was omitted to monitor the absence of genomic DNA.

The stage-dependent PFD1160w expression was analyzed in PCRs with cDNA samples obtained from 3D7S8 ring stages (~6 h), trophozoites (20–24 h), and schizonts (>40 h) using forward primer P1 (CTT CCC CTT TAC AAA TGA ATG CTC) and reverse primer P2 (AAC ATC AAC ACC TCT ACG CCG G). The cycling conditions were 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s. Further exon 1 and 2 sequences of the PFD1160w gene were amplified with the following primer sets and conditions: forward primer P1 and reverse primer P5 (CTT CAA ACC ATT TTT CTC CTT); and forward primer P6 (TGG CAA GAT GTA GAA ATA AGT TGG) and reverse primer P7 (CCA TTG ATT CCA CAA AAA GGC). Cycling conditions were 40 cycles of 94°C for 15 s, 50°C for 60 s, and 72°C for 4.5 min.

A PFD1160w homologue was amplified from cDNA derived from synchronized cultures of the parasite FCR3S1.2 (24–28 h a.i.). Two primer sets were used, forward primer P3 (TCA CCT GTA TCA CGA ACG CTT) and reverse primer P8 (TTT TTA TGG AAA CCA CTA TGA GAC); and forward primer P6 and reverse primer P7. The cycling conditions were 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s. Amplification products were cloned and sequence analyzed as described previously (8).

For the Northern blot analysis, primers p2 and p3 were used to RT-PCR amplify a 594-bp segment of the SURFIN transcript, which was cloned into Topo TA dual promoter vector pCRII (Invitrogen). Digestion-labeled surf$_{4.2}$ antisense RNA was produced using the Dig RNA labeling Kit (Roche) according to the manufacturer’s instructions. 3D7S8 total RNA was prepared as described previously (8) and 1 µg was transferred to nylon membranes (Roche) using a PosiBlot pressure blower (Stratagene). Blots were prehybridized and probed with 100 ng/ml digitonin-labeled antisense-RNA in Dig Easy Hyb buffer (Roche) at 62°C for 6 h, and then washed twice with 2× SSC, 0.1% SDS and twice with 0.5× SSC, 0.1% SDS at 62°C. Detection of hybridized RNA was performed with the Dig Luminescent detection kit (Roche).

**Sera and specific antibodies.** The SURFIN$_{4.2}$-directed antibodies anti-S1.3 and anti-S1.4 were raised in rabbits by a commercial supplier (INOVADEG AB) using KLH-conjugated synthetic peptides designed on the basis of the PFD1160w gene sequence. For the production of anti-recombinant SURFIN$_{4.2}$ (anti-rSURFIN$_{4.2}$) rabbit sera, an 813-bp segment of the
amino-terminal PFD1160w domain was cloned in pGEX-6P-1 vector (GE Healthcare). rSURFIN4.2 was expressed as a glutathione s-transferase fusion protein and then bound to glutathione sepharose. To remove the glutathione s-transferase, the bound fusion protein was cleaved with PreScission protease (GE Healthcare) and the eluted protein was used for the immunizations. The relative location of the SURFIN4.2-derived immunogens in the PFD1160w gene, including the sequences of the synthetic peptides, are shown in Fig. 3 A. The peptide antibody anti-S1.4 was used after affinity purification over rSURFIN4.2 coupled to cyanogen bromide–activated Sepharose–4B (GE Healthcare) using standard procedures. Rat and rabbit anti-PIE1 antibody sera directed against the conserved COOH-terminal–ATS domain of Plasmodium falciparum and transiently stained with 0.1% Ponceau S in 1% acetic acid. acrylamide gels. Proteins were transferred onto nitrocellulose membrane by centrifugation. Extracts and a remaining precipitate were boiled in re-sample buffer was added and boiled for 5 min. Alternatively, to obtain protein subfractions, cells were extracted with 1% Triton X-100 in PBS for 30 min on ice, centrifuged at 20,000 g for 20 min, and the Triton-insoluble material was reextracted with 2% SDS in PBS for 10 min at 10°C, followed by centrifugation. Extracts and a remaining precipitate were boiled in reducing SDS-PAGE sample buffer and separated in 5% and 12% SDS–polyacrylamide gels. Proteins were transferred onto nitrocellulose membrane and transiently stained with 0.1% Ponceau S in 1% acetic acid.

For immunostaining, membranes were blocked with 5% milk powder/PBS at room temperature for 1 h, and then incubated with anti-SURFIN4.2 antibodies diluted at 1:250 in 5% milk powder/PBS. For the detection of PIE1, a purified rabbit anti-PIE1–ATS immunoglobulin fraction was used (5 μg/ml in 5% milk powder/PBS). After 1 h at room temperature, membranes were washed three times, incubated with alkaline phosphatase–conjugated anti-rabbit antibody for 1 h at room temperature, and developed as described previously (8).

Surface labeling of IE. Surface biotinylation of the IEs of synchronized cultures of 3D7S8 were harvested at various points of development. Cells were processed as a whole cell culture mix or after separating trophozoite-stage parasites (20–24 h a.i.) from rings and uninfected cells on a MACS magnetic cell sorter (25). For cell lysis reduction, SDS sample buffer was added and boiled for 5 min. Alternatively, to obtain protein subfractions, cells were extracted with 1% Triton X-100 in PBS for 30 min on ice, centrifuged at 20,000 g for 20 min, and the Triton-insoluble material was reextracted with 2% SDS in PBS for 10 min at 10°C, followed by centrifugation. Extracts and a remaining precipitate were boiled in reducing SDS-PAGE sample buffer and separated in 5% and 12% SDS–polyacrylamide gels. Proteins were transferred onto nitrocellulose membrane and transiently stained with 0.1% Ponceau S in 1% acetic acid.

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Indirect immunofluorescence and immunoelectron microscopy. Indirect immunofluorescence on air-dried monolayers of IEs at various stages of development was performed as described previously (12, 23). To examine antigen expression in late schizonts and merozoites, synchronized cultures containing rupturing schizonts, merozoite clusters, and free merozoites were carefully harvested without washing, added to poly-L-lysine–coated wells, and left for sedimentation for at least 2 h. The supernatant was aspirated and the cells/parasites were air dried before incubation with antibodies.

For immunoelectron microscopy, parasites were fixed in 4% paraformaldehyde/0.1 M phosphate buffer overnight at room temperature. The cells were washed with phosphate buffer, embedded in 2% low melting point agarose, cut into pieces, and step-wise dehydrated with increasing concentrations of ethanol (50, 70, 95, and 100%). Each dehydration step was performed three times for 10 min at room temperature. Dehydrated cells were incubated in LR–White (London Resin Company Ltd.) for 3 h at room temperature, followed by overnight incubation in fresh LR–White. Polymerization was done at 58 to 60°C for 24 h.

Embedded cell pellets were cut into thin sections, placed on nickel grids, blocked in 0.5% milk/PBS, and incubated with primary antibodies overnight at 4°C. The grids were rinsed in PBS and incubated with anti-rabbit IgG antibodies conjugated with 10-nm gold particles (Sigma–Aldrich) for 2 h at room temperature. The sections were stained with 5% aqueous uranyl acetate for 40 min at room temperature and then examined in a transmission electron microscope (model CM12; Philips) at 80 kV.

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