Variation in the Expression of a *Plasmodium falciparum* Protein Family Implicated in Erythrocyte Invasion

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The PfrH protein family of *Plasmodium falciparum* is implicated in erythrocyte invasion. Here we report variations in the sequence, transcription, and protein expression of four different members of this family in three parasite lines, 3D7, T996, and FCB1. There are sequence polymorphisms in *PfRH1*, *PfRH2a*, *PfRH2b*, and *PfRH3*, ranging from variations across repeat regions to a 585-bp deletion in the 3' end of *PfRH2b* in T996. Not all the genes are transcribed: although all members of the family are transcribed in 3D7 and T996, *PfRH2a* and *PfRH2b* are not transcribed in FCB1. The PfrH1, PfrH2a, and PfrH2b proteins are expressed in late schizonts and merozoites and are located in apical organelles and on the apical surface. However, the PfrH1 protein does not appear to be correctly targeted to the apex in 3D7 and T996. In contrast, the PfrH1 protein is present at the apical end of FCB1 merozoites, but the PfrH2a and PfrH2b proteins are undetectable. The apparent redundancy in the PfrH family of proteins at the level of gene number and sequence and the variations in transcription and protein expression may allow the parasite to use alternative invasion pathways.

The human malaria parasite *Plasmodium falciparum* grows and replicates within host red blood cells for part of its life cycle. This erythrocytic stage is responsible for all of the pathology associated with the disease. The invasive merozoite stage of the parasite has a specialized set of apical organelles, the micronemes, rhoptries, and dense granules, whose contents are released at the time of invasion (reviewed in references 6, 11, 12, and 43). Several of the parasite ligands binding to host receptors and involved in invasion are located in the apical organelles. Parasite protein interactions with erythrocytes are thought to mediate a tightly regulated series of steps that lead to the internalization of the parasite within the parasitophorous vacuole. The ligands include, among others, members of the erythrocyte binding protein (EBP) family (1, 9, 29, 51), the apical membrane antigen 1 (AMA-1) family (15, 41), and a superfamily of proteins represented by the *P. yoelii* 235-kDa rhoptry protein family (Py235) (22, 33, 35) and *P. vivax* reticulocyte binding proteins (PvRBP) 1 and 2 (16). Recently, genes coding for members of this superfamily were described for *P. falciparum* (44, 49, 52). Two proteins from this family, referred to here as PfrH2a and PfrH2b (*P. falciparum* rhoptry protein homolog and reticulocyte binding protein homolog, also known as PfrBP2-Ha and PfrBP2-Hb, respectively), are implicated in *P. falciparum* invasion of erythrocytes; they appear to be localized to the rhoptries of *P. falciparum*, and antibodies to these proteins inhibit parasite invasion (44, 52). *P. falciparum* possesses a third related gene, *PfRH3*, although this sequence appears to be a transcribed pseudogene in some parasite lines (49).

In this study, we demonstrate variations in the sequence and expression of the PfrH family in laboratory lines of *P. falciparum*. We characterize an additional member of the family,

PfrH1, which is localized to the apical end of merozoites in the FCB1 line but not in the 3D7 and T996 lines. In contrast, the FCB1 line does not express PfrH2a or PfrH2b.

MATERIALS AND METHODS

Bioinformatics. TblastN searches of preliminary *P. falciparum* sequence data at The Institute for Genomic Research, The Sanger Institute, and the Stanford University websites (<http://www.tigr.org/>, <http://www.sanger.ac.uk/>, and <http://sequence-www.stanford.edu/>) and sequence data submitted to EMBL (<http://www.ncbi.nlm.nih.gov/Malaria/>) were performed by using the translated protein sequences from *PfRH2a*, *PfRH2b*, *PfRH3*, *PvRBP*, and *Py235*. Sequence data for *P. falciparum* chromosome 4 were obtained from The Sanger Institute website (http://www.sanger.ac.uk/Projects/P_falciparum/). Sequencing of *P. falciparum* chromosome 4 was accomplished as part of the Malaria Genome Project with support from The Wellcome Trust. N-terminal signal sequence, transmembrane domain, hydrophobicity, and topology predictions were performed by using TM-HMM and iPSORT (<http://www.expasy.ch/>). ClustalX was used for alignment of the protein homology regions (50).

Parasite preparation, metabolic labeling, and treatment with brefeldin A (BFA). *P. falciparum* line 3D7, T996, and FCB1 parasites were cultured in vitro as previously described (7) and used to prepare DNA, RNA, and proteins. For tight synchronization of developmental stages, a combination of sorbitol treatment (27), centrifugation over 70% Percoll (39), and parasite collection with a magnetic separator was used. Large amounts of highly purified schizonts were obtained by an adaptation of the method of Staalsøe et al. (48). Briefly, a MACS type D depletion column was used in conjunction with a SuperMacs II magnetic separator (Miltenyi Biotec). The column was washed with 100% ethanol followed by distilled water and then equilibrated with warm RPMI 1640 plus 2% fetal calf serum (RPMI-FCS) (Invitrogen Life Technologies). *P. falciparum*-infected erythrocytes were harvested by centrifugation and resuspended in an equal volume of RPMI-FCS. The cell suspension was passed through the column under gravity, and then the column was washed with approximately four column volumes of RPMI-FCS until no erythrocytes were seen in the eluate. The column was removed from the magnet and washed with 50 ml of RPMI-FCS to allow collection of schizonts. Merozoites were prepared as described previously (5).

For collection of parasites throughout an intraerythrocytic developmental cycle, 25 flasks containing 50 ml of a tightly synchronized 3D7 parasite culture (1.4% hematocrit and 10% parasitemia) were gassed and placed at 37°C, and the contents were harvested at 4-h intervals over a 48-h period, beginning with cultures up to 2 h postinvasion. The parasites were pelleted by centrifugation, washed once in phosphate-buffered saline (PBS), and resuspended in TRIZOL (Invitrogen Life Technologies) for RNA preparation. Thin smears for microscopy were made at each time point and stained with Giemsa reagent.

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FIG. 1. PfRH1 is a member of the PfRH protein family. (A) Diagrammatic representation of PfRH1, PfRH2a, and PfRH2b. One centimeter represents 250 amino acids. hr, homology region (hatched boxes); ss, signal sequence (light grey boxes); tm, transmembrane region (black box). In PfRH2a and PfRH2b, the boxes with wavy grey lines represent the degenerate repeat region, and the boxes with dots and checks represent the unique parts of PfRH2a and PfRH2b, respectively. Constructs used to raise GST fusion proteins are marked RH1.1, RH1.2, RH2.HR, RH2a.1, and RH2b.1. (B) Alignment of the homology region of PfRH1 with those previously described. Sequences were aligned by using ClustalX (50) and the following regions of the proteins (GenBank accession numbers): E8 (U36927), 1669 to 1837; E3 (L27838), 1175 to 1343 (incomplete sequence); PvRBP1 (M88097), 1717 to 1885; PvRBP2 (AF184623), 1680 to 1848; PfRH1, 1898 to 2072; PfRH2 (AF312916 and AF312917), 1688 to 1857; and PfRH3 (predicted protein correcting the frameshifts in AF324831), 1751 to 1920. Shading of conserved (black) and semiconserved (grey) residues was done by using BioEdit (18) with the Blosum 62 matrix. (C) Alignment of portions of the predicted protein sequences from region PfRH1.2, starting at amino acid 2840, in 3D7, T996, and FCB1.

Tightly synchronized late schizonts, estimated to be at 40 to 42 h postinvasion, were radiolabeled by growth in the presence of [35 S] methionine and cysteine (Promix; Amersham Pharmacia) (49). Similar schizonts were also labeled in the presence of BFA essentially as described previously (34). Briefly, late schizonts were treated with BFA in methanol (final concentration, 5 μ g/ml) or 25 μ l of methanol alone and then cultured for 1 h prior to 35 S labeling for 1 h as described above in the continuing presence of BFA or methanol (control). To obtain radiolabeled culture supernatants, schizonts at 44 h postinvasion were labeled with [35 S]methionine and cysteine for either 4 h or overnight and allowed to release merozoites in the absence of erythrocytes. Culture supernatants were centrifuged at 40,000 rpm in a Beckman TL-100 ultracentrifuge prior to use.

Genotyping of parasite lines with polymorphic markers. DNA from lines 3D7, FCB1, and T996 was typed for the polymorphic loci merozoite surface protein 1 (MSP1) and MSP2 by using PCR primers as described previously (24).

DNA cloning and sequencing and preparation of antisera to recombinant proteins. PCR primers RH1.2f (GGA TCC ATC TAA TTC ATG TTA AGA AAC AAT TTG AAC ACA CC) and RH1.2r (GGA TCC GTG TAG ATA TAT CTT GTT CCT GTA ATT TTG TTG) were used to amplify the region of *PfRH1*

that contains an apparent frameshift in the database sequence with the proof-reading polymerase *Pfx* (Invitrogen Life Technologies) and genomic DNA derived from lines 3D7, T996, and FCB1 of *P. falciparum*. The PCR products were sequenced directly by using ABI dRhodamine-terminator cycle sequencing (PE Applied Biosystems).

PCR of the unique regions in *PfRH2a* and *PfRH2b* was performed by using primers RH2a.1f (GGA TCC TAA AAA GTA AAC TAG AAT CTG ATA TGG TG) and RH2a.1r (GGA TCC GGT ATT ATC ATC AGT AGT ACT TTC CGA) and primers RH2b.1f (GGA TCC GTC CAC AAA CTA GTC ATA GAA GTA ACA CC) and RH2b.1r (GGA TCC CCA TGT GTT TCC ATA GGT TCA TCA AGT G), respectively. PCR across the repeat regions was performed by using primers RH2repeatF (TAG TAC ATT AAC ACT TGA ATC AAT TCA AAC G) and RH2arepeatR (GTG ATT TCA ATG ATT TCA TCC TTC TCC) or RH2b.1r.

The following primer pairs were used to amplify regions of *PfRH1* and *PfRH2* for the preparation of recombinant proteins: the homology region of PfRH1 (RH1.1f, GGA TCC TGC AAA ACG AAA TAA GAA ACA TGA ATC TAG; RH1.1r, GGA TCC GTT ATA GTC CTC TTT TAT ATT GTG TAC ATC G),

FIG. 2. *PfrH2b* contains a large deletion in T996 parasites. (A) PCR across the repeat regions of *PfrH2a* (left) and *PfrH2b* (middle) demonstrating the size polymorphisms in 3D7 (lanes 2), T996 (lanes 3), and FCB1 (lanes 4). Lane 1, 1-kb marker (Invitrogen Life Technologies). The large size polymorphism in *PfrH2b* (right) seen with T996 is due to a 585-bp deletion in fragment *PfrH2b.1*. Lane 1, 1-kb marker; lane 2, 3D7; lane 3, T996. (B) Alignment of region *PfrH2b.1* in 3D7 and T996, showing the 195-amino-acid deletion in T996.

region *PfrH1.2* (RH1.2f and RH1.2r), the homology region of *PfrH2a* and *PfrH2b* (RH2.HRf, GGA TCC TGA ATG ATG TAT CAA AAT CTG ACC AGA TTG; RH2.HRr, GGA TCC CAC ATC TTC AAT TTT AAT ATA CTG TT), and unique parts of *PfrH2a* (RH2a.1f and RH2a.1r) and *PfrH2b* (RH2b.1f and RH2b.1r). The products were cloned into the TA vector (Invitrogen Life Technologies) and subcloned into *Bam*HI-restricted pGEX-3X (Amersham Pharmacia). Glutathione *S*-transferase (GST) fusion proteins were made and purified on glutathione-agarose (Sigma). The fusion proteins were used to raise antisera in rabbits (RH2.HR and RH2a.1) and BALB/c mice (RH1.1, RH1.2, RH2.HR, RH2a.1, and RH2b.1).

RNA preparation and analysis. RNA was prepared from *P. falciparum* by using TRIZOL and was analyzed by hybridization of Northern blots as described previously (25, 49). DNA fragments for hybridization were labeled by using a PrimeIt II kit (Stratagene). 5' Rapid amplification of cDNA ends (RACE) (Invitrogen Life Technologies) was carried out with 3D7 schizont-stage RNA by using primers 5race1RH1 (AAC ATC AAA TTT ATA AGA GGA ATC ATT TC) and 5race2RH1 (TAA TAC CGT TTT CTC TTC CTC GAT AGG TC).

Immunoprecipitation of metabolically labeled parasites. Metabolically labeled parasites and culture supernatants were used for immunoprecipitation. Two different extraction methods were used to prepare labeled parasite lysates for immunoprecipitation. In the first protocol, frozen parasite pellets were thawed in 100 μ l of sodium dodecyl sulfate (SDS) denaturing buffer (1% SDS, 50 mM Tris-HCl, 5 mM EDTA [pH 8.0]) and boiled for 5 min, and then 900 μ l of deoxycholate (DOC) buffer (0.5% sodium deoxycholate, 50 mM Tris-HCl, 5 mM EDTA, 5 mM EGTA) was added. Alternatively, the pellets were thawed in 1 ml of NP-40 buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA, 5 mM EGTA [pH 8.0]) containing a complete protease inhibitor mix

(Pharmacia). Following antibody addition, the samples were left on ice overnight and then spun at 15,000 rpm and 4°C (Sigma 1K15 centrifuge). Antigen-antibody complexes were precipitated from the supernatants by using protein G-Sepharose for 2 h at 4°C. The beads were washed four times in wash buffer I (50 mM Tris-HCl [pH 8.2], 5 mM EDTA, 0.5% [wt/vol] Triton X-100, 1 mg of bovine serum albumin/ml, 0.5 M NaCl) and four times in wash buffer II (50 mM Tris-HCl [pH 8.2], 5 mM EDTA, 0.5% [wt/vol] Triton X-100) (for SDS-extracted material, the first wash was carried out with wash buffer II). Proteins were resolved with either SDS-polyacrylamide gel electrophoresis (PAGE) (26) or precast NuPAGE gels (Invitrogen Life Technologies).

Western blotting of parasite extracts. Uninfected erythrocytes, tightly synchronized late schizonts (greater than 80% parasitemia), or purified merozoites were lysed directly into sample buffer for SDS-PAGE or NuPAGE. Either 5% polyacrylamide or precast 3 to 8% NuPAGE gradient gels were used. Following electrophoresis, gels were blotted overnight onto Protran nitrocellulose membranes (Schleicher & Schuell). Specific proteins were detected by using polyclonal mouse or rabbit sera followed by horseradish peroxidase-linked secondary antibodies (ICN) and enhanced chemiluminescence (Pierce).

Immunofluorescence assays (IFAs) and microscopy. Smears of *P. falciparum*-infected red blood cells were fixed with 1% formaldehyde in PBS for 5 min at room temperature. Slides were rinsed in PBS and immersed in blocking buffer (1% bovine serum albumin and 0.1% Triton X-100 in PBS) at 37°C for 30 min in a humid chamber. In addition to the antisera raised to *PfrH1*, *PfrH2a*, and *PfrH2b*, the following were used: rat monoclonal antibody [MAb] 4G2dc1 to AMA-1 (23) (a gift from Alan Thomas), polyclonal rabbit antiserum to EBA-175 (MR4 reagent MRA-2) and rat antiserum to EBA-175 (MR4 reagent MRA-15) (gifts from John Adams), and polyclonal rabbit serum to RhopH2 (19). All

FIG. 3. Transcription of the *PfrH* gene family is stage specific. (A) Giemsa-stained smears of 3D7 parasites harvested during their developmental cycle for RNA analysis. Each smear is labeled with the time of harvesting in hours after invasion. (B) Northern analysis of RNA from 3D7 parasites throughout their developmental cycle. Tightly synchronized 3D7 parasites were harvested at 4-h intervals over a 48-h period, and RNA was extracted at each time point and analyzed by hybridization of a Northern blot. Each lane is labeled with the time of harvesting in hours after invasion. The blot was hybridized with probes for *PfrH1* (region PfrH1.1), *PfrH2a* (region PfrH2a.1), *PfrH2b* (region PfrH2b.1), *PfrH3* (homology region) (49), and C-341 (a constitutively expressed sequence) (4). Each probe hybridized to a different transcript which was larger than the highest-molecular-mass marker (9.49 kb), with the exception of C-341, which was 2.5 kb.

antisera were diluted in blocking buffer. Slides were incubated with primary antibodies for 30 min, followed by two 5-min washes in PBS. They were then incubated with fluorescein isothiocyanate isomer (FITC)-, tetramethylrhodamine isothiocyanate (TRITC)-, or Texas red-conjugated secondary antibodies (Sigma) for 30 min. Slides were washed in PBS again, dipped in diamidinophenylindole (DAPI, 0.5 μ g/ml), and washed in PBS for a further 5 min. For double-labeling experiments, each of the two primary and two secondary antibodies was applied sequentially, with washing in between applications. Citifluor was applied to the slides, and the coverslips were sealed. Slides were visualized by using a Deltavision cooled charge-coupled device imaging system (Applied Precision Inc.). Images from the fluorescence microscope were collected and analyzed with Softworx and were prepared for publication with Adobe Photoshop.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this study are available in the GenBank database under accession numbers AJ430086 to AJ430089.

RESULTS

Variations in the sequences of the PfrH family in different *P. falciparum* lines. A sequence coding for an additional member of the PfrH family was identified on chromosome 4 in the *P. falciparum* genome sequence database (chr4_P19325, as of 21 December 2001). Following the removal of a single intron, this sequence potentially codes for a 358-kDa type I membrane protein, PfrH1. It has sequence similarity to the other mem-

bers of the superfamily and contains the motifs within the previously defined homology region that distinguish it as a member of the family (17, 22, 49) (Fig. 1A). Figure 1B shows the alignment of this region in PfrH1, PfrH2a, PfrH2b, and PfrH3; PvRBP1 and PvRBP2; and E8 and E3, members of the Py235 family. However, the sequence contained a reading frame shift (at about amino acids 2764 to 2766 of the predicted sequence for PfrH1) in the database contig. To determine whether this sequence was correct or was a sequencing artifact, primers spanning this region were used to PCR amplify the sequence from 3D7 DNA. The PCR was performed with the proofreading polymerase *Pfx*. The sequences of three independent PCR products were identical and demonstrated an open reading frame across this region. This sequence result was confirmed by using DNA derived from three 3D7 lines: the current batch growing at the National Institute for Medical Research; a batch from 1998 derived from a recloned parasite obtained from The Weatherall Institute for Molecular Medicine, Oxford, United Kingdom; and a batch from The Walter and Eliza Hall Institute, Melbourne, Victoria, Australia. All three 3D7 stocks were identical at the polymorphic loci MSP1 and MSP2. Both FCB1 and T996 parasites had the same open reading frame across this region of *PfrH1*. There were, how-

FIG. 4. Transcription of the *PfrH* gene family varies between parasite lines. (A) Giemsa-stained smears of late 3D7, FCB1, and T996 schizonts harvested for RNA analysis. (B) Northern analysis of RNA from late 3D7 (lanes 3), FCB1 (lanes F), and T996 (lanes T) schizonts. RNA on four identical blots was hybridized to probes for *PfrH1* (region PfRH1.1), *PfrH2a* (region PfRH2a.1), *PfrH2b* (region PfRH2b.1), and *PfrH3* (homology region). The blots were stripped and probed with *msp7* to check for equal loading (38). All probes were derived from 3D7 DNA. The probe for *PfrH2b* spans the region of the gene in T996 which has undergone a 585-bp deletion; hence, the relative amounts of *PfrH2b* mRNA in 3D7 and T996 cannot be directly compared. Size markers are in kilobases.

ever, differences in the nucleotide sequence coding for a run of His-Asn and Gln-Asn (HN and QN) repeats (Fig. 1C).

Sequence variations in *PfrH2a* and *PfrH2b* between different parasite lines were previously described for a region of degenerate repeats near the 3' end of the gene (44, 52). In addition, a parasite line lacking the *PfrH2b* gene was also described (52). The *P. falciparum* 3D7, FCB1, and T996 lines differ across the region of degenerate repeats in the sequences of both *PfrH2a* and *PfrH2b* (Fig. 2A). In addition, *PfrH2b* in T996 has a 585-bp deletion in the 3' end downstream of the repeats but upstream of the region coding for the transmembrane domain (Fig. 2). A polymorphism in the sequence of *PfrH3* was previously described (49).

3D7 and T996 parasites transcribe all the members of the family, while FCB1 does not transcribe *PfrH2a* or *PfrH2b*. To confirm that *PfrH1* was transcribed and to identify the 5' end of the gene, 5' RACE was carried out with RNA prepared from 3D7 schizonts. Sequencing of the RACE products revealed that a 98-bp intron is removed in the mRNA, after a 55-bp first exon. This is the same basic gene structure as that identified in all other members of the superfamily. The first exon codes for a hydrophobic region that is predicted by iPSORT to contribute to an N-terminal signal sequence, with the cleavage site between N30 and E31 (<http://www.HypothesisCreator.net/iPSORT>).

To determine the precise timing of transcription of the *PfrH* gene family, RNA was isolated from tightly synchronized cul-

tures of 3D7 parasites at 4-h intervals. Northern analysis of RNA at these time points revealed that the transcription of all four genes (*PfrH1*, *PfrH2a*, *PfrH2b*, and *PfrH3*) is tightly regulated, with the greatest message abundance in late schizonts, just prior to merozoite release (Fig. 3). Probing the Northern blot with the constitutively expressed sequence C-341 (4) demonstrated that although the lanes for the later stages were relatively overloaded compared to those for the ring stages, there were few loading differences in the samples from 20 h onward. The lack of transcription of the *PfrH* genes in ring stages was confirmed by using an additional Northern blot with equal amounts of ring-, trophozoite-, and schizont-stage RNA (data not shown). Hence, the signal intensities obtained with the *PfrH* probes are not accounted for by the presence of different amounts of total RNA.

Late schizonts (40 h onward postinvasion) were collected from cultures of 3D7, T996, and FCB1. Figure 4A shows Giemsa-stained smears of each of these cultures, confirming that all three are at the same stage. A Northern blot of RNA from these parasites was probed with each of *PfrH1* to *PfrH3* and with *msp7*, a gene which has been shown to be transcribed in schizonts (38) (Fig. 4B). The message for *PfrH1* is much more abundant in FCB1 than in 3D7 or T996 parasites. In contrast to the 3D7 and T996 lines, the FCB1 line makes no message for either *PfrH2a* or *PfrH2b*. The signal obtained with the probe for *PfrH2b* in T996 is lower than that obtained in 3D7, but for this gene the relative amounts of message cannot be

FIG. 5. Expression of PfrRH1, PfrRH2a, and PfrRH2b in 307 and FCB1 schizonts. (A) Immunoprecipitation of metabolically labeled FCB1 and 3D7 schizont extracts with antibodies to PfrRH1, PfrRH2a, and PfrRH2b. Proteins were immunoprecipitated from late-schizont lysates (extracted with SDS and DOC) by using normal mouse serum (lanes N), antiserum RH1.1 (lanes 1), antiserum RH2a.1 (lanes 2a), antiserum RH2b.1 (lanes 2b), or antiserum RH2.HR (lane 2a/b) and then run on 5% polyacrylamide gels. Size markers are shown in kilodaltons. The proteins immunoprecipitated with antiserum RH1.1 are marked with arrows on the left side of each panel, and those immunoprecipitated with antisera RH2a and RH2b are marked with arrows on the right side. The same bands were present when parasites were extracted with NP-40. (B) Immunoprecipitation of metabolically labeled 3D7 schizonts treated or not treated with BFA. 3D7 schizonts (40 to 42 h postinvasion) were treated with BFA (+) or methanol (control) (-) for 1 h and then metabolically labeled in the continuing presence of BFA or methanol. Proteins were immunoprecipitated from parasite lysates (extracted with SDS and DOC) by using antiserum RH1.1 (lanes 1), antiserum RH2a.1 (lanes 2a), antiserum RH2b.1 (lanes 2b), or antiserum RH2.HR (lanes 2a/b) and then run on 3 to 8% NuPAGE Tris-acetate gels. Size markers are shown in kilodaltons.

determined, as the probe spanned the region of *PfRH2b* that contains the 585-bp deletion in T996. The 3D7 line makes less transcript for the pseudogene *PfRH3* than the other two parasite lines. Approximately the same amounts of total RNA were loaded in the lanes, as demonstrated by probing the filter with *msp7* and another rhoptry protein gene (data not shown).

The expression of PfrRH1 differs in 3D7 and T996 compared to FCB1 parasites. Polyclonal antisera were raised in mice and rabbits to GST fusion proteins containing different regions of

PfrRH1, PfrRH2a, and PfrRH2b (Fig. 1A). The specificity of the antibodies was confirmed by immunoprecipitating in vitro-translated proteins derived either from the same region of the gene or from control sequences as previously described (49) (data not shown).

Antiserum RH1.1 immunoprecipitated high-molecular-mass proteins from metabolically labeled late schizonts. In FCB1 schizonts, a predominant band of about 195 kDa was seen, along with a high-molecular-mass band (>240 kDa) (Fig. 5A). Proteins of similar sizes were also immunoprecipitated from 3D7 and T996 schizonts (Fig. 5A and data not shown), although they were much less abundant in lysates of these parasite lines.

The predicted molecular mass of PfrRH1 is 358 kDa, much larger than that of the most predominant protein immunoprecipitated with antiserum RH1.1. However, the 195-kDa protein was shown to be derived from the high-molecular-mass protein following treatment of 3D7 and FCB1 schizont cultures with BFA. BFA reversibly blocks protein translocation from the endoplasmic reticulum (ER) to the Golgi complex. Biosynthetic labeling of late-schizont cultures in the presence of BFA led to an accumulation of the high-molecular-mass band in both 3D7 (Fig. 5B) and FCB1 (data not shown) schizonts. The same high-molecular-mass band was seen in BFA-treated 3D7 parasites after immunoprecipitation with antiserum RH1.2 (data not shown). This process was fully reversible following BFA removal before labeling. These results demonstrated that the larger band is full-length PfrRH1 and that PfrRH1 is processed by proteolysis after trafficking through the ER. Immunoblotting (see below) confirmed that the smaller band in FCB1 was the processed product of PfrRH1. We were unable to detect any soluble PfrRH1 in culture supernatants from 3D7, T996, or FCB1 parasites.

Antiserum RH2.HR was raised to a region common to both PfrRH2a and PfrRH2b and hence should recognize both proteins. The antiserum recognized a series of high-molecular-mass bands in lysates of 3D7 and T996 but not FCB1 schizonts (Fig. 5A and data not shown). The protein-specific antisera RH2a.1 and RH2b.1 each recognized a subset of these bands (Fig. 5A). Again, the minor high-molecular-mass bands were shown to be precursors of the smaller proteins by radiolabeling the cultures in the presence of BFA (Fig. 5B). These results also suggested that the processing of PfrRH2a and PfrRH2b occurs at the N-terminal end of these proteins, in a fashion similar to the processing of Py235 (34), because antisera RH2a.1 and RH2b.1 were raised to constructs derived from the C-terminal end of the proteins.

Antisera to PfrRH1, PfrRH2a, and PfrRH2b were used to probe Western blots of parasite extracts (both late schizonts and purified merozoites). Antiserum RH1.1 reacted with an ~195-kDa band in FCB1 schizonts and merozoites and with a minor high-molecular-mass band corresponding in size to the proteins immunoprecipitated from the metabolically labeled cultures. In contrast, no obvious bands of reactivity were seen with antiserum RH1.1 when 3D7 or T996 schizonts or merozoites were probed (Fig. 6). The most likely explanation for the different results obtained with the Western blotting and immunoprecipitation methods is that the PfrRH1 protein was present below the level of detection of Western blotting in 3D7 and

FIG. 6. Immunoblots of parasite extracts probed with antisera to PfrRH1, PfrRH2a, and PfrRH2b. Late T996 schizonts (lanes Ts), 3D7 schizonts (lanes 3s), and FCB1 schizonts (lanes Fs) and purified merozoites from 3D7 (lanes 3m) and FCB1 (lanes Fm) were lysed directly into NuPAGE or SDS-PAGE loading buffer and run on 3 to 8% NuPAGE Tris-acetate gels (left and middle panels) or 5% polyacrylamide gels (right panel). Lanes U, uninfected erythrocytes. Gels were blotted onto nitrocellulose membranes and probed with polyclonal mouse antisera RH1.1, RH2a.1, and RH2b.1; detection was done with enhanced chemiluminescence. Size markers are shown in kilodaltons.

T996 but was detected with the more sensitive immunoprecipitation method, particularly in the presence of BFA.

Antiserum RH2.HR cross-reacted very strongly with spectrin from uninfected red blood cells and gave a high nonspecific background with purified merozoites on Western blots. However, the two specific antisera to PfrRH2a and PfrRH2b, RH2a.1 and RH2b.1, reacted with high-molecular-mass bands in lysates of both 3D7 and T996 but not of FCB1 (Fig. 6). The

proteins recognized by antiserum RH2b.1 (and, to a lesser extent, RH2a.1) were larger in 3D7 than in T996, in agreement with the differences in the sizes of the genes in the two parasite lines. Interestingly, for both PfrRH1 in FCB1 and PfrRH2a and PfrRH2b in 3D7, the high-molecular-mass forms of the proteins were detected in merozoites, suggesting that this stage of the parasite was actively making these proteins. There was almost no schizont contamination of the merozoite preparations, discounting the possibility that the high-molecular-mass forms of the proteins were carried over from these stages.

Despite demonstrating the presence of PfrRH1 in 3D7 and T996 parasites by immunoprecipitation, we did not detect any staining when antibodies to this protein were used in IFAs with 3D7 or T996 schizonts (Fig. 7). In contrast, strong apical staining was seen with both antiserum RH1.1 and antiserum RH1.2 for FCB1 parasites. Only late schizonts or free merozoites were stained with these antisera. The staining pattern with free merozoites suggested that PfrRH1 moves from the apical organelles in the maturing schizont to the apical surface of the merozoite. In some merozoites, staining was seen around the anterior half of the merozoite surface (Fig. 7).

Antibodies to PfrRH2 were also used in IFAs. Due to its strong cross-reaction with spectrin, neither preabsorbing antiserum RH2.HR with uninfected red blood cells nor affinity selecting it on recombinant protein PfrRH2.HR was able to reduce the background with this antiserum in IFAs. However, each of the antisera RH2a.1 and RH2b.1 gave a punctate pattern of staining with late schizonts, sometimes giving the characteristic double dot typical of a location in the rhoptries (Fig. 7). In free merozoites, apical staining was seen (Fig. 8). As expected, FCB1 parasites were not stained with these antisera.

Double staining and fluorescence microscopy were used to try to establish whether the PfrRH proteins were located in the rhoptries of *P. falciparum*. Antisera to a known rhoptry protein, RhopH2, to AMA-1 (thought to be located in the rhoptries), and to micronemal protein EBA-175 were used in conjunction with antisera to PfrRH1 and PfrRH2 in double-staining IFAs. In late FCB1 schizonts, there was a large degree of overlap between the staining obtained not only with antisera

FIG. 7. Single-staining IFA of 3D7, T996, and FCB1 parasites. Primary antibodies were polyclonal mouse antisera RH1.1, RH1.2, RH2a.1, and RH2b.1. Secondary antibodies were FITC-conjugated anti-mouse immunoglobulin G (Sigma). The parasite nuclei are stained with DAPI. Scale bar, 10 μ m.

FIG. 8. Double-staining IFA of FCB1 and 3D7 parasites. (A and B) FCB1 parasites. (C to H) 3D7 parasites. All panels show late schizonts, except for panels F and H, which show released merozoites. The following primary antibodies were used: mouse polyclonal antisera RH1.1, RH2a.1, and RH2b.1; rabbit polyclonal antiserum RH2a.1 and antiserum to RhopH2 (a rhoptry protein); rat polyclonal antiserum to EBA-175 (a microneme protein); and rat MAb 4G2dc1 to AMA-1 (thought to be located in the rhoptries). Parasite nuclei are stained with DAPI (blue). For each panel, the FITC-conjugated sec-

ondary antibodies (green) are to mouse polyclonal sera, and the TRITC-conjugated secondary antibodies (red) are to rabbit polyclonal sera, with the following exceptions. (E and F) Rabbit antiserum RH2a.1 was labeled with FITC-conjugated anti-rabbit immunoglobulin G (IgG) (Sigma), and rat MAb 4G2dc1 was labeled with Texas red-conjugated anti-rat IgG (Sigma). (H) Rabbit antiserum RH2a.1 was labeled as described above, and rat antiserum to EBA-175 was labeled with Texas red-conjugated anti-rat IgG. In the merged images, areas of overlap between the red and the green signals are shown in yellow. Scale bar, 10 μ m.

DISCUSSION

In this report, we demonstrate that different laboratory lines of *P. falciparum* vary in their expression of members of a protein family implicated in parasite invasion. The three laboratory lines, 3D7, FCB1, and T996, differ in the sequences and levels of transcription of different members of the *PfPRH* family and in the amounts and locations of the *PfPRH* proteins. FCB1 expresses *PfPRH1* at the apical end of merozoites within late schizonts but does not express either *PfPRH2a* or *PfPRH2b*. In contrast, 3D7 and T996 make *PfPRH1*, but the protein is not seen in the apical organelles by IFAs. 3D7 and T996 parasites express both *PfPRH2a* and *PfPRH2b*, and both proteins appear to be initially located in the rhoptries. In T996, the large deletion toward the C terminus of *PfPRH2b* does not affect the location of this protein.

The resolution of the images produced by IFAs with a fluorescence microscope was insufficient for a definitive assignment of these proteins to specific organelles. Even sequential image acquisition with confocal microscopy did not provide an absolutely clear result, because the size of the rhoptry and microneme organelles and the space between them are close to the resolution of the microscope. Nevertheless, the data suggest that in late schizonts, the *PfPRH2* proteins are located in the rhoptries, but at schizont rupture, the proteins move apically to the tip of free merozoites. These suggestions are in agreement with previously published work on *PfPRH2* (44, 52). The data for *PfPRH1* are less clear, but it appears that this protein also moves from apical organelles, relocating to the merozoite apex and around the surface, distributing backward from the tip. There is a precedent for this type of capping in *Plasmodium* merozoites: a processed form of AMA-1 has been shown to cap backward over the merozoite surface during

invasion (32). It has been suggested that this process may occur by interaction with the actin-myosin motor of the parasite (20), as has been described for some microneme proteins in other apicomplexans (8, 10). As none of the three parasite lines examined expressed all three proteins at the apical end of the merozoite, it was not possible from these experiments to determine whether PFRH1, PFRH2a, and PFRH2b are in the same compartment. Definitive experiments to localize these proteins to cellular compartments will require a combination of subcellular fractionation, electron microscopy, and more molecular markers for specific compartments (6).

The contig on which we found *PfPRH1* contains a frameshift in the gene sequence, and it has been suggested that 3D7 makes a truncated version of PFRH1 (45). We sequenced several PCR products covering this region from 3D7 parasites cultured at different times and recently originating from different laboratories, showing conclusively that there is no frameshift in the 3D7 parasites used in the experiments described in this study. The 3D7 line makes both the full-length transcript and the full-length PFRH1 protein. However, the relatively low level of PFRH1 immunoprecipitated from 3D7 and T996 schizonts and our inability to detect the protein by Western blotting and IFA analysis suggest that the protein is made in a small quantity and/or rapidly turned over. Either or both of these two explanations would fit with these data. For example, a very small amount of protein may be made and correctly located at the apical end of the parasite but be below the level of detection of an IFA. Furthermore, the protein may be rapidly degraded after passage beyond the ER and may or may not be transported to the apical organelles. We favor the explanation of rapid turnover, because more PFRH1 was immunoprecipitated from BFA-treated 3D7 parasites than from the untreated control parasites. However, no antigen was detected by antisera to PFRH1 in IFAs with BFA-treated 3D7 parasites, suggesting that for detection by IFAs, this protein must be locally concentrated.

It is interesting that FCB1, which does not make PFRH2a and PFRH2b, may compensate by making increased amounts of *PfPRH1* transcript and PFRH1 protein. However, this may be only part of the story. During preparation of this manuscript, Rayner and coworkers described *P. falciparum* normocyte binding protein 1 (PfNBP1) (45). PfNBP1 is the same protein as that described here as PFRH1. The reported location of PfNBP1 matches that of PFRH1. Interestingly, Rayner et al. (45) demonstrated apical expression of PFRH1 and either PFRH2a or PFRH2b or both in one parasite line, FVO. As the PFRH2-specific antibody used recognized both PFRH2a and PFRH2b, it is possible, but not certain, that FVO parasites express all three proteins at the apex of the merozoite. Rayner et al. (45) suggested that PfNBP1 and PFRH2b form a complex, based on the presence in the immunoprecipitate obtained with antibodies to PfNBP1 of an additional high-molecular-mass doublet which is similar in size to that recognized by antibodies to PFRH2b. However, PfNBP1 did not appear to be present in the reciprocal immunoprecipitation. Our results obtained with parasites treated with BFA suggest a more likely explanation—that the doublet is actually a processed product of PfNBP1 or PFRH1. In the 3D7 and FCB1 lines, a complex between PFRH1 and PFRH2 is unlikely to be seen, because 3D7 expresses only

a low level of PFRH1 which cannot be detected at the end of the merozoite and FCB1 does not express PFRH2a or PFRH2b.

The three PFRH proteins described thus far have been shown to have a role in erythrocyte invasion (44, 45, 52). PfNBP1 or PFRH1 binds to a trypsin-resistant uncharacterized erythrocyte ligand, and antibodies to PFRH1, PFRH2a, and PFRH2b have been shown to inhibit invasion. No erythrocyte binding activity has been demonstrated for PFRH2. However, not all proteins involved in erythrocyte invasion will necessarily bind to the erythrocyte surface. Other possible roles include intracellular signaling, interaction with the actin-myosin motor of the parasite, or restructuring of the host cell cytoskeleton (6, 42). These processes are well recognized in other invasive microorganisms (reviewed in references 14, 28, and 47).

It is clear that some lines of *P. falciparum* maintained in vitro have mutant phenotypes of the RH protein family (if an apical location of PFRH1 and PFRH2 is the wild type), and these parasites do not require all three proteins to invade at least some erythrocytes. It has yet to be shown definitively that all three proteins can be expressed apically in the same merozoite. This phenotypic diversity highlights a potential difficulty in interpreting the results of experiments designed to disrupt members of this family. The background phenotype of the parasite must be characterized, even in parasites that make transcripts for all family members.

Diversity in the RH protein family may allow the parasite to invade erythrocytes with structurally diverse receptors. This variability is at the levels of sequence variation and expression of the proteins. *P. vivax* parasites are able to invade only reticulocytes and require selection of the correct cells by the PvRBP (16). Moreover, invasion is dependent on an interaction between the erythrocyte Duffy blood group antigen and the parasite Duffy binding protein (a member of the EBP family) (2, 30). In contrast, multiple invasion pathways are available for *P. falciparum* parasites (9, 13, 36, 37, 46). The apparent redundancy in the PFRH protein family may allow the parasite to use alternative invasion pathways. An added level of complexity is the presence of several EBPs in *P. falciparum* (1, 29, 31, 40, 51, 53). How the PFRH proteins interact with each other and with other proteins known to be involved in invasion is a crucial question. As more merozoite proteins are identified from the *P. falciparum* genome sequencing project and the related proteomics project (3) (<http://www.ebi.ac.uk/parasites/proteomes.html>), it is certain that additional proteins involved in invasion will be identified. For example, there is at least one other *P. falciparum* sequence that shares some similarity with PFRH family sequences (PFRH4) (21; unpublished data). It will be important to determine whether there is a requirement for all the PFRH proteins to be functional in parasites isolated from natural infections or whether there is a level of redundancy within the family, as shown in laboratory lines.

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