Identification of Proteins from *Plasmodium falciparum* That Are Homologous to Reticulocyte Binding Proteins in *Plasmodium vivax*

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*Plasmodium falciparum* infections can be fatal, while *P. vivax* infections usually are not. A possible factor involved in the greater virulence of *P. falciparum* is that this parasite grows in red blood cells (RBCs) of all maturities whereas *P. vivax* is restricted to growth in reticulocytes, which represent only approximately 1% of total RBCs in the periphery. Two proteins, expressed at the apical end of the invasive merozoite stage from *P. vivax*, have been implicated in the targeting of reticulocytes for invasion by this parasite. A search of the *P. falciparum* genome databases has identified genes that are homologous to the *P. vivax* *rhp-1* and *-2* genes. Two of these genes are virtually identical over a large region of the 5′ end but are highly divergent at the 3′ end. They encode high-molecular-mass proteins of >300 kDa that are expressed in late schizonts and localized to the apical end of the merozoite. To test a potential role in merozoite invasion of RBCs, we analyzed the ability of these proteins to bind to mature RBCs and reticulocytes. No binding to mature RBCs or cell preparations enriched for reticulocytes was detected. We identified a parasite clone that lacks the gene for one of these proteins, showing that the gene is not required for normal in vitro growth. Antibodies to these proteins can inhibit merozoite invasion of RBCs.

A number of *Plasmodium* species cause malaria in humans. *Plasmodium falciparum*, the most virulent form that causes human malaria, invades both reticulocytes and mature red blood cells (RBCs), while *P. vivax* and *P. ovale*, which cause less severe disease, are both restricted to reticulocytes. *Plasmodium* species that infect rodents also show a preference for RBCs of different stages of development and maturity. For example, virulent strains of *P. yoelii* invade both mature and immature RBCs, while nonlethal strains show a preference for reticulocytes (9). Hence, members of the *Plasmodium* species can be divided into two groups: those that predominantly invade reticulocytes, and those which invade RBCs at all stages of maturity. The basis of this RBC specificity is presumably the presence of different ligands at the apical end of the invasive merozoite stage of the various species.

A 235-kDa rhoptry protein from *P. yoelii* has been suggested to be important in the ability of this parasite to invade mature RBCs (8). Passive transfer of monoclonal antibodies (MAbs) specific to this protein protect mice infected with the virulent YM strain, by restricting invasion of reticulocytes (4). In *P. vivax*, proteins termed *PvRBP* have been identified by immunofluorescence assay (IFA) experiments (5). *PvRBP-1* and *PvRBP-2* form a protein complex through noncovalent interactions and colocalize to the apical end of the merozoite. The *PvRBP-1* and *PvRBP-2* proteins have calculated molecular masses of 325 and 330 kDa, respectively, and share similar structures with a signal sequence at the N terminus and a putative transmembrane domain and cytoplasmic tail at the C terminus (6). Interestingly, the full sequence of one member of the *Py235* family recently deposited in GenBank (accession no. U36927) encodes a protein with a predicted molecular mass of 325 kDa and the same structure as *PvRBP-1* and *-2* (7). *PyRBP-2* and members of the *Py235* family share a 500-amino-acid region which shows significant homology (9). The *Py235* proteins are encoded by a multigene family of up to 50 members, with at least 11 distinct genes spread across different chromosomes of the *P. yoelii* genome (2). At least one member of this protein family has been shown to bind both mature and immature RBCs (11), a finding consistent with the fact that a *Py235* MAb can restrict parasite invasion of reticulocytes (4). More recently, it has been found that individual merozoites within a single developing schizont can have different *Py235* genes transcribed in *P. yoelii* (14). It is not known if each *Py235* protein has a distinct target cell specificity, but it is likely that the proteins are antigenically distinct. This would ensure that even with host anti-*Py235* antibodies, some merozoites would be free to invade new RBCs at each cycle.

In this study, we describe two genes initially identified from the *P. falciparum* genome databases (*P. falciparum* sequencing group at the Sanger Centre [ftp://ftp.sanger.ac.uk/pub/databases/P.falciparum_sequences], the Stanford DNA Sequencing and Technology Centre [http://sequence-www.stanford.edu/group/malaria], and The Institute for Genomic Research [ftp://ftp.tigr.org]) that are homologous to *PvRbp-2* and the *Py235* family. We have analyzed the expression of these genes, and results of immunofluorescence assay (IFA) experiments are consistent with a subcellular localization at the apical end of the merozoite. By analogy with the role of the other members of this family, these proteins may be involved in...
the targeting of particular RBC subpopulations for invasion by *P. falciparum* merozoites.

**MATERIALS AND METHODS**

**Parasites and nucleic acids.** Parasites were maintained (20) and synchronized by standard procedures. Genomic DNA (gDNA) was obtained from trophozoites as described elsewhere (21). Southern blotting was carried out using standard procedures. Poly(A)^+ RNA was obtained from synchronized late-stage schizont cultures (Ambion Inc.) and then converted to cDNA using Superscript II (Gibco-BRL).

**Antibodies.** Two Pf2h fragments were amplified by PCR from 3D7 genomic DNA, subcloned into pGEX, and fusion protein affinity purified on glutathione-agarose. The fusion proteins were used to immunize both rabbits and mice. The primers used for production of the 2A9 antibody were 5'-GGATGATGCAGGCAGG-3' and 5'-TATCTGGGGAGCTC-3'. The primers used for the 2A11 antibody were 5'-GACGATTTTCCTATGACGCT-3' and 5'-GTACGTCGGCTTCAAGTTTTTTCTGTTAGATGTACT-3'. Note that the latter primer pair could amplify only the Pf2h gene, but part of the product overlaps the Pf2h gene. Locations of the expressed fragments in relation to the complete Pf2H2 proteins are shown in Fig. 2C. Other antibodies used in the IFA experiments were a mouse MAb specific to *P. falciparum* apical membrane antigen 1 (PfAMA1), 2C5 (22), a mouse MAb specific to *P. falciparum* rhoptry-associated antigen 1 (PfRAPI), 7H8/50 (17), and a mouse anti-EBA175 (erythrocyte binding antigen 175) serum (16).

**Erythrocyte binding.** Metabolic labeling of parasites and erythrocyte binding assays were performed as described elsewhere (3). Synchronized parasite cultures at the trophozoite stage were enriched to greater than 80% parasitemia on Percoll gradients consisting of 80, 60, and 40% steps. Purified trophozoites for production of unlabeled supernatant were put in a culture dish containing only one-fourth of the original volume of complete medium. For [35S]Met-labeled supernatant, purified trophozoites were washed in methionine- and cysteine-deficient medium and cultured in the same medium containing [35S]methionine-[35S]cysteine mixture (Trans 35S; 200 μCi/ml; ICN Radiochemicals). Parasites were incubated for 16 h, and the supernatant was harvested by centrifugation at 12,000 × g for 20 min at 4°C. The supernatant was stored in aliquots at −70°C.

Erythrocyte binding assays were performed using 500 μl of unlabeled or radiolabeled culture supernatant which was mixed with 100 μl of packed RBCs for 30 min at 23°C. The cells were centrifuged at 12,000 × g for 30 s through 500 μl of silicone oil (Dow Corning 550). The supernatant depleted of RBC binding structures of Py235, PvRBP-1, and PvRBP-2 (7).

**Structurally, Py235 (325 kDa [accession no. U36927]) and PvRBP-2 (330 kDa masses of 370 and 383 kDa, respectively, comparable in size to the homologous region were identified. The homologous re-

**RESULTS**

**The *P. falciparum* genome encodes at least four genes related to the *Pvrpb-1* and *Py235* gene families.** The *PVRBP-2* and *Py235* protein families have been demonstrated to bind reticu-

**Immunofluorescence.** Synchronized late-stage D10 schizonts were smeared and air dried followed by fixation with 100% methanol for 2 min at −20°C. Smears were incubated with mouse antibodies to either PfAMA1, PfRAPI, or EBA175 and coincubated with the rabbit anti-PfR2Ha- and -Hb antibodies (2A9 and 2A11). Secondary antibodies were a mixture of rhodamine anti-mouse IgG (Chemicon), and fluorescein isothiocyanate (FITC) anti-rabbit IgG (Silenus Laboratories). Dual-color fluorescence images were captured using a digital camera (Zeiss, Jena, Germany).

**Invasion inhibition assay.** Synchronized trophozoites were purified by passage over a Percoll gradient and counted in a hemocytometer; 8 × 10^9 schizonts were added to each well. A final volume of 180 μl of medium with 4 × 10^6 uninfected human erythrocytes at 2% hematocrit. The anti-PfR2Ha- and -Hb antibodies (2A9 and 2A11) or normal rabbit serum (NRS) control were purified by affinity chromato-

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**Identification of a *P. falciparum* parasite line that lacks the Pf2h gene.** Comparison of the nucleotide sequences of Pf2h and -hb suggested that they were identical over most of the sequences but diverged significantly at the 3' end (Fig. 2C). To confirm that they represented two genes, we used PCR of

**Western blotting.** Synchronized D10 parasites were sampled at 8-h intervals throughout the asexual life cycle. Parasite samples of each time point and from erythrocyte binding assays were analyzed by SDS-PAGE on 6% polyacrylamide gels and transferred to nitrocellulose as described elsewhere (21). The antibodies to *P. falciparum* hsp70 (Phsp70) have been previously described (1). Bound antibody was detected with horseradish peroxidase-coupled sheep anti-rabbit immunoglobulin G (IgG; Silenus Laboratories, Boronia, Victoria, Australia) and developed by the enhanced chemiluminescence method (Amersham International).

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genomic DNA from 3D7 with a 5' primer (P1), contained within both genes, and two different 3' primers specific to Pfr2ha and -hb (P2 and P3, respectively). These primers amplified DNA fragments of the expected lengths, and results of sequencing analysis were consistent with the presence of two genes that differed at the 3' end (data not shown). To confirm these results, we hybridized a probe from the common region (C2 in Fig. 2A and B) to Southern hybridization filters containing gDNA from D10, 3D7, and HB3 digested with different restriction enzymes (Fig. 3A). Two fragments were detected in 3D7 and HB3 gDNA for three different restriction enzymes, which is consistent with the presence of two genes in these cloned lines.

Interestingly, hybridization of the C2 probe to D10 genomic DNA revealed only one hybridizing fragment with the different restriction enzymes, suggesting the presence of only one gene (Fig. 3A). This possibility was further analyzed by PCR using primers to the unique regions of Pfr2ha and -hb genes with genomic DNA from D10 and 14 other P. falciparum isolates. PCR products of the expected sizes were obtained for both the Pfr2ha and -hb genes for all parasite DNA samples tested except D10 (data not shown). D10 genomic DNA gave a PCR product for Pfr2ha but not for Pfr2hb. The absence of Pfr2hb in D10 was confirmed by Southern hybridization of genomic DNA from D10 and 3D7 probed with gene-specific probes U1 and U2 (Fig. 3B). The Pfr2hb-specific probe (U1) failed to hybridize to D10 but did hybridize to 3D7 genomic DNA, whereas the Pfr2ha-specific probe (U2) hybridized to both parasite DNAs. The presence of only one gene in D10 with the common 5' end of Pfr2ha and -hb was also shown by quantitation of the gene copy number in D10 and 3D7 compared to the single-copy dhps (dihydropteroate synthase) gene (21). Restriction enzyme-digested genomic DNA from both D10 and 3D7 was probed with both the common region probe C1 and a dhps probe (Fig. 3C). Quantitation of the signal intensity of each hybridizing fragment using a PhosphorImager showed that 3D7 had two genes that shared the 5' end whereas D10 had only a single copy. These results are consistent with the absence of Pfr2hb from the D10 genome.

Characterization of PfR2Ha and -Hb. To characterize the proteins encoded by Pfr2ha and -hb, antibodies to two different regions of the 3D7 Pfr2ha gene were made. The 2A9 antibody was made to a region common to PfR2ha and -hb; the 2A11 antibody was to a region mostly within the unique 3' end of Pfr2ha, although it did overlap into the common 5' end (Fig. 2C). To confirm the specificities of both antibodies, total parasite proteins and culture supernatants from HB3 and D10 parasites were separated by SDS-PAGE, transferred to nitrocellulose, and incubated with the two antisera. Both 2A9 and 2A11 antibodies detected three or more bands of the same size (≈200 kDa) in the parasite pellet and the supernatant; however, the intensities of these bands varied with the different regions of the sequence data shown: Py235, L27838; Pyr2p-2, Q00799; Pf2ha/hb (AL049181, AF312916, and AF312917). Sequences were aligned using CLUSTAL V software. Boxes represent positions which have >50% identity; dots represent spaces inserted into the sequence to provide optimal homology.
FIG. 2. 3D7 parasites have both PfR2Ha and PfR2Hb genes, while D10 has only PfR2Ha. (A) Schematic representation of the PfR2hb gene in 3D7 parasites. The short signal peptide (exon 1) is followed by an intron and then exon 2. The C1 probe common to both PfR2ha and PfR2hb was amplified from D10 gDNA using primers 5'-ACAGGAAATATGTGAAAAACGG-3' and 5'-TTATTATATTAGTTGTTTTTAC-3'. The C2 probe common to both PfR2ha and PfR2hb was amplified from 3D7 gDNA using primers 5'-CACCAAGATCCTTTATATCTA3' and 5'-CTTTATATAATTATTTATGAAT-3'. The C2 probe, unique to the PfR2hb gene, was amplified from 3D7 gDNA using primers 5'-GAATTGAGTACTGACCAACGT-3' and 5'-TTATTATTATTAGTGTTTTTAC-3'. The 5' and 3' XmnI (X) sites is shown expanded below the PfR2hb gene. The Hinfl (H) site is shown together with fragment sizes in base pairs. (B) Schematic representation of the PfR2ha gene in D10 and 3D7 parasites. The C1 and C2 probes are as in panel A. The U2 probe was amplified from 3D7 gDNA using primers 5'-GAATTGATAGTACTGACCAACGT-3' and 5'-ACAGGAAATATGTGAAAAACGG-3'. A region of 2,037 bp bounded by the RsaI (R) and XmnI (X) sites is shown expanded below the PfR2hb gene. The Hinfl (H) site is shown together with fragment sizes in base pairs. (C) Schematic representation of the PfR2ha and -Hb proteins in 3D7 and the PfR2Ha protein in D10 parasites. The three sequences are presumed to be nearly identical (see Results) but differ markedly from amino acid(aa) 2776 onward even though they are structurally similar, with a putative transmembrane domain (TM) and a short cytoplasmic tail at the C terminus. The unique regions are shown as diagonally hatched in R2Hb and horizontally hatched in R2Ha. The 500-amino-acid region showing some conservation in PvRBP-2 and Py235 as shown in Fig. 1 is indicated. The DNA corresponding to approximately 1,100 amino acids at the C terminus of the 3D7 r2ha and r2hb genes and approximately 800 amino acids at the C terminus of the D10 r2hb gene was sequenced. This encompassed the regions to which the 2A9 and 2A11 antibodies were made. For PCR amplification of the D10 and 3D7 r2ha genes, primers P1 (5'-AAATACGTGAATGGTCAACGC-3') and P2 (5'-GATCATGTTTCCCTTTTTATGTTGAT-3') were used. For amplification of the 3D7 r2hb gene, primers P1 and P3 (5'-AAACAAACAGTACACATCAGCATTG-3') were used. The approximate locations of primers P1, P2, and P3 are shown. The PCR products were fully sequenced using internal primers. The amino acid differences within the regions of the three genes which are nearly identical are indicated by asterisks. The 5'-most change (amino acid 2546) is A (Ala) in 3D7 R2Ha but D (Asp) in the other proteins. The next change (position 2635) is E (Glu) in both R2Ha proteins but K (Lys) in 3D7 R2Hb. The 3'-most change (beginning at position 2719) is EEE1RKK in 3D7 R2Ha but EALKKQ in the other proteins. The portions of Pir2Ha and -Hb used for production of rabbit antibodies 2A9 and 2A11 are also shown. The lengths of the signal sequence (S) and transmembrane domain (TM) are not shown to scale. The checkered shading represents the 500-amino-acid conserved region. The diagonal and horizontal shaded regions represent the unique regions of Pir2Ha and Pir2Hb, respectively.
cronemes, and IFA experiments with antibodies to PfR2Ha and EBA175 show that the two proteins do not exactly colocalize, suggesting that PfR2Ha is not located in the micronemes. PfRAP1 has been localized to the body of the rhoptries by immunoelectron microscopy. Localization of PfR2Ha and PfRAP1 shows that they overlap to some extent, although there is significant labeling of PfR2Ha that is more apical than PfRAP1. This is especially evident in Fig. 5D, which shows that PfR2Ha is located at the apical end of the merozoite and separate from PfRAP1, which is present within the body of the rhoptries.

Do PfR2Ha and Hb bind human RBCs? The protein encoded by \textit{PvRBP-2} has been demonstrated to bind reticulocytes (5), and a member of the Py235 protein family can bind to mature RBCs (11). Therefore, we wished to determine if PfR2Ha and b could bind mature RBCs and/or reticulocytes. Purified trophozoites were grown to schizonts either in the presence of [35S]methionine or with no radiolabel; after schizont rupture, the supernatant was collected and used for erythrocyte binding assays. The [35S]Methionine-labeled total supernatant contained a large number of labeled proteins that included PfR2Ha and -Hb, as demonstrated by immunoprecipitation using both 2A9 and 2A11 antibodies (Fig. 6A). Similarly, anti-EBA175 antibodies precipitated a 175-kDa protein corresponding to EBA175 from the radiolabeled supernatant as has previously been shown. When the total supernatant was used in an erythrocyte binding assay, we detected a subset of proteins including EBA175, as shown by immunoprecipitation.
Neither the 2A9 nor 2A11 antibody detected any PfR2Ha or -Hb in the proteins eluted from RBCs in binding assays, suggesting that the proteins released into the supernatant do not bind RBCs. *P. falciparum* can preferentially invade younger RBCs and reticulocytes (10, 13), and it was possible that PfR2Ha and -Hb bind reticulocytes. To test this, we used unlabeled supernatants containing PfR2Ha/Hb and EBA175 to test binding to reticulocytes that had been enriched to 10% compared to RBCs depleted of reticulocytes. Bound proteins were salt eluted, separated by SDS-PAGE, transferred to nitrocellulose, and then probed with antibodies to EBA175 and PfRh (2A11). Two microfilters of the supernatant (S) was also used as a positive control. SE, salt eluted. (C) Unlabeled supernatant from 3D7 parasites (100 μl) was bound to 20 μl of RBC and then eluted with 300 mM NaCl (SE). The supernatant was further depleted either two times (D2) or eight times (D8) by repeated addition of 20 μl of RBC for 30 min. Either 2 μl of the original supernatant (S) or between 2.5 and 3.2 μl (to allow for volume increases during repeated RBC additions) of the depleted supernatants, together with the salt-eluted proteins, was separated by SDS-PAGE, transferred to nitrocellulose, and then probed with antibodies to EBA175 and PfRh (2A11). (D) Unlabeled supernatant from 3D7 parasites (100 μl) which had been ultracentrifuged at 100,000 rpm for 30 min was bound to 20 μl of RBC. The supernatant was depleted six times (D6) by repeated addition of 20 μl of RBC for 30 min. Either 2 μl of the original supernatant (S) or 2.6 μl of the depleted supernatant was separated by SDS-PAGE, transferred to nitrocellulose, and probed with the 2A11 antibody.

Neither the 2A9 nor 2A11 antibody detected any PfR2Ha or -Hb in the proteins eluted from RBCs in binding assays, suggesting that the proteins released into the supernatant do not bind RBCs.

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To assess the possibility that PfR2Ha and -Hb were able to bind to RBCs but could not be eluted under the conditions used, we increased the NaCl concentration stepwise up to 1.5 M for elution. However, we could not detect any PfR2Ha/Hb binding to RBCs at any NaCl concentration (data not shown). To confirm that PfR2Ha and -Hb could not bind RBCs, we did sequential depletion experiments where the same supernatant was incubated with fresh RBCs up to eight times to deplete any binding proteins (Fig. 6C). As expected, EBA175 was depleted...
ozoite invasion was observed with the 3D7 parasite in two
(Fig. 7A and B). However, more significant inhibition of mer-
the second experiment there was a small level of inhibition
merozites with either the 2A9 or 2A11 antibody, although in
In two identical experiments, there was little inhibition of D10
of invading merozoites, and the ability to invade RBCs was
and 2A11 antibodies and NRS were incubated in the presence
inhibit merozoite invasion of RBCs. Protein G-purified 2A9
test.
PfR2Ha and -Hb do not bind RBCs under the conditions
detected (Fig. 6D). These results suggest that the soluble
membrane-associated forms. About 10% of the PfR2Ha/Hb
ultracentrifugation to remove PfR2Ha/Hb complexes and
have depleted the PfR2Ha/Hb content. To test this, we used
with each addition of fresh RBCs or each centrifugation may
wells containing NRS. Standard errors are shown except those less
than 4% (e.g., panel D); the standard errors ranged from 0 to 14%.
from the supernatant after two cycles, and no protein was
detected after eight rounds of incubation with RBCs; in con-
trast, PfR2Ha/Hb was still present after eight rounds of deple-
tion on RBCs, although the amount, especially of the higher-
molecular-weight protein, appeared to decrease (Fig. 6C).
This finding suggested that either very little binding occurred
with each addition of fresh RBCs or each centrifugation may have
depleted the PfR2Ha/Hb content. To test this, we used
ultracentrifugation to remove PfR2Ha/Hb complexes and
membrane-associated forms. About 10% of the PfR2Ha/Hb
protein was lost from the supernatant (data not shown). When
this supernatant, which now contained truly soluble PfR2Ha/
Hb, was used in depletion experiments with six rounds of RBC
incubation, no decrease in the presence of PfR2Ha/Hb was
detected (Fig. 6D). These results suggest that the soluble
PfR2Ha and -Hb do not bind RBCs under the conditions tested.

Antibodies to PfR2Ha and -Hb inhibit merozoite invasion.
We tested the ability of anti-PfR2Ha/Hb rabbit antibodies to
inhibit merozoite invasion of RBCs. Protein G-purified 2A9
and 2A11 antibodies and NRS were incubated in the presence
of invading merozoites, and the ability to invade RBCs was
assessed microscopically following Giemsa staining of smears.
In two identical experiments, there was little inhibition of D10
merozoites with either the 2A9 or 2A11 antibody, although in
the second experiment there was a small level of inhibition
(Fig. 7A and B). However, more significant inhibition of mer-
ozoite invasion was observed with the 3D7 parasite in two
independent experiments (Fig. 7C and D). There was a
marked dose response for 3D7 as the concentrations of both
2A9 and 2A11 antibodies were increased to 0.5 mg/ml com-
pared to the same antibody concentration for NRS (Fig. 7).
Both antisera were able to inhibit merozoite invasion in 3D7 to
levels between 40 and 55% of control values with NRS. This
suggests that PfR2Ha and -Hb are important for the invasion
of merozoites into RBCs.

It was interesting that the 2A9 and 2A11 antibodies were
able to inhibit invasion to some extent in 3D7 but showed little
effect for D10. To determine if there were polymorphisms that
may reduce the ability of the antisera raised to 3D7 PfR2Ha to
inhibit the D10 parasite line, we sequenced the region of the
Pfr2ha and -hb genes in D10 and 3D7 used to raise the 2A9 and
2A11 antibodies. There were six nucleotide changes resulting
in amino acid differences between 3D7 and D10 Pfr2ha genes,
and these occurred in the region used to raise the 2A11 anti-
bodies (Fig. 2C). There were no polymorphisms in the region
used to raise the 2A9 antibody between the 3D7 Pfr2ha and -hb
genes and the D10 Pfr2ha gene (Fig. 2C).

DISCUSSION

Erythrocyte invasion by the merozoite form of P. falciparum
is an ordered process requiring sequential steps that involve
specific adhesive interactions of parasite ligands with receptors
on the host cell. In P. vivax, PvrBP-1 and PvrBP-2 form a
complex that binds to reticulocytes, and it has been hypothe-
sized that this confers specificity for this cell type (6). Members
of the Py235 protein family in P. yoelii are homologous to
PvrBP-1 and -2, and one of these proteins has been shown to
bind to erythrocytes (11). The identification of related protein
families in P. vivax and P. yoelii involved in adhesion to reticu-
locytes and erythrocytes suggested that P. falciparum may
express homologous proteins with similar functions. A search of
the P. falciparum genome databases has identified two genes
coding proteins, which we have called PfR2Ha and PfR2Hb,
that are related to the PvrBP and Py235 families.
Comparisons of gene structure, timing of expression, and
subcellular localization suggest that PfR2Ha and -Hb are func-
tional homologues of the PvrBP and Py235 protein families.
Pfr2ha and -hb, Pvrbp-1 and -2, and Py235 genes have the same
exon/intron structure (6, 7). The short intron at the 5’ end
encodes a putative signal sequence region, and the second
large exon contains a putative transmembrane domain near the
C terminus of each protein with a very short cytoplasmic tail.
This structure is found in all three gene families of the different
Plasmodium species. Additionally, subcellular localization of
PfR2Ha/Hb suggests that it is located at the apical end of the
merozoite, similar to that found for the homologous proteins
in P. vivax and P. yoelii.

Because of the similarity of PfR2Ha/Hb with PvrBP-1/2 and
Py235, it was expected that the P. falciparum proteins would be
able to bind to erythrocytes or reticulocytes. Experiments with
both mature erythrocytes and enriched reticulocytes, using
PfR2Ha/Hb protein released into the supernatant, failed to
detect binding, suggesting that these proteins are unable to
bind directly to either erythrocytes or reticulocytes. This ob-
servation was further confirmed by incubating an ultracentri-
fuged extract multiple times with erythrocytes, in which case no

![Antibodies to PfR2Ha and -Hb result in some invasion inhibition.](image)

FIG. 7. Antibodies to PfR2Ha and -Hb result in some invasion inhibition. Purified schizonts from D10 and 3D7 parasites were plated in human erythrocytes to determine the ability of released merozoites to invade in the presence or absence of protein G-purified 2A9 and 2A11 antibodies (0.125, 0.25, and 0.50 mg/ml) for each parasite, results of two independent experiments done in duplicate are shown.
depletion of these proteins was detected. It is possible that the proteins found in the supernatant were processed and the RBC binding domains had been removed. This would be consistent with the multiple forms of PfR2Ha and -Hb seen in Western blots. This inference suggested that the full-length protein at the apical end of the merozoite could bind to RBCs but subsequent cleavage would remove this binding domain. Interestingly, we have identified in the P. falciparum genome databases a further three homologues of the Pf2ha and -hb genes described here, some of which may be able to interact with erythrocytes (unpublished results). Second, it is possible that the proteins found in the supernatant were not processed but that the PfR2Ha/Hb complex had been disrupted such that it could no longer bind RBCs. Third, it is possible that both proteolytic processing and disruption of a binding complex occur rapidly in P. falciparum merozoites in the absence of fresh RBCs for invasion. It will be important to test the ability of the other proteins encoded by genes homologous to PfR2ha and -hb for binding to RBCs and reticulocytes.

The ability of anti-PfR2Ha/Hb antibodies to inhibit merozoite invasion in 3D7 suggests that these proteins play some role in this process. MAbs to Py235 are able to specifically inhibit invasion of reticulocytes in P. yoelii (4). It was surprising that the antibodies to PfR2Ha were not able to inhibit merozoite invasion in the D10 cloned line, and sequencing of the equivalent gene showed some polymorphism that may reduce the ability of antibodies raised to the 3D7 protein to bind and inhibit. However, there were no polymorphisms in the 2A9 portion of the protein, and it is possible that specific PfRH proteins function in different P. falciparum parasite lines. The demonstration in P. yoelii that different Py235 genes are expressed in individual merozoites within a schizont provides some support for this possibility (14).

It is clear that the D10 parasite genome does not contain the PfR2hb gene, showing that this gene is not essential for parasite invasion and growth. However, this parasite does express the PfR2ha gene, which is nearly identical to PfR2hb throughout most of its sequence. PfR2ha and -hb appear to lie next to each other on chromosome 13 (data not shown), suggesting they have arisen by a recent gene duplication. It is known for the P. yoelii parasite that individual merozoites express a different Py235 gene (14). From analysis of the two Py235 genes for which sequence is available (e3 and e8 types), it appears that there are extensive differences at the protein level with approximately 25% sequence variation (18). In P. falciparum, the much more closely related proteins PfR2Ha and -Hb may be expressed differentially in individual merozoites. The explanation for the absence of the PfR2hb gene in D10 is unknown. The PfR2ha/hb locus is in an internal location on the chromosome, and it is unlikely that loss of the gene has occurred by removal of the subtelomeric region of the chromosome that is common in P. falciparum. It is apparent that the other three Pfhb homologues in the D10 parasite are sufficient to compensate for the lack of the PfR2Hb protein.

Here we have described in detail two high-molecular-weight proteins (PfR2Ha and -Hb) which are expressed at the apical end of the P. falciparum merozoite. These proteins belong to a family of proteins found in other plasmodia which are involved in targeting of RBC populations prior to invasion by the merozoite. Following review of this report, work from another group describing analysis of this gene family was published (15). The conclusions drawn by these authors are in broad agreement to the work presented here. Importantly, they also have not been able to demonstrate interaction of PfR2Ha and -Hb with the RBC surface. A P. falciparum database search has identified a further three genes potentially belonging to this family (Pfhr3, Pfhr1h, and Pfhr4). Pfhr3 appears to be a pseudogene (H. M. Taylor, T. Triglia, J. Thompson, M. Sajid, R. Fowler, M. E. Wickham, A. F. Cowman, and A. A. Holder, unpublished data). Pfhr1h appears to be more closely related to Pvrhp-1 and may be the P. falciparum homologue. Pfhr4 has some features of other Py235/Pvrhp-2/Pfhr family members but appears to be more distantly related. The generation of specific antibodies to individual PfRH proteins should enable dissection of the roles of different members in RBC targeting by P. falciparum.

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