Two Plasmodium falciparum genes express merozoite proteins that are related to Plasmodium vivax and Plasmodium yoelii adhesive proteins involved in host cell selection and invasion

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Two related Plasmodium falciparum genes and their encoded proteins have been identified by comparative analyses with Plasmodium vivax reticulocyte binding protein 2 (PvRBP-2). The P. falciparum genes have a structure which suggests that they may be the result of an evolutionary duplication event, as they share more than 8 kb of closely related nucleotide sequence but then have quite divergent unique 3’ ends. Between these shared and unique regions is a complex set of repeats, the nature and number of which differs between the two genes, as well as between different P. falciparum strains. Both genes encode large hydrophilic proteins, which are concentrated at the invasive apical end of the merozoite and are predicted to be more than 350 kDa, with an N-terminal signal sequence and a single transmembrane domain near their C termini. Importantly, they also share gene structure and amino acid homology with the Plasmodium yoelii 235-kDa rhoptry protein family, which is also related to PvRBP-2. Together these Plasmodium proteins define an extended family of proteins that appear to function in erythrocyte selection and invasion. As such, they may prove to be essential components of malaria vaccine preparations.

Parasites of the genus Plasmodium are estimated to cause between 300 and 500 million cases of malaria, the majority of which are caused by Plasmodium vivax and Plasmodium falciparum (1). Plasmodium parasites have a complex life cycle involving a series of developmental stages in both mosquitoes and mammals, but the clinical manifestations of malaria are all caused by the asexual blood stage. Merozoites, ovoid cells with an apical prominence at one end, invade red blood cells (RBCs), wherein they undergo a growth and multiplication phase (schizogony). The resulting schizont eventually ruptures the RBC, releasing newly formed merozoites for subsequent rounds of invasion.

How merozoites identify and invade RBCs has long been a focus of research (2, 3). The merozoite first attaches to a RBC at any point on its surface, and then reorients to bring its apical end into contact with the RBC. The initial attachment stages are reversible, and merozoites can dissociate and attach to a new potential target cell. The subsequent steps are irreversible, and involve the formation of an electron-dense adhesion zone between the apical end of the merozoite and the RBC. This zone then moves around the merozoite toward its posterior end, with a concurrent invagination of the RBC membrane and entry of the merozoite. This cascade of molecular events also involves release of proteins from the rhoptries and micronemes, specialized apical organelles central to the invasion process.

The molecular adhesion details behind this tantalizing outline are sketchy. The merozoite surface proteins (MSPs), several of which have been described in a number of species of Plasmodium, together make up a structurally complex coat around the outer membrane of the merozoite and may have a role in the initial reversible adhesive interaction between the merozoite and the RBC (4). The P. vivax and Plasmodium knowlesi Duffy binding proteins (DBPs) and their P. falciparum orthologue, EBA-175 (erythrocyte binding antigen 175) have been shown to bind well-defined glycoprotein motifs on the RBC membrane (5–7). Studies of the P. knowlesi DBP have implicated these related microneme proteins in the formation of the adhesion zone that characterizes the beginning of the entry phase of invasion (8). Apical membrane antigen 1 (AMA-1) and MAEBL, a chimeric protein containing features of both the DBP/EBA-175 proteins and AMA-1, may also play a role in RBC invasion, but their precise functions are as yet unclear (9, 10).

Further complexity is due to the fact that some proteins recognize subpopulations of RBCs. In P. vivax, the reticulocyte binding proteins (PrRBP-1 and PrRBP-2) were identified through their ability to bind reticulocytes, the immature subpopulation of the RBC pool that P. vivax merozoites preferentially invade (11, 12). PrRBP-2 is distantly related to a 235-kDa rhoptry protein of the rodent parasite Plasmodium yoelii, which adheres to mouse erythrocytes, and is encoded by a multigene family (13–15). Passive transfer of specific monoclonal antibodies (16) or active immunization with purified protein (17) restricts parasites of a normally virulent strain to a self-limiting infection of reticulocytes, suggesting that, like the PrRBPs, the P. yoelii 235-kDa proteins play a role in host cell selection.

Here we present two P. falciparum orthologues of PrRBP-2, which we have termed Plasmodium falciparum RBP-2 homologues a and b (PfRBP2-Ha and -Hb). We propose that together with the RBP-2 of P. vivax and the P. yoelii 235-kDa proteins, they constitute an important Plasmodium family that plays a pivotal role in the early phases of merozoite invasion.

Materials and Methods

Parasites. The FVO strain of P. falciparum was obtained from a line adapted to Aotus monkeys and then established in in vitro culture (18, 19). Material from the P. falciparum 3D7 strain was obtained from in vitro culture in human O+ erythrocytes.

DNA Cloning and Analysis. P. falciparum FVO or 3D7 genomic DNA (gDNA) was prepared as described (11). DNA probes were...
labeled with [α-32P]dATP by random priming (Prime-it Kit; Stratagene). A λZAPII (Stratagene) gDNA library was constructed from *P. falciparum* FVO strain DNA by using mung bean nuclease methodology (20) with a range of formamide concentrations (30–45%) to allow digestion around (~30%) as well as within (~35–45%) genes. This library was screened with a PCR fragment amplified from *P. falciparum* FVO gDNA by using primers 5'-TACCCCTTTATGAACGG-3' and 5'-TACCCTTTTAATGAAACGG-3' (S1); 5'-ATCCGGTTATTA AAAGGGG-3' and 5'-AGGATTTTCTGGATTTT TCCG-3' (S3); 5'-AGAAAGAAACTGATTTAGCAGC-3' and 5'-CTATTAGTGATGCTTGTCTG-3' (A); and 5'-ATCCAGAATAGAAGACCA-3' and 5'-TTGTTGATG GTTGAATACC-3' (B). Fusion proteins were purified from *Escherichia coli* by using standard methods on glutathione-Sepharose 4B (Pharmacia) and injected into New Zealand White rabbits in Freund’s complete and incomplete adjuvant. The antisera were checked for cross-reactivity, and all reacted only with the fusion protein against which they were raised.

Gene and deduced protein sequences were analyzed and compared by using the MacVECTOR 6.5 DNA/Protein analysis module (Oxford Molecular). Potential signal peptide sequences were analyzed by using the weight matrix method of von Heijne (21) and a neural network method (22) accessible at http://www.cbs.dtu.dk/services/SignalP.

**Results**

**Identification of *P. falciparum* PVRBP-2 homologues.** The 3’ portion of the PVRBP-2 gene, clone 16-6 (12), hybridizes to *P. falciparum* (FVO strain) gDNA on Southern blots under reduced stringency (data not shown). Subsequent BLASTp searching of the GenBank database with 16-6 translated sequence revealed a 2.4-kb *P. falciparum* (3D7 strain) DNA sequence that includes an open reading frame (ORF) of 500 amino acids designated *P. falciparum* unknown membrane protein (PIUMP, accession no. L04159). Reprobing gDNA Southern blots with a corresponding *P. falciparum* FVO-strain PCR-amplified fragment revealed the same pattern of hybridization bands as was obtained with 16-6 and also suggested the presence of two homologous genes (data not shown). This PCR fragment was therefore used to screen a *P. falciparum* (FVO strain) gDNA library in search of possible PVRBP-2 homologues.

Two initial positive clones, 6A1 and 4D1, and subsequent related clones (1.1 and 18.2) were sequenced in their entirety (Fig. 1A). The latter clones were isolated by using the 5’-most 500 bp of 6A1 as a probe. 6A1 and 1.1 include a long ORF spanning all of the sequence, whereas 18.2 and 4D1 both have stop codons near their 3’ ends. The clones fell into two groups, encoding two related genes with an unusual relationship to each other, being almost identical at their 5’ ends (subsequently called the shared region), but divergent at their 3’ ends (subsequently called the unique regions). In 1.8 kb of sequence shared by clones 1.1 and 18.2, there is only a single nucleotide difference, causing a V→A substitution in the predicted amino acid sequence. Intriguingly, where the sequences diverge, both genes encode a mixture of amino acid repeat motifs, which are discussed further below. The C termini after the repeats are clearly divergent (Fig. 1B). However, CLUSTAL alignment suggests that they are still related and both have homology to the PVRBP-2 protein and the *P. yoelii* 235-kDa family member E-3 (see below). The hybridization data, sequence homologies, and further supporting data described below have lead us to term these two genes *Plasmodium falciparum* PvRBP2-Ha and PvRBP2-Hb, corresponding to the 6A1/1.1 and 4D1/18.2 sequences, respectively.

**The PVRBP2-Ha and PVRBP2-Hb Genes Share >8 kb of Closely Related Sequence Before Diverging.** The presence of an extensive shared region made it difficult to identify the full PVRBP2-Ha and PVRBP2-Hb gene sequences by conventional library screening, as any clone that did not include some 3’ unique sequence was impossible to assign to one gene or the other. An example is clone 20.2b (Fig. 1A). BLAST searching of the Sanger Centre database facilitated this process, revealing several contigs from *P. falciparum* (3D7 strain) chromosome 13 that contained...
Fig. 1. \textit{PfRBP2-H} genes are composed of shared, repetitive, and unique domains. (A) Schematic diagram of PfRBP2-H DNA clones and the PfUMP GenBank sequence. Sequences are grouped according to their relationship to PfRBP2-Ha (PfUMP, 6A1, 1.1) or -Hb (4D1, 18.2). Clone 20.2b could be a fragment of either. Regions shared by all sequences (gray), repeat regions (black), and regions unique to PfRBP2-Ha or -Hb (hatched) are noted. (B) \textsc{clustal} alignment of predicted amino acid sequence from clones 6A1 and 4D1. Areas of identity and similarity are boxed, with identical residues highlighted in gray. Not all of the 6A1 or 4D1 sequences are shown. Solid lines indicate the boundaries of repeated motifs.

\textit{PfRBP2-H} sequences. Contig 08813 (2.2 kb) contains the \textit{PfRBP2-Ha} unique region, whereas contig 04278 (11.5 kb) contains the \textit{PfRBP2-Hb} unique region (Fig. 2A). Comparisons of these 3D7 contigs with our FVO gDNA clones showed only one nucleotide difference in the unique region of \textit{PfRBP2-Hb} and none in the unique region of \textit{PfRBP2-Ha}.

As discussed above, our FVO sequence data revealed only one difference between \textit{PfRBP2-Ha} and \textit{-Hb} in 1.8 kb of closely related sequence 5' to the repeat region and divergent 3' unique domains. Comparing the shared region from our largest FVO \textit{PfRBP2-Ha} clone, 1.1, with the corresponding region of the 3D7 \textit{PfRBP2-Hb} contig 04278 revealed only seven nucleotide differences in over 3.2 kb. It remains to be determined whether these are true allelic differences or sequencing errors not yet detected in the preliminary genome project data. One such difference introduces a stop codon into the ORF of \textit{PfRBP2-Hb} (contig 04278) before the repeat region, and we have determined by reverse transcription–PCR (data not shown). The stop codon Initiates a stop codon into the ORF of \textit{PfRBP2-Hb} (contig 04278) before the repeat region, and we have determined by reverse transcription–PCR (data not shown). The stop codon will be clarified further as the genome project is finalized.

The Southern blot data suggest that the region shared by \textit{PfRBP2-Ha} and \textit{-Hb} extends past the 5' end of contig 04278 for 5.5 kb and is at least 14.5 kb in total, while the long range PCR confirms at least 8 kb of overlap. The precise sequence of this shared region will be clarified further as the genome project is finalized.

\textit{PfRBP2-Ha} and \textit{-Hb} Are Homologous to \textit{PvRBP-2} and the \textit{P. yoelii} p235 Proteins. The 04278 contig contains a major ORF of over 9 kb (once the database sequencing error noted above is corrected). Upstream of this ORF we noticed a potential intron of 214 bp and initial exon of 57 bp (Fig. 3B). The presence of the intron and transcription of the short exon 1 were confirmed by using reverse transcription–PCR (data not shown). The SF7 primer used in reverse transcription–PCR (data not shown). The SF7 primer used in reverse transcription–PCR (data not shown). The SF7 primer used in reverse transcription–PCR (data not shown). The SF7 primer used in reverse transcription–PCR (data not shown). The SF7 primer used in reverse transcription–PCR (data not shown). The SF7 primer used in reverse transcription–PCR (data not shown). The SF7 primer used in reverse transcription–PCR (data not shown). The SF7 primer used in reverse transcription–PCR (data not shown).
In TBLASTN searches suggests that it is more closely related to and Py 235 proteins (14). Using the 04278 translated sequence region previously reported to show homology between PvRBP-2 (data not shown).

**Fig. 3.** Structure of the PRBP2-H genes and their relationship with each other and with other Plasmodium proteins. (A) Schematic of the PRBP2-H genes, highlighting structural features as marked. Shared regions (gray), repeats (black), and unique regions (hatched) are also noted. (B) Sequence of the 5’ end of the PRBP2-H genes, showing the boundaries of the intron (arrow) and sequence of the short exon 1 that encodes a signal peptide. (C) Clustal alignment of the C terminus of the PRBP2-H proteins, the P. vivax reticulocyte-binding proteins, and the E3-member of the P. yoelii p35 family. Identical residues are highlighted in gray and similar residues in light gray.

a short exon 1, a short intron, and then a large exon 2 encoding the majority of the protein (Fig. 3A). This is highly reminiscent of the PVRBP-1-2 genes (11, 12). Like the PVRBP genes, the PRBP2-H (04278) exon 1 encodes a hydrophobic core flanked by charged amino acids, which yields high probability scores for signal peptide assessment (21, 22). The remaining large portion of the protein is highly hydrophilic, with a putative TMD and cytoplasmic domain at the C terminus. This deduced model also predicts that both of the PRBP2-H genes encode similar-sized large proteins, with estimated molecular masses of 370 kDa and 383 kDa for PRBP2-Ha and -Hb, respectively.

There is low but significant amino acid homology between the PRBP2-Hb (04278) ORF deduced sequence and both the P. yoelii 235-kDa rhoptry proteins, with Clustal analysis showing homology scores (identity plus similarity) of 38% for PRBP-1, 44% for PRBP-2, and 42.5% for the E3 and E8 P. yoelii 235-kDa rhoptry proteins. However, individual regions show a much stronger homology (~50%), in particular at the C terminus (Fig. 3C) and over a 500 amino acid region previously reported to show homology between PRBP-2 and Py 235 proteins (14). Using the 04278 translated sequence in tBLASTN searches suggests that it is more closely related to PRBP-2 and the P. yoelii 235-kDa proteins than it is to PRBP-1 (data not shown).

The repeated region of PRBP2-Ha and -Hb differs between these two proteins and their counterparts in various P. falciparum strains. The repeated region that marks the boundary between the highly conserved 5’ shared domain and the divergent 3’ unique domains was the only aspect of the sequencing projectcontigs that differed markedly from our gDNA clones. When a code is used for the consensus repeat motifs (Fig. 4), all sequences began with a conserved pattern of repeats, ABCAD. The FVO PRBP2-Ha and -Hb genes are transcribed, cDNA specific to each was generated by reverse transcription of total schizont stage RNA from FVO parasites by using reverse transcription primers designed from their unique regions. Nested PCR was then performed with these cDNA as templates and reverse primers specific to one or the other unique region (Fig. 5A). A PCR product could be generated with PRBP2-Ha unique region reverse primers on cDNA synthesized with a primer specific to PRBP2-Hb (Fig. 5B, lane 3). Similarly, a PCR product was generated with PRBP2-Hb unique region reverse primers on cDNA specific to PRBP2-Ha (lane 6). Crucially, no PCR product was produced with PRBP2-Ha reverse primers on PRBP2-Hb cDNA (lane 4), or with PRBP2-Hb primers on PRBP2-Ha cDNA (lane 5), indicating the absence of gDNA contamination. No PCR product was produced if reverse transcriptase was omitted at the cDNA synthesis step (data not shown). Both PRBP2-Ha and PRBP2-Hb are therefore clearly transcribed in schizonts.

Antisera were raised against several regions of PRBP2-Ha and -Hb expressed as glutathione S-transferase (GST) fusion proteins. Two GST fusion proteins, S1 and S3, contained fragments of the shared region, while two others, A and B, contained the unique regions of PRBP2-Ha and -Hb, respectively (Fig. 5A). Immunoblot analyses using a number of parasite protein preparations suggested that the PRBP2-H proteins are subject to extensive and rapid proteolytic degradation, as several bands were detected that were not present in uninfected erythrocyte controls (Fig. 5C and data not shown). The sizes of these bands were not consistent between preparations, but antisera against both unique regions and the shared S3 sequence recognize high molecular mass bands of approximately 370 kDa in all samples (arrow in Fig. 5C). This size corresponds with the molecular mass predicted for each of the PRBP2-H proteins.

To localize the PRBP2-H proteins, immunofluorescence...
two foci were visible in a single merozoite (marked by arrows in Fig. 5D Upper). This double-dot pattern is reminiscent of proteins such as the Pf140 protein (24), which are localized to the paired rhoptry organelles (Fig. 5D Lower Left). It should be noted, however, that the appearance of paired foci predominates with antisera against the Pf140 protein, but is not so typical with the PfRBP2-H antisera. Higher magnification (Fig. 5D Lower Center) reveals that the staining is concentrated at the apical end of the merozoites. Costaining infected cells with rabbit anti-A serum and rat anti-B serum suggests that single merozoites express both PfRBP2Ha and -Hb, because individual merozoites were labeled with antisera against both unique domains (Fig. 5D Lower Right).

Discussion

We have identified and characterized P. falciparum homologues (PfRBP2-Ha and -Hb) of the P. vivax RBP-2 gene. Like the PfRBP-2 gene, the PfRBP2-H genes are expressed in the merozoite stage of the parasite and are predicted to encode large type I membrane-anchored proteins that localize to the apical pole.

The structure of the PfRBP2-Ha and -Hb genes, with 5′ shared sequence, junctional repeated motifs, and 3′ unique regions, is unusual and suggests that the two genes may have arisen as a result of a duplication event, followed by evolutionary drift of the 3′ ends. The nature and extent of this duplication will become more evident with the completion of the P. falciparum genome project, but it would appear that it is an evolutionarily distant event, because both genes are present in P. falciparum strains from distinct geographical locations (J.C.R. and J.W.B., unpublished observations). Regions of almost identical nucleotide sequence have been observed previously in Plasmodium—for example, several Plasmodium species contain two identical copies of the EF-1α gene (25). Repeated sequences within genes are also commonplace in Plasmodium, although those observed here have a unique cadence of alternating consensus motif units. What is unusual is that these repeats are strikingly placed between the strongly conserved predominant part of these genes and the more loosely maintained unique region that includes the TMD and cytoplasmic domains.

The general structure of the PfRBP2-H proteins, with a signal sequence and an extensive extracellular domain followed by a putative TMD and short cytosolic domain is the same as that of PvRBP-2 and the two P. yoelii 235-kDa rhoptry proteins so far characterized (11–13, 26). Their two-exon gene structures are also similar. Although the overall amino acid homology between these proteins is not high, there are blocks of outstanding homology between the PfRBP2-H and PvRBP-2 proteins that are suggestive of a familial relationship. The identification here of domains with notable significant homology amidst divergent sequence mimics the familial relationship of the P. knowlesi and P. vivax DBPs and the P. falciparum EBA-175 (5).

The rodent and each of the human malaria parasite species form evolutionarily distant clades, so considerable differences between members of the family could be expected, especially if proteins are under immune pressure (27). However, the similarities in gene structure, amino acid homology, and biology noted here together support the premise that the PfRBP2-Hs, PvRBP-2, and the Py235 family members are related. We have not included PVRBP-1 in this grouping, although it does show some similarity to both PfRBP2-Hs, as we have detected and characterized a distinct PVRBP-1 homologue in P. falciparum (J.C.R., M.R.G., and J.W.B., unpublished observations).

There are clearly some features of this gene family that differ between the human parasites P. falciparum and P. vivax and the rodent parasite P. yoelii. There are up to 50 members of the 235-kDa protein gene family in P. yoelii (14), and the two so far reported are in fact very similar (26). Cross-hybridization suggests a large family of related genes is also present in the rodent
malaria 

Plasmodium berghei (14). Furthermore, each merozoite originating from a single 
P. yoelii schizont apparently expresses a distinct member of the family (28), a mechanism that may help the parasite evade the host immune response or facilitate the use of different host cell niches (29). Does a similar mechanism operate in 
P. vivax and 
P. falciparum? It would appear not, because both 
PpRBP-1 and -2 hybridize as single-copy genes and only two 
PpRBP2-H genes have been detected. We certainly do not rule out the possibility that the 
P. vivax and 
P. falciparum genomes may contain other more distantly related homologues, but such a scenario would clearly be different from the large closely related families found in 
P. yoelii and 
P. berghei. Given the apparent lack of a large multigene family in 
P. falciparum, the mechanism of transcriptional control described for the 
P. yoelii 235-kDa proteins would be of less relevance. Besides, our immunofluorescence assay data indicate that a single merozoite expresses both 
PpRBP2-Ha and -Hb.

What then is the function of 
PpRBP-2 and its orthologues? The 
PpRBPs were identified in 
P. vivax by their ability to bind to reticulocytes and are thus predicted to function in selecting reticulocytes for invasion by 
P. vivax merozoites (11). The 
P. yoelii 235-kDa protein family has also been shown to play a role in host cell selection (16, 17). In this case, however, the 235-kDa ligands appear to select mature normal erythrocytes, because neutralizing antibodies confer 
P. yoelii merozoite invasion to young RBCs (16). The 
PpRBPs have also been implicated in regulating invasion (30). Merozoites can dissociate from potential host cells up to the point that an electron-dense junction forms between the apical pole and the RBC. The case of 
P. vivax and its preferential invasion of reticulocytes implies the existence of a control step between initial random attachments and the irreversible cascade of events leading to merozoite entry. In this way junction formation and the later stages of invasion would be triggered only after a correct host cell has been selected, and unproductive invasion attempts into inappropriate host cells would be avoided (11, 30). The 
PpRBPs are prime candidates for such a triggering role, given their location at the apical end of the merozoite, their extracellular structures that are predicted to bind target cells, and their short intracellular domains that could undergo conformational change upon binding and thus transmit a signal to the merozoite cytoplasm. It follows that the 
PpRBP2-Hs may participate in a similar scheme in 
P. falciparum invasion of its primary host cell, the mature normocyte population of erythrocytes. The presence of two closely related 
PpRBP2-H proteins with almost identical extracellular domains but differences in their TMD and cytoplasmic domains raises the possibility that they may bind the same ligand but communicate different biological signals to the merozoite cytoplasm.

Given this hypothesis, and the localization of the 
PpRBP2-Hs at the invasive end of merozoites, we carried out extensive erythrocyte binding assays (EBAs), using labeled 
P. falciparum culture supernatants to establish whether the 
PpRBP2-Hs bind to RBCs, but with inconclusive results (data not shown). This work however, did corroborate our immunoblot data, that the 
PpRBP2-Hs are extensively and rapidly degraded, as full-length 
PpRBP2-H product was not detectable in any of the culture supernatants we obtained, although fragments of various sizes were detected. Such cleavage could easily be removing adhesive domains or disrupting tertiary structure, so our lack of consistent results in no way rules out the possibility that the 
PpRBP2-Hs bind to RBCs.

In summary, we have identified two related 
P. falciparum proteins ( 
PpRBP2-Ha and -Hb), which are expressed at the invasive apical end of the merozoite, and which, on the basis of gene structure and amino acid homology, are related to the 
P. vivax 
PpRBP-2 and 
P. yoelii 235-kDa proteins. We propose that these proteins, as previously shown for their orthologues, play an important adhesion function and possibly also a signaling role in the early stages of merozoite invasion. Comparative studies across species will continue to provide insights toward assessing the essential features of these and other critical components of the invasion cascade, which should be considered for inclusion in malaria vaccines or as possible drug targets.

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Rayner et al.
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