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Short communication

# *Plasmodium vivax* reticulocyte binding protein-2 (PvRBP-2) shares structural features with PvRBP-1 and the *Plasmodium yoelii* 235 kDa rhoptry protein family☆

Mary R. Galinski *, Mengyao Xu, John W. Barnwell [1]

*Department of Medical and Molecular Parasitology, New York University School of Medicine, 341 East 25th Street, New York, NY 10010, USA*

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*Plasmodium vivax* merozoites are known to invade reticulocytes, rather than mature red blood cells [1–3]. The molecular basis responsible for the specificity of this host cell selection is important for understanding the invasion process of this human malaria parasite species. We previously identified two high molecular mass *P. vivax* proteins and predicted that they target the reticulocyte host cells for invasion by merozoites [4]. These two proteins, PvRBP-1 and PvRBP-2, are co-expressed at the apical pole of *P. vivax* merozoites and bind to reticulocytes in an erythrocyte binding assay.

In the initial report of the reticulocyte binding proteins, the full 8.69 kb gene and deduced protein sequence of PvRBP-1 (325 000 Da) was presented, along with a partial, 3.7 kb gene fragment and deduced protein information for PvRBP-2 [4]. While the full-length *Pvrbp-1* gene and flanking sequences were readily obtained from several λ DASH II clones, a few λ DASH II clones containing *Pvrbp-2* gene sequence had obvious rearrangements. This may be related to the fact that the *Pvrbp-2* gene has a higher A/T content (72%), closer to that of *P. falciparum* genes (up to 80%), which have been known to be difficult to clone by traditional means. *Pvrbp-1* and other *P. vivax* genes analyzed have A/T contents between 50 and 70%.

*Abbreviations:* gDNA, genomic DNA; PvRBP, *P. vivax* reticulocyte binding protein.

☆ *Note*: The nucleotide sequence reported in this paper is available in the EMBL, GenBank™ and DDJB databases under the accession number AF184623.

* Corresponding author. Present address: Department of Medicine, Division of Infectious Diseases, Emory University School of Medicine, Emory Vaccine Center at Yerkes, 954 Gatewood Road NE, Atlanta, GA 30329, USA. Tel.: +1-770-7277214; fax: +1-770-7277845.

*E-mail address:* galinski@rmy.emory.edu (M.R. Galinski)

[1] Present address: Division of Parasitic Diseases, Center for Disease Control and Prevention, 4770 Buford Highway NE, Atlanta, GA 30341, USA.



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We thus proceeded to obtain the remaining *Pvrbp-2* gene sequence by sequential hybridization screenings of cDNA and genomic DNA (gDNA) λ ZAP libraries containing smaller inserts where rearrangements would likely be less of a concern (Fig. 1A). This scheme proved to be readily successful and also facilitated rapid sequencing and verification by the dideoxy methodology [5]. The numerous small cDNA clones obtained reflect the fact that during synthesis of

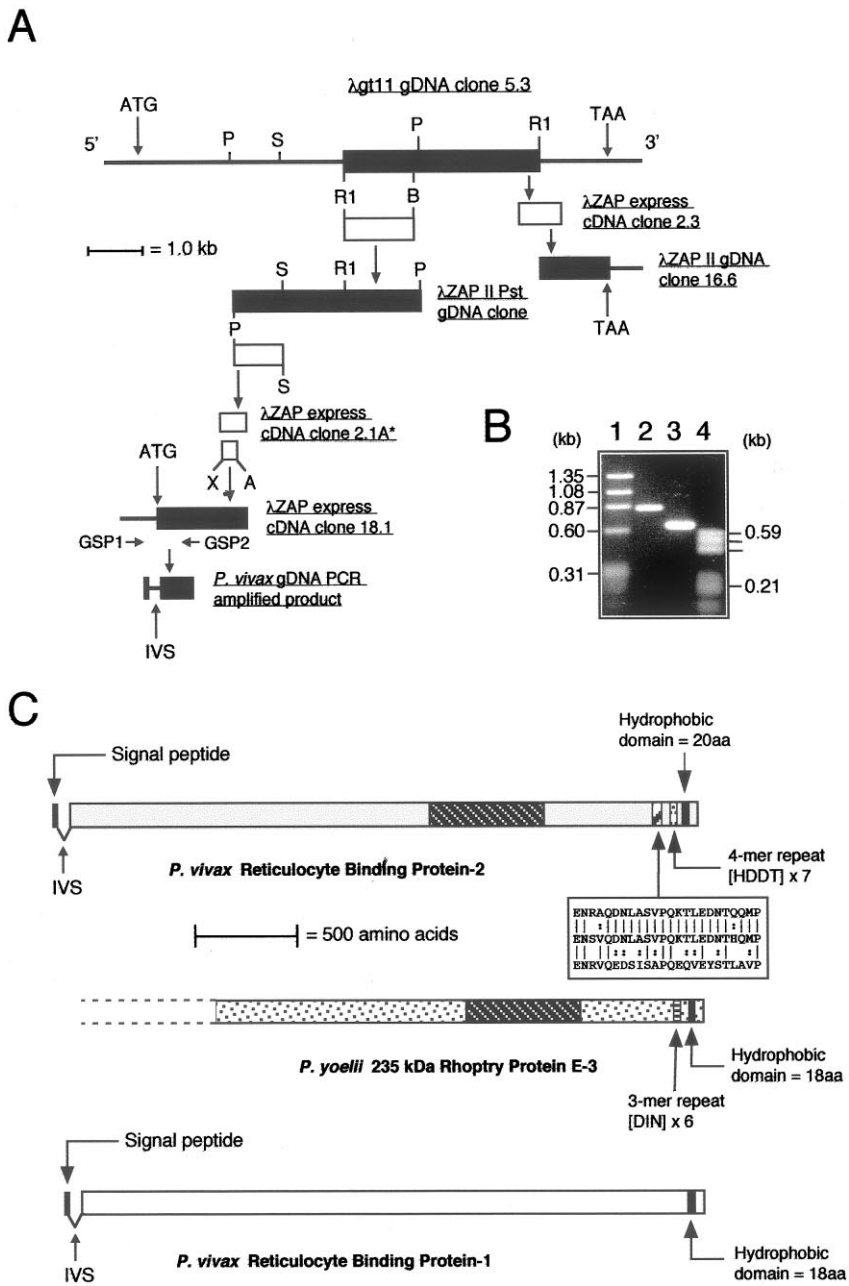


Fig. 1.

the cDNA oligo dT priming had occurred frequently along this A/T rich gene, even with as few as five contiguous adenine residues. New downstream sequence includes ~1300 additional bases of coding sequence, a TAA stop codon, and ~600 bases of 3′ untranslated region. cDNA clone 18.1 is predicted to encode the ATG initiation codon and signal peptide sequence of PvRBP-2. The ATG codon is preceded by an adenine residue in the -3 position, which is typical for eukaryotic initiation codons including *Plasmodium* [7,8]. Given that *Pvrbp*-1 [4] and members of the erythrocyte binding protein gene family [9] have a small intron immediately after the signal peptide coding sequence, we questioned if this was also the case for *Pvrbp*-2. We therefore designed two gene specific primers (GSP-1, 5′-gttgtccctttta-gagcgg-3′; and GSP-2, 5′-cgaaggcattgaggtagtc-3′) flanking the predicted junction of the signal peptide coding sequence and used these primers in a polymerase chain reaction on *P. vivax* gDNA (Fig. 1B). These primers amplified an intervening sequence, which is 202 bp, with 5′ and 3′ splice sites of GTAA and CAG, respectively. Similar small introns following signal peptide encoding domains have since been noted for a variety of *Plasmodium* genes (for some examples, see [10]).

The full determined coding region of *Pvrbp-2* is ~8.6 kb with a deduced protein of 330 000 Da. The predicted gene size and protein mass are very close to that determined previously for PvRBP-1, which migrates closely on reducing SDS-PAGE gels and has similar biochemical and basic structural characteristics [4,11]. Both proteins are largely hydrophilic with a predicted propensity to form α-helical secondary structure and possibly coiled-coil motifs. Additionally, each of these proteins has only two significant hydrophobic stretches, which define their presumptive N-terminal signal peptides and the C-terminal transmembrane domains that precede a short cytoplasmic tail region (Fig. 1C). Genomic DNA and cDNA analyses also indicate that both are encoded by a similar two-exon structure; although additional small introns would seem unlikely given current gDNA Southern blot and mRNA analyses [4], we note that the possibility of other small introns cannot be entirely excluded without the full comparative analysis of the cDNA, which is beyond the scope of this study.

Although quite similar in basic structure and characteristics, the two proteins show little overall amino acid sequence identity (~23%) and low similarity (~45%). Two specific noteworthy distinctions, however, are that PvRBP-1 has a greater content of cysteines, which tend to be clustered, and result in the dimerization of this molecule via disulfide bond formation [4,11]. PvRBP-2, on the other hand, has a repeated amino acid motif (HDDT) near its C-terminus

Fig. 1. Schematic representation for the ~10 kb locus containing the *P. vivax rbp-2* gene and characterization of its deduced product. (A) Cloning strategy and basic restriction map deduced from cDNA and gDNA analyses. The *Eco*RI gDNA insert of the original 3.7 kb *Pvrbp-2* λgt11 clone 5.3, which had been obtained by immunoscreening [4], was first used as a probe on a mixed-stage λ ZAP express *P. vivax* (Belem) cDNA library. Clone 2.3 (0.8 kb) was obtained, which extended the 3′ end of the gene by a few hundred bases. This cDNA was then utilized to again screen the cDNA library and a *P. vivax* λ ZAP II gDNA library prepared using mung bean nuclease methodology [6] followed by *Eco*R1 digestion prior to cloning of gene fragments. Several small cDNA clones and one gDNA clone of 1.9 kb called 16.6 were identified. To obtain upstream sequence, gDNA Southern blot experiments (not shown) indicated that the 5′ portion of clone 5.3 hybridized to a 3.5 kb *Pst*I fragment. A λ ZAP II *Pst*I gDNA library was thus prepared and screened with an *Eco*RI/*Ban*II 5′ fragment of clone 5.3. The sought after *Pst*I fragment was obtained and its open reading frame extended the 5′ DNA sequence by 2.1 kb. A 0.8kb *Pst*I/*Sca*I fragment was then used to probe the λ ZAP express cDNA library. The resulting cDNA clone 2.1* contains a 0.3 kb region of additional upstream sequence defined by *Xmn*I and *Acc*I sites, which was used to re-screen the cDNA library, and yielded 2.2 kb clone #18.1. GSP-1 and -2 primers were used in a PCR to identify the noted IVS. Restriction enzymes are *Acc*I (A); *Ban*II (B); *Eco*RI (RI), *Pst*I (P), *Sca*I (S), and *Xmn*I (X). IVS, intervening sequence (intron); PCR, polymerase chain reaction; ATG, translation initiation codon; and TAA, translation termination codon. (B) PCR amplification using GSP-1 and -2 with gDNA (lane 2) and cDNA (lane 3) demonstrates ~200 bp intron in the PvRBP-2 gene. Lanes 1 and 4 contain size marker DNA from *Hae*III restricted ϕX174 and pBR322, respectively. (C) Comparison of PvRBP-2 features with the partial amino acid sequence deduced from the E-3 gene of the *P. yoelii* 235 kDa rhoptry protein family and with PvRBP-1. Dark diagonal hatched box (▨) represents the region between PvRBP-2 and the *P. yoelii* 235 kDa E-3 protein with 29.6% amino acid identity and 50% similarity.

repeated seven times, whereas there are no repeated motifs in PvRBP-1. Also, a second degenerate 24 amino acid unit repeated three times is situated upstream of the HDDT repeat in PvRBP-2.

Keen et al., have noted that the 3.7 kb partial gene fragment of PvRBP-2 clone 5.3 originally reported has a low but significant level of homology (29% identity and 50% similarity) over a region of 500 amino acids with the E-3 member of

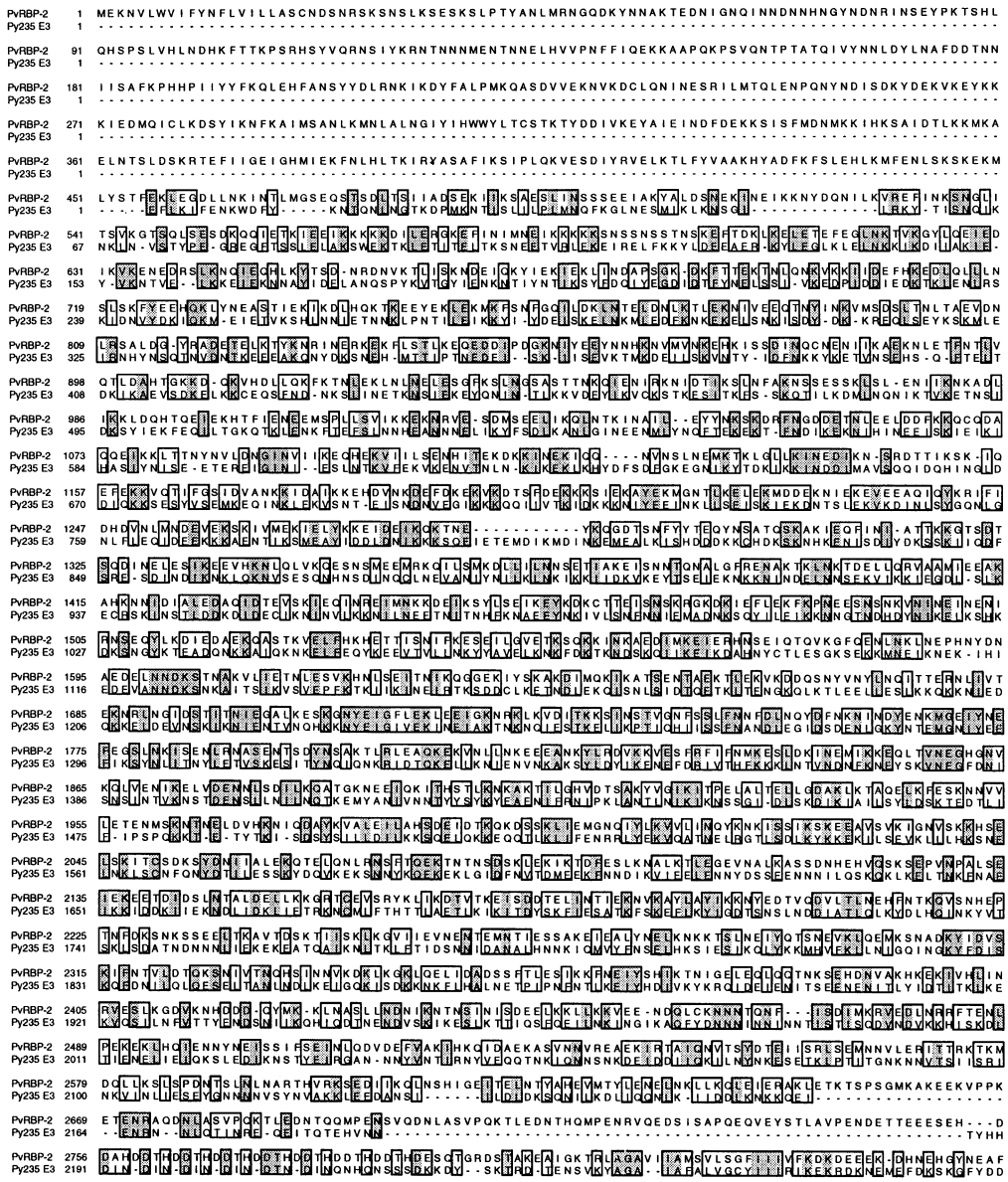

Fig. 2. Clustal W based best fit alignment between the complete amino acid sequence of *P. vivax* RBP-2 (PvRBP-2) and a partial amino acid sequence of the *P. yoelii* 235 kDa rhoptry protein encoded by the E-3 gene (Py235 E3). Clustal alignment and manual adjustments presented through the SeqVu 1.1.0 program. Gray shading indicates identity and similar amino acids are boxed.

the ~235 000 Da rhoptry protein family of the rodent-malaria parasite, *P. yoelii* ([12], Fig. 1C). A comparative clustal-based alignment of the near full-length amino acid sequences, however, from either the E-8 [13] or E-3 *P. yoelii* 235 000 Da rhoptry protein with PvRBP-2 (Fig. 2), produces an identity parameter of only ~20%, which is similar to the overall limited amino acid identity between PvRBP-1 and 2. Nevertheless, there are similar characteristics held in common between the *P. yoelii* 235 proteins and PvRBP-2 that suggest a common origin. Like PvRBP-1 and -2, *P. yoelii* 235 proteins have a large hydrophilic α-helical extracellular domain followed by a predicted transmembrane domain and a short cytoplasmic tail. Additionally, like PvRBP-2 (yet unlike PvRBP-1), the *P. yoelii* 235 proteins have a small repeated amino acid motif just preceding their transmembrane domain (the E-3 member has a 'DIN' motif repeated six times). Furthermore, although not present in the 235 000 Da sequences deposited in Genbank, it has been reported that similar to PvRBP-1 and -2, the 235 000 Da rhoptry proteins are encoded by a similar two exon gene structure (Holder et al., a *P. yoelii* rhoptry protein family and erythrocyte invasion, ICOPA 1998, Japan, Abstract S-M2-2).

Curiously, the same PvRBP-2 500 amino acid region noted above also shows the highest homology in comparison to PvRBP-1 (27.6% identity and 49.3% similarity), suggesting these two distinct proteins may have arisen at some point long ago as a result of a gene duplication. Although they share some significant homology in this particular region, however, PvRBP-1 does not have the repeated domain feature noted in both PvRBP-2 and the *P. yoelii* 235 proteins, and is also differentiated by its greater cysteine content and apparent dimeric structure [4,11]. Each of these *P. vivax* proteins has been shown to exhibit erythrocyte-binding activity, the precise nature and specificities of which remain to be sorted out along with a further unraveling of the functional roles of these proteins [4,14]. Reduction in parasitemia by passive antibody transfer and active immunization with the *P. yoelii* 235 proteins, and the erythrocyte binding specificity shown for one, indicate an important functional role in invasion by this class of merozoite proteins [15,16]. Though widely divergent, the PvRBPs and the *P. yoelii* 235 proteins appear to belong to a biologically related protein family, which may include analogues from *P. falciparum* and other malaria species [4], and share the common function of targeting host cells. However, the recent finding that a family of as many as 50 235-protein genes has been noted in *P. yoelli* [12,17–19], which appear to be differentially expressed in individual merozoites within the same schizont [20], while additional PvRBP-1 and -2 genes are not evident, suggests a different degree of host cell selection operative between *P. vivax* and *P. yoelii* parasites. Whether or not some of the *P. yoelii* 235 family members are actually counterparts of the PvRBP-1 protein and form an erythrocyte-binding complex as has been predicted for the PvRBPs [4,11] also remains an open question. Continued investigation across these and other diverse species of malaria parasites will aid in the further discovery and analysis of these mysteries and better enable the development of effective vaccine or receptor-mediated blockade strategies to prevent merozoite entry into red blood cells.

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