Host-Parasite Interaction: Selective Pv-fam-a Family Proteins of *Plasmodium vivax* Bind to a Restricted Number of Human Erythrocyte Receptors

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**Background.** *Plasmodium vivax* synthesizes the largest number of 36 tryptophan-rich proteins belonging to the Pv-fam-a family. These parasite proteins need to be characterized for their biological function because tryptophan-rich proteins from other *Plasmodium* species have been proposed as vaccine candidates.

**Methods.** Recombinant *P. vivax* tryptophan-rich antigens (PvTRAgS) were used to determine their erythrocyte-binding activity by a cell-based enzyme-linked immunosorbent assay, flow cytometry, and a rosetting assay.

**Results.** Only 4 (PvTRAg26.3, PvTRAg34, PvTRAg36, and PvTRAg36.6) of 21 PvTRAgS bind to host erythrocytes. The cross-competition data indicated that PvTRAg36 and PvTRAg34 share their erythrocyte receptors with previously described proteins PvTRAg38 and PvTRAg33.5, respectively. On the other hand, PvTRAg26.3 and PvTRAg36.6 cross-compete with each other and not with any other PvTRAg, indicating that these 2 proteins bind to the same but yet another set of erythrocyte receptor(s). Together, 10 of 36 PvTRAgS possess erythrocyte-binding activity in which each protein recognizes >1 erythrocyte receptor. Further, each erythrocyte receptor is shared by >1 PvTRAg.

**Conclusions.** This redundancy may be useful for the parasite to invade red blood cells and cause disease pathogenesis, and it can be exploited to develop therapeutics against *P. vivax* malaria.

**Keywords.** receptor-ligand interaction; tryptophan-rich proteins; malaria parasite; host cell invasion.

Malaria caused by *Plasmodium vivax* is very common in tropical world. It remains uncontrolled and requires newer antimalarial drugs and vaccines. This in turn requires the identification of newer targets that play important roles in parasite survival inside the human host. The most sought after target molecules of the parasite are those that are involved in host cell invasion. In this regard, a large number of molecules on merozoites have been identified that are involved in host-parasite interaction during red cell invasion [1–4]. Yet we do not have full knowledge about these parasite molecules. Therefore, there is a need to identify and characterize these additional parasite molecules that are involved in host-parasite interaction during red cell invasion.

Patients with *P. vivax* malaria may also undergo complications such as organ failure, leading to death [5–10]. Such complications may arise by the ability of the parasitized erythrocyte to bind to different host cells [11–13]. Although this is a well-known knob-mediated cytoadherence phenomenon in *Plasmodium falciparum* malaria, in which the mature form of the parasite can sequester in postcapillary venules of internal organs, leading to complications [14–16], the mechanisms behind these complications in the case of *P. vivax* remain elusive. This is because *P. vivax* does not induce knobs on the surface of infected erythrocytes for cytoadherence. Yet the *P. vivax*-infected erythrocytes are known to bind to different types of host cells for cytoadherence, causing complications [8, 12].
**METHODS**

P. vivax ligands recognizing host cell receptors involved in cytoadherence are not defined and need to be investigated.

The genome of P. vivax encodes 36 tryptophan-rich proteins belonging to the Pv-fam-a family [17]. The number of tryptophan-rich proteins encoded by the P. vivax genome is much higher than that of P. falciparum. In the latter case, some of these proteins have been shown to play important role in red-cell invasion and thus are proposed as potential vaccine candidates against P. falciparum [18, 19]. The question arises as to why such a large number of tryptophan-rich proteins are being expressed by P. vivax and whether they play different roles than P. falciparum during the host-parasite interaction. The stage-specific expression of these P. vivax tryptophan-rich protein genes is indicative of their different roles in the parasite’s life cycle [20]. Elsewhere, we described the immunological responses against these P. vivax tryptophan-rich proteins: all were found to induce the humoral and cellular immune responses during P. vivax infection, and most were found to contain conserved sequences in the parasite population [21] (Zeeshan et al, unpublished data). Previously, we also investigated the erythrocyte-binding activity of a group of 15 PvTRAGs, with only 6 possessing this activity [22]. Here, we investigate the erythrocyte-binding activity of the remaining 21 P. vivax tryptophan-rich proteins of the Pv-fam-a family and report that only 4 exhibiting this activity [22]. These 4 proteins, 2 (expressed by the merozoites) recognized the same erythrocyte receptors that have been described for other P. falciparum and report that only 4 exhibiting this activity. Of the remaining 21 tryptophan-rich proteins: all were encoded by the Pv-fam-a family [17].

The number of tryptophan-rich proteins encoded by the P. vivax genome is much higher than that of P. falciparum [21]. Previously, we also investigated the erythrocyte-binding activity of a group of 15 PvTRAGs, with only 6 possessing this activity [22]. Here, we investigate the erythrocyte-binding activity of the remaining 21 P. vivax tryptophan-rich proteins of the Pv-fam-a family and report that only 4 exhibiting this activity. Of these 4 proteins, 2 (expressed by the merozoites) recognized the same erythrocyte receptors that have been described for other tryptophan-rich proteins, whereas the remaining 2 (expressed by the ring/ trophozoite stages) recognized a set of red cell receptor(s) that differ from those described so far. Together, 10 of 36 PvTRAGs possess erythrocyte-binding activity, with 5 different erythrocyte receptors likely recognized.

**ETHICS STATEMENT**

Heparinized blood was collected from healthy individuals and P. vivax–infected patients in accordance with institutional ethical guidelines. Written consent was obtained from participants prior to blood collection. The ethics committee of All India Institute of Medical Sciences, New Delhi, approved the study via approval numbers IEC/NP-342/2012 and RP-11/2012.

**Expression and Purification of Recombinant PvTRAGs**

Exon 2 of 21 PvTRAGs in blood specimens obtained by finger prick from P. vivax–infected patients was amplified by polymerase chain reaction (PCR), using primers and conditions given in Supplementary Table 1. The amplified products were cloned into the expression vector pET28a, except for PvTRAG39.8a, PvTRAG34.9, and PvTRAG56.2, which were cloned into pET32a. Expression of the recombinant proteins was induced by adding 1 mM of IPTG and purified by immobilized metal affinity chromatography on Ni²⁺-NTA agarose (Qiagen, Hilden, Germany) as described earlier [21, 23–28]. Purified recombinant proteins were analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by Western blot analysis using anti-His₆ monoclonal antibody.

**Expression of PvTRAGs in CHO-K1 Cells**

Inserts from the above cloned plasmids of PvTRAG36.6, PvTRAG34, P. falciparum, and PvTRAG53.7 were amplified by PCR, using specific primers and conditions described in Supplementary Table 1. The amplified products were cloned into the PvU1 and ApaI sites of the pRE4 vector in frame with the signal sequence and transmembrane segment of herpes simplex virus glycoprotein D [29, 30]. CHO-K1 cells (American Type Culture Collection) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium with 10% fetal calf serum (Invitrogen Life Technologies, Carlsbad, California), 4 mM glutamine, and 1× penicillin-streptomycin (Sigma-Aldrich, St. Louis, Missouri) at pH 7.4 in a humidified incubator containing 5% CO₂ at 37°C. The transfection of CHO-K1 cells with PvTRAg-RE4 plasmid was done using the cationic lipid Lipofectamine 2000, following the manufacturer’s protocol (Invitrogen Life Technologies). Transfected CHO-K1 cells were grown over a coverslip and stained for 1 hour with DL6 mouse monoclonal antibodies (Santa Cruz, Dallas, Texas) directed against the C-terminus of the herpes simplex virus glycoprotein D sequences. For secondary antibody, Alexa Fluor 488–conjugated goat anti-mouse immunoglobulin G was used, and for nuclei staining, DAPI (4′,6-diamidine-2-phenylindole) was used. Images were captured under UV light at 400 times the original magnification, using an Olympus microscope.

**Erythrocyte-Binding Assay**

**Flow Cytometry**

The flow cytometry–based erythrocyte-binding assay was performed as described earlier [22]. Briefly, approximately 1 million human erythrocytes were incubated with 1 μM of histidine-tagged recombinant PvTRAGs at room temperature for 4 hours. After centrifugation at 2000g for 5 minutes, the pellet was washed with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and incubated in the dark at 4°C for 1 hour with mouse anti-penta-His Alexa Fluor 647–conjugated monoclonal antibody (Qiagen). Cells were pelleted and washed again (with 1% BSA in PBS) and incubated at 25°C for 45 minutes with thiazole orange Retic-COUNT reagent (Becton-Dickinson, San Jose, California). After washing, the cells were suspended in 1% BSA (in PBS) for acquisition. Two hundred thousand total events were acquired per sample, using FACSDiva software on a BDLSRII flow cytometer (Becton-Dickinson Immunocytometry Systems, Palo Alto, California).

For competitive inhibition, an equimolar concentration of respective histidine-tagged and untagged PvTRAG proteins (1 μM each) were added to the (approximately 1 million) erythrocytes
and incubated for 4 hours at room temperature. Competition was not allowed for normal binding but was used as a control. The rest of the steps were the same as those described above. The histidine-tagged recombinant thioredoxin from Desulfovibrio desulfuricans was used as a negative control [31].

**Cell-Based Enzyme-Linked Immunosorbent Assay (ELISA)**

An ELISA-based erythrocyte-binding assay was performed as described elsewhere [22, 24, 32, 33]. Briefly, approximately 1 million erythrocytes were added to each well of a 96-well microtiter plate and incubated overnight at 4°C. The cells were fixed with 0.3% glutaraldehyde by incubating the plate for 30 minutes at 25°C. The wells were washed and blocked with 5% BSA for 2 hours at 37°C. After washing, the plates were incubated at room temperature for 4 hours with different concentrations (0–2 µM) of histidine-tagged recombinant PvTRAGs. Histidine-tagged recombinant thioredoxin from *D. desulfuricans* was used as a negative control [31]. Plates were developed with mouse monoclonal anti-His6 antibody (Serotech, Raleigh, North Carolina) as described earlier [22].

For saturation assay, the ELISA plate was coated with approximately 1 million red blood cells and incubated with 1 µM of untagged PvTRAGs, followed by incubation with 1 µM of respective histidine-tagged recombinant proteins for 4 hours at room temperature. The plate was developed with anti-His6 monoclonal antibodies as described above.

For competitive inhibition studies, a fixed amount of histidine-tagged recombinant PvTRAg (200 nM) was mixed with increasing concentrations (0–2 µM) of respective untagged PvTRAg (the histidine tag of the recombinant PvTRAGs was removed by the treatment of AcTEV protease [Invitrogen Life Sciences], using the manufacturer’s protocol). The mixture was then added to the wells of the 96-well microtiter plate, which had been coated with approximately 1 million erythrocytes, and incubated for 4 hours at room temperature. After washing with PBS containing 0.1% tween 20, the plate was developed with monoclonal anti-His6 antibody as described above. For a positive control, no untagged PvTRAG (only PBS) was allowed to compete with the histidine-tagged recombinant M-PvTRAGs.

For the cross-competition study, each of the untagged PvTRAg was added at a concentration of 1 µM to the wells of the microtiter plate, which had been coated with approximately 1 million erythrocytes. The plate was incubated for 4 hours at room temperature and washed 3 times with PBS. Then the plate was incubated for 3 hours at room temperature with different histidine-tagged PvTRAGs (concentration, 1 µM). For a positive control, erythrocytes were not preincubated with untagged PvTRAGs. The plate was incubated with anti-His6 monoclonal antibody and processed for color development as described above.

**Rosetting**

An erythrocyte-binding assay with CHO-K1 cells expressing PvTRAGs on their surface was performed as described elsewhere [29]. Briefly, erythrocytes were collected in 10% citrate phosphate dextrose and washed 3 times in RPMI 1640 medium, pH 7.4, containing 0.36 mM hypoxanthine. Hematocrit at 1% was added to CHO-K1 cells expressing PvTRAGs, which were then incubated for 1 hour in a humidified incubator containing 5% CO2 at 37°C. Cells were washed 4 times with incomplete RPMI 1640 medium, pH 7.4. Numbers of rosettes were scored in 20 fields at 200 times the original magnification and were normalized to a transfection efficiency of 10%. The CHO-K1 cells transfected with pRE4-PvTRAG53.7 (nonbinder), and plasmid pHVDR22, which contains thePVRII region of *P. vivax* Duffy binding protein [29] were used as negative and positive controls, respectively.

**Binding of *P. vivax* Tryptophan-Rich Proteins to Enzyme-Treated Human Erythrocytes**

Human blood samples were adjusted to 5% hematocrit and treated separately either with chymotrypsin, trypsin, or neuraminidase, as described earlier [22]. The ELISA plate was then coated with approximately 1 million enzyme-treated or untreated erythrocytes, and the binding assay was performed with a 1 µM concentration of histidine-tagged PvTRAGs, as described above.

**Inhibition of *P. vivax* Tryptophan-Rich Protein Binding to Erythrocytes by Sera Obtained From *P. vivax*-Infected Patients**

Each of the histidine-tagged PvTRAGs (250 nM) was incubated overnight at 4°C with different dilutions of pooled sera from *P. vivax*-infected patients. This reaction mixture was then allowed to bind to approximately 1 million human erythrocytes in a 96-well ELISA plate and processed for color development as described above after exposure to anti-His6 monoclonal antibody.

The inhibition of erythrocyte binding to CHO-K1 cells expressing PvTRAGs by pooled sera from *P. vivax*-infected patients was performed as described by Wickramarachchi et al [34]. Briefly, transfected CHO-K1 cells were preincubated with different dilutions of sera before binding to erythrocytes under the conditions described above. Serum specimens from uninfected healthy individuals were used as negative controls. Results are expressed as relative binding to positive control (RPMI 1640 medium only).

**Statistical Analysis**

Unpaired and paired Student *t* tests and one-way analysis of variance were used to evaluate the statistical significance of these experiments, as appropriate. A *P* value of < .05 was considered statistically significant. Calculations were performed using GraphPad and STATA software.

**RESULTS**

**Limited Number of Proteins From the Pv-fam-a Family Bind to Host Erythrocytes**

Earlier, we reported that 6 of 15 PvTRAGs from the Pv-fam-a family bound to human erythrocytes [22]. Since the parasite...
Genome encodes 36 of these proteins, the remaining 21 PvTRAGs were expressed, purified (Supplementary Figure 1), and used here to determine their erythrocyte-binding activity by flow cytometry and cell-based ELISA. Of these 21 PvTRAGs, only 4 (PvTRAG36.6, PvTRAG26.3, PvTRAG36, and PvTRAG34) showed significant ($P < .05$) binding to human erythrocytes (Figure 1). The remaining PvTRAGs showed binding activity similar to that of the negative control, bacterial thioredoxin. The binding activity of these PvTRAGs was reduced to approximately 50% if the equimolar concentrations of each

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**Figure 1.** Erythrocyte-binding activity of 21 Plasmodium vivax tryptophan-rich antigens (PvTRAGs). **A**, Flow cytometry. Total erythrocytes were used for the binding assay. Dot plot and histogram of erythrocytes gates were used for the analysis. Bar diagram shows the binding of different PvTRAGs with the erythrocytes. One million erythrocytes were incubated with a 1-µM concentration of histidine-tagged recombinant proteins. Histidine-tagged PvTRAG38 and bacterial recombinant thioredoxin were used as positive and negative controls, respectively. Data are mean values (± SD) of at least 3 independent experiments. The difference between PvTRAGs and thioredoxin binding to erythrocytes was significant ($P < .05$). “Background” denotes that no PvTRAG was added. Representative dot plots for the binding of PvTRAGs to erythrocytes are shown in the upper panel: a, unstained erythrocytes; b, background control without proteins; c, PvTRAG34.9; d, PvTRAG36; e, PvTRAG36.6; f, PvTRAG26.3; g, PvTRAG34; and h, thioredoxin. **B**, Cell-based enzyme-linked immunosorbent assay (ELISA). An ELISA plate was coated with approximately 1 million erythrocytes and reacted with different concentrations (0–2 µM) of histidine-tagged recombinant proteins. The plate was developed with monoclonal anti-His6 antibody as described elsewhere[22]. Data are mean values (± SD) of at least 3 independent experiments. Recombinant bacterial thioredoxin was used as a negative control. Abbreviation: SD, standard deviation.
histidine-tagged and untagged protein were mixed together before they were added to erythrocytes and subjected to flow cytometry (Figure 2A). Similarly, the binding sites were blocked when erythrocytes were preincubated with untagged PvTRAg (1 µM) prior to their reaction with the respective histidine-tagged PvTRAg at the same concentration in a saturation assay in cell-based ELISA (Figure 2B). The specificity of binding was also confirmed by the competition assay, in which binding was reduced with increased concentrations of the untagged protein competing with histidine-tagged protein in cell-based ELISA (Figure 3). Furthermore, after heat-denaturation at 70°C for 10 minutes, the mean erythrocyte-binding activity (±SD) of PvTRAg34, PvTRAg26.3, PvTRAg36, and PvTRAg36.6 was reduced to 65.33% ± 5.06%, 78.68% ± 3.69%, 74.50% ± 3.74%, and 59.77% ± 4.26%, respectively, in comparison to the nondenatured protein, in cell-based ELISA. On the contrary, heat denaturation had no effect on the erythrocyte-binding activity of the nonbinder protein PvTRAg38.7 and thioredoxin, thus confirming the binding activity of these PvTRAgS. Moreover, these 4 PvTRAgS, expressed on the surface of CHO-K1 cells, were found to bind the erythrocytes (Figure 4). To compare the erythrocyte-binding activity of all proteins of the P.v-fam-a family, we analyzed all 36 PvTRAgS together by flow cytometry and cell-based ELISA and found that 10 PvTRAgS showed this activity (Supplementary Figure 2).

Sharing of Erythrocyte Receptors by PvTRAgS

We performed cross-competition analysis of these 4 PvTRAgS to determine their erythrocyte-binding capacity. We included the representatives of previously described PvTRAgS from each category as a control [22]. We found that PvTRAg36.6 and PvTRAg26.3 compete with each other for their erythrocyte receptor(s) (Figure 5). But they did not compete with the other 2 PvTRAgS or with the previously described antigens PvTRAg38 and PvTRAg33.5 [22], indicating that they may be binding to a different set of erythrocyte receptor(s). However, PvTRAg36 competed with PvTRAg38 and PvTRAg34 competed with PvTRAg33.5 for their binding to erythrocytes, indicating that PvTRAg36 and PvTRAg34 share their erythrocyte receptors with PvTRAg38 and PvTRAg33.5, respectively (Figure 5). There was partial competition between PvTRAg36 and PvTRAg34 for erythrocyte receptors, indicating that these 2 PvTRAgS may share one of the 2 erythrocyte receptors (Figure 5). This is similar to our earlier observations for PvTRAg38 and PvTRAg33.5, in which the proteins also partially compete with each other for these erythrocyte receptors [22]. Earlier, we identified 2 erythrocyte receptors for each of these proteins, and PvTRAg33.5 and PvTRAg38 shared one of the erythrocyte receptors [22].

Enzymatic treatment of erythrocytes prior to their use in binding assay proved that the erythrocyte receptors for these 4 PvTRAgS were resistant to trypsin and neuraminidase (Figure 6). Except for PvTRAg36, the erythrocyte receptors for the other 3 PvTRAgS were also resistant to chymotrypsin. The chymotrypsin sensitivity and cross-competition data further confirms that PvTRAg38 and PvTRAg36 share same 2 erythrocyte receptors where one of the receptor is chymotrypsin sensitive.

**Figure 2.** Specificity of *Plasmodium vivax* tryptophan-rich antigen (PvTRAg) binding. A, Competition assay by flow cytometry. Equimolar concentrations of respective histidine-tagged and untagged PvTRAg proteins (1 µM each) were added to 1 million erythrocytes. Competition was not allowed for normal binding but was used as a control. Thioredoxin was used as a negative control. Data are mean values (± SD) of at least 3 independent experiments. “Background” denotes that no PvTRAg was added. B, Saturation assay performed by cell-based enzyme-linked immunosorbent assay (ELISA). An ELISA plate was coated with approximately 1 million erythrocytes and incubated with 1 µM of untagged PvTRAgS, followed by incubation with 1 µM of histidine-tagged recombinant proteins. The plate was developed with monoclonal anti-His6 antibody as described elsewhere [22]. Data are mean values (± SD) of at least 3 independent experiments. Abbreviation: SD, standard deviation.
Sera From *P. vivax*–Infected Patients Inhibit the Erythrocyte-Binding Activity of PvTRAg

The *P. vivax*–infected patients were producing antibodies against these antigens (Zeeshan et al, unpublished data) that inhibited the binding of each of the 4 PvTRAg to human erythrocytes in a dilution-dependent manner in cell-based ELISA, as well as in rosetting assays (data not shown).

**DISCUSSION**

The significance of *Plasmodium* tryptophan-rich proteins to the survival and growth of malarial parasites has been described in literature for the murine malaria parasite *Plasmodium yoelii* and the human malaria parasite *P. falciparum* [18, 19, 35, 36]. The results of the present study are important because we identified 4 additional biologically significant tryptophan-rich proteins from a very common but noncultivable human malaria parasite, *P. vivax*. If we combine the results of the present study with that of our previous study [22], it completes the information needed about the number of erythrocyte-binding proteins in the Pv-fam-a family.

The combined erythrocyte-binding data for all 36 PvTRAg of the Pv-fam-a family revealed that only 10 possess erythrocyte-binding activity (Supplementary Figure 2). This prompts questions about why *P. vivax* synthesizes so many erythrocyte-binding PvTRAg and about the possible advantageous roles they might play for the parasite. We hypothesize that their biological function is correlated with their stage-specific expression profile, as described by Bozdech et al [20]. We found transcriptome data for 8 of these 10 erythrocyte-binding PvTRAg in this data set (the transcriptome data for PvATRAG74 and PvTRAg33.5 were not available). Of these 8 erythrocyte-binding PvTRAg, 4 (PvTRAg35.2, PvTRAg38, PvTRAg36, and PvTRAg34) were expressed at the late schizont/merozoite stage, and other 4 (PvTRAg, PvTRAg26.3, PvTRAg36.6, and PvTRAg69.4) were expressed at the ring/trophozoite stage of the

**Figure 3.** Competitive inhibition of *Plasmodium vivax* tryptophan-rich antigen (PvTRAg) binding to erythrocytes by cell-based enzyme-linked immunosorbent assay. A mixture of histidine-tagged recombinant PvTRAg (200 nM) and different concentrations (0–2 µM) of respective untagged PvTRAg was added to approximately 1 million erythrocytes in a 96 well microtiter plate. After incubation, the plates were developed with monoclonal anti-His6 antibodies as described earlier [22]. Data are mean values (± SD) of at least 3 independent experiments. Panels denote data for PvTRAg36 (A), PvTRAg34 (B), PvTRAg36.6 (C), and PvTRAg26.3 (D). PBS, phosphate-buffered saline. Abbreviation: SD, standard deviation.
parasite. This suggests that PvTRAg, PvTRAg26.3, PvTRAg36.6, and PvTRAg69.4, expressed at the early stage of the parasite (the ring/trophozoite stage), may be involved in the rosetting phenomenon, in which several uninfected erythrocytes can attach to a single parasitized erythrocyte (Table 1). On the other hand, PvTRAg35.2, PvTRAg38, PvTRAg36, and PvTRAg34, expressed at late schizont/merozoites stage, may be helping the parasite to invade the host erythrocytes (Table 1). The latter possibility is supported by our observations in the heterologous system, in which PvTRAg36 and PvTRAg34 (expressed at the late schizont/merozoite stage), not PvTRAg36.6 and PvTRAg26.3 (expressed at the early stage), were partially blocking *P. falciparum* infection of erythrocytes in *in vitro* culture (Zeeshan et al, unpublished data).

In our earlier studies, we described the cross-competition data for 6 of these 10 PvTRAs and concluded that each PvTRAg binds to 2 erythrocyte receptors [22]. Four of these 6 PvTRAs (PvTRAg33.5, PvTRAg35.2, PvTRAg69.4, and PvTRAg) competed with each other to recognize the same 2 erythrocyte receptors, termed receptor A and receptor B (Table 1). The remaining 2 PvTRAs (PvTRAg38 and PvATRAg74) recognized a common chymotrypsin-sensitive erythrocyte receptor, termed receptor C. The other erythrocyte receptor recognized by these 2 PvTRAs was receptor A, for PvTRAg38, and receptor B, for PvATRAg74. Thus, there are 3 erythrocyte receptors (A, B, and C) recognized by these 6 PvTRAs [22]. The cross-competition data for the remaining 4 PvTRAs are described in the present study along with the representative of 6 previously described PvTRAs to cover already described erythrocyte receptors (Figure 5). Among the 4 new PvTRAs, PvTRAg34 cross-competed with PvTRAg33.5 and PvTRAg36 cross-competed with PvTRAg38, indicating that PvTRAg34 recognized erythrocyte receptors A and B, whereas PvTRAg36 recognized erythrocyte receptors A and C. We noticed that PvTRAg36.6 and PvTRAg26.3 competed with each other but not with any other PvTRAg (Figure 5). This indicates that both share the same erythrocyte receptor(s). But this receptor(s) is not shared by the 8 remaining erythrocyte-binding PvTRAs, as they did not show any cross-competition with these 2 proteins for erythrocyte binding. The erythrocyte receptor(s) for these 2 PvTRAs were resistant to trypsin, chymotrypsin, and neuraminidase (Figure 6). Since each PvTRAg recognizes 2 erythrocyte receptors, these 2 PvTRAs may also be recognizing 2 erythrocyte receptors, termed receptor D and receptor E.

Therefore, we predict that there may be 5 erythrocyte receptors for these 10 PvTRAs (Table 1). Results show that each PvTRAg recognizes >1 erythrocyte receptor and that each receptor is recognized by >1 PvTRAg (Table 1). If we correlate this receptor recognition and the stage-specific expression profile of these PvTRAs, we hypothesize that the parasite is using the same
Figure 5. Erythrocyte receptor sharing by *Plasmodium vivax* tryptophan-rich antigens (PvTRAgs). Plates coated with 1 million erythrocytes were incubated with 1 µM of untagged PvTRAg36, PvTRAg26.3, PvTRAg34, PvTRAg36.6, PvTRAg38, and PvTRAg33.5. After washing with phosphate-buffered saline (PBS), 1 µM of histidine-tagged PvTRAg36 (A), PvTRAg34 (B), PvTRAg36.6 (C), and PvTRAg26.3 (D) were added, and binding was detected by monoclonal anti-His6 antibody as described earlier [22]. Mean values (± SD) of 3 different experiments are shown. Binding of PvTRAgs without competitor (PBS) was considered as 100%. Abbreviation: SD, standard deviation.

Figure 6. Effect on the capacity of *Plasmodium vivax* tryptophan-rich antigens (PvTRAgs) to bind enzyme-treated erythrocytes. Human erythrocytes were pretreated with trypsin, chymotrypsin, or neuraminidase prior to their addition to the microtiter plate. The plate was coated with approximately 1 million enzyme-treated and untreated human erythrocytes and then reacted with histidine-tagged recombinant PvTRAgs (concentration, 1 µM). The plates were developed with monoclonal anti-His6 antibodies as described earlier [22]. Mean values (± SD) of at least 3 different experiments are shown. Abbreviation: SD, standard deviation.
Table 1. Probable Function of Erythrocyte-Binding Proteins From the Pv-fam-a Family and Their Hypothetical Receptors on Human Erythrocytes

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Abbreviation: PvTRAg, Plasmodium vivax tryptophan-rich antigen.

* Cross-competition data for 6 of these PvTRAgS are described elsewhere [22].
* Based on transcriptome data described elsewhere [20].
* These receptors were resistant to trypsin, neuraminidase, and chymotrypsin, except receptor C, which was susceptible to chymotrypsin.
* This is based on the stage-specific expression of PvTRAgS.

erythrocyte receptor for 2 different purposes. For example, erythrocyte receptors A and B are recognized by PvTRAgS that are expressed by an early stage of the parasite and thus could be involved in the rosetting phenomenon. The same erythrocyte receptors (ie, A and B) are also recognized by PvTRAgS that are expressed by merozoites and thus could be involved in red blood cell invasion (Table 1). However, the combination of receptors A and C seems to be specific for parasite ligands expressed by merozoites and thus could be involved in erythrocyte invasion. Similarly, receptors D and E seem to be used only for rosetting, as these receptors recognize PvTRAg26.3 and PvTRAg36.6, which are expressed by ring/trophozoite stages. The binding of each protein to 2 different receptors and the recognition of each receptor by >1 parasite ligand could be advantageous for the parasite. *P. vivax* may be using this redundancy in the receptor-ligand interaction as an alternate invasion pathway or for tightly binding to its host cell during the invasion/rosetting process.

The biological significance of non–erythrocyte-binding PvTRAgS remains unknown, except that they are highly immunogenic in humans with limited genetic variation (Zeeshan et al, unpublished data) [21]. However, we observed that the majority interacted with spectrin (Alam et al, unpublished data). This interaction could be important for various biological functions of the parasite that are similar to those of *P. falciparum*, such as protein transport, egress of merozoites, and erythrocyte cytoskeleton modulation to provide rigidity to the infected erythrocyte [37–42]. Furthermore, patients with spectrin deficiency are also poor hosts for malaria parasites [43]. Thus, the nonbinder PvTRAgS may also be playing an important role in parasite growth, but this requires further investigation.

It is interesting that the receptor-ligand interaction was blocked by antibodies from *P. vivax*-infected patients. This indicates not only that there is some overlap between B-cell epitopes and binding domains in these proteins, but also that the host produces inhibitory antibodies against these antigens. This holds significance for the host-parasite interaction. Further studies on these PvTRAgS and their erythrocyte receptors might lead to the development of drugs and vaccines against this most common human malaria parasite.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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**References**


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