

Host–parasite interaction: multiple sites in the *Plasmodium vivax* tryptophan-rich antigen PvTRAg38 interact with the erythrocyte receptor band 3

Mohd S. Alam, Sumit Rathore, Rupesh K. Tyagi and Yagya D. Sharma

Department of Biotechnology, All India Institute of Medical Sciences, Ansari Nagar, New Delhi, India

Correspondence

Y. D. Sharma, Department of Biotechnology, All India Institute of Medical Sciences, Ansari Nagar, New Delhi 110029, India

Fax: +91 11 26589286

Tel: +91 11 26594609

E-mail: ydsharma_aaims@yahoo.com

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Tryptophan-rich antigens of malarial parasites interact with host molecules and play an important role in parasite survival. Merozoite expressed *Plasmodium vivax* tryptophan-rich antigen PvTRAg38 binds to human erythrocytes and facilitates parasite growth in a heterologous *Plasmodium falciparum* culture system. Recently, we identified band 3 in human erythrocytes as one of its receptors, although the receptor-ligand binding mechanisms remain unknown. In the present study, using synthetic mutated peptides of PvTRAg38, we show that multiple amino acid residues of its 12 amino acid domain (KVVQWKNDKIRS) at position 197–208 interact with three different ectodomains of band 3 receptor on human erythrocytes. Our findings may help in the design of new therapeutic approaches for malaria.

Keywords: alanine scanning; band 3 ectodomains; erythrocyte binding; malaria parasite; peptide mapping; receptor-ligand interactions

Plasmodium vivax is a very common human malaria parasite affecting millions of people each year in the tropical world. In general, this parasite causes benign malaria and is sensitive to most anti-malarial drugs. However, there are reports indicating that *P. vivax* can also cause complications in humans leading to disease severity and deaths [1–3]. Furthermore, it is also developing resistance towards commonly used anti-malarial drugs [3,4]. Thus, there is a need to focus on this neglected human malaria parasite, as well as develop newer therapeutics against it.

Plasmodium tryptophan-rich proteins that are involved in the host–parasite interaction are potential drug or vaccine targets [5–9]. These proteins are present in all the *Plasmodium* species sequenced so far (www.plasmodb.org). Their number varies from only one in *Plasmodium gallinaceum* to 36 in *P. vivax* and *Plasmodium cynomolgi*, highlighting their significance for the parasite. Tryptophan-rich proteins of *P. vivax* belong to ‘Pv-fam-a’ family [9] and there is a stage-

specific expression of these proteins in the parasite, probably to perform different functions [10]. Previously, we immunobiologically characterized large numbers of these proteins and hypothesized that some of them may be involved in red cell invasion [11–20]. These proteins have exceptionally high tryptophan contents and the tryptophan residues are positionally conserved with a certain spatial pattern. The exact role of tryptophan residues in these proteins is not known.

One of the *P. vivax* tryptophan-rich antigens, PvTRAg38, is expressed by the merozoites [10], binds to host erythrocytes, is highly immunogenic in humans, has a conserved sequence in parasite population, and is probably involved in red cell invasion [18,19,21,22]. On the erythrocytes, band 3 acts as one of the host receptor for this parasite protein [22]. Interaction of PvTRAg38 with band 3 was mapped to its three different ectodomains (loops 1, 3 and 6) and the binding region of PvTRAg38 to band 3 was mapped to the KVVQWKNDKIRSWLSEW

Abbreviations

RBC, red blood cell; SPR, surface plasmon resonance.

sequence at amino acid position 197–214 [22]. We aimed to investigate the binding mechanisms of this parasite ligand to the host erythrocyte receptor band 3 and also to determine whether the tryptophan residues of this domain play any role in erythrocyte binding. The results obtained show that there are multiple sites in this 12 amino acid long peptide domain of PvTRAg38, including two tryptophan residues, which interact differentially with band 3 ectodomains, and the replacement of any of these amino acids with alanine affected the erythrocyte binding activity of the peptide.

Materials and methods

Ethics statement

Heparinized blood was collected from healthy individuals in accordance with Institutional Ethical Guidelines. Written consent was obtained from the individuals prior to blood collection. The ethics committee of the All India Institute of Medical Sciences, New Delhi, approved the study via approval number IEC/NP-342/2012 & RP-11/2012.

Materials

The purified histidine-tagged PvTRAg38, bacterial thioredoxin and band 3 fragments (B3F1, B3F3 and B3F6) were available in the laboratory [18,22,23]. *O*-phenyldiamine, RPMI 1640, hypoxanthine, penicillin-streptomycin, poly-L-lysine and monoclonal antibodies against His₆ (Sigma-Aldrich, St Louis, MO, USA), CM5 chips and other coupling reagents (GE Healthcare, Uppsala, Sweden), FBS, Lipofectamine 2000, Alexa flour 488 conjugated goat anti-mouse or anti-rabbit IgG (Invitrogen Life Technologies, Carlsbad CA, USA) and horseradish peroxidase conjugated anti-mouse IgG secondary antibody (Pierce Biotechnology Inc., Rockford, IL, USA) were obtained commercially.

Synthetic peptides

Mutated 18-mer peptides where three consecutive amino acid residues were replaced by alanine residues, and mutated 12-mer peptides where single amino acid residue was replaced by an alanine residue, were synthesized commercially (Thermo Fischer Scientific GmbH, Ulm, Germany) with or without six histidine residues attached to their C-terminal end.

Procedures

Erythrocyte binding by cellular ELISA

This assay was performed as described previously [21,24] where approximately 1 million human erythrocytes, fixed

on each well of a 96-well microtiter plate, were blocked with 5% BSA, and incubated with 1 μ M mutated or wild-type peptide for 4 h at room temperature. Recombinant *Desulfovibrio desulfuricans* thioredoxin was used as negative control [23]. Plates were washed with PBS containing 0.05% Tween 20, incubated with mouse monoclonal anti-His₆ antibody (Serotech, Raleigh, NC, USA) at a dilution of 1 : 2000, followed by horseradish peroxidase conjugated anti-mouse IgG secondary antibody, and developed with *O*-phenyldiamine substrate.

Erythrocyte binding assay by rosetting

This assay was performed as described previously [21,22]. Briefly, the CHO-K1 cell line (American Type Culture Collection, Manassas, VA, USA) was transfected with PvTRAg38-RE4 plasmid using cationic lipid Lipofectamine 2000 [22]. For rosetting, erythrocytes at 1% hematocrit were added to CHO-K1 cells expressing PvTRAg38 and incubated for 1 h in a humidified 5% CO₂ incubator at 37 °C. After 1 h of incubation, cells were washed four times with incomplete RPMI 1640 medium (pH 7.4) and the number of rosettes (more than five erythrocytes bound to transfected CHO-K1 cells) was scored in 20 fields at \times 200 magnification and normalized to a transfection efficiency of 10%. For competition assays, erythrocytes at 1% hematocrit were pre-incubated with peptides (20 μ M, in incomplete RPMI 1640, pH 7.4) for 1 h at 37 °C and then added to transfected CHO-K1 cells, and the number of rosettes was scored as described above.

Solid-phase binding assay

This assay with purified band 3 was performed as described previously [22]. Briefly, each well of the ELISA plate was coated with 50 nm of band 3 protein, blocked with 5% skimmed milk and then incubated with 0.5 μ M histidine-tagged peptides (wild-type or mutated form) for 4 h at room temperature. Plates were developed with mouse monoclonal anti-His₆ antibody as described above.

For binding of band 3 fragments to synthetic peptides, each well of a 96-well ELISA plate was separately coated with 0.5 μ M synthetic peptides. After blocking with 5% skimmed milk in PBS, the plates were incubated with 0.5 μ M Trx-tagged band 3 fragments (B3F1, B3F3 or B3F6) and developed with mouse monoclonal anti-Trx-tag antibody as described above.

Surface plasmon resonance analysis

The kinetics of binding of peptides to band 3 fragments (B3F1, B3F3 and B3F6) were determined on the surface plasmon resonance (SPR) based biosensor BIACORE 2000 (Biacore AB, Uppsala, Sweden). All experiments were per-

formed in 10 mM Hepes (pH 7.4) containing 150 mM NaCl, 3 mM EDTA and 0.005% P20 surfactant at 25 °C. The addition of 0.05% P20 blocked the nonspecific adsorption of analytes to the sensor chips. In each experiment, ligands were coupled either to Fc-2 or Fc-4 and Fc-1 or Fc-3 served as control flow cells. Experiments were performed either by immobilizing band 3 fragments onto the sensor chip CM5 using amine-coupling chemistry in accordance with the protocol described previously [22,25]. Binding was measured at 50 $\mu\text{L}\cdot\text{min}^{-1}$ to avoid the mass transport effect. Binding was measured for 120 s and dissociation was monitored for an additional 180 s. The sensor chips were regenerated with 30 s pulses of 10 mM glycine (pH 3.0). Sensograms obtained for the control flow cell were subtracted from the data for the flow cell immobilized with a ligand. SPR data were analyzed using BIAEVALUATION, version 3.2 (GE Healthcare) with global fitting. The apparent equilibrium dissociation constant (K_D) was calculated from $K_D = k_d/k_a$, where k_d is the dissociation rate constant and k_a is the association rate constant.

Results

Erythrocyte binding activity in the P-4 domain of PvTRAg38 resides in a 12 amino acid region

We have recently reported on the 18 amino acid long P-4 domain of PvTRAg38, which binds to human erythrocytes, as well as to the purified band 3 protein [22]. To further narrow down the erythrocyte binding activity of the P-4 domain, mutated peptides were synthesized where three consecutive amino acid residues were systematically replaced by three alanine residues and were then used in erythrocyte binding assays. Cellular ELISA results (Table 1) showed that replacing first three amino acids of peptide P-4 with three alanine residues resulted in the abolition of its erythrocyte binding activity. Restoring these first three amino acid residues to wild-type but mutating the next three amino acid residues to alanines did not restore its binding activity. This abolition of erythrocyte binding

activity continued until the 12th amino acid residue of P4 (Fig. 1 and Table 1). However, replacement of three amino acid residues at positions 13–15th or thereafter resulted in the restoration of its erythrocyte binding activity. These results therefore eliminated the possibility of the last six amino acids of P-4 being involved in erythrocyte binding. Similar results were obtained when these mutated peptides were allowed to compete with PvTRAg38 expressed on the surface of CHO-K1 cells for erythrocyte binding by the rosetting assay (Fig. 1A). These results obtained from two different assays indicate that the first 12 amino acid residues (amino acid residues 197–208) of this P-4 peptide domain are important for erythrocyte binding activity.

Multiple sites within the 12 amino acid (TP-4) domain of PvTRAg38 are involved in erythrocyte binding

To map the critical amino acid residues of the 12 amino acid TP-4 domain of PvTRAg38 involved in erythrocyte binding, we synthesized the wild-type and mutated peptides where a single amino acid residue was replaced with alanine in a stepwise manner and used these in erythrocyte binding assays. Cellular ELISA results showed that the erythrocyte binding activity was reduced when certain amino acid residues within this 12 amino acid region were replaced by alanine (Table 2). Analogous to the binding experiment by cellular ELISA, we performed a binding inhibition experiment where binding of erythrocytes (rosetting) to CHO-K1 cells expressing PvTRAg38 competed with the wild-type peptide and its mutated forms. Here, mutated peptides showed trends similar to those observed in cellular ELISA (Fig. 1B). Out of 12 amino acid residues in this peptide domain, erythrocyte binding activity was markedly affected (40–50% reduction) by five amino acids (W198, V199, K202, N203, R207). The maximum effect on erythrocyte binding activity was observed when an asparagine residue was replaced

Table 1. Binding activity of P-4 of PvTRAg38 and its mutated peptides with human erythrocytes and purified band 3 protein. Mutated amino acids are underlined. Numbers at the beginning and end of a peptide sequence indicate the amino acid position.

Sequence number	Peptide name	Peptide sequence	% Binding with erythrocyte	% Binding with band 3
1	P4	¹⁹⁷ KWVQWKNDKIRSWLSSEW ²¹⁴	100 ± 0.0	100 ± 0.0
2	P4-AS1	<u>AAA</u> QWKNDKIRSWLSSEW	11.3 ± 2.9	12.8 ± 3.5
3	P4-AS2	KWV <u>AAA</u> NDKIRSWLSSEW	9.8 ± 0.5	22.9 ± 3.2
4	P4-AS3	KWVQWK <u>AAA</u> IRSWLSSEW	11.0 ± 1.7	18.4 ± 4.8
5	P4-AS4	KWVQWKNDK <u>AA</u> AWLSSEW	11.2 ± 2.6	16.3 ± 2.7
6	P4-AS5	KWVQWKNDKIR <u>SA</u> AASEW	91.8 ± 3.0	92.4 ± 2.6
7	P4-AS6	KWVQWKNDKIRSWL <u>SAAA</u>	92.7 ± 1.9	94.6 ± 2.3
8	Thioredoxin		5.1 ± 2.4	5.8 ± 2.0

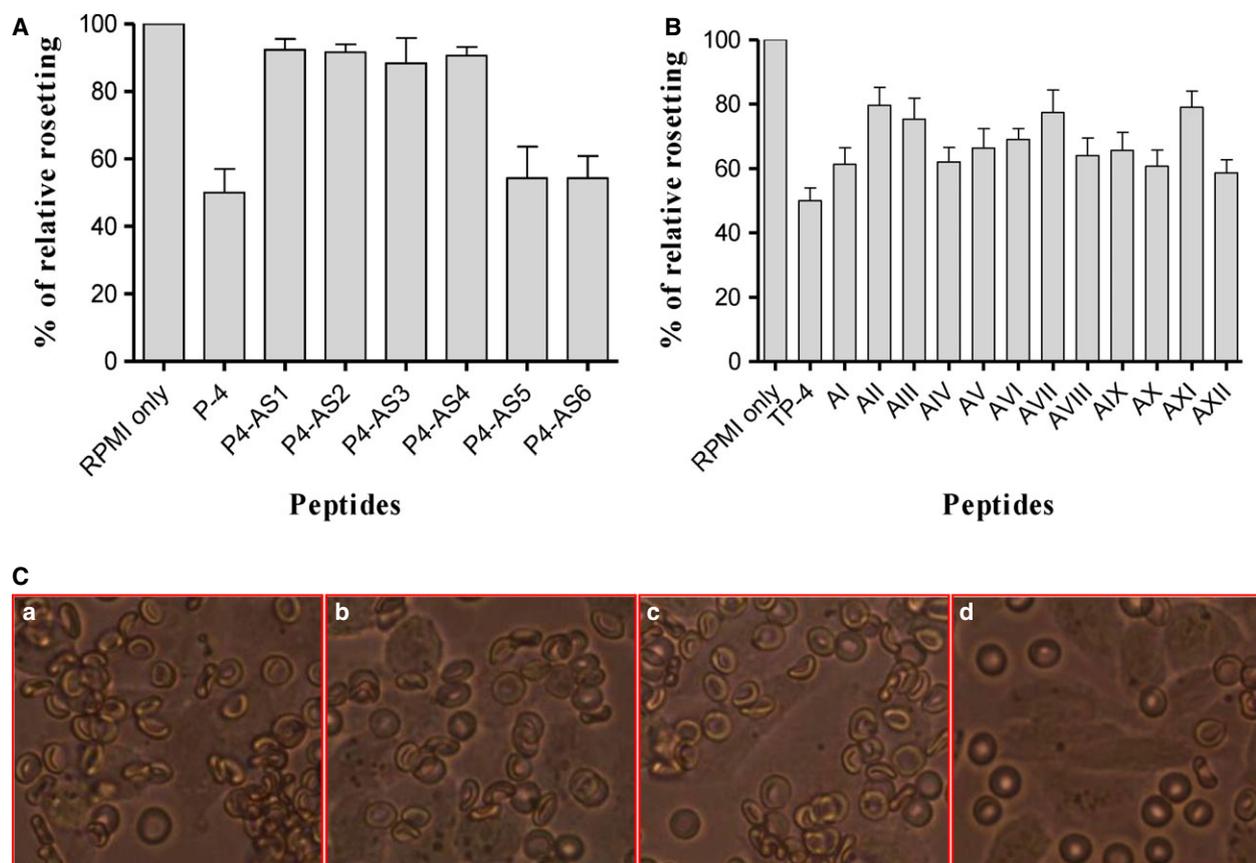


Fig. 1. Inhibition of erythrocyte binding to CHO-K1 cells expressing PvTRAg38 on its surface by the rosetting assay. (A) Synthetic 18 amino acid long P-4 wild-type peptide or its mutants with triple mutations or (B) a synthetic 12 amino acid long TP-4 wild-type peptide or its mutants with a single mutation were used to compete for binding of erythrocytes to CHO-K1 cells expressing PvTRAg38 in the rosetting assay. Erythrocytes (1% hematocrit) were separately pre-incubated with 20 μM of peptides for 1 h at 37 °C. The erythrocytes were then allowed to bind to RE4-PvTRAg38 transfected CHO-K1 cells at 37 °C for 1 h. The number of rosettes was scored in 20 fields at $\times 200$ magnification. The results are expressed as relative binding to positive control (binding of PvTRAg38 transfected cells with untreated RBCs, i.e. RPMI only). Error bars indicate the SD of three experiments. The transfection efficiency for each experiment was normalized to 15%. A single CHO-K1 cell transfected with PvTRAg-RE4 construct and attached to more than five RBCs was considered as a rosette. (C) Representative images of rosette formation by CHO-K1 cells expressing PvTRAg38 with erythrocytes pretreated with (a) RPMI only, (b) native peptide TP-4, (c) mutant peptide TP-4 (AII) and (d) untransfected CHO-K1 cells (negative control).

with alanine (N203A). Replacement of the first and last amino acids of this 12 amino acid TP-4 peptide with alanine had a minimal effect on its erythrocyte binding activity.

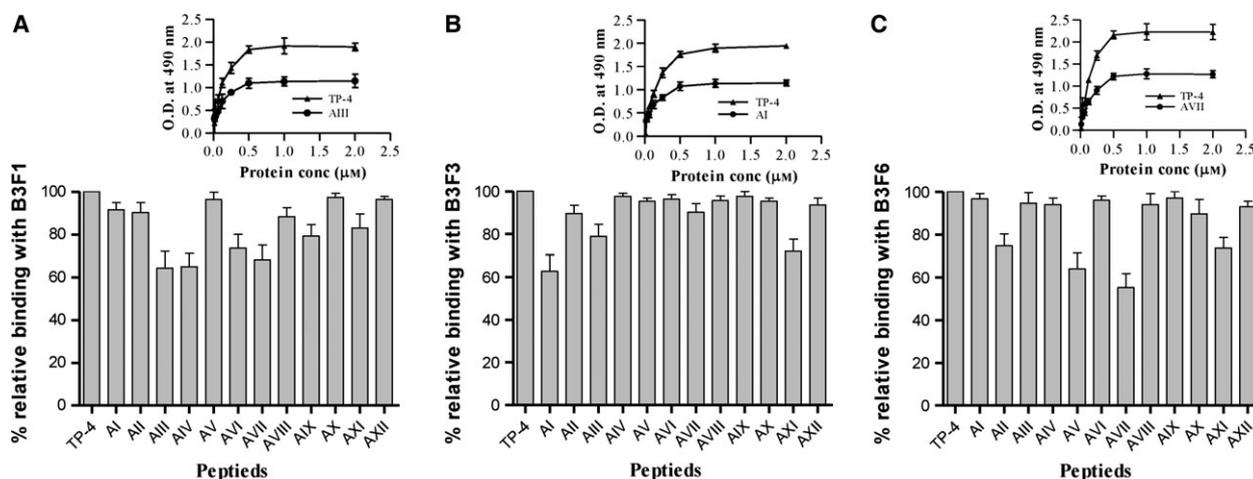
The same multiple sites in the 12 amino acid (TP-4) region of PvTRAg38 also interact with purified erythrocyte band 3 protein

We used the mutated peptides described above in a solid-phase binding assay with the purified band 3 protein instead of erythrocytes. Here, the last six amino acid residues of the 18 amino acid long P-4 domain of PvTRAg38 were not involved in binding to band 3 (Table 1). Similarly, multiple amino acid residues of

the 12 amino acid TP-4 domain were also interacting with the purified band 3 protein (Table 2). This pattern was similar to that of the erythrocyte binding activity of these mutated peptides. Replacement of asparagine with alanine (peptide AVII) also showed a maximum reduction in the binding activity of this mutated peptide with respect to the purified band 3 protein compared to the wild-type peptide. Band 3 binding activity of mutated peptides AIII and AXI, where valine and arginine were replaced by alanine, respectively, was also maximally affected compared to the wild-type peptide. These results confirmed that band 3 on the erythrocyte surface interacts with multiple sites of the 12 amino acid TP-4 domain of PvTRAg38.

Table 2. Binding activity of truncated P-4 (TP-4) of PvTRAg38 and its mutated peptides with human erythrocytes and purified band 3 protein. Mutated amino acids are underlined. Numbers at the beginning and end of a peptide sequence indicate the amino acid position.

Sequence number	Peptide name	Peptide sequence	% Binding with erythrocyte	% Binding with band 3
1	TP-4 (Wild)	¹⁹⁷ KVVQWKNDKIRS ²⁰⁸	100.00 ± 0.0	100 ± 0.0
2	TP-4 (AI)	<u>A</u> VVQWKNDKIRS	81.06 ± 8.2	83.0 ± 6.2
3	TP-4 (AII)	K <u>A</u> VQWKNDKIRS	53.50 ± 8.8	63.6 ± 3.5
4	TP-4 (AIII)	KV <u>A</u> QWKNDKIRS	50.46 ± 4.6	49.0 ± 4.6
5	TP-4 (AIV)	KVV <u>A</u> WKNDKIRS	75.46 ± 8.7	78.6 ± 8.7
6	TP-4 (AV)	KVVQ <u>A</u> KNDKIRS	66.86 ± 4.7	64.0 ± 6.6
7	TP-4 (AVI)	KVVQW <u>A</u> NDKIRS	58.10 ± 8.9	60.7 ± 9.0
8	TP-4 (AVII)	KVVQWK <u>A</u> DKIRS	45.70 ± 5.3	45.6 ± 7.8
9	TP-4 (AVIII)	KVVQWKNA <u>K</u> IRS	74.43 ± 4.2	80.3 ± 6.0
10	TP-4 (AIX)	KVVQWKND <u>A</u> IRS	68.06 ± 6.9	68.1 ± 7.2
11	TP-4 (AX)	KVVQWKNDK <u>A</u> RS	75.66 ± 5.0	79.0 ± 7.9
12	TP-4 (AXI)	KVVQWKNDKI <u>A</u> S	53.63 ± 12.7	50.3 ± 5.5
13	TP-4 (AXII)	KVVQWKNDKIR <u>A</u>	83.73 ± 7.2	83.8 ± 6.6
14	Thioredoxin		4.70 ± 1.8	5.3 ± 1.16

**Fig. 2.** Binding of truncated P-4 (TP-4) of PvTRAg38 and its mutated peptides with band 3 fragments by ELISA. The 0.5 μM synthetic peptides were separately coated in each well of a 96-well ELISA plate and blocked with 5% skimmed milk in PBS. The plate was incubated with 0.5 μM Trx-tagged band 3 fragments (A) B3F1, (B) B3F3 or (C) B3F6 and developed with mouse monoclonal anti-Trx-tag antibody. The results are expressed as percentage binding relative to positive control (binding of TP-4 to the respective band 3 fragment). Mean ± SD values of three experiments are plotted. Inset: saturation binding of wild-type peptide TP-4 and representative mutant peptide AIII, AI or AVII was separately coated in each well of a 96-well ELISA plate and blocked with 5% skimmed milk. The plates were incubated with different concentrations (0–2 μM) of Trx-tagged band 3 fragments (A) B3F1, (B) B3F3 or (C) B3F6 and developed with mouse monoclonal anti-Trx-tag antibody.

Differential recognition of band 3 ectodomains by multiple sites in the 12 amino acid region of PvTRAg38

The results reported above show that PvTRAg38 binds to band 3 of the host erythrocyte via multiple sites of its 12 amino acid peptide domain. Previously, we have shown that peptide P-4 from PvTRAg38 interacts with three different ectodomains of band 3 (fragments B3F1, B3F3 and B3F6) [22]. Here, we performed an

interaction between these three band 3 fragments and the mutated peptides by solid phase-ELISA, as well as by SPR, aiming to identify the critical amino acid residues involved in the interaction with band 3 ectodomains. We observed that majority of the amino acids of this 12 amino acid domain were interacting with the specific individual loops of band 3 in both the assays [i.e. by solid phase ELISA (Fig. 2) and by SPR (Table 3)]. The binding of mutated peptides with B3F1

Table 3. Binding activity of truncated P-4 (TP-4) of PvTRAg38 and its mutated peptides with band 3 fragments by surface plasmon resonance.

Binding kinetics K_D (M)				
Sequence number	Peptide name	B3F1	B3F3	B3F6
1	TP-4 (Wild)	$4.7 \pm 0.23 \times 10^{-9}$	$1.07 \pm 0.27 \times 10^{-9}$	$6.57 \pm 0.47 \times 10^{-7}$
2	TP-4 (AI)	$3.6 \pm 0.45 \times 10^{-9}$	$9.65 \pm 0.95 \times 10^{-6}$	$3.89 \pm 0.55 \times 10^{-7}$
3	TP-4 (AII)	$1.04 \pm 0.65 \times 10^{-8}$	$1.05 \pm 0.23 \times 10^{-8}$	$2.4 \pm 0.88 \times 10^{-5}$
4	TP-4 (AIII)	$1.7 \pm 0.32 \times 10^{-5}$	$1.14 \pm 0.12 \times 10^{-7}$	$1.01 \pm 0.71 \times 10^{-7}$
5	TP-4 (AIV)	$2.7 \pm 0.22 \times 10^{-5}$	$1.44 \pm 0.11 \times 10^{-9}$	$1.25 \pm 0.65 \times 10^{-7}$
6	TP-4 (AV)	$8.2 \pm 1.20 \times 10^{-9}$	$6.46 \pm 0.65 \times 10^{-10}$	$3.08 \pm 0.41 \times 10^{-4}$
7	TP-4 (AVI)	$3.7 \pm 0.89 \times 10^{-6}$	$9.69 \pm 1.50 \times 10^{-9}$	$4.25 \pm 0.42 \times 10^{-7}$
8	TP-4 (AVII)	$1.8 \pm 0.54 \times 10^{-5}$	$1.38 \pm 0.22 \times 10^{-8}$	$9.40 \pm 1.20 \times 10^{-4}$
9	TP-4 (AVIII)	$1.5 \pm 0.12 \times 10^{-8}$	$7.47 \pm 1.03 \times 10^{-10}$	$5.01 \pm 0.38 \times 10^{-7}$
10	TP-4 (AIX)	$2.1 \pm 0.32 \times 10^{-6}$	$1.01 \pm 0.21 \times 10^{-9}$	$1.07 \pm 0.65 \times 10^{-7}$
11	TP-4 (AX)	$1.8 \pm 0.24 \times 10^{-9}$	$1.01 \pm 0.11 \times 10^{-9}$	$2.10 \pm 0.78 \times 10^{-6}$
12	TP-4 (AXI)	$1.4 \pm 0.22 \times 10^{-7}$	$8.78 \pm 1.54 \times 10^{-7}$	$1.01 \pm 0.32 \times 10^{-5}$
13	TP-4 (AXII)	$8.1 \pm 1.40 \times 10^{-9}$	$9.64 \pm 1.23 \times 10^{-9}$	$3.20 \pm 0.67 \times 10^{-8}$

was maximally affected when amino acids at positions 199 (V199A, peptide AIII), 200 (Q200A, peptide AIV) or 203 (N203A, peptide AVII) of the P4 domain were replaced by alanine (Fig. 2). Mutation K202A (peptide AVI), K205A (peptide AIX) and R207A (peptide AXI) in TP-4 also affected its interaction with B3F1. Similar results were obtained by SPR analysis where mutated peptides AIII, AIV and AVII showed an approximately 10 000-fold reduction in binding affinity towards B3F1, whereas AVI, AIX and AXI showed an approximately 100–1000-fold reduction in this binding affinity compared to the wild-type TP-4 (Table 3). In the case of B3F3, the interaction of mutated peptide AI (K197A) was affected more compared to amino acid mutations at positions 199 (V199A, peptide AIII) or 207 (R207A, peptide AXI) by solid phase ELISA (Fig. 2). However, the SPR data showed almost similar binding affinity for these three peptides to B3F3, which was reduced to approximately 100-fold compared to the wild-type peptide TP-4 (Table 3). Binding of mutated peptides with B3F6 was maximally affected at the amino acids positions 198 (W198A, peptide AII), 201 (W201A, peptide AV), 203 (N203A, peptide AVII) or 207 (R207A, peptide AXI) by solid phase ELISA (Fig. 2). Similar results were obtained by SPR where these mutated peptides showed an approximately 100–1000-fold reduction in binding affinity to B3F6 compared to wild-type TP-4 (Table 3). Mutation I206A (peptide AX) had also slightly affected this binding in both of the assays. These results indicate that each ectodomain of band 3 was recognized by specific amino acids located at certain positions of PvTRAg38, except V1199 and N203, which bind to two loops, and R207, which interacts to all three ecto-

domains of Band 3. This one-to-one interaction indicated by the SPR data also correlates well with the erythrocyte binding activity of these mutated peptides.

Discussion

Interfering with the host–parasite molecular interaction required for parasite survival and growth can lead to the development of anti-malarial therapeutics. Therefore, the parasite molecules involved in host-cell invasion hold great importance. The human erythrocyte band 3 interacts with a number of *P. falciparum* proteins and plays an important role in host cell invasion [26–31]. Recently, we have shown that the *P. vivax* tryptophan-rich antigen PvTRAg38 [18,21,22] is involved in the host–parasite interaction, and band 3 present on the human erythrocytes also acts as one of its receptors [22]. We had also defined an 18 amino acid long P-4 peptide domain with sequence KWVQWKNDKIRSWLSEW at amino acid positions 197–214 of PvTRAg38, which interacted with three different ectodomains of Band 3. Here, we have further defined this domain to 12 amino acid residues (peptide TP-4) by alanine scanning and showed that the last six amino acids of the P-4 peptide are not essential for binding (Table 1). The binding activities of wild-type P-4 and TP-4 were almost comparable. However, changing any triplet in TP-4 caused a drastic reduction in the binding activity of this mutated peptide compared to the binding activity displayed by the mutant peptide with a single amino acid change in the same triplet. This appears to be the result of an additive effect. Furthermore, we show here that five amino acid residues of PvTRAg 38, located within the TP-4

domain, were more critical for binding to erythrocytes and were interacting differently with three ectodomains of band 3 (Fig. 2 and Table 3). An asparagine residue (N203) of this 12 amino acid domain was more critical for binding than any other amino acid residue. This is because mutation of this residue (peptide AVII) resulted in a loss of the maximum erythrocyte binding activity (Fig. 1 and Table 2). The probable reason for this could be attributed to its stronger interaction observed with two different ectodomains (loop 1 and loop 6) of band 3 protein in SPR (Table 3). Although peptide AIII with the V199A mutation also interacts with two loops (loop 1 and loop 3), its erythrocyte binding activity was affected to a lesser extent than that of the peptide AVII with the N203A mutation. This difference in erythrocyte binding activity could be attributed to the weaker interaction of this peptide (AIII) with loop 3 than loop 1 of band 3 compared to the mutated peptide AVII, which interacts strongly with both loops (loop 1 and loop 6) of band 3 (Table 3). The glutamine residue of this 12 amino acid peptide domain was interacting with loop 1 of band 3 because its mutation caused a drastic reduction in K_D values in SPR, although this did not affect erythrocyte binding activity proportionately. The other mutated peptides interacting with the single loop also showed a minimum effect on erythrocyte binding activity, except for AII (W198A), which also interacts with single loop (loop 6) but had affected the erythrocyte binding activity more than the other mutated peptides binding to a single loop. Indeed, the effect of the W198A mutation on the erythrocyte binding activity of this mutated peptide (AII) was same as in the case of the R207A mutation (peptide AVI), although the latter was interacting with all three loops. Mutant peptides where the above mentioned five amino acids were replaced with alanine showed a reduced affinity for at least one of the band 3 loops, thus accounting for the corresponding reduced red blood cell (RBC) binding. The only mutant peptide (AI) that had a reduced affinity for loop 3 only in SPR was unable to affect RBC binding.

Tryptophan residues in several proteins play important role in protein–protein interactions [32–35]. At the binding interface of the protein–protein interaction, the probability of having tryptophan residues is maximal compared to other amino acids [36]. There are a number of studies where tryptophan residues play an important role in biochemical and biological function. In *Plasmodium*, the PHIST domains of PHISTa family members contain two positionally conserved tryptophan residues, and one of them plays an important role in its interaction with host erythrocyte cytoskeletal protein band 4.1 [37]. In members of class I cytokine

receptor superfamily, the tryptophan rich motif WXXW/WSXWS is involved in various functions, including ligand binding [38]. The binding region of TP-4 of PvTRAg38 also contains the WXXW motif, which could have provided an anchoring role for its binding with band 3. In host–parasite interactions, conserved tryptophan rich motifs are involved in membrane infusion of HIV virus [39,40], SARS virus [41] and Ebola virus [42]. In the present context, we do not know whether the tryptophan residues of PvTRAg38 are involved in such a function, although we may speculate that they help the parasite to fuse with erythrocyte membrane during invasion of the host cell by the merozoite.

In the presence of the Duffy independent pathway of invasion, an effective vaccine against *P. vivax* requires a multiple epitope subunit vaccine. In this regard, the 12 amino acid region of TP-4 of PvTRAg38 could be used as part of the subunit vaccine. This is because, during the natural course of *P. vivax* infection, patients elicit good humoral and cellular immune responses against PvTRAg38 [19] and these natural antibodies inhibit the erythrocyte binding activity of this protein, as well as its peptide P-4 [18]. Furthermore, the sequence of this P-4 domain is highly conserved in the parasite population [19].

We have seen that PvTRAg38 facilitates the parasite growth in a heterologous *P. falciparum* culture system [22]. The same finding needs to be confirmed for *P. vivax* as and when a more robust culture system becomes available for this parasite. Adding this protein to the *P. falciparum* culture leads to parasite growth inhibition, which could result from PvTRAg38 binding to band 3 of the human erythrocyte, which is a receptor for merozoite proteins of this parasite [22]. Considering band 3 as a common receptor for *P. falciparum* and *P. vivax* merozoite proteins, this 12 amino acid peptide or any other similar molecule could inhibit parasite growth and be developed as a therapeutic agent. The small organic molecules or peptides that bind to a protein–protein interface and subsequently inhibit the host–parasite interaction have been developed as therapeutics for *Plasmodium* [43–46].

In conclusion, the 12 amino acid residues stretch of TP-4 of PvTRAg38 binds to erythrocyte band 3 via multiple amino acids. The critical amino acids present at the interface of the hot spot of binding would prove helpful in the design of therapeutics for malaria.

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Author contributions

YDS designed the study and wrote the paper. MSA performed solid phase assays with band 3 and its fragments, as well as rosetting assays and SPR, and wrote the paper. RKT performed the erythrocyte binding assay. SR performed SPR.

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