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Article

Targeting *Plasmodium falciparum* Invasion via Antibodies against Merozoite Surface Enolase that Binds to Band 3 of Human Red Blood Cells

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Abstract: The antibodies targeting invasive proteins of *Plasmodium falciparum* (Pf) merozoites is a clever strategy against malaria parasite. The recent finding that *Plasmodium* merozoites surface enolase is a target for growth neutralizing antibodies has generated interest in identifying its receptor (s) on erythrocytes. In the present study, several biochemical experiments were undertaken involving recombinant Pf enolase and antibody against it to search for its cognate receptor. The binding of rPfeno to erythrocytes was selectively sensitive to trypsin but resistant to neuraminidase. Failure of yeast enolase binding to human red blood cells under similar conditions indicated high specificity of parasite Pfeno. The mass spectrometric and immunological analyses of proteins obtained in pull downs and co-immunoprecipitation samples led to identification of band 3 as an interactor/receptor for *Plasmodium* enolase. Structural characterization of sequences of band 3 - rPfeno interacting regions revealed C-terminal exocytic regions of band 3 to be binding to Pfeno. Similarly, band 3 binding regions of rPfeno were also identified. Identification of receptor-ligand (band 3: Pfeno) pair paves the way for antimalarial anti-Pf enolase-based vaccine, anti-Pf enolase antibodies as drug and also for developing novel small molecule-based invasion inhibitory therapeutics including peptidomimetics that could disrupt the band 3: Pfeno protein-protein interactions.

Keywords: Malaria; *Plasmodium* enolase; Band 3; antibodies; hRBCghosts

1. Introduction

Malaria is one of the most devastating parasitic infections, associated with a huge burden of morbidity and mortality [1]. *P. falciparum*, the causative agent for the deadly disease is an obligatory intracellular parasite. In its blood stage of infection, it invades erythrocytes making erythrocyte invasion an attractive target for malaria intervention. Invasion of host cells require interaction between parasite ligands and host cell receptors. In a bid to evade challenges, *P. falciparum* dabbles in extensive functional redundancy by deploying multiple invasion pathways that use different proteins as parasite ligands pairing with different receptors on erythrocytes. Parasite proteins which act as ligands come from two major families: Duffy binding like or erythrocyte binding like (DBL/EBL) and the reticulocyte binding like (RBL) proteins [2–4]. In *P. falciparum*, there are four and five known ligands of EBL class (EBL-175, EBL-1, EBA-140 and EBA-181) and RBL class (Rh1, Rh2a, Rh2b, Rh4 and Rh5) respectively [5]. The erythrocyte receptors that *P. falciparum* engages for invasion could be placed in two categories: the ones with sialic acid (SA) and the others without SA [6–9]. Accordingly, *P. falciparum* invasion pathways are either SA-dependent (SAD) or SA-independent (SAID). Interestingly, the malaria parasite has an uncanny ability to spontaneously switch from one invasion phenotype to another presumably to circumvent the host immune response [10]. Erythrocyte Glycophorins A-C are sialylated glycoproteins that serve as receptors for EBA-175, EBA-

140 and EBL-1 respectively [7,11–13]. The other receptors that have been shown to be used by the parasite include complement receptor 1 (CR1) [14–16], basigin [17] and band 3 [18]. CR1 and basigin serve as receptors for Rh4 [4,15] and Rh5 [17] respectively. Apart from these, there are a host of other merozoite cell surface proteins that are believed to participate in the complex events of host cell invasion [5,19]. These include MSP1 that forms protein-protein complex with band 3 of erythrocyte [18], and *Pf*MTRAP that binds to erythrocyte Semaphorin 7A [20]. However, there may be many more parasite ligands that are yet to be identified [21,22]. Thus, the parasite proteins expressed on the cell surface of invasive stages (i. e. sporozoites, merozoites and ookinetes) deserve to be examined for their functional involvement in host cell invasion. *P. falciparum* enolase (*Pf*eno), a moonlighting glycolytic enzyme has emerged as one such antigen. Surface expression of this protein has been observed in all three invasive forms of the parasite [23–26]. Exposure to antibodies against recombinant *Pf*eno (*rPf*eno) shows potent growth/invasion inhibitory effect on *in vitro* blood stage cultures presumably due to the blockade of merozoite surface enolase [26–28]. Likewise, antibody blockade of ookinete surface enolase is known to bring about inhibition of the ookinete traversal across the mosquito midgut epithelium [24]. Indeed, an enolase binding protein (EBP) has been isolated from mosquito gut epithelium that acts as host cell receptor for binding to ookinete cell surface enolase [29]. Molecular mechanisms of membrane bound enolase function in merozoite invasion of erythrocyte are yet to be explored. Recent observations about the ability of a monoclonal antibody directed against a unique pentapeptide (⁻¹⁰⁴EWGWS¹⁰⁸-) epitope of *Pf*eno to bring about potent invasion inhibition [28] indicate *Pf* enolase to serve as an invasion ligand. This would imply the presence of a receptor for merozoite enolase on host cell surface. In the present study, a series of experiments were undertaken in an attempt to identify and characterize the erythrocyte receptor for *Pf*eno. Multiple binding assays were performed to affinity pull down or co-immuno-precipitate the *Pf*eno binding human red blood cells (hRBC) membrane protein (s) and identify the pulled-out proteins by mass spectrometry and western blotting. Our results indicate that band 3 of hRBC is a *Plasmodium* merozoite cell surface enolase binding protein. In the present study we have also identified the peptide sequences in *Pf* enolase and band 3 that make the band 3 -*Pf* enolase interface which seems to facilitate invasion.

2. Materials and Methods

All the chemicals used were of analytical grade. The sources of different reagents were as given: Protein A-Sepharose (Pierce), Sepharose beads coupled to glutathione (GE Healthcare , Uppsala Sweden), Yeast enolase , anti-yeast enolase antibodies (α -Yeno), anti-band 3 antibodies (α -band 3), anti-spectrin antibody (α -spectrin), anti MSP1₁₉ antibody and Integrin alpha 7 antibodies (Sigma-Aldrich), Protease inhibitor cocktail (Roche Applied Science, Indianapolis, USA), HRP conjugated anti-mouse IgG secondary antibody (Molecular Probes, NJ, USA), Vectashield-mounting medium (Vector laboratories CA, USA), Biotin-labelled peptide library (15-mer peptides with five overlapping amino acids) covering the full sequence of *Pf*eno (Mimotopes, Australia) , synthetic band 3 peptides (GenScript, USA Inc). Maxi-pro ELISA plates (Nunc), ready to use ABTS (, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid) (Sigma Aldrich, St. Louis, MO, USA), PVDF membrane (Millipore), Complete RPMI (cRPMI: RPMI with 0.5% Albumax II), Dibutyl phthalate, Iodoacetamide, Trypsin, Chymotrypsin, Neuraminidase and Coomassie stain (Sigma Aldrich). Sequencing grade Trypsin (Promega Madison, WI, USA).

2.1. Purification of recombinant *P. falciparum* enolase (*rPf*eno), preparation of Human RBC ghost, packed RBCs, and raising anti-*rPf*eno antibodies (α -*rPf*eno):

Recombinant *P. falciparum* enolase (*rPf*eno) [30,31], mouse anti-enolase antibodies (α -*rPf*eno) [31] and Human Red Blood Cell ghosts (hRBC^G) were prepared as described earlier [32]. Starting with human blood taken in heparinized tube, packed RBCs were prepared by centrifuging the sample at 14000 × g for 20 min. Cellular pellet was washed thrice with incomplete RPMI medium. The washed hRBCs were stored at 4°C and used within 48 hr after collection. The α -*rPf*eno antibody was raised in Swiss mice in the animal house facility at TIFR and obtained as described earlier [31].

2.2. Erythrocytes binding Assays:

Packed hRBCs (100 μ l) were incubated with varying concentrations of rPfeno or Yeast enolase (Yeno as control samples) in RBC binding buffer (50 mM Tris -HCl pH 8, 200 mM NaCl, 1mM EDTA, 2.5 mM $MgCl_2$, 2 mM dithiothreitol and 1% glycerol) for 2 hours (hrs) at 37 $^{\circ}$ C. Samples were centrifuged and hRBC pellet was washed twice with the binding buffer. This pellet was then layered on 100 μ l of dibutyl phthalate and centrifuged (14000 \times g, 5 min). The aqueous supernatant and the organic liquid layers were removed by aspiration. The RBC pellet was washed with phosphate buffer saline (PBS :10 mM Na-Phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4). Recombinant Pfeno bound to the hRBCs was eluted using 25 μ l of 2M-NaCl in PBS. The desalted eluate was run on 10% SDS-PAGE and the bound rPfeno / Yeno were visualized using western blot with α -rPfeno and α -Yeno antibodies respectively. In another experiment 100 μ l of packed hRBCs in PBS were treated with trypsin, chymotrypsin (0.1 and 0.5 mg/ml) and neuraminidase (25 and 66 mU/ml) for 12 hours at 37 $^{\circ}$ C. Post treatment the enzymes were inactivated by incubating with 1mM PMSF for 1 hr. Samples were centrifuged (2100 \times g, 10 min) and the pellet was washed five times with PBS containing 0.1 mM PMSF. The enzyme treated hRBCs were then used for binding assay [33] as described above. Protein samples were analyzed on 10% SDS-PAGE. Western blotting was performed to detect the bound enolase using appropriate antibodies (α -Pfeno / α -Yeno). For binding with human RBC ghosts (hRBC^G), ~100 μ g protein equivalent ghost membranes were incubated with varying amounts of rPfeno (0, 5 and 25 μ g) or Yeno (0, 5 and 100 μ g) in PBS for 1 hr at 37 $^{\circ}$ C. Post incubation, hRBC^G membranes were collected by centrifugation and washed with PBS to remove unbound rPfeno. After each wash, absorbance at 280 nm was checked and wash step was repeated till the absorbance dropped to zero. Samples were analyzed on 10% SDS-PAGE and bound rPfeno was visualized by western blotting.

2.3. Blot Overlay assay:

The blot overlay assay or Far-western blot was done in accordance with the published protocol [34]. The blot bearing hRBC^G proteins was incubated with varying concentrations of rPfeno (or Yeno as negative control) in protein binding buffer (PBB) and washed with PBST. The presence of hRBC^G protein bound enolase was detected by probing the blot with α -rPfeno or α -Yeno antibodies.

2.4. Pull Down assay:

In a scouting experiment, purified hRBC^G were solubilized using different detergents (CHAPS, NP-40 and Triton-X100) to identify the detergent for maximal extraction of hRBC^G proteome [35]. In brief, the solubilized hRBC^G proteins were incubated with purified glutathione S transferase tagged rPfeno (GST-rPfeno) for overnight at 4 $^{\circ}$ C. For pulling down the GST-rPfeno bound with hRBC^G protein(s), GSH- Sepharose 4B resin was added and allowed to incubate for 1 hr. In another set of experiments (for detection of non-specific binding), a control sample consisting of solubilized hRBC^G were incubated with the Glutathione S-Sephadex-beads (GSH-B). The glutathione beads from the two sets of samples were separated by centrifugation (2000 rpm, 2 min), six washes of PBS were given, washed beads were suspended in SDS-PAGE sample buffer, boiled and proteins in the supernatant were resolved on 10% SDS PAGE, followed by silver staining. The bands (other than GST-rPfeno) were cut and processed for protein identification by mass spectroscopy.

2.5. Immuno-precipitation (IP) and reverse IP using Protein-A (PrA):

Protein A beads (PrA-B) (20 μ l) were incubated with 2 μ l α -Pfeno polyclonal antibody (1:200) for 6-8 hrs at room temperature (RT) and PrA- α -Pfeno beads were washed six times with PBS to remove unbound α -Pfeno. In a separate set, solubilized hRBC^G proteins were incubated with rPfeno (in PBS, pH 7.4) overnight at 4 $^{\circ}$ C. The solubilization was performed using solubilization buffer (20 mM Tris HCl pH-8, 137 mM NaCl, 10% glycerol and 1% NP-40 in presence of protease inhibitor cocktail). PrA- α -Pfeno beads prepared as above were added to the solubilized hRBC^G -rPfeno sample and incubated for 2 hrs at RT. After incubation, beads were washed with PBS and collected by

centrifugation (2000 rpm, 2 minutes) and then analyzed for rPfeno-bound proteins on 10% SDS-PAGE. Western blotting was done using α -band 3, α -Spectrin and α -integrin alpha-7 antibodies. In reverse IP experiments, 20 μ l of PrA-B were pre-incubated with antibodies against band 3/ Spectrin / Integrin alpha-7. These pre-incubated PrA-B were then mixed with solubilized hRBC^G proteins that were pre-incubated with rPfeno overnight at 4°C and for 2 hr at RT. The PBS washed beads were collected by centrifugation, solubilized with Laemmli buffer, run on 10% SDS-PAGE and transferred to a blot which was then probed using α -Pfeno to detect rPfeno bound hRBC^G proteins. In an IP coupled with blot-overlay experiment, the band 3 protein from hRBC^G extract was pulled out using PrA- α -band 3 as antibody-antigen complex (PrA- α - band 3-band 3). The beads were washed and mixed with Laemmli buffer, run on 10% SDS-PAGE and proteins were transferred to a blot. This was followed by overlay with rPfeno. The washed blot was then probed with α -Pfeno to confirm direct binding of immuno purified and immobilized band 3 with purified rPfeno.

2.6. Two-dimensional Gel Electrophoresis (2DE) of hRBC ghost and blot overlay:

hRBC^G (120 μ g protein) were solubilized in 100 μ l of rehydration buffer (7M urea, 2M thiourea, 2% CHAPS, 50 mM DTT and 2% IPG buffer). The IPG strips (11 cm, 4 - 7 pH) were hydrated overnight and used for iso-electric focusing (IEF) with the following program, step 1: 250 V for 30 mins, step 2: 4000 V for 1hr and final step 3: 15000 V hrs. The strips were equilibrated with 2% DTT followed by 2.5% IAA (Iodoacetamide) (10 minutes each) before proceeding to second dimensional 10% PAGE. Three replicate gels were used for this sample. First gel was silver stained to profile the rehydration buffer solubilized proteome of hRBC^G. Second gel was used for a western blot using α - band 3 to visualize band 3 and its variants. Third gel was used to blot the proteins on to a PVDF membrane, followed by blocking and overlay with rPfeno (10 μ g) in protein binding buffer (10 ml) overnight at 4°C, washing and then probing with α -Pfeno. The method used for blot overlay with rPfeno followed by western blot was the same as described above.

2.7. In-gel Digestion of 2DE protein spots and Mass spectrometric analysis:

Spots corresponding to rPfeno binding proteins observed in gel overlay assay were excised from silver-stained gel and In-gel digestion was carried out using trypsin [36]. The gel pieces were treated with 25 mM ammonium bicarbonate (ABC): acetonitrile (ACN) 1:1 v/v). This was followed by reduction using 10 mM DTT at 56 °C for 1 hr and alkylation with 55 mM iodoacetamide at room temperature for 45 mins. Gel pieces were washed with 25 mM ABC and dried in vacuum evaporator. Finally, trypsin (400 ng) was added to gel pieces and the mixture was incubated for 16 hrs at 37°C for digesting proteins to peptides. The peptides were eluted with 70% ACN containing 5% formic acid. Eluted solutions were dried in speed-vac evaporator at 30°C. Peptides were dissolved in 0.1% formic acid (5 μ l). Extracted peptides were analyzed on a Nano LC-ESI-MS/MS (Agilent 6250-Q-TOF) as described elsewhere [37]. Briefly, the extracted peptides dissolved in 0.1 % formic acid were applied to an Agilent HPLC chip (G4240-62002) which had a compact 40 nl C18 RP enrichment column and a 75 μ m x 150 mm separation column that was packed with Zorbax 300 SB-C18 (5 μ m) resin. Mobile phase solvents used were, solvent A: 0.1% formic acid in water and Solvent B: 0.1% formic acid in 90% ACN. After injection of the sample, column was washed with a 3 -12% gradient of solvent B for 3 min and the peptides were eluted with linear gradients of varying slopes, viz. 12-60% B for 3 to 23 min, and 60-95% B from 23 to 27 min. The mass spectrometric analysis was performed in extended dynamic mode in 50-1700 m/z range with active exclusion 0.5 min after each MS/MS spectrum. The scan rates (spectra/sec) for MS and MS/MS were 3.66 and 2.19 respectively. The acquired data were analyzed using Mass hunter software (Agilent technologies version B.07.00). For database search Mascot (Matrix sciences version 2.3.2) in-house server was used. The mascot generating files (mgf) were submitted to updated NCBI and Swiss-Prot database with parameter taxonomy set as *Homo sapiens*, fixed modification carbamidomethyl (cysteine), missed cleavage max 2, peptide mass tolerance 50 ppm and MS/MS mass tolerance of 0.6 Da.

2.8. *P. falciparum* and *P. berghei* parasite preparations:

P. falciparum 3D7 were maintained in culture. Briefly, human blood from healthy adults was collected in acid citrate dextrose (ACD) as the anticoagulant. After removing the leukocytes, the erythrocytes were washed and suspended in RPMI with 0.5% Albumax (cRPMI). Parasites were maintained at 5% haematocrit in cRPMI at 37°C in a humidified chamber containing 5% CO₂. The ring stage synchronized *P. falciparum* culture was allowed to grow for 48 hrs at which time when the egressed merozoites would be maximum in the medium, the merozoites from the supernatant were harvested (at a parasitemia of ~ 5%) by centrifugation (1500xg, 10 min), and used for binding experiments. *P. berghei* ANKA infected RBCs (~10⁶ cells) were injected into Swiss mice intraperitoneally. Parasitemia was monitored every day and when it reached to ~40%, infected blood (1-2 ml) was collected in equal volume of ACD solution. For the preparation of merozoites, blood samples were centrifuged and cellular pellet was washed thrice with cRPMI. Cell pellet was suspended in 60% ice cold percoll and spun at 1500xg in a swing-out rotor at 4°C for 15 min. Cellular layer at the interphase of aqueous and percoll media was collected. The infected RBCs in this layer were rich in schizonts and trophozoites. The interphase layer cells were repeatedly passed through a 25-gauge needle to release merozoites. Cellular debris from this suspension was removed by centrifugation at 600xg and the supernatant was centrifuged at 18,000xg for 15 min. to pellet down the merozoites [38]. Both the *P. falciparum* and the *P. berghei* merozoite pellets were washed with PBS and suspended in 100 µl of non-denaturing lysis buffer (20 mM Tris-HCl, pH 8 containing 137 mM NaCl, 10% glycerol, 1% NP-40 and protease inhibitor cocktail) and incubated on ice for 30 min. Suspension was centrifuged at 20,000xg (15 min, 4°C) and supernatant was used as merozoite cellular extract. These native parasite preparations and hRBC ghost preparations (as the source for band 3 protein) were used for blot overlay, IP and reverse IP experiments.

2.9. ELISA for monitoring protein-protein interaction:

A library of biotin labelled fifteen-mer peptides (overlapping by five amino acids) covering the entire sequence of *Pfeno* {Accession No. Q27727, purchased from Mimotopes (Australia)} was immobilized on a streptavidin coated ELISA plate. The purity of the peptides was > 80% and the amino acid composition of each peptide was confirmed by mass spectrometric analysis. The custom ELISA plates consisted of 2 wells (wells 89 and 90) coated with control peptides. The methodology followed for ELISA was according to manufacturer's protocol. In brief the *Pfeno* peptide coated wells were blocked with sodium caseinate (1%w/v) for 2 hrs at 22°C, washed with PBST (phosphate buffer saline containing 0.05% Tween 20, pH 7.4) followed by incubation with solubilized hRBC^G proteins at 37°C. Plates were developed with α -band 3 monoclonal antibody (1:5000). This was followed by addition of HRP conjugated mouse secondary antibody at a dilution of 1:1000 and incubation at room temperature for 45 min. The solution was discarded, and wells were washed thrice with PBS. Ready-to-use ABTS substrate (100 µl) was added. The color was developed for 10-15 min and the OD was measured at 405 nm on a Tican Plate Reader. ELISA values for control peptides were subtracted from value of each well before plotting the data.

For the converse experiment, i. e. to monitor the r*Pfeno* - band 3 peptide interaction, the ELISA plate wells were coated with different synthetic peptides representing various regions of band 3. Binding of these peptides to r*Pfeno* was probed by co-incubating with 1 µg of purified r*Pfeno* in PBS (100 µl) for 6 hrs at 37°C. The wells were washed with PBST followed by incubation with α -*Pfeno* (1:2000) for 1 hour, washing three times with PBST, adding HRP conjugated mouse secondary antibody (1:1000) into the wells and incubating it at room temperature for 45 minutes. The supernatant was discarded, wells were washed with PBS and ready to use ABTS (200 µl) added into each well. The color was developed for 10-15 min. and absorbance was measured at 405 nm on a Tican Plate Reader.

2.10. Molecular Modelling:

The UniProt database was used to obtain the amino acid sequence of *Pfeno* (UniProt Accession no. Q27727; 446 residues). The structure was visualized using PyMOL 2.1.0. The 3D structure of *Plasmodium falciparum* enolase (*Pfeno*) was modelled using the crystal structure of *Toxoplasma gondii*

enolase 1 (PDB 3OTR) [39] as a template, with the help of the homology modeller SWISS MODEL (<http://swissmodel.expasy.org/>). *Toxoplasma gondii* enolase 1 was used for modelling since it shows >90% sequence identity with *Pfeno*.

3. Results:

3.1. Binding of *rPfeno* to hRBCs and hRBC ghosts:

To test for the binding of *rPfeno* to human red cells, *rPfeno* was incubated with hRBCs and the washed red cells were then run on SDS-PAGE. Western blot of such a gel was probed with α -*rPfeno* (Fig 1A). Lanes 2-4 showed a concentration dependent binding of *rPfeno* to hRBCs. The ligand concentration dependent binding isotherm for *rPfeno* showed a typical hyperbolic saturation response with increasing amounts of *rPfeno* (Fig. 1B) suggesting that binding is most likely to a specific receptor.

Specific nature of this binding was further supported by the failure of yeast enolase to bind to the erythrocytes under similar experimental conditions (Fig. 1C). Effect of prior treatment of hRBCs with trypsin and chymotrypsin on binding of *rPfeno* revealed that treatment with low concentrations of trypsin eliminated the *rPfeno* binding (Fig 1D, lanes 3 and 4) while it required high concentrations of chymotrypsin to observe a similar effect (Fig. 1D, lanes 5 and 6). A negative control showing that hRBCs did not have any cross-reacting proteins to α -*rPfeno* is shown in lane 1 (Fig.1D). Untreated hRBCs incubated with *rPfeno* showed the normal binding as expected (positive control lane 2). The Western blots (Fig. 2) show the specificity of direct binding of *rPfeno* to hRBC^G.

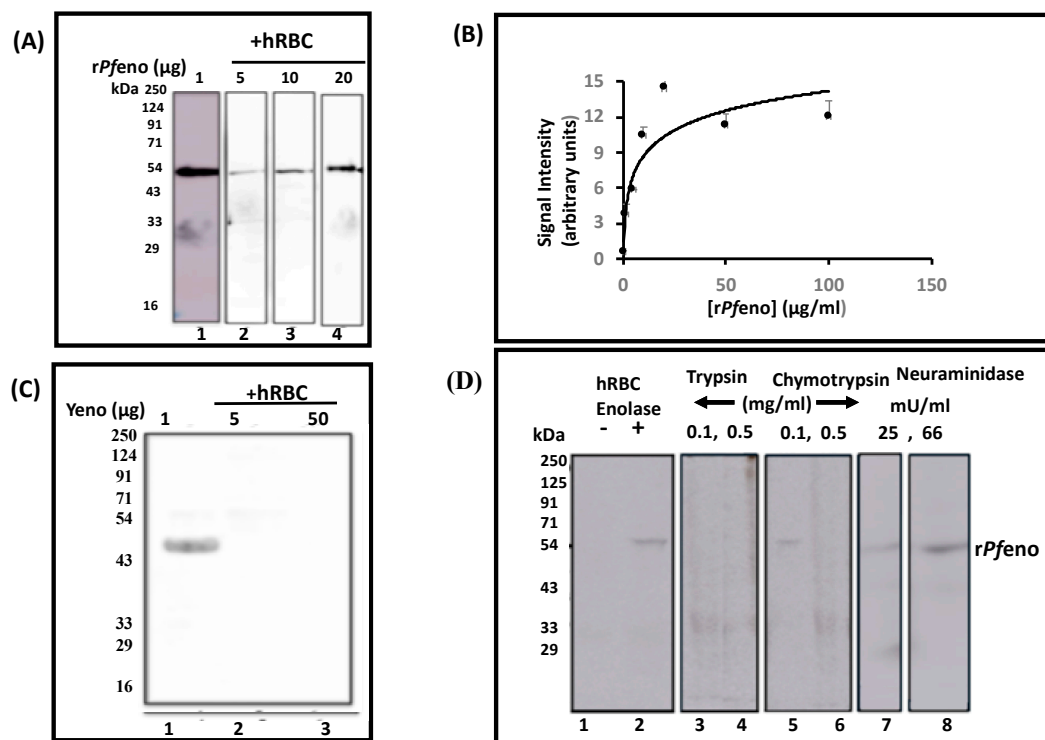


Figure 1. Binding of *Pf* enolase (*Pfeno*) with intact hRBCs: **(A)** 100 μ l packed hRBCs were incubated with various concentrations of recombinant *Pfeno* (*rPfeno*). Following washing with PBS, bound *rPfeno* was eluted with NaCl (2M) and analyzed on a 10% SDS-PAGE and western blots were performed using α -*rPfeno* antibodies: lane 1: Purified *rPfeno* alone; lanes 2-4: eluted samples where 5, 10 and 20 μ g respectively of *rPfeno* was incubated with hRBCs. **(B)** Variation in bound *rPfeno* as detected by western blot with increasing concentration of *rPfeno*. **(C)** Western blots of Yeno with hRBCs., lane 1: pure Yeno (1 μ g); lanes 2 and 3: eluted samples where Yeno (5 and 50 μ g) was incubated with hRBCs and then probed against α -Yeno. **(D)** Effect of neuraminidase and protease treatment of hRBC on binding of *rPfeno*. Western blots of *rPfeno* eluted samples. Lane 1: hRBC eluate

where no rPfeno was added; In remaining samples (lanes 2-8), eluted samples from variously treated 100 μ l hRBCs were incubated with 15 μ g rPfeno. Lane 2: untreated; lanes 3 and 4: pre-treated with trypsin; lanes 5 and 6: pre-treated with chymotrypsin, lanes 7 and 8: pre-treated with neuraminidase.

The concentration dependent interaction of parasite enolase with the ghost membranes was evident from the greater binding of rPfeno in 25 μ g/ml sample as compared to the 5 μ g/ml sample (Fig. 2A). In a control sample where rPfeno was replaced with yeast enolase and the western blot was probed with α -yeast enolase antibody (α -Yeno), there was no detectable binding of Yeno to hRBC even at a concentration as high as 100 μ g/ml (Fig. 2B).

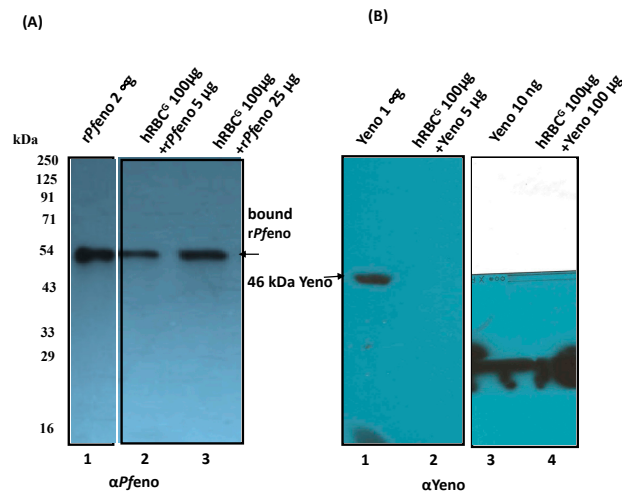


Figure 2. Specificity of direct binding of rPfeno to hRBC ghosts (hRBC^G) proteins. hRBC^G (100 μ g protein) were used in each sample. hRBC^G were incubated with rPfeno (or Yeno), pelleted by centrifugation, washed and analyzed on a 10% SDS-PAGE. (A) Western blot of a gel visualized using α -rPfeno. Lane 1: 2 μ g rPfeno (control); lanes 2 and 3: hRBC^G incubated with 5 and 25 μ g rPfeno respectively. (B) Western blot of Yeno samples visualized using α -Yeno. Lanes 1 and 3: 1 μ g and 10 ng Yeno (controls). Lanes 2 and 4: samples where hRBC^G were incubated with 5 and 100 μ g of Yeno respectively. Absence of Yeno in lanes 2 and 4 indicates failure of Yeno to bind to hRBC^G. Arrows mark the position of rPfeno and Yeno protein bands.

3.2. Pfeno interacting membrane proteins in hRBC:

Blot overlay assay (Method section - 2.3) was used to identify the likely interactors of rPfeno in hRBC^G proteins. hRBC^G proteins separated on a gel were allowed to interact with rPfeno and binding was detected by western blot analysis using rPfeno. Fig. 3A (lane 2) shows the profile of proteins in hRBC^G proteome. Western blot of a replicate of lane 2 did not show any cross reactivity with α -rPfeno (Fig. 3A, lane 4, a negative control) while it was able to detect purified rPfeno (Fig 3A, lane 3, a positive control). Results showed the binding of rPfeno to hRBC^G proteins at molecular masses ~91, 65, 48, 35 and 25 kDa (Fig. 3B, lane 2; marked with arrowheads). Further, similar experiment with yeast enolase yielded negative (no binding) results (Fig. 3C, lanes 2 and 3) indicating that binding of rPfeno to hRBC^G proteins was specific to parasite enolase.

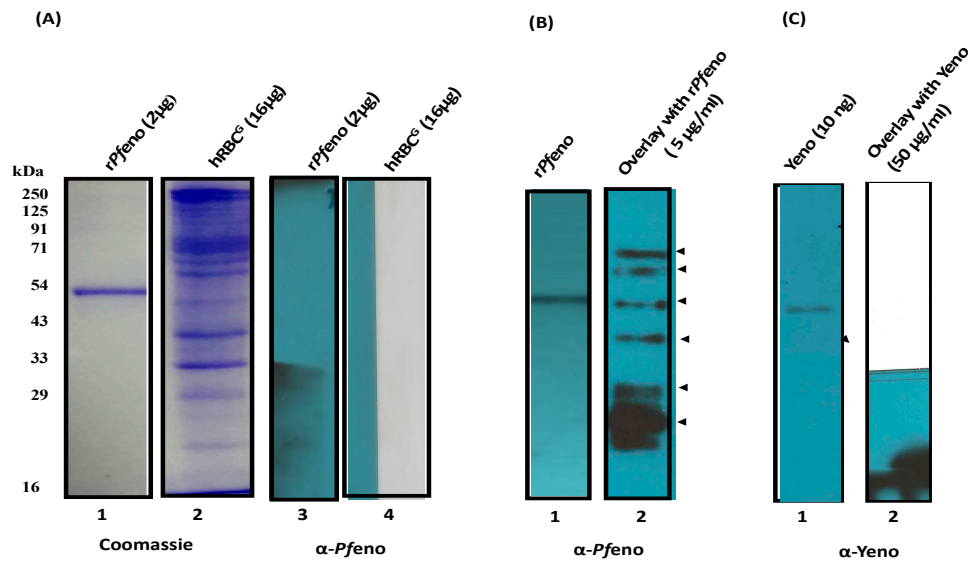


Figure 3. Blot-overlay assays to identify potential interactor(s) of Pfeno among hRBC membrane proteins. 16 μ g hRBC^G membrane proteins were separated on 10% SDS-Page. **(A)** Coomassie stained gel. Lane 1, rPfeno; lane 2, hRBC^G membrane proteins. Lanes 3 and 4: Western blot of replicates of lane 1 and 2 probed with α -rPfeno. **(B)** Blot of hRBC^G proteins was incubated with 5 μ g/ml (lane 2) rPfeno. After washing, α -rPfeno antibody was used to detect bound rPfeno. **(C)** Lane 1, 10 ng of Yeno, lane 2, blot of hRBC^G proteins was incubated with 50 μ g/ml of Yeno and anti-Yeno (α -Yeno) was used for the detection of bound Yeno.

3.3. Molecular Identification of rPfeno binding proteins in hRBC ghosts:

GST-tagged rPfeno was immobilized on GSH-B and used to pull-down rPfeno binding protein(s) from hRBC^G extracts. A silver-stained gel of the hRBC^G proteins captured using different detergents is shown in Fig. 4A (lanes 2 - 4). Lane 5 shows a control sample where GSH-Beads (GSH-B) were incubated with hRBC^G extract to determine any non-specific adsorption of proteins by the beads. The bands marked with rectangular boxes (A-F) shown in various detergent solubilized hRBC^G pull-down samples represented the potential Pfeno binding proteins. The proteins identified in various bands using Mass spectrometry are listed in Table 1 along with their Protein scores. Two major proteins identified in these bands were spectrin and band 3. Interestingly, two other proteins ankyrin and integrin- α 7 were also detected. This was understood in the background of the fact that for the binding of merozoite cell surface enolase with a receptor on hRBC, it would be essential that binding domain of the receptor be exposed on the cell surface. Among the above mentioned four proteins, it is interesting to note that band 3 is the only one that has an extracellular domain [18,32]. Ankyrin and spectrin are peripheral membrane proteins associated with cytoskeletal structures and are likely to be captured in pull-down along with band 3.

In a separate experiment a pull-down assay was performed on a Triton-X100 solubilized sample of hRBC^G that were pre-treated with alkaline solution to eliminate peripheral proteins [33]. Lane 1 in Fig. 4B shows the protein profile of alkali treated hRBC^G proteome. For the pull-down, immobilized GST-rPfeno was incubated with the solubilized extract of alkali treated hRBC^G. A 91 kDa protein bound to the parasite enolase was detected in this assay (Fig. 4B, lane 3). Sepharose beads (without rPfeno) did not show any non-specific binding in hRBC^G extract (Fig. 4B, lane 2; negative control). A western analysis of the three samples using α -band 3 to probe the blot showed the presence of band 3 in hRBC^G extract (Fig. 4C, lane 3) as well as in the pull-down sample (lane 1). Thus, in this assay where peripheral membrane proteins were removed from hRBC^G, only band 3 was identified as an interactor of Pfeno (Fig. 4C). The major protein band in the pull-down sample was of ~91 kDa besides which some low molecular weight proteins were also identified by band 3 antibody.

The pull-down protein profiles in samples solubilized using different detergents (CHAPS, NP-40 and Triton X100) showed variation in the isoforms of band 3 captured by *rPfeno* indicating differential solubilization properties of each detergent (Fig. 4A, Table 1). CHAPS and NP-40 solubilized samples yielded lower molecular mass (25 and 48 kDa) variants of band 3 while Triton-X100 solubilized sample captured band 3 at ~91 kDa. Results presented in Fig. 4B and 4C provide antibody-based identification of band 3 as the most likely candidate for *Pfeno* binding to hRBC.

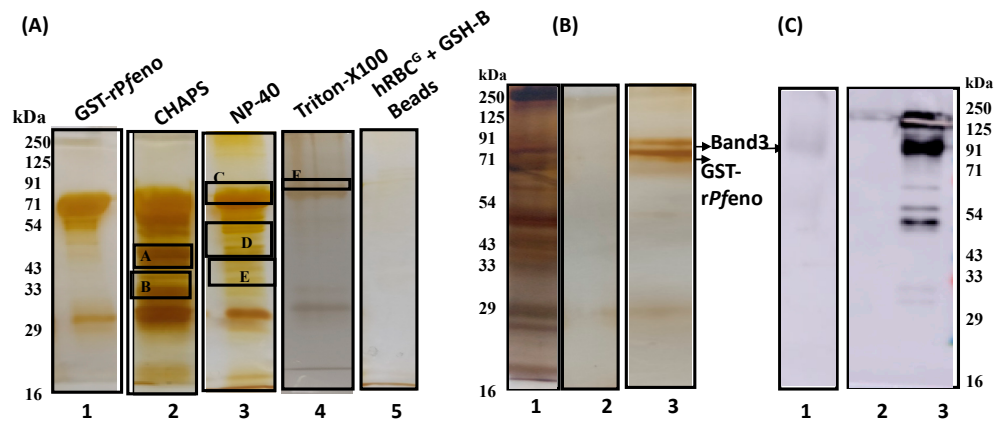


Figure 4. Pull down assay for capturing *rPfeno* binding protein(s) in solubilized hRBC ghost. **(A)** Detergent solubilized hRBC^G proteins were incubated with GST-tagged *rPfeno* immobilized on GSH beads (GSH-B). Post washing, the supernatants of Laemmli buffer treated bead bound protein(s) were analyzed on 10% SDS-PAGE (silver stained). Lane 1: purified GST-tagged *rPfeno*-GSH-B (GST-*rPfeno*). Profile of *rPfeno* bound hRBC^G proteins solubilized with CHAPS (lane 2); NP-40 (lane 3) and Triton-X100 (lane 4). Lane 5: GSH-B incubated with hRBC^G protein extracts to identify any non-specific interaction with GSH-B. Bands marked with rectangular boxes (A-F) were cut out and proteins identified using mass spectrometry. Note the absence of any bands in lane 5 indicating absence of non-specific interactions with GSH beads. Results of proteins identified are presented in Table 1. **(B)** Silver stain of hRBC^G treated with 0.1 M NaOH for removal of peripheral membrane proteins. The ghosts were then solubilized in Triton-X100, and a pull-down assay was performed. Lane 1: alkali treated hRBC^G; lane 2: GSH-B incubated with alkali treated hRBC^G (control); lane 3: GSH-B incubated with alkali treated hRBC^G extract + GST-tagged *rPfeno*. **(C)** Western blot using monoclonal α -band 3 antibody of three lanes from (B). Note the presence of band 3 in hRBC^G extract (lane 3) and pull-down of band 3 with *rPfeno* (lane 1). Lack of band 3 in lane 2 indicates absence of non-specific binding of hRBC^G extract proteins with beads. The arrow indicates the position of pull-down band 3. Various protein bands were sliced out from the gel lanes shown in Fig. 4A. These were subjected to trypsin digestion and the extracted peptides were analyzed using LC-MS/MS. Major proteins identified are listed in Table 1.

Table 1. Mass spectrometric analysis of *rPfeno* interactor proteins in different detergent solubilized hRBC^G. Various protein bands were sliced out from the gel lanes shown in Fig. 4A. These were subjected to trypsin digestion and the extracted peptides were analysed using LC-MS/MS. Major proteins identified are listed below.

S.No.	Detergents	Protein Bands	Proteins Identified	Protein Score
1	CHAPS	A	Spectrin	49
		B	Band 3	81
		C	Spectrin	51
2	NP-40	D	Band 3	71
		E	Band 3	83

3	Triton X100	F	Band 3	89
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3.4. The band 3 of human erythrocytes interacts with rPfeno:

The co-immuno-precipitate obtained using Protein A Beads (PrA-B) bound to α -Pfeno (PrA-B- α Pfeno) along with the two control samples was analyzed on SDS-PAGE [Fig. 5A(i)]. A western blot of the gel probed with α -band 3 showed three different forms of band 3 in hRBC^G detergent extract (Fig. 5 A(ii) lane 1). A control sample where PrA-B were incubated with hRBC^G extract (lane 2) indicated that agarose beads did not bind any band 3 protein. In the sample where hRBC^G extract was incubated with rPfeno, prominent bands were observed at ~64 and 91 kDa [Fig. 5A (ii) lane 3]. In the PrA-B- α Pfeno system, the pull-down of band 3 with anti-rPfeno antibody implied the formation of a 'band 3-rPfeno complex'. Similarly, α -band 3 antibody was immobilized on Protein A beads (PrA-B- α -band 3) and used to pull-down band 3 protein from a pre-incubated mixture of solubilized hRBC^G and rPfeno. Further the western blot analysis of co-immuno-precipitate (probed with α -Pfeno) showed the presence of rPfeno at ~50 kDa [Fig. 5B (ii) lane 3]. As expected, there were no α -Pfeno cross-reacting proteins in hRBC^G (lane 1, control) and absence of any non-specific binding of rPfeno to PrA-B (lane 2, control). Also, the Western blot analysis of a pull-down sample obtained using α -rPfeno antibody bound Protein-A beads and probed with α -Spectrin antibody did not show spectrin in co-immunoprecipitate (data not shown). The pull-down sample was analyzed on SDS-PAGE. The Coomassie staining of the gel showed protein bands at molecular masses of 25, 64 and 91 kDa [Fig. 5C (i) lane 2]. A blot of this immunoprecipitated sample was over laid with rPfeno and subsequently probed to detect the bound rPfeno using α -Pfeno antibody. The rPfeno showed specific binding to all three isoforms of the band 3 [Fig. 5C (ii) lane 2 marked with arrowheads]. Protein bands at 91 and 64 kDa showed more pronounced binding of rPfeno as compared to the 25 kDa form of band 3 protein. Co-immunoprecipitation of rPfeno and band 3 in the reciprocal pull-down assays revealed direct interaction of rPfeno with band 3 providing unequivocal evidence for the direct interaction of parasite enolase to band 3 protein on human RBC plasma membrane.

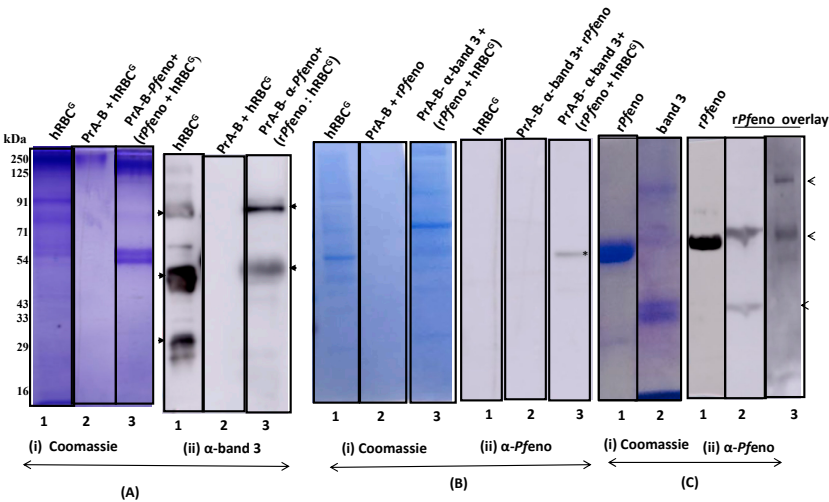


Figure 5. Co-immuno-precipitation of rPfeno and band 3 from solubilized extract of hRBC ghost (hRBC^G) incubated with rPfeno. **(A)** hRBC^G were solubilized in detergent and the extracted proteins were incubated with rPfeno allowing the formation of rPfeno-interactor complex. For co-precipitation, α -rPfeno or α -band 3 was first immobilized on PrA forming PrA-B- α -rPfeno or PrA-B- α -band 3. Two sets of beads were incubated in two separate samples of hRBC^G + rPfeno. **(i):** Coomassie stained gel; lane 1, detergent extracted hRBC^G proteins; lane 2, PrA beads incubated with hRBC^G extract (control); lane 3, PrA-B- α -rPfeno incubated with hRBC^G and rPfeno sample. Proteins bound to the beads were directly eluted in gel loading buffer. **(ii)** Western blot of gel in (i) probed with α -

band 3. Lanes 1 and 3 are positive for band 3. **(B)** Pull-down of band 3 protein from solubilized extract of hRBC^G and rPfeno sample. PrA- α -band 3 was used to pull down band 3 along with the associated proteins. The beads were separated from hRBC^G and rPfeno sample, washed and bound proteins were analysed. **(i)** Coomassie stained gel and **(ii)** Western blot was probed with α -Pfeno. Lanes 1 and 2 did not show any reactivity to α -Pfeno while lane 3 was positive (rPfeno band marked with *) indicating association with hRBC^G band 3. **(C)** **(i)** Coomassie stained rPfeno (lane 1) and PrA-B- α -band 3 pull down band 3 from hRBC^G extract. (lane 2) **(ii)** Lanes 1 and 3 are a blot of lanes 1 and 2 from Fig 5C (i); lane 3 is a pull down of band 3 from the second independent sample of hRBC^G. All three lanes were overlaid with rPfeno and probed with α -Pfeno. Lanes 2 and 3 reflect the observed variability in band 3 pattern in different hRBC^G samples.

3.5. Native enolases from *P. falciparum* and *P. berghei* merozoites bind to hRBC band 3:

Since all the earlier binding studies were carried out with recombinant Pfeno protein, we wanted to confirm whether native *Plasmodium* enolase also interacts with hRBC band 3 protein. Hence blot overlay and pull-down experiments were carried out using *P. falciparum* culture supernatant (as a source of *P. falciparum* merozoites) and *P. falciparum* infected RBCs (iRBC). Figure 6, Panel A, lane 2 shows a blot of hRBC ghost preparation, overlaid with merozoite extract, and then probed with α -Pfeno antibodies. The bands that light up are shown with arrows and some of them correspond to degradation products of band 3 protein. In lane 3 we show a pull-down experiment using Protein A beads coupled with α -band 3 antibodies, incubated with *P. falciparum* iRBC extract. The pulled-down proteins were run on a 10% SDS-PAGE and the Western blot was probed with α -band 3 antibodies. The band 3 protein was indeed seen to be pulled down from the *P. falciparum* iRBC extract. This pull down and the blot overlay showed band patterns similar to each other and was commensurate with canonical human band 3 protein fragments on SDS-PAGE. Lane 1 is a control lane to show that the α -Pfeno antibodies reacted with rPfeno protein specifically. Our *P. falciparum* cultures typically had a parasitemia of about 5-10%, and we realized that an iRBC preparation would contain uninfected RBCs as well. Therefore, the band 3 protein pull down in lane 3 could be due to pull down of the protein from uninfected RBCs. In order to ascertain whether band 3 protein did interact with native Pfeno present in iRBC, pull-down experiments were carried out with Protein beads coupled to α -band 3 antibodies, incubated with *P. falciparum* iRBCs, which were probed with α -Pfeno antibodies (Fig. 6, Panel B). The α -band 3 antibodies did pull-down Pfeno from *P. falciparum* iRBCs as observed in lane 3. A similar result was observed when the rodent malaria parasite *P. berghei* infected RBCs were incubated with protein A beads coupled with α -band 3 antibodies (Panel B, lane 4). Lane 1 shows the molecular weight markers labelling the blot, while lanes 2 and 5 are controls to show that α -Pfeno antibodies are specific, since they react with rPfeno, but not with proteins from uninfected RBCs.

It is envisaged that the human RBC band 3 protein is a relevant binding partner for parasite enolase present on the merozoite surface. Since the *P. falciparum* culture did not provide us with adequate amounts of purified merozoites, we decided to use *P. berghei* merozoites purified as described in the Methods section. Protein profiles of the merozoite (M, lane 1) and hRBC^G extracts (G, lane 2) are shown in a Coomassie stained gel in Fig. 6C. Western blots of M and G preparations were made and probed with α -MSP-1₁₉, α -Pfeno and α -band 3 (Fig. 6C lanes 3, 5 and 7). Absence of band 3 (lane 5) and the presence of MSP-1₁₉ (lane 3) and enolase (lane 7) in these blots validated the merozoite preparation. The cross-reactivity of our α -Pfeno antibodies to murine *Plasmodium* parasites has been demonstrated by us earlier. Immunoblots of hRBC^G proteins showed no cross reactivity with α -MSP-1₁₉ (Fig 6C, lane 4) or α -Pfeno (Fig. 6C, lane 8), but as expected, showed positive signals when probed with α -band 3 (Fig 6C, lane 6). In pull-down experiments, Pfeno was pulled out from *Pb* merozoite extract by incubation with PrA-B- α -Pfeno. The beads (PrA-B- α -Pfeno-Pfeno) were then incubated with hRBC^G extract. Presence of band 3 in this pull-down was detected in a Western blot probed with α -band 3 (Fig. 6 Panel D, lane 2). Conversely, PrA-B- α -band 3 was used to pull down band 3 from hRBC^G extract as PrA-B- α -band 3-band 3 complex and then incubated with merozoite extract. Presence of band 3 protein in this pull-down was detected in a Western blot probed with α -

Pfeno (Fig. 6 Panel D, lane 4). The possibility of non-specific binding of band 3 or *Pbeno* proteins to PrA-B was ruled out by incubating PrA+ merozoite extract + hRBC^G extract and probing with α -band 3 antibodies (lane 1); PrA-B- α *Pfeno* + hRBC^G ghost extract and probing with α -band 3 antibodies (lane 3) and PrA + merozoite extract and probing with α -*Pfeno* antibodies (lane 5). These controls showed an absence of nonspecific adsorption of band 3 or *Pbeno* on PrA beads under the experimental conditions used here. The sample where PrA- α *Pfeno*-*Pbeno* incubated with hRBC^G extract was probed with α -band 3 showed two bands at ~25 and 65 kDa (Fig. 6D, lane 2) indicating the binding of band 3 to immobilized *Pbeno*. The blot where PrA-B- α -band 3 was incubated with merozoite extract and probed with α -*Pfeno*, showed an enolase band at ~50 kDa and a proteolyzed product at a lower molecular weight (Fig. 6D, lane 4). Presence of band 3 in lane 2 and *Pbeno* in lane 4 (marked by arrows) indicated the formation of PrA-B- α -band 3-band 3-*Pbeno* and PrA-B- α -*Pfeno*-*Pbeno*-band 3 complexes in the two samples. Observation of low molecular weight forms of *Pbeno* (Fig 6D lane 4) and band 3 (Fig 6A lane 3) are due to possible proteolysis of native forms. The experiments shown in the Figure 6 confirm the binding of native *Plasmodium* enolases to human band 3 protein.

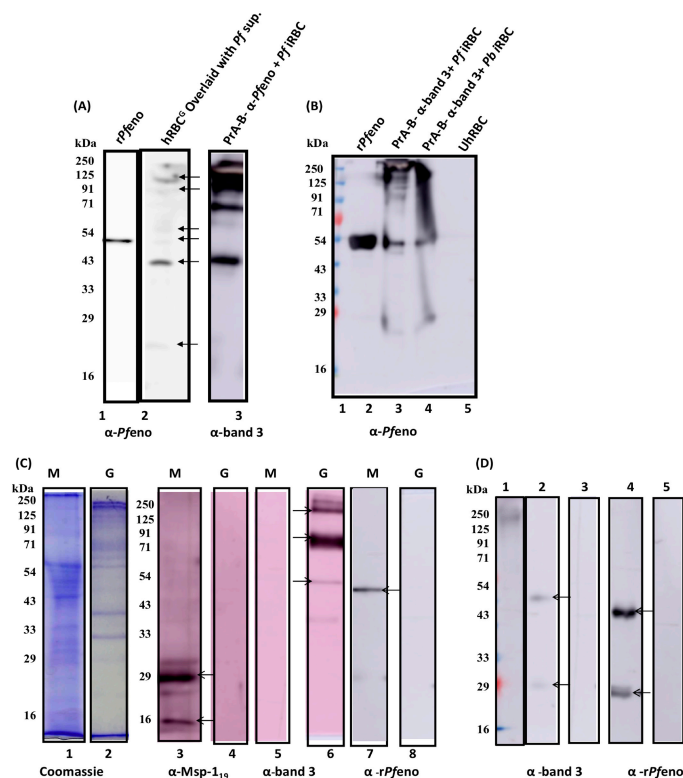


Figure 6. Binding of native *Plasmodium* enolase to band 3 protein. (A) Blot-overlay assay: Lanes 1 and 2 contain 1 μ g rPfeno and 16 μ g hRBC ghost preparation (hRBC^G) run on a 10% SDS-PAGE, the corresponding blot overlaid with supernatant from *P. falciparum* culture, and then probed with α -Pfeno antibodies. Arrows mark the position of hRBC^G proteins which interact with native *P. falciparum* enolase. Lane 3: Western blot of a pull-down assay using Protein A beads coupled with α -band 3 antibodies incubated with *P. falciparum* infected RBC (iRBC), probed with α -band 3 antibodies. (B) Pull down experiment using *P. falciparum* and *P. berghei* iRBC. Protein A beads were bound to α -band 3 antibodies and then incubated with *P. falciparum* (Lane 3) and *P. berghei* (Lane 4) iRBC, washed and run on 10% SDS-PAGE and then probed with α -rPfeno antibodies. Lane 5 shows the control of the pull-down experiments using uninfected RBC (uRBC) extract. Lanes 1 and 2 contain molecular weight markers and 1 μ g rPfeno protein, respectively. (C) Lanes 1 and 2 show protein profiles of 20 μ g *P. berghei* merozoite extract (M) and 16 μ g of solubilized hRBC^G (G), run on 10% SDS-PAGE and stained with Coomassie Blue. Blots of the same samples were probed with α -MSP-119 (Lanes 3 and 4), α -band 3 (Lanes 5 and 6) and α -Pfeno (Lanes 7 and 8) antibodies, respectively. Arrows mark the position of each protein (and variants arising due to proteolysis). α -MSP-119 and α -Pfeno

antibodies were specific and exhibited no cross reactivity with hRBC^G proteins (Lanes 4 and 8). Similarly, α -band 3 antibodies showed no cross reactivity with the *P. berghei* merozoite extract (Lane 5). (D) Pbeno protein was pulled down from *P. berghei* merozoite extract by incubation with Protein A beads (PrA-B) bound to α -Pbeno antibodies. The beads (PrA-B- α -Pbeno-Pbeno) were then incubated with solubilized hRBC^G extract. Presence of band 3 protein in this pull down was detected in a Western blot probed with α -band 3 antibodies (Lane 2). Reciprocally, PrA-B- α -band 3 was used for pulling down band 3 protein from hRBC^G extract, and the resultant PrA-B- α -band 3-band 3 complex was then incubated with *P. berghei* merozoite extract. The Western blot analysis of PrA-B- α -band 3-band 3 interactor proteins, using α -Pbeno antibodies, showed the presence of enolase (Lane 4). The possibility of non-specific binding of band 3 or Pbeno proteins to PrA-B was ruled out by incubating PrA+ merozoite extract + hRBC^G extract and probing with α -band 3 antibodies (Lane 1); PrA-B- α -Pbeno + hRBC^G ghost extract and probing with α -band 3 antibodies (Lane 3) and PrA + merozoite extract and probing with α -Pbeno antibodies (Lane 5). These controls showed an absence of nonspecific adsorption of band 3 or Pbeno on PrA beads under the experimental conditions used here.

3.6. Post translationally modified variants of band 3 exhibit differential binding to rPbeno:

In a two-dimensional gel electrophoresis (2DE) experiment (Method section-2.6), we attempted to separate the different variants of band 3. A silver stained 2DE of hRBC^G is shown in Fig 7A. Western blot of a replicate of this where band 3 was detected using α -band 3 showed two clusters of spots (Fig. 7B). 25 kDa form of band 3 got resolved into at least five different spots with pIs ranging from 4.8-5.3 while the 64 kDa form showed two spots (pI~5.18). Intriguingly, the 91 kDa form was not observed in the western blot, presumably because it did not get solubilized in 2DE sample buffer. Using a replicate blot, the binding ability of band 3 variants to rPbeno was examined in an overlay assay. Interestingly, while rPbeno binding was observed with both forms at 64 kDa, the 25 kDa forms of band 3 exhibited binding to the three variants (pI~4.93, 5.03, 5.18). Band 3 variants with highest pI~5.31 and lowest pI~4.81 failed to bind *Pf* enolase (Fig. 7C). The individual spots corresponding to band 3 (as seen in western blot Fig. 7B) were excised from the silver-stained gel and subjected to Mass-spectrometry for the identification of the proteins. Two proteins that could be identified with high Prot Score were Spectrin and band 3. The protein spot at pI~5.18 and MW~64 kDa had both band 3 and spectrin while the ~25 kDa spot with pI~5.31 showed the presence of three proteins viz. diacylglycerol kinase, chromosome 20 ORF and cadherin 12 (Table 2). Although in Western blot using band 3, there was a positive identification of band 3 protein, mass spectrometry failed to confirm that. This can be due to missing the spot of interest on the silver-stained gel. Amounts of protein present in other spots with MW~25 kDa were too small to be identified by mass spectrometry.

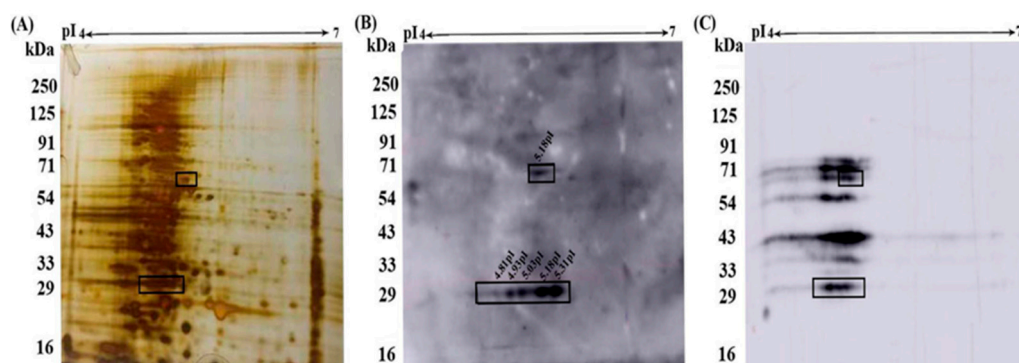


Figure 7. Two-dimensional gel analysis of hRBC ghost proteome and identification of Pbeno binding protein(s). (A) Silver-stained gel of hRBC^G. (B) Western blot of 2DE probed with α -band 3 antibodies. Proteins in boxes at near around 25 kDa and 64 kDa represent band 3 and its variants. (C) A 2DE blot of hRBC^G proteome was overlaid with rPbeno protein. After removal of unbound rPbeno, blot was probed with α -rPbeno antibody. Binding of rPbeno to band 3 (rectangles) is quite evident and was marked in silver stain gel of hRBC (Panel A) to excise the bands for Mass spectrometry.

Table 2. Identification of proteins in the rectangles in panel A by mass spectrometry. Two spots were cut out from the silver-stained gel, trypsin digested and analyzed by mass spectrometry. Proteins identified are listed.

Protein Spot	Proteins Identified	Protein Score	Mol.Wt (kDa)
A (pI~ 5.31 MW~25kDa)	Diacylglycerol Kinase	42	102
	Chromosome 20 ORF	30	125
	Cadherin 12	30	110
B (pI~ 5.18, MW~64 kDa)	Spectrin α chain	50	281
	Spectrin β chain	31	247
	Band 3 chain P	33	102

As shown in Fig 8, band 3 has 911 residues (NCBI Reference Sequence Accession NP_000333.1). Table 3 lists the sequences of ten different band 3 peptides that were sequenced in mass spectrometry experiments covering ~15% (136 residues) of band 3. The residues that could be sequenced in peptides, led to identification of band 3.

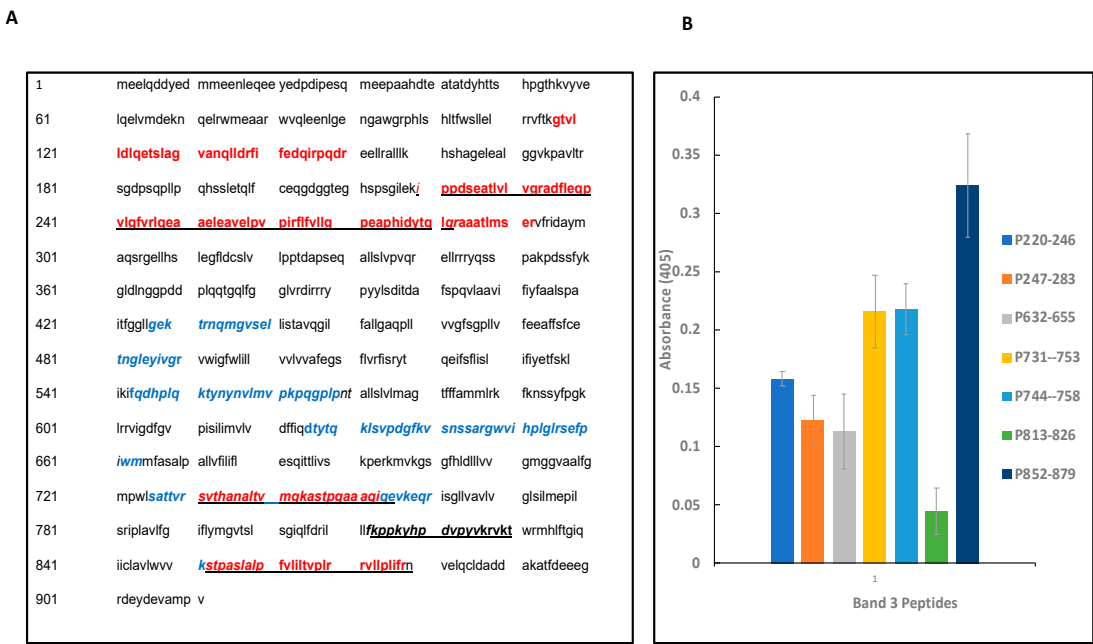


Figure 8. (A) Amino acid sequence of band 3 Erythrocyte anion exchanger protein (NCBI Reference Sequence: NP_000333.1). Peptide sequences obtained from mass spectrometric analysis of band 3 in various pull-down assays (Figure 4, Tables 3 and 4) are shown in red color. Regions of the protein that are exocytic in hRBC as defined by Fuginaga et al. [36] are in blue italics. **(B)** ELISA for the binding of certain band 3 synthetic peptides (Peptide sequences obtained from mass spectrometric analysis of the band 3 in various pull-down assays as shown in Figure 4 and Tables 3 and 4) to rPfen. Plates were coated with different band 3 peptides in triplicates and rPfen was added to each well. After washing, the bound rPfen was estimated using α Pfen based assay. The mean \pm SD value of absorbance from three experiments is shown. The sequences and locations of various peptides (also shown in Table 4) that bind to Pfen have been underlined in panel A.

Table 3. Amino acid sequences of band 3 peptides obtained in Mass spectrometric analysis of tryptic digests of protein spots. These sequences . cover ~15% of band 3.

S. No.	Peptide positions and Sequences*
1	G ¹¹⁷ TVLLDLQETSLAGVANQLLDR ¹³⁸
2	F ¹³⁹ IFEDQIRPQDR ¹⁵⁰
3	I ²²⁰ PPDSEATVLVGR ²³³
4	A ²³⁴ DFLEQPVLGFR ²⁴⁶
5	L ²⁴⁷ QEAAELEAVELPVPIR ²⁶³
6	F ²⁶⁴ LFVLLGPEAPHIDYTQLGR ²⁸³
7	A ²⁸⁴ AATLMTER ²⁹²
8	S ⁷³¹ VTHANALTVMGKASTPGAAAQ ⁷⁵²
9	S ⁸⁵¹ TPASLALPFVLILTVPLR ⁸⁷⁰
10	V ⁸⁷² LLPLIFR ⁸⁷⁹

3.7. Synthetic peptide towards mapping the binding sites of band 3 and Pfeno:

Band 3 is a trans membrane protein that has its N-terminal globular region on the cytosolic face while the membrane spanning and exocytic loop regions are at the C-terminal end [35,36]. Thus, the site for parasite enolase that binds to band 3 on extra cellular surface must map on the C-terminal region of band 3. For mapping the *Pfeno* binding region on band 3, a set of seven different peptides belonging to the exocytic domains of band 3 were custom synthesized (Table 4).

Table 4. Amino acid sequence of custom synthesized band 3 peptides. Some of these peptides cover the sequences detected in Mass-spectrometry analysis of tryptic digests obtained from r*Pfeno* binding protein samples.

S. No.	Amino acid sequence of band 3 peptides
1	I ²²⁰ PPDSEATLVLVGRADFLEQPVLGFR ²⁴⁶
2	L ²⁴⁷ QEAAELEAVELPVPIRFLFVLLGPEAPHIDYTQLGR ²⁸³
3	L ⁶³² SVPDGPVKVSNSSARGWVIHPLGL ⁶⁵⁵
4	S ⁷³¹ VTHANALTVMGKASTPGAAAQ ⁷⁵³
5	A ⁷⁴⁴ STPGAAAQIQEVKE ⁷⁵⁸
6	F ⁸¹³ KPPKYHPDVPYVK ⁸²⁶
7	S ⁸⁵² TPASLALPFVLILTVPLRRVLLPLIFRN ⁸⁷⁹

Four of these peptides (P⁶³²⁻⁶⁵⁵, P⁷³¹⁻⁷⁵³, P⁷⁴⁴⁻⁷⁵⁸ and P⁸⁵²⁻⁸⁷⁹) localize in exocytic regions of band 3. Peptide P⁸¹³⁻⁸²⁶ is from a trans membrane region while the peptides P²²⁰⁻²⁴⁶ and P²⁴⁷⁻²⁸³ are from the endocytic domain of the band 3 [36]. Sequence positions of all the seven synthetic peptides are underlined (red) in Fig. 8A. C-terminal region of band 3 (aa 361-911) forms the trans-membrane domains and exocytic regions of the protein (highlighted in turquoise blue in Fig. 8A). Using these peptides as baits, their ability to capture r*Pfeno* was further tested in an ELISA experiment. The results of the assay (Fig. 8B) indicated that while the peptide P⁸¹³⁻⁸²⁶ that localizes in trans-membrane region had no interaction with r*Pfeno*, the three exocytic peptides (P⁷³¹⁻⁷⁵³, P⁷⁴⁴⁻⁷⁵⁸ and P⁸⁵²⁻⁸⁷⁹) showed significant binding and are likely to form the binding region for the parasite enolase. The remaining three peptides (P²²⁰⁻²⁴⁶, P²⁴⁷⁻²⁸³ and P⁶³²⁻⁶⁵⁵) showed intermediate level of reactivity (Fig. 8B).

Interaction of r*Pfeno* with the two N-terminal peptides (P²²⁰⁻²⁴⁶ and P²⁴⁷⁻²⁸³) is of unclear physiological relevance as these regions are located on the cytoplasmic face of the hRBC membrane. However, the possibility of post invasion interactions between enolase and the two N terminal peptides cannot be ruled out. Another peptide P⁶³²⁻⁶⁵⁵ that showed an intermediate level of binding is also likely to be a part of enolase binding site. Thus, the data presented here imply that r*Pfeno* binding

region of band 3 includes sequences P⁶³²⁻⁶⁵⁵, P⁷³¹⁻⁷⁵³, P⁷⁴⁴⁻⁷⁵⁸ and P⁸⁵²⁻⁸⁷⁹. It is important to realize that the method used here is largely limited to identifying sequentially continuous binding sites. Protein-protein interactions that are stabilized by secondary and/or tertiary structural features formed by non-contiguous sequence would not be identified by this approach.

For mapping the *Pfeno* sites that bind to band 3, a 'Peptide Scanning' strategy was used. The method involved synthesis of entire enolase protein sequence as short biotin tagged 15 mer overlapping peptides and testing them individually for binding to band 3 [37]. Binding of band 3 with the *Pfeno* peptide library was monitored using an ELISA format. As shown (Fig. 9A), band 3 showed binding with peptides 16, 40, 49, 50 and 57-64. The sequences of these band 3 binding *Pf* enolase peptides as also their two neighboring peptides that failed to bind band 3 are shown in Fig. 9B. Sequence alignment among peptides 15 - 17 indicated that the peptide 16 shares ten residues with these two non-binding peptides. Thus, if any stretch of five or ten residues in peptide 16 formed a binding site, one or the other neighboring peptides could also show some binding. Absence of such recognition with flanking peptides 15 and 17 was indicative of nonspecific binding of peptide 16. Similar is the case with peptide 40 which also showed no binding for the flanking peptides. Peptides 49 and 50 have ten residues in common (Panel B, shown in green) and both showed interaction with band 3 indicating that these residues are part of the binding domain. The N-terminal five residues of peptide 49 and the C-terminal five residues of peptide 50 are not involved in the binding to band 3. This may be the reason why the flanking peptides 48 and 51 that did not have adequate number of binding domain residues failed to interact with band 3. Thus, from these data we infer that *Pf* Enolase region ²⁴⁶GKVKIAMDAV²⁵⁵ is involved in binding interactions with band 3. Similar analysis of the other cluster of peptides (peptides 57-64) yielded a binding sequence of ²⁸⁶QLVDLYIDL³²⁵VKKYPIVSIEDPFDQDDWENYAKLTAAIGKD³²⁵ as the band 3 binding domain in *Pfeno*. To visualize the location of this sequence in 3D structure of *Pfeno*, a model structure of *Pfeno* was generated [38] using the crystal structure of *T. gondii* enolase (pdb: 3OTR) [39] as template (Fig. 9 C) as the two proteins have >90% sequence homology. In this structure, location of these sequences is shown in blue and green. Enolase is also known to bind plasminogen that facilitates invasion of mosquito mid gut epithelium [24]. The plasminogen interacting domain is shown in red. All apicomplexan enolases differ from host enolases in having an insert of five amino acids (¹⁰⁴EWGWS¹⁰⁸) (Fig. 9 C, pink). This epitope has been shown to be a target for potent growth inhibiting antibodies [27,28]. It is interesting to note that the domains of *Pfeno* that are involved in executing one or the other moonlighting function are clustered on the same face of the molecule in the 3D model structure (Fig. 9 C).

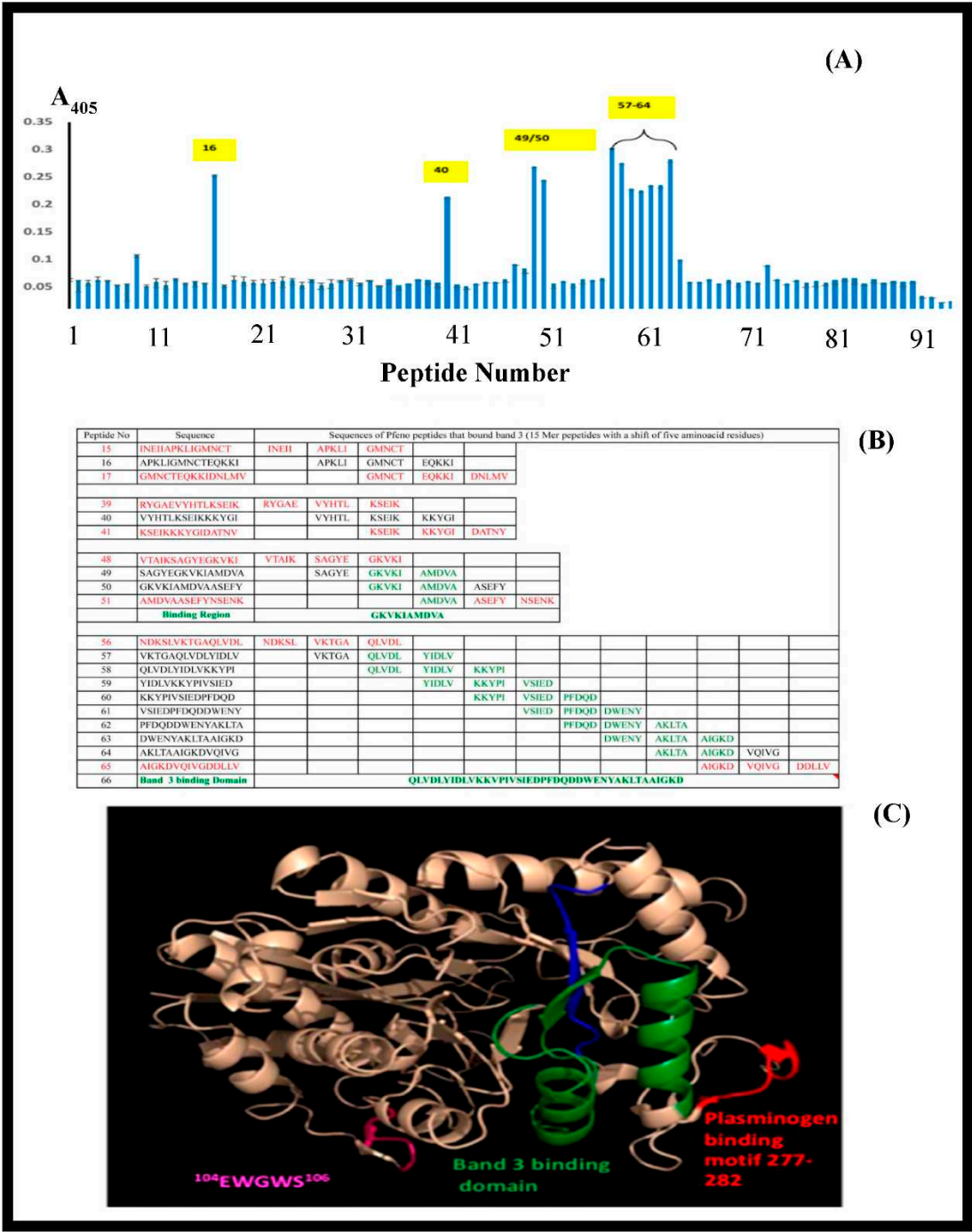


Figure 9. Mapping of the binding domain in *P. falciparum* enolase for hRBCghost band 3 protein. (A) A library of biotin labelled fifteen-mer peptides covering full sequence of Pfen was immobilized on a streptavidin coated ELISA plate. This was incubated with solubilized hRBC^G proteins. Binding of band 3 to Pfen peptides was determined using a-band 3 in standard ELISA. Peptide numbers 16, 40, 49, 50 and 57-64 (Highlighted yellow) showed affinity for band 3. The data shown are mean + SD for triplicate experiments. Single peptide showing binding in a given region (peptide no. 16 and 40) were considered nonspecific, as the two neighboring peptides having overlap of 10 residues failed to bind band 3. (B) Sequences of the Pfen peptides involved in binding to band 3 are shown in black. Sequences of neighboring peptides that did not show any interaction with band 3 are shown in red. Comparison of the overlapping regions of binding and non-binding peptides suggested that a sequence of ten amino acid residues (shown in green) was essential for binding to band 3. (C) Modelled 3D structure showing the locations of the unique EWGWS insert (pink), the two band 3 binding regions (green & blue) and the Plasminogen binding domain (red) in Pfenolase.

4. Discussion:

Plasmodium falciparum enolase (*Pfeno*) is a highly conserved glycolytic enzyme that is expressed on the surface of the merozoites. Involvement of enolase in merozoite invasion of erythrocytes was evident from the observed strong inhibition of the *P. falciparum* growth in blood cell cultures by α -*Pfeno* and reduction of parasitemia in *P. yoelii* infected mice immunized with α -*Pfeno* [26–28,30]. The target epitope for the inhibitory α -*Pfeno* antibodies was identified as the pentapeptide insert $^{104}\text{EWGWS}^{108}$ present in all apicomplexan parasite enolases but absent in host enolases [46]. As the initial invasion event requires interaction between a relevant parasite ligand with host receptor, an attempt was made to identify and characterize the *Pfeno* binder/receptor on hRBC surface. Several experiments presented here indicated that r*Pfeno* bound specifically to both hRBC and hRBC^G in a ligand concentration dependent manner. In the past, the effect of treatment of hRBC with proteases (trypsin and chymotrypsin) and neuraminidase on the binding of the parasite ligands have been used to define sialic acid dependent (SAD) or independent (SAID) pathways [6–9]. We found that the binding of r*Pfeno* was sensitive to trypsin but resistant to neuraminidase suggesting that the r*Pfeno* binding with the receptor is of the SAID kind. Results from a host of pull-down and overlay assays combined with western blotting and/or mass spectrometry (MS/MS) (Figs. 4-7) analysis led to identification of band 3 as the enolase binding protein in hRBC cell membrane. Band 3 is an anion exchanger that mediates the exchange of the cellular HCO_3^- with Cl^- in plasma [42,47,48]. Structurally, it has 911 amino acid residues (Fig. 8A) with a glycosylation site at N⁶⁴². It is a trans-membrane protein that coimmunoprecipitates with RhopH3 and MSP1 [18,49]. Two extracellular domains of band 3, viz 5C ($^{744}\text{ASTPGAAAQIGEVKE}^{758}$) and 6A ($^{807}\text{DRILLFLKPPKYHPDVYPYVK}^{826}$) form the binding site for MSP1. Interestingly, MSP1 mediated invasion pathway has also been shown to be of the SAID kind [18]. The enolase binding sequences identified here consisted of four exocytic peptides viz $\text{P}^{632-655}$, $\text{P}^{731-753}$, $\text{P}^{744-758}$ and $\text{P}^{852-879}$ (Fig. 8A and 8B). Thus, there is quite a bit of overlap in the binding sites for MSP1 and *Pfeno* on band 3. Topographically, N-terminal part of band 3 is globular and located on cytosolic side while C terminal domain forms its transmembrane and exocytic regions [42,47]. As expected, the *Pfeno* binding site on band 3 mapped in C-terminal exocytic domain (Fig. 8). Conversely, the binding site for band 3 on *Pfeno* was also mapped using a peptide scanning approach. The results showed that the *Pfeno* regions that bind band 3 are constituted by $^{246}\text{GKVKIAMDAV}^{255}$ and $^{286}\text{QLVDLYIDL VKKYPIVSIEDPFDQDDWENYAKLTAAIGKD}^{325}$ (Fig. 9B). *Pfeno* appears to have multiple distinct sites that relate to its diverse physiological functions. In order to visualize the spatial disposition of various functional regions on the molecular surface of *Pfeno*, its 3D-structure was modelled using X-ray crystallographic structure of *T. gondii* enolase (Fig. 9 C). Apart from the catalytic site (not shown), there is a plasminogen binding site constituted by $^{277}\text{DKSLVK}^{282}$ residues [24], band 3 binding site formed by $^{246}\text{GKVKIAMDAV}^{255}$ and $^{286}\text{QLVDLYIDL VKKYPIVSIEDPFDQDDWENYAKLTAAIGKD}^{325}$ and an invasion inhibitory antibody binding site $^{104}\text{EWGWS}^{108}$ [27,28,30]. Presence of distinct *Pfeno* binding regions in band 3 and parasite growth inhibitory *Pfeno* antibodies indicates that band 3 enolase binding site may facilitate initial adhesion of merozoite to hRBC. The unique pentapeptide insert in *Pfeno* ($^{104}\text{EWGWS}^{108}$) is likely to serve as a docking port for some (yet to be identified) molecular factor of host origin whose interaction must be critical for merozoite invasion of hRBC in an enolase mediated pathway. In many pathogens as also in malignant cells, cell surface enolase is known to act as plasminogen binder which upon activation cleaves inter-cellular matrix protein facilitating the establishment of infection [25,50–53]. However, plasminogen binding function of *Pfeno* is unlikely to be of any relevance in blood stage merozoite invasion of hRBC as there is no barrier of matrix proteins between the host and the parasite.

Pfeno is an unusual surface antigen that expresses on cell surface of all the three invasive stages (sporozoites, merozoites and ookinetes) in *Plasmodium*'s life cycle [23,24]. In a series of elegant experiments, the physiological functions of ookinete cell surface enolase were demonstrated [23–25,29]. In mosquito mid gut, ookinete has to cross the peritrophic matrix barrier to reach to mid gut epithelium. This task is accomplished by binding of human plasminogen (derived from the blood meal of the mosquito) to ookinete cell surface enolase [24,54]. Disruption of this inter molecular interaction between *Pfeno* and plasminogen by anti-*Pfeno* antibodies or by feeding plasminogen depleted blood resulted in drastic reduction in traversal of ookinetes across the mid gut epithelium [24,29]. Most of the ookinete traversal inhibitory activity could be accounted for by the plasminogen receptor function of *Pfeno*. In addition, an enolase binding protein (EBP) that expresses on the luminal surface of the mosquito midgut epithelium was also identified [29]. Although physiological significance of *Pfeno*-EBP interaction is not well understood, it is likely to be important in mediating host cell recognition by the parasite (similar to merozoite cell surface enolase binding to band 3 of hRBC). Peritrophic matrix in mosquito mid gut is rich in proteins that contain PWWP, a protein-protein interaction motif [55]. Recently, this motif was shown to bind to ¹⁰⁴EWGWS¹⁰⁸ [56] raising an interesting possibility of aromatic interactions-based assembly of a ternary complex formed by *Pfeno*, plasminogen and PWWP containing matrix proteins, leading to high efficiency proteolysis of peritrophic matrix. Physiological functions of *Pfeno* on merozoite and ookinete cell surfaces are different and well adapted for host cell invasion needs of the parasite. Thus, it appears that enolase functions very much depend on when and where this versatile protein is present. This has been seen in several organisms where enolase has multiple moonlighting functions [57]. So far, the role of *Pfeno* on sporozoite cell surface has not been examined. It will be interesting to examine whether it has any functional role in sporozoite invasion of hepatocytes.

In conclusion, the identification of band 3 as a major erythrocyte receptor for the highly conserved glycolytic protein *Pfeno* should provide new insights in understanding the role of *Pfeno* on merozoite cell surface. More *in-vivo* and *in-vitro* studies such as RBC invasion inhibition assay using different laboratory strains and real time interaction using Surface Plasmon Resonance (SPR), are required involving both *rPfeno* and band 3 for better understanding of the host parasite interaction. It is hoped that the identification of erythrocyte band 3 as a receptor for *Plasmodium* enolase will provide a window of opportunity to develop new peptide/small molecule-based therapeutics and possible vaccine component(s) to combat the deadly disease of malaria.

Abbreviations:

ABTS, 2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid; α *Pfeno*, anti-*rPfeno* antibodies; α Band 3, anti-Band 3 antibodies; GSH, Glutathione S-Sephadex beads; GST-*rPfeno*, glutathione S transferase tagged *rPfeno*; IP, immune-precipitation; α MSP 1₁₉, anti MSP1 antibody; α Spectrin, anti spectrin antibody; α Yeno, anti-yeast enolase antibody; 2DE, Two dimensional gel electrophoresis; hRBC, human red blood cells; hRBCG, human RBC ghosts; hrs, hours; *Pbeno*, *Plasmodium berghei* enolase; PrAB, Protein A agarose beads; *rPfeno*, recombinant *P. falciparum* enolase; RT, room temperature. iRBC, infected RBC, uRBC, uninfected RBC

Author Contributions: GKJ, conceived, planned and designed the study, planned laboratory work, participated in data analysis and interpretation, data presentation and manuscript preparation; RV, planned and executed most experiments, analyzed data and manuscript writing; CB, performed mass-spectrometry of proteins, data analysis; CB and RV performed 2-D gel electrophoresis and overlay experiments; SS, contributed to experimental design, data interpretation and manuscript editing, DS found new perspectives in the work and contributed to uplifting the manuscript.

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Ethical Statement: Human red blood cells (hRBC) were collected from volunteers, after obtaining their written consents, for all the RBC experiments including *in-vitro* cultures of *Plasmodium falciparum*. The procedure for such collection, details of informed consent and the frequency of samples to be

collected were in accordance with a detailed proposal approved by the Institutional Human Ethics Committee (IHEC) of Tata Institute of Fundamental Research (TIFR). The IHEC of TIFR functions as per the guidelines of Indian Council of Medical Research (ICMR), Govt. of India. TIFR Animal House is registered under CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Govt. of India (registration no. 56/1999/CPCSEA) for breeding and experiments on animals. *Plasmodium berghei* were maintained in mice and the study was carried out under strict accordance with the guidelines of CPCSEA, with prior approval.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data is contained within the article.

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