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# Proteome Analysis Reveals a Large Merozoite Surface Protein-1 Associated Complex on the *Plasmodium falciparum* Merozoite Surface

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*Plasmodium* merozoite surface protein-1 (MSP-1) is an essential antigen for the merozoite invasion of erythrocytes. A key challenge to the development of an effective malaria vaccine that can block the erythrocyte invasion is to establish the molecular interaction(s) among the parasite surface proteins as well as with the host cell encoded receptors. In the present study, we applied molecular interactions and proteome approaches to identify PfMSP-1 associated complex on the merozoite surface. Proteomic analysis identified a major malaria surface protein, PfRhopH3 interacting with PfMSP-$1_{42}$. Pull-down experiments with merozoite lysate using anti-PfMSP-1 or anti-PfRhopH3 antibodies showed 16 bands that when identified by tandem mass spectrometry corresponded to11 parasite proteins: PfMSP-3, PfMSP-6, PfMSP-7, PfMSP-9, PfRhopH3, PfRhopH1, PfRAP-1, PfRAP-2, and two RAP domain containing proteins. This MSP-1 associated complex was specifically seen at schizont/merozoite stages but not the next ring stage. We could also identify many of these proteins in culture supernatant, suggesting the shedding of the complex. Interestingly, the PfRhopH3 protein also showed binding to the human erythrocyte and anti-PfRhopH3 antibodies blocked the erythrocyte invasion of the merozoites. These results have potential implications in the development of PfMSP-1 based blood stage malaria vaccine.

**Keywords:** Malaria • *Plasmodium falciparum* • MSP-1 • RhopH3 • rhoptry • cell surface protein • erythrocyte • parasitology • protein−protein interactions

## 1. Introduction

Apicomplexan parasites represent an important group of eukaryotic pathogens that includes *Plasmodium*, the causative agent of human malaria, responsible for about one million deaths per year.[1] Host cell invasion by apicomplexan pathogens is mediated by a succession of conserved and highly specialized molecular events. The first step in merozoite invasion of red blood cells (RBCs), involves low affinity binding of merozoites to the RBCs followed by their reorientation so that its apical prominence is in close association with the host cell surface. Next step involves the high affinity attachment between the apical end of the merozoites and erythrocytes, resulting in the formation of a moving junction. Finally the merozoite invagination into the erythrocyte occurs via formation of a parasitophorous vacuole.[2−4] Merozoite invasion is orchestrated by proteins released from its apical secretory organelles — micronemes, rhoptries, and dense granules including exonemes.[5] At the time of invasion, these proteins are released in a timely and spatially coordinated manner to facilitate parasite release, its invasion into the erythrocyte and formation of parasitophorous vacuole in which the parasite resides after invasion.[6−8] Studies in *Toxoplasma* have demonstrated that micronemes secrete their contents first, followed by secretion from rhoptries and dense granules.[9]

Merozoite invasion has been shown to involve multiple ligand — receptor interactions with the erythrocyte.[10] Initial attachment of the parasite to the host cell occurs via glycosylphosphatidylinositol (GPI) anchored surface proteins such as Merozoite surface proteins (MSP-1, -2, -4, -5, -8, and -10). Other MSPs such as MSP-3, MSP-6, MSP-7, and MSP-9 which do not possess transmembrane or GPI domains also get associated with the merozoite surface.[11] The next step in the invasion is the reorientation of the merozoites after which the apical end of merozoite faces the erythrocyte surface and apical membrane antigen-1 (AMA-1) appears to be involved in this process.[12] This is followed by secondary interactions that involve two invasion ligand families, the erythrocyte-binding

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antigens (EBA-175, -140, and -181) and *Plasmodium falciparum* reticulocyte binding homologue (PfRh) proteins (PfRh1, -2a, -2b, and -4). Many of the molecules secreted at the time of erythrocyte invasion are being considered as vaccine targets with the aim of blocking the invasion. For example, passive immunization with monoclonal antibodies specific for a 235 kDa erythrocyte binding protein in rhoptry or active immunization with the same protein protected mice against blood stage challenge with *P. yoelii*.[13−15] A monoclonal antibody 4G2 raised against AMA-1, a micronemal protein inhibited erythrocyte invasion by all strains of *P. falciparum*.[16,17]

Although there has been much progress in our understanding of host cell invasion at the ultrastructural level in *Plasmodium*, the precise molecular interactions, their structure - function make up and specific roles of the surface/secretory proteins are still not understood. A few molecular complexes such as the high molecular weight complex (HMW) composed of RhopH1, RhopH2, and RhopH3 and the low molecular weight complex (LMW) composed of RAP1, RAP2, and RAP3 identified on the merozoite surface have been shown to be crucial for malaria parasite invasion.[18−20] Rhoptry associated membrane antigen (RAMA) has been suggested to be in close association with RAP as well as Rhop complexes.[21] Likewise, protein complexes consisting of MSP-1/MSP-6 as well as MSP-1/MSP-6 and MSP-7 have been reported in the supernatant of *P. falciparum*.[22,23] PfMSP-1 has also been shown to form a coligand complex with PfMSP-9 that binds to erythrocyte invasion receptor Band 3.[24] Recently, a large AMA-1 associated complex has been reported with essential rhoptry neck protein RON4 and two additional proteins in *Toxoplasma* as well as in *Plasmodium*.[2,17,25−28]

In the present study, we performed protein−protein interaction studies using PfMSP-1 as a bait and identified its interacting partners that may be involved in the merozoite invasion of human RBCs. Interestingly, our results using a bacterial two hybrid *P. falciparum* library identified PfRhopH3 as an interacting partner to PfMSP-1. This interaction was subsequently confirmed by an in vitro interaction study as well as by immunoprecipitation and matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) analysis. These results showed that PfMSP-1 exists in a complex with 10 different proteins including PfRhopH3. We next demonstrated that PfRhopH3 binds to intact RBCs and anti-PfRhopH3-C antibodies inhibited the merozoite invasion suggesting a role of this complex in RBC binding. These results thus provide an important clue for the existence of a large MSP-1 associated ligand complex on the merozoite surface that may be involved in the invasion process.

## 2. Materials and Methods

**2.1. In Vitro Parasite Culture.** *P. falciparum* strain 3D7 was cultured on human erythrocytes (4% hematocrit) in RPMI 1640 media (Invitrogen) supplemented with 10% $O^+$ human serum using standard protocol described by Trager and Jensen.[29] Parasite cultures were synchronized by two consecutive sorbitol treatments 4 h apart following the protocol described by Lambros and Vanderberg.[30]

**2.2. DNA Manipulation Techniques.** Plasmid isolation, restriction enzyme digestion, ligation, and competent cell preparation were carried out by standard procedures.[31]

**2.3. *P. falciparum* cDNA Library Construction and Bacterial Two-Hybrid Screen for PfMSP-1.** *P. falciparum* cDNA library was constructed in pTRG vector as described in our previous study.[32] To identify the putative PfMSP-1 interacting proteins, a fragment (1.8 kbp) corresponding to C-terminal region of PfMSP-1 was cloned in a plasmid, pBT (Stratagene) using primers MSP1B/YF and MSP1B/YR (Supplemental Table 4, Supporting Information). Recombinant pBT plasmid (pBT-PfMSP1-C) and cDNA plasmid library (in pTRG plasmid) were cotransformed in a BacterioMatch II two-hybrid system reporter strain (Stratagene). The transformants obtained were selected via three cycles of screening following the manufacturer's instructions. The interacting recombinant pBT plasmids were isolated and PCR was done using pTRG specific primers (supplied with the kit) and the amplified products were sequenced. Sequences were analyzed using BLASTX program at NCBI Web site (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and PlasmoDB Web site (http://plasmodb.org/plasmo/).

**2.4. Cloning of PfRhopH3-C and PfRhopH3-N Terminal Fragment in pGEX-4T-3, pGEX-4T-1, and pMAL-c2X.** The C-terminal and N-terminal regions of PfRhopH3 gene were amplified by PCR from *P. falciparum* 3D7 cDNA using primer sets, RhopexpF2/RhopexpR2 and RhopN_For/RhopTM1_Rev, respectively (Supplemental Table 4, Supporting Information). The amplified and purified PfRhopH3-C gene fragment was digested with *Bam*HI/*Sal*I and cloned in pGEX-4T-3, while PfRhopH3-N gene fragment was digested with *Eco*RI/*Xho*I and ligated in pGEX-4T-1, an *Escherichia coli* expression vector (GE Healthcare) to get recombinant plasmid constructs, pGEX-PfRhopH3-C and pGEX-PfRhopH3-N.

To clone PfRhopH3-C and PfRhopH3-N gene fragments into the pMAL-c2X plasmid, gene fragments were amplified using primer sets Rhop-C-pMal-For/RhopexpR2 and Rhop-N-TM1-pMal-For/Rhop-N-TM1-pMal-Rev, respectively (Supplemental Table 4, Supporting Information). The amplified products were ligated using the *Eco*RI/*SalI* restriction sites of the expression vector pMAL-c2X (New England Biolabs) to generate expression vectors, pMal-PfRhopH3-C and pMal-PfRhopH3-N.

**2.5. Expression and Purification of Recombinant Proteins: GST-PfRhopH3-C, GST-PfRhopH3-N, MBP-PfRhopH3-C, MBP-PfRhopH3-N, PfMSP-$1_{19}$, and PfMSP-$1_{42}$.** GST fused PfRhopH3-N and PfRhopH3-C proteins and MBP-PfRhopH3-C and MBP-PfRhopH3-N proteins were expressed and purified using Glutathione Sepharose 4 Fast Flow resin (GE Healthcare) and amylose resin (New England Biolabs), respectively, according to the manufacturer's instructions (Detailed protocol available as Supporting Information). The purified proteins were analysed on SDS-PAGE in Laemmli's Buffer.[33] Cloning, expression, and purification of PfMSP-$1_{19}$ and PfMSP-$1_{42}$ were performed as described earlier by Lalitha et al.[34] and Sachdeva et al.,[35] respectively.

**2.6. Generation of Antibodies against GST-PfRhopH3-C, GST-PfRhopH3-N, GST, and MBP.** The animals were housed and handled in accordance with the institutional and national guidelines. The institutional animal ethical committee at ICGEB, New Delhi, India, approved the animal use protocol describing our studies. The animals were bred under the guidelines of the authorizing committee and BALB/c inbred mice were used for our studies. 4−6 week old nonpregnant BALB/c mice were immunized with 20 $\mu$g of antigen (90−95% pure) in the presence of complete/incomplete Freund's adjuvant employing the intraperitoneal mode of injection. Antibody titer in serum samples were quantified by enzyme-linked immunosorbent assay (ELISA). The animals were euthanized

after the third and/or last bleed. Antibodies against $(His)_6$-PfMSP-$1_{42}$ was generated as described earlier.[35]

**2.7. In vitro Protein Binding Assay of Recombinant Proteins.** The in vitro protein−protein binding assays were done as previously described,[36] with slight modification. Briefly, 2 $\mu$g of MBP-PfRhopH3-C were allowed to interact with either $(His)_6$-PfMSP-$1_{19}$ or $(His)_6$-PfMSP-$1_{42}$ proteins in a reaction volume of 100 $\mu$L containing 1× binding buffer pH 7.0 (50 mM phosphate buffer pH 7.0, 75 mM NaCl, 2.5 mM EDTA pH 8.0 and 5 mM $MgCl_2$), 0.1% Nonidet P40, 10 mM DTT at room temperature for 45 min. Subsequently, 20 $\mu$L of Ni-NTA$^+$ resin (pre-equilibrated) was added and protein was allowed to bind at room temperature. The beads were washed with 200 $\mu$L of wash buffer (1× binding buffer and 400 mM NaCl). The bound proteins, eluted in SDS−PAGE reducing dye, were electrophoresed on SDS−PAGE, immunoblotted, and probed with anti-MBP antibody. In a control reaction, MBP was used instead of PfRhopH3-C protein.

**2.8. Immunoprecipitation Analysis of PfMSP-1 or PfRhopH3 Associated Proteins.** Immunoprecipitation reactions were performed using a protocol described,[28] with slight modifications. Approximately $2 \times 10^{11}$ mature stage *P. falciparum* infected human erythrocytes (late schizonts) were separated by Percoll gradient centrifugation. The culture was kept for 4−5 h to allow the release of the merozoites. The separation of the merozoites was done by centrifugation at 3300*g* for 5 min. Parasite pellet was washed thrice with 1 × PBS and resuspended in 10 volumes of ice-cold TNET buffer (50 mM Tris-HCl pH 8.0, 400 mM NaCl, 5 mM EDTA pH 8.0, and 2% Triton X-100) in the presence of protease inhibitor cocktail (Roche Diagnostics). Parasites were lysed by freeze−thaw using liquid nitrogen. The clarified extract was incubated with Protein-A Sepharose beads (100 $\mu$L of 50% suspension) coupled with anti-PfMSP-$1_{42}$ or anti-PfRhopH3 antibodies for overnight at 4 °C. Beads were washed with TNET buffer containing 0.5% BSA, with TNET buffer alone and subsequently with high-salt TNET buffer (600 mM NaCl) and low-salt TNET buffer (50 mM NaCl + 0.1% Triton X-100). Finally, proteins were extracted from the Protein-A conjugated beads by boiling at 100 °C with 2× reducing dye and subjected to SDS−PAGE in reducing conditions followed by either silver nitrate staining or Western blot analysis. Membranes were probed with anti-PfAMA-1, anti-PfRhopH1, anti-PfRAP-1, anti-PfMSP-$1_{42}$, or anti-PfRhopH3 antibodies.

**2.9. Protein Identification by Peptide Mass Fingerprinting with MALDI-TOF/TOF.** To identify the proteins associated with PfMSP-1 or PfRhopH3 proteins, immunoprecipitations were performed after treating the merozoites with 3,3′-dithiobis(sulfosuccinimidylpropionate) [DTSSP], a thiol-cleavable membrane impermeable cross-linker. Parasite extract thus obtained was treated with anti-PfMSP-1 or PfRhopH3 antibodies bound to protein-A Sepharose beads. The beads were washed and the bound proteins were eluted in SDS−PAGE sample buffer. The eluted proteins were resolved by SDS−PAGE and gel was silver stained. The resolved protein bands were excised and were subjected to in-gel trypsin digestion using TPCK treated bovine Trypsin (Roche) at a final concentration of 0.01 mg/mL in 25 mM $NH_4HCO_3$ pH 8.0, for 16 h at 37 °C. The peptides were extracted twice with 50 $\mu$L of 1% trifluoroacetic acid (TFA) in 60% acetonitrile (ACN) and the extract was concentrated in a speed-vac. The pellet was dissolved in 50% ACN/0.5% TFA and were loaded onto a MALDI target plate by mixing 1 $\mu$L of each sample with the same volume of matrix solution (20 mg/mL α-cyanohydroxycinnamic acid, 5 mg/mL nitrocellulose in 7:7:2:2 acetone/ACN/isopropanol/0.1% TFA) mixed well and allowed to dry. The dried samples were washed with 0.1% TFA and then with Milli-Q water. Samples and calibration standards with the same matrix composition were spotted adjacent to each other on the target plate for optimal calibration. Spectra were externally calibrated with the calibration standard containing the following peptide mass $(M + H)^+$ − angiotensin II (1046.54180), angitensin I (1296.684780), substance P (1347.735430), bombesin (1619.822350), renin substrate (1758.932610), adrenocorticotropic hormone (ACTH) fragment (1−17) (2093.086170), ACTH fragment (18−39) (2465.198340), ACTH fragment (1−24) (2932.590000), insulin $\beta$-chain (3494.650780).

Spectral measurements were performed using an Ultraflex III MALDI TOF/TOF instrument (Bruker Daltonics, Germany), in the positive ion reflector mode with the accelerating voltage of 25 kV (Ion source 1) and 21.85 kV (Ion source 2), respectively. The spectra were processed and internally calibrated with Trypsin autolysis peaks (804.41, 905.50, 1019.50, 1110.55, 1152.57, 1432.71, 2162.05, 2272.15, 2551.24), and keratin peaks (1066.44, 1179.59, 1232.62, 1277.70, 1475.78, 1993.97, 2383.93). Erythrocyte membrane protein peaks if found were removed manually before processing. For peptide mass fingerprinting based identification of proteins, the tryptic peptide mass maps (monoisotopic) were searched using MASCOT peptide mass fingerprint (PMF) against MSDB (3,239,079 sequences; 1,079,594,700 residues) and PlasmoDB (5460 sequences; 4087155 residues) using the Biotools version 3.2 (build 1.31) (Bruker Daltonics, Germany). The searching parameters were set as follows enzyme-trypsin (cleavage at the C-terminal side of Lys and Arg unless the next residue was Pro), mass tolerance of 100 ppm, 1 missed tryptic cleavage, fixed modification-carbamidomethylation (C), variable modification-oxidation (M), taxonomy - *P. falciparum* with no restriction in protein molecular weight. Only those proteins with MOlecular Weight SEarch (MOWSE) score >40 ($p < 0.05$) were accepted as identified.

**2.10. Immunoblot Analysis of *P. falciparum* Culture Supernatant.** Culture supernatant was collected using the protocol described by Haynes et al.[37] Briefly, schizonts infected human erythrocytes ($1.5 \times 10^7$ parasites/mL) were enriched, and the parasites were cultured for 6−10 h to allow the maturation of schizonts and release of merozoites. A control culture of uninfected erythrocytes was similarly prepared. Culture supernatants were collected from both the cultures and were centrifuged at 1000*g* for 15 min and then 10000*g* for 20 min before freezing at −70 °C. Culture supernatant was subsequently analyzed for the presence of parasite-specific protein by immunoblot analysis.

**2.11. Immunofluorescence Assay on Unpermeabilized Merozoites.** The assay was performed on the merozoite stage of *P. falciparum* 3D7 parasite as described earlier.[38] Briefly, 10 $\mu$L of the purified merozoites were resuspended in a 100 $\mu$L solution of any two of the primary antibodies (mice anti-PfRhopH3-C, 1:100; rabbit anti-PfMSP-1, 1:250; rabbit anti-PfMSP-3, 1:100; mice anti-PfRAP-1, 1:250; rabbit anti-Pf-Fal-2, 1:100 antibodies) in 1% (wt/vol) BSA/PBS, at 4 °C for 30 min. The merozoites were pelleted and washed twice in 100 $\mu$L of 1% (wt/vol) BSA/PBS. Cells were further incubated with appropriate secondary antibody [fluorescein isothiocyanate (FITC) or Cy3 labeled; dilution 1:100] in 1% (wt/vol) BSA/PBS at 4 °C for 30 min. The stained merozoites were subsequently washed in 1% BSA/PBS and stained with 10 ng/mL DAPI for 5 min. The merozoites were subsequently washed with PBS, resuspended in 30 $\mu$L of PBS and mounted on a slide under a

coverslip. Images were acquired on Nikon TE 2000-U fluorescence microscope and processed using Adobe Photoshop.

**2.12. Immunofluorescence Assay.** Indirect immunofluorescence assays were performed on schizont and merozoite stages of *P. falciparum* 3D7 parasite as described earlier.[35] Thin smears were made on glass slide and fixed with a mixture of methanol/acetone. Slides were blocked in blocking buffer (PBS, 10% FBS) for 2 h at 37 °C. Immunostaining was performed using primary antibodies (mice anti-PfRhopH3-C, 1:100; rabbit anti-PfClag3.1, 1:250) and appropriate secondary antibody conjugated to fluorescent dye (FITC or Cy3; dilution 1:100) as previously described in section 2.11. Immunofluorescence assay using preimmune mouse serum and anti-GST (1:100 dilution) were used as negative controls. Images were acquired on Nikon TE 2000-U fluorescence microscope and processed using Adobe Photoshop.

**2.13. Erythrocyte Binding Assay with Recombinant Protein.** Erythrocyte binding assays were carried out using recombinant MBP-PfRhopH3-C, MBP-PfRhopH3-N, and MBP.[39,40] About 100 $\mu$L of packed cell volume of normal human erythrocytes, and erythrocytes treated with neuraminidase, chymotrypsin, and trypsin were used to analyze the binding of two PfRhopH3 protein fragments. Briefly, human erythrocytes were washed with RPMI1640 media and incubated with 1 U of neuraminidase (*Clostridium perfringens*; Roche Diagnostics) for 1 h at 37 °C. For chymotrypsin and trypsin treatment, washed human erythrocytes were incubated with 1 mg/mL TLCK treated α-chymotrypsin (Sigma) and 1 mg/mL TPCK-treated trypsin (Sigma) for 1 h at 37 °C, respectively. The erythrocytes were suspended in RPMI1640 containing 10% FCS and ∼2.5−5 $\mu$g of recombinant protein to a final volume of 700 $\mu$L. This suspension was incubated for 1 h at room temperature with constant shaking on a nutator. The reaction mixture was layered over 600 $\mu$L of dibutyl phthalate (Sigma) and centrifuged to collect erythrocytes. Proteins bound to the untreated and treated erythrocytes was eluted with 300 mM NaCl and separated by SDS−PAGE and detected by Western blot using anti-MBP antibody. *P. vivax* Duffy binding antigen region II (PvRII)[41] was taken as a positive control for binding reaction.

**2.14. COS7 Cell Expression Plasmid Constructs, COS7 Cell Culture, Transfection, Immunofluorescence Assay and Erythrocyte Binding Assay on COS7 Cells.** Gene fragments corresponding to C terminal and N-terminal regions of PfRhopH3 gene were amplified using primer sets: Rhop-C-cos_For/Rhop-C-cos_Rev and Rhop-N-cos_For/Rhop-N-cos_Rev respectively (Supplemental Table 4, Supporting Information). The amplified fragments were digested with *Pvu*II and *Apa*I restriction enzymes and ligated in pRE4 vector in frame with the signal sequence and transmembrane segment of *Hepes simplex virus* glycoprotein D (HSV gD).[41]

COS7 cell binding assay was performed according to the protocol described by Chitnis and Miller.[42] Briefly, COS7 cells were transfected with 2 $\mu$g of recombinant plasmid DNA using Lipofectamine Plus reagent (Invitrogen). The transfected cells were grown for 24 h and split into two fresh 35 mm diameter, six well plates and were again grown for 24 h at 37 °C. Expression of the fusion proteins on the cell surface was analyzed by immunofluorescence assays after 24−30 h using anti-PfRhopH3-C and anti-PfRhopH3-N antibodies. COS7 cells transfected with plasmid pHVDR22 were used as positive control and anti-PvRII antibody was used for immunofluorescence assay.[42] For the erythrocyte binding assay, COS7 cells expressing PfRhopH3-C and PfRhopH3-N on the surface were incubated with untreated erythrocytes. The number of rosettes of erythrocytes bound to transfected COS7 cells were scored in 20 fields at 200× magnification for each set. A cluster of eight or more erythrocytes bound to a COS7 cell was scored as a rosette. The number of rosettes observed was normalized for transfection efficiency of 5% for all the sets.

**2.15. Invasion Inhibition Assay.** Invasion inhibition assay was performed as previously described.[35] Briefly, ring-stage parasites were synchronized by sorbitol lysis and allowed to mature through to the schizont stage. The hematocrit and parasitemia were adjusted to 0.8 and 0.5%, respectively. Mouse sera (preimmune and immune) were heat inactivated at 56 °C for 30 min and were added to the parasite cultures in 96-well plates at final concentrations of 10 and 20%. The cultures were incubated for 26 h to allow for schizont rupture and merozoite invasion. For microscopic analysis, smears were made from duplicate wells, stained with Giemsa, and the numbers of ring stage parasites per 1000−1500 RBCs were determined for each well. Percentage inhibition of the parasite invasion was calculated.

**2.16. Protein−Protein Interaction Network Construction.** The protein−protein interaction network was developed using STRING 8.1 database at http://string81.embl.de/. The protein identity (IDs) obtained from MALDI TOF/TOF analyses were entered in the database and were searched for possible interactions and interacting proteins. The confidence score was set to 0.150 (low confidence) with not more than 10 interacting proteins.

## 3. Results

**3.1. Identification of PfRhopH3 as a PfMSP-1 Interacting Protein.** MSP-1 is one of the most abundant and essential protein on the surface of *Plasmodium* merozoites.[43] To get an insight about the MSP-1 associated complex on the *Plasmodium* merozoite surface, we generated a bacterial *P. falciparum* cDNA library in a pTRG vector and screened it with a pBT plasmid carrying a fragment of PfMSP-1 gene (1.8 kbp) corresponding to 65 kDa C-terminal region of PfMSP-1. Plasmids were isolated from the positive clones and the cloned fragments were PCR amplified and sequenced (Supplemental Figure 1A, Supporting Information). Sequences were analyzed at PlasmoDB and NCBI database, using BLASTX program. On the basis of sequence homology, a number of *Plasmodium* proteins were identified; however, among these proteins best *e*-value ($1.1e^{-16}$) was observed for a protein, PfRhopH3 [accession number PF10265c] (Supplemental Figure 1B, Supporting Information). Sequencing data showed that the C-terminal region of PfRhopH3 (amino acids 727−847) interacted with PfMSP-1 fragment (Figure 1). A previous large-scale yeast interactome study carried out by LaCount et al.,[44] using yeast two hybrid libraries, identified a similar interaction between C-terminal region of PfRhopH3 [amino acids 734−865] and C-terminal [amino acids 1385−1441] region of PfMSP-1 (Figure 1).

**3.2. PfMSP-$1_{19}$/PfMSP-$1_{42}$ Proteins Interacts with the C-Terminal Region of PfRhopH3.** PfMSP-1 and PfRhopH3 interaction was further confirmed by an in vitro interaction study between recombinant PfMSP-1 protein fragments $(His)_6$-PfMSP-$1_{19}$/$(His)_6$-PfMSP-$1_{42}$ and MBP-PfRhopH3-C protein (Figure 2A). As shown in Figure 2B, MBP-PfRhopH3-C protein interacted strongly with both $(His)_6$-PfMSP-$1_{19}$ as well as $(His)_6$-PfMSP-$1_{42}$ proteins, while MBP alone did not show any binding.

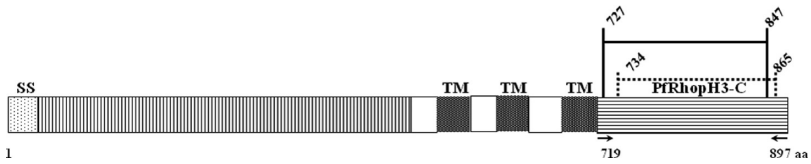



**Figure 1.** Schematic representation of PfRhopH3 protein (Gene ID: PFI0265c) indicating the positions of predicted signal sequence (white square with black dots), N-terminal region (white square with vertical black lines), C-terminal region (white square with horizontal black lines) and predicted transmembrane region (black square with white dots). The solid line (—) indicates interaction identified by bacterial two-hybrid screen (present study), and the dotted line (···) indicates interaction identified by yeast two-hybrid screen (see ref 44).

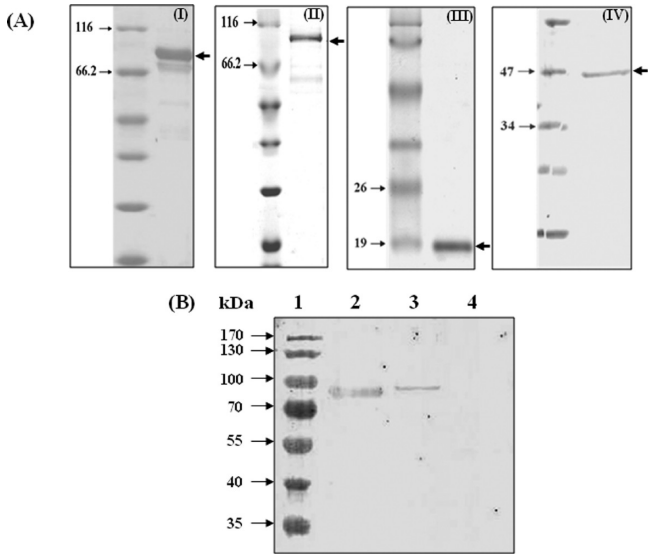



**Figure 2.** PfRhopH3-C terminal fragment interacts with PfMSP-1. (A) Coomassie-stained SDS polyacrylamide gel of affinity purified protein fragments of PfRhopH3 and PfMSP-1. (I) MBP-PfRhopH3-C, (II) MBP-PfRhopH3-N, (III) $(His)_6$-PfMSP-$1_{19}$, (IV) $(His)_6$-PfMSP-$1_{42}$. (B) In vitro protein−protein pull-down assay to study interaction of MBP-PfRhopH3-C and MBP alone with PfMSP-$1_{19}$ and PfMSP-$1_{42}$. Western blot developed using anti-MBP antibody to detect MBP fusion protein in eluates. Lane 1: marker; lane 2: MBP-PfRhopH3-C & $(His)_6$-PfMSP-$1_{19}$; lane 3: MBP-PfRhopH3-C & $(His)_6$-PfMSP-$1_{42}$; lane 4: MBP & $(His)_6$-PfMSP-$1_{19}$.

These results provided additional evidence for the PfMSP-1 interaction with PfRhopH3.

**3.3. Analysis of PfMSP-1 and PfRhopH3 Interactions by Immunoprecipitation followed by Mass Spectrometry-Based Proteome Analysis.** To determine whether such an interaction between PfMSP-1 and PfRhopH3 takes place on the *Plasmodium* merozoite surface, immunoprecipitation studies were performed with the cultured parasites enriched in schizonts and merozoites stages using either preimmune mouse sera or anti-PfMSP-$1_{42}$ or anti-PfRhopH3 sera bound to protein-A Sepharose beads. Western blot analysis of anti-PfMSP-$1_{42}$ immunoprecipitated extract showed the presence of PfRhopH3 as well as PfMSP-1 in the same sample. Similar association was observed when parasite extract was immunoprecipitated with anti-PfRhopH3 sera (Figure 3). However, neither PfMSP-1 nor PfRhopH3 were detected in parasite extract immunoprecipitated with preimmune sera. PfAMA-1, a known micronemal surface protein, was not detected in either of the immunoprecipitated extracts showing the specificity of the reaction (Figure 3). Even an internal parasite protein, falcipain-2, could not be detected in these immunoprecipitated extracts (data not shown). Together, these results suggested that PfRhopH3 specifically associates with PfMSP-1 on the surface of *Plasmodium* merozoites.

To know whether PfMSP-1 interacts with other surface proteins besides the PfRhopH3, gel slices corresponding to each resolved protein band were excised, digested with trypsin, and the eluted peptides were analyzed using MALDI-TOF/TOF (Figure 4A, Table 1, Supplemental Table 1, Supporting Information). A total of 16 distinct proteins were identified by peptide mass fingerprint analysis of the spectral plots against PlasmoDB as well as MSDB. Among these proteins, band numbers -8, -14, and -15 represented the mouse immunoglobin chains, while rest of the 13 bands belonged to the proteins of parasite origin. Number of peptides sequences corresponding to each band (protein) and their sequence coverage is depicted in Figure 4B,C. Identified peptides sequences corresponding to these bands are presented in Supplemental Tables 2 and 3, Supporting Information. They represented PfMSP-1 (three fragments; ∼200 kDa, ∼185 kDa, and ∼19 kDa), PfMSP-3, PfMSP-6, PfMSP-7, PfMSP-9, PfRhopH1/Clag9, PfRhopH3, PfRAP-1, PfRAP-2, and two RAP domain containing proteins. Interestingly, both immunoprecipitation reactions obtained using either anti-PfMSP-1 or anti-PfRhopH3 antibodies showed similar protein profiles as identified by MALDI-TOF/TOF (Figure 4B,C, Table 1, Supplemental Table 1, Supporting Information). None of the parasite-specific proteins except for mouse IgG bands were identified in preimmune precipitated fraction. These results were further confirmed by Western blot studies performed on anti-PfMSP-1 or anti-PfRhopH3 immunoprecipitated merozoite extract. Both immunoprecipitated reactions showed the presence of PfRAP-1 as well as PfRhopH1 as determined by their respective antibodies in these parasites extracts (Figure 3). Taken together, results of immunoprecipitation experiments followed by Western blot as well as MALDI-TOF/TOF analysis showed that PfMSP-1 and PfRhopH3 interact with each other, and nine other proteins are associated with these proteins, thereby forming a large complex not identified so far.

**3.4. Localization of PfRhopH3 at the Apical Tip of Merozoites Showing its Association with PfMSP-1.** Next, we sought to define the association between these proteins on the unpermeabilized merozoites surface by performing colocalization studies using specific antibodies against PfMSP-1, PfMSP-3, PfRAP-1, and PfRhopH3-C proteins. As shown in Figure 5, PfMSP-1 and PfMSP-3 staining was detected on the entire surface of merozoites, while PfRhopH3 and PfRAP-1 staining localized at the apical end of merozoites. Co-localization was observed between PfMSP-1 and PfRAP-1, PfMSP-1 and PfRhopH3, PfMSP-3 and PfRhopH3 as well

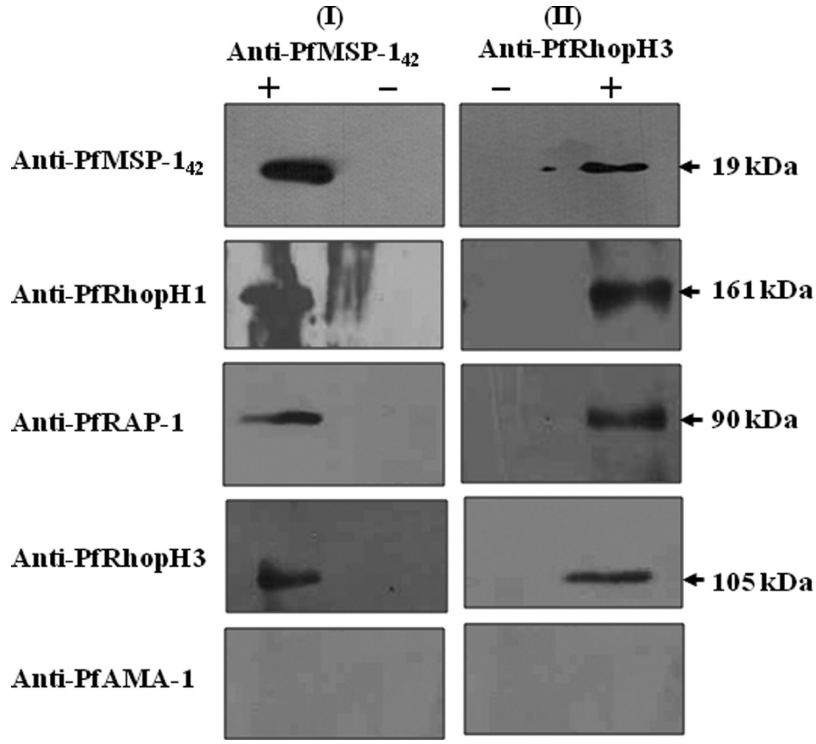



**Figure 3.** Immunoblot analysis of immunoprecipitated *P. falciparum* merozites extract. Anti-PfMSP-1$_{42}$ or anti-PfRhopH3 immunoprecipitated merozoite extract were probed with anti-PfMSP-1$_{42}$, anti-PfRhopH1, anti-PfRAP-1, anti-PfRhopH3, or anti-PfAMA-1 antibodies. (+) indicates immunoprecipitation with anti-PfMSP-1$_{42}$ (I) or anti-PfRhopH3 antibody (II). (−) Indicates immunoprecipitation with preimmune sera.

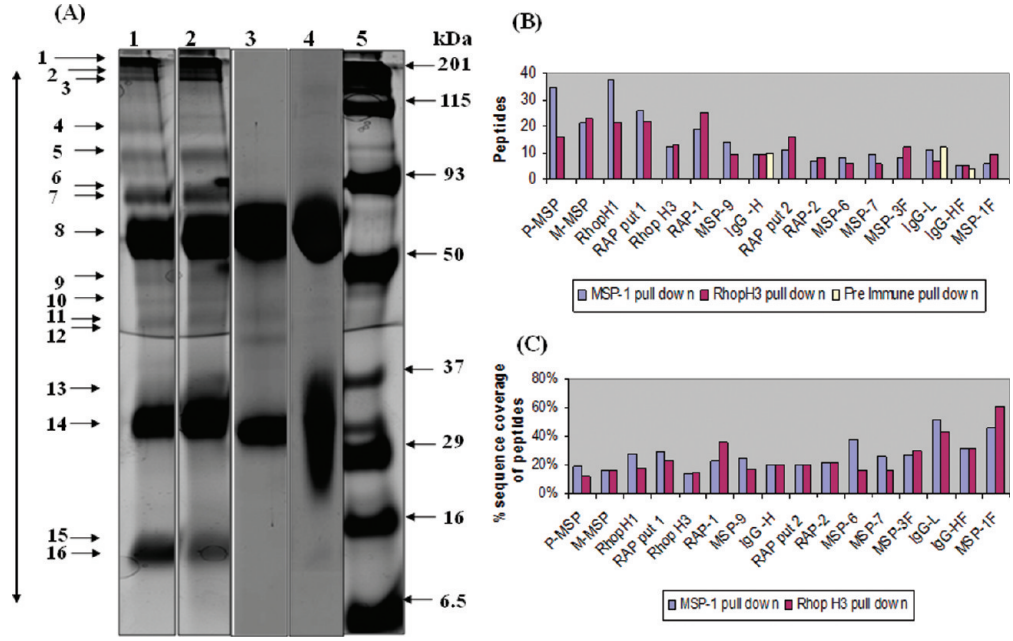



**Figure 4.** Isolation and characterization of 11-member PfMSP-1 associated invasion complex. (A) Silver stained SDS−PAGE gels of proteins eluted from immunoprecipitations performed using anti-PfMSP-1 (lane 1), anti-PfRhopH-3 (lane 2) antibodies on parasite lysates prepared from late schizonts and merozoites stages. Lane 3: immunoprecipitation performed using preimmune sera. Lane 4: immunoprecipitaion performed using anti-PfMSP-1 antibody on parasite lysate prepared from ring stage. Lane 5: molecular weight marker. (B) The total number of peptides present in all excised bands from lanes 1 and 2. (C) Sequence coverage for each of the identified proteins from lanes 1 and 2.

as between PfMSP-3 and PfRAP-1, mainly at the apical end of the merozoites. To know whether any permeabilization occurred during the staining process, we performed colocalization studies using anti-PfMSP-1 and antifalcipain-2 (Pffal-2) antibodies. As shown in Figure 5 (panel V), MSP-1 staining was seen on the surface of merozoite, but no falcipain-2 staining was detected, thus confirming the intactness of merozoites. We also carried out colocalization studies using anti-PfRhopH3 and anti-PfClag3.1 in permeabilized merozoites to know about the specificity of anti-PfRhopH3

**Table 1.** Identification of Malarial Proteins Immunoprecipitated Using Anti-PfMSP-$1_{42}$ Antibody by MALDI-TOF/TOF

| band ID | protein name | accession number MSDB | accession number PLASMODB | apparent molecular weight (kDa) | sequence coverage (%) | masses matched | MOWSE Score | source |
|---|---|---|---|---|---|---|---|---|
| 1 | precursor of the major merozoite surface antigens precursor | Q25922_PLAFA | psu\|PFI1475w | 196.746 | 19 | 35 | 153 | *P. falciparum* |
| 2 | major merozoite surface antigen precursor | S05603 | psu\|PF14_0236 | 188.643 | 16 | 21 | 84 | *P. falciparum* |
| 3 | RhopH1/Clag9 | Q4AE83_PLAFA | psu\|PFI1730w | 161.069 | 28 | 38 | 96 | *P. falciparum* |
| 4 | RAP protein, putative | Q8IAY8_PLAF7 | psu\|PF08_0070 | 116.788 | 29 | 25 | 201 | *P. falciparum* |
| 5 | rhoptry complex polypeptide RhopH3 | A45554 | psu\|PFI0265c | 105.560 | 14 | 12 | 67 | *P. falciparum* |
| 6 | rhoptry-associated protein 1 | Q8ILZ1_ PLAF7 | psu\|PF14_0102 | 90.452 | 23 | 19 | 143 | *P. falciparum* |
| 7 | 101 K malaria antigen precursor-merozoite surface protein 9 | ABRA_PLAFG | psu\|PFL1385c | 69.308 | 24 | 11 | 64 | *P. falciparum* |
| 8 | immunoglobulin gamma-3 heavy chain precursor | BAA03476 | | 52.171 | 20 | 9 | 82 | *Mus musculus* |
| 9 | RAP protein, putative | Q8IJA7_ PLAF7 | psu\|PF10_0291 | 52.993 | 20 | 11 | 66 | *P. falciparum* |
| 10 | rhoptry-associated protein 2 (fragment) | Q9U432_PLAFA | psu\|PFE0080c | 47.051 | 20 | 6 | 74 | *P. falciparum* |
| 11 | merozoite surface protein 6 (fragment) | Q6R1M1_PLAFA | psu\|PF10_0346 | 42.250 | 29 | 7 | 57 | *P. falciparum* |
| 12 | merozoite surface protein 7 | Q8IDX8_PLAF7 | psu\|PF13_0197 | 41.308 | 26 | 9 | 93 | *P. falciparum* |
| 13 | merozoite surface protein 3 (fragment) | Q9NFV9_PLAFA | psu\|PF10_0345 | 36.952 | 27 | 8 | 90 | *P. falciparum* |
| 14 | monoclonal antibody 13-1 light chain | JC5810 | | 24.144 | 52 | 11 | 153 | *M. musculus* |
| 15 | immunoglobulin gamma-1 chain precursor (fragment) | AAA60441 | | 13.081 | 32 | 5 | 71 | *M. musculus* |
| 16 | merozoite surface protein 1 (fragment) | Q208F4_PLAFA | | 12.549 | 46 | 6 | 101 | *P. falciparum* |

antibody and the specific location of PfRhopH3 in rhoptries. As shown in Supplemental Figure 2, Supporting Information, anti-PfClag3.1 antibodies showed punctate staining in schizonts and stained the rhoptries specifically in the free merozoites. PfRhopH3 colocalized with PfClag3.1, a known rhoptry protein at the schizont as well as merozoite stages.

**3.5. PfMSP-1 Associated Complex Is Shed off after the Invasion of Merozoite.** To know whether the large MSP-1 complex remains associated with the ring stage parasite after invasion, colocalization studies were carried out at the ring stage of the parasite using anti-PfMSP-$1_{19}$, anti-PfRhopH3, and anti-PfRAP-1 antibodies. We could not detect either PfRhopH3 or PfRAP-1 proteins at the ring stage of *P. falciparum*; however, a weak staining for PfMSP-1 protein was seen at this stage (Supplemental Figure 3, Supporting Information). Loss of MSP-1 associated complex after invasion was also confirmed by immunoprecipitation performed with the *P. falciparum* ring stage extract using anti-PfMSP-1 antibody (Figure 4, lane 4). Except for PfMSP-$1_{19}$ and the two IgG moieties, no other surface protein was identified by MALDI-TOF/TOF analysis.

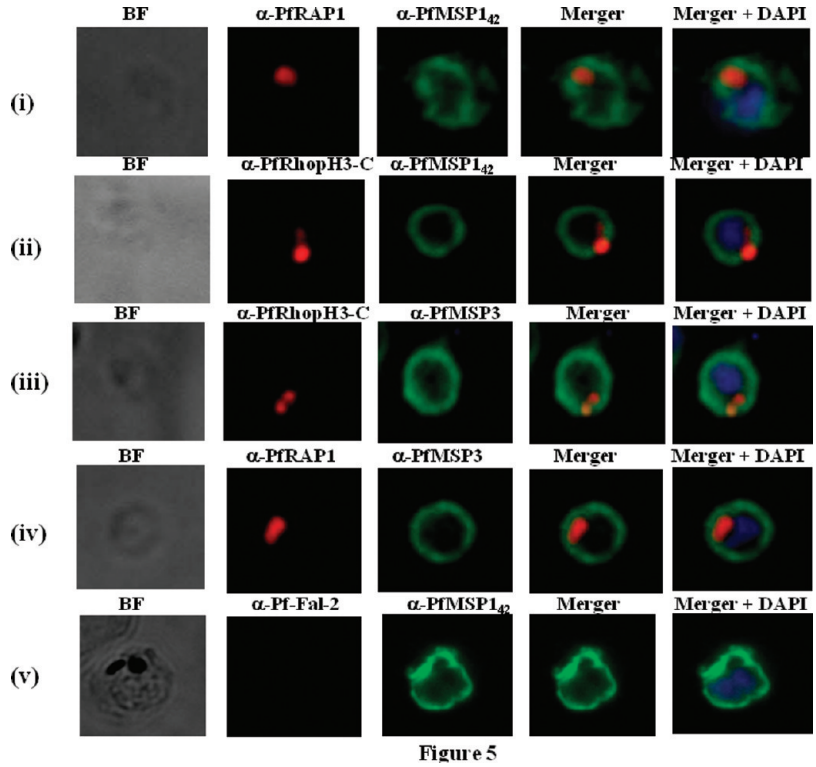



Figure 5

**Figure 5.** Localization of proteins of PfMSP-1 associated complex by immunofluorescence assay. Unpermeabilized *P. falciparum* merozoites were coimmunostained with anti-PfRAP-1 and anti-PfMSP-$1_{42}$ antibodies (i), with anti-PfRhopH3-C and anti-PfMSP-$1_{42}$ antibodies (ii), with anti-PfRhopH3-C and anti-PfMSP-3 antibodies (iii), anti-PfRAP-1 and anti-PfMSP-3 antibodies (iv), and anti-Pf-falcipain-2 and anti-PfMSP-$1_{42}$ antibodies (v).

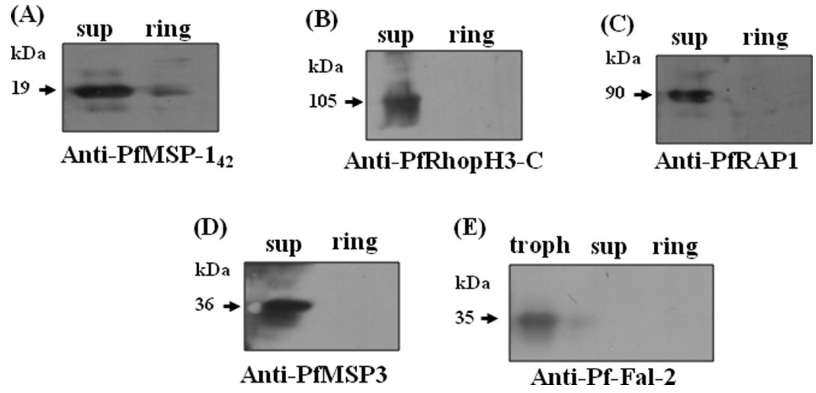



**Figure 6.** Immunoblot analyses to show the shedding of components of PfMSP-1 associated complex. Western blot analysis of culture supernatant (sup) and ring stage *P. falciparum* extract (ring) using anti-PfMSP-1$_{42}$ (A), anti-PfRhopH3-C (B), anti-PfRAP-1 (C), anti-PfMSP-3, (D) and anti-Pf-falcipain-2 (E) antibodies.

We next performed Western blot analysis of culture supernatant and of parasite extract at ring stage of *P. falciparum*. As seen in Figure 6, we could detect proteins of the PfMSP-1 associated complex, and MSP-3, RhopH3, and RAP-1 in the culture supernatant. However, none of these surface proteins except for MSP-1 were detected in the ring stage parasite extract suggesting that MSP-1 associated complex is shed off after the invasion. Falcipain-2, an internal food vacuole protein, was neither detected in the culture supernatant nor at the ring stage but was found to be expressed at the trophozoite stage of the parasite, confirming the specificity of the staining reaction.

**3.6. PfRhopH3 Binds with Human RBCs through its C-terminal and Anti-PfRhopH3-C Antibody Inhibits Erythrocyte Invasion.** We next characterized PfRhopH3, a component of MSP-1 associated complex, for RBC binding. In vitro RBC binding assay was performed with two recombinant fragments of PfRhopH3: MBP-PfRhopH3-C and MBP-PfRhopH3-N. *P. vivax* Duffy binding antigen region-II protein (PvRII) was taken as a positive control. Purified MBP-PfRhopH3-C and PvRII proteins showed significant binding to human erythrocytes [Figure 7A (I and II)], whereas MBP-PfRhopH3-N and MBP showed weak or no binding to RBCs [Figure 7A (III and IV)], suggesting that PfRhopH3 especially its C-terminal region is involved in binding to human RBCs. MBP-PfRhopH3-C failed to bind to trypsin treated RBCs suggesting that PfRhopH3-C binds to a trypsin sensitive receptor on the RBC surface [Figure 7A (I)].

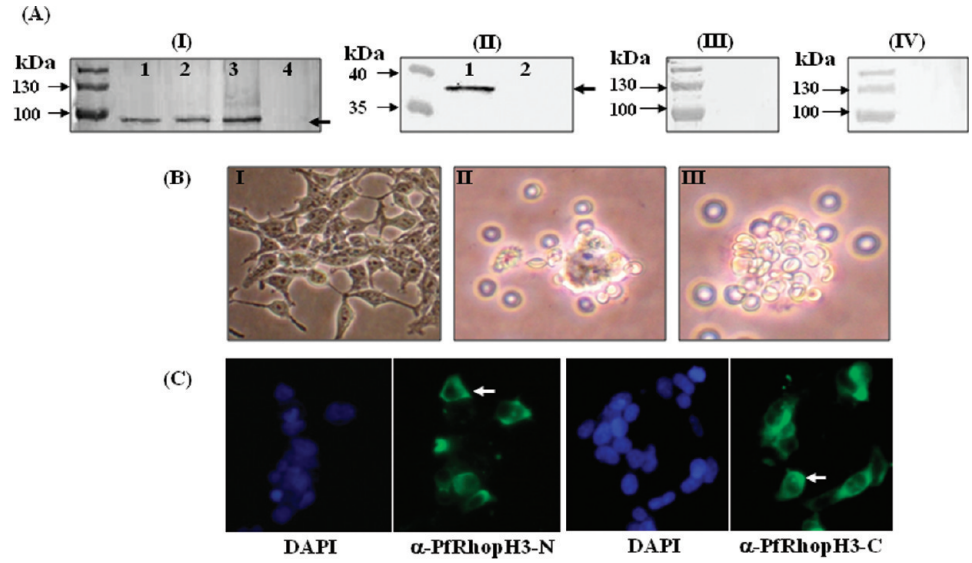
To further confirm the binding of PfRhopH3-C to RBCs, COS-7 cell expressing N- and C-terminal fragments of PfRhopH3 were analyzed for RBCs binding. Expression of the proteins on the surface of COS-7 cells was determined by their respective antibodies and the binding of RBC was determined by number of rosette seen under the microscope (Figure 7B,C). COS7 cells expressing PfRhopH3-C bound RBCs with almost similar efficiency as observed for PvRII protein, a known RBC binding protein, whereas low level of RBC binding was seen in the case of COS7 cells expressing PfRhopH3-N fragment (Figure 7D).

We next assessed the ability of anti-PfRhopH3 antibodies to inhibit the parasite invasion by in vitro parasite growth assay. As shown in Table 2, anti-PfRhopH3 antibodies significantly inhibited the parasite growth in a concentration-dependent manner. The invasion inhibition by anti-PfRhopH3 antibody was comparable to the inhibition by anti-PfMSP-1$_{42}$ antibodies. Taken together, results of our studies suggested that the C-terminal region of PfRhopH3 is involved in RBC binding as well as erythrocyte invasion. Together, these results are in agreement with a number of previous reports that have shown binding of PfRhopH complex or PfRhopH3 to erythrocytes and suggested its possible role in invasion.[45−48]

## 4. Discussion

The invasion in the apicomplexan is a unique and complex process that involves a number of surface/secretory antigens present or released on the merozoite surface.[27] It is believed that many merozoite surface proteins form complexes at the time of merozoite invasion and elucidating such interaction(s) may provide insights into the exact molecular basis of invasion. The surface coat of *P. falciparum* merozoites is largely comprised of nine GPI anchored proteins, of which MSP-1 is essential and most predominant.[8,43] Even though these proteins have been studied in detail as malaria vaccine candidate antigens, a definitive function of these molecules remain elusive. Although a large-scale yeast interactome analysis for *P. falciparum* proteins has suggested an interaction link between MSP-1/MSP-9 to other surface proteins especially the PfRhopH family of proteins, no experimental evidence has been provided yet.[44] To gain insights into such MSP-1 associated molecular interactions on the merozoite surface, a *P. falciparum* bacterial two hybrid cDNA library was cotransformed with PfMSP-1 and a fragment corresponding to the C-terminal region of PfRhopH3 was found to be interacting with PfMSP-1. Interaction between PfRhopH3 and Pf MSP1 was further confirmed by in vitro interaction and colocalization studies. These interactions appeared to be on the surface of merozoites as we could colocalize these proteins in unpermeabilized merozoites. It appeared that PfMSP-1 is present on the merozoite surface continuously while PfRhopH3 is secreted out around the time of invasion to enable it to form a complex with MSP-1 and other MSPs. We could not localize PfRhopH3 at the ring stage suggesting that this complex is formed only for a short period during merozoite invasion.

**Identification of PfMSP-1 and Rhoptry Proteins Complex.** PfRhopH3 protein has been previously shown to be a part of a heavy molecular weight RhopH complex.[4,46,49] Since a novel interaction between PfMSP-1$_{42}$ and PfRhopH3 was identified by bacterial as well as yeast two hybrid screening and MSP-1 has also been shown to interact with other merozoite surface proteins,[22−24] it appeared of interest to us to look for a large MSP-1 associated complex on the merozoite surface. To do so, merozoite surface proteins were cross-linked as described by




(D)

| Construct | Number of Rosettes[1] |
|---|---|
| PfRhopH3-C | 66 ± 9 |
| PfRhopH3-N | 29 ± 2 |
| pHVDR22[2] | 67 ± 1 |

**Figure 7.** Erythrocyte and COS7 cell binding assays to show binding of recombinant MBP-PfRhopH3-C with RBCs. (A) [I] Western blot analysis of eluates from the RBC binding assay using anti-RhopH3-C antibodies. Lane 1: eluates from untreated RBC, lane 2: eluates from α-chymotrypsin treated RBC, lane 3: eluates from neuraminidase treated RBC, lane 4: eluates from trypsin treated RBC (II) Western blot using monoclonal antipenta histidine antibodies to detect recombinant proteins in elutes from the RBC binding assays of protein PvRII, using untreated human RBC (lane 1), human RBCs treated with chymotrypsin (lane 2), (III) and (IV) Western blot assay using anti-PfRhopH3-N and anti-MBP antibodies to detect respective recombinant proteins in elutes from the RBC binding assays. (B) PfRhopH3-C binds to RBC in COS7 binding assay. Cells expressing PfRhopH3-N or PfRhopH3-C terminal fragments were incubated with normal erythrocytes and number of rosettes was counted. (I) Normal COS7 cells (II) RBCs bound to COS7 cells expressing PfRhopH3-N and (III) RBCs bound to COS7 cells expressing PfRhopH3-C. Note the rossette in panel III (C) Immuno-fluorescence assay to show the expression of C-terminal fusion protein on the surface of the COS7 cells. Arrow indicates transfected cells. (D) Table showing the binding of human erythrocytes with COS7 cells expressing PfRhopH3-C and PvRII fusion proteins. [1]The number of COS7 cells with rosettes of bound erythrocytes was scored in 20 fields at 200× magnification. Number of rosettes of each experiment was normalized according to respective transfection efficiency to 5%. Mean value of two independent experiments is reported with standard deviation. [2]Construct designed to express *P. vivax* Duffy binding protein region II (see ref 40).

**Table 2.** Invasion-Inhibition of Erythrocytes by *P. falciparum* in the Presence of Anti-PfRhopH3 Antibodies[a]

| | conc of antibody 10%[b] | | conc of antibody 20%[b] | |
|---|---|---|---|---|
| group | % parasitemia | % inhibition | % parasitemia | % inhibition |
| adjuvant | 1.54 ± 0.13 | 0.0 | 1.97 ± 0.24 | 0.0 |
| preimmune | 1.51 ± 0.43 | 1.95 | 1.65 ± 0.35 | 16.24 |
| anti-GST | 1.32 ± 0.03 | 4.28 | 1.74 ± 0.05 | 11.68 |
| anti-PfRhopH3-C | 1.06 ± 0.03 | 31.17 | 0.94 ± 0.05 | 52.28 |
| anti-PfMSP$1_{42}$ | 0.83 ± 0.31 | 46.1 | 1.00 ± 0.16 | 49.23 |

[a] Anti-GST and anti-PfMSP-$1_{42}$ antibodies were used as a control. ± represents the SD values in the % parasitemia data. [b] Final percentage of sera in the culture.

Sanders et al.[52] and immunoprecipitated with anti-PfMSP-$1_{42}$ or anti-PfRhopH3 antibodies. Importantly, each antiserum was able to pull-down PfMSP-1, PfRhopH3, and a number of other proteins. Western blot analysis identified two other merozoite surface proteins, PfRhopH1 and PfRAP-1 in the immunoprecipitates, indicating the presence of a large PfMSP-1 associated complex. These associations appeared to be highly specific as PfAMA-1 was not detected in any of the immunoprecipitated extracts. Stafford et al. previously used a similar approach and identified a number of parasite-derived polypeptides associated with MSP-1.[50] Also reports by LaCount et al.[44] and Suthram et al.[51] have indicated PfRhopH1 and PfRAP-1 as a part of MSP-1 interaction network. To unravel proteins associated with PfMSP-1, we performed a mass spectrometry based proteome analysis for anti-PfMSP-1 and anti-PfRhopH3 immunoselected polypeptides. Similar approach has been recently tried to identify protein complexes such as AMA-1/AMA1-associating protein (AAP) complexes from *Toxoplasma gondii*.[17,25] In total, 11 different proteins, PfMSP-1, PfMSP-3, PfMSP-6, PfMSP-7, PfMSP-9, PfRhopH3, PfRhopH1/Clag, PfRAP-1, PfRAP-2, and two putative RAP domain containing proteins were identified in the immunoprecipitated merozoite extract using anti-PfMSP-$1_{42}$ antibody. Nine of these proteins are the known merozoite surface proteins, while two other proteins are the RAP domain containing proteins. Figure 8 reveals the network of PfMSP-1 and PfRhopH3 associated proteins based on the proteome analysis performed in the present study and in a number of previous studies.[23,44] Association of PfRAP and PfRhop complexes with PfMSP-1 is intriguing as these complexes have been shown to be present in detergent-resistant membrane fraction.[46,48,52,53] However, a number of reports have also shown the role of PfRhopH3 in erythrocyte binding and merozoite invasion.[47−50] It may be possible that these protein complexes are secreted out for a short time and form a complex with MSP-1 at the time of invasion. The presence of two RAP domain containing proteins in PfMSP-1 complex is also surprising as

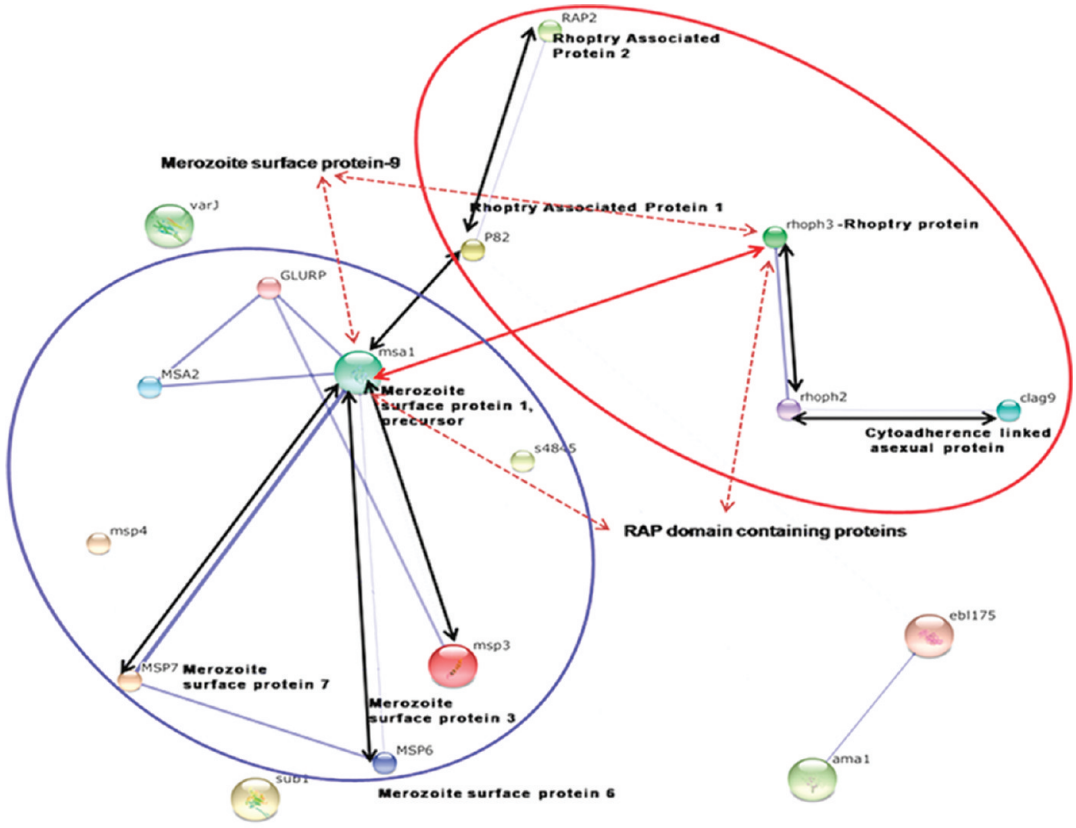



**Figure 8.** STRING 8.1 generated network of PfMSP-1 and PfRhopH3 associated proteins. The map shows the confidence view of the network with stronger associations being represented by thicker (blue) lines. The black lines depict the proteins identified using MALDI TOF/TOF analysis, the blue and red circles show the PfMSP-1 and PfRhopH3 protein complexes, respectively. PfMSP-9 and putative RAP domain containing proteins were not recognized by the database search and they are represented as separate entries with (red) dotted lines.

the RAP domain is a putative RNA binding motif and is particularly abundant in apicomplexan.[54] Interestingly, both RAP domain containing proteins (Pf08_0070 and Pf10_0291) are conserved among different *Plasmodium spp.*, and one of the RAP domain containing protein homologues in *P. vivax* (PVX_111235) has been listed as a putative secretory protein in PlasmoDB. Strikingly, the same proteins were identified when merozoite extract was immunoprecipitated with anti-PfRhopH3 antibody. It will be interesting to further study the role of these putative RAP proteins in the invasion process. Importantly, the proteome data identified PfMSP-6, PfMSP-7, and PfMSP-9 in the MSP-1 associated complex, thus confirming the previous reports which showed PfMSP-1 interaction with PfMSP-9 and with PfMSP-6/PfMSP-7.[23,24,36] We could not detect PfRhopH2 and PfRAP-3 in this complex.[46,55] This could be due to transient association of these proteins with the complex or these proteins may form an independent complex in the rhoptries. The large PfMSP-1 complex identified on the merozoite surface could not be detected at the ring stage of *P. falciparum*. Instead, we could detect the components of this in the culture supernatant, indicating shedding of this complex. Together, results from the present study provide strong evidence for the existence of a PfMSPs and rhoptry proteins complex on the merozoite surface of *P. falciparum* which presumably is involved in erythrocyte invasion and is shed off after the invasion process.[8]

Many of the MSP-1 associated proteins described in the present study have been shown to be involved in erythrocyte binding as well as its invasion. For example, a coligand complex of PfMSP-1 and PfMSP-9 binds to human erythrocyte via Band 3 protein.[24] Even a high molecular weight complex of rhoptry proteins present on *P. falciparum* merozoites and high-activity binding peptides corresponding to PfRhopH3 have been shown to bind erythrocytes.[45,48] Recently, antibodies targeting MSP-1/6/7 complex have been shown to inhibit erythrocyte invasion.[56] In the present study, we demonstrate that PfRhopH3-C domain binds to intact erythrocyte with high affinity in an in vitro as well as COS7 cell binding assays, and antibodies against PfRhopH3 efficiently block the entry of merozoites into erythrocytes. These results provide support to a number of previous reports that showed the protective effects of Rhop-3 immunization in mice against lethal homologous and heterologous rodent *Plasmodium* challenge infections.[57] Together, these results along with a number of previous studies[22−24,44] advocate the existence of a large MSP-1 associated ligand complex on the *P. falciparum* merozoites which is involved in multiple interactions with the erythrocyte.

## 5. Conclusions

In conclusion, this is one of the few studies that unambiguously show the cooperation between major merozoite surface proteins (MSPs) and rhoptry proteins on the *P. falciparum* merozoite surface. It identifies PfRhopH3 as another RBC binding protein. The study significantly extends our understanding of host−parasite interactions and highlights the complexity driving the active invasion of erythrocytes by *Plasmodium* merozoites. The study also advocates the use of

a multiprotein malaria vaccine based on complexes that exist on the merozoite surface.

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**Supporting Information Available:** Supplemental figures and table. This information is available free of charge via the Internet at http://pubs.acs.org.

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