Chapter-2

Materials and Methods
2.1 Parasite culture

*P. falciparum* laboratory strain 3D7, HB3, Dd2 and MCamp were cultured using methods described by Trager and Jensen [151]. Parasite cultures were grown in complete medium i.e. RPMI 1640 medium supplemented with 0.5 % Albumax I (Invitrogen, USA), 25mM sodium bicarbonate (Sigma, USA), 50 mg/L hypoxanthine (Sigma, USA) and 10 ml/L gentamycin (Gibco, USA). The cultures were maintained at 4% hematocrit and 5-10% parasitemia. Parasite stage and parasitemia was checked by making a thin smear on glass slide, fixing with methanol and staining with 5-10% Giemsa stain (Sigma, USA) for 5-10 minutes.

2.2 Sorbitol synchronization of the parasite

*P. falciparum* cultures were synchronized using sorbitol method [152]. Culture with majority at the early ring stage was centrifuged at 500xg for 5 minutes. After washing the culture once with incomplete RPMI-1640 (RPMI-1640 without albumax) (iRPMI), it was incubated with 7-10 pellet volumes of 5% sorbitol at 37°C for 10 minutes with intermittent mixing. Sorbitol lyses the mature trophozoite and schizonts, leaving rings unaffected. After incubation with 5% sorbitol, it was centrifuged at 500 g for 5 min. The culture was washed once with iRPMI and the pellet was re-suspended in complete RPMI-1640 (cRPMI) at a hematocrit of 4%. This was further allowed to grow at 37°C under mixed gas.

2.3 Enrichment of the parasite at the trophozoite or schizont stage by Percoll method

*P. falciparum* cultures with majority of infected erythrocytes at schizont stage were washed with incomplete RPMI. Parasite pellet was resuspended in iRPMI and layered onto equal volume of 65% Percoll (Sigma, USA). Tube was centrifuged at 800xg for 20 minutes. Schizonts obtained at the interface were
collected and washed twice with iRPMI. The culture was again re-suspended in cRPMI and allowed to grow further. The purity was estimated by Giemsa staining.

2.4 Enzyme treatments

2.4.1 Neuraminidase treatment:

100 µl of erythrocytes were washed twice with iRPMI. Erythrocytes were then incubated with 300mU of neuraminidase (Roche) in 14 ml of iRPMI (pH 6.8) at 37°C for 2 hrs. Erythrocytes were washed twice with iRPMI and further used for erythrocyte invasion assays.

2.4.2 Chymotrypsin/Trypsin treatment:

For Chymotrypsin/Trypsin treatment, 10⁹ erythrocytes in 10 ml of iRPMI were incubated with TPCK-treated trypsin (Sigma) or TLCK-treated chymotrypsin (Sigma, USA) at a final concentration of 1 mg/ml with rocking at 37°C for 1 hour. Cells were then washed twice with iRPMI. The erythrocytes were then treated with 1 mg/ml of trypsin-chymotrypsin inhibitor (Sigma, USA) at room temperature for 15 minutes. Cells were washed with iRPMI and stored at 4°C.

2.5 Invasion inhibition assay

In this assay, 6x10⁴ schizont-infected erythrocytes were incubated with 2x10⁹ target erythrocytes along with the purified total IgG (described in section 2.14) in a reaction volume of 100 µl. The efficacy of the antibodies was compared with pre-immune IgG control purified from the pre-bleeds of the same rabbits immunized with respective proteins. Parasitemia in cultures was estimated after an incubation of 40 hrs (one cycle of invasion in target erythrocytes) by flow cytometry. The whole sample was collected and washed twice with phosphate
buffer saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) and subjected to staining with ethidium bromide (10 μM) for 15 minutes at room temperature in dark. The cells were washed with PBS, and analyzed by flow cytometry on FACSCalibur (Becton Dickinson, USA) using CellQuest software. Fluorescence signal (FL-2) was detected with the 590 nm band pass filter using an excitation laser of 488 nm collecting 100,000 cells per sample. Following acquisition, data was analyzed for % parasitemia of each sample by determining the proportion of FL-2-positive cells using Cell Quest. The % inhibition was calculated by applying the formula:

\[
% \text{Inhibition} = \left[1 - \frac{\text{Invasion (PfMA IgG)}}{\text{Invasion (Pre-immune IgG)}}\right] \times 100
\]

Three independent experiments were done in duplicate. The inhibition data with untreated and enzyme treated erythrocytes was analyzed statistically using Student’s t-test. Statistical analyses were performed using Graph Pad Prism software (version 5.01; Graph Pad Software Inc. USA). Significant difference between two groups was calculated in terms of exact P-values by two-tailed non parametric Mann–Whitney test. A p value < 0.05 was considered statistically significant.

2.6 Preparation of genomic DNA.

Parasite culture (P. falciparum and P. berghei) was centrifuged at 500 g for 5 min and resuspended in iRPMI. Saponin (Sigma, USA) was added to a final concentration of 0.15% to lyse the erythrocyte membrane. Following centrifugation at 4000 g for 5 min, the parasite was resuspended in lysis buffer (50 mM Tris-Cl pH-8.0, 5 mM EDTA pH-8.0, 100 mM NaCl, 1% SDS, 0.1 mg/ml Proteinase K (Roche, USA) and incubated at 37°C for 3 hours with intermittent shaking. Equal volume of phenol (Sigma) was added, mixed well and centrifuged at 8000 g for 10 mins. Upper aqueous layer was transferred to a fresh tube. Extraction was
performed similarly with chloroform (Sigma). RNase (Sigma) was added at a final concentration of 10 μg/ml and incubated for 1 hr at 37°C. Extraction was repeated again in a similar manner with phenol:chloroform. Genomic DNA was precipitated from upper aqueous layer by addition of 1/10 volume of sodium acetate (pH-5.2) and 2.5 volumes of absolute ethanol. After overnight incubation at -20°C, genomic DNA was pelleted by centrifugation at 8000 g for 30 min at 4°C. Genomic DNA was washed once with 70% ethanol and air-dried. The pellet was dissolved in 25-100 μl of DNase-RNase free water. Concentration of the genomic DNA was measured by spectrophotometer by taking O.D. at 260 nm.

2.7 Preparation of total RNA and cDNA from *P. falciparum*

*P. falciparum* culture at different stages i.e. ring, trophozoite and schizonts were grown to a parasitemia of ~10%. Infected erythrocytes were washed with diethyl pyrocarbonate (DEPC) treated PBS and further lysed using 0.5% saponin as described above. After lyses of infected erythrocytes, parasites were collected by centrifugation at 4000 xg for 5 min at 4°C. Pellet was re-suspended in 1 ml of TRIzol reagent (Invitrogen, USA) and incubated for 5 minutes at 15-30°C to permit complete dissociation of nucleoprotein complexes. Subsequently, 0.2 ml of chloroform (Merck) per ml of TRIzol reagent was added and the tubes were shaken vigorously for 15 seconds followed by incubation at room temperature for 2-3 minutes. The tubes were centrifuged at 15000 g for 15 minutes at 2-8°C and the upper layer containing RNA was transferred to a fresh tube. RNA was precipitated with 0.5 ml of isopropanol (Sigma, USA) per ml of TRIzol. Samples were incubated at 15-30°C for 10 minutes and centrifuged at 15,000 g for 10 minutes at 2-8°C. RNA pellet was washed with 75% ethanol (Merck), centrifuged at 9,000 g for 5 minutes at 4°C and dried. RNA pellet was finally resuspended in 200 μl of RNase free water. Equal RNA from each stage (~500 ng) was used for
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cDNA preparation using SuperScript First-Strand Synthesis System (Invitrogen, USA) following manufacturer’s instructions.

### 2.8 RT-PCR amplification of cDNA

cDNA obtained was used for RT-PCR using primers specific for PfMA, PfARNP, EBA-175, 18SrRNA and intron region. Primers used for PCR amplification are as follows:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PfMA Fwd</td>
<td>5'-AAC ATT CTG AGT AGC CCG TT-3'</td>
</tr>
<tr>
<td>PfMA Rev</td>
<td>5'-GGT TCA TAT TCC TGT TCT TCG-3'</td>
</tr>
<tr>
<td>PfARNP Fwd</td>
<td>5'-TACGACCCCATGGGCAGAAAATATGATAAA AATAAGAATAAG-3'</td>
</tr>
<tr>
<td>PfARNP Rev</td>
<td>5'-CATATACTCGAGTCAATGGTGATGGTGATGGTGTTCCTAGTGATTTTTTCCTCTCTCTCT-3'</td>
</tr>
<tr>
<td>EBA-175 Fwd</td>
<td>5'-AAT TTCTGTAAAATATTGTGACATATG-3'</td>
</tr>
<tr>
<td>EBA-175 Rev</td>
<td>5'-GATACTGCAACAACACAGATTTCTTG-3'</td>
</tr>
<tr>
<td>18SrRNA Fwd</td>
<td>5'-CCGCCCGTCGCTCTACCGG-3'</td>
</tr>
<tr>
<td>18SrRNA Rev</td>
<td>5'-CCTTGTACAGACTTCTCTCTCC-3'</td>
</tr>
<tr>
<td>Intronic Fwd</td>
<td>5'-GACTTCCACCCTATATTTCCATG-3'</td>
</tr>
<tr>
<td>Intronic Rev</td>
<td>5-TATAAGCCG TAGTTTATCCCTA-3'</td>
</tr>
</tbody>
</table>
Target DNA was denatured for 5 minutes initially followed by 30 cycles of PCR using PCR master mix (Fermentas, USA) with conditions optimized for each gene. PCR conditions for amplification are as follows:

**PfMA**

- 94°C - 5min
- 94°C - 30sec
- 45°C - 1min
- 68°C - 1min
- 68°C - 10min

30 cycles

**PfARNP**

- 94°C - 5min
- 94°C - 30sec
- 56°C - 1min
- 68°C - 1min
- 68°C - 10min

30 cycles

**PfEBA-175**

- 94°C - 5min
- 94°C - 30sec
- 52°C - 1min
- 68°C - 1min
- 68°C - 10min

30 cycles

**18SrRNA**

- 94°C - 5min
- 94°C - 30sec
- 56°C - 1min
- 68°C - 1min
- 68°C - 10min

30 cycles

**Intron**

- 94°C - 5min
- 94°C - 30sec
- 55°C - 1min
- 68°C - 1min
- 68°C - 10min

30 cycles

25µl of the PCR amplified product was analyzed on 1% agarose gel.
2.9 Cloning, expression and purification of PfMA

The region of the native PF3D7_0316000 gene encoding the amino acid sequence of (Met 1 to Lys 244) was PCR amplified from *P. falciparum* 3D7 genomic DNA using the respective PfMA Fwd 2 (5'-TACGACCCATGGGCATGCACGATTTTTTTAAAATC-3') and PfMA Rev 2 5'(CACGTACTCGAGTCAATGGTGATGGTGATGGTGTTCTAGTTTTTTTAAC AAAATATACATGGTT-3') primers. PCR was performed using PCR Master Mix (Fermentas) at following PCR conditions:

**PfMA**

94°C - 5min  
94°C - 1min  
56°C - 1min  
68°C - 1min  
68°C - 10min

The amplified DNA fragment was purified using PCR purification kit (Qiagen). The PCR product encoding PfMA and vector pET-28a(+) (Novagen, Germany) were digested with Ncol and Xhol (Fermentas, USA) and inserted downstream of the T7 promoter in a *E. coli* expression vector pET-28a(+). The digested insert and the vector were ligated overnight with T4 DNA Ligase (New England BioLabs, UK) at 16°C. The ligation mixture was transformed into *E. coli* DH5α competent cells. Positive clones containing recombinant plasmid pPfMA-pET28a(+) were confirmed by colony PCR using gene specific primers. The plasmid was sequenced and transformed into *E. coli* BL21 (DE3) codon plus cells (Stratagene). The recombinant *E. coli* cells were grown overnight in LB media (Difco, USA) and subjected to induction by secondary inoculation (0.1% of...
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primary overnight culture) in autoinduction medium (Formedium, UK) for 22 hrs. The bacterial cells were harvested by centrifugation at 4000 g in Sorvall Evolution RC centrifuge at 4°C for 15 minutes. Harvested cell pellets were lysed by sonication and the protein was found in inclusion bodies. The inclusion bodies were collected by centrifugation at 15000 g. The inclusion bodies were washed twice with buffer W1 (20 mM Tris, 2 M Urea, 2% Triton X 100, pH 8.0). The pellet obtained was washed with Wash buffer; W2 (20 mM Tris, 250 mM NaCl) and solubilized in 6 M guanidine hydrochloride buffer (20 mM Tris, 150 mM NaCl, 6 M GuHCl, pH 7.8). The rPfMA with a 6x-His-tag was purified by Ni-NTA (nitritoltriacetic acid) affinity chromatography by pH based elution method (Wash buffer: 20 mM Tris, 150 mM NaCl, 6 M GuHCl, pH 5.6, Elution buffer: 20 mM Tris, 150 mM NaCl, 6 M GuHCl, pH 4.3). Metal-affinity purified rPfMA was diluted 25-fold in an L-Arginine based buffer (20 mM Tris pH 7.4, 10 mM L-Arginine, 2 M urea, 150 mM NaCl). The solution was incubated for 24 hours at 4°C with continuous stirring, dialyzed using snake skin dialysis tubing (Thermo Scientific, USA) against Tris Buffer Saline (TBS, pH 7.4) and concentrated. The purified protein was processed for in gel trypsin digestion followed by MALDI TOF/TOF described in section 2.15.

2.10 Cloning, Expression and purification of PfARNP.

The region of the native PF3D7_0511600 gene encoding the amino acid sequence of (Arg 21 to Ala 179) was PCR amplified from P. falciparum 3D7 genomic DNA using the following primers: PfARNP Fwd 2 (5'-TACGACCCATGGGCAGAAAATATGATAAAAATAAGAATAAG-3'), PfARNP Rev 2 (5'-CATATACTCGAGTCA
ATGGTGATGGTGATGGTGTTCTAGTGCATTTTTCCTCTCTCTCT-3'). The PCR
was carried for 30 cycles at following conditions:

\[
\begin{align*}
\text{PfARNP} & \\
94^\circ\text{C} & - 5\text{min} \\
94^\circ\text{C} & - 1\text{min} \\
54^\circ\text{C} & - 1\text{min} \\
68^\circ\text{C} & - 1\text{min} \\
68^\circ\text{C} & - 10\text{min} \\
\end{align*}
\]

The amplified DNA fragment was purified using PCR purification kit. The
PCR product encoding PfARNP and vector pET-28a(+) were digested with NcoI
and XhoI and inserted downstream of the T7 promoter in a E. coli expression
vector pET-28a(+). The digested insert and the vector were ligated overnight with
T4 DNA Ligase at 16°C. The ligation mixture was transformed into E. coli DH5α
competent cells. Positive clones containing plasmid pPfARNP-pET28a(+) were
confirmed by colony PCR using gene specific primers. The plasmid was
sequenced and transformed into E. coli BL21 (DE3) codon plus cells. The
recombinant E. coli cells were grown overnight in LB media and subjected to
induction by secondary inoculation (0.1% of primary overnight culture) in
autoinduction medium for 22 hours. Harvested cell pellets were lysed by
sonication and the protein was found in inclusion bodies. The inclusion bodies
were washed and solubilized by the protocol explained in section 2.9. The
protein was purified by Ni-NTA affinity chromatography by similar protocol
section explained in section 2.9. The dilution buffer composition was 20 mM Tris
pH 7.8, 10 mM, 2 M urea, 150 mM NaCl) and final protein was dialyzed in TBS,
pH 7.8. The purified protein was processed for in gel trypsin digestion followed
by MALDI TOF/TOF and MS analysis by standard protocols explained in section 2.15.

2.11 Immunization and generation of antisera against PfMA and PfARNP.

Group of five Balb/c mice were immunized intra-peritoneally with 25 μg protein emulsified with complete Freund’s adjuvant, CFA (Sigma, USA) followed by three boosts emulsified with incomplete Freund’s adjuvant. One New Zealand white rabbit was immunized sub-cutaneously with 150 μg protein emulsified with complete and incomplete Freund’s adjuvant and boosted thrice intramuscularly. The following immunization schedule was followed.

The final sera were collected on day 70. Antibody levels in sera were measured by ELISA.

2.12 Enzyme linked immunosorbent assay (ELISA)

Antibody response in mice, rabbits was quantified by ELISA. Briefly, wells of flat bottom micro titer plates (Nunc, Denmark) were coated with 200 ng of the recombinant protein in 0.06 M carbonate-bicarbonate buffer (pH 9.6) (Sigma). The plates were washed thrice with 0.05% Tween in PBS (PBST) for 5
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minutes each and blocked with 5% skimmed milk (Difco) in PBS for 1 hour at room temperature. The antigen-coated wells were sequentially incubated with the appropriate dilutions (1:1000-1024000) of the respective test sera followed with optimal dilutions of anti-mouse (1:2500) or anti-rabbit (1:3000) horse radish peroxidase (HRP) conjugated secondary antibody (Sigma, USA). The enzyme reaction was developed with o-phenylenediamine (Sigma, USA) as a chromogen and hydrogen peroxide (Merck) as a substrate prepared in citrate phosphate buffer, pH 5.0 (Sigma, USA). The reaction was stopped with 2N H2SO4 and the Optical density was measured at 490 nm using an ELISA micro-plate reader (Molecular Devices, USA). The experiments were performed twice in triplicate. End point titer was defined as the dilution of the test sera at which OD490 nm was greater than the mean OD490 of the pre-immune sera plus two times the standard deviation.

2.13 Western blot

For Western blot analyses, parasites were isolated from tightly synchronized culture by lyzing infected erythrocytes with 0.15% saponin. Parasite pellets were resuspended in RIPA buffer (100 mM Phosphate buffer pH 7.2, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM EDTA and 1 mM PMSF) and resultant lysate was quantified by BCA protein estimation method (Thermo Scientific, USA). Equal amount of total protein (50 μg) from each stage was boiled with SDS buffer and separated on a 12% SDS-PAGE gel. The fractionated proteins were transferred from gel onto the PVDF membrane (Millipore) and blocked in blocking buffer (PBS, 5% milk powder) for 2 hrs. The blot was washed twice with PBST (0.10%Tween-20) followed by PBS and incubated for 1 hour with primary antibody diluted in dilution buffer (PBS, and 1% milk powder). Later, the blot was washed and incubated for 1 hour with
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appropriate secondary antibody conjugated to HRP (Sigma). Bands were visualized by using the Super Signal West Pico ECL detection kit (Thermo Scientific, USA).

2.14 IgG purification

Total IgG was purified from the sera of rabbits immunized with PfMA and PfARNP. Briefly, sera samples were loaded on a protein G-Sepharose column (GE Healthcare, Sweden) equilibrated with the binding buffer (100 mM Tris-HCl, pH 8.0). The column was washed with 10-column volumes of the binding buffer. The bound IgG was eluted with 0.2 M glycine-HCl, pH 3.0 and the fractions were analyzed on SDS-PAGE. The fractions containing purified IgG were pooled and dialyzed against iRPMI.

2.15 Immunoprecipitation and protein identification by tandem mass spectrometry (MS/MS) with MALDI-TOF/TOF.

Late schizonts (46-48 hr, purified by percoll gradient) were used for immunoprecipitation of PfARNP with anti-PfARNP rabbit sera using Pierce co-immunoprecipitation kit (Thermo Scientific). The eluted protein was then subjected to SDS-PAGE under reducing conditions, followed by colloidal coomassie staining and Western blot analysis. A ~22 kDa polypeptide of PfARNP was excised from colloidal Coomassie stained SDS-PAGE gels, trypsinized [153] and extracted peptides were analyzed by MALDITOF/TOF. The purified recombinant proteins i.e. PfMA, PfARNP, PbAARP-N were also excised from SDS-PAGE gel and trypsinized. Briefly, equal volumes of extracted peptide and matrix solution (10 mg/ml α-cyanohydroxycinnamic acid in ACN/0.1%TFA (1:2) were spotted onto MALDI ground steel target plate (BrukerDaltonics, USA) and acquired using Ultraflex -III MALDI TOF/TOF platform (BrukerDaltonics, USA).
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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, Angiotensin_I-1296.684780, Substance_P-1347.735430, Bombesin-1619.822350, Renin_Substrate-1758.932610, ACTH (1-17)- 2093.086170,ACTH(18-39)-2465.198340, ACTH(1-24)- 2932.590000, Insulin bchain- 3494.650780. Spectrum annotation and processing were performed through Flexanalysis (version 3.3) and identified using Mascot search engine (version 2.3) (www.matrixscience.com) through Biotools (version 3.2). Search parameters were set as follows: Enzyme specificity: Trypsin, Precursor mass tolerance: + 100 ppm, Fragment mass tolerance: + 0.75da, Max missed cleavage: 1, Protein mass: Unrestricted, Database: NCBInr, Taxonomy: Plasmodium falciparum, Fixed modifications: Carbamidomethyl (Cysteine), Variable modifications: Oxidation (Methionine). Minimum 2 unique peptides were sequenced through MS/MS to confirm the protein identity. Only those peptides with individual ion score >30 (p<0.05) were considered for protein identification.

2.16 Immunofluorescence assay (IFA)

IFAs were performed on the P. falciparum 3D7 clone as described earlier [59]. Thin smears of schizont, trophozoite, and ring stage parasites were made on glass slides, air dried and fixed with methanol (ice cold) for 45 minutes and blocked overnight at 4°C in 3% (wt/vol) BSA (Sigma, USA) in PBS. After blocking, the slides were washed twice with PBS containing 0.05% Tween-20 (PBST) and PBS. Following washing, slides were incubated with respective antibodies against test antigen (PfMA (1:50), PfARNP (1:150)) and marker proteins (EBA-175(1:100), PfRH2 (1:100), PfMSP-1α(1:100), PfASP (1:100), AMA-1
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(1:100), RON-2 (1:50)) at 37°C for 1 hour.Slides were washed and incubated with an Alexa Fluor 488 conjugated anti-mouse IgG at dilution of 1:200 (Molecular probes, Invitrogen) and Alexa Fluor 594 conjugated rabbit antibody at a dilution of 1:500 for 1 hour. The slides were washed and mounted in ProLong Gold antifade reagent with 4, 6 di-aminidino 2, phenyindole DAPI (Invitrogen, USA) and were viewed on a Nikon TE 2000-U fluorescence microscope. To detect the localization during erythrocyte invasion, *P. falciparum* merozoites were isolated as described earlier, treated with 1 µM cytochalasin D for 10 minutes, incubated with uninfected erythrocytes for 5 minutes and fixed as described earlier [30]. After fixation and washing the IFA protocol mentioned above was followed.

2.17 Confocal, Structured Illumination Microscopy and Image Analysis

For Confocal and Structured Illumination Microscopy A1 and N-SIM Nikon microscope was used. All images were collected as 3D data sets (z-stacks) with a step size of 0.1 µm between the 21 successive optical sections. De-convolved images were saved and analyzed through Imaris image analysis software (version Imaris x 64 7.2.1, Bitplane Scientific). For the ease of presentation, images in this study are displayed as maximum projection of the 3D image stacks. SIM was also performed as described previously [30,154]. Co-localization analysis was performed by using "Coloc" module of Imaris which provides functionality for the visualization, segmentation and interpretation of 3D microscopy datasets. The result of co-localization analysis is expressed as a Pearson’s correlation coefficient generated for each co-localization experiment.

2.18 Immunoelectron microscopy (IEM)

For IEM, schizont stage of parasite was fixed for 15 minutes on ice in a mixture of 1% paraformaldehyde–0.1% glutaraldehyde in 0.1 M phosphate buffer
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(pH 7.4). Fixed specimens were washed, dehydrated, and embedded in LR White resin (Polysciences) and subjected to sectioning. Ultra thin sections were blocked at 37°C for 30 minutes containing 2% non-fat milk in water. The grids were then incubated at 4°C overnight with anti-PfMA mouse sera and anti-EBA-175 mouse sera in 1% fish gelatin (TABB) prepared in 10mM PB (pH 7.2). After washing with 1% fish gelatin, the grids were incubated at 37°C for 2 hrs with goat anti-mouse IgG conjugated to 15 nm gold particles diluted 1:50 (TABB). The grids were stained with aqueous uranyl acetate for 2 minutes and examined with a transmission electron microscope (Tecnai).

2.19 Erythrocyte binding assay (EBA)

EBA were performed with culture supernatant and recombinant proteins in a standard assay. Soluble parasite proteins were obtained from *P. falciparum* 3D7 culture supernatants of schizont-infected erythrocytes by incubating 5*10^8 percoll purified parasites per ml in iRPMI for 10 hrs. Briefly, culture supernatants/recombinant proteins (2.5-10 μg) were incubated with human erythrocytes (100 μl packed volume) at 37°C following which the suspension was centrifuged through dibutyl phthalate (Sigma, USA). The supernatant and oil were removed by aspiration. Bound parasite proteins were eluted from the erythrocytes with 1.5 M NaCl. The eluate fractions were analyzed for the presence of the proteins by immunoblotting.

2.20 Cloning, expression and purification of PbAARP-N

The region of the PBANKA_052380 gene encoding the amino acid sequence of (24 to 179) was PCR amplified from *P. berghei* genomic DNA using the following primers: PbAARP-N Fwd (5'GGCGATCCATGGGCCAAATTAGTTCAAGAGAAAATG-3'), PbAARP-N Rev
(5'-ATGTCGACCTCGAGTTCATGATCGTGCTCATGGGC-3'). The PCR was carried for 30 cycles at following conditions:

\[
\begin{align*}
&94°C - 5min \\
&94°C - 1min \\
&56°C - 1min \\
&68°C - 1min \\
&68°C - 10min
\end{align*}
\]

30 cycles

The amplified DNA fragment was purified using PCR purification kit (Qiagen, USA). The PCR product encoding PbAARP-N and vector pQE30 (Qiagen, USA) was digested with BamHI and SalI (New England Biolabs, UK). The digested insert and the vector were ligated overnight with T4 DNA Ligase (New England BioLabs, UK) at 16°C. The ligation mixture was transformed into *E. coli* DH5α competent cells. Positive clones containing recombinant plasmids with insert were confirmed by colony PCR using gene specific primers. The recombinant expression plasmid was sequenced and transformed into *E. coli* M15 cells (Qiagen, USA). Recombinant protein with N-terminus 6x-His-tag was expressed in *E. coli*, following induction in LB medium (Difco) with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma, USA) for 4 hrs. Harvested cell pellets were lysed by sonication and the protein was found in the soluble fraction. The soluble lysate from *E. coli* cells was collected by centrifugation at 15000g. The protein was purified from by Ni-NTA (nitrilotriacetic acid) affinity chromatography by imidazole elution (Buffer A 20 mM Tris, 200 mM NaCl, pH-8.0; Elution buffer: 20 mM Tris, 200 mM NaCl, 200 mM Imidazole, pH-8.0). Metal-affinity purified PbAARP-N protein was subjected to anion exchange chromatography using Q Sepharose column. The Ni-NTA eluate was diluted 10 fold in Buffer A1 (20 mMTris, pH 7.0) and subjected to step elution of NaCl gradient of 200 mM and
500 mM NaCl (Buffer B1: 20 mM Tris, 200 mM /500 mM NaCl, pH 7.0). The purified protein was processed for in gel trypsin digestion followed by MALDI TOF/TOF and MS analysis by standard protocols as described in section 2.15.

2.21 Cloning, expression and purification of PbAARP-F

The region of the native PBANKA_052380 gene encoding the amino acid sequence of (24 to 142) was PCR amplified from *P. berghei* genomic DNA using the following primers: PbAARP-F Fwd (5'-GGGATCCATGGGCCAAATTAGTTCAAGAGAAATG-3'), PbAARP-F Rev (5'-TAAGTCGACCTCGAGAGGAAGTGGAGGTGGAACTAC-3'). The PCR was carried for 30 cycles at following conditions:

PbAARP-F
94°C - 5min
94°C - 1min
56°C - 1min
68°C - 1min
68°C - 10min

The amplified DNA fragments were purified using PCR purification kit (Qiagen, USA). The PCR product encoding PbAARP-F and vector pETM11 (A kind gift from Dr. Amit Sharma, ICGEB, New Delhi) was digested with Ncol and XhoI (New England Biolabs). Similar cloning strategy was followed as explained in section 2.10. The plasmid was transformed in *E.coli* BL21 DE3 codon plus cells (Stratagene). Recombinant protein with N-terminus 6x-His-tag was expressed in *E.coli*, following induction in LB medium (Amresco) by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 hours. Harvested cell pellets were lysed by sonication and the protein was found in soluble fraction. The soluble lysate from *E. coli* cells was collected by centrifugation at 15000g. The protein was purified
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from by Ni-NTA (nitrilotriacetic acid) affinity chromatography by imidazole elution (20 mM Tris, 200 mM NaCl, pH-8.0). Metal-affinity purified PbAARP-F protein was subjected to anion exchange chromatography using Q Sepharose column. The Ni-NTA eluate was dilute 10 fold in Buffer A (20 mM Tris, pH 7.2) and subjected to step elution of NaCl gradient of 200 mM and 500 mM NaCl (Buffer B; 20 mM Tris, 200 mM /500 mM NaCl, pH 7.2). The purified protein was processed for in gel trypsin digestion followed by MALDI TOF/TOF and MS analysis by standard protocols as described in section 2.15.

2.22 Immunization of mice with PbAARP-N and PbAARP-F

Group of ten Balb/c female mice were immunized intra-peritoneally with 25 µg protein emulsified with CFA (Sigma) followed by three boosts emulsified with incomplete freund’s adjuvant. The immunization schedule was same as described in section 2.11. The titer of antibodies was determined by ELISA as described in section 2.12. The titer of antibodies against PbAARP-N and PbAARP-F were also tested a day before the challenge of mice with P. berghei.

2.23 Parasite maintenance and challenge

P. berghei strain ANKA was maintained in BALB/c mice by injection of cryopreserved infected blood stocks, before passage into experimental mice. When the parasitemia of donor mice was 10-12% blood from tail vein was collected and total erythrocyte counting was done by hemocytometer. A dose of $10^5$ infected erythrocytes in phosphate buffer saline was introduced into mice by intra peritoneal route. Parasitemia was monitored by Giemsa-staining of smears of blood drawn from tail vein of mice. The percent parasitemia was determined by counting 1000-1500 cells per slide. The survival of mice was monitored each day.