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2.1 Plasmid Construct for Recombinant PvRII Expression in Insect Cells

DNA fragments encoding PvRII (amino acids 194 to 521 of the *Plasmodium vivax* Duffy-binding protein (PvDBP) containing 12 cysteines) fused to six-histidine (6xHis) tag at the C'-terminal end were amplified by polymerase chain reaction (PCR) as follows: A plasmid, p192B, containing the PvDBP gene (Figure 10, Chitnis and Miller, 1994), and primers BVR2U 5'- ATA gTT Tag Cgg Cgg CTC CCT ggT gAT ggT gAT ggT gTg TCA CAA CTT CTT gAg T -3' and BVR2D 5'- gCg ggA TCC ACg ATC TCT AgT gCT -3' (Integrated DNA Technology) were used. PCR was performed in a 50 µl reaction volume containing 50 pmol of each primer, 100 ng of template plasmid DNA, 200 µM of each dNTP, 1 U of high fidelity Vent DNA polymerase (New England Biolabs), having 3' to 5' proof-reading exonuclease activity, and 5 µl of 10x PCR buffer (containing MgCl₂ as supplied with the enzyme). PCR was carried out in a thermal cycler (Perkin Elmer-2400) under the following conditions: 94°C/2 min for one cycle; 94°C/30 sec, 40°C/30 sec, and 72°C/2 min 30 sec for 5 cycles; followed by 25 cycles of 94°C/30 sec, 48°C/30 sec, and 72°C/2 min 30 sec.

The PCR product was resolved on 1% agarose gel, and a DNA fragment of the expected size (~1 kb) was excised and extracted using Qiagen gel extraction column according to manufacturer's protocol (Qiagen). Negative controls, lacking either the plasmid DNA or one of the primers (single primer controls) all showed no amplification. This ruled out the possibility of non specific amplification at low annealing temperatures.

The baculovirus expression vector systems makes use of transfer vectors constructed with AcNPV DNA to carry the gene of interest. Like most other transfer vectors intended for use in an insect cell system, pAcGP67B (Becton Dickenson, PharMingen, Figure 11) depends on the strong, very late promoter from the AcNPV polyhedrin gene, placed upstream from the cloning site. pAcGP67B contains an *E. coli* origin of replication (ColE ori) for propagation and storage of the vector in bacteria, ampicillin resistance (Amp⁻) for selection of recombinant bacteria, and a cloning region (MCS) for ligation of the foreign gene of interest.
Figure 10a. Gene structure of the *Plasmodium vivax* Duffy antigen Binding Protein (PvDBP). PvDBP has a signal sequence (SS) at the N-terminus and a transmembrane segment (TM) with a cytoplasmic domain (CYT) at the C-terminus. The exon (named with Roman numerals) and intron (limited by arrows) boundaries are conserved in the gene. The two conserved cysteine-rich regions are regions II and VI. The N’-terminal *P. vivax* DBP cysteine rich domain, region II, is the functional binding domain (PvRII). The plasmid p192B contains the putative gene encoding for the PvDBP within *HindIII* restriction enzyme sites, and was the template for the region II used for expression of PvRII {Chitnis and Miller, 1994}.

Figure 10b. pHVDR22 contains the gene encoding for PvRII fused with the signal sequence (SS) and transmembrane (TM) segments of herpes simplex virus glycoprotein D (HSVgD). The fusion proteins are targeted to the surface of mammalian cells with the HSVgD SS and are anchored to the surface with the TM segment {Chitnis and Miller, 1994}. The vector pRE4 contains the complete HSVgD gene between the *HindIII* restriction sites. {Cohen *et al.*, 1988}.
Figure 11. Baculovirus transfer vector pAcGP67B, used for expression of PvRII. The expressed gene, PvRII was cloned within BamHI and NotI cut sites at the Multi-Cloning Site (MCS) of the baculovirus transfer vector pAcGP67B. The vector contains both a polyhedrin promoter and gp67 secretion signal sequence for protein expression into insect cell supernatant, as well as a ColE origin of replication and ampicillin resistance for propagation of the plasmid in E. coli {From Becton Dickenson, PharMingen Baculovirus Expression Manual, 2001}. 

**pAcGP67A**

9761 bp

unique sites underlined
The transfer vector with gene of interest and Baculogold® are co-transfected into insect cells to generate recombinant virus. The regions flanking the cloning site in pAcGP67B contain large tracts of AcNPV sequences to facilitate the homologous recombination with Baculogold® that takes place during co-transfection.

The purified DNA fragment (~10 μg) was subjected to digestion with restriction enzymes BamHI and NotI (10 U each, Roche) for two hours at 37°C. The reaction was then subjected to agarose gel electrophoresis and the desired DNA fragment (~1kb) was excised and extracted as above. The BamHI and NotI digested DNA fragment was cloned downstream of the polyhedrin promoter in the baculovirus transfer vector pAcGP67B, previously digested with BamHI and NotI and treated with shrimp derived alkaline phosphatase (SAP), to yield plasmid pAcR2H.

The ligation reaction was carried out with 100 ng of pAcGP67B vector DNA and 40 ng of the PCR product in 20 μl of total reaction mixture, the volume was made up with 10 units of T4 DNA ligase (New England Biolabs), 2 μl of 10x T4 ligase buffer, and 0.2 mM ATP (Sigma). The reaction proceeded at 16°C for 16 hours. The ligation mixture containing pAcR2H was ethanol precipitated under aseptic conditions and reconstituted in 10 μl of ddH2O. 5 μl of reconstituted ligation mix was added to 50 μl thawed electro-competent DH10B E. coli cells (Gibco) in a precooled microcentrifuge tube and incubated on ice for 15 minutes. The ligation mix and cells were transferred into a 0.1 cm electroporation cuvette and electroporation was performed with a Biorad electroporator (model no. 165-2107) at 1.8 kV, 25 μF, and 200 Ω for approximately 2 seconds. The resultant time constant is an index of the efficiency of the procedure and is ideally just above 4. The transformed cells were recovered by adding 350 μl of SOC medium (Gibco) and grown on a rotary shaker at 200 rpm, at 37°C for 1 hour. 100 μl of culture was plated on LB agar medium supplemented with 25 μg / ml ampicillin and incubated at 37°C for 16 hours. E. coli transformants were screened by colony PCR and plasmid constructs were confirmed by restriction digest using BamHI and NotI.

2.1.1 Preparation of plasmid DNA for use in insect cells

Plasmid DNA is usually purified from bacteria using the method of alkaline lysis (Sambrook et al., 1989). Many commercial kits are also available for this purpose. The procedure is invariably the same, where an overnight culture of bacteria is pelleted, the pellet resuspended, lysed in alkaline detergent solution that is then neutralized and then, where the DNA usually undergoes phenol/chloroform extraction, these kits have replaced the phenol step with a more
"user-friendly" perforated glass filter that retains the DNA until it can be eluted by treatment with Tris-EDTA buffer or with double distilled water. Transformation of insect cells requires a high degree of purity from the DNA being used since Spodoptera frugiperda (Sf) insect cells are highly sensitive to contaminants found in impure preparations of plasmid DNA. This may cause cell lysis after transfection, thus resulting in an apparently lower level of production of proteins or a lower viral titer. For electroporation, it is sufficient to ethanol precipitate with a final 70% ethanol wash to remove superfluous salts. Transformation requires pure and sterile plasmid DNA that is obtained by ethanol precipitation under aseptic conditions of the ligation products.

2.2 Generation of Recombinant Baculovirus for Expression of PvRII

2.2.1 Insect cell line used for generation of recombinant virus and protein expression

Frozen S. frugiperda insect cells (Sf21 cells) were revived from liquid nitrogen and plated in cell culture treated flasks (Falcon) in TNM-FH (Trichoplusia ni medium formulation Hink, B.D., PharMingen) medium at 27°C, as prescribed by the manufacturer (B.D., PharMingen). Their health was ascertained by checking viability by the trypan blue exclusion test (0.1 ml trypan blue stain in 1 ml of cells). A maximum of 10% cells should have blue colour when counted on a hemocytometer (Bright-Line, Hausser Scientific) averaged over three counts, but the percentage of blue cells should go down to 3% at stages of co-transfection or protein production. When they had grown to a sufficient quantity (over one half million cells per ml) they were transferred to shake flask culture at 60 rpm and 27°C. The insect cells were slowly adapted to growth in Sf-900 medium (serum free insect cell medium, Gibco BRL) gradually over a period of 10 days.

2.2.2 Generation of recombinant baculovirus by co-transfection

In order to transfect insect cells, a stock was kept growing in suspension culture at a density of approximately two million cells per ml and plated for surface culture during transfection and for assays (recall Figure 9 for a schematic view). Two wells of a 6 well culture plate
(COSTAR™ PS, 3.5 cm diameter wells) were used, one for the co-transfection reaction, and one for transfection with plasmid DNA alone (as a negative control as well as to check for contamination in the DNA). Insect cells were transfected at just over 50% confluency. 2.5 µg of plasmid DNA (pAcR2H diluted to 100 µg/ml in sterile ddH2O) was added to 0.5 µg Baculogold™ DNA (B.D., PharMingen) in a microcentrifuge tube, mixed and incubated at room temperature for 5 minutes. 1 ml transfection buffer B (125 mM Hepes, pH 7.1, 125 mM CaCl₂, and 140 mM NaCl) was added to the mixture and allowed to rest for 15 minutes. The medium in the wells was replaced with 1ml transfection buffer A (complete insect media, pH 6.0 to 6.2). The DNA mix in transfection buffer B was added drop by drop to the cells while gently rocking the plate. Transfection buffer B with pAcR2H DNA alone was added to the control well. A pasty white precipitate of calcium phosphate / DNA was visible in the medium. The reaction was incubated for 5 hours at 27°C, after which the medium was removed from the wells, and cells were washed once with 3 ml Sf-900 medium. The two wells were incubated for 5 days at 27°C in 3 ml Sf-900 medium. From the third day, the transfected well was examined to see if infection had begun by comparing it to the control well (see Figure 9, point 6). Nuclei of the infected cells enlarge and the cells become rounded and bloated. The supernatant containing recombinant virus was collected after day 5 and used for end point dilution assay (EPDA) and for plaque assay (PA) to screen for recombinant virus.

2.2.3 Plaque assay

The plaque assay is the ideal manner to purify recombinant virus. It allows the user to isolate a single recombinant virus (measured in plaque forming units, or pfu, i.e. a colony grown from a single virus) and to determine the viral titer so that known quantities of virus are used in subsequent experiments (Figure 9, point 7). Insect cell monolayers in 6 well culture plates (Falcon) were grown to 70% confluency, and used for infection when they were healthy (a maximum of 10% should colour blue with trypan blue stain) and in log phase growth. Medium was replaced before infection to remove cells that were dead or adhering improperly to the culture plate. Cells were then infected with serial log-scale dilutions (10⁻³, 10⁻⁴, to a maximum of 10⁻⁸) of the recombinant virus. High dilutions ensure that only isolated cells become infected and that the viral plaques do not mix. The insect cells were incubated with the recombinant virus at 27°C for 1 hour. The medium was removed and the cells were very slowly overlaid by a 1% low-melting point agarose solution. 2% Agarplaque Plus™ (B.D., PharMingen) was prepared in serum-free medium (SFM), heated until clear and cooled to
45°C. It was then mixed with an equal amount of pre-warmed TNM-FH to obtain a final concentration of 1%. The agarose overlay was left immobile to dry for 20 minutes and then placed in a humid chamber at 27°C for a week. The agarose overlay stabilizes cells and limits spreading of virus through the head space. Several cycles of infection pass before infected cells lyse and the plaque becomes visible. Each plaque is derived from one single virus. After the third day the search for plaques began, but only after 7 to 9 days, according to the size of the plaques, were they picked. Plaques can be seen as small clearings among uninfected cells visible to the naked eye when viewed inverted against a lamp or by light microscopy. From each well 20 plaques were picked using sterile micropipette tips and transferred aseptically to 1 ml of TNM-FH media in 1.5 ml microcentrifuge tubes. To avoid cross-contamination, only isolated plates were picked. Virus particles were eluted by rotating the tubes O/N at 4°C. 200µl of this solution was added to separate wells of 12 well plates seeded with 2x10^5 Sf21 cells in 800µl medium and incubated for 3 days at 27°C. The recombinant virus supernatant from these wells was collected and spun down to remove cellular debris (this is the first passage recombinant virus and can be stored at 4°C for up to 6 months). 200µl of this recombinant virus was used to infect a well of a 6 well plate, incubated at 27°C for 4 days, and harvested (this is the second passage recombinant viral stock). It was centrifuged to remove debris and used to determine viral titer with the end point dilution assay (if titer falls below 2x10^8 pfu / ml it is reamplified using the methodology briefly outlined above).

2.2.4 End point dilution assay

In order to screen for recombinant virus there are two methods. The plaque assay primarily serves to identify, ideally, a single recombinant virus. The end point dilution assay (EPDA) amplifies the recombinant virus from the transfection reaction to produce a high-titer stock solution to use for expression of recombinant protein. More importantly, the higher dilutions of recombinant virus that were used here, in comparison to the plaque assay, allow for an accurate evaluation of higher viral titer. The EPDA can also serve to isolate a single colony, working under the assumption that the infection in the well with the highest dilution of virus derives from a single viral plaque. Here, this assay was used only to amplify virus and for the determination of viral titer.

Insect cell monolayers in 12 well culture plates (COSTARTM PS, 1 cm diameter wells) were grown in the same manner as in the plaque assay above, then, infected with serial log-scale dilutions of recombinant virus (10^3, 10^4, and so forth to 10^9 or higher dilutions if necessary,
one well was always left uninfected as a control.). The insect cells were incubated at 27°C and after one hour the virus was replaced with fresh medium and allowed to grow for 4-5 days. Wells were inspected regularly after the third day post-infection (p.i.) to watch for signs of infection. The well with the highest dilution that showed signs of infection was used to calculate the viral titer. This method was repeated until the viral titer came to be at least $10^9$ pfu/ml, which is to say that if a well has one million cells, 1 µl of viral supernatant is sufficient to provide one virus to infect each cell in the well.

2.3 Expression and Characterization of Recombinant PvRII from Insect Cells

2.3.1 Preliminary screening for best PvRII producing virus

From each well of the plaque assay (using log-scale dilutions of $10^{-3}$, $10^{-4}$, $10^{-5}$, and $10^{-6}$) a maximum of ten plaque picks were selected and analyzed by eluting virus overnight under slow rotation in 1 ml SF-900 medium and then each pick was used to reinfect another well of a 6 well plate of cultured insect cells. The supernatant from these wells were tested by Western blot until positive clones were found. With a claim of nearly 100% transfection efficiency from the manufacturer, it is likely that any negative samples occurred only on account of an error in lifting the plaque pick. The supernatants containing recombinant virus from the positives clones at the highest dilutions (i.e. positive clones from the $10^{-6}$ dilution were preferred as these represented the purest samples of virus) were used at high multiplicity of infection (MOI is defined as the number of cells divided by the viral titer, here an MOI of ~10 was used) to re-infect 1 well each of a 6 well plate in order to express a small quantity of recombinant protein. In order to ascertain the protein was being correctly secreted and not retained within the cell, starting at 3 days p.i., the cells were dislodged and harvested separately from the supernatant (or a small sample was collected from shake flask). These samples were run on SDS-PAGE and analyzed as described below.
2.3.2 Screening recombinant virus and production of PvRII

For a quick initial confirmation of the presence of PvRII protein in the cell culture supernatant, small aliquots were taken from 10 ml monolayer cultures in T75 flasks (Falcon) daily after day 3 of infection and tested for the presence of PvRII. Recombinant PvRII was purified from these supernatants using Ni-NTA (Nickel nitritoltriacetic acid, Qiagen) spin columns as follows.

A spin column is equilibrated with 600 μl binding buffer (50 mM phosphate buffer, pH 8.0 with 150 mM NaCl), and spun at 2,000 rpm for 2 minutes. A maximum of 5 ml supernatant was centrifuged to remove cellular debris and passed through the columns in 600 μl batches, taking into consideration that the binding limit of the column is approximately 150 μg of 6xHis-tagged protein. The column was then washed twice with wash buffer (binding buffer with 20 mM imidazole) and elution was done twice with 200 μl of elution buffer (binding buffer and 250 mM imidazole) each time. Samples were analyzed for expression of PvRII by Western blot and compared by Coomasie brilliant blue stain (CBB). The viral clone that yielded the best expression levels for PvRII based on detection by Western blot was selected for large scale expression. The selected virus was re-amplified in T175 flasks (Nunc) and found, by end point dilution assay, to have a titer of \( \sim 1 \times 10^9 \) pfu / ml.

Sf21 insect cells were grown to a density of two million cells / ml in 1.2 litres in a 4 litre flask at 27°C, under constant shaking at 60 rpm. Cells were over 98% viable when stained with trypan blue. 30 ml recombinant pAcR2H virus with a titer of \( \sim 1 \times 10^9 \) pfu / ml were used to infect 1.2 litres, for a multiplicity of infection (MOI) of 10. The infected cells were left to incubate a further 3 days in the same conditions, taking daily samples to check the progress of the infection under a microscope.

2.3.3 Purification of recombinant PvRII using metal affinity chromatography

In order to obtain the cleanest purification of baculovirus produced 6xHis-tagged PvRII, an adaptation of the standard Ni-NTA procedure was adopted. 1.2 litres of insect cell supernatant containing the recombinant PvRII were collected 5 days p.i. and dialysed at 4°C under gentle agitation for 48 hours against dialysis buffer (50 mM phosphate buffer, pH 8, 150 mM NaCl). The supernatant was centrifuged at 4°C, at 10,000 rpm for 5 minutes, and was then filtered on ice with a low protein binding bottle-top filter (\( \Phi = 0.45 \mu m \)). The pH was adjusted to 8.0 with the addition of Ni-NTA binding buffer. 200 μl of Ni-NTA (Qiagen) agarose beads stored in 50% ethanol were rinsed with water and equilibrated with binding
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buffer. A small diameter column with perforated glass disk to retain the beads was washed and mounted on a column holder. The beads were added to 1 litre supernatant in a 2 litre glass bottle and incubated 20 minutes at 4°C under constant but gentle rotation. A sufficient amount of rinsed and equilibrated beads were added to cover the column’s glass retaining disk and the culture supernatant was passed through the column two times under the flow of gravity, allowing the beads to be retained. The column was washed with 10 column volumes of binding buffer. For more stringent washing, the column was then washed twice with one column volume of binding buffer with 20 mM imidazole. The rest of the bound protein was eluted with elution buffer (binding buffer and 100 mM imidazole). Concentration of protein was estimated from OD$_{280nm}$ (using an extinction coefficient estimated from *E. coli* produced PvRII, Singh *et al.*, 2001). PvRII was concentrated and imidazole was removed using an Amicon™ rotary filtration unit. Purified PvRII was characterized and used to raise antibodies in mice as described below.

2.3.4 Western blot analysis of PvRII

Ni-NTA purified PvRII was resolved by SDS-PAGE on 10% acrylamide gel and transferred to PVDF or nitrocellulose (BioRad) membrane using semi-dry transfer apparatus (140 mA for 45 minutes or 110 mA for 1 h, Pharmacia). PVDF was pre-activated for 5 minutes in methanol and rinsed in transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS, 20% methanol) before use. After transfer, the membrane was blocked by incubating with 3% BSA and 0.5% fat free skimmed milk powder solution overnight at 4°C, then washed thrice with phosphate buffer saline containing 0.05% Tween 20 (PBST) at 2 minute intervals. The membrane was incubated with monoclonal mouse antibody to 6xHis (Qiagen) at a dilution of 1:1000 in 50% blocking solution and 50% PBST for 60 minutes at room temperature. After three washes of 10 minutes each in PBST, the membrane was incubated with anti-mouse IgG horseradish-peroxidase (HRP)-conjugate (Sigma) at a dilution of 1:2000 in 50% blocking solution and 50% PBST for 60 minutes at room temperature, and finally washed with PBST (four times, 10 minutes each). The membrane was developed with 3,3'-diaminobenzidine (DAB) as the chromogen (10 ml of PBS containing 10 mg DAB and 10 μl of H$_2$O$_2$) in order to detect recombinant PvRII on the membrane. Once colour develops the reaction is stopped by rinsing with abundant washes of double distilled water.
2.3.5 Mobility of native and reduced PvRII on SDS-PAGE

Purified PvRII was reduced by adding 50 mM β-mercaptoethanol (β-ME, in a solution of 10 mM Tris-HCl pH6.8). The tube was mixed gently and incubated 15 minutes at room temperature. 50 µl of iodoacetamide (IAA, from a stock solution of 20% w/v, Pierce, Rockford, Illinois), which reacts with free cysteines forming S-carboxamido-methylcysteine) was added to alkylate the reduced protein. The tube was promptly flushed with nitrogen gas and incubated in dark for 30 minutes at 37°C. PvRII was then precipitated with chilled ethanol and dissolved in SDS-PAGE reducing buffer (100 mM dithiothreitol (DTT) 50 mM Tris-HCl, 2% SDS, 0.1% bromophenol blue, 10% glycerol, pH 6.8). For non-reduced and non-alkylated PvRII, non-reducing SDS-PAGE buffer (50mM Tris-HCl, 2% SDS, 0.1% bromophenol blue, 10% glycerol, pH 6.8) was used. Both samples were resolved by SDS-PAGE on 10% acrylamide gels with Tris-Tricine electrophoresis buffer and visualized by Coomasie blue staining or Silver stain.

2.3.6 Analysis of purified PvRII by reverse phase chromatography

The technique of reverse phase high performance chromatography (RP-HPLC) is used to separate different conformers of the same protein that may be present in the purified sample according to differences in surface hydrophobicity. The purified recombinant PvRII was loaded on a reverse phase C8 column (at a flow rate of 1 ml/min). The gradient used for elution was developed using buffer A (0.05% trifluoroacetic acid (TFA) in water and buffer B (0.05% TFA in 90% acetonitrile, and 10% water). The column was equilibrated initially with 95% buffer A and 5% buffer B and reached the composition of 90% buffer A and 10% of buffer B in 10 minutes. A final composition of 5% buffer A and 95% buffer B was reached in another 45 minutes (Waters HPLC Millennium chromatography system).

2.3.7 Chymotrypsin treatment of erythrocytes

Human blood was collected in 10% citrate phosphate dextrose (CPD) and washed three times in RPMI 1640 (Life Technologies) before use. Duffy antigen was removed by treating 100 µl packed cell volume (PCV) Duffy-positive human erythrocytes in 7.9 ml of incomplete RPMI 1640 (iRPMI) with 2 ml chymotrypsin (from a stock solution of 1 mg/ml in iRPMI, Sigma) for one hour at 37°C with gentle shaking. Cells were washed thrice with iRPMI and
resuspended to the desired hematocrit (to 1% hemocrit (HC) for erythrocyte binding assay (EBA) on plated cells, or 33.3% HC for EBA using soluble protein).

2.3.8 Erythrocyte binding assay using soluble recombinant PvRII

Purified PvRII (10 μg in 250 μl iRPMI) was incubated with normal and chymotrypsin-treated human erythrocytes [100 μl PCV in 200 μl iRPMI and 50 μl fetal bovine serum (FBS)] for 1 hour at room temperature on nutator to allow binding (Figure 12). The reaction mixture was layered over 400 μl dibutylphthalate (DBT, Sigma) and centrifuged to collect erythrocytes after removal of the oil and aqueous residue layers. The bound protein was then eluted with 300 mM NaCl separated by SDS-PAGE and detected by Western blotting using a commercially available mouse monoclonal antibody raised against 6xHis-tag (Qiagen) as described above.

2.4 Immunization of Mice with Recombinant PvRII

Mice used in this study were procured from the Animal Facility of the International Center for Genetic Engineering and Biotechnology (ICGEB), New Delhi, India. Animals were housed, fed, and used in experiments according to the guidelines set forth in the National Institutes of Health (NIH) manual titled 'Guide for the Care and Use of Laboratory Animals' (National Institutes of Health Publication No. 86-23, US Department of Health and Human Services, Washington D.C. USA).

Five four-week BALB/C female mice were immunized intraperitoneally with 25 μg of recombinant PvRII emulsified in complete Freund's adjuvant. The mice were boosted on days 28, and 56 with 25 μg of recombinant PvRII formulated in incomplete Freund's adjuvant and delivered by the same route. Three mice were immunized with adjuvant alone according to the schedule described above to obtain control antiserum. Sera were collected by bleeding 14 days after each immunization, pre-bleed sera was taken one day before the first injection with antigen. Serum was prepared for storage by first incubating the blood at 37 °C for 2 h, then overnight at 4 °C. The coagulated blood was spun at 3000 rpm for 10 minutes at 4 °C and the clear supernatant, that is the serum, was stored at 4°C in 50 μl aliquotes.
Figure 12. Erythrocyte binding assay on soluble PvRII protein. Figures represent step-by-step procedure for EBA on soluble PvRII protein: 1. in separate 1.5ml eppendorf tubes put 100µl Fy⁺ and 100µl Fy⁻ RBCs, each with 200µl iDMEM, 50µl FBS and incubate 10' at RT, 2. add 250µl iDMEM containing 10µg protein (~700µl supernatant) and inc. on nutator for 60'. 3. Overlay RBC/PvRII mixture on 400 µl of Dibutylphthalate (DBPT) and, 4. centrifuge at 13,000 rpm for 30' at RT. Remove everything above the RBC pellet, being very careful not to disturb it. 5. Add 20µl of 1.5 M NaCl, and mix gently, 6. spin at 13,000 rpm for 1', remove and keep supernatant for 7. SDS-PAGE and 8. Western Blot.
2.4.1 Determination of antibody titer of anti-PvRII mouse sera by ELISA

Mouse sera were tested for recognition of recombinant PvRII by ELISA as described earlier (Singh et al., 2001). In summary, wells were coated with 2 µg recombinant E. coli PvRII in 100 µl of coating buffer (0.5M carbonate buffer pH 9.6) and incubated 2 hours at 37°C. Washing was carried out with PBST, 3 times at intervals of 3 minutes each. The plate was blocked for 2 hours at RT (or may be done O/N at 4°C) in PBS containing 2.5% BSA. Washing was repeated as above. Mouse sera were diluted in PBS containing 0.1% BSA as required. 100µl of diluted sera were incubated with PvRII coated ELISA plate at 37°C for 1 hour. A primary antibody blank was prepared using 100 µl of pre-immune sera added against PBS with 0.1% BSA in each control well. Plates were washed 5 times as above. Secondary, anti-mouse IgG-HRP conjugate was diluted (1:2000) with 0.1% BSA in PBS, 100 µl of this was added to each well and incubated at RT for 1h. The plates were washed 5 times as above, and 2 times with PBS. 100 µl developer was added per well (0.2% OPD, 0.05% H2O2, in 0.1 M citrate buffer). The reaction was allowed to proceed until the colour stabilized before termination with 25 µl of 2 N H2SO4 after about five minutes. The OD490nm was measured using an ELISA microplate reader (Molecular Devices). As controls, both the pre-immune sera as well as sera raised against adjuvant alone were used at similar dilutions. To determine end-point titers, the last dilution of test sera yielding an OD490nm of 2.5 times that obtained with pre-immune serum was determined. All samples were tested in triplicate.

2.5 Inhibition of Erythrocyte Binding

2.5.1 Transfection of mammalian cells with recombinant PvRII

Mammalian cells (COS7 or 293T, American Type Cell culture collection (ATCC), Rockville, MD, USA) were cultured in DMEM with 10% heat inactivated FCS (both from Gibco, Gaithersburg, MD, USA) in a humidified CO2 (5%) incubator at 37°C. Fresh monolayers of mammalian cells were seeded at 10-20% confluence in 6 well tissue culture plates and allowed to settle at 37°C, 5% CO2 for 10 minutes. Fresh medium was exchanged and the cells were grown to 60% confluence and washed with incomplete medium.

Transfection mix was prepared by gently mixing plasmid DNA and Lipofectamine™ (a liposome formulation of the polycationic lipid DOSPA (2,3-dioleyl oxy N
[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propaninium trifluoro acetate) with the neutral lipid DOPE (dioleoyl phosphatidyl ethanol-amine) in water, Gibco BRL) and incubating for 30 minutes at RT.

2 µg plasmid DNA was used for the transfection mix consisting of 2 µg per well of 6 well culture plate of plasmid DNA (pHVDR22, Chitnis and Miller, 1994) in solution A (iDMEM) and 12 µL Lipofectamine™ in solution B (iDMEM) and allowed to stand for 30 min. 1 µg plasmid DNA per well of 12 well culture plate in solution A was mixed with 4 µL Lipofectamine in solution B as above. These transfection mixes were added, drop by drop, while slowly rocking the plates with the cells. After 5 hours, the transfection supernatant was replaced with 3 ml of fresh iDMEM.

The plasmid pHVDR22 is designed to express region II, the 5’ cysteine-rich region from the P. vivax Duffy binding protein (PvRII), on the surface of mammalian cells. The plasmid pHVDR22 contains DNA encoding the PvRII sequence fused to the signal sequence of Herpes simplex virus glycoprotein D (HSVgD) at the N’-terminus and to the transmembrane region and cytoplasmic domain of HSVgD at the C’-terminus in a mammalian expression vector. The signal sequence and transmembrane segment of HSVgD target the fusion protein to the surface of the cells and the transmembrane region anchors it to the surface of the cell. Expression of PvRII on the cell surface is confirmed by immunofluorescence assay using a murine monoclonal antibody, DL6, directed against a proline-rich region of the HSVgD sequences in the fusion protein (Chitnis and Miller 1994, Cohen et al., 1988).

2.5.2 Detection of PvRII on COS7 cells by immunofluorescence assay

Cells were transfected as above and after a minimum of 40 h post-transfection they were rinsed in phosphate-buffered saline (PBS) and fixed in cold 2% formaldehyde (5.4 ml Formalin (Sigma) in 100 ml PBS) for 10 minutes at 4°C. Cells were then washed thrice in PBS and then incubated with 1.5 ml of DL6 primary antibody (diluted 1:1000 in PBS and 0.5% BSA for surface staining, and with additional 0.2% saponin for internal staining) for 1.5 hours at room temperature. Cells were washed again and incubated with anti-mouse fluorescein isothiocyanate (FITC)-conjugated secondary antibody (diluted 1:500 as above) for a further 90 minutes. Transfection efficiency was measured as the percentage of cells that showed green fluorescence under inverted fluorescence microscope.
2.5.3 Erythrocyte binding assay on COS7 cells expressing PvRII

The pHVDR22 plasmid used for transfection was tested to ascertain binding activity using the erythrocyte binding assay in the COS7 cell system (Cohen et al., 1988). Erythrocytes used for the erythrocyte binding assay were human Duffy-positive and chymotrypsin treated human erythrocytes. Chymotrypsin treatment of human Duffy-positive erythrocytes cleaves the Duffy antigen and gives the functional equivalent of Duffy-negative erythrocytes (Ranjan and Chitnis, 1999).

In order to optimize the parameters for the inhibition of binding assay, COS7 cells were used in a preliminary erythrocyte binding assay. COS7 cells have been previously shown to successfully express proteins targeted to the surface using the signal sequence and transmembrane region of HSVgD (Cohen et al., 1988), and maintain the characteristic binding phenotype of PvRII (Chitnis and Miller, 1994). COS7 cells were plated at 20-30% confluence in 6 well cell culture plates and grown O/N to 75-80% confluence. 48 to 60 hours post-transfection, transfected and untransfected control cells were incubated with 200μl RBC suspension (20μl PCV red blood cells in 160μl iDMEM and 20μl FBS) of normal human Duffy-positive and chymotrypsin-treated human erythrocytes for 2 hours at 37°C, 5% CO2. Washing was done six times with PBS and 2ml fresh PBS was left in the wells. Rosettes were scored positive when erythrocytes were stacked covering more than 50% of the cell surface and the entire well was scored at a magnification of 200x. Figure 13i shows a COS7 cell covered with a rosette of erythrocytes, counted in the entire well under medium magnification. Figure 13i shows a binder, erythrocytes are either flat against the cell or upright, and not stacked on each-other or lying on top. Figure 13ii shows a wide field with only a few sparse erythrocytes and no binding COS7 cells.

2.5.4 Detection of PvRII on 293T cells by immunofluorescence assay

In order to obtain a sufficient number of mammalian cells that express PvRII on the surface, it is important to optimize the efficiency of transfection. Trasfection efficiency was tested in the human 293T cell line. The protocol described above for COS7 cells was used for 293T cells, while taking into consideration the faster growth rate of 293T cells.
Figure 13. Erythrocyte Binding Assay. Mammalian cells expressing PvRII will attach to Duffy positive erythrocytes, binding in the form of a rosette (i.), and will not attach to Duffy negative erythrocytes. Photo shows large field of non-binding cells (ii.). A positive rosette must have at least half of the plated cell covered with a single layer of adhering erythrocytes. Immunofluorescence Assay. PvRII expressing mammalian cells are detected with DL6 mouse antibodies followed by anti-mouse FITC-conjugated secondary antibodies, (iii.), shows surface staining under high magnification (400x) and, (iv.), internal staining is obtained by adding saponin to the antibody solution.
293T cells were plated at a 10% confluence in 12 well cell culture plates and grown overnight to 50-60% confluence. Transfection with 12 well plates was carried out as outlined above using ~1μg per well. Expression of PvRII was tested using IFA as described above for COS7 cells. Transfection efficiency was determined by scoring of fluorescent cells per 100 293T cells in each well.

2.5.5 Inhibition of erythrocyte binding by anti-PvRII sera in EBA on plated 293T cells

Inhibition of erythrocyte binding to PvRII expressed on the surface of 293T cells with antisera raised against recombinant PvRII. 293T cells were cultured and transfected as described above in 12 well tissue culture plates. Erythrocyte binding assay was then performed in the presence of different dilutions of mouse sera raised against recombinant PvRII (the final bleed from mouse 5 was used). Pre-immune sera and mouse sera raised against adjuvant alone (from the final bleed of a control mouse injected with adjuvant alone) were used as controls. Scoring was performed as described above.