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Biochemical, Biophysical, and Functional Characterization of Bacterially Expressed and Refolded Receptor Binding Domain of *Plasmodium vivax* Duffy-binding Protein*

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Invasion of erythrocytes by malaria parasites is mediated by specific molecular interactions. *Plasmodium vivax* is completely dependent on interaction with the Duffy blood group antigen to invade human erythrocytes. The *P. vivax* Duffy-binding protein, which binds the Duffy antigen during invasion, belongs to a family of erythrocyte-binding proteins that also includes *Plasmodium falciparum* sialic acid binding protein and *Plasmodium knowlesi* Duffy binding protein. The receptor binding domains of these proteins lie in a conserved, N-terminal, cysteine-rich region, region II, found in each of these proteins. Here, we have expressed *P. vivax* region II (PvRII), the *P. vivax* Duffy binding domain, in *Escherichia coli*. Recombinant PvRII is incorrectly folded and accumulates in inclusion bodies. We have developed methods to refold and purify recombinant PvRII in its functional conformation. Biochemical, biophysical, and functional characterization confirms that recombinant PvRII is pure, homogeneous, and functionally active in that it binds Duffy-positive human erythrocytes with specificity. Refolded PvRII is highly immunogenic and elicits high titer antibodies that can inhibit binding of *P. vivax* Duffy-binding protein to erythrocytes, providing support for its development as a vaccine candidate for *P. vivax* malaria. Development of methods to produce functionally active recombinant PvRII is an important step for structural studies as well as vaccine development.

The invasion of erythrocytes by malaria parasites is mediated by specific molecular interactions between host receptors and parasite ligands (1). *Plasmodium vivax* and the related simian malaria parasite *Plasmodium knowlesi* require interaction with the Duffy blood group antigen to invade human erythrocytes (2, 3). *P. knowlesi* can also invade rhesus erythrocytes using alternative Duffy-independent receptors (4). *P. falciparum* commonly uses sialic acid residues of glycophorin A as invasion receptors (5–9). Like *P. knowlesi*, *P. falciparum* also invades erythrocytes by multiple pathways and is not completely dependent on sialic acid residues of glycophorin A (8, 10, 12, 13).

Parasite ligands that bind host receptors to mediate erythrocyte invasion include *P. vivax* and *P. knowlesi* Duffy-binding proteins, *P. knowlesi* β and γ proteins, which bind Duffy-independent receptors on rhesus erythrocytes, and *P. falciparum* sialic acid-binding protein (also known as EBA-175), which binds sialic acid residues on glycophorin A (4, 14–18). These parasite ligands share similar features and belong to a family of erythrocyte-binding proteins (19). The extracellular domain of each erythrocyte-binding protein contains two conserved cysteine-rich regions, regions II and VI, at the amino and carboxyl ends, respectively. *P. falciparum* EBA-175 contains a tandem duplication (F1 and F2) of the N-terminal, conserved, cysteine-rich region. The functional receptor binding domain of each erythrocyte-binding protein lies in region II (20, 21). In the case of EBA-175, region F2 was found to have receptor binding activity (21). *P. vivax* region II (PvRII)[1] specifically binds the human Duffy antigen, and *P. falciparum* region F2 specifically binds sialic acid residues of glycophorin A. Region II of the *P. knowlesi* Duffy-binding protein binds both human and rhesus Duffy antigens. *P. knowlesi* β region II binds sialic acid residues on rhesus erythrocytes, and *P. knowlesi* γ region II binds as yet unidentified receptors on rhesus erythrocytes (20, 22). These conserved, receptor binding domains are referred to as Duffy binding-like (DBL) domains after the binding domain of *P. vivax* Duffy-binding protein.

DBL domains are also found in the extracellular region of the *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) family of proteins that are expressed on the surface of *P. falciparum*-infected trophozoites and schizonts (23–25). Some members of the PfEMP-1 family bind endothelial receptors such as CD36, ICAM-1, vascular cell adhesion molecule, E-selectin, CD31, chondroitan sulfate A, and hyaluronic acid to mediate cytoadherence, which is responsible for sequestration of *P. falciparum* trophozoites and schizonts in the deep vasculature of various host organs (26–31). Binding to ICAM-1 is thought to be important for sequestration in brain capillaries and is implicated in the pathology of cerebral malaria (32). Binding to hyaluronic acid and chondroitan sulfate A is important for

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[1] The abbreviations used are: PvRII, *P. vivax* region II; DBL, Duffy binding-like; PfEMP-1, *P. falciparum* erythrocyte membrane protein-1; ICAM-1, intercellular adhesion molecule 1; GdnHCl, guanidine hydrochloride; DTT, dithiothreitol; CD, circular dichroism; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; *Herpes simplex* virus glycoprotein D; VCAM, vascular cell adhesion molecule.

sequestration in the placenta, which often leads to complications in pregnancy (30, 33). DBL domains derived from PfEMP-1 have been shown to bind ICAM-1, chondroitan sulfate A, CD31, and uninfected erythrocytes (34–39).

DBL domains are thus used by different *Plasmodium* species to bind diverse host receptors to mediate erythrocyte invasion and cytoadherence, two mechanisms that are central to malaria pathogenesis. It is important to understand the structural basis of the interaction of DBL domains with host receptors. This may allow the development of receptor-blocking agents that inhibit invasion or reverse cytoadherence. Receptor binding DBL domains are also attractive vaccine candidates because antibodies directed against such functional domains may block their interaction with erythrocytes or endothelial receptors.

Here, we describe methods to produce milligram quantities of correctly folded recombinant PvRII, the binding domain of *P. vivax* Duffy-binding protein, which contains around 350 amino acids, including 12 cysteines. Recombinant PvRII has been expressed in *Escherichia coli* purified from inclusion bodies under denaturing conditions and refolded *in vitro* into its native conformation. We demonstrate that refolded PvRII is highly immunogenic and elicits high titer antibodies that can inhibit the binding of *P. vivax* Duffy-binding protein to erythrocytes, providing support for the development of recombinant PvRII as a vaccine candidate for *P. vivax* malaria. Large scale production of functional DBL domains is now possible, opening avenues for more extensive biochemical, structural, and immunological studies.

MATERIALS AND METHODS

Plasmid Constructs and E. coli Strains Used for Recombinant Expression of PvRII—DNA encoding PvRII (amino acids 194–521 of *P. vivax* Duffy-binding protein) fused to hexa-histidine (6-His) at the C-terminal end was amplified by polymerase chain reaction using primers 5′-GCA TGC CAT GGA TCA TAA GAA AAC GAT CT-3′ and 5′-CGA GTG TCG ACT CAG TGA TGG TGA TGG TGA TGT GTC ACA ACT TCC TGA GT-3′ and a plasmid containing the gene encoding *P. vivax* Duffy-binding protein as template (18). The polymerase chain reaction product was digested with *Nco*I and *Sal*I and cloned as a *Nco*I-*Sal*I fragment downstream of the T7 promoter in expression vector pET28a+ (Novagen) to yield plasmid pVPET1. *E. coli* BL21 (DE3) strain (Novagen) was transformed with plasmid pVPET1 and used for expression of recombinant PvRII.

Expression of PvRII in E. coli—Luria broth containing kanamycin (50 μg/ml) was inoculated with *E. coli* BL21(DE3)pVPET1 and cultured overnight at 37 °C. Fresh Luria broth containing kanamycin (25 μg/ml) was inoculated with the overnight culture at a dilution of 1:50 and cultured at 37 °C to an $A_{600\ nm}$ of 0.6–0.8. Expression of PvRII was induced by adding isopropyl-1-thio-β-galactopyranoside to the culture at a final concentration of 1 mM. Induced cultures were allowed to grow for 4 h at 37 °C.

Isolation of Inclusion Bodies and Purification of Recombinant PvRII by Metal Affinity Chromatography under Denaturing Conditions—After induction, *E. coli* cells were harvested by centrifugation, washed in chilled wash buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 mM NaCl), resuspended in chilled lysis buffer (10 mM Tris, pH 8.0, 5 mM benzamidine-HCl, 2 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, 100 mM NaCl, 200 μg/ml lysozyme), and lysed by sonication. Inclusion bodies were collected by centrifugation of lysed cells at 4 °C, solubilized in 10 mM Tris buffer, pH 8.0, containing 6 M guanidine hydrochloride (GdnHCl), and treated with 10 mM dithiothreitol (DTT) for 2 h at 37 °C. After removal of DTT by ultrafiltration using filters with 10-kDa cutoff, recombinant PvRII was purified from solubilized inclusion bodies by metal affinity chromatography under denaturing conditions using a nickel nitrilotriacetic acid column as described by the manufacturer (Qiagen). Solubilized inclusion bodies were loaded on a nickel nitrilotriacetic acid column previously equilibrated with equilibration buffer (10 mM Tris, pH 8.0, 100 mM NaH_2PO_4, 6 M GdnHCl). The column was washed with equilibration buffer at pH 6.3, and bound protein was eluted using a pH gradient starting at pH 6.3 and ending at pH 4.3. The final concentration of the purified protein was adjusted to ~4.5 mg/ml with equilibration buffer.

Refolding PvRII by Rapid Dilution—Purified PvRII was refolded by 100-fold dilution in refolding buffer containing 50 mM phosphate buffer, pH 7.2, 1 mM reduced glutathione, 0.1 mM oxidized glutathione, 1 M urea, and 0.5 M arginine so that the final protein concentration was ~45 μg/ml. Refolding was allowed to proceed at 10 °C for 36 h with stirring. At the end of 36 h, the refolding solution was dialyzed for 48 h against dialysis buffer (50 mM phosphate buffer, pH 6.5, 1 M urea) to remove arginine before proceeding with purification by ion-exchange chromatography.

Purification of Refolded PvRII by Ion Exchange and Gel Filtration Chromatography—After removal of arginine by dialysis, the refolded protein was loaded on an SP-Sepharose column equilibrated with 50 mM phosphate buffer, pH 6.5. The bound protein was eluted with a linear gradient of NaCl (100 mM NaCl to 1.5 M NaCl). Fractions containing refolded PvRII were pooled, and PvRII was further purified by gel filtration chromatography using a Superdex 75 column (Amersham Pharmacia Biotech). For gel filtration chromatography, 50 mM phosphate buffer, pH 7.2, containing 200 mM NaCl was used.

Analysis of Refolded, Purified PvRII by Reverse Phase Chromatography—Refolded PvRII was loaded on a reverse phase C8 column. The gradient used for elution was developed using Buffer A (0.05% trifluoroacetic acid in water) and Buffer B (0.05% trifluoroacetic acid in 90% acetonitrile, 10% water). The column was initially equilibrated with 90% Buffer A and 10% Buffer B and reached a composition of 10% Buffer A and 90% Buffer B in 40 min.

N-terminal Sequencing and Mass Spectral Analysis of Refolded, Recombinant PvRII—Automated Edman degradation was carried out using an Applied Biosystems 491 protein sequencer using standard methods. Approximately 100 pmol of purified refolded PvRII was used for the N-terminal sequencing reaction. Mass spectral analysis of refolded, purified PvRII was performed by electron spray ionization mass spectroscopy using a Hewlett Packard HP100 Series LC/MSD mass spectrometer by standard procedures.

Detection of Free Thiols in Refolded, Recombinant PvRII—Analysis of free protein thiol was carried out using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) according to the method of Ellman (40). The sensitivity of the assay was determined using known amounts of cysteine. Free thiol could be clearly detected at a concentration of 30 μM. The presence of free thiols in refolded, purified PvRII was determined in the presence of 6 M GdnHCl to promote side chain availability.

Circular Dichroism (CD) Spectra—CD spectra were recorded on a Jasco-J720 spectropolarimeter. Spectra of purified, refolded PvRII in 10 mM phosphate buffer, pH 7.2, were recorded as the average of 10 individual spectral scans in the far-UV region from 184 to 260 nm using a cuvette with a path length of 0.1 cm and the following instrument parameters: instrument sensitivity, 1 millidegrees; response time, 2 s; scan speed, 50 nm/min. Concentration of PvRII used for CD spectroscopy was measured by the BCA protein assay method (Pierce) using bovine serum albumin as a standard. Deconvolution of the CD spectra was performed using the method of Bohm *et al.* (41).

Erythrocyte Binding Assay—Blood collected in 10% citrate phosphate dextrose was stored at 4 °C for up to 4 weeks and washed three times in RPMI 1640 (Life Technologies, Inc.) before use. Duffy phenotypes of erythrocytes were determined by standard blood-typing methods using two antisera (anti-Fya and anti-Fyb) (Ortho-Clinical Diagnostics). Duffy negative erythrocytes, Fy(a−b−), did not react with either anti-Fya or anti-Fyb. Duffy-positive erythrocytes used in the erythrocyte binding assays had the phenotype Fy(a+b+). Refolded PvRII was incubated with Duffy-positive and Duffy-negative human erythrocytes at room temperature for 1 h to allow binding. The reaction mixture was layered over dibutylpthalate (Sigma) and centrifuged to collect erythrocytes. Bound protein was eluted from the erythrocytes with 300 mM NaCl, separated by SDS-PAGE, and detected by Western blot using rabbit serum raised against a 42-amino acid peptide derived from PvRII (amino acids 378–420 of *P. vivax* Duffy-binding protein).

Detection of Inactive PvRII by Immuno-precipitation and Western Blotting after Preabsorption of Refolded PvRII with Duffy-positive Erythrocytes—Different quantities of refolded PvRII (100, 200, 400, 800, 2000 ng) were immuno-precipitated as described below using polyclonal rabbit serum raised against refolded PvRII, separated by SDS-PAGE, and detected by Western blotting using a commercially available mouse monoclonal antibody raised against penta-histidine (5-His) (Qiagen) to determine the sensitivity of detection. Immunoprecipitation was performed by incubating refolded PvRII with a rabbit polyclonal serum raised against PvRII at a dilution of 1:200 for 1 h on ice and with protein A-Sepharose beads (Amersham Pharmacia Biotech) at room temperature for 1 h. The beads were collected by centrifugation and washed once with 0.5% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 50 mM Tris, pH

7.4 (NETT) containing 0.5% bovine serum albumin and twice with NETT buffer without bovine serum albumin. Bound proteins were eluted by boiling, separated by SDS-PAGE, and detected by Western blot using a mouse monoclonal antibody directed against 5-His (Qiagen). 100 ng of refolded PvRII can be clearly detected by this method. Refolded PvRII (8 μg) was preabsorbed four times with 800 μl of packed Duffy-positive human erythrocytes to remove active PvRII before immunoprecipitation. Preabsorption with an equal number of Duffy-negative human erythrocytes was used as a control. Residual PvRII left after preabsorption was detected by immunoprecipitation and Western blotting as described.

Animals and Immunization—Rabbits used in this study were procured from the Animal Facility of the International Center for Genetic Engineering and Biotechnology, New Delhi, India. Animals were housed, fed, and used in experiments according to the guidelines set forth in the National Institutes of Health manual titled Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication 86–23, United States Department of Health and Human Services, Washington D. C.). Two 8-week-old New Zealand White rabbits were immunized with 200 μg of recombinant PvRII emulsified in complete Freund's adjuvant and delivered by the subcutaneous route. The rabbits were boosted on day 28 with 100 μg of PvRII formulated in incomplete Freund's adjuvant delivered by the subcutaneous route. One rabbit was immunized with adjuvant alone according to the schedule described above to provide control antiserum. Rabbits were bled on days 0 (preimmune), 27, and 42, and the sera were used for immunoassays.

ELISA—Rabbit sera were tested for recognition of recombinant PvRII using an ELISA. Briefly, wells of flat-bottom Immulon-2 plates (Dynatech Laboratories) were coated with 0.1 μg of PvRII and blocked with 5% milk powder solution in phosphate-buffered saline (blocking buffer). Antigen-coated wells were incubated for 90 min at 37 °C with 100 μl of rabbit serum diluted in blocking buffer. Serial dilutions (1:2-fold) of sera starting with a 1:1000-fold dilution were tested. After washing with phosphate-buffered saline and 0.05% Tween 20, 100 μl of horseradish peroxidase-labeled anti-rabbit IgG antibody (Sigma) diluted 1:2000-fold was added to each well and incubated for 60 min at 37 °C. The enzyme reaction was developed with *o*-phenylenediamine dihydrochloride as the chromogen and hydrogen peroxide as the substrate. The reaction was terminated by the addition of sulfuric acid, and the absorbance at 490 nm (A_{490}) was recorded in each well using an ELISA microplate reader (Molecular Devices). Preimmune sera as well as adjuvant-alone control sera were used at similar dilutions. To determine end point titers, the last dilution of test sera yielding an A_{490} value 1.5 times that obtained with preimmune serum was determined.

Inhibition of Erythrocyte Binding to PvRII Expressed on the Surface of COS Cells with Antisera Raised against Refolded PvRII—COS7 cells were cultured as described previously (20). COS7 cells growing in 35-mm-diameter wells at 40–60% confluency were transfected using Lipofectin with 2.5 μg of plasmid pHVDR22 according to the manufacturer's instructions (Life Technologies, Inc.). Plasmid pHVDR22 was designed to express PvRII on the surface of mammalian cells (20); it contains DNA encoding PvRII fused to the signal sequence of *Herpes simplex* virus glycoprotein D (HSV gD) at the N terminus and the transmembrane region and cytoplasmic domain of HSV gD at the C terminus in a mammalian expression vector (20, 42). The signal sequence and transmembrane segment of HSV gD target the fusion protein to the surface of COS cells. Expression of PvRII on the COS cell surface was confirmed by immunofluorescence assays, as described previously using a monoclonal antibody, DL6, directed against HSV gD sequences in the fusion protein (20, 42). Binding of erythrocytes to transfected COS cells expressing PvRII on the surface was tested using an erythrocyte binding assay, as described earlier (20). Briefly, 200 μl of a 10% suspension of Duffy-positive human erythrocytes was added to 2 ml of media in wells containing transfected COS cells. Erythrocytes were allowed to settle for 2 h at 37 °C. Non-adherent erythrocytes were removed by washing COS cells three times with RPMI. Erythrocyte binding assays were performed in the presence of different dilutions of rabbit sera raised against refolded PvRII. Preimmune sera and rabbit sera raised against adjuvant alone were used as controls. The number of COS cells covered with rosettes of adherent erythrocytes was scored in 50 fields at a magnification of 40.

RESULTS

Expression, Purification, and Refolding of Recombinant PvRII—Recombinant PvRII accumulates in inclusion bodies as a misfolded, insoluble aggregate when expressed in *E. coli*. Misfolded PvRII was solubilized in 6 M GdnHCl, purified by metal affinity chromatography under denaturing conditions, and refolded by rapid dilution. Oxidized and reduced glutathione were used during refolding to enable disulfide bond formation. Conditions such as final concentration of the recombinant protein after dilution, pH, temperature, duration of refolding, and concentrations of arginine, urea, glutathione, and oxidized glutathione were optimized to maximize yield of soluble PvRII after refolding. Refolded PvRII was purified by ion-exchange chromatography and gel filtration chromatography as described.

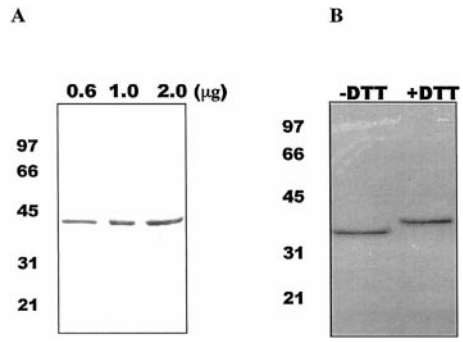

FIG. 1. **Refolded and purified recombinant PvRII.** *A*, silver-stained SDS-PAGE gel of refolded and purified PvRII. Different quantities (0.6 μg, 1.0 μg, and 2.0 μg) of refolded and purified PvRII were reduced, denatured, separated by SDS-PAGE, and detected by silver-staining. *B*, mobility of refolded and purified PvRII by SDS-PAGE before and after reduction. Refolded PvRII has slower mobility by SDS-PAGE after reduction with dithiothreitol (+*DTT*), indicating the presence of disulfide linkages. Molecular mass markers in kDa are shown.

Refolded, purified PvRII was separated by SDS-PAGE and detected by silver staining (Fig. 1*A*). Densitometry scanning of silver-stained gels indicates that the purity of recombinant PvRII is greater than 98%. Yields of purified, refolded PvRII are ~2 mg/liter *E. coli* culture. Recombinant PvRII migrates with the expected mobility of ~39 kDa.

Biochemical, Biophysical, and Functional Characterization of Refolded and Purified PvRII—Refolded and purified PvRII was characterized using a variety of biophysical, biochemical, and functional assays. N-terminal sequencing of recombinant PvRII yields the sequence MDHKKTISSAINHA, which is identical to the expected sequence. No other sequence is detected. The molecular mass of recombinant PvRII measured by electron spray ionization mass spectroscopy is 39,803 Da. The predicted mass of PvRII with a 6-His fusion if all 12 cysteines are disulfide-linked is 39,802 Da. N-terminal sequencing and mass spectroscopic data confirm the identity of recombinant PvRII. The mobility of refolded PvRII by gel filtration chromatography on a Superdex 75 column is consistent with an apparent molecular mass of ~39 kDa, indicating that purified PvRII does not form aggregates or multimers (data not shown).

Refolded PvRII migrates slower on SDS-PAGE gels after reduction with DTT, indicating that disulfide linkages are present in the refolded protein (Fig. 1*B*). The homogeneity of refolded PvRII was analyzed by reverse phase chromatography, a method that can be expected to separate different conformers of the same protein based on differences in surface hydrophobicity. Refolded PvRII elutes as a single symmetric peak by reverse phase chromatography on a C-8 column, suggesting that the final purified product is homogeneous (Fig. 2). Reduction of refolded PvRII with DTT results in an increase in elution time by 1 min, confirming the presence of disulfide linkages in the refolded protein (Fig. 2). Free thiol content in refolded PvRII was assayed using the method of Ellman (40) to further assess the oxidation state of the refolded protein. Free thiol could be clearly detected at a concentration of 30 μM in this assay. No

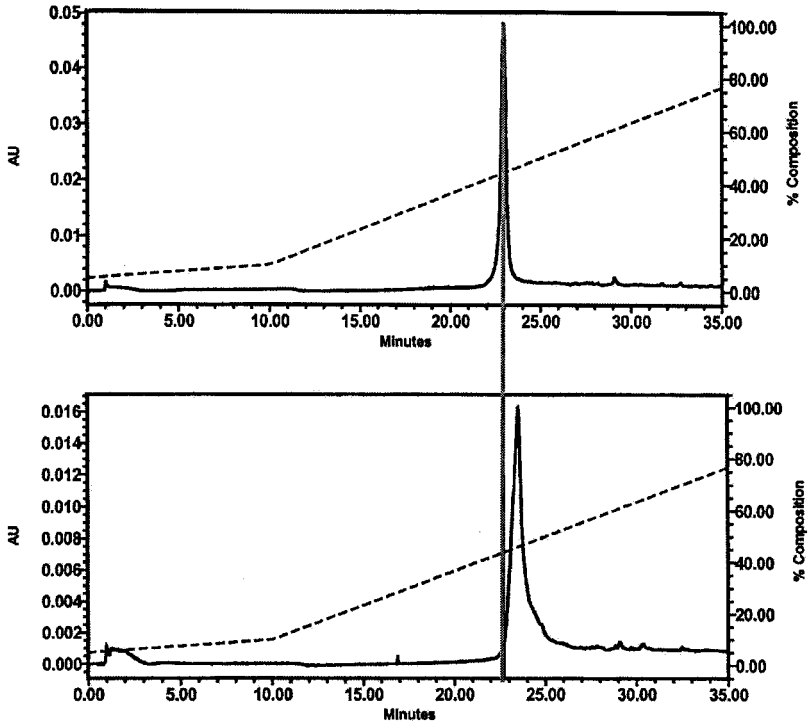

FIG. 2. **Reverse phase high performance liquid chromatography profile of refolded and purified PvRII before and after reduction.** Refolded, purified PvRII was analyzed by reverse phase chromatography on a C8 column. The gradient used for elution was developed using Buffer A (0.05% trifluoroacetic acid in water) and Buffer B (0.05% trifluoroacetic acid in 90% acetonitrile, 10% water). The column was initially equilibrated with 90% Buffer A and 10% Buffer B and reached a composition of 10% Buffer A and 90% Buffer B in 40 min. Refolded PvRII elutes as a single, symmetric peak, indicating that it contains a single, homogeneous population of conformers. Reduction of refolded PvRII results in a shift in elution time by 1 min. *AU*, absorbance units.

free thiols were detected in recombinant PvRII at a concentration of 50 μM. Considering that PvRII contains 12 cysteines, this indicates that greater than 95% of cysteines are disulfide linked.

CD spectroscopy was used to probe the secondary structure of refolded PvRII. The CD spectrum of PvRII shows characteristic α-helical signature minima at 208 and 222 nm (Fig. 3). Deconvolution of the CD spectrum by the method of Bohm *et al.* (41) indicates the following distribution of secondary structure components for PvRII: 52.6% α-helices, 9.8% β-sheets, 14.0% β-turns, and 21.8% random-coils. The sum of secondary structural elements calculated from the CD spectrum totals 98.2%, showing confidence in the measurement and deconvolution. Spectra recorded on three different batches of refolded PvRII are identical. Denaturation of refolded PvRII with 6 M GdnHCl results in loss of minima at 208 and 222 nm, indicating disruption of α-helical structures.

An erythrocyte binding assay was used to test whether refolded PvRII is functional. Recombinant PvRII was incubated with Duffy-positive and Duffy-negative human erythrocytes to allow binding. Erythrocytes with bound protein were collected by centrifugation. Bound protein was eluted with 300 mM NaCl, separated by SDS-PAGE, and detected by Western blot. Rabbit serum raised against a 42-amino acid peptide derived from PvRII was used to detect bound PvRII. Refolded PvRII binds Duffy-positive human erythrocytes but not Duffy-negative human erythrocytes (Fig. 4*A*). Refolded PvRII thus binds erythrocytes with the same specificity as *P. vivax* Duffy-binding protein, indicating that it is correctly folded in its functional conformation. Rabbit serum reacts with a high molecular weight erythrocyte-derived protein that is released due to red cell lysis upon the addition of NaCl for elution of bound protein. Preimmune rabbit serum collected before immunization with PvRII also reacts with this erythrocyte-derived protein (data not shown).

What fraction of refolded PvRII is functionally active and correctly folded? Preabsorption of refolded PvRII with erythrocytes followed by detection of residual PvRII by immunoprecipitation and Western blotting was used to detect incorrectly folded PvRII. Approximately 100 ng of PvRII is clearly detected

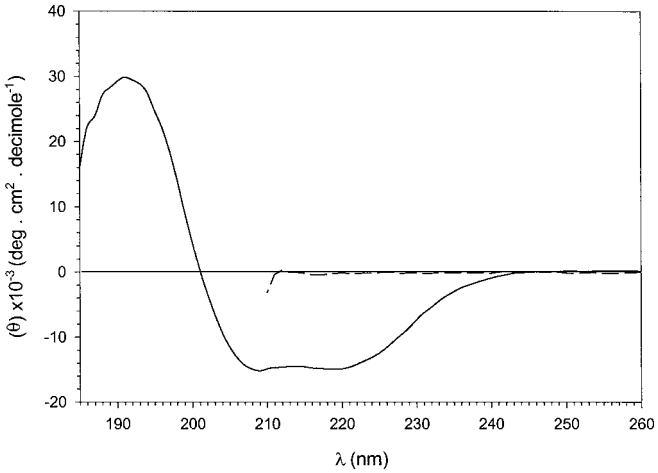

FIG. 3. **CD spectra of refolded and purified PvRII.** CD spectra of recombinant PvRII are shown before (*solid line*) and after (*broken line*) denaturation with 6 M GdnHCl. Minima near 208 and 222 nm and a maximum near 190 nm indicate the presence of significant α-helical content in the refolded protein. Denaturation of refolded PvRII with 6 M GuHCl results in loss of minima at 208 and 222 nm.

by this method. Duffy-positive and Duffy-negative human erythrocytes were used to preabsorb 8 μg of refolded PvRII. No residual PvRII was detected when Duffy positive erythrocytes were used for preabsorption (Fig. 4*B*). On the other hand Duffy-negative erythrocytes failed to absorb PvRII. Considering that sensitivity of detection of PvRII is 100, and 8 μg of refolded PvRII was used, the inability to detect residual protein after preabsorption indicates that at least 98.75% of refolded PvRII is removed by preabsorption and is functional.

Immunogenicity of Refolded PvRII and Inhibition of Erythrocyte Binding to PvRII with Antisera Raised against Refolded PvRII—Refolded PvRII formulated in Freund's adjuvant was used to immunize rabbits. Titers for reactivity of rabbit sera with PvRII were determined by ELISA. Sera from two immunized rabbits had titers of 1:64,000 and 1:32,000, indicating that PvRII is highly immunogenic.

The ability of rabbit sera to block binding of PvRII to eryth-

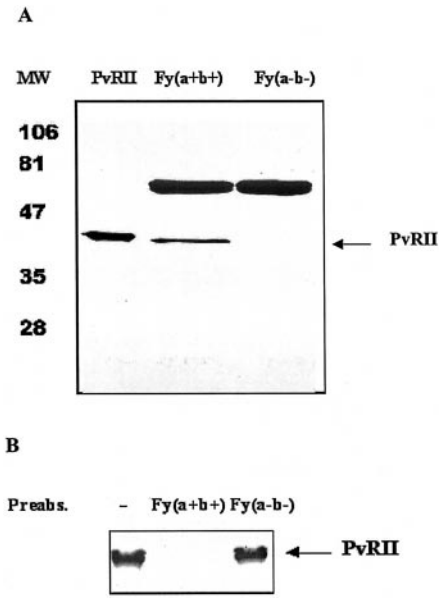

FIG. 4. **Erythrocyte binding assay with refolded and purified PvRII.** *A*, erythrocyte binding assay. Refolded PvRII was incubated with Duffy-positive (Fy(a+b+)) and Duffy-negative (Fy(a−b−)) erythrocytes to allow binding. Erythrocytes and bound protein were collected by centrifugation. Bound PvRII was eluted with 300 mM NaCl, separated by SDS-PAGE, and detected by Western blotting using a rabbit antiserum raised against a 42-amino acid peptide derived from PvRII. Refolded PvRII specifically binds Duffy-positive (Fy(a+b+)) but not Duffy-negative ((Fy(a−b−)) human erythrocytes. *B*, detection of inactive PvRII by immunoprecipitation and Western blotting after preabsorption with Duffy-positive erythrocytes. Residual PvRII was immunoprecipitated, separated by SDS-PAGE, and detected by Western blot either after preabsorption with Duffy-positive (Fy(a+b+)) or Duffy-negative (Fy(a−b−)) human erythrocytes or without preabsorption (−). MW, M_r.

rocytes was tested in an erythrocyte binding assay. COS cells were transfected with constructs designed to express PvRII fused with the signal sequence and transmembrane domain of HSV gD at the amino and carboxyl ends, respectively, to target the *P. vivax* domain to the cell surface (20). Monoclonal antibody DL6 directed against HSV gD sequences in the fusion protein confirmed expression of PvRII on the COS cell surface (20, 42). Transfection efficiencies were in the range of 15–20%. Erythrocyte binding assays were performed 48–60 h after transfection. COS cells were incubated with Duffy-positive human erythrocytes in the presence of either preimmune serum, serum from a rabbit immunized with adjuvant alone, or different dilutions of serum from a rabbit immunized with PvRII. The number of COS cells covered with rosettes of erythrocytes was scored in 50 fields at a magnification of 40. Results of one of three similar experiments are shown in Table I. Rabbit serum raised against refolded PvRII completely blocks binding of erythrocytes to COS cells expressing PvRII up to a dilution of 1:2500. These data indicate that refolded PvRII can be used to elicit high titer antibodies that are capable of inhibiting binding of *P. vivax* Duffy-binding protein to erythrocytes.

TABLE I
Inhibition of erythrocyte binding to PvRII expressed on the surface of COS cells with rabbit serum raised against refolded, purified PvRII

Antisera	Dilution	No. of rosettes in 50 fields[a]
None	NA	++++
Preimmune	1:10	++++
Adjuvant control	1:10	+++
Anti-PvRII	1:250	−
Anti-PvRII	1:500	−
Anti-PvRII	1:1,000	−
Anti-PvRII	1:2,500	−
Anti-PvRII	1:5,000	+
Anti-PvRII	1:7,500	++
Anti-PvRII	1:10,000	+++

[a] Numbers of COS cells covered with rosettes of red blood cells were scored in 50 fields at a magnification of 40. The average number of rosettes found in two wells is reported. +, 25–50 rosettes; −, no rosettes seen. NA, not applicable. Results from one of three similar experiments are reported.

DISCUSSION

The functional binding domains of erythrocyte-binding proteins of *Plasmodium* sp. map to region II, the conserved, N-terminal, cysteine-rich regions that are also referred to as DBL domains (20, 21). Functional domains of PfEMP-1 that mediate rosetting as well as binding to endothelial receptors such as ICAM-1, chondroitan sulfate A, and CD31 have also been mapped to DBL domains (34–39). DBL domains thus play central roles in two important pathogenic mechanisms, namely erythrocyte invasion and cytoadherence. To dissect the structural basis for the interaction of DBL domains with host receptors, it is necessary to obtain the three-dimensional structures of DBL domains and map receptor-binding sites within these functional domains. We have previously shown that binding residues map to a central 170-amino acid stretch between the fourth and seventh cysteines of PvRII (22). Here, we describe methods to produce milligram amounts of recombinant PvRII in its functional conformation and present biochemical, biophysical, and functional characterization of the recombinant domain.

Recombinant PvRII was expressed in *E. coli*, purified from inclusion bodies under denaturing conditions, refolded *in vitro* by the method of rapid dilution, and further purified by ion exchange chromatography and gel filtration chromatography. The final product was shown to be pure, homogenous, and functional. Purified PvRII is greater than 98% pure as determined by densitometry scanning of silver-stained SDS-PAGE gels. The mobility of purified PvRII by gel filtration chromatography is consistent with an apparent molecular weight of ~39 kDa, indicating that purified PvRII does not form aggregates or multimers. Moreover, refolded PvRII elutes as a single, symmetrical peak by reverse phase chromatography, suggesting that it contains a single, homogeneous population of conformers. Importantly, refolded PvRII binds Duffy-positive human erythrocytes but not Duffy-negative human erythrocytes, indicating that it is folded in its functional conformation. No residual PvRII is detected after preabsorption of refolded PvRII with Duffy-positive human erythrocytes. Considering that the detection limit for PvRII is 100 ng, and 8 μg of refolded PvRII was used, the inability to detect residual PvRII after preabsorption with Duffy-positive human erythrocytes indicates that greater than 98.75% of refolded PvRII is functional. The CD spectrum of refolded PvRII indicates the presence of significant α-helical content, as has been predicted for DBL domains (43). It remains to be experimentally determined if other DBL domains also contain high α-helical content. Importantly, CD spectra recorded for different batches of refolded PvRII are identical, implying conformational consistency in the refolded product produced by the method described.

The binding domain of *P. vivax* Duffy-binding protein, PvRII, is a promising vaccine candidate since antibodies directed against this functional domain may block erythrocyte binding and invasion by *P. vivax*. Rabbit sera raised against refolded PvRII recognize PvRII up to dilution of 1:64,000 by ELISA, indicating that refolded PvRII is highly immunogenic. Sera from immunized rabbits completely inhibit erythrocyte binding to COS cells expressing PvRII on the surface up to a dilution of 1:2500. These data indicate that recombinant PvRII can elicit

high titer binding inhibitory antibodies, providing support for the development of recombinant PvRII as a vaccine for *P. vivax* malaria. Due to the difficulty in culturing *P. vivax*, it is not possible to test antibodies raised against PvRII for inhibition of erythrocyte invasion by *P. vivax*. Antibodies raised against the binding domain of *P. falciparum* EBA-175 have been shown to inhibit erythrocyte invasion by *P. falciparum in vitro* (44).[2] By analogy, antibodies directed against PvRII can be expected to effectively block erythrocyte invasion by *P. vivax*.

Individuals living in endemic areas develop binding inhibitory antibodies directed against PvRII after repeated exposure to *P. vivax* infection (45). However, naturally acquired binding inhibitory antibodies are of low titer. Moreover, human sera from individuals residing in endemic areas completely block binding of erythrocytes to COS cells expressing PvRII only up to a dilution of 1:10 (45). Here, we have shown that rabbit sera raised against refolded PvRII inhibit erythrocyte binding to COS cells expressing PvRII up to a dilution of 1:2500. It remains to be determined if the presence of high titer antibodies that inhibit binding of *P. vivax* Duffy-binding protein to erythrocytes will confer protection against *P. vivax* malaria.

Recombinant PvRII has been previously expressed in its functional form as a secreted protein in insect cells using baculoviral vectors (46). Recombinant proteins expressed as secreted proteins in eukaryotic cells are commonly glycosylated, which is often a disadvantage for crystallographic studies. Previous attempts to express PvRII in its functional form using bacterial expression systems have been unsuccessful (11, 46). This report is the first description of methods developed for refolding and purification of functional PvRII produced in a bacterial expression system. These methods should be applicable to DBL domains derived from other erythrocyte-binding proteins and from extracellular regions of PfEMP-1. Indeed, we have used similar methods to express, purify, and refold *P. falciparum* region F2, the binding domain of *P. falciparum* EBA-175. Refolded *P. falciparum* region F2 specifically binds sialic acid residues of human glycophorin A.[2] The availability of methods for large scale production of correctly folded DBL domains is an important step for structural studies as well as for malaria vaccine development efforts based on these functional receptor binding domains.

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REFERENCES

1. Chitnis, C. E., Sinnis, P., and Miller, L. H. (1999) in *Malaria: Molecular and Clinical Aspects* (Perlmann P. and Wahlgren M., eds) pp. 249–285, Harwood Academic Publishers Gmbh, London
2. Miller, L. H., Mason, S. J., Clyde, D. F., and McGinnis, M. H. (1976) *N. Eng. J. Med.* **295,** 302–304
3. Miller, L. H., Mason, S. J., Dvorak, J. A., McGinnis, M. H., and Rothman, I. K. (1975) *Science* **189,** 561–563
4. Haynes, J. D., Dalton, J. P., Klotz, F. W., McGinniss, M. H., Hadley, T. J., Hudson, D. E., and Miller, L. H. (1988) *J. Exp. Med.* **167,** 1873–1881
5. Pasvol, G., Wainscoat, J. S., and Weatherall, D. J. (1982) *Nature* **297,** 64–66
6. Breuer, W. V., Ginsburg, H., and Cabantchik, Z. I. (1983) *Bioch. Biophys. Acta* **755,** 263–271
7. Friedman, M. J., Blankenburt, T., Sensabaugh, G., and Tenforde, T. S. (1984) *J. Cell Biol.* **98,** 1682–1687
8. Perkins, M. E., and Holt, E. A. (1988) *Mol. Biochem. Parasitol.* **27,** 23–28
9. Miller, L. H., Haynes, J. O., and McAuliffe, F. M., Shiroishi, T., Durocher, J., and McGinnis, M. H. (1977) *J. Exp. Med.* **146,** 277–281
10. Mitchell, G. H., Hadley, T. J., McGinniss, M. H., Klotz, F. W., and Miller, L. H. (1986) *Blood* **67,** 1519–1521
11. Fraser, T., Michon, P., Barnwell, J. W., Noe, A. R., AlYaman, F., Kaslow, D. C., and Adams, J. H. (2000) *Infect. Immun.* **65,** 2772–2777
12. Dolan, S. A., Proctor, J. L., Alling, D. W., Okubo, Y., Wellems, T. E., and Miller, L. H. (1994) *Mol. Biochem. Parasitol.* **64,** 55–63
13. Okoyeh, J. N., Pillai, C. R., and Chitnis, C. E. (1999) *Infect. Immun.* **67,** 5784–5791
14. Camus, D., and Hadley, T. J. (1985) *Science.* **230,** 553–555
15. Wertheimer, S. P., and Barnwell, J. W. (1989) *Exp. Parasitol.* **69,** 340–350
16. Adams, J. H., Hudson, D. H., Torii, M., Ward, G. E., Wellems, T. E., Aikawa, M., and Miller, L. H. (1990) *Cell* **63,** 141–153
17. Sim, B. K. L., Orlandi, P. A., Haynes, J. D., Klotz, F. W., Carter, J. M., Camus, D., Zegans, M. E., and Chulay, J. D. (1990) *J. Cell Biol.* **111,** 1877–1884
18. Fang, X., Kaslow, D. C., Adams, J. H., and Miller, L. H. (1991) *Mol. Biochem. Parasitol.* **44,** 125–132
19. Adams, J. H., Sim, B. K. L., Dolan, S. A., Fang, X., Kaslow, D. C., and Miller, L. H. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 7085–7089
20. Chitnis, C. E., and Miller, L. H. (1994) *J. Exp. Med.* **180,** 497–506
21. Sim, B. K. L., Chitnis, C. E., Wasniowska, K., Hadley, T. J., and Miller, L. H. (1994) *Science* **264,** 1941–1944
22. Ranjan, A., and Chitnis, C. E. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 14067–14072
23. Baruch, D. I., Pasloske, B. L., Singh, H. B., Xiahui, B., Ma, X. C., Feldman, M., and Howard, R. J. (1995) *Cell* **82,** 77–88
24. Smith, J. D., Chitnis, C. E., Craig, A. G., Roberts, D. J., Hudson-Taylor, D. E., Peterson, D. S., Pinches, B., Newbold, C. I., and Miller, L. H. (1995) *Cell* **82,** 101–110
25. Su, X., Heatwole, V., Wertheimer, S. P., Guinet, F., Herrfeldt, J. A., Peterson, D. S., and Wellems, T. (1995) *Cell* **82,** 89–100
26. Ockenhouse, C. F., Tandon, N. N., Magowan, C., Jamieson, G. A., and Chulay, J. D. (1989) *Science* **243,** 1469–1471
27. Berendt, A. R., Simmons, D. L., Tansey, J., Newbold, C. I., and Marsh, K. (1989) *Nature* **341,** 51–57
28. Ockenhouse, C. F., Tegoshi, T., Maeno, Y., Benjamin, C., Ho, M., Kan, K. E., Thway, Y., Win, K., Aikawa, M., and Lobb, R. R. (1992) *J. Exp. Med.* **176,** 1183–1189
29. Rogerson, S. J., Chaiyaroj, S. C., Ng, K., Reeder, J. C., and Brown, G. V. (1995) *J. Exp. Med.* **182,** 15–20
30. Beeson, J. G., Rogerson, S. J., Cooke, B. M., Reeder, J. C., Chai, W., Lawson, A. M., Molyneux, M. E., and Brown, G. V. (2000) *Nat. Med.* **6,** 86–90
31. Treutiger, C. J., Heddini, A., Fernandez, V., Muller, W. A., and Wahlgren, M. (1997) *Nat. Med.* **3,** 1405–1408
32. Turner, G. D., Morrison, H., Jones, M., Davis, T. M., Looareesuwan, S., Buley, I. D., Gatter, K. C., Newbold, C. I., Pukritayakamee, S., and Nagachinta, B. (1994) *Am. J. Pathol.* **145,** 1057–1071
33. Fried, M., and Duffy, P. (1996) *Science* **272,** 1502–1504
34. Smith, J. D., Craig, A. G., Kriek, N., Hudson-Taylor, D., Kyes, S., Fagan, T., Pinches, R., Baruch, D. I., Newbold, C. I., and Miller, L. H. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 1766–1771
35. Buffet, P. A., Gamain, B., Scheidig, C., Baruch, D., Smith, J. D., Hernandez-Rivas, R., Pouvelle, B., Oishi, S., Fujii, N., Fusai, T., Parzy, D., Miller, L. H., Gysin, J., and Scherf, A. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 12743–12748
36. Reeder, J. C., Cowman, A. F., Davern, K. M., Beeson, J. G., Thompson, J. K., Rogerson, S. J., and Brown, G. V. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 5198–5202
37. Rowe, A. J., Moulds, J. M., Newbold, C. I., and Miller, L. H. (1997) *Nature* **398,** 292–295
38. Chen, Q., Barragan, A., Fernandez, V., Sundstrom, A., Schlichtherle, M., Sahlen, A., Carlson, J., Datta, S., and Wahlgren, M. (1998) *J. Exp. Med.* **187,** 15–23
39. Chen, Q., Heddini, A., Barragan, A., Fernandez, V., Pearce, S. F., and Wahlgren, M. (2000) *J. Exp. Med.* **192,** 1–10
40. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82,** 70–77
41. Bohm, G., Muhr, R., and Jaenicke, R. (1992) *Protein Eng.* **5,** 191–195
42. Cohen, G. H., Wilcox, W. C., Sodora, D. L., Long, D., Levin, J. Z., and Eisenberg, R. J. (1988) *J. Virol.* **62,** 1932–1940
43. Smith, J. D., Subramanian, G., Gamain, B., Baruch, D. I., and Miller, L. H. (2000) *Mol. Biochem. Parasitol.* **110,** 293–310
44. Narum D. L., Haynes, J. D., Fuhrmann, S., Moch, K., Liang, H., Hoffman, S. L., and Sim, B. K. (2000) *Infect. Immun.* **68,** 1964–1966
45. Michon, P., Fraser, T., and Adams, J. H. (2000) *Infect. Immun.* **68,** 3164–3171
46. Dutta, S., Daugherty, J. R., Ware, L. A., Lanar, D. E., and Ockenhouse, C. F. (2000) *Mol. Biochem. Parasitol.* **109,** 179–184

[2] K. C. Pandey, S. Singh, and C. E. Chitnis, unpublished results.