# Identification of the Erythrocyte Binding Domains of *Plasmodium vivax* and *Plasmodium knowlesi* Proteins Involved in Erythrocyte Invasion

By Chetan E. Chitnis and Louis H. Miller

*From the Laboratory of Malaria Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892*

## Summary

*Plasmodium vivax* and the related monkey malaria, *P. knowlesi*, require interaction with the Duffy blood group antigen, a receptor for a family of chemokines that includes interleukin 8, to invade human erythrocytes. One *P. vivax* and three *P. knowlesi* proteins that serve as erythrocyte binding ligands in such interactions share sequence homology. Expression of different regions of the *P. vivax* protein in COS7 cells identified a cysteine-rich domain that bound Duffy blood group–positive but not Duffy blood group–negative human erythrocytes. The homologous domain of the *P. knowlesi* proteins also bound erythrocytes, but had different specificities. The *P. vivax* and *P. knowlesi* binding domains lie in one of two regions of homology with the *P. falciparum* sialic acid binding protein, another erythrocyte binding ligand, indicating conservation of the domain for erythrocyte binding in evolutionarily distant malaria species. The binding domains of these malaria ligands represent potential vaccine candidates and targets for receptor-blockade therapy.

The human malaria, *Plasmodium vivax*, and the related monkey malaria, *P. knowlesi*, use the Duffy blood group antigen as a receptor to invade human erythrocytes (1, 2). The Duffy blood group antigen has recently been shown to be a receptor for a family of chemokines that includes IL-8 and melanoma growth–stimulating activity (MGSA)[1] (3). Duffy blood group–negative (Duffy negative) human erythrocytes lack the Duffy blood group antigen and are refractory to invasion by *P. vivax* and *P. knowlesi*. The invasion of erythrocytes by malaria merozoites is a multi-step process that is mediated by several molecular interactions between the invading merozoite and the target erythrocyte. The binding of parasite ligands to the Duffy blood group antigen is required for the formation of an irreversible junction between *P. knowlesi* merozoites and Duffy-positive human erythrocytes and is followed by the entry of the merozoite into the erythrocyte (4). A 140-kD *P. vivax* protein and a 135-kD *P. knowlesi* protein, which bind Duffy-positive but not -negative human erythrocytes, probably serve as ligands for junction formation during invasion (5, 6). Affinity-purified Abs to the 135-kD *P. knowlesi* protein were used to clone the putative gene ($\alpha$) encoding this protein from *P. knowlesi* (7). Two other related *P. knowlesi* genes ($\beta$ and $\gamma$) and the homologous *P. vivax* gene were cloned by cross-hybridization to the *P. knowlesi* $\alpha$ gene (8, 9). Independent of these studies, the gene encoding a sialic acid binding protein of *P. falciparum*, the other major human malaria, was also cloned (10). These genes from *P. knowlesi*, *P. vivax*, and *P. falciparum* have similar structures and share regions of amino acid sequence homology suggesting that they belong to a gene family encoding erythrocyte binding proteins (8). The extracellular domains of these proteins can be divided into six regions based on amino acid sequence homology (8). Each protein has two cysteine-rich regions (regions II and VI, also referred to as the 5′ and 3′ cysteine-rich regions, respectively) which contain highly conserved cysteines and aromatic amino acid residues (8). In this paper, we identify one of these cysteine-rich regions (region II) as the binding domain of the *P. vivax* and *P. knowlesi* ligands. The three *P. knowlesi* ligands have different binding specificities that may provide redundancy in invasion pathways resulting in a survival advantage in case of mutations in host receptors.

## Materials and Methods

### *Construction of Recombinant Plasmids for Surface Expression in COS7 Cells*

The plasmid pRE4 (provided by Drs. Gary Cohen and Roselyn Eisenberg, University of Pennsylvania, Philadelphia, PA), has been described previously (11). It contains a SV40 origin of replication (SV40 *ori*), a Rous sarcoma virus LTR (RSV LTR) as a promoter and the SV40 early polyadenylation signal (SV40 polyA). The HSV glycoprotein D gene (HSV *gD*) was cloned in the HindIII cloning site downstream of the RSV LTR. HSV gD has a signal peptide of 25 amino acids at the $NH_2$-terminal end and a 24 amino acid hydrophobic transmembrane stretch with a 30 amino acid cytoplasmic tail at the COOH-terminal end (12). HSV *gD* has unique

[1] *Abbreviations used in this paper:* HSV gD, HSV glycoprotein D; MGSA, melanoma growth–stimulating activity; PDI, protein disulfide isomerase.

ApaI and PvuII restriction sites (12). The PvuII site is 27 amino acids downstream of the signal peptide cleavage site and the ApaI site is 71 amino acids upstream of the COOH-terminal hydrophobic stretch. We have used the signal sequence and hydrophobic transmembrane stretch of HSV gD to target different regions of the malarial erythrocyte binding proteins to the surface of mammalian COS7 cells. The plasmid pRE4 was restricted with PvuII and ApaI to excise the central region of HSV *gD* encoding amino acids 33 to 248. The restriction fragments were separated by gel electrophoresis and the vector was recovered using Geneclean II (Stratagene, La Jolla, CA). DNA fragments encoding different regions of the *P. vivax* and *P. knowlesi* proteins were cloned in the vector using the PvuII and ApaI sites to make the following constructs:

*pHVDR11.* This construct contains DNA sequences encoding region I (amino acids 27–214) of the *P. vivax* protein. The PCR was used to amplify the DNA sequence encoding this region. The plasmid p192B which contains the putative gene encoding the *P. vivax* Duffy antigen binding protein, was used as a template, and the primers, 5'TCT CGT CAG CTG GAT GCA GAA GGA CAT GAC3' and 5'ACG AGT GGG CCC CAT TAC AGT ATT TTG AAG3', were used as the 5' and 3' primers, respectively, in the PCR reaction. The 5' and 3' primers contain 18 bases that are homologous to the putative gene encoding the *P. vivax* Duffy antigen binding protein and the restriction sites for PvuII and ApaI, respectively. The product of the PCR reaction was restricted with PvuII and ApaI, electrophoresed on a 1% agarose gel, recovered using Geneclean II, and ligated with the vector. The ligation created a chimeric gene in which the HSV *gD* sequence was in frame with the malarial gene sequence. The method described above was used to make the constructs given below, except where stated otherwise.

*pHVDR22.* This construct contains DNA sequences encoding region II (amino acids 198–522) of the *P. vivax* protein. The primers, 5'TCT CGT CAG CTG ACG ATC TCT AGT GCT ATT3' and 5'ACG AGT GGG CCC TGT CAC AAC TTC CTG AGT3', and the plasmid p192B were used to amplify region II by PCR.

*pHVDR35.* This construct contains DNA sequences encoding regions III–V (amino acids 516–922) of the *P. vivax* protein. The primers, 5'TCT CGT CAG CTG ACT CAG GAA GTT GTG ACA3' and 5'ACG AGT GGG CCC TGC TTT GAC ATC TCT GTA3', and the plasmid p192B were used to amplify regions III–V by PCR.

*pHVDR66.* This construct contains DNA sequences encoding region VI (amino acids 864–1000) of the gene encoding the *P. vivax* protein. The primers, 5'TCT CGT CAG CTG GAT GCA GAA GGA CAT GAC3' and 5'ACG AGT GGG CCC CGT AAA GCA CTT GTA GGA3', and the plasmid p192B were used to amplify region VI by PCR.

*pHKBDR22.* This construct contains DNA sequences encoding region II (amino acids 199–517) of the *P. knowlesi* $\beta$ protein. The primers, 5'TCT CGT CAG CTG AGT GGT GTT ATT AAT CAA3' and 5'ACG AGT GGG CCC CAA GTG TTC CTG AGT ATT3', and the plasmid p10g which contains the *P. knowlesi* $\beta$ gene were used to amplify region II by PCR.

*pHKADR22.* This construct contains DNA sequences encoding region II (amino acids 189–525) of the *P. knowlesi* $\alpha$ protein. The *P. knowlesi* $\alpha$ gene contains an internal EcoRI site and was cloned as two EcoRI fragments on the plasmids p1cg and p6D. A DNA fragment encoding amino acids 189–276 of the *P. knowlesi* $\alpha$ protein was amplified by PCR using the primers 5'CTG CGT ATG AAT CGA AAG GAA3' and 5'ACG AGT GGG CCC CAG GTC GAA TTC TTT GTT ATA TTG3', and the plasmid P1cg, and was restricted with EcoRI. A DNA fragment encoding amino acids 276–525 of the *P. knowlesi* $\alpha$ protein was amplified by PCR using the primers 5'AGC ACG GAA TTC TGT AAG GAT ATA AGA TGG3' and 5'ACG AGT GGG CCC TTC AAC ACC ACT TCC TAC3', and the plasmid p6D, and was restricted with EcoRI and ApaI. A three-way ligation was performed with the two restricted PCR products and the vector.

*pHKGDR22.* This construct contains DNA sequences encoding region II (amino acids 196–529) of the *P. knowlesi* $\gamma$ protein. The *P. knowlesi* $\gamma$ gene contains an internal EcoRI site and was cloned as two EcoRI fragments on the plasmids p1Bg and p4.5. A DNA fragment encoding amino acids 196 to 399 of the *P. knowlesi* $\gamma$ protein was amplified by PCR using the primers, 5'TCT CGT CAG CTG ATG TCT AGT GGT GCT ATT3' and 5'ACG AGT GGG CCC CAG GTC GAA TTC TGA CAT ATA ATC3', and the plasmid p1Bg, and was restricted with PvuII and EcoRI. Another DNA fragment encoding amino acids 399–529 of the *P. knowlesi* $\gamma$ protein was amplified by PCR using the primers 5'AGC ACG GAA TTC CGC GAA CAA CGA CGG AAA3' and 5'ACG AGT GGG CCC ACT GGG AGC CTT AGA TTC3', and the plasmid p4.5, and was restricted with ApaI and EcoRI. A three-way ligation was performed with the two restricted PCR products and the vector.

### *Cell Culture and Transfection of COS7 Cells*

COS7 cells (American Type Culture Collection [ATCC] CRL 1651; Rockville, MD) were cultured in DMEM with 10% heat inactivated FCS (both from GIBCO BRL, Gaithersburg, MD) in a humidified $CO_2$ (5%) incubator at 37°C. Fresh monolayers of COS7 cells (30–50% confluent) in 3.5-cm diameter wells were transfected with 5 $\mu$g of plasmid DNA by the calcium phosphate precipitation method (13). Cells were grown on coverslips for use in immunofluorescence assays. The cells were washed three times with media 12–16 h after the transfection. The transfected cells were used for immunofluorescence or erythrocyte binding assays 40–60 h after transfection.

### *Immunofluorescence Assay for Expression in COS7 Cells*

Transfected COS7 cells were assayed for expression 40–60 h after transfection. To check for expression of the chimeric proteins on the surface, the transfected COS7 cells were washed in PBS, fixed in 2% formaldehyde (in PBS) for 15 min at room temperature, washed in PBS, and stained with a primary antibody (1 h at room temperature) to amino acid sequences of HSV gD in the chimeric protein. Ascites fluid containing mAbs, ID3 or DL6 (provided by Drs. Gary Cohen and Roselyn Eisenberg; 11), which map to amino acids 11–19 and 272–279 of the mature HSV gD protein, respectively, were used as the primary antibodies (at a dilution of 1:2000 in PBS containing 0.1% BSA). The cells were washed once in an excess of PBS for 10 min and incubated with fluorescein-conjugated goat anti-mouse antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) at a dilution of 1:100 in PBS containing 0.1% BSA for 30 min. The cells were washed in an excess of PBS for 10 min, mounted with Fluoromount (Southern Biotechnology Associates Inc., Birmingham, AL) and scored for surface expression using a fluorescence microscope. The mAbs, ID3 and DL6, were tested against untransfected COS7 cells to check for background fluorescence. Sera from an unimmunized mouse was used with transfected COS7 cells as a negative control. A mouse mAb, HP13 (provided by Dr. Charlotte Kaetzel, University of Kentucky, Lexington, KY; 14), raised against human protein disulfide isomerase (PDI), which is localized in the endoplasmic reticulum, was used as a control to ensure that fixing with formaldehyde did not permeabilize the cells. Ascites fluid containing mAb HP13 was used

as a control to ensure that fixing with formaldehyde did not permeabilize the cells. Ascites fluid containing mAb HP13 was used as a primary antibody at a dilution of 1:500 in PBS containing 0.1% BSA. The reactivity of HP13 against PDI in COS7 cells was tested by using it in the presence of 0.2% saponin, which permeabilizes the cells and allows access to PDI. To visualize the internal distribution of the chimeric proteins, the mAbs DL6 and ID3 were also used in the presence of 0.2% saponin. Immunofluorescence assays for surface expression were also performed at 4°C (antibodies are not internalized by capping at this temperature) as described above but without fixing the cells. The cells were fixed in 2% formaldehyde for 15 min after staining with the antibodies, just before mounting with Fluoromount. In this method, since the cells are not fixed before staining with antibodies, there is no concern about permeabilization of the cells due to any fixing procedure.

### *Erythrocytes and Pretreatments with Enzymes*

Blood was collected in 10% citrate phosphate dextrose (CPD) (Baxter, Deerfield, IL) and stored at 4°C for up to 4 wk. The Duffy phenotypes of the erythrocytes were determined by standard blood banking methods using two antisera (anti-Fya and anti-Fyb). Duffy-negative erythrocytes, Fy($a^-b^-$), did not react with either antiserum. The Duffy-positive erythrocytes used in the erythrocyte binding assays had the phenotype Fy($a^+b^+$). Erythrocytes were washed three times in RPMI 1640 (GIBCO BRL) and resuspended to a hematocrit of 10% in RPMI 1640 for use in the erythrocyte binding assay described below. Washed human and monkey erythrocytes were treated with chymotrypsin, trypsin, and neuraminidase as described previously (15).

### *Erythrocyte Binding Assay*

COS7 cells were transfected in 3.5-cm diameter wells and used for erythrocyte binding assays 40–60 h after transfection. 200 $\mu$l of a 10% erythrocyte suspension was added to 2 ml of media in wells containing the transfected cells, the plate was swirled to mix the erythrocytes well, and the erythrocytes were allowed to settle over 2 h at 37°C. The COS7 cells were then washed three times with 2 ml of PBS to remove nonadherent erythrocytes. Transfected COS7 cells with rosettes of adherent erythrocytes (see Fig. 4 for an example) were scored. The number of rosettes was scored in either 10 or 20 fields at a magnification of 40 using an inverted microscope. Binding was scored as negative when no rosettes were seen in the entire well.

The erythrocyte binding assay was followed by an immunofluorescence assay to ensure that COS7 cells with rosettes of adherent erythrocytes were transfected and expressed the chimeric proteins. After the erythrocyte binding assay, coverslips with COS7 cells and adherent erythrocytes were used in the immunofluorescence assay as described earlier.

To study the effect of the chemokines MGSA and IL-8 on binding, the erythrocytes were resuspended to a hematocrit of 1% in 1 ml of complete DMEM and incubated for 1 h at room temperature with the chemokines at the required concentrations (stock solutions of purified MGSA and IL-8 at concentrations of 125 $\mu$M were provided by Dr. Richard Horuk, Genentech Inc., South San Francisco, CA). The erythrocytes were then used in erythrocyte binding assays as described above. The number of COS7 cells with rosettes of adherent erythrocytes was scored in 20 randomly chosen fields at a magnification of 40 in each well and the percent inhibition was determined as follows: percent binding = 100 × (no. of bound COS7 cells in the presence of chemokines)/(no. of bound COS7 cells in absence of chemokines); percent inhibition = 100 − percent binding; and percent inhibition = 0 if binding (%) $\geqslant$ 100.

## Results

*The System for Expressing Different Regions of the P. vivax Protein to Study Erythrocyte Binding.* Different regions (I, II, III–V, and VI) of the *P. vivax* protein were expressed on the surface of COS7 cells. The secretory signal sequence and transmembrane segment of HSV gD were used to target different regions of the malarial proteins to the COS7 cell surface. The HSV *gD* gene contains unique PvuII and ApaI sites, 27 amino acids downstream of the signal peptide cleavage site and 72 amino acids upstream of the transmembrane segment, respectively (11, and Fig. 1). Different regions of the *P. vivax* gene were cloned between the PvuII and ApaI sites to create chimeric genes.

COS7 cells were transfected with these constructs which were designed to express regions I, II, III–V, and VI of the *P. vivax* protein. The transfected COS7 cells were fixed with formaldehyde and a pair of mAbs, DL6 and ID3, to amino acid sequences of HSV gD flanking the cloning sites (11, and Fig. 1) were used to assay for surface expression of the chimeric proteins by immunofluorescence microscopy. Fig. 2, *A* and *E* show the results of the immunofluorescence assays using the mAb DL6 with formaldehyde-fixed COS7 cells that had been transfected with pHVDR22 and pHVDR35, respectively. The edges and protruding processes of these cells were stained indicating that the chimeric proteins were expressed on the cell surface. The mAbs DL6 and ID3 did not react with untransfected COS7 cells.

To ensure that the formaldehyde-fixing procedure did not permeabilize the COS7 cells, a mAb, HP13, raised against PDI, which is localized to the endoplasmic reticulum, was used. HP13 did not react with formaldehyde-fixed COS7 cells (Fig. 2, *C* and *G*), indicating that the cells have not been permeabilized and internal proteins are not accessible. When the formaldehyde-fixed COS7 cells were stained with HP13 in the presence of saponin, which permeabilizes the cells, every cell was stained (Fig. 2, *D* and *H*). These experiments clearly showed that the formaldehyde-fixing procedure does not permeabilize the cells and the staining of formaldehyde-fixed cells with DL6 and ID3 indicates that the chimeric proteins are expressed on the surface of the COS7 cells. The internal dis-

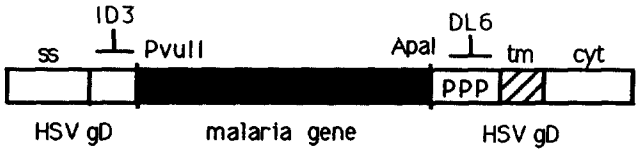


**Figure 1.** Chimeric constructs of HSV glycoprotein D (*HSV gD*) and malaria sequences for the expression of different regions of the *P. vivax* and *P. knowlesi* erythrocyte binding proteins on the COS7 cell surface. HSV *gD* has a signal sequence (*ss*) of 25 amino acids at the $NH_2$-terminal end and a 24 amino acid hydrophobic transmembrane segment (*tm*) with a 30 amino acid cytoplasmic tail (*cyt*) at the COOH-terminal end (12). HSV *gD* has a unique PvuII restriction site 27 amino acids downstream of the signal peptide cleavage site and a unique ApaI restriction site 71 amino acids upstream of the transmembrane segment (12). HSV *gD* has a proline-rich region (*ppp*) adjacent to the transmembrane segment. The mAb ID3 reacts with amino acids 11–19, and the mAb DL6 reacts with amino acids 272–279 of the mature HSV gD protein (11).

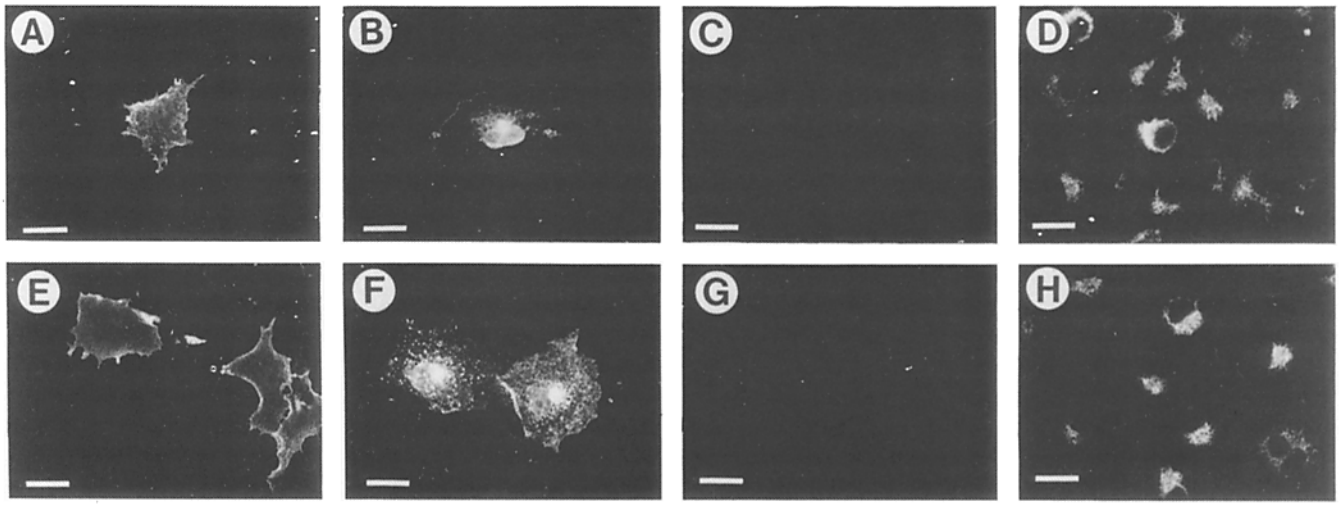


**Figure 2.** Immunofluorescence assays showing surface expression of chimeric proteins containing regions of the *P. vivax* protein in transfected COS7 cells. Immunofluorescence assays on COS7 cells transfected with pHVDR22 (*A–D*) and pHVDR35 (*E–H*), which contain region II and regions III–V of the *P. vivax* protein, respectively. The mAb DL6, which reacts against amino acid sequences of HSV gD in the chimeric proteins, was used with formaldehyde-fixed cells in the absence (*A* and *E*) or presence (*B* and *F*) of saponin, which permeabilizes the cells. The mAb HP13, which reacts against human PDI, was used in the absence (*C* and *G*) or presence (*D* and *H*) of saponin to test if the formaldehyde-fixing procedure permeabilizes COS7 cells. HP13 stained formaldehyde-fixed cells only in the presence of saponin, indicating that formaldehyde fixing did not permeabilize the cells. Note the immunofluorescence patterns with DL6 in the absence (surface pattern, *A* and *E*) or presence (internal pattern, *B* and *F*) of saponin. Bar, 75 μM.

tribution of the chimeric proteins is seen when the transfected cells are stained with DL6 in the presence of saponin (Fig. 2, *B* and *F*). COS7 cells transfected with each construct shown in Fig. 3 gave immunofluorescence patterns with the mAbs DL6 and HP13, that were similar to those shown in Fig. 2, indicating that each chimeric protein tested was targeted to the cell surface. The transfection efficiency (the percentage of COS7 cells with surface immunofluorescence) varied from ~0.5 to 10% depending on the construct and transfection conditions.

Expression of the chimeric proteins on the surface of transfected COS7 cells was also tested by a different immunofluorescence assay. In this assay, the transfected COS7 cells were stained with the primary and secondary antibodies at 4°C before fixing with formaldehyde. The results of this assay with the mAbs DL6 and HP13 were identical to those described with formaldehyde-fixed cells, in that, DL6 stained transfected cells in the absence of saponin, and HP13 stained cells only in the presence of saponin. The results from these assays indicate that all the chimeric proteins were targeted to the surface of COS7 cells.

*Region II of the P. vivax Protein Is the Erythrocyte Binding Domain.* COS7 cells transfected with constructs that expressed the different regions of the *P. vivax* protein were tested for their ability to bind Duffy-positive and -negative human erythrocytes. The transfection efficiency for each construct was determined by the immunofluorescence assay described above. The transfection efficiency and the number of COS7 cells with rosettes of adherent erythrocytes seen in 20 fields at a magnification of 40 is shown in Table 1 for each construct in three separate experiments. In cases where the binding of erythrocytes is reported as negative, no rosettes were seen in the entire well. COS7 cells expressing region II of the *P. vivax* protein bound Duffy-positive, but not -negative, human erythrocytes (Fig. 3, and Fig. 4, *A* and *B*). When the erythrocyte binding assay was followed by an immunofluorescence assay on the same cells, it was found that whereas all COS7 cells with rosettes of adherent erythrocytes were transfected and expressed region II, not all the COS7 cells that expressed region II bound erythrocytes. This is probably due to different levels of expression in these cells. No rosettes of either Duffy-positive or -negative erythrocytes were seen in wells containing COS7 cells expressing regions I, III–V, and VI of the *P. vivax* protein (Fig. 3, and Fig. 4,

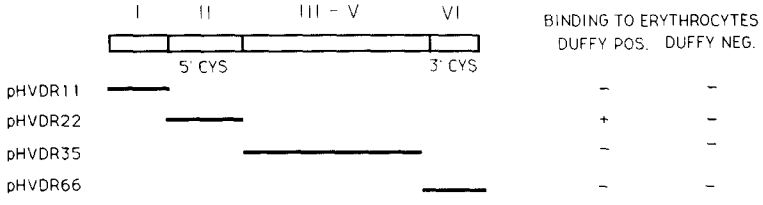


**Figure 3.** Identification of region II of the *P. vivax* protein as an erythrocyte binding domain. The extracellular domain of the *P. vivax* protein was divided into six regions as described previously (8). COS7 cells were transfected with the plasmids shown and expressed different regions (regions *I*, *II*, *III–V*, and *VI*, each indicated by a *line*) of the *P. vivax* protein on the COS7 cell surface. The transfected cells were tested for their ability to bind Duffy-positive or -negative human erythrocytes. (+) Rosettes of adherent erythrocytes were seen on the COS7 cells. (−) No rosettes were seen.

**Table 1.** *Binding of Duffy-positive and -negative Human Erythrocytes to Regions of the P. vivax Protein*

| Region of *P. vivax* protein* | Transfection efficiency‡ | Binding of Duffy-positive human erythrocytes§ | Binding of Duffy-negative human erythrocytes§ |
|---|---|---|---|
| | % | | |
| I | 0.5 | – | – |
| | 3.0 | – | – |
| | 2.5 | – | – |
| II | 2.1 | 225 | – |
| | 3.0 | 238 | – |
| | 5.1 | 180‖ | – |
| III-V | 5.0 | – | – |
| | 1.3 | – | – |
| | 1.0 | – | – |
| VI | 1.0 | – | – |
| | 2.5 | – | – |
| | 2.5 | – | – |

* COS7 cells were transfected with constructs designed to express chimeric proteins containing different regions of the *P. vivax* protein and the signal sequence and transmembrane segment of HSV gD.
‡ The transfection efficiency was determined by an immunofluorescence assay using the mAb DL6, which recognizes amino acid sequences of HSV gD in the chimeric proteins. Results from three independent experiments are shown for each construct.
§ The number of COS7 cells with rosettes of adherent erythrocytes seen in 20 fields at a magnification of 40. Minus signs (–) indicate no rosettes were seen in the entire well. Results from three independent experiments are shown for each construct.
‖ The number of COS7 cells with rosettes of adherent erythrocytes seen in 10 fields at a magnification of 40.

*C* and *D*). These experiments identified region II, the 5′ cysteine-rich region of the *P. vivax* protein, as an erythrocyte binding domain.

It has been shown previously that a 140-kD protein in *P. vivax* culture supernatants binds Duffy-positive but not -negative human erythrocytes (5). The binding of region II of the *P. vivax* protein to enzyme-treated human erythrocytes and rhesus erythrocytes paralleled the known binding profile of the 140-kD protein (Table 2). Chymotrypsin treatment of Duffy-positive human erythrocytes destroys the Duffy blood group antigen and these enzyme-treated erythrocytes did not bind region II of the *P. vivax* protein (Table 2). Trypsin treatment, which does not cleave the Duffy blood group antigen, or neuraminidase treatment, which removes only sialic acid residues, did not affect the binding of *P. vivax* region II (Table 2). Rhesus erythrocytes, which are Duffy positive, did not bind *P. vivax* region II, indicating that the *P. vivax* binding domain specifically binds the human Duffy blood group antigen. The correlation of the binding profiles indicates that the binding of region II to human erythrocytes was specific and that the cloned *P. vivax* gene indeed encodes the 140-kD *P. vivax* Duffy antigen binding protein. The binding of region II and the 140-kD protein to the erythrocytes described correlates with their susceptibility to invasion by *P. vivax* (Table 2).

*Erythrocyte Binding Specificities of Region II of the P. knowlesi α, β, and γ Proteins.* The simian malaria, *P. knowlesi*, invades both Duffy-positive human erythrocytes and erythrocytes from rhesus monkeys, the host in which this parasite is maintained in the laboratory. *P. knowlesi* has three genes (α, β, and γ) that are homologous to the single copy gene that encodes the *P. vivax* Duffy antigen binding protein (8). COS7 cells expressing region II of the *P. knowlesi* α, β, and γ proteins were tested for binding to erythrocytes. Whereas region II of all three *P. knowlesi* proteins (α, β, and γ) bound rhesus erythrocytes, region II of only the α protein bound

**Table 2.** *Comparison of Erythrocyte Invasion by P. vivax to the Binding of P. vivax Region II and the Binding of the 140-kD P. vivax Protein to these Erythrocytes*

| Erythrocytes | Enzyme treatment | *P. vivax* 140-kD binding* | *P. vivax* region II binding‡ | *P. vivax* invasion§ |
|---|---|---|---|---|
| Human Duffy positive | None | + | + | + |
| Human Duffy negative | None | – | – | – |
| Human Duffy positive | Chymotrypsin | – | – | – |
| Human Duffy positive | Trypsin | + | + | + |
| Human Duffy positive | Neuraminidase | + | + | + |
| Rhesus | None | – | – | – |
| Mouse | None | – | – | – |

* Binding of the 140-kD Duffy antigen binding protein from *P. vivax* culture supernatants to erythrocytes. (+) Binding; (–) no binding. Data from Wertheimer and Barnwell (5).
‡ Binding of erythrocytes to COS7 cells expressing region II of the *P. vivax* protein. (+) Binding; (–) no binding.
§ Invasion of erythrocytes by *P. vivax* from Barnwell et al. (29). (+) Erythrocytes invaded; (–) erythrocytes not invaded.

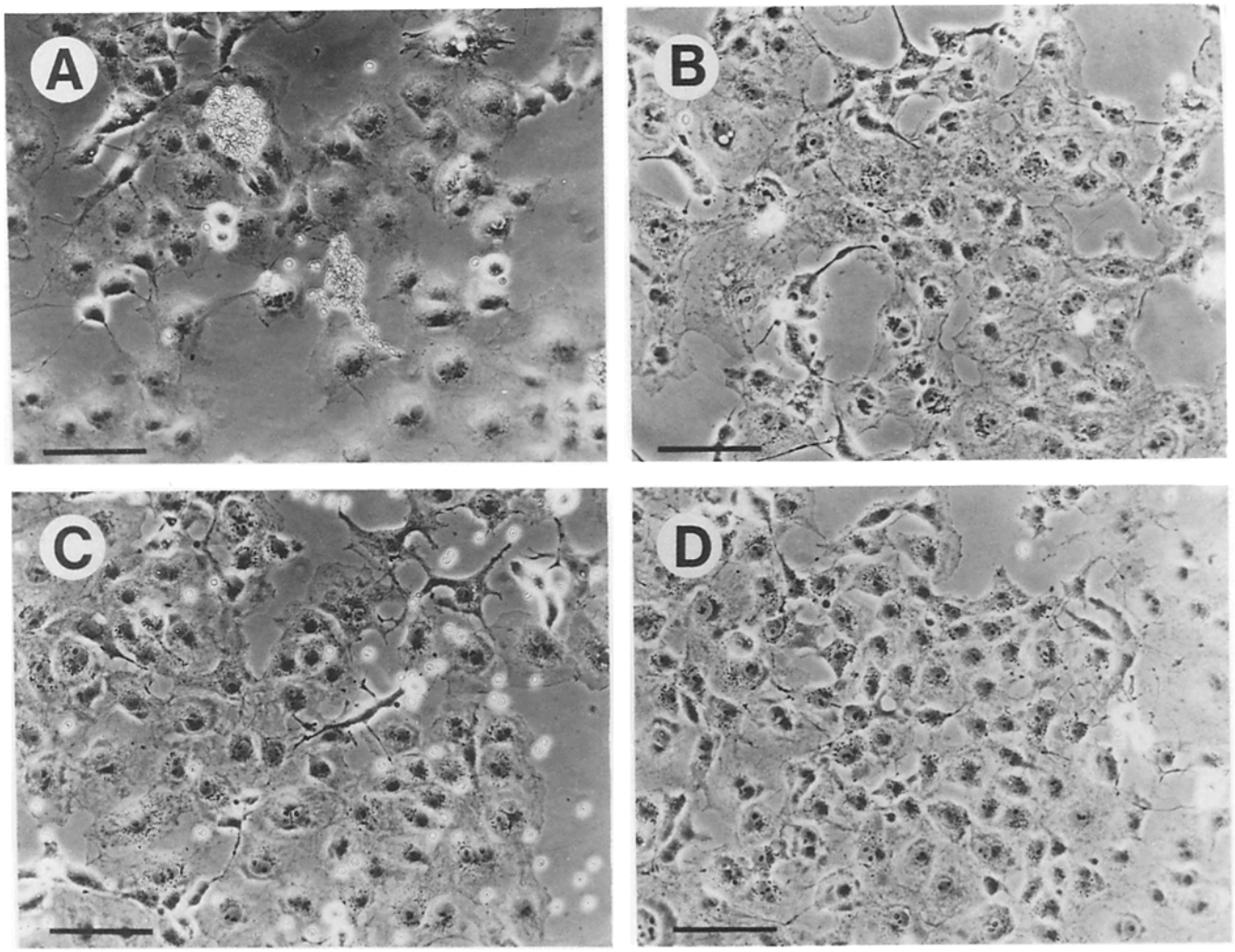


**Figure 4.** Erythrocyte binding assay. Erythrocyte binding assays with transfected COS7 cells expressing either *P. vivax* region II (*A* and *B*) or expressing *P. vivax* regions III–V (*C* and *D*). Duffy-positive (*A* and *C*) or -negative (*B* and *D*) human erythrocytes were used in the assay. Note the two cells in *A* covered with Duffy-positive human erythrocytes. Bar, 50 $\mu$M.

**Table 3.** *Comparison of Erythrocyte Invasion by P. knowlesi to the Binding of Region II of the P. knowlesi α, β, and γ Proteins and the Binding of the 135-kD P. knowlesi Protein to these Erythrocytes*

| Erythrocytes | Enzyme treatment | *P. knowlesi* 135-kD binding* | *P. knowlesi* α region II binding‡ | *P. knowlesi* β region II binding‡ | *P. knowlesi* γ region II binding‡ | *P. knowlesi* invasion§ |
|---|---|---|---|---|---|---|
| Rhesus | None | + | 56 | >100 | >100 | + |
| Human Duffy positive | None | + | 20 | – | – | + |
| Human Duffy negative | None | – | – | – | – | – |
| Human Duffy positive | Chymotrypsin | – | – | – | – | – |
| Rhesus | Chymotrypsin | – | – | >100 | >100 | + |
| Mouse | None | – | – | – | – | – |

* Binding of the 135-kD Duffy antigen binding protein from *P. knowlesi* culture supernatants to erythrocytes from Haynes et al. (6). (+) Binding; (–) no binding.

‡ Binding of erythrocytes to COS7 cells expressing region II of the *P. knowlesi* α, β, or γ protein. The number of COS7 cells with rosettes of adherent erythrocytes seen in 10 fields (α) or 5 fields (β, γ) at a magnification of 40 is shown. The transfection efficiencies for the α, β, and γ genes were 4, 1.9, and 5.5% respectively. (–) No rosettes were seen in the entire well. Three independent experiments gave similar results.

§ Invasion of erythrocytes by *P. knowlesi* from Haynes et al. (6). (+) Erythrocytes invaded; (–) erythrocytes not invaded.

both rhesus and Duffy-positive human erythrocytes (Table 3). The binding profile of region II of the $\alpha$ protein paralleled the known binding profile of the 135-kD *P. knowlesi* protein (Table 3), indicating that the $\alpha$ gene encodes the 135-kD *P. knowlesi* Duffy antigen binding protein, the parasite ligand that mediates erythrocyte invasion by the Duffy antigen–dependent pathway.

The binding domains (region II) of the *P. knowlesi* $\beta$ and $\gamma$ proteins have different binding specificities compared with the $\alpha$ protein. Region II of the $\beta$ and $\gamma$ proteins bound rhesus erythrocytes but not human erythrocytes (Table 3). In addition, region II of the $\beta$ and $\gamma$ proteins bound chymotrypsin-treated rhesus erythrocytes (Table 3). As chymotrypsin destroys the Duffy blood group antigen on rhesus erythrocytes, the attachment of these erythrocytes to region II of the $\beta$ and $\gamma$ proteins indicates that the Duffy blood group antigen is not the receptor for these ligands. The *P. knowlesi* ligands encoded by the $\beta$ and $\gamma$ genes bind as yet undefined chymotrypsin-resistant receptors on rhesus erythrocytes.

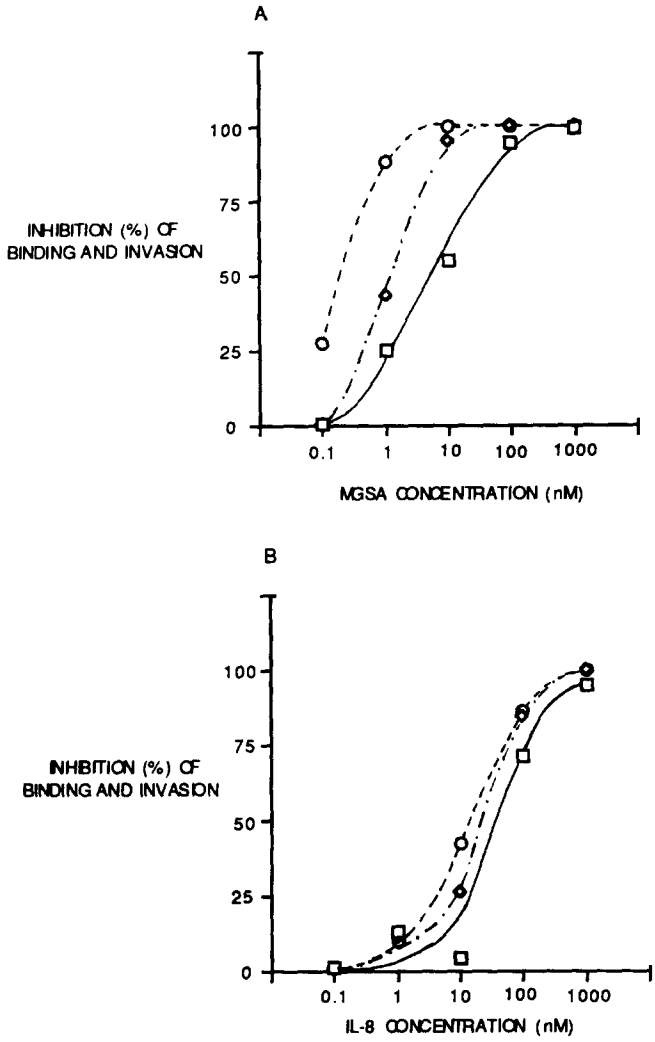


**Figure 5.** Inhibition by chemokines (*MGSA* and *IL-8*) of erythrocyte binding to region II of the *P. knowlesi* $\alpha$ and *P. vivax* proteins. Duffy-positive human erythrocytes were preincubated with different concentrations of MGSA (*A*) or IL-8 (*B*) and were used in binding assays with transfected COS7 cells that expressed region II of the *P. knowlesi* $\alpha$ (O---O) or *P. vivax* (◇- · -◇) proteins on their surface. The inhibition of erythrocyte invasion by *P. knowlesi* (□–□) with MGSA (*A*) or IL-8 (*B*) is adapted from Horuk et al. (3).

*MGSA and IL-8 Block the Binding of Duffy-positive Human Erythrocytes to Region II of the P. vivax and P. knowlesi Duffy Antigen Binding Proteins.* We have shown recently that the Duffy blood group antigen is a receptor for a family of chemotactic and proinflammatory peptides that includes MGSA and IL-8 (3). The chemokines, MGSA and IL-8, block the binding of the 135-kD *P. knowlesi* Duffy antigen binding ligand to Duffy-positive human erythrocytes and inhibit the invasion of these erythrocytes by *P. knowlesi* (3). It is difficult to do similar studies with *P. vivax* because of technical problems in culturing *P. vivax*. The method developed here allows direct studies on the inhibition of this receptor–ligand interaction of *P. vivax*. Duffy-positive human erythrocytes were preincubated with the chemokines, MGSA and IL-8, and used in erythrocyte binding assays. MGSA and IL-8 blocked the binding of Duffy-positive human erythrocytes to COS7 cells expressing region II of the *P. vivax* and *P. knowlesi* Duffy antigen binding proteins on their surface (Fig. 5). The number of COS7 cells with rosettes of bound erythrocytes was determined in 20 randomly chosen fields at a magnification of 40 for each well. In one experiment, in the absence of any chemokines, 75 and 102 rosettes of Duffy-positive human erythrocytes were seen with COS7 cells expressing regions II of the *P. vivax* and *P. knowlesi* Duffy antigen binding proteins, respectively. These values were used to determine the percent inhibition of binding in the presence of the chemokines (Fig. 5). Data on the inhibition of erythrocyte invasion by MGSA and IL-8 was reported previously (3) and are shown in Fig. 5 for comparison. MGSA inhibited erythrocyte binding more efficiently than IL-8. An approximately 10-fold higher concentration of IL-8 than MGSA was required to achieve 50% inhibition of erythrocyte binding to region II of the *P. vivax* and *P. knowlesi* proteins (Fig. 5). This difference between MGSA and IL-8 is similar to that reported previously for the inhibition of erythrocyte invasion by *P. knowlesi* (Fig. 5).

## Discussion

The invasion of erythrocytes by malarial merozoites is a multi-step process that involves several molecular interactions. The early steps of erythrocyte invasion include: (*a*) initial attachment, (*b*) apical reorientation, and (*c*) junction formation that initiates entry into the vacuole (16). *P. vivax* and *P. knowlesi* require the Duffy blood group antigen for invasion of human erythrocytes. When *P. knowlesi* merozoites interact with Duffy-negative human erythrocytes that lack the Duffy blood group antigen, initial attachment and apical reorientation take place normally but a junction between the invading merozoite and target erythrocyte does not form and invasion is aborted (4). These studies showed that the formation of a junction is necessary for erythrocyte invasion and *P. knowlesi* uses the Duffy blood group antigen as a receptor for junction formation. It is not possible to do similar ex-

periments with *P. vivax* because of the technical difficulties in isolating invasive *P. vivax* merozoites. However, by analogy, it appears likely that *P. vivax* also uses the Duffy blood group antigen as a receptor for junction formation during invasion.

Previous studies have identified a 135-kD *P. knowlesi* protein and a 140-kD *P. vivax* protein that bind Duffy-positive but not -negative human erythrocytes (5, 6). These studies used an erythrocyte binding assay in which the erythrocytes were first incubated with metabolically labeled parasite culture supernatants. The erythrocytes with bound parasite proteins were then centrifuged through oil. The bound parasite proteins were eluted with salt, separated by gel electrophoresis, and visualized by autoradiography. This erythrocyte binding assay is limited in that it only detects those parasite proteins that are released into culture supernatants in a form capable of binding erythrocytes. For example, *P. knowlesi* proteins from culture supernatants did not bind chymotrypsin-treated rhesus erythrocytes, even though these treated erythrocytes are invaded normally (6). In addition, the molecular characterization of the binding domains of the erythrocyte binding proteins identified was not possible with this assay.

*Region II of the P. vivax and P. knowlesi Proteins Is the Erythrocyte Binding Domain.* The cloning of the putative genes encoding the erythrocyte binding proteins of *P. vivax* and *P. knowlesi* enabled us to take a molecular approach to study their interactions with erythrocytes. In this study, we identified region II of these proteins as an erythrocyte binding domain and studied the binding specificity of this domain. Region II of the *P. vivax* protein bound Duffy-positive, but not -negative, human erythrocytes. In addition, the binding of *P. vivax* region II to normal and enzyme-treated human and rhesus erythrocytes paralleled the known binding profile of the 140-kD *P. vivax* Duffy antigen binding protein (Table 2). The binding of region II to erythrocytes also correlated with the susceptibility of these erythrocytes to invasion by *P. vivax*. *P. vivax* invades all erythrocytes that bound to region II of the *P. vivax* protein and does not invade erythrocytes from rhesus monkeys and mice which did not bind to region II of the *P. vivax* protein (Table 2).

We have demonstrated that when expressed in the COS7 cell expression system developed here, region II of the *P. vivax* protein binds erythrocytes. Moreover, we have demonstrated that region II of the *P. vivax* protein binds erythrocytes with the same specificity as the parasite protein it is derived from. The perfect correlation between binding of region II and the 140-kD *P. vivax* protein identified region II as a binding domain. None of the other regions bind erythrocytes when expressed in COS7 cells using this system. However, we cannot rule out a role for these regions in erythrocyte binding based on these data.

Region II of the *P. knowlesi* $\alpha$, $\beta$, and $\gamma$ proteins also contains erythrocyte binding domains, but these proteins have different binding specificities. Region II of all three *P. knowlesi* proteins ($\alpha$, $\beta$, and $\gamma$) bound erythrocytes from rhesus monkeys, the laboratory host for *P. knowlesi* (Table 3). In addition, region II of the *P. knowlesi* $\alpha$ protein also bound Duffy-positive human erythrocytes which are invaded by *P. knowlesi* (Table 3). The binding profile of region II of the *P. knowlesi* $\alpha$ protein paralleled the known binding profile of the 135-kD *P. knowlesi* protein (Table 3), indicating that the $\alpha$ gene encodes the 135-kD Duffy antigen binding protein, the parasite ligand that mediates erythrocyte invasion by the Duffy antigen–dependent pathway.

The genes for the *P. vivax* and *P. knowlesi* proteins belong to a gene family encoding erythrocyte binding proteins that also includes the *P. falciparum* sialic acid binding protein (8). These genes have similar structures and share regions of amino acid homology, suggesting a common evolutionary origin (8). Region II, the binding domain of the *P. vivax* and *P. knowlesi* erythrocyte binding proteins, is one of the two regions of these proteins that contains conserved cysteines and aromatic amino acid residues (8). The cysteines probably form structurally important disulfide bonds and the hydrophobic residues may play a role in the folding of the binding domain through hydrophobic interactions. Conservation of the cysteines and hydrophobic amino acid residues in region II suggests that the binding domains of these proteins share a common structural motif that is used for erythrocyte binding. A computer search of the protein and nucleic acid data bases using the BLAST algorithm (17) revealed that these erythrocyte binding proteins are not homologous to any other known proteins. The spacing of the conserved cysteines and aromatic residues in region II may define a unique structural motif that is used for erythrocyte binding. The differences in the amino acid sequences found in region II of the *P. knowlesi* and *P. vivax* proteins probably confer different binding specificities to these proteins. The binding domains of the *P. vivax* and *P. knowlesi* erythrocyte binding proteins lie in a region of homology with the sialic acid binding protein of *P. falciparum*. The approach described here has been used to demonstrate that the domain responsible for the sialic acid–dependent binding of the *P. falciparum* protein also lies in the homologous cysteine-rich region (region II) (18). Sequence analysis of rRNA genes has demonstrated that the primate malarias *P. vivax* and *P. knowlesi* are evolutionarily distant from *P. falciparum* which is more closely related to the avian malarias (19). Conservation of functional domains in evolutionarily distant malaria species was also seen in the case of the circumsporozoite protein where the only conserved regions (regions I and II) between *P. knowlesi* and *P. falciparum* were shown to bind hepatocytes (20, 21). A recent study (22) used soluble recombinant polypeptides from different regions of the *P. falciparum* sialic acid binding protein in erythrocyte binding assays and found that a polypeptide spanning amino acids 869–982, which lie outside region II, bound erythrocytes in a sialic acid–independent manner. Whether the binding of this polypeptide to erythrocytes plays a role in erythrocyte invasion, however, remains to be demonstrated.

*Multiple Erythrocyte Binding Ligands Provide Redundancy in Erythrocyte Invasion Pathways.* The binding domains (region II) of the *P. knowlesi* $\beta$ and $\gamma$ proteins have different binding specificities compared to the *P. knowlesi* $\alpha$ protein. Region II of the $\beta$ and $\gamma$ genes bound rhesus erythrocytes but did not bind either Duffy-positive or -negative human erythrocytes (Table 3). In addition, region II of the $\beta$ and $\gamma$ genes bound chymotrypsin-treated rhesus erythrocytes which have

lost their Duffy blood group antigen (Table 3). *P. knowlesi* is known to invade normal and chymotrypsin-treated rhesus erythrocytes at the same rate, indicating that *P. knowlesi* has alternate pathways for the invasion of rhesus erythrocytes that do not depend on the Duffy blood group antigen (6). The ligands encoded by the *P. knowlesi* $\beta$ and $\gamma$ genes may mediate junction formation with chymotrypsin-treated rhesus erythrocytes allowing the invasion of these erythrocytes by Duffy antigen–independent pathways. Although these ligands evidently do not occur in culture supernatants in a form that binds chymotrypsin-treated rhesus erythrocytes, the mRNAs for these proteins are expressed as measured by reverse transcription with gene-specific primers (8). *P. knowlesi* can also invade trypsin-treated human Duffy–negative erythrocytes (6). Trypsin treatment may uncover receptors on the erythrocyte surface that are used for invasion by *P. knowlesi*. Parasite ligands that bind these receptors have also not yet been found. Region II of the *P. knowlesi* $\alpha$, $\beta$, and $\gamma$ genes did not bind these erythrocytes. Although the possibility that other regions of the *P. knowlesi* $\alpha$, $\beta$, and $\gamma$ genes may contain the binding domain for the trypsin-treated Duffy-negative human erythrocytes cannot be ruled out, it appears likely that *P. knowlesi* uses an as yet undefined ligand to invade these treated Duffy-negative erythrocytes by a pathway that is independent of the Duffy blood group antigen.

This apparent redundancy in invasion pathways for *P. knowlesi* may be an important survival strategy. The lack of such redundancy in *P. vivax* may explain the absence of *P. vivax* from West Africa where almost 95% of the people have the Duffy-negative phenotype and are resistant to *P. vivax*. Invasion by alternate pathways has also been described for *P. falciparum* (23–25). For example, the *P. falciparum* clone Dd2 uses sialic acid residues on glycophorins for invasion, but can switch its phenotype to invade erythrocytes by a sialic acid–independent pathway at a frequency of $10^{-4}$ (Dolan, S. A. and L. H. Miller, unpublished data). When these rare parasites were selected by continuous culture in neuraminidase-treated erythrocytes, the selected parasite line could invade neuraminidase-treated erythrocytes at the same efficiency as normal erythrocytes (26). The redundancy in invasion pathways may permit parasite survival in case of mutations in host receptors. For example, although deletion of glycophorin A or B occurs in various parts of Africa (27, 28), *P. falciparum* has not been eliminated, presumably because of its ability to invade by alternate pathways.

*Applications of the New Erythrocyte Binding Assay.* It has been shown previously that the Duffy blood group antigen is the erythrocyte chemokine receptor (3). The chemokines, IL-8, and MGSA, block the binding of the 135-kD *P. knowlesi* Duffy antigen binding ligand and block invasion of Duffy-positive human erythrocytes (3). It is difficult to do the same studies with *P. vivax* because of technical problems in culturing *P. vivax*. The method developed here allows direct studies on the inhibition of this receptor–ligand interaction of *P. vivax*. MGSA and IL-8 blocked the binding of Duffy-positive human erythrocytes to COS7 cells expressing region II of the *P. vivax* and *P. knowlesi* Duffy antigen binding proteins on their surface. This method can now be used to study the *P. vivax* receptor–ligand interaction at the molecular level and develop chemokine analogues for receptor-blockade therapy. If antibodies to the binding domain are effective at blocking the receptor–ligand interaction, it may also be possible to use the binding domains as a vaccine against malaria. The efficacy of antibodies to such an immunogen in blocking the receptor–ligand interaction can be followed with the in vitro erythrocyte binding assay.

We thank Drs. Gary Cohen and Roselyn Eisenberg for providing the plasmid pRE4, and the mAbs, DL6 and ID3, which were crucial for this work. We also thank Dr. Charlotte Kaetzel for providing the mAb HP13; Richard Horuk for gifts of IL-8 and MGSA; Jeff Murphy for advice on transfection and immunofluorescence techniques; Eugene Koonin for the computer search; and Juan Bonifacino, David Kaslow, Genevieve Milon, Kim Lee Sim, and Gary Ward for comments on this manuscript.

Address correspondence to Dr. Louis H. Miller, Laboratory of Malaria Research, Building 4, Room B126, National Institutes of Health, Bethesda, MD 20892.

*Received for publication 2 February 1994 and in revised form 29 April 1994.*

*Note added in proof:* A recent publication describes the use of fusions with HSV gD to express regions of the heterologous HSV gE protein on the surface of mammalian cells in order to identify Fc binding activity (Dubin, G., S. Basu, D. L. P. Mallory, M. Basu, R. Tal-Singer, and H. M. Friedman. 1994. Characterization of domains of herpes simplex virus type 1 glycoprotein E involved in Fc binding activity for immunoglobulin G aggregates. *J. Virol.* 68:2478.)

## References

1. Miller, L.H., S.J. Mason, J.A. Dvorak, M.H. McGinnis, and I.K. Rothman. 1975. Erythrocyte receptors for (*Plasmodium knowlesi*) malaria: Duffy blood group determinants. *Science (Wash. DC).* 189:561.
2. Miller, L.H., S.J. Mason, D.F. Clyde, and M.H. McGinnis. 1976. The resistance factor to *Plasmodium vivax* in Blacks: Duffy blood group genotype, FyFy. *N. Engl. J. Med.* 295:302.
3. Horuk, R., C.E. Chitnis, W.C. Darbonne, T.J. Colby, A.

Rybicki, T.J. Hadley, and L.H. Miller. 1993. A receptor for the malaria parasite *Plasmodium vivax*: the erythrocyte chemokine receptor. *Science (Wash. DC).* 261:1182.

4. Miller, L.H., M. Aikawa, J.G. Johnson, and T. Shiroishi. 1979. Interaction between cytochalasin B–treated malarial parasites and erythrocytes. Attachment and junction formation. *J. Exp. Med.* 149:172.
5. Wertheimer, S.P., and J.W. Barnwell. 1989. *Plasmodium vivax* interaction with the human Duffy blood group glycoprotein: identification of a parasite receptor-like protein. *Exper. Parasitol.* 69:340.
6. Haynes, J.D., J.P. Dalton, F.W. Klotz, M.H. McGinnis, T.J. Hadley, D.E. Hudson, and L.H. Miller. 1988. Receptor-like specificity of a *Plasmodium knowlesi* malarial protein that binds to Duffy antigen ligands on erythrocytes. *J. Exp. Med.* 167:1873.
7. Adams, J.H., D.E. Hudson, M. Torii, G.E. Ward, T.E. Wellems, M. Aikawa, and L.H. Miller. 1990. The Duffy receptor family of *Plasmodium knowlesi* is located within the micronemes of invasive merozoites. *Cell.* 63:141.
8. Adams, J.H., B.K.L. Sim, S.A. Dolan, X. Fang, D.C. Kaslow, and L.H. Miller. 1992. A family of erythrocyte binding proteins of malaria parasites. *Proc. Natl. Acad. Sci. USA.* 89:7085.
9. Fang, X., D.C. Kaslow, J.H. Adams, and L.H. Miller. 1992. Cloning of the *Plasmodium vivax* Duffy receptor. *Mol. Biochem. Parasitol.* 44:125.
10. Sim, B.K.L., P.A. Orlandi, J.D. Haynes, F.W. Klotz, J.M. Carter, D. Camus, M.E. Zegans, and J.D. Chulay. 1990. Primary structure of the 175K *Plasmodium falciparum* erythrocyte binding antigen and identification of a peptide which elicits antibodies that inhibit malaria merozoite invasion. *J. Cell Biol.* 111:1877.
11. Cohen, G.H., W.C. Wilcox, D.L. Sodora, D. Long, J.Z. Levin, and R.J. Eisenberg. 1988. Expression of *Herpes simplex* virus type 1 glycoprotein D deletion mutants in mammalian cells. *J. Virol.* 62:1932.
12. Watson, R.J., J.H. Weis, J.S. Salstrom, and L.W. Enquist. 1982. *Herpes simplex* virus type 1 glycoprotein D gene: nucleotide sequence and expression in *Escherichia coli*. *Science (Wash. DC).* 218:381.
13. Graham, F.L., and A.J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology.* 52:456.
14. Kaetzel, C.S., C.K. Rao, and M.E. Lamm. 1987. Protein disulfide-isomerase from human placenta and rat liver. Purification and immunological characterization with monoclonal antibodies. *Biochem. J.* 241:39.
15. Camus, D., and T.J. Hadley. 1985. A *Plasmodium falciparum* antigen binds to host erythrocytes and merozoites. *Science (Wash. DC).* 230:553.
16. Dvorak, J.A., L.H. Miller, W.C. Whitehouse, and T. Shiorishi. 1975. Invasion of erythrocytes by malaria merozoites. *Science (Wash. DC).* 187:748.
17. Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403.
18. Sim, B.K.L., C.E. Chitnis, K. Wasniowska, T.J. Hadley, and L.H. Miller. 1994. Receptor and ligand domains for *Plasmodium falciparum* malaria invasion of erythrocytes. *Science (Wash. DC).* In press.
19. Waters, A.P., D.G. Higgins, and T.F. McCutchan. 1991. *Plasmodium falciparum* appears to have arisen as a result of lateral transfer between avian and human hosts. *Proc. Natl. Acad. Sci. USA.* 88:3140.
20. Aley, S.B., M.D. Bates, J.P. Tam, and M.R. Hollingdale. 1992. Synthetic peptides from the circumsporozoite proteins of *Plasmodium falciparum* and *Plasmodium knowlesi* recognize the human hepatoma cell line HepG2-A16 in vitro. *J. Exp. Med.* 164:1915.
21. Cerami, C., U. Frevert, P. Sinnis, B. Takacs, P. Clavijo, M.J. Santos, and V. Nussenzweig. 1992. The basolateral domain of the hepatocyte plasma membrane bears receptors for the circumsporozoite protein of *Plasmodium falciparum* sporozoites. *Cell.* 10:1021.
22. Kain, K.C., P.A. Orlandi, J.D. Haynes, B.K.L. Sim, and D.E. Lanar. 1993. Evidence for two-stage binding by the 175-kD erythrocyte binding antigen of *Plasmodium falciparum*. *J. Exp. Med.* 178:1497.
23. Mitchell, G.H., T.J. Hadley, F.W. Klotz, and L.H. Miller. 1986. Invasion of erythrocytes by *Plasmodium falciparum* malaria parasites. Evidence for receptor heterogeneity and two receptors. *Blood.* 67:1519.
24. Hadley, T.J., F.W. Klotz, G. Pasvol, J.D. Haynes, M.H. McGinniss, Y. Okubo, and L.H. Miller. 1987. *Flaciparum* malaria parasites invade erythrocytes that lack glycophorin A and B (MkMk). Strain differences indicate receptor heterogeneity and two pathways for invasion. *J. Clin. Invest.* 80:1190.
25. Perkins, M.E., and E.H. Holt. 1988. Erythrocyte receptor recognition varies in *Plasmodium falciparum* isolates. *Mol. Biochem. Parasitol.* 27:23.
26. Dolan, S.A., L.H. Miller, and T.E. Wellems. 1990. Evidence for a switching mechanism in the invasion of erythrocytes by *Plasmodium falciparum*. *J. Clin. Invest.* 86:618.
27. Fraser, G.R., E.R. Gilbert, and A.G. Motulski. 1966. Population genetic studies in the Congo III. Blood groups (ABO, MNSs, Rh, $Js^a$). *Am. J. Hum. Genet.* 18:546.
28. Unger, P., J.L. Procter, J.J. Moulds, D. Blanchard, M.C. Guizzo, L.A. McCall, J.P. Cartron,and W. Dahr. 1987. The Dantu erythrocyte phenotype of the NE variety. II. Serology, immunochemistry, genetics and frequency. *Blut.* 55:33.
29. Barnwell, J.W., M.E. Nichols, and P. Rubenstein. 1989. In vitro evaluation of the role of the Duffy blood group in erythrocyte invasion by *Plasmodium vivax*. *J. Exp. Med.* 169:1795.