MAJOR ARTICLE

# Functional and Immunological Characterization of a Duffy Binding–Like–γ Domain from *Plasmodium falciparum* Erythrocyte Membrane Protein–1 Expressed by a Placental Isolate

**Yu-Shan Chia,[1] Cyril Badaut,[2] Nicaise G. Tuikue Ndam,[3] Ayman Khattab,[1] Sebastien Igonet,[2] Nadine Fievet,[3] Graham A. Bentley,[2] Philippe Deloron,[3] and Mo-Quen Klinkert[1]**

[1]Department of Molecular Medicine, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany; [2]Unité d'Immunologie Structurale, Centre National de la Recherche Scientifique—Unité de Recherche Associée 2185, Département de Biologie Structurale and Chimie, Institut Pasteur, and [3]Institut de Recherche pour le Développement, UR010, Laboratoire de Parasitologie, Faculté des Sciences, Pharmaceutiques et Biologiques, Paris, France

**A recombinant Duffy binding–like (DBL)–γ domain from a previously identified placental isolate, 732, was expressed by use of the baculovirus/insect cell system and was purified in milligram quantities. The recombinant protein binds specifically to chondroitin sulfate A (CSA) and inhibits CSA binding by placental infected erythrocytes (IEs). Polyclonal antibodies raised against the domain recognized the surfaces of live IEs from CSA-adherent clinical placental isolates. These antibodies also abrogated the in vitro binding of IEs to CSA. The 732 DBL-3γ domain was specifically recognized by plasma from pregnant women but not by plasma from control subjects. In addition, the protein was, comparatively, significantly more reactive with plasma from women with infected placentas, strongly suggesting that the 732 DBL-3γ domain carries preferentially IE-expressed immunogenic epitopes. High levels of plasma antibodies to the recombinant domain were associated with reduced placental parasite density. This is the first report of a recombinant DBL-γ domain derived from a placental isolate that shows CSA-binding properties.**

The expression of *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP-1) on the surfaces of infected erythrocytes (IEs) is associated with distinct adhesive properties [1, 2]. The protein, which is encoded by *var* genes, binds cellular receptors such as CD36 [3], intercellular adhesion molecule–1 (ICAM-1) [4], and chondroitin sulfate A (CSA) [5]. PfEMP-1 variants with the CSA-binding phenotype appear to be constantly expressed by parasites infecting pregnant women but are less commonly expressed by parasites infecting children and nonpregnant adults. The inability of the mother to control parasites in the placenta probably contributes to the pathogenesis of pregnancy-associated malaria (PAM) [6].

Several *var* genes have been shown to fulfil at least 1 of the criteria necessary to qualify as a PAM-causing gene, such as up-regulation in CSA-binding parasites, more frequent transcription in placental parasites than in parasites from children, surface expression, CSA binding, induction of antibodies capable of blocking CSA binding, sex-specific and parity-dependent recognition, and domain/epitope conservation in field isolates [7]. The first domain implicated in PAM was a Duffy binding–like (DBL)–γ domain from the *var*1CSA gene of the FCR3CSA laboratory strain [8] and the CS2*var* gene of the ItGCS2 laboratory strain [9]. These induce blocking antibodies but are not expressed in placental isolates. The leading candidate gene, *var*2CSA, is up-regulated in CSA-binding parasites, but it lacks DBL-γ

Received 1 March 2005; accepted 25 April 2005; electronically published 25 August 2005.

Potential conflicts of interest: none reported.

Financial support: European Commission (grants QLK2-CT-2001-01302 and QLK2-CT-2002-01197); Fondation pour la Recherche Médicale; French Ministry of Education and Research (grant to N.G.T.N.).

Reprints or correspondence: Dr. Mo-Quen Klinkert, Bernhard Nocht Institute for Tropical Medicine, Bernhard-Nocht-Str. 74, 20359 Hamburg, Germany (mo.klinkert@bni-hamburg.de).

**The Journal of Infectious Diseases 2005; 192:1284–93**



0022-1899/2005/19207-0023$15.00

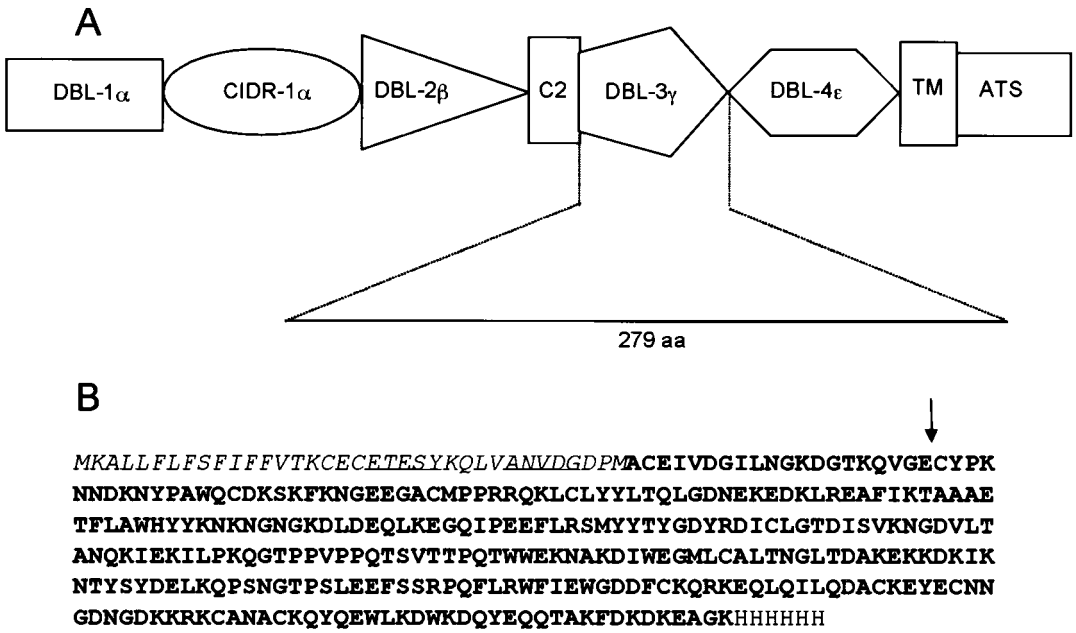


**Figure 1.** *A,* Domain organization of the full-length 732*var* gene. Starting from the Duffy binding–like (DBL)–3γ domain sequence, the full-length 732*var* sequence was obtained in 2 steps. The first step was polymerase chain reaction (PCR) amplification with a specific reverse primer that was designed on the basis of the most variable region of the DBL-3γ sequence and that, therefore, was unique to isolate 732 (it is not found in GenBank). The second primer was a degenerate primer designed on the basis of DBL-1α, which is present in all *var* genes. The second step was another PCR amplification with a primer designed on the basis of the obtained DBL-1α sequence and a degenerate primer designed on the basis of the conserved acidic terminal sequence (ATS) region. *B,* Deduced amino acid sequence of the 732 DBL-3γ domain. The amino acids representing the merozoite surface protein 1 signal are shown in italics, and the expressed DBL-3γ domain (indicated by the arrows) and flanking amino acids are shown in boldface and are followed by the histidine tag. The underscored sequences represent the N-terminal sequencing of 2 cleavage sites of the expressed 732 DBL-3γ domain in insect cells. C2, constant 2; CIDR, cysteine-rich interdomain region; TM, transmembrane.

domains, and surface-expressed protein remains to be demonstrated [10].

Five distinct DBL-γ domains that share 39%–44% amino acid sequence identity, collectively termed "*var*PAM DBL-γ domain types," were previously characterized from placental isolates and were shown to bind to CSA when expressed on the surfaces of COS cells [11, 12]. These domains exhibit 44%–50% and 37%–44% identity with the DBL-γ domains from *var*1CSA and CS2*var*, respectively. The 720/734*var*PAM gene is very similar to the *var*1-like 3D7*chr5var* gene, which is commonly found in placental isolates [13]. The amino acid sequences in their DBL-γ domains are 82% identical, whereas the other *var*PAM DBL-γ domains, compared with that of 3D7*chr5var*, show only 35%–47% identity. On the basis of the overall lower homology and its distinct domain organization, 732*var* was chosen for further study. Insect cell–produced 732 DBL-3γ domain was analyzed functionally and immunologically, as a first step toward understanding the structural requirements for CSA interaction and to investigate the contribution of these genotypes to the pathogenesis of PAM.

## PARTICIPANTS, MATERIALS, AND METHODS

***Cell lines.*** Insect cells (*Spodoptera frugiperda* 9 [Sf9]) were maintained at 27°C in TC-100 medium (Invitrogen) containing 50 μg/mL gentamicine (Invitrogen), 2 g/mL glucose, yeastolate (Invitrogen), and 5% fetal calf serum (FCS; Eurobio). High Five (HF) cells (Invitrogen) were grown in Insect XPress medium (Cambrex), also at 27°C. CHO cells were grown in Ham's medium (PAA) supplemented with 10% FCS (PAA), 2 mmol/L glutamine (PAA), and 100× penicillin-streptamycin (PAA). Wild-type CHO-K1 cells express glycosaminoglycans (GAGs) on the cell surface; CHO pgsA-745 cells are defective with respect to xylosyltransferase and do not express GAGs. CHO CD36 and CHO ICAM-1 are stable transfectants thereof, expressing the corresponding receptors on a GAG-free background (CHO transfectants were gifts from T. Staalsoe, Copenhagen University Hospital).

***Construction, expression, and purification of the recombinant 732 DBL-3γ domain.*** The 732 DBL-3γ domain sequence (GenBank accession no. AF334807) was cloned from a Cameroonian placental isolate [12], and full-length 732*var* (Gen-

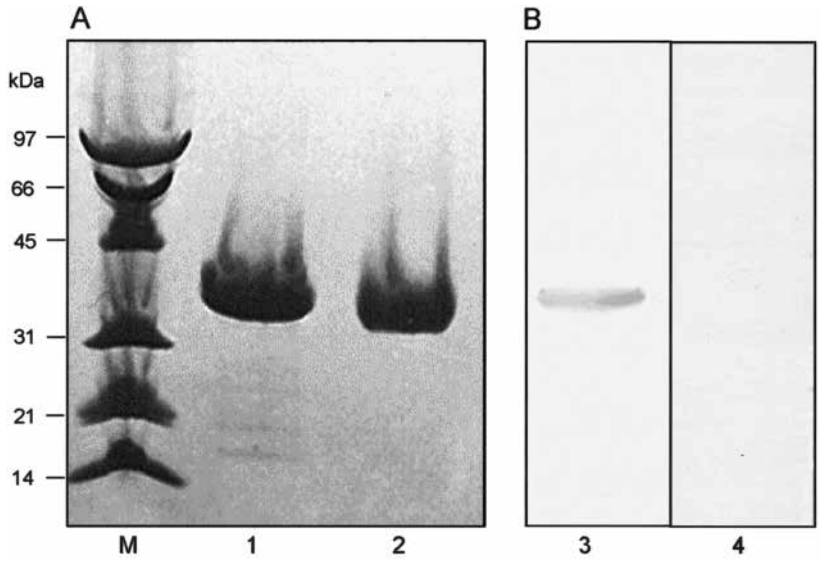


**Figure 2.** Analysis of the recombinant 732 Duffy binding–like (DBL)–3γ domain. *A,* Migration of purified 732 DBL-3γ domain is as expected for a protein with a molecular mass of 38.3 kDa. The 732 DBL-3γ domain was separated on a polyacrylamide gel and was stained with Coomassie blue. Under reducing conditions, in addition to the major band, 2 weak bands are seen in the gel *(lane 1);* however, under nonreducing conditions, only a single band is observed *(lane 2). B,* Western blot analysis. A pool of human plasma obtained from 10 randomly selected multiparous women recognized the 732 DBL-3γ domain *(lane 3),* but the protein was not reactive with a pool of human plasma obtained from 3 exposed men living in the same area *(lane 4).* Molecular mass markers (M) are indicated in kilodaltons.

Bank accession no. AY679117) was obtained by polymerase chain reaction (PCR) with unique and degenerate primers [14]. Arginine residues at potential N-glycosylation sites (N156 and N214) were mutated to glutamine by site-directed mutagenesis (Quikchange; Stratagene). The mutated gene fused to the signal sequence plus the 17 N-terminal residues of merozoite surface protein (MSP) 1 from *P. vivax,* and a C-terminal hexahistidine tag (gift from S. Longacre; Institut Pasteur) was ligated into the pBlueBac4.5 Topo plasmid (Invitrogen). The resultant construct was cotransfected with Bac-N-Blue DNA (Invitrogen) into Sf9 cells, and the recombinant viral clones were confirmed by PCR.

HF cells were used for protein expression. The XPress medium, into which the recombinant protein was secreted, was harvested and dialyzed 3 times (8–14 h/step) against 20 mmol/L Tris (pH 7.5) and 500 mmol/L NaCl. Purification steps were performed at 4°C as rapidly as possible, to minimize protein degradation. After incubation with Talon gel (Clontech; Becton Dickinson), the protein was eluted with an imidazole gradient (0–200 mmol/L), and the fractions were pooled and concentrated in a YM-10 Centricon column (Amicon; Millipore) and were further purified by gel filtration chromatography (16/60 Superdex 200; Amersham Pharmacia). The recombinant product was verified by N-terminal sequencing.

***Antibody production.*** One hundred micrograms of 732 DBL-3γ domain in 300 μL of phosphate buffer suspended with Freund's complete adjuvant (Sigma) was used for immunization. Chicken antibody was used to overcome nonspecific binding of mouse or rat antibodies to placental isolates. IgY, the functional equivalent of mammalian IgG, was purified from egg yolk by polyethylene glycol precipitation [15, 16]. Antibody specificity was tested by immunoblot with goat anti–chicken IgY (diluted 1:15,000; Sigma).

***Affinity of the recombinant 732 DBL-3γ domain for CSA on CHO cells.*** CHO cells were seeded at a concentration of $3 \times 10^5$ cells/mL on coverslips, were fixed with 2% paraformaldehyde in PBS for 30 min at room temperature (RT), and were washed and blocked with 3% bovine serum albumin (BSA) in PBS (BSA-PBS) for 30 min at RT. Cells were incubated with 0.5 μmol/L of recombinant protein (732 DBL-3γ or rifin50) in BSA-PBS for 40 min at RT. Protein binding was detected by use of primary antibody (1:500) and then Alexa-Fluor 594–conjugated secondary antibody (1:1000; Molecular Probes), each incubated for 30 min at 4°C. CSA, CSB, and CSC (100 μg/mL each) were used to demonstrate specificity. Cell nuclei were visualized by use of 5 μg/mL 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI; Roth). The cells were mounted in MOWIOL 4-88 (Calbiochem) and were observed under 20× magnification (Leitz DMRB; Leica).

***Parasite collection and cultivation.*** Experiments were conducted after informed consent was received from participants, in accordance with the human experimentation guidelines of the institutions involved; the study was approved by the ethics committees of the institutions. Placental parasites of Gabonese origin were extracted from blood collected into EDTA mono-

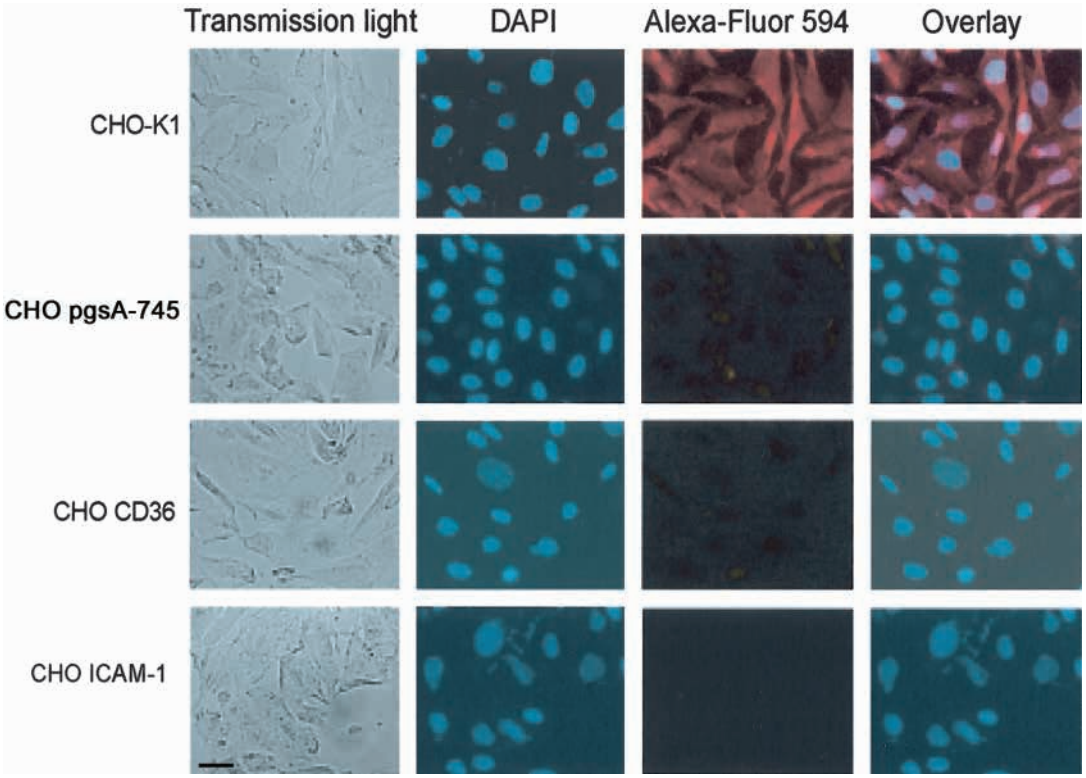


**Figure 3.** Binding of the 732 Duffy binding–like (DBL)–3γ domain to chondroitin sulfate A (CSA) expressed on CHO cells. Normal light images *(first column)* and 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI)–stained cell nuclei images *(second column)* allow easy visualization of the cells. Binding of the recombinant protein to CHO-K1 cells expressing CSA *(first row)* is detected with the help of specific primary antibodies and secondary antibodies coupled to the Alexa-Fluor 594 fluorophore *(third column).* No recognition is observed for the other cell lines (*second, third,* and *fourth rows*). An overlay *(fourth column)* shows the colocalization of the recombinant protein with the cells. The scale bar represents 90 μm. ICAM, intercellular adhesion molecule.

vettes without the use of needles from incisions made in the placenta (maternal side) [17]. Parasites of Senegalese origin were collected by flushing the placentas of O blood group women with 0.1% sodium heparin in PBS [18]. Placental thick-blood smears were analyzed microscopically. Parasites were used directly in flow cytometry or were frozen after 2 cycles for further culture. *P. falciparum* isolates were grown at 37°C at 5% hematocrit in RPMI 1640 medium containing 200 mmol/L glutamine (PAA), 10% human serum ($AB^+$; PAA), and 5% Albumax II (Gibco). The CSA-adherent population of the isolates was enriched by use of CSA immobilized on plastic petri dishes, as described elsewhere [5]. Midtrophozoite stage cultures of Gb337, VIP43, Gb03, and FCR3 were harvested (parasitemia of >2%) and were enriched in MACS columns (Miltenyi Biotec) for further downstream applications.

***Static in vitro inhibition assay.*** CSA was immobilized on plates, and IE binding was inhibited [19]. Inhibitory activities of polyclonal antibody or human plasma (diluted at concentrations of 1:25, 1:50, 1:100, and 1:200) were determined by preincubating IEs (5% hematocrit; 2% parasitemia) for 1 h at 37°C with antibody. BSA (5%) and CSC (100 μg/mL each in PBS) served as controls for CSA specificity. To investigate the effect of the recombinant protein, the above assay was modified. IEs ($3 \times 10^7$) were pipetted into a 1-cm circle that was drawn by use of a DakoCytomation pen (Dako Denmark) and that contained 100 μL of 732 DBL-3γ domain (1–20 μmol/L). The parasite-protein mix was left for 1 h at RT. Rifin50 protein, which has no known role in CSA binding, was the control for protein specificity. CSA binding is expressed as percentages, comparing the number of bound IEs per millimeter in the test sample (with protein or antibody) with the number of bound IEs per millimeter in the reference sample (with neither protein nor antibody).

***Immunofluorescence assay.*** The CSA-enriched and the isogenic non–CSA-enriched strains were tested in parallel. Nuclear DNA staining was visualized by incubation with DAPI (4 μg/mL) for 30 min at RT. IEs were stained with anti–732 DBL-3γ domain antibodies (1:100) and were counterstained with rabbit anti–chicken IgY conjugated to Alexa-Fluor 488 (6 μg/mL; Molecular Probes); each step was for 30 min at 4°C. In control experiments, IEs were treated with 0.1 mg/mL trypsin at 37°C for 10 min before staining. Cells were resuspended with 10 μL of 2% FCS-PBS and were counted under 40× magnification (Axioskop 2 plus; Zeiss).

***Plasma sample collection and patient data.*** The pregnant-

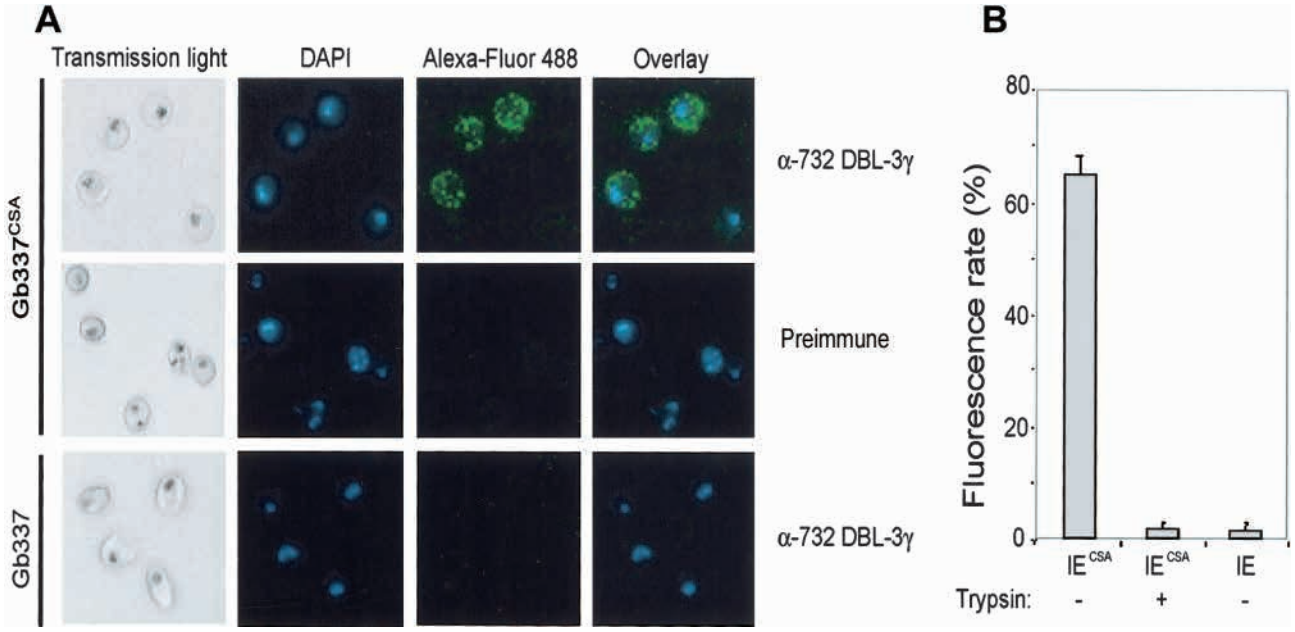


**Figure 4.** *A,* Recognition of surfaces of live infected erythrocytes (IEs). Indirect immunofluorescence analysis was conducted on cells infected with Gb337 and Gb337$^{\text{chondroitin sulfate A (CSA)}}$, by use of Duffy binding–like (DBL)–3γ domain antiserum. IEs were first observed under normal transmission light *(first column),* and DNA from IEs was visualized by use of 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI; *second column*). Binding of DBL-3γ domain–specific antibodies to CSA-selected cells was observed with Alexa-Fluor 488–labeled anti–chicken IgY *(third column, first row),* whereas no binding was observed with preimmune serum *(third column, second row)* or when the cells were not selected for CSA binding *(third column, third row).* Recognition of DBL-3γ domain–specific antibodies was confirmed by overlays of DAPI and Alexa-Fluor 488 images *(fourth column). B,* Determination of fluorescence rates for Gb337$^{\text{CSA}}$. The florescence rate was calculated as (no. of fluorescent cells/total no. of late trophozoites) × 100% [25]. The rates of CSA-selected IE (IE$^{\text{CSA}}$) and nonselected IE in the presence of the antibody with (+) and without (−) trypsin treatment were calculated on the basis of 3 independent assays; SDs are shown as vertical lines above the bars.

women cohort study was conducted in Thiadiaye, Senegal, where malaria is seasonally transmitted (15 infectious mosquito bites/person/year) during the rainy season, from September 2001 to May 2002. The cohort comprised 257 women, whose mean ± SD age was 26.1 ± 6.5 years. In the cohort were 60 primiparous, 49 secundiparous, and 148 multiparous women; 66 and 41 women presented with *P. falciparum* parasites in the placental blood and the peripheral blood, respectively, when examined microscopically. Samples were collected at delivery, were coded, and were tested in a blinded fashion. Control plasma samples were collected from 17 Senegalese adults living in the same area as the pregnant women (9 men and 8 nulligravid women) and from 55 nonexposed European adults.

***ELISAs.*** ELISAs were used to analyze human responses to the purified recombinant 732 DBL-3γ domain [20]. Plates were coated with 2.5 μg/mL concentrations of antigen. Wells were incubated with 100 μL of human plasma (1:200), followed by horseradish peroxidase–conjugated anti–human IgG (1:15,000). The optical density (OD) was obtained by subtracting the average OD of duplicate wells from that of the corresponding blank wells. Values were converted into arbitrary units (AUs), as follows [21]:

$$\text{AU} = 100 \times \left[\frac{\ln(\text{OD test sample}) - \ln(\text{OD negative control})}{\ln(\text{OD positive control}) - \ln(\text{OD negative control})}\right].$$

Participants with an antibody response were defined as those with an AU value >2 SDs above the mean AU value of the 55 negative control plasma samples (i.e., those from the nonexposed European adults).

***Flow cytometry.*** Antibodies (IgG) to variant surface antigens (VSAs) were measured by flow cytometry [22] with 7 isolates. IEs from the placentas of blood type O women ($10^5$ IEs/μL; 40% parasitemia; 2.5% hematocrit) were labeled with ethidium bromide (0.1 mg/mL) in the presence of test plasma samples (1:20) in 96-well round-bottom plates. Cells were sequentially incubated with goat anti–human IgG (1:250; Dako) and fluorescein isothiocyanate–conjugated rabbit anti–goat IgG (1:100; Dako) and were analyzed by use of a FACScalibur flow cytometer (Bec-

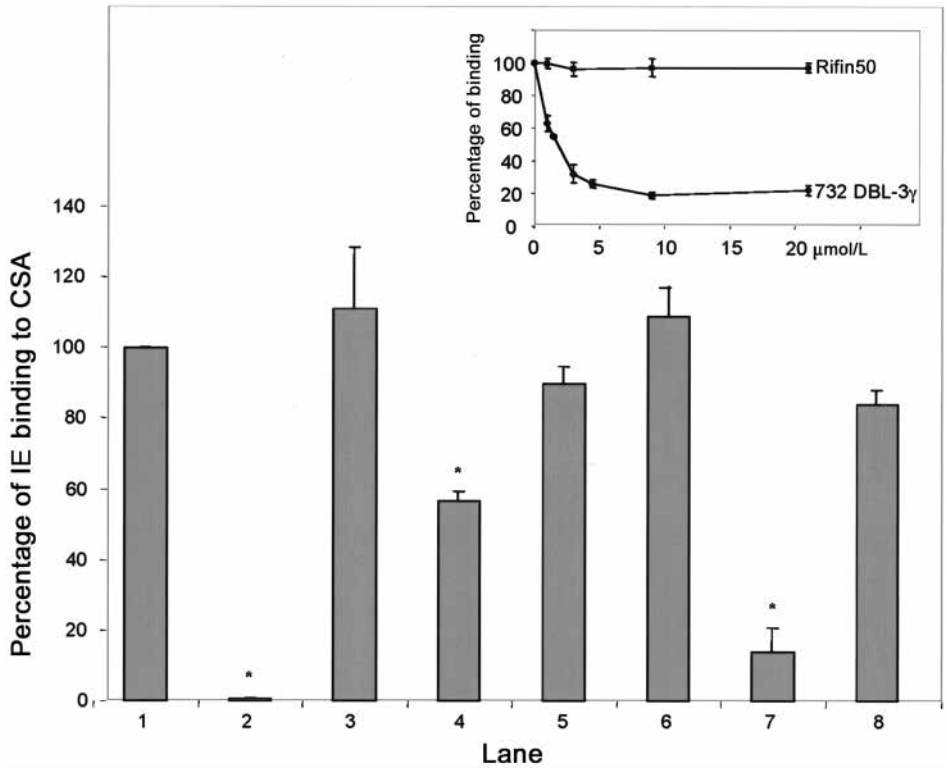


**Figure 5.** Effect of antibodies to the Duffy binding–like (DBL)–3γ domain on chondroitin sulfate A (CSA) binding of infected erythrocytes (IEs). IEs were counted under 40× magnification in 10 randomly selected fields, to give the total no. of bound IEs per square millimeter, from which the background number of IEs (i.e., those that bound to bovine serum albumin–coated controls) was subtracted. CSA binding is expressed as percentages, comparing the no. of bound IEs per millimeter in the test sample (with protein or antibody) to the number of bound IEs per millimeter in the reference sample (with neither protein nor antibody). The results shown here are for the parasite isolate VIP43$^{CSA}$ and serum samples used at a 1:100 dilution. The total number of bound IEs per square millimeter in the reference sample after background subtraction is given as 100% *(lane 1).* IEs were pre-incubated with CSA (100 μg/mL; *lane 2*), CSC (100 μg/mL; *lane 3*), polyclonal antibodies to the 732 DBL-3γ domain *(lane 4),* antibodies to recombinant rifin50 *(lane 5),* preimmune serum *(lane 6),* plasma from multiparous women *(lane 7),* and plasma from men living in the same area as the multiparous women *(lane 8).* The asterisks above the bars indicate significant binding inhibition, compared with the reference sample. The inset shows the results when the 732 DBL-3γ domain was incubated with IEs at the concentrations indicated. Rifin50 was included in the assay as a negative control at similar concentrations. Three independent binding-inhibition experiments were conducted; vertical lines denote the SDs.

ton Dickinson). For each sample, the level of IgG that recognized VSAs was expressed as the median fluorescence intensity (MFI) of the labeled IE, gated according to ethidium bromide fluorescence. For each isolate, the threshold for positivity was defined as 2 SDs above the mean MFI obtained with plasma samples from 30 nulligravid women from Senegal.

***Statistical analysis.*** Differences between groups were tested by Student's unpaired *t* test. Correlations were examined by the Pearson test. The relationship between placental parasite density (after log transformation) and antibody levels was assessed by a multiple linear regression model that included parity. The significance limit was $P < .05$. Statistical analysis was performed by use of Statview (version 5.0; SAS Institute).

## RESULTS

***Comparison of the 732var gene with known 3D7var genes.*** The domain organization of the 732*var* gene (figure 1*A*) is close to that of the type 15 *var* gene [23], differing only by an additional DBL-4ε domain before the transmembrane region of 732*var*. The 732 DBL-3γ gene shares 41%–51% amino acid sequence identity with annotated 3D7 DBL-γ domains (PlasmoDB). This is comparable to the observed identity between the 5 *var*PAM genes we have analyzed (39%–55%) [11, 12], which were cloned with degenerate UNIEBP5′ and UNIEBP3′ primers [24]. These partial *var*PAM DBL-γ genes covered the domain sufficiently to retain CSA-binding properties when expressed on the surfaces of CHO cells, showing that the binding site is contained, at least in significant part, within this region [12].

***Expression and purification of the recombinant 732 DBL-3γ domain.*** To study the antigenicity and functionality of the DBL-3γ domain of the 732*var* gene, it was expressed by use of the baculovirus/insect cell system. The recombinant protein covered the entire DBL-3γ domain of 279 aa, as well as 20 residues upstream and 8 residues downstream of the sequence

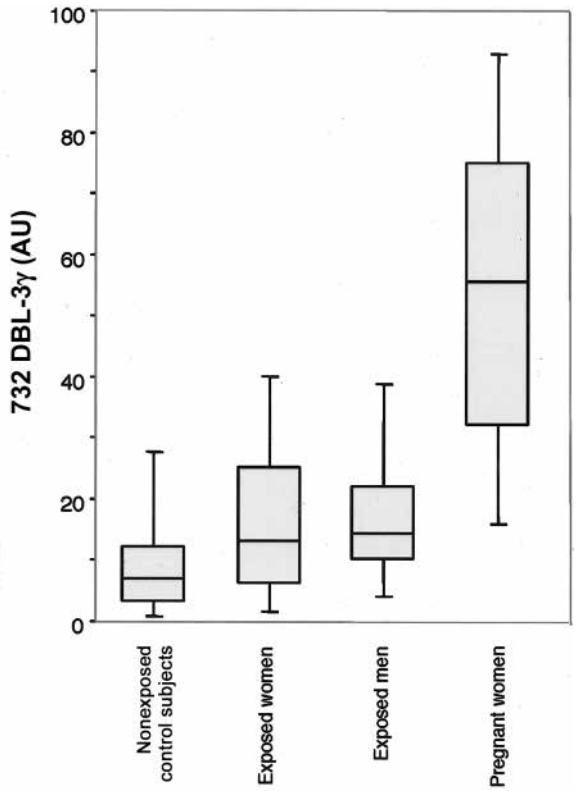


**Figure 6.** Assessment, by ELISA, of levels of plasma IgG that reacted with the recombinant 732 Duffy binding–like (DBL)–3γ domain. The graph shows levels of IgG, expressed as arbitrary units (AUs), in control groups of nonexposed European adults, malaria-exposed nulligravid women, malaria-exposed men, and in pregnant women. The horizontal lines indicate the medians, the boxes indicate the interquartile ranges, and the vertical lines indicate the 10%–90% ranges. Recognition of the 732 DBL-3γ domain by plasma from the pregnant women was significantly higher than that by plasma from each of the 3 control groups ($P < .0001$, Student's *t* test).

(figure 1*A*). The DBL-3γ domain sequence and the flanking amino acids are boldfaced in figure 1*B*. N-terminal sequencing of the recombinant product gave 2 sequences, ETESY and ANVDG (underscored in figure 1*B*), indicating that the MSP1 signal sequence (shown in italics in figure 1*B*), cloned upstream of the DBL-3γ domain, was cleaved in 2 places.

The purified protein was observed in a denaturing gel as 1 major band and 2 very weak bands in the presence of reducing agent; however, only 1 band was observed under nonreducing conditions (figure 2*A*). The weak bands indicate that the protein was slightly cleaved but remained covalently linked by cysteine bridges in the nonreduced form. Yields of up to 7 mg/L of culture medium were obtained.

***Affinity of the recombinant 732 DBL-3γ domain for CSA.*** The conformational integrity of the recombinant 732 DBL-γ domain was examined by assessment of its ability to bind to CHO-K1 cells expressing CSA. The protein adhered to CHO-K1 cells (figure 3, top row); by contrast, it showed virtually no binding either to CHO pgsA-745 cells (second row), to CHO cells expressing CD36 (third row), or to CHO cells expressing ICAM-1 (fourth row). Protein-CSA interaction was competed out by CSA but not by CSB or CSC (data not shown). When an unrelated soluble control protein (rifin50) was used, no binding was observed (data not shown). Therefore, the recombinant 732 DBL-3γ domain interacts specifically with CSA.

***Characterization of antibodies raised against the recombinant 732 DBL-3γ domain.*** As was expected, the chicken polyclonal antibody recognized the recombinant immunogen, whereas preimmune antibody showed no reactivity (data not shown). Surface staining of heterologous CSA-adherent parasites (labeled "$Gb337^{CSA}$" in figure 4*A*), but not the corresponding nonadherent populations, was observed (figure 4*A*). This pattern underscores the panreactive property of the polyclonal antibodies. CSA-adherent IEs (labeled "$IE^{CSA}$" in figure 4*B*) showed a fluorescence rate of ~65%; no reactivity was observed in non–CSA adherent and trypsin-treated CSA-adherent IEs (figure 4*B*).

***Inhibition of binding of IEs to CSA by the recombinant 732 DBL-3γ domain and the antibodies raised against it.*** Both the recombinant 732 DBL-3γ domain and the antibodies raised against it showed a concentration-dependent CSA-binding inhibition of all tested isolates. A representative graph of the isolate $VIP43^{CSA}$ is shown in figure 5. The mean number of bound IEs per square millimeter after background subtraction in the reference sample was used as a reference for 100% binding. In this representation, the mean number of bound IEs was 953 ± 73; however, 953 was given as 100% (lane 1). As expected, CSA completely inhibited binding (lane 2), in contrast to CSC (lane 3). Polyclonal antibodies to the 732 DBL-3γ domain had an inhibitory activity of 45% ($P < .05$) (lane 4). This incomplete inhibition could have been due to the expression

**Table 1. Measurement of levels of antibodies in plasma to the recombinant Duffy binding–like (DBL)–3γ domain, by ELISA.**

| Group | Antibody level, mean ± SD, AUs[a] |
|---|---|
| Control subjects ($n = 70$) | 12.22 ± 13.91 |
| Pregnant women ($n = 257$) | 53.52 ± 28.14[b] |
| Placenta infected ($n = 66$) | 60.25 ± 27.74 |
| Placenta not infected ($n = 178$) | 51.26 ± 28.42[c] |
| Peripheral blood infected ($n = 41$) | 58.39 ± 29.01 |
| Peripheral blood not infected ($n = 214$) | 52.38 ± 27.92 |

**NOTE.** Plasma samples were collected from pregnant women in Thiadiaye, Senegal; placenta slides were not available for 13 women, and peripheral blood slides were not available for 2 women.

[a] See Participants, Materials, and Methods for an explication of arbitrary units (AUs).

[b] Significantly different from control subjects ($P < .0001$, Student's *t* test).

[c] Significantly different from the placenta-infected pregnant women ($P < .05$, Student's *t* test).

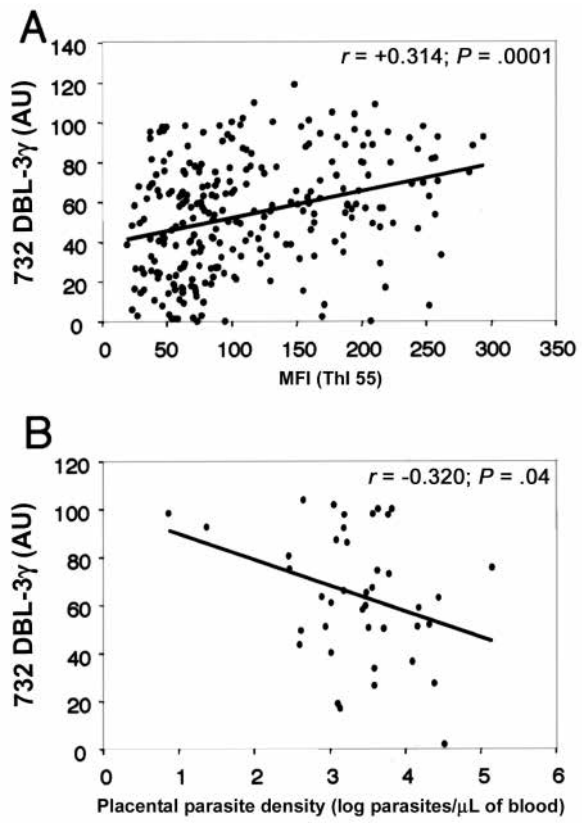


**Figure 7.** Correlation between levels of antibodies to the recombinant Duffy binding–like (DBL)–3γ domain and (1) variant surface antigen (VSA) recognition *(A)* and (2) placental parasite density at delivery *(B)*. Antibody levels are expressed as arbitrary units (AUs). VSA recognition was determined by flow cytometry; Pearson regression analysis yielded $r = 0.314$ and $P = .0001$. Antibody levels correlated negatively with parasite density in infected placentas ($r = -0.320$; $P = .04$).

of another CSA-binding ligand in the heterogeneous IE population, or the recognized epitopes may only partially overlap with the binding site. Antibodies to recombinant rifin50 (lane 5) and preimmune serum (lane 6), used as controls, produced negligible binding inhibition. Plasma from multiparous women (lane 7) strongly inhibited binding, but plasma from men living in the same area did not (lane 8).

As can be seen in the figure 5 inset, the 732 DBL-3γ domain inhibited IE binding in a dose-dependent manner. Maximal inhibition (80%) was obtained at 5 μmol/L. Binding was not affected by rifin50, demonstrating the specificity of the interaction between the 732 DBL-3γ domain and CSA.

***Recognition of the recombinant 732 DBL-3γ domain by human immune plasma.*** In immunoblots, the recombinant 732 DBL-3γ domain was recognized by a pool of plasma obtained from pregnant women and was not recognized by a pool of plasma obtained from men (figure 2*B*). This seropositivity was further characterized by ELISA, to evaluate antibody responses during natural malaria.

Plasma samples from the pregnant women showed sex-specific recognition, with a mean level that was significantly higher than those for plasma samples from the control groups of nonexposed European adults, malaria-exposed nulligravid women, and malaria-exposed men ($P < .0001$) (figure 6). To examine whether antibody recognition was placental-infection specific, the pregnant women were categorized by whether their placentas were infected ($n = 66$) or not infected ($n = 178$) at delivery. Levels of antibodies to the recombinant protein in the plasma samples from the women with infected placentas were higher than those in the plasma samples from the women with noninfected placentas (table 1). This recognition appears to be specific to placental infections only, because no such difference was observed when the women were categorized by whether their peripheral blood was infected.

Although levels of antibodies to the 732 DBL-3γ domain, as determined by ELISA, did not correlate with parity ($r < -0.065$; $P > .30$), they did correlate with levels of antibodies that recognized VSAs expressed by 7 different fresh placental isolates ($r$ values between +0.217 and +0.338; $P < .005$, for all), as determined by flow cytometry. A representative plot of such a correlation is shown for placental isolate Thi55 in figure 7*A*.

***Control of parasite density in the placenta.*** The most significant finding of the present study is that the levels of antibodies to the recombinant 732 DBL-3γ domain correlated negatively with parasite density in the placenta ($r = -0.320$; $P = .04$) (figure 7*B*). This correlation was not present when parasites in the peripheral blood were analyzed. To investigate the combined influence of placental parasite density and parity on antibody levels, these 2 variables were included in a multiple linear regression model. Parity was included because, although other studies have shown a strong association between this parameter and PAM, our study indicated only a borderline correlation with antibody levels. This analysis confirmed that only placental parasite density was correlated with the levels of antibodies to the recombinant protein ($P = .04$).

## DISCUSSION

The first understanding of PAM came from the observation that placental isolates bound to CSA [5] and that the parasite ligand implicated in CSA interaction belonged to the DBL-γ domain subtype [8, 9]. Further work on DBL-γ domains from placental isolates supported this observation [12]. Interestingly, the sequences for these domains were also conserved in placental parasites that had been isolated several years apart and that were from different geographic locations [11].

The cloned and purified DBL-3γ domain from the 732*var* gene has been shown here to be correctly folded and functional, on the basis of its ability to bind to CSA expressed on CHO cells. Antibodies raised against the recombinant protein stained the surfaces of placental isolates and laboratory strains that had

been selected for their CSA-binding properties. Therefore, CSA-binding parasites probably display surface antigens that share common conserved epitopes with the 732 DBL-3γ domain. Furthermore, not only was the recombinant protein able to compete with IEs for CSA binding, but 732 DBL-3γ domain–specific antibodies were also capable of inhibiting the binding of IEs to CSA. Thus, we conclude that the recombinant protein expressed in insect cells resembles the domain expressed on the surfaces of IEs and possesses a functionally active CSA-binding site.

By flow cytometry, antibodies in plasma from pregnant women recognized CSA-adherent IEs in a sex- and parity-dependent manner. These responses were measured with human-plasma antibodies that recognized the entire repertoire of VSAs present on IEs. Recognition correlated with protection of mothers against PAM [6] as well as other against clinical consequences of PAM, such as maternal anemia, low-birth-weight babies, and prematurity [26–28]. To obtain further insight into VSA reactivities, the recognition of individual domains of PfEMP-1 has been investigated. Domains such as DBL-1α, cysteine-rich interdomain region (CIDR)–α, and DBL-2β (cloned from the CSA-selected parasite line 2O2CSA) do not show sex- or parity-specific antibody recognition, which is in line with observations that do not implicate these domains in PAM [29].

Our study extends investigations that were based on a single PfEMP-1 domain and show, for the first time, a link with immune control of placental parasitemia. We have demonstrated that antibodies in human plasma that react with the 732 DBL-3γ domain are correlated with decreased parasite density in malarial parasite–infected placentas; moreover, the protein domain exhibits the characteristics of a pregnancy-specific antigen, being specifically recognized by plasma from pregnant women and, in particular, by plasma from women with infected placentas. The levels of antibodies recognizing the antigen by ELISA correlated with the levels of antibodies reacting with VSAs expressed by 7 different fresh placental isolates. On the basis of these observations, we suggest that antibodies in human plasma that recognize the 732 DBL-3γ domain form a subset of the antibodies against IE that could affect placental parasite replication. Involvement of these antibodies in the control of placental parasitemia raises hope for a DBL-γ domain–based vaccine against PAM.

To date, all *var* genes that have been implicated in PAM belong to different *var* gene types with different domain orders. The recombinantly expressed DBL-5ε domain of the unique *var*2CSA gene was recently found to be recognized by human plasma in a sex- and parity-dependent manner, and the level of antibodies to the DBL-5ε domain was reported to correlate with birth weight [30]. An as-yet unidentified 13.2–14.0-kb transcript [31, 32] and 4 novel variants identified by proteomics analysis [33] have also been published.

CSA-binding function has now been attributed to several DBL domains, such as DBL-γ, -ε, and -X, as well as to CIDR-α. Gamain et al. have shown that a 67-aa segment located in the C-terminal region of the FCR3CSA DBL-3γ domain binds to CSA; accordingly, they have suggested that it contains the minimal CSA-binding motif [34]. This region has significant sequence identity with the equivalent segments of the IT-R29*var* and 3D7*chr5var* DBL-γ domains, which also bind to CSA. Furthermore, the *var*2CSA DBL-2X and −6ε domains also bind to CSA, and regions on these domains show some homology to the segment [35]. However, this minimal binding motif is not found in the DBL-5ε domain, which is associated with sex- and parity-specific recognition [30]. The significance of this stretch of amino acids remains unclear, because such stretches are also present on DBL-γ domains that do not bind to CSA [34]. The 5 *var*PAM DBL-γ domains from placental isolates that we had examined were truncated and lacked the apparent CSA-binding motif but were, nonetheless, capable of binding to CSA. These results would indicate the presence of additional CSA-binding sites within the central two-thirds of the DBL-γ domain. The 732 DBL-3γ domain is the only *var*PAM CSA-binding domain that we have completely sequenced to date; however, sequence identity with the 67-aa CSA-binding segment from FCR3CSA DBL-3γ is only 44%, which is not significantly different from the 42% identity for the whole of the domain. The structural correlate of CSA binding may, therefore, not be unique, and there is a clear need to define the structural characteristics of *var*PAM DBL-γ domains and their interaction with CSA ligands. This will provide better insight into their role in PAM and their capacity to induce a protective immune response.

## Acknowledgments

We thank all of the individuals who participated in this study; Allioune Gaye (Centre de Santé Roi Baudouin, Guediawaye) and Sokhna Sow (Hôpital de Thiadiaye), for their support in sample collection; and Jean Yves Le Hesran (Institut de Recherche pour le Développement), for the follow-up of pregnant women.

## References

1. Chen Q, Fernandez V, Sundstrom A, et al. Developmental selection of *var* gene expression in *Plasmodium falciparum*. Nature **1998**; 394:392–5.
2. Scherf A, Hernandez-Rivas R, Buffet P, et al. Antigenic variation in malaria: in situ switching, relaxed and mutually exclusive transcription of *var* genes during intra-erythrocytic development in *Plasmodium falciparum*. EMBO J **1998**; 17:5418–26.
3. Ockenhouse CF, Tandon NN, Magowan C, Jamieson GA, Chulay JD. Identification of a platelet membrane glycoprotein as a falciparum malaria sequestration receptor. Science **1989**; 243:1469–71.
4. Berendt AR, Simmons DL, Tansey J, Newbold CI, Marsh K. Intercellular adhesion molecule-1 is an endothelial cell adhesion receptor for *Plasmodium falciparum*. Nature **1989**; 341:57–9.
5. Rogerson SJ, Chaiyaroj SC, Ng K, Reeder JC, Brown GV. Chondroitin sulfate A is a cell surface receptor for *Plasmodium falciparum*-infected erythrocytes. J Exp Med **1995**; 182:15–20.

6. Fried M, Nosten F, Brockman A, Brabin BJ, Duffy PE. Maternal antibodies block malaria. Nature **1998**; 395:851–2.
7. Rowe JA, Kyes SA. The role of *Plasmodium falciparum var* genes in malaria in pregnancy. Mol Microbiol **2004**; 53:1011–9.
8. Buffet PA, Gamain B, Scheidig C, et al. *Plasmodium falciparum* domain mediating adhesion to chondroitin sulfate A: a receptor for human placental infection. Proc Natl Acad Sci USA **1999**; 96:12743–8.
9. Reeder JC, Cowman AF, Davern KM, et al. The adhesion of *Plasmodium falciparum*-infected erythrocytes to chondroitin sulfate A is mediated by *P. falciparum* erythrocyte membrane protein 1. Proc Natl Acad Sci USA **1999**; 96:5198–202.
10. Salanti A, Staalsoe T, Lavstsen T, et al. Selective upregulation of a single distinctly structured *var* gene in chondroitin sulphate A-adhering *Plasmodium falciparum* involved in pregnancy-associated malaria. Mol Microbiol **2003**; 49:179–91.
11. Khattab A, Kremsner PG, Klinkert MQ. Common surface-antigen *var* genes of limited diversity expressed by *Plasmodium falciparum* placental isolates separated by time and space. J Infect Dis **2003**; 187:477–83.
12. Khattab A, Kun J, Deloron P, Kremsner PG, Klinkert MQ. Variants of *Plasmodium falciparum* erythrocyte membrane protein 1 expressed by different placental parasites are closely related and adhere to chondroitin sulfate A. J Infect Dis **2001**; 183:1165–9.
13. Rowe JA, Kyes SA, Rogerson SJ, Babiker HA, Raza A. Identification of a conserved *Plasmodium falciparum var* gene implicated in malaria in pregnancy. J Infect Dis **2002**; 185:1207–11.
14. Khattab A. The molecular basis of pathogenesis of *Plasmodium falciparum* malaria during pregnancy [PhD thesis]. Tübingen, Germany: Eberhard-Karls-Universität, **2003**.
15. Polson A, von Wechmar MB, van Regenmortel MH. Isolation of viral IgY antibodies from yolks of immunized hens. Immunol Commun **1980**; 9:475–93.
16. Polson A, von Wechmar MB, Fazakerley G. Antibodies to proteins from yolk of immunized hens. Immunol Commun **1980**; 9:495–514.
17. Mayengue PI, Rieth H, Khattab A, et al. Submicroscopic *Plasmodium falciparum* infections and multiplicity of infection in matched peripheral, placental and umbilical cord blood samples from Gabonese women. Trop Med Int Health **2004**; 9:949–58.
18. Moore JM, Nahlen B, Ofulla AV, et al. A simple perfusion technique for isolation of maternal intervillous blood mononuclear cells from human placentae. J Immunol Methods **1997**; 209:93–104.
19. Khattab A, Reinhardt C, Staalsoe T, et al. Analysis of IgG with specificity for variant surface antigens expressed by placental *Plasmodium falciparum* isolates. Malar J **2004**; 3:21.
20. Abdel-Latif MS, Khattab A, Lindenthal C, Kremsner PG, Klinkert MQ. Recognition of variant rifin antigens by human antibodies induced during natural *Plasmodium falciparum* infections. Infect Immun **2002**; 70:7013–21.
21. Rasheed FN, Bulmer JN, De Francisco A, et al. Relationships between maternal malaria and malarial immune responses in mothers and neonates. Parasite Immunol **1995**; 17:1–10.
22. Staalsoe T, Giha HA, Dodoo D, Theander TG, Hviid L. Detection of antibodies to variant antigens on *Plasmodium falciparum*-infected erythrocytes by flow cytometry. Cytometry **1999**; 35:329–36.
23. Gardner MJ, Hall N, Fung E, et al. Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature **2002**; 419:498–511.
24. Peterson DS, Miller LH, Wellems TE. Isolation of multiple sequences from the *Plasmodium falciparum* genome that encode conserved domains homologous to those in erythrocyte-binding proteins. Proc Natl Acad Sci USA **1995**; 92:7100–4.
25. Scholander C, Treutiger CJ, Hultenby K, Wahlgren M. Novel fibrillar structure confers adhesive property to malaria-infected erythrocytes. Nat Med **1996**; 2:204–8.
26. O'Neil-Dunne I, Achur RN, Agbor-Enoh ST, et al. Gravidity-dependent production of antibodies that inhibit binding of *Plasmodium falciparum*-infected erythrocytes to placental chondroitin sulfate proteoglycan during pregnancy. Infect Immun **2001**; 69:7487–92.
27. Duffy PE, Fried M. Antibodies that inhibit *Plasmodium falciparum* adhesion to chondroitin sulfate A are associated with increased birth weight and the gestational age of newborns. Infect Immun **2003**; 71:6620–3.
28. Staalsoe T, Shulman CE, Bulmer JN, Kawuondo K, Marsh K, Hviid L. Variant surface antigen-specific IgG and protection against clinical consequences of pregnancy-associated *Plasmodium falciparum* malaria. Lancet **2004**; 363:283–9.
29. Jensen AT, Zornig HD, Buhmann C, et al. Lack of gender-specific antibody recognition of products from domains of a *var* gene implicated in pregnancy-associated *Plasmodium falciparum* malaria. Infect Immun **2003**; 71:4193–6.
30. Salanti A, Dahlbäck M, Turner L, et al. Evidence for the involvement of VAR2CSA in pregnancy-associated malaria. J Exp Med **2004**; 200: 1197–203.
31. Duffy MF, Brown GV, Basuki W, et al. Transcription of multiple *var* genes by individual, trophozoite-stage *Plasmodium falciparum* cells expressing a chondroitin sulphate A binding phenotype. Mol Microbiol **2002**; 43:1285–93.
32. Kyes SA, Christodoulou Z, Raza A, et al. A well-conserved *Plasmodium falciparum var* gene shows an unusual stage-specific transcript pattern. Mol Microbiol **2003**; 48:1339–48.
33. Fried M, Wendler JP, Mutabingwa TK, Duffy PE. Mass spectrometric analysis of *Plasmodium falciparum* erythrocyte membrane protein-1 variants expressed by placental malaria parasites. Proteomics **2004**; 4: 1086–93.
34. Gamain B, Smith JD, Avril M, et al. Identification of a 67-amino-acid region of the *Plasmodium falciparum* variant surface antigen that binds chondroitin sulphate A and elicits antibodies reactive with the surface of placental isolates. Mol Microbiol **2004**; 53:445–55.
35. Gamain B, Trimnell AR, Scheidig C, Scherf A, Miller LH, Smith JD. Identification of multiple chondroitin sulfate A (CSA)–binding domains in the *var2CSA* gene transcribed in CSA-binding parasites. J Infect Dis **2005**; 191:1010–3.