

# 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

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## Summary

The complications of malaria in pregnancy are caused by the massive sequestration of parasitized erythrocytes (PE) in the placenta. Placental isolates of *Plasmodium falciparum* are unusual in that they do not bind the primary microvasculature receptor CD36 but instead bind chondroitin sulphate A (CSA). Pregnant mothers develop antibodies that recognize placental variants worldwide, suggesting that a vaccine against malaria in pregnancy is possible. Some members of the Duffy binding-like  $\gamma$  (DBL- $\gamma$ ) domain of the large and diverse *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) family, when expressed on Chinese hamster ovary (CHO) cells, bind CSA. To characterize better the molecular requirements for DBL- $\gamma$  adhesion to CSA, we determined the binding of various DBL- $\gamma$  domains. Most DBL- $\gamma$  did not bind CSA, and no conserved region was identified that strictly differentiated binders from non-binders. Structure–function analysis of the FCR3-CSA DBL- $\gamma$  domain localized the minimal CSA binding region to a 67-residue fragment. This region was partially conserved among some binding sequences. Serum from a rabbit immunized with the minimal domain reacted with CSA-binding parasite lines, but not with non-CSA-

adherent PE lines that adhered to CD36 and other receptors. The identification of a minimal binding region from a highly variable cytoadherent family may have application for a vaccine against malaria in pregnancy.

## Introduction

In *Plasmodium falciparum*, cytoadherence and immune evasion properties are determined primarily by the expressed members of a variant surface antigen family known as *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1) (Baruch *et al.*, 1995; Smith *et al.*, 1995; Su *et al.*, 1995). The antigenic variation and sequestration properties attributed to these proteins provide a competitive advantage for the parasite by allowing parasitized erythrocytes (PE) to evade host immunity and spleen-dependent killing mechanisms, thereby facilitating chronic infection and parasite transmission (Miller *et al.*, 2002). The various binding properties of PfEMP-1 proteins are also virulence determinants implicated in the different pathogenic syndromes of *P. falciparum*. Although most *P. falciparum* infections do not require hospitalization or even treatment in endemic regions, several forms of severe and even fatal disease are associated with PE accumulation in specific organs such as the brain and placenta.

In high-transmission regions, protective clinical immunity to *P. falciparum* develops during the early years of life, limiting serious complications of malaria to young children (World Health Organization, 2003). Pregnant women are an exception and become especially susceptible to severe *P. falciparum* infections during their first pregnancy (primigravidas) (Duffy, 2003). Malaria during pregnancy is associated with the massive sequestration of PE in the placental intervillous blood spaces. Placental sequestration affects both mother and fetus (Menendez, 1995; Duffy, 2003; Duffy and Fried, 2003) but, until recently, it was not clear why high parasitaemia infections occurred in pregnant women who otherwise possess significant clinical immunity to malaria. It has recently been discovered that the parasite variants that infect pregnant women are structurally, antigenically and functionally distinct from those that infect children and non-pregnant adults (Fried

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and Duffy, 1996; Beeson *et al.*, 1999; Gamain *et al.*, 2002). Whereas sequestration in the peripheral microvasculature is associated with PE that bind CD36 and variably to several other host receptors (Baruch, 1999), the placental variants do not bind CD36 but instead bind to chondroitin sulphate A (CSA) and, to a lesser extent, to hyaluronic acid and non-immune IgG (Fried and Duffy, 1996; Beeson *et al.*, 2000; Flick *et al.*, 2001). Hence, the placenta supports the clonal expansion of a unique subset of variants to which immunity has not yet developed. Significantly, during the course of pregnancy and in subsequent pregnancies, broadly strain-transcendent antibodies develop that recognize placental PE from different geographical regions and correlate with protection from malaria during pregnancy (Fried and Duffy, 1996; Ricke *et al.*, 2000; O'Neil-Dunne *et al.*, 2001; Staalsoe *et al.*, 2001). These findings suggest that the surface molecule(s) expressed by placental variants have conserved antigenic determinants and have spurred efforts to characterize these protective antibodies and to induce them by vaccination.

Recent work demonstrated that the PfEMP-1 proteins have an important role in PE binding to CD36 and CSA and therefore regulate PE sequestration to microvasculature or placenta (Baruch *et al.*, 1997; Buffet *et al.*, 1999; Reeder *et al.*, 1999; Gamain *et al.*, 2002). PfEMP-1 proteins are expressed on the surface of PE and possess multiple adhesive modules: the cysteine-rich interdomain regions (CIDR) and the Duffy binding-like (DBL) domains. On the basis of their sequences, these adhesion domains group into different sequence types (Smith *et al.*, 2001; Gardner *et al.*, 2002). The CIDR1- $\alpha$  domain mediates binding to CD36 (Baruch *et al.*, 1997), while some DBL- $\gamma$ -type domains bind CSA (Buffet *et al.*, 1999; Gamain *et al.*, 2002). PfEMP-1 proteins from CSA-binding parasites have a combination of a CSA-binding DBL- $\gamma$  domain and a non-CD36-binding CIDR1 (Buffet *et al.*, 1999; Gamain *et al.*, 2001; 2002). Recombinant DBL- $\gamma$  proteins are able to elicit antibodies reactive with CSA-binding parasite isolates from different endemic regions (Costa *et al.*, 2003). It remains to be determined how many different PfEMP-1 variants are involved in placental malaria and the targets of the variant transcending response to the placental isolates. This knowledge will be crucial to designing a broad-spectrum vaccine to protect pregnant women.

In this study, we performed binding experiments to determine the range of different DBL- $\gamma$  sequences that bind CSA. We compared binding and non-binding DBL- $\gamma$  sequences to identify features that may contribute to the variant transcending immunity. We also defined the minimal CSA binding sequence as a 67-residue fragment of the FCR3-CSA DBL- $\gamma$ . Serum from a rabbit immunized with this fragment reacted with CSA-binding PE collected

from various parts of the world but not with those that expressed CD36-binding variants or other non-CSA-binding phenotypes.

## Results

### *CSA-binding phenotypes of different DBL- $\gamma$ sequences*

PE that accumulate in the placenta bind CSA, but not the closely related chondroitin sulphate C (CSC) (Fried and Duffy, 1996). To investigate further the requirements for adhesion, DBL- $\gamma$  recombinant proteins were expressed at the surface of CHO-745 cells and tested for CSA binding. In the present study, 10 different DBL- $\gamma$  sequences were analysed; only the R29-var DBL2- $\gamma$  and the 3D7 chr5var DBL3- $\gamma$  domains bound to Biot-CSA (Table 1). Although cells expressing the 3D7 chr5var DBL3- $\gamma$  recombinant protein bound CSA at a lower level than the R29var DBL2- $\gamma$  and the control FCR3-CSA DBL3- $\gamma$  recombinant proteins (Table 1), we have shown separately that chr5var DBL3- $\gamma$  recognizes CSA expressed on the proteoglycan thrombomodulin (Vazquez-Macias *et al.*, 2002). As controls for binding, none of the recombinant proteins bound Biot-CSC (data not shown), and adhesion of Biot-CSA was inhibited by soluble CSA but not by soluble CSC (Table 1). Although the shark cartilage CSC contains around 10% CSA, this glycosaminoglycan does not inhibit PE binding to CSA (Alkhalil *et al.*, 2000). This failure to inhibit binding may relate to the level of sulphatation of the CSA present in shark cartilage CSC (Achur *et al.*, 2003).

DBL- $\gamma$  domains are not common in PfEMP-1 proteins. Of the 59 predicted PfEMP-1 proteins from the 3D7 genome, only 12 have a DBL- $\gamma$  domain plus the 3D7 chr5var sequence (accession number CAD51686; gene name PFE1640w), which is a truncated pseudogene. These 13 genes contain 15 DBL- $\gamma$  domains. From the present and previous studies, 10 out of 15 3D7 DBL- $\gamma$  sequences have been tested for CSA binding (Table 1; Fig. 1A). Of these, only the 3D7 chr5 pseudovar and the identical, duplicated genes PFD1235w (on chromosome 4) and MAL7P1.1 (on chromosome 7) bound CSA (Gamain *et al.*, 2002). PFD1235w and MAL7P1.1 were previously referred to as AL010226 in a study before publication of the *P. falciparum* genome (Gamain *et al.*, 2002; Gardner *et al.*, 2002). Thus, few DBL- $\gamma$  domains from a single parasite genome bind CSA. Altogether, six out of 17 DBL- $\gamma$  sequences tested from different parasite genotypes bound CSA (Fig. 1A), indicating that most DBL- $\gamma$  domains do not bind CSA.

CD36 and CSA binding are mutually exclusive parasite adhesion traits (Gamain *et al.*, 2002). We have shown previously that one element in this dichotomy is that

**Table 1.** Binding characteristics of different DBL $\gamma$  to Biot-CSA.

Construct expressed	Binding of Biot-CSA to CHO-745 cells <sup>a</sup>					
	No inhibition		Inhibition with CSA		Inhibition with CSC	
	Positive cells <sup>b</sup> (%)	Beads/100 cells <sup>c</sup>	Positive cells <sup>b</sup> (%)	Beads/100 cells <sup>c</sup>	Positive Cells <sup>b</sup> (%)	Beads/100 cells <sup>c</sup>
FCR3-CSA DBL3	98 $\pm$ 2	1324 $\pm$ 71	2 $\pm$ 1	ND	94 $\pm$ 4	1298 $\pm$ 29
IT-R29Var-DBL2	84 $\pm$ 4	1023 $\pm$ 44	6 $\pm$ 2	ND	76 $\pm$ 6	981 $\pm$ 17
3D7-PF13_0003-DBL3	2 $\pm$ 1	ND	1 $\pm$ 0	ND	1 $\pm$ 0	ND
3D7-chr2T.2-DBL2	4 $\pm$ 1	ND	1 $\pm$ 0	ND	1 $\pm$ 0	ND
3D7-PFD0020c-DBL3	3 $\pm$ 3	ND	1 $\pm$ 1	ND	0 $\pm$ 0	ND
3D7-PFD0020c-DBL4	3 $\pm$ 1	ND	2 $\pm$ 2	ND	1 $\pm$ 1	ND
3D7-PFD0005w-DBL2	1 $\pm$ 1	ND	0 $\pm$ 0	ND	1 $\pm$ 1	ND
3D7-PF08_0140-DBL3	0 $\pm$ 0	ND	0 $\pm$ 0	ND	0 $\pm$ 0	ND
3D7-Chr5var-DBL3	37 $\pm$ 8	632 $\pm$ 41	4 $\pm$ 2	ND	31 $\pm$ 4	608 $\pm$ 37
3D7-Chr5var-DBL5	2 $\pm$ 2	ND	0 $\pm$ 0	ND	2 $\pm$ 1	ND
3D7-PF11_0008-DBL2	2 $\pm$ 1	ND	2 $\pm$ 2	ND	1 $\pm$ 0	ND

a. Cells were incubated with Biot-CSA without (control) or after preincubation with 200  $\mu$ g ml<sup>-1</sup> CSA or CSC.

b. One hundred cells expressing the recombinant protein were evaluated for the presence of CSA-coated Dynal beads on their surface. Cells with four or more beads attached were considered to be positive for binding. Results are expressed as the mean and SD of three independent experiments.

c. The number of beads was counted on 100 cells determined to be positive for binding to CSA-coated Dynal beads. Results are expressed as the mean and SD of three independent experiments.

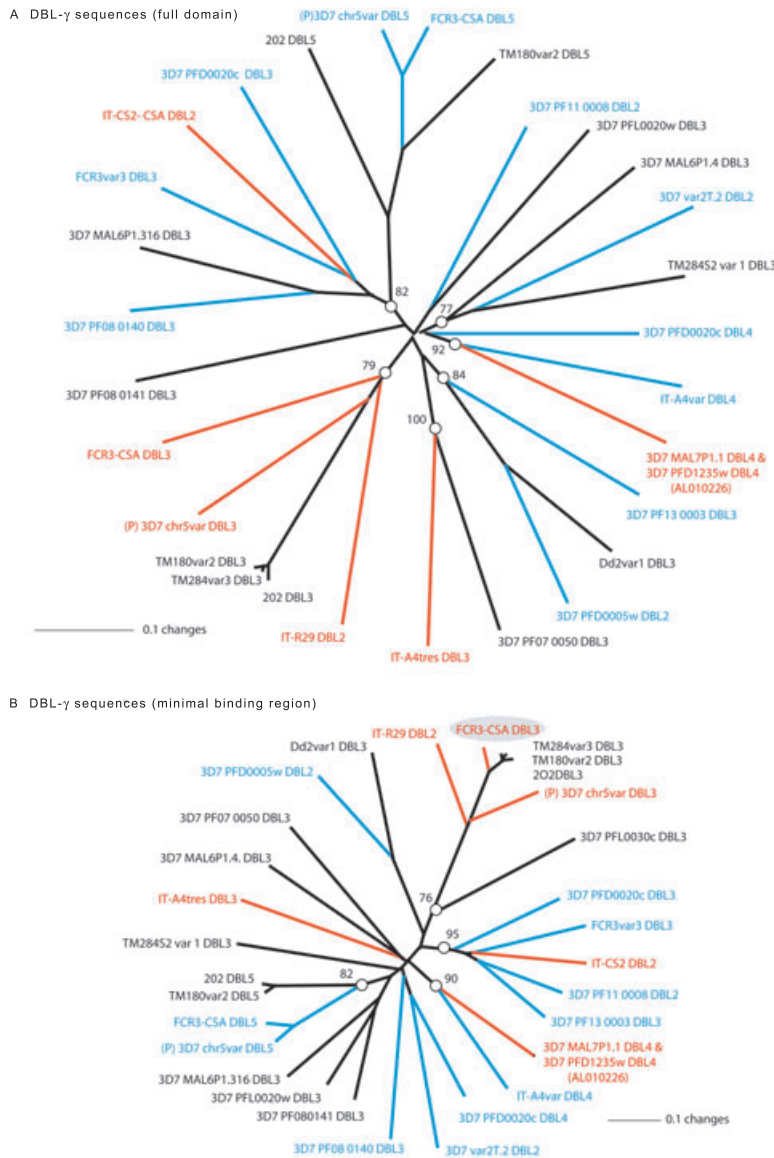
CD36-binding CIDR- $\alpha$  domains can inhibit the ability of downstream DBL- $\gamma$  domains to bind CSA (Gamain *et al.*, 2002). As a general rule, only CIDR- $\alpha$  binds CD36 (Robinson *et al.*, 2003). However, there is one example of a CIDR- $\alpha$  domain from the IT-CS2 variant that does not bind CD36 (Gamain *et al.*, 2002). Of interest, nearly all the DBL- $\gamma$  domains that bound CSA, including IT-CS2, are preceded by CIDR1 domains that are demonstrated or predicted to be non-CD36-binding types (Robinson *et al.*, 2003). The only exception, A4tres, has a CD36-binding CIDR- $\alpha$  domain and a DBL- $\gamma$  domain that, when expressed on the surface of CHO-745, binds CSA although the PE do not adhere to CSA (Gamain *et al.*, 2002).

#### *A 67-amino-acid fragment from FCR3-CSA DBL- $\gamma$ binds specifically to CSA*

The DBL- $\gamma$  sequences tested were diverse ( $\approx$  30–50% amino acid identity), and the neighbour-joining tree analysis did not define common features present only in CSA-binding domains (Fig. 1A); however, at least three out of six binding sequences (3D7 chr5var DBL3- $\gamma$ , FCR3-CSA DBL3- $\gamma$  and It-R29 DBL2- $\gamma$ ) grouped together and did not include any non-binding sequences (Fig. 1A). This group also included three FCR3-CSA homologues (TM180var2, TM284var3 and 202) that were cloned in separate studies. The TM isolates were collected from patients with cerebral malaria, and the 202 line was selected on CSA (Rowe *et al.*, 2002; Salanti *et al.*, 2002). To identify features and residues critical for CSA binding, we generated deletion mutants

from the FCR3-CSA DBL3 $\gamma$  domain and subjected them to structure–function analysis to identify the minimal CSA-binding domain. Seven FCR3-CSA DBL3- $\gamma$  partial domain fragments (F2 to F8) were expressed on the surface of CHO-745 cells. Of these seven truncations, only the F5 and F6 fragments, localized to the C-terminus of the DBL3 $\gamma$  domain, bound Biot-CSA (Fig. 2; Table 2). Ninety-eight per cent of cells expressing the full-length FCR3-CSA DBL- $\gamma$  domain bound Biot-CSA-coated beads, and 73% of the cells expressing the F6 peptide bound to Biot-CSA. Curiously, only 9% of the cells expressing the F5 fragments bound CSA-coated beads, despite the fact that it was slightly larger than the F6 fragment. It should be noted that the region in the F5 and not in the F6 fragments contains a cysteine residue that may have caused misfolding. In addition, the F4 peptide, which encompassed F5 and F6, did not bind CSA (Fig. 2; Table 2). The larger peptide may have adopted a different conformation that does not support binding. For both F5 and F6 domain fragments, binding was specific for Biot-CSA but not for biotin alone or Biot-CSC (data not shown). Moreover, binding was blocked by soluble CSA but not by CSC (Table 2).

To localize the minimum number of residues for CSA adhesion, six smaller fragments (F6.1 to F6.6) were derived from fragment F6. Two of these fragments (F6.3 and F6.4) exhibited binding to Biot-CSA but at a somewhat reduced level compared with the full-length FCR3-CSA DBL- $\gamma$  domain (68% and 56% of the cells, respectively, compared with 98% for full-length DBL- $\gamma$ ) (Fig. 2; Table 2). The fragments retained the binding specificity of the full-length DBL- $\gamma$ , and addition of CSA, but not of CSC,



**Fig. 1.** DBL- $\gamma$  domains from PfEMP-1 protein comparisons.

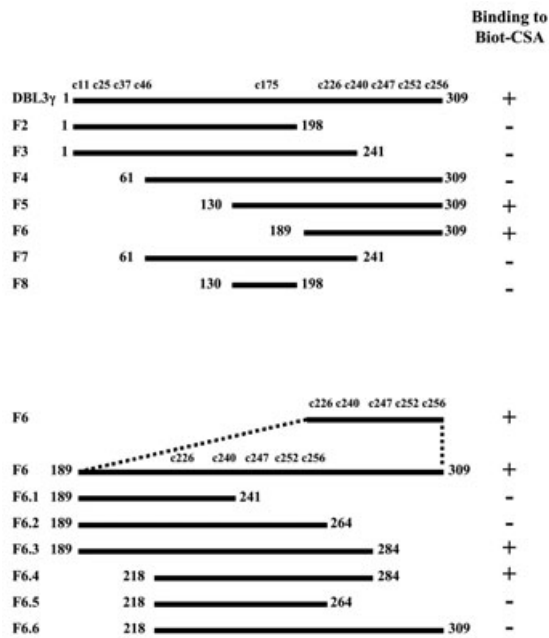
A. A neighbour-joining tree comparing full-length DBL- $\gamma$  sequences cloned from different *P. falciparum* strains. Sequences are identified by the parasite strain from which they were cloned followed by gene name and domain position (IT stands for Ituxi strain). The PFD1235w (chromosome 4) and the MAL7P1.1 (chromosome 7) are identical, duplicated genes that have been referred to separately by their provisional accession number (AL010226) in an earlier publication (Gamain *et al.*, 2002). The 3D7 chr5var gene (gene name PFE1640w) is a truncated, partial (P) gene presumed to be non-functional. Sequences highlighted in red bound CSA-coated beads, blue sequences did not, and black sequences were not tested for binding. Sequence groupings with bootstrap support above 70 are indicated by circles and percentage support.

B. Comparison of the same sequences as in (A) but confined to the 67 residues defined as the minimal CSA-binding region from FCR3-CSA DBL3- $\gamma$ . The region of the PFL0030c DBL3-X domain that had BLAST identity to the minimal CSA-binding region was added to the comparison. Sequences in (B) are coloured red, blue or black based upon CSA binding of the full-length domains tested in Table 1 or earlier studies. The FCR3-CSA sequence is shadowed in grey because it was the only sequence tested as a minimal binding region for CSA binding.

blocked the binding of Biot-CSA to both fragments. No binding to Biot-CSC was observed. Thus, from this analysis, a 67-amino-acid fragment was identified that is able to mimic the binding properties of the 309-amino-acid FCR3-CSA DBL- $\gamma$  domain (Table 2) and *P. falciparum*-infected erythrocytes.

The sequence representing the minimal binding region from various DBL- $\gamma$  sequences was compared. Although not highly conserved between all CSA-binding DBL- $\gamma$  sequences (Fig. 1B), it contains a concentration of residues shared between the three CSA binders that grouped together (Figs 1B and 3A). This identity is striking because it occurs in a region that is otherwise highly divergent among all DBL- $\gamma$  sequences (Fig. 1B).

When the minimal binding region was PSI-BLASTED against the database, the top scoring BLAST hits with *E*-values better than threshold all derived from the FCR3-CSA-like or the 3D7 chromosome 5 *var*-like homologues cloned from different parasite strains, or the R29var (*E*-values ranging between 1e-32 and 6e-17). The next BLAST hit and the last sequence with *E*-value above the default PSI-BLAST threshold (*E*-value of 0.001) was a DBL3-X domain from the PFL0030c *var* gene (Figs 1B and 3B) that was shown recently to be transcriptionally upregulated in parasite lines selected to bind CSA (Salanti *et al.*, 2003). This may provide some support to the idea that the PFL0030c *var* gene is involved in CSA binding.



**Fig. 2.** Schematic representation and binding properties of the FCR3-CSA DBL-γ fragments. FCR3-CSA DBL-γ fragments were cloned in the pSRα5 vector and transfected into CHO-745 cells. Cloned CHO cell lines expressing these various constructs were tested for binding to Biot-CSA, and binding was controlled with Biot-CSC (detailed data are shown in Tables 1 and 2). Construct boundaries are indicated. Conserved cysteines and their respective positions are indicated above the top line.

*Antiserum to the FCR3-CSA minimal binding region recognizes a broad range of CSA-binding PE*

To test whether the FCR3-CSA minimal binding region would elicit antibodies reactive with the surface of PE that bind CSA and placental isolates, a rabbit was immunized with a synthetic peptide corresponding to the 67-residue minimal binding domain. Immunization was performed without any conjugation of the peptide but with the use of Freund's adjuvant. To exclude the possibility of non-specific IgM and non-immune IgG binding to CSA-adherent PE (Flick *et al.*, 2001; Creasey *et al.*, 2003), the preimmune serum was used as a control. As additional controls, rabbit sera raised against other antigens were used (data not shown). The rabbit antiserum tested in L-IFA reacted only with the CSA-adherent PE but not with several different non-CSA-adherent PE lines that adhered to CD36 and other undefined receptors (Table 3; Fig. 4). No labelling was observed using the corresponding preimmune or unrelated rabbit sera. It is important to note that the adhesion of CSA-adherent PE to Sc1D cells was almost completely inhibited by the addition of 100 μg ml<sup>-1</sup> CSA, whereas the FA6-152 monoclonal antibody against CD36 was only able to inhibit partially the adhesion of CD36-adherent PE to Sc1D. These results show that the CD36-adherent PE used in this study are able to bind to other receptors on the surface of the Sc1D cells. In total, the rabbit antipeptide serum reacted with each of seven different CSA adherent lines that were collected from different geographical regions, including three placental isolates (Table 3).

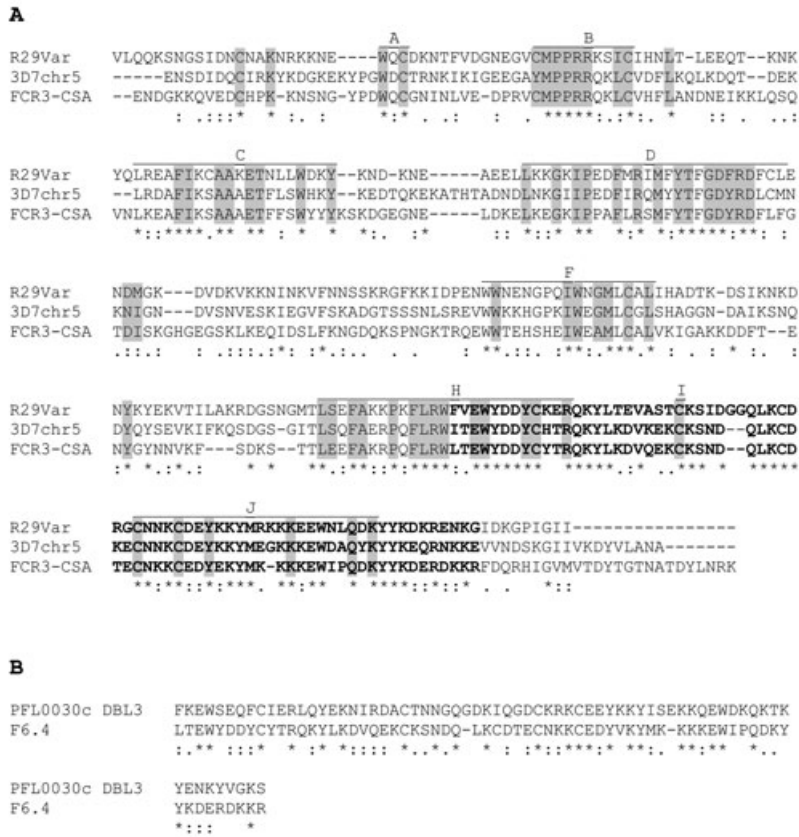
**Table 2.** Binding characteristics of FCR3-CSA DBL-γ fragments to Biot-CSA.

Construct expressed	Binding of Biot-CSA to CHO-745 cells <sup>a</sup>					
	No inhibition		Inhibition with CSA		Inhibition with CSC	
	Positive cells <sup>b</sup> (%)	Beads/100 cells <sup>c</sup>	Positive cells <sup>b</sup> (%)	Beads/100 cells <sup>c</sup>	Positive cells <sup>b</sup> (%)	Beads/100 cells <sup>c</sup>
FCR3-CSA DBL3γ	98 ± 2	1324 ± 71	2 ± 1	ND	94 ± 4	1298 ± 29
F2	0 ± 0	ND	0 ± 0	ND	0 ± 0	ND
F3	2 ± 1	ND	1 ± 0	ND	1 ± 0	ND
F4	3 ± 1	ND	1 ± 1	ND	1 ± 1	ND
F5	9 ± 3	451 ± 21	1 ± 0	ND	10 ± 2	429 ± 21
F6	73 ± 3	1156 ± 52	2 ± 1	ND	76 ± 9	1085 ± 83
F7	1 ± 1	ND	0 ± 0	ND	1 ± 0	ND
F8	0 ± 0	ND	0 ± 0	ND	0 ± 0	ND
F6.1	0 ± 0	ND	0 ± 0	ND	0 ± 0	ND
F6.2	2 ± 2	ND	0 ± 0	ND	1 ± 1	ND
F6.3	68 ± 3	905 ± 49	2 ± 2	ND	71 ± 6	923 ± 54
F6.4	56 ± 4	755 ± 32	1 ± 1	ND	59 ± 3	725 ± 28
F6.5	2 ± 2	ND	0 ± 0	ND	2 ± 2	ND
F6.6	0 ± 0	ND	0 ± 0	ND	1 ± 1	ND

**a.** Cells were incubated with Biot-CSA without (control) or after preincubation with 200 μg ml<sup>-1</sup> CSA or CSC.

**b.** One hundred cells expressing the recombinant protein were evaluated for the presence of CSA-coated Dynal beads on their surface. Cells with four or more beads attached were considered to be positive for binding. Results are expressed as the mean and SD of three independent experiments.

**c.** The number of beads was counted on 100 cells determined to be positive for binding to CSA-coated Dynal beads. Results are expressed as the mean and SD of three independent experiments.



**Fig. 3.** A. Conservation of the FCR3-CSA minimal binding region among DBL- $\gamma$  sequences. A sequence alignment comparing the FCR3-CSA DBL3- $\gamma$ , 3D7 chr5 var DBL3- $\gamma$  and IT-R29Var-DBL2- $\gamma$  recombinant proteins that bound CSA. The minimal binding region in FCR3-CSA is indicated by bold letters. \*Identical or conserved residues; ;, conserved substitutions; ., semi-conserved substitutions. The position of semi-conserved homology blocks characteristic of DBL domains A to J are indicated above the alignment. Grey-shaded amino acids are signature-type residues conserved between  $\geq 80\%$  of the DBL- $\gamma$  sequences from Fig. 1 (data not shown). B. CLUSTAL alignment of the FCR3-CSA minimal binding region, labelled F6.4, with the homologous region of the PFL0030c DBL3-X.

**Discussion**

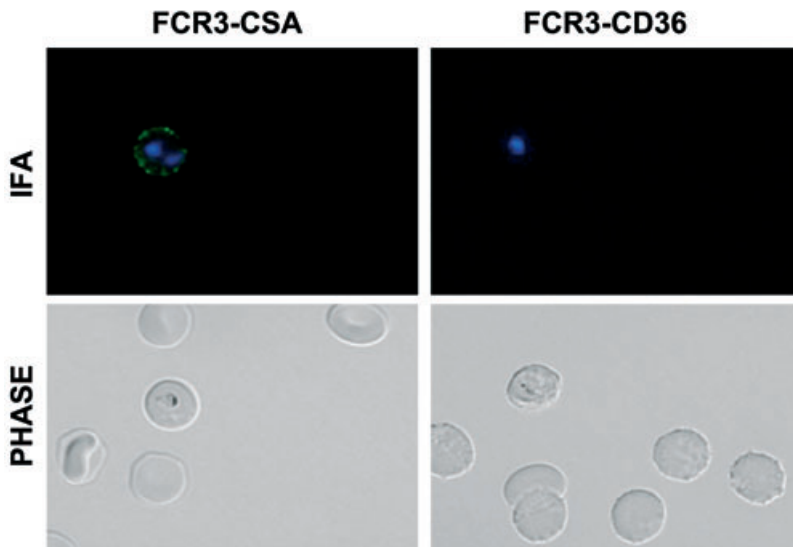
Malaria in pregnancy is a special complication of *P. falciparum* infections associated with PE sequestration in the placenta. A low-sulphated form of CSA acts as a placental

receptor and is expressed by the syncytiotrophoblasts (Achur *et al.*, 2003; Agbor-Enoh *et al.*, 2003). With successive pregnancies, women develop antibodies that recognize the placental parasite variants and block PE adhesion to CSA (Fried *et al.*, 1998), suggesting that it

**Table 3.** Reactivity of sera raised against the minimal binding domain to parasitized erythrocytes.

Parasite <sup>a</sup>	Origin	Parasite source	Sera reactivity <sup>b</sup>		Inhibition assay on Sc1D <sup>c</sup>	
			D0	D73	CSA 100 $\mu\text{g ml}^{-1}$	FA6-152
FCR3 CSA <sup>Sc17</sup>	West Africa	Peripheral	1%	90%	85%	0%
B358 CSA <sup>Sc17</sup>	Brazil	Peripheral	1%	90%	76%	0%
SUK CSA <sup>Sc17</sup>	West Africa	Peripheral	3%	98%	92%	0%
IPL/BRE1 <sup>Sc17</sup>	Brazil	Peripheral	0%	98%	91%	0%
24 CSA <sup>Sc17</sup>	Cameroon	Placenta	2%	99%	98%	7%
42 CSA <sup>Sc17</sup>	Cameroon	Placenta	1%	96%	96%	0%
42DJ CSA <sup>Sc17</sup>	Cameroon	Placenta	2%	84%	90%	0%
FCR3 <sup>ScC2</sup>	West Africa	Peripheral	0%	0%	5%	78%
B358 <sup>ScC2</sup>	Brazil	Peripheral	0%	2%	0%	12%
BXII <sup>ScC2</sup>	Brazil	Peripheral	0%	2%	1%	12%
IPL/BRE1 <sup>ScC2</sup>	Brazil	Peripheral	0%	0%	8%	73%

a. Parasites were selected on *Saimiri* endothelial Sc17 cells for CSA phenotype or on *Saimiri* endothelial ScC2 cells for CD36 and other non-CSA-binding phenotypes.  
b. Rabbit sera before (D0) or 10 days after the fourth immunization (D73) were tested against different parasite lines. Results are expressed as the percentages of PE recognized by the sera.  
c. CD36 and CSA parasite phenotypes were reassessed by inhibiting adhesion of PE on Sc1D expressing CSA, CD36, ICAM-1 and other undefined receptors on their surface by either CSA at 100  $\mu\text{g ml}^{-1}$  or a monoclonal antibody against CD36 (FA6-152) at 12  $\mu\text{g ml}^{-1}$ . Results are expressed as the percentages of adhesion inhibition compared with control.



**Fig. 4.** Surface labelling of PE with an antiserum raised against the 67-residue minimal CSA-binding domain. Typical surface staining of live FCR3-CSA PE in liquid-phase IFA. No labelling was detected for FCR3-CD36 PE. The nuclei of parasitized erythrocytes are visualized by DAPI staining.

may be possible to vaccinate against malaria during pregnancy. Although the PE surface determinants targeted by these antibodies have still to be better characterized, binding to CSA has been shown to be mediated by the DBL- $\gamma$  of some PfEMP-1 variants (Buffet *et al.*, 1999; Gamain *et al.*, 2002).

To determine whether all DBL- $\gamma$  domains bind CSA and to examine whether binding sequences have conserved features, DBL- $\gamma$  domains were expressed at the surface of CHO cells. Our analysis showed that CSA binding is uncommon in these domains. Only six out of 17 distinct DBL- $\gamma$  sequences bound CSA. Moreover, among the *var* genes from the 3D7 strain tested for binding, only three out of 10 had a CSA-binding DBL- $\gamma$ . Of these, 3D7 chr5*var* is truncated in the extracellular binding region and presumably non-functional, while the other two, MAL7P1.1 and PFD1235w, are identical genes located on chromosomes 4 and 7. From this study, we were unable to identify conserved residues characteristic of DBL- $\gamma$  domains that bind CSA (Fig. 1A).

As a complementary approach to localize critical binding residues in a CSA-binding DBL- $\gamma$ , a 67-residue minimal binding region was defined from the FCR3-CSA DBL3- $\gamma$  sequence, which grouped with two other binding sequences in the analysis (Fig. 1A and B). This binding group also included three FCR3-CSA *var* homologues that have not yet been tested for CSA binding (Rowe *et al.*, 2002; Salanti *et al.*, 2002). Sequence analysis showed that the minimal binding region was highly conserved among the three binding sequences compared with their full-length domains (Figs 1B and 3A), but was otherwise divergent among all DBL- $\gamma$  sequences (Fig. 1B). Intriguingly, the minimal CSA-binding region from the DBL- $\gamma$  domain was also related to a DBL-X domain in the 3D7 PFL0030c PfEMP-1 protein (Figs 1B

and 3B). This sequence similarity could have biological relevance, as PFL0030c-like *var* messages (*var2csa*) are transcriptionally upregulated in PE selected to bind CSA (Salanti *et al.*, 2003; A. Scherf *et al.*, data not shown). Interestingly, the PFL0030c PfEMP-1 has a distinctive protein architecture that lacks a CIDR1- $\alpha$  domain that might otherwise interfere with CSA binding (Gamain *et al.*, 2002) and does not contain a DBL- $\gamma$  domain (Gardner *et al.*, 2002). It will be important to determine whether the PFL0030c is present on the erythrocyte surface of CSA-binding PE.

Recently, it was discovered that FCR3-CSA-like *var* gene transcription (*var1csa*) is distinct from other *var* loci and does not correlate with the PE binding phenotype (Kyes *et al.*, 2003). This unexpected finding has cast doubt on the role of *var1csa* in CSA binding. The constitutive transcription of *var1csa* is obviously not sufficient to predict expression of the corresponding PfEMP1 on the PE surface, and it is possible that some mechanisms, possibly post-transcriptional or hormonal from the pregnant woman to the parasite, could occur to allow the expression of *var1csa* on the surface of CSA-binding PE.

Despite its unusual transcriptional regulation, additional experiments have suggested that *var1csa* may be involved in CSA adhesion. First, genetic disruption of *var1csa* in FCR3-CSA PE led to a phenotypic switch from a CSA-binding phenotype to CD36 binding (Andrews *et al.*, 2003). The FCR3-CSA KO parasite line, reselected to bind CSA, expressed on their surface a high-molecular-weight protein detected by surface iodination, slightly distinct in molecular weight from the original FCR3-CSA parasite line (Andrews *et al.*, 2003). This suggests a role for an additional protein in CSA binding. Secondly, recombinant DBL- $\gamma$  proteins have been brought forward as potential vaccine antigens because a recombinant protein

based upon the full-length DBL3- $\gamma$  domain from FCR3-CSA binds CSA and elicits monoclonal antibodies against CSA-binding parasite lines that inhibit PE binding to CSA (Costa *et al.*, 2003). These antibodies may be cross-reactive, and their binding does not prove that the domain expressed on the PE surface is identical to the immunizing antigen.

Thirdly, as further evidence that DBL- $\gamma$  domains may be involved in binding to CSA, we note that five out of six DBL- $\gamma$  recombinant proteins that bind CSA are associated with PfEMP1 proteins that have a non-CD36-binding CIDR1 domain (Robinson *et al.*, 2003). The only exception comes from the CD36 and ICAM-1 binding parasite clone A4tres that does not bind to CSA, despite the fact that the corresponding PfEMP1 contains a CSA-binding DBL- $\gamma$  (Gamain *et al.*, 2002). As CD36-binding CIDR1 domains block the ability of downstream DBL- $\gamma$  domains to bind CSA (Gamain *et al.*, 2002), this interaction between two domains may reflect a structural and functional adaptation that has occurred in PfEMP-1 proteins selected for CSA binding and explain the dichotomous binding of PEs to CD36 or CSA. Although indirect, the lack of a CD36-binding CIDR1 domain suggests that some of these DBL- $\gamma$  domains may be involved in CSA binding. This discussion, however, underscores the difficulties of studying the protein on the erythrocyte surface involved in binding and emphasizes the need for other techniques (e.g. proteomics) to define the basis for CSA binding.

Placental isolates are antigenically and phenotypically distinct from those that characterize children's infections (Fried and Duffy, 1996; Beeson *et al.*, 1999) and appear to express relatively strain-transcendent or conserved epitopes (Fried *et al.*, 1998). Pregnant women develop antibodies, lacking in men and nulligravid women, which recognize placental isolates or CSA-binding parasite lines (Fried *et al.*, 1998; Beeson *et al.*, 1999; Ricke *et al.*, 2000; O'Neil-Dunne *et al.*, 2001). In general, there is a gravidity-dependent increase in the kinetics, level or prevalence of these antibodies (Fried *et al.*, 1998; Beeson *et al.*, 1999; Ricke *et al.*, 2000; O'Neil-Dunne *et al.*, 2001), which could explain the decreasing susceptibility to malaria in pregnancy with increasing gravidity. However, it is not unusual to detect CSA adhesion-inhibitory or surface-reactive antibodies in primigravidas as early as the second trimester (O'Neil-Dunne *et al.*, 2001; Staalsoe *et al.*, 2001), even though the women are infected at term. Serological studies have relied on one or a few CSA-binding lines or placental isolates, which may not reflect the full diversity of CSA-binding variants.

More work is needed to understand the role of specific antibodies in protection. It has been suggested that anti-adhesion antibodies could block PE sequestration in the placenta (Fried *et al.*, 1998). In addition, it is possible that

cytophilic antibodies bind to monocytes that are heavily recruited to infected placentas (Walter *et al.*, 1982), and these have a role in controlling infection. Taken together, these findings indicate that antibodies are produced during pregnancy in response to placental infection. However, the antibody response may need to be broadened to recognize the full diversity of placental isolates for complete protection.

Using an FCR3-CSA DBL- $\gamma$  recombinant protein, polyclonal antisera and monoclonal antibodies have been raised against the surface of CSA-binding lines or placental isolates (Lekana Douki *et al.*, 2002; Costa *et al.*, 2003). The same monoclonal antibodies react on PEs reselected to bind CSA after genetically disrupting the FCR3-CSA *var* gene (Andrews *et al.*, 2003), indicating that cross-reactivities are possible. The cross-reactive epitope(s) have not yet been defined; however, *var2csa* is transcriptionally upregulated in the CSA-reselected FCR3 line (A. Scherf *et al.*, unpublished). Given the uncertainties about FCR3-CSA protein translation (Kyes *et al.*, 2003) and the complete lack of knowledge about what is expressed at the surface of placental isolates, the precise targets of these antibodies are still not known. Nevertheless, they highlight the potential utility of the FCR3-CSA DBL- $\gamma$  recombinant protein as a vaccine antigen.

A vaccine based on a minimal binding region may focus the antibody response to functionally conserved determinants and perhaps diversify the immune response to different CSA-binding PfEMP-1 domains, including the *var2csa* and non-DBL- $\gamma$  domains that could be involved in CSA binding. For this study, rabbit serum was generated to a chemically synthesized 67-residue minimal DBL- $\gamma$ -binding peptide. This serum specifically recognized CSA-binding PE from different endemic regions and did not react with CD36-binding PE lines or those that recognized other receptors. However, the serum was unable to inhibit PE binding to CSA (B. Gamain *et al.*, data not shown). This failure to block binding may not exclude efficacy of the vaccine. First, infection may rapidly boost immunity. Secondly, cytophilic antibody may lead to phagocytosis of PE by macrophages. It is evident that trials may be needed to resolve these questions, either in an animal model, if available, or in women immunized before pregnancy.

In conclusion, this study identifies a small CSA-binding region, unusually conserved among a subset of CSA-binding DBL- $\gamma$  domains and showing similarity to the DBL3-X from PFL0030c. Peptide immunization supports the feasibility of using minimal binding peptides to raise antibodies to CSA-binding lines. Although the optimal antigens required for a pregnancy malaria vaccine remain to be better characterized, it is possible that a combination of antigens will be required to generate a broadly neutral-

izing response. An increased understanding of binding and protective immunity to placental isolates may clarify the pathogenic mechanisms of pregnancy malaria and lead to interventions that prevent disease.

## Experimental procedures

### *Construction of recombinant plasmids for surface expression in mammalian cell lines*

Constructs were amplified from genomic DNA by polymerase chain reaction (PCR) and cloned into either the pSR $\alpha$ 5 or the pSR $\alpha$ 5-12CA5 vector (Affymax Research Institute) (Whitehorn *et al.*, 1995). Both vectors supply a signal sequence and a glycosylphosphatidylinositol anchor for cell surface expression, a selectable marker for stable integration and an epitope tag recognized by the 179 monoclonal antibody (mAb) to monitor surface expression. In addition, the pSR $\alpha$ 5-12CA5 vector has an upstream haemagglutinin (HA) epitope tag recognized by mAb 12CA5. The following domains were used (amino acid boundaries of each clone and GenBank accession numbers are given): IT-R29Var-DBL2 (776–1069, CAA73831); 3D7-PF13-0003-DBL3 (1223–1540, CAD52145); 3D7-chr2T.2-DBL2 (836–1147, AAC71792); 3D7-PFD0020c-DBL3 (1212–1526, CAD49096); 3D7-PFD0020c-DBL4 (1581–1895, CAD49096); 3D7-PFD0005w-DBL2 (878–1180, CAD49094); 3D7-PF08-0140-DBL3 (1217–1535, CAD51361); 3D7-Chr5var-DBL3 (1248–1551, CAD51686); 3D7-Chr5var-DBL5 (2072–2382, CAD 51686); and 3D7-PF11-0008-DBL2 (874–1166, AAN35605). Partial domain fragments of the FCR3-CSA DBL3 $\gamma$  (1270–1578, accession number AJ133811) were expressed in pSR $\alpha$ 5. Amino acid boundaries for the fragments were: F2 (1270–1467); F3 (1270–1510); F4 (1330–1578); F5 (1399–1578); F6 (1458–1578); F7 (1330–1510); F8 (1399–1467); F6.1 (1458–1510); F6.2 (1458–1533); F6.3 (1458–1553); F6.4 (1487–1553); F6.5 (1487–1533); and F6.6 (1487–1578).

### *Surface expression of various domains in Chinese hamster ovary cells*

Chinese hamster ovary PgsA-745 (CHO-745) cells deficient in glycosaminoglycans (American Type Culture Collection) were grown in RPMI-1640 medium (Life Technologies) supplemented with 10% heat-inactivated fetal calf serum (Life Technologies). Cells were transfected with 2.5  $\mu$ g of plasmid DNA using the Superfect transfection reagent (Qiagen) according to the manufacturer's recommendations and selected with 1 mg ml<sup>-1</sup> geneticin (Life Technologies). Stable transfectants expressing the various domains on the surface of CHO-745 cells were selected by single-cell cloning using a fluorescence-activated cell sorter (FACS) as described previously (Smith *et al.*, 2000).

### *Binding assays with CSA linked to biotin*

Binding assays with CSA (Sigma) or CSC (Seikagaku) linked to biotin (Biot-CSA or Biot-CSC) were performed as

described previously (Buffet *et al.*, 1999). Briefly, sheep anti-mouse IgG M-450 Dynabeads ( $2 \times 10^6$ ) were incubated overnight at 4°C with 2  $\mu$ g of mouse anti-biotin mAb (Jackson Immunoresearch Laboratories). The beads were washed three times with RPMI 1640 (Sigma) containing 1% BSA (BMB) and resuspended with 45  $\mu$ l of BMB to  $4 \times 10^7$  beads ml<sup>-1</sup>. One hundred thousand CHO-745 cells expressing the different constructs were grown for 48 h on four glass coverslips in a six-well plate. Coverslips were transferred into a 12-well plate containing 1 ml of BMB and 50  $\mu$ g of Biot-CSA and incubated 1 h. For inhibition assays, the cells were incubated for 1 h with 200  $\mu$ g ml<sup>-1</sup> CSA or CSC (Sigma) before the addition of Biot-CSA. The coverslips were washed three times with BMB and incubated for 1 h at room temperature with the anti-biotin mAb-coated Dynal beads (45  $\mu$ l of  $4 \times 10^7$  beads ml<sup>-1</sup>). The coverslips were then flipped cell side down on to a stand and incubated for 3 min to allow unbound beads to settle by gravity. Coverslips were washed three times with BMB, fixed with 2% paraformaldehyde in PBS, and the degree of bead association with cells was examined.

### *Parasite selection on CSA and CD36*

*Plasmodium falciparum* strains (FCR3, B358, SUK and IPL/BRE1) and Cameroonian placental isolates (24, 42 and 42DJ) were cultured and maintained under standard conditions as described previously (Pouvelle *et al.*, 1998) replacing 10% human serum with 5% albumax (Life Technologies). Parasites including placental isolates were selected for CSA binding on Sc17 *Saimiri* brain microvascular endothelial cells as described elsewhere (Gay *et al.*, 1995; Pouvelle *et al.*, 1997). Parasites were selected for CD36 adhesion phenotype on chondroitinase ABC-treated ScC2 cells (Gay *et al.*, 1995; Pouvelle *et al.*, 1997). CD36 and CSA parasite phenotypes were reassessed by inhibiting PE adhesion on SC1D cells expressing CSA, CD36, ICAM-1 and other receptors on their surface (Gay *et al.*, 1995; Pouvelle *et al.*, 1997) by either CSA at 100  $\mu$ g ml<sup>-1</sup> or the FA6-152 anti-CD36 mAb (Immunotech) at 12  $\mu$ g ml<sup>-1</sup>.

### *Immunization of a rabbit with the minimal binding domain*

To raise serum against the minimal binding domain, a synthetic peptide corresponding to the entire minimal binding domain was chemically synthesized. A rabbit was immunized subcutaneously (10 sites in the back) by four injections of 250  $\mu$ g each of unconjugated peptide on days 0, 21, 42 and 63, formulated with Freund's complete adjuvant for the first injection and incomplete adjuvant for subsequent injections.

### *Immunofluorescence assay in liquid phase (L-IFA)*

Rabbit serum was assessed for reactivity by L-IFA at 4°C on PE selected for binding to CSA or CD36 as described previously (Lekana Douki *et al.*, 2002). In short, to prevent non-specific staining, 200  $\mu$ l of each rabbit serum diluted 1:5 in RPMI 1640 was absorbed twice on 100  $\mu$ l pellets of fresh normal human O<sup>+</sup> red blood cells during 30 min at 37°C. Pellets (10  $\mu$ l) of different parasite cultures (4–6% of mature forms) were incubated for 45 min at 4°C with 100  $\mu$ l of serum

and 40 µg ml<sup>-1</sup> 4'-diaminido-2-phenylindole (DAPI). After washing with PBS, pH 7.2, bound antibodies were revealed by a second incubation of 30 min at 4°C with a goat F(ab')<sub>2</sub> Alexa Fluor 488-labelled anti-rabbit IgG (Molecular Probes). Immunofluorescence staining was analysed with a Nikon E800 microscope, and images were acquired with a Nikon DXM 1200 camera.

### Sequence analysis

Multiple sequence alignments were obtained using CLUSTALW software available at <http://www.ebi.ac.uk/clustalw/>. Neighbour-joining trees were constructed using CLUSTAL X1.81 for multiple alignments and PAUP\*4.0b8 (\*Phylogenetic analysis using parsimony and other methods) for tree generation accompanied by bootstrap analysis with 1000 replicates. In addition to the DBL-γ sequences that were tested directly for CSA binding, the following sequences (gene name, accession number) were also analysed: TM180var2, CAD20867; Dd2var1, L40608; TM284var3, AJ420412; 2O2, AF411601; TM284S2 var1, AF366567. Nucleotide and amino acid sequences for all 3D7 var genes can be accessed at the PlasmoDB website (<http://plasmodb.org/PlasmoDB.shtml>) using the gene name identified in the figures.

The percentage of amino acid identity between sequences was calculated using the formula in MEGALIGN 5.0 (DNASar). Consensus residues were identified using the consensus program at <http://www.bork.embl-heidelberg.de/Alignment/consensus.html>.

### Acknowledgements

The authors thank Erik Whitehorn of the Affymax Research Institute for providing the pSRα5 vectors and mAb 179. J.D.S. thanks Joy Zartman for assistance in cloning DBL-γ inserts. J.D.S. is supported by an Ellison Medical Foundation New Investigator Award in Infectious Diseases and an award from the National Institutes of Health (grant RO1 AI47953-01A1). A.S. and J.G. are supported by grants from the Ministère de l'Éducation Nationale de la Recherche et de la Technologie (MENRT) Paludisme (PAL) PAL + 2000 programme, PAL + 2002 programme, and the European Union for Research and Technical Development (contract no. QLK2-CT2000-00109). M.A. holds a PhD fellowship (no. 6414-2002) from MRT.

### References

Achur, R.N., Valiyaveetil, M., and Gowda, D.C. (2003) The low sulfated chondroitin sulfate proteoglycans of human placenta have sulfate group-clustered domains that can efficiently bind *Plasmodium falciparum*-infected erythrocytes. *J Biol Chem* **278**: 11705–11713.

Agbor-Enoh, S.T., Achur, R.N., Valiyaveetil, M., Leke, R., Taylor, D.W., and Gowda, D.C. (2003) Chondroitin sulfate proteoglycan expression and binding of *Plasmodium falciparum*-infected erythrocytes in the human placenta during pregnancy. *Infect Immun* **71**: 2455–2461.

Alkhalil, A., Achur, R.N., Valiyaveetil, M., Ockenhouse, C.F., and Gowda, D.C. (2000) Structural requirements for the

adherence of *Plasmodium falciparum*-infected erythrocytes to chondroitin sulfate proteoglycans of human placenta. *J Biol Chem* **275**: 40357–40364.

Andrews, K.T., Pirrit, L.A., Przyborski, J.M., Sanchez, C.P., Sterkers, Y., Ricken, S., et al. (2003) Recovery of adhesion to chondroitin-4-sulphate in *Plasmodium falciparum* varCSA disruption mutants by antigenically similar PfEMP1 variants. *Mol Microbiol* **49**: 655–669.

Baruch, D.I. (1999) Adhesive receptors on malaria-parasitized red cells. *Baillieres Best Pract Res Clin Haematol* **12**: 747–761.

Baruch, D.I., Pasloske, B.L., Singh, H.B., Bi, X., Ma, X.C., Feldman, M., et al. (1995) Cloning the P. falciparum gene encoding PfEMP1, a malarial variant antigen and adherence receptor on the surface of parasitized human erythrocytes. *Cell* **82**: 77–87.

Baruch, D.I., Ma, X.C., Singh, H.B., Bi, X., Pasloske, B.L., and Howard, R.J. (1997) Identification of a region of PfEMP1 that mediates adherence of *Plasmodium falciparum* infected erythrocytes to CD36: conserved function with variant sequence. *Blood* **90**: 3766–3775.

Beeson, J.G., Brown, G.V., Molyneux, M.E., Mhango, C., Dzinjalama, F., and Rogerson, S.J. (1999) *Plasmodium falciparum* isolates from infected pregnant women and children are associated with distinct adhesive and antigenic properties. *J Infect Dis* **180**: 464–472.

Beeson, J.G., Rogerson, S.J., Cooke, B.M., Reeder, J.C., Chai, W., Lawson, A.M., et al. (2000) Adhesion of *Plasmodium falciparum*-infected erythrocytes to hyaluronic acid in placental malaria. *Nat Med* **6**: 86–90.

Buffet, P.A., Gamain, B., Scheidig, C., Baruch, D., Smith, J.D., Hernandez-Rivas, R., et al. (1999) *Plasmodium falciparum* domain mediating adhesion to chondroitin sulfate A: a receptor for human placental infection. *Proc Natl Acad Sci USA* **96**: 12743–12748.

Costa, F.T., Fusai, T., Parzy, D., Sterkers, Y., Torrentino, M., Douki, J.B., et al. (2003) Immunization with recombinant duffy binding-like-gamma3 induces pan-reactive and adhesion-blocking antibodies against placental chondroitin sulfate A-binding *Plasmodium falciparum* parasites. *J Infect Dis* **188**: 153–164.

Creasey, A.M., Staalsoe, T., Raza, A., Arnot, D.E., and Rowe, J.A. (2003) Nonspecific immunoglobulin M binding and chondroitin sulfate A binding are linked phenotypes of *Plasmodium falciparum* isolates implicated in malaria during pregnancy. *Infect Immun* **71**: 4767–4771.

Duffy, P.E. (2003) Maternal immunization and malaria in pregnancy. *Vaccine* **21**: 3358–3361.

Duffy, P.E., and Fried, M. (2003) *Plasmodium falciparum* adhesion in the placenta. *Curr Opin Microbiol* **6**: 371–376.

Flick, K., Scholander, C., Chen, Q., Fernandez, V., Pouvelle, B., Gysin, J., and Wahlgren, M. (2001) Role of nonimmune IgG bound to PfEMP1 in placental malaria. *Science* **293**: 2098–2100.

Fried, M., and Duffy, P.E. (1996) Adherence of *Plasmodium falciparum* to chondroitin sulfate A in the human placenta. *Science* **272**: 1502–1504.

Fried, M., Nosten, F., Brockman, A., Brabin, B.J., and Duffy, P.E. (1998) Maternal antibodies block malaria. *Nature* **395**: 851–852.

Gamain, B., Smith, J.D., Miller, L.H., and Baruch, D.I. (2001)

- Modifications in the CD36 binding domain of the *Plasmodium falciparum* variant antigen are responsible for the inability of chondroitin sulfate A adherent parasites to bind CD36. *Blood* **97**: 3268–3274.
- Gamain, B., Gratepanche, S., Miller, L.H., and Baruch, D.I. (2002) Molecular basis for the dichotomy in *Plasmodium falciparum* adhesion to CD36 and chondroitin sulfate A. *Proc Natl Acad Sci USA* **99**: 10020–10024.
- Gardner, M.J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R.W., *et al.* (2002) Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* **419**: 498–511.
- Gay, F., Robert, C., Pouvelle, B., Peyrol, S., Scherf, A., and Gysin, J. (1995) Isolation and characterization of brain microvascular endothelial cells from Saimiri monkeys. An *in vitro* model for sequestration of *Plasmodium falciparum*-infected erythrocytes. *J Immunol Methods* **184**: 15–28.
- Kyes, S.A., Christodoulou, Z., Raza, A., Horrocks, P., Pinches, R., Rowe, A.J., and Newbold, C.I. (2003) A well-conserved *Plasmodium falciparum* var gene shows an unusual stage-specific transcript pattern. *Mol Microbiol* **48**: 1339–1348.
- Lekana Douki, J.B., Traore, B., Costa, F.T., Fusai, T., Pouvelle, B., Sterkers, Y., *et al.* (2002) Sequestration of *Plasmodium falciparum*-infected erythrocytes to chondroitin sulfate A, a receptor for maternal malaria: monoclonal antibodies against the native parasite ligand reveal pan-reactive epitopes in placental isolates. *Blood* **100**: 1478–1483.
- Menendez, C. (1995) Malaria during pregnancy: a priority area of malaria research and control. *Parasitol Today* **11**: 178–183.
- Miller, L.H., Hudson-Taylor, D., Gamain, B., and Saul, A.J. (2002) Definition of the minimal domain of CIDR1alpha of *Plasmodium falciparum* PfEMP1 for binding CD36. *Mol Biochem Parasitol* **120**: 321–323.
- O'Neil-Dunne, I., Achur, R.N., Agbor-Enoh, S.T., Valiyaveetil, M., Naik, R.S., Ockenhouse, C.F., *et al.* (2001) Gravity-dependent production of antibodies that inhibit binding of *Plasmodium falciparum*-infected erythrocytes to placental chondroitin sulfate proteoglycan during pregnancy. *Infect Immun* **69**: 7487–7492.
- Pouvelle, B., Meyer, P., Robert, C., Bardel, L., and Gysin, J. (1997) Chondroitin-4-sulfate impairs *in vitro* and *in vivo* cytoadherence of *Plasmodium falciparum* infected erythrocytes. *Mol Med* **3**: 508–518.
- Pouvelle, B., Fusai, T., Lepolard, C., and Gysin, J. (1998) Biological and biochemical characteristics of cytoadhesion of *Plasmodium falciparum*-infected erythrocytes to chondroitin-4-sulfate. *Infect Immun* **66**: 4950–4956.
- Reeder, J.C., Cowman, A.F., Davern, K.M., Beeson, J.G., Thompson, J.K., Rogerson, S.J., and Brown, G.V. (1999) 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* **96**: 5198–5202.
- Ricke, C.H., Staalsoe, T., Koram, K., Akanmori, B.D., Riley, E.M., Theander, T.G., and Hviid, L. (2000) Plasma antibodies from malaria-exposed pregnant women recognize variant surface antigens on *Plasmodium falciparum*-infected erythrocytes in a parity-dependent manner and block parasite adhesion to chondroitin sulfate A. *J Immunol* **165**: 3309–3316.
- Robinson, B.A., Welch, T.L., and Smith, J.D. (2003) Widespread functional specialization of *Plasmodium falciparum* erythrocyte membrane protein 1 family members to bind CD36 analysed across a parasite genome. *Mol Microbiol* **47**: 1265–1278.
- Rowe, J.A., Kyes, S.A., Rogerson, S.J., Babiker, H.A., and Raza, A. (2002) Identification of a conserved *Plasmodium falciparum* var gene implicated in malaria in pregnancy. *J Infect Dis* **185**: 1207–1211.
- Salanti, A., Jensen, A.T., Zornig, H.D., Staalsoe, T., Joergensen, L., Nielsen, M.A., *et al.* (2002) A sub-family of common and highly conserved *Plasmodium falciparum* var genes. *Mol Biochem Parasitol* **122**: 111–115.
- Salanti, A., Staalsoe, T., Lavstsen, T., Jensen, A.T., Sowa, M.P., Arnot, D.E., *et al.* (2003) Selective upregulation of a single distinctly structured var gene in chondroitin sulphate A-adhering *Plasmodium falciparum* involved in pregnancy-associated malaria. *Mol Microbiol* **49**: 179–191.
- Smith, J.D., Chitnis, C.E., Craig, A.G., Roberts, D.J., Hudson-Taylor, D.E., Peterson, D.S., *et al.* (1995) Switches in expression of *Plasmodium falciparum* var genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. *Cell* **82**: 101–110.
- Smith, J.D., Craig, A.G., Kriek, N., Hudson-Taylor, D., Kyes, S., Fagen, T., *et al.* (2000) Identification of a *Plasmodium falciparum* intercellular adhesion molecule-1 binding domain: a parasite adhesion trait implicated in cerebral malaria. *Proc Natl Acad Sci USA* **97**: 1766–1771.
- Smith, J.D., Gamain, B., Baruch, D.I., and Kyes, S. (2001) Decoding the language of var genes and *Plasmodium falciparum* sequestration. *Trends Parasitol* **17**: 538–545.
- Staalsoe, T., Megnekou, R., Fievet, N., Ricke, C.H., Zornig, H.D., Leke, R., *et al.* (2001) Acquisition and decay of antibodies to pregnancy-associated variant antigens on the surface of *Plasmodium falciparum*-infected erythrocytes that protect against placental parasitemia. *J Infect Dis* **184**: 618–626.
- Su, X.Z., Heatwole, V.M., Wertheimer, S.P., Guinet, F., Herfeldt, J.A., Peterson, D.S., *et al.* (1995) The large diverse gene family var encodes proteins involved in cytoadherence and antigenic variation of *Plasmodium falciparum*-infected erythrocytes. *Cell* **82**: 89–100.
- Vazquez-Macias, A., Martinez-Cruz, P., Castaneda-Patlan, M.C., Scheidig, C., Gysin, J., Scherf, A., and Hernandez-Rivas, R. (2002) A distinct 5' flanking var gene region regulates *Plasmodium falciparum* variant erythrocyte surface antigen expression in placental malaria. *Mol Microbiol* **45**: 155–167.
- Walter, P.R., Garin, Y., and Blot, P. (1982) Placental pathologic changes in malaria. A histologic and ultrastructural study. *Am J Pathol* **109**: 330–342.
- Whitehorn, E.A., Tate, E., Yanofsky, S.D., Kochersperger, L., Davis, A., Mortensen, R.B., *et al.* (1995) A generic method for expression and use of 'tagged' soluble versions of cell surface receptors. *Biotechnology (NY)* **13**: 1215–1219.
- World Health Organization (2003) Malaria kills 3000 children a day in Africa. *Bull WHO* **81**: 472.