Biochemical and Immunological Characterization of *Plasmodium falciparum* (Welch, 1897) Erythrocyte Membrane Protein (PfEMP) -1 Domains

Dissertation

Submitted in partial fulfillment of the requirements for the doctoral degree from the Department of Biology, Faculty of Mathematics, Informatics and Natural Sciences, University of Hamburg, Germany

By

Yu-Shan Chia

from Kuala Lumpur, Malaysia

Hamburg, 2006

List of Abbreviations

List of Abbreviations:

ATS	Acidic terminal sequence
bp	base-pair
BSA	Bovine serum albumin
BVH	Bovine vitreous humor
C2	Constant 2
Ca^{2+}	Calcium
CHO	Chinese hamster ovary cell
CIDR	Cysteine-rich interdomain regions
CR-1	Complement receptor-1
CSA/ CSB/ CSC	Chondroitin sulfate A, B or C
d	day
DBL	Duffy-binding like
DEAE	Diethylaminoethyl
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
ds / ss DNA	Double or single stranded DNA
DTT	Dithiothreitol
EDTA	Ethylene-diamine-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium bromide
FCS	Fetal calf serum
GAG	Glycosaminoglycan

h	hour
HA	Hyaluronic acid
HCl	Hydrochloride acid
HRP	Horse-radish peroxidase
HUC	Human umbilical cord
ICAM-1	Intercellular adhesion molecule-1
IE	*Plasmodium falciparum*-infected erythrocytes
Ig G / M	Immunoglobulin G / M
IPTG	Isopropyl β-D-1-thiogalactopyranoside
K / Na PO_4	Potassium or sodium phosphate
KAHRP	Knob-associated histidine-rich protein
kD	kilodalton
LB	Luria Bertani
MESA	*Plasmodium falciparum* mature erythrocyte surface antigen
mg	milligram
min	minutes
ml	milliliter
NaOH	sodium hydroxide
NTS	N-terminal sequence
OD	Optical density
PAM	Pregnancy-Associated Malaria
PBS	phosphate-buffered saline
PCR	Polymerase chain reaction

PFA	Paraformaldehyde
PfEMP-1	*Plasmodium falciparum* erythrocyte membrane protein-1
RNA	Ribonucleic acid
RT	Room temperature
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	second
TBE	Tris-borate buffer with EDTA
TBS	Tris-buffered saline
TE	Tris EDTA
UV	Ultraviolet
V	Volts
VSA	Variant surface antigen
μg	microgram

1. Introduction

1.1 Malaria - the disease

Malaria remains one of the major infectious diseases in tropical and subtropical areas, causing high mortality and morbidity. Of the four species of *Plasmodium* that naturally infect humans, *Plasmodium vivax, P. ovale* and *P. malaria* cause severe morbidity, whereas *P. falciparum* is responsible for nearly all malaria-specific mortality. It has been recently estimated that 2.2 billion people are exposed to the threat of *P. falciparum* malaria; there are around 300 and 600 million clinical attacks per year, and 1.1 million deaths of children under the age of five (Snow et al., 2005).

1.2 The life cycle of Plasmodium falciparum

The life cycle of *P. falciparum* in the human host starts with the sporozoites being injected through the bite of an infected female anopheline mosquito. The sporozoites are transported via the bloodstream to infect the hepatocyte cells of the liver. Within the hepatocytes, the parasite matures, differentiates, and undergoes several rounds of asexual division. Thousands of infective merozoites are formed and released into the bloodstream. The time taken for the exoerythrocytic cycle to occur is approximately 15 d and during this pre-patent period, the infected individual is asymptomatic (Taylor, Strickland, 2000; White, 2003). Merozoites released from the hepatocytes invade host erythrocytes within seconds, and again undergo a process of growth and asexual division to produce 6 – 32 daughter merozoites over a period of 48 h (Taylor, Strickland, 2000; White, 2003). When

the daughter merozoites fully mature, the infected erythrocyte (IE) bursts and merozoites are released to invade other erythrocytes (Figure 1).

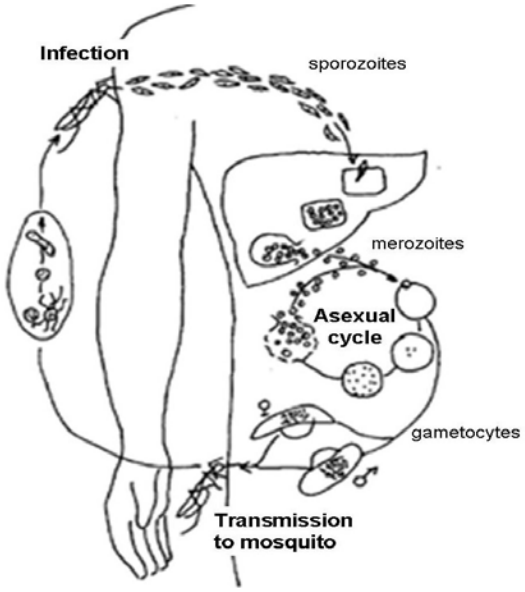


Figure 1 The life cycle of Plasmodium falciparum within the insect vector (sexual) and the human host (asexual). (taken from www.tigr.org/tdb/edb/pfdb/lifecycle.html)

A small proportion of the infecting merozoites undergoes an alternative pathway of differentiation producing micro- and macrogametocytes, which are taken up by the female anopheline mosquito. The sexual life cycle starts in the mid-gut of the mosquito when the gametocytes differentiate into gametes. They then fuse and form zygotes, which then transforms into ookinetes. Sporogony starts when the ookinetes penetrate the wall of the mid-gut and develop into oocysts. Mature sporozoites exit the oocysts, enter the hemolymph, and invade the salivary glands (Taylor, Strickland, 2000; White, 2003). The sporozoites are now ready to infect another human host, thereby completing the life cycle.

The process of exponential growth in the asexual cycle within the human host erythrocytes is responsible for all the clinical features of malaria. The asexual cycle of the *P. falciparum* will continue until the host dies unless intervened by chemotherapy or

immune response. The growth of the parasite within the erythrocyte is roughly synchronous. The rupture of the IE and the concomitant release of material that induces pyrogenic cytokines tend to occur at the same time of the day and coincide with the onset of periodic fever and chills, which are notable features of the disease.

1.3 The P. falciparum *infected erythrocytes*

The mature erythrocytes are terminally differentiated, devoid of all intracellular organelle and incapable of *de novo* protein or lipid synthesis (Haldar et al., 2002). As *P. falciparum* develops in the host erythrocyte, a number of changes that are important for parasite survival take place, including: (1) modification of the host cell membrane to form knob-like structures, leading to an increase in the rigidity of the cells, (2) alteration in metabolite transport, and (3) insertion of parasitic proteins onto the surface of the erythrocyte membrane (Craig, Scherf, 2001).

1.3.1 Knob formation

The most obvious modification at the IE surface is knob formation around 16-18 h post-infection (Nagao et al., 2000; Horrocks et al., 2005). Knobs are electron-dense, cup-shaped structure (~100 nm diameter) that underlie a protrusion of the erythrocyte's membrane and act as attachment points of sequestered parasites (Figure 2). Knobs consist of a number of parasite-encoded proteins, including knob-associated histidine-rich protein (KAHRP), *P. falciparum* erythrocyte membrane protein-3 (PfEMP-3) and *P. falciparum* mature erythrocyte surface antigen (MESA). Although exported to the IE plasma membrane, these proteins are not exposed on the external face (Deitsch, Wellems, 1996). KAHRP is known to be a major structural component of knobs since the deletion

of the gene that encodes this protein results in knobless (K^-) IE (Crabb et al., 1997). Spontaneous deletion of the KAHRP gene located on one arm of chromosome 2 typically occurs in parasites kept in culture (Biggs et al., 1989; Lanzer et al., 1994). K^- IE exhibit a reduced level of adhesion to endothelial cell lines or purified host ligands.

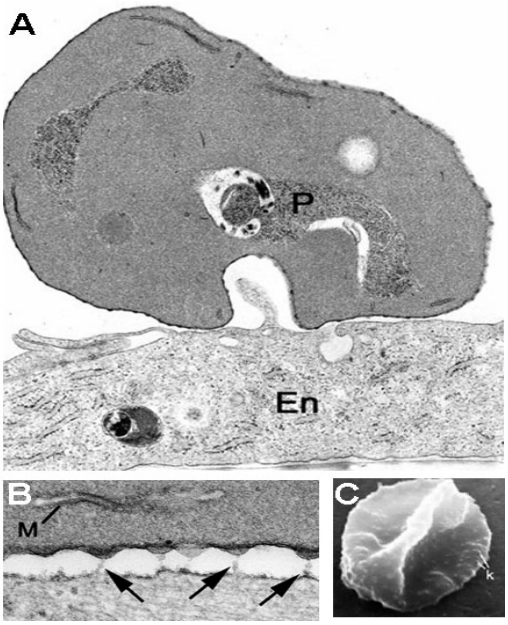


Figure 2 Electron micrographs of P. falciparum-infected erythrocytes (IE). (A) Low-power transmission micrograph of an IE (P) adhering to an endothelial cell (En). (B) Upon higher magnifications. Strands of connecting material (indicated by arrows) between knobs and the surface of the endothelial cell can be seen. Note the presence of a Maurer's cleft (M). (C) A low power scanning micrograph of an IE illustrating its irregular shape and the presence of knobs (k) on the surface. (Taken from Horrocks et al., 2005)

1.3.2 Expression of var gene encoded Plasmodium falciparum erythrocyte membrane protein (PfEMP)-1 proteins

The *P. falciparum* erythrocyte membrane protein (PfEMP) -1 is the most well known and the best-characterized variant surface antigen (VSA). PfEMP-1 molecules are expressed on the surface of knobs and interact with proteins within the knobs such as KAHRP and

PfEMP-3. PfEMP-1 molecules are multi-domain proteins of 200 – 400 kDa. The genes that encode these proteins have been identified as *var* genes.

The *var* genes are rich in A/T content and are located predominantly in the subtelomeric regions of all fourteen chromosomes (Rubio et al., 1996). There are ~ 60 copies of the *var* genes in the haploid 3D7 genome (Gardner et al., 2002). Each gene consists of a long, highly variable exon I, a relatively conserved intron, and a short conserved exon II (Figure 3A). Exon I encodes the N-terminal sequence (NTS), cysteine-rich Duffy-binding like domains (DBL), cysteine-rich interdomain regions (CIDR) and constant 2 (C2) domains. Both DBL and CIDR domains have been grouped into different sequence types based on the presence of key sequence motifs (Smith et al., 2000; Robinson et al., 2003). The six different DBL subtypes are numbered α to ε together with the unclassified X types, and the three different subtypes of CIDR are α, β and γ. Some of the DBL and CIDR domains have been attributed with adhesion functions (Figure 3B). Different subtypes, order, and number of DBL and CIDR domains make up the different PfEMP-1 molecule variants (Figure 3C).

Exon II codes for a highly conserved acidic terminal sequence (ATS). The ATS segment interacts with proteins within the knobs to anchor the PfEMP-1 molecule onto the surface of the erythrocyte membrane. The intron sequence of the *var* genes varies in length from 170 to ~1200 bp. It has been suggested from experimental data that a single member of the *var* gene family is expressed at the surface of a given IE and determines the host adhesion receptor preference of the IE (Rowe et al., 1997; Chen et al., 1998; Scherf et al., 1998).

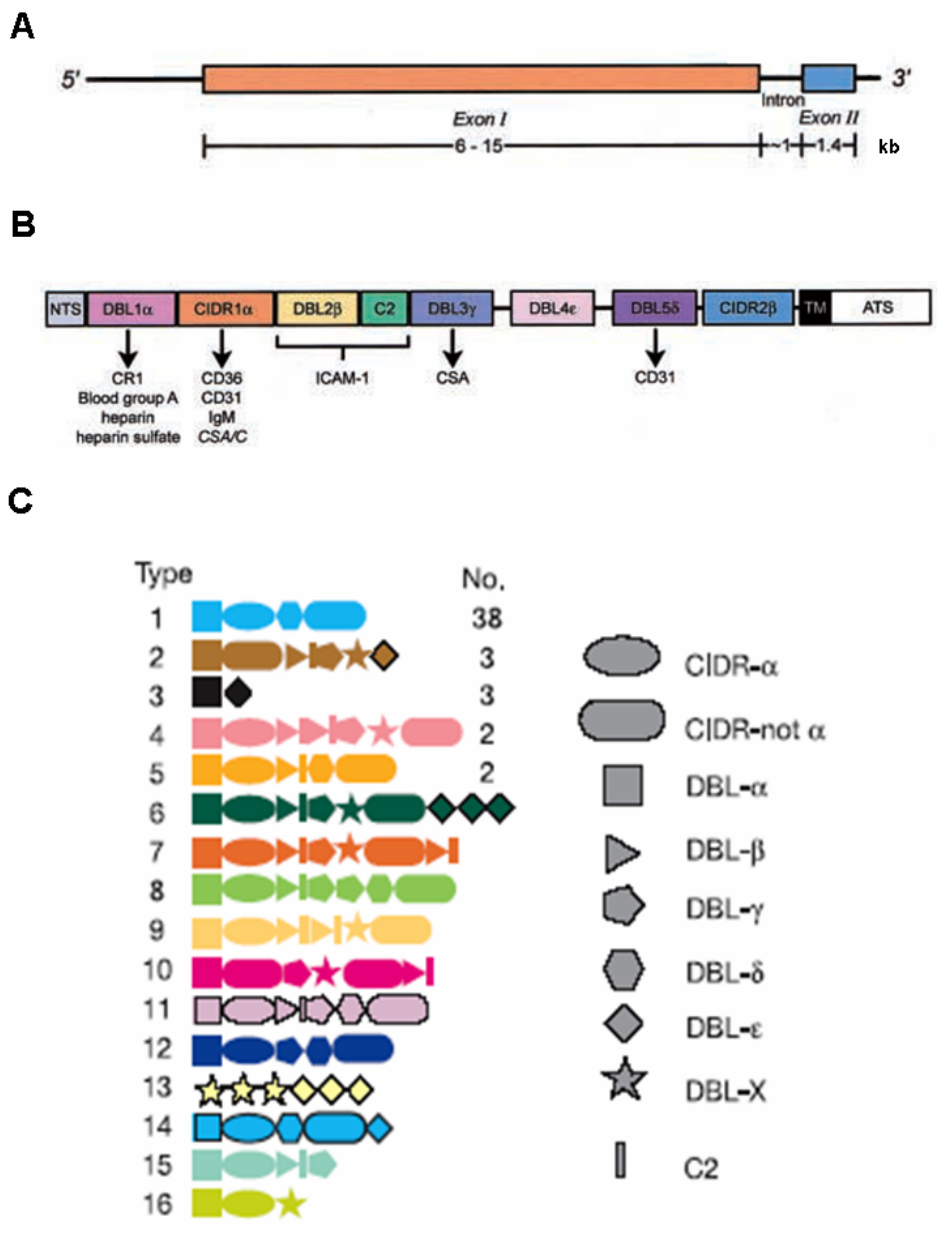


Figure 3 *var* gene and its domain architecture. (A) Schematic representation of the structural organization of *var* genes. Exons are represented by rectangles. (B) Schematic representation of the domain organization of PfEMP-1. Domains mediating particular adhesive interactions are indicated together with the host receptor-binding partners. (C) The domain organization of 3D7 *var* genes. The relative order of the domains in each gene is indicated. *var* genes with the same domain types in the same order have been colour coded as an identical class and given an arbitrary number for their type and the total number of members of each class in the genome of the *P. falciparum* clone 3D7. CR1 Complement receptor 1; ICAM-1 intercellular adhesion molecule-1; CSA chondroitin sulfate A; IgM immunoglobulin M. (Taken from Andrews, Lanzer, 2002; Gardner et al., 2002)

1.4 Cytoadhesion to host endothelial cells and parasite sequestration

Erythrocytes infected with young forms of the parasite circulate freely, whereas erythrocytes infected with the more mature forms of the parasite "disappear" from the circulation, in a process called sequestration. It is a means that the IE has evolved to escape splenic clearance. The result of sequestration is the obstruction of the capillary, a major cause of malaria pathogenesis. Several molecules on the surface of endothelial cells, such as CD36 (Barnwell et al., 1989), the intercellular adhesion molecule-1 (ICAM-1) (Berendt et al., 1989), and chondroitin sulfate A (CSA) (Rogerson et al., 1995) have been identified to be able to support IE binding (Tab. 1) (for review see Smith, Craig, 2005).

Table 1 Adhesion phenotypes of *P. falciparum*-infected erythrocytes

Target cells	Receptors involved	Process
Endothelial	Thrombospondin, CD36, ICAM-1, VCAM-1, E-Selectin, CD31, CSA, P-Selectin	Microvasculature sequestration
Syncytiotrophoblasts	CSA, HA	Placental sequestration
Uninfected erythrocytes	CR1, Blood group antigen ABO, CD36, heparin sulfate	Rosetting

(Adapted from Smith, Craig, 2005)

IE display a range of different binding properties and individual IE differs in their receptor specificity depending on the surface-expressed PfEMP-1 molecule. Parasite tropism for different tissues can have an impact on disease outcome. However, the mechanism that influences IE-receptor-specificity in different organs is unknown. It is likely that a combination of binding events acting synergistically brings about severe disease. Increased binding of IE to the ICAM-1 receptors and the formation of rosettes in the brain have been associated with higher risks of cerebral malaria (Turner et al., 1994;

Fernandez-Reyes et al., 1997; Rowe et al., 1995). Severe malaria patient isolates have been reported to bind multiple receptors (Heddini et al., 2001).

1.5 Pregnancy-associated malaria (PAM)

Pregnancy-associated malaria (PAM) is a major global health problem. An estimated 50 million women living in malaria endemic regions become pregnant annually and thousands of them will die from malaria. PAM-related complications cause the death of 62,000 – 363,000 African infants annually (Beeson, Duffy, 2005). Adults living in malaria endemic areas acquire protective immunity against malaria (Figure 4). However, pregnant women apparently lose this immunity and become susceptible to malaria infections, especially during her first pregnancy. For women living in high transmission areas, this susceptibility diminishes over successive pregnancies (Brabin, 1983; Hviid, 1998; Brabin et al., 2004). Accumulation of IE in the placenta is the key feature of PAM and can lead to a range of complications such as maternal anaemia, low birth-weight infant, infant anaemia, spontaneous abortions, stillbirth, and prematurity (McGregor, 1984; Fleming, 1989). Intermittent treatment with anti-malarial drugs during pregnancy has been shown to be an effective preventive measure against PAM. However, due to the advent of drug resistance parasites coupled with the issues of teratogenicity and embryotoxicity that complicate the effort to identify new treatments and prophylaxis strategies for pregnant women, other preventive measures such as vaccination may be a more promising aspect of disease control.

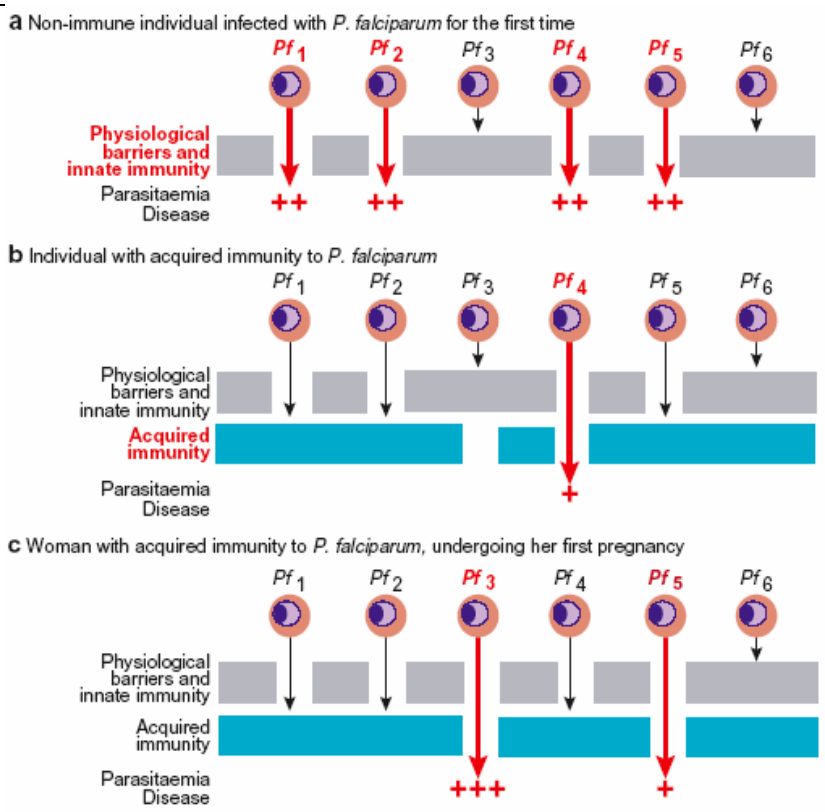


Figure 4 Schematic representation of the resistance to *P. falciparum*-infection. The different levels of immunity to malaria are shown for (A) non-immune individuals infected for the first time, (B) individuals with acquired immunity to *P. falciparum*, and (C) women with acquired immunity undergoing her first pregnancy. Different antigenic variants of IE have been designated $Pf_{1\text{-}6}$ in the figure. (Taken from Hviid, 1998)

1.5.1 Placental sequestration and binding to CSA

Sequestration of IE occurs in the intervillous space of the placenta. It is believed that IE bind to chondroitin sulfate A (CSA) (Rogerson et al., 1995; Fried, Duffy, 1996), a glycosaminoglycan (GAG) present on the cells lining the placenta (Figure 5). In vitro binding assays show that most placental-derived IE can bind CSA immobilized on plastic plates, but exhibit no binding affinity to either CD36 or ICAM-1 (Rogerson et al., 1995). In addition, these IE do not form rosettes (Rogerson et al., 2000). Moreover, soluble CSA was shown to have the in vivo capacity to de-sequester IE from the microvasculature in *P.*

falciparum-infected Saimiri monkeys (Pouvelle et al., 1997). A switch to CSA binding by the IE may contribute to the change of tissue tropism and is probably the key molecular event in the disease process observed during PAM. Indeed, the magnitude of CSA-binding exhibited by placental isolates can be correlated with the severity of PAM (Tuikue Ndam et al., 2004).

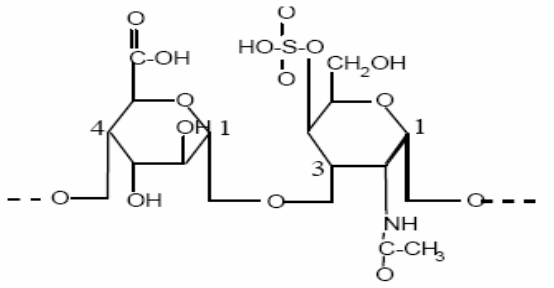


Figure 5 Disaccharide unit of chondroitin sulfate A (CSA)

The IE-CSA interaction depends on structural motifs specified by the degree of sulfation and chain length of the CSA molecule. IE do not bind to two other known GAG molecules, CSB and CSC, nor can these molecules inhibit IE binding to CSA. CSB and CSC differ from CSA in their sugar moiety and sulfation, respectively. The optimal CSA motif for interaction with IE comprises dodecasaccharide sequence formed by mixed non-sulfated and 4-O-sulfated N-acetyl-galactosamine alternating with glucuronic acid (Muthusamy et al., 2004).

Recently, it was shown that hyaluronic acid (HA) (Beeson et al., 2000; Beeson et al., 2004) might also be involved in parasite binding in the placenta (Figure 6)). However, the role of HA, a non-sulfated GAG, in parasite placental sequestration is still questionable. CSA is often present as a contaminant in HA preparations (Achur et al., 2000) and so far, highly purified preparations have failed to support binding of placental

isolates in vitro (Valiyaveettil et al., 2001). Moreover, HA is only present in low amount in the placental intervillous space, amounting to only 1 – 2 % of the total GAG content (Achur et al., 2000).

Another new adhesive interaction involving non-immune IgG has been described (Flick et al., 2001), postulated to act as a bridge between neonatal Fc receptors on the placenta and the IE. However, neonatal Fc receptors are normally localized to vesicles in the syncytiotrophoblast, where they bind IgG taken up by pinocytosis (Kristoffersen, Matre, 1996; Duffy, Fried, 2001; Lyden et al., 2001). It is not understood how the IgG-IE binding takes place only with the neonatal Fc receptors and not with other Fc receptors expressed in vascular beds of other organs. It is not known whether many of these interactions are artifacts, caused by the preparation of ex vivo placental cryosections used for binding assays, which may result in the exposure of host molecules that are otherwise not accessible for binding (Duffy, Fried, 2001).

The importance of placental sequestration was emphasized by the discovery that during pregnancy, malaria infected women develop antibodies that inhibit IE-CSA binding. Such antibodies are associated with protection against placental infection and PAM complications (Fried et al., 1998; Ricke et al., 2000; O'Neil-Dunne et al., 2001; Duffy, Fried, 2003; Staalsoe et al., 2004). These adhesion-blocking antibodies were shown to be produced in a strain-independent manner (Fried et al., 1998; Ricke et al., 2000). The identification of the parasite ligand that mediates CSA-binding and is the target of adhesion-blocking antibodies would have great implications in the development of a vaccine against PAM.

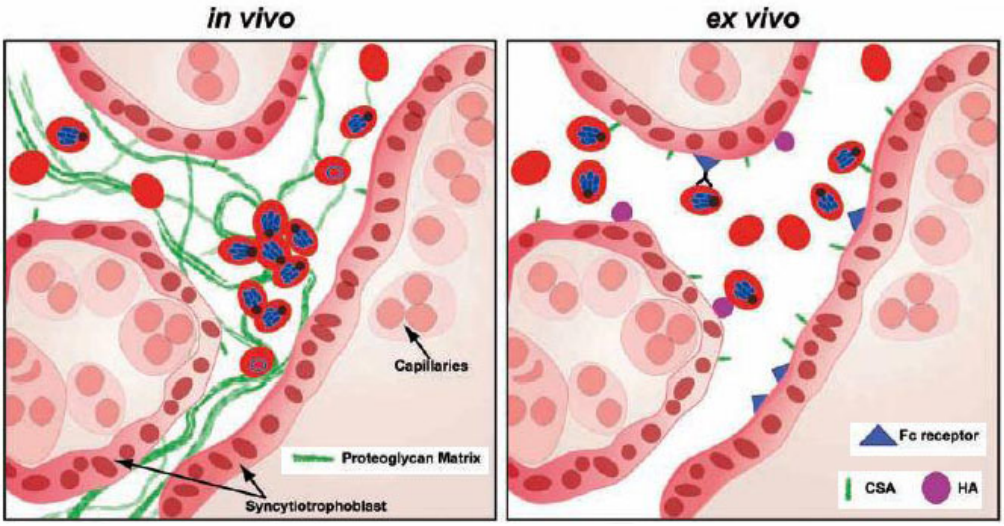


Figure 6 Schematic representation of cytoadhesive events in the placenta under in vivo and ex vivo conditions. Under in vivo conditions, IE sequester in the intervillous space by binding to an extracellular proteoglycan matrix. Under ex vivo conditions, the IE adhere directly to otherwise non-exposed receptors on the syncytiotrophoblast surface. HA hyaluronic acid, CSA chondroitin sulfate A, Fc neonatal receptors. (Taken from Andrews, Lanzer, 2002)

1.5.2 DBL-γ domains as CSA-binding ligands

The extensive study of PfEMP-1 since its identification on the IE surface has led to the recognition of its role in mediating cytoadhesion endothelial cells. Reeder et al. first showed that antibodies to recombinant DBL-γ inhibited CSA binding of a cloned cell line (Reeder et al., 1999). At the same time, Buffet et al. demonstrated that a CHO cell line expressing a DBL-γ domain of PfEMP-1 directly bound CSA (Buffet et al., 1999). Since then, several DBL-γ domains from different *var* genes have been shown to bind CSA in vitro (Vazquez-Macias et al., 2002; Gamain et al., 2002; Gamain et al., 2004). Antibodies raised against recombinant DBL-γ domains are adhesion blocking and capable of recognizing surface epitopes on heterologous CSA-adherent IE (Reeder et al., 1999; Lekana Douki et al., 2002; Costa et al., 2003; Chia et al., 2005).

Furthermore, DBL-γ domains cloned from placental *var* genes demonstrated CSA binding capacities and conservation in placental isolate in a spatial and temporal manner (Khattab et al., 2001; Khattab et al., 2003). These placental DBL-γ domains were termed *var*PAM DBL-γ and members of this group exhibited sequences with 39 – 55 % amino acid identities between each other (Khattab et al., 2001). Together these observations provided first clues that the DBL-γ domain may be the elusive conserved antigen recognized by immune pregnant women antibodies, thereby raising hopes to develop a potential vaccine candidate based on information on the DBL-γ domain.

1.5.3 *var* genes implicated in PAM

The DBL-γ domains identified in 1999 were encoded by two different *var* genes, the FCR3*var*CSA (Buffet et al., 1999) and CS2*var* (Reeder et al., 1999). Detailed studies have shown that the FCR3*var*CSA gene was equally transcribed in all isolates (in both CSA-binding and non-CSA-binding IE), so that a role of this *var* gene in causing PAM has become questionable (Fried, Duffy, 2002; Winter et al., 2003; Fried et al., 2004). A similar argument against a role of CS2*var* gene is such that the gene was transcribed at low or undetectable levels, and levels do not increase in CSA-binding parasites (Duffy et al., 2002; Fried, Duffy, 2002; Fried et al., 2004). In contrast, a third PAM-related *var* gene, termed *var*2csa, was shown to be up-regulated in CSA-binding parasites (Salanti et al., 2003). Moreover, maternal antibody recognition to protein domains of the *var*2csa gene correlated to parity (Salanti et al., 2004), an observation that the FCR3*var*CSA and CS2*var* domains were lacking. However, an analysis of IE membrane proteins by mass spectrometry did not show a preferential expression of either *var*2csa or FCR3*var*CSA

proteins by CSA-binding or placental isolates. Interestingly, several novel PfEMP-1 sequences were reported to be preferentially expressed but as yet their link to causing disease awaits to be demonstrated (Fried et al., 2004).

These observations highlight the *var* gene diversity between different parasites. The need to extend the study of *var* genes, particularly those originating from wild isolates, is great. Clearly, work carried out so far on laboratory-adapted clones cannot provide a true picture of the natural situation.

1.6 Objectives of this study

1. To clone, express and purify *var*PAM DBL-γ domains identified from placental isolates as histidine-tagged fusion proteins in *Escherichia coli,*
2. To biochemically characterize the recombinant proteins for their capacity to bind receptor molecules expressed on CHO cell surface and to inhibit in vitro IE binding to CSA,
3. To evaluate their immunogenicities in a cohort of plasma samples collected from pregnant women in ELISA and to correlate their antibody levels to disease states,
4. To clone and express in *E. coli* individual domains of the full-length 732*var* gene of a placental isolate,
5. To investigate the in vitro binding properties of the recombinant proteins,
6. To compare their immune recognition by pregnant women sera, aimed at understanding a possible involvement in PAM.

2. Materials

2.1 Chemicals and reagents

1,4-dithio-DL-threitol (DTT)	Roth, Germany
Acetic acid	Roth, Germany
Acrylamide/bis solution	Merck, USA
Agar	Roth, Germany
Albumax	Gibco, USA
Ammonium persulfate (APS)	Roth, Germany
Bacto-tryptone	Roth, Germany
Bacto-yeast extracts	Roth, Germany
Borate	Roth, Germany
Chondroitin sulfate A, B and C (CSA, CSB, CSC)	Sigma, USA
Coomassie Brilliant Blue	Roth, Germany
DMSO	Sigma, USA
Ethanol	Roth, Germany
Freund's complete/incomplete adjuvant	Sigma, Germany
Glucose	Roth, Germany
Glycine	Roth, Germany
HEPES	Roth, Germany
Hyaluronic acid from bovine vitreous humour	Sigma, USA
Hyaluronic acid from human umbilical cord	Sigma, USA
Hypoxanthine	Sigma, USA

Imidazole	Sigma, USA
Isopropyl β-D-thiogalactopyranoside (IPTG)	Roth, Germany
KCl	Roth, Germany
KH_2PO_4	Roth, Germany
L-glutamine	PAA, Austria
Low-fat dried milk powder	Roth, Germany
Methanol	Roth, Germany
Na lactate	Sigma, USA
Na_2 EDTA	Roth, Germany
Na_2HPO_4-$2H_2O$	Roth, Germany
Na_2HPO_4-$7H_2O$	Roth, Germany
NaCl	Roth, Germany
NaH_2PO_4-H_2O	Roth, Germany
sodium bicarbonate	Sigma, USA
Ponceau S	Roth, Germany
Sodium dodecyl sulfate (SDS)	Roth, Germany
Trichloracetic acid	Sigma, USA
Trizma base	Roth, Germany

2.2 Instruments and apparatuses

Beckmann JA 12 centrifuge	Beckmann, USA
Centrifuge 5415 D and 5810 R	Eppendorf, Germany
Leitz inverted microscope	Leitz, Germany

Leitz fluorescence microscope	Leitz, Germany
Mini Protean II Gel Electrophoresis Chamber	Bio-Rad, Germany
Power Pac 300	Bio-Rad, Germany
Primus Thermal Cycler	MWG Biotech, Germany
*vario*MACS	Miltenyi Biotec, Germany

2.3 Laboratory consumables

0.2 ml PCR reaction tubes	Sarstedt, Germany
15 ml Falcon tubes	Greiner, Germany
50 ml Falcon tubes	Greiner, Germany
Aluminium foils	Roth, Germany
96-well EIA/RIA Flat Bottom Plate	Corning, USA
Cell culture flasks (25 cm^2, 75 cm^2)	Sarstedt, Germany
Combitips (10 ml)	Eppendorf, Germany
Coverslips	Sarstedt, Germany
Cryovials	Sarstedt, Germany
Gloves	Kimberley Clark, USA
Immersion oil	Roth, Germany
Immunoblot cellulose membrane	Bio-Rad, Germany
MACS column CS	Becton Dickinson, Germany
Microfuge tubes (0.5 ml, 1.5 ml, 2.0 ml)	Sarstedt, Germany
Microscope glass slides	Roth, Germany
Petri dishes (Cat. Num. 351016)	Becton Dickinson, Germany

Petri dishes	Roth, Germany
Pipette tips (10 µl, 200 µl, 1000 µl)	Sarstedt, Germany
Saran wraps	Roth, Germany
Sterile filter units (0.45 µm, 0.2 µm)	Roth, Germany
Sterile filter units 0.2 µm (250 ml, 500 ml)	Millipore, Germany
Whatmann™ 3MM paper	Roth, Germany

2.4 Bacteria cells, mammalian cells and Plasmodium falciparum*-infected erythrocytes*

2.4.1 *Escherichia coli* cells

TOP10

F$^-$*mcr*AΔ(*mrr-hsd*RMS-*mcr*BC)Φ80*lac*ZΔM15Δ*lac*X74*rec*A1*deo*R*ara*D139Δ(*ara-leu)*7697*gal*U*gal*K*rps*L(Str^R)*end*A1*nup* (Invitrogen)

2.4.2 Mammalian Chinese hamster ovary (CHO) cells

Chinese ovary cell (CHO) K1

Chinese hamster ovary wild type cell expresses a wide repertoire of glcosaminoglycans (GAG) including chondroitin sulfates and hyaluronic acids on the cell surface. (Invitrogen)

CHO-pgsa-745 (CHO-745)

CHO mutant cell line with a defect in xylosyltransferase, which is involved in the early initiation step of GAG *de novo* synthesis, and do not express GAG molecules. (Gift from T. Staalsoe, Copenhagen)

CHO-CD36

CHO-745 stable transfectant expressing human CD36 receptor on the cell surface. (Gift from T. Staalsoe, Copenhagen)

CHO-ICAM-1

CHO-745 stable transfectant expressing the human intracellular adhesion molecule (ICAM)-1 on the cell surface. (Gift from T. Staalsoe, Copenhagen)

2.4.3 *P. falciparum*-infected erythrocytes (IE)

Laboratory strains

FCR3: FCR3CSA, FCR3CD36, FCR3ICAM-1 and non-selected FCR3

Wild isolates

(Gabonese placental isolate)	Gb337 / Gb337CSA
(Gabonese cerebral isolate)	Gb03 / Gb03CSA
(Senegalese placental isolate)	VIP43 / VIP43CSA

2.5 GenBank accession numbers

482 DBL-γ	AF334803
498 DBL-γ	AF334804
701 DBL-γ	AF334805
3D7*chr*5*var*	AL929354
732*var*	AY679117
A4*tres*	AF193424
A4*var*	L42244

FCR3*var*CSA	AJ133811
Gb23aDBL-1α	AF366357
MC*var*	AAB60251
*var*2csa	NP_701371

2.6 Plasma sample collection and patient data

The pregnant women cohort collection was conducted in Thiadiaye, Senegal from September 2001 to May 2002. These samples were provided for use in this thesis by the courtesy of P. Deloron and N. Fievet, Paris. The mean age of the 247 women was 26 ±7 years. Amongst them were 59 primiparous women, 52 secundiparous, and 136 multiparous. 36 pregnant women showed placental infection and 21 showed peripheral infection upon microscopical examination of thick blood smear. Placental slides from 12 women and peripheral blood slides from 2 women were not available. An additional 16 plasma samples were collected from 8 men and 8 nulliparous women living in the same endemic area. 66 non-exposed European plasmas were collected as controls.

2.7 Plasmids

pTrcHis2	Invitrogen
pTrcHis482	Available in laboratory
pTrcHis498	Available in laboratory
pTrcHis701	Available in laboratory
pTrcHis732	Available in laboratory
pRIG	Courtesy of W. Hol, Seattle

2.8 Oligonucleotides

The following oligonucleotides (Table 2) were ordered from Qiagen, Germany:

Table 2 Primers used to amplify PfEMP-1 domains and their sequences

Primer name	Sequence
732 DBL-3γ 5’	GCGTGTGAAATAGTGGAT
732 DBL-3γ 3’	CTTACCTGCTTCTTTATC
3D7*chr*5 DBL-5γ 5’	CCTAAATGGAGTTGTATTGTAGGT
3D7 DBL-5γ 3’	GTTATCTTTATCCTTTTTGAACTTTTC
732 DBL-1α 5’	CCCCCCCTCGAGTGGATATCTGCAGAATTCGCCC
732 DBL-1α 3’	CCCCCCAAGCTTCGTTGTCATACTATTAGATGC
732 CIDR-1α 5’	CCCGGGATAAATAACAAAAAA
732 CIDR-1α 3’	GTCGACTGGTCCACCGTTATT
732 DBL-2β 5’	CCCCCCCCCCAGATCTGCTAGTCGTGCTGGTAG
732 DBL-2β 3’	CCCCCCCTCGAGATAAGCATCAATACCATGAGC
732 DBL-4ε 5’	CCCCCCCCCCAGATCTCAAATAGCAAAACATTTACGTG
732 DBL-4ε 3	CCCCCCCTCGAGTTCATGATATAAATAATCTGTGC

2.9 Enzymes

Taq polymerase	Qiagen, Germany
Pwo polymerase	Peqlab, Germany

2.10 Antibiotics

Antibiotics used are listed in Table 3.

Table 3 List of antibiotics used in this study

Antibiotics	Stock concentration	Working concentration	Source
Ampicillin	50 mg/ml dissolved in water	50 µg/ml	Sigma, USA
Chloramphenicol	34 mg/ml dissolved in ethanol	34 µg/ml	Roth, Germany
Gentamycin	80 mg in 2 ml ampoule	40 µg/ml	Gibco, USA
Penicillin-Streptomycin	100 x	1 x	PAA, Austria

2.11 DNA and protein standards

100 bp DNA Ladder	Roth, Germany

1 kb DNA Ladder	Roth, Germany
Prestained Protein Marker, Broad Range	New England Biolabs, USA
Prestained Protein Marker	Roth, Germany
Molecular Protein Marker	Biorad, USA

2.12 Antibodies

Antibodies used are listed in Table 4.

Table 4 List of primary and secondary antibodies and the conjugated enzyme or fluorophore

Antibody	Conjugation	Source
Chicken anti-732 DBL3γ	-	Self-made
Rat anti-732 DBLγ	-	Self-made
Rat anti-482 DBLγ	-	Self-made
Mouse anti-482 DBLγ	-	Self-made
Goat anti-human IgG	-	Dako
Rabbit anti-chicken IgY	Peroxidase	Dianova
Rabbit anti-mouse IgG	Peroxidase	Dianova
Goat anti-rabbit IgG	Peroxidase	Dianova
Goat anti-rat IgG	Peroxidase	Dianova
Rabbit anti-mouse IgG	Alkaline phosphatase	Pierce
Goat anti-rat IgG	Alkaline phosphatase	Sigma
Goat anti-human IgG	Alkaline phosphatase	Sigma
Goat anti-chicken IgY	Alkaline Phosphatase	Dianova
Rabbit anti-chicken IgY	Biotin	Pierce
Rabbit anti-rat IgG	Biotin	Pierce
Rabbit anti-goat IgG	FITC	Dako
Rabbit anti-rat IgG	Alexa-fluor 488	Molecular Probes
Goat anti-chicken IgY	Alexa-fluor 594	Molecular Probes
Goat anti-mouse IgG	Cy3	Jackson
Avidin	Alexa-fluor 488	Molecular Probes
Avidin	Alexa-fluor 488	Molecular Probes

2.13 Buffers

1 M HEPES (500 ml, pH 7.2)

119.16 g of HEPES

10x phosphate-buffered saline (PBS) (1 L, pH 7.2

80 g NaCl

2 g KCl

26.8 g Na_2HPO_4-$7H_2O$

2.4 g KH_2HPO_4

**Osmolarity: 0.3 mol/kg

Tris buffer (500 ml)

1.5 M Tris, pH 8.8: 90.86 g of Trizma base

1.0 M Tris, pH 6.8: 60.57 g of Trizma base

1x Tris-buffered saline (TBS) (1 L, pH 7.5)

6.05 g of Tris

8.76 g of NaCl

**TBS is stable at 4ºC for 3 months.

1x TBS - 0.1 % Tween (1 L)

1 ml Tween-20 was dissolved in 1 L of TBS buffer

**TBS-Tween is stable at 4°C for 3 months

10x Tris-Borate EDTA buffer (TBE) buffer (1 L, pH 8)

108 g Tris

55 g Borate

40 ml of 0.5 M of Na_2EDTA

8x phosphate buffer (100 ml, pH 7.4)

1.42 g Na_2HPO_4-$2H_2O$

1.11 g NaH_2PO_4-H_2O

23.38 g NaCl

10x SDS-PAGE running buffer (1 L)

10 g SDS

30.3 g Tris

144.1 g glycine

**1x SDS-PAGE running buffer was prepared fresh prior to use.

5x Western blot transfer buffer (for wet blots)(1 L)

72.06 g glycine

15.14 g tris

1.87 g SDS

**1x transfer buffer was completed with 20 % methanol prior to use and discarded after 5 times of use.

0.1 M Na Phosphate ($NaPO_4$) buffer (200 ml, pH6.5)

27.6 g NaH_2PO_4-H_2O (MW=138) dissolved in 1 L of water

53.62 g Na_2HPO_4-7 H_2O (MW=268.1) dissolved in 1 L of water

68.5 ml of Na mono-salt solution was mixed with 31.5 ml of Na di-salt solution and the volume adjusted to 200 ml with water.

1 M K Phosphate (KPO_4) buffer (200 ml, pH7.2)

179.25 ml K_2HPO_4

70.75 ml KH_2PO_4

KPO_4 / 7 % PEG 6000 buffer (500 ml)

35 g PEG 6000 in 500 ml KPO_4 buffer

KPO_4 / 24 % PEG 6000 buffer (500 ml)

120 g PEG 6000 in 500 ml KPO_4 buffer

2.14 Media and plates

All culture media and sera were purchased from PAA, Austria unless stated otherwise.

2.14.1 *E. coli* cultures

Luria-Bertani (LB) Medium (1 L, pH 7.2)

10 g bacto-tryptone

5 g bacto-yeast extract

10 g NaCl

Luria-Bertani (LB) agar (1 L, pH 7.2)

10 g bacto-tryptone

5 g bacto-yeast extract

10 g NaCl

20 g agar

**The agar was cooled to 55ºC after autoclaving and poured into Petri dishes. Approximately 25 ml of liquid agar was poured into one 100 mm Petri dish.

LB-Ampicillin agar (1 L, pH 7.2)

10 g bacto-tryptone

5 g bacto-yeast extract

10 g NaCl

20 g agar

**25 mg of filter-sterilized ampicillin was added to the cooled liquid agar before pouring in to Petri dishes. For double antibiotic selection with ampicillin and

chloramphenicol, 50 µl of chloramphenicol (100µg/ml) was plated into the agar prior to use.

Super Medium (1 L, pH 7.2)

25 g bacto-tryptone

10 g bacto-yeast extract

5 g NaCl

2.14.2 P. falciparum *cultures*

Supplemented RPMI-1640 Medium (500 ml)

500 ml of RPMI-1640 medium

25 mM of HEPES (pH 7.2)

50 ml 10x Albumax

10 ml human serum (blood group AB+)

50 mg/ml gentamycin

10x Albumax (500 ml, pH 7.2)

5.2 g RPMI-1640 powder

2.98 g HEPES

1.67 g sodium bicarbonate

1 g glucose

0.1 g hypoxanthine

25 g Albumax II

50 mg/ml gentamycin

Glycerolyte freezing solution (100 ml, pH 6.8)

57 % v/v glycerol

1.6 g Na-lactate (Sigma)

30 mg KCl

100 ml of 25 mM sodium phosphate buffer (pH6.8)

Thawing solution

12 % and 1.6 % sterile saline

Binding medium (500 ml, pH 6.8)

5.2 g RPMI powder (without bicarbonate)

1.44 g Glucose

3.75 g HEPES (35.7 mM)

2.5 g BSA

2.14.3 Chinese Hamster Ovary (CHO) cell cultures

Supplemented Ham's Medium

500 ml of Hank's medium

10 % heat-inactivated FCS

100x penicillin-streptomycin

2 mM L-glutamine

Freezing solution

Complete Hank's medium

20 % heat-inactivated FCS

10 % DMSO

2.15 Solutions

Glycosaminoglycan (GAG) solutions

Chondroitin sulfate A, B, and C

Hyaluronic acid from bovine vitreous humor and human umbilical cord

**Stock solutions of 10 mg/ml were prepared by dissolving the GAG powder in PBS and stored at -20ºC. Working solutions were prepared by diluting the stock in PBS to the appropriate concentration.

10 % Sodium dodecyl sulfate (SDS)

10 g SDS was dissolved in 100 ml of water by heating to 68°C.

10 % Ammonium persulfate (APS) (10 ml)

1 g ammonium persulfate

The solution was aliquoted and kept at -20°C, at 4°C it is stable for 2 weeks.

Coomassie blue stain (1 L)

2.5 g Coomassie blue powder

450 ml methanol

100 ml acetic acid

Coomassie destaining buffer (1 L)

450 ml methanol

100 ml acetic acid

Ponceau S Solution (100 ml)

2 g Ponceau S

30 g trichloracetic acid

30 g sulfosalicylic acid

Western blot blocking solution

3 g of low-fat dried milk was dissolved in 100 ml of TBS buffer (3 %)

** The blocking solution are prepared fresh prior to use

1 M isopropyl β-D-thiogalactopyranoside (IPTG) (5 ml)

1.19 g IPTG (238.3 g/mol) was dissolved in 5 ml water

The IPTG was kept at -20°C in 1 ml aliquots

1 M 1,4-dithio-DL-threitol (DTT) (20 ml)

3.085 g DTT (154.25 g/mol) was dissolved in 20 ml water.

The solution was kept in 1 ml aliquots at -20°C.

3. Methods

3.1 Handling of cultures

3.1.1 Bacteria culture

Escherichia coli cells were cultured in Luria Bertani (LB) medium. The culture was supplemented with ampicillin (for pTrcHis2 plasmid) and/or chloramphenicol (for pRIG plasmid) to maintain the recombinant plasmids within the transformed bacterial cells (see Section 3.2.3.1). *E. coli* strains were grown in shaking cultures at 37ºC. Transformed bacteria clones were streaked on LB agar and kept at 4ºC. Glycerol (1:1 ratio to the culture) was added to the bacterial culture and kept at -70ºC.

3.1.2 *Plasmodium falciparum* culture

Plasmodium falciparum cultures were cultivated in T-25 and T-75 tissue culture flasks at 5 % haematocrit in 5 ml and 20 ml of completed RPMI-1640 medium, respectively. The cultures were kept in a special gas environment consisting of 5 % carbon dioxide, 5 % oxygen and 90 % nitrogen. Daily change of the culture medium was performed. Thin-blood films were prepared daily on glass slides to check the parasitaemia. Sub-cultures were prepared when the parasitaemia exceeds 5 %.

Frozen vials were removed from liquid nitrogen storage and thawed quickly in a 37°C water bath. The blood was transferred into a 50 ml Falcon tube and the volume within the vial was estimated (V). One-tenth of the blood volume ($^{1}/_{10}$ V) of 12 % warm NaCl was added to the blood slowly drop-wise while gently swirling the tube. The

solution was left standing at RT for 5 min. Following this, 10 V of 1.6 % warm NaCl was added to the solution slowly and drop-wise. The blood-saline solution was centrifuged at 1500 rpm for 5 min to pellet the blood cells. The supernatant was aspirated and the pellet was washed with 10 volumes of complete medium. The suspension was centrifuged and the medium removed. The pellet was resuspended in 5 ml complete medium and the haematocrit adjusted to 5 % before putting into culture.

Cultures containing erythrocytes parasitized by ring-stages of the parasite were centrifuged at 2000 rpm for 5 min. The supernatant was removed and the pellet volume of the packed cells, V, was estimated. One-third V ($^1/_3$ V) of glycerolyte was added slowly into the pellet and mixed gently. The suspension was left to stand for 5 min at RT. Then $^4/_3$ V of glycerolyte was added drop by drop and mixed gently. Aliquots of 1 ml of blood were frozen at -70ºC for a minimum of 18 h before putting into liquid nitrogen storage tanks for long-term storage.

3.1.2.1 Giemsa staining of thin blood films

A thin blood film was prepared from 40 µl of erythrocyte suspension, air-dried and fixed by immersing it in methanol for 30 sec. 10 % Giemsa solution was prepared in a staining jar. The glass slide was immersed in the Giemsa solution for 20 min then washed and dried.

3.1.2.2 Estimation of parasitaemia

The Giemsa-stained thin-blood film was observed under 100x magnifications. An area where the erythrocytes are evenly distributed was chosen for estimation of parasitaemia.

All erythrocytes and infected erythrocytes (IE) within the field were counted. This was done for 10 fields. Parasitaemia is calculated as:

Parasitaemia = number of IE/uninfected erythrocyte x 100%

3.1.2.3 Selection of cytoadherent **P. falciparum-***infected erythrocytes (IE)*

3.1.2.3.1 Selection of CSA-binding IE

Three-ml of CSA (10 µg/ml in PBS) was used to coat Petri dishes overnight at 4ºC. The CSA solution was aspirated and the plates blocked with filtered sterile 2 % BSA (in PBS) for 1 h at RT. Three-ml of parasite culture (at 5 % haematocrit, >10 % parasitaemia) was added after the removal of the blocking solution and incubated for 1 h at 37ºC. The plate was gently agitated to resuspend the erythrocytes every 15 min. After this, the parasite culture was aspirated and the plate was washed 3-5 times with binding medium. Thereafter 3 ml of completed medium at 5 % haematocrit was added into the plate. Seven such plates were used for every 20 ml parasite culture.

3.1.2.3.2 Selection of CD36- and ICAM-1-binding IE

CHO-CD36 and CHO-ICAM-1 cells were seeded and grown in T-25 flasks. The parasite (at 5 % haematocrit, > 5 %. parasitaemia) was washed once with binding medium and resuspended in 5 ml of binding medium. CHO cells were washed once with binding medium to remove growth medium and overlaid with the erythrocyte suspension. The parasite was incubated with CHO cells at 37ºC for 1-1.5 h. The flask was gently shaken every 15 min to resuspend the erythrocytes. Thereafter the flask was gently washed three times with binding medium to remove unbound erythrocytes. Binding of erythrocytes

was checked using an inverted microscope. Completed malaria medium and blood were added to the flask at 5 % haematocrit. The next day, the culture was collected and cultured in a new flask with fresh medium.

3.1.2.4 Sorbitol synchronization of IE

Synchronization was performed with young ring-stages of the IE. Four-ml of parasite culture was centrifuged at 2000 rpm for 5 min and the medium (supernatant) was removed. The cell pellet was resuspended in 4 ml of 5 % sorbitol (dissolved in water and filtered through a 0.2 μm membrane) and incubated for 10 min at RT. The suspension was shaken 2-3 times. The suspension was centrifuged and the supernatant removed. The pellet was washed 3 times with RPMI-1640 medium. The pellet was resuspended in complete medium (at 5 % haematocrit) after the last wash and put into culture. Synchronization was performed in four individual tubes for a 20 ml parasite culture.

3.1.2.5 Enrichment of late-stage IE by magnetic cell sorting (MACS)

A MACS column was first washed and flooded with 2 % FCS –PBS before insertion into the *vario*MACS. The flow-rate of the buffer from the column was adjusted until it is drop-wise. When the buffer was only a thin layer above the column bed, the parasite culture (at > 5 % haematocrit, containing mainly late-stage IE) was added into the column. The late-stage IE were retained in the column and washed with 30 ml of buffer before elution from the column. The IE was eluted from the column by flushing the column with 50 ml of buffer and was collected in a 50 ml Falcon tube. The IE was pelleted by centrifugation and the pellet resuspended in the appropriate volume of buffer. The enriched late-stage IE is now ready for downstream application.

3.1.2.6 Removal of surface expressed PfEMP-1 by trypsin treatment

100 µg/ml of trypsin was used to remove IE surface expressed PfEMP-1 molecules. Treatment was performed at 37ºC for 10 min followed by extensive washes with buffer to remove traces of trypsin.

3.1.3 Mammalian cell culture

Chinese hamster ovary (CHO) cells were detached from the surface of a T-25 culture flask with 1 ml of trypsin. The complete removal of medium before addition of trypsin is essential, as the Ca^{2+} in the medium will inactivate the trypsin. The cells were incubated with the trypsin buffer for 10-15 min at 37ºC until all cells have detached from the surface. Fresh culture medium was added into the flask to stop the reaction. For routine sub-culturing, a new culture was initiated with 1:20 dilution.

To freeze CHO cells, the cells were detached as described and pelleted. The cells were resuspended in pre-chilled freezing solution at $1x10^7$ cells/ml. Aliquots of the cell suspension was put on ice until transferred into -70ºC freezer or in liquid nitrogen for long-term storage.

CHO cells were thawed in a warm water bath quickly and transferred to fresh warm medium to initiate a new culture. The medium was replaced with fresh medium to remove traces of the cryo-preservatives.

3.2 Molecular biology

3.2.1 Polymerase chain reaction (PCR)

Routine PCR reactions were performed in 50 µl reaction volumes as following:

PCR reaction:	1 µl	DNA template
	5 µl	10x PCR buffer
	1 µl	2 mM dNTPs
	1 µl	Forward primer (10 µM)
	1 µl	Reverse primer (10 µM)
	40.5 µl	Sterile water
	0.5 µl	*Taq* polymerase (1 unit/µl)

The following cycling profile was used:

	95ºC	3 min
(5 cycles):	95ºC	30 sec
	55ºC	1 min
	68ºC	3 min
(5 cycles):	95ºC	30 sec
	50ºC	1 min
	68ºC	3 min
(25 cycles):	95ºC	30 sec
	45ºC	1 min
	68ºC	3 min
	68ºC	10 min

3.2.2 Purification of PCR product

The QIAquick PCR Purification Kit (Qiagen, Germany) was used to purify DNA fragments (100 bp to 10 kb) after PCR or enzymatic reactions. Five volumes of Buffer PB was added to 1 volume of the PCR sample and mixed. A QIAquick spin column was

placed in a 2 ml collection tube. The column was briefly centrifuged to bind the DNA to the column. The flow-through was discarded and the column was placed in the same collection tube. 0.75 ml of Buffer PE was added to wash the DNA bound on the column and centrifuged for 1 min. The flow-through was discarded and the column centrifuged for another 1 min to remove all traces of Buffer PE. Fifty-µl of Buffer EB (10 mM Tris Cl, pH 8.5) was added to the membrane and incubated for 1 min and then centrifuged for 1 min to elute the DNA.

3.2.3 TOPO TA Cloning

Five-µl of TOPO cloning reaction was set up consisting of 4 µl of PCR product and 1 µl of TOPO vector from the TOPO TA Cloning Kit (Invitrogen, USA). The reaction was mixed gently and incubated for not more than 5 min at RT or the transformation efficiencies may decrease. The TOPO cloning reaction may be kept at -20°C for 24 h if necessary although this will lower the transformation efficiency.

3.2.3.1 Transformation

This was carried out according to the manufacturer's instructions. Two µl of TOPO cloning reaction was added into a vial of TOP10 bacterial competent cells (Invitrogen, USA) and mixed gently. The suspension should not be mixed by pipetting up and down. The mixture was incubated on ice for 30 min and then heat shocked for 45 sec at 42°C without shaking. The tube was transferred immediately to ice before adding 250 µl of SOC medium (pre-warmed to RT). The tube was closed tightly and incubated at 37°C for 30 min with vigorous shaking. The transformation reaction was spread out on a pre-

warmed LB-Amp selection plate. Two different volumes were plated, i.e. at 250 µl and at a smaller volume of 50 µl to ensure well-spaced colonies. The plates were incubated at 37°C overnight. Colony screening for positive colonies were performed on the following morning.

For transformation with plasmid DNA, 1-10 ng of DNA was used for 0.1 ml of competent bacterial cells. The plasmid DNA stock was diluted with water to the appropriate concentration. In the case, that RIL plasmid is present in the bacterial cell, the selection plate contains chloramphenicol in addition to ampicillin.

3.2.3.2 Analysis of positive clones

PCR screening procedure was used to identify transformed *E. coli* carrying the recombinant plasmids. A PCR was first performed using vector-specific forward and reverse primers (available with the cloning kit from the manufacturer) to identify clones that carry the recombinant plasmid. A second PCR was then performed using vector-specific forward primer and the insert-specific reverse primer to determine the orientation of the inserted DNA fragment within the plasmid. The identified plasmid was confirmed by nucleotide sequencing (Agowa, Germany).

Positive clones were analyzed directly by PCR. A PCR cocktail was prepared and dispensed in 50 µl aliquots. Ten colonies were picked with a clean toothpick and dipped into the PCR reaction. The toothpick was then used to inoculate 2 ml of LB medium containing the appropriate antibiotics overnight at 37ºC with vigorous shaking. The amplified PCR product was visualized by agarose gel electrophoresis. Positive clones were then tested for recombinant protein expression.

3.2.4 DNA isolation

Bacterial plasmid DNA isolation was performed using the QIAprep Miniprep or Maxiprep kits (Qiagen, Germany) depending on the concentration of DNA required for downstream application of the plasmid. For nucleotide sequencing and PCR analysis, plasmid was isolated by miniprep. However, for higher concentration of plasmid, Maxiprep was used to prepare the DNA plasmid.

3.2.4.1 Miniprep

One ml of bacterial culture was centrifuged at maximum speed for 1 min and resuspended in 250 µl of Buffer P1. 250 µl of Buffer P2 was added and the tube gently inverted for 4-6 times to mix. The suspension left standing at RT for 3 min before adding 350 µl of Buffer N3. The tube was inverted 4-6 times immediately after adding Buffer N3 and then centrifuged for 10 min at maximum speed. The supernatant was then transferred into a fresh QIAprep column in a 2 ml collection tube and then centrifuged again for 1 min. The flow-through was discarded. The QIAprep column was washed with 0.75 ml of Buffer PE and centrifuging again for 1 min. The flow-through was discarded and centrifuged for an additional 1 min. The QIAprep column was placed in a clean 1.5 ml microfuge tube. The DNA was eluted by adding 50µl of Buffer EB (10 mM TrisCl, pH 8.5) to the centre of the column and incubating it for 1 min before centrifugation. The miniprep yield was analyzed in agarose gel.

3.2.4.2 Maxiprep

A single colony was picked and inoculated into a 100 ml of selective LB medium. The culture was grown at 37°C overnight with vigorous shaking. The bacterial cells were harvested by centrifugation at 6000 g for 15 min at 4°C. The bacterial pellet was completely resuspended in 4 ml Buffer P1 by vortexing and pipetting until no cell clumps were visible. Ten ml of Buffer P2 was added and mixed gently by inverting the tube 4-6 times before incubating it for 5 min at RT. Ten ml of chilled Buffer P3 was added and mixed immediately by inverting the tubes. The suspension was incubated on ice for 20 min. Following this, the suspension was centrifuged at 12000 g for 30 min at 4°C. The supernatant containing the plasmid DNA was removed immediately. The supernatant was centrifuged again. In the meantime, a QIAGEN-tip 500 was equilibrated by applying 10 ml of Buffer QBT and the column was allowed to empty by gravity flow. The plasmid containing supernatant was added into the column. The QIAGEN-tip was washed twice with 30 ml of Buffer QC. The DNA was eluted with 15 ml Buffer QF and precipitated with 10.5 ml (0.7 volumes) of isopropanol by centrifuging immediately after addition of isopropanol at 12000 g for 30 min at 4°C. The supernatant was decanted carefully without disturbing the DNA pellet. The DNA pellet was washed with 70 % ethanol and centrifuged at 12000 g for 10 min. The supernatant was decanted carefully. The pellet was air-dried and the DNA redissolved in a suitable volume of sterile buffer (TE, ph 8.0, sterile filtered). The yield was determined by both quantitative agarose gel electrophoresis and by UV spectrophotometry.

3.2.5 DNA purity and concentration determination

Five µl of DNA was diluted in 95 µl of water (1/50 dilution) and the absorbance measured at 260 nm and 280 nm. The integrity of the DNA was analysed by gel electrophoresis. The purity of the DNA was determined from the A_{260}/A_{280} ratio.

$A_{260}/A_{280} \geq 1.8$ indicates pure DNA

$A_{260}/A_{280} > 2.0$ indicates possible contamination with RNA

$A_{260}/A_{280} < 1.8$ indicates contamination with proteins and aromatic substances

The following formula was used to estimate the DNA concentration:

1 A_{260} Unit of dsDNA = 50 µg/ml in water

3.2.6 DNA agarose gel electrophoreses

Thirty-five-ml of 1.5 % agarose solution in 1x TBE buffer containing 1µg/ml EtBr was used. The electrophoresis was performed with 1x TBE running buffer at 100 V until separation is complete. The agarose gel was removed from the chamber and viewed with a UV lamp. A picture was taken for documentation.

3.2.7 DNA extraction from agarose gel

The QIAquick Gel Extraction Kit (Qiagen, Germany) was used to extract DNA from a low-melting agarose gel after gel electrophoresis. The DNA fragment was excised from the agarose gel with a clean sharp scalpel. The gel slice was weighed in a microfuge. Three volumes of Buffer QG was added to 1 volume of gel (100 mg = 100 µl) and incubated at 50ºC for 10 min or until the gel has completely dissolved. The tube was vortexed every 2-3 min to help dissolve the gel. One gel volume of isopropanol was

added to the mix if the DNA fragment was <500 bp or >4kb. The mixture was placed in a QIAquick spin column and centrifuged for 1 min at maximum speed to bind the DNA to the column. To wash the DNA, 0.75 ml of Buffer PE was added and the column centrifuged for 1 min. The flow-through containing the Buffer PE was centrifuged for another 1 min to remove all traces of the buffer. The column was placed in a clean tube and 50 µl of Buffer EB was added, the column was left to stand for 1 min and centrifuged for another min. Alternatively, for a more concentrated DNA, only 30 µl of the buffer was used to elute the DNA.

3.2.8 Protein expression in bacterial cells

Two-ml of LB medium containing the necessary antibiotics were inoculated with a single recombinant *E. coli* colony and grown overnight at 37°C under agitation. Ten ml of Super medium containing 50 µg/ml ampicillin (and 34 µg/ml chloramphenicol if the pRIG plasmid is present) was inoculated with 0.2 ml of the overnight culture. The culture was grown at 37°C with vigorous shaking to an optical density (OD_{600}) of 0.9. One-ml aliquot of cells was kept as zero time point sample. IPTG was added to the culture to a final concentration of 1 mM (9µl of a 1 M IPTG stock to 9 ml of culture) to initiate protein expression. The induced culture was incubated at 37°C with vigorous shaking for a further 5 hours. One-ml sample was taken at the end of the induction period. The 8 ml culture was pelleted and frozen for further analysis.

The 1 ml pre- and post-induction samples were centrifuged at maximum speed and the supernatant aspirated. The cell pellet was boiled at 95°C for 5 min with 50 µl of 2x SDS-PAGE loading buffer. The buffer was transferred to a fresh tube and stored at -20°C. Fifteen µl of the sample was loaded into and analyzed in 12 % SDS-PAGE gel.

The gel was stained with Coomassie blue and a band with increased intensity in the post- and not in the pre-induction sample corresponding to the expected size range of the recombinant protein was sought. Western blot analysis was performed and probed with anti-6xHis antibody to confirm the identity of the expressed recombinant protein band.

For scale-up expression, the conditions determined previously were used to grow and induce 500 ml of cell culture. The induced cell culture was centrifuged and the cell pellets frozen at -20ºC if protein purification was performed at a later date.

3.2.9 Purification of recombinant protein

Frozen pellets of the induced *E. coli* cells were thawed on ice. Twenty-ml of lysis buffer provided by the Ni-NTA Agarose kit (Qiagen, Germany) was used to resuspend the cell pellets from 400 ml of culture. The bacteria cells were lysed by repeated freeze-thaw cycles with liquid nitrogen. The cell suspension was then briefly sonicated to ensure complete lysis of the bacterial cells. The cell debris was collected by centrifuging at maximum speed for 40 min at 4ºC. The supernatant was collected and filtered through a 0.45 µm membrane to remove cell debris. The pellet was dissolved in lysis buffer containing 8 M urea. Twenty-µl from the supernatant fraction and the dissolved pellet fraction was then analysed by SDS-PAGE and Western blot to confirm in which fraction is the protein compartmentalised.

The supernatant containing the recombinant protein was used for purification. The supernatant was incubated with 500 µl of 50 % Ni-NTA resin, which was equilibrated beforehand with lysis buffer (20 mM Phosphate buffer, 0.5 M NaCl and 10 mM imidazole), for 1 h at 4ºC. Following the 1 h incubation, the Ni-NTA resin was loaded into a column and washed with 30 ml of lysis buffer. The protein was eluted in a stepwise

increase of imidazole concentration (100, 300 and 500 mM imidazole) in 1 ml fractions. The protein was stored at 4ºC for immediate use. The resin was washed with water, followed by 5 ml of 0.5 M NaOH, 10 ml of water and stored in 1 ml of water at 4ºC. The recombinant protein is unstable and has a tendency to precipitate after having undergone freeze-thawing. Therefore, proteins were purified and stored at 4ºC for a maximum period of 2 weeks or used immediately. All protein fractions obtained during the purification procedure, including the flow-through and wash, were analyzed by SDS-PAGE and Western blot.

3.3 Immunological methods

3.3.1 Western blot

Four pieces of Whatmann 3MM paper and one piece of nitrocellulose membrane was cut to the exact size of the SDS-polyacrylamide gel. The filter papers and the membrane were pre-wet in transfer buffer. Two of the Whatmann papers that have been soaked in transfer buffer were placed onto the plastic cover. The sheets was stacked one on top of the other so that they are exactly aligned. The membrane was placed on the stack of Whatmann paper making sure that the filter is exactly aligned. A glass pipette was used to roll on the stack of Whatmann papers and membrane to remove any trapped air bubbles. The gel was transferred on top of the membrane. The final two sheets of Whatmann paper was placed on the gel. Any trapped air bubbles were squeezed out using a glass pipette. The protein was wet blotted at 400 mA for 1 h, taking care that the orientation of the sandwich within the chamber is correct. The membrane was removed from the chamber and incubated briefly with a Ponceau-S stain to visualize the protein bands. Relevant protein bands were

marked on the membrane before transferring to the blocking buffer (3 % milk in TBS). After 1 h of blocking, the membrane was incubated with the specific primary antibody for 1 h at RT or overnight at 4°C. This was followed with an appropriate secondary antibody for 1 h at RT. The membrane was washed extensively with TBS containing 0.1 % Tween-20 after antibody incubation. The western blot was detected with NBT/BCIP solution. The substrate reaction proceeded for 2-30 min with AP developing mixture. The membrane was washed briefly in water and dried.

Alternatively, detection was performed using chemiluminescence reagents, such as the ECL system. The immunoblot was probed with an appropriate secondary that is conjugated with a peroxidase enzyme. The washed blot was placed protein-side up on a clean plastic sheet on a flat surface. The ECL substrate was prepared as instructed by the product manual. The substrate was applied at 100 μl/cm^2 of membrane so that the blot is completely covered and incubated at RT for 1 min. The blot was carefully removed and excess reagents removed by blotting. The blot was sealed in Saran wrap. At this point the blot should be kept in the dark before imaging. The chemifluorescence signal intensity typically increases exponentially during the first 30 min of incubation and then begins to plateau. The blot was exposed to film for the desired time (typically between 30 sec to 20 min depending on the antibody). The membrane was washed several times in TBS-Tween and stored at 4°C if the membrane is stripped within the next day or two.

3.3.2 Stripping membrane of bound antibodies

The immunoblot can be stripped of antibody complexes and re-probed for up to five times if using the ECL system. The blot was incubated in 10 ml of stripping solution (0.2

M Glycine-HCl, pH 2.5, 0.05 % Tween-20 and 100 mM DTT) at 60°C for 60 min in a sealed plastic container in a shaking water bath. The stripping procedure was performed twice to ensure a cleaner strip. The blot was rinsed with TBS-Tween and blocked before re-probing with antibody. Alternatively, the blot can be stored at 4°C for 1 week.

3.3.3 Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to analyse human responses to the various purified recombinant proteins. Plates were coated with 100 µl of 0.5 µg/ml of antigen in coating buffer overnight at 4ºC. The wells were then blocked with 100 µl of blocking buffer (3 % milk in PBS). Wells were then incubated with 100 µl of human serum (1:200), followed by HRP-conjugated anti-human IgG (1:15000). The wells were washed extensively in washing buffer (PBS containing 0.5 % Tween-20) after the antibody incubations. The wells were incubated in the developing solutions for approximately 10 min and the reaction stopped by adding 100 µl of phosphoric acid. The optical density (OD) of each well was measured in a spectrophotometer at 450 nm. The OD was obtained by subtracting the average OD of duplicate wells from the control well. Values were converted into arbitrary units (AU) as follows:

$$AU = 100 * \frac{Ln(ODtest) - Ln(ODneg)}{Ln(ODpos) - Ln(ODneg)};$$

Whereby, ***neg*** *is negative serum control,* ***pos*** *is positive serum control and* ***test*** *is the test serum.*

Antibody responders were those having an AU value greater than two standard deviations above the mean AU of the negative control (non-exposed European) samples.

3.3.4 Animal immunization

3.3.4.1 Mouse

Serum from non-immunized BALB mice was first tested by Western blot for reactivity against parasitic and human erythrocytic antigens prior to immunization. Non-reactive BALB mouse was injected subcutaneously with 150 μl of purified recombinant protein (30 μg) emulsified in an equal volume of Freund's complete adjuvant. A second injection was performed with 750 μl of purified recombinant protein (33 μg) emulsified in an equal volume of Freund's incomplete adjuvant two weeks later. After another two-week interval venous blood was collected from the tail and tested in immunoblot against the recombinant protein for the presence of specific antibodies in the antiserum. A third booster was performed similarly to the second injection and blood was collected at the end of two weeks post-injection. Blood was collected by cardiac puncture into a sterile microfuge tube and left to stand for 30 min at RT. Serum was collected by centrifuging the clotted blood for 15 min at high speed and stored at -20ºC in aliquots.

3.3.4.2 Chicken

One hundred μg of recombinant 732 DBL-3γ protein in 300 μl of phosphate buffer suspended with Freund's complete adjuvant was used for immunization. Each chicken was given 2 further boosts with the same amount of protein. After six weeks after the initial immunization, the polyclonal immunoglobulin (Ig) Y antibody can be detected in the egg yolk of the eggs. IgY antibody was purified from the egg yolk by polyethylene

glycol precipitation. Specificity of antibodies was tested by immunoblot using goat anti-chicken IgY antibodies (diluted 1:15000) (Sigma).

3.3.4.3 Immunoglobulin G (IgG) purification

One-ml of animal antiserum was centrifuged at 3000 g for 30 min to remove large protein aggregates that will precipitate at low ammonium sulfate concentration. The supernatant was transferred to a small clean glass beaker and gently stirred. 0.5 ml of saturated ammonium sulfate (761 g in 1 L of distilled water) was added slowly drop-wise into the beaker over a span of 30 min. The ammonium sulfate must be added slowly and in small volumes while constantly stirring the supernatant to prevent changes in the local ammonium sulfate concentration in the suspension. The suspension was stirred continuously overnight at 4ºC. On the next day, the suspension was centrifuged to precipitate the proteins. The supernatant was transferred to another fresh beaker and another 0.5 ml of ammonium sulfate was added drop-wise slowly and stirred for an additional 6 h at 4°C. The suspension was then centrifuged and the supernatant carefully discarded. The precipitated protein, which contains the IgG, was carefully dissolved in 0.3 ml of 5 mM $NaPO_4$ buffer and transferred into a clean dialysis tubing. The ammonium sulfate-purified antibodies were dialyzed against three changes of 5 mM $NaPO_4$ buffer. The dialysed IgG suspension was then purified by DEAE-cellulose affinity chromatography.

The DEAE-cellulose matrix (Amersham Pharmacia, Sweden) was washed extensively prior to use as instructed by the product manual. The matrix was then equilibrated with 20 volumes of 5 mM $NaPO_4$ buffer (pH 6.5). The matrix was washed until the pH is 6.5. The dialyzed antibodies were combined with the washed DEAE-

matrix. The slurry was agitated for 1 h at RT. For most DEAE matrix, 2 ml of wet matrix will bind the proteins found in 1 ml of serum. The slurry was gently centrifuged and the antibodies-containing supernatant carefully collected. The concentration and purity of the antibodies were analysed by SDS-PAGE. The antibodies will yield polypeptides of approximately 25 and 55 kD. The pure antibodies were stored at 4ºC as working stock or at -20 ºC in 20 % glycerol for long term storage. The DEAE-matrix was regenerated by sequential washes with 0.5 M HCl and 0.5 NaOH.

3.3.4.4 Immunoglobulin Y (IgY) purification

The egg yolk was separated from the egg white. The cords were cut with a sharp scalpel without damaging the egg yolk sac. The egg yolk was rinsed with water to remove traces of egg white. The yolk sac was sniped and the yolk drained into a clean measuring cylinder. The volume of the egg yolk was estimated (V). The yolk was mixed with equal volumes (V) of KPO_4 buffer and then transferred into a 50 ml Falcon tube. Double volumes (2V) of KPO_4/7 % PEG 6000 was added into the suspension and mixed for 30 min at 4ºC. The suspension was centrifuged at 10000 rpm for 10 min 4ºC in a Beckmann JA 12 centrifuge. The pellet was discarded. The supernatant was carefully filtered through double layered gauze and funnelled into a fresh 50 ml Falcon tube. Following this, 10 % w/v PEG 6000 powder was dissolved in the solution to give a final PEG concentration of 12 %. The solution was centrifuged and the supernatant discarded. The pellet was resuspended in 10 ml of KPO_4 buffer and mixed for 30 min at 4ºC. Ten-ml of KPO_4/24 % PEG 6000 buffer was added into the solution thus giving a PEG final concentration of 12 %. The suspension was centrifuged. The pellet containing the IgY was washed with 5 ml of KPO_4 buffer. The suspension was centrifuged at 12000 rpm for

30 – 60 min at 4ºC. The supernatant was carefully transferred into 1 ml aliquots and stored at -20ºC with 0.02 % Na-azide.

3.4 Imaging techniques

3.4.1 Light microscopy

3.4.1.1 Binding of antibodies to IE cell surface

Late stages of CSA-binding and the isogenic non-CSA-binding strains were enriched and tested in parallel. Nuclear DNA staining was visualized by incubating with DAPI (4 μg/ml) at RT for 30 min. IE were stained with specific primary antibodies (1:100) and counterstained with a appropriate secondary antibody conjugated to Alexa Fluor 488 or 594 (6 μg/ml). Each incubation was performed for 30 min at 4°C. In control experiments, IE were treated with 0.1 mg/ml trypsin at 37°C for 10 min prior to staining. Cells were resuspended with 10 μl of 2 % FCS-PBS and observed under 40x magnification.

3.4.1.2 Binding of recombinant proteins to CHO cells

CHO cells were seeded on coverslips at a cell density of $3x10^5$ cells/ml. The cells were fixed with 2 % paraformaldehyde (PFA) in PBS for 30 min at RT, washed and blocked with 3 % BSA in PBS (BSA-PBS) for 30 min at RT. Cells were incubated with 0.5 μM of recombinant protein in BSA-PBS for 40 min at RT. Protein binding was detected using a specific primary antibody (1:500 in BSA-PBS), followed by a secondary antibody conjugated to Alexa-Fluor 594 or Alexa-Fluor 488 (1:1000), each incubated for 30 min at 4°C. Cell nuclei were visualized by DAPI (5 μg/ml). The cells were mounted in

MOWIOL and observed under 20x magnification. To demonstrate the specificity of the binding of the recombinant protein to the CHO cells expressing CSA, soluble CSA, CSB and CSC (100 μg/ml) were co-incubated with the protein on the cells.

3.4.2 Image processing

Adobe Photoshop, USA or Volocity, USA were used to process the images. The images were further processed using Adobe Illustrator, USA.

3.5 Biochemical methods

3.5.1 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

The glass plates, spacers and combs were assembled following the manufacturer's instructions. The appropriate volume of the components for the desired concentration of acrylamide was prepared and mixed together by swirling. The acrylamide solution was poured into the gap between the glass plates leaving sufficient space for the stacking gel (the length of the teeth of the comb plus 0.5 cm). The acrylamide solution was overlaid with a thin layer of isopropanol. The isopropanol was poured off and the gel rinsed with water after polymerization was completed (approximately 30 minutes). The stacking gel containing the appropriate amount of acrylamide was prepared and poured into the surface of the separating gel. The combs were placed into the stacking gel and left to polymerize (approximately 15 min). The gel can be kept at 4^{o}C wrapped in a wet tissue and Saran wrap. The gel was placed into the electrophoresis chamber and the chambers filled with 1x running buffer. The combs were removed from the stacking gel and the sample wells were washed with the running buffer by pipetting the buffer up and down

into the wells. The protein sample was dissolved in equal volume of 2x Laemmli sample buffer, heated at 95^{o}C for 5 minutes and the samples loaded into the bottom of the wells. The chamber was connected to the power supply and a voltage of 60 V was applied until the samples reach the separating gel. At this time, the voltage was increased to 120 V. When the dye front reaches the bottom of the gel the power supply was turned off. The glass plates were removed from the electrophoresis chamber.

The gel was fixed and stained with Coomassie staining solution for 1 h to overnight. The gel was incubated successively in Coomassie destaining solution to visualize the protein bands.

3.5.2 MALDI-tof peptide mass fingerprinting analysis

This technique was a service provided by the institute. In-gel digests were performed using the In-Gel-Digest-Kit-ZP (Millipore, USA), following a modified protocol, but using the solutions provided with the kit. Polyacrylamide gel plugs from Coomassie Blue stained bands representing the candidate proteins were punched out of the gel, using a cut-off, thin walled pipette tip and deposited in the wells of a ZIPplate, together with 100 µl of Solution 1. After 20 min, the ZIPplate was placed on a vacuum manifold, and the Solution 1 was filtered through the bottom resin of the plate. A hundred µl of Solution 2 (destaining solution) was added and incubation at RT was continued for 20 min. Vaccum was applied to remove the destaining solution, and the step was repeated. The destained gel plugs were dehydrated with 200 µl each of acetonitrile at RT for 10 min. The acetonitrile was removed by vacuum filtration, and 15 µl of Trypsin solution (30 µg/ml in 40 mM NH_4HCO_3, 5% acetonitrile) were added to the dehydrated gel plugs. The ZIPplate,

with lid and collection plate were placed inside a sealed plastic bag and incubated at 37°C for 4 h.

Eight-µl of acetonitrile was added and incubation at 37°C was continued for 15 min. After addition of 130 µl of Solution 6 (extraction/wash solution) and 30 min incubation at RT, the extract was filtered through the C18 resin at the bottom of the wells by applying a gentle vacuum. The bound peptides were then washed with another 100 µl of Solution 6. The wells were emptied completely using full vacuum. Eight-µl of Solution 7 (Elution) was added to each well, and the ZIPplate was placed on the collection plate. Extraction was performed by centrifugation of the plate assembly at 2000 rpm for 30 sec. Four-µl of the eluate was then spotted onto an AnchorPlate600 mass spectrometry target. The dried spots were then overlaid with 1.5 µl of matrix solution, i.e. saturated (RT) alpha-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile, 0.1 % trifluor acetic acid with 0.2 pM each of angiotensin II, angiotensin I, and ACTH clip 1-17.

Mass spectra were recorded on a Bruker Autoflex MALDI-TOF unit, using the internal peptide standards for calibration. Peptide masses were then compared with theoretical trypsin digest products of the expressed recombinant proteins, obtained with the MS-Digest algorithm (http://prospector.ucsf.edu/ucsfhtml4.0/msdigest.htm).

3.5.3 In vitro static binding assays of IE

3.5.3.1 Binding assays using immobilized receptors

Circles were drawn at the back of Petri dish to indicate where the receptors will be immobilized. Circles should be large enough to accommodate 10 µl of parasite suspension. Six-µl of CSA (100 µg/ml), HA (100 µg/ml) or BSA (5 % BSA in PBS, as

control) was dropped on the interior surface of the Petri dish at sites designated by the marked circles. The CSA, HA and BSA were allowed to adsorb to the surface of the dish for 1 h at RT. The dishes were covered with wet paper towels to avoid evaporation. The receptor solution was removed by suction through a yellow tip and the whole Petri dish was blocked with 3 ml of 2 % BSA in PBS for 2 hours at RT. The blocking solution was removed and 3 ml of parasite culture (2 % parasitaemia and 5 % haematocrit) was added to the dish. The dish was incubated for 1 h at 37°C with gentle shaking every 15 min to resuspend the culture. After the incubation time, the dish was washed 3-5 times with PBS to remove unbound infected erythrocytes. The PBS solution was gently added into the dish at the edge of the dish to prevent dislodging of bound IE. After addition of PBS the plate was swirled gently to wash unbound IE. The PBS was collected by suction at the side of the dish. Care was taken to prevent the dish from drying in between washes. Bound cells were fixed with 0.5 % glutardialdehyde for 10 min and stained with 1 % Giemsa solution for 15 min. The dish was washed repeatedly with water and allowed to dry. IE were counted under 40x magnification with an inverted microscope (Leitz, Germany). 10 randomly selected fields were counted to give the total number of bound IE/mm^2, from which the background number of IE bound to BSA-coated control spots was subtracted.

3.5.3.1.1 Co-incubation with polyclonal antibody or human plasma

CSA was immobilized on plates as described before (see Section 3.1.2.3). Inhibitory activities of polyclonal antibody or human serum (both diluted at 1:100 in the parasite suspension) were determined by pre-incubating IE (5 % haematocrit, 2% parasitaemia) for 1 h at 37°C with antibody prior to addition in prepared dish. BSA (5 %) and CSC

(100 μg/ml in PBS) served as controls. The pre-treated parasite culture was then added into the prepared dish and the assay proceeded for 1 h at 37°C. Bound IE were counted under 40x magnification in 10 randomly selected fields to give the total number of bound IE/mm^2, from which the background number of IE bound to BSA-coated control spots was subtracted. Adhesion results were presented as a percentage of bound IE/mm^2 in the test sample (with CSC or antibody) to bound IE/mm^2 in the control sample (without CSC or antibody).

3.5.3.1.2 Co-incubation with recombinant proteins

To investigate the inhibitory activities of recombinant protein, the assay was slightly modified as described earlier. Six μl of CSA (100 μg/ml) and BSA (5 % BSA in PBS) were dropped to the interior surface of the Petri dish within the 1 cm circles drawn by a DakoCytomation pen. The CSA and BSA were adsorbed to the surface for 1 h at RT. The solution were aspirated and blocked with BSA. In the meanwhile, IE were enriched and resuspended to 3x10^8 IE/ml. One hundred-μl of IE was mixed with the recombinant protein at various concentrations (1-20 μM final concentration) before overlaying on the CSA within the circles. The protein Rifin that has no known role in CSA binding was incorporated in the assay as a negative control. Ten randomly selected fields were counted to give the total number of bound IE/mm^2, from which the background number of IE bound to BSA-coated control spots was subtracted. Inhibition results are presented as the difference of bound IE/mm^2 in the test sample (with recombinant protein) to bound IE/mm^2 in the control sample (without recombinant protein) in percentage

3.5.3.2 Binding assays using CHO cell lines

CHO cells were grown in 6-well plates as described in Sect. 3.1.2.3. Late-stage IE were enriched and resuspended to $3x10^8$ IE/ml. The IE suspension was incubated with the CHO cells for 1 h at RT. The culture plate was gently shaken every 15 min to resuspend the IE. The wells were washed gently for three times with binding medium to remove unbound IE. Binding of IE to CHO cells were observed with an inverted microscope. The number of IE bound to 300 CHO cells were counted under 40x magnification. IE binding was expressed as the number of IE bound to 100 CHO cells.

3.5.3.2.1 Co-incubation with recombinant proteins

CHO cells were grown in 6-well plates as described previously. IE were enriched and resuspended to $3x10^8$ IE/ml. The IE was mixed with the recombinant proteins at various concentrations to a final volume of 100 μl. 2.5 – 250 nM recombinant DBL-γ protein concentration and 20 – 2000 nM 732 recombinant domains was used in this assay. The IE-protein suspension was then overlaid on the CHO cells. The binding assay then proceeds as described above.

3.6 Statistical analysis

Differences between groups were tested by the Student unpaired t-test. Correlations were examined by the Pearson test. A multiple linear regression model that included parity was used to assess the relationship between placental parasite density (after log transformation) and antibody levels. The significance limit (P) was set at 0.05. Statistical analysis was performed using JMP 5 program.

3.7 Supplementary data

3.7.1 Amino acid alignment

The amino acid alignments were performed at http://www.ncbi.nlm.nih.gov/blast/bl2seq /wblast2.cgi. Multiple sequence alignments were performed by Clustal W at http://align.genome.jp.

3.7.2 Protein secondary structure prediction

Protein secondary structures were predicted by Predator at http://bioweb.pasteur.fr/seqanal/interfaces/predator-simple.html

4. Results

4.1 Establishment of protocols

4.1.1 Mammalian Chinese Hamster Ovary cells

The commercially available wild-type Chinese hamster ovary (CHO) cell line, from which the CHO-K1 cell line is derived, is one of the most common mammalian cell lines kept in culture. These cells have a smooth surface and grow in a "fibroblast-like" fashion, in elongated structures that tend to align in parallel fashion to form colonies with rough edges (Figure 7 first row first column). CHO-K1 cells readily express glycosaminoglycan (GAG) molecules, in particular chondroitin sulfate A (CSA) on their surface. CHO-CD36 and CHO-ICAM-1 cells are stable cell lines derived from a mutant CHO cell line, CHO-745, which is devoid of surface GAG. As shown in Figure 7 (first row second and third column) they grow in a manner similar to CHO-K1.

Surface expression of CSA, CD36 and intercellular adhesion molecule -1 (ICAM-1) receptors on the three CHO cell lines were controlled by indirect immunofluorescence assays (IFA) in the presence of commercially available antibodies raised against the corresponding surface receptors (Figure 7 second row). The expression of CSA receptors were observed only on CHO-K1 cells and not on CHO-CD36 or CHO-ICAM-1 cells (Figure 7 second row first column). CHO-CD36 expresses only CD36 receptors but not CSA or ICAM-1 (Figure 7 second row second column). Similarly, CHO-ICAM-1 expresses only ICAM-1 receptors (Figure 7 second row third column).

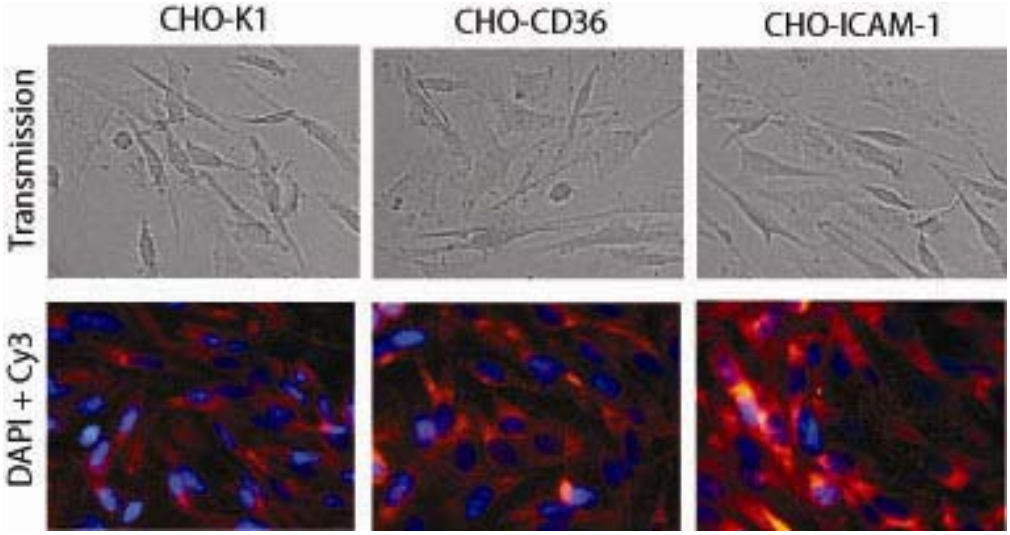


Figure 7 Chinese Hamster Ovary (CHO) cell morphology. CHO-K1 (first column), CHO-CD36 (second column) and CHO-ICAM-1 (third column) were observed under transmission light (first row). An overlay of the fluorescent images obtained from DAPI stained CHO cells (blue) and those stained with specific antibodies that recognize the receptors (red) co-localizes the expression of CSA, CD36 and ICAM-1 receptors on the CHO cells (second row).

4.1.2 Enrichment of late-stages infected erythrocyte (IE)

P. falciparum-infected erythrocytes (IE) were maintained as asynchronized culture. Trophozoites and schizonts (late-stage parasites) were isolated from culture by magnetic cell sorting (MACS) for further downstream applications (Figure 8). Parasites from an asynchronized culture were introduced into the MACS column to enrich for the late-stage parasites. While early-stage (ring stage) parasites and uninfected erythrocytes are found in the flow-through fraction, late-stage parasites are retained on and eluted off the column. The MACS technique is capable of enriching for approximately 90 % late-stage parasites independent of the volume and parasitaemia of the starting culture. One of the drawbacks of this method however, is that the enrichment process is harsh and risk “traumatizing”

the parasites. For applications that require further culturing of enriched parasites, other enrichment methods, such as gelatin flotation, may be more suitable.

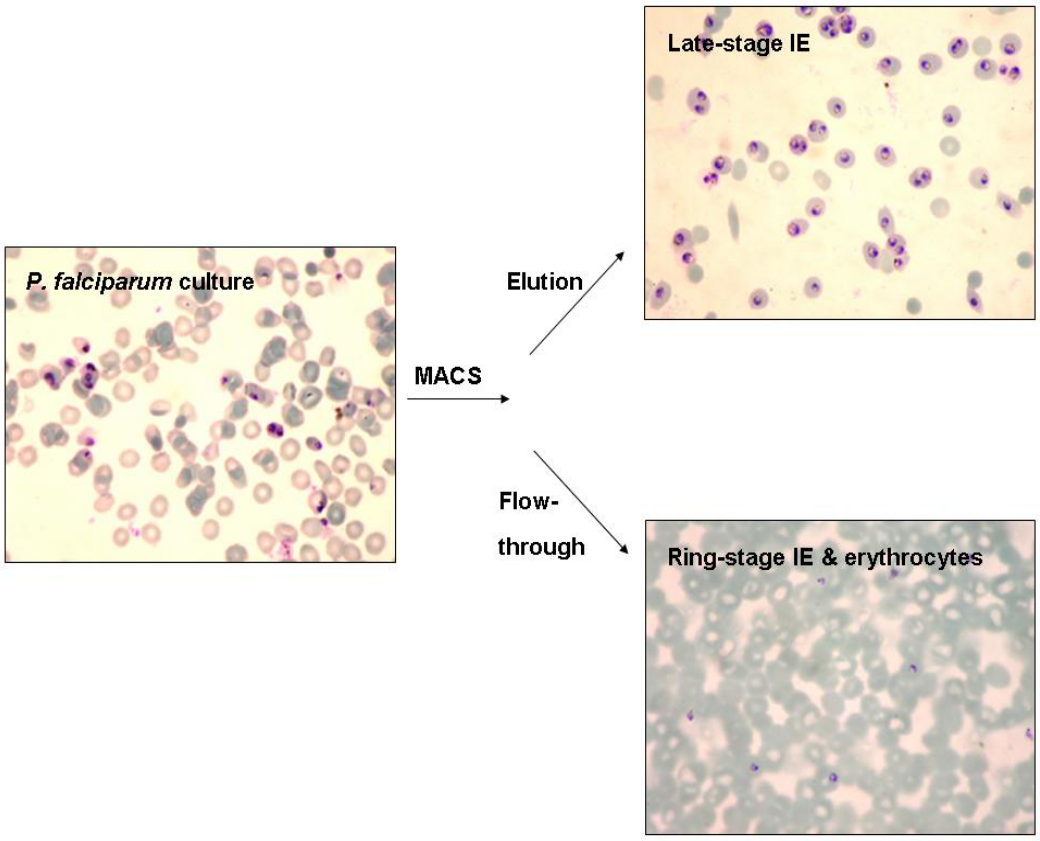


Figure 8 Enrichment of late-stage IE by MACS. The ring-stage IE and uninfected erythrocytes are collected in the flow-through while the late-stage IE were retained in the column, and later eluted.

4.1.3 *P. falciparum* infected-erythrocyte receptor binding phenotype

The isogenic lines of the *P. falciparum* cultures expressing different binding phenotypes (IE^{CSA}, IE^{CD36} and IE^{ICAM-1}) were established. Gb337CSA, VIP43CSA, Gb03CSA and FCR3CSA are parasites lines that bind CSA. The FCR3CD36 and FCR3ICAM-1 are FCR3 isogenic lines that bind CD36 and ICAM-1, respectively. The binding phenotype of each parasite line was maintained by repeated enrichment with the receptors every 2-3 weeks. Binding of the three FCR3 isogenic lines to CSA, CD36 and ICAM-1 receptors

are shown (Figure 9 left column). To determine that the IE-CHO binding was not due to unspecific interaction between the erythrocytes and the CHO cells, uninfected erythrocytes and CHO-745 cells were included as controls. Uninfected erythrocytes do not bind to any of the CHO cells tested (Figure 9 right column). CHO-745 cells do not support binding of IE or uninfected erythrocytes (data not shown).

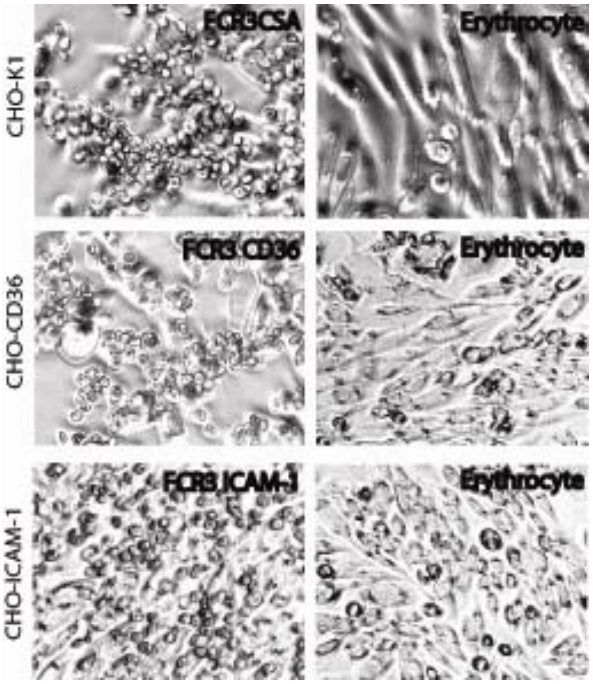


Figure 9 Binding of FCR3CSA, FCR3CD36 and FCR3ICAM-1 to CSA, CD36 and ICAM-1 expressed on CHO cells. CHO-K1 (first row), CHO-CD36 (second row) and CHO-ICAM-1 (third row) cells were observed under normal transmission light. IE, seen as small "bead-like" structures on CHO cells, adhere to the CHO cells (left column), i.e. FCR3CSA binds to CHO-K1, FCR3CD36 to CHO-CD36 and FCR3ICAM-1 to CHO-ICAM-1. Uninfected erythrocytes do not bind CHO cells (right column).

4.1.4 Specificity of the binding of IE to glycosaminoglycans (GAG)

IE^{CSA} bind to CHO-K1, as shown previously. Further definition of this binding was performed using soluble CSA, CSB, CSC, and hyaluronic acid (HA) (purified from human umbilical cord (UC) and bovine vitreous humour (BVH)) receptors. IE^{CSA} were tested with the immobilized GAG in binding assays and the number of IE bound per mm^2 to each kind of GAG was counted. The binding assay for FCR3CSA and Gb337CSA are shown in Figure 10. FCR3CSA is a laboratory strain that has been selected for CSA binding whereas Gb337CSA is a placental isolate, whose CSA-binding phenotype has

been maintained in culture. Large numbers of IE from both strains bound CSA, but not to CSB, CSC or HA from BVH (Figure 10). HA from UC was found to support limited IE-binding, believed to be due to contaminating CSA in the HA preparation as stated by the manufacturer.

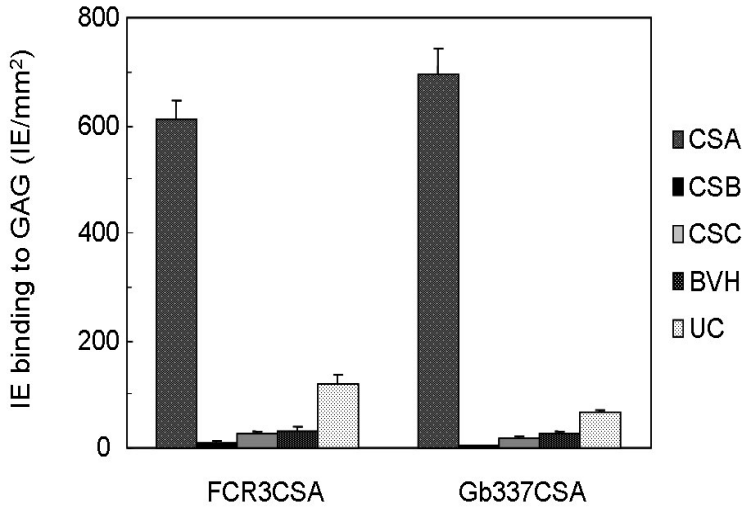


Figure 10 Binding of the IE to glycosaminoglycans (GAG). The binding of two *P. falciparum* strains, the FCR3CSA laboratory strain and a placental isolate, Gb337CSA, to chondroitin sulfate A (CSA), chondroitin sulfate B (CSB), chondroitin sulfate C (CSC), hyaluronic acid (HA) obtained from bovine vitreous humor (BVH) and human umbilical cord (UC) are shown for comparison. Binding of IE to each GAG is expressed as number of bound IE/mm^2.

4.2 Analysis of recombinant PfEMP-1 DBL-γ domains from placental isolates

A major phenomenon in pregnancy-associated malaria (PAM) is the sequestration of IE in the placenta. Recently, two recent major findings have been reported, (1) the host cell receptor, CSA, supports binding of parasites in the placenta, and (2) DBL-γ domains of *var* genes possess CSA-binding properties. These findings implicated an involvement of DBL-γ domains in parasite-host interaction. So far, DBL-γ domains have not been as

extensively characterized as the DBL-1α and CIDR-1α domains, which together form the "head structure" of PfEMP-1 and represent the most conserved region in PfEMP-1 molecules compared to the other DBL domains.

A group of DBL-γ domains, cloned from expressed *var* genes of placental isolates 482, 498, 701 and 732, have been identified in earlier works and collectively termed *var*PAM domains. In this study, the biochemical properties of these DBL-γ domains were characterized and their immunogenicities evaluated. The 3D7*chr*5 DBL-5γ (subsequently called the 3D7*chr*5 DBL-γ) and the Gb23aDBL-1α served as DBL domain controls in this study. The 3D7*chr*5 DBL-γ was cloned from a *var* gene on chromosome 5 of the 3D7 laboratory strain whereas Gb23aDBL-1α was from a field isolate of a Gabonese child with mild malaria.

4.2.1 Cloning, expression and purification of recombinant proteins

Six recombinant domains, 482, 498, 701, 732 and 3D7*chr*5 DBL-γ and Gb23aDBL-1α domains were expressed in this work. The 482, 498 and 701 DBL-γ domains were available in the expression vector, pTrcHis2, in the laboratory and could be used directly for recombinant protein expression. The 732 DBL-3γ, 3D7*chr*5 DBL-γ and Gb23a DBL-1α had to be first cloned into expression vectors. All DBL domains were expressed as recombinant protein fused with a C-terminal 6xHis tag. The presence of 6xHis tag enables a direct one-step affinity purification of the recombinant protein. Furthermore, the recombinant protein can be easily detected using specific anti-6xHis antibody in Western blots and immunofluorescence assays (IFA). Specific primers covering the

entire domain (see Sec 2.8) were used and PCR amplification gave a DNA fragment of 921 bp, 897 bp and 606 bp in size respectively (Figure 11A and Table 5).

The 6xHis-tagged recombinant proteins were affinity purified from *E. coli* lysate using commercially available Ni-NTA resin. The protein was eluted and analyzed by SDS-PAGE. A yield of 0.1-0.5 mg/ml of protein per liter of induced culture were obtained for the recombinant proteins.

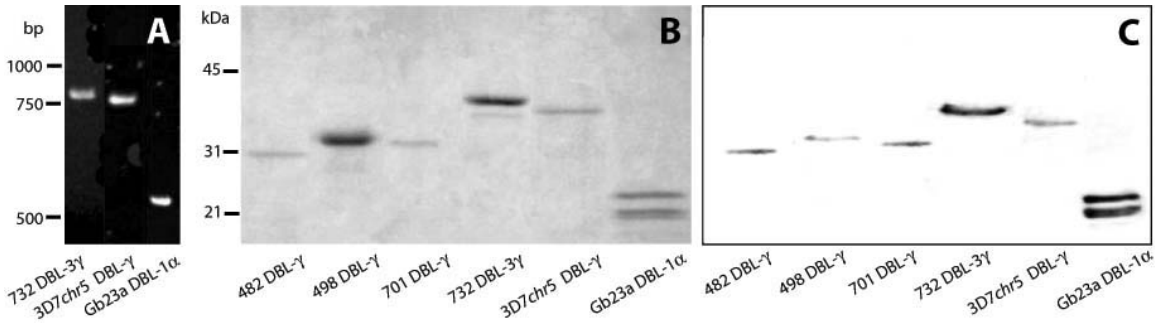


Figure 11 Recombinant PfEMP-1 DBL domains. (A) The DNA fragment of 732 BDL-3γ, 3D7chr5 DBL-γ and Gb23a DBL-1α obtained from PCR. The domains were amplified using specific primers and were cloned into bacterial expression vector for protein expression. (B) Purified recombinant domains were analyzed by SDS-PAGE. (C) The identity of the recombinant proteins were confirmed by Western blot using an anti-6xHis antibody.

Each of the recombinant 482, 498, 701, and 3D7*chr*5 DBL-γ domains was seen as a single protein band. The recombinant 732 DBL-3γ and Gb23a DBL-1α domains, appear as double protein bands in the gel (Figure 11B). Proteins were analyzed by Western blot (Figure 11C). The calculated and observed molecular masses of each recombinant DBL domain are summarized in Table 5. All recombinant *var*PAM DBL-γ domains produced were somewhat larger than their calculated molecular masses, even after taking into account vector-specific amino acids, estimated to add 3 - 4 kD to the protein core. Peptide-fingerprinting analysis of the double bands from 732 DBL-γ

showed that both bands share matching peptide species, thus indicating that both proteins were derived from the same gene. This was also found for the two Gb23a DBL-1α bands.

Table 5 DNA and protein sizes of the recombinant DBL domains

Domains	DNA size (bp)	Calculated molecular mass (kD)	Observed molecular mass (kD)
482 DBL-γ	529	20.870	31
498 DBL-γ	480	18.764	32
701 DBL-γ	531	21.209	32
732 DBL-3γ	921	35.560	43 (41*)
3D7*chr*5 DBL-γ	897	35.646	40
Gb23a DBL-1α	606	23.784	25 (21*)

* Lower band from protein double bands

4.2.2 Biochemical characterizations of PfEMP-1 DBL-γ domains

4.2.2.1 Static in vitro binding assay using CHO-cells

The recombinant proteins were first characterized for CSA-binding in IFA. All *var*PAM DBL-γ domains were observed to bind CSA. The proteins were detected on CHO-K1 cells (Figure 12 first and second column), as evidenced by the green fluorescence. They did not bind either CHO-CD36 or CHO-ICAM-1 cells (Figure 12 third and fourth column). On the other hand, recombinant 3D7*chr* DBL-γ (Figure 12 fifth row) and Gb23a DBL-1α (Figure 12 sixth row) domains did not bind to any of the CHO cells tested. CHO-745 cells did not support binding of the recombinant domains (data not shown).

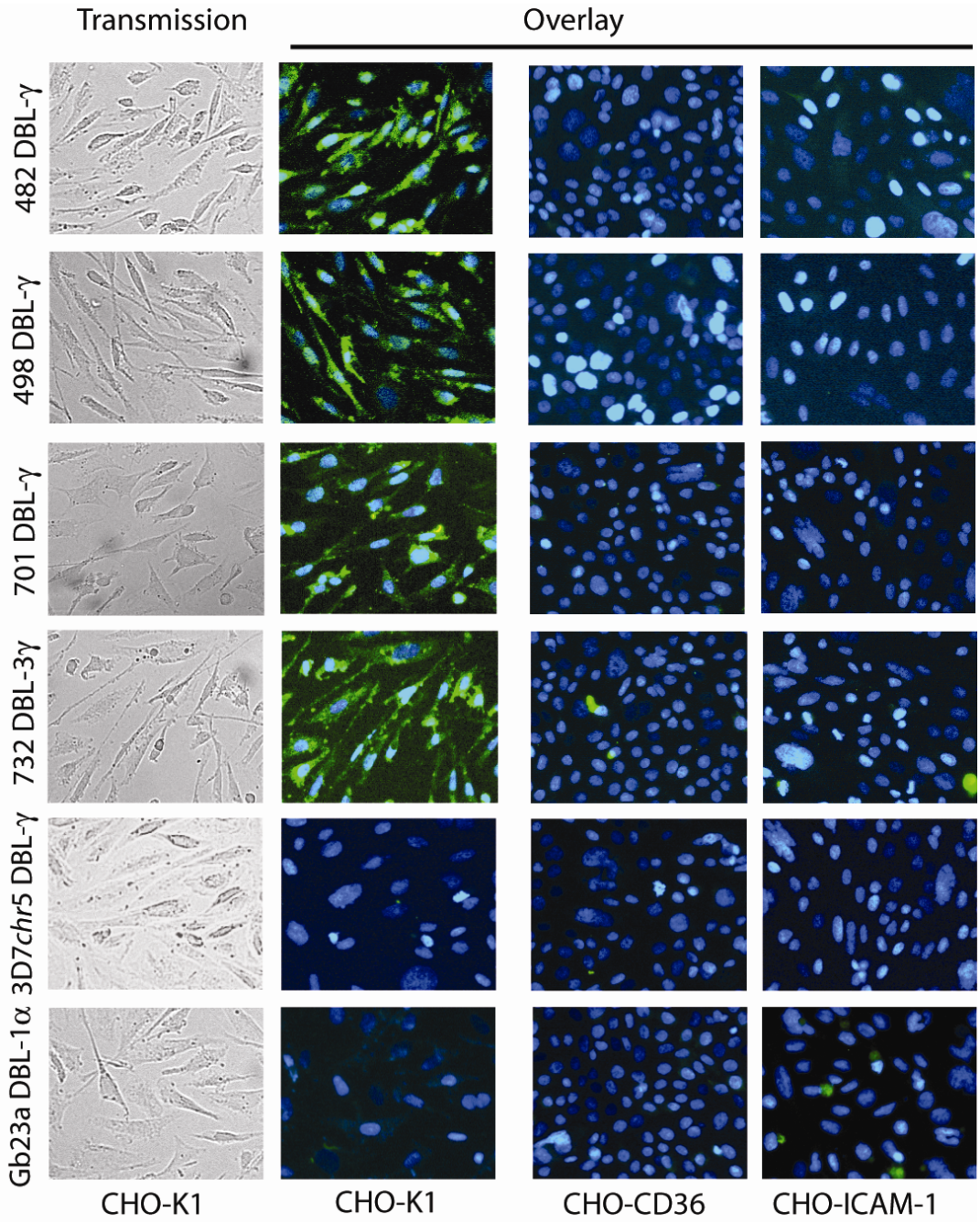


Figure 12 Assessment of the binding capacities of recombinant DBL domains to CSA, CD36 and ICAM-1 receptors in IFA. 482 (first row), 498 (second row), 701 (third row), 732 (fourth row), and 3D7*chr*5 (fifth row) DBL-γ domains and Gb23a DBL-1α (sixth row) domain were tested with CHO-K1 (second column), CHO-CD36 (third column) and CHO-ICAM-1 (fourth column) cells. Co-localization of recombinant proteins bound on CHO cells were visualized with an overlay of Alexa-flor 488 (green) and DAPI (blue) fluorescent images (second-fourth columns). Transmission light images are shown in the first column.

4.2.2.2 Competitive inhibition of IE-CSA binding to CHO-K1 cells

Recombinant proteins were further characterized in a static IE binding assay to see if they can competitively inhibit the IE-CSA binding. To this end, CSA-receptor immobilized on plastic Petri dishes was used. Recombinant DBL domains were incubated (at 10 nM) with IE prior to the binding assay. A protein Rifin, which has no known relevant function in IE binding, was included in the assay to rule out unspecific protein interactions. The results of the binding assay for VIP43CSA are shown in Figure 13A. Similar results were obtained for the other CSA-binding isolates. The DBL-γ domains were able to inhibit the binding of VIP43CSA to CSA when compared to control (calculated as 100 %), where no recombinant protein was included in the assay. The 732 DBL-3γ was able to reduce the IE-CSA binding by 80 %, whereas 482 and 498 DBL-γ were able to inhibit up to 62 % and 55 %, respectively. The 701 DBL-γ was only able to reduce IE binding by 40 %. As expected, the 3D7*chr*5 DBL-γ did not bind CSA and did not inhibit IE-CSA binding. Additionally, the proteins Gb23a DBL-1α and Rifin had no affect on IE-CSA binding.

Incubating the IE^{CSA} with increasing concentrations (2.5 – 250 nM) of *var*PAM DBL-γ domains showed that 482, 498 and 732 DBL-γ competitively inhibited IE–binding in a concentration-dependent manner (shown for FCR3CSA in Figure 13B). In contrast, 701 DBL-γ did not affect the IE-binding even at high concentrations. 3D7*chr*5 DBL-γ, Gb23a DBL-1α and Rifin were not included in this assay because they have been shown earlier that they neither bound CSA (see Figure 12) nor had an effect on IE-CSA binding (see Figure 13A). At the highest 482, 498 and 732 DBL-γ concentration tested (250 nM);

the percentage of IE^{CSA} binding to CSA was 0.1 %, 26 %, and 19 % of the binding observed in controls, respectively.

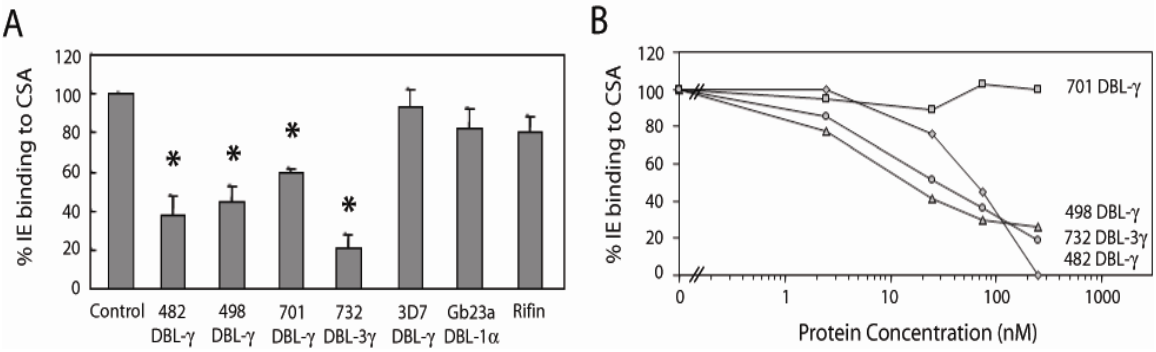


Figure 13 Inhibition of IE-CSA binding to CSA. (A) A representative result obtained using parasite isolate VIP43CSA is shown here. The IE were incubated with recombinant proteins. IE-CSA binding in the absence of protein (control) was calculated to 100 %. The results are shown as a percentage of the IE-binding when incubated with the recombinant protein compared to the control. The binding assay was performed with the recombinant *var*PAM DBL-γ domains. 3D7*chr*5 DBL-γ, Gb23a DBL-1α and Rifin were included in the assay to rule out unspecific protein-protein interaction. (B) Concentration-dependent IE-CSA-binding inhibition of recombinant *var*PAM DBL-γ domains was observed. Recombinant DBL-γ proteins were tested at concentrations of 2.5 - 250 nM with the parasite FCR3CSA strain. * Significantly different from control, $P<0.05$

4.2.3 Immunological characterizations of PfEMP-1 DBL-γ domains

4.2.3.1 Immunogenicity of the recombinant DBL domains

A cohort of plasma samples from African adults was used to evaluate the human antibody responses to *var*PAM DBL-γ domains during natural malaria infections. The recombinant 3D7*chr*5 DBL-γ and Gb23a DBL-1α domains were included as protein controls. Samples were grouped into two groups, that of malaria-exposed non-pregnant adults (n=16) and malaria-exposed pregnant women (n=247). European control samples were also included in the assay. The most notable result is the finding that of the four *var*PAM domains

analyzed, antibody levels to the 482 and 732 DBL-γ domains in the pregnant women group were significantly higher compared to the non-pregnant group (P<0.05) (Table 6). Unexpectedly, this was also the case for the control proteins. Whether and how the 3D7*chr*5 DBL-γ domain is related to placental infections remains to be clarified. The DBL-1α domain reactivity could be attributed to this domain as being part of the more conserved "head structure" present in the majority of PfEMP-1 molecules, and is hence more commonly seen by (or frequently exposed to) the immune system. However, a specific role of this domain in PAM infections is not known.

Table 6 Measurement of level of antibodies in plasma to recombinant DBL domains in ELISA

Group	n	482 DBL-γ	498 DBL-γ	701 DBL-γ	732 DBL-3γ	3D7 DBL-γ	Gb23a DBL-1α
European controls	50	40.37 ± 3.66	45.08 ± 9.89	20.08 ± 5.05	8.70 ± 3.65	14.74 ± 4.12	4.35 ± 3.69
Non-pregnant adults	16	6.78 ± 6.46	50.27 ± 17.48	38.27 ± 2.34	16.64 ± 6.46	22.22 ± 7.28	8.16 ± 6.24
Pregnant women	247	40.53 ± 1.98‡	84.50 ± 4.33*	40.76 ± 8.93*	52.90 ± 1.63*‡	40.95 ± 1.91*‡	23.08 ± 1.52*‡
Placenta infected	36	47.32 ± 4.06	94.33 ± 12.25	42.13 ± 6.84	59.78 ± 1.43#	52.68 ± 5.96#	27.13 ± 4.74
Placenta non-infected	199	38.54 ± 2.00	83.58 ± 4.94	36.22 ± 2.57	7.99 ± 3.47	38.29 ± 2.24	23.31 ± 1.93
Peripheral blood infected	21	42.11 ± 5.88	113.55 ± 15.48	46.64 ± 8.44	61.99 ± 4.47	49.18 ± 7.10	27.59 ± 5.92
Peripheral blood non-infected	224	40.09 ± 1.97	82.39 ± 4.60	37.36 ± 2.52	60.41 ± 1.35	40.08 ± 2.12	23.30 ± 1.77

Plasma samples were collected from pregnant women in Guediawaye, Senegal and European adults
n indicates number of individuals from whom plasma were collected (placenta slides were not available for 12 women; peripheral blood slides were not available for 2 women).
§ Data are expressed as mean of Arbitrary Units (AU) followed by the standard deviation
***** Significantly different from European controls, Student t-test P<0.05
‡ Significantly different from non-pregnant adults, Student t-test P<0.05
# Significantly different from non-infected .placenta, Student t-test P<0.05

4.2.3.2 Status of placental infection and antibody recognition

The pregnant women group was segregated into those who presented with an infected placenta or those with a healthy placenta during delivery. Levels of antibodies from pregnant women who had an infected placenta during childbirth to 732 DBL-γ domains

were higher when compared to women whose placentas were healthy during delivery (P=0.0296). Antibody titres against 482 DBL-γ showed a similar trend as 732 DBL-γ but the difference was not significant (P=0.0542). The observation as to why the antibody titre to 3D7*chr*5 DBL-γ was higher in women who had a healthy placenta compared to those with an infected placenta during delivery (P=0.0250) cannot be easily explained. There were no differences in the antibody recognition level of the remaining DBL domains by both groups of pregnant women (P>0.05). The mean levels of antibodies to the domains are summarized in Table 6.

4.2.3.3 Correlation of antibodies with parasitaemias

A correlation between antibody levels and parasitaemia was sought. Antibodies recognizing 482 and 732 DBL-γ domains were found to negatively correlate with placental parasite density (R= -0.4479, P= 0.0090 and R= -0.34695, P= 0.0412, respectively). Such an association was not observed with either 498 and 701 DBL-γ domains or with the protein controls (Figure 14). To investigate whether this observation was restricted to the placenta, a similar analysis was performed with maternal peripheral parasitaemia during delivery. None of the DBL domains showed any significant correlation between antibody titre and maternal peripheral parasite density (all P>0.05) (Figure 14).

4.2.3.4 Correlation of antibodies with maternal haematocrit

Antibody recognition was also compared to maternal haematocrit levels. From the panel of DBL domains, antibody titres against 482 and 732 DBL-γ domains showed a

correlation that was of statistical significance (Figure 14). The pregnant women anti-482 antibody titre showed a negative association to haematocrit (R=-0.1578, P=0.0399) and similarly for anti-732 DBL-γ antibody titres (R=-0.1756, P=0.0058).

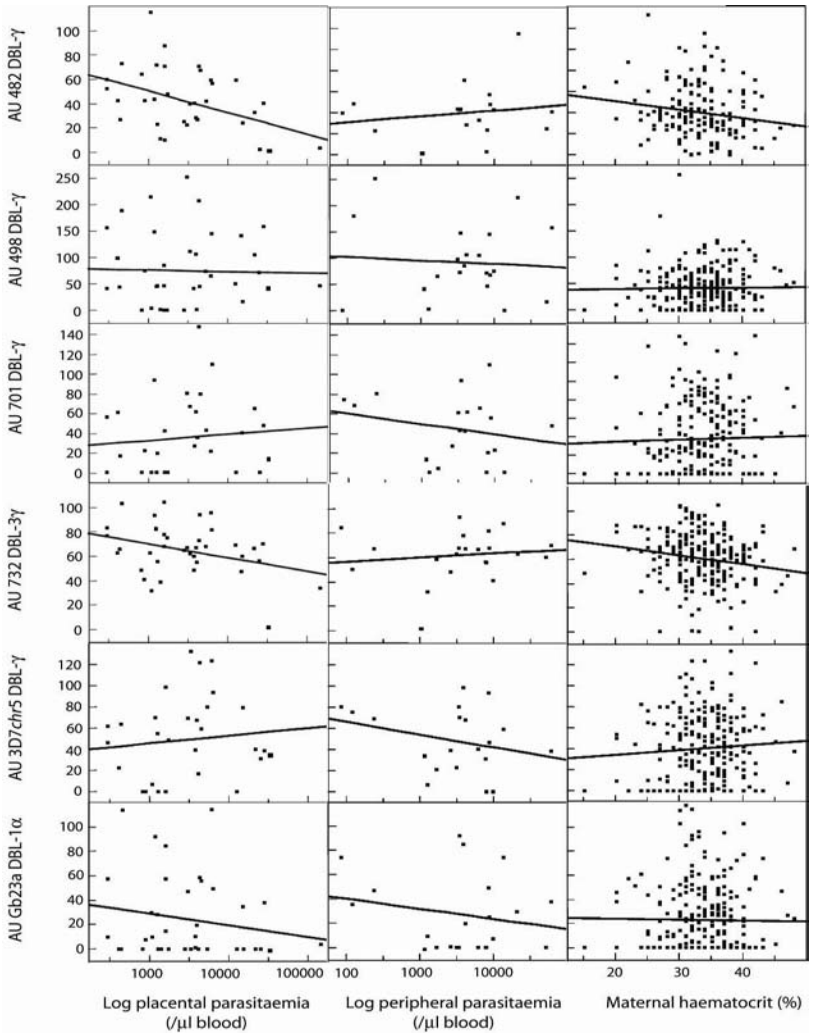


Figure 14 Correlation between antibody titres to *var*PAM DBL-γ domains (first-fourth rows), 3D7*chr*5 DBL̃γ (fifth row) and Gb23a DBL-1α (sixth row) and log placental parasitaemia (left column), log peripheral parasitaemia (middle column) and maternal haematocrit (right column). Level of antibodies to the 482 and 732 DBL−γ domain correlates negatively to log placental parasitaemia (Pearson's regression analysis yielded R=-0.4479, P=0.009 and R=-0.34695, P=0.0412, respectively) and not to log peripheral parasitaemia (both P>0.05). Anti-482 and 732 DBL-γ domain antibody titres also correlated negatively to maternal haematocrit (R=-0.1578, P=0.0399 and R=-0.1756, P=0.0058, respectively). The other DBL domains did not show any correlation to log placental parasitaemia, log peripheral parasitaemia or maternal haematocrit (all P>0.05).

4.2.3.5 Correlation of antibodies with parity

Flow cytometry measurements for antibody recognition of variant surface antigens expressed by IE have demonstrated a correlation with parity. Whether such a correlation between the levels of antibodies to the recombinant domains (as measured in ELISA) and parity also exist was sought. The reactivities of all parity groups for individual DBL domains were similar to each other and showed no correlation (P values >0.05, data not shown).

4.2.3.6 Correlation of antibodies between var*PAM DBL-γ domains*

A multivariate analysis of the levels of antibodies to *var*PAM DBL-γ domains showed two pairs of proteins that correlated to each other. A correlation between 482 and 732 DBL-γ domains was observed (Spearman's Rho=0.4885, P<0.0001), as also for the 498 and 701 DBL-γ domain pair (Spearman's Rho=0.5812, P<0.0001). Taken into consideration that levels of antibodies to both 482 and 732 DBL-γ correlate with placental parasitaemia as well as with maternal haematocrit levels, and the absence of such correlations in the case of 498 and 701 DBL-γ, may allow their tentative division in terms of their immunoreactivities into two groups, for example, those that are 482/732 DBL-γ–like and those that are 498/701 DBL-γ-like.

4.2.4 Assessment of anti-482 and anti-732 DBL-γ polyclonal antibodies

The 482 and 732 DBL-γ domains were shown above to be recognized by human antibodies in a pregnancy- and infected placenta- specific manner. Antibody titres also correlated with decreasing placental parasitaemia and decreasing maternal haematocrit. These observations together advance the hypothesis that the human antibodies that recognize 482 and 732 DBL-γ domains may contribute towards protecting pregnant women from PAM. To further investigate this possibility, polyclonal antibodies were raised against the 482 and 732 DBL-γ domains in experimental animals and analyzed for functions related to IE binding to CSA and IE surface recognition.

4.2.4.1 Specificity of recognition

The polyclonal α-482 and α-732 DBL-γ antibodies were first examined for their capacities to recognize their own immunizing antigens as well as for cross-reactivities with other *var*PAM domains. As expected, the α-482 DBL-γ polyclonal antibody recognized the 482 DBL-γ antigen well, but was also reactive with heterologous 701 and 498 DBL-γ domains and, to a lesser extent, to 732 DBL-γ (Figure 15 upper panel), indicating some cross-reactivities between the domains. On the other hand, with α-732 DBL-3γ antibody, the level of cross-reactivity with heterologous domains was lower. The antibody reacted with the homologous antigen, but was comparatively less reactive with the heterologous DBL-γ domains (Figure 15 lower panel). The Gb23a DBL-1α and 3D7*chr*5 DBL-γ domains were not recognized by both antibodies. The reason for the weak recognition of the Rifin protein by α-732 DBL-γ polyclonal antibody is not known.

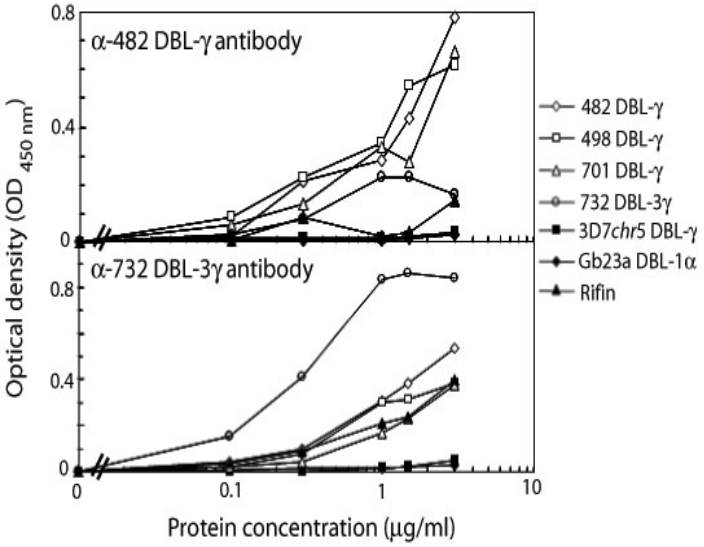


Figure 15 Reactivity of the α-482 and α-732 DBL-γ antibodies to recombinant DBL domains. The α-482 antibody (upper panel) showed a higher recognition to heterologous DBL-γ domains compared to α-732 DBL-γ antibody (lower panel). The reactivity of the antibodies is expressed as optical density (OD).

4.2.4.2 Inhibition of IE binding to CSA by the α-DBL-γ antibody

To test if the polyclonal antibodies can inhibit IE-CSA binding, the antibodies were first incubated with the IE prior to the static binding assay. The level of binding of IE to CSA in the absence of either human plasma or animal antiserum was calculated as 100 % binding (control), and used as a basis of comparison. A representative result obtained using the isolate Gb337CSA is shown in Figure 16. The α-482 DBL-γ antibody was able to reduce the CSA-binding of Gb337CSA to 30 % and α-732 DBL-3γ antibody to 45 %. The inhibition by α-482 DBL-γ antibody was comparable to those achieved by plasma from a multiparid woman. Soluble CSA and not CSC abolished binding of the Gb337CSA thus showing that the IE binding was CSA specific. On the other hand, α-

rifin antibody, used as a negative control, and the pre-immune plasma did not affect IE-CSA binding. Plasma from African men did not reduce the binding of IE^{CSA} significantly.

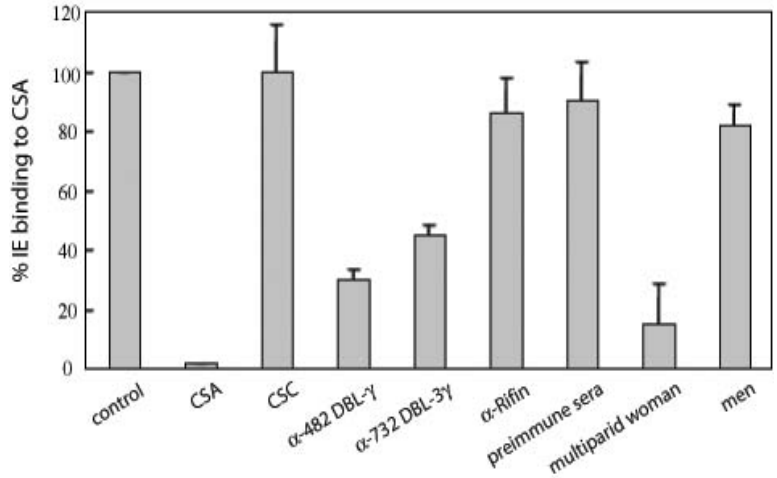


Figure 16 Inhibition of IE-CSA binding by specific α-482 and α-732 DBL-γ polyclonal antibody in a static binding assay. The results for Gb337CSA is shown here.

4.2.4.3 Surface staining of live IE

The antibodies were also examined for surface recognition of live IE in IFA (Figure 17). Both α-DBL-γ antibodies were visualized with a rim-like surface on live heterologous IE^{CSA} but not the non-CSA-binding isogenic lines (Gb337CSA and Gb337 shown in Figure 17A). This fluorescent rim is interpreted to correspond to the staining of the erythrocyte-membrane. No staining was seen within the IE indicating that the antibody-complex was not internalized by endocytosis or diffused through permeabilised membrane. When the α-DBL-γ polyclonal antibodies were used to stain non-CSA-binding IE, no fluorescence was detected (Figure 17A). Pre-immune sera did not recognize any IE (Figure 17A). To quantify the fluorescence staining, fluorescent rates were calculated (Figure 17B). Anti-482 and α-732 DBL-γ polyclonal antibodies showed

a fluorescence rate of 75 % and 66 %, respectively for the heterologous IE^{CSA} (Figure 17B left panel). This was the average fluorescence rate of Gb337CSA, VIP43CSA, Gb03CSA and FCR3CSA. When the IE^{CSA} was treated with trypsin, the fluorescence rate drops significantly to values that were comparable to those of pre-immune sera stained IE^{CSA}. Fluorescence rate for IE (the Gb337, VIP43, Gb03 and FCR3 non-CSA-binding isogenic lines) by the α-DBL-γ polyclonal antibodies and pre-immune sera was less than 5 % (Figure 17B right panel).

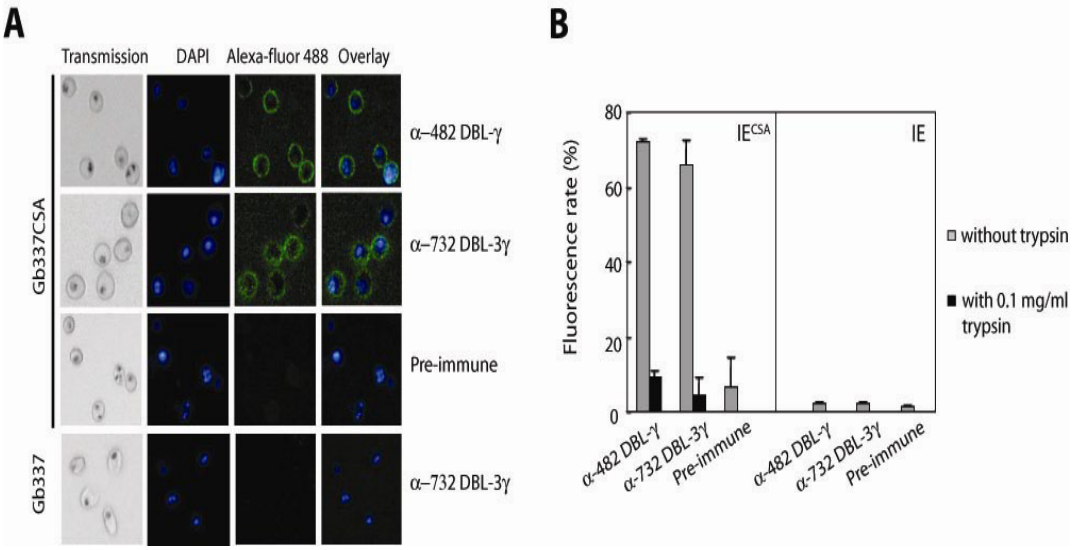


Figure 17 Recognition of live IE surface by anti-DBL-γ antibodies. (A) IFA was carried out on live Gb337 and the isogenic line Gb337CSA IE using α-DBL-γ antiserum. Cells were first observed under normal transmission light (left column) and the DNA from IE was visualized by DAPI (blue, second column). Binding of α-DBL-γ antibodies to CSA-binding IE was detected with Alexa-Flor 488-labelled secondary antibody (green, third column, first and second rows), whereas no binding was observed using pre-immune serum (third column, third) nor when the cells were not selected for CSA binding (third column, fourth row shown using α-732 DBL-3γ antibody). Specific anti-DBL-γ antibody recognition was confirmed by overlays of DAPI and Alexa-Flor 488 images (fourth column). (B) Determination of fluorescence rates of IE. Florescence rate was calculated as (number of fluorescent cells/total number of late trophozoites) x 100 % (Scholander *et al.*, 1996). The rates of IE^{CSA} and IE in the presence of the antiserum (without and with trypsin treatment) were calculated based on three independent assays, with the standard deviation shown as vertical lines above the bars.

4.3 Analysis of a placental var *gene: 732*var

4.3.1 732*var* and the PfEMP-1 domains

The 732*var* gene represents a full-length gene, first cloned from a placental isolate (Khattab et al., 2001). The first exon of the 732*var* gene codes for the extracellular variable region of the PfEMP-1 molecule. The second exon encodes the intracellular conserved acidic terminal sequence (ATS) (Figure 18). The variable region of the PfEMP-1 molecule is of interest because domains within this region have been implicated in the pathogenesis of malaria. In the 732*var* gene, the extracellular region consists of four Duffy-binding like (DBL) domains, one cysteine-rich inter-domain region (CIDR) and one conserved 2 (C2) region. The extracellular region is flanked by a transmembrane (TM) region at the C-terminal end. The 732*var* DBL domains are of the α, β, γ and ε sub-types, and the CIDR of the α subtype (Figure 18).

The 732 DBL-3γ is a member of the panel of *var*PAM DBL-γ domains that was analyzed above. For comparison purposes, the results for the DBL-3γ are also included in the results shown here for the other 732*var* domains, namely the CIDR-1α and DBL-1α, -2β and -4ε domains.

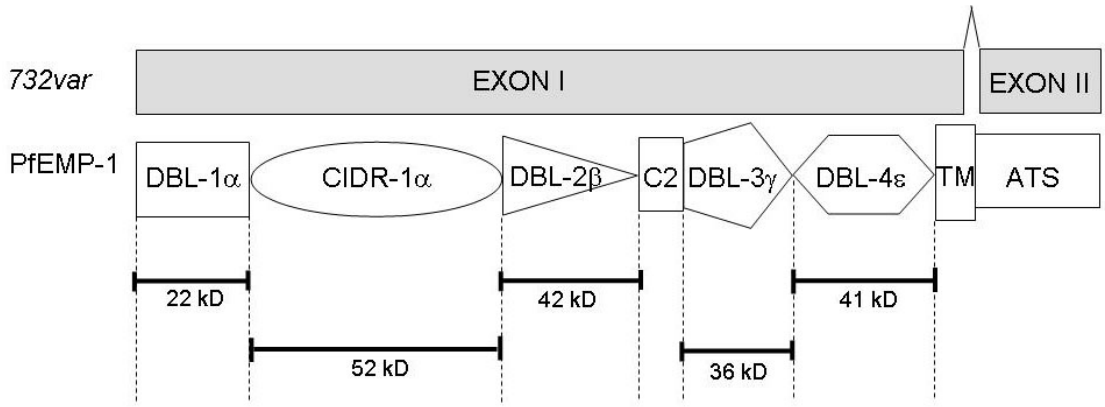


Figure 18 Domain organization of the 732*var*. The DBL and CIDR domains delineated above by dotted lines were cloned and expressed as recombinant proteins. The estimated molecular weights of each of the individual recombinant domains are given in kilodalton (kD).

4.3.1.1 Comparisons of CIDR-1α and DBL-3γ domains

Other workers have mapped the minimal receptor-binding regions of a number of *var* genes from different parasite in attempts to understand the functional requirements for receptor binding. In particular, they have looked at the CIDR-1α domains of A4*var*, A4*tres*, MC*var* CIDR-α regions and the DBL-3γ domain of FCR3*var*CSA. To see if the 732 CIDR-1α and DBL-3γ domains contain these minimal binding regions, Clustal alignments were made with the known sequences of the minimal receptor-binding regions.

4.3.1.1.1 Comparison of the 732 CIDR-1α domain with other CIDR-α domains

A stretch of 159-179 amino acid residues located at the central region of the A4*var*, A4*tres* and MC*var* CIDR-α domains has been identified to contain the region required for CD36-binding. Thirty-three residues have been reported to be conserved within these three CIDR domains. The 732 CIDR-1α domain was compared to reported minimal

binding regions. Out of the 33 identified conserved residues, 31 residues were found in the 732 CIDR-1α (Figure 19).

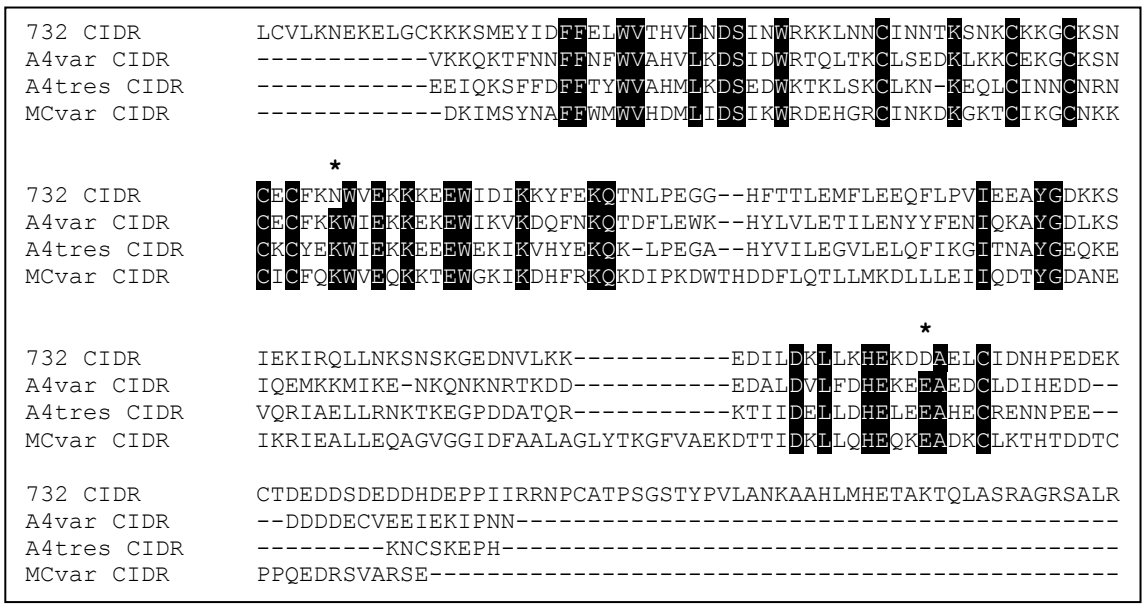


Figure 19 Conservation of the minimal binding region for CD36 in 732 CIDR-1α. A Clustal alignment of the central region of 732 CIDR-1α, A4*var* CIDR-α, A4*tres* CIDR-α and MC*var* CIDR-α was made. Thirty-three 100 % identical residues (shaded black) were identified between the latter three *var* genes, 31 of which are present in 732 CIDR-1α. The two non-identical amino acids are marked with an asterisk. The alignment was made with Clustal W (http://align.genome.jp).

4.3.1.1.2 Comparison of the 732 DBL-3γ domain with the FCR3CSA DBL-γ domain

Recently, a 67 amino acid residue region was identified in the FCR3CSA DBL-γ domain to be the minimal CSA-binding region. This region has been shown to be conserved in ItR29 DBL-2γ and 3D7*chr*5 DBL-3γ. Out of the 41 conserved residues between FCR3*var*CSA, It29 and 3D7*chr*5 DBL-γ domains, only 16 were found in 732 DBL-3γ (Figure 20).

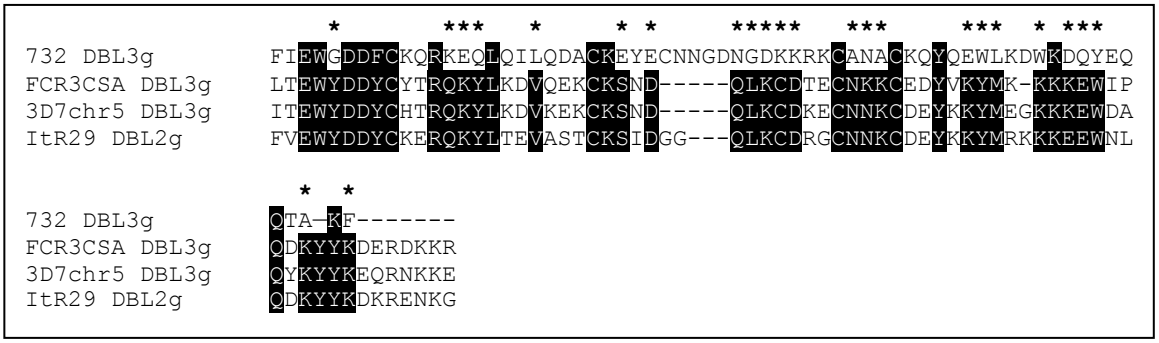


Figure 20 Conservation of the minimal binding region for CSA in 732 DBL-3γ. A Clustal alignment of the regions close to the C-terminal end of 732 DBL-3γ, FCR3CSA DBL-3γ, 3D7*chr*5 DBL-3γ and ItR29 DBL-2γ was made. 100 % identical residues are shaded in black; residues in the 732 sequence differing from those in the other sequences are marked with an asterisk. The alignment was made with Clustal W (http://align.genome.jp).

4.3.2 Cloning, expression and purification of recombinant 732*var* domains

Prediction of the secondary protein structures (See appendix 1) was performed using the program Predator available at the server hosted by the Pasteur Institute, Paris, France (http://bioweb.pasteur.fr/seqanal/interfaces/predator-simple.html). All four 732*var* protein domains were expressed in *E. coli* cells and the purified fraction was separated by SDS-PAGE are shown (Figure 21). DNA sequencing, Western blot analysis, and peptide fingerprinting analysis verified the identities of the proteins.

The 732 DBL-1α domain was produced as a recombinant protein with the size of approximately 28 kD, which was within the expected range of the recombinant protein taking account vector-specific amino acids estimated to add 3 – 4 kD to the protein core. A 40 kD sized recombinant CIDR-1α domain was observed in the SDS-PAGE gel, which is smaller than the expected 52 kD. The DBL-2β and DBL-3γ recombinant proteins were observed with sizes of 40 and 43 kD, respectively, rather than 42 and 36 kD, respectively.

The unexpected size difference between observed and calculated molecular masses of the recombinant CIDR–1α, DBL-2β and DBL-3γ domains could be due to anomalous migration in the SDS-polyacrylamide gel. The recombinant DBL-4ε domain, with a size of 43 kD, was purified together with two smaller protein bands (with a protein size of 35kD and 30 kD). The two smaller bands were found by Western blot analysis and peptide fingerprinting analysis to be breakdown products of the larger.

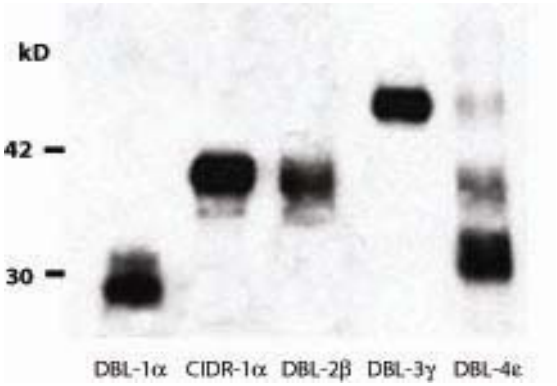


Figure 21 Analysis of the recombinant 732*var* domains. Purified recombinant domains were separated in a 12 % polyacrylamide gel under reducing conditions and stained with Coomassie Blue.

4.3.3 Biochemical characterizations of different 732*var* domains

The 732*var* gene in being cloned from a placental isolate is unique and novel. The domains from this *var* gene were extensively analyzed for the first time in this thesis. Based on reports by others that the DBL-β, DBL-γ and CIDR-α domains from various *var* genes can bind ICAM-1, CSA, and CD36 receptors, respectively and since these domains are also present in the 732*var* gene, the next most logical step therefore was to test their binding properties.

4.3.3.1 Static in vitro binding assay using CHO-cells

CHO cells expressing the receptors CSA (CHO-K1), CD36 (CHO-CD36) and ICAM-1 (CHO-ICAM-1) were used in this assay. Recombinant 732 CIDR-1α and DBL-3γ domains were able to bind CHO-CD36 and CHO-K1 cells, respectively, but not CHO-ICAM-1 (Figure 22 second and fourth row). The 732 DBL-2β did not bind to the ICAM-1 receptor (CHO-ICAM-1) or to any other receptors (Figure 22 third row), similarly for the 732 DBL-1α protein (Figure 22 first row). Further controls were included in this preliminary binding assay to prevent detection of non-specific protein interaction with the surface of the CHO cells. Rifin, a *P. falciparum* recombinant protein with no known relevant function in receptor binding, was included in the assay as a protein control. As expected, CHO-745 cells, which is devoid of surface expression of all three receptors, did not support the binding of any of the recombinant proteins tested (data not shown).

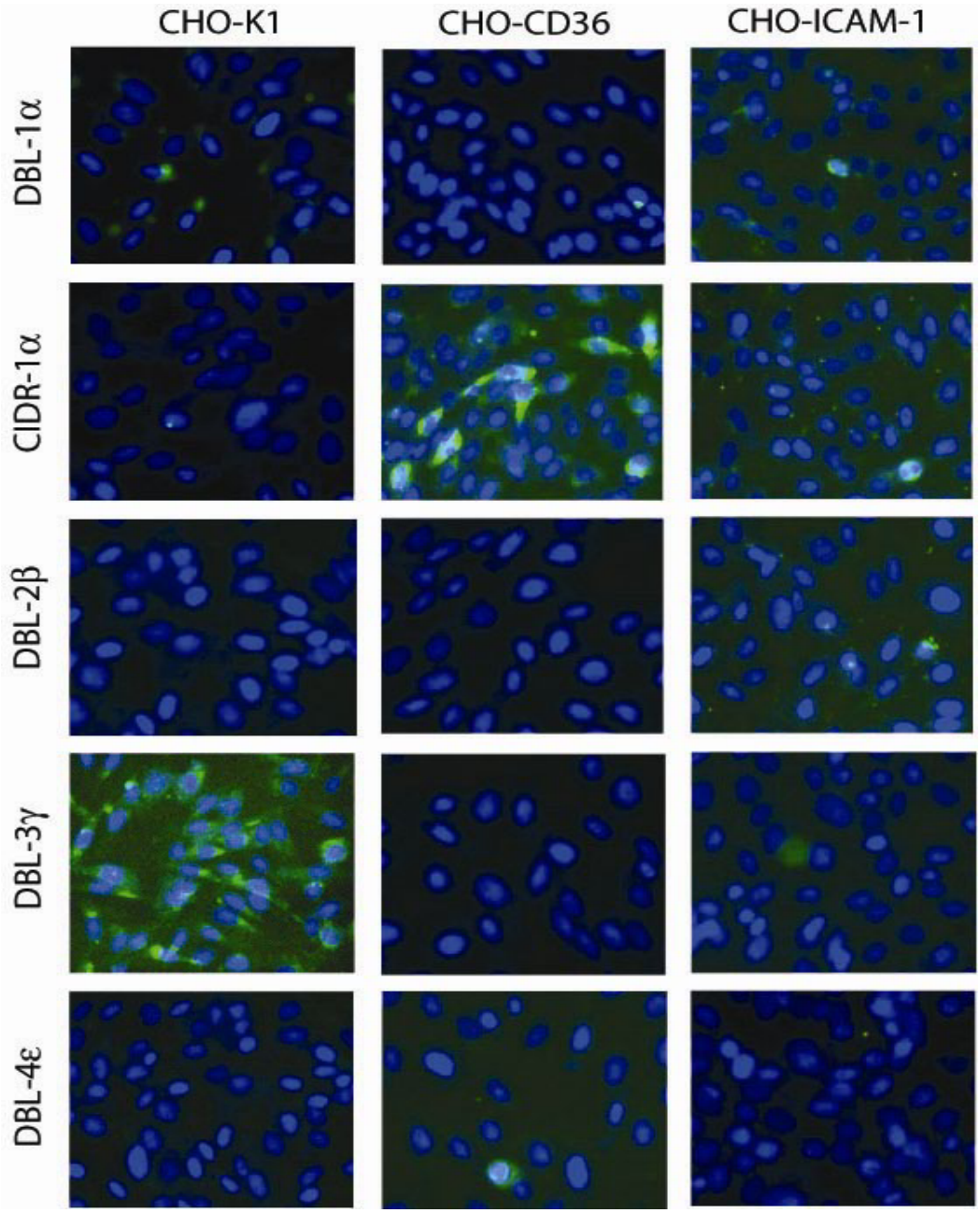


Figure 22 Binding of 732*var* domains to CSA, CD36 and ICAM-1 receptors expressed on CHO cells. The recombinant DBL-1α (first row), CIDR-1α (second row), DBL-2β (third row), DBL-3γ (fourth row) and DBL-4ε (last row) domains were assessed for binding to CHO-K1 (first column), CHO-CD36 (middle column) and CHO-ICAM-1 (last column) cells. Co-localization of recombinant proteins bound on CHO cells was visualized with an overlay of Alexa-flor 488 (green) and DAPI (blue) fluorescent images.

4.3.3.2 Specificity of the binding of 732 DBL-3γ to glycosaminoglycans

Chondroitin and heparan sulfate are glycosaminoglycan (GAG) receptors that are expressed on CHO-K1 cells. To characterize the binding specificity of the 732 DBL-3γ, soluble GAGs were used to compete for the binding of recombinant proteins to CHO-K1 cells. CSA, CSB, CSC and HA from BVH were included in the test. Recombinant DBL-3γ was shown to bind CHO-K1 cells, as evidenced by the red fluorescence around the cells. This binding was inhibited by soluble CSA in a concentration-dependent manner, detected as a reduction in fluorescence intensity in IFA (Figure 23 upper row). Competition for binding was less efficient using the other three GAGs. There was some weak inhibition by CSC and HA at high GAG concentrations, whereas CSB did not seem to affect the binding of DBL-3γ to CHO-K1 cells (Figure 23 lower row).

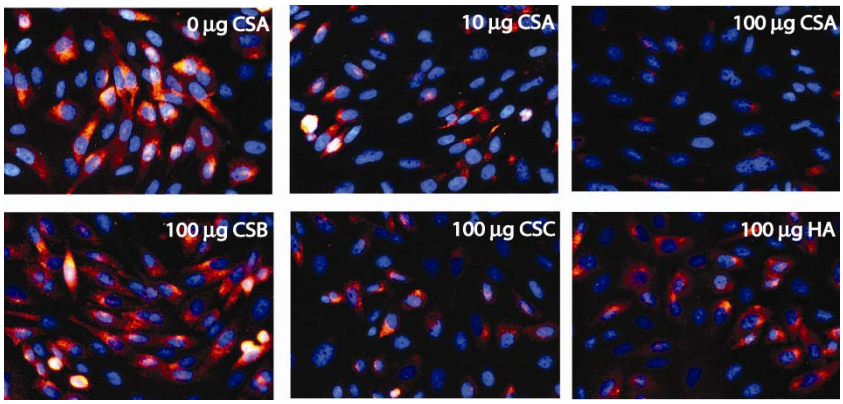


Figure 23 Binding specificity of the 732 DBL-3γ to GAG receptors. The recombinant DBL-3γ domain was incubated with increasing concentrations of soluble CSA receptors (10 µg and 100 µg of CSA) (upper row). The protein was incubated with 100 µg of CSB, CSC and HA (lower row). Co-localization of recombinant proteins bound on CHO-K1 cells were visualized with an overlay of Alexa-flor 594 (red) and DAPI (blue) fluorescent images.

4.3.3.3 Competitive inhibition of IE binding to different CHO-cells

The binding of recombinant 732 CIDR-1α and DBL-3γ domains to CD36 and CSA receptors, respectively, was further characterized in IE-receptor binding. FCR3CSA parasite-IE bound CHO-K1 cells and recombinant DBL-3γ domain was found to inhibit this binding (Figure 24A). When the recombinant CIDR-1α was incubated with FCR3CSA and CHO-K1, the binding of FCR3CSA to CHO-K1 was not affected. In a similar fashion, FCR3CD36 bound CHO-CD36 cells. However, incubation of FCR3CD36 with recombinant CIDR-1α domain reduced the number of IE bound to CHO-CD36 but not with DBL-3γ (Figure 24B). Both recombinant domains exhibited concentration-dependent inhibition of binding. Recombinant DBL-1α, -2β and -4ε domains were not incorporated in this assay because they do not possess any binding activity to any of the three receptors tested as shown earlier (see Sect. 4.3.3.1)

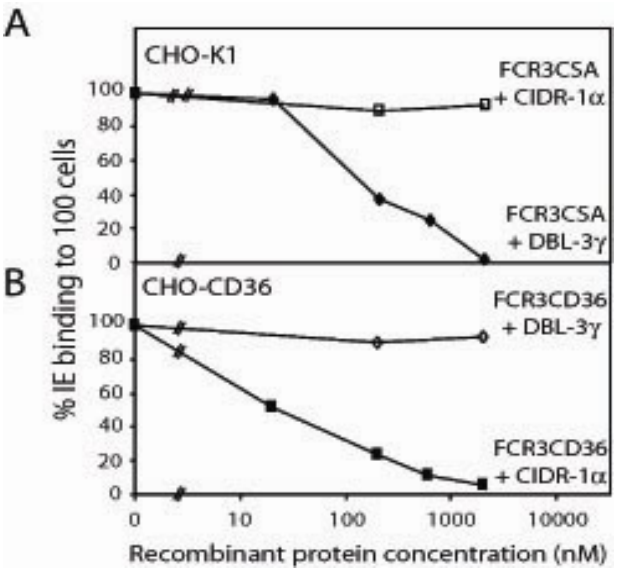


Figure 24 Competitive inhibition of IE binding to CSA and CD36 by recombinant 732 DBL-3γ and CIDR-1α domains. (A) Incubating FCR3CSA with DBL-3γ inhibited the binding of IE and CHO-K1 cells in a concentration-dependent manner. This inhibition was not observed with CIDR-1α domain. (B) Similarly, the presence of CIDR-1α inhibited binding of FCR3CD36 to CHO-CD36 in a concentration-dependent manner. 732 DBL-3γ did not affect FCR3CD36-CHO-CD36 binding.

4.3.4 Immunological characterizations of the recombinant 732*var* domains

4.3.4.1 Immunogenicity of recombinant proteins

To evaluate the immunogenicity of the 732*var* domains in ELISA, the same cohort of plasma samples as used above was employed. The number of plasma samples in the present investigation was lower than the previous one described in Sect. 4.2.3, due to depletion of samples.

As above, the cohort was divided into two groups; namely the malaria-exposed non-pregnant African adults and malaria-exposed pregnant women. The most significant result is the comparison of the reactivities of DBL-1α and -3γ domains between non-pregnant adults and pregnant women. Here, a higher mean level of antibody recognition was achieved in pregnant women compared to non-pregnant adults (both $P<0.05$) (Table 7). The three remaining domains showed no significant difference in antibody levels between the pregnant and non-pregnant malaria exposed individuals. While the DBL-3γ domain may have an implication in PAM, like those reported from the FCR3*var*CSA and the CS2*var* genes, a role for DBL-1α remains to be clarified.

Table 7 Measurement of plasma antibody titres to the recombinant DBL domains by ELISA.

Group	*n*	DBL-1α	CIDR-1α	DBL-2β	DBL-3γ	DBL-4ε
European controls	39	3.96 ± 3.78	18.41 ± 5.95	24.17 ± 5.54	9.54 ± 4.04	40.09 ± 31.85
Non-pregnant adults	16	8.16 ± 6.28	41.25 ± 9.26	28.50 ± 8.64	16.64 ± 6.51	49.47 ± 49.44
Pregnant women	235	24.01 ± 1.56*‡	56.94 ± 2.44*	38.91 ± 2.24*	52.90 ± 1.64*‡	132.48 ± 13.01*

Plasma samples were collected from Africans living in Guediawaye, Senegal and malaria naïve Europeans.
n indicates the number of individuals from whom plasma were collected.
§ Data are expressed as mean of Arbitrary Units (AU) followed by the standard deviation
* Significantly different from European controls, Student t-test $P<0.05$
‡ Significantly different from non-pregnant adults, Student t-test $P<0.05$

4.3.4.2 Correlation of antibodies with parasitaemias

The antibody responses of the pregnant women were further analyzed for clinical associations. Interestingly, level of antibodies to 732 DBL-3γ domain correlated with a decreasing placental parasitaemia in the pregnant women (R= -0.3366, P=0.0447) (Figure 25 left column). This negative correlation could indicate that the antibodies are capable of controlling parasite growth. While the levels of antibodies to DBL-1α, -2β and -4ε did not show any correlation to placental parasitaemia (all P>0.05), there was a positive correlation of placental parasite density with anti-CIDR-1α antibody titres (R= +0.49929, P=0.0080) (Figure 25 left column). The significance of the reactivity of anti-CIDR-1α antibodies in PAM is not yet clear, but possibly these antibodies could serve as an indicator of infection. To investigate whether these observations are restricted to the placenta, a similar analysis was performed with maternal peripheral parasitaemia during delivery. None of the antibody titres to the 732*var* domains was observed to significantly correlate with maternal peripheral parasitaemia (all P>0.05) (Figure 25 middle column).

4.3.4.3 Correlation of antibodies with maternal haematocrit

Further analysis of the ELISA reactivities showed that anti-DBL-3γ antibody titres correlated negatively to maternal haematocrit (R= -0.1756, P=0.0058). No correlation was observed between levels of antibodies to the 732*var* domains and maternal haematocrit (P>0.05) (Figure 25 right column).

4.3.4.4 Correlation of antibodies with parity and age

Further analysis was performed to investigate if levels of antibodies to the 732*var* domains correlated to the pregnant women's parity and age. The level of antibodies were similar when the women were categorized by parity and age being investigated ($P>0.05$) (data not shown). This was observed in all 732 DBL and CIDR domains. Further multiple linear regression analysis showed that antibody recognition to 732*var* domains is not influenced by parity or age ($P>0.05$).

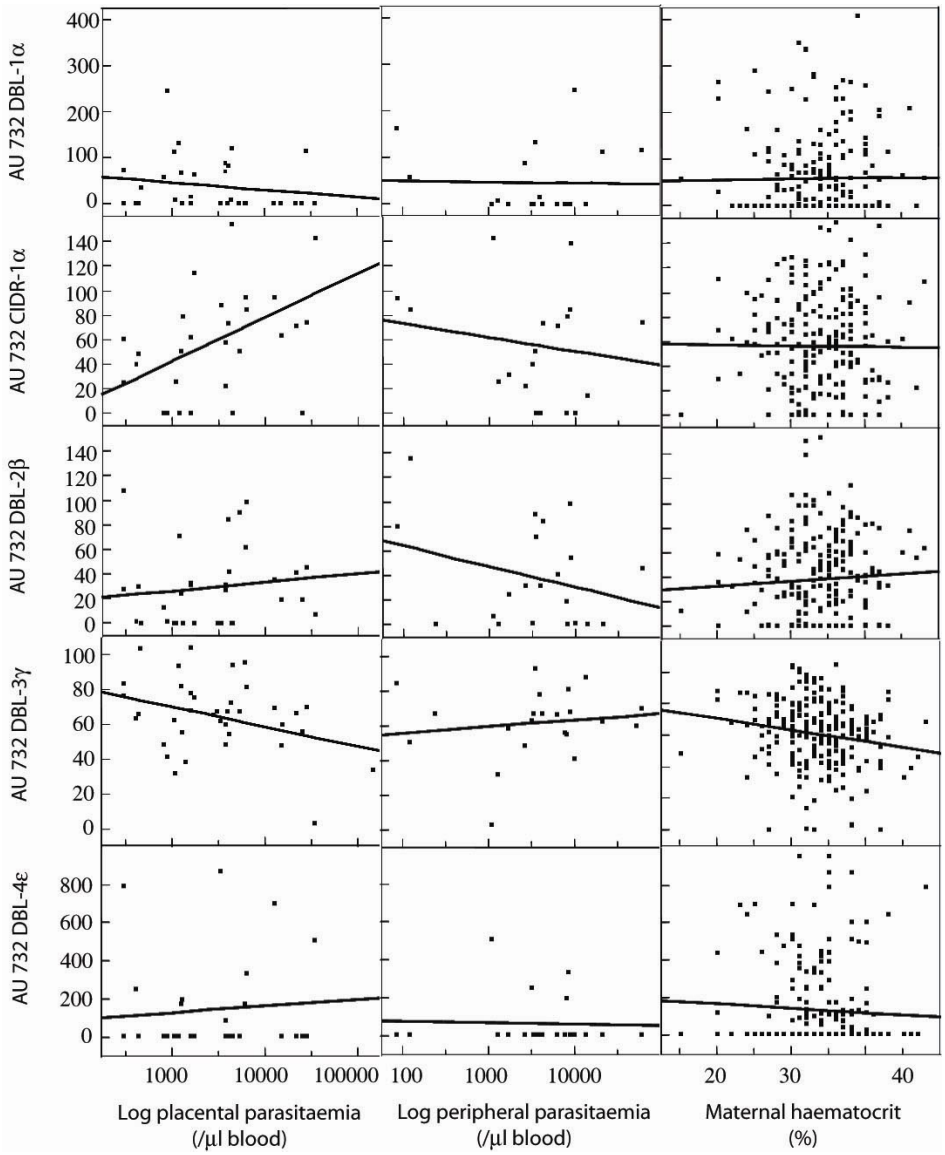


Figure 25 Correlations between of levels of antibodies to 732*var* domains and placental parasitaemia (left column), peripheral parasitaemia (middle column) and maternal haematocrit (right column). Levels of antibodies to DBL-3γ correlated negatively to log placental parasitaemia (R= -0.3366, P= 0.0447) and positively to maternal haematocrit (R= -0.1756, P= 0.0058). Anti-CIDR-1α antibodies highly correlated positively to log placental parasitaemia (R= +0.49929, P= 0.0080) but not to maternal haematocrit (P>0.05). Levels of antibodies to DBL-1α, -2β and -4ε did not show any correlation to log placental parasitaemia or maternal haematocrit (all P>0.05). None of the antibody titres correlated to log peripheral parasitaemia (all P>0.05).

5. Discussion

5.1 Materials and methods used in this study

In order to investigate the biochemical characteristics of the various *P. falciparum* erythrocyte membrane-1 (PfEMP-1) domains, three essential starting materials were required: (a) the host-receptor molecules as binding partners to parasite ligands, (b) the isogenic lines of *P. falciparum*-infected erythrocytes (IE) that bind to different host receptors, and (c) various PfEMP-1 domains in recombinant proteins to function as receptor-binding ligands. Efforts were therefore taken to acquire these materials as outlined below.

5.1.1 Host receptors for biochemical characterization of PfEMP-1 domains

Various receptors have been implicated with a role in host-parasite interactions in malaria, amongst them CD36, intercellular adhesion molecule-1 (ICAM-1) and CSA. Substantial evidence has supported the role of CSA as the binding partner of placental IE in PAM.

CSA is a glycosaminoglycan (GAG), and shares structural similarities with chondroitin sulfate B and C (CSB and CSC) and hyaluronic acid (HA). The GAG molecules are commercially available as purified receptors. These purified receptors can be immobilized on plastic surfaces for binding assays. GAG molecules are also expressed naturally on the surface of Chinese hamster ovary (CHO) cells, CHO-K1 (Tjio, Puck, 1958; Esko et al., 1985) which serve in this work as an alternative source of CSA and HA for binding studies.

Since CD36 and ICAM-1 receptors were not readily available as purified receptors, CHO cells that express these receptors on their surface were used instead. These CHO-CD36 and -ICAM-1 cells are stable transfectants of a mutant CHO cell line, CHO-pgsA-745 cells (referred to as CHO-745 in this thesis), that is devoid of endogenous CSA expression. CHO-745 cells lack an active xylosyltransferase enzyme that participates in the concomitant first sugar transfer in the GAG biosynthesis pathway, which is common for CSA and HA (Esko et al., 1985). As a result, the CHO-745 cell is unable to synthesize GAG and therefore the cell surface is devoid of CSA and HA. CSA/HA-expressing CHO-K1 cells can thus be compared with transfectant CD36- and ICAM-1-expressing CHO lines for their interaction with IE.

5.1.2 Establishment of IE isogenic lines that bind CSA, CD36 and ICAM-1

IE that sequesters in the placenta has been shown to bind CSA only but not CD36 or ICAM-1 (Rogerson et al., 1995; Fried, Duffy, 1996). The study of placental isolates (or laboratory strains that have been selected to bind to CSA) requires the inclusion of IE that bind receptors other than CSA as controls in binding assays. For the purpose of this study, three isogenic lines of FCR3were used: FCR3CSA that binds CSA receptor, FCR3CD36 to CD36, and FCR3ICAM-1 to ICAM-1 (see Fig 9).

5.1.2.1 Specificity of the CSA-binding phenotype of the IE

All CSA-binding IE (IE^{CSA}) strains used in this study (the Gb337CSA and VIP43CSA placental isolates, the Gb03CSA cerebral isolate and the FCR3CSA laboratory strain) bind specifically to CSA and only marginally to HA (see Fig 10). These strains show no affinity to CSB and CSC. It has been reported that IE strains that were selected to bind to

CSA can also bind to HA, an alternative receptor reported to be involved in IE binding (Beeson et al., 2000; Beeson, Brown, 2004). However, this was not found to be the case here.

5.1.3 Expression of PfEMP-1 recombinant domains in *Escherichia coli* cells

In order to characterize the DBL and CIDR domains, they were first produced as soluble recombinant proteins. Proteins synthesized in this form are expected to retain properties related to their biological function. However, it is not uncommon that proteins produced by *E. coli* do not fold properly, post-translational processing does not occur normally, and they tend to aggregate in inclusion bodies within the bacterial cell. Proteins that are produced within bacterial inclusion bodies require extraction under denaturing conditions and subsequent tedious "re-folding" steps. The baculovirus/insect cell system is ideal for producing correctly folded and post-translational modified proteins, but overall is slow and difficult to use, involving multiple steps to produce the recombinant baculovirus and to infect insect cells with the virus. Consequently, it was decided to express the proteins in *E. coli* cells, given that a number of PfEMP-1 domains have been successfully extracted from the bacterial cytoplasm as soluble and functional proteins (Smith et al., 1998 ;Reeder et al., 1999; Degen et al., 2000).

5.1.3.1 Gb23a DBL-1α domain as control

The Gb23a DBL-1α domain was included in this study as a control. This domain has been previously characterized using sera from semi-immune children in an earlier work (Oguariri et al., 2001). The DBL-1α exhibits adhesive features associated with severe

malaria including blood group antigen A, heparin sulfate A and complement receptor (CR) 1 binding. It shows no affinity to CD36, ICAM-1 and CSA (Smith, Craig, 2005).

*5.1.3.2 3D7*chr5var *DBL-5γ domain as control*

The 3D7*chr*5*var* gene has 62 % amino acid identity with the FCR3*var*CSA gene, and has been shown to be highly conserved in various IE (Rowe et al., 2002; Winter et al., 2003). The FCR3*var*CSA is one of the first *var* genes to be implicated in PAM (Buffet et al., 1999). The 3D7*chr*5*var* sequence is truncated at the end of DBL-7ε and does not contain the expected intron and exon II sequence (Rowe et al., 2002; Winter et al., 2003). The resulting protein is consequently not exported to the surface and is therefore non-functional (Winter et al., 2003). The 3D7*chr*5*var* contains two DBL-γ domains, DBL-3γ and -5γ (Vazquez-Macias et al., 2002; Kyes et al., 2003). The latter has been shown to have no CSA-binding property (Vazquez-Macias et al., 2002; Gamain et al., 2005) and therefore used in this study as a negative control for the *var*PAM DBL-γ domain binding assays.

5.2 Analysis of recombinant var*PAM DBL-γ domains*

Pregnant women, particularly those in their first and second pregnancies, are vulnerable to PAM. The observation that multiparious women are less affected has been linked to the development of strain-transcendent antibodies in these individuals that prevent the binding of IE to the placenta (Fried et al., 1998; Beeson, Duffy, 2005). These anti-adhesion antibodies probably recognize conserved parasite antigens that are essential for CSA-binding in a subpopulation of parasites. The corresponding DBL-γ domains from

the FCR3*var*CSA and CS2*var* genes have been established to bind specifically to CSA (Buffet et al., 1999; Reeder et al., 1999), as shown also for placental-derived isolates (Rogerson et al., 1995). Work on DBL-γ domains from placental isolates has supported this observation (Khattab et al., 2001). These DBL-γ domains, known as *var*PAM DBL-γ domains bind CSA and are conserved among wild isolates that infect placenta from different geographical regions and sampled at different times (Khattab et al., 2001; Khattab et al., 2003). This observation agrees with the consensus that CSA-binding placental IE express conserved PfEMP-1 variants.

5.2.1 Biochemical characterization of recombinant *var*PAM DBL-γ domains

5.2.1.1 Production of functional recombinant var*PAM DBL-γ domains*

In this study, the four *var*PAM DBL-γ domains together with the control 3D7*chr*5 DBL-γ and Gb23a DBL-1α domains were produced as correctly folded and functional proteins. They have been in-part characterized by their specific binding to CSA but not to CD36 or ICAM-1 receptors expressed on CHO cells (see Fig. 12). The observed binding of the recombinant *var*PAM proteins to CSA was also shown to be specific. Neither the 3D7*chr*5 DBL-γ nor Gb23a DBL-1α recombinant domains were found to bind CSA.

5.2.1.2 Inhibition of IE-CSA binding by the recombinant var*PAM DBL-γ domains*

Recombinant 482, 498 and 732 DBL-γ domains not only bound CSA in vitro, but they also competitively inhibited the binding of IE to CSA in a concentration-dependent manner (see Fig 13). The differences in the level of inhibition by the recombinant domains may be attributed to the differences in amino acid sequence of the proteins.

Costa et al. had earlier obtained similar observations where the recombinant proteins of the FCR3CSA and 3D7 DBL-3γ proteins could bind CSA, but showed differences in their capabilities to inhibit IE-CSA binding in vitro (Costa et al., 2003). It is not understood why the 701 DBL-γ domain did not compete out IE-CSA binding despite its affinity to CSA in vitro. Presumably, it only binds CSA weakly and is thus unable to compete with the stronger IE-CSA interaction. Binding assays performed here are only qualitative measurements and an interaction analysis using a surface resonance biosensor, such as a BIAcore system, could be employed in future experiments to provide quantitative information on the rate and affinity of each recombinant domain binding to CSA (Horan et al., 1999; Maeda et al., 2003).

5.2.3 Immunological characterization of the recombinant *var*PAM DBL-γ domains

5.2.3.1 Pregnancy-specific of var*PAM DBL-γ domains*

Variant surface antigens (VSA), mainly PfEMP-1 variants encoded by *var* genes, are targeted by the human immune system. Adults gradually become immune to clinical malaria after repeated infections through the acquisition of a broad repertoire of anti-VSA antibodies (Bull et al., 1998). One of the rationales behind this thesis was that *var* gene products from placental isolates were similarly the target of immunity in the pregnant woman. One first step was the evaluation of the immunogenicity of the cloned *var*PAM DBL-γ domains in ELISA. Overall, some antigens analyzed were found to induce a pregnancy-specific antibody response in the study cohort of African adults.

5.2.3.2 Contribution of human antibodies in placenta infection control

Further analysis of the antibody responses in pregnant women showed that antibodies to two of the domains, 482 and 732 BDL-γ, associated negatively with placenta parasite density (see Fig. 14). This association was observed only with placental parasites but not with parasites from peripheral blood. This finding implies a role of the antibodies in controlling placental infection. For the first time, a link between a defined parasite CSA-binding ligand and a decrease in placental parasitaemia is shown, thereby raising hopes in the development of a subunit PAM vaccine based on a recombinant molecule.

Earlier works have shown an association between low placental parasitaemia and high levels of antibodies that blocked adhesion of IE to CSA (Tuikue Ndam et al., 2004; Taylor et al., 2004; Fried et al., 1998). Reported ELISA observations have currently only been able to show an association between antibody response to DBL-6ε (a domain encoded by *var*2csa gene) and low-birth-weight babies, a PAM complication (Salanti et al., 2004). Human antibody response to 2O2CSA*var* DBL-1α, CIDR-1α and DBL-2β domains was not correlated with PAM complications (Jensen et al., 2003). Thus far, decreasing placental parasitaemia has only been associated with anti-RESA and anti-MSP1-19 antibody titers (Mvondo et al., 1992; Taylor et al., 2004; Astagneau et al., 1994). The antibodies to RESA and MSP1-19 contribute to immune protection against blood-stage parasite multiplication in the pregnant women but are not PAM-specific.

An unexpected observation worthy of discussion is the association of anti-482 and anti-732 DBL-γ antibody titers with decreasing maternal haematocrit. In the first instance, this appears to be contradictory to a protective role of the anti-482 and anti-732 DBL-γ antibodies. One likely explanation would be that women in the study group have become

infected by malaria parasites over a prolonged period, the consequence of which is a low haematocrit level, but in being constantly exposed, the women are also simultaneously mounting a sturdy antibody response to the antigens.

5.2.3.3 Absence of parity-dependent recognition

Human antibodies that recognize 482 and 732 DBL-γ domains do not display the parity-dependent antibody recognition pattern. Flow cytometry measurements have shown that antibodies from pregnant women recognize placental isolates as well as IE selected to bind CSA in a gender- and parity-dependent manner. The level of recognition of these anti-VSA antibodies correlated with protection of mothers from PAM as well as other clinical complications of PAM such as maternal anaemia, low-birth-weight and premature babies (O'Neil-Dunne et al., 2001; Duffy, Fried, 2003; Tuikue Ndam et al., 2004; Staalsoe et al., 2004; Khattab et al., 2004).

However, the existence of adhesion blocking antibody that are independent of parity and VSA recognition has been reported (Beeson et al., 2004; Tuikue Ndam et al., 2004). These anti-adhesion antibodies appear to target distinct and overlapping epitopes that are different from those recognized by anti-VSA antibodies measured in flow cytometry (Beeson et al., 2004; Tuikue Ndam et al., 2004). Based on these findings, it is postulated that anti-482 and anti-732 DBL-γ antibodies form a discrete population of antibodies within those found in human plasma. They exist in small quantities in the human plasma and are likely to be detected only in the presence of specific antigens.

5.2.3.4 Generation of adhesion blocking antibodies in experimental animals

The recombinant 482 and 732 DBL-γ domains induced in experimental animals adhesion-blocking antibodies that were IE strain independent. In IFA, these antibodies recognize conserved epitopes on the surface of heterologous IE^{CSA} (see Fig. 17). These results are consistent with those observed using antibodies generated against the FCR3*var*CSA and 3D7*chr*5 DBL-3γ domains (Costa et al., 2003). Hence, these experimentally induced anti-DBL-γ antibodies are probably similar in function to those protective antibodies that are present in multiparous women.

The anti-482 DBL-γ polyclonal antibody can inhibit IE-CSA binding better than the anti-732 DBL-γ polyclonal antibody (see Fig. 16) even though both antibodies stain the IE^{CSA} surface efficiently (see Fig. 17). The anti-482 DBL-γ polyclonal antibody shows a higher reactivity to heterologous DBL-γ domains compared to anti-732 DBL-3γ polyclonal antibody, which was more specific to its homologous recombinant DBL-3γ protein. These observations can be interpreted in the following way: the anti-482 DBL-γ polyclonal antibody recognizes epitopes that are more conserved in DBL-γ domains, whereas anti-732 DBL-3γ polyclonal antibody recognizes specific overlapping CSA-binding epitopes. The different properties of the antibodies can alternatively be explained by differences per se in the anti-DBL-γ antibody content (such as antibodies with different specificities) defined by the individual host factors rather than being antigen-dependent.

5.2.4 Putative CSA-binding ligands

PAM is a complicated and not well-understood phenomenon. Several *var* genes have been implicated to play a role in causing PAM (Buffet et al., 1999; Reeder et al., 1999; Rowe et al., 2002; Salanti et al., 2003), however, to date conclusive evidence is still lacking. In an attempt to resolve some of these issues, Rowe and Kyes recently suggested a number of criteria that a PAM causing *var* gene product should fulfill, as listed in Table 1 (Rowe, Kyes, 2004). Properties of the different *var*PAM DBL-γ domains in relation to the listed criteria are summarized in Table 8. Accordingly, the two 482 and 732 DBL-γ domains appear to display most of the characteristics of a PAM ligand.

On the other hand, the 498 and 701 DBL-γ domains bind CSA in vitro but antibodies to them do not associate with malaria complications. The fact that 498 and 701 DBL-γ domains were cloned from expressed *var* genes in placental isolates could mean that these *var* genes are involved in PAM in other ways. It is likely, for example, that there are multiple CSA-binding domains on the *var* genes. It has been shown that CIDR-1α and DBL-X domains can bind CSA (Reeder et al., 2000; Degen et al., 2000; Gamain et al., 2005). Evidence is also accumulating that there are other host receptors involved in the host-parasite interaction.

Table 8 Criteria for identification of *P. falciparum* CSA-binding ligand.

Criterion	*var*PAM DBL-γ domains			
	482	498	701	732
The protein should bind CSA.	+	+	+	+
(Inhibition of IE-CSA binding by protein, this study:)	+	+	-	+
The protein, or epitopes within it, should be present on the surface of IE^{CSA} and conserved in IE from different geographical regions.	+	ND	ND	+
Antibodies raised against it should inhibit IE-CSA binding.	+	ND	ND	+
The protein should be recognized by pregnant women in a pregnancy-specific manner.	+	-	-	+
The gene should be recognized by pregnant women in a parity-specific manner.	-	-	-	-
The gene should be up-regulated in IECSA (both Laboratory strains and placental isolates) compared to non-IECSA.	ND	ND	ND	ND
Association of human antibody recognition to a clinical outcome/pathology of PAM.	+	-	-	+
(Decreasing placental parasitaemia, this study:)	+	-	-	+
(Decreasing maternal haematocrit, this study:)	+	-	-	+

Note: + indicates positive result, - indicates negative result, and **ND** not done

Clearly, these criteria only serve as a guideline to identify putative PAM parasite ligands and PAM-causing *var* genes. So far, the three leading *var* genes, the FCR3*var*CSA (Buffet et al., 1999), CS2*var* (Reeder et al., 1999) and *var*2csa (Salanti et al., 2003), have not been able to fulfill all of the criteria outlined above. Domains of all three *var* genes have been shown to be capable of binding CSA (Reeder et al., 1999; Buffet et al., 1999; Gamain et al., 2005). The recombinant FCR3*var*CSA DBL-3γ elicits strain independent anti-adhesion antibodies that recognize surface epitopes of IE^{CSA} in experimental animals (Lekana Douki et al., 2002; Costa et al., 2003). However, human antibody recognition of the FCR3*var*CSA DBL-3γ protein failed to display pregnancy- and parity-specific recognition. The FCR3*var*CSA has been shown to be conserved in IE, but the transcription pattern of the gene does not coincide with the CSA-binding phenotype (Rowe et al., 2002; Fried, Duffy, 2002; Winter et al., 2003). The CS2*var* gene has received little attention compared to the FCR3*var*CSA. Antibodies raised to the

CS2*var* DBL-2γ domain inhibit IE-CSA binding (Reeder et al., 1999). Transcription of CS2*var* gene consistently up-regulated in all IE strains regardless of their binding phenotype (Rowe, Kyes, 2004). In contrast, the most recently characterized *var*2csa gene has been shown to be up-regulated in IE^{CSA}. Moreover, human antibody recognition of the DBL-6ε domain is pregnancy- and parity-specific. An equally important finding is that pregnant women who have antibodies recognizing the *var*2csa DBL-6ε are at a lower risk of giving birth to an underweight infant (Salanti et al., 2003; Salanti et al., 2004). Despite these PAM-linked characteristics, to date there is no experimental evidence that the *var*2csa encoded protein is on the surface of IE and inhibition of IE-CSA-binding by anti-*var*2csa antibodies has not been shown. To further complicate matter, analyses of IE membrane proteins by mass spectrometry have also indicated that CSA-binding or placental isolates do not preferentially express FCR3*var*CSA, CS2*var* or *var*2csa gene products, but rather novel PfEMP-1 sequences (Fried et al., 2004). In summary, there are probably at least two possibilities to explain why none of the *var* genes characterized to date has been successfully identified as the gene involved in causing PAM. The responsible *var* gene (if indeed a *var* gene plays an active role) has so far eluded identification. Alternatively, there are more than one *var* gene product linked to the disease.

*5.3 Analysis of 732*var *gene originating from a placental isolate*

The 732*var* gene described in this thesis is a novel *var* gene. Most importantly, it originates from a placenta-infecting isolate in contrast to FCR3*var*CSA, CS2*var* and *var*2CSA, which were isolated from laboratory-adapted strains. It has a unique domain organization not seen before in any of the *var* genes described in the 3D7 reference strain. This is indicative that differences in *var* genes between laboratory strains and field isolates exist, thereby emphasizing the importance of extending *var*-related studies beyond laboratory strains.

5.3.1 732*var* domain organization as type 15 *var* gene

The first exon of the 732*var* encodes four DBL and one CIDR domains in the following order: DBL-1α, CIDR-1α, DBL-2β, -3γ and -4ε domains (see Fig 18). The domain organization of the 732*var* gene is most similar to that of the type 15 *var* gene (Gardner et al., 2002), differing only by the presence of an additional DBL-4ε domain in the 732*var* gene. An analysis of the 732*var* gene showed that it has relatively low homology with the FCR3*var*CSA and CS2*var* genes, with overall identities of 42 % and 28 %, respectively (Chia et al., 2005).

5.3.2 Conservation of the minimal CD36-binding region in 732*var*

Some DBL and CIDR domains of the PfEMP-1 molecule have been assigned adhesive roles, possibly related to cytoadhesion of the IE (Smith, Craig, 2005). Examples of host receptors to which the DBL and CIDR domains bind are CR1, CD36, ICAM-1 and CSA (Smith et al., 2001). Miller et al. have shown that the CD36-binding minimal region of

the CIDR-α domains of A4*var,* A4*tres* and MC*var* genes lies within the central region of the domain (Miller et al., 2002). A recombinant protein consisting of the CD36-minimal binding region produced from MC*var* has been shown to be able to inhibit adhesion of IE to CD36 under flow condition (Yipp et al., 2003). This was interpreted to mean that the region of the MC*var* CIDR-1α (by inference, also the other two CIDR-1α) is essential for CD36 binding. This region is conserved in 732 CIDR-1α.

Gamain et al. have mapped the minimal CSA-binding region of the FCR3CSA DBL-3γ to a 67-amino acid stretch located in the C-terminal region (Gamain et al., 2004). This 67-amino acid stretch is not conserved in the 732 DBL-3γ, even though it has significant amino acid identity to the equivalent segments of the IT-29*var* and the 3D7*chr*5 DBL-3γ domains, both known to bind CSA. However, significance of this stretch of amino acids remains unclear particularly since such stretches are also present in other DBL-γ domains that do not bind CSA (Gamain et al., 2004; Gamain et al., 2005). The *var*PAM DBL-γ domains from placental isolates that were examined by Khattab et al. were capable of binding CSA, even though they were truncated in the C-terminal ends and hence did not encompass the apparent CSA-binding motif (Khattab et al., 2001). These observations indicate the presence of additional CSA-binding sites within the central two-thirds of the DBL-γ domains. Further structural characteristics of DBL-γ domains and their interaction with the CSA-receptor remain to be defined.

5.3.3 Biochemical characterization of the recombinant 732*var* domains

5.3.3.1 Production of functional CIDR-1α and DBL-3γ domains

CD36-, ICAM-1- and CSA-binding properties of several different PfEMP1 molecules has been localized to the CIDR-1α, DBL-β and DBL-γ domains, respectively. In an initial approach to identify adhesive interactions on the 732*var* gene, recombinant domains were tested on receptors expressed on CHO cells. From binding assays, it was observed that CIDR-1α and DBL-3γ bound to CD36- and CSA-receptors, respectively, but DBL-2β did not bind any receptors tested (see Fig. 22). The binding of CIDR-1α can be explained by the presence of the CD36-binding minimal region, as illustrated in Section 4.3.2.1. Even though the DBL-3γ recombinant protein does not contain the postulated minimal CSA-binding region it can still bind CSA. This is probably through the presence of alternate binding sites. The DBL-2β did not bind ICAM-1 receptors. The missing C2 region downstream of the DBL-2β domain is believed to restrict ICAM-1-binding, as an interdependence between the two has been previously reported (Chattopadhyay et al., 2004; Springer et al., 2004). It is also likely that the 732*var* gene product does not contain an active ICAM-1-binding DBL-2β domain, as the *var* gene was isolated from a placental strain (Khattab et al., 2001; Chia et al., 2005), and in line with the observation that the majority of placental isolates has no ICAM-1 binding phenotype. The lack of ICAM-1-binding capacity has been reported in the DBL-2β domain of the A4*var* gene (Smith et al., 1998). Neither DBL-1α nor -4ε bound any of the three receptors tested. Since other receptors such as heparan sulfate A and CR1 were not tested in the framework of this thesis, the binding activity of the recombinant 732 DBL-1α remains unknown. To date no

known interacting partner has been demonstrated for DBL-ε domains (Smith, Craig, 2005).

*5.3.3.2 Inhibition of IE binding by the recombinant 732*var *domains*

Results described in this thesis have demonstrated that the CIDR-1α and DBL-3γ domains are not only capable of binding to CD36 and CSA receptors, respectively, but that the proteins can also compete with IE for receptor binding (see Fig. 24). This IE-receptor inhibition exerted by the recombinant domains was not due to unspecific protein-protein interaction. For example, DBL-3γ did not affect the binding of FCR3CD36 to CHO-CD36, nor did CIDR-1α interfere with the binding of FCR3CSA to CHO-CSA. Similar observations were reported for the 179-amino acid MC*var* CIDR-1α where the recombinant protein under flow conditions not only inhibited binding of IE to CD36 but also was able to detache previously adhered clinical isolates from CD36 under flow conditions (Yipp et al., 2003).

5.3.4 Immunological characterization of the recombinant 732*var* domains

*5.3.4.1 Pregnant specificity of 732*var *recombinant domains*

The immunogenicity of the panel of recombinant proteins was analyzed in ELISA. The levels of antibodies to DBL-1α and -3γ were higher in pregnant women when compared to African adults (men but also nulliparous women). DBL-γ domain has been implicated with a role in PAM; therefore, it was not surprising to observe a stronger reactivity to the 732 DBL-3γ domain by pregnant women antibodies. The pregnancy-specific antibody

recognition to DBL-1α is not so clear, since the function of this domain in placental infection and the role of the antibodies in PAM are still unknown.

5.3.4.2 Association of antibodies with parasitaemia

In the analysis of placental parasitaemia, anti-DBL-3γ and CIDR-1α antibody titres showed contradictory correlations (see Fig. 25). While the former is negatively associated with parasite density, the latter is positively associated. It is speculated that anti-DBL-3γ antibodies help to curb the progression of the disease by controlling placental parasite infection. While a direct protective role of human antibodies that recognise CIDR-1α is not obvious, the simplest explanation would be that these antibodies are part of the general repertoire of antibodies produced by women in response to malaria infection but are not specifically induced during PAM.

5.3.5 IE surface expression of 732 DBL-3γ domains

Results of live IFA presented here show that anti-DBL-3γ polyclonal antibodies raised in experimental animals are pan reactive, that is to say they are able to recognize the surface of IE from different parasite strains (see Fig. 17). Based on this observation, it can be postulated that the DBL-3γ domain generally has a surface localization and the antibodies recognize one or more epitopes on this protein domain that appear to be conserved on the surface of heterologous IE^{CSA} strains.

5.3.6 732*var* and the dichotomy in CSA and CD36 binding

The 732*var* codes for two active domains, namely the CIDR-1α and DBL-γ domains exhibiting active binding properties. So far, all placental isolates have been reported to bind to CSA. To date, none of the PAM implicated *var* genes known to possess CIDR-1α domains with functional CD36-binding properties (Reeder et al., 2000; Robinson et al., 2003). The *var* genes instead have been reported to express multiple adhesive domains, such as CIDR-α and DBL-X, that has been reported to bind CSA (Reeder et al., 2000; Robinson et al., 2003; Gamain et al., 2005). Therefore, the in vivo function of the 732*var* encoded CIDR-1α domain that binds CD36 in PAM is unknown. In general, the syncytiotrophoblast cells lining the placenta lack the capacity of CD36 expression. Moreover, ex vivo desequestration of placental IE using anti-CD36 antibodies in lieu of soluble CSA was not successful (Gysin et al., 1999). CIDR-1α is a multiple-adhesive domain and has been shown to bind CD36 (Gamain et al., 2002; Smith et al., 1998) and CSA (Reeder et al., 2000; Degen et al., 2000). However, CIDR-1α domains of placental origins mostly bind CSA (Reeder et al., 2000; Degen et al., 2000), although there are also some with no CSA-binding property (Robinson et al., 2003).

The binding capacities of 732*var* domains are similar to those of the A4*tres* gene. The CIDR-1α and DBL-3γ domains of the A4*tres var* gene bind to CD36 and CSA, respectively (Smith et al., 1998; Gamain et al., 2002). The parasite from which the A4*tres* gene was isolated from binds CD36. The presence of a DBL-3γ domain in the gene has no apparent influence on the binding phenotype of the parasite and current observations appear to support the hypothesis that two different binding phenotypes cannot co-exist within the same PfEMP-1 molecule. The exclusive CD36-binding capacity of the A4*tres*

gene product has been explained by a direct domain-domain interaction of the DBL-2β with either CIDR-1α or DBL-3γ domains to discriminate the binding phenotype. Alternatively, it can also be explained by the modification of one of the active domains to suppress the adhesive properties of the other (Smith et al., 1998). This may hold true for the 732*var* gene to favour a CSA-binding phenotype.

*5.3.6.1 Implication of 732*var *in PAM*

In this study, recombinant 732*var* gene domains have been extensively characterized, and two of the domains, CIDR-1α and DBL-3γ, have been shown to bind CD36 and CSA, respectively. Pregnant women antibodies that recognize the former domain are hypothesized to indicate the presence of an infection. Iin contrast, antibodies that recognize the latter domain appear to help control placental infection and hence the disease. These observations suggest that both domains are involved in the pathogenesis of PAM.

The discovery of new *var* genes, such as *var*2CSA (Salanti et al., 2003; Salanti et al., 2004), and new binding phenotypes of PfEMP-1 domains, such as binding of CSA by CIDR-α, DBL-X and DBL-ε domains (Degen et al., 2000), have underscored the limited understanding of *var* genes and their functional constraints for adhesion to receptors. Studies of additional *var* genes especially those originating from field isolates, such as the 732*var,* is important for the basic understanding of the pathogenesis of malaria in the pregnant woman and her newborn. This in turn, contributes to a broader picture of adhesive interactions of parasites with host receptors, all of which will have implications for the ongoing efforts in the design of a PAM vaccine.

6. Summary

The accumulation of infected erythrocytes (IE) in the placenta is a key feature of pregnancy-associated malaria (PAM). The first receptor molecule shown to be responsible for cytoadhesion in the syncytiotrophoblast lining of the placenta is chondroitin sulfate A (CSA). When a woman is infected during pregnancy, she develops anti-adhesion antibodies that are strain-independent of the infecting IE, indicating that a conserved parasite ligand is in effect mediating the binding to CSA. The first putative parasite ligand proposed in the host-parasite interaction was a domain present on the protein PfEMP-1, a major variant surface antigen encoded by the multicopy *var* gene family. The ligand in play was the DBL-γ domain, first identified from two *var* genes, the FCR3*var*CSA gene and CS2*var* gene.

The first part of this thesis concerns the study of four different DBL-γ protein domains (482, 498, 701 and 732 DBL-γ domains), named after the numbers given to each of the placental isolates from which they originate. These domains, collectively called *var*PAM DBL-γ were previously demonstrated to bind CSA. As an extension of this study, my work here showed that immune recognition of 482 and 732 DBL-γ domains by plasma samples from a pregnant women cohort is pregnancy-specific. Pregnant women who have higher antibody reactivities to these two domains appear to be less vulnerable to placental infections. Based on these findings, it is postulated that 482 and 732 DBL-γ domains contain some important epitopes for CSA-binding, that are probably also the target of protective maternal antibodies. So far, evidence has also accumulated to show that other PfEMP-1 domains like CIDR-α and DBL-X can interact with CSA. This

supports the notion that CSA-binding motifs are probably structurally very similar to each other, differing only in their primary amino acid sequences. Studying the physico-chemical behaviour of these domains, eventually leading to an elucidation of their crystal structures, will help to unfold basic structural requirements for CSA binding.

The different domains from the full-length 732*var* gene, which comprises four DBL domains and one CIDR domain were characterized in the second part of this thesis. Interestingly, two of these domains, CIDR-1α and DBL-3γ, were shown to bind CD36 and CSA receptors, respectively. Given that the majority of placental parasites are generally known to bind CSA only, the observed CD36 recognition was an unexpected finding. The molecular mechanism that suppresses the adhesive characteristic of CIDR-1α within a CSA-binding PfEMP-1 molecule is unknown. Therefore, future work could be directed at understanding this mechanism responsible for the dichotomy in adhesion.

When analyzed for their reactivities with pregnant women sera in ELISA, the two domains showed differential antibody recognition. Specifically, the level of antibodies to recombinant 732 DBL-3γ correlated with a decrease of parasite density in the placenta. In contrast, anti-CIDR-1α antibody titres increased with placental parasite density. From this result, it is tempting to conclude that antibodies to DBL-3γ may play a role in protecting a woman from placental infections. However, what remains a riddle is the immunological significance of the presence of antibodies to CIDR-1α in PAM

In summary, results presented in this thesis provide a basis for further understanding molecular mechanisms of CSA-cytoadhesion and development of immunity in pregnant women, and may have important implications in the search for a PAM vaccine. .

Reference List

Achur RN, Valiyaveettil M, Alkhalil A, Ockenhouse CF, Gowda DC. 2000. Characterization of proteoglycans of human placenta and identification of unique chondroitin sulfate proteoglycans of the intervillous spaces that mediate the adherence of Plasmodium falciparum-infected erythrocytes to the placenta. *J Biol Chem 275*:40344-40356.

Andrews KT, Lanzer M. 2002. Maternal malaria: Plasmodium falciparum sequestration in the placenta. *Parasitol Res 88*:715-723.

Astagneau P, Steketee RW, Wirima JJ, Khoromana CO, Millet P. 1994. Antibodies to ring-infected erythrocyte surface antigen (Pf155/RESA) protect against P. falciparum parasitemia in highly exposed multigravidas women in Malawi. *Acta Trop 57*:317-325.

Barnwell JW, Asch AS, Nachman RL, Yamaya M, Aikawa M, Ingravallo P. 1989. A human 88-kD membrane glycoprotein (CD36) functions in vitro as a receptor for a cytoadherence ligand on Plasmodium falciparum-infected erythrocytes. *J Clin Invest 84*:765-772.

Beeson JG, Brown GV. 2004. Plasmodium falciparum-infected erythrocytes demonstrate dual specificity for adhesion to hyaluronic acid and chondroitin sulfate A and have distinct adhesive properties. *J Infect Dis 189*:169-179.

Beeson JG, Duffy PE. 2005. The immunology and pathogenesis of malaria during pregnancy. *Curr Top Microbiol Immunol 297*:187-227.

Beeson JG, Mann EJ, Elliott SR, Lema VM, Tadesse E, Molyneux ME, Brown GV, Rogerson SJ. 2004. Antibodies to variant surface antigens of Plasmodium falciparum-infected erythrocytes and adhesion inhibitory antibodies are associated with placental malaria and have overlapping and distinct targets. *J Infect Dis 189*:540-551.

Beeson JG, Rogerson SJ, Cooke BM, Reeder JC, Chai W, Lawson AM, Molyneux ME, Brown GV. 2000. Adhesion of Plasmodium falciparum-infected erythrocytes to hyaluronic acid in placental malaria. *Nat Med 6*:86-90.

Berendt AR, Simmons DL, Tansey J, Newbold CI, Marsh K. 1989. Intercellular adhesion molecule-1 is an endothelial cell adhesion receptor for Plasmodium falciparum. *Nature 341*:57-59.

Biggs BA, Kemp DJ, Brown GV. 1989. Subtelomeric chromosome deletions in field isolates of Plasmodium falciparum and their relationship to loss of cytoadherence in vitro. *Proc Natl Acad Sci U S A 86*:2428-2432.

Brabin BJ, Romagosa C, Abdelgalil S, Menendez C, Verhoeff FH, McGready R, Fletcher KA, Owens S, D'Alessandro U, Nosten F, Fischer PR, Ordi J. 2004. The sick placenta-the role of malaria. *Placenta 25*:359-378.

Brabin BJ. 1983. An analysis of malaria in pregnancy in Africa. *Bull World Health Organ 61*:1005-1016.

Buffet PA, Gamain B, Scheidig C, Baruch D, Smith JD, Hernandez-Rivas R, Pouvelle B, Oishi S, Fujii N, Fusai T, Parzy D, Miller LH, Gysin J, Scherf A. 1999. Plasmodium falciparum domain mediating adhesion to chondroitin sulfate A: a receptor for human placental infection. *Proc Natl Acad Sci U S A 96*:12743-12748.

Bull PC, Lowe BS, Kortok M, Molyneux CS, Newbold CI, Marsh K. 1998. Parasite antigens on the infected red cell surface are targets for naturally acquired immunity to malaria. *Nat Med 4*:358-360.

Chattopadhyay R, Taneja T, Chakrabarti K, Pillai CR, Chitnis CE. 2004. Molecular analysis of the cytoadherence phenotype of a Plasmodium falciparum field isolate that binds intercellular adhesion molecule-1. *Mol Biochem Parasitol 133*:255-265.

Chen Q, Fernandez V, Sundstrom A, Schlichtherle M, Datta S, Hagblom P, Wahlgren M. 1998. Developmental selection of var gene expression in Plasmodium falciparum. *Nature 394*:392-395.

Chia YS, Badaut C, Tuikue Ndam NG, Khattab A, Igonet S, Fievet N, Bentley GA, Deloron P, Klinkert MQ. 2005. Functional and Immunological Characterization of a Duffy Binding-Like- gamma Domain from Plasmodium falciparum Erythrocyte Membrane Protein-1 Expressed by a Placental Isolate. *J Infect Dis 192*:1284-1293.

Costa FT, Fusai T, Parzy D, Sterkers Y, Torrentino M, Douki JB, Traore B, Petres S, Scherf A, Gysin J. 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.

Crabb BS, Cooke BM, Reeder JC, Waller RF, Caruana SR, Davern KM, Wickham ME, Brown GV, Coppel RL, Cowman AF. 1997. Targeted gene disruption shows that knobs enable malaria-infected red cells to cytoadhere under physiological shear stress. *Cell 89*:287-296.

Craig A, Scherf A. 2001. Molecules on the surface of the Plasmodium falciparum infected erythrocyte and their role in malaria pathogenesis and immune evasion. *Mol Biochem Parasitol 115*:129-143.

Degen R, Weiss N, Beck HP. 2000. Plasmodium falciparum: cloned and expressed CIDR domains of PfEMP1 bind to chondroitin sulfate A. *Exp Parasitol 95*:113-121.

Deitsch KW, Wellems TE. 1996. Membrane modifications in erythrocytes parasitized by Plasmodium falciparum. *Mol Biochem Parasitol 76*:1-10.

Duffy MF, Brown GV, Basuki W, Krejany EO, Noviyanti R, Cowman AF, Reeder JC. 2002. Transcription of multiple var genes by individual, trophozoite-stage Plasmodium falciparum cells expressing a chondroitin sulphate A binding phenotype. *Mol Microbiol 43*:1285-1293.

Duffy PE, Fried M. 2001. Biomedicine. Turncoat antibodies. *Science 293*:2009-2010.

Duffy PE, Fried M. 2003. Antibodies that inhibit Plasmodium falciparum adhesion to chondroitin sulfate A are associated with increased birth weight and the gestational age of newborns. *Infect Immun 71*:6620-6623.

Esko JD, Stewart TE, Taylor WH. 1985. Animal cell mutants defective in glycosaminoglycan biosynthesis. *Proc Natl Acad Sci U S A 82*:3197-3201.

Fernandez-Reyes D, Craig AG, Kyes SA, Peshu N, Snow RW, Berendt AR, Marsh K, Newbold CI. 1997. A high frequency African coding polymorphism in the N-terminal domain of ICAM-1 predisposing to cerebral malaria in Kenya. *Hum Mol Genet 6*:1357-1360.

Fleming AF. 1989. Tropical obstetrics and gynaecology. 1. Anaemia in pregnancy in tropical Africa. *Trans R Soc Trop Med Hyg 83*:441-448.

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

Fried M, Duffy PE. 1996. Adherence of Plasmodium falciparum to chondroitin sulfate A in the human placenta. *Science 272*:1502-1504.

Fried M, Duffy PE. 2002. Two DBLgamma subtypes are commonly expressed by placental isolates of Plasmodium falciparum. *Mol Biochem Parasitol 122*:201-210.

Fried M, Nosten F, Brockman A, Brabin BJ, Duffy PE. 1998. Maternal antibodies block malaria. *Nature 395*:851-852.

Fried M, Wendler JP, Mutabingwa TK, Duffy PE. 2004. Mass spectrometric analysis of Plasmodium falciparum erythrocyte membrane protein-1 variants expressed by placental malaria parasites. *Proteomics 4*:1086-1093.

Gamain B, Gratepanche S, Miller LH, Baruch DI. 2002. Molecular basis for the dichotomy in Plasmodium falciparum adhesion to CD36 and chondroitin sulfate A. *Proc Natl Acad Sci U S A 99*:10020-10024.

Gamain B, Smith JD, Avril M, Baruch DI, Scherf A, Gysin J, Miller LH. 2004. 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 53*:445-455.

Gamain B, Trimnell AR, Scheidig C, Scherf A, Miller LH, Smith JD. 2005. Identification of Multiple Chondroitin Sulfate A (CSA)-Binding Domains in the var2CSA Gene Transcribed in CSA-Binding Parasites. *J Infect Dis 191*:1010-1013.

Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, Carlton JM, Pain A, Nelson KE, Bowman S, Paulsen IT, James K, Eisen JA, Rutherford K, Salzberg SL, Craig A, Kyes S, Chan MS, Nene V, Shallom SJ, Suh B, Peterson J, Angiuoli S, Pertea M, Allen J, Selengut J, Haft D, Mather MW, Vaidya AB, Martin DM, Fairlamb AH, Fraunholz MJ, Roos DS, Ralph SA, McFadden GI, Cummings LM, Subramanian GM, Mungall C, Venter JC, Carucci DJ, Hoffman SL, Newbold C, Davis RW, Fraser CM, Barrell B. 2002. Genome sequence of the human malaria parasite Plasmodium falciparum. *Nature 419*:498-511.

Gysin J, Pouvelle B, Fievet N, Scherf A, Lepolard C. 1999. Ex vivo desequestration of Plasmodium falciparum-infected erythrocytes from human placenta by chondroitin sulfate A. *Infect Immun 67*:6596-6602.

Haldar K, Mohandas N, Samuel BU, Harrison T, Hiller NL, Akompong T, Cheresh P. 2002. Protein and lipid trafficking induced in erythrocytes infected by malaria parasites. *Cell Microbiol 4*:383-395.

Heddini A, Pettersson F, Kai O, Shafi J, Obiero J, Chen Q, Barragan A, Wahlgren M, Marsh K. 2001. Fresh isolates from children with severe Plasmodium falciparum malaria bind to multiple receptors. *Infect Immun 69*:5849-5856.

Horan N, Yan L, Isobe H, Whitesides GM, Kahne D. 1999. Nonstatistical binding of a protein to clustered carbohydrates. *Proc Natl Acad Sci U S A 96*:11782-11786.

Horrocks P, Pinches RA, Chakravorty SJ, Papakrivos J, Christodoulou Z, Kyes SA, Urban BC, Ferguson DJ, Newbold CI. 2005. PfEMP1 expression is reduced on the surface of knobless Plasmodium falciparum infected erythrocytes. *J Cell Sci 118*:2507-2518.

Hviid L. 1998. Clinical disease, immunity and protection against Plasmodium falciparum malaria in populations living in endemic areas. *Expert Rev Mol Med 1998*:1-10.

Jensen AT, Zornig HD, Buhmann C, Salanti A, Koram KA, Riley EM, Theander TG, Hviid L, Staalsoe T. 2003. Lack of gender-specific antibody recognition of products from domains of a var gene implicated in pregnancy-associated Plasmodium falciparum malaria. *Infect Immun 71*:4193-4196.

Khattab A, Kremsner PG, Klinkert MQ. 2003. Common surface-antigen var genes of limited diversity expressed by Plasmodium falciparum placental isolates separated by time and space. *J Infect Dis 187*:477-483.

Khattab A, Kun J, Deloron P, Kremsner PG, Klinkert MQ. 2001. 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 183*:1165-1169.

Khattab A, Reinhardt C, Staalsoe T, Fievet N, Kremsner PG, Deloron P, Hviid L, Klinkert MQ. 2004. Analysis of IgG with specificity for variant surface antigens expressed by placental Plasmodium falciparum isolates. *Malar J 3*:21.

Kristoffersen EK, Matre R. 1996. Co-localization of the neonatal Fc gamma receptor and IgG in human placental term syncytiotrophoblasts. *Eur J Immunol 26*:1668-1671.

Kyes SA, Christodoulou Z, Raza A, Horrocks P, Pinches R, Rowe JA, Newbold CI. 2003. A well-conserved Plasmodium falciparum var gene shows an unusual stage-specific transcript pattern. *Mol Microbiol 48*:1339-1348.

Lanzer M, Wertheimer SP, de Bruin D, Ravetch JV. 1994. Chromatin structure determines the sites of chromosome breakages in Plasmodium falciparum. *Nucleic Acids Res 22*:3099-3103.

Lekana Douki JB, Traore B, Costa FT, Fusai T, Pouvelle B, Sterkers Y, Scherf A, Gysin J. 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.

Lyden TW, Robinson JM, Tridandapani S, Teillaud JL, Garber SA, Osborne JM, Frey J, Budde P, Anderson CL. 2001. The Fc receptor for IgG expressed in the villus endothelium of human placenta is Fc gamma RIIb2. *J Immunol 166*:3882-3889.

Maeda N, He J, Yajima Y, Mikami T, Sugahara K, Yabe T. 2003. Heterogeneity of the chondroitin sulfate portion of phosphacan/6B4 proteoglycan regulates its binding affinity for pleiotrophin/heparin binding growth-associated molecule. *J Biol Chem 278*:35805-35811.

McGregor IA. 1984. Epidemiology, malaria and pregnancy. *Am J Trop Med Hyg 33*:517-525.

Miller LH, Hudson-Taylor D, Gamain B, Saul AJ. 2002. Definition of the minimal domain of CIDR1alpha of Plasmodium falciparum PfEMP1 for binding CD36. *Mol Biochem Parasitol 120*:321-323.

Muthusamy A, Achur RN, Valiyaveettil M, Madhunapantula SV, Kakizaki I, Bhavanandan VP, Gowda CD. 2004. Structural characterization of the bovine tracheal chondroitin sulfate chains and binding of Plasmodium falciparum-infected erythrocytes. *Glycobiology 14*:635-645.

Mvondo JL, James MA, Sulzer AJ, Campbell CC. 1992. Malaria and pregnancy in Cameroonian women. Naturally acquired antibody responses to asexual blood-stage antigens and the circumsporozoite protein of Plasmodium falciparum. *Trans R Soc Trop Med Hyg 86*:486-490.

Nagao E, Kaneko O, Dvorak JA. 2000. Plasmodium falciparum-infected erythrocytes: qualitative and quantitative analyses of parasite-induced knobs by atomic force microscopy. *J Struct Biol 130*:34-44.

Oguariri RM, Borrmann S, Klinkert MQ, Kremsner PG, Kun JF. 2001. High prevalence of human antibodies to recombinant Duffy binding-like alpha domains of the Plasmodium falciparum-infected erythrocyte membrane protein 1 in semi-immune adults compared to that in nonimmune children. *Infect Immun 69*:7603-7609.

O'Neil-Dunne I, Achur RN, Agbor-Enoh ST, Valiyaveettil M, Naik RS, Ockenhouse CF, Zhou A, Megnekou R, Leke R, Taylor DW, Gowda DC. 2001. Gravidity-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, Gysin J. 1997. Chondroitin-4-sulfate impairs in vitro and in vivo cytoadherence of Plasmodium falciparum infected erythrocytes. *Mol Med 3*:508-518.

Reeder JC, Cowman AF, Davern KM, Beeson JG, Thompson JK, Rogerson SJ, Brown GV. 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 U S A 96*:5198-5202.

Reeder JC, Hodder AN, Beeson JG, Brown GV. 2000. Identification of glycosaminoglycan binding domains in Plasmodium falciparum erythrocyte membrane protein 1 of a chondroitin sulfate A-adherent parasite. *Infect Immun 68*:3923-3926.

Ricke CH, Staalsoe T, Koram K, Akanmori BD, Riley EM, Theander TG, 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 BA, Welch TL, Smith JD. 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.

Rogerson SJ, Beeson JG, Mhango CG, Dzinjalamala FK, Molyneux ME. 2000. Plasmodium falciparum rosette formation is uncommon in isolates from pregnant women. *Infect Immun 68*:391-393.

Rogerson SJ, Chaiyaroj SC, Ng K, Reeder JC, Brown GV. 1995. Chondroitin sulfate A is a cell surface receptor for Plasmodium falciparum-infected erythrocytes. *J Exp Med 182*:15-20.

Rowe JA, Obeiro J, Newbold CI, Marsh K. 1995. Plasmodium falciparum rosetting is associated with malaria severity in Kenya. *Infect Immun 63*:2323-2326.

Rowe JA, Kyes SA, Rogerson SJ, Babiker HA, Raza A. 2002. Identification of a conserved Plasmodium falciparum var gene implicated in malaria in pregnancy. *J Infect Dis 185*:1207-1211.

Rowe JA, Kyes SA. 2004. The role of Plasmodium falciparum var genes in malaria in pregnancy. *Mol Microbiol 53*:1011-1019.

Rowe JA, Moulds JM, Newbold CI, Miller LH. 1997. P. falciparum rosetting mediated by a parasite-variant erythrocyte membrane protein and complement-receptor 1. *Nature 388*:292-295.

Rubio JP, Thompson JK, Cowman AF. 1996. The var genes of Plasmodium falciparum are located in the subtelomeric region of most chromosomes. *EMBO J 15*:4069-4077.

Salanti A, Dahlbäck M, Turner L, Nielsen MA, Barfod L, Magistrado P, Jensen ATR, Lavstsen T, Ofori MF, Marsch K, Hviid L, Theander TG. 2004. Evidence for the involvement of var2CSA in pregnancy-associated malaria. *J Exp Med 200*.

Salanti A, Staalsoe T, Lavstsen T, Jensen AT, Sowa MP, Arnot DE, Hviid L, Theander TG. 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.

Scherf A, Hernandez-Rivas R, Buffet P, Bottius E, Benatar C, Pouvelle B, Gysin J, Lanzer M. 1998. Antigenic variation in malaria: in situ switching, relaxed and mutually exclusive transcription of var genes during intra-erythrocytic development in Plasmodium falciparum. *EMBO J 17*:5418-5426.

Scholander C, Treutiger CJ, Hultenby K, Wahlgren M. 1996. Novel fibrillar structure confers adhesive property to malaria-infected erythrocytes. *Nat Med 2*:204-208.

Smith JD, Craig AG. 2005. The surface of the Plasmodium falciparum-infected erythrocyte. *Curr Issues Mol Biol 7*:81-93.

Smith JD, Gamain B, Baruch DI, Kyes S. 2001. Decoding the language of var genes and Plasmodium falciparum sequestration. *Trends Parasitol 17*:538-545.

Smith JD, Kyes S, Craig AG, Fagan T, Hudson-Taylor D, Miller LH, Baruch DI, Newbold CI. 1998. Analysis of adhesive domains from the A4VAR Plasmodium falciparum erythrocyte membrane protein-1 identifies a CD36 binding domain. *Mol Biochem Parasitol 97*:133-148.

Smith JD, Subramanian G, Gamain B, Baruch DI, Miller LH. 2000. Classification of adhesive domains in the Plasmodium falciparum erythrocyte membrane protein 1 family. *Mol Biochem Parasitol 110*:293-310.

Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI. 2005. The global distribution of clinical episodes of Plasmodium falciparum malaria. *Nature 434*:214-217.

Springer AL, Smith LM, Mackay DQ, Nelson SO, Smith JD. 2004. Functional interdependence of the DBLbeta domain and c2 region for binding of the Plasmodium falciparum variant antigen to ICAM-1. *Mol Biochem Parasitol 137*:55-64.

Staalsoe T, Shulman CE, Bulmer JN, Kawuondo K, Marsh K, Hviid L. 2004. Variant surface antigen-specific IgG and protection against clinical consequences of pregnancy-associated Plasmodium falciparum malaria. *Lancet 363*:283-289.

Taylor DW, Zhou A, Marsillio LE, Thuita LW, Leke EB, Branch O, Gowda DC, Long C, Leke RF. 2004. Antibodies that inhibit binding of Plasmodium falciparum-infected erythrocytes to chondroitin sulfate A and to the C terminus of merozoite surface protein 1 correlate with reduced placental malaria in Cameroonian women. *Infect Immun 72*:1603-1607.

Taylor TE, Strickland GT. 2000. Malaria. In: Strickland GT, ed. *Hunter's tropical medicine and emerging infectious diseases*. Pennsylvania: W.B. Saunder's Company. pp 614-643.

Tjio JH, Puck TT. 1958. Genetics of somatic mammalian cells. II. Chromosomal constitution of cells in tissue culture. *J Exp Med 108*:259-268.

Tuikue Ndam NG, Fievet N, Bertin G, Cottrell G, Gaye A, Del P. 2004. Variable adhesion abilities and overlapping antigenic properties in placental *Palsmodium falciparum* isolates. *J Infect Dis In Press*.

Turner GD, Morrison H, Jones M, Davis TM, Looareesuwan S, Buley ID, Gatter KC, Newbold CI, Pukritayakamee S, Nagachinta B, . 1994. An immunohistochemical study of the pathology of fatal malaria. Evidence for widespread endothelial activation and a potential role for intercellular adhesion molecule-1 in cerebral sequestration. *Am J Pathol 145*:1057-1069.

Valiyaveettil M, Achur RN, Alkhalil A, Ockenhouse CF, Gowda DC. 2001. Plasmodium falciparum cytoadherence to human placenta: evaluation of hyaluronic acid and chondroitin 4-sulfate for binding of infected erythrocytes. *Exp Parasitol 99*:57-65.

Vazquez-Macias A, Martinez-Cruz P, Castaneda-Patlan MC, Scheidig C, Gysin J, Scherf A, 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.

White NJ. 2003. Malaria. In: Cook GC, Zumla AI, eds. *Manson's tropical diseases*. London: Elsevier Science Limited. pp 1205-1295.

Winter G, Chen Q, Flick K, Kremsner P, Fernandez V, Wahlgren M. 2003. The 3D7var5.2 (var COMMON) type var gene family is commonly expressed in non-placental Plasmodium falciparum malaria. *Mol Biochem Parasitol 127*:179-191.

Yipp BG, Baruch DI, Brady C, Murray AG, Looareesuwan S, Kubes P, Ho M. 2003. Recombinant PfEMP1 peptide inhibits and reverses cytoadherence of clinical Plasmodium falciparum isolates in vivo. *Blood 101*:331-337.

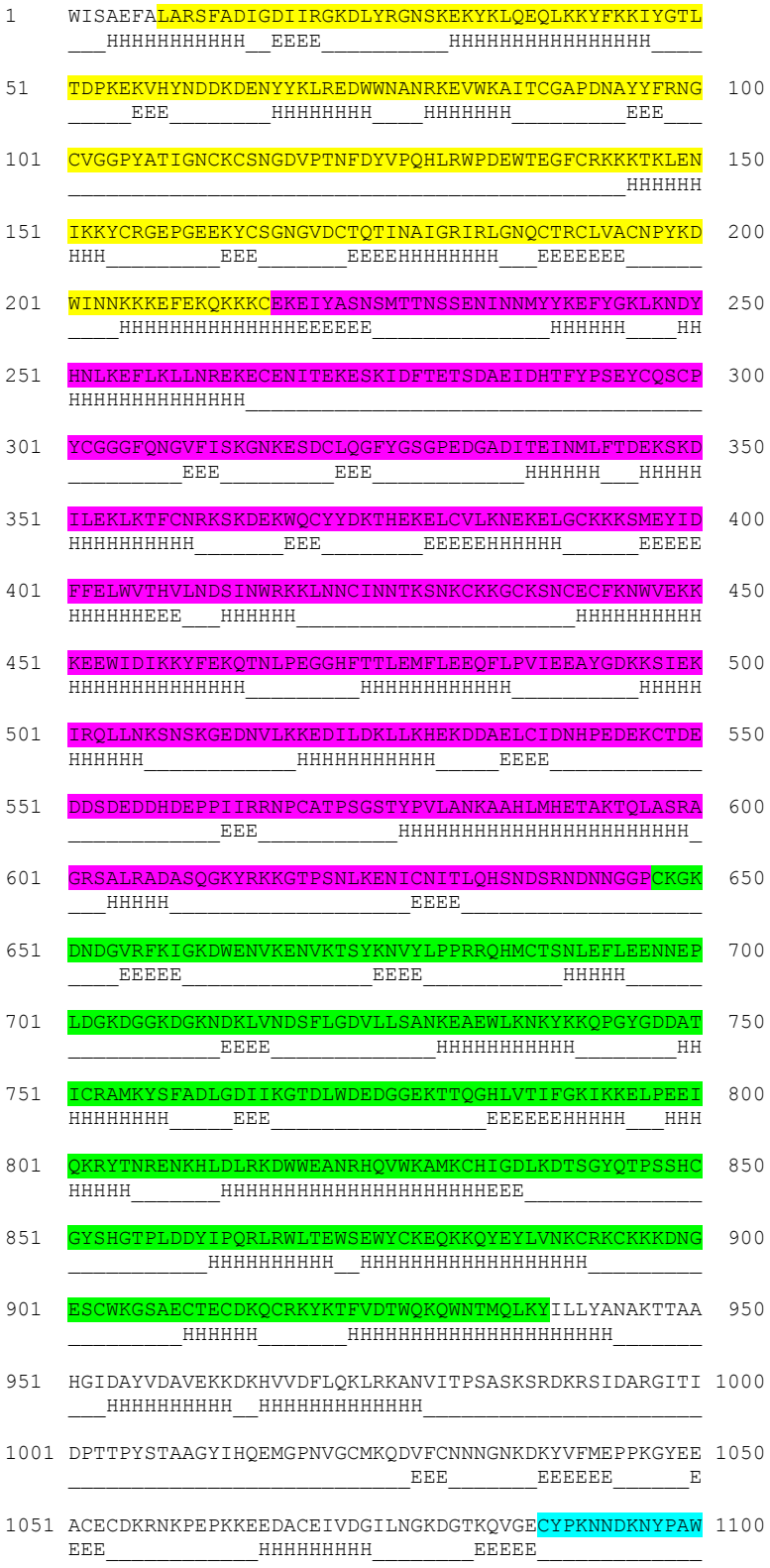
Acknowledgements

I wish to express my deepest appreciation for my thesis supervisor, PD Dr. Mo-Quen Klinkert for providing me with the opportunity to work on this very interesting research topic, for her excellent support, continuous encouragement and enthusiastic commitment throughout the thesis and last but not least, for her assistance in editing the dissertation. I am also greatly indebted to Dr. Ayman Khattab for his generous and incisive advice on laboratory techniques and his valuable insights during all stages of the work. The many useful discussions have led to fruitful developments. My special thanks extend to Michaela Petter and Insa Bonow for their patience and consideration for bearing with me in the laboratory. Particular acknowledgements go also to Prof. Dr. Rolf Horstmann for demonstrating interest in my research and for providing me the benchspace to work in his department and to PD Dr. Joachim Clos for performing the MALDI-tof analysis. There are also many from "behind the scenes" at the institute, whose friendship and help I value and appreciate, but I hesitate to name them here, in case I leave anyone out. I am grateful to Dr. P. Deloron and Dr. N. Fievet for allowing me to use the plasma samples from their pregnant women cohort, Dr. G. Bentley for his feedback on protein expression protocols and to Dr. T. Staalsoe for the kind gifts of CHO cell lines.

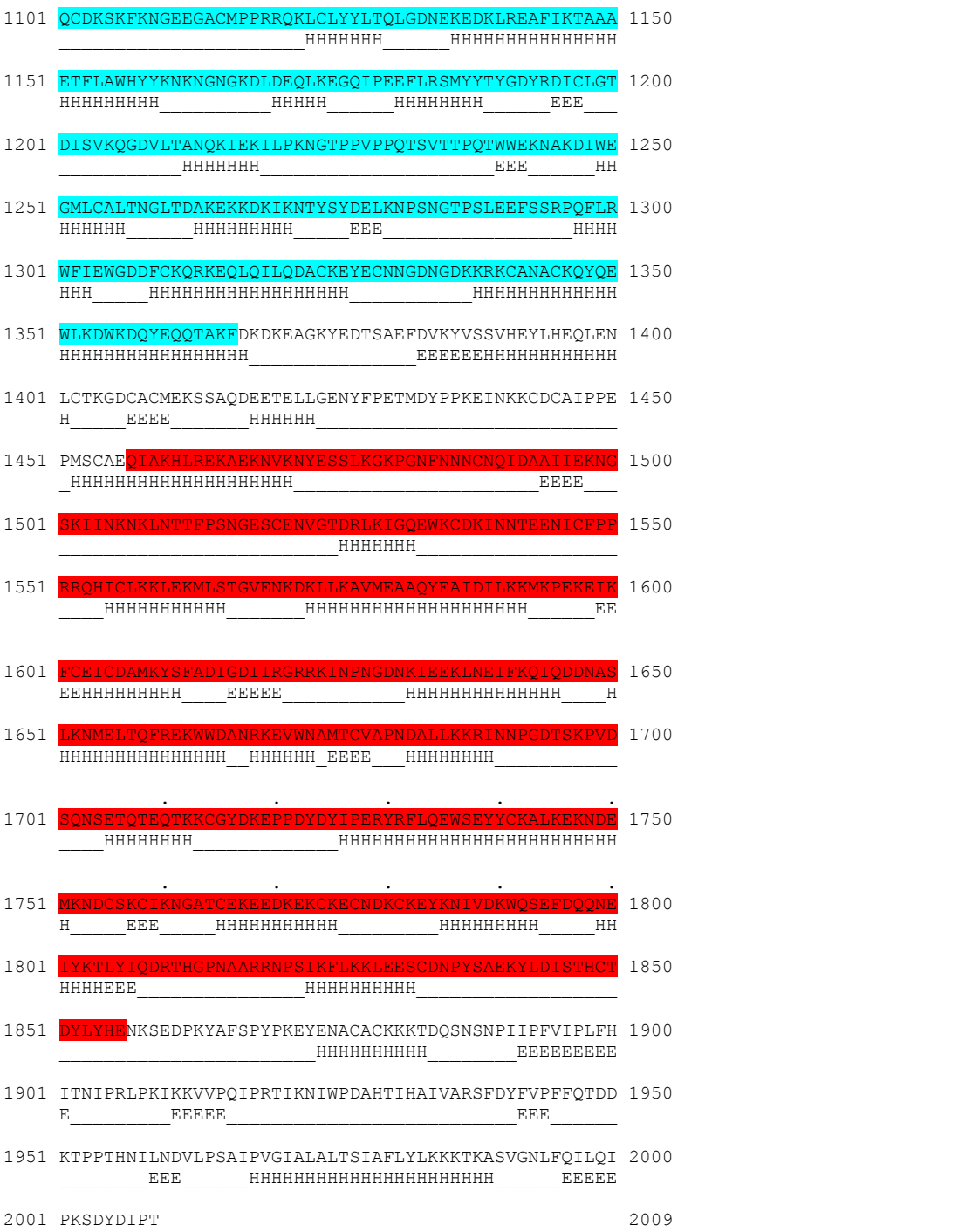
I would not have been able to complete the thesis without the love and tireless support of Hilmar. Thank you for putting up with lost weekends and odd working hours. I am also grateful to Sigrid and Helmut for their encouragement and those wonderful home cooked meals. Finally, I most fully thank my family for inspiring me on with their seemingly unlimited belief in me. A big hug and a big thank you to my father for critical

reading of my dissertation drafts; to my mother for her efforts to cheer me up and her wise advices; and to my siblings for lending a long-distance sympathetic ear to listen to my complaints. Thank you, all of you.

Predicted secondary protein structure of the PfEMP-1 encoded by 732*var*



Appendix



(Predator, http://bioweb.pasteur.fr/seqanal/interfaces/predator-simple.html)

N.B. H: helix structure
E: beta sheets
Amino acid residues are represented by their single letter code
DBL-1α domain is shaded in yellow, CIDR-1α domain in purple, DBL-2β in green, DBL-3γ in blue and DBL-4ε in red