

Structural and Functional Insight into How the *Plasmodium falciparum* VAR2CSA Protein Mediates Binding to Chondroitin Sulfate A in Placental Malaria*

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Thomas M. Clausen^{†1,2}, Stig Christoffersen^{§1,3}, Madeleine Dahlbäck[‡], Annette Eva Langkilde[¶], Kamilla E. Jensen[‡], Mafalda Resende[‡], Mette Ø. Agerbæk[‡], Daniel Andersen[‡], Besim Berisha[‡], Sisse B. Ditlev[‡], Vera V. Pinto[‡], Morten A. Nielsen[‡], Thor G. Theander[‡], Sine Larsen[§], and Ali Salanti^{‡4}

From the [‡]Department of Infectious Diseases, Centre for Medical Parasitology and Department of International Health, Immunology, and Microbiology, Copenhagen University Hospital (Rigshospitalet), Copenhagen, Denmark and the Departments of [§]Chemistry and [¶]Drug Design and Pharmacology, University of Copenhagen, Copenhagen DK-1014, Denmark

Background: VAR2CSA expressing *Plasmodium falciparum* parasites cause placental malaria by interacting with chondroitin sulfate A (CSA) on placental syncytiotrophoblasts.

Results: The CSA-binding site in VAR2CSA lies within the N-terminal DBL2X domain, which maps to the center of the compact VAR2CSA structure.

Conclusion: VAR2CSA fragments based on the CSA-binding region are potent vaccine candidates.

Significance: The data presented has important implications for vaccine development.

Malaria is a major global health problem. Pregnant women are susceptible to infection regardless of previously acquired immunity. Placental malaria is caused by parasites capable of sequestering in the placenta. This is mediated by VAR2CSA, a parasite antigen that interacts with chondroitin sulfate A (CSA). One vaccine strategy is to block this interaction with VAR2CSA-specific antibodies. It is a priority to define a small VAR2CSA fragment that can be used in an adhesion blocking vaccine. In this, the obvious approach is to define regions of VAR2CSA involved in receptor binding. It has been shown that full-length recombinant VAR2CSA binds specifically to CSA with nanomolar affinity, and that the CSA-binding site lies in the N-terminal part of the protein. In this study we define the minimal binding region by truncating VAR2CSA and analyzing CSA binding using biosensor technology. We show that the core CSA-binding site lies within the DBL2X domain and parts of the flanking interdomain regions. This is in contrast to the idea that single domains do not possess the structural requirements for specific CSA binding. Small-angle x-ray scattering measurements enabled modeling of VAR2CSA and showed that the CSA-binding DBL2X domain is situated in the center of the structure. Mutating classic sulfate-binding sites in VAR2CSA, along with testing dependence of ionic interactions, suggest that the CSA binding is not solely dependent on the sulfated CSA structure.

Based on these novel PfEMP1 structure-function studies, we have constructed a small VAR2CSA antigen that has the capacity to induce highly adhesion-blocking antibodies.

There are an estimated 225 million cases of malaria each year leading to nearly a million deaths (1). Of the five malaria parasite species that can infect humans *Plasmodium falciparum* is by far the most virulent (1). The *P. falciparum* parasite escapes the immune system by inserting antigens, functioning as adhesins, into the membrane of the infected erythrocyte (IE).⁵ This allows it to sequester in the host microvasculature, thereby avoiding clearance in the spleen. People living in endemic regions develop partial immunity to *P. falciparum* infection as a function of age (2). However, pregnant women are especially susceptible to infection despite previously acquired immunity. This is due to a distinct parasite subtype that is capable of sequestering in the placenta (3). The microvasculature of the placenta provides a new environment for adhesion not present in children, males, or nonpregnant women. Placental malaria (PM) confers severe clinical complications for both mother and fetus, by elevating the risk of severe maternal anemia, abortion, pre-eclampsia, low birth weight, and death (4, 5).

The *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) proteins are important adhesins, mediating binding to the host endothelium (6, 7). One of these is the unique PfEMP1 protein VAR2CSA (8), which is responsible for the

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¹ Both authors contributed equally to this work.

² To whom correspondence may be addressed: Centre for Medical Parasitology, Dept. of International Health, Immunology, and Microbiology, University of Copenhagen, CSS, Øster Farimagsgade 5A, DK-1014 Copenhagen K, Denmark. Tel.: 4535456090; E-mail: tmc@sund.ku.dk.

³ To whom correspondence may be addressed. E-mail: Stig@chem.ku.dk.

⁴ To whom correspondence may be addressed. E-mail: Salanti@sund.ku.dk.

⁵ The abbreviations used are: IE, *P. falciparum*-infected erythrocyte; CIDR, cysteine-rich inter-domain region; CSA, chondroitin sulfate A; CSPG, chondroitin sulfate proteoglycan; DBL, Duffy binding-like domain; DSM, dimorphic sequence motif in DBL2X; FV2, full-length ectodomain of the VAR2CSA protein without N-terminal segment; GAG, glycosaminoglycan; HSPG, heparan sulfate proteoglycan; ID, inter-domain; PfEMP1, *Plasmodium falciparum* erythrocyte membrane protein 1; PM, placental malaria; SAXS, small-angle x-ray scattering; HS, heparan sulfate; PDB, Protein Data Bank.

binding to placental tissue (9, 10). VAR2CSA interacts with an unusually low-sulfated form of chondroitin sulfate A (CSA), attached to proteoglycans in the intervillous spaces of the placenta (11–14). Immunity to placental malaria is acquired over multiple pregnancies (15). Evidence suggests that VAR2CSA is the main target of this protective immunity (9). This, and the fact that VAR2CSA is unusually well conserved (16), suggests that it is possible to obtain a VAR2CSA-based vaccine against PM.

VAR2CSA is a large multidomain protein (350 kDa) consisting of six Duffy binding-like domains (three DBLX followed by three DBL ϵ domains), a cysteine-rich inter-domain region (termed CIDR_{PAM}) between DBL2X and DBL3X, and a number of interdomains (8, 17, 18). The large size and complex structure, along with genetic variability among isolates complicates current large scale production strategies. The focus in vaccine development is therefore to produce a small fragment of VAR2CSA that has the ability to induce functional cross-inhibitory antibodies (19, 20). In this, the obvious approach is to define the binding regions within VAR2CSA and examine the mechanism underlying the interaction.

The expression of the full-length VAR2CSA ectodomain (FV2) from FCR3 (13, 19) and 3D7 (14, 20) type parasites has enabled us to study both the binding properties and the structural characteristics of the VAR2CSA protein. Such studies revealed that the full-length VAR2CSA ectodomain binds specifically to CSA with an affinity in the nanomolar range (13, 14, 19). In addition, we have recently shown that the core CSA-binding site lies within the DBL2X-CIDR_{PAM} region. Specifically, we found two overlapping VAR2CSA fragments (DBL1X-CIDR_{PAM} and ID1-DBL3X) showing CSA affinity comparable with that of the full-length protein (19). Srivastava *et al.* (20) showed the same binding characteristics for DBL1X-DBL3X. This, and small-angle x-ray scattering (SAXS) data proposing that VAR2CSA exhibits an overall compact structure (14), suggested a CSA-binding site dependent on multiple domain and interdomain regions.

Structural information about full-length VAR2CSA is scarce because there is no available crystal structure. The crystal structures of several PfEMP1 single DBL domains and a single CIDR domain (21) are available from the Protein Data Bank (PDB). This includes DBL3X (22, 23) and DBL6 ϵ (24), from VAR2CSA. Furthermore, the crystal structure of the two DBL-domain EBA-175 protein (PDB 1ZRO) has been solved (25). This showed structural similarities to a SAXS structure of a homologous, but functionally distinct, two DBL domain protein (26), indicating conservation of the DBL domain structural arrangements. The currently available high-resolution crystal structures of DBL domain proteins and EBA-175 has allowed the prediction of all VAR2CSA DBL domain structures (17). Yet, the overall fold and spatial orientation of individual domains in the VAR2CSA structure is not known.

In this study we address a number of key questions related to the molecular mechanism behind placental adhesion in PM: 1) what are the exact minimal structural requirements for VAR2CSA binding to CSA; 2) where is the minimal binding region positioned in the VAR2CSA structural envelope; 3) what type of chemical interaction exists between VAR2CSA and

CSA; and finally 4) can this information be used to design an optimal vaccine antigen?

We demonstrate that the high CSA binding affinity is retained in several shorter fragments of VAR2CSA and that DBL2X, including small regions from the flanking interdomains, forms a compact core that contains the high affinity CSA-binding site. To predict the overall arrangement of structural domains in VAR2CSA we performed SAXS analysis on several VAR2CSA fragments. The available structural information from the DBL domain crystal structures allowed the construction of an overall model for VAR2CSA. Furthermore, we show that the VAR2CSA-CSA interaction is only partially dependent on ionic interactions by mutating classic sulfate-binding sites in VAR2CSA, along with testing dependence of ionic interactions. Finally, we show that several short VAR2CSA fragments are capable of inducing the production of adhesion-blocking antibodies and that the anti-adhesive antibodies target the proposed CSA-binding region. These data provide the first detailed insight into the biochemical nature of the interaction between a PfEMP1 protein and its ligand and show how structure function studies can aid vaccine development.

EXPERIMENTAL PROCEDURES

Cloning and Protein Expression—VAR2CSA sequence fragments were amplified from codon optimized FCR3 (GenBankTM accession no. GU249598) or 3D7 (GenBank accession no. JQ247428) VAR2CSA genes using specific primers. Simple fragments were amplified in a one-step PCR. Amino acid substitution constructs were made in a two-step PCR. First we PCR amplified two fragments from the codon-optimized FCR3 template, containing overlapping complementary ends. Second we PCR amplified the total construct, using the two overlapping fragments as template with primers specific for the outer borders. All fragments were sequenced for verification. Fragments were cloned into the baculovirus vector pAcGP67-A (BD Biosciences), modified to contain a V5 and His tag at the C-terminal. The proteins were expressed in baculovirus-infected insect cells as soluble proteins secreted into the cell culture supernatant. Briefly, the linearized Bakpak6 Baculovirus DNA (BD Biosciences) was co-transfected with pAcGP67-A plasmids, into Sf9 insect cells for generation of recombinant virus particles. 10 ml of the second amplification was used to infect High-Five cells in 400 ml of serum-free medium (10486, Invitrogen) at a density of 1×10^6 cells/ml. The secreted recombinant protein was harvested from the supernatant 3 days after the initial infection. The supernatant was filtered (0.2 μ m), dialyzed, and concentrated before protein purification.

Protein Purification and SDS-PAGE—The filtered supernatant containing the secreted recombinant protein was dialyzed using an ÄKTA cross-flow (GE Healthcare). The dialysis was performed in 10 mM NaH₂PO₄ (pH 7.4, Sigma) and 500 mM NaCl. The resulting solution was filtered (0.2 μ m) and imidazole was added to a final concentration of 15 mM. The protein was then purified on a 1-ml HisSelect column (H8286, Sigma). Bound protein was eluted with 10 mM NaH₂PO₄ (pH 7.4), 500 mM NaCl, and 500 mM imidazole. Proteins needed for Quartz Crystal Microbalance measurements and SAXS were further

purified to obtain monomers by size exclusion chromatography using a HiLoad 16/60 Superdex 200 column (GE Healthcare) in 20 mM Tris (pH 8) and 200 mM NaCl. The purity and structural integrity of the protein was verified by SDS-PAGE.

Solid Phase Binding Assay (ELISA)—The solid phase binding assay was performed as previously described (19). Briefly, microtiter plates were incubated with chondroitin sulfate proteoglycan (CSPG) (bovine decorin, D8428, Sigma) and heparan sulfate proteoglycan (HSPG) (H4777, Sigma) overnight at 4 °C. The plates were then blocked with TSM binding buffer (20 mM Tris, 150 mM NaCl, 2 mM CaCl_2 , 0.05% Tween 20, 1% BSA, pH 7.4, at 25 °C). A 2-fold dilution series (1.56–100 mM) of protein was prepared in TSM binding buffer and added to the plates. All measurements were performed in triplicates. Proteins were detected by incubating with 1:3000 anti-V5-HRP antibody (R96125, Invitrogen) in TSM binding buffer. Finally, plates were developed with *o*-phenylenediamine substrate (DAKO) for 15 min. The reaction was quenched with 2.5 M H_2SO_4 . Absorbance was measured at 490 nm.

Quartz Crystal Microbalance (Attana A100)—Experiments were performed as previously described on an Attana A100 (Attana AB) (19). In brief, the ligand CSPG (D8428, Sigma) or HSPG (H4777, Sigma) was coated at a concentration of 100 $\mu\text{g}/\text{ml}$. Coating was done in steady state by adding ligand solution and incubating for 30 min at room temperature. This was followed by blocking the plate with PBS containing 0.1% Ig-free BSA (BSA-50, Rockland) for 30 min at room temperature. Prior to sample injection PBS was injected as a blank. Analyte (VAR2CSA protein) was injected in a 1:3 dilution series (0.25–60 $\mu\text{g}/\text{ml}$) starting with the lowest concentration. Association time was set to 84 s and disassociation time to 5 min. Due to the high affinity of binding it was not possible to regenerate the binding surface following injections. The data collected was processed in Attester Evaluation software (Attana AB). Curves were fitted in a simple 1:1 model. k_{on} and k_{off} were determined by curve fitting and K_D was calculated as $K_D = k_{\text{off}}/k_{\text{on}}$.

Animal Immunizations and Serum Extraction—All animal immunizations complied with National and European regulations. Wistar rats were injected subcutaneously with 30 μg of recombinant protein in Freund's complete adjuvant (F5881, Sigma). The immunization was boosted three times at 3-week intervals with 15 μg of protein in Freund's incomplete adjuvant (F5506, Sigma). Blood samples were taken 1 week after each boost, and serum was extracted by centrifugation.

Ig Affinity Purification—Pools of sera from rats immunized with full-length FCR3 VAR2CSA (FV2) were affinity purified on a 1-ml NHS-activated HP column (HiTrap NHS-activated HP, 17-0716-01, GE Healthcare), containing immobilized multidomain FCR3 proteins (DBL1X-DBL2Xa, DBL1X-ID2a, ID1-ID2a, or ID1-DBL4e) and full-length FV2. Purification was done according to the manufacturer's protocol. In short, coupling of ligand to column was done by adding 1 ml of 1:1 solution of coupling buffer (0.2 M NaHCO_3 , 0.5 M NaCl, pH 8.3) and ligand (concentration 0.5–10 mg/ml) to the column. The column was sealed and incubated for 30 min at room temperature, followed by incubation at 4 °C overnight. The column was washed with 6 ml of Buffer A (0.5 M ethanolamine, 0.5 M NaCl,

pH 8.3), 6 ml of Buffer B (0.1 M acetate, 0.5 M NaCl, pH 4), and finally 6 ml Buffer A. After an incubation period of 30 min at room temperature, the washing was repeated in reverse order (Buffer B, A, B). 8–10 ml of PBS was injected to adjust the pH before purifying the serum. The sample was passed through the column 3–5 times. The column was washed with 10 ml of PBS before antibodies were eluted with 10 ml of elution buffer (0.1 M citric acid, pH 2.7).

***P. falciparum* Parasite Cultures**—*P. falciparum* FCR3-type parasites were maintained in culture using 5% hematocrit (human blood group 0 Rh+) in parasite medium RPMI 1640 (BE12115F, Lonza) supplemented with 25 mM NaHCO_3 , 0.125 $\mu\text{g}/\text{ml}$ of gentamycin sulfate (BE02012E, Lonza), 0.125 $\mu\text{g}/\text{ml}$ of AlbuMAX II (11021029, Invitrogen), and 2% normal human serum. IEs were repeatedly panned on BeWo cells (CCL98, ATCC) to maintain the CSA adhering phenotype. Furthermore, isolates were tested to be mycoplasma negative and regularly genotyped by PCR using nested GLURP (glutamate-rich protein) and MSP-2 (merozoite surface protein 2) primers.

Purification of Late Stage Trophozoites—Parasite cultures were enriched for late trophozoite and schizont stage in a strong magnetic field using a MACS CS-column (130-041-305, Miltenyi Biotec) and a Vario-MACS magnet (Miltenyi Biotec). In brief, the parasite culture suspension was applied to the column. The column was then washed with 2% fetal calf serum (F6178, Sigma) in PBS. Late stage infected erythrocytes were eluted from the column after separation from the magnet, spun down, and resuspended in 2% fetal calf serum in PBS and diluted to a concentration of 2×10^6 IEs/ml.

Flow Cytometry—Antibody binding to native VAR2CSA on the purified late-stage trophozoites infected erythrocytes, was measured by flow cytometry. 100 μl of purified late stage parasites at a concentration of 2×10^5 IEs/ml in PBS with 2% FCS were labeled with serum (depleted for nonspecific binding by preincubation with noninfected erythrocytes) in a final concentration of 1:10. The cells were washed three times in PBS with 2% FCS. The cells were then further labeled with ethidium bromide (15585011, Invitrogen) in a final concentration of 2 $\mu\text{g}/\text{ml}$ and a 1:100 dilution of FITC-labeled secondary anti-rat IgG antibody (62-9511, Invitrogen). As negative controls, late stage parasites were also incubated with serum from rats immunized with an antigen other than VAR2CSA and with secondary antibodies alone. Data from 5000 ethidium bromide-positive IEs were collected using a FC500 flow cytometer (Beckman Coulter). Finally the median fluorescence intensity was determined using the WinList 5.0 software (Verify Software House).

Inhibition of Parasites Binding CSPG—Serum antibodies were analyzed for their ability to inhibit IE binding to CSPG. This was done in a 96-well plate format using a robot-standardized washing method. Wells were coated with 2 $\mu\text{g}/\text{ml}$ of CSPG (D8428, Sigma). A total of 2×10^5 tritium-labeled (hypoxanthine monohydrochloride, PerkinElmer Life Sciences, NET177005MC) late stage IEs in 100 μl were added in triplicates to the wells. The labeled IEs were then incubated with serum for 90 min at 37 °C. Unbound IEs were washed away by a pipetting robot (Beckman Coulter). The proportion of adhering IEs was determined by liquid scintillation counting on a Topcount NXT (PerkinElmer Life Sciences).

SAXS—The homogeneity of samples subjected to SAXS analysis was checked by gel filtration and gel electrophoresis at reducing and nonreducing conditions. The protein was kept in a buffer containing 20 mM Tris-HCl (pH 7.0) and 200 mM NaCl for size exclusion chromatography, to minimize unspecific ionic interactions between the protein and column material. In cases where proteins eluted as dimers or multimers, only fractions containing monomeric protein were used for further analysis. All the proteins studied retained their C-terminal His₆ purification tags. Molecular weight determinations on proteins DBL1X-ID2a and ID1-DBL4 ϵ were conducted on a Bruker MALDI-TOF mass spectrometer. The level of glycosylation was quantified as the difference between the average determined mass and theoretical mass. Sinapic acid matrix solution used for high molecular weight proteins was purchased from Sigma.

SAXS data were collected at the BioSAXS beamline ID14-3 at the European Synchrotron Radiation Facility (ESRF). The beamline was equipped with a PILATUS 1M detector set 1.668 meters from the sample collecting data in the q -range 0.004 to 0.6 Å⁻¹. Samples of 25 μ l were loaded, using the automated sample changer and passed through the beam with continuous flow as 10 frames of 10-s exposure times were collected, processed, and averaged automatically by the BsxCuBE software, developed at the beamline. Prior to data collection, the proteins were concentrated using 10-kDa cutoff concentration units, and the flow-through was used for buffer measurements recorded both before and after the protein sample. Data were collected on solutions of VAR2CSA proteins in the 0.5–7 mg/ml concentration range (A_{280}) and the scattering from the protein was obtained, after subtracting the scattering from the buffer, using PRIMUS (27). In cases where concentration-dependent attraction or repulsion effects were observed, scattering curves were merged with low concentration data to counter the effects. Extrapolation of the Guinier region to obtain the radius of gyration (R_g) and the forward scattering intensity ($I(0)$) was done using AutoRg implemented in PRIMUS. Values of $I(0)$ for each protein construct were converted to estimates of protein mass by comparing $I(0)$ values from measurements on protein standards bovine serum albumin (BSA) and Lysozyme, available at the beamline. From the scattering curves, the pair distance distribution functions, used in subsequent *ab initio* shape reconstructions, were calculated using AUTOPOROD (28), which includes GNOM (29) and also evaluates the excluded particle volume and from that the molecular weight protein content. The program also ensures the truncation of data for use in subsequent *ab initio* reconstructions.

Ab Initio Shape Reconstructions—For each protein construct, 10 *ab initio* shape reconstructions were generated to fit the scattering data using DAMMIF (30) run in interactive mode using 50 spherical harmonics in the process. Data extending to $q_{\max} = 8/R_g$ was included in the *ab initio* shape reconstruction. Molecular envelopes for each VAR2CSA construct were created by aligning, averaging, and filtering the 10 reconstructions using DAMAVER (31).

Modeling of VAR2CSA Domain Structure Using Structural Envelopes and Docked Crystal Structures—The VAR2CSA DBL domain crystal structure homologue EBA-175 (PDB 1ZRO)

(25) describes the orientation of two consecutive DBL domains. This structure was used to represent the DBL1X-DBL2X, DBL3X-DBL4 ϵ , and DBL5X-DBL6 ϵ two-domain regions of VAR2CSA. Inter-domain linker regions separating homologous DBL domains in VAR2CSA were defined as regions with poor sequence homology to available single DBL domain crystal structures. These regions model as the linker-part DBL domain in modeling DBL-ID-DBL structures according to the two-DBL crystal structure template EBA-175 (PDB 1ZRO). This suggests that the interdomains do not form actual domains but function as short structural linkers. In the protein sequence of VAR2CSA, the ID2a region links DBL2X to the CIDR-like ID2b region. There is currently no crystal structure that indicates the structure of this region. A homologous crystal structure (PDB 3C64) of a CIDR domain (21) was included to represent the VAR2CSA ID2b domain. The length of the ID2b sequence of VAR2CSA is 182 amino acids and the length of a standard DBL domain is 300 amino acids. For comparison, the lengths of the ID regions are ID1, 79 amino acids; ID2a, 141 amino acids; ID2b-DBL3X, 9 amino acids; DBL3X-DBL4 ϵ (ID3), 18 amino acids; DBL4 ϵ -DBL5 ϵ (ID4), 106 amino acids; and DBL5 ϵ -DBL6 ϵ (ID5), 68 amino acids. The crystal structures were initially positioned in the *ab initio* shape envelopes by hand. Optimizations of the initial positions were done using collage supplied in the Situs program package version 2.7 (32) for each individual envelope. In optimization of the ID1-DBL4 ϵ envelope, the C-terminal single DBL domain was extracted from PDB 1ZRO to represent the ID1-DBL2X structure. All envelope optimizations were re-run from their optimized domain positions using single DBL structures, to check if changes in domain orientations were introduced.

Salt Titration Assay—The ionic dependence of VAR2CSA-CSA binding was tested in an ELISA-based binding assay. CSPG was coated at 3 μ g/ml. A 1:2 dilution series (400–1.56 nM) of protein was added in several different NaCl concentrations (150, 200, 250, and 300 mM). All experiments were performed in triplicates. The K_D values were calculated for each titration series in GraphPad Prism using nonlinear regression (least squares fit with Hill slope).

RESULTS

Production of Truncated Recombinant VAR2CSA Proteins—All protein truncations were produced according to previously defined domain borders (19). Intra-domain truncations were based on conserved cysteines and predictions of the intra-domain disulfide bridge structure (33). For the purpose of simplification we have divided the CIDR_{PAM} domain (19) into two domains ID2a and ID2b, where ID2a is the N-terminal part of CIDR_{PAM} not containing the CIDR-like sequence and ID2b corresponds to the CIDR-like sequence (17). Srivastava *et al.* (20) showed CSA binding for a DBL1X-DBL2X fragment using a DBL2X C-terminal border incorporating 93 amino acids of what we call ID2a. For simplification we call this border DBL2Xb, whereas our border will be referred to as DBL2Xa. Fragments were expressed in baculovirus-infected insect cells as soluble proteins. Most proteins were produced based on the FCR3 genotype. Some FCR3 fragments did not express and these were instead made based on the 3D7 genotype. The pro-

Protein	Minimal Binding Region										K _D (nM)
	NTS	DBL1X	ID1	DBL2X	ID2a	ID2b	DBL3X	DBL4ε	DBL5ε	DBL6ε	
FV2		DBL1X	ID1	DBL2X	ID2a	ID2b	DBL3X	DBL4ε	DBL5ε	DBL6ε	5.2
DBL1X-ID2b		DBL1X	ID1	DBL2X	ID2a	ID2b					1.5
DBL1X-ID2a		DBL1X	ID1	DBL2X	ID2a						8.0
ID1-ID2a			ID1	DBL2X	ID2a						7.6
ID1-DBL2Xb			ID1	DBL2X							21.8
DBL1X-DBL2Xa		DBL1X	ID1	DBL2X							N/A ^a
ID1-DBL2Xa			ID1	DBL2X							N/A ^a

^aKinetic fit could not be obtained due to a lack of binding to CSPG

FIGURE 1. **Schematic representation of produced VAR2CSA fragments.** ID2a-ID2b corresponds to CIDR_{PAM} in Ref. 19. The figure shows the tested fragments along with K_D values determined in the kinetic analysis. The kinetic analysis was performed on a Quartz Crystal Microbalance (Attana A100). The sensorgrams are shown in Fig. 3. ID1-DBL2Xb and ID1-DBL2Xa were produced based on the 3D7 genotype. The minimal binding region is ID1-DBL2Xb.

teins were used interchangeably in the analysis because we have shown that recombinant VAR2CSA from both genotypes bind equally to CSA (data not shown). All proteins showed a shift in gel mobility when comparing reduced and nonreduced samples by SDS-PAGE (data not shown). This is consistent with the formation of intramolecular disulfide bridges. Some proteins formed high molecular weight complexes detected by nonreduced SDS-PAGE. This is probably due to the formation of inter-molecular disulfide bridges between unpaired cysteines. This was confirmed by reducing the complexes to monomeric protein using DTT (data not shown).

Core CSA-binding Site Lies within DBL2X Domain—It has been suggested that the minimal CSA-binding region in VAR2CSA lies within DBL2X-ID2b, with the need of flanking domains for full affinity binding (19, 20). Here we have analyzed shorter fragments of VAR2CSA to further map the regions required for CSA binding (Fig. 1).

The truncated proteins were first screened for binding to a CSPG in ELISA (Fig. 2) and then further purified to obtain monomers for examination on the Quartz Crystal Microbalance (Fig. 3). The minimal binding region is ID1-DBL2Xb (Figs. 2 and 3). This region showed a binding affinity of 21.8 nM, which is comparable with that of full-length VAR2CSA.

Placental IEs are highly selective for low-sulfated placental CSPG (11). They do not adhere to any other glycosaminoglycans (GAG), such as heparan sulfate (HS) (3). The same is true for the full-length recombinant VAR2CSA protein (13, 19). The solid state binding assay showed that the VAR2CSA fragments, containing the minimal CSA-binding region, bound specifically to CSA (Fig. 2). To confirm this, the minimal binding fragments were further tested for binding to a HSPG on the Quartz Crystal Microbalance (data not shown). None of the fragments bound HSPG. FV2 bound HSPG with a K_D value comparable with that of CSPG. However, as seen before, it bound with a low peak response (19). This indicates that a subfraction of the protein truncations in solution interacts with HSPG, but that the majority does not.

Antibodies Induced against Novel Minimum Binding Regions Induce a Potent Parasite Anti-adhesive Immune Response—A VAR2CSA-based vaccine against PM must be able to induce a strong protective immune response. In this, the most important aspect is the formation of anti-VAR2CSA IgG antibodies capable of inhibiting placental sequestration. We have examined the molecular mechanism underlying the VAR2CSA-CSA interac-

tion for the purpose of designing optimal vaccine antigens. To test whether our produced VAR2CSA recombinant fragments showed the capacity to induce an adhesion blocking immune response, they were used in rat immunizations.

VAR2CSA fragment-specific serum was tested for ability to inhibit IE adhesion to CSPG (Fig. 4). Antibodies raised against all CSA-binding fragments were very potent inhibitors of binding. In fact binding was inhibited nearly 100% in all cases. DBL1X-DBL2Xa and ID1-DBL2Xa were not good inhibitors, consistent with the lack of CSA binding of these fragments. The data implies that the CSA-binding proteins are properly folded and support the localization of the above defined minimal binding region.

Epitopes Responsible for Induction of Anti-adhesive Antibodies Lies within the Minimal Binding Region—It has been shown that full-length VAR2CSA induces potent inhibitory antibodies in rats (13). The same has been shown for several CSA-binding multidomain fragments, as well as single domain non-CSA binding fragments (DBL4ε) (19, 34, 35). To examine if the inhibitory anti-FV2 response is directed toward the minimal binding region, we affinity purified FV2 antibodies on four of the previously described VAR2CSA fragments. The fragment-specific antibodies were then tested for the capacity to inhibit the VAR2CSA expressing parasite binding to CSPG (Fig. 5). Antibodies purified on immobilized ID1-DBL4ε, DBL1X-ID2a, and ID1-ID2a fully inhibited parasite adhesion. Furthermore, the depleted FV2 samples lost a significant portion of their inhibitory capacity. This indicates that epitopes inducing anti-adhesive antibodies are present within these fragments. Antibodies purified on DBL1X-DBL2Xa show a reduced inhibitory capacity, consistent with the lack of CSA binding of this fragment. The data suggest that epitopes responsible for induction of inhibitory antibodies are located within the minimal binding region (here illustrated by ID1-ID2a).

Structural Model of VAR2CSA from SAXS Envelopes—There is currently no crystal structure of a high affinity CSA-binding VAR2CSA protein. To obtain information about the location of the core CSA-binding region (DBL2X) in the structural envelope of VAR2CSA, we analyzed a panel of full-length and truncated VAR2CSA proteins by SAXS.

The statistics from the primary analysis of the SAXS data are listed in Table 1. Scattering curves and pair-distance distribution functions of the analyzed VAR2CSA proteins are shown in Fig. 6. The maximum particle sizes (*D*_{max}) and the radius of

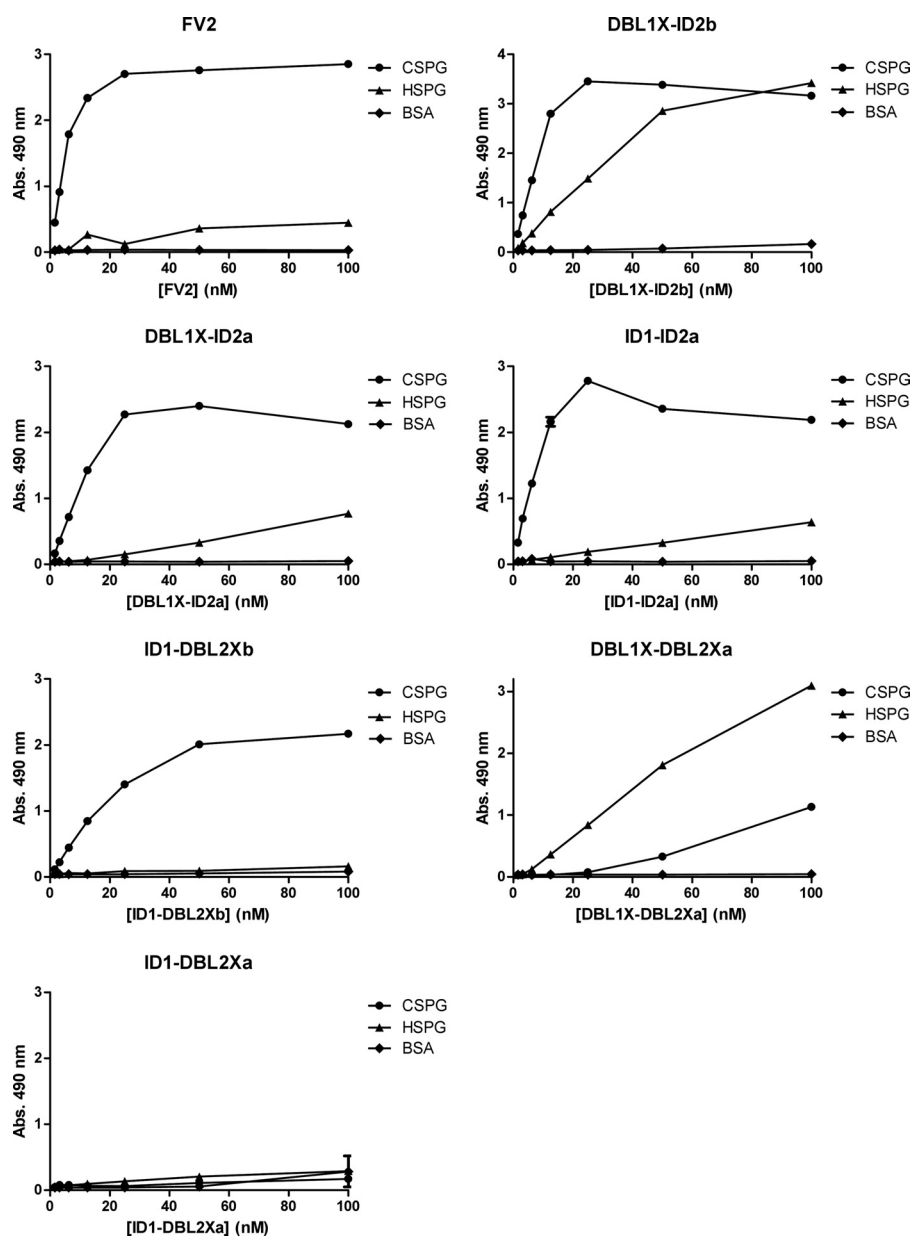
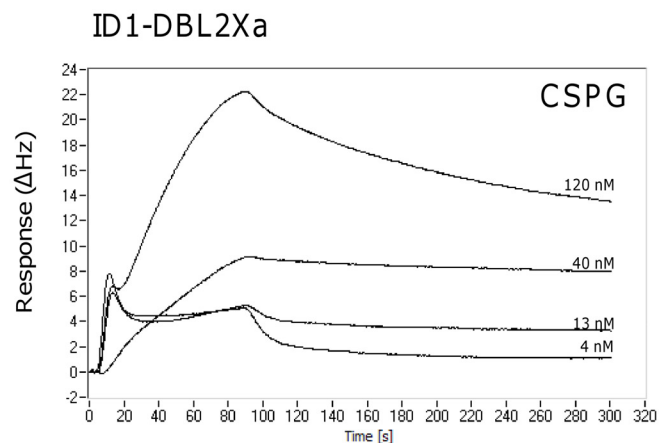
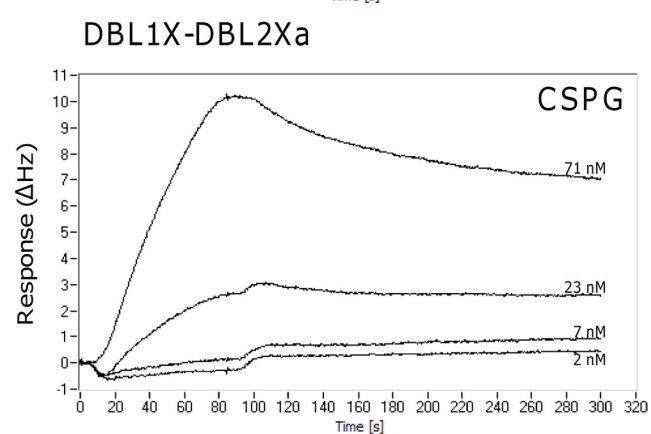
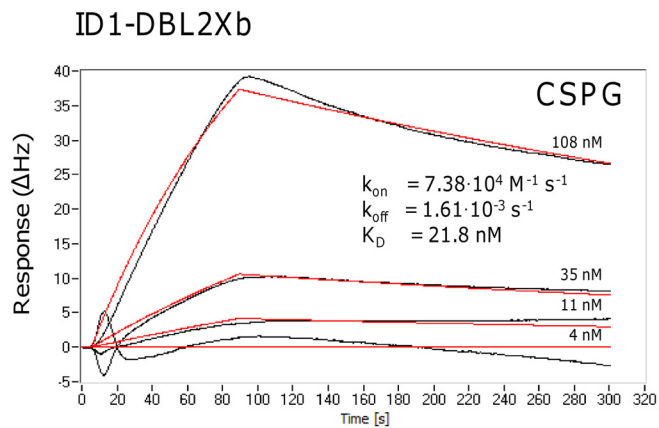
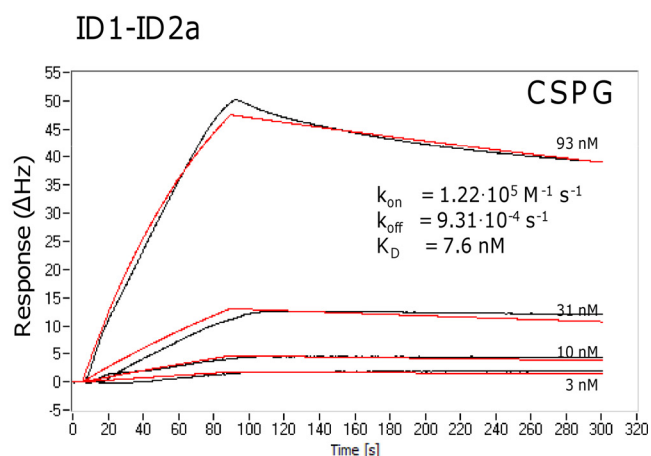
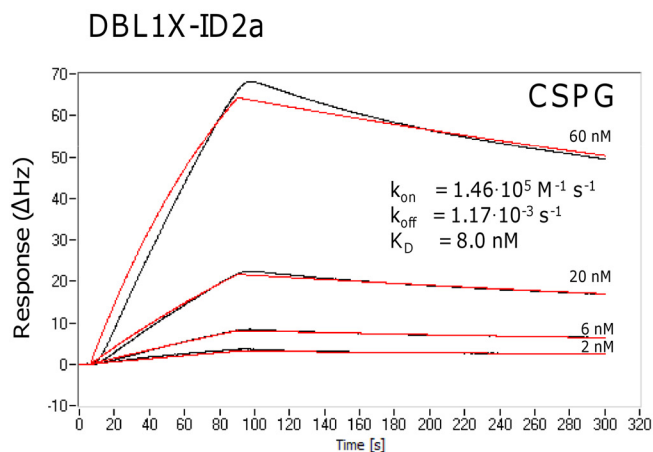
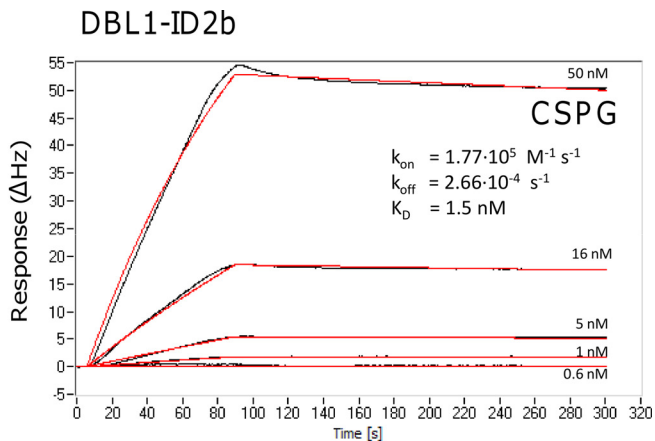
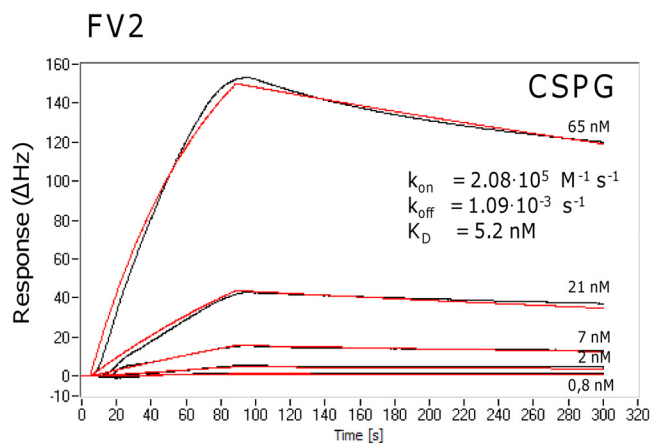


FIGURE 2. **Screening for CSPG binding VAR2CSA fragments in a solid phase binding assay (ELISA).** The protein tested is given at the top of each graph. For schematic representation of fragments, see Fig. 1. Microtiter plates were coated with 3 μ g/ml of CSPG, 3 μ g/ml of HSPG, and 1% BSA. A 2-fold dilution series of protein (1.56–100 nM) was added to the wells. Bound protein was detected using anti-V5 HRP-conjugated antibody. The plates were developed with OPD and absorbance was detected at 490 nm. The minimal CSPG-binding protein is ID1-DBL2Xb.

gyration (R_g) reflected the difference in size between the four tested proteins: DBL1X-ID2a, ID1-DBL4 ϵ , DBL1X-DBL4 ϵ , and DBL1X-DBL6 ϵ . The molecular weight estimates ($M_{w(0)}$ and $M_{w_{\text{Porod}}}$), were generally higher than the theoretical values (Table 1). The percentile deviations of $M_{w_{\text{Porod}}}$ compared with theoretical molecular weight values were largest for the DBL1X-ID2a and DBL1X-DBL4 ϵ data. To explain this difference and further check the protein quality, we determined the exact molecular weights of DBL1X-ID2a and ID1-DBL4 ϵ by mass spectrometry (Table 1). This showed glycan levels of 12 and 5% by mass, respectively.

The *ab initio* shapes, interpreted as molecular envelopes, were reconstructed based on the SAXS data. These are shown in Fig. 6 as the dummy atoms used by DAMMIF. The shape

reconstructions include the contribution from possible glycosylation and represent the averaged structures. The glycan substitution sites in VAR2CSA are scattered throughout the sequence, with the relative majority (6 of 20) mapping to the N-terminal DBL1X domain. Because the level of glycosylation at these sites was not known, it was not possible to account for the glycan contribution. The q -range used in the envelope shape reconstruction, and the resulting fit to the experimental data is plotted alongside the envelopes in Fig. 6. χ^2 values were 2.5, 1.3, 1.5, and 1.1 for DBL1X-ID2a, ID1-DBL4 ϵ , DBL1X-DBL4 ϵ , and DBL1X-DBL6 ϵ , respectively. The normalized spatial discrepancy values between reconstructions for each VAR2CSA construct averaged between 0.72 and 0.8.



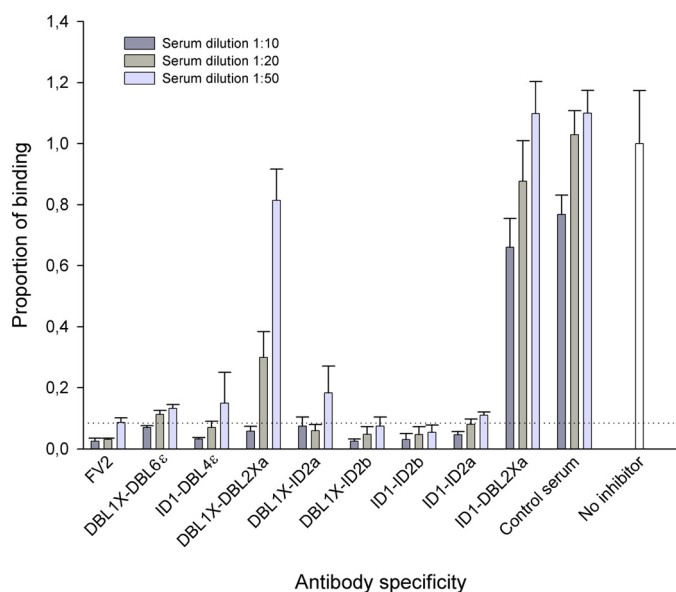


FIGURE 4. Testing the inhibitory capacity of serum extracted in VAR2CSA fragment immunizations. The figure shows inhibition of VAR2CSA-expressing FCR3-infected erythrocytes binding to CSPG using VAR2CSA-specific serum. Serum was tested at several dilutions (1:10, 1:20, and 1:50). The fraction of binding parasites (tritium labeled) was measured by liquid scintillation. Inhibition is given relative to the positive control (binding without inhibitor). The dotted line indicates the mean + 2 S.D. of binding to bovine serum albumin. We show that all CSA-binding fragments induce a strong anti-adhesive immune response in rats.

The DBL1X-ID2a molecular envelope (Fig. 6B) is elongated, resembling the elongated EBA-175 crystal structure. This confirms the use of the EBA-175 protein as representative for the structure of two consecutive DBL domains.

The maximum particle sizes (D_{max}) obtained for the ID1-DBL4 ϵ and DBL1X-DBL4 ϵ fragments are compatible with the value obtained for the DBL1X-ID2a fragment (Table 1). This indicates a compact domain arrangement in the truncated VAR2CSA fragments. The DBL1X-DBL4 ϵ envelope must describe a structure containing two EBA-175-like (two-DBL domain) structural units, separated by the ID2a-ID2b region. This suggested that ID2a-ID2b(CIDR_{PAM}) acts as a structural turn, allowing formation of the compact structure. This was also the case in the docking of two EBA-175 structures and one CIDR structure into the DBL1-DBL4 ϵ envelope, as illustrated in Fig. 7C. The docking was done with the program Situs version 2.7. This program is able to refine multiple structures to the envelope features avoiding steric clashes between the positioned structures. Homologues crystal structure data from the PDB were chosen over homology modeled VAR2CSA structures to prevent poorly predicted regions from sterically interfering with the domain position optimizations. Fig. 7A shows the docked structure of EBA-175 inside the DBL1X-ID2a envelope and reveals an extension of the envelope at the EBA-175 C-terminal. This probably corresponds to the structurally

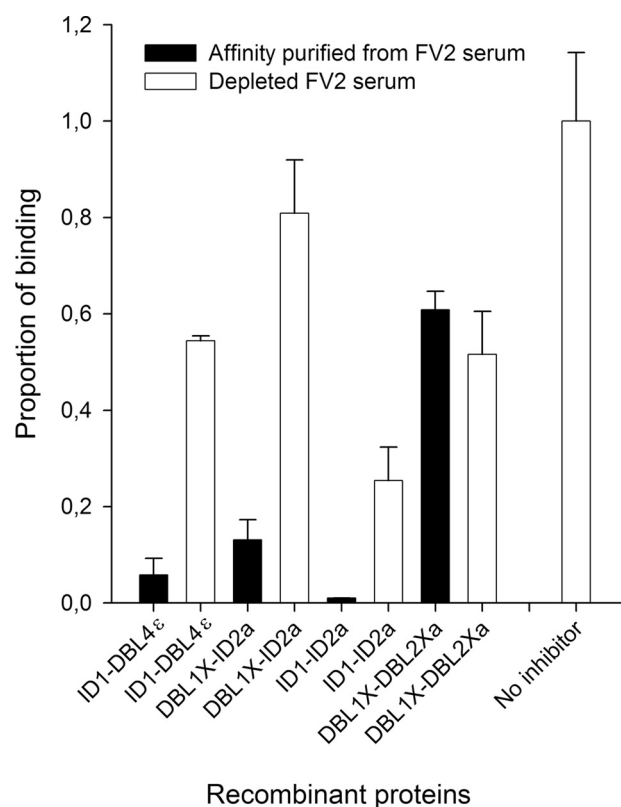


FIGURE 5. Testing the inhibitory capacity of anti-FV2 Ig, affinity purified on minimal binding fragments. The figure shows inhibition of VAR2CSA-expressing FCR3-infected erythrocytes binding to CSPG using anti-FV2 Ig affinity purified on VAR2CSA fragments. The inhibitory capacity was tested for both affinity purified samples and the depleted run-through. The samples were tested at a 1:10 dilution. The fraction of binding parasites (tritium labeled) was measured by liquid scintillation. Inhibition is given relative to the positive control (binding without inhibitor). We show that inhibitory antibodies target the minimal binding region.

unknown ID2a region and the His₆ purification tag. The program consistently resulted in the compact domain organization seen from DBL1X-DBL4 ϵ upon docking into the remaining ID1-DBL4 ϵ and DBL1X-DBL6 ϵ envelopes (Fig. 7, B and D). This provides a strong indication of the actual VAR2CSA domain arrangement. To verify the use of EBA-175 as representative for the DBL-DBL structure, the program was re-run using EBA-175-derived single DBL domains only. This did not give rise to significant reorientations of the domains (data not shown). The molecular weight statistics obtained from the ID1-DBL4 ϵ SAXS data show the best agreement with the theoretical value. However, a region of the envelope, extending from the C-terminal of DBL4 ϵ , which supposedly was not sufficient to hold the docked structures, was left empty after the docking process. This extension may originate from the 106-amino acid ID4 inter-domain linker region, connecting DBL4 ϵ to DBL5 ϵ , together with the retained purification tag. The correlation coefficients obtained from Situs for each of these multidomain

FIGURE 3. Examining binding kinetics of minimal binding fragments on the Quartz Crystal Microbalance (Attana A100). The protein tested is given at the top of each graph. For schematic representation of fragments, see Fig. 1. Sensorgrams show VAR2CSA fragments binding to immobilized CSPG. The binding is illustrated as a change in frequency over time. The CSPG was coated at 100 μ g/ml. The proteins were injected at different concentrations in a 1:3 dilution series (0.25–60 μ g/ml). The association and disassociation phases are shown. The black curve corresponds to the actual data collected and the red is the fitted 1:1 binding model. Values for k_{on} , k_{off} , and the calculated K_D values are shown in each graph. It was not possible to fit a 1:1 binding model onto DBL1X-DBL2Xa or ID1-DBL2Xa. The minimal CSPG-binding protein is ID1-DBL2Xb.

TABLE 1
Basic statistics from SAXS analysis

Basic statistics from SAXS. From left: the highest protein concentration, at which data were recorded. Molecular masses calculated from the cloned protein sequence, with the value obtained by mass spectrometry shown in parentheses. Molecular mass calculations derived from comparing $I(0)$ values of the Guinier analysis, against values obtained from ESRF provided protein standards. Estimates of the particle radius of gyration (R_g), were also from the Guinier analysis. Values of maximum particle size ($D_{\max}$), particle volumes (Porod), and molecular masses were calculated from the pair-distance distribution functions. Experimental scattering curves and corresponding $P(r)$ functions are shown in Fig. 6.

	Concentration	Mw _{Protein}	Mw _{I(0)}	R_g	$D_{\max}$	Volume _{Porod}	Mw _{Porod}
	mg/ml	kDa		nm		nm ³	kDa
DBL1X-ID2a	3	111 (124)	167	4.8	16.6	245	153
ID1-DBL4ε	1.3	183 (192)	207	4.9	16.9	398	249
DBL1X-DBL4ε	3.1	229	307	5.5	18.8	483	302
FV2	4	310	360	6.3	21.9	690	431

envelope refinements were between 0.49 and 0.58. The docking process conveniently placed the ID2b region into a void in the DBL3X domain, leaving space for ID2a inside the cleft formed by the V-shaped ID2b positioned between DBL2X and DBL3X (Fig. 7, left panels).

The docked VAR2CSA structural model (Fig. 7D) indicates that the DBL4ε and DBL5ε domains can be superimposed onto the EBA-175 crystal structure. The length of a 4-DBL domain structure, manually modeled by continuation of the EBA-175 crystal structure, is 21 nm. This agrees with the obtained $D_{\max}$ for VAR2CSA, implying that the 4-domain model for DBL3X-DBL6ε spans the entire VAR2CSA structure. Provided that DBL3X-DBL6ε follows the conserved DBL-DBL structure, as indicated in this study, alternative options for the positioning of the remaining DBL1X, DBL2X, and ID2b structures are very limited.

Mutating Putative GAG-binding Sites in the Minimal Binding Region Has No Effect on CSPG Binding—Characterizing the nature of the interaction between VAR2CSA and CSA is important for the design of multivalent PM vaccines. In this, a major part is identification of the specific CSA-binding site and characterization of the underlying chemical interactions. Sequence analysis of the minimal CSA-binding region revealed two conserved putative GAG-binding sites. One is located in the ID1 region and has the classic Cardin-Weintraub XBBBXXBX motif (36) (⁴⁵⁸NKKKECKD⁴⁶⁵). Another, in DBL2X, has the same motif in reverse (⁶²⁵GKNLKKRY⁶³²). It has also been hypothesized that a dimorphic sequence motif (DSM), found in the N-terminal part of DBL2X, is involved in binding CSA (37). To test whether these putative sites had a function in CSA binding, we substituted basic amino acids in the classic GAG-binding sites with alanines and made a 10-amino acid (⁵⁹⁰KLENVCEDVK⁶⁰³) deletion in the middle of a surface-exposed loop within the DSM region (17). All mutations were performed in the DBL1X-ID2a fragment.

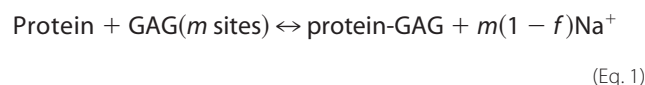
Substituting basic amino acids in the putative ID1 (alanine substitution with Lys (459, 460, 461, 464)) and DBL2X (alanine substituting with Lys(626, 629, 630), Arg(631)) GAG-binding sites, with alanines had no effect on CSPG binding. Neither did deletion of the DSM region. No decrease in CSPG binding was seen compared with the wild-type protein in ELISA or in the kinetic analysis as illustrated by similar K_D values and peak responses (data not shown).

VAR2CSA Binding to CSPG Does Not Depend on Ionic Interactions—Mutation of the classic Cardin-Weintraub GAG binding motifs had no effect on CSPG binding. This indicates

that the VAR2CSA-CSA binding mechanism differs from the general mode of sulfate binding in classic GAG binding models. There are examples of GAG-binding proteins showing little dependence on ionic interactions with the sulfated GAG structure (38). To test if this was the case, we examined ionic dependence according to the polyelectrolyte theory (39–41).

Glycosaminoglycans, like DNA, are highly charged polymers often referred to as polyelectrolytes (39). The negatively charged groups incur a high degree of repulsive energy within each polymer. Monovalent cations, such as Na⁺, interact with the negatively charged groups to minimize the repulsive energy. Binding of basic amino acids to the sulfate groups displaces the bound cations and leads to the release of free energy. The favorable release of bound Na⁺ ions is referred to as the polyelectrolyte effect (39).

The theory states that the binding of a protein to a GAG can be described by,

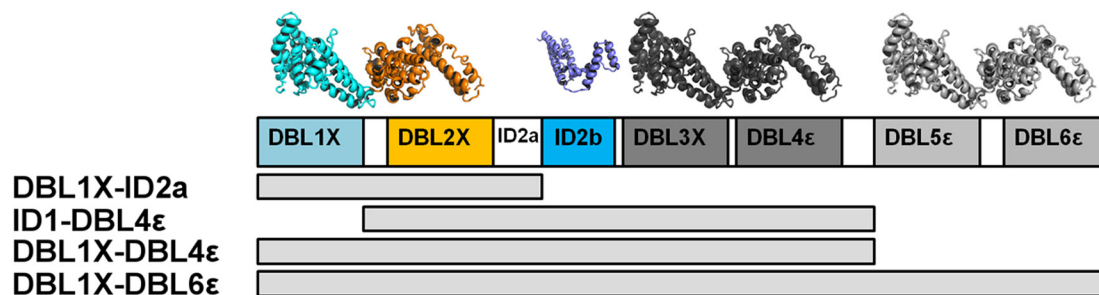
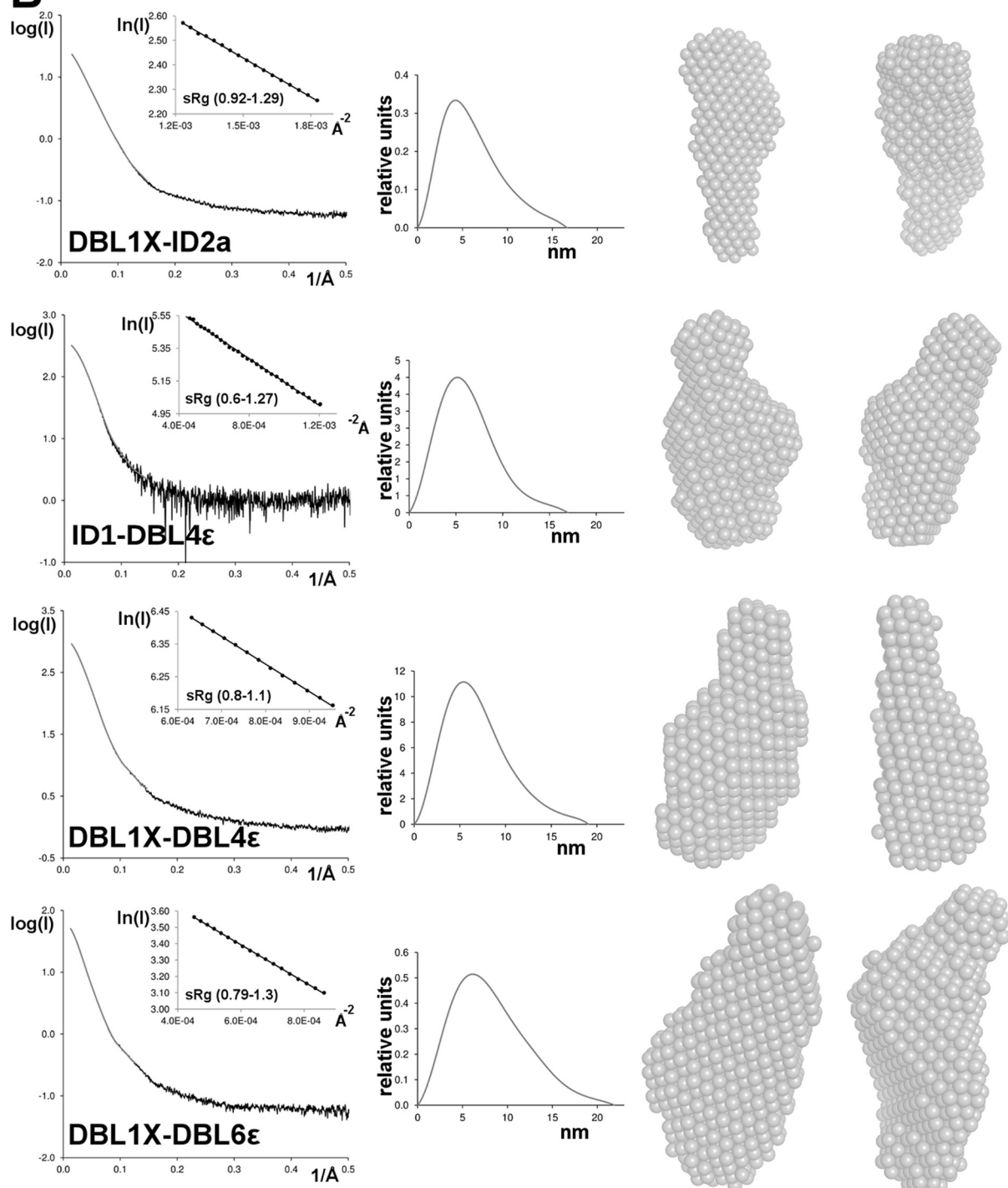


where m is the number of Na⁺ ions released upon binding of a single protein and f is the fraction of anions not shielded by Na⁺ ions. According to the theory the observed K_D value is related to ionic and nonionic contributions by (40, 41),

$$\log K_{D,\text{observed}} = \log K_{D,\text{nonionic}} + m(1 - f)\log[\text{Na}^+] \quad (\text{Eq. 2})$$

where $K_{D,\text{nonionic}}$ is the disassociation constant in the absence of ionic interactions. A plot of $\log K_{D,\text{observed}}$ versus $\log [\text{Na}^+]$ is linear with a slope of $m(1-f)$. Thus, if the fraction of unshielded anions (f) is known, the number of ionic interactions involved in the binding can be determined. For heparin, $(1-f)$ is 0.8 (40). The value is not known for CSA, but $(1-f)$ cannot exceed 1. We can therefore estimate the maximal number of ionic interactions involved. Furthermore, when $[\text{Na}^+] = 1 \text{ M}$, $\log [\text{Na}^+] = 0$, which means that at this Na⁺ concentration $\log K_{D,\text{observed}} = \log K_{D,\text{nonionic}}$.

We tested the binding of FV2, DBL1X-ID2a, and ID1-ID2a to CSPG in a solid state binding assay at different concentrations of NaCl (150, 200, 250, and 300 mM), by performing titrations of binding from 400 to 1.65 nM protein in a 1:2 dilution series. The observed K_D values were determined as the protein concentration giving half-maximum ($B_{\max}$) response. This was done using nonlinear regression (least squares fit with Hill slope)

A

B


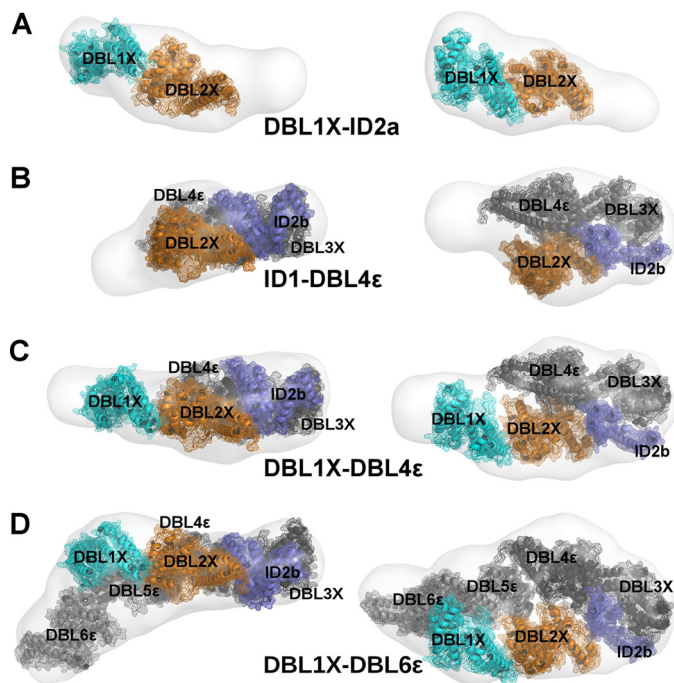


FIGURE 7. Docking of crystal structure homologues into the molecular envelopes obtained from SAXS. The molecular envelopes are depicted as a mesh. The docked crystal structure homologues are shown as domains with a surface representation. A, DBL1X-ID2a; B, ID1-DBL4 ϵ ; C, DBL1X-DBL4 ϵ ; D, DBL1X-DBL6 ϵ . The left panel shows a side view and the right panel shows the top view after a 90° rotation.

in GraphPad Prism. Higher salt concentrations were not included in the analysis as binding was almost completely inhibited. This is probably due to a change in protein structure. This notion is supported by the fact that $\log K_{D, \text{observed}}$ versus $\log [\text{Na}^+]$ was linear only between 150 and 300 mM, suggesting that other factors play a role at higher concentrations of NaCl.

$\log K_{D, \text{observed}}$ versus $\log [\text{Na}^+]$ shows a linear relationship (Fig. 8). The slope $m(1-f)$ ranges between 2.7 for ID1-ID2a and 3.4 for full-length (FV2). We do not know the value for f , but the maximal number of ionic interactions involved in the binding must be between 2 and 3. It is interesting that the value for the full-length protein is higher than for the short fragments, indicating that this protein makes an extra ionic interaction with CSPG. The K_D values at 150 mM NaCl serves as our reference point, as this is the physiological NaCl concentration. By extrapolating the linear relationship and finding the y intercept we find that $K_{D, \text{nonionic}} = 5.9 \mu\text{M}$ for FV2, $K_{D, \text{nonionic}} = 3.4 \mu\text{M}$ for DBL1X-ID2a, and $K_{D, \text{nonionic}} = 0.7 \mu\text{M}$ for ID1-ID2a. Comparing the logarithmic values of these and the reference point (150 mM NaCl), we estimate that between 25 and 35% of the VAR2CSA binding can be accounted for by ionic interactions. This suggests that the high CSA affinity for VAR2CSA cannot

be explained by ionic interactions with the sulfated GAG structure alone. The high affinity may be achieved through a complex binding site making a multivalent interaction with the CSA carbohydrate backbone.

DISCUSSION

Malaria is one of the most common infectious diseases and one of the largest global health problems. Pregnant women are especially vulnerable to infection, despite previously acquired immunity. In this study we have addressed key questions related to the molecular mechanism behind the VAR2CSA-CSA interaction in PM.

Previous work has suggested that the minimal CSA binding region in VAR2CSA is DBL2X-ID2b, with the need for DBL1X or DBL3X for full affinity binding (19, 20). In continuation of this work we made further truncations of VAR2CSA, focusing on the DBL2X region. We show that the core CSA-binding site lies within the DBL2X domain including small parts of the flanking interdomain regions. The binding does not depend on the ID2b region, or on the DBL1X or DBL3X flanking domains, as previously suggested (19). This is evident by the specific CSPG binding of ID1-ID2a and ID1-DBL2Xb (Figs. 2 and 3). The minimal binding region is ID1-DBL2Xb, which bound CSPG with characteristics comparable with that of full-length VAR2CSA.

It is interesting that these new data maps the core CSA-binding site onto a single domain. Binding of DBL2X (and any other single DBL domain) to CSA has previously been shown to be nonspecific and of weak affinity (42). It is clear that the ID1 and ID2a interdomains are essential for CSA binding. However, data showing that DBL2X-DBL4 ϵ binds CSA (19), suggests that ID1 is not essential for binding but rather needed for the formation of a functional CSA-binding protein. DBL1X-DBL2Xa and ID1-DBL2Xa did not bind CSPG. The two C-terminal DBL2X borders (DBL2Xa and DBL2Xb) differ by 93 amino acids. Because deletion of these amino acids eliminates binding they must be important for CSA binding.

The ID1-DBL2Xb minimal binding region is much smaller than full-length VAR2CSA, having a molecular mass of only 62 kDa. It is unlikely that further substantial truncations of VAR2CSA will be functional in binding CSA. Our data redefines DBL2X as a larger functional domain, incorporating parts of the flanking ID1 and ID2a interdomains.

A VAR2CSA-based vaccine against PM must be able to induce a strong anti-adhesive immune response. Rat sera raised against all fragments containing the CSA-binding site inhibited parasite adhesion to CSA (Fig. 4). Importantly, sera raised against ID1-ID2a resulted in almost complete inhibition. This suggests that the minimal CSA-binding fragments retain the capacity for inducing a strong antiadhesive immune response.

FIGURE 6. Experimental SAXS curves, pair-distance distribution functions $P(r)$, and *ab initio* shape reconstructions. A, a schematic representation of VAR2CSA fragments used in the SAXS analysis together with color-coded VAR2CSA crystal structure homologues (PDB codes 1ZRO (25) and 3C64 (21)) used in the docking processes for the presentation in Fig. 7. White boxes refer to poorly homologous inter-domain linker regions of VAR2CSA (ID's) separating single DBL domains. B, left, SAXS curves for DBL1X-ID2a, ID1-DBL4 ϵ , DBL1X-DBL4 ϵ , and FV2 (DBL1X-DBL6 ϵ). The logarithm of the scattering intensity ($\log(I)$) is shown as a function of the momentum transfer q in units of $\text{\AA}^{-1}$. Gray solid lines show the fit of the shape reconstructions to the experimental data. χ^2 values were 2.5, 1.3, 1.5, and 1.1, respectively. The Guinier fit are shown as the natural logarithm of the scattering intensity as a function of the squared momentum transfer. Center, $P(r)$ functions calculated from each scattering curve are plotted in relative units as a function of the distance in nanometers. Right, the average *ab initio* shape reconstructions are shown with spheres representing the dummy-atoms used by DAMMIF. The orientation on the right is after a 90° right-hand rotation.

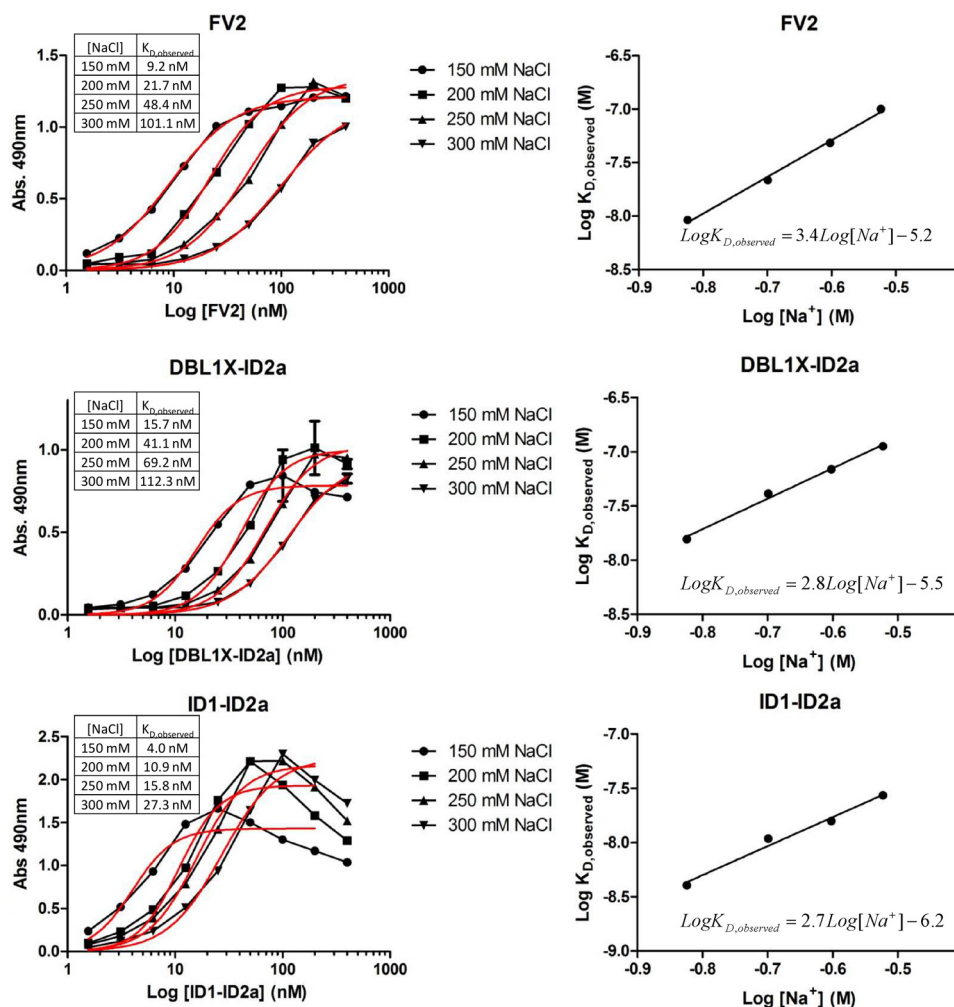


FIGURE 8. Influence of ionic strength on VAR2CSA binding to CSPG. Proteins tested were FV2, DBL1X-ID2a, and ID1-ID2a from 3D7. Left panel, ELISA-based binding assay. CSPG was coated at 3 μ g/ml. A 1:2 dilution series (400 to 1.56 nM) of protein was added in several different NaCl concentrations (150, 200, 250, and 300 mM). The K_D observed values were calculated for each titration series in GraphPad Prism using nonlinear regression (least squares fit with Hill slope). Fit is shown in red. Right column, $log K_{D,observed}$ versus $log [Na^+]$. The graph is linear between 150 and 300 mM [NaCl]. The linear relationship is given as $y = ax + b$. The slope (a) corresponds to $m(1-f)$ and b corresponds to $log K_{D,nonionic}$ as illustrated in Equation 2.

This conclusion was further supported by the fact that antibodies purified from anti-FV2 serum on ID1-ID2a retained most of the adhesion blocking activity, and that the anti-ID1-ID2a antibody-depleted anti-FV2 sample lost most of its activity (Fig. 5). This indicates that epitopes required for the induction of adhesion blocking antibodies are located within this region.

In this study we have tested anti-VAR2CSA sera in homologous inhibition of FCR3 parasites binding to CSA. It is important that a vaccine is capable of inhibiting placental adhesion regardless of parasite strain origin. A major concern in vaccine development is therefore the high interclonal diversity among parasite variants. Although recombinant full-length VAR2CSA is very immunogenic the antibodies produced are not cross-inhibitory (43). A recent study shows that DNA vaccination with ID1-DBL2X from FCR3 induces antibodies that are cross-inhibitory, inhibiting CSA adhesion of other laboratory strains as well as parasites isolated in the field (34). This supports the use of this small fragment in a PM vaccine.

We have generated a model showing the structural organization of VAR2CSA. All six DBL domains together with the ID2b(CIDR_{PAM}) domain were mapped by analyzing the full

extracellular part of VAR2CSA and three truncated VAR2CSA proteins using SAXS. The model was based on the interpretation of SAXS-derived molecular envelopes and the subsequent positioning of the available homologous crystal structures within them. The volumes of the *ab initio* reconstructed envelopes were increased above what was expected from the protein content. This was most likely due to protein glycosylation. Despite this discrepancy, the domain positions in the SAXS envelopes provides a consistent and reasonable image of the overall VAR2CSA structure. Provided that the four C-terminal DBL domains (DBL3X–DBL6e) follow an EBA-175-like structural arrangement, little ambiguity is left for the positioning of the remaining ID2b(CIDR_{PAM}) and N-terminal DBL2X and DBL1X domains in the VAR2CSA envelope. In the model, ID2b(CIDR_{PAM})-DBL3X forms a structural turn resulting in the compact VAR2CSA structure.

The CSA-binding DBL2X maps to an exposed central region of the structure (Fig. 7). In the model, space for the unmodeled ID2a region is available in the cleft formed by the V-shaped ID2b domain, between DBL2X and DBL3X (Fig. 7, left panels). It is likely that the shape of ID2a together with the DBL2X

domain is of great importance to the structure-function properties of the VAR2CSA/CSA interaction. We have previously shown that CSA adhesion blocking antibodies can be induced against recombinant DBL4 ϵ -ID4 (35). DBL4 ϵ -ID4 is not part of the CSA-binding region, but in our model it lies in close proximity to DBL2X. It is therefore likely that the anti-DBL4 ϵ -ID4 antibodies block the CSA-binding site by steric hindrance.

Cardin and Weintraub (36) predicted that a GAG-binding site would take one of two forms. These are XBBXB and XBBBXXB, where X is any hydrophobic residue and B is any basic residue, with a preference for arginine (38). Both of these describe a binding site for a sulfated disaccharide. We mutated two such sites within the minimal binding region: ⁶²⁵GKNLK-KRY⁶³² in DBL2X and ⁴⁵⁸NKKKECKD⁴⁶⁵ in ID1. We also deleted a large region within a DSM located in the N-terminal part of DBL2X, as this has been suggested to have a function in binding (37). None of these mutations had an effect (data not shown). This is a clear indication that these sites have little or no function in CSA binding.

It is remarkable that the VAR2CSA expressing parasites, *in vivo*, are very specific for CSA carrying only 2–8% C4-sulfated disaccharide units. To examine if the VAR2CSA-CSA complex formation is dependent on ionic interactions, we tested binding at different salt concentrations. Binding of ID1-ID2a, DBL1X-ID2a, and FV2 in 150–300 mM NaCl show a linear relationship when plotting log($K_{D,observed}$) versus log[Na⁺] (Fig. 8). We find that binding depends on a maximum of 2–3 ionic interactions. It is interesting that the value for the full-length protein is higher than for the shorter fragments, indicating that this protein makes an additional ionic interaction with CSA. We have in this study screened for fragments containing the CSA-specific high-affinity binding region. It is possible that more interactions occur in downstream regions of the protein, but that the core site lies within DBL2X. Extrapolating and finding the y intercept ([Na⁺] = 1 M, log[Na⁺] = 0) tells us that $K_{D,nonionic}$ = 5.9 μ M for FV2, $K_{D,nonionic}$ = 3.4 μ M for DBL1X-ID2a, and $K_{D,nonionic}$ = 0.7 μ M for ID1-ID2a. This indicates that only 25–30% of the VAR2CSA-CSA binding can be accounted for by ionic interactions (41). This is in contrast to other GAG-binding proteins, which have shown up to 80–90% dependence on ionic interactions in similar assays (38, 41).

Our data suggest that the VAR2CSA-CSA interaction does not conform to conventional GAG-protein interactions. We hypothesize that the high CSA affinity is achieved through a multivalent interaction, which may include multiple binding sites making nonionic interactions with the CSA carbohydrate backbone. Some of the interactions are ionic and some degree of sulfation is needed for VAR2CSA binding (12, 44). It is therefore likely that there is an interaction between basic amino acids and sulfates, but that this is not the determining factor in the affinity.

In this study we have defined a small single-domain VAR2CSA fragment that can be produced in eukaryotic cells as a functional CSA-binding protein, and has the capacity to induce highly adhesion-blocking antibodies. This fragment has the potential to be a powerful candidate for a vaccine against PM. In conclusion, this paper provides the first insight into the biochemical nature of the interaction between a PfEMP1 mol-

ecule and its ligand and shows how structure-function studies can aid vaccine development.

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REFERENCES

1. WHO (2010) *World Malaria Report 2010*, WHO, Geneva, Switzerland
2. Doolan, D. L., Dobaño, C., and Baird, J. K. (2009) Acquired immunity to malaria. *Clin. Microbiol. Rev.* **22**, 13–36
3. Fried, M., and Duffy, P. E. (1996) Adherence of *Plasmodium falciparum* to chondroitin sulfate A in the human placenta. *Science* **272**, 1502–1504
4. Menendez, C. (1995) Malaria during pregnancy. A priority area of malaria research and control. *Parasitol. Today* **11**, 178–183
5. McGregor, I. A. (1987) Thoughts on malaria in pregnancy with consideration of some factors that influence remedial strategies. *Parasitologia* **29**, 153–163
6. Smith, J. D., Chitnis, C. E., Craig, A. G., Roberts, D. J., Hudson-Taylor, D. E., Peterson, D. S., Pinches, R., Newbold, C. I., and Miller, L. H. (1995) Switches in expression of *Plasmodium falciparum* var genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. *Cell* **82**, 101–110
7. Su, X. Z., Heatwole, V. M., Wertheimer, S. P., Guinet, F., Herrfeldt, J. A., Peterson, D. S., Ravetch, J. A., and Welles, T. E. (1995) The large diverse gene family var encodes proteins involved in cytoadherence and antigenic variation of *Plasmodium falciparum*-infected erythrocytes. *Cell* **82**, 89–100
8. Salanti, A., Staalsøe, T., Lavstsen, T., Jensen, A. T., Sowa, M. P., Arnot, D. E., Hviid, L., and Theander, T. G. (2003) Selective up-regulation of a single distinctly structured var gene in chondroitin sulfate A-adhering *Plasmodium falciparum* involved in pregnancy-associated malaria. *Mol. Microbiol.* **49**, 179–191
9. Salanti, A., Dahlbäck, M., Turner, L., Nielsen, M. A., Barfod, L., Magistrado, P., Jensen, A. T., Lavstsen, T., Ofori, M. F., Marsh, K., Hviid, L., and Theander, T. G. (2004) Evidence for the involvement of VAR2CSA in pregnancy-associated malaria. *J. Exp. Med.* **200**, 1197–1203
10. Magistrado, P., Salanti, A., Tuikue Ndam, N. G., Mwakalinga, S. B., Resende, M., Dahlbäck, M., Hviid, L., Lusingu, J., Theander, T. G., and Nielsen, M. A. (2008) VAR2CSA expression on the surface of placenta-derived *Plasmodium falciparum*-infected erythrocytes. *J. Infect. Dis.* **198**, 1071–1074
11. Achur, R. N., Valiyaveetil, M., Alkhalil, A., Ockenhouse, C. F., and Gowda, D. C. (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
12. Alkhalil, A., Achur, R. N., Valiyaveetil, M., Ockenhouse, C. F., and Gowda, D. C. (2000) Structural requirements for the adherence of *Plasmodium falciparum*-infected erythrocytes to chondroitin sulfate proteoglycans of human placenta. *J. Biol. Chem.* **275**, 40357–40364
13. Khunrae, P., Dahlbäck, M., Nielsen, M. A., Andersen, G., Ditlev, S. B., Resende, M., Pinto, V. V., Theander, T. G., Higgins, M. K., and Salanti, A. (2010) Full-length recombinant *Plasmodium falciparum* VAR2CSA binds specifically to CSPG and induces potent parasite adhesion-blocking antibodies. *J. Mol. Biol.* **397**, 826–834
14. Srivastava, A., Gangnard, S., Round, A., Dechavanne, S., Juillerat, A., Raynal, B., Faure, G., Baron, B., Ramboarina, S., Singh, S. K., Belrhali, H., England, P., Lewit-Bentley, A., Scherf, A., Bentley, G. A., and Gamain, B. (2010) Full-length extracellular region of the var2CSA variant of PfEMP1 is required for specific, high-affinity binding to CSA. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 4884–4889
15. Brabin, B. J. (1983) An analysis of malaria in pregnancy in Africa. *Bull. World Health Organ* **61**, 1005–1016
16. Bockhorst, J., Lu, F., Janes, J. H., Keebler, J., Gamain, B., Awadalla, P., Su,

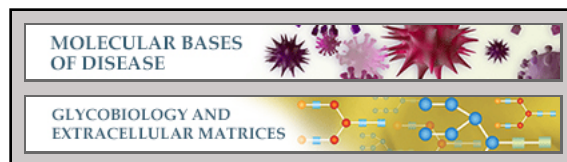
- X. Z., Samudrala, R., Jojic, N., and Smith, J. D. (2007) Structural polymorphism and diversifying selection on the pregnancy malaria vaccine candidate VAR2CSA. *Mol. Biochem. Parasitol.* **155**, 103–112
17. Andersen, P., Nielsen, M. A., Resende, M., Rask, T. S., Dahlbäck, M., Theander, T., Lund, O., and Salanti, A. (2008) Structural insight into epitopes in the pregnancy-associated malaria protein VAR2CSA. *PLoS Pathog.* **4**, e42
 18. Smith, J. D., Subramanian, G., Gamain, B., Baruch, D. I., and Miller, L. H. (2000) Classification of adhesive domains in the *Plasmodium falciparum* erythrocyte membrane protein 1 family. *Mol. Biochem. Parasitol.* **110**, 293–310
 19. Dahlbäck, M., Jørgensen, L. M., Nielsen, M. A., Clausen, T. M., Ditlev, S. B., Resende, M., Pinto, V. V., Arnot, D. E., Theander, T. G., and Salanti, A. (2011) The chondroitin sulfate A-binding site of the VAR2CSA protein involves multiple N-terminal domains. *J. Biol. Chem.* **286**, 15908–15917
 20. Srivastava, A., Gangnard, S., Dechavanne, S., Amirat, F., Lewit Bentley, A., Bentley, G. A., and Gamain, B. (2011) Var2CSA minimal CSA-binding region is located within the N-terminal region. *PLoS One* **6**, e20270
 21. Klein, M. M., Gittis, A. G., Su, H. P., Makobongo, M. O., Moore, J. M., Singh, S., Miller, L. H., and Garboczi, D. N. (2008) The cysteine-rich interdomain region from the highly variable *Plasmodium falciparum* erythrocyte membrane protein-1 exhibits a conserved structure. *PLoS Pathog.* **4**, e1000147
 22. Higgins, M. K. (2008) The structure of a chondroitin sulfate-binding domain important in placental malaria. *J. Biol. Chem.* **283**, 21842–21846
 23. Singh, K., Gittis, A. G., Nguyen, P., Gowda, D. C., Miller, L. H., and Garboczi, D. N. (2008) Structure of the DBL3x domain of pregnancy-associated malaria protein VAR2CSA complexed with chondroitin sulfate A. *Nat. Struct. Mol. Biol.* **15**, 932–938
 24. Khunrae, P., Philip, J. M., Bull, D. R., and Higgins, M. K. (2009) Structural comparison of two CSPG-binding DBL domains from the VAR2CSA protein important in malaria during pregnancy. *J. Mol. Biol.* **393**, 202–213
 25. Tolia, N. H., Enemark, E. J., Sim, B. K., and Joshua-Tor, L. (2005) Structural basis for the EBA-175 erythrocyte invasion pathway of the malaria parasite *Plasmodium falciparum*. *Cell* **122**, 183–193
 26. Grüber, A., Gunalan, K., Ramalingam, J. K., Manimekalai, M. S., Grüber, G., and Preiser, P. R. (2011) Structural characterization of the erythrocyte binding domain of the reticulocyte-binding protein homologue family of *Plasmodium yoelii*. *Infect. Immun.* **79**, 2880–2888
 27. Konarev, P. V., Volkov, V. V., Sokolova, A. V., Koch, M. H. J., and Svergun, D. I. (2003) PRIMUS. A Windows PC-based system for small-angle scattering data analysis. *J. Appl. Crystallogr.* **36**, 1277–1282
 28. Petoukhov, M. V., Konarev, P. V., Kikhney, A. G., and Svergun, D. I. (2007) ATSAS 2.1. Towards automated and web-supported small-angle scattering data analysis. *J. Appl. Crystallogr.* **40**, s223–s228
 29. Svergun, D. (1992) Determination of the regularization parameter in indirect-transform methods using perceptual criteria. *J. Appl. Crystallogr.* **25**, 495–503
 30. Franke, D., and Svergun, D. I. (2009) DAMMIF, a program for rapid ab initio shape determination in small-angle scattering. *J. Appl. Crystallogr.* **42**, 342–346
 31. Volkov, V. V., and Svergun, D. I. (2003) Uniqueness of ab initio shape determination in small-angle scattering. *J. Appl. Crystallogr.* **36**, 860–864
 32. Wriggers, W., Milligan, R. A., and McCammon, J. A. (1999) Situs. A package for docking crystal structures into low-resolution maps from electron microscopy. *J. Struct. Biol.* **125**, 185–195
 33. Avril, M., Hathaway, M. J., Cartwright, M. M., Gose, S. O., Narum, D. L., and Smith, J. D. (2009) Optimizing expression of the pregnancy malaria vaccine candidate, VAR2CSA in *Pichia pastoris*. *Malar. J.* **8**, 143
 34. Bordbar, B., Tuikue-Ndam, N., Bigey, P., Doritchamou, J., Scherman, D., and Deloron, P. (2012) Identification of Id1-DBL2X of VAR2CSA as a key domain inducing highly inhibitory and cross-reactive antibodies. *Vaccine* **30**, 1343–1348
 35. Nielsen, M. A., Pinto, V. V., Resende, M., Dahlbäck, M., Ditlev, S. B., Theander, T. G., and Salanti, A. (2009) Induction of adhesion-inhibitory antibodies against placental *Plasmodium falciparum* parasites by using single domains of VAR2CSA. *Infect. Immun.* **77**, 2482–2487
 36. Cardin, A. D., and Weintraub, H. J. (1989) Molecular modeling of protein-glycosaminoglycan interactions. *Arteriosclerosis* **9**, 21–32
 37. Sander, A. F., Salanti, A., Lavstsen, T., Nielsen, M. A., Magistrado, P., Lusingu, J., Ndam, N. T., and Arnot, D. E. (2009) Multiple *var2csa*-type PfEMP1 genes located at different chromosomal loci occur in many *Plasmodium falciparum* isolates. *PLoS One* **4**, e6667
 38. Hileman, R. E., Fromm, J. R., Weiler, J. M., and Linhardt, R. J. (1998) Glycosaminoglycan-protein interactions. Definition of consensus sites in glycosaminoglycan-binding proteins. *Bioessays* **20**, 156–167
 39. Record, M. T., Jr., Lohman, M. L., and De Haseth, P. (1976) Ion effects on ligand-nucleic acid interactions. *J. Mol. Biol.* **107**, 145–158
 40. Olson, S. T., Halvorson, H. R., and Björk, I. (1991) Quantitative characterization of the thrombin-heparin interaction. Discrimination between specific and nonspecific binding models. *J. Biol. Chem.* **266**, 6342–6352
 41. Faller, B., Mely, Y., Gerard, D., and Bieth, J. G. (1992) Heparin-induced conformational change and activation of mucus proteinase inhibitor. *Biochemistry* **31**, 8285–8290
 42. Resende, M., Ditlev, S. B., Nielsen, M. A., Bodevin, S., Bruun, S., Pinto, V. V., Clausen, H., Turner, L., Theander, T. G., Salanti, A., and Dahlbäck, M. (2009) Chondroitin sulfate A (CSA) binding of single recombinant Duffy binding-like domains is not restricted to *Plasmodium falciparum* erythrocyte membrane protein 1 expressed by CSA-binding parasites. *Int. J. Parasitol.* **39**, 1195–1204
 43. Avril, M., Hathaway, M. J., Srivastava, A., Dechavanne, S., Hommel, M., Beeson, J. G., Smith, J. D., and Gamain, B. (2011) Antibodies to a full-length VAR2CSA immunogen are broadly strain-transcendent but do not cross-inhibit different placental-type parasite isolates. *PLoS One* **6**, e16622
 44. Chai, W., Beeson, J. G., and Lawson, A. M. (2002) The structural motif in chondroitin sulfate for adhesion of *Plasmodium falciparum*-infected erythrocytes comprises disaccharide units of 4-O-sulfated and non-sulfated N-acetylglucosamine linked to glucuronic acid. *J. Biol. Chem.* **277**, 22438–22446

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Thomas M. Clausen, Stig Christoffersen,
Madeleine Dahlbäck, Annette Eva Langkilde,
Kamilla E. Jensen, Mafalda Resende, Mette
Ø. Agerbæk, Daniel Andersen, Besim Berisha,
Sisse B. Ditlev, Vera V. Pinto, Morten A.
Nielsen, Thor G. Theander, Sine Larsen and
Ali Salanti

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