Var2CSA Minimal CSA Binding Region Is Located within the N-Terminal Region

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Abstract

Var2CSA, a key molecule linked with pregnancy-associated malaria (PAM), causes sequestration of Plasmodium falciparum infected erythrocytes (PEs) in the placenta by adhesion to chondroitin sulfate A (CSA). Var2CSA possesses a 300 kDa extracellular region composed of six Duffy-binding like (DBL) domains and a cysteine-rich interdomain region (CIDRpm) module. Although initial studies implicated several individual var2CSA DBL domains as important for adhesion of PEs to CSA, new studies revealed that these individual domains lack both the affinity and specificity displayed by the full-length extracellular region. Indeed, recent evidence suggests the presence of a single CSA-binding site formed by a higher-order domain organization rather than several independent binding sites located on the different domains. Here, we search for the minimal binding region within var2CSA that maintains high affinity and specificity for CSA binding, a characteristic feature of the full-length extracellular region. Accordingly, truncated recombinant var2CSA proteins comprising different domain combinations were expressed and their binding characteristics assessed against different sulfated glycosaminoglycans (GAGs). Our results indicate that the smallest region within var2CSA with similar binding properties to those of the full-length var2CSA is DBL1X-3X. We also demonstrate that inhibitory antibodies raised in rabbit against the full-length DBL1X-6e target principally DBL3X and, to a lesser extent, DBL5e. Taken together, our results indicate that efforts should focus on the DBL1X-3X region for developing vaccine and therapeutic strategies aimed at combating PAM.

Introduction

Pregnancy-associated malaria (PAM) causes adverse pregnancy outcomes, including anemia and hypertension in first-time pregnant women, and low birth weight due to premature delivery and fetal growth restriction, which are associated with a higher risk of fetal and neonate morbidity and mortality [1,2]. Complications arising from PAM have been attributed to massive accumulation of Plasmodium falciparum-infected erythrocytes (PEs) in the placenta mediated by adhesion to chondroitin sulfate A (CSA) [3]. Significantly, after one or two pregnancies, women acquire transient antibodies recognizing placental PEs from different geographic regions that inhibit placental adhesion, thus correlating with protection against malaria [4,5].

Several lines of evidence support var2CSA, a member of the Plasmodium falciparum Erythrocyte Membrane Protein 1 (PfEMP1) adhesins encoded by the var gene family [6,7,8], as the leading PAM vaccine candidate. Indeed, var2CSA is the only var gene transcribed in CSA-binding laboratory isolates and placental PEs [9,10,11,12,13,14]. Importantly, gene disruption studies have clearly demonstrated that var2CSA is the primary var gene responsible for the CSA-binding phenotype, as Δvar2CSA mutant clones either did not recover the CSA-binding phenotype [15] or otherwise switched to low affinity CSA binders that no longer reacted in a gender-specific manner with multigravid sera [16]. These mutant parasites were unable to express any other ligand that promoted extensive sequestration in placental tissue [16,17]. Finally, antibodies to var2CSA-expressing isolates and to var2CSA recombinant proteins have been consistently associated with protection against malaria during pregnancy [11,18,19,20]. World-wide parasite isolates analysed so far contain at least one var2CSA ortholog with an amino acid identity ranging from 54% to 94% and distinct, as well as conserved, epitopes [13,21,22,23].

Var2CSA is a 350 kDa transmembrane protein with a 300 kDa extracellular region composed of six Duffy-binding-like (DBL) domains and a cysteine-rich interdomain region (CIDRpm) module, as well as short interdomain regions (Fig. 1A) [24,25]. From in vitro binding assays, the CSA-binding properties have been mapped to several var2CSA domains, namely DBL2X, DBL3X, DBL5e, and DBL6e [14,26,27]. These studies suggested that the various CSA-binding domains might function independently of each other by forming multivalent interactions that together cause
Var2CSA Minimal Binding Region

Plasmodium falciparum (Pf) var2csa minimal binding region is a crucial component in the invasion of red blood cells (RBCs) by the parasite and is a potential target for vaccine development. We previously reported that a domain DBL3X in var2csa, which differs from DBL1X and DBL2X in the extracellular region of PfEMP1, is the principal target for inhibitory antibodies and that strategies aimed at blocking PE adhesion to CSA should focus on the N-terminal segment of var2CSA to create a rational basis for accelerating vaccine and therapeutic developments.

Methods

Expression and Purification of Recombinant Protein

(i) HEK 293-F cell Recombinant Protein Expression and Purification. Synthetic var2csa genes for 3D7-DBL1X-3X (residues 59–1577) and 3D7-DBL4e-6e (residues 1578–2630) were designed with optimized codons for human cell expression, as previously described [34], and were cloned into the pTT3 vector [36]. pTT3-3D7-DBL1X-6e (residues 59–2630) were prepared by incorporation of 3D7-DBL4e-6e into pTT3-3D7-DBL1X-3X using compatible restriction sites. Genes encoding 3D7-DBL1X (residues 59–430) and 3D7-DBL1X-CIDR (residues 59–1209) were PCR amplified from pTT3-3D7-DBL1X-3X and cloned into the pTT3 vector using EcoRI/HindIII restriction sites for expression in FreeStyle 293-F cells, as previously described [InVitrogen] [34]. The oligonucleotide primers used for amplification were as follows.
(restriction sites are represented in lower case): 3D7-DBL1X: 5′-AGC gattc ATG GATG ACC GAC ACC G-3′ and 3D7-DBL1XR 5′-CAC aagct TCG GATG ATG ACC C-3′ and 3D7-DBL1X-AGC 5′-AGC gattc ATG GATG ACC GAC ACC G-3′ and 3D7-DBL1X-CIDR 5′-CTG gattct GTG CAC ACC G-3′ and 3D7-DBL1X-CIDR 5′-CTG aagct TCG GATG ATG ACC C-3′. Genes encoding 3D7-DBL1X-4e (residues 59-1905) and 3D7-DBL1X-5e (residues 39-2296) were obtained by PCR amplification of 3D7-DBL1X and 3D7-DBL1X-6e from pH3-3D7-DBL1X-6e prior to cloning into pH73-3D7-DBL1X-3X, using HindIII/XbaI restriction sites. The oligonucleotide primers used to amplify were as follows (restriction sites are represented in lower case): DBL1X 5′-AAAC gattc ATG GATG ACC GAC ACC G-3′ and the DBL1X primer 5′-GAT TCC GGC ACC GAT TTT GCA CTG G-3′ used to amplify the DBL1X primer 5′-CAG GAT TTC CTG CGC ATT CTG-3′. The gene encoding DBL1X using the following oligonucleotide primers FCR3-DBL3X (5′-CTG gattct GTG CAC ACC G-3′) and DBL3X (5′-GGCAG gtcgac TTA ATG ATG ATG ATG ATG ATG ATT GAT TCC GGC ACA AAT GAT CCT TG-3′) were PCR amplified and cloned into a modified pET21b vector in frame with a C-terminal hexa-His tag using the NdeI/XhoI restriction sites, in frame with the N-terminal hexa-His tag using the following oligonucleotide primers FCR3-DBL3X (5′-CTG gattct GTG CAC ACC G-3′) and DBL3X (5′-GGCAG gtcgac TTA ATG ATG ATG ATG ATG ATG ATT GAT TCC GGC ACA AAT GAT CCT TG-3′) and the DBL3X primer 5′-CAG GAT TTC CTG CGC ATT CTG-3′. Similarly, genes encoding 3D7-DBL2X and 3D7-DBL2X-2X were PCR amplified and cloned into a modified pET21b vector in frame with a C-terminal hexa-His tag using the following oligonucleotide primers FCR3-DBL3X (5′-CTG gattct GTG CAC ACC G-3′) and DBL3X (5′-GGCAG gtcgac TTA ATG ATG ATG ATG ATG ATG ATT GAT TCC GGC ACA AAT GAT CCT TG-3′) and the DBL3X primer 5′-CAG GAT TTC CTG CGC ATT CTG-3′. All proteins were expressed either in the Rosetta-Gami, Origami B or Shuffle strains of E. coli (Novagen) as soluble proteins at 20°C for 20 h after IPTG induction. Post-induction cells were centrifuged, resuspended in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5 and lysed with an Emulsiflex homogeniser (Avestin). The proteins were purified using a metal affinity column (TALON, Clontech). All proteins were further purified using a heparin affinity column (GE Healthcare) and were eluted in 20 mM Tris-HCl pH 7.5, 1 M NaCl, followed by gel filtration (Sephacryl 75 16/60, GE Healthcare) in 20 mM Tris-HCl 150 mM NaCl, pH 7.5.

ELISA binding assays of recombinant proteins to various sulfated glycosaminoglycans

ELISA binding assays were performed as previously described [34]. Briefly, ELISA plates were coated overnight at 4°C with different sulfated glycosaminoglycans (GAG): 5 µg/mL for decorin (Sigma, D8429); 50 µg/mL for chondroitin sulfate A (CSA) (Sigma, C8529), chondroitin sulfate C (CSC) (Seikagaku, 400670) and heparan sulfate (HS) (Sigma, H7640) in PBS (Gibco, NaCl 155 mM pH 7.2), using 100 µL per well. BSA at 1% in PBS was used as background measurement. After coating, the wells were blocked with 150 µL of dilution buffer per well (PBS 1% BSA, 0.05% Tween20) for 1 h at 37°C. After removal of the blocking solution, each recombinant protein (3D7-DBL1X, 3D7-DBL2X, FCR3-DBL3X, 3D7-DBL5e, 3D7-DBL1X-2X, FCR3-DBL3X-4e, 3D7-DBL1X-CIDR, 3D7-DBL1X-3X, 3D7-DBL1X-4e, 3D7-DBL1X-5e and 3D7-DBL2X-6e) at serial dilutions of 0.3125–20 µg/mL in the dilution buffer, was added per well and incubated for 1 h at 37°C with gentle shaking. After washing three times with PBST (PBS containing 0.05% Tween 20), 100 µL anti-His HRP conjugated antibody (diluted 1/2000 in dilution buffer) was added to each well and incubated for 1 h at 37°C. After washing three times with PBST, the interaction was quantified with TMB (3,3′,5,5′-tetramethylbenzidine) substrate (Biorad) using 100 µL per well for 20 min or until saturation was reached. Absorbance was measured at 655 nm.

Adhesion inhibition assays of recombinant proteins to CSPG

Inhibition assays were performed using a protocol similar to that described above for ELISA, with decorin coated on the plate. Recombinant proteins (3D7-DBL1X-2X, 3D7-DBL1X-CIDR, 3D7-DBL1X-3X, 3D7-DBL1X-4e, 3D7-DBL1X-5e and 3D7-DBL1X-6e) at a concentration of 1 µg/mL were premixed with increasing amounts of BSA, CSA, CSC, or HS (0.156–100 µg/mL) and incubated for 30 min at room temperature with gentle shaking before addition to the coated ELISA plate.

Surface Plasmon Resonance

Interaction between the recombinant proteins and human placental CSPG was studied by surface plasmon resonance (SPR) using a Biacore® 2000 system (GE Healthcare) as previously described [34]. Human placental CSPG (MR4 Reagents Resource) was covalently coupled via primary amino groups of the protein moiety to the sensor chip (CM5 chip, GE Healthcare) surface using amine coupling kit (GE Healthcare) as described previously [38]. The amount of immobilized CSPG corresponded to 240 response units (RU). A separate flow channel on the same sensor chip without CSPG was used for control runs. For all SPR measurements, the recombinant domains were dialyzed against PBS buffer (Gibco), 0.005% P20 (GE Healthcare), and centrifuged immediately before the runs to minimize possible effects from nonspecific aggregation. The association was monitored by injecting different concentrations of the DBL analytes at 25°C with the flow rate of 20 µL/min for 300 s to achieve steady-state binding. Between each injection, surfaces were regenerated using 10 µL of 2 M NaCl followed by 10 µL of SDS 0.05%. All curves
were corrected for nonspecific binding by subtraction of control curves obtained from injection of the corresponding protein through the blank flow channel. $K_D$ was determined from the concentration dependence of steady-state SPR response (after corrections for nonspecific binding) using the Biacore BIAevaluation 3.1 software (Biacore AB).

Animal immunization

Immunizations with 3D7-DBL1X-6e VAR2CSA recombinant protein were performed at Proteogenix, France, according to animal immunization guidelines. Briefly, a New Zealand white rabbit was immunized with recombinant protein in TiterMax Gold Adjuvant (Sigma) intradermally with 50 μg of immunogen in subsequent injections. IgG were purified from fractions, and incubated for 30 min at room temperature with Gold Adjuvant (Sigma) intradermally with 50 μg of immunogen in subsequent injections. IgG were purified from rabbit plasma using Hitrap protein G (GE Healthcare) according to manufacturer’s instructions.

IgG purification and depletion

The recombinant proteins (3D7-DBL1X, 3D7-DBL2X, FCR3-DBL2X, 3D7-DBL5e, 3D7-DBL6e, 3D7-DBL1X-2X, 3D7-DBL1X-3X, FCR3-DBL3X-4e, 3D7-DBL1X-4e, 3D7-DBL1X-5e, and 3D7-DBL1X-6e) were coated on M-280 tosyl-activated Dynabeads (Invitrogen) according to manufacturer’s instructions. Briefly, 66 μL of M-280 tosyl-activated Dynabeads were washed three times in 900 μL PBS, pH 7.4, and resuspended in the same buffer. Beads were then mixed with 20 μg of each recombinant protein containing 1.2 M ammonium sulfate and incubated for 12 h at 37°C with slow rocking. Following the incubation, the beads were washed five times with 1 mL PBS, incubated 1 h at room temperature with PBS, 0.5% BSA and washed 2 times in PBS and stored at 4°C in the same buffer.

For antibody depletion, 50 μL of purified rabbit anti 3D7-DBL1X-6e IgG at 160 μg/mL diluted in PBS were incubated for 1 h at 37°C with the different protein-coated beads. Unbound antibodies were collected and beads were washed three times with 1 mL PBS prior to elution of bound antibodies using 50 μL 0.1 M glycine pH 2.5. Eluted fractions were subsequently neutralized with Tris-HCl 1 M pH 8.0.

Inhibition assays were performed using a protocol similar to that described above for ELISA using decorin-coated plates. Recombinant proteins at a concentration of 0.25 μg/mL were premixed with either the unbound antibody fractions or the eluted antibody fractions, and incubated for 30 min at room temperature with gentle shaking before addition to the coated ELISA plate.

Results

Recombinant DBL domains were successfully purified to high purity

Var2CSA is a 350 kDa protein possessing a large extracellular region, a transmembrane region and a short, conserved intracellular domain (Fig. 1A). While individual domains of var2CSA have been expressed and purified in various heterologous expression systems [29,30,33,39,40], it has been difficult to produce multiple PfEMP1 domains due to their large size and the presence of many cysteine bridges. Recently, we reported the expression of the complete extracellular region of var2CSA using the human embryonic kidney cell (HEK293) heterologous expression system [34]. In the present study, we attempted to express in the HEK293 heterologous expression system various single and multiple domain recombinant proteins from the 3D7 var2CSA ortholog (3D7-DBL1X, 3D7-DBL1X-CIDR, 3D7-DBL1X-3X, 3D7-DBL1X-4e, 3D7-DBL1X-5e and 3D7-DBL1X-6e). In addition, single (3D7-DBL2X, FCR3-DBL3X, 3D7-DBL5e) and double (3D7-DBL1X-2X, FCR3-DBL5X-4e) DBL domains were expressed as soluble proteins in E. coli. All these proteins were successfully expressed and purified to better than 95% homogeneity (Fig. 1B). Some domains, such as DBL1e, could not be obtained due to poor expression yields. In SDS-PAGE under reducing conditions, all proteins migrated according to their expected molecular weights: 3D7-DBL1X (45 kDa), 3D7-DBL2X (53 kDa), FCR3-DBL3X (43 kDa), 3D7-DBL5e (37 kDa), 3D7-DBL1X-2X (105 kDa), FCR3-DBL3X-4e (86 kDa), 3D7-DBL1X-CIDR (154 kDa), 3D7-DBL1X-3X (176 kDa), 3D7-DBL1X-4e (215 kDa), 3D7-DBL1X-5e (259 kDa), and 3D7-DBL1X-6e (300 kDa) (Fig. 1C), while under non-reducing conditions a shift in the migration of all proteins confirmed the presence of disulfide bridges (Fig. 1B). terminal sequencing and Western blots using anti-His antibodies confirmed that the proteins were not degraded (data not shown).

The yields after purification varied from 0.1 to 20 mg per litre of culture media: 3D7-DBL1X (1 mg/L), 3D7-DBL2X (5 mg/L), FCR3-DBL3X (20 mg/L), 3D7-DBL5e (1 mg/L), 3D7-DBL1X-2X (1 mg/L), FCR3-DBL3X-4e (3 mg/L), 3D7-DBL1X-CIDR (0.25 mg/L), 3D7-DBL1X-3X (0.25 mg/L), 3D7-DBL1X-4e (0.15 mg/L), 3D7-DBL1X-5e (0.15 mg/L), and 3D7-DBL1X-6e (0.5 mg/L).

Minimal CSA-binding region lies within 3D7-DBL1X-3X

The adhesion properties of the different recombinant proteins were tested by direct ELISA using plates coated with different sulfated glycosaminoglycans (GAG), including chondroitin sulfate A (CSA), chondroitin sulfate C (CSC), decorin (a proteoglycan containing CSA), and heparan sulfate (HS) (Fig. 2A-C). Among the single DBL domains tested 3D7-DBL1X did not bind to any of the sulfated GAGs (data not shown) and 3D7-DBL3X bound weakly to CSA and decorin at higher concentrations (Fig. 2B), like DBL6e as previously reported [34]. Interestingly, 3D7-DBL2X and 3D7-DBL5e bound to decorin and CSA in a dose-dependent manner but these proteins also bound to BSA and heparan sulfate at higher concentrations (Fig. 2A and C), indicating some non-specific interactions. For 3D7-DBL2X, a much stronger signal was observed in binding to decorin in comparison to CSA, reaching saturation at very low concentrations. Binding properties of the double domain recombinant proteins were also tested. While DBL1X-2X bound with high affinity to decorin, the apparent affinity to CSA was lower (Fig. 2E). DBL3X-4e bound to the different GAGs in a weak, non-specific manner (Fig. 2D). Similarly, recombinant proteins possessing more than two domains (3D7-DBL1X-CIDR, 3D7-DBL1X-3X, 3D7-DBL1X-4e and 3D7-DBL1X-5e) were tested for their binding behavior with CSA and were compared with the full-length 3D7-DBL1X-6e protein. All multiple domain proteins bound to decorin and CSA in a dose-dependent manner with various degrees of affinity, except for DBL1X-4e which did not bind to CSA (Fig. 2F-J). DBL1X-3X and DBL1X-5e exhibited an affinity and specificity quite similar to the full-length 3D7-DBL1X-6e protein while 3D7-DBL1X-CIDR had lower affinity to CSA and decorin.

Our results indicate that the high affinity CSA-binding site lies within the DBL1X-3X recombinant protein.

To further assess the binding specificity of the multidomain recombinant proteins, we tested whether different sulfated GAGs could compete with decorin in binding to these proteins. Decorin-coated plates were incubated with a constant concentration of recombinant protein premixed with increasing concentrations of CSA, CSC, or HS. Binding of all the recombinant proteins (3D7-DBL1X-2X, 3D7-DBL1X-CIDR, 3D7-DBL1X-3X, 3D7-DBL1X-4e,
DBL1X-5e and DBL1X-6e to decorin was inhibited by CSA in a dose-dependent manner. Significant inhibition with the other sulfated GAGs, was also observed at higher concentrations for 3D7-DBL1X-CIDR and 3D7-DBL1X-4e (Fig. 3B and D). However, the binding of 3D7-DBL1X-2X and 3D7-DBL1X-3X (Fig. 3A and C) to decorin was specifically and efficiently inhibited by CSA and most closely mimicked the binding inhibition of DBL1X-5e and DBL1X-6e (Fig. 3E and F).

Taken together, our results indicate that the minimal region within var2CSA for specific and high affinity binding to CSA lies within DBL1X-3X and that the binding site is mainly centred on the DBL2X domain. Although 3D7-DBL2X binds with high affinity to decorin and CSA, it shows poor specificity as it also
binds to other GAGs at higher concentrations (Fig. 2A). Thus, with our observations that DBL1X does not bind to any GAG (data not shown) and that DBL3X binds weakly to HS and CSA (Fig. 2B), we propose that the main CSA-binding residues are likely to be present on DBL2X and that the surrounding domains, DBL1X, CIDR and DBL3X, contribute to the affinity and specificity of this interaction by a higher-order organisation of the multidomain protein.

N-terminal multidomain constructs bind placental CSPG with high affinity

The binding of the recombinant proteins 3D7-DBL2X, 3D7-DBL1X-2X, 3D7-DBL1X-CIDR, 3D7-DBL1X-3X, 3D7-DBL1X-5X and 3D7-DBL1X-6X to placental CSPG was examined more quantitatively by real-time SPR. Data from the kinetic association and dissociation curves did not fit a simple 1:1 binding model, thus precluding the use of kinetic constants kon and koff to estimate the binding constant KD. We therefore performed real-time SPR. Data from the kinetic experiments were performed with placental CSPG immobilized on a sensor chip and soluble proteins as analyte. The variation in the binding experiments were performed with placental CSPG immobilized on beads to deplete rabbit IgG; the depleted and protein-purified antibodies were then tested for their capacity to inhibit the interaction of the full-length protein with decorin. As expected, immobilized DBL1X-6e was able to deplete the inhibitory activity of the var2CSA-purified IgG fraction very efficiently, as inhibition dropped from 85% to less than 20% (Fig. 5). Furthermore, antibodies eluted from the beads were able to inhibit the interaction between DBL1X-6e and decorin by 72%, almost completely recapitulating the initial level of inhibition. Among the single and multidomain var2CSA proteins, only DBL1X-CIDR, DBL1X-3X, DBL1X-4e and DBL1X-5e were able to significantly deplete an important fraction of inhibitory antibodies (Fig. 5A).

Table 1. KD for var2CSA recombinant proteins binding to placental CSPG.

| Protein | KD (µM) |
|---------|---------|
| 3D7-DBL2X | 5.3     |
| FCR3-DBL3X | 344*    |
| CYK39-DBL5e | 150*    |
| 3D7-DBL6e | 92*     |
| 3D7-DBL1X-2X | 0.77    |
| 3D7-DBL1X-CIDR | 1.58    |
| 3D7-DBL1X-3X | 0.58    |
| 3D7-DBL1X-5e | 0.115   |
| 3D7-DBL1X-6e | 0.127   |

KD values were determined from the concentration dependence of steady-state SPR response using the Biacore BIAEVALUATION 3.1 software.

*: from reference [34].
+: from reference [41].
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DBL3X and DBL5e possess epitopes for inhibitory antibodies

Recently, we showed that immunization of rabbits with full-length var2CSA generated inhibitory antibodies capable of blocking var2CSA and PEs adhesion to CSA [42]. In order to determine which domains of var2CSA are preferentially targeted by these inhibitory antibodies, we used our var2CSA recombinant proteins immobilized on beads to deplete rabbit IgG; the depleted antibody fractions were then tested for their capacity to inhibit the interaction of the full-length protein with decorin. As expected, immobilized DBL1X-6e was able to deplete the inhibitory activity of the var2CSA-purified IgG fraction very efficiently, as inhibition dropped from 85% to less than 20% (Fig. 5A). Furthermore, antibodies eluted from the beads were able to inhibit the interaction between DBL1X-6e and decorin by 72%, almost completely recapitulating the initial level of inhibition. Among the single and multidomain var2CSA proteins, only DBL1X-CIDR, DBL1X-3X, DBL1X-4e and DBL1X-5e were able to significantly deplete an important fraction of inhibitory antibodies (Fig. 5A).

No significant inhibitory activity was observed in the fractions eluted from beads carrying DBL1X, DBL2X, DBL6e, DBL2X-2X or DBL1X-CIDR (Fig. 5B). However, antibody fractions eluted from the single domains DBL5X and DBL5e, as well as those eluted from the multidomains DBL3X-4e, DBL1X-3X, DBL1X-4e and DBL1X-5e, had significant inhibitory activity ranging from 35% for DBL1X-4e up to 59% for DBL1X-3X (Fig. 5B). Of note, although we were not able to express DBL4e domain alone to assess its contribution, DBL3X-4e was more effective than DBL3X and almost as effective as DBL1X-3X in retaining inhibitory antibodies, suggesting that DBL4e could also be a target of inhibitory antibodies, as previously reported [32]. Taken together, these results indicate that although the DBL1X-DBL2X region is central in forming the high affinity CSA-binding site, no inhibitory antibodies were generated against these domains, while most of the inhibitory antibodies targeted DBL3X and, to some extent, DBL5e.

Discussion

Pregnancy-associated malaria is the consequence of Plasmodium falciparum PE sequestration in the intervillous space of placenta [1]
Recent work suggests that a high-affinity, CSA-specific binding site is formed by the higher-order domain organization of the var2CSA extracellular region. Indeed, unlike individual DBL domains, the full-length var2CSA extracellular region binds with high affinity and specificity to CSA [34,35]. Structural characterization by analytical ultracentrifugation and small angle X-ray scattering has revealed a compact organization of the full-length var2CSA extracellular region (most likely governed by specific inter-domain interactions) rather than an extended structure [34]. Furthermore, animals immunized with DBL1X-6e generate antibodies that are broadly strain-transcendent but do not cross-inhibit different placental-type parasite isolates [42]. Although it is now possible to express the full-length var2CSA, difficulties due to polymorphism and to scaling up of the production make it of limited utility as a vaccine candidate. Hence there is a need to identify the minimal binding region of var2CSA that retains the high affinity and specificity of the full-length protein, but also generates a broad inhibitory antibody response.

In this study, we have expressed various truncated var2CSA recombinant proteins in order to map the minimal binding region within the full-length protein that can mimic its high affinity and specificity. As reported previously, none of the single domains tested in this study bind specifically to CSA. Among these single domains, DBL2X had a strong interaction with decorin but it also bound other GAGs in a non-specific manner. Although the affinity of DBL1X-DBL2X for human placental CSPG was about five fold lower than for the full-length protein, it remained in the nanomolar range and displayed a specificity similar to the full-length protein. Our results indicate that DBL2X forms the central core of the binding site and that the high affinity and specificity of the var2CSA recombinant proteins are only achieved when DBL1 at least, is added as flanking region to the construct. Furthermore, the addition of the CIDR-DBL3X domain tends to increase even further the affinity and specificity of DBL1X-3X. This increase could be due to a stabilizing effect of DBL3X on the recombinant protein and the binding site. In addition, this domain could also contain residues directly involved in the interaction with CSA. However, DBL1X-4e (which includes DBL1X-3X) did not bind to CSA, suggesting that the added DBL4e domain, in the absence of the remaining C-terminal domains, may not adopt the correct higher-order configuration to form the native CSA-binding site. Our data also support a role for DBL3e in stabilizing the CSA binding site, which could indicate that this domain is in close proximity to the binding site. Taken together, our results suggest that the high affinity CSA binding site lies within DBL1X-3X and that interactions between these domains and the domains outside of it are important for its stability.

The depletion/elution experiments indicate that DBL3X is one of the main targets of inhibitory antibodies, as only constructs carrying this domain were able to significantly deplete an important fraction of these antibodies. More importantly, purified antibodies eluted from these constructs, including the DBL3X single domain, possess significant adhesion-inhibitory properties ranging from 45% to 60%. Surprisingly, DBL1X-4e and DBL1X-5e were less efficient in retaining inhibitory antibodies than DBL1X-3X and even DBL1X alone, but this may reflect subtle effects of inter-domain interactions on the higher-order structure of the various multi-domain proteins, which, in the absence of structural data, we are unable to assess. Although the CSA binding site lies within the DBL1X-DBL2X region, no significant inhibitory activity was observed using eluted fractions from DBL1X, DBL2X and DBL1X-2X. This might indicate that, in the context of the full-length protein, the central binding core is not sufficiently exposed to induce an antibody response against

![Figure 5](image-url)
important residues involved in CSA adhesion and that the inhibitory antibodies may be acting by steric hindrance. This hypothesis is supported by our observations that the single domain DBL5ε also retained inhibitory antibodies and that DBL5X-4ε was more effective than DBL5X, and almost as effective as DBL1X-3X, in retaining inhibitory antibodies, suggesting that DBL4ε is also a target of inhibitory antibodies, as previously reported [32]. In line with this, human monoclonal antibodies isolated from affinity-matured memory B cells of *P. falciparum*-exposed women were shown to recognize the DBL3X and DBL5ε domains and also inhibit var2CSA binding to CSA [45]. Similarly, it was recently reported that animals immunized with DBL5ε could induce adhesion blocking antibodies [43].

In conclusion, our study clearly indicates that the minimal CSA-binding region of var2CSA lies within DBL1X-3X and that inhibitory antibodies mainly target DBL3X and, to a lesser extent, DBL5ε. Studies of the immunogenic properties of DBL1X-3X and DBL3X-DBL5ε are in progress to provide further information on the role of these domains in CSA interaction and in generating inhibitory antibodies. Structural studies on the DBL1X-3X domain organization would provide additional insight into critical residues involved in CSA adhesion, permitting rational vaccine development strategies. This study is a step further in the development of effective drugs and vaccine strategies aiming to fight PAM.

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Author Contributions
Conceived and designed the experiments: AS SG SD AB BG. Performed the experiments: AS SG SD FA. Analyzed the data: AS SG SD AB GB BG. Wrote the paper: AS SG SD AB BG.

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