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Research

Identification of glycosaminoglycan binding regions in the *Plasmodium falciparum* encoded placental sequestration ligand, VAR2CSA

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Abstract

Background: Pregnancy malaria is caused by *P   a a * -infected erythrocytes binding the placental receptor chondroitin sulfate A (CSA). This results in accumulation of parasites in the placenta with severe clinical consequences for the mother and her unborn child. Women become resistant to placental malaria as antibodies are acquired which specifically target the surface of infected erythrocytes binding in the placenta. VAR2CSA is most likely the parasite-encoded protein which mediates binding to the placental receptor CSA. Several domains have been shown to bind CSA   *t* ; and it is apparent that a VAR2CSA-based vaccine cannot accommodate all the CSA binding domains and serovariants. It is thus of high priority to define minimal ligand binding regions throughout the VAR2CSA molecule.

Methods: To define minimal CSA-binding regions/peptides of VAR2CSA, a phage display library based on the entire *a 2 a* coding region was constructed. This library was screened on immobilized CSA and cells expressing CSA resulting in a limited number of CSA-binding phages. Antibodies against these peptides were affinity purified and tested for reactivity against CSA-binding infected erythrocytes.

Results: The most frequently identified phages expressed peptides residing in the parts of VAR2CSA previously defined as CSA binding. In addition, most of the binding regions mapped to surface-exposed parts of VAR2CSA. The binding of a DBL2X peptide to CSA was confirmed with a synthetic peptide. Antibodies against a CSA-binding DBL2X peptide reacted with the surface of infected erythrocytes indicating that this epitope is accessible for antibodies on native VAR2CSA on infected erythrocytes.

Conclusion: Short continuous regions of VAR2CSA with affinity for multiple types of CSA were defined. A number of these regions localize to CSA-binding domains and to surface-exposed regions within these domains and a synthetic peptide corresponding to a peptide sequence in DBL2 was shown to bind to CSA and not to CSC. It is likely that some of these epitopes are involved in native parasite CSA adhesion. However, antibodies directed against single epitopes did not inhibit parasite adhesion. This study supports phage display as a technique to identify CSA-binding regions of large proteins such as VAR2CSA.

Background

PAM (Pregnanc Associated Malaria) is a major health problem in malaria-endemic areas and on a world basis it affects millions of pregnant women and their offspring. The presence of parasites in the placenta of pregnant women can have serious consequences for both mother and child including: maternal anaemia, premature deliver , low birth weight and increased infant mortalit [1]. In malaria endemic areas, children acquire clinical immunit after multiple infections, and adults are in general protected against malaria. Women who have acquired immunit against malaria during childhood become susceptible to malaria during pregnanc due to novel parasite phenot pes expressing unique antigens not encountered during childhood infections [2,3]. In areas of high parasite transmission PAM mainl affects primigravidae since immunit is acquired as a function of gravidit [1]. Protective antibodies target proteins expressed on the surface of infected er throc tes (IE), which mediate binding to s nc tiotrophoblasts. B this process, the parasite is not filtered through the spleen and thus avoids exposure to effector mechanisms, which clear er throc tes infected with late blood stage parasite from circulation [4]. The best characterized surface protein is the *Plasmodium falciparum* er throc te membrane protein 1 (PfEMP1) [5,6], which is encoded b the pol morphic *var* gene famil containing 50–60 copies per parasite genome [7]. The PfEMP1 famil constitutes high-molecular proteins of 200–400 kDa, which are highl pol morphic. Different PfEMP1 molecules have different receptor specificities, therefore switching between expression of various *var* genes in a mutuall exclusive manner allows the parasite to modif its adhesion properties (reviewed in [8]). PfEMP1 proteins include three to seven Duff -binding-like (DBL) domains, which belong to a parasite adhesion-domain super-famil present in er throc te invasion ligands called: er throc te-binding ligands (EBL). Antibodies against PfEMP1 can interfere with parasite binding and the successive acquisition of a broad range of PfEMP1 antibodies is important for the acquisition of immunit during childhood [9-13]. Several molecules such as ICAM-1 [14], VCAM-1 [15], thrombospondin [16], CD36 [17], and chondroitin sulfate A (CSA) [18,19] have been identified as host receptors for PfEMP1. In the placenta IE exclusivel bind to the gl cosaminogl can CSA [19,20]. The parasite protein mediating IE adhesion to CSA in the placenta is a distinct member of the PfEMP1 protein famil , named VAR2CSA [21]. High levels of anti-VAR2CSA antibodies are correlated with favourable birth outcome and the are acquired as a function of parit [22]. Disruption of the *var2csa* gene causes the loss of the IE's abilit to bind CSA [23]. VAR2CSA is a large IE surface-expressed antigen consisting of six DBL domains with a total estimated molecular mass of 350 kDa. The ultimate aim of PAM vaccine development is to define a VAR2CSA construct capable of eliciting antibodies that inhibit binding of IEs to CSA. However, several of the VAR2CSA domains have *in vitro* affinit to CSA [24-26] and this complicates vaccine design. It is thus of high priorit to define the minimal epitopes within each domain and inter-domains that have affinit to CSA.

Phage displa is a strong and widel used tool for mapping protein ligand interactions and has in several studies been used to define adhesive parts of proteins present on the surface of different organisms causing infectious diseases (reviewed in [27]). Phage displa has also been extensivel used in malaria research. For vaccine development Case and others [28], used phage displa to isolate a phage-derived peptide that mimic an important epitope of AMA-1 and had the abilit to induce functionall protective antibodies. Lanzillotti and others [29], used a phage displa librar to search for *P. falciparum* encoded motifs involved in er throc te invasion, and identified regions in EBA-175 and Ebl-1 like proteins binding to receptors on the human er throc te. EBA and Ebl belong to the same super famil of Duff -binding-like proteins as the DBL domains from VAR2CSA. We were thus encouraged to use this technique to search for CSA-binding motifs in VAR2CSA. In this stud a phage displa librar was constructed based on the exon1 coding region of VAR2CSA. The librar was biopanned on different sources of gl cosaminogl cans (GAG) including: immobilized bovine CSA; immobilized proteogl cans purified from placentas – CSPG; CSA-expressing CHO cells, and BeWo cells derived from the human placental s nc tiotrophoblasts. Five regions of VAR2CSA potentiall involved in *in vivo* parasite sequestration were identified and are thus potential candidate components of a multivalent PAM vaccine.

Methods

Cells

The human choriocarcinoma cell line BeWo was purchased from the American T pe Culture Collection [30]. The cells were maintained in Ham's F12 with L-glutamine culture medium (Lonza, Verviers, Belgium) supplemented with 10% FCS and 1% penicilin/streptavidin (Sigma-Aldrich, A shire, UK). Chinese Hamster ovar cells (CHO) were maintained in RPMI1640 (with L-glutamine and 25 mM HEPES) (Lonza, Verviers, Belgium) suplemented with 10% FCS and 1% penicilin/streptavidin.

Construction of the **var2csa** *phage display library*

The exon1 of *var2csa* was digested with DNase1 (Sigma-Aldrich, Brøndb , Denmark) and fragments of 100 bp–150 bp were ligated into the T7select 415-1b vector (Novagen Inc., Madison, USA) at *EcoRI* and *HindIII* sites.

Phage packaging amplification and titration were performed according to the manufacturer's instructions [31].

Selection of the GAG binding phages from T7var2csa library

Different approaches to select for phages binding to CSA were used. Common to these approaches was the general procedure for selection of clones, which consisted in four rounds of biopanning, followed by infection of *E. coli* (BL21) with the selected phages. The bacteria were plated and individual plaques were amplified and sequenced.

(1) Phage selection on human choriocarcinoma cell line BeWo or CHO cell line:

BeWo/CHO cells were cultured in Nuclon™ delta surface (Nunc, Roskilde, Denmark) 6 well-plates until confluent. The plates were incubated for 15 minutes at 4°C before adding 1 × 10⁹ plaque forming units (pfu) T7*var2csa* phages to the cells for 1 hour at 37°C. The cells were then washed carefully four times in pre-warmed 1% Bovine Serum Albumin (BSA) in PBS for five minutes per wash. The bound phages were eluted in 1 ml of 0.2 M glycin pH 6 for 10 minutes. The eluted phages were amplified to ensure a titer of 10^9 pfu for input phages at the start of each successive round.

(2) Phage selection on biotinylated CSA

CSA (C9819 Sigma-Aldrich, Brøndby, Denmark) was biotinylated using EZ-Link Sulfo-NHS-LC-Biotinylation kit (Pierce, Bonn, Germany). 100 l of 50 g/ml biotinylated CSA in PBS was added to pre-coated avidin plates (Nunc, Roskilde, Denmark) for 1 hour at room temperature and the wells were subsequently blocked with 200 l of 1% BSA in PBS for 1 hour. After washing the plates three times with PBS, 1 × 10⁹ pfu of T7*var2csa* phages were added to the plate for 1 hour at 37°C. The wells were then washed twice in round 1, three times in round 2, and five times in subsequent rounds with TBS 0.5 % Tween-20 for 3 minutes. The phages were eluted in 100 l of 0.1 M glycin pH 2.2 for 10 minutes, neutralized immediately with

6 l of 2 M Tris base pH 8 and added to fresh log-phase BL21 cells.

(3) Phage selection on human placenta derived chondroitin sulfate proteoglycans (CSPG) [32] or bovine CSA (Sigma). Proteoglycan (5 g/ml in PBS) was coated to microtiter plates (Nunc, Roskilde, Denmark) overnight at 4°C. The following steps were done as described for biotinylated CSA.

PCR amplification of plaques

A portion of top agarose of an individual plaque of interest was scrapped by a pipette tip, dispersed in a tube containing 100 l of 10 mM EDTA, and heated at 65°C for 10 min. The plaques collected served as templates in a PCR reaction with primers for the T7 vector supplied by the manufacturer (Novagen, Madison, USA). 15 l of the reaction product was analysed on a 1% agarose gel. For sequencing, the PCR product was purified by Minielute purification kit (Qiagen) and 4 l of pure PCR product was used in the sequencing reaction.

Peptide synthesis

Peptides were synthesized by Schafer-N, Århus, Denmark (P2c, P3, P4 and P5) or Sigma (P1, P2a, P2b) (Table 1). Peptides P1, P2a, b, c and P5 were synthesized with the addition of a terminal cystein for increased stability. P2 region is very long and difficult to synthetize and was therefore divided into two peptides P2a and P2c. P2b correspondes to the P2a region and has the FCR3 isolate sequence. PC is a non-VAR2CSA peptide and it was used as a negative control (Table 1). Peptide identity and purity were analysed by MALDI-TOF mass spectrometry and high-performance liquid-chromatography (HPLC). The synthesized peptide sequences are shown in one-letter code (Table 1).

Biotinylation of peptides

All peptides were biotinylated using EZ-link Sulfo-NHS-LC-Biotin (Pierce, Bonn, Germany) following the instructions for preferentially biotinylation of N-terminal -amino groups in peptides [33].

Table 1: Amino acid sequence of the synthetic peptides identified by phage display

Peptide ID	Protein ID	Sequence
P1	3D7 – DBL1X	ENQKNKYTELYQQNKC
P2a	3D7 – DBL2X	GKKTQELKNIRTNSELLKEWIIAAFHEGKC
P2b	FCR3 – DBL2X	EDVKDINFDTKEKFLAGCLIVSFHEGKC
P2c	3D7 – DBL2X	LKPSHEKKNDDNGKKLCKAC
P3	3D7 – DBL3X	IKKIIEKGTTKQNGKTVGSGAEN
P4	3D7 – DBL4	IKNKNDITNAKKELLETLQIVAERE
P5	3D7 – DBL5	ILKGAQSEGKFLGNYYNEDKDKEKALEAMC
PC	*P.a* a Potassium pump	WRRDTYNMIWGFNKIPTYIYNMLLILLSTSYID

GAG binding ELISA

Flat-bottomed, 96 well plates (Falcon 351172) were coated for 2 hours at 37°C either with CSA (C9819 Sigma-Aldrich, Brøndb , Denmark) 50 g/ml in PBS; CSC (shark cartilage, Seikagaku, Tok o, Japan) 50 g/ml in PBS; or onl PBS (as a negative control). The plates were then blocked for 1 hour at 37°C with 1% BSA, 0.05% Tween 20, in PSM buffer (PBS, 2 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7). After two times washing with 0.05% Tween 20 in PSM buffer, 50 l of a dilution series (0.4 – 25 g/ml) of peptide in blocking buffer was added per well and incubated overnight at 4°C. After three times washing, 50 l of a 1/2000 dilution of a streptavidin-conjugated horseradish peroxidase antibod (Dako, Glostrup, Denmark) in blocking buffer was added to each well and incubated at 37°C for one hour. The binding assa was finalized with three times washing and developing with 100 l/well of o-phen lenediamine substrat for 10 minutes. Plates were read at 490 nm. The curves (Figure 3A) are t pical of four independent experiments, and each absorbance value is the mean of two experiments. Figure 3B absorbance values are the result of one experiment.

Affinity purification of peptide-specific rabbit antibodies

Affinit purification of antibodies was done according to manufacturer's instructions. In brief, 0,5 mg of peptide was dial zed against 0.2 M $NaHCO_3$, 0.5 M NaCl (pH 8.3), and applied to a NHS-activated HiTrap 1-ml column (GE Healthcare) that had been equilibrated with 3 × 2 ml 1 mM HCl. After coupling, the column was washed with 0.5 M ethanolamine, 0.5 M NaCl (pH 8), 0.1 M acetate, 0.5 M NaCl (pH 4), and a final wash with PBS (pH 7.4). 3 ml of rabbit antisera against the specific domain [34] was subsequentl added to the column. After washing in 10 ml PBS, affinit -bound antibodies were eluted b CH_3COONH_4 (pH 3) and neutralized in 1 M Tris (pH 7.5). The specificit of the purified antibodies was tested in ELISA against (1) the peptide used for affinit purification, and (2) other VAR2CSA peptides. The affinit -purified antibodies were onl positive against the VAR2CSA peptides on which the had been affinit purified.
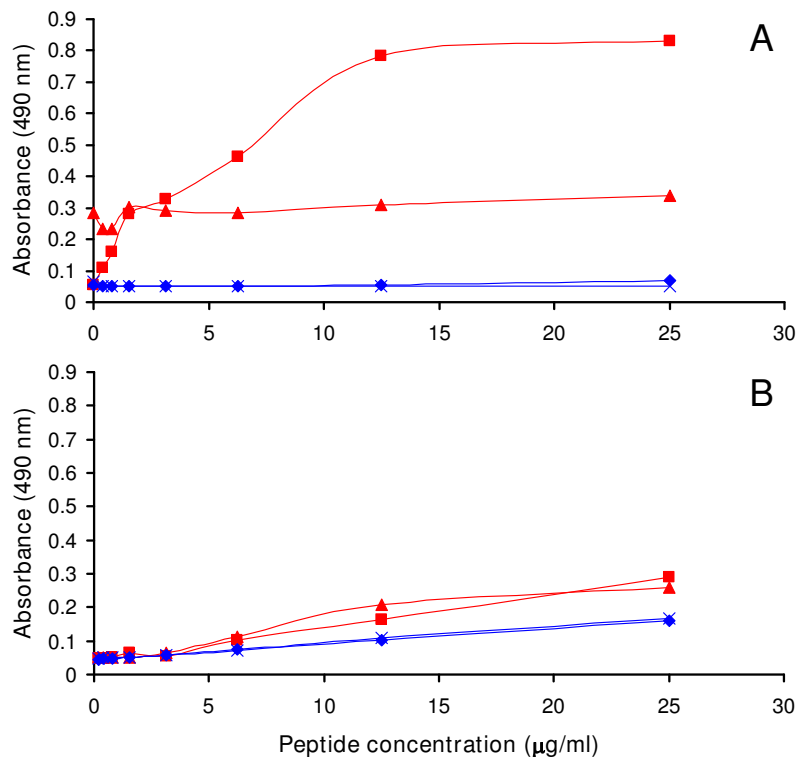

Figure 3
A DBL2 peptide binds to CSA and not to CSC. (A) Peptide binding assay to CSA: P2b (red) binds to CSA (n) in a peptide concentration-specific manner and not to the plate (s). The control peptide (blue) does not bind to neither the plate (×) nor CSA (◆). (B) Peptide binding assay to CSC: P2b (red) does not bind to either CSC (n) or to the plate (s). The control peptide (blue) does not bind to neither the plate (×) nor CSC (◆).

Parasite culture and selection

The NF54 laborator strain of *P. falciparum* was used. Parasite culture was done as previousl described [34]. The genot pe of the parasite was regularl checked b GLURP and MSP2-specific primers in a single PCR step.

To create a VAR2CSA-expressing parasite population, the NF54 parasites were repeatedl panned on VAR2CSA-specific antibodies creating the NF54var2csa parasite line. Briefl , late stage trophozoites were obtained b gelatin purification and washed twice in RPMI1640. The IE were then incubated for 0.5 hour at 37°C with VAR2CSA-DBL5 -specific rabbit antiserum and washed three times with medium to remove unbound antibodies. IE expressing VAR2CSA were isolated b the use of protein G-coupled magnetic beads (D nal, Invitrogen) on a MACS magnet (Milten i Biotec). The suspension of IE and beads was washed three times and added to a culture flask with 4% hematocrit of uninfected er throc tes in 5 ml culture medium. After 24 hours, the beads were removed b magnetic separation. This was repeated until parasites specifi-call expressed VAR2CSA on the surface. Expression of the VSA$_{PAM}$ phenot pe was confirmed b specific binding of parasites to CSA in static binding assa s, and exclusive recognition of the surface of the IE b antibodies from women exposed to *P. falciparum* during pregnanc .

Flow cytometry and IFA

Flow c tometr was used to test if the peptide-specific antibodies recognize the surface of CSA-binding IEs. Parasite cultures (NF54^{VAR2CSA}) were enriched to contain >75% er throc tes infected b late trophozoite and schizont stage parasites b exposure to a strong magnetic field [35]. Aliquots (2×10^5 IE) were labeled b ethidium bromide (to allow exclusion of remaining uninfected er throc tes). For IE surface staining with rabbit anti-peptide IgG, samples were sequentiall exposed to 20 l sera followed b 1 l biotin lated sheep-anti-rabbit IgG (The Binding Site, Birmingham, UK) and to 0.5 l streptavidin-FITC (BD Pharmingen, San Diego, US). All incubations were performed in a total volume of 100 l PBS with 2% FCS for 30 minutes. Samples were washed two times with 3 ml PBS, 2% FCS between each incubation. Data from a minimum of 5000 IE were acquired using a FC500 flow c tometer (Beckman Coulter, Ramcon, Denmark). For each sample, the mean fluorescence index (MFI) was recorded as a measure of VSA-specific IgG reactivit . Each assa was repeated three times on separate da s with similar results. A batch of uninfected er throc tes was ana-l sed to exclude IgG binding to er throc te antigens.

The wet immuno-fluorescent preparations were performed following the staining procedure described for the flow c tometr assa s with a few modifications: DNA was labelled with 10 ng/ml DAPI and in the final staining step

0.5 l Streptavidin-Alexa®488 was used instead of FITC (Invitrogen, Paisle , UK). Images were taken using a TE 2000-E Nikon Eclipse confocal microscope, using a 100× Apoplan oil immersion objective. Images were captured using the EZ-C1 Gold imaging s stem (version 3.30). Images of each parasite preparation stained with VAR2CSA-specific antibodies, peptide-specific antibodies or control antibodies were captured without adjusting pixel dwell time, laser-power nor photomultiplier gain.

Results

Multiple linear VAR2CSA regions have affinity for proteoglycans

The exon1 of *var2csa* has 9171 bp and was PCR amplified and cloned into the T7select 415-1b phage vector. The plasmid was propagated and the insert containing the whole *var2csa* exon1 was cut out and digested with DNase1 to generate 200 bp fragments. The *var2csa* fragments were used to create a T7 Phage displa librar as described in the material and methods section. This vector is described to displa 415 copies of peptides on the surface of the T7 capsid [31].

To ensure that all parts of VAR2CSA exon1 were present in this constructed librar a number of clones were sequenced before biopanning. No sequences were over-represented and sequences belonging to all six DBL domains were present in the librar (Figure 1A, black). The *var2csa* phage displa librar was biopanned four rounds on: CHO cells (two independent assa s); human placental choriocarcinoma cell line BeWo (four independent assa s); bovine CSA (two independent assa s); biotin lated CSA (two independent assa s) and chondroitin sulfate proteogl cans of human placenta (CSPG) (two independent assa s). Control biopannings were done on ELISA plates coated with BSA (Figure 1A, Blocking Bf, blue). From each assa 15 clones were sequenced. Figure 1 shows the frequenc b which different phages expressing particular VAR2CSA regions were identified after the different t pes of biopanning. Biopanning on CSA resulted in enrichment of a single region (20% of all phages) corresponding to a peptide sequence in DBL3 (Figure 1A, green). CSA coated directl on ELISA plates might not be ver efficient; therefore the biopanning was subsequentl repeated using biotin lated CSA (bCSA). This resulted in enrichment of phages representing sequences present in DBL1, DBL2 and DBL4 (Figure 1A, red). In addition, biopanning on human placental CSPG resulted in enrichment of phages representing DBL4 and DBL5 sequences (Figure 1A, ellow). However, the DBL4 sequence was also present in 18% of the control BSA bio-pannings (Figure 1A, blue). To further identif proteogl -can-binding regions, the VAR2CSA phage displa librar was biopanned on BeWo and CHO cells expressing CSPG and CSA, respectivel (Figure 1B). Enrichment of phages

expressing peptides from DBL2, DBL3, DBL4 and DBL5

DBL2, DBL3 and DBL5 there was a high degree of overlap between the CSA-binding regions and the surface-exposed regions (Figure 2, green). Peptides residing in DBL1 and DBL4 did not map to the predicted surface exposed regions. All mapped regions, except the DBL1 region, mapped to the S2 subdomain of the DBL domains. The CSA-binding regions mapped in DBL2 and DBL5 are in close vicinit to the chemokine-binding site region of Pk -DBL Duff [37].

Synthetic peptides representing P1-P5

Seven peptides, corresponding to VAR2CSA regions P1-P5 (Figure 1 and Table 1), were s nthetized and biotin lated to evaluate their abilit to bind CSA in an ELISA assa . For the ELISA based CSA-binding assa : plates were coated with CSA and binding was measured using a streptavidin-conjugated peroxidase-antibod . In order to determine the background binding and detect the true signal, half of the plate was not coated with CSA but all the subsequentl steps were performed equall in the whole plate. Using
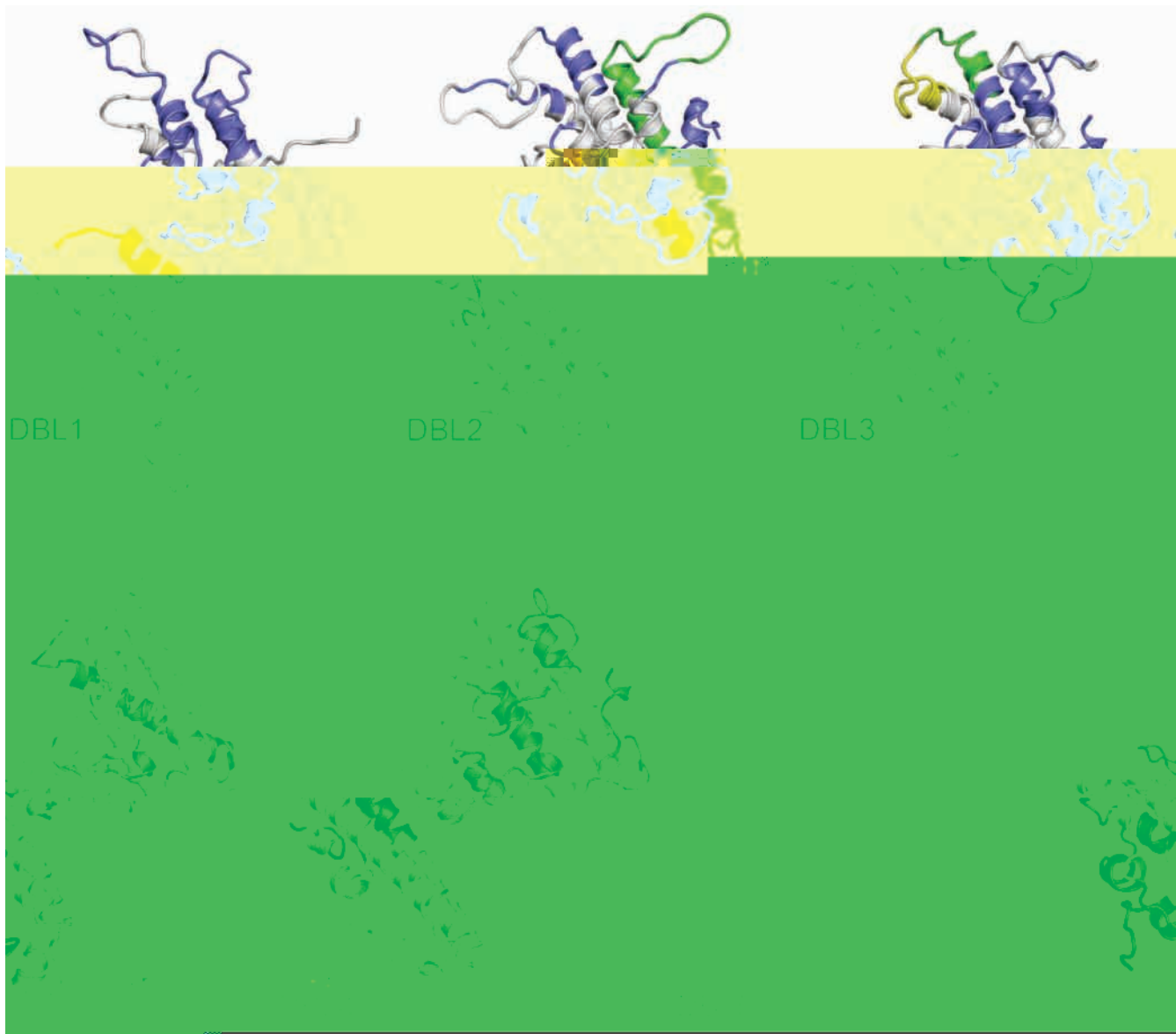

Figure 2
Structural models of VAR2CSA DBL domains showing surface-exposed regions and GAG binding regions. The surface-exposed epitopes previously determined by depleting female IgG plasma on parasites expressing VAR2CSA are shown in blue. The GAG binding regions identified by phage display assays are shown in yellow. The overlap of surface-exposed regions and GAG binding regions is shown in green.

this assa P2b (DBL2) showed specific binding to CSA compared to the background and additionall did not bind to CSC (Figure 3, red curves). A non-VAR2CSA control peptide (Table 1, PC) was used (Figure 3, blue curves) and didn't show binding to an of the GAGs.

Antibodies specific to the DBL2X peptide react with native VAR2CSA on the surface of infected erythrocytes

Sera from rabbits immunized with recombinant VAR2CSA DBL domains [34] were tested for reactivit against the six peptides. Rabbits immunized with recombinant whole domain DBL1 and DBL4 did not react with the peptides from these domains (P1 and P4). Rabbits immunized with recombinant whole domain DBL2, DBL3 and DBL5 reacted with P2, P3, and P5, respectivel . Peptide-specific antibod reagents were then produced b affinit purif ing rabbit antibodies on the peptides. The affinit -purified antibodies were tested in flow c tometr for reactivit with native VAR2CSA expressed on er thro-c tes infected with the NF54 parasite strain (Figure 4A). Anti-P2c peptide specific antibodies stained the surface of the infected CSA-binding er throc tes. In addition, the reactivit was observed b wet IFA (Figure 4B). The surface staining determined b IFA showed a dotted pattern t pi-cal of PfEMP1 staining. We were unable to detect binding with P2a, P2b, P3 and P5 specific antibodies.

Discussion

Malaria parasites causing PAM have been shown to bind to gl cosaminogl cans in the intevillous space of the placenta. These parasites also bind specificall to bovine CSA [18], CSPG [38] as well as BeWo [39] and CHO cells having CSPG on the surface [18]. The binding between the parasite and the placental CSPG is most likel mediated through the parasite expressed protein, VAR2CSA. VAR2CSA is a large multidomain protein and for vaccine development it is important to define which regions of VAR2CSA are responsible for the interaction with CSPG. In the present stud a *var2csa* phage displa librar was biopanned on five different CSA containing targets (bovine CSA, bovine bCSA, human placental CSPG, BeWo and CHO cells) in 12 independent experiments. Five regions of VAR2CSA repeatedl showed affinit for the different CSA preparations. The CSA-binding peptides identified with the phage displa approach were based on the linear sequence of VAR2CSA. However, the CSA binding region might be conformational and involve peptides from several domains. It is preferable that results obtained b phage displa assa s are confirmed b showing that peptides corresponding to the identified regions also possess binding capacit . The *var2csa* phage displa librar used in this stud was constructed from DNA fragments of 100–150 bp, and the corresponding peptides are thus 34–75 aa. These long peptides were difficult to s nthetize and were unstable in solution and we thus had to divide some

of the phage regions into several s nthetic peptides. Furthermore, the structure of peptides in solution might be ver different from peptides bound to a phage. These factors could explain wh onl one out of seven s nthetic peptides could have its binding to CSA confirmed.

DBL2, DBL3 and DBL5 domains of VAR2CSA have previ-ousl been shown to bind to CSA [24-26] and the surface-exposed regions within these domains have been mapped [26,36]. Three of the five peptides are located on surface-exposed parts of the previousl described CSA-binding domains and two of these peptides map to regions on the DBL domains, which are in close proximit to the ligand-binding region of Pk-alfa-DBL [37]. These findings show an agreement between two independent approaches, which strengthens the present results. No CSA-binding epitopes were found in the highl pol morphic DBL6 domain, which previousl has been shown to bind CSA indicating the presence of conformational CSA binding regions in this domain [25].

Region P2 was ver long and had to be divided into two peptides for s nthesis. The first part of the region showed specific CSA binding (P2b, Figure 3) and the second part of the region (P2c) using peptide affinit purified antibodies in IFA, confirmed that the CS- binding peptide region present in DBL2 was in fact surface-exposed in native VAR2CSA expressed on IE (Figure 4).

The affinit -purified antibodies did not inhibit parasite binding to CSA (data not shown), but this is not surprising as multiple epitopes/domains of VAR2CSA are suggested to be independentl involved in CSA binding [24-26]. However, it is possible that all domains of VAR2CSA contribute to a common CSA binding groove, either in a monomeric form or a di-pol meric form, such a gl can binding groove has been shown to be present in a reverse-hand shake dimer of EBA-175 [40]. Individual peptides and domains ma have low affinit for binding to CSA, whereas high affinit is onl achieved b the assembl of the entire binding site. Chr stallograph and structure elucidation of an entire VAR2CSA molecule is not techni-cal feasible with current technolog , therefore a complex multi-domain CSA-binding site is difficult to identif . Current methods of identif ing the CSA-binding regions of VAR2CSA primaril emplo the abilit of peptide-specific antibod reagents to inhibit parasite adhesion. If multiple sites are involved in adhesion, screening with single specificit antibodies might not suffice to identif a vaccine construct. B combining the knowledge from: recombinant DBL binding assa s; parasite anti-adhesion assa s using domain-specific antibodies; epitope mapping approaches and phage displa approaches, we might be able to construct a chimeric vaccine, encompassing different CSA binding regions and important antibod
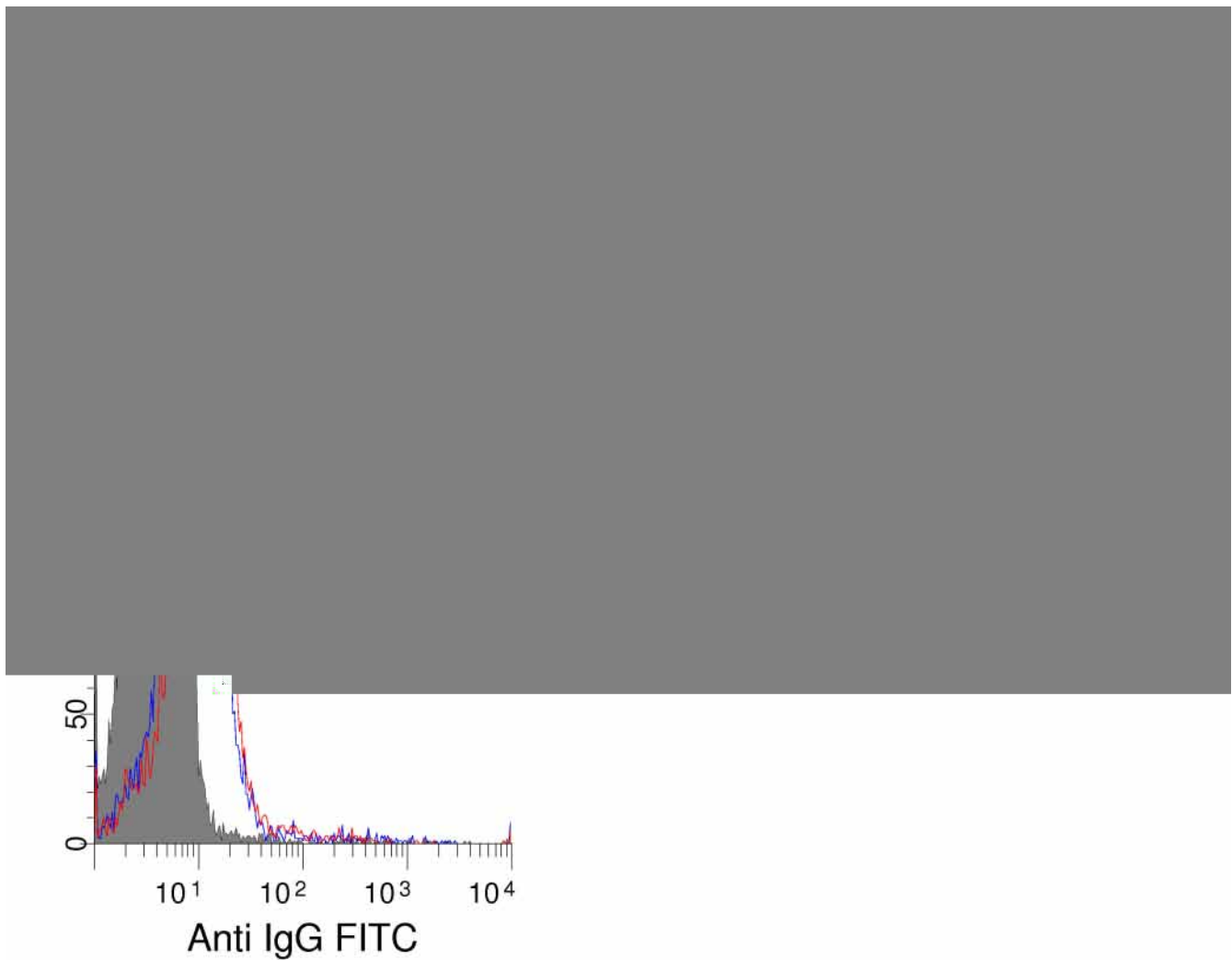
Figure 4
Anti-P2 antibodies react with native VAR2CSA expressed on the surface of infected erythrocytes. The histogram (A) shows staining of red blood cells infected with late stages of NF54^var2csa. The IE reacted with rabbit affinity purified antibodies against P2c peptide (red) and DBL5 (blue). The rabbit prebleed is shown in solid grey. The picture (B) shows an IFA image of IE double stained with anti-P2c antibodies (green) and DNA (DAPI) staining (blue).

epitopes. The phage displa approach described here is the first stud that attempts to find smaller CSA binding regions within the DBL domains of the vaccine candidate VAR2CSA.

Conclusion
Phage displa was used to identif GAG binding linear regions of VAR2CSA. Five regions located in five different domains were found to have affinit for both immobilized CSA and CSA expressed on the surface of cells. The most frequentl observed GAG binding phages mapped to DBL2, 3, 4 and DBL5, and except DBL4 all these domains have been shown to bind CSA *in vitro*. These results are supported b data published b Andersen and others [36], demonstrating that the phage displa defined CSA-binding regions in DBL2, 3, 5 all locate to areas of VAR2CSA that appear to be exposed on the native molecule. The DBL2 CSA binding peptide showed specific binding to CSA and affinit -purified antibodies against the same phage displa identified region reacted with the surface of infected er throc tes. This work is the first step in defining small regions of VAR2CSA, which can be used in an adhesion blocking sub-unit vaccine protecting pregnant women against PAM.

Abbreviations
aa: amino acid; bp: base pair; BSA: bovine serum albumin; CSA: chondroitin sulfate A; DARC: Duff Antigen Recep-

tor for Chemokines; DBL: Duff -binding-like; EBA: Er th-roc te Binding Antigen; Ebl: Er throc te binding ligand; ELISA: Enz me-Linked Immunosorbent Assa ; FCS: Fetal calf serum; ID1: Inter-Domain region 1 of PfMP1; IFA: Immunofluorescence assa ; IgG: immunoglobulin gamma; LBW: low birth weight; NTS: N-terminal segment of PfMP1; PAM: pregnanc -associated malaria; PfEMP1: *Plasmodium falciparum* Er throc te Membrane Protein 1.

Authors' contributions

MR, MAN, MD and SD performed the experiments, PHA plotted GAG binding regions onto the VAR2CSA models, NTN purified the human CSPG used for this stud , MR, MAN, NTN, AFS, MD, TGT, and AS were responsible for the stud design and the interpretation of data. All authors contributed to writing of the manuscript and approved the final version.

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