Identification of Multiple Chondroitin Sulfate A (CSA)–Binding Domains in the *var2CSA* Gene Transcribed in CSA-Binding Parasites

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Malaria in pregnancy is a serious complication associated with parasitized erythrocyte (PE) sequestration in the placenta. Recent work suggests that *var* genes could play an important role in PE binding to chondroitin sulfate A (CSA), a primary placental adherence receptor. Here, we confirm that *var2CSA* is transcriptionally up-regulated in CSA-binding parasites and define CSA-binding domains in *var2CSA*. The identification of multiple binding domains in *var2CSA* strengthens the evidence for their involvement in malaria during pregnancy and may have applications for the development of a vaccine against malaria in pregnancy.

Plasmodium falciparum infection during pregnancy is associated with parasitized erythrocyte (PE) sequestration in the placenta and contributes to low birth weight and neonatal mortality [1]. Placental isolates are functionally distinct, because they do not bind to CD36 but instead bind to chondroitin sulfate A (CSA) [2] and hyaluronic acid [3]. During pregnancy, antibodies to the PE surface develop that are broadly strain transcendent and have been suggested to play a role in protective immunity [4]. These observations suggest it may be possible to vaccinate against malaria during pregnancy, if the parasite adhesion ligands can be defined.

Received 3 September 2004; accepted 19 October 2004; electronically published 11 February 2005.

Financial support: Bill & Melinda Gates Foundation (award to J.D.S. and A.S.); National Institutes of Health (grant RO1 AI47953-01A1 to J.D.S.); MJ Murdock Charitable Trust (Seattle Biomedical Research Institute) (support to J.D.S.).

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The Journal of Infectious Diseases 2005; 191:000–000

0022-1899/2005/19106-00XX$15.00

P. falciparum–infected erythrocytes employ a variable family of erythrocyte surface adhesion ligands called "*var*" genes, which encode *P. falciparum* erythrocyte membrane protein–1 (PfEMP-1) [5], to sequester in different microvasculature sites. PfEMP-1 proteins have multiple adhesion domains, called "Duffy binding–like" (DBL) domains and cysteine-rich interdomain regions (CIDRs), which determine PE binding specificity [5]. Of importance to placental sequestration, CD36 and CSA are mutually exclusive PE adhesion phenotypes [2, 6] that are functionally incompatible in the same PfEMP-1 protein [7]. Mechanistically, this may have arisen because CD36 binding and nonbinding *var* genes localize to distinct chromosomal regions and are transcribed in opposite orientations [8–10]. This genetic organization may cause them to recombine separately and evolve different structures and functions under distinct selective pressures. Since placental isolates do not bind to CD36 [2, 3], the maternal placenta selects for non–CD36-binding PfEMP-1 proteins that ensure localization in placenta and not on vascular endothelium.

Within the group of PfEMP-1 proteins predicted not to bind to CD36, a small subset, including *var1CSA,* have DBL-γ domains that bind to CSA [7, 11, 12]. Another gene within this group, *var2CSA,* does not contain DBL-γ domains but was recently found to be up-regulated in PEs that bind to CSA [13]. *var2CSA* is considered to be an additional candidate for CSA binding and placental adhesion, because of its expression profile and lack of a typical CD36-binding domain. Of interest, *var2CSA* contains sequence homology to the minimal CSA-binding region from *var1CSA* [12] and is unusually conserved between parasite strains [10, 13], which are features that might be expected of a placental adhesion ligand. The aim of the present study was to determine whether *var2CSA* contains CSA-binding domains.

Materials and methods. For *var2CSA* Northern blot analysis, total RNA was prepared from ring- and trophozoite-stage cultures ~10 h and ~24 h after invasion, respectively. RNA preparation, electrophoresis, membrane transfer (Hybond N+; Amersham), and hybridization with radiolabeled A4 *var2CSA* DBL3 probe (3660–4623 bp) or a probe based on the *var* semiconserved exon 2 (*var* T11.1 gene; GenBank accession no. U67959; 7930–9147 bp) generated using the Megaprime Labeling Kit (Amersham) were performed as described elsewhere [14]. Membranes were hybridized at high-stringency conditions overnight at 60°C and were washed twice with 0.2× saline sodium citrate and 0.1% SDS for 30 min at 60°C.

For the determination of surface expression of various do-

The figure is available in its entirety in the online edition of the *Journal of Infectious Diseases*.

Figure 1. Northern blot analysis of *var1CSA* genetically disrupted parasites before and after reselection on chondroitin sulfate A. The figure and legend are available in their entirety in the online edition of the *Journal of Infectious Diseases*.

mains in CHO-745 cells, constructs were amplified from genomic DNA by polymerase chain reaction and were cloned into the pSRα5-12CA5 vector (Affymax Research Institute), as described elsewhere [12]. The following domains from 3D7 PFL0030c *var2CSA* (GenBank accession no. NP_701371) and from A4 *var2CSA* (GenBank accession no. AAQ73926) were used (amino acid boundaries of each clone are given in parentheses): 3D7 DBL1 (aa 57–382), 3D7 DBL2 (aa 542–853), 3D7 DBL3 (aa 1213–1523), 3D7 DBL3b (aa 1213–1571), 3D7 DBL4 (aa 1576–1883), 3D7 DBL5 (aa 2001–2272), 3D7 DBL6 (aa 2318–2589), A4 DBL1 (aa 52–383), A4 DBL2 (aa 543–858), A4 DBL3 (aa 1220–1541), and A4 DBL3b (aa 1220–1580). Chinese hamster ovary PgsA-745 (CHO-745) cells deficient in glycosaminoglycans (American Type Culture Collection) were transfected and selected by single-cell cloning by use of a fluorescence-activated cell sorter (Becton Dickinson), as described elsewhere [12].

Binding assays with bovine trachea CSA (Sigma) or shark cartilage chondroitin sulfate C (CSC) (Sigma or Seikagaku) linked to biotin ("biot-CSA" and "biot-CSC," respectively) were performed as described elsewhere [11, 12]. In brief, 50 μg of biot-CSA or biot-CSC was incubated for 1 h with stably transfected CHO-745 clones grown on coverslips. Binding was visualized with Dynabeads (Dynal) coated with anti-biotin monoclonal antibody (Jackson Immunoresearch Labs). For inhibition assays, the cells were incubated for 1 h with 200 μg/mL CSA or CSC (Sigma) before addition of biot-CSA.

Results and discussion. To determine the role of *var2CSA* in CSA adhesion, Northern blot analysis was performed on a parasite line in which *var1CSA* had been genetically disrupted (C1 mutant) [15]. C1 parasites bind to CD36 [15] and predominantly express an 8–9-kb *var* product (figure 1). Upon CSA selection, the C1-CSA parasite line switches to the larger, *var2CSA* transcript (figure 1), confirming previous results showing that *var2CSA* is transcriptionally up-regulated in different parasite strains selected to bind to CSA [13].

3D7 *var2CSA* contains 3 DBL-X–type and 3 DBL-ε–type domains [13]. For determination of the CSA-binding domain(s) from *var2CSA,* recombinant proteins corresponding to the different 3D7 *var2CSA* individual domains were expressed on the surface of CHO-745 cells and tested for binding. 3D7 *var2CSA* DBL2-X and DBL6-ε bound to biot-CSA (table 1). The binding was specific to CSA, since the adhesion of biot-CSA was inhibited by soluble CSA but not by soluble CSC (table 1). We also tested the DBL2-X domain of A4 *var2CSA*. Like 3D7, it bound to biot-CSA (table 1). None of the domains we tested bound to biot-CSC (data not shown).

Interestingly, the 3D7 *var2CSA* DBL3-X domain tested negative for CSA binding, although this domain had the closest sequence similarity to the minimal CSA-binding region from the FCR3 *var1CSA* DBL3-γ domain [12]. This same domain still did not bind to biot-CSA when expressed as a slightly longer domain (3D7 DBL3b-X; table 1). Sequence comparison of *var2CSA* from the A4, MC, 3D7, and Dd2 strains [10] showed that DBL3-X domains were highly similar (80% homology) but contained a 12-residue deletion in the 3D7 DBL3-X domain

Table 1. Binding characteristics of domains from *var2CSA* to chondroitin sulfate A (CSA) linked to biotin (biot-CSA).

	No inhibition		Inhibition with CSA		Inhibition with CSC	
Construct expressed	Positive cells, %[a]	No. of beads/ 100 cells[b]	Positive cells, %[a]	No. of beads/ 100 cells[b]	Positive cells, %[a]	No. of beads/ 100 cells[b]
3D7-DBL1-X	1 ± 2	ND	2 ± 1	ND	1 ± 1	ND
3D7-DBL2-X	88 ± 7	1217 ± 73	4 ± 2	ND	82 ± 4	1179 ± 89
3D7-DBL3-X	2 ± 1	ND	1 ± 0	ND	1 ± 0	ND
3D7-DBL3b-X	0 ± 1	ND	1 ± 1	ND	1 ± 1	ND
3D7-DBL4-ε	2 ± 2	ND	1 ± 0	ND	1 ± 2	ND
3D7-DBL5-ε	1 ± 1	ND	0 ± 0	ND	1 ± 0	ND
3D7-DBL6-ε	75 ± 6	1156 ± 52	19 ± 8	ND	70 ± 9	1027 ± 21
A4-DBL1-X	1 ± 2	ND	1 ± 1	ND	0 ± 0	ND
A4-DBL2-X	93 ± 5	1398 ± 79	3 ± 1	ND	91 ± 6	1239 ± 56
A4-DBL3-X	87 ± 4	1306 ± 67	2 ± 2	ND	85 ± 11	1269 ± 78
A4-DBL3b-X	84 ± 7	1253 ± 92	4 ± 3	ND	81 ± 6	1125 ± 48

NOTE. Data are mean ± SD of 3 independent experiments. CSC, chondroitin sulfate C; ND, not determined. Results are for binding of biot-CSA to CHO-745 cells; cells were incubated with biot-CSA without (control) or after preincubation with 200 μg/mL CSA or CSC.

[a] One hundred cells expressing the recombinant protein were evaluated for the presence of CSA-coated Dynal beads on their surface. Cells with $\geqslant$4 beads attached were considered to be positive for binding.

[b] The no. of beads was counted on 100 cells determined to be positive for binding to CSA-coated Dynal beads.

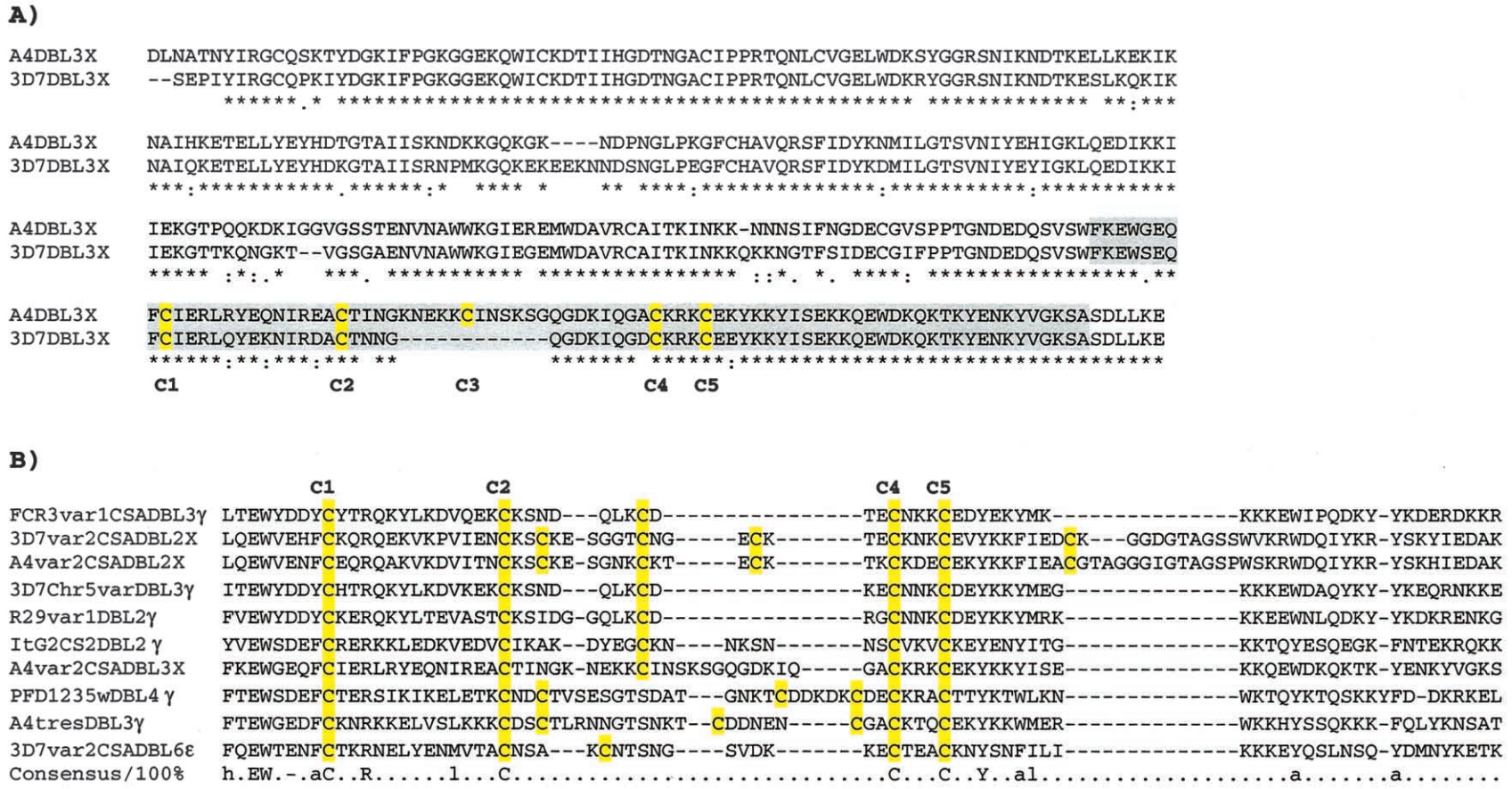

Figure 2. Comparison of chondroitin sulfate A (CSA)–binding and -nonbinding domains from *var2CSA*. *A,* Alignment of the A4 and 3D7 DBL3-X expression constructs. The A4 sequence bound to CSA, whereas the 3D7 sequence did not. The region corresponding to the minimal binding region from the FCR3 *var1CSA* Duffy binding–like (DBL)–γ minimal binding domain is highlighted in gray. Conserved cysteines present in that region have been highlighted in yellow and numbered by reference to the 5 cysteines present in the FCR3 *var1CSA* DBL3-γ minimal binding domain. Multiple sequence alignments were obtained using ClustalW software, available at http://www.ebi.ac.uk/clustalw/. Asterisks (*) indicate identical or conserved residues, colons (:) indicate conserved substitutions, and periods (.) indicate semiconserved substitutions. *B,* Alignment of the FCR3 *var1CSA* DBL-γ minimal binding domain with the corresponding regions from DBL-γ type [12] and DBL2X, DBL3-X, and DBL-ε in *var2CSA* domains (present study) that bind to CSA. Cysteines are highlighted in yellow and numbered from C1 to C5, on the basis of the *var1CSA* DBL3-γ minimal binding domain. The code for the consensus residues is as follows: −, negative charge; a, aromatic; capital letters, conserved amino acids; h, hydrophobic; l, aliphatic.

(figure 2*A*). This modification eliminated, among other things, a cysteine residue (figure 2*A* and 2*B*) present in the *var1CSA* DBL3-γ minimal binding domain and might explain the inability of the 3D7 DBL3-X domain to bind to CSA.

To examine this hypothesis, the DBL3-X and DBL3b-X domains from A4 *var2CSA* were expressed on CHO-745 cells and tested for binding (table 1). The A4 DBL3-X and DBL3b-X domains bound specifically to biot-CSA (table 1) but not to biot-CSC (data not shown). Considered with the previous mapping of the minimal binding domain in *var1CSA* [12], this *var2CSA* binding comparison additionally highlights the potential importance of the C-terminal region in CSA-binding DBL domains. Proof that the C-terminal region defines a general CSA-binding pocket must await further studies to confirm the minimal domain(s) of DBL2-X, DBL3-X, and DBL6-ε responsible for the CSA-binding phenotype.

Since the initial report that maternal antibodies recognize placental isolates from geographically dispersed regions and block infected erythrocyte binding to CSA [4], there has been intense interest in defining the parasite adhesion ligands, since these may form the basis of a vaccine against malaria during pregnancy. A leading candidate has been the parasite variant surface antigens responsible for cytoadhesive activities, but questions have arisen as to whether these are too divergent between parasite strains to explain the cross-reactive antibody response. Unlike the majority of the *var* gene family, *var2CSA* sequences are unusually conserved between parasite strains [10, 13]. In the present study, we demonstrate that 2 slightly distinct *var2CSA* sequences, which are transcriptionally up-regulated in different CSA-adherent parasite strains, contain multiple CSA-binding domains. This is the first evidence, to our knowledge, that *var2CSA* sequences bind to CSA, and it raises the possibility that multivalency is important for infected erythrocyte sequestration in the placenta. In the present study and others [12], several different DBL domains, including DBL-γ, -ε, and -X types, have now been shown to specifically bind to CSA (figure 2*B*). Binding sequences are highly diverse, although a consensus sequence based on the minimal binding region from FCR3 *var1CSA* DBL3-γ and other CSA-binding domains (figure 2*B*) would be defined as follows (using the single-letter code, with "X" meaning any amino acid and conserved cysteine residues in bold font): (hydrophobic)XEWX(E/D)X(F/Y)(**C1**)X_2RX_6(aliphatic)X_3**C2**(variable length with 1 or 3 cysteines)**C4**X_3**C5**X_2YX_2(aromatic)(aliphatic)(variable length)(aromatic)$X_{6/7}$(F/Y)X_8. These consensus residues can also be detected in DBL-γ domains that do not bind to CSA [12], suggesting that they are necessary but not sufficient for binding. Evidence for this will require studies of the effects of mutations of critical residues on binding to CSA.

In conclusion, the *var2CSA* is a strain-transcendent member of the parasite variant antigen family, which is transcriptionally up-regulated in infected erythrocytes selected to bind to CSA. Identification of CSA-binding domains in *var2CSA* strengthens the evidence for their involvement in malaria during pregnancy and is important for future design(s) of a broad-spectrum vaccine against malaria during pregnancy.

Acknowledgments

We thank Erik Whitehorn of the Affymax Research Institute for providing the pSRα5 vectors and mAb179, Susan Kraemer for discussions, Eric Flamoe for assistance in generating some of the expression constructs, Yvon Sterkers for helping with the figures, and Michael Lanzer for providing the FCR3 *var1CSA* genetically disrupted parasites.

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