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ORIGINAL ARTICLE

Molecular dissection of placental malaria protein VAR2CSA interaction with a chemo-enzymatically synthesized chondroitin sulfate library

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Abstract Placental malaria, a serious infection caused by the parasite *Plasmodium falciparum*, is characterized by the selective accumulation of infected erythrocytes (IEs) in the placentas of the pregnant women. Placental adherence is mediated by the malarial VAR2CSA protein, which interacts with chondroitin sulfate (CS) proteoglycans present in the placental tissue. CS is a linear acidic polysaccharide composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-galactosamine that are modified by sulfate groups at different positions. Previous reports have shown that placental-adhering IEs were associated with an unusually low sulfated form of chondroitin sulfate A (CSA) and that a partially sulfated dodecasaccharide is the minimal motif for the interaction. However, the fine molecular structure of this CS chain remains unclear. In this study, we have characterized the CS chain that interacts with a recombinant minimal CS-binding region of VAR2CSA (rVAR2) using a CS library of various defined lengths and sulfate compositions. The CS library was chemo-enzymatically synthesized with bacterial chondroitin polymerase and recombinant CS sulfotransferases. We found that C-4 sulfation of the N-acetyl-D-galactosamine residue is critical for supporting rVAR2 binding, whereas no other sulfate modifications showed effects. Interaction of rVAR2 with CS is highly correlated with the degree of C-4 sulfation and CS chain length. We confirmed that the minimum structure binding to rVAR2 is a tri-sulfated CSA dodecasaccharide, and found that a highly sulfated CSA eicosasaccharide is a more potent inhibitor of rVAR2 binding than the dodecasaccharides. These results suggest that CSA derivatives may potentially serve as targets in therapeutic strategies against placental malaria.

The original version of this article was revised: Tatsumasa Shioiri's name was incorrectly presented as "Tatsuasa Shioiri".

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Abbreviations

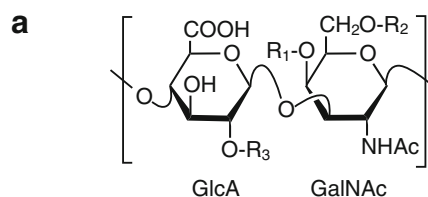|	
CS	chondroitin sulfate
GlcA	D-glucuronic acid
GalNAc	N-acetyl-D-galactosamine
dp	degree of polymerization (number of monosaccharides)
DS	degree of sulfation per disaccharide unit
D4S	degree of C-4 sulfation of GalNAc per disaccharide unit
IE	infected erythrocyte by malaria parasite
PM	placental malaria
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
VAR2CSA	a PfEMP1 protein

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rVAR2	recombinant minimal CS-binding region of VAR2CSA
SPR	surface plasmon resonance
ELISA	enzyme-linked immunosorbent assay

Introduction

Malaria is a global health problem worldwide, causing nearly 200 million infections and leading to more than 500,000 deaths every year [1]. More than 90 % of the mortality is caused by *Plasmodium falciparum*, the most virulent of the five *Plasmodium* species infecting humans [2]. The parasite infects the host erythrocytes. During this stage, the parasite inserts antigens into the membrane of the infected erythrocyte (IE). Some of these proteins, including *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), function as adhesins, allowing the IE to adhere to endothelial receptors in the host microvasculature [3–5]. This selective sequestration is part of an immune evasion strategy allowing the IE to avoid filtration and immune surveillance in the spleen [3, 6]. Pregnant women are especially susceptible to infection despite previously acquired immunity. This is due to infection by a serologically distinct parasite causing an accumulation of IEs in the placenta [2, 7]. Placental malaria (PM) parasites express a unique PfEMP1 protein called VAR2CSA that enables specific sequestration in the placenta through its interaction with chondroitin sulfate (CS) proteoglycan, which is present on the placental syncytium [8–10].

VAR2CSA is a large multidomain protein (350 kDa) consisting of six Duffy binding-like (DBL) domains (three DBLX domains followed by three DBLe domains) and several inter-domains (ID) [11]. We previously demonstrated that the minimal CS-binding site lies within ID1-DBL2X-ID2a region (114 kDa). Specifically, we found that several protein fragments containing this region retain full affinity and specificity for CS compared to the full-length VAR2CSA protein [12, 13]. VAR2CSA is known to bind CS proteoglycan in the placenta, even though CS is present throughout the vasculature of the human host. This suggests that placental CS is distinct and that VAR2CSA interactions are limited to placental CS only. It has been reported that placental-adhering IEs interact with an unusually low-sulfated form of chondroitin sulfate A (CSA) [14] and that the minimal binding requirement is a CS dodecasaccharide [15, 16].

CS is a glycosaminoglycan composed of repeating disaccharide units of [−4-D-glucuronic acid (GlcA)-β-1-3-N-acetyl-D-galactosamine (GalNAc)-β-1-] and modified with sulfate groups at various positions on the sugar residues [17–19]. These units (Fig. 1 a) are categorized as follows: a nonsulfated unit (O unit, GlcA-GalNAc), a monosulfated unit at the C-4 position of the GalNAc residue (A units, GlcA-GalNAc4S), a monosulfated unit at the C-6 position of the
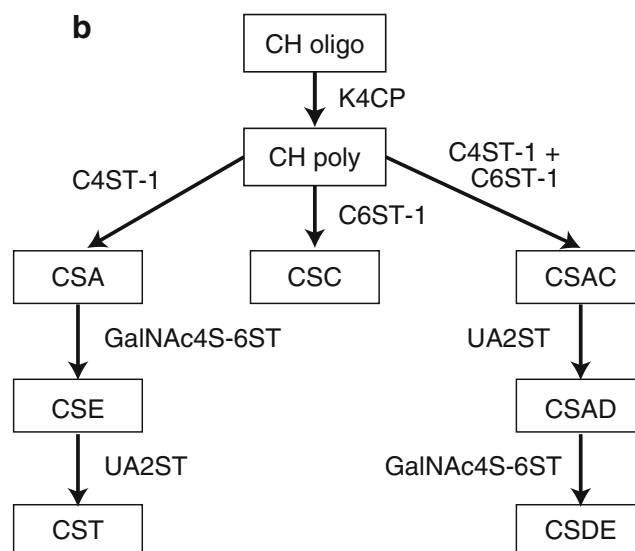

Fig. 1 Structures of chondroitin sulfate disaccharides units (**a**) and scheme of enzymatic synthesis of CS library (**b**). R_1, R_2, and R_3 are O-substituted groups at the C-4 and C-6 positions of the GalNAc residue, and the C-2 position of the GlcA residue, respectively

GalNAc residue (C unit, GlcA-GalNAc6S), a disulfated unit at the C-4 and C-6 positions of the GalNAc residue (E unit, GlcA-GalNAc4S6S), a disulfated unit at the C-2 position of GlcA and the C-6 position of the GalNAc residues (D unit, GlcA2S-GalNAc6S), and a trisulfated unit at the C-2 position of GlcA and at the C-4 and C-6 positions of the GalNAc residue (T unit, GlcA2S-GalNAc4S6S).

CS chains are synthesized onto a specific linkage tetrasaccharide covalently bound to a serine residue of a core protein through the alternating addition of GalNAc and GlcA monomers. During polymerization, the chondroitin chain is modified with sulfate groups at various positions of the sugar residues, mediated by sulfotransferases [20]. Members of the chondroitin 4-O-sulfotransferase (C4ST) family generate the

A unit by sulfating the C-4 position of GalNAc. Chondroitin 6-*O*-sulfotransferases (C6STs) generate the C unit. *N*-Acetylgalactosamine 4-sulfate 6-sulfotransferase (GalNAc4S-6ST) catalyzes the further sulfation of the A unit to generate the E unit. Uronosyl 2-*O*-sulfotransferase (UA2ST) sulfates the C-2 position of GlcA to generate the D unit from the C unit. The T unit is generated with UA2ST from the E unit. Using bacterial chondroitin polymerase K4CP [21] and mammalian CS sulfotransferases, we previously constructed a CS library containing oligo- and polysaccharides with defined lengths and sulfate compositions (Fig. 1 b) [22]. While these studies provide valuable insight into the structural requirements of the VAR2CSA placental CS receptor and CS composition in general, the defined structure of the CS specifically binding to the VAR2CSA protein remains unclear.

In this study, we aimed to examine the interaction of recombinantly expressed minimal CS binding region of VAR2CSA (rVAR2) [13] with synthesized CS oligo- and polysaccharides of various lengths and different sulfate modifications from our published CS library. Interactions were analyzed using surface plasmon resonance (SPR), enzyme-linked immunosorbent assay (ELISA), and inhibition ELISA. The work outlined here will provide further information to determine how placental CS is targeted by VAR2CSA and to improve our understanding of CS derivatives as potential PM therapeutic strategies.

Materials and methods

Materials

Chondroitin (CH), produced by desulfation of CS from shark cartilage, chondroitinase ABC, and chondroitinase AC II were obtained from Seikagaku (Tokyo, Japan). Chondroitin polymerase from *Escherichia coli* strain K4 (K4CP) and point mutants of the enzyme were prepared as described previously [23]. Recombinant chondroitin sulfate sulfotransferases C4ST-1, C6ST-1, GalNac4S-6ST, and UA2ST were prepared as described previously [22]. Adenosine 3′-phosphate 5′-phosphosulfate (PAPS) and hyaluronidase from sheep testis were purchased from Sigma (St. Louis, MO). Uridine 5′-diphospho-α-D-*N*-acetylgalactosamine (UDP-GalNAc) and uridine 5′-diphospho-α-D-glucuronate (UDP-GlcA) were from Yamasa (Choshi, Japan). Streptavidin-conjugated sensor chips (SA chips) for the Biacore biosensor were from GE Healthcare (Piscataway, NJ). Horseradish peroxidase (HRP)-conjugated anti-V5 antibody was from Invitrogen (Carlsbad, CA). Streptavidin-coated 96-well microplates were from Thermo Fisher Scientific (Waltham, MA). The 3,3′,5,5′-tetramethylenbenzidine (TMB) peroxidase substrate (SureBlue) was from KPL (Gaithersburg, MD).

Production of recombinant VAR2CSA proteins

The recombinant ID1-DBL2X-ID2a region of VAR2CSA protein (rVAR2) and the control protein (rDBL4) containing V5 tag at the C-terminals were prepared in *E. coli* as described previously [24]. Briefly. The expression strain SHuffle T7 Express Competent *E. coli* (NEB) was transformed with a plasmid carrying the gene encoding the VAR2 domains DBL1-ID2a and a C terminal V5 and His tag. The cells were grown in shake flasks at 37 °C. When mid-exponential growth phase was reached the temperature was decreased to 20 °C, followed by induction with 0.1 mM isopropyl thio-β-D-galactoside and incubated for 16 h. The cells were harvested by centrifugation and the cell pellet was either stored at −20 °C. The cells were lysed by sonication in 10 mM sodium phosphate buffer, pH 7.2, containing 500 mM NaCl and 60 mM Imidazole (IMAC binding buffer) supplemented with Complete Mini EDTA-free protease inhibitor. After centrifugation, the soluble fraction was filtered and loaded on a His Trap column (GE Healthcare). Column bound material was eluted with IMAC elution buffer (IMAC binding buffer containing 300 mM imidazole), peak fractions were pooled and loaded on a HiLoad 16/600 Superdex 200 pg column (GE Healthcare) equilibrated in phosphate buffered saline. Fractions containing the monomeric protein were pooled, flash frozen in liquid nitrogen and stored at −80 °C. Purified protein was analyzed using SDS page where 0.8 μg was incubated in loading buffer either with DTT or without DTT, the proteins were visualized using Coomassie Brilliant Blue. Binding profiles of rVAR2 protein with chondroitin sulfate proteoglycan (CSPG) and heparan sulfate proteoglycan (HSPG) were analyzed by ELISA as described previously [13].

Preparation of chondroitin sulfate species and their biotin conjugates

The chemo-enzymatically synthesized CS library was constructed as described previously [22]. Briefly, chondroitin hexasaccharide (CH6, degree of polymerization (dp) 6) was prepared from a CH polymer digested with testicular hyaluronidase [23]. Chondroitin dodecasaccharide (CH12) was synthesized from CH6 with two immobilized enzyme mutants of K4CP and UDP-sugar (UDP-GalNAc or UDP-GlcA) by an alternative elongation reaction. Chondroitin polymers CH20, CH6k, CH10k, CH30k, and CH130k, whose average molecular weights (Mr) were 4.5 k, 6 k, 10 k, 30 k, and 130 k, respectively, were prepared with chondroitin polymerase K4CP, UDP-GalNAc, and UDP-GlcA from CH6 and then purified by gel filtration chromatography. CS species with different sulfation patterns were synthesized with the elongated CH polymers as acceptor substrates, various recombinant chondroitin sulfotransferases as catalytic enzymes, and PAPS as sulfate donor substrate. For example, CH species with diverse chain lengths were sulfated at C-4 positions of the

GalNAc residues with C4ST-1 in different conditions to obtain various sulfated CSA species. CSC10k was synthesized with C6ST-1 from CH10k. CSAC10k was synthesized by simultaneous reaction with C4ST-1 and C6ST-1 from CH10k. CSAD10k was synthesized with UA2ST from CSAC10k. CSE10k was synthesized with GalNAc4S6ST from CSA10k. CSDE10k was synthesized with GalNAc4S6ST from CSAD10k. CST10k was synthesized with UA2ST from CSE10k. The CH and CS oligo- and polysaccharides were conjugated with hexamethylenediamine (HMDA) at the reducing ends by the reductive amination method, and then modified with sulfo-NHS-activated biotin reagent (sulfo-NHS-LC-biotin, Pierce, Rockford, IL) at the amine group of the HMDA residue [22].

SPR analysis

The interaction between the CS-biotin conjugates and the rVAR2 or the rDBL4 control protein was analyzed with an SPR biosensor (Biacore 1000 and T200; GE Healthcare) as described previously with slight modifications [22]. Briefly, the biotin-conjugated CS library (10 μg/ml, 70 μl) was immobilized to an SA sensor chip at a flow rate of 5 μl/min. Binding assays were performed at 25 °C at a constant flow rate of 30 μl/min. A titration of rVAR2 (0–150 nM) in 10 mM HEPES-NaOH buffer, pH 7.4, containing 0.15 M NaCl, 3 mM EDTA, and 0.005 % Tween 20 (HBS-EP) was then flushed over the surface, and the interaction was recorded in real-time. Dissociation was observed for 180 s. The equilibrium dissociation constants (K_D) and maximum response units (R_{max}) were determined by a 1:1 (Langmuir) binding model using BIAevaluation 4.1 software (GE Healthcare).

ELISA

The interaction between the rVAR2 protein and the biotin-conjugated CS species was analyzed by ELISA as described previously with modifications [22]. Briefly, the solutions of CS-biotin conjugates (3 μg/ml) in 20 mM Tris-HCl, pH 7.5 containing 0.15 M NaCl, 2 mM $CaCl_2$, and 2 mM $MgCl_2$ (TBS+) were applied to a streptavidin-coated 96-well microplate (Thermo Fisher Scientific, Waltham, MA) and incubated at room temperature for 1 h. After washing with TBS+ containing 0.05 % (*v*/v) Tween 20 (TBST+), TBST+ containing 1 % bovine serum albumin was applied as a blocking agent. After washing, various concentrations (0–100 nM) of rVAR2 protein were added to the plate. After 1 h incubation at room temperature, the plate was washed 3 times with TBST+. The plate was developed using an HRP-conjugated anti-V5 antibody (1:3000 dilution with TBST+) and TMB peroxidase substrate solution. Absorbance was measured at 450 nm using a microplate reader (VERSA max; Molecular Devices, Sunnyvale, CA) to determine the rVAR2 adhesion.

Inhibition ELISA

The interaction between rVAR2 and the CSA species was further analyzed by inhibition ELISA. Briefly, the CSA10k-biotin conjugates (0.5 μg/ml) in TBS+ were added to a streptavidin-coated 96-well microplate and incubated at room temperature for 1 h for CSA immobilization. After washing, TBST+ containing 1 % bovine serum albumin was applied for blocking. After washing, the rVAR2 protein (25 nM) mixed with a titration of the CSA species (0–400 μg/mL) was added to the plate. After 1 h incubation at room temperature, the plate was washed three times to remove unbound protein and saccharide. The plate was developed using an HRP-conjugated anti-V5 antibody (1:3000 dilution with TBS+) and TMB peroxidase substrate solution. The absorbance was measured at 450 nm to determine the degree of rVAR2 binding inhibition by the CSA species.

Compositional analysis of CS derivatives

The disaccharide composition of the CS derivatives was determined as described previously [25]. Briefly, the synthesized CS derivatives were digested with chondroitinase ABC or AC II (10 mU) at 37 °C for 1 h. The unsaturated disaccharide products were analyzed using a fluorometric post-column HPLC system.

Measurement of molecular weight of CS derivatives

Average molecular weights of the synthetic CS polysaccharides were estimated by gel filtration chromatography using polysaccharide standards of which the absolute average molecular weights were determined with a laser light scattering photometer (Wyatt Technology, Santa Barbara, CA) [22]. The molecular weights of CH12 and CH20 were confirmed with a MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) as described previously [26].

Results

Recombinant expression of rVAR2

The recombinant minimal CS-binding region of VAR2CSA protein (rVAR2) and the control protein (rDBL4) were produced in *E. coli* cells as soluble protein. The proteins were initially purified from the cell lysates using the incorporated His tag, and then further purified by size exclusion chromatography. The proteins showed a little shift in gel mobility when comparing the formation of intramolecular disulfide bonds with reduced and nonreduced conditions of SDS-PAGE (Supplementary Fig. S1 a). The rVAR2 protein was fully functional in binding to CSPG in ELISA and did not

bind HSPG, confirming the protein specificity (Supplementary Fig. S1 b). This is consistent with the recombinant proteins expressed in baculovirus-infected insect cells as described previously [13].

Preparation and characterization of CS poly- and oligosaccharides

A summary of the synthesized CS species used herein is provided in Table 1. We initially prepared CH polysaccharides exhibiting *Mr* of 10 k (CH10k) by the alternating addition of GlcA and GalNAc monosaccharides onto CH6, using UDP-GlcA and UDP-GalNAc and K4CP. Then we synthesized CS10k species containing various sulfation types and degrees with different recombinant chondroitin sulfotransferases (C4ST-1, C6T-1, GalNAc4S-6ST, and UA2ST). Following chondroitinase digestion, the disaccharide compositions of the CS products were determined by fluorometric post-column HPLC (Table 1). The products were designated as CSA10k (98 % A unit), CSC10k (98 % C unit), CSAC10k (45 % A unit and 54 % C unit), CSAD10k (45 % A unit and 12 % D unit), CSE10k (10 % A unit and 87 % E unit),

Table 1 Synthesized chondroitin sulfate library

Synthetic CS	*Mr*	dp	O%	A%	C%	E%	D%	T%	DS	D4S
						Disaccharide compositions				
CH10k	9.6 k	46	100.0						0.00	0.00
CSA10k	10.5 k	46	1.9	98.1					0.98	0.98
CSC10k	10.5 k	46	1.6		98.4				0.98	0.00
CSAC10k	10.5 k	46	1.0	44.7	54.3				0.99	0.45
CSAD10k	11.9 k	46	1.3	44.5	12.2		41.9		1.41	0.45
CSE10k	13.4 k	46	2.6	10.0		87.3			1.85	0.97
CSDE10k	12.1 k	46	2.0	48.3	0.9	7.7	41.1		1.47	0.56
CST10k	13.9 k	46	1.1	9.5		71.1		18.3	2.07	0.99
CSA10k-I	9.7 k	46	88.0	12.0					0.12	0.12
CSA10k -II	9.8 k	46	79.4	20.6					0.21	0.21
CSA10k -III	10.2 k	46	38.3	61.7					0.62	0.62
CSA10k -IV	10.2 k	46	28.3	71.7					0.72	0.72
CSA10k -V	10.3 k	46	20.8	79.2					0.79	0.79
CH6k	5.8 k	28	100.0						0.00	0.00
CSA6k-I	5.9 k	28	90.5	9.5					0.09	0.09
CSA6k-II	6.1 k	28	55.8	44.2					0.44	0.44
CSA6k-III	6.3 k	28	7.1	92.9					0.93	0.93
CH30 k	29 k	140	100.0						0.00	0.00
CSA30 k-I	29 k	140	96.8	3.2					0.03	0.03
CSA30 k-II	30 k	140	63.7	36.3					0.36	0.36
CSA30 k-III	32 k	140	3.5	96.5					0.97	0.97
CH130 k	130 k	620	100.0						0.00	0.00
CSA130 k-I	130 k	620	96.2	3.8					0.04	0.04
CSA130 k-II	135 k	620	57.8	42.2					0.42	0.42
CSA130 k-III	141 k	620	2.1	97.9					0.98	0.98
CH12	2.5 k	12	100.0						0.00	0.00
CSA12-I	2.5 k	12	80.1	19.9					0.20	0.20
CSA12-II	2.6 k	12	60.4	39.6					0.40	0.40
CSA12-III	2.7 k	12	41.2	58,8					0.59	0.59
CSA12-IV	2.7 k	12	29.3	70.7					0.71	0.71
CH20	4.2 k	20	100.0						0.00	0.00
CSA20	4.5 k	20	30.1	69.9					0.70	0.70

Mr: average molecular weight without biotin component

dp: degrees of polymerization (numbers of monosaccharide)

DS: degrees of sulfation per disaccharide unit

D4S: degrees of C-4 sulfation of GalNAc per disaccharide unit

CSDE10k (48 % A unit, 1 % C unit, 8 % E unit, and 41 % D unit), and CST10k (10 % A unit, 71 % E unit, and 18 % T unit).

Next, we synthesized CSA species with various molecular weights (6 k, 10 k, 30 k, and 130 k) using K4CP. These were sulfated at the C-4 position of the GalNAc residue to different degrees by optimizing the reaction time as well as the amounts of enzyme (C4ST-1) and donor substrate (PAPS). This included five Mr: 10 k compounds (CSA10k-I, −II, −III, −IV, and −V containing 12 %, 21 %, 62 %, 72 %, and 79 % A units, respectively), three Mr: 6 k compounds (CSA6k-I, −II, and −III containing 10 %, 44 %, and 93 % A units, respectively), three Mr: 30 k compounds (CSA30 k-I, −II, and −III containing 3 %, 36 %, and 97 % A units, respectively), and three Mr: 130 k compounds (CSA130 k-I, −II, and −III containing 4 %, 42 %, and 98 % A units, respectively). We further synthesized differentially sulfated chondroitin sulfate dodecasaccharides (CS12). These included CSA12-I, −II, −III, and -IV containing 20 %, 40 %, 59 %, and 71 % A units, respectively. We also synthesized an eicosasaccharide CSA20 containing 70 % A units. The different CS species were conjugated with biotin at the reducing end following HMDA conjugation before or after sulfate modification. The disaccharide composition of these CS species remained unaltered after biotin conjugation.

In the CS disaccharides, the O unit has no sulfate group, the A and C units each have one, the D and E units have two, and the T unit has three sulfate groups. The A, E, and T units all have a sulfate group at the C-4 position of the GalNAc residue, whereas the other units have no sulfate group at the C-4 position (Fig. 1 a). The degree of sulfation (DS), which is the ratio of sulfation per disaccharide unit, and the degree of GalNAc C-4 sulfation (D4S), which is the degree of GalNAc C-4 sulfation per disaccharide unit, were estimated by the following formulas:

$$DS = [A\% + C\% + 2 \times E\% + 2 \times D\% + 3 \times T\%] \times 100$$

$$D4S = [A\% + E\% + T\%] \times 100$$

The DS, and the D4S values of the synthesized CS species are summarized in Table 1.

SPR analysis

Having constructed our CS library, we first analyzed the biomolecular kinetics of rVAR2 binding to the CS species with equal lengths and different sulfation patterns using SPR (Biacore) analysis. The sensorgrams revealed rVAR2 interactions with different immobilized CS derivatives (Supplementary Fig. S2). The K_D and R_{max} values calculated using the 1:1 (Langmuir) binding model are provided in Supplementary Table S1.

While the rVAR2 protein showed no binding to the non-sulfated CH10k, the protein bound to all sulfated CS species in a dose-dependent manner with different binding kinetics. The K_D and R_{max} values obtained from the kinetic analysis between rVAR2 protein and the CS10k species showed a close correlation with the D4S values (Fig. 2). The binding capacity (R_{max}) was strongest to almost fully C-4 sulfated CSA10k, CSE10k, and CST10k. CSC10k with almost full C-6 sulfation but no C-4 sulfation showed the lowest binding activity in the CS10k species. CSAD10k and CSDE10k were highly sulfated, but the corresponding 4S ratios were lower than that of CSA10k and similar to that of CSAC10k. The R_{max} values for CSAD10k and CSDE10k were lower than for CSA10k. Together, these data indicate that C-4 sulfation of the GalNAc residue is critical for supporting rVAR2 adherence to CS, whereas other sulfate modifications do not affect the interaction.
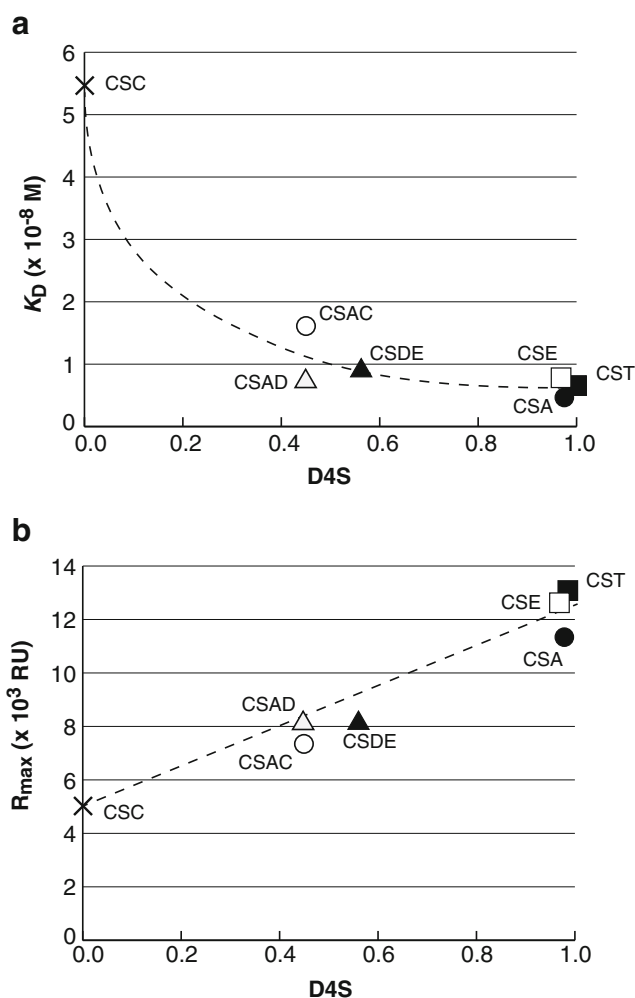

Fig. 2 Kinetic analysis of rVAR2 interactions with CS10k species using SPR. K_D (**a**) and R_{max} (**b**) values from the analysis of rVAR2 interacting with different sulfated 10 k CS species were determined and plotted against D4S. Data are shown as the average ± S.D. of three independent experiments

Next, we analyzed the interaction between rVAR2 and immobilized CSA10k species with different degrees of C-4 sulfation (Supplementary Fig. S3 and Table S1). The K_D and R_{max} values obtained from the CSA10k species indicated exponential and linear correlations, respectively, to the D4S values (Fig. 3 a and b). This means that the affinity of rVAR2 for CS is directly related to the degree of C-4 sulfation (A unit).

We then investigated the dependence of CS chain length, as well as 4S ratio, in supporting rVAR2 adhesion. Biotin-conjugated *Mr.* 6 k saccharides (CH6k, CSA6k-I, −II, and -III, Supplementary Fig. S4), *Mr.* 30 k saccharides (CH30k, CSA30 k-I, −II, and-III, Supplementary Fig. S5), *Mr.* 130 k saccharide chains (CH130k, CSA130k-I, −II, and -III, Supplementary Fig. S6), and dodecasaccharides (CH12, CSA12-I, −II, −III, and -IV, Supplementary Fig. S7), were immobilized and analyzed for interaction with rVAR2 (Supplementary Table S2). The rVAR2 protein showed no binding to the non-sulfated CH species with different chain lengths. The relationships between the kinetic parameters (K_D and R_{max} values) and the D4S ratio for the CSA species having different chain lengths were similar to those of CSA10k, while CSA12 species showed lower affinity than longer CS species; moreover, longer CSA (*Mr* 30 k and 130 k) species with high degrees of C-4 sulfation showed lower R_{max} values than shorter CSA (*Mr* 6 k and 10 k) species (Fig. 3 c and d).

The control protein (rDBL4) showed no binding to any of the immobilized CS species (data not shown).

ELISA

To analyze the interaction between rVAR2 and the CS species further, we used the ELISA method to measure the amount of bound rVAR2 to various CS10k-Biotin conjugates (Supplementary Fig. S8) and CSA species of different lengths (Supplementary Fig. S9). The half maximum effect (ED_{50}) values for the binding of rVAR2 to the CS species were estimated from the dose response profiles (Table 2). The rVAR2 protein showed no binding to the non-sulfated CH-biotin species with different chain lengths. The protein bound to sulfated CS-biotin species according to C-4 sulfation degree in a dose-dependent manner. CSC10k with no C-4 sulfation showed lower binding than CSA10k and other A unit-rich CS10k species. Furthermore, CSA species with low D4S values (D4S < 0.2) showed low rVAR2 binding ($ED_{50} \geq 200$ nM), whereas CSA species with relatively high D4S values (D4S $\geq$ 0.2) displayed higher binding activity ($ED_{50} \leq 9$ nM). The longest and high 4S CSA (CSA130 k-III) was found to have the strongest binding interaction of all CS species examined. The rDBL4 control protein did not bind to CSA-biotin species in the ELISA (data not shown).

Fig. 3 Kinetic analysis of rVAR2 interaction with CSA species using SPR. K_D (**a**, **c**, and **e**) and R_{max} (**b**, **d**, and **f**) values from the analysis of rVAR2 interacting with synthetic CSA species with various lengths and degrees of sulfation. K_D (**a**) and R_{max} (**b**) values of 10 k CSA species (CH10k; CSA10k-I, −II, −III. -IV, −V; and CSA10k) plotted against the D4S. K_D (**c**) and R_{max} (**d**) values of dp12 CSA species (CSA12-II, −III, and -IV, closed circle), 6 k CSA species (CSA6k-I, −II, and -III, open circle), 30 k CSA species (CSA30 k-I, −II, and -III, open square), and 130 k CSA species (CSA130 k-I, −II, and -III, closed square) plotted against the D4S
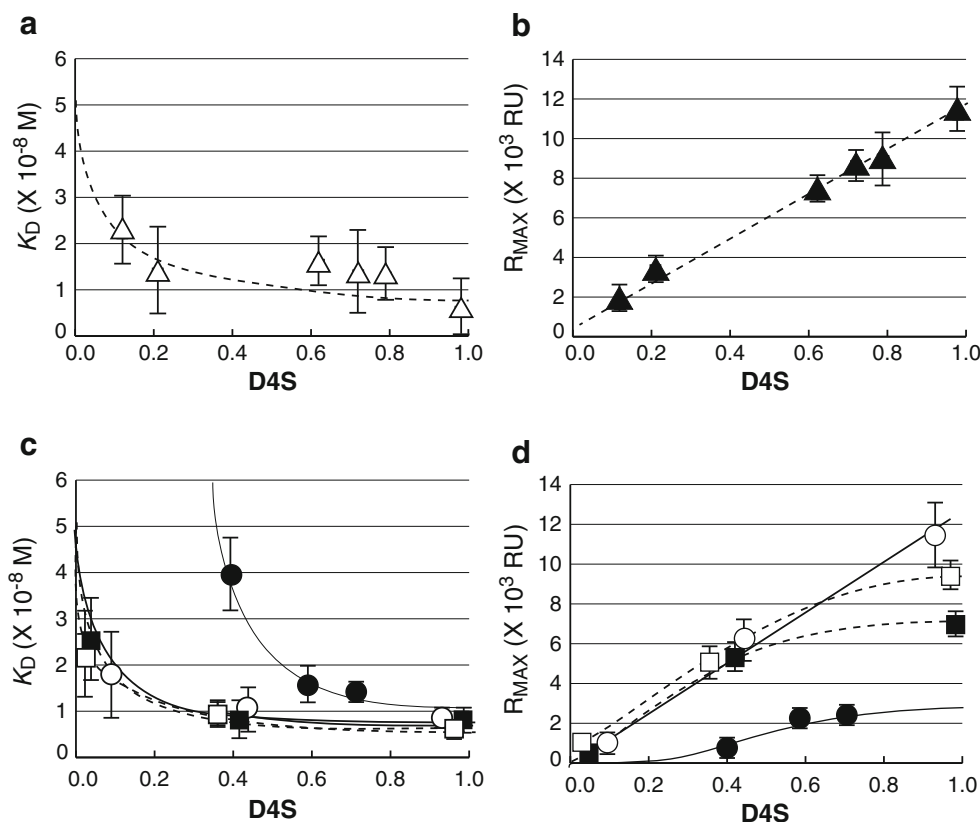
Table 2 ED_{50} values of rVAR2 binding to immobilized CS species in ELISA

CS species	ED_{50} of rVAR2 (nM)
CH10k	nd
CSA10k	4.2
CSC10k	12.0
CSAC10k	6.4
CSAD10k	5.6
CSE10k	5.9
CSDE10k	5.2
CST10k	6.0
CSA10k-I	12.0
CSA10k-II	6.3
CSA10k-III	5.7
CSA10kIV	4.3
CSA10k-V	4.2
CH6k	nd
CSA6k-I	≈ 200
CSA6k-II	8.0
CSA6k-III	6.6
CH30 k	nd
CSA30 k-I	≈ 200
CSA30 k-II	9.0
CSA30 k-III	6.0
CH130 k	nd
CSA130 k-I	≈ 200
CSA130 k-II	3.7
CSA130 k-III	2.2

nd not determined

Inhibition ELISA

To further validate the dependence of rVAR2 interaction on CS chain length and C-4 sulfation, and to rule out any steric constraints that may occur when immobilizing CSA10k and adding rVAR2 and CS oligosaccharides, we tested the efficiency of increasing concentrations of dodeca- and eicosasaccharides with various C-4 sulfations in inhibiting binding of rVAR2 to a CSA polysaccharide using ELISA (Fig. 4). Whereas non- and low-sulfated dodecasaccharides (CH12, CSA12-I, and -II) and non-sulfated eicosasaccharide (CH20) showed almost no inhibition activity, more highly sulfated dodecasaccharides (CSA12-III, and -IV) and eicosasaccharide (CSA20) demonstrated dose-dependent inhibition of rVAR2 binding. From the dose response curves, we estimated half maximal (50 %) inhibitory concentration (IC_{50}) values of added CSA (Table 3). The CSA dodecasaccharides containing three and four continuous A units (CSA12-III and -IV) were more potent inhibitors (IC_{50} = 120 and 100 μg/ml) than the lower sulfated dodecasaccharide containing two A units (CSA12-II). The eicosasaccharide CSA20, which has a longer chain length
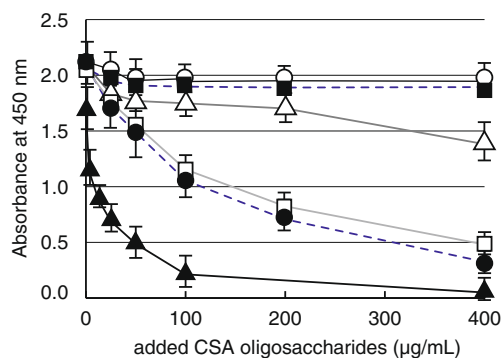

Fig. 4 Inhibition of rVAR2 binding to immobilized CSA10k with CH and CSA oligosaccharides analyzed by ELISA. Oligosaccharides used for inhibition were CH12 (open circle), CSA12-I (closed square), CSA12-II (open triangle), CSA12-III (open square), CSA12-IV (closed circle), and eicosasaccharide CSA20 (closed triangle). Data are shown as the average ± S.D. of three independent experiments

than CSA12 and contains seven A units (D4S = 0.70), showed higher inhibition activity (IC_{50} = 17 μg/ml) than the highly sulfated dodecasaccharides.

Discussion

The placenta-specific adhesion of *P. falciparum* IEs in PM represents a unique phenomenon of organ-specific sequestration. The PM parasite expresses a distinct PfEMP-1 protein called VAR2CSA that allows the IEs to adhere to distinct CS proteoglycans present on the placental syncytiotrophoblasts. It is remarkable that VAR2CSA-expressing IEs only adhere to CS chains in the placenta and not elsewhere in the human host. To provide a therapeutic strategy for PM, it is essential to define the fine molecular structure of the CS chain interacting with VAR2CSA. We previously found that rVAR2 protein containing minimal CS-binding region of VAR2CSA specifically bound placental tissue and the binding was inhibited by CSA chain or by chondroitinase [24].

Table 3 IC_{50} values of oligosaccharide inhibition of rVAR2–CSA adhesion in ELISA

CS inhibitor	IC_{50} of CSA (μg/mL)	Numbers of A units
CH12	nd	0
CSA12-I	nd	1
CSA12-II	600	2
CSA12-III	120	3
CSA12-IV	100	4
CH20	nd	0
CSA20	17	7

IC_{50}: half maximal (50 %) inhibitory concentration of free CS inhibitor added

nd not determined

In this study, we defined the CS structure that rVAR2 protein interacts with using a chemo-enzymatically synthesized CS library and SPR analysis, ELISA, and inhibition ELISA. We found that sulfation at the C-4 position of the GalNAc residue of the CS chain, containing A, E, and T disaccharide units, is critical for the interaction with rVAR2, whereas other sulfate modifications have negligible effects. Binding of rVAR2 to CS is correlated with the degree of A unit modifications and the molecular weight of the sugar chain. Here, we show that CS molecules having chain lengths longer than 6 kDa and C-4 sulfation higher than the D4S value 0.2 are required for optimal interaction with rVAR2. The quite long (Mr 130 k) and highly C-4 sulfated CSA showed higher binding activity than intermediate-length (Mr 6–30 k) CSA species in ELISA, although binding capacity of long and highly C-4 sulfated CSA species indicated weaker interactions than with intermediate-length CSA in the SPR assay. These differences may depend on the different reaction systems of the analytical methods, as ELISA is a static end-point reaction and SPR is a dynamic real-time reaction.

Previous studies have shown that a low sulfated CSA dodecasaccharide [15, 16] was the minimum structure necessary to inhibit adherence of VAR2CSA to a placental CS motif. Here, we confirmed that three and four C-4 sulfated CSA dodecasaccharides show significant binding and inhibition activity towards rVAR2. Indeed, the low-sulfated dodecasaccharides with only one or two A units have almost no inhibitory activity. Recently, we determined the sequence structures of chemo-enzymatically synthesized CS dodecasaccharides [27], including CSA12 species prepared with C4ST-1, which preferentially sulfates the middle region of the dodecasaccharide. The major sequences of mono-sulfated CSA12-I, di-sulfated CSA12-II, tri-sulfated CSA12-III, and tetra-sulfated CSA12-IV were identified as O-O-A-O-O-O, O-O-A-A-O-O, O-A-A-A-O-O, and O-A-A-A-A-O, respectively. It is possible that VAR2CSA requires at least three continuous A units for interaction, because CSA12-III having three continuous A units and CSA12-IV having four continuous A units indicated high inhibitory activity and dodecasaccharides having no, one, or two A units showed no or low activity (Fig. 4).

We recently found that the VAR2CSA binding epitope in placental CS is larger than a tetradecasaccharide using a footprint analysis (Pereira and Clausen *et al.*, in review). Thus, we produced synthetic eicosasaccharide resembling this epitope. The eicosasaccharide (CSA20) was far more potent in inhibiting rVAR2 binding to CSA polysaccharides than the dodecasaccharides (CSA12) in ELISA (Fig. 4 and Table 3). Although a CSA dodecasaccharide with continuous three A units may be the minimum structure for the interaction with VAR2, longer lengths (dp $\geq$ 20) with higher C-4 sulfation (D4S $\geq$ 0.7) provide higher inhibition and binding activity. This correlates with previous studies showing that longer

oligosaccharides were more potent in inhibiting parasite binding to CS, but that the core binding site had the length of a dodecasaccharide [15, 16, 28, 29]. It is possible that the higher capacity of longer oligosaccharides for inhibiting rVAR2 adhesion to CS is due to the interaction between CS and amino acids outside the core CS binding site in rVAR2. Our result supports the observation here that longer CS oligosaccharides with high numbers of A units are required to inhibit the adhesion of VAR2CSA and IEs to the placental CS proteoglycan (Pereira and Clausen *et al.*, in review).

In this study, we used a fragment of the VAR2CSA protein, produced in *E. Coli* cells, to determine the CS structure. We have previously shown that this fragment of VAR2CSA retains full binding specificity and affinity for CSA as compared to the full-length protein [13], which was based on recombinant proteins expressed in a eukaryotic insect cell system. The VAR2CSA protein contains many disulfide bridges than need to be correctly formed [30]. We have recently shown that the rVAR2 protein, expressed in the T7 shuffle E. coli cells, form disulfide bonds and is fully functional in binding to placental CS [24]. However it is important to note that the disulfide bond formation in the recombinant proteins may differ from that in native VAR2CSA, thereby impacting the CS specificity.

While these data do not completely resolve the VAR2CSA binding placental CS epitope and provide an explanation for how this type of CS is different from CS in other organs, we determined the minimum required chain lengths and degree of sulfation for CSA oligosaccharides binding to the rVAR2 protein. Although rVAR2 protein is not the full sequence of VAR2CSA and there may be some differences between VAR2CSA and rVAR2 behavior *in vivo*, these data suggest that highly sulfated CSA oligosaccharide derivatives may inhibit parasite adhesion in PM. Thus, synthetic CSA derivatives could provide an effective biological tool to investigate malaria infection, thereby aiding vaccine development and the development of anti-PM intervention strategies.

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