Structural Basis for Binding of \textit{Plasmodium falciparum} Erythrocyte Membrane Protein 1 to Chondroitin Sulfate and Placental Tissue and the Influence of Protein Polymorphisms on Binding Specificity*\textsuperscript{S}

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Chondroitin sulfate (CS) A is a key receptor for adhesion of \textit{Plasmodium falciparum}-infected erythrocytes (IEs) in the placenta and can also mediate adhesion to microvascular endothelial cells. IEs that adhere to CSA express \textit{var2csa}-type genes, which encode specific variants of the IE surface antigen \textit{P. falciparum} erythrocyte membrane protein 1 (PfEMP1). We report direct binding of native PfEMP1, isolated from IEs and encoded by \textit{var2csa}, to immobilized CSA. Binding of PfEMP1 was dependent on 4-O-sulfated disaccharides and glucuronic acid rather than iduronic acid, consistent with the specificity of intact IEs. Using immobilized CS oligosaccharides as neoglycolipid probes, the minimum chain length for direct binding of PfEMP1 was eight monosaccharide units. Similarly for IE adhesion to placental tissue there was a requirement for 4-O-sulfated GalNAc and glucuronic acid mixed with non-sulfated disaccharides; 6-O-sulfation interfered with the interaction between placental CSA and IEs. The minimum chain length for maximal inhibition of adhesion was 10 monosaccharide residues. Partially 4-O-sulfated CS oligosaccharides (45–55% sulfation) were highly effective inhibitors of placental adhesion (IC\textsubscript{50}, 0.15 µg/ml) and may have potential for therapeutic development. We used defined \textit{P. falciparum} isolates expressing different variants of \textit{var2csa} in adhesion assays and found that there were isolate-specific differences in the preferred structural motifs for adhesion to CSA that correlated with polymorphisms in PfEMP1 encoded by \textit{var2csa}-type genes. This may influence sites of IE sequestration or parasite virulence. These findings have significant implications for understanding the pathogene-
sis and biology of malaria, particularly during pregnancy, and the development of targeted interventions.

Adhesion of parasite-infected erythrocytes (IEs)\textsuperscript{3} to the microvascular endothelium of various organs is an important pathogenic feature of \textit{Plasmodium falciparum} (1, 2), the major cause of malaria accounting for up to 3 million deaths annually (3). Sequestration of IEs is thought to contribute to the survival of parasites by aiding replication and evasion of splenic clearance and can lead to severe consequences when large numbers of IEs accumulate in vital organs. Several cell adhesion molecules have been reported for \textit{P. falciparum}-IEs, including CD36 (4), intercellular adhesion molecule 1 (ICAM-1) (5), and the glycosaminoglycans (GAGs) chondroitin sulfate A (CSA) (6), heparan sulfate (7), and hyaluronic acid (8).

Evidence from several studies demonstrates that CSA is an important receptor for sequestration of IEs in the placenta (9–11). \textit{P. falciparum} isolates from the placenta typically adhere to CSA, and IEs can adhere to \textit{ex vivo} placental tissue in a CSA-dependent manner \textit{in vitro} (10, 12). CSA has also been identified as a cell surface receptor for adhesion to endothelial cells (13) and may therefore contribute to parasite sequestration in other organs (14). IEs can also bind via CSA side chains to the proteoglycan thrombomodulin (15) that is present on endothelial cells throughout the vasculature (16). Other CSPGs that support IE adhesion \textit{in vitro} have been isolated from the placenta, \textit{Saimiri} brain endothelium, and other cells (supplemental Table S1) (17–20).

\textit{P. falciparum} erythrocyte membrane protein 1 (PfEMP1) has been identified as the principle adhesive ligand of IEs and is encoded by the \textit{var} multigene family (up to 60 copies per

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\textsuperscript{S} The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1 and S2.

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3 The abbreviations used are: IE, infected erythrocyte; GAG, glycosaminoglycan; CSPG, chondroitin sulfate proteoglycan; PfEMP1, \textit{P. falciparum} erythrocyte membrane protein 1; CS, chondroitin sulfate; NGL, neoglycolipid; ICAM-1, intercellular adhesion molecule-1; HexUA, hexuronic acid; GlcUA, glucuronic acid; IdoUA, iduronic acid; de6S, de-6-O-sulfated; de4S, de-4-O-sulfated; ATS, acidic terminal segment; BSA, bovine serum albumin; ΔUA, 4,5-unhydroxysulfonic acid; CSA-PR, CSA from porcine rib cartilage; RPMI, RPMI 1640 medium; PBS, phosphate-buffered saline.
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var genes are highly diverse both within and between different genomes, and different variants encode proteins with different receptor binding properties. IEs selected for adhesion to CSA up-regulate var2csa-type genes, which appear to be the predominant var gene encoding CSA-binding domains (21–24) and show increased expression in placental malaria (25). Recombinant domains of var2csa bind CSA (22). However, binding of full-length or native PfEMP1 to CSA or inhibition of IE adhesion by antibodies to var2csa domains has not been demonstrated to confirm a direct role of var2csa in adhesion. Some Duffy-binding-like γ-type domains from other var genes have also been shown to bind CSA in vitro when expressed as recombinant proteins (26–28), but their significance in mediating IE adhesion to CSA remains unclear (29, 30). Although var2csa genes are relatively conserved, there is considerable polymorphism among different variants (21, 31–33). These polymorphisms can substantially influence the binding and adhesion-inhibitory activity of acquired antibodies suggesting that they have evolved to facilitate immune evasion (34). Polymorphisms also influence sensitivity of var2csa PfEMP1 to trypsin (34, 35), but it is not known whether they influence specificity of interactions with CSA.

CS comprises repeating disaccharide units of hexuronic acid (HexUA) β1–3 linked to GalNAc, i.e. -(4HexUAβ1–3GalNAc1)α-. Typically GalNAc is mono-O-sulfated at either the 4- or the 6-O position and this differentiates the principal CSA and CSC disaccharide units, respectively. CSB (or dermatan sulfate) is similar in sulfation to CSA but has iduronic acid (IdoUA) rather than glucuronic acid (GlcUA) as the predominant uronic acid. Other variations in sulfation pattern also frequently occur. CS chains show heterogeneity in sulfation patterns and uronic acid compositions, and these features determine their biological properties.

Several studies have demonstrated the importance of 4-O-sulfation for IE binding to CS (6, 36). The minimum chain length for inhibition of adhesion to CSA is 12 monosaccharide residues (36–38), and the optimal motif for interaction with IEs is formed by mixed 4-O-sulfated and non-sulfated GalNAc alternating with GlcUA (37, 38). IdoUA and 6- or 2-O-sulfation interfere with the interaction (38). In the vasculature, the CSPGs, to which IEs may bind, are present as diverse structures that differ significantly in their level and pattern of sulfation (supplemental Table S1) (17–20, 39–41). CSPGs isolated from placental intervillosus blood are particularly low in sulfate content (17, 42), containing 2–8% 4-O-sulfation; there are also regions of higher levels of sulfation (20–28%) (43). Cell-associated placental CSPGs present in detergent extracts have a higher level of sulfation with ~30% 4-O- and 13–15% 6-O-sulfation (17). Thrombomodulin, which is widely expressed in the vasculature, is typically composed of ~87% 4-O-sulfated disaccharides balanced with non-sulfated disaccharides (39), and CSPGs from Saimiri brain endothelial cells were composed predominantly of 4-O-sulfated disaccharides with ~37% non-sulfated disaccharides (18).

GAGs play essential roles in a range of biological functions and have been increasingly recognized as important molecules for attachment, adhesion, and invasion of infectious pathogens (44). Interactions between CS and other microbes, such as human immunodeficiency virus (45), have been reported, and P. falciparum also interacts with heparan sulfate during invasion of hepatocytes (46). Despite its widespread distribution and expression on many cell surfaces there is relatively little known about the structure-function relationship of CS. Furthermore it is not known how polymorphisms, which are common among antigens of microbial pathogens, influence the specificity and function of interactions with GAGs, which may influence the clinical manifestations of infection.

In this study, we aimed to further define the molecular basis of the interactions among IEs, CSA, and placental tissue and to identify key features common to different CSA-binding variants. To date, the structural requirements of CS for interactions with P. falciparum have been defined only by inhibition of IE adhesion rather than through direct PfEMP1 binding studies and have predominantly used purified immobilized CSA preparations for adhesion. Here we examined direct binding of native PfEMP1 to oligosaccharides and polysaccharides, as neoglycolipid (NGL) (47) and biotin probes, respectively; adhesion of IEs from different P. falciparum isolates to polysaccharides with different degrees of sulfation; and inhibition of IE adhesion to CSA and placental tissue using various types of defined oligosaccharides and polysaccharides. Additionally we hypothesized that the requirement for P. falciparum to interact with diverse CS structures in the vasculature will have favored the evolution of CS-binding PfEMP1 variants with differing fine specificities for CS structural motifs. More broadly, we used PfEMP1 as a model to investigate strain-specific differences in microbial interactions with GAGs and whether polymorphisms in GAG-binding proteins may alter the fine specificity of interactions with structural motifs.

**EXPERIMENTAL PROCEDURES**

**Preparation of Desulfated and Biotinylated CS Polysaccharides**—CSA (from bovine trachea), CSB (from porcine intestinal mucosa), and CSC (from shark cartilage) were obtained from Sigma. Chondroitinase ABC (EC 4.2.2.20, from Proteus vulgaris; Sigma) digestion and strong anion-exchange HPLC disaccharide composition analysis (38) indicated mixed sulfation patterns in all three CS preparations. They all contained three disaccharide units: ΔUA-GalNAc (0S), ΔUA-GalNAc4S (4S), and ΔUA-GalNAc6S (6S). CSA contained 4.9% 0S, 55.3% 4S, and 39.8% 6S; CSB contained 0.5% 0S, 85.6% 4S, and 4.8% 6S; and CSC contained 0.8% 0S, 15.5% 4S, and 76.7% 6S (Table 1). Selective de-6-O-sulfation of CS poly- and oligosaccharides and partial de-4-O-sulfation of CSB were performed as described previously (38). De-6-O-sulfation involved conversion of poly- and oligosaccharides to pyridinium salts and desulfation in a mixture of anhydrous pyridine and N,O-bis(trimethylsilyl)acetamide. CSB polysaccharide was converted to pyridinium salt, dissolved with 90% Me2SO in H2O, and heated to 80 °C. The reaction was stopped at 70- and 100-min intervals by cooling and neutralization with NaOH. The desulfation products were confirmed by strong anion-exchange HPLC disaccharide composition analysis as described previously (38) (Table 1). Results indicated that all 6-O-sulfate groups were selectively removed from CSA and CSC poly- and oligosaccharides, and 30 and 46% of sulfates were removed.
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from the two partially de-4-O-sulfated (de4S) CSB preparations, CSB-de4S-1 and CSB-de4S-2, respectively (Table 1).

Biotinylated CSA and CSC (biotin-CSA and biotin-CSC, respectively) were prepared and purified as described previously (48) with some modification. The level of biotin introduced was one for every 50 carboxyl groups. In brief, 50 mg of CSA or CSC polysaccharides were dissolved in 10 ml of 0.1 M MES buffer (pH 5.5) before addition of 109 μl of 25 mM biotin-LC-hydrazide (Pierce, Perbio Science, Tattenhall, UK) solution in MeOH and 130 μl of 25 mg/ml 1-ethyl-2-(2-dimethylaminopropyl)carbodiimide solution in 0.1 M MES buffer. The reaction mixtures were stirred at room temperature for 24 h and then dialyzed extensively against deionized water. A short Sephadex G-10 column (1.6 × 30 cm) was used to remove remaining biotin and other reagents. Quantitation of CS poly- and oligosaccharides and their modified forms was carried out by carbazole assay (38).

Preparation of CS Oligosaccharide Fragments and Their NGL Probes—Oligosaccharides were prepared as described previously (36, 38) from CSA, CSC, and de-6-O-sulfated CSA (CSA-de6S) by partial depolymerization with chondroitinase ABC (49) followed by size fractionation using a Bio-Gel P-6 column (1.6 × 90 cm) with elution by ammonium acetate (0.2 M). The 12-mer and 14-mer fractions isolated from partially digested CSA-de6S were each further fractionated by strong anion-exchange HPLC into six subfractions. All six 12-mer subfractions (containing zero to six 4-O-sulfates, designated as subfractions CSA-12-0S to -6S, respectively) and two 14-mer subfractions containing five and seven sulfates (designated as CSA-14D and CSA-14F, respectively) were used in inhibition assays after quantitation by carbazole assay. The chain length and sulfate content of the fractions and subfractions were determined by electrospray mass spectrometry using optimized conditions to minimize potential sulfate loss (38, 50). HPLC disaccharide composition analysis was performed as described previously (38).

NGL probes of CSA and CSC oligosaccharide fragments were prepared by conjugation to 1,2-dihexadecyl-sn-glycero-3-phosphoethanolamine (Fluka, Dorset, UK) as described previously (47). In brief, lyophilized oligosaccharide fragments (typically 100 nmol) were mixed with 5 μl of water, 100 μl of 1,2-dihexadecyl-sn-glycero-3-phosphoethanolamine stock solution (7 nmol/μl in CHCl3/MeOH, 1:3, v/v), and 10 μl of freshly prepared tetraethylammonium cyanoborohydride solution (20 μg/μl in MeOH). The mixture was incubated at 60 °C for 96 h. NGLs of CS oligosaccharides were purified on a minicolumn and analyzed by high performance TLC and mass spectrometry (47).

Parasite Culture—P. falciparum was maintained in continuous culture (9, 36) using human group O positive erythrocytes. Parasite isolate E8B (or FAF-EA8) (51) is a clone of Brazilian isolate ItG2. CS2 was derived from E8B by selection for adherence to CSA (6). HCS3 was originally isolated from a traveler to Asia and was generated by selection for adherence to immobilized CSA (35, 36). 3D7 was selected multiple times for adhesion to immobilized CSA to generate isolate 3D7-CSA (34, 35). Isolates CS2, HCS3, and 3D7-CSA are genetically distinct and were reselected for adherence to CSA prior to use in assays. The specificity of adhesion of the different isolates to CSA has been established elsewhere (35, 36). Isolate 3CI was generated from 3D7 by selection for adhesion to ICAM-1 (52). The phenotypes and properties of the different isolates are shown in supplemental Table S2.

Parasite Adhesion Assays—Adhesion assays were performed using trophozoite-infected erythrocytes at 3–5% parasitemia and 1% hematocrit, resuspended in RPMI-HEPES with 5% pooled serum from non-exposed donors (9). Adhesion receptors used were CS polysaccharides and various desulfated analogs described above together with CSA from porcine rib cartilage (CSA-PR, Sigma), CD36, and recombinant human ICAM-1 (Bender MedSystems). Oligo- and polysaccharides were tested for inhibitory activity by preincubating with parasite suspensions for 15 min prior to testing for adhesion (36, 38). Receptor-bound IEs were stained with Giemsa stain and counted by microscopy. Parasite adhesion was also examined using receptor-coated beads. Streptavidin-coated beads (M288, Dynal, Melbourne, Australia) were incubated with biotin-CSA or biotin-CS (100 μg/ml in PBS) for 30 min and then blocked with 1% bovine serum albumin (BSA) or 1% casein in PBS for 1 h or overnight. IEs, labeled with 10 μg/ml ethidium bromide, were incubated with receptor-coated beads at 3–5% parasitemia and 5% hematocrit in RPMI-HEPES with 5% pooled human serum on a rotating wheel for 60–120 min at room temperature. Cell suspensions (10 μl) were examined by combined light and fluorescence microscopy.

Adhesion assays with ex vivo placental tissue were performed as described previously (12). Briefly trophozoite stage IEs were purified using gelatin and resuspended at 5 × 10⁷ IEs/ml in RPMI (pH 6.8) containing 10% human serum. Test oligosaccharides and polysaccharides were preincubated with IE suspensions for 15 min before incubating on unfixed sets of three consecutive 5-μm cryosections of normal human placenta. IEs were allowed to adhere to placental sections for 1 h with agitation every 20 min followed by gentle washing in PBS to remove unbound IEs. Following fixing in 2% glutaraldehyde and staining with Giemsa the average number of adherent IEs (±S.E.) was determined for at least three independent cryosections. Binding was compared with untreated controls present on each slide. Placental tissue was obtained after informed written consent and with approval from the Mater Human Research Ethics Committee, Brisbane, Australia (approval number 751M).

Extraction of Parasite Protein PfEMP1 and Its Detection by Western Blotting—PfEMP1 was extracted from pigmented trophozoite stage IEs in culture at 10–15% parasitemia. IEs were lysed with cold 1% Triton X-100 in PBS containing protease inhibitors. Insoluble proteins were resuspended in 2% SDS in PBS by vigorous mixing over several minutes to achieve a saturating concentration of proteins (34, 53). After centrifugation at high speed, the supernatant containing PfEMP1 (confirmed by Western blotting) was diluted 1:20 in cold RPMI-HEPES with 0.5% Triton X-100, 1% BSA (or 0.1% casein), and protease inhibitors and then passed through a 0.45-μm-pore size filter before use in binding assays.
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Western blotting for detection of PfEMP1 was performed as described previously (34, 53) using Triton X-100-insoluble proteins isolated from pigmented trophozoite stage IEs and resolubilized in 2% SDS. Proteins on nitrocellulose membranes were probed with an affinity-purified antiseraum raised in rabbits against a conserved sequence in the acid terminal segment (ATS) of PfEMP1 or a monoclonal antibody against ATS (26, 34).

Binding of PfEMP1 to CS Polysaccharides and Oligosaccharide NGL Probes—For PfEMP1 binding to receptor-coated beads, biotin-CSA or biotin-CSC (prepared as described above) or biotinylated BSA (Sigma) were incubated at 100–200 μg/ml in PBS with streptavidin-coated beads (Dynal) for 1 h at room temperature and then blocked with 1% casein in PBS overnight at 4 °C. Beads were incubated with PfEMP1 extract for 1 h at room temperature or 4 h at 4 °C and then washed three times with RPMI-HEPES with 0.5% Triton X-100 and three times with RPMI-HEPES. Bound proteins were eluted with SDS-PAGE sample buffer at 100 °C and detected by SDS-PAGE and Western blotting with PfEMP1-specific anti-ATS antibody.

To perform plate-based binding assays, 96-well microtiter plates (Falcon 3072, BD Biosciences; or Maxisorb, Nunc) were incubated with polysaccharides (50 μg/ml in PBS) or NGLs of oligosaccharides (1–5 μM in water or PBS) overnight at 4 °C. Polysaccharides used were CSA, CSB, CSC, and heparan sulfate (from bovine kidney; Sigma). Oligosaccharide NGLs of 2–20 monosaccharide units prepared from CSA and CSC were used. After coating, wells were washed and blocked for 2 h at 37 °C or overnight at 4 °C with 4% BSA in PBS with 0.05% Tween 20 or with 1% casein in PBS (Pierce). After washing, wells were incubated with IE membrane extract for 2 h at 37 °C and then washed with RPMI-HEPES containing 0.5% Triton X-100 and 1% BSA. To detect bound PfEMP1, wells were incubated with affinity-purified rabbit antibodies or a mouse monoclonal antibody generated against the ATS of PfEMP1, diluted in RPMI-HEPES with 1% BSA and 0.5% Triton X-100, for 1 h at room temperature; then washed; and incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:500 in the same buffer) for 45 min. After washing with RPMI-HEPES with 0.5% Triton X-100, color was developed with azino-bis(3-ethylbentihazoline-6-sulfonic acid) liquid substrate system (Sigma-Aldrich), and absorbance was read by spectrophotometry. Binding was measured as absorbance less background levels in control wells that were coated with BSA or PBS only, and results from each experiment were standardized and expressed in arbitrary units where levels for CSA binding were designated a value of 100. Prior studies have established the specificity of anti-ATS antibody for PfEMP1 and that the antibody does not label other parasite proteins or erythrocyte proteins by Western blot (26, 34). To confirm that PfEMP1 is responsible for the interaction, bound proteins were eluted from the surface of multiple CSA-coated wells by sequential incubation with 50 μl of SDS-PAGE sample buffer. Heparan sulfate-, CSB-, and BSA-coated wells were also used and eluted in the same way as controls. The eluted proteins were fractionated by SDS-PAGE followed by Western blotting for detection of PfEMP1.

In alternate assays, microtiter plates (96-well, Maxisorb, Nunc) were incubated with an anti-ATS monoclonal antibody (2 μg/ml) overnight at 4 °C. Wells were washed and blocked for 1–2 h with 1% casein in PBS (Pierce). After washing, wells were incubated with the IE membrane extract containing PfEMP1 diluted in PBS with 0.1% casein and 0.5% Triton X-100 for 1 h at room temperature. After washing with 0.5% Triton X-100 in PBS, wells were blocked with 1% casein in PBS for 30 min and then incubated with biotin-CSA or biotin-CSC (10 μg/ml) or buffer only followed by streptavidin-horseradish peroxidase (1 μg/ml; Dako) in PBS with 0.1% casein and 0.05% Tween 20 for...
1 h at room temperature for each step. After washing with PBS, color was developed with azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), and absorbance was read by spectrophotometry.

RESULTS

 Extraction and Identification of Native PfEMP1 and Its Specific Binding to Immobilized CSA Polysaccharides—We first sought to determine whether native PfEMP1 isolated from parasite cultures binds specifically to immobilized CSA in a manner similar to the characteristics of IE adhesion. Membrane-bound proteins were isolated from mature stage IEs of the CSA-binding isolate CS2 and tested for binding to biotinylated CSA, CSC, and BSA immobilized on streptavidin-coated beads. Proteins eluted from the beads were probed with anti-ATS antibodies to detect PfEMP1 by Western blotting. A single PfEMP1 band was observed in proteins eluted from CSA-coated beads, but only a faint band from CSC-coated beads was observed (Fig. 1A). No band from BSA-coated beads was detected. The size of the eluted PfEMP1 (~300 kDa) corresponded to the dominant PfEMP1 band detected in Western blots of CS2 IE extracts. When we probed membranes with pooled serum from exposed pregnant women, no additional bands were found that were specific to the CSA-coated beads (data not shown). We confirmed that intact CS2 IEs bind to biotin-CSA immobilized on beads (Fig. 1B). Most (>90%) CS2 IEs bound CSA-coated beads, whereas there was little or no binding of IEs to CSC- or BSA-coated beads or to uncoated beads (Fig. 1C) or of uninfected erythrocytes to CSA-coated beads (Fig. 1B).

We next aimed to develop an assay that would allow further investigations to define the specificity of interactions between PfEMP1 and CS. Direct binding assays were performed with CSA and other molecules coated in wells of microtiter plates, and bound PfEMP1 was detected by enzyme immunoassays with anti-ATS antibodies. Across repeated experiments, PfEMP1 extracted from CS2 IEs bound to immobilized CSA (Fig. 1D) but not to CSB or CSC in agreement with the adhesion characteristics of intact IEs (6, 36). Positive binding to CSA, but not CSC, was observed when using affinity-purified polyclonal anti-ATS or monoclonal anti-ATS antibodies. Specific binding of PfEMP1 was also observed with CSA covalently linked to plastic surfaces using Nunc CovaLink or amino Immobilizer plates (data not shown). PfEMP1 extracted from isolate E8B did not show significant binding to CSA (data not shown); E8B IEs do not adhere to CSA (35). To further validate the assay and establish that PfEMP1 was the protein detected in the assays, proteins bound in wells were eluted and examined by Western blotting. PfEMP1 was detected in elutes from CSA-coated wells, whereas little or no protein was detected from control wells coated with heparan sulfate (Fig. 1E), CSB, or BSA (data not shown). Using an alternate approach, PfEMP1 was captured in wells coated with an anti-ATS monoclonal antibody and tested for binding of biotinylated CS in solution. We observed specific binding of biotin-CSA to PfEMP1 compared with biotin-CSC (Fig. 1F).

Direct Binding of Native PfEMP1 to CS Oligosaccharides Depends on Their Chain Length and Sulfation Pattern—We further examined interactions between PfEMP1 and CS oligosaccharides by direct binding assays using oligosaccharide NGL probes to determine the specificity of CS binding to PfEMP1, including the chain length and sulfation pattern. Oligosaccharide fragments of CSA and CSC were converted into NGL probes by conjugation to an aminolipid for immobilization on solid matrices. The importance of sulfation pattern of CS for interaction with PfEMP1 isolated from CS2 IEs was demonstrated with the largest NGLs (20-mers) derived from CSA and CSC (Fig. 2A). We observed higher levels of binding of PfEMP1 probes to determine the specificity of CS binding to PfEMP1, including the chain length and sulfation pattern. Oligosaccharide fragments of CSA and CSC were converted into NGL probes by conjugation to an aminolipid for immobilization on solid matrices. The importance of sulfation pattern of CS for interaction with PfEMP1 isolated from CS2 IEs was demonstrated with the largest NGLs (20-mers) derived from CSA and CSC (Fig. 2A). We observed higher levels of binding of PfEMP1...
from CS2 IEs to the CSA NGLs compared with CSC NGLs. In additional experiments, we observed similar findings using CSA and CSC 18-mer NGLs (data not shown). As a reflection of specificity, PfEMP1 isolated from CS2 IEs bound CSA 18-mer and 20-mer NGLs, whereas PfEMP1 from the ICAM-1-binding isolate 3CI did not bind to CSA NGLs (Fig. 2B).

Testing NGLs of CSA oligosaccharides with 2–18 monosaccharide units in length suggests significant binding occurs with 8-mer or longer chain oligosaccharide NGLs (Fig. 2C). This pattern is similar to size-dependent inhibition of IE adhesion to CSA by oligosaccharides with which 12-mer or larger fragments were required for maximum inhibition of adhesion to immobilized CSA (36–38).

Structural Requirements for IE Adhesion to Placental Tissue—Having defined structural requirements of CS for binding PfEMP1 and confirming that PfEMP1 is a ligand for binding CS, we next investigated the specificity of IE adhesion to placental tissue to further define the molecular basis of parasite sequestration. This was studied by inhibition of the interaction using various sequence-defined CS oligo- and polysaccharides (Table 1) (38) that may have potential for therapeutic development.

Oligosaccharide fractions of 4–18 monosaccharide residues in length derived from either CSA or CSC were tested for inhibition of adhesion equivalent to that observed with the polysaccharide and shown to have the capacity to inhibit the adhesion of IEs to fresh placental cryosections using the parasite line CS2 (Fig. 3A) to determine the minimum chain length for interaction. The inhibitory effects of the CSA oligosaccharides were highly dependent on length. Maximum inhibition of adhesion equivalent to that observed with the polysaccharide was found with the CSA-derived 10-mer or larger oligosaccharides at 10 μg/ml. Substantial inhibition (83%) was also observed with CSA 8-mer at 20 μg/ml (data not shown) but not at 10 μg/ml. By comparison, inhibition by the 10-mer was 79% (Fig. 3A) and 98% (data not shown) at 10 and 20 μg/ml, respectively. Little inhibition was observed with shorter CSA oligosaccharides or CSC-derived oligosaccharides of the same length.

The inhibitory activities of partially desulfated polysaccharides and oligosaccharides demonstrated the significance of the extent and pattern of sulfation of CS for parasite adhesion to placental tissue (Fig. 3B and Table 1). CSA-derived 14-mers effectively inhibited adhesion. CSA 14-mer subfraction CSA-14F was composed of 13% 0S, 46% 4S, and 41% 6S, and CSA-14F was composed of 1% 0S, 55% 4S, and 44% 6S (38). Their inhibitory activity was enhanced by selective de-6-sulfation (Fig. 3B), indicating that 6-O-sulfation inhibits parasite-CS interactions even when a sufficient number of 4-O-sulfate groups are present. CSA-14F-de6S (55% 4S) was somewhat more inhibitory than CSA-14D-de6S (45% 4S). CSC-de6S (16% 4S) was not inhibitory, whereas CSA-de6S (55% 4S and 45% 0S) was highly inhibitory, indicating the significance of the extent of 4-O-sulfation for placental adhesion.

To evaluate the importance of GlcUA versus IdoUA, we compared the inhibitory activity of CSA and CSB. However, CSB has a much higher level of 4-O-sulfation than CSA (Table 1). Therefore, CSB was partially de-4-O-sulfated to enable a more direct comparison with CSA. CSB-de4S was composed of 50% 4S and 46% 0S disaccharides, similar to the sulfation level of CSA-de6S, but has IdoUA rather than GlcUA (36, 38). CSB-de4S was not inhibitory compared with CSA-de6S, indicating the importance of uronic acid type for adhesion (Fig. 3B). Furthermore a CSA 14-mer was relatively non-inhibitory compared with CSA 14-mers. CSB with or without various degrees of desulfation (30–50% 0S) did not inhibit adhesion of IEs to immobilized CSA (data not shown).

In earlier experiments we found that partially 4-O-sulfated CSA oligosaccharides, generated by selective de-6-O-sulfation, were the most effective inhibitors of adhesion to immobilized CSA polysaccharide (38). We demonstrated here that these are also highly effective inhibitors of placental adhesion. The inhibitory activity of the partially sulfated CSA 14-mers, CSA-14F-de6S, was equivalent to that shown with CSA-de6S (Fig. 3C); both preparations (CSA-de6S and CSA-14F-de6S) contained around 55% 4S and 45% 0S disaccharides, which is similar to the level of 4-O-sulfation of optimal 12-mer inhibitors (Fig. 4A). The IC$_{50}$ of CSA-14F-de6S was 0.15 μg/ml.

### Table 1

| Composition | Activity in parasite assays$^b$ | 0S  | 2S  | 4S  | 6S  | 2,6DIS | 4,6DIS | 2,4DIS |
|-------------|--------------------------------|-----|-----|-----|-----|-------|-------|-------|
| CSA$^c$     |                                | 4.9 | —   | 55.3| 39.8| —     | —     | —     |
| CSR         |                                | 0.5 | —   | 85.6| 4.8 | —     | 6.4   | 2.3   |
| CSC         |                                | 0.8 | —   | 15.5| 76.7| 7.0   | <0.5  | —     |
| CSA-PR      |                                | 4.2 | —   | 66.8| 29.0| —     | —     | —     |
| CSA-de6S$^d$|                                | 45.3| —   | 54.7| <0.5| —     | —     | —     |
| CSA-de4S    |                                | 74.5| 7.8 | 15.9| 1.8 | —     | 1.8   | —     |
| CSA-de4S-1$^e$|                              | 30.1| 2.1 | 64.7| 3.1 | —     | <0.5  | —     |
| CSA-de4S-2  |                                | 45.6| 2.2 | 50.1| 2.2 | —     | <0.5  | —     |
| CSA-14F     |                                | 1.3 | —   | 54.9| 43.7| —     | —     | —     |
| CSA-14F-de6S|                                | 46.6| —   | 55.0| 0.4 | —     | —     | —     |
| CSA-14D     |                                | 13.3| —   | 45.6| 41.1| —     | —     | —     |
| CSA-14D-de6S|                                | 53.5| —   | 44.9| 1.6 | —     | —     | —     |
| CSB         |                                | 5.3 | —   | 88.4| 6.2 | —     | —     | —     |

$^a$ 2S, dUA(2S1–3GalNAc; 2,6DIS, dUA(2S1–3GalNAc(6S); 4,6DIS, dUA1–3GalNAc(4,6S); 2,4DIS, dUA(2S1–3GalNAc(4S). Trisulfated disaccharides were not detected. —, not detected.

$^b$ Compounds inhibited adhesion of P. falciparum-infected erythrocytes to immobilized CSA and/or placental tissue. —, little or no inhibition; +/−, some inhibition with some P. falciparum isolates only; +, inhibitory; ++, highest inhibitory activity. See text for details.

$^c$ CSA and CSC contained undetectable levels of IdoUA; CSB contained predominantly IdoUA (36, 38).

$^d$ de6S, prepared by regioselective de-6-O-sulfation (38); de4S, prepared by partial solvolytic desulfation (38).
Strain-specific Variation in the Specificity of Interactions Relates to Polymorphisms in PfEMP1—We sought to identify oligosaccharides with optimal inhibitory activity against different isolates and evaluate differences between strains in the specificity for CS structural motifs for adhesion that relate to polymorphisms in var2csa PfEMP1. We used three genetically distinct CSA-binding isolates, CS2, HCS3, and 3D7-CSA (supplemental Table S2) (6, 35, 36). Analysis by Northern blots and real time PCR demonstrated that the three isolates express var2csa-type genes as the dominant var gene transcripts (23, 54), and Western blots showed that each expresses a single surface-exposed PfEMP1 variant of apparently the same Mr (34). Isolates were recognized in a parity- and gender-associated manner by sera from exposed donors (34, 55), suggesting that they are representative of CSA-binding variants expressed in malaria during pregnancy.

By comparison of the inhibitory activities of defined CS oligo- and polysaccharides (Table 1) on adhesion of the different isolates, several common features were observed (Fig. 4). For all three isolates inhibition of adhesion was dependent on 4-O-sulfated GalNAc and GlcUA as the predominant uronic acid rather than iduronic acid, whereas 6-O-sulfation interfered with the binding interaction. Additionally using defined 12-mers with varying levels of 4-O-sulfation, we demonstrated that inhibition was greater with oligosaccharides containing mixed 4-O-sulfated and non-sulfated disaccharides for all three isolates. CSA-de6S (54.7% 4S and 45.3% 0S) more effectively inhibited adhesion of all three isolates than did unmodified
CSA (composition: 4.9% 0S, 55.3% 4S, and 39.8% 6S). CSC and partially de-4-O-sulfated CSB had much less inhibitory activity. Partially desulfated CSB (64.7 or 50.1% 4-O-sulfation for CSB-de4S-1 and CSB-de4S-2, respectively) were used for comparison with the activity of CSA-de6S; the limited inhibitory activity of CSB-de4S can therefore be attributed to the presence of IdoUA in CSA-de6S.

The isolates differed in the pattern and extent of inhibition by defined oligo- and polysaccharides and varied in the level of sulfation of test inhibitors needed for optimal inhibition of adhesion. The level of sulfation required for optimal inhibition by oligo- and polysaccharides was greater for CS2 than HCS3 and 3D7-CSA and somewhat higher for HCS3 than 3D7-CSA (Fig. 4). The optimal sulfate content of 12-mer for inhibition of IE adhesion was four or five sulfate groups (67–83% sulfation) for CS2, three or four sulfate groups (50–67% sulfation) for HCS3, and three sulfate groups for 3D7-CSA. The 12-mer fraction with an average of two sulfate groups (33% sulfation) was relatively inhibitory against 3D7-CSA but not CS2 or HCS3. Those with zero or one sulfate group (0–17% sulfation), on average, were relatively non-inhibitory for all isolates. The 12-mer fraction with five sulfate groups was most effective with CS2 IEs but had less activity with HCS3 or 3D7-CSA. Some inhibition was also observed with fully 4-O-sulfated 12-mers, but this was less than the inhibition with the partially 4-O-sulfated 12-mers for all three isolates. Additionally HCS3 and 3D7-CSA IEs were substantially inhibited by CSC-de6S polysaccharide, which has a lower level of 4-O-sulfation (16%) than CSA-de6S (55%), whereas adhesion of CS2 IEs was not substantially inhibited by CSC-de6S. We found no major differences between isolates in the minimum oligosaccharide chain length for maximum inhibition of IE adhesion to CSA (data not shown).

The activity of different inhibitors was not influenced by the degree and pattern of sulfation of the CS receptor used in adhesion assays. To enable the most direct evaluation, we used CSA compared with CSA-de6S as the receptors. Inhibition of adhesion of CS2 IEs to both receptors was maximal with 12-mers composed of four or five 4-O-sulfated disaccharide units balanced with non-sulfated disaccharide units (Fig. 5A). There was no substantial difference in the pattern of inhibitory activity of the oligosaccharides when using CSA compared with CSA-de6S. Furthermore inhibition of adhesion to CSPGs isolated from placental blood, which have very low levels of sulfation, was also maximal with 12-mers having four or five sulfate groups (data not shown). These findings suggest that the specificity of parasite-CS interactions is determined by parasite factors such as the PfEMP1 type expressed.

Differences between isolates in their fine specificity for CS interaction were also observed in direct adhesion assays of IEs to immobilized CSA polysaccharides (Fig. 5B). For all isolates, adhesion was substantially higher with CSA-de6S than the parent CSA, consistent with observations of greater inhibitory activity of partially 4-O-sulfated oligosaccharides. There was a large difference between CS2 and 3D7-CSA IEs in binding to unmodified CSA compared with CSA-de6S. Adhesion of 3D7-CSA to CSA was only 12% of adhesion to CSA-de6S, whereas adhesion of CS2 IEs to unmodified CSA was 58% of adhesion to CSA-de6S. These findings reflect the preference for higher levels of CS sulfation by CS2 versus 3D7-CSA that were observed in inhibition assays (Fig. 4) and indicate that CS2 is less affected by the presence of 6-O-sulfation. CS2 IEs also bound well to CSA-PR (64% of CSA-de6S binding), whereas 3D7-CSA bound very poorly to this preparation (2% of CSA-de6S levels). The disaccharide composition of CSA-PR was determined as 4.2% 0S, 66.8% 4S, and 29% 6S, which is very similar to the composition of CSA from bovine trachea used in our assays (4.9% 0S, 55.3% 4S, and 39.8% 6S). However, the 4-O-sulfated disaccharides of CSA-PR contain equal amounts of GlcUA and IdoUA, whereas CSA from bovine trachea has no detectable IdoUA (38, 56). None of the isolates adhered to CSC, CSB, or partially de-4-O-sulfated CSB (Fig. 5B and data not shown).

**DISCUSSION**

These studies establish several important principles regarding the molecular basis of interactions among PfEMP1, CSA, and placental tissue that are highly relevant to understanding malaria pathogenesis and biology. We demonstrated that native PfEMP1, isolated from CS2 IEs and encoded by var2csa, can bind to immobilized CSA but not CSC or CSB, consistent with the specificity observed with intact IEs. We successfully adapted this to an assay method using a 96-well plate format that can be used to probe CS-PfEMP1 interactions in further detail. Although recombinant var2csa PfEMP1 domains have been shown to bind to CSA (22), it was important to establish...
that native PfEMP1 also binds in a specific manner because expression of single recombinant domains may not accurately reflect the binding activities of full-length PfEMP1 (30, 57, 58). This is highlighted by studies reporting that several recombinant Duffy-binding-like y domains can bind CSA, but their role in mediating IE adhesion or PfEMP1 binding to CSA remains unclear (30, 59). Our findings further support var2csa PfEMP1 as the key ligand for adhesion to CSA and its role in placental malaria. It remains possible that other proteins could be involved with PfEMP1 as part of a receptor-binding complex (60); however, no other genes have yet been identified. Our approach using native PfEMP1 may be valuable for complementing future studies of the CSA binding activity of recombinant PfEMP1 domains expressed in other systems (22, 27) and to identify effective inhibitors.

We demonstrated direct binding of native PfEMP1 to CS oligosaccharides as NGL probes, which were derived from conjugation of CS oligosaccharide fractions to an aminophospholipid. The NGLs were immobilized on solid matrices and probed with PfEMP1. The NGL approach has been used to study many other carbohydrate-protein interactions (61, 62) but has not been applied previously either to protein-GAG interactions or to malaria research. In previous investigations, the minimum motif for interactions between IEs and CSA was determined by inhibition of IE adhesion to purified immobilized CSA or CSPGs (36–38). In the present study, we found that the minimum chain length for direct binding of PfEMP1 was eight monosaccharide units, whereas prior studies found that 12-mers were required for inhibition of IE adhesion. These differences may reflect real differences in requirements for PfEMP1 binding compared with requirements for inhibition of cellular adhesion. Alternatively the clustered presentation of oligosaccharides immobilized as NGLs (62) may have increased the protein binding activity of shorter chain oligosaccharides. Furthermore we demonstrated the importance of 4-O-sulfated GalNAc and GlcUA rather than IdoUA for PfEMP1 binding. PfEMP1 bound CSA oligosaccharide NGLs at higher levels than NGLs derived from CSC; the difference between oligosaccharide NGLs from CSA and CSC of the same length was their pattern of sulfation (predominantly 4-O-sulfated versus 6-O-sulfated for CSA and CSC, respectively) (36). The lack of binding to CSB indicated the importance of GlcUA rather than IdoUA for the interaction. These findings have important implications for understanding the molecular basis of adhesion, including structural studies that may identify critical residues for binding.

The structural features for IE adhesion to placental tissue were similar to those determined for direct binding of PfEMP1. There was a requirement for 4-O-sulfated GalNAc mixed with non-sulfated GalNAc, GlcUA rather than IdoUA was important, and 6-O-sulfation inhibited the interaction with IEs. Candidate CSA receptors in the placenta, including CSPGs of the intervillous blood, cell-associated CSPGs, and thrombomodulin, vary in their level of sulfation, but all contain substantial amounts of non-sulfated disaccharides in addition to 4-O-sulfated disaccharides (supplemental Table S1). The minimum chain length for maximal inhibition of adhesion was 10-mer. This is shorter than that required for inhibition of IE adhesion to immobilized CSA and was similar to results from PfEMP1 binding assays.

The structural basis of placental adhesion has not been defined previously. Prior placental adhesion studies showed that CSA, but not CSC, polysaccharides inhibited adhesion (10, 12). Overall results from placental adhesion studies were remarkably similar to our previous results obtained by testing inhibition of adhesion to immobilized purified CSA (38). However, the difference in minimum chain length for inhibition highlights the need for studies using placental tissue, or other cell types, to confirm results obtained using purified CS preparations. Our findings that IEs adhere better to CSA-de6S than to unmodified CSA and that there is variation among isolates in the preference for undersulfated motifs may partly explain prior observations that placental isolates vary in the efficiency with which they adhere to immobilized CSA preparations (9).

We identified partially 4-O-sulfated CSA 14-mers and polysaccharides, generated by de-6-O-sulfation of commercially available CSA, as highly effective inhibitors of placental adhesion that may have potential for therapeutic development. The IC50 values are the lowest reported for any placental adhesion inhibitor to date and are within a range that has been achieved previously in vivo by administration of CS to humans (63). CSA polysaccharide has an average molecular mass of 45 kDa, equivalent to ~200 monosaccharide residues in length. It is not surprising that, on a molar basis, CSA polysaccharides are several-fold more active in inhibition assays than 14-mers (average molecular mass, 3.135 kDa) due to multiple recognition motifs present along the polysaccharide chain. Although there were differences between isolates, we identified common features of CS–parasite interactions. These include a consistent preference or requirement for 4-O-sulfated mixed with non-sulfated GalNAc together with GlcUA rather than IdoUA, whereas 6-O-sulfation of GalNAc inhibited CS interaction with IEs. These requirements correspond with the composition of known CSPGs available for adhesion in the vasculature (supplemental Table S1). Oligosaccharides with 50–67% sulfation would be effective against many isolates as they are active against the three isolates tested here. Variation between isolates in the level of 4-O-sulfation required for maximum inhibition may limit the ability to design and synthesize a single oligosaccharide sequence that effectively inhibits adhesion of all isolates; oligosaccharide mixtures may be required.

Isolate-specific variation among P. falciparum IEs in the preferred structural motifs for adhesion to CSA correlates with polymorphisms in PfEMP1 encoded by var2csa-type genes. Three different isolates expressing polymorphic variants of var2csa varied in their sensitivity to inhibition of adhesion by oligo- and polysaccharides with different levels of sulfation and varied in their levels of direct binding to immobilized CS polysaccharides with differing disaccharide compositions. These findings have major implications for the potential development of therapies or vaccines against maternal malaria and for structural studies of receptor-ligand interactions. Structural and functional studies established with a single variant will need to be extended to different variants. The apparent effect of ligand polymorphism on specificity is likely to apply to other pathogens that use GAGs for adhesion and host cell invasion.
and warrants further investigation. We have recently shown that polymorphisms in var2csa influence antibody binding and are a likely mechanism of immune evasion (34). It is also possible that the evolution of polymorphisms in CSA-binding ligands may have partly evolved as an adaptive response to facilitate parasite interactions with the diverse range of CS structural motifs in the placenta and wider vasculature. An ability to bind a broad range of different CSPGs in the vasculature would be a survival advantage for parasites through evasion of splenic clearance from the circulation.

Differences in the specificity of CS interaction by isolates may be relevant to disease pathogenesis by influencing the sites and extent of parasite sequestration and the structures to which CSA-binding IEs adhere in the vasculature. There is substantial diversity in the level and pattern of sulfation of CSPGs in the vasculature and placenta (supplemental Table S1), which may influence the ability of these molecules to support adhesion of different variants. *P. falciparum* infections in pregnancy are typically polyclonal (64), so it is likely that circulating IEs of different genotypes each have a different polymorphic var2csa variant that may preferentially adhere to different CS structural motifs in the placenta and other vascular beds. An ability to sequester, to some extent, outside the placenta may contribute to inefficient clearance of IEs by local placental immune responses and facilitate the maintenance of chronic parasitemia. Evidence suggests some CSA-binding variants are not restricted to malaria in pregnancy and may establish infection, to some extent, in non-pregnant individuals (6, 9, 65, 66) supporting the idea that some CSA-binding variants can adhere in vascular beds other than the placenta.

These findings have significant implications for understanding the pathogenesis and biology of malaria, particularly during pregnancy, and for the development of targeted interventions. The results presented here are, to our knowledge, the first indication of potential influence of protein polymorphisms on the fine specificity of GAG binding interactions and have broad relevance to other systems and invasive pathogens that interact with host cell GAGs.

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