The Low Sulfated Chondroitin Sulfate Proteoglycans of Human Placenta Have Sulfate Group-clustered Domains That Can Efficiently Bind Plasmodium falciparum-infected Erythrocytes*

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Plasmodium falciparum infection in pregnant women results in the chondroitin 4-sulfate-mediated adhesion of the parasite-infected red blood cells (IRBCs) in the placenta, adversely affecting the health of the fetus and mother. We have previously shown that unusually low sulfated chondroitin sulfate proteoglycans (CSPGs) in the intervillous spaces of the placenta are the receptors for IRBC adhesion, which involves a chondroitin 4-sulfate motif consisting of six disaccharide moieties with ~30% 4-sulfated residues. However, it was puzzling how the placental CSPGs, which have only ~8% of the disaccharide 4-sulfated, could efficiently bind IRBCs. Thus, we undertook to determine the precise structural features of the CS chains of placental CSPGs that interact with IRBCs. We show that the placental CSPGs are a mixture of two major populations, which are similar by all criteria except differing in their sulfate contents: 2–3% and 6–14% of the disaccharide units of the CS chains are 4-sulfated, and the remainder are nonsulfated. The majority of the sulfate groups in the CSPGs are clustered in CS chain domains consisting of 6–14 repeating disaccharide units. While the sulfate-rich regions of the CS chains contain 20–28% 4-sulfated disaccharides, the other regions have little or no sulfate. Further, we find that the placental CSPGs are able to efficiently bind IRBCs due to the presence of 4-sulfated disaccharide clusters. The oligosaccharides corresponding to the sulfate-rich domains of the CS chains efficiently inhibited IRBC adhesion. Thus, our data demonstrate, for the first time, the unique distribution of sulfate groups in the CS chains of placental CSPGs and that these sulfate-clustered domains have the necessary structural elements for the efficient adhesion of IRBCs, although the CS chains have an overall low degree of sulfation.

A distinctive feature of Plasmodium falciparum compared with the other three human malaria parasites is its ability to express adherent protein(s) on the surfaces of the infected red blood cells (IRBCs) and thereby sequester in the microvasculature of various organs by adhering to endothelial cell surfaces (1–5). The extensive accumulation of IRBCs in vital organs causes capillary blockage with deprivation of oxygen and nutrients and production of toxic levels of proinflammatory cytokines (3, 6–10), damaging the endothelial lining and causing organ dysfunction and severe pathological conditions. A number of studies have shown that the adherent protein expressed on the surfaces of IRBCs to be P. falciparum erythrocyte membrane protein 1 (EMP1), a multidomain, antigenic var gene family protein (11–17). P. falciparum EMP1 can bind, in a domain-specific manner, CD36, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, E-selectin, platelet endothelial cell adhesion molecule-1/CD31, and thrombospondin on vascular endothelial cell surface (18–24). In addition, P. falciparum EMP1 can also bind complement receptor (25), heparan sulfate (26), and chondroitin 4-sulfate (C4S) (22–28). Thus, the parasite, by its ability to express divergent P. falciparum EMP1s using the var gene repertoire, can adhere to various organs. However, over a period of time, the host develops antibodies against the exposed P. falciparum EMP1 that are able to inhibit adhesion of IRBC adhesion and aid clearance of infection (29–34). To overcome this defensive mechanism, the parasite constantly switches, at low frequency, to various adherent phenotypes by expressing P. falciparum EMP1s with different receptor specificity (35, 36). This ability of the parasite to express P. falciparum EMP1, for which the host has not yet developed adhesion-inhibitory antibodies, enables it to selectively adhere through a different receptor. In this manner, when one adherent phenotype of parasite is eliminated by the host, another phenotype continues to thrive. In endemic areas, people by adulthood acquire a broad spectrum protective immunity against P. falciparum, including antibodies to P. falciparum EMP1s (37, 38). Therefore, in immune-protected people, the IRBCs cannot adhere in the vascular capillaries, limiting the parasite growth.

In pregnant women, however, the placenta provides a new opportunity for IRBC adhesion, because women lack immunity against placenta-adherent parasites prior to pregnancy (39). Extensive adherence of IRBCs in the placenta and infiltration of mononuclear cells in response to the infection results in impaired placental function, leading to poor fetal outcome and maternal morbidity and mortality (40–46). However, women acquire placental malaria-specific immunity, including adhesion-inhibitory antibody response, during the first and second
pregnancies (29–34). Therefore, primigravidas are at highest risk of placcental malaria, and the susceptibility diminishes with increasing gravidity (44, 45).

C4S mediates the adhesion of IRBCs in the human placenta (39, 47–49). Previously, we have shown that the C4S motif localized in the intervillous spaces of the placenta are the receptors for the adherence of IRBCs in the placenta (50). These C4S motifs were found to be unusually low sulfated; on an average, only 8% of the disaccharide repeating units of the CS chains of placental C4Ss are 4-sulfated, and the remainder are nonsulfated (50). In previous studies, we have also shown that IRBC adhesion involves the participation of both nonsulfated and 4-sulfated disaccharide repeating units and the optimal binding requires ~30% 4-sulfated and ~70% nonsulfated disaccharide repeats (51). Further, we have established that a C4S motif having six disaccharide repeating units (6-mer) with two 4-sulfated and four nonsulfated disaccharide units is the minimum structural motif required for optimal binding of IRBCs (51). A recent study confirmed most of our findings (52), except that four or five rather than two of the disaccharide repeating units of the binding motif containing six-disaccharide repeating units needs to be 4-sulfated for effective IRBC binding. However, it should be noted that these investigators measured C4S-IRBC interactions by immobilizing the commercially available bovine trachea C4S/C6S copolymer (52), a nonrelevant glycosaminoglycan, rather than the placental C4Ss, the natural receptor used in our study (50).

Regardless of this discrepancy, it remained a puzzle how IRBCs are able to efficiently bind the unusually low sulfated CS chains of the placental intervillous space C4Ss. A full understanding of the structural requirements for IRBC binding to placenta is important for developing therapeutics or vaccine for placental malaria (53). Therefore, in this study, we investigated in detail the structure of the CS chains of placental C4Ss, particularly the pattern of sulfate group distribution and its correlation to IRBC binding. The placental C4Ss were fractionated into two differentially sulfated proteoglycan populations and their CS chains isolated. The polysaccharide chains were degraded with an endoenzyme that specifically cleaves the nonsulfated regions of the CS into disaccharides, and the oligosaccharide products thus obtained were purified and examined for their ability to inhibit IRBC binding to intact C4Ss. The data demonstrate that, although the overall sulfate content of the CS chains of placental C4Ss are markedly lower than that required for optimal binding, the sulfate groups in the CS chains are clustered in uniquely size-defined domains. These sulfate-rich CS domains have the requisite structural features for the efficient binding of IRBCs.

**EXPERIMENTAL PROCEDURES**

**Materials—**Proteus vulgaris chordinitase ABC (120 units/mg), Streptococcus dysgalactiae hyaluronidase (0.5 units/vial) and Streptomyces hyalurolyticus hyaluronidase (2000 turbidity-reducing units/mg), Pseudomonas aeruginosa heparinase (115 units/mg), chordinitase, and C4S (sturgeon notochord) were purchased from Seikagaku America (Falmouth, MA); ovine testicular hyaluronidase (2160 units/mg) was from ICN Biomedicals; C4S (bovine trachea) was from Sigma; Sepharose CL-6B, Sepharose CL-4B, DEAE-Sephalac, DEAE-Sepharose, and blue dextran were from Amersham Biosciences; Bio-Gel P-6 was from BioRad; HPLC grade 6 M HCl, trifluoroacetic acid, and micro-BCA reagent were prepared for the solvolysis desulfation of a fully 4-sulfated C4S from sturgeon notochord as described previously (51).

**Isolation of CSPGs from Human Placenta—**The low sulfated CSPGs of the placental intervillous spaces were isolated as described previously with minor modification (50). Briefly, the placentas were cut into small pieces and extracted with PBS, pH 7.2, containing protease inhibitors and the extract was applied onto DEAE-Sephalac columns (2.5 × 22 cm). The columns were washed with 25 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, pH 8.0, and then equilibrated with 50 mM NaOAc, 100 mM NaCl, pH 5.5. The bound material was eluted with a linear gradient of 0.1–0.9 mM NaCl in 50 mM NaOAc, pH 5.5. 10-ml fractions were collected, and aliquots were analyzed for uronic acid content (54). The acid-containing fractions corresponding to BCSPG-2, the major CSPG of the placental intervillous spaces (50), were pooled and dialyzed against water. The dialysates were adjusted to 50 mM NaOAc, 100 mM NaCl, pH 5.5, and applied onto DEAE-Sepharose columns (2.5 × 17 cm). The columns were washed with 50 mM NaOAc, 0.15 mM NaCl, pH 5.5, and eluted with a linear gradient of 0.15–0.55 mM NaCl in 50 mM NaOAc, pH 5.5. Fractions of 10-ml were collected, absorption at 280 and 280 nm was measured, and aliquots were analyzed for uronic acid content (54).

**Cesium Bromide Density Gradient Centrifugation of CSPGs—**The crude CSPG fractions obtained by DEAE-Sepharose chromatography were dissolved (1 mg/ml) in 25 mM sodium phosphate, pH 7.2, containing 50 mM NaCl, 0.02% NaN3, 4 mM guanidine hydrochloride, and 42% (w/v) CsBr. The solutions were centrifuged in a Beckman 50 TI rotor at 44,000 rpm for 65 h (55). Gradients were collected from the bottom of the centrifuge tubes into 15 equal fractions and analyzed for uronic acid content (54) and for proteins by measuring the absorption at 260 and 280 nm.

**Size Exclusion Chromatography of CSPGs—**The CSPG fractions obtained from the CsBr density gradient centrifugation step were further purified by chromatography on columns of Sepharose CL-6B (1 × 49 cm) and/or Sepharose CL-4B (1 × 48 cm) in 20 mM Tris-HCl, 150 mM NaCl, pH 7.6, containing 4 mM guanidine hydrochloride. Fractions were collected and monitored for absorption at 280 and 280 nm and for uronic acid content (54).

**Analysis of CSPGs for Purity—**The purified CSPGs (0.3 mg) were treated with S. hyalurolyticus hyaluronidase and heparitinase as described previously (50). The enzyme-incubation mixtures were chromatographed on Bio-Gel P-6 (1 × 47 cm) in 0.1 M acetic acid, 0.1 M pyridine. Fractions (0.67 ml) were collected and analyzed for uronic acid content (54). The enzyme digests were heated at 100 °C for 30 min, centrifuged, and the extracts were applied onto a DEAE-Sepharose column (1 × 10 cm) in 20 mM Tris-HCl, pH 7.8, washed with 20 mM Tris-HCl, 0.15 mM NaCl, pH 7.8, and then eluted with a linear gradient of 0.15–0.6 mM NaCl in the same buffer. Fractions (2.5 ml) were collected, and aliquots were assayed for uronic acid content (54). Uronic acid-positive fractions were pooled, dialyzed against distilled water, and lyophilized.

**Digestion of CS with S. dysgalactiae Hyaluronidase—**The placentals CS chains and chordiniton (1.4–1.8 mg each) were treated with S. dysgalactiae hyaluronidase (300 units/ml) in 250 μl of 100 mM sodium phosphate buffer, pH 6.2, containing 0.02% BSA at 37 °C for 24 h (57). The enzyme digests were heated at 100 °C for 5 min, centrifuged, and the digests were applied onto Bio-Gel P-6 (1 × 15 cm) in 0.1 M pyridine, 0.1 M acetic acid. Fractions (0.67 ml) were collected, and aliquots were analyzed for uronic acid content (54). The gel electrophoresis—The CS oligosaccharides were dissolved in 100 mM Tris base, 0.5 mM boric acid, 2 mM EDTA, pH 8.3, containing 5% glycerol. The solutions were electrophoresed on 10% polyacrylamide gel (15 × 16 cm) in 100 mM Tris base, 0.5 mM borate, 2 mM EDTA, pH 8.3. The gels were stained with 0.03% Alcian Blue in 25% ethanol, 10% aqueous acetic acid for 4 h and destained with 25% ethanol, 10% aqueous acetic acid. For silver staining, the gels were treated with 10% aqueous glutaraldehyde for 30 min and washed with water three times for 30 min each. The gels were then treated with 1% aqueous ammonium molybdate for 15 min, washed twice for 30 s each, developed with 0.005% citric acid and 0.019% formaldehyde in water, and then washed with water (59).

**DEAE-Sepharose Chromatography of Oligosaccharides—**The oligosaccharides (fractions 20–35, 50 μg) obtained by the Bio-Gel P-6 chro-
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matography of the *S. dysgalactiae* hyaluronidase digest of chondroitin were digested in 20 mM NaOAc, 50 mM NaCl, pH 5.0, and applied onto a DEAE-Sepharose microcolumn (0.1-ml bed volume). After washing with 0.5 ml of the above buffer, the bound oligosaccharides were eluted stepwise with buffer containing 0.1, 0.2, 0.4, and 0.6 M NaCl (0.5 ml each). Fractions (0.1 ml) were collected, and aliquots were analyzed for uronic acid (54). The oligosaccharide-containing fractions were pooled and digested with chondroitinase ABC, and the disaccharides formed were analyzed by HPLC.

Disaccharide Composition Analysis—The C4Ss or C4S oligosaccharides (10–15 μg) were digested with chondroitinase ABC (10–20 milliunits/ml) as well as by competitive inhibition with various sulfated CSPGs of human placenta by DEAE-Sepharose chromatography of the isotonic buffer extract of placentas. This method was used to determine the uronic acid contents in various column chromatography fractions. The columns were eluted with a linear gradient of 16–530 mM NaH2PO4 over 70 min at room temperature at a flow rate of 1 ml/min. The elution of disaccharides was monitored by measuring the absorbance at 232 nm using a Waters 484 variable wavelength UV detector. The data were processed with the Millennium 2010 chromatography manager using NEC PowerMate 433 data processing system.

Carbohydrate Composition Analysis—The CSPGs or CS chains (5–10 μg) were hydrolyzed with 4 M HCl at 100 °C for 6 h. The hydrolysates were dried in a Speed-Vac and analyzed on a CarboPac PA1 high pH anion exchange HPLC column (4 × 250 mm; Dionex) (62). The column was eluted with 20 mM sodium hydroxide, elution of sugars was monitored by pulsed amperometric detection, and the response factors for sugars were determined using standard sugar solutions.

Other Analytical Procedures—The uronic acid contents in various column chromatography fractions were determined by the carbazole-sulfuric acid method (54). Protein contents were measured using the Micro BCA Protein Assay Reagent Kit from Pierce (63). *P. falciparum Cell Culture*—The CSPGs or CS chains were digested with chondroitinase ABC (10–20 milliunits/ml) and assayed by competitive inhibition with various CSPGs of human placenta by DEAE-Sepharose chromatography of the isotonic buffer extract of placentas. This method was used to determine the uronic acid contents in various column chromatography fractions. The columns were eluted with a linear gradient of 16–530 mM NaH2PO4 over 70 min at room temperature at a flow rate of 1 ml/min. The elution of disaccharides was monitored by measuring the absorbance at 232 nm using a Waters 484 variable wavelength UV detector. The data were processed with the Millennium 2010 chromatography manager using NEC PowerMate 433 data processing system.

IRBC Adhesion and Adhesion-Inhibition Assays—The adherence of IRBCs was performed by coating solutions (10–15 μl) of purified CSPGs as circular spots on 15-mm plastic Petri dishes as described previously (51). The specificity of IRBC binding to CSPGs was ascertained by incubating the CSPG-coated plates with chondroitinase ABC (50 milliunits/ml) as well as by competitive inhibition with various CSPGs.

For adhesion-inhibition assays, IRBCs were incubated with various C4Ss or C4S oligosaccharides at the indicated concentrations in PBS, pH 7.2, in 96-well microtiter plates at room temperature for 30 min with intermittent mixing (51). The IRBC suspension was then layered on CSPG-coated spots on Petri dishes. After 40 min at room temperature, the unbound cells were washed, and the bound cells were fixed with 2% glutaraldehyde, stained with Giemsa, and counted using a light microscope.

RESULTS

Fractionation and Characterization of Differentially Sulfated CSPGs of Human Placental Intervillous Spaces—The major low sulfated CSPG fraction (previously designated as BCSPG-2) (50) was isolated by one-step DEAE-Sephalochromatography of the isotonic buffer extract of placenta. This CSPG fraction represents about 93–94% of the total low sulfated CSPGs in the intervillous spaces (50). When subjected to DEAE-Sepharose chromatography, using a 0.15–0.55 M NaCl gradient, the CSPG was partially resolved into two fractions (designated BCSPG-2a and BCSPG-2b) that are distinct in their sulfation levels (Fig. 1). The proportions of BCSPG-2a and BCSPG-2b varied considerably from one placenta to another, in the range 40–65% and 35–60%, respectively. These CSPG fractions were further purified and characterized, and their ability to bind IRBCs was studied.

On CsBr density gradient centrifugation, BCSPG-2a and BCSPG-2b sedimented to the middle of the gradients (average ρ = 1.43 g/ml) separating from protein and nucleic acid contaminants (not shown). The sedimentation patterns of BCSPG-2a and BCSPG-2b were indistinguishable from each other and very similar to that previously reported for the total BCSPG-2 (50). In both cases, fractions of the gradient containing significant levels of CSPGs (ρ = 1.35–1.5 g/ml density regions) were pooled, and the material was recovered.

BCSPG-2a and BCSPG-2b were further purified by Sepha-rose CL-6B and Sepharose CL-4B chromatography, which removed any residual protein contaminants. In each case, the CSPG fraction eluted as a single nonsymmetrical peak, and the chromatograms were similar to that previously reported for the total BCSPG-2 (not shown) (50). The yields and compositions of the purified BCSPG-2a and BCSPG-2b are shown in Table I. As in the case of total BCSPG-2, BCSPG-2a and BCSPG-2b each contained high and low molecular mass (570-kDa, respectively) proteoglycan species (50).

The uronic acid-containing fractions corresponding to the major CSPG fraction (designated BCSPG-2 in Ref. 50) were pooled, dialyzed, and chromatographed on DEAE-Sepharose (2.5 × 17 cm) in 50 mM NaOAc, pH 5.5. The column was washed with the same buffer, and the bound CSPGs were eluted with a linear gradient of 0.15–0.55 M NaCl in 50 mM NaOAc, pH 5.5. 10-ml fractions were collected, absorptions at 280 and 260 nm were measured, and aliquots were assayed for uronic acid (54). The CSPG peaks were pooled as indicated by horizons. Note that the previously reported minor BCSPG-1 (50), which represents 6–7% of the total CSPGs of the intervillous spaces of the placenta, was not studied here.

BCSPG-2b sedimented to the middle of the gradients (average ρ = 1.43 g/ml) separating from protein and nucleic acid contaminants (not shown). The sedimentation patterns of BCSPG-2a and BCSPG-2b were indistinguishable from each other and very similar to that previously reported for the total BCSPG-2 (50). In both cases, fractions of the gradient containing significant levels of CSPGs (ρ = 1.35–1.5 g/ml density regions) were pooled, and the material was recovered.

BCSPG-2a and BCSPG-2b were further purified by Sepha-rose CL-6B and Sepharose CL-4B chromatography, which removed any residual protein contaminants. In each case, the CSPG fraction eluted as a single nonsymmetrical peak, and the chromatograms were similar to that previously reported for the total BCSPG-2 (not shown) (50). The yields and compositions of the purified BCSPG-2a and BCSPG-2b are shown in Table I. As in the case of total BCSPG-2, BCSPG-2a and BCSPG-2b each contained high and low molecular mass (570-kDa, respectively) proteoglycan species (50). The proportions of –1000- and ~570-kDa species in BCSPG-2a and BCSPG-2b were similar (not shown). Chondroitinase ABC degraded the glycosaminoglycan chains of both BCSPG-2a and BCSPG-2b completely into unsaturated disaccharides, as assessed by chromatography of the enzyme digests on Bio-Gel P-6 column (not shown), indicating the absence of hyaluronic acid and/or heparan sulfate in these fractions. Consistent with these results, the CS chains of the proteoglycan fractions retain predominantly galactosamine (Table II), and they were completely resistant to the action of *S. hyalurolyticus* hyaluroni- dase and heparitinase (not shown). As evident from the disaccharide compositions of the CS chains (see below; Table II), BCSPG-2a and BCSPG-2b differ significantly in their sulfate contents. Thus, the differences in the overall charge density of the proteoglycan fractions, as indicated by their elution at different salt concentrations from DEAE-Sepharose columns (Fig. 1), is mainly due to the differences in sulfate contents of the CS chains.

SDS-PAGE analysis of the core proteins released after chondroitinase ABC treatment revealed that BCSPG-2a and BCSPG-2b each contain two distinct core proteins, a high
Adhesion of *P. falciparum* IRBCs to BCSPG-2a and BCSPG-2b—The purified BCSPG-2a and BCSPG-2b were assessed for their abilities to adhere IRBCs by an *in vitro* cytoadherence assay (Fig. 2). Both CSPGs efficiently bound IRBCs in a concentration-dependent manner. Significant levels of IRBC binding were observed at coating concentration as low as 12 ng/ml. At 50 ng/ml, both fractions efficiently bound IRBCs, and at 100–200 ng/ml they exhibited saturated levels of binding. Thus, despite significant difference in the sulfate contents of the CS chains, BCSPG-2a and BCSPG-2b were indistinguishable from one another with regard to the number of IRBCs adhering when immobilized on solid surfaces (Fig. 2A). It is possible that similar IRBC binding capacities of BCSPG-2a and BCSPG-2b might merely reflect the number of IRBCs bound, even if the IRBCs adhered to these proteoglycans with different affinities. Therefore, to determine the relative affinities of IRBC binding by BCSPG-2a and BCSPG-2b, we performed adhesion inhibition assays. BCSPG-2a and BCSPG-2b were coated on plastic plates and tested in parallel for competitive inhibition of IRBC binding by a regioselectively 6-O-desulfation of bovine trachea 4S fraction with 36% 4-sulfate and a 4S 6-mer with 36% 4-sulfate groups. Both compounds were marginally (10–20%) more inhibitory to IRBC binding to BCSPG-2a compared with BCSPG-2b, suggesting that BCSPG-2b binds IRBCs with slightly higher affinity than BCSPG-2a (Fig. 2B). This agrees with the difference in the sulfate content and the number of IRBC binding sites in the CS chains of BCSPG-2a and BCSPG-2b. However, the difference in the levels of inhibition is not as much as that expected based on the number of IRBC binding sites in the CS chains of BCSPG-
The CS-2 chains of placental CSPG fractions were isolated by the alkaline NaOH/NaBH₄ digestion of the purified placental CSPGs. BCSPG-2a and BCSPG-2b, were chromatographed on DEAE-Sepharose columns (1 × 10 cm) equilibrated with 20 mM Tris-HCl, pH 7.8. The columns were washed with 20 mM Tris-HCl, 0.15 M NaCl, pH 7.8, and the bound glycosaminoglycans eluted with a linear gradient of 0.15–0.6 M NaCl in 20 mM Tris-HCl, pH 7.8. Fractions (2.5 ml) were collected, and 50-μl aliquots were analyzed for uronic acid (530 nm). The CS chain fractions were pooled as shown by the horizontal bars and recovered for further studies. The CS chains (CS-2a) of BCSPG-2a and CS-2b of BCSPG-2b. The inset shows the elution pattern of CS chains obtained from the total BCSPG-2 (corresponding to fractions 22–49 in Fig. 1) from another placenta.

2b; the CS chains of BCSPG-2b has 4–5 times more binding sites than those of BCSPG-2a (see below).

The Level and Distribution Pattern of Sulfate Groups in the CS Chains of Placental CSPGs—The CSPGs of the intervillous spaces can efficiently bind IRBCs despite markedly lower total sulfate contents of their CS chains than that required for the optimal IRBC binding (51). This suggested that the sulfate groups may be clustered in their CS chains and that these sulfate-rich regions could efficiently bind IRBCs. To investigate this further, we performed a detailed structural characterization of the CS chains of placental CSPGs. The CS chains of BCSPG-2a and BCSPG-2b were isolated by the alkaline β-elimination of the proteoglycans followed by chromatography on DEAE-Sepharose columns (Fig. 3). In the case of BCSPG-2a, about 68% of CS chains (designated as CS-2a) eluted as a single peak at 0.28 m NaCl, and the remainder eluted as heterogeneous peaks at a mean NaCl concentration of 0.36 m. In the case of BCSPG-2b, on the other hand, only 28% of the CS chains eluted at 0.28 m NaCl, and the remainder (CS-2b) eluted as a single peak at 0.37 m NaCl. The presence of additional CS population (Fig. 3) in both BCSPG-2a and BCSPG-2b was probably due to the overlapping separation of the CSPG fractions on DEAE-Sepharose columns (see Fig. 1). Thus, these results demonstrate that BCSPG-2a and BCSPG-2b carry differentially sulfated CS chains. The presence of two intervillous space CSPGs with distinctively sulfated CS chains was also evident from the elution pattern of the CS chains released by alkaline β-elimination of the total placental intervillous space CSPGs (BCSPG-2) on the DEAE-Sepharose column (see inset in Fig. 3).

The CS chains of BCSPG-2a and BCSPG-2b, CS-2a and CS-2b, fractionated by DEAE-Sepharose chromatography, were recovered by pooling the fractions as shown in Fig. 3. The molecular sizes of the CS chains were assessed by chromatography on a Sepharose CL-6B column calibrated with glycosaminoglycans of known molecular weights (50). The CS-2a and CS-2b were eluted as single symmetrical peaks, indistinguishable from one another, with an estimated molecular weight of ~60,000 (not shown). Hexosamine compositional analysis indicated that both CS-2a and CS-2b have predominantly N-acetylgalactosamine (Table II). HPLC analysis of the unsaturated disaccharides released by the digestion with chondroitinase ABC revealed that CS-2a and CS-2b, obtained from CSPGs of various placentas, consist of, respectively, 2–3% and 9–14% 4-sulfated and 97–98% and 86–91% nonsulfated disaccharide repeating units. Together, these results suggest that CS-2a and CS-2b are similar in molecular sizes but differ in the levels of sulfate groups.

To determine the distribution of sulfate groups, the CS chains of placental CSPG fractions were digested with S. dysgalactiae hyaluronidase, an endo-β-N-acetylgalactosaminyl lyase. This enzyme degrades hyaluronic acid and chondroitin, but not chondroitin sulfate, to produce disaccharides with nonreducing 4,5-unsaturated uronic acid residue (57). Chromatography of the enzyme digests on Bio-Gel P-6 columns indicated that ~95 and 80%, respectively, of CS-2a and CS-2b were degraded predominantly into disaccharides with minor amount of tetrasaccharides. The remainders of the CS chains were converted into oligosaccharides larger than decasaccharide (5-mer) (Fig. 4). The disaccharide composition of the oligosaccharide fractions (see Fig. 4), determined by HPLC after digestion with chondroitinase ABC, are given in Table III. In both cases, the larger oligosaccharides (Fractions I and II), formed by the action of S. dysgalactiae hyaluronidase, contained significant levels of sulfate groups. Fractions I and II from CS-2a comprised 20–22% 4-sulfated

![Image 346x496 to 534x737](https://example.com/image_url)
The oligosaccharides, formed by treatment of the CS chains, CS-2a and CS-2b, of the placental CSPGs with *S. dysgalactiae* hyaluronidase, were isolated (see Fig. 4) and degraded with chondroitinase ABC. The unsaturated disaccharides formed were analyzed by HPLC on an amine-bonded Microsorb-MV column (4.6 × 250 mm; Varian) using a linear gradient of 16–530 mM NaH2PO4 (61).

and 78–80% nonsulfated disaccharide repeating units, whereas Fractions I and II from CS-2b had 25–28% 4-sulfated and 72–75% nonsulfated disaccharide repeating units. The tetrasaccharides (Fraction III) from CS-2a were ~97% nonsulfated and ~3% sulfated, whereas those from CS-2b were ~95% nonsulfated and ~5% sulfated. As expected, based on the specificity of *S. dysgalactiae* hyaluronidase, the disaccharides (Fraction IV), formed by the action of this enzyme, were exclusively nonsulfated. Interestingly, however, hexa- to decasaccharides (3- to 5-mers) were not formed in appreciable amounts from either CS-2a or CS-2b. These results indicate that, in the CS chains of both BCSPG-2a and BCSPG-2b, the majority of sulfate groups are clustered such that these regions have 20–28% or more of 4-sulfated disaccharide repeating units. The yields of the larger oligosaccharides (Fractions I and II) from CS-2a and CS-2b should correspond to the number of sulfate group-rich domains in the CS chains. Since the oligosaccharide Fractions I and II (Fig. 4) obtained by the digestion of the CS chains of placental CSPGs with *S. dysgalactiae* hyaluronidase contained only 20–28% sulfated disaccharides, it is possible that the oligosaccharides are a mixture of sulfated and nonsulfated species. Because the placental CS chains were available only in limited amounts, the specificity of the enzyme, under the conditions used for the placental CS chains, was studied using a commercially available chondroitin, which consisted of ~4% 6- and 4-sulfated disaccharide moieties and ~96% nonsulfated. Bio-Gel P-6 chromatography showed that the enzyme degraded ~90% of chondroitin into disaccharides and ~10% (~6% in fractions 20–25 and ~4% in fractions 26–35; the pattern is similar to that of CS-2a in Fig. 4, not shown) into a mixture of oligosaccharides with >5 disaccharide repeating units. The oligosaccharides in fractions 20–25 and 26–35 had 12–15% 6-sulfated and 6–8% 4-sulfated disaccharide moieties and the remainder nonsulfated. Ion exchange chromatography on DEAE-Sepharose using stepwise elution with NaCl and compositional analysis of the fractions showed the presence of predominantly sulfated oligosaccharides. These results suggested that *S. dysgalactiae* hyaluronidase readily degrades the nonsulfated regions of CS and slowly acts at the sulfated domains, forming partially sulfated oligosaccharides.

### Table III

| Disaccharide | Yielda | % | mol proportionb |
|-------------|--------|---|-----------------|
| FI          | 3.0    | 20 | 80              |
| FII         | 3.5    | 22 | 78              |
| FIII        | 21.1   | 3  | 97              |
| FIV         | 72.4   | 0  | 100             |
| CS-2b       |        |    |                 |
| FI          | 8.9    | 25 | 75              |
| FII         | 10.0   | 28 | 72              |
| FIII        | 35.4   | 5  | 88              |
| FIV         | 42.7   | 100|                 |

a By the area of the chromatographic peaks obtained by plotting uronic acid contents of the fractions (see Fig. 4).
b The values were calculated from the areas of HPLC peaks by assuming that the different unsaturated disaccharides have similar molar extinction coefficients.

To determine the exact sizes of the larger oligosaccharides formed by the digestion of CS-2a and CS-2b with *S. dysgalactiae* hyaluronidase, Fractions I and II (see Fig. 4) were analyzed by polyacrylamide gel electrophoresis (Fig. 5). In both the cases (CS-2a and CS-2b), the oligosaccharides in Fractions I and II ranged in size from 8 to 14 and from 6 to 11 disaccharide units, respectively (Fig. 5). These results indicate that the sulfate groups in the CS chains of BCSPG-2a and BCSPG-2b are clustered in CS chain motifs composed of 6–14 disaccharide units.

**Inhibition of *P. falciparum* IRBC Adherence to the Placental CSPGs by the CS Chains of Placental CSPGs and Their Oligosaccharides**—The intact CS chains, CS-2a and CS-2b, and the oligosaccharides obtained by the digestion of CS chains with *S. dysgalactiae* hyaluronidase (see Fig. 4) were assessed for their ability to inhibit the adhesion of IRBCs to placental CSPGs. The CS chains as well as their oligosaccharide Fractions I and II inhibited the IRBC adhesion to BCSPG-2a and BCSPG-2b in a dose-dependent manner (Fig. 6). Consistent with the prediction based on the level of 4-sulfated disaccharide clustered domains (see Table II), the inhibition of IRBC binding by CS-2b was 2–3-fold higher than that by CS-2a. Further, oligosaccharide Fractions I and II, obtained from CS-2a and CS-2b, were significantly better inhibitors than the corresponding intact chains (Fig. 6 and data not shown). The inhibitory capacity of Fractions I and II of CS-2a was only marginally lower than those of Fractions I and II from CS-2b. The inhibitory ability of the oligosaccharide from CS-2b was comparable with that of C4S, with 36% 4-sulfated disaccharide residues prepared by the regioselective 6-O-desulfation of bovine
The plastic Petri plates were coated overnight with 200 ng/ml solution of placental CSPG fractions and oligosaccharides of the CS chains. Shown is the inhibition of IRBC binding to BCSPG-2b-coated plates. Fixed, stained with Giemsa, and measured using light microscopy. The unbound cells were washed. The bound cells were outlined in the legend to Fig. 2. The CS chains and their oligosaccharides were incubated at the indicated concentrations with a 2% IRBC suspension in PBS, pH 7.2, for 30 min at room temperature and then overlaid onto the CSG-coated spots. After a 40-min incubation at room temperature, the unbound cells were washed. The bound cells were fixed, stained with Giemsa, and measured using light microscopy. Shown is the inhibition of IRBC binding to BCSPG-2b-coated plates. FII from CS-2b; CS-2a; CS-2b; oligosaccharide FII from CS-2b; I, I, bovine trachea C4S/C6S copolymer; CS4S with 36% 4-sulfate prepared by the regioselective 6-O-desulfation of bovine trachea C4S followed by DEAE-Sepharose fractionation; CS4S with 36% 4-sulfate prepared by solvolytic partial desulfation of sturgeon notochord CS4S (51); oligosaccharide. The inhibition capacities of oligosaccharide Fract. I and II from CS-2a were either similar or only marginally lower compared with those from CS-2b (not shown). Inhibition of IRBC binding to BCSPG-2a was similar to that observed in the case of BCSPG-2b, except that a 10–20% higher inhibition was observed for each inhibitor used. Note that the inhibitory capacity of the 6-mer prepared by the testicular hyaluronidase digestion of C4S with 36% 4-sulfated disaccharide was similar to that by the intact C4S with 36% 4-sulfate.

trachea C4S/C6S copolymer (Fig. 6). In contrast, the C4S with 3 and 11% 4-sulfate groups prepared by solvolytic desulfation of a fully 4-sulfated sturgeon notochord C4S were markedly less inhibitory compared with the CS chains of BCSPG-2a, which have only 2–3% sulfated disaccharide repeating units. These results agree with the finding described above that the sulfate groups in the CS chains of BCSPG-2a and BCSPG-2b are clustered in CS chain motifs consisting of 6–14-disaccharide repeating units. The results also agree with our previous finding that optimal binding of IRBCs requires a 6-mer motif in which two of six disaccharide repeating units sulfated on C-4 of N-acetylglalactosamine. Since the oligosaccharides obtained by the digestion of CS-2a and CS-2b with S. dysgalactiae hyaluronidase are larger than six disaccharide repeating units, the sulfate content in the 6-mer IRBC-binding motif is likely to be ~30% or more. This satisfies the level of 4-sulfation required for optimal IRBC binding. These results indicate that the sulfate groups in the CS chains of placental CSPGs are uniquely distributed and provide the necessary structural elements for the efficient adhesion of IRBCs.

**DISCUSSION**

Recently, we showed that unusually low sulfated CSPGs of the intervillous spaces of human placenta can efficiently support the adherence of *P. falciparum* IRBCs in the placenta (50). The IRBC binding involves critical interactions by both 4-sulfated and nonsulfated repeating units of the CS chains, and the optimal binding requires a 6-mer motif with two 4-sulfated disaccharide repeating units (51). In this study, the placental intervillous space CSPGs and the structural features of their CS chain motifs that bind IRBCs were investigated. The new findings are as follows. 1) The placental CSPGs are a mixture of two distinct populations, which are similar with regard to proteoglycan type and sizes but different with respect to the levels of sulfation. 2) The majority of the sulfate groups in the glycosaminoglycan chains of both CSPGs are clustered in size-defined domains that comprised 6–14 disaccharide repeating units. Within these domains, about 20–28% of the disaccharide repeating units are 4-sulfated, whereas the other regions of the CS chains have essentially no sulfate groups. 3) The oligosaccharides corresponding to these sulfate group-rich domains can efficiently inhibit IRBC binding to placental CSPGs. Thus, our data define, for the first time, the distribution of sulfate groups in the CS chains of the low sulfated placental CSPGs and establish that *P. falciparum* uses the sulfate group-clustered domains of the CS chains for IRBC adhesion in the placenta.

The data show that the low sulfated CSPGs of the intervillous spaces of placenta consist of two major, differentially sulfated proteoglycan populations, BCSPG-2a and BCSPG-2b. These CSPG populations resemble one another very closely with respect to their physical properties, including hydrodynamic size, buoyant density, and the core protein type, proportion, and composition. The CSPG species are also very similar with respect to the number and molecular sizes of the CS chains, but they differ significantly in the sulfate contents of the CS chains (2–3% and 9–14%, respectively, depending on placentas).

A CSPG population with 2% sulfate groups in the CS chains has been previously identified as a minor CSPG in the placental intervillous spaces (50). This CSPG species, designated as BCSPG-1, accounted for only 6–7% of the total CSPGs and was eluted from the DEAE-Sephalac column as a distinct peak, completely separated from the remainder of the intervillous space CSPG molecules, BCSPG-2 (50). A comparison of the data from this study with those of the previous study indicates that BCSPG-1 is very similar to BCSPG-2a by all criteria including overall charge density, core protein types, and the content of sulfate groups in CS chains. Therefore, BCSPG-1 and BCSPG-2a appear to represent similar CSPG populations. The results of the present study clearly show that these proteoglycan species represent 40–65% of the total CSPGs of the placental intervillous spaces. Based on the results of the previous study, it is evident that only a portion of BCSPG-2a aggregates with the low amount of hyaluronic acid present in the intervillous spaces and eluted as a distinct peak during DEAE-Sephalac chromatography (50).

The results of this study conclusively establish that the majority of sulfate groups of the CS chains of the CSPGs of placental intervillous spaces are clustered in size-defined motifs, consisting of 6–14 disaccharide repeating units. The sizes of the oligosaccharides formed by the digestion of the CS chains of BCSPG-2a and BCSPG-2b with *S. dysgalactiae* hyaluronidase support this conclusion. The enzyme converted >90% of the CS chains of BCSPG-2a and ~80% of the CS chains of BCSPG-2b to predominantly nonsulfated disaccharides and a minor amount of tetrasaccharides; only a small portion of the latter appears to be 4-sulfated (Table III). Based on the specificity of the enzyme (degrades chondroitin but not chondroitin sulfate), the amount of sulfate groups in the tetrasaccharide fraction must represent the level of 4-sulfated disaccharide units that occur as single residues randomly distributed in relatively large nonsulfated regions of the CS chains. This accounts for 3–5% of the total sulfate groups in the CS chains of BCSPG-2a and BCSPG-2b. These results clearly show that the majority of the sulfate groups in CS chains of placental CSPGs are clus-
tered in domains consisting of 6–14 disaccharide repeating units.

Our data demonstrate that *P. falciparum* IRBCs bind to the sulfate group-clustered motifs but not to the nonsulfated regions of the CS chains of placental intervillous space CSPGs. Consistent with this conclusion, chondroitin, the nonsulfated glycosaminoglycan, has no inhibitory effect on the adhesion of IRBCs to placental CSPGs. The C4Ss with 3 and 11% 4-sulfate content, prepared by partial desulfation of fully sulfated stur-geon notochord C4S, could only marginally inhibit the IRBC binding (Fig. 6). In these C4Ss, the majority of the sulfate groups are likely to be distributed as single residues separated by a number of nonsulfated moieties. In contrast, the CS chains of BCSPG-2a and BCSPG-2b, containing clustered sulfate residues, are severalfold more inhibitory. The oligosaccharides corresponding to the sulfate group-clustered domains (Fractions I and II), obtained by the *S. dysgalactiae* hyaluronidase digestion of the CS chains of the placental CSPGs, are superior inhibitors compared with the corresponding intact chains (Fig. 6). This is especially evident in the case of the CS chains of BCSPG-2a. This is not surprising, because the major portion of CS-2a and significant amounts of CS-2b have no sulfate groups, and thus these portions are not able to bind IRBCs. Therefore, on weight basis, the oligosaccharide Fractions I and II should be much more active in inhibiting IRBC binding than the intact CS chains, CS-2a and CS-2b, from which they were obtained.

We have previously shown that two of the six disaccharide residues of the CS 6-mer motif must be 4-sulfated for optimal IRBC binding (51). This translates to 33% 4-sulfated residues in the oligosaccharide. The level of 4-sulfate determined in this study for the sulfate group-clustered domains of the CS chains of placental CSPGs is 20–28%. However, it should be noted that the sizes of the predominant oligosaccharides (Fractions I and II in Fig. 4) formed by the action of *S. dysgalactiae* hyaluronidase are larger than the 6-mer, the minimum chain length required for optimal inhibition. Therefore, it is possible that the 6-mer binding motifs in the CS chains of placental CSPGs have the level of 4-sulfation required for optimal IRBC adhesion.

Although the capacity of the CS chains of BCSPG-2a to inhibit the IRBC binding to placental CSPGs is relatively low, immobilized BCSPG-2a efficiently binds IRBCs. When coated on plastic plates, the density of IRBC binding by BCSPG-2a is remarkably similar to that by BCSPG-2b. Further, the CS chains of BCSPG-2b contain significantly higher 4-sulfate, and the inhibitory capacities of these chains were 4–5-fold higher compared with those of BCSPG-2a. Based on the sulfate contents, size of the CS chains, and requirements of two sulfate groups per 6-mer IRBC-binding motif, it can be calculated that the CS chains of BCSPG-2a and BCSPG-2b have about 1–2 and 4–10 binding sites, respectively. Thus, the difference in the inhibitory capacities of the CS chains of these proteoglycan species agree with that expected based on the number of available IRBC binding sites in the CS chains. However, when IRBC binding and inhibitory data considered together, it appears that the density of IRBCs adhered to CSPG-coated plastic plates merely represents the number of cells bound and does not reflect the binding strength. When IRBC binding affinity to immobilized BCSPG-2a and BCSPG-2b was measured by adhesion inhibition using C4S with 30% 4-sulfate, marginal differences were observed, only at very low inhibitor concentra-tions (Fig. 6). This was also the case with C4S 6-mer having 36% 4-sulfate or oligosaccharide Fractions I and II from the CS chains of BCSPG-2b. Therefore, it appears that, when CSPGs are immobilized on solid surface, because of clustering of mole-cules, the CS chains of BCSPG-2a, despite containing fewer binding sites, can provide a sufficient number of binding sites for IRBCs using adjacent CS chains. Thus, they are able to bind IRBCs with capacity almost similar to that of the CS chains of BCSPG-2b. Alternatively, it is possible that because of the steric constraints due to the one-dimensional disposition of IRBCs with respect to the immobilized CS chains, IRBCs might not be able to interact with all available binding sites in the CS chains of BCSPG-2b. Therefore, the CS chains of both proteoglycan fractins could provide a single number of binding sites to interact with IRBCs, and thus the CS chains of both BCSPG-2a and BCSPG-2b bind with almost comparable capacity. In solution phase, on the other hand, the disposition of IRBCs to binding by CS chains is multidirectional, which might allow for effective interactions with all of the available binding sites in the CS chains. Therefore, the CS chains of BCSPG-2b can inhibit the IRBC adhesion more effectively than the CS chains of BCSPG-2a. However, in the placenta, since the CSPGs are immobilized in the intervillous spaces, it is likely that both proteoglycans are equally efficient in binding IRBCs.

The clustering of almost all of the sulfate groups present in CS chains of the placental CSPGs in size-defined oligosacchar-ide domains suggests a well regulated mechanism of biosyn-thesis. This clustering of sulfate groups in the CS chains of the CSPGs might have important roles in the function of placenta. Clearly, *P. falciparum* has evolved to exploit these sulfated group-clustered motifs of the CS chains for adherence in the placenta. Recent studies have demonstrated that versican, a high molecular weight CSPG expressed in a variety of cells and tissues, including fibroblasts, arterial smooth muscle cells, keratinocytes, kidney, brain, aorta, and skin, can bind L-selectin, P-selectin, CD44, and a number of chemokines (64–66). The last class of proteins include secondary lymphoid chemokines, macrophage inflammatory protein, stromal cell-derived factor 1, monocyte chemoattractant protein, regulated on activation normal T-cell-expressed and secreted proteins, γ-interferon-inducible protein 10, platelet factor 4, and liver and activation-regulated chemokine (64–66). It has been shown that CS-de-pendent binding of these proteins to versican is important in leukocyte trafficking, signal transduction to trigger inflamma-tory responses, and the regulation of chemokine functions (64– 66). These proteins have been shown to bind different struc-tural features within the CS chains of versican. CD44, a hyaluronic acid-binding protein, was found to bind to the non-sulfated regions of the CS chains of versican, whereas L- and P-selectins and various chemokines have been shown to inter-act with motifs consisting of specific sulfated residues (66). Therefore, analogous to this, the nonsulfated regions of the CS chains of placental CSPGs may bind CD44, whereas sulfate group-clustered regions support binding of other functional proteins. The placental CSPGs have been identified as members of the aggrecan family of proteoglycans (50). Since versi-can and aggrecan exhibit many similarities with respect to both core protein and CS chain structural features, it is possi-ble that the CS chains of placental CSPGs also have diverse biological functions.

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