Rat Spongiotrophoblast-specific Protein Is Predominantly a Unique Low Sulfated Chondroitin Sulfate Proteoglycan*

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We have previously demonstrated that the human placenta contains a uniquely low sulfated extracellular aggrecan family chondroitin sulfate proteoglycan (CSPG). This CSPG is a major receptor for the adherence of Plasmodium falciparum-infected red blood cells (IRBCs) in placentas, causing pregnancy-specific malaria. However, it is not known whether such low sulfated CSPGs occur in placentas of other animals and, if so, whether IRBCs bind to those CSPGs. In this study, we show that rat placenta contains a uniquely low sulfated extracellular CSPG bearing a chondroitin sulfate (CS) chains, which comprise only ~2% 4-sulfated and the remainder nonsulfated disaccharides. Surprisingly, the core protein of the rat placental CSPG, unlike that of the human placental CSPG, is a spongiotrophoblast-specific protein (SSP), which is expressed in a pregnancy stage-dependent manner. The majority of rat placental SSP is present in the CSPG form, and only ~10% occurs without CS chain substitution. Of the total SSP-CSPG in rat placenta, ~57% is modified with a single CS chain, and ~43% carries two CS chains. These data together with the previous finding on human placental CSPG suggest that the expression of low sulfated CSPG is a common feature of animal placentas. Our data also show that the unique species-specific difference in the biology of the rat and human placentas is reflected in the occurrence of completely different CSPG core protein types. Furthermore, the rat SSP-CSPG binds P. falciparum IRBCs in a CS chain-dependent manner. Since IRBCs have been reported to accumulate in the placentas of malaria parasite-infected rodents, our results have important implications for exploiting pregnant rats as a model for studying chondroitin 4-sulfate-based therapeutics for human placental malaria.

Chondroitin sulfate proteoglycans (CSPGs) are abundantly present in the connective tissues, such as cartilage, skin, and tendon, and in cornea, bone, umbilical cord, and blood vessels (1–3). CSPGs are also ubiquitous in tissues and cell types of mammalian and other vertebrate animals as components of extracellular matrices as well as cell surface molecules. The CSPGs of cartilage and those from a wide variety of tissues and cell types of different animals have been extensively characterized with respect to their core proteins and the structural characteristics of the constituent CS chains (1–6). However, very little is known about the CSPGs in the maternal blood space of the placentas of various animals. Previously, we have shown that human placenta contains a major extracellular, unusually low sulfated aggrecan family CSPG, localized predominantly in the intervillous space (7, 8). The CS chains of the placental CSPG consist of, on average, ~8% 4-sulfated and ~92% nonsulfated disaccharide residues (7). The human placental CSPG is a mixture of two major distinctively sulfated aggrecan species, one with 2–3% and the other with 9–14% sulfated disaccharide moieties (9). Although the biological function of the low sulfated aggrecan CSPG in human placenta is not known, it is possible that the placental low sulfated CS chains are involved in mobilizing nutrients, cytokines, hormones, and growth factors required for placental function and for the development of the fetus. The low sulfated CS chains are ideally suited for this function, since they are likely to reversibly and readily release the bound factors for their function.

We have previously shown that the low sulfated CSPG is the receptor for the adherence of Plasmodium falciparum IRBCs in the placentas of pregnant women and that the sulfate-glycosaminoglycan regions of the C4S chains are the IRBC-binding sites (7–9). Previous studies have also shown that P. falciparum IRBC binding to C4S involves the participation of both sulfated and nonsulfated disaccharide residues, and a dodecasaccharide motif with two 4-sulfated disaccharides is the minimal structural motif for optimal binding of IRBCs (9–11). Recently, we have defined the role of the key functional groups, including the acetamido and carboxyl groups of C4S for IRBC binding. Furthermore, although the data from these studies offer strategies for developing C4S oligosaccharide-based therapy for placental malaria, animal models for studying the therapeutic potentials of such compounds have not been defined.

In pregnant rats infected with P. berghei, as in the case of...
human placent al malaria, IRBCs and monocytes accumulate in high density in the placenta, causing severe placent al pathology (12–14). However, the molecular interactions involved in the observed IRBC sequestration in the rat placenta are not known. As a part of our effort to determine whether pregnant rats can be useful as a model for studying pregnancy-specific malaria, we studied, in detail, the extracellular CSPG of the rat placenta. Interestingly, we found that although the rat placenta synthesizes very low sulfated CSPG, this proteoglycan is totally unrelated to aggrecan, and the sulfate content of the CS chains of rat placent al CSPG is much more lower than that of the low sulfated human placent al CSPG. The core protein of the rat placent al CSPG is SSP, which is expressed in a pregnancy stage-specific manner (15). The expression of similarly low sulfated CS chains both in humans and rats, although the core proteins of CSPGs from these species are entirely different, points to important biological functions for the unusually low sulfated CS chains of placentas. Here, we report the structural characterization and IRBC-binding properties of the rat placent al SSP-CSPG.

EXPERIMENTAL PROCEDURES

Materials—Proteus vulgaris chondroitinase ABC, protease-free P. vulgaris chondroitinase ABC (120 units/mg), Arthrobacter aurescens chondroitinase AC II (87 units/mg), and super special grade C6S (shark cartilage) were purchased from Seikagaku America (Falmouth, MA). Bovine tracheal chondroitin sulfate A and Streptococcus species hyaluronic acid were from Calbiochem. Phenylmethylsulfonyl fluoride, N-ethylmaleimide, and benzamidine were from Sigma. TLCK and TPCK were from Roche Applied Science. Sepharose CL-6B, DEAE-Sephalc chromatography of the placental extract was dissolved (2 mg/ml) in 25 mM sodium phosphate, pH 7.2, containing 50 mM NaCl, 0.02% NaN3, 4 M GdnHCl, and 42% (w/w) CsBr. The solutions were centrifuged (8 ml/tube) in a Beckmann 50 T1 rotor at 44,000 rpm for 65 h at 14 °C (17). Fractions (2 ml) were collected from the bottom of the tubes into 15 equal portions, absorption at 260 and 280 nm was measured, and aliquots were analyzed for uronic acid content (16). The uronic acid-containing fractions were pooled, dialyzed, and lyophilized; yield was 7.5 mg.

Purification of the CSPG by Size Exclusion Chromatography—The proteoglycan partially purified by CsBr density centrifugation was chromatographed on a column of Sepharose CL-6B (2 × 65 cm) in 50 mM NaOAc, 150 mM NaCl, pH 6.0, containing 4 M GdnHCl. Fractions (2 ml) were collected and monitored for proteins by measuring absorption at 280 nm, and aliquots were analyzed for uronic acid contents (16); yield was 4.9 mg.

Isolation of the CS Chains of Placent al CSPG—The purified CSPG (0.6 mg) was dissolved in 0.5 ml of 0.1 M NaOH, 1 M NaH4 and incubated at 45 °C for 24 h under nitrogen (18). The solution was cooled in an ice bath, neutralized with 1 M HClOAc, and dried in a rotary evaporator. Boric acid was removed by repeated evaporation with 0.1% acetic acid in methanol. The residue was dissolved in 0.2 M NaCl and chromatographed on a Sepharose CL-6B column (1 × 49 cm) in 0.2 M NaCl. Fractions (0.67 ml) were collected, and aliquots were assayed for uronic acid content (16). Fractions containing the CS chains were combined, dialyzed (molecular weight cut-off 3,500) against distilled water, and lyophilized.

Carbohydrate Composition Analysis—The purified proteoglycan or the CS chains (5–10 μg each) released by alkaline β-elimination of the CSPG were hydrolyzed with 400 μl of 4 M HCl at 100 °C for 4 h, and the hydrolysates were dried in a SpeedVac and analyzed for hexosamines. For analysis of neutral sugar in the core proteins, the purified CSPG was treated with protease-free chondroitinase ABC (see below) and analyzed by SDS-PAGE using 15% gels, and the protein bands in the gels were transferred onto PVDF membranes, stained for 30 s with 0.1% Coomassie Blue in 40% MeOH and 1% AcOH, and destained with 50% MeOH. The membrane portions corresponding to the protein bands were cut into pieces, suspended in 400 μl of 2.5 M trifluoroacetic acid and hydrolyzed at 100 °C for 5 h, and the hydrolysates were dried as above. The hydrolysates from both procedures were analyzed on a CarboPac PA1 high pH anion exchange column (4 × 250 mm) using a Dionex BioLC HPLC system with a pulsed amperometric detector (19).
The elution was performed with 20 mm sodium hydroxide, and the response factors for the monosaccharides were determined using standard sugar solutions.

**Treatment with Chondroitinases**—For GAG chain analysis, the purified CSPG or GAG chains obtained by alkaline β-elimination (100 µg) were treated with chondroitinase ABC (20 milliunits) in 50 µl of 100 mM Tris–HCl, pH 8.0, containing 30 mM NaOAc and 0.01% BSA at 37 °C for 5 h (20). The CSPG (50 µg) was also treated with chondroitinase AC II (200 milliunits) in 50 µl of 100 mM NaOAc, pH 6.0, containing 0.01% BSA at 37 °C for 30 min (21).

**Disaccharide Compositional Analysis of GAGs**—The GAGs (10–15 µg), obtained by the treatment of the purified proteoglycan with 0.1 M NaOH, 1 M NaBH₄, followed by Sepharose CL-6B chromatography, were digested with chondroitinase ABC (5 milliunits) as above. The released unsaturated disaccharides were analyzed on a 4.6 × 250-mm amine-bonded silica PA03 column (YMC Inc., Milford, MA), using a Waters 600E HPLC system (Milford, MA), and eluted with a linear gradient of 16–530 mM NaH₂PO₄ over 70 min at room temperature at a flow rate of 1 ml/min (22). The elution of disaccharides was measured by recording the absorption at 232 nm with a Waters 484 variable wavelength UV detector. The data were processed with the Millennium 2010 chromatography manager using NEC PowerMate 433 data processing system.

**Analysis of the CSPG Core Protein by SDS-PAGE and Western Blotting**—The purified CSPG (100 µg) was treated with protease-free chondroitinase ABC (20 milliunits; protease inhibitors were also added to the incubation mixture as a precaution) in 100 µl of 100 mM Tris–HCl, 30 mM NaOAc, pH 8.0, at 37 °C for 6 h (20). The enzyme digest corresponding to 20 µg of CSPG and 30 µg of untreated CSPG was electrophoresed on 4–15% polyacrylamide gradient gels under reducing conditions using 2-mercaptoethanol. The gels were stained sequentially with Coomassie Blue followed by Alcian Blue. For Western blot analysis, the protein bands (corresponding to 6 µg of CSPG) on gels were transferred onto PVDF membranes, and the membranes blocked with 1% BSA in 50 mM Tris–HCl, 150 mM NaCl, pH 8.0, containing 0.1% Tween 20. The membranes were incubated with 1:1000 diluted rabbit anti-SSP antiserum, and the bound antibodies were detected using alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibodies and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate color-developing reagent.

**NH₂-terminal Sequencing of the CSPG Core Proteins**—The core proteins released from the purified CSPG (100 µg) by protease-free chondroitinase ABC (20 milliunits) were electrophoresed (20 µg CSPG/well) on 15% gels under reducing conditions. The protein bands on gels were transferred onto PVDF membranes using 10 mM 3-(cyclohexylamino)propane sulfonic acid buffer, pH 11.0, containing 10% methanol. The membranes were stained with Ponceau S, and the protein bands were cut out and sequenced by the Edman degradation method at the protein sequencing facility of the Penn State University College of Medicine using Applied Biosystems Procise 491 peptide sequencer.

**Analysis of Nonglycosylated and CSPG Form of SSP in Rat Placenta**—The isotonic buffer extracts of rat placentas were analyzed on 15% polyacrylamide minigels before and after treatment with protease-free chondroitinase ABC, and the protein bands in the gels were transferred onto PVDF membranes. The membranes were treated with 1:1000-diluted rabbit anti-SSP antiserum followed by 1:2000 diluted horseradish peroxidase-conjugated goat anti-rabbit IgG. The bound secondary antibody was detected using chemiluminescence substrate.

**Other Analytical Procedures**—The uronic acid contents were determined by the Dische method (16). Protein contents were estimated by using the micro-BCA protein estimation kit from Pierce (23).

**P. falciparum Culture**—The C4S adherent *P. falciparum* were panselected from a 3D7 parasite clone as described earlier (24). Parasites were cultured in RPMI 1640 medium supplemented with 25 mM HEPES, 29 mM sodium bicarbonate, 0.005% hypoxanthine, p-aminobenzoic acid (2 mg/liter), gentamicin sulfate (50 mg/ml), and 10% O⁺ human serum using type O⁺ human red blood cells at 3% hematocrit (24, 25). The cultures were incubated at 37 °C in an atmosphere of 90% nitrogen, 5% oxygen, and 5% carbon dioxide. Parasites with 20–30% parasitemia at the early trophozoite stage were used for the IRBC binding and inhibition assays.

**IRBC Adherence Assay**—The assay was performed as described previously (24). Briefly, the purified rat placental CSPGs at various concentrations were coated onto plastic Petri dishes as 0.4-cm circular spots, blocked with 2% BSA in PBS, pH 7.2, and then overlaid with a 2% suspension of parasite culture in PBS, pH 7.2. The unbound IRBCs and RBCs were washed with PBS, and the bound IRBCs were fixed with 2% glutaraldehyde. The bound IRBCs were stained with Giemsa and examined under the light microscope.

**IRBC Adherence Inhibition Assay**—Solutions (1 µg/ml) of the purified rat placental CSPG were coated onto plastic plates as 4-mm circular spots, and the spots were blocked with 2% BSA. The IRBC suspensions in PBS, pH 7.2, were preincubated with the indicated amounts of partially sulfated C4S (40% 4-sulfate, 59% nonsulfated, and 1% 6-sulfate), C6S, chondroitin, or hyaluronic acid at room temperature for 30 min with occasional mixing. The cell suspensions were overlaid onto the CSPG-coated spots and allowed to stand at room temperature for 30 min. After washing the unbound cells, the bound cells were fixed, stained, and counted by light microscopy.

**RESULTS**

**Isolation and Purification of the Extracellular Proteoglycan of Rat Placenta**—DEAE-Sepharose chromatography of the isotonic buffer extracts of the placenta from 20- or 21-day-old pregnant rats using a 0.15–0.75 M NaCl gradient eluted the uronic acid-containing material (proteoglycan) as a single homogeneous peak at 0.35 M NaCl (Fig. 1). Final elution of the column with 1.2 M NaCl did not elute any uronic acid-containing material (not shown). Upon CsBr gradient centrifugation, the proteoglycan was fractionated as a broad peak to the middle of the density gradient, separating from most of the associated proteins, which remained...
at the top of the gradient (Fig. 2). Upon further purification by size exclusion chromatography on Sepharose CL-6B column, the proteoglycan was eluted as a single but somewhat asymmetrical peak (\(K_{av} = 0.67\)) with an estimated average molecular weight of \(\sim 120,000\) (Fig. 3). The yield and composition of the proteoglycan is given in Table 1. The presence of protein, uronic acid, and hexosamine in high amounts is consistent with the proteoglycan nature of the purified material from rat placentas.

Characterization of the GAG Chains of Rat Placental Blood Space Proteoglycan—The carbohydrate compositional analysis revealed that the purified proteoglycan contains >99% GalN and a trace amount of GlcN (Table 1). Upon treatment with chondroitinase ABC or chondroitinase AC II, the GAG chains were quantitatively converted into disaccharides (not shown). These results indicated that the proteoglycan is a CSPG. The CSPG was subjected to alkaline \(\beta\)-elimination using NaOH/NaBH\(_4\), and the released CS chains were purified by gel filtration on a Sepharose CL-6B column calibrated with chondroitin sulfates of known molecular weight (Fig. 4). The CS chains of the CSPG were eluted as a single symmetrical peak with an estimated molecular weight of \(\sim 40,000\). Digestion of the CSPG with chondroitinase ABC and HPLC analysis of the disaccharides formed showed that the CS chains consist of 2% 4-sulfated and 98% nonsulfated disaccharides, demonstrating

![Image](image_url)
that the CS chains of the CSPG are extremely low sulfated, resembling nonsulfated chondroitin chains (Table 1).

**Characterization of Core Proteins of the Rat Placental CSPG**—Upon SDS-PAGE analysis, the purified CSPG was electrophoresed as two partially separated broad bands with mobility corresponding to molecular weights of 60,000–80,000 (average ~70,000) and 80,000–180,000 (~130,000) (Fig. 5A). The core proteins released by the treatment of the purified CSPG with chondroitinase ABC were electrophoresed as a doublet corresponding to molecular mass of ~17 and ~18 kDa (Fig. 5A). The relative abundance of the 17- and 18-kDa core protein bands in gels, as assessed by the densitometric scan, was ~57% and ~43%, respectively (Fig. 5B). The NH2-terminal sequence analysis of the two core protein bands on PVDF membranes revealed, in each case, an AILPDTTLYAELQEQ sequence for the first 15 amino acids. These results indicated that the 17- and 18-kDa doublets of the rat placental CSPG represent a single protein but differ either in posttranslational modifications or differentially processed at the COOH terminus.

Blast search analysis of the NCBI protein data base indicated that the NH2-terminal amino acid sequence of the core protein of the rat placental CSPG is identical to the NH2-terminal sequences of the rat placental SSP (GenBankTM accession number AB0099890), which has been shown to be a 107-amino acid secretory protein with the theoretically calculated molecular weight of 12,210 (15) (Fig. 6). Iwatsuki et al. (15) have previously shown that SSP is expressed specifically during the late stage of the placental development. To confirm whether the core protein of the rat placental CSPG is SSP, the CSPG was treated with chondroitinase ABC, and the released core proteins were analyzed by Western blotting using anti-rabbit antiserum raised against the recombinant SSP expressed in E. coli. The 17- and 18-kDa core protein bands were specifically reactive to the anti-SSP polyclonal antibodies (see Fig. 5C), demonstrating that both core proteins of the CSPG represent rat placental SSP.

SSP contains two potential GAG attachment sites, one at Ser74 and the other at Ser96 (see Fig. 6). Therefore, we reasoned that the two distinct SSP-CSPG species, 60,000–80,000 and 80,000–180,000 (see Fig. 5A), observed upon SDS-PAGE represent SSP bearing one and two CS chains, respectively, and that the 17- and 18-kDa core proteins of SSP-CSPG correspond to SSP carrying, respectively, one and two core carbohydrate moieties. The ~1-kDa difference in molecular mass between 17- and 18-kDa core proteins of SSP-CSPG probably corresponds to the difference in the amount of sugars that remain at the CSP attachment sites on the core proteins after treatment of the CSPG with chondroitinase ABC. To determine whether this is the case, the core proteins released by chondroitinase ABC were separated by SDS-PAGE and blotted onto PVDF membranes, and the carbohydrate composition of protein bands was determined (Table 2). The results indicated that the 18-kDa core protein band has about twice the amount of galactose and xylose per mol of protein compared with those present in 1 mol of the 17-kDa protein band. Since the rat CSPG has no N-glycans, the difference in the amounts of galactose to xylose in the 18- and 17-kDa core proteins must correspond to the difference in the number of CS chains attached to the core proteins. It is known that the common core glycan moiety at the GAG attachment regions of the proteoglycans is a tetrasaccharide consisting of GlcA-Gal-Gal-Xyl and that chondroitinase ABC treatment of the CSPGs removes all of the GAG chain disaccharide repeat moieties except the one that is attached directly to the tetrasaccharide glycan core. Since the residual disaccharide moiety remaining on the core glycan moiety is present as an unsaturated stub, and because the GAG chains of rat SSP-CSPG are predominantly nonsulfated, the structure of the residual glycan left on the core protein after chondroitinase ABC treatment probably corresponds to ΔGlcA-GalNAc-GlcA-Gal-Gal-Xyl. The calculated mass of the GAG chain core glycan with one attached unsaturated nonsulfated disaccharide stub is 1017 Da. This value is comparable with the observed difference in the molecular mass of the 17- and 18-kDa core proteins released from SSP-CSPG. From these results, it is
likely that the 60,000–80,000 and 80,000–180,000 CSPG species observed on SDS-PAGE (see Fig. 5A) represent SSP-CSPG with one and two CS chains, respectively.

Analysis of the Relative Levels of Free and CSPG Form of SSP—Analysis of the PBS extract of rat placentas by Western blotting indicated the presence of a protein band at a mobility slightly faster than that of the 17-kDa core protein band and a diffused high molecular mass, chondroitinase ABC-susceptible proteoglycan band (Fig. 7). These bands correspond, respectively, to SSP lacking the CS chains and SSP-CSPG. Western blotting of the placental extract after treatment with chondroitinase ABC showed the presence of 17- and 18-kDa SSP bands (not well resolved, probably because high levels of other components in the total placental extract interfering with the electrophoretic mobility) with concomitant abolition of high molecular weight CSPG band (Fig. 7). These data taken together indicate that SSP is present predominantly in the CSPG form. Based on the intensity of the SSP bands in Western blots, before and after treatment with chondroitinase ABC, SSP without the GAG chains and the CSPG form of SSP are present in the approximate proportions of 10 and 90%, respectively.

Adherence of P. falciparum-IRBCs to the Purified Rat Placental CSPG—To determine whether the purified rat placental CSPG binds IRBCs, the CSPG was coated onto plastic plates at different concentrations and tested with IRBCs selected for binding to human placental CSPGs (24). Despite the presence of very low level of sulfate in the chondroitin chains, the rat placental CSPG could efficiently bind IRBCs in a concentration-dependent manner similar to IRBC binding to human placental aggrecan CSPG (Fig. 8A) (7). The CSPG exhibited a significant level of IRBC adherence at coating concentrations of 50–500 ng/ml and a saturated level of binding at the coating concentration of 1 µg/ml. The IRBC binding was totally abolished when the CSPG-coated spots were treated with chondroitinase ABC (not shown). The binding of IRBCs to the CSPG-coated plates was inhibited efficiently by partially sulfated C4S but not by chondroitin, C6S, hyaluronic acid, or heparin, indicating that the adhesion is C4S-specific and that the IRBCs bind to the chondroitin sulfate chains of the SSP-CSPG (Fig. 8B) (data not shown).

The IRBC-binding strength of the rat placental CSPG was assessed by measuring the inhibition of IRBC binding by a C4S containing 40% 4-sulfated disaccharide moieties. The C4S could inhibit the binding of IRBCs to rat CSPG in a dose-dependent manner with an IC_{50} of 1.25 µg/ml C4S (Fig. 8B).

DISCUSSION

This paper describes the biochemical characterization of a novel uniquely low-sulfated secretory CSPG of rat placenta. The CSPG can be extracted readily by isotonic buffer from rat placentas, and, as indicated by the SDS-PAGE analysis, it consists of two distinct species with electrophoretic mobility corresponding to the molecular masses of ~70,000 and ~130,000 Da. The CS chains of the rat CSPGs are extremely low sulfated; on an average, two disaccharide moieties of the CS chains per 100 disaccharides are sulfated, and the sulfation is exclusively at C-4. The occurrence of very low sulfated CSPGs in animal tissues is very rare. Previously, only the aggrecan type CSPG of the human placental intervillous space and a subset of bovine corneal decorin CSPG have been identified as very low sulfated proteoglycans with ~8 and ~15%, respectively, of the disaccharide moieties of the CS chains being 4-sulfated (9, 26). Compared with the CS chains of human placental and bovine corneal low sulfated CSPGs, the degree of sulfation in the CS chains of rat placental CSPG is even lower. Thus, the rat placental CSPG is the most undersulfated among the CSPGs of various animal tissues that have been characterized to date.
Rat Spongiotrophoblast-specific CSPG

A previous study from our laboratory has demonstrated that the extracellular low sulfated CSPG of the human placental intervillosus layer has a high molecular weight (0.57–1 × 10⁶) aggrecan family proteoglycan with 6–8 low sulfated CS chains of ~60 kDa per molecule (7, 9). In this study, interestingly, we found that the low sulfated rat placental CSPG is completely different, and actually it is SSP bearing one or two chondroitin sulfate chains of ~40 kDa. The following evidence supports this finding. (i) The rat placental CSPG consists of two distinct core proteins with a molecular mass of ~17 and ~18 kDa as revealed by SDS-PAGE analysis before and after treatment with chondroitinase ABC. The NH₂-terminal amino acid sequence of each core protein is identical to that of rat SSP. Based on the 40-kDa size of the CS chains, it appears likely that the 17- and 18-kDa core proteins are derived from ~70- and ~130-kDa CSPG species bearing, respectively, one and two CS chains. (ii) The exclusive presence of galactose and xylose as neutral sugars, in a molar ratio of ~2:1 in both 17- and 18-kDa core proteins strongly suggests that these sugars represent the core sugar residues at the linkage region of the GAG chains. Further, the overall stoichiometry of these two sugars in the core proteins (i.e. ~1.8 mol of galactose and ~1 mol of xylose/mol of ~17-kDa core protein and ~3.7 mol of galactose and ~2 mol of xylose/mol of 18-kDa core protein) confirms that the 17- and 18-kDa core proteins are substituted with one and two CS chains, respectively. This conclusion also agrees with the observed ~1-kDa difference in the molecular mass of the two core proteins released by the chondroitinase ABC-treatment of the rat placental CSPG. As described under “Results,” the observed ~1-kDa mass difference between 17- and 18-kDa core proteins is due to the presence of one and two ΔGlaGalNAc-GlcA-Gal-Gal-Xyl-carbohydrate moieties, respectively (where ΔGla is 4,5-unsaturated glucuronyl residue), in the former and the latter core proteins released by chondroitinase ABC. (iii) Based on the amino acid sequence of rat SSP (see Fig. 6), it is logical to conclude that the ~70-kDa CSPG species represent SSP with one CS chain at either Ser⁷⁴ or Ser⁹⁶, and the ~130-kDa CSPG species represent SSP carrying CS chains on both Ser⁷⁴ and Ser⁹⁶. (iv) Finally, the deduced amino acid sequence of rat SSP lacks potential N-glycosylation sites. Consistent with this information, the carbohydrate compositional analysis showed only a trace amount of GlcNAc in the intact CSPG as well as in the 17- and 18-kDa core proteins obtained by chondroitinase ABC treatment of the CSPG. Thus, the low sulfated rat placental CSPG is the proteoglycan form of SSP carrying one and two chondroitin sulfate chains.

Previously, SSP has been reported as nonglycosylated secretory molecule. Although this is true with regard to N-glycosylation, the results of this study clearly show that SSP occurs predominantly in the CSPG form and that only a minor portion of SSP is present without GAG chain substitution. In rat placentas, the expression of SSP has been reported to start on the 14th day of the pregnancy with the maximum level of expression at day 16 and remains at this level until the term. Our results show that throughout its expression from day 14 until the term, SSP is present predominantly in the CSPG form. The expression of SSP appears to correspond to the period of full establishment of spongiotrophoblast layer at the maternal and fetal interface between the trophoblast giant cell layer of the junctional zones and the labyrinth (15). The strategic location of SSP-CSPG expression in the placenta and the pregnancy stage- and placental developmental stage-specific expression of SSP-CSPG are likely to have important biological relevance. Placenta produces pregnancy-specific hormones and a multitude of chemokines, growth factors, cytokines, and other factors that are essential for the maintenance and progression of pregnancy, modulation of immune and endocrine functions, development of the fetus, and delivery (27–31). For example, prostaglandins and endothelins produced in response to certain cytokines have been reported to function as the key myometrial contratile agents essential for delivery (27, 32). Placental hormones and growth factors are likely to enter fetal compartments, where they promote fetal development. Thus, based on the ability of GAG chains to interact with a variety of cytokines and other factors (33), we speculate that the low sulfated SSP-CSPG, secreted by the spongiotrophoblast cells to the maternal blood that bathes the labyrinth, is involved in reversibly capturing and mobilizing cytokines, hormones and growth factors produced by placenta, creating concentrated
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pools of these molecules in the maternal and fetal interface and modulating the functions of placenta. The low sulfated CSPGs are ideally suited for this purpose, because the low net negative charge allows capturing and mobilizing nutrients, cytokines, hormones, and growth factors by low affinity interactions and nonetheless readily release the bound factors for their functions. It is anticipated that the results reported here will facilitate efforts toward understanding the functional role of SSP-CSPG in the placenta.

Recently, it has been found that *P. chabaudi* AS also sequesters in the parasite-infected mouse placenta, and this system has been suggested as a suitable mouse model for studying maternal malaria (34). If the low sulfated SSP-CSPG is found to have a role in the sequestration of IRBCs in the placentas of infected rodents, then pregnant rats would be useful as a model to study the therapeutic benefits of CS oligosaccharides and/or their mimetics. Further *in vivo* studies are required to validate the model.

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