Malaria Circumsporozoite Protein Binds to Heparan Sulfate Proteoglycans Associated with the Surface Membrane of Hepatocytes

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Summary

During feeding by infected mosquitoes, malaria sporozoites are injected into the host's bloodstream and enter hepatocytes within minutes. The remarkable target cell specificity of this parasite may be explained by the presence of receptors for the region II-plus of the circumsporozoite protein (CS) on the basolateral domain of the plasma membrane of hepatocytes. We have now identified these receptors as heparan sulfate proteoglycans (HSPG). The binding of CS to the receptors is abolished by heparitinase treatment, indicating that the recognition of region II-plus is via the glycosaminoglycan chains. We have purified and partially characterized the CS-binding HSPGs from HepG2 cells. They have a molecular weight of 400,000–700,000, are tightly associated with the plasma membrane, and are released from the cell surface by very mild trypsinization, a property which the CS receptors share with the syndecan family of proteoglycans.

Previous observations indicate that a hepatocyte ligand for malaria sporozoites is contained in the COOH terminus of the circumsporozoite protein (CS)[1] (1, 2). This ligand is evolutionarily conserved among the CS of all malaria parasites. Here we designate the conserved motif as region II-plus (EWXXCXVTCGXGXXXRXK/R) because it encompasses part of the previously defined region II (3), and because recognition by the liver receptor requires the presence of the cluster of positively charged amino acids RXK/R at the 3' end (Sinnis, P., B. Chait, P. Clavijo, and V. Nussenzweig, manuscript in preparation). Synthetic peptides representing portions of this sequence specifically inhibit host cell invasion by sporozoites, and binding of CS to its liver receptor (2).

The nature of the sporozoite hepatocyte receptors is unknown, but several lines of evidence suggest that they are sulfated molecules. Sporozoites of Plasmodium berghei bind specifically to sulfatide (4). CS has binding affinity for sulfatide, and for other sulfated macromolecules, such as heparin, fucoidan, and dextran sulfate (1, 4). CHO cells defective in genes affecting xylosyl-transferase and glycosaminoglycan biosynthesis bind CS less effectively than the parental cells (4).

Moreover, thrombospondin, properdin, and other proteins that bind sulfatide contain the region II-plus motif (5–9).

For these reasons, in the experiments to be described, we have attempted to reveal specific interactions between sulfated, biosynthetically labeled hepatocyte molecules and recombinant CS constructs.

Materials and Methods

Materials. For the coimmunoprecipitation studies, we used either rat (Brown Norway) liver, kidney tissue, or HepG2 cells (HB8065; American Type Culture Collection, Rockville, MD) metabolically labeled with carrier-free $Na_2^{35}SO_4$ (Amersham Corp., Arlington Heights, IL) or with [^{35}S]methionine and [^{35}S]cysteine (Tran^{35}S-label; ICN, Costa Mesa, CA). As ligands for the putative receptors, we used four recombinant Plasmodium falciparum CS proteins (2). Two of them are Escherichia coli derived (F. Hoffmann-La Roche Ltd.): CS27IVC, which contains region I, region II-plus, and a full representation of the repeat domain; and CSFZ (Cys), which is identical to CS27IVC except that it contains only a single copy of the repetitive sequence NANP. The other two recombinants, Falc-1 and Falc-2, are yeast derived and were kindly provided by Dr. I. Bathurst (Chiron Corporation, Emeryville, CA). Both Falc-1 and Falc-2 contain region I and the repeat domain, but only Falc-2 contains region II-plus. The HepG2 binding assays were performed in tissue culture plates (Removawell; Dynatech Laboratories, Chantilly, VA). For the immunoelectron microcopy studies, glutaraldehyde (grade I; Sigma Immunochemicals,

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[1] Abbreviations used in this paper: CS, circumsporozoite protein; HSPG, heparan sulfate proteoglycan; PI-PLC, phosphatidylinositol-specific phospholipase C.

St. Louis, MO) and paraformaldehyde (Eastman Kodak, Rochester, NY); Lowicryl K4M and London Resin Co. (LR) White (Polysciences Inc., Warrington, PA) and protein A gold 15 nm (PAG15; Amersham Corp.) were used. For proteoglycan purification, G-25 columns (PD-10; Pharmacia Fine Chemicals, Piscataway, NJ) and Centricon-10 units (Amicon, Beverly, MA) were used. Other reagents were Ham's F-12 medium (Sigma Immunochemicals); methionine-free DMEM (Gibco, Grand Island, NY); the mAb 2A10 (10) detecting the repeat domain of *P. falciparum* CS; prefixed *Staphylococcus aureus* cells (*S. aureus* cells; Pansorbin; Calbiochem-Novabiochem Corp., La Jolla, CA); heparinase II (Sigma Immunochemicals) and heparitinase (ICN; this enzyme does not degrade heparin), chondroitinase ABC (Boehringer Mannheim, Indianapolis, IN; ICN), pronase (Boehringer Mannheim), heparin and chondroitin sulfate (both from Sigma Immunochemicals); and phosphatidylinositol-specific phospholipase C (PI-PLC; Boehringer Mannheim).

Immunoelectron Microscopy. Normal rat kidney tissue was fixed with 4% paraformaldehyde and 1% glutaraldehyde in PBS and embedded in LR White. Normal human liver was fixed similarly and embedded in Lowicryl K4M (2). Ultrathin sections were sequentially labeled with 10–50 μg/ml CS27IVC, 15 μg/ml mAb 2A10, and a 1:30 dilution of PAG15. Before immunolabeling, part of the sections were incubated for 120 min at 37°C with heparitinase or chondroitinase ABC (both from ICN) using concentrations of 20 and 2 U/ml, respectively. Control specimens were incubated only with the gold markers or with mAb 2A10 and PAG15.

Biosynthetic Labeling. Rats received two intraperitoneal injections of 1 mCi carrier-free $Na_2^{35}SO_4$, 24 and 12 h before they were euthanized. The livers and kidneys were chilled on ice, minced and homogenized in lysis buffer A (1% Triton X-100, 100 mM NaCl, 50 mM Tris-HCl, pH 7.2, 1% BSA; [Sigma Immunochemicals] containing 1 mM PMSF, 5 μg/ml each of leupeptin, pepstatin, and antipain [all from Boehringer Mannheim] as protease inhibitors). After shaking for 2 h, the lysates were centrifuged at 16,000 g for 30 min. The supernatants were then used for coimmunoprecipitation with the CS recombinant proteins.

Semiconfluent HepG2 cell monolayers were incubated with 100 μCi/well $Na_2^{35}SO_4$ in Ham's F-12 medium supplemented with 1–2% FCS equilibrated with PBS by Sephadex G-25 passage. The incubation was performed in 6-well plates (Costar Corp., Cambridge, MA) for 6 h. After washing with PBS, the cells were scraped off the plate, and treated with lysis buffer A for 30 min on ice. Supernatants for coimmunoprecipitation were obtained by spinning the lysates for 30 min at 16,000 g.

A separate sample of the cells was metabolically labeled with 100 μCi/well [^{35}S]methionine and [^{35}S]cysteine for 3 h in methionine-free MEM containing 1% FCS equilibrated in PBS by Sephadex G-25 passage.

Release of Proteoglycans from the Surface of HepG2 Cells. Adherent HepG2 cells labeled with $Na_2^{35}SO_4$ as described above were washed three times with cold PBS, and then incubated for 5 min at 4°C with PBS containing 0, 2.5, 5, 10, 20, or 40 μg/ml trypsin (type XIII; Sigma Immunochemicals). The supernatants were removed and 200 μg/ml soybean trypsin inhibitor (Sigma Immunochemicals) was added. The trypsin-treated cells were then scraped off in PBS/BSA and extracts were prepared in lysis buffer A as above.

Separate samples of HepG2 cells labeled with sulfate were treated with 0, 31.25, 62.5, 125, 250, or 500 μg/ml heparin in medium for 10 min at 25°C, or incubated with 0, 6.25, 12.5, 25, 50, and 100 U/ml or PI-PLC for 60 min at 37°C. The supernatants of the treated cells were analyzed by SDS-PAGE and by coprecipitation with the CS. Before coimmunoprecipitation with the recombinant

CS proteins, the supernatants were mixed with 1:5 vol of lysis buffer A. The cell pellets were scraped off and lysed with buffer A as above.

CS/Proteoglycan Coimmunoprecipitation. All extracts and supernatants were first precleared with normal rabbit serum and *S. aureus* cells. Then, 32 μg/ml of CSFZ (Cys) or 64 μg/ml of CS27IVC, Falc-2, or Falc-1 were added for 30 min, followed by 10.8 μg/ml mAb 2A10 for 30 min, and 1% *S. aureus* cells (final concentrations). The suspensions were shaken for 1–5 h at 4°C. The *S. aureus* cells were washed three times with the lysis buffer A, two times with lysis buffer B (10 mM Tris-HCl, pH 7.2, containing 100 mM NaCl and 0.5% NP-40), one time with 50 mM Tris-HCl, pH 7.2, and processed in this buffer for electrophoresis under reducing and nonreducing conditions.

Samples of the immunoprecipitations were incubated with: 0.1 U/ml heparitinase (ICN) in 50 mM Tris-HCl, pH 7.0, containing 0.1% BSA; 1 U/ml chondroitinase ABC (ICN) in 50 mM Tris-HCl, pH 8.0, containing 0.1% BSA and 50 mM sodium acetate for 3 h at 37°C in the presence of protease inhibitors; or 100 μg/ml pronase for 1 h at 37°C. After this treatment, the *S. aureus* cells were washed and processed as above.

SDS-PAGE. $^{35}SO_4$-labeled immunoprecipitates were examined on either 5% or 3–20% gradient polyacrylamide gels (11). The gels were fixed with 10% glacial acid and 30% methanol, impregnated for 30 min in 1 M salicylic acid, dried, and exposed to Kodak X-Omat AR film at −70°C.

Purification of HepG2 Cell Proteoglycans. Trypsin-released and $^{35}SO_4$-labeled proteoglycans were equilibrated in 50 mM Tris-HCl, pH 6.5 by passage in a Sephadex G-25 column, and concentrated by Centricon-10 centrifugation. Urea (Boehringer Mannheim) was added to a final concentration of 7 M, and the sample subjected to anion exchange chromatography in a Mono-Q column using a FPLC apparatus (Pharmacia Fine Chemicals). Elution was performed with 50 ml of a 0–2 M NaCl gradient in 50 mM Tris-HCl, pH 6.5, containing 7 M urea. 1-ml fractions were collected and radioactivity counted in 20-μl aliquots. Positive fractions were pooled, equilibrated in 50 mM Tris-HCl, pH 7.2, and 7 M urea, and concentrated as above. A 200-μl sample (22,500 cpm) was subjected to a Superose 6 column and eluted with 50 mM Tris-HCl, pH 6.5, and 7 M urea. Fractions (0.5 ml) were collected, and the cpm were counted in 20-μl aliquots. Thyroglobulin (669 kD), apoferritin (443 kD), β-amylase (200 kD), and alcohol dehydrogenase (150 kD) were used as molecular weight markers (all from Sigma Immunochemicals). The combined positive fractions from three column runs were pooled and concentrated to a final volume of 1 ml. Part of this preparation was subjected to hydrolysis and amino acid and amino sugar analysis by Dr. S. Mische (Rockefeller University, New York). For amino acid analysis, the hydrolysis was with 6 N HCl, 110°C, 22 h, and for amino sugars, 4 N HCl, at 100°C, for 7 h. Analysis was performed using Maxima software, a model 510 pump, and a model 490 detector. Novapak C8, 15-cm column was used (all from Waters, Bedford, MA).

Inhibition of Binding of CS to HepG2 Cells. 10^5 HepG2 cells were deposited in 96-well Removawell tissue culture plates and allowed to grow overnight in MEM (Gibco) containing 10% FCS, 1 mM L-glutamine (all from Gibco), 3 mg/ml glucose (Sigma Immunochemicals) and 1× nonessential amino acids (Gibco). The HepG2 cells were fixed with 4% paraformaldehyde in TBS (50 mM Tris-HCl, pH 7.5, 137 mM NaCl, 2 mM KCl), washed three times with TBS, and stored at 4°C in BSA/TBS until use. CSFZ (Cys) at 5 μg/ml was incubated with increasing amounts of the presumed inhibitors at 37°C for 15 min. 50 μl of these mixtures were added to the cells, incubated for 1 h at 37°C, washed three times with TBS/0.05% Tween, and then sequentially incubated with 50 μl

of mAb 2A10 at a concentration of 10 μg/ml in TBS/BSA buffer for 30 min at 37°C, and 1:50 dilution of goat anti–mouse IgG conjugated to alkaline phosphatase (Boehringer Mannheim) for 30 min. Bound enzyme was revealed by the addition of the fluorescent substrate, 4-methylumbelliferyl-phosphate (Sigma Immunochemicals) in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM MgCl₂. After 15 min, the fluorescence was measured in a plate reader (Fluoroskan II; ICN).

In some experiments, the HepG2 cells were treated with either heparitinase (Sigma Immunochemicals) or chondroitinase ABC (ICN) before incubation with the CS. Heparitinase treatment was performed in 0.05 M acetate buffer, pH 6.0, containing 1 mg/ml BSA and 1 mM PMSF, and 5 μg/ml leupeptin and pepstatin. Cells were treated for 3 h at 37°C and then washed three times with TBS before protein was added. Chondroitinase ABC treatment was performed overnight at 37°C in 0.1 M Tris/HCl, pH 8.0, 0.03 M sodium acetate, and 0.1% BSA, and washed three times with TBS. Enzyme-treated cells were then incubated with CS as outlined above.

Results

Heparitinase Digests the Plasma Membrane Receptors for CS. To explore the possibility that the hepatocyte plasma membrane molecules that bind CS are proteoglycans, we incubated human liver sections with heparitinase or chondroitinase ABC for 2 h at 37°C, before immunolabeling with recombinant CS. The previously described, patchy pattern of CS-labeled hepatocyte microvilli within the space of Disse (2), was still present in the chondroitinase-treated sections (Fig. 1 *a*). However, heparitinase treatment abolished the Disse staining, and left only a faint labeling of the hepatocyte lysosomes (Fig. 1 *b*).

As shown previously, CS binds to paraformaldehyde-fixed HepG2 cells in a dose-dependent and saturable manner (2). The experiment illustrated in Fig. 2 shows that the CS binding to HepG2 cells is inhibited by heparin, heparan sulfate, dextran sulfate, and fucoidan, but not by chondroitin sulfate and dextran. In other experiments, the fixed HepG2 cells were treated with different concentrations of heparitinase or chondroitinase ABC, and then incubated with CS. As shown in Fig. 3, heparitinase treatment prevented CS binding, whereas chondroitinase ABC treatment had no effect.

CS Binds to Kidney Tubular Epithelia and Basement Membranes and to Mast Cell Granules. We previously reported that recombinant CS does not bind to sections of several organs, including spleen, brain, heart, and lung, although proteoglycans are present in all these organs. We have now extended these studies, and detected a highly selective CS staining in mast cell granules and in the rat kidney, in a pattern consistent with proteoglycan involvement. In the kidney, CS binds specifically to the basement membranes of the glomerular Bowman capsule, proximal tubules, Henle loop, distal and collecting tubules, but not to the glomerular basement membrane (Fig. 4, *a* and *b*). In addition, there was labeling on the basolateral domain of the proximal tubular epithelia, whereas the apical microvilli and all the other kidney epithelia were negative. The proximal epithelia showed intracellular CS staining of the lysosomes. To verify that proteoglycans in these locations were involved in CS binding, we treated

the kidney sections either with heparitinase or with chondroitinase ABC before CS staining as above. Again, only heparitinase abolished the staining (data not shown).

Connective tissue mast cell granules were also intensely labeled by the CS protein (Fig. 5). In this case, however, the staining was affected neither by previous incubation with heparitinase (which does not cleave heparin), nor by chondroitinase ABC treatment.

CS Binds to Heparan Sulfate Proteoglycans (HSPGs). In the next series of experiments we attempted to identify the CS receptors in rat liver, rat kidney, and HepG2 cells. Our approach was to biosynthetically label the cellular proteoglycans with ³⁵SO₄, incubate cell extracts with recombinant CS, and immunoprecipitate the complexes with a mAb directed against the CS repeats. Fig. 6 demonstrates that in these various locations the recombinant protein CSFZ (Cys) binds to sulfated macromolecules migrating as a smear on top of the SDS-PAGE gel, in a pattern typical of proteoglycans. Identical results were obtained when two other CS recombinants containing region II-plus (CS27IVC and Falc-2) were used, except that the immunoprecipitation was less efficient. Negative results were obtained with recombinant Falc-1, which does not contain region II-plus.

Several additional observations indicate that the sulfated macromolecules that bind CS are HSPGs. The high molecular weight bands on SDS-PAGE disappear after treatment of the immunoprecipitated material with heparitinase, but chondroitinase has no effect. Digestion of the immunoprecipitates with pronase results in a significant decrease of the apparent molecular weight of the sulfated molecules, which now migrate as a broad 200,000-M_r band in SDS-PAGE. Also, the addition of 100 μg/ml of heparin to the lysates inhibits the immunoprecipitation reaction, presumably by competing with the labeled proteoglycans for CS (Fig. 7).

The Nature of Membrane Attachment of the CS Cellular Receptors. HepG2 cells metabolically labeled with ³⁵SO₄, were incubated with increasing concentrations of trypsin for 5 min at 4°C. In a dose-dependent manner, labeled molecules were released from the cell surface. A plateau was reached at 20 μg/ml of trypsin, when 50% of the total incorporated label was released. Regardless of the trypsin concentration, about 80% of the released counts were coimmunoprecipitated with the CS recombinant CSFZ (Cys) (Fig. 8). Coimmunoprecipitation of the trypsin-released receptor with CS27IVC and Falc-2 was less efficient, reflecting perhaps the degree and/or type of aggregate formation in the different recombinants (2). Nevertheless, all region II-plus containing CS recombinants bound sulfated molecules which migrated as smears on top of SDS-PAGE gels (Fig. 9).

From the total HepG2 cell extracts before trypsin treatment, and from those after trypsin treatment, 54 and 37% of the counts, respectively, were coimmunoprecipitated by CSFZ (data not shown). The trypsin-insensitive, immunoprecipitable counts most likely represent HSPGs which are either intracellular or are associated with the plasma membrane in areas of the cells that are attached to the plastic and are inaccessible to trypsin. On SDS-PAGE gels, both cell associated, and trypsin-released molecules ran similarly as high
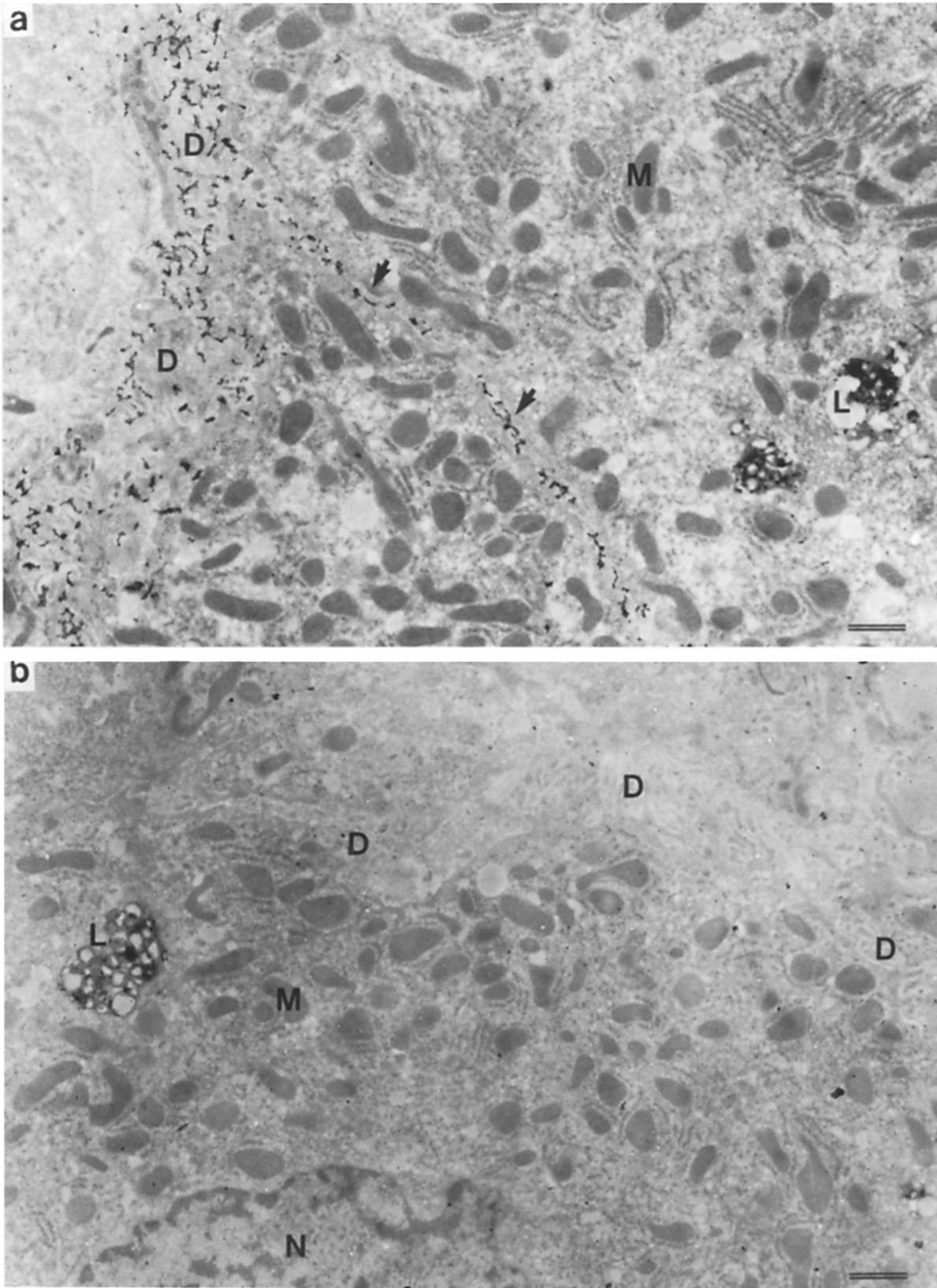
Figure 1. CS receptors are heparitinase sensitive. Electron micrographs of Lowicryl K4M-embedded human liver labeled with CS27IVC, followed by mAb 2A10 and PAG15. (a) This section was treated with 2 U/ml chondroitinase ABC (ICN) for 2 h at 37°C before the immunolabeling. The
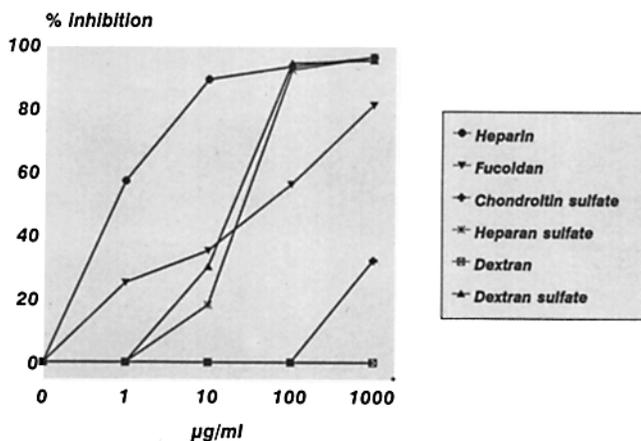
% Inhibition

Figure 2. Inhibition by glycosaminoglycans of CS binding to HepG2 cells. Proteoglycans were preincubated with 5 µg/ml of CSFZ (Cys) and then added to paraformaldehyde-fixed HepG2 cells. Binding of CS to the HepG2 cells was revealed by mAb 2A10, followed by anti–mouse alkaline phosphatase–conjugated IgG. Bound enzyme was revealed by a fluorescent substrate, 4-methylumbelliferyl-phosphate. Each point represents the mean of triplicates. Percent inhibition was calculated by comparison to CSFZ (Cys) preincubated in medium alone.

% Inhibition
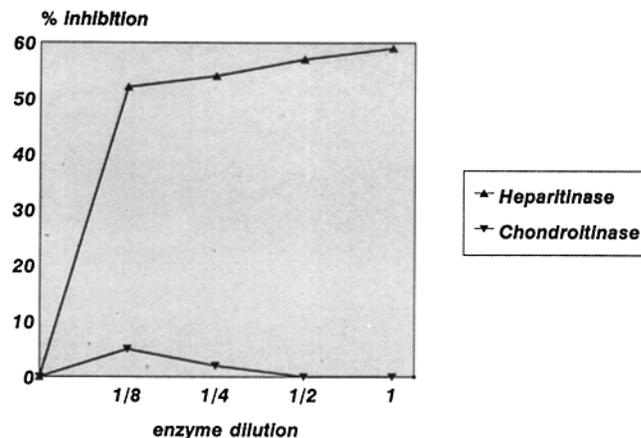
Figure 3. CS binding to HepG2 cells is inhibited by treatment of the cells with heparitinase, but not with chondroitinase ABC. Paraformaldehyde-fixed HepG2 cells were preincubated with varying amounts of enzyme, washed, and then incubated with 5 µg/ml of CSFZ (Cys). CS binding was revealed as described in Fig. 2. Heparitinase and chondroitinase ABC were used at initial concentrations of 5 and 1 U/ml, respectively. Each point represents the mean of triplicates, and percent inhibition was calculated by comparison to cells that were not treated with enzyme.

molecular weight smears on top of the gel, indicating that mild trypsinization of the HepG2 cells does not lead to extensive degradation of the proteoglycan core proteins (compare patterns in Fig. 7 and in the inset of Fig. 10).

To further analyze the mode of association of the CS receptor to the membrane of the $^{35}SO_4$-labeled HepG2 cells, the cells were treated with 0–100 U/ml PI-PLC for 60 min at 37°C or with 0–500 µg/ml heparin for 10 min at 25°C, respectively. Neither treatment lead to the specific release of sulfate-labeled molecules.

In an attempt to identify the core protein of the malarial protein HSPG receptor, HepG2 cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine for 3 h. As revealed by SDS-PAGE, the HepG2 cell extracts contained a very large number of radiolabeled proteins. However, after mild trypsinization as above (20 µg/ml, for 5 min, at 4°C), no counts were released in the supernatant above the background (data not shown). As shown below, this observation greatly facilitated the isolation of the CS receptor from the supernatants.

Purification and Properties of the CS Receptor from HepG2 Cell Membranes. Subconfluent sulfate-labeled HepG2 cells were incubated with 20 µg/ml trypsin as above, and the supernatants subjected to anion exchange chromatography on a Mono-Q column in buffers containing 7 M urea. The radiolabel was eluted from the column in a sharp peak (Fig. 10), and about 80% of the counts in this peak were coimmunoprecipitated by CSFZ (Cys). Heparin, but not chondroitin sulfate, inhibited coimmunoprecipitation. In addition,

heparitinase and pronase, but not chondroitinase ABC, degraded the CS receptors (inset of Fig. 10).

By molecular sieving chromatography on a Superose 6 column, the purified HSPG eluted in a broad peak with an apparent molecular weight between 400,000 and 700,000 (Fig. 11). The fractions in this peak were pooled and subjected to compositional analysis (Table 1). The results indicate the presence of large amounts of glucosamine, presumably originating from the heparan sulfate chains, and among the amino acids, a relatively high proportion of serine and glycine.

Discussion

We have previously shown that hepatocytes bear on their plasma membranes specific receptors for region II-plus of the CS protein. Here we demonstrate that the hepatocyte receptors are HSPGs. This conclusion is based on observations made at the tissue, cellular, and molecular levels. The binding of CS constructs to tissue sections and to HepG2 cells is specifically inhibited by heparitinase treatment of the cells, and by the presence of heparin in the incubation medium. In hepatocyte extracts, the CS receptors are sulfated molecules of 400,000–700,000 daltons, which migrate as a smear on SDS polyacrylamide gels and are digested by heparitinase. The size of the individual GAG chains, and of the core protein, remain to be determined. After pronase digestion, the sulfated molecules are significantly reduced in size to about 200,000 daltons, perhaps representing remnants of the core protein linked to multiple GAG chains.

typical CS label on the space of Disse (D), on the lateral domain of the hepatocyte plasma membrane (*arrows*), and on lysosomes (L) is unaltered. (b) This section was treated with 20 U/ml heparitinase (ICN) under identical conditions, and subsequent CS staining was not observed in the spaces of Disse (D). Only a faint label was left on the lysosomes (L). (M) Mitochondrion; (N) nucleus. Bars, 1 µm.
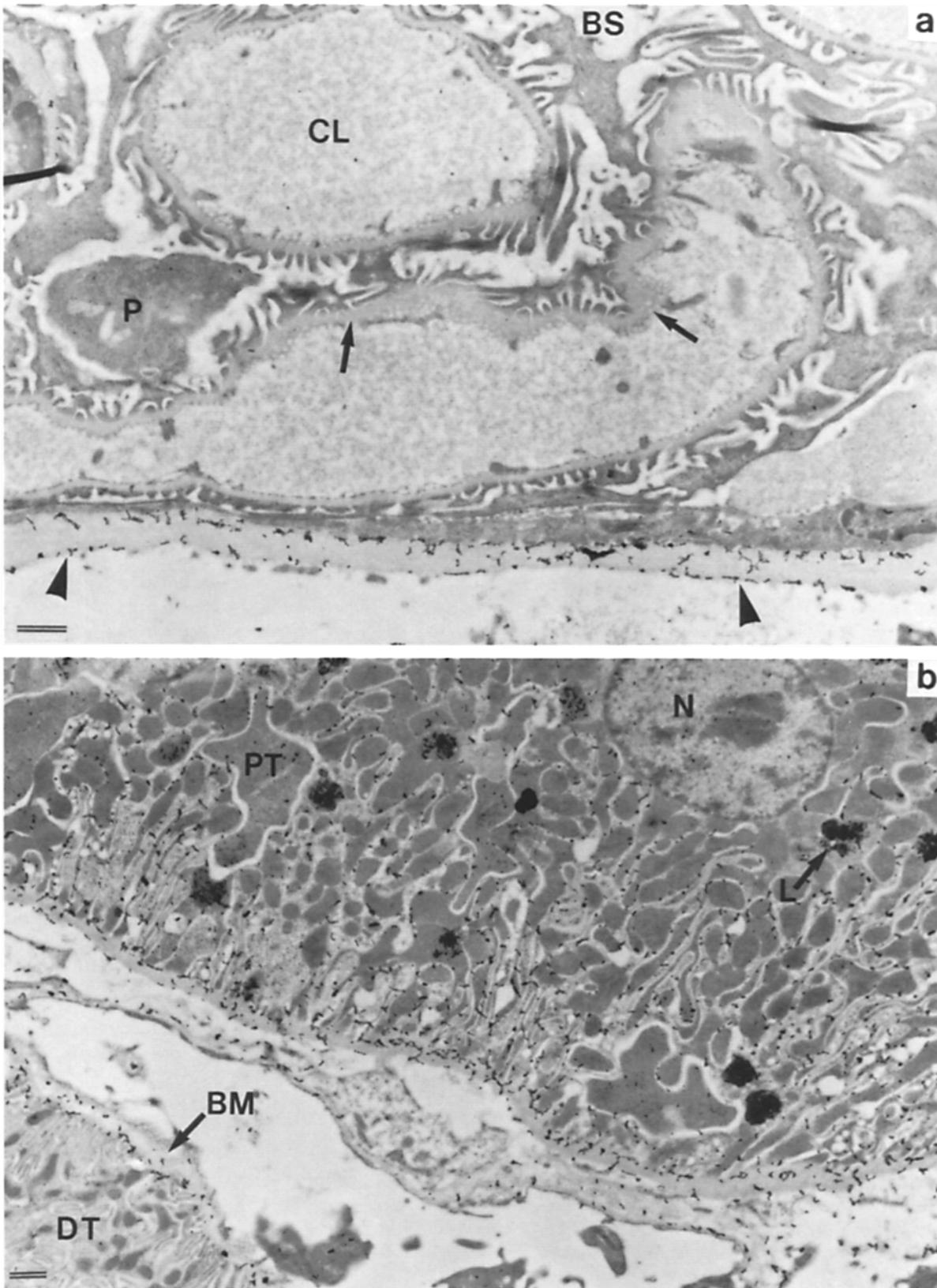
Figure 4. In the kidney, CS binds to the basolateral domains of proximal tubular epithelia, and to the basement membrane of the Bowman capsule. LR White-embedded rat kidney sections labeled with CS27IVC, mAb 2A10, and PAG15. (*a*) In the glomerulus, the CS binding is restricted to the basement membrane of the Bowman capsule, predominantly to the laminae rarae (*arrowheads*). The glomerular basement membrane below the fenestrated
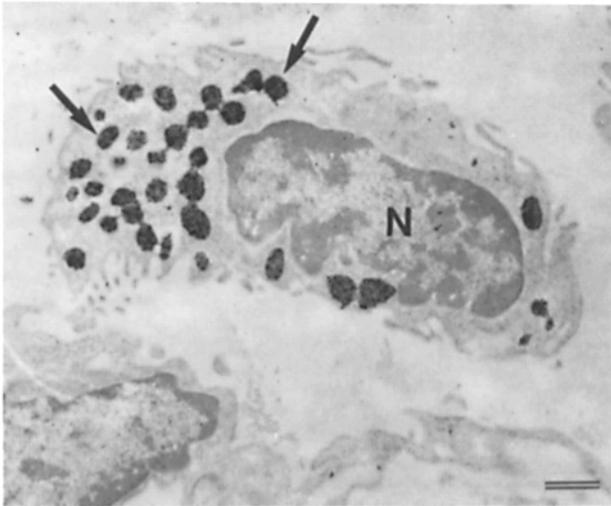
Figure 5. CS binds to connective tissue mast cell granules. Electron micrograph of an LR-White-embedded mast cell labeled with CS27IVC, mAb 2A10, and PAG15. The intracellular granules are intensely stained. Bar, 1 μm.
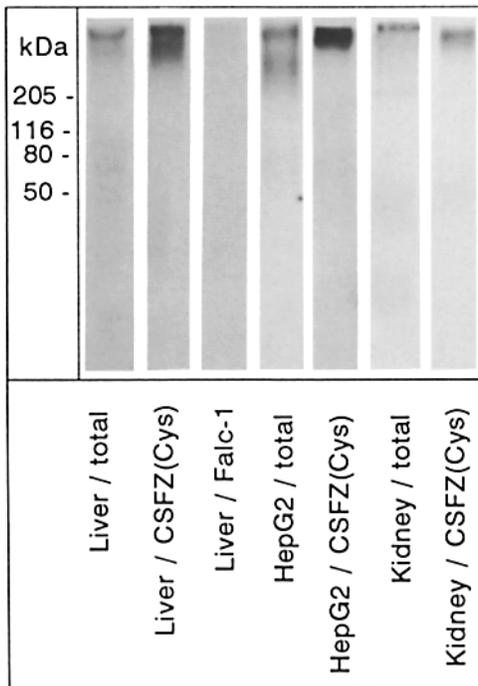
Figure 6. CS binds to sulfate-labeled molecules with high molecular weight in extracts of liver, HepG2 cells, and kidney. Extracts of cells metabolically labeled with $^{35}SO_4$ were incubated for 30 min at 4°C with 32 μg/ml of recombinant CSFZ (Cys) or 64 μg/ml Falc-1, and immunoprecipitated with mAb 2A10 and S. aureus cells. From the total liver lysate, CSFZ (Cys) coimmunoprecipitates sulfated molecules migrating as a smear on top of the SDS-PAGE. Immunoprecipitation with Falc-1 was negative. The SDS-PAGE patterns of the total lysate of HepG2 cells, and of the
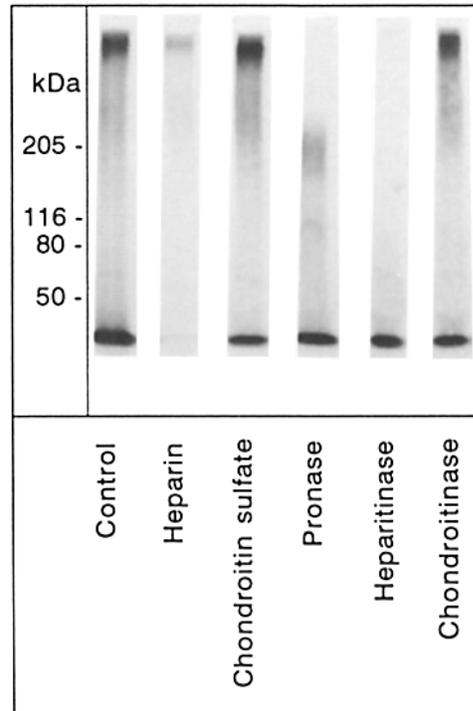

Figure 7. Susceptibility of the CS receptor to heparitinase and pronase treatments. Inhibition by heparin of the binding of the CS receptor to CSFZ(Cys). Coimmunoprecipitation of the CS receptor by CSFZ(Cys) was prevented by previous addition of 100 μg/ml heparin to $^{35}SO_4$-labeled HepG2 cell extracts (control lane), whereas the same concentration of chondroitin sulfate had no effect. Digestion of the proteoglycans by 100 μg/ml pronase for 1 h at 37°C after coimmunoprecipitation, results in a shift of the labeled smear to about 200 kD. Treatment of the immunoprecipitated material with 0.1 U/ml heparitinase for 3 h at 37°C led to complete degradation of the high molecular weight smear, whereas 1 U/ml chondroitinase ABC had no effect.

Biosynthetically labeled, sulfated molecules are rapidly removed from the surface membrane of HepG2 cells by low concentrations of trypsin, and more than 80% of the labeled released molecules are precipitated with the CS. The $^{35}SO_4$-labeled molecules from the membrane of HepG2 cells were purified by two chromatographic steps. By molecular sieving chromatography in the presence of 7 M urea, their apparent molecular weight is between 400,000 and 700,000 daltons, and on a molar basis they contain 40% glucosamine, the amino sugar found in heparan sulfate GAG chains. The smaller amounts of galactosamine may be derived from O-linked oligosaccharides, or from chondroitin sulfate GAG chains. The amino acid analysis, which reflects only the composi-

molecules coimmunoprecipitated with CSFZ (Cys) resemble the corresponding liver SDS-PAGE patterns. In kidney lysates, CSFZ (Cys) selectively immunoprecipitates a band of slightly lower molecular weight than the total kidney-sulfated molecules.

capillary endothelium (arrows) is unstained. (CL) Capillary lumen; (P) podocyte; (BS) Bowman space. (b) The epithelia of the distal tubule (DT) show CS label only on the laminae rarae of the basement membrane (BM). In contrast, the epithelia of the proximal tubule (PT) are also stained on their basolateral domains and on lysosomes (L). Nucleus. Bars, 1 μm.
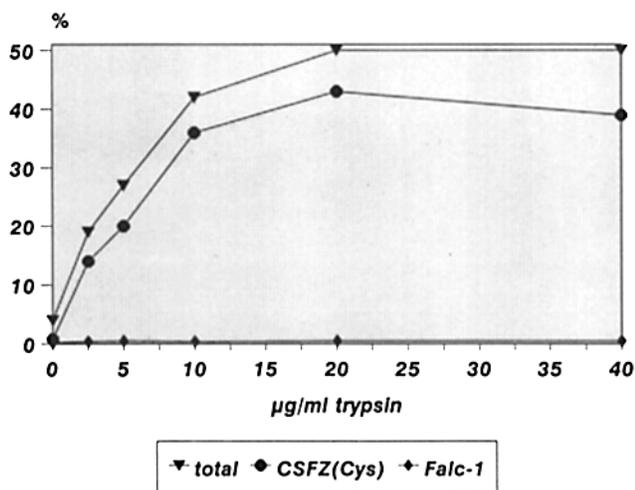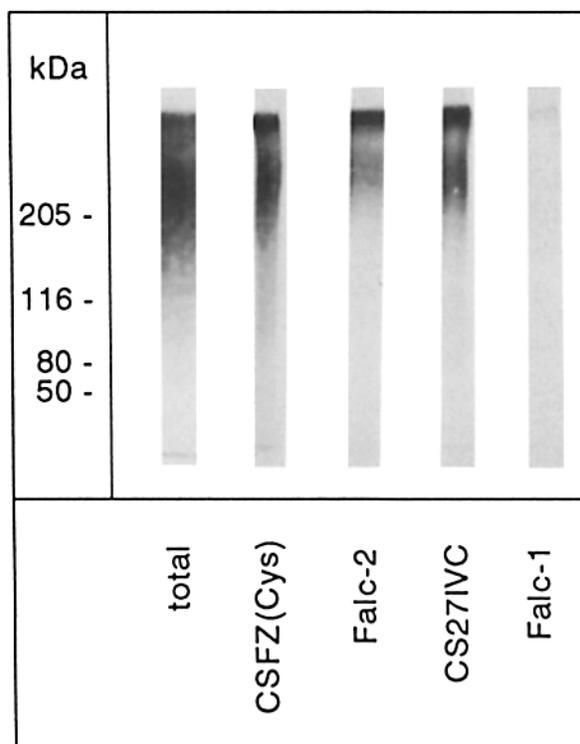
Figure 8. Trypsin sensitivity of the CS receptors from the surface of HepG2 cells. Subconfluent HepG2 cells were incubated with various concentrations of trypsin for 5 min at 4°C. Sulfate label is released in the supernatant in a dose-dependent manner, and maximum release (50% of the total incorporated cpm) is reached at 20 μg/ml trypsin. Independent of the trypsin concentration, about 80% of the released HSPG is coimmunoprecipitated by 32 μg/ml CSFZ (Cys) and 10.8 μg/ml mAb 2A10. Under the same conditions, Falc-1 is negative.

Figure 9. Binding of various CS constructs to proteoglycans released from the surface of HepG2 cells by 20 μg/ml trypsin. $^{35}SO_4$-labeled trypsin-released proteoglycan (total supernatant) was precipitated by CSFZ (Cys), Falc-2, CS27IVC, and Falc-1. All constructs containing region II-plus coimmunoprecipitated sulfated molecules migrating as smears on top of SDS-PAGE gels. The high molecular weight of the immunoprecipitated material shows that trypsin did not lead to extensive degradation of the CS receptors.

tion of the trypsin-resistant portion of the core protein, is not informative, except that it reveals the presence of a high content of serine and glycine. GAGs are known to be assembled on serine residues of the core protein, frequently within the amino acid sequence acidic-X-Ser-Gly-acidic (12, 13).

Heparitinase digestion or the presence of heparin in the incubation medium hinders CS binding to the receptor. The GAG chains are therefore involved in CS recognition, a conclusion further supported by the observation that the invasion of hepatocytes by sporozoites in vitro is inhibited by heparin, dextran sulfate, and fucoidan (2, 4). Nevertheless, a role for the core protein(s) in the interaction with the CS cannot be excluded.

HSPGs are ubiquitous constituents of mammalian cell surfaces. They may behave as integral membrane proteins, or peripheral membrane proteins that can be released from the cell membrane by treatment with heparin or high ionic strength (14–19). Both types of HSPGs have been reported to be present on the hepatocyte surface exposed to the space of Disse. A third possible mode of membrane attachment is through a PI anchor (20). However, this is unlikely to be the mode of attachment of the CS receptors, since in epithelial cells, PI-anchored molecules are sorted to their apical surface (21), whereas CS receptors are restricted to the basolateral cell membrane of hepatocytes. In fact, CS receptors are not cleaved from the membrane of HepG2 cells by PI-PLC, and are not eluted by treatment of the cells with heparin. As shown in Fig. 8, most sulfate-labeled CS receptors are released from subconfluent HepG2 cells by very mild trypsinization. These results indicate that the core protein of the HSPG is tightly attached to the membrane of HepG2 cells (and of hepatocytes [2]), most likely through a hydrophobic peptide anchor.

Table 1. *Compositional Analysis of the Purified Trypsin-released HepG2 Cell Proteoglycan*

| Component	Percent molar	Component	Percent molar
Gal-NH$_2$	5.2	Pro	4.4
Glc-NH$_2$	40.5	Tyr	0.7
Asp or Asn	2.3	Val	2.4
Glu or Gln	4.5	Met	1.2
Ser	8.7	Cys	0.0
Gly	12.8	Ile	1.9
His	0.3	Leu	3.4
Arg	0.9	Phe	1.1
Thr	3.4	Lys	1.6
Ala	4.8		

Fractions 13–22 from Fig. 10 (19,000 cpm, derived from 7 × 10^7 HepG2 cells) were pooled and subjected to amino acid and hexosamine analysis. On the basis of the results of the amino acid analysis, the sample contained 2.8 μg of the proteoglycan.
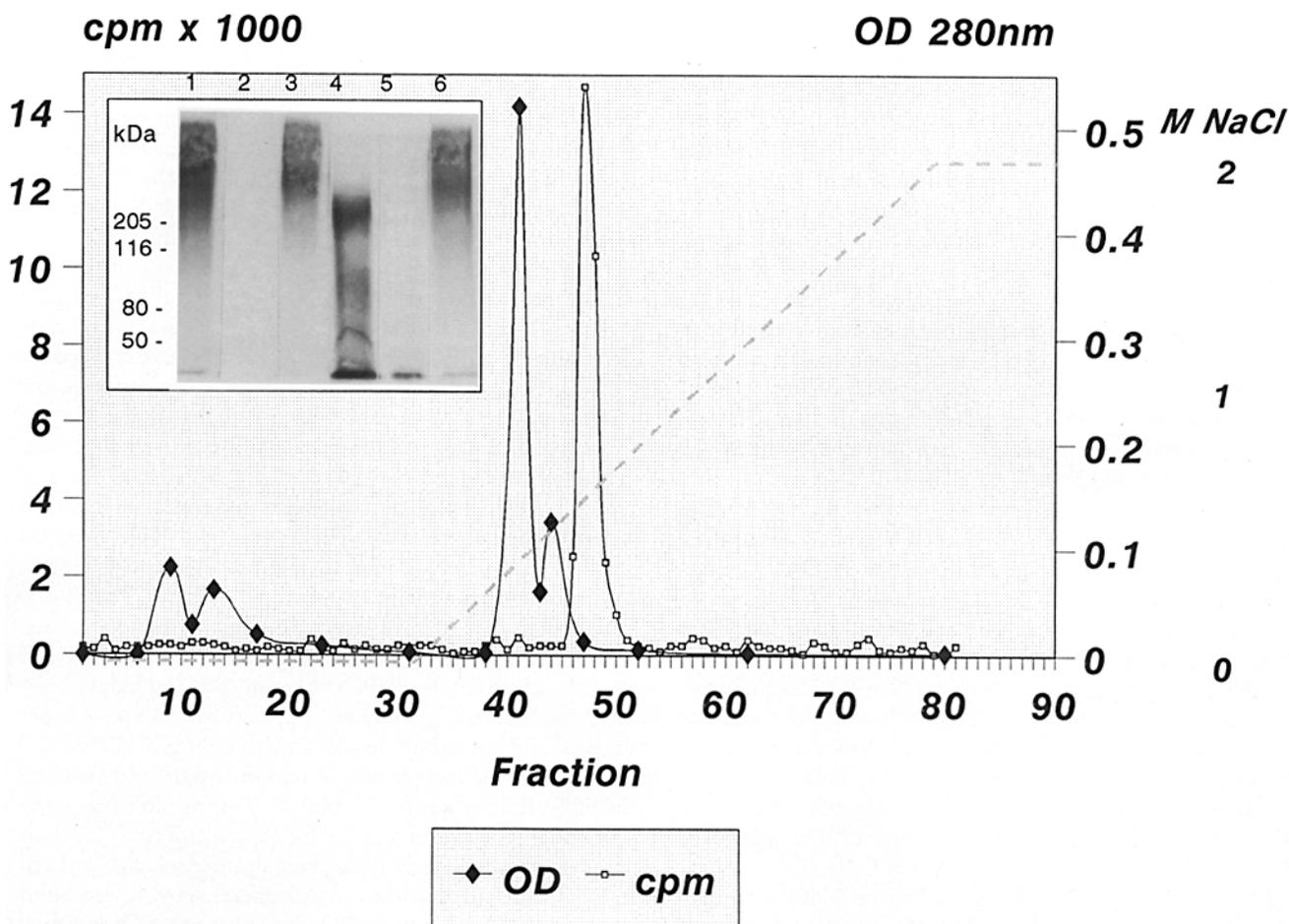
Figure 10. Ion exchange chromatography of trypsin-released HepG2 cell proteoglycans. Concentrated, trypsin-released (20 μg/ml, 5 min at 4°C) proteoglycans from HepG2 cells were loaded onto a Pharmacia Mono-Q column and eluted with a 0–2 M NaCl gradient in the presence of 7 M urea. Sulfate-labeled proteoglycans were eluted as a single sharp peak at a NaCl concentration of 0.6–0.8 M. Note that protein was not detectable in the labeled peak. 1-ml fractions were collected and counted. *(Inset)* CSFZ (Cys) (32 μg/ml) precipitates the purified proteoglycans as a high molecular weight smear (lane *1*). Heparin (lane *2*), but not chondroitin sulfate (lane *3*) inhibits the precipitation. Pronase (lane *4*), and heparitinase (lane *5*), but not chondroitinase ABC (lane *6*), degrade the high molecular weight band.

CS receptors share some properties with the syndecan family of HSPGs. Syndecans are a gene family of integral membrane proteins (22), which bear both heparan sulfate and chondroitin sulfate chains. They are mainly synthesized by epithelial cells, and like the CS receptor, many are targeted to the basolateral side of the cell membrane (23). The GAG composition varies in a cell-specific fashion, and can be modulated by cytokines (24). Another characteristic feature of syndecans is that they contain a single dibasic site immediately upstream of the anchor sequence which is very sensitive to proteolysis (25, 26). As shown here, the CS receptors can be released from the membrane of HepG2 cells by treatment for 5 min at 4°C with 10–20 μg/ml of trypsin, and may contain chondroitin sulfate GAG chains. Several matrix proteins are syndecan ligands, including thrombospondin, which shares region II-
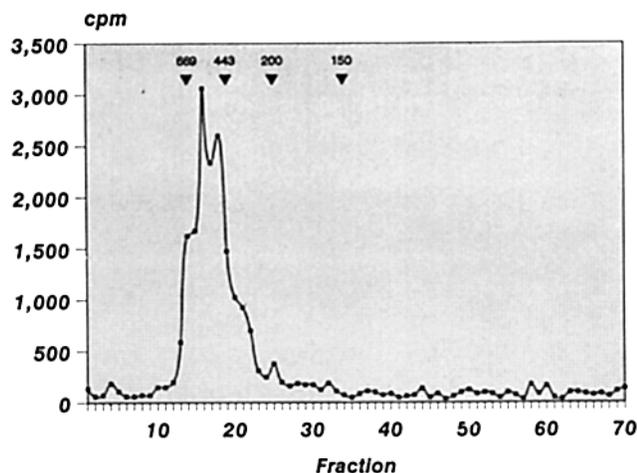
Figure 11. Molecular sieving chromatography of purified proteoglycans released by trypsin from HepG2 cells. The peak labeled fractions from the anion exchange chromatography were pooled (22,500 cpm) and loaded onto a Superose 6 column in 50 mM Tris buffer pH 6.5 containing 7 M urea. The $^{35}SO_4$ label eluted in a broad peak between 400 and 700 kD. Thyroglobulin (669 kD), apoferritin (443 kD), β-amylase (200 kD), and alcohol dehydrogenase (150 kD) were used as molecular weight markers. Fractions (500 μl) were collected and counted. Fractions 13–22 were pooled and used for the analysis shown in Table 1.

plus motifs with CS. By immunohistochemistry, Corless et al. (27) demonstrated the coexpression and identical tissue localization of syndecan and thrombospondin during murine embryonic development. Although these observations provide some support for the hypothesis that the CS receptors are syndecans, the known syndecans have much lower molecular weights than the CS receptors (22, 26, 28, 29).

Microorganisms may take advantage of the widespread distribution of proteoglycans on cell surfaces to use them as ligands for the attachment phases of infection. In recent years, there have been several examples of the utilization by viruses (herpes simplex, bovine herpes, and pseudorabies), and bacteria (*S. aureus*, *Bordetella pertussis*, *Streptococcus pyogenes*) of the GAG chains of HSPG as cellular adhesion sites (30–35). *Chlamydia trachomatis* appears to bear heparan sulfate GAGs on its surface, which serve as a bridge to attach to GAG receptors on the host cell surface membrane (36). Among the protozoa, *Trypanosoma cruzi* attachment and penetration of host cells involves a surface molecule that specifically recognizes HSPG (37).

It is unlikely that sporozoites and these various microbes bind to identical GAG receptors. Heparan sulfate consists mainly of repeating disaccharide units [GlcA-GlcNAc]n, where GlcA is glucuronic acid and GlcNAc is N-acetyl-glucosamine (38). However, these saccharides undergo N-deacetylation and N-sulfation of the GlcNAc residues, O-sulfation at various positions, and epimerization of GlcA to iduronic acid. These secondary modifications give rise to an enormous structural diversity throughout the length of each GAG chain. For example, a specific pentasaccharide in heparin binds with high affinity to antithrombin III (39), a distinctive dermatan sulfate hexasaccharide activates heparin co-factor II (40), and a specific heparan sulfate oligosaccharide is recognized by basic fibroblast growth factor (41). There is, in addition, increasing evidence of ordered aggregation and supramolecular organi-

zation of GAG chains in solution (42). In fact, the GAG receptors for CS need not be of high affinity; if they are abundant on the hepatocyte membrane, the overall binding capacity for sporozoites within the liver will be very high. It is conceivable that the retained sporozoites subsequently associate with high affinity to receptors for other regions of the CS, or for other sporozoite ligands. Such a dual receptor system involving initial low affinity GAG binding is required for fibroblast growth factor activity (43).

Another consideration is that the biological properties of structurally identical GAG chains in different tissues may vary, depending on whether the GAG chains are accessible to specific ligands. For example, CS-binding HSPGs are found in the laminae rarae of the tubular basement membranes in the kidney, and in mast cell granules which contain a high concentration of heparin. Renal tubular epithelia are separated from the blood circulation by fenestrated endothelia, but the fenestrae are closed with a diaphragm (44). Therefore, the kidney HSPGs cannot compete with the hepatocyte HSPGs for the circulating malaria sporozoites. Likewise, the intracellular mast cell granules are inaccessible to the sporozoites.

The relationship between the structure of heparin, and of the GAG chains of the HSPGs of hepatocytes and kidney basement membranes is not known. The elucidation of these structures should contribute to the understanding of the molecular basis of the recognition of region II-plus, and facilitate the rational development of inhibitors for possible use in the immunoprophylaxis of malaria. Of particular interest for drug development are heparin derivatives, some of which have been injected in humans without toxic effects. It will also be of interest to verify whether HSPGs analogous to the CS receptors also bind F-spondin (45), Unc-5 (46), and other proteins bearing the region II-plus motif, and to determine the physiological significance of this interaction.

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