

Characterization of a Novel Heparan Sulfate Proteoglycan Found in the Extracellular Matrix of Liver Sinusoids and Basement Membranes

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Abstract. A novel heparan sulfate proteoglycan (HSPG) present in the extracellular matrix of rat liver has been partially characterized. Proteoglycans were purified from a high salt extract of total microsomes from rat liver and found to consist predominantly (~90%) of HSPG. A polyclonal antiserum raised against this fraction specifically recognized HSPG by immunoprecipitation and immunoblotting. The intact, fully glycosylated HSPG migrated as a broad smear (150–300 kD) by SDS-PAGE, but after deglycosylation with trifluoromethanesulfonic acid only a single ~40-kD band was seen. By immunocytochemistry this HSPG was localized in the perisinusoidal space of Disse associated with irregular clumps of basement membrane-like extracellular matrix material, some of which was closely associated with the hepatocyte sinusoidal cell surface. It was also localized in biosynthetic compartments (rough ER and Golgi cisternae) of

hepatocytes, suggesting that this HSPG is synthesized and deposited in the space of Disse by the hepatocyte. The anti-liver HSPG IgG also stained basement membranes of hepatic blood vessels and bile ducts as well as those of kidney and several other organs (heart, pancreas, and intestine). An antibody that recognizes the basement membrane HSPG found in the rat glomerular basement membrane did not precipitate the 150–300-kD rat liver HSPG. We conclude that the liver sinusoidal space of Disse contains a novel population of HSPG that differs in its overall size, its distribution and in the size of its core protein from other HSPG (i.e., membrane-intercalated HSPG) previously described in rat liver. It also differs in its core protein size from HSPG purified from other extracellular matrix sources. This population of HSPG appears to be a member of the basement membrane HSPG family.

HEPARAN sulfate proteoglycans (HSPG)¹ are important extracellular matrix components that appear to have multiple functions among which are regulation of cell adhesion (24, 35, 36), cell migration and differentiation (8, 23), and basement membrane permeability (18). Considerable structural diversity exists among HSPG with two major families having been described: one found in basement membrane (9, 12, 42) and another that is associated with the plasma membrane of many cells (6, 31, 32, 34).

Two populations of HSPG have been extracted from rat liver: a detergent-soluble, membrane-intercalated HSPG (22, 32) and a heparin-releasable HSPG which binds to the cell surface via its glycosaminoglycan (GAG) side chains (21).

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1. *Abbreviations used in this paper:* BM, basement membrane; EHS, Engelbreth-Holm-Swarm; GAG, glycosaminoglycan; HSPG, heparan sulfate proteoglycan; PLP, periodate-lysine-paraformaldehyde; TFMS, trifluoromethanesulfonic acid; TM, total microsome.

Previously a polyclonal antibody was raised against proteoglycans purified from a detergent extract of rat liver microsomes. It proved to recognize the presumptive membrane-intercalated population of HSPG, which were found to be associated with the sinusoidal domain of the hepatocyte plasmalemma and with biosynthetic and endocytic compartments of the hepatocyte (41). The nature of the heparin-releasable population of HSPG is not yet established, but it has been suggested that these HSPG may represent a proteolytically cleaved form of the membrane-intercalated HSPG (21).

The purpose of this study was to characterize the heparin-releasable, salt-extractable population of HSPG from rat liver (22, 32). Toward this end, we raised an antibody against proteoglycans obtained by extraction of rat liver microsomes with high salt. Using the antibody, we have partially characterized a novel population of HSPG which was found to be associated with the liver perisinusoidal extracellular matrix and basement membranes, rather than with the hepatocyte cell membrane. This HSPG appears to represent a member of the family of basement membrane HSPG.

Materials and Methods

$\text{Na}_2^{35}\text{SO}_4$ (carrier free) was obtained from ICN Radiochemicals (Radioisotope Division, Irvine, CA). Chondroitinase ABC was from Miles Scientific Division (Naperville, IL); Superose 6, Q Sepharose, and Protein A-Sepharose were from Pharmacia Fine Chemicals (Piscataway, NJ), and Freund's complete and incomplete adjuvants were from Gibco Laboratories (Grand Island, NY). Ultrapure urea was obtained from United States Biochemicals (Cleveland, OH), and ultrapure guanidine hydrochloride (gdn-HCl) was obtained from Bethesda Research Laboratories (Gaithersburg, MD). Aprotinin was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN); autofluor and Ecoscint from National Diagnostics (Manville, IL); BCA protein assay reagents from Pierce Chemical Co. (Rockford, IL); and Immobilon polyvinylidene difluoride (PVDF) transfer membrane from Millipore Corp. (Bedford, MA). Mouse Engelbreth-Holm-Swarm (EHS) tumor laminin was purchased from Collaborative Research Inc. (Bedford, MA); human plasma fibronectin from Bethesda Research Laboratories; and NBT/BCIP (alkaline phosphatase substrate) from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD). All other chemicals were supplied by Sigma Chemical Co. (St. Louis, MO).

Antibodies

Anti-basement membrane HSPG (anti-BM HSPG) which was raised against proteoglycans purified from rat glomeruli was characterized elsewhere (Pietromonaco, S. F., and M. G. Farquhar. *J. Cell Biol.* 107:909; 40, 42, 43). It specifically precipitates only HSPG from glomerular proteoglycans (42) and from cultured glomerular epithelial cells (43) and stains all basement membranes by immunofluorescence. TRITC-conjugated goat anti-rabbit F(ab')₂ was purchased from Tago, Inc. (Burlingame, CA), and Fab fragments of sheep anti-rabbit IgG conjugated to HRP were obtained from Dr. J. Kim (Tago, Inc.). Goat anti-rabbit IgG conjugated to 5 nm colloidal gold was purchased from Janssen Pharmaceuticals, Inc. (Piscataway, NJ), and alkaline phosphatase-conjugated goat anti-rabbit IgG from Fisher Scientific (Springfield, NJ).

Biosynthetic Labeling

For analytical characterization of liver HSPG, rats (125–150 g) were given two injections of 1 mCi [³⁵S]sulfate (at $t = 0$ and 8 h), and the animals were killed 4 h after the last injection.

Preparation and Extraction of Rat Liver Total Microsomes

Rats were perfused via the descending aorta with PBS, and the livers were quickly removed and placed on ice after which a total microsomal fraction (TM) was prepared from the livers (11). All buffers contained protease inhibitors (1 mM PMSF, 5 mM benzamidine, 100 mM ϵ -aminocaproic acid, and 5 $\mu\text{g}/\text{ml}$ aprotinin). The method for extraction of liver TM was based upon that used previously to differentially solubilize HSPG associated with the hepatocyte cell surface (21, 22, 32). Briefly, the TM pellet was gently homogenized and extracted with 2 M NaCl in Tris-buffered sucrose (250 mM sucrose, 100 mM Tris, pH 7.4) for 30 min at 4°C. The extract was centrifuged at 100,000 g for 90 min, and the supernatant was diluted to 0.2 M NaCl with urea buffer (8 M urea, 0.05 M sodium acetate, pH 6.0, and 0.5% Triton X-100) for subsequent ion exchange chromatography.

Ion Exchange Chromatography

Proteoglycans were isolated by ion exchange chromatography using Q Sepharose, eluting with a 0.2–1.0 M NaCl linear gradient at a flow rate of 15 ml/h (43). 1 ml fractions were collected and aliquots were analyzed for radioactivity. Fractions containing [³⁵S]sulfate-labeled proteoglycans were pooled, diluted to <0.2 M NaCl with urea buffer and subjected to a second, concentrating ion exchange step over a 200- μl Q Sepharose column. For samples to be analyzed directly by gel filtration chromatography, the column was eluted with 4 M gdnHCl, 0.05 M sodium acetate, pH 6.0, 0.5% Triton X-100. For samples to be subjected to enzymatic or chemical treatments before gel filtration chromatography or immunoprecipitation, the column was eluted with 100 mM Tris-HCl, pH 8.0, 1 M NaCl.

Gel Filtration Chromatography

Analytical gel filtration chromatography was performed using a Superose

6 FPLC column equilibrated in 4 M gdnHCl, 0.05 M sodium acetate, pH 6.0, 0.5% Triton X-100 (47). Proteoglycan samples (0.2 ml) containing 2,000–20,000 cpm were injected onto the column, and 0.4-ml fractions were collected at 24 ml/h. Aliquots were diluted with 70% ethanol and analyzed for radioactivity. The void volume (V_0) and total volume (V_t) of the column were determined with blue dextran and [³H]serine, respectively.

Enzymatic and Chemical Treatments

Chondroitinase ABC digestion (0.1 U/0.1-ml sample) was carried out at 37°C for 1 h in the presence of 50 mM sodium acetate and protease inhibitors (2 mM *N*-ethylmaleimide, 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ pepstatin). If the samples were to be separated by gel filtration (to determine the relative amounts of heparan sulfate to chondroitin/dermatan sulfate proteoglycan), an equal volume of 8 M gdnHCl, 1% Triton X-100 was added to the digested material, and the sample was loaded directly onto the Superose 6 column. For immunoprecipitation, an equal amount of 2 \times RIPA buffer (50 mM Tris-HCl, pH 7.3, 0.15 M NaCl, 1% Triton X-100, 0.1% SDS, and 10 mM EDTA) was added to the samples.

Nitrous acid deamination was conducted (20°C for 1 h) according to Cifonelli and King (7). Briefly, 0.1 ml of ion exchange purified proteoglycan in 100 mM Tris-HCl, pH 8.0, 1 M NaCl, was lyophilized and reconstituted with 0.1 ml 0.2 M NaNO_2 in 3.5 M acetic acid. The reaction was terminated by neutralization with 1 M Tris, pH 10, and the digested material was diluted with an equal volume of either 8 M gdnHCl, 0.05 M sodium acetate, pH 6.0, 1% Triton X-100 for gel filtration, or with 2 \times RIPA buffer for immunoprecipitation.

Alkaline β -elimination of *O*-linked GAG chains was conducted with 0.05 N NaOH and 1 M NaBH_4 according to Carlson (5). After digestion for 24 h at 45°C, the sample was adjusted to 4 M gdnHCl, 0.05 M sodium acetate, pH 6.0, 0.5% Triton X-100, and analyzed by gel filtration.

Chemical deglycosylation of the proteoglycan was carried out with trifluoromethanesulfonic acid (TFMS) according to Edge and Spiro (10), with minor modifications. In brief, ~10–25 μg ion exchange purified proteoglycan from unlabeled rat liver was dialyzed against 10 mM ammonium bicarbonate, pH 8.6, and lyophilized in Reacti-vials (Pierce Chemical Co.). Anisole (20 μl) and TFMS (30 μl) were added to the dried samples on ice, the Reacti-vials sealed and purged with nitrogen, and the reaction carried out at 0°C with constant stirring for 4–6 h. The sample was transferred to -70°C (10–15 min), after which ice-cold pyridine:H₂O (1:1) (150 μl) was added dropwise, while the sample was kept cool in an ice bath. It was then dialyzed against 10 mM Tris, pH 6.8, for 12–24 h with many changes, extracted with anhydrous ether (three times), lyophilized, and finally resolubilized in SDS sample buffer (0.15 M Tris, pH 6.8, 10% SDS, 25% glycerol, 5% β -mercaptoethanol, and bromophenol blue) before electrophoresis.

Preparation of Antigen

Total microsomes were prepared from unlabeled rat livers and extracted as

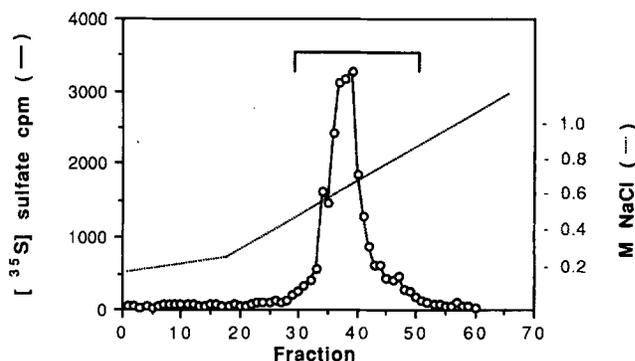


Figure 1. Ion exchange chromatography of [³⁵S]sulfate-labeled proteoglycans purified from rat liver microsomes. Total microsomes were prepared from biosynthetically labeled rat liver as described in Materials and Methods, extracted with 2 M NaCl, applied to a Q Sepharose column, and eluted with a 0.2–1.0 M NaCl gradient (40 ml each). 1-ml fractions were collected, and aliquots (20 μl) were counted for radioactivity. Liver proteoglycans elute in a single peak between 0.5 and 0.9 M NaCl (bracket).

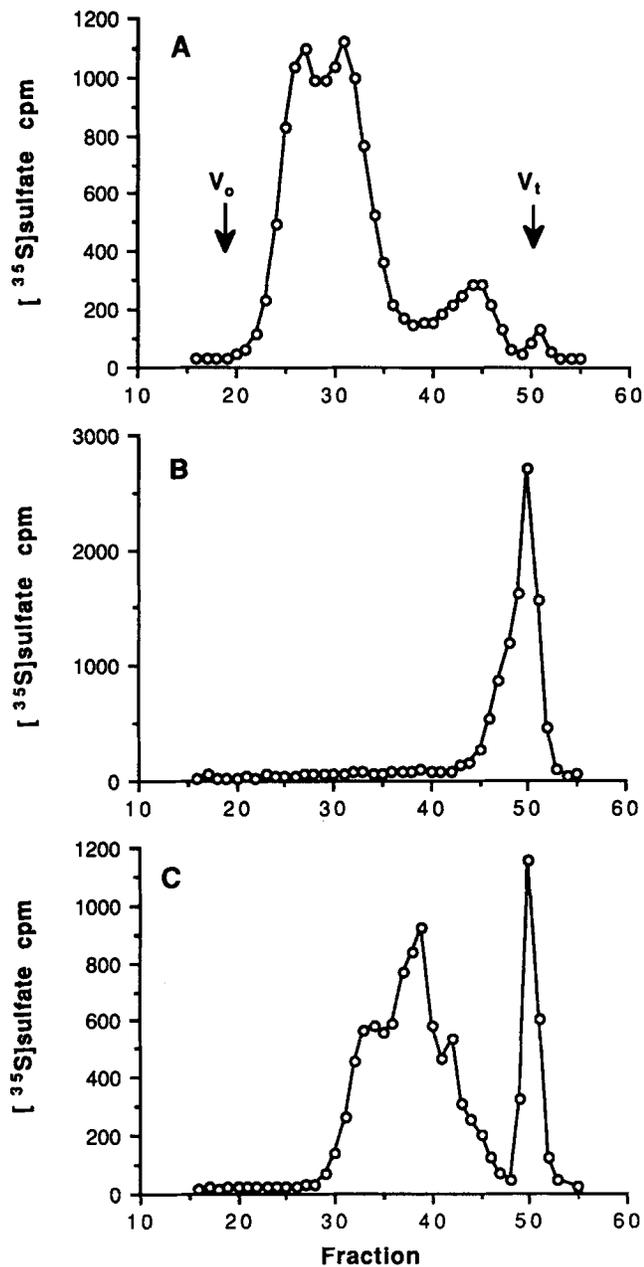


Figure 2. Gel filtration chromatography of proteoglycans from rat liver microsomes. Radiolabeled proteoglycans were purified by two passes over Q Sepharose, and gel filtration chromatography performed under FPLC conditions (<170 psi, 24 ml/h) using a Superose 6 column. Proteoglycans (200 μ l, \sim 20,000 cpm) were applied to the column in gdnHCl buffer, and 0.4 ml fractions collected. (A) Approximately 88% of the proteoglycan elutes early from the column in two poorly resolved peaks (K_{av} 0.26 and 0.39). The remainder of the counts are found in a small peak (K_{av} 0.81). (B) Nitrous acid deamination of the proteoglycan reduces most of the material to small fragments which elute in the V_t . (C) Alkaline β -elimination results in a shift to a broad peak (K_{av} 0.65), indicating extensive heterogeneity of GAG chains.

described above. Tracer [³⁵S]sulfate-labeled proteoglycan was added to the extract which was separated by ion exchange chromatography. Fractions from the Q Sepharose column containing the proteoglycan were pooled (see Fig. 1) and precipitated with ice-cold 95% ethanol (5–6 vol). Protein content of the precipitate was determined by the Pierce BCA assay. The yield was \sim 10 μ g proteoglycan/g liver tissue.

Antibody Preparation

A rabbit was immunized via the popliteal lymph nodes following precisely the procedure of Louvard et al. (26). The initial injection consisted of 30 μ g protein emulsified in CFA. Subsequent boosts (20 μ g) were given at days 22, 32, and 33, and the animal was bled 1 wk later. IgG was purified from serum by ammonium sulfate precipitation.

Immunocytochemistry

Rat tissues were fixed by retrograde perfusion with periodate-lysine-para-

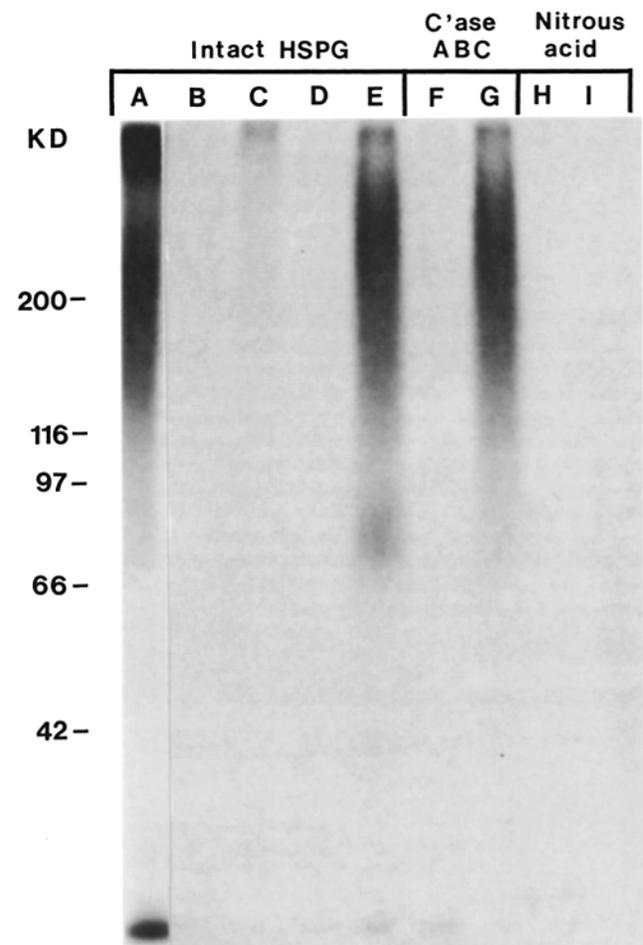


Figure 3. Immunoprecipitation of [³⁵S]sulfate-labeled HSPG by anti-liver HSPG IgG. Proteoglycans were purified by ion exchange chromatography from a 2 M NaCl extract of biosynthetically labeled rat liver microsomes and separated by SDS-PAGE (lane A). Immunoprecipitation was carried out as described in Materials and Methods using anti-liver HSPG IgG (lanes E, G, I), anti-BM HSPG IgG (lane C), or preimmune IgG (lanes B, D, F, H). Proteoglycan (\sim 50,000 cpm) was either precipitated directly (lanes B–E) or subjected to chondroitinase ABC (lanes F and G) or nitrous acid (lanes H and I) treatment prior to precipitation. Ion exchange purified proteoglycans migrate on SDS-PAGE as a broad, diffuse smear, 150–300 kD, or barely enter the resolving gel (lane A). Anti-liver HSPG IgG precipitates a heterogeneous group of proteoglycans that appear as a broad smear 150–300 kD and a weaker, heterogeneous band at \sim 82 kD (lane E). Precipitation of the upper broad band is not affected by treatment with chondroitinase ABC (lane G), but is eliminated by nitrous acid deamination (lane I). Anti-BM HSPG IgG precipitates HSPG, which barely enters the resolving gel (lane C). Preimmune IgG does not precipitate [³⁵S]sulfate-labeled macromolecules (lanes B, D, F, H).

formaldehyde (PLP) fixative (2% paraformaldehyde, 0.75 M lysine, 0.01 M sodium periodate in phosphate buffer [30]), followed by immersion (6 h) in the same fixative. For immunofluorescence, semithin frozen sections (0.5 μm) were prepared on a Reichert Ultracut OMU-4 ultramicrotome equipped with a cryoattachment as described elsewhere (39). Sections were incubated sequentially with preimmune or immune serum (1:200 in PBS, 0.1% ovalbumin, pH 7.4 for 2 h), followed by TRITC-anti-rabbit F(ab')₂ conjugate (1:200 for 2 h). The sections were examined by epifluorescence and photographed in a Zeiss Photomicroscope III (Kodak Tri-X Pan ASA 400).

For immunoperoxidase, cryostat sections (20–30 μm) cut from PLP-fixed, fasted rat liver were incubated with immune IgG (1:100) overnight, followed by incubation with Fab fragments of sheep anti-rabbit IgG conjugated to HRP (1:50) for 2 h. They were then fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, reacted with DAB, and processed for electron microscopy as detailed elsewhere (4).

Immunogold labeling on ultrathin frozen sections was performed as described by Tokuyasu (45). Ultrathin cryosections were cut from rat liver tissue at -110°C on a Reichert OMU-4 equipped with a cryoattachment. Sections were incubated for 1 h with immune IgG (diluted 1:30 in PBS containing 10% FBS), followed by goat anti-rabbit IgG conjugated to 5 nm colloidal gold (diluted 1:50). They were then postfixed in 2% glutaraldehyde in PBS (10 min), stained with 2% OsO₄ (10 min), followed by 2% acidic uranyl acetate (20 min), and finally absorption stained (5 min) with 0.002% lead citrate in 3% polyvinyl alcohol (46).

Immunoprecipitation

Ion exchange-purified proteoglycans (10,000–50,000 cpm) obtained from [³⁵S]sulfate-labeled rat liver TM were incubated with 20 μl preimmune serum in 1 ml RIPA buffer for 30 min at 20°C, after which 5 mg Protein A-Sepharose beads were added (1 h). After centrifugation, the cleared supernatants were incubated (1 h) with 20 μl of immune IgG, 5 mg Protein A-Sepharose beads were added, and the antigen-antibody mixture incubated overnight at 4°C. After extensive washing (four changes of RIPA buffer over 4 h, one change PBS, two changes distilled water), the immunoprecipitates were solubilized in SDS-PAGE sample buffer (0.15 M Tris, pH 6.8, 10% SDS, 25% glycerol, 5% β -mercaptoethanol, bromophenol blue) and separated on 5–10% gradient Laemmli polyacrylamide gels. Fluorography was conducted using Autofluor, and the gels exposed to Kodak X-Omat AR x-ray film in the presence of an intensifier screen.

Immunoblotting

Ion exchange-purified proteoglycans were obtained from 2 M NaCl extracts of unlabeled rat liver TM. Intact and TFMS deglycosylated proteoglycans (10–25 μg), mouse EHS laminin (1 μg), and human plasma fibronectin (1 μg) were separated by SDS-PAGE under denaturing conditions, and the separated proteins were electrophoretically transferred to Immobilon membranes (150 mA for 15–18 h at 4°C). Unbound sites on the membrane were blocked by incubation in 5% nonfat dry milk, 1% Tween 20 in PBS (1 h at 20°C). Immunoreactive bands were detected by sequential incubation with immune IgG (1:1,000), alkaline phosphatase-conjugated anti-rabbit IgG, and NBT/BCIP substrate.

Results

Chromatographic Analysis of Liver HSPG Extracted by 2 M NaCl

When a TM fraction prepared from rat liver was extracted with 2 M NaCl, $\sim 25\%$ of the total ³⁵S radioactivity was solubilized. 80–95% of these counts bound to, and were eluted from the ion exchange column in a single peak between 0.5 and 0.9 M NaCl (Fig. 1). Analysis of the proteoglycans pooled from this peak by gel filtration chromatography indicated that most of the counts ($\sim 88\%$) eluted as two poorly resolved peaks, K_{av} 0.26 and 0.39, while $\sim 12\%$ eluted in a minor peak, K_{av} 0.81 (Fig. 2 A). Pretreatment of the sample with chondroitinase ABC removed only 10–15% of the ³⁵S radioactivity from the major peak (data not shown). The counts were depleted equally from the K_{av} 0.26 and 0.39

peaks but the minor peak at K_{av} 0.81 was unchanged. Nitrous acid deamination before chromatography reduced the majority of the high molecular weight material to small fragments that eluted in the V_i (Fig. 2 B). The lack of susceptibility of the fraction to chondroitinase ABC, together with its sensitivity to nitrous acid deamination, indicate that most of the sample consists of HSPG. Alkaline β -elimination released GAG chains with a complex elution profile, the major peak being K_{av} 0.65 (Fig. 2 C). The complex profile suggests a bimodal pattern with the trailing edge representing the original K_{av} 0.81 peak, which most likely consists of intracellular degradation products. The large peak at the V_i probably represents free sulfate released by alkaline treatment. The complexity of the pattern is most likely due to extensive heterogeneity of GAG chains which is known to occur in other proteoglycans.

Characterization of the Anti-Liver HSPG IgG by Immunoprecipitation and Immunoblotting

When polyclonal IgG generated against liver proteoglycans purified from a high salt extract of TM was used to precipitate biosynthetically labeled proteoglycan, a broad, smeared band (150–300 kD) was precipitated along with high molecular weight material at the top of the resolving gel (Fig. 3, lane E). The predominant smeared band centered near the 200-kD molecular weight marker had the typical diffuse profile characteristic of proteoglycans, which is due to the attached GAG chains. Sometimes a minor band at 82 kD (not seen in the original starting material) was also seen, but this

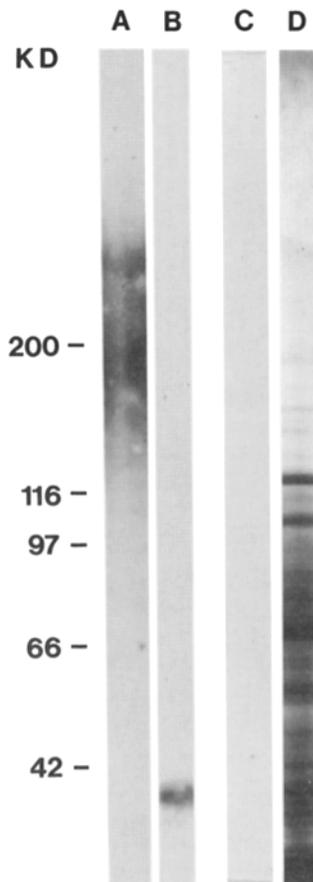


Figure 4. Immunoblotting of liver proteoglycans before and after TFMS deglycosylation. Proteoglycans purified by ion exchange chromatography from a 2 M NaCl extract of unlabeled rat liver microsomes were separated by SDS-PAGE under denaturing conditions and transferred to Immobilon membrane. Anti-liver HSPG IgG reacts with intact proteoglycans of heterogeneous size visualized as a broad smear, 150–300 kD (lane A). After TFMS treatment, the antibody recognizes a 40-kD core protein (lane B). The unbound fraction of the Q Sepharose column contains many proteins demonstrated by Coomassie blue staining (lane D); none reacts with the polyclonal IgG (lane C).

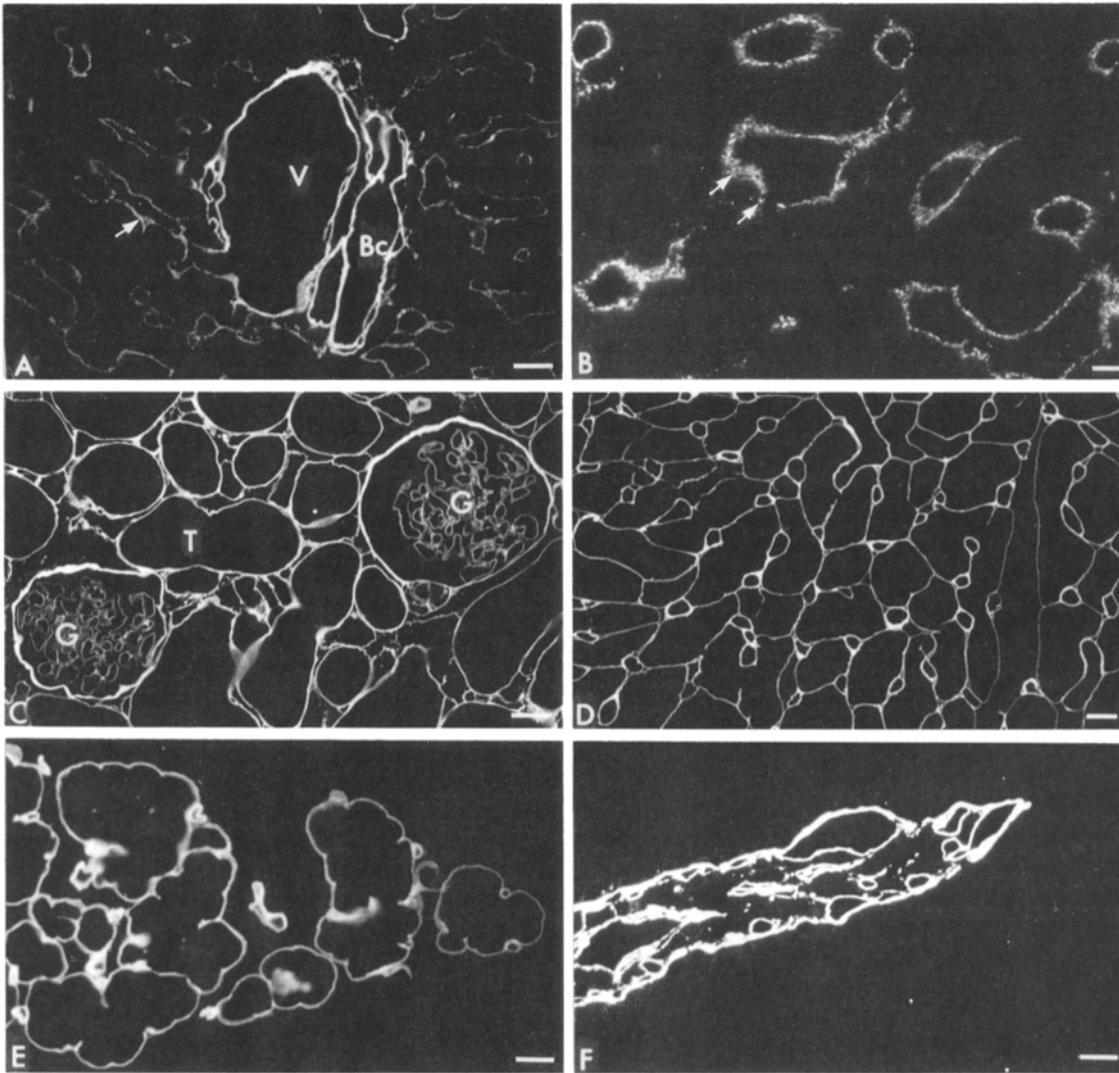


Figure 5. Indirect immunofluorescence staining of semithin ($0.5\ \mu\text{m}$) cryosections prepared from various PLP-fixed rat tissues with anti-liver HSPG. (A and B) Liver: strong staining is seen of the perisinusoidal regions (arrows) and of basement membranes of the blood vessels (V) and bile capillaries (Bc) in the portal triad. Staining of the perisinusoidal extracellular matrix (arrows) is shown at higher magnification in B. (C) Kidney: the glomerular basement membranes (G), Bowman's capsule, and basement membranes of tubules (T) and peritubular capillaries are stained. (D) Heart: cross-section of cardiac muscle from the left ventricle. The antibody reacts strongly with the basement membranes surrounding the myocytes and capillaries. (E) Pancreas: the capillary basement membranes and those surrounding the pancreatic lobules at the basal surface of the acinar cell are stained. (F) Small intestine: the basement membranes underlying the epithelial cells and surrounding the capillaries and lymphatics in the lamina propria are stained. Bars: (A, C, E, and F) $20\ \mu\text{m}$; (B and D) $10\ \mu\text{m}$.

was an inconsistent finding; it may represent a breakdown product generated during the immunoprecipitation procedure. Pretreatment of the sample with chondroitinase ABC had no effect on the precipitation of the broad 150–300-kD band (Fig. 3, lane G), whereas the latter was not seen after treatment with nitrous acid (Fig. 3, lane I), which degrades HSPG. No radiolabeled material was precipitated by preimmune IgG (Fig. 3, lanes B, D, F, and H). When an antibody (anti-BM HSPG) raised against glomerular proteoglycans that recognizes kidney and other basement membranes (42) was used for immunoprecipitation, only material of high molecular mass which barely entered the resolving gel was precipitated (Fig. 3, lane C). This indicates that the anti-liver HSPG, but not the anti-BM HSPG recognized and precipi-

tated a population of HSPG which migrates at $\sim 150\text{--}300\ \text{kD}$ on SDS gels.

The anti-liver HSPG also detected a similar broad 150–300 kD band by immunoblotting of purified liver proteoglycans (Fig. 4, lane A), and, similarly, prior treatment with chondroitinase ABC did not alter the reactivity (data not shown). When purified liver proteoglycans were chemically deglycosylated with TFMS before SDS-PAGE (to remove GAG chains and *N*- and *O*-linked oligosaccharides), the antibody recognized a $\sim 40\text{-kD}$ core protein (Fig. 4, lane B). The flowthrough (unbound fraction) from the Q Sepharose column was also tested for immunoreactivity with anti-liver HSPG. As anticipated, many Coomassie blue-stained bands were present in the flow-through (Fig. 4, lane D), but none

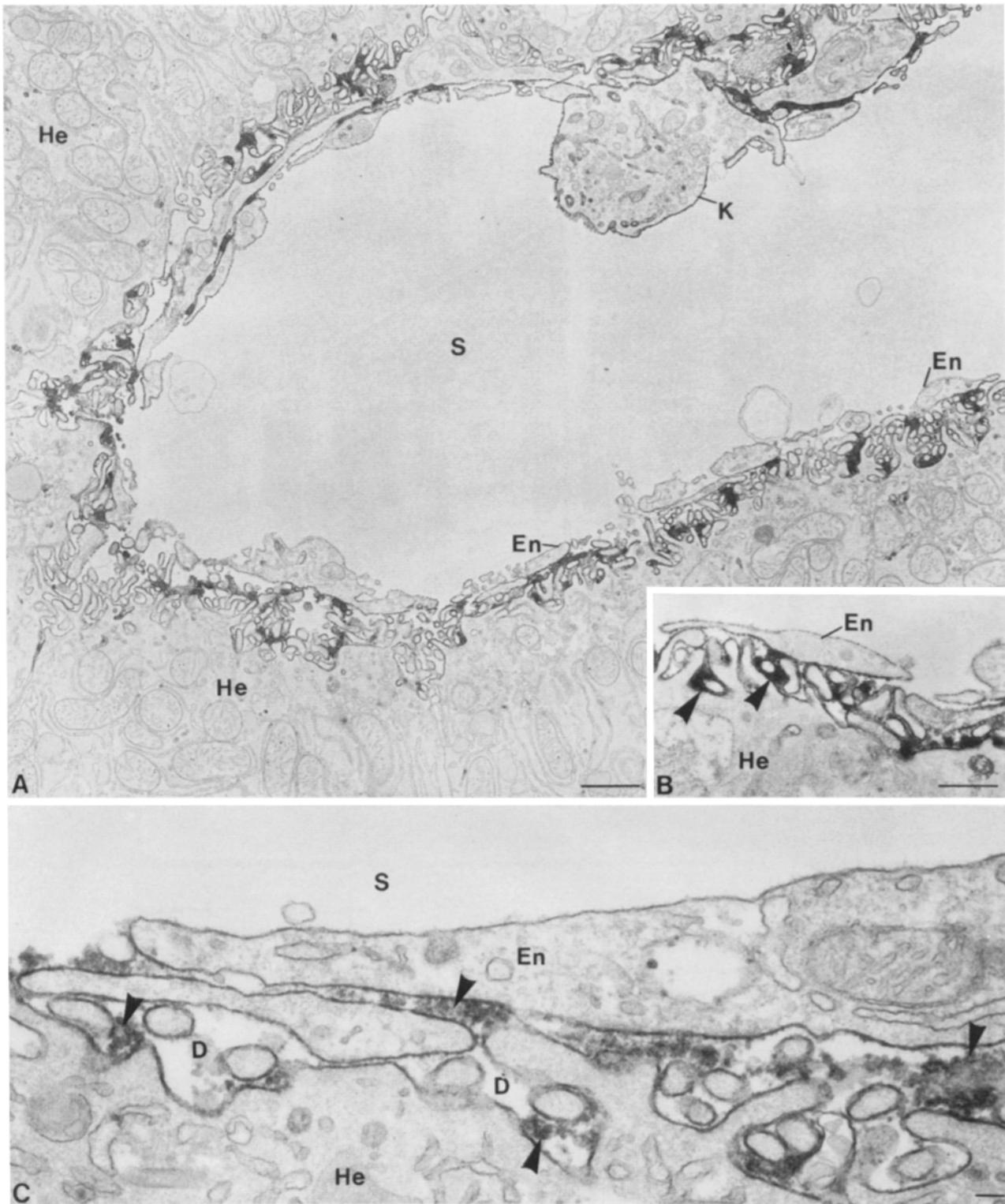


Figure 6. Immunoperoxidase localization of liver HSPG. Cryostat sections were incubated with anti-liver HSPG IgG followed by Fab fragments of sheep anti-rabbit IgG and processed as described in Materials and Methods. (A) Peroxidase reaction product is detected around the sinusoids (S) in the space of Disse (D), where it is associated with clumps of extracellular matrix material located between the endothelium (En) and the sinusoidal surface of the hepatocyte (He). (B) Enlargement of the space of Disse showing reaction product (arrowheads) located between processes of the sinusoidal endothelium (En) and the hepatocyte (He) microvilli. Reaction product is also seen in association with the hepatocyte and endothelial plasma membranes. (C) In a less strongly reacted specimen, clumps of reactive extracellular matrix material (arrowheads) are seen to be located in close proximity to the adjacent endothelial and hepatocyte cell membranes in the space of Disse (D). K, Kupffer cell. Bars: (A and C) 1 μ m; (B) 0.5 μ m.

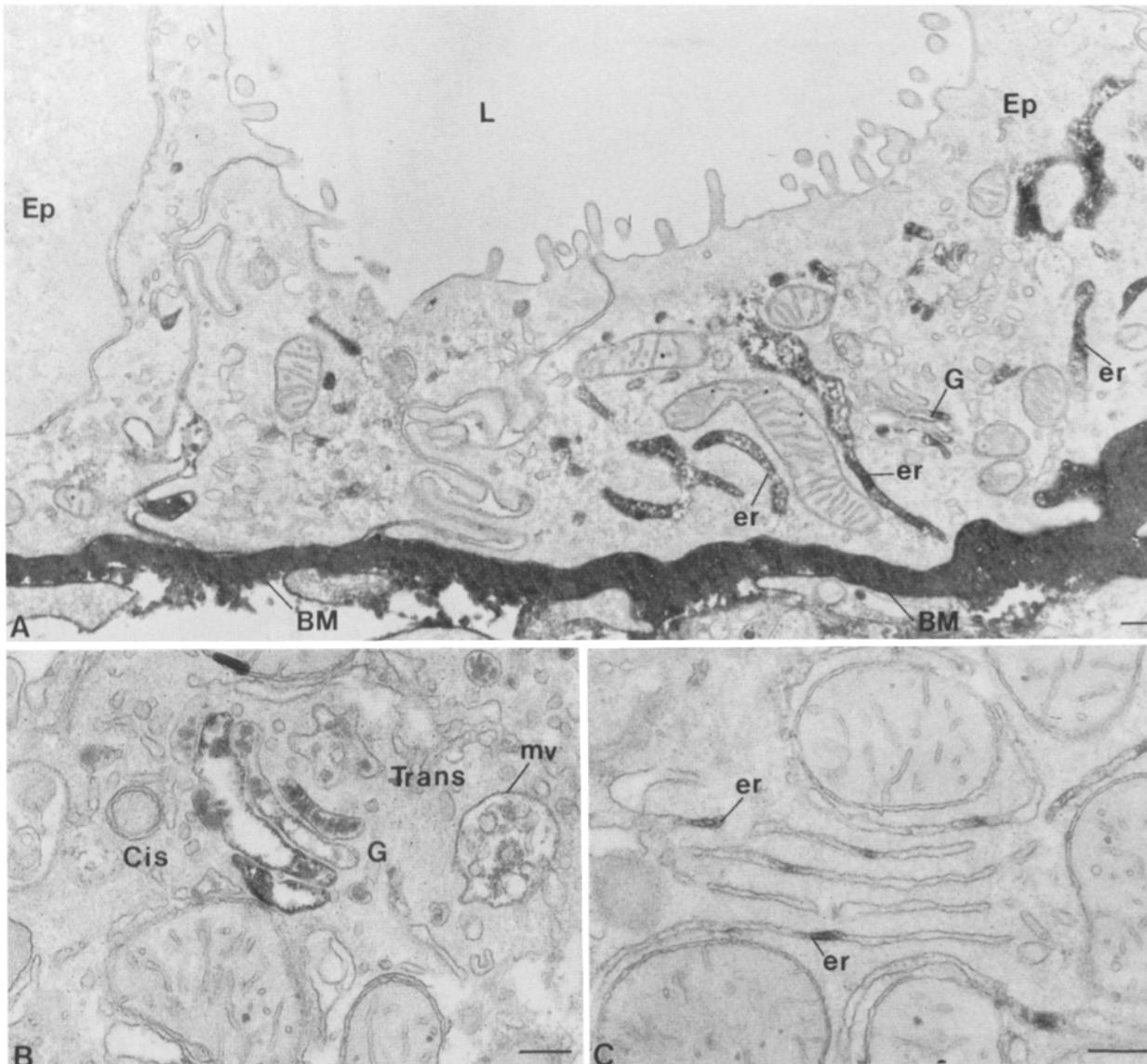


Figure 7. Immunoperoxidase localization of liver HSPG in the bile duct epithelium (A) and in hepatocytes (B and C). Preparation similar to that in Fig. 6. (A) The basement membrane (BM) surrounding a bile duct in the portal triad is strongly reactive for this HSPG. The core protein of this HSPG is also detected within the rough ER (er) and Golgi (G) cisternae of the bile duct epithelium (EP). (B and C) Small fields demonstrating reaction product in the stacked Golgi cisternae (G) and rough ER (er) of hepatocytes. L, lumen of the bile duct; Cis, cis side of Golgi; Trans, trans side of the Golgi; mv, multivesicular endosome. Bars: (A and B) 1 μ m; (C) 0.5 μ m.

of them stained with the anti-liver HSPG IgG (Fig. 4, lane C), thus confirming the specificity of the antibody for the tightly bound proteoglycan fraction. No reactivity with laminin or fibronectin was detected by immunoblotting or by ELISA (data not shown).

These data indicate that the antibody raised against the proteoglycans extracted by high salt from liver microsomes specifically recognizes by both immunoprecipitation and immunoblotting a population of HSPG (150–300 kD) with a 40-kD protein core. This population of HSPG is not recognized by the anti-BM HSPG raised against glomerular proteoglycans.

Immunocytochemical Localization of Liver HSPG

When the anti-liver IgG was used for indirect immunofluorescence on semithin (0.5 μ m) sections from rat liver, there

was strong staining of the perisinusoidal region, as well as of basement membranes of the vessels of the portal triad (Figs. 5, A and B). The sinusoidal staining appeared as an interrupted, punctuate fluorescence pattern surrounding the sinusoids (Fig. 5 B). Staining of basement membranes was also seen in all organs examined, including kidney (Fig. 5 C), heart (Fig. 5 D), pancreas (Fig. 5 E), and intestine (Fig. 5 F). Interestingly, when the anti-BM HSPG antibody prepared from glomerular proteoglycans was used to stain rat liver, only the basement membranes of the bile ducts and large blood vessels of the portal triad were stained. No reactivity was seen with the extracellular matrix material in the space of Disse.

When the HSPG recognized by the anti-liver HSPG IgG was localized at the electron microscopic level by immunoperoxidase, the perisinusoidal staining was seen to be due to

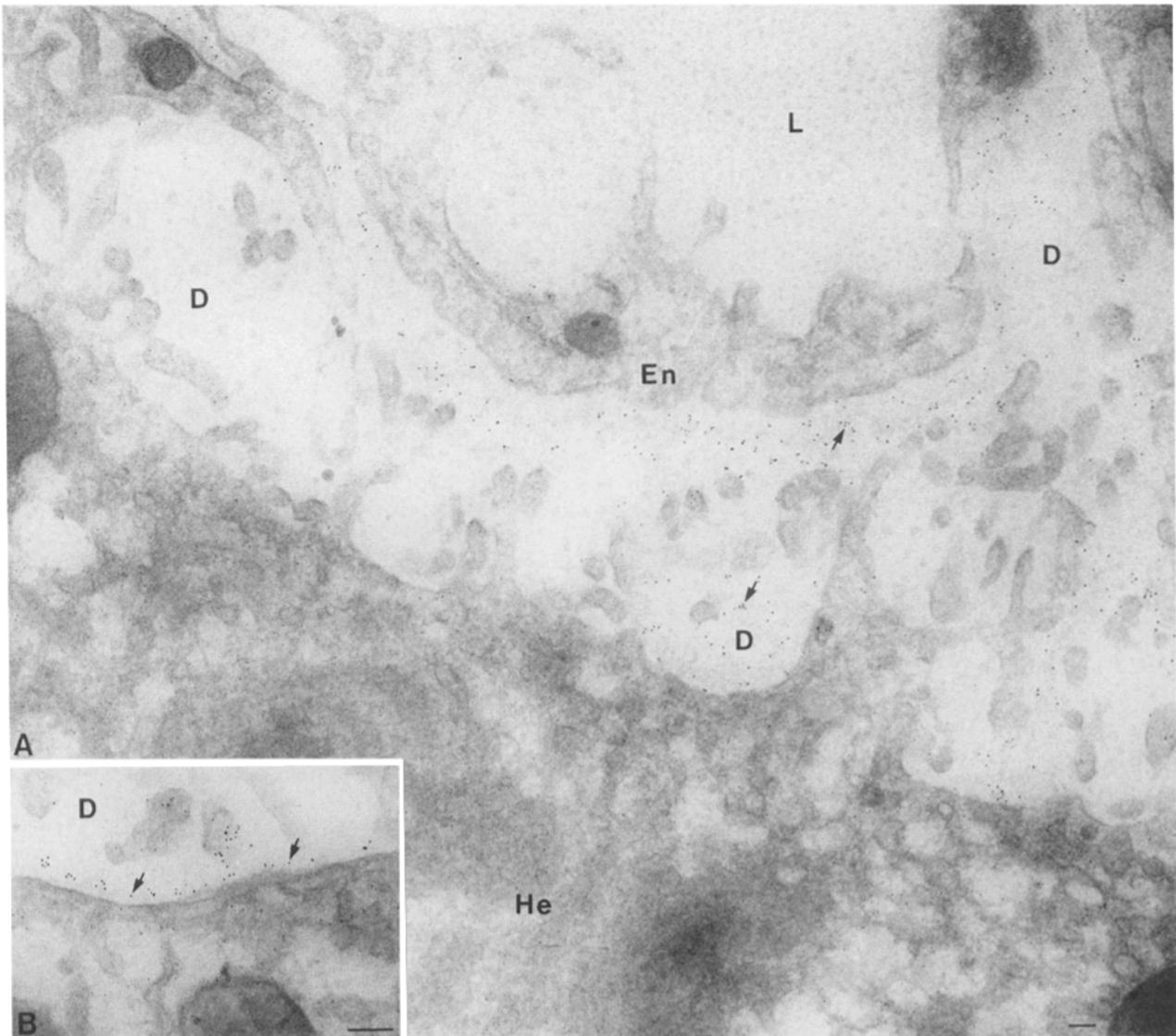


Figure 8. Immunogold localization of liver HSPG. Ultrathin cryosections of rat liver were incubated with anti-liver HSPG followed by a 5-nm gold-anti-rabbit IgG conjugate. (A) Most of the gold particles (*arrows*) are associated with clumps of barely discernable extracellular matrix material located in the space of Disse (*D*) between the endothelium (*En*) and the sinusoidal plasma membrane domain of the hepatocyte (*He*). (B) Another field of a similar preparation showing the proximity of the HSPG to the sinusoidal domain of the hepatocyte plasma membrane (*arrows*) facing the space of Disse (*D*). Bar, 0.1 μ m.

the presence of this HSPG in the space of Disse. Aggregates of reaction product were located between the abluminal surface of the endothelium and the sinusoidal plasmalemma of the hepatocyte (Fig. 6, A-C). In lightly reacted specimens (Fig. 6 C), reaction product was seen to be associated with ill-defined clumps of extracellular matrix material in the space of Disse. Sometimes small amounts of reaction product were closely associated with the epithelial and endothelial plasma membranes adjacent to the reactive extracellular matrix. However, little or no reaction product was seen on the bile canalicular membrane or lateral surfaces of the hepatocyte below the junctional complexes. The basement membranes of the blood vessels and bile ducts of the portal triad (Fig. 7 A) were also heavily reactive. Moreover, reaction product was found within the ER and Golgi cisternae of both the hepatocytes (Figs. 7, B and C) and the bile duct epi-

thelium (Fig. 7 A). The presence of reaction product within biosynthetic compartments indicates that both these cell types synthesize HSPG recognized by the antibodies.

Results of immunogold localization of this HSPG on ultrathin cryosections confirmed results obtained by immunoperoxidase. Gold particles were mainly found in association with extracellular matrix material in the space of Disse (Fig. 8 A). They were also found in clusters along the hepatocyte plasmalemma (Fig. 8 B), suggesting close association of the extracellular matrix material with the surface of the hepatocyte.

Thus, the anti-liver HSPG IgG we generated recognizes a population of HSPG found in the perisinusoidal extracellular matrix and basement membranes of the rat liver. It also reacts with HSPG present in basement membranes of many other rat tissues.

Discussion

We have previously shown that two antigenically distinct populations of HSPG can be distinguished in liver, kidney and other cells: one type of HSPG is associated with cell membranes and the other with basement membranes. These two types of proteoglycans are found both in situ in intact liver (41) and kidney (14, 42) and in cultured liver and kidney cell lines (40, 43). In the present study we set out to characterize the heparin/salt-releasable type of HSPG described in rat liver which was suggested to represent a proteolytically cleaved form of the membrane-associated HSPG. An antibody raised against proteoglycans purified from a high salt extract of rat liver microsomes proved to recognize exclusively HSPG by immunoprecipitation and immunoblotting and to stain irregular clumps of basement membrane-like extracellular matrix material deposited in the liver sinusoidal space of Disse and with basement membranes of the blood vessels and bile duct epithelium of the portal triads. Thus, the immunocytochemical results indicate that the HSPG recognized are associated with the extracellular matrix rather than with cell membranes. Moreover, the anti-liver HSPG stained basement membranes of kidney and other tissues (heart, pancreas, intestine). The pattern was comparable to that obtained with antibodies raised against HSPG purified from other basement membrane sources, e.g., renal glomeruli (42) and the EHS sarcoma (17, 20) and was quite different from that obtained with an antibody that recognizes membrane intercalated HSPG (40, 41). The anti-liver HSPG showed no reactivity by immunoblotting or ELISA with laminin or fibronectin. Moreover, the antibody precipitates from a rat liver proteoglycan fraction a population of HSPG with a mobility of 150–300 kD (by SDS-PAGE) with an ~40-kD core protein (after TFMS deglycosylation). The overall size of this population of HSPG and its core protein size differ from those of the membrane-intercalated HSPG previously found to be associated with plasma membranes of rat liver (41) which have an overall size of ~80 kD with an ~20-kD protein core (22). Thus, the immunochemical data we obtained indicate that the antibody recognizes the core protein of a population of extracellular matrix-associated HSPG found in rat liver that differs in its properties from the membrane-intercalated HSPG from rat liver. It also differs in its overall size and core protein size from HSPG purified from other extracellular matrix sources, especially kidney basement membranes.

A good deal of size diversity has been reported among HSPG from different extracellular matrix sources (28, 44). For example, the EHS sarcoma which makes a basement membrane-like matrix contains two (high and low density) HSPG, 650 and 130 kD, with core proteins of 450 and 21–34 kD, respectively (19, 25), whereas HSPG from the bovine (10) and rat (33) glomerular basement membrane have been reported to be 200–400 kD, with a core protein of 130 kD (determined by TFMS treatment). Further evidence suggesting differences between the liver and glomerular basement membrane HSPG is that an antibody we raised previously against glomerular proteoglycans fails to precipitate the 150–300 kD HSPG from rat liver, and it fails to stain the clumps of basement membrane-like matrix in the space of Disse (although it does stain basement membranes of liver arterioles, venules and bile ducts). Similar immunostaining results

were obtained by Couchman (9) and Schleicher et al. (38) with antibodies raised against HSPG purified from PYS-2 cell cultures and porcine glomeruli, respectively. Previously it has been shown that the BM-1 antibody raised against EHS sarcoma HSPG does stain the perisinusoidal matrix (15, 16). These results suggest that the anti-liver HSPG IgG we have generated recognizes a novel HSPG present in the liver perisinusoidal extracellular matrix that shares some epitopes in common with other basement membrane HSPG but has one or more antigenic determinants which are recognized by some but not all antibodies raised against basement membrane HSPG.

Cellular Sources of Basement Membrane HSPG in Rat Liver

The hepatocyte most likely represents the main cellular source of the HSPG we have used as immunogen because the hepatocyte is by far the predominant liver cell type and a total microsome fraction from rat liver would be expected to be composed of vesicles derived primarily from this cell type. Other cell types present (Kupffer cells, endothelial cells, bile duct epithelium, fat-storing cells) contribute <5% of the total microsome fraction (2), which was the starting material for the salt extraction. Microsomes contain vesicles derived from both intracellular compartments (rough and smooth ER, Golgi, vesicular carriers, endosomes) and from the plasma membrane. Therefore, the HSPG extracted from liver microsomes would be expected to be derived primarily from hepatocytes and would include not only those from ER and Golgi vesicles containing biosynthetic precursors in transit through hepatocytes, but also those from plasmalemmal vesicles presumably with amorphous clumps of extracellular matrix still attached. That the proteoglycans can be extracted from microsomes with 2 M NaCl is compatible with the earlier assumption (21) that these HSPG are bound to the cell membrane or to other extracellular matrix components via electrostatic interactions.

Previous studies conducted on primary cultures of isolated hepatocytes and lipocytes have reported that the primary source of sinusoidal extracellular matrix components (laminin, collagen IV, and HSPG) in the liver is the lipocyte (perisinusoidal stellate cell or fat-storing cell) (1, 15, 27, 37). However, in this study we have demonstrated by immunoelectron microscopy the presence of the core protein of basement membrane HSPG in biosynthetic compartments (ER and Golgi cisternae) of both the hepatocyte and the bile duct epithelium. This suggests that the hepatocyte most likely synthesizes and deposits HSPG in the perisinusoidal matrix, whereas the bile duct epithelium synthesizes and deposits HSPG in their basement membranes. Furthermore, in an earlier study, we demonstrated the presence of membrane-associated HSPG in the intracellular compartments of hepatocytes (41) using an antibody that recognizes only the membrane-associated type of HSPG. These data indicate that the hepatic parenchymal cell synthesizes both cell membrane and basement membrane-type HSPG which are the only HSPG from liver that have been characterized to date. Interestingly, Brandan and Hirschberg (3) have shown that there is a temporal (precursor-product?) relationship between a hydrophobic, Golgi-associated form of HSPG and hydrophilic, plasma membrane-associated HSPG. Our results demonstrate that the basement membrane and cell mem-

brane-type HSPG we have characterized have distinctive sizes and different distributions once released from the cell. Whether or not the HSPG studied by Brandan and Hirschberg corresponds to the 150–300-kD HSPG with the 40-kD protein core identified in this study remains to be determined.

Diversity among Basement Membrane HSPG

Our data add to the growing evidence for the existence of heterogeneity in HSPG populations associated with different basement membrane sources. The size difference and the existence of unique epitope(s) in the perisinusoidal population of liver HSPG suggests that there may be differences in its amino acid sequence or in its conformation as compared to HSPG derived from other sources. That there should be structural differences between HSPG derived from different types of basement membranes is logical, because there are vast differences in the organization and functions of basement membranes in different locations. This is especially notable when comparing the renal glomerular basement membrane with the loosely organized basement membrane-like matrix found in the space of Disse in the liver. The former is a uniform and compact basement membrane layer which is organized into a continuous sheet (in three dimensions) made up of a tight, well-defined latticework that functions as a refined macromolecular filter restricting passage of anionic molecules >70 Å effective molecular radius (13). By contrast, in the liver, where the sinusoids form an open network with a discontinuous endothelium, there is no typical basement membrane layer. The surface of the hepatocyte is in direct contact with the blood plasma, and the endothelium is separated from the hepatocyte plasmalemma by the space of Disse, which contains occasional collagen fibrils and irregular clumps of extracellular matrix material (29). The latter is known to contain collagen III and basement membrane components, such as collagen IV, fibronectin, and laminin (16, 27, 29), as well as basement membrane-type HSPG (15, 16). The function of this loosely organized matrix composed largely of basement membrane proteins is unknown, but, since it is discontinuous, it seems unlikely that it has a major filtration function.

HSPG are known to differ widely in their hydrodynamic properties due in large part to the number and size of their GAG chains. That genuine differences also exist in the size of their core proteins is also now generally accepted (28, 44). However, at present it is not clear how much of the size diversity is generated by differential proteolytic processing of a common precursor, by differential splicing of mRNA, or whether they represent separate gene products. The explanation will become apparent only when DNA sequence data is available on the core proteins of basement membrane HSPG derived from a variety of sources.

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