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Heparan Sulfate Proteoglycans Are Concentrated on the Sinusoidal Plasmalemmal Domain and in Intracellular Organelles of Hepatocytes

JENNIFER LEA STOW, LENA KJÉLLEN,* ERIC UNGER,* MAGNUS HÖÖK,‡ and MARILYN GIST FARQUHAR
*Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510; *Department of Medical and Physiological Chemistry, Swedish University of Agricultural Sciences, The Biomedical Centre, Uppsala, Sweden; and ‡Connective Tissue Laboratory, Diabetes Hospital, University of Alabama in Birmingham, Birmingham, Alabama 35294.*

ABSTRACT The distribution of cell surface heparan sulfate proteoglycans (HSPGs) was determined in rat liver by immunocytochemistry. A polyclonal antibody was raised against HSPGs purified from rat liver microsomes which specifically immunoprecipitated liver membrane HSPGs. It was shown to recognize both the heparin-releasable and membrane-intercalated form of membrane HSPGs and to recognize determinants on the core protein of these HSPGs. By immunocytochemistry membrane HSPGs were localized to hepatocytes. The distribution of HSPGs at the cell surface of the hepatocyte was restricted to the sinusoidal domain of the plasmalemma; there was little or no staining of the lateral or bile canalicular domains. Intracellularly, HSPGs were occasionally detected in cisternae of the rough endoplasmic reticulum and were regularly found in Golgi cisternae—usually distributed across the entire Golgi stack. HSPGs were also localized in some endosomes, lysosomes, and cytoplasmic vesicles of hepatocytes. We conclude that the HSPGs recognized by this antibody have a restricted distribution in rat liver: they are largely confined to the sinusoidal plasmalemmal domain and to biosynthetic and endocytic compartments of hepatocytes.

Heparan sulfate proteoglycans (HSPGs)[1] are widely distributed in mammalian tissues and cell cultures. They have been found in association with cell surfaces (1–5), intracellular organelles (6, 7), and extracellular matrices (especially basement membranes) (8–11), and have been proposed to participate in a variety of functions such as cell-cell interactions (12), cell migration (13), and cell adhesion (14, 15). HSPGs isolated from different sources have distinct structures in terms of their overall size (70–400 kD) and the size of their glycosaminoglycan chains (14–70 kD).

One of the best characterized populations of HSPGs are those isolated from rat liver membranes (3, 15). These HSPGs are relatively small (75 kD) and consist of a core protein with about four attached heparan sulfate (HS) side chains (14 kD). Three forms of HSPG have been purified from rat liver membranes (3, 16) and from primary hepatocyte cultures (15, 17)—one that behaves as a peripheral membrane protein in that it can be displaced from cells by exogenous heparin, another that behaves as an integral membrane protein in that its core protein is intercalated into the lipid bilayer, and a third form, recently characterized, which consists of HS oligosaccharides and is believed to be associated with lysosomes (Kjéllen, L., H. Pertoft, A. Oldberg, M. Höök, submitted for publication). The heparin-displacable and membrane-intercalated forms are known to be present in rat liver microsomal and plasma membrane fractions, but their precise localization in rat liver in situ is unknown.

In this study, an antibody was generated against rat liver membrane HSPGs and used to determine the distribution of these HSPGs in rat liver.

MATERIALS AND METHODS

Materials: Molecular weight standards for SDS PAGE and Sepharose 4B-Protein A beads were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Iodobeads were obtained from Pierce Chemical Co. (Rockford, IL), and Na[^{125}I] was obtained from Amersham Corp. (Arlington Heights, IL). Fab fragments of sheep anti-rabbit IgG conjugated to horseradish peroxidase were obtained from the Institute Pasteur Productions (Marnes La Coquette, France). 3,3 diaminobenzidine and pepstatin A were obtained from Sigma Chemical Co. (St. Louis, MO). Fluorescein isothiocyanate-conjugated rabbit anti-goat IgG and goat anti-rabbit F(ab')$_2$ were obtained from Cappel Laboratories, Inc. (Cochranville, PA), and 10 nm colloidal gold coupled to sheep anti-rabbit IgG was obtained from Janssen Life Science Products (Piscataway, NJ). Platelet-derived heparitinase was prepared as previously described (3).

[1] *Abbreviations used in this paper:* ER, endoplasmic reticulum; HS, heparan sulfate; HSPGs, heparan sulfate proteoglycans; PMs, plasma membrane fractions.

Published March 1, 1985

Preparation of Antiserum: HSPGs were purified from rat liver microsomes by gel chromatography, ion exchange chromatography, and density gradient ultracentrifugation (3). Purity was assessed by enzymatic degradation, analytical centrifugation, and amino acid analysis (3). An amount corresponding to 25 μg protein (in 100 μl) was emulsified in complete Freund's adjuvant (100 μl) and injected (50-μl aliquots) into the popliteal lymph nodes of a rabbit. Booster injections in incomplete Freund's adjuvant were given twice at 2-wk intervals. The rabbit was bled 10, 20, and 30 d after the last injection, and the antiserum was screened for reactivity to HSPGs by immunoprecipitation as described below. IgG was isolated from the antiserum by affinity chromatography on Protein A-Sepharose (18).

Preparation of ^{125}I-HSPGs and ^{125}I-PMs: Plasma membrane fractions (PMs) (19) and membrane HSPGs (16) were prepared from rat livers as detailed previously, and radioiodinated by the Chloramine T method using Iodobeads. ^{125}I-PMs were solubilized in 1% Triton X-100, in phosphate-buffered saline (PBS).

Preparation of [^{35}S]Sulfate HSPG: Primary cultures of rat hepatocytes were radiolabeled with 100 μCi/ml of [^{35}S]sulfate for 24 h as previously described (15). Protease inhibitors, phenylmethylsulfonyl fluoride (1 mM), *n*-ethylmaleimide (2 mM), EDTA (2 mM), and pepstatin (10 μg/ml), were present in all buffers used for isolation of HSPGs. The radiolabeled cells were incubated with 0.5 mg/ml heparin to displace receptor-bound HSPGs (15) which were collected, and the cells were further incubated with 1% Triton X-100 in 0.05M Tris buffer (pH 8.0) for 30 min at 25°C to release membrane-intercalated HSPGs and HS polysaccharide. After centrifugation (to pellet unsolubilized material), HSPGs were purified from the detergent extract and from heparin-displaced fractions by DEAE-ion exchange chromatography and gel chromatography on Sepharose CL-4B (16). The membrane-intercalated HSPGs and HS oligosaccharide are separated in the latter step.

Immunoprecipitation: ^{125}I-HSPGs (3 μg protein) or ^{125}I-PMs (2 μg) were incubated at 4°C overnight with 100 μl of normal or immune rabbit serum in PBS containing 0.1% Triton X-100 and 5 mg/ml bovine serum albumin in a total volume of 1 ml. ^{35}S-HSPGs or HS (5,000 cpm) were incubated under similar conditions with varying amounts of IgG. After addition of 100 μl of a 1/1 suspension of Protein A-Sepharose in PBS and further incubation for 1 h at room temperature, the beads were pelleted by centrifugation, washed with 4 × 1 ml of PBS containing 0.5 M NaCl and 0.1% Triton X-100, and assayed for ^{125}I- or ^{35}S-radioactivity.

SDS PAGE: Immunoprecipitates obtained from ^{125}I-labeled samples were solubilized and analyzed on 7–15% polyacrylamide gradient gels with a 4.8% stacking gel in a Maisel buffer system as described by Blobel and Dobberstein (20). After fixation in 12% trichloroacetic acid, gels were stained with Coomassie Blue, and autoradiographed using Kodak X-Omat AR films. Exposure was at −70°C for 3 d.

Preparation of Tissue for Immunocytochemistry: Procedures used for the fixation and incubation of rat liver were as described in an earlier paper (21). In brief, fixation was initiated by perfusion with periodate-lysine-paraformaldehyde, and blocks of tissue were excised and immersed in the same fixative for an additional 4–6 h at 25°C.

Immunofluorescence: Frozen sections (0.5 μm) were prepared from periodate-lysine-paraformaldehyde–fixed rat liver on a Reichert Ultracut OMU-4 ultramicrotome equipped with a cryoattachment. Sections were mounted on glass slides and incubated sequentially in (*a*) normal rabbit serum or anti-HSPG antiserum or IgG, (*b*) fluorescein isothiocyanate goat anti-rabbit F(ab')2, and (*c*) fluorescein isothiocyanate rabbit anti-goat IgG (to enhance the fluorescent signal); sections were then examined in a Zeiss Photomicroscope II equipped with epifluorescence.

Immunoperoxidase: Procedures used for indirect immunoperoxidase staining were given elsewhere (21–23). In brief, cryostat sections (15–20 μm) were prepared from periodate-lysine-paraformaldehyde–fixed liver and incubated in (*a*) normal rabbit or immune serum (diluted 1/5) or IgG (3 μg/ml) for 12–16 h and (*b*) peroxidase-conjugated sheep anti-rabbit Fab (2 h). Sections were then fixed in 1.5% glutaraldehyde, incubated in diaminobenzidine medium, postfixed in ferrocyanide–reduced OsO_4 and embedded in Epon.

Immunogold: Cryostat sections were incubated in immune or preimmune serum as above and then in sheep anti-rabbit-gold conjugate (24–48 h), postfixed in 1% OsO_4 in acetate-veronal buffer, stained in block with uranyl acetate, and embedded in Epon.

RESULTS

Immunoprecipitation of ^{125}I-HSPGs

When fractions of ^{125}I-HSPGs purified from rat liver plasma membranes (composed of a mixture of membrane-intercalated and heparin-releasable HSPGs) were incubated with the antibody, 30% of the ^{125}I counts were precipitated, whereas only 2% were precipitated with control serum. When PMs were iodinated and the solubilized proteins were used for immunoprecipitation, only background amounts of radioactivity were precipitated with either immune or normal rabbit serum. Previous experiments have established that this is because no detectable amount of ^{125}I-radioactivity is incorporated into HSPGs when intact PMs are iodinated (Kjéllen, L., and M. Höök, unpublished observations). When ^{125}I-PMs and ^{125}I-HSPGs (constituting 40% of the total protein or 10% of the total ^{125}I-radioactivity) were mixed, the precipitation of ^{125}I-HSPGs was as efficient as with ^{125}I-HSPGs alone, indicating that plasma membrane proteins do not inhibit antibody binding.

Digestion of the ^{125}I-HSPGs with a purified platelet heparitinase (3) and the presence of unlabeled heparin did not affect the amount of HSPGs precipitated, suggesting that the antibody recognizes determinants on the core protein—not the polysaccharide chains—of these HSPGs.

By SDS PAGE (Fig. 1), immunoprecipitates obtained from ^{125}I-HSPGs or a mixture of the latter and ^{125}I-PMs with

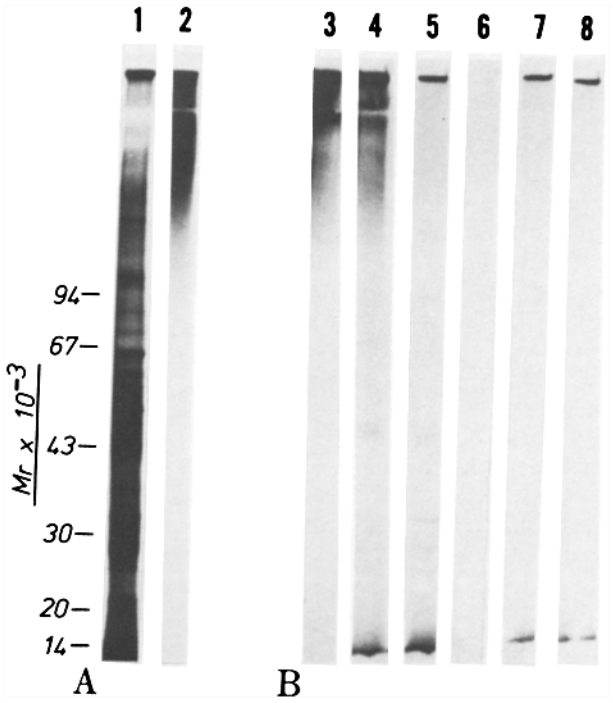

FIGURE 1 (*A*) Samples of ^{125}I-labeled rat liver plasma membrane fractions (^{125}I-PMs) (80,000 cpm; lane *1*) and purified ^{125}I-HSPGs (20,000 cpm; lane *2*) were separated by SDS PAGE and exposed for autoradiography. ^{125}I-PMs contain numerous labeled protein bands, and ^{125}I-HSPGs appear as a typical broad band concentrated at the interface of the stacking and running gels. (*B*) Autoradiograms of immunoprecipitates obtained when anti-HSPG serum (lanes *3–5*) or normal rabbit serum (lanes *6–8*) were incubated with ^{125}I-HSPGs alone (lanes *3* and *6*), with a mixture of ^{125}I-HSPGs and ^{125}I-PMs (lanes *4* and *7*), or with ^{125}I-PMs alone (lanes *5* and *8*). A smeared band at the top of the gel corresponding to HSPGs was precipitated by the anti-HSPG serum from ^{125}I-HSPGs (lane *3*) and from the mixture of ^{125}I-HSPGs and ^{125}I-PMs (lane *4*), but not from ^{125}I-PMs alone (lane *5*). No such bands were precipitated with normal rabbit serum (lanes *6–8*). The narrow band at the top of the stacking gel (lanes *5*, *7*, and *8*) is considered to be nonspecific since it is present with both normal and immune sera.

Published March 1, 1985

immune serum were identical to that of purified ^{125}I-HSPGs—i.e., the labeled material accumulated at the interface between the stacking and running gel, and no other protein bands were precipitated. (Compare lanes 2, 3, and 4). No labeled material was precipitated from ^{125}I-HSPGs with normal rabbit serum (lanes 6–8) or from ^{125}I-PMs with immune serum (lane 5).

Enzyme-linked immunosorbent assays analyses showed no reactivity of the antiserum with laminin, fibronectin, or Type IV collagen (data not shown).

Collectively, these data indicate that the antiserum generated against liver membrane HSPGs recognizes the core protein of membrane HSPGs but does not react with plasma membrane proteins or with common extracellular matrix constituents.

Immunoprecipitation of ^{35}S-HSPGs

To establish which forms of cell-associated HSPGs are recognized by the antibody, we carried out immunoprecipitations with ^{35}S-HSPGs isolated from primary cultures of rat hepatocytes. Up to 50% of the membrane-intercalated ^{35}S-HSPGs but only ~12% of the heparin-releasable ^{35}S-HSPGs was precipitated by the antibody (Fig. 2). There was no reactivity with the ^{35}S-HS oligosaccharide (Fig. 2) as antigen or when the antiserum was substituted with normal rabbit serum (not shown).

Localization of HSPGs in Rat Liver

When 0.5-μm ultracryomicrotome sections were incubated with anti-HSPG serum or IgG for immunofluorescence, staining was seen around the hepatic sinsuoids (Fig. 3*A*). A similar distribution of reaction product was seen in 0.5-μm sections of plastic-embedded immunoperoxidase preparations (Fig. 3*B*). Some foci of intracellular staining were also seen in the hepatocyte cytoplasm in the latter. No staining was detected in sections similarly reacted with normal rabbit serum.

When immunoperoxidase preparations were examined by electron microscopy (Figs. 4–6 and 8–9), it was evident that the staining seen around the hepatic sinusoids by light microscopy was due to its presence on the hepatocyte cell surface facing the space of Disse (Figs. 4 and 8), and that there was little staining of the endothelial and Kupffer cell surfaces. In

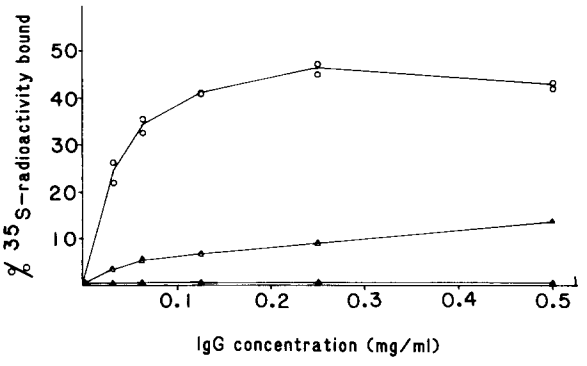

FIGURE 2 Different forms of ^{35}S-HSPGs or HS oligosaccharides (5,000 cpm) were incubated with indicated amounts of anti-HSPG IgG. After incubation with protein A-Sepharose, the percentage of ^{35}S-radioactivity bound to the gel was determined. Precipitation of intercalated membrane ^{35}S-HSPGs (○) is more efficient than that of peripheral (heparin-released) ^{35}S-HSPGs (△). The HS-oligosaccharide (▲) is not recognized.

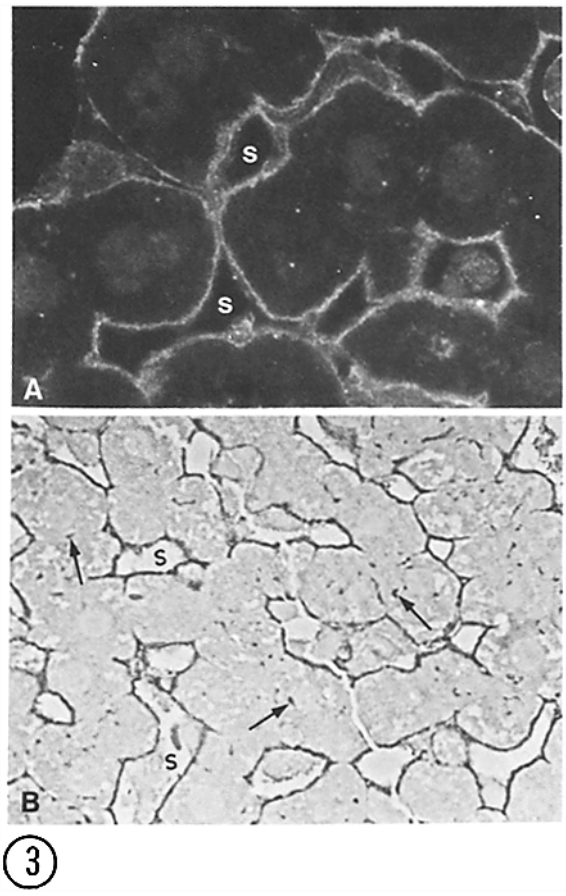

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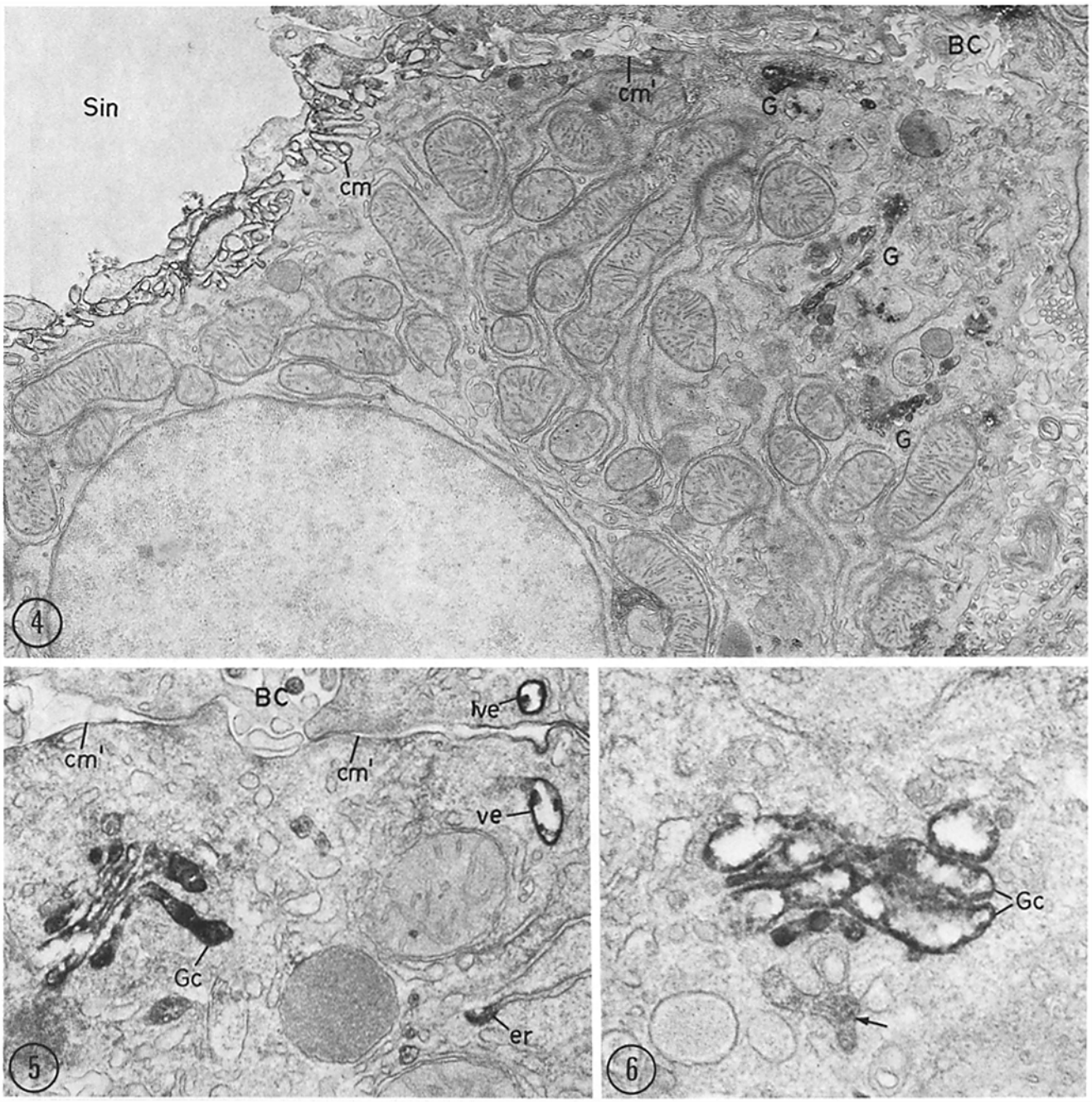
FIGURE 3 Localization of HSPGs in rat liver by immunofluorescence (*A*) (0.5-μm ultracryomicrotome section) and by immunoperoxidase (*B*) (0.5-μm plastic-embedded section). Staining is concentrated around the hepatic sinusoids (*S*) in both preparations. Staining is also seen intracellularly (arrows) in sites corresponding to the location of Golgi complexes (see Figs. 4 and 5). (*A*) × 150; (*B*) × 98.

hepatocytes, a continuous layer of reaction product was present all along the microvilli and coated pits at the sinusoidal domain, but there was little or no staining of the lateral or bile canalicular plasmalemmal domains (Figs. 4 and 5). The same concentration of HSPGs on the sinusoidal surface of hepatocytes was seen in sections reacted by the immunogold procedure,—i.e. gold particles were present in large numbers on the sinusoidal cell surface of hepatocytes (Fig. 7), but few were seen on endothelial or Kupffer cells or on the lateral or bile canalicular surfaces of hepatocytes.

Within the hepatocyte, staining was present in Golgi and endoplasmic reticulum (ER) cisternae (Figs. 4–6) as well as in some cytoplasmic vesicles (Fig. 5) and in some endosomes and lysosomes (Figs. 8–9). The most regular and prominent site of intracellular staining was the Golgi complex where reaction product was consistently found in the stacked cisternae (Figs. 5–6). In the majority of cases all the cisternae in a given stack were stained, but there was no staining of GERL-like structures. Staining of the rough ER was less frequent and more patchy; when present, reaction product was typically concentrated at the tips of the reactive cisternae (Fig. 5). The number of stained endosomes and lysosomes was quite variable from one cell to another.

In controls incubated with normal rabbit serum, no reaction product was detected in any location.

Published March 1, 1985



FIGURES 4–6 Electron micrographs demonstrating the localization of HSPGs in rat liver by immunoperoxidase. Fig. 4 is a low magnification view of a hepatocyte showing staining of the sinusoidal domain of the hepatocyte cell membrane (*cm*) facing the sinusoid (*Sin*). The lateral (*cm'*) domain (between the sinusoid and the bile canaliculus [*BC*]) and the bile canalicular domain (facing the bile canaliculus) of the hepatocyte show little or no staining. Staining is also present intracellularly in several Golgi complexes (*G*). Figs. 5 and 6 illustrate the localization of HSPGs in the stacked Golgi cisternae (*Gc*). Reaction product is seen distributed across all of the cisternae in each stack. It is not present in a GERL- or lysosome-like structure (Fig. 6, arrow). Also illustrated (Fig. 5) is the presence of reaction product at the tip of an ER cisternae (*er*) and in several cytoplasmic vesicles (*ve*), and the absence of reaction product from the bile canalicular (*BC*) and lateral (*cm'*) domains of the plasmalemma. (Fig. 4) × 13,500; (Fig. 5) × 39,000; (Fig. 6) × 50,000.

DISCUSSION

In this study, we produced a specific antibody to rat liver membrane HSPGs and used it to determine the localization of HSPGs in the rat liver. Immunoprecipitation analyses established that the antibody generated specifically recognizes HSPGs purified from rat liver plasma membrane fractions, and it preferentially recognizes the membrane-intercalated form of HSPGs. It was concluded that the antibody recognizes determinants on the core protein but not on the polysaccharide chains of these HSPGs because it does not react with HS oligosaccharide and because release of the polysaccharide chains (by heparitinase digestion) did not affect immunoprecipitation.

By immunocytochemistry, we found that the HSPGs rec-

Published March 1, 1985

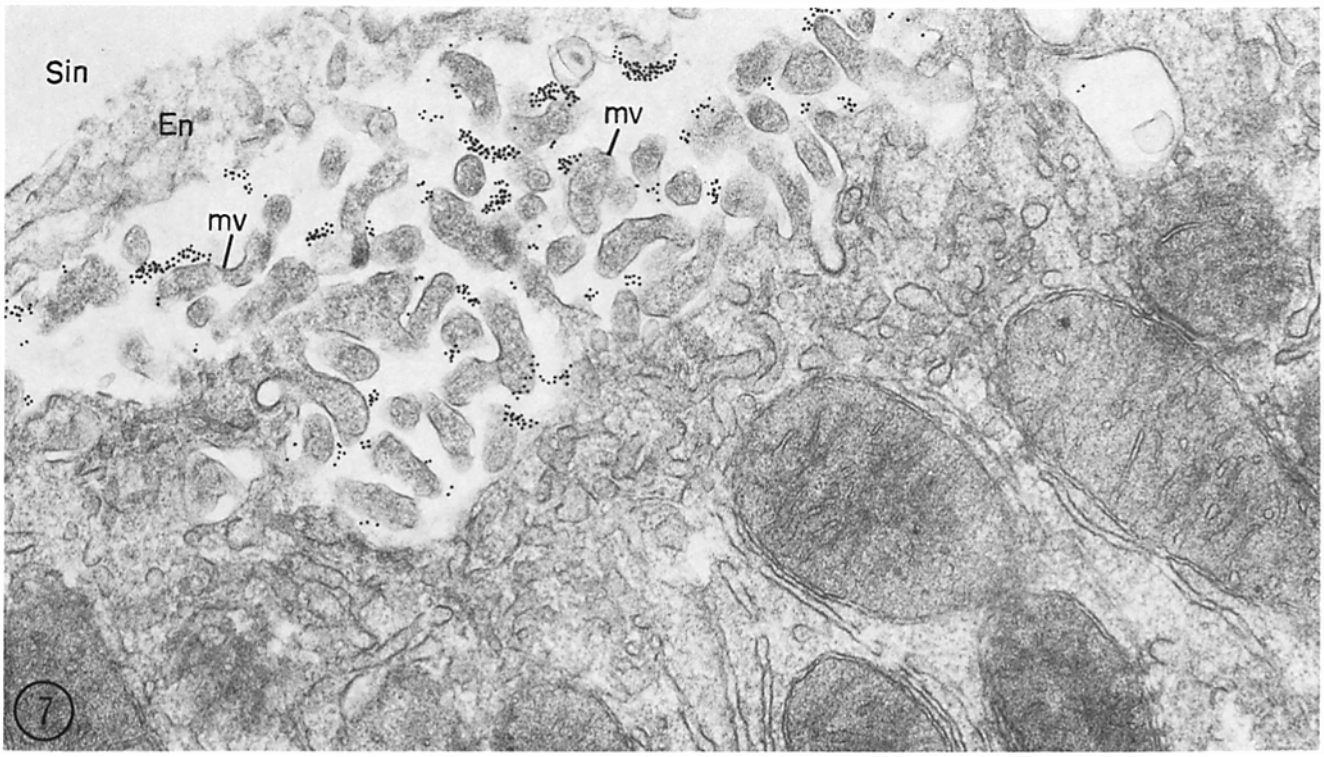


FIGURE 7 Portion of a hepatocyte from a section incubated with anti-HSPG IgG and labeled by an indirect immunogold procedure. Clumps of gold particles are concentrated on the microvilli (*mv*) at the sinusoidal domain of the hepatocyte and few are bound to the endothelial (*en*) plasmalemma. × 40,000.

ognized by this antibody are restricted in their distribution to hepatocytes where they are concentrated along the sinusoidal domain of the plasmalemma. In addition, they are regularly found intracellularly associated with Golgi cisternae and some rough ER cisternae, as well as with some of the cytoplasmic vesicles, endosomes, and lysosomes.

Previous biochemical results established that HSPGs are present in rat liver plasma membrane fractions (3) prepared by the method of Ray (19). Such fractions are assumed to be derived primarily from the bile canalicular and lateral domains of the hepatocyte plasmalemma, but they also contain variable amounts of sinusoidal plasma membrane and other (e.g., Golgi) membranes as well (24). The present immunocytochemical results confirm that HSPGs are associated with the hepatocyte plasmalemma, and demonstrate further that (*a*) they are largely restricted to the sinusoidal plasmalemmal domain of this cell, (*b*) they are missing or present in very low concentrations on the lateral and bile canalicular domains, and (*c*) they are not restricted to the plasma membrane but are found in intracellular compartments of hepatocytes as well.

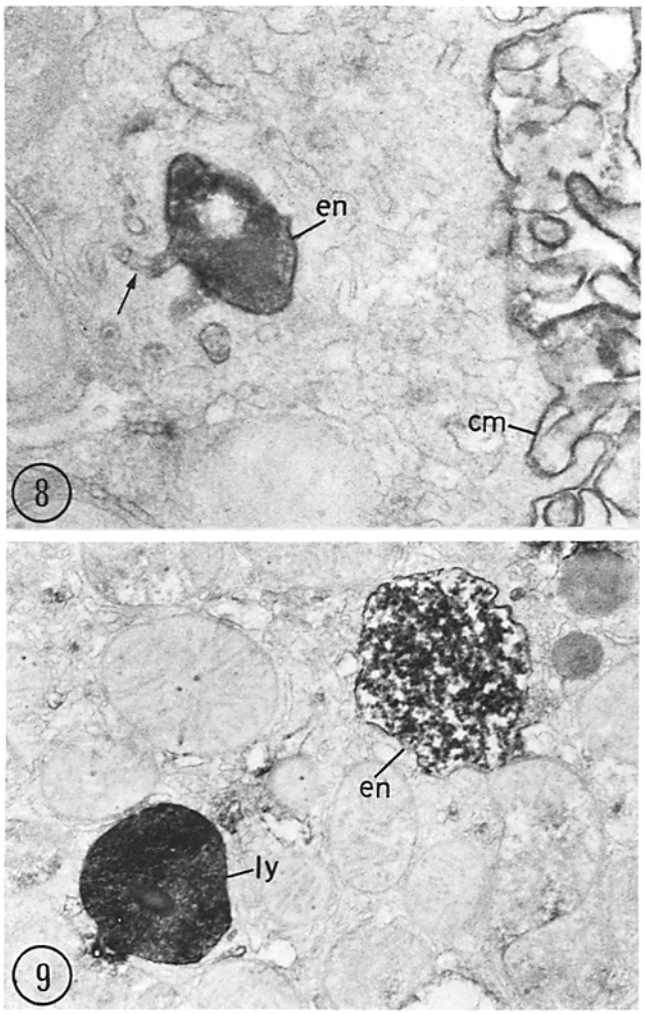
At present there are a number of examples of membrane constituents which are highly concentrated or located exclusively on a particular domain of the hepatocyte plasmalemma. Some membrane constituents, such as receptors for insulin (25), asialoglycoproteins (26), and mannose-6-phosphate (21) are found mainly if not exclusively on the sinusoidal domain, whereas others, such as (Na^+K^+)-ATPase (27) and adenylate cyclase (24) are found on both the sinusoidal and lateral domains. Still other components, such as phosphatases and leucine aminopeptidase (24), and three membrane glycoproteins (105, 110, and 160 kD) of unknown function (28), are restricted to the bile canalicular domain.

The function of the HSPGs concentrated at the vascular surface of the hepatocyte is not known. Previous studies have demonstrated that HSPGs on the surfaces of endothelial cells, which, like the sinusoidal surface of the hepatocyte are exposed to the blood plasma, are able to bind molecules from the circulation such as lipoprotein lipase (29) and antithrombin (30). A role for heparin in modulating binding of low density lipoproteins to their receptors has also been postulated (31).

The HSPGs present within the ER and Golgi cisternae of hepatocytes most likely represent a biosynthetic pool of HSPGs destined for the cell surface since it is known that newly synthesized proteoglycans, like glycoproteins, are segregated in the rough ER and transported to the Golgi complex where they undergo extensive posttranslational modification (e.g., glycosylation and sulfation) (32). The HSPGs detected in endosomes and lysosomes could be destined for degradation or could be in transit during recycling (33). The vesicles located near the cell surface in which we detected HSPGs are probably involved in the transport of HSPGs either from the Golgi to the cell surface or from the latter to endosomes and lysosomes. However, the possibility that the HSPGs are integral components of the membranes of these compartments cannot be ruled out at present. Kinetic and cell fractionation studies are required to determine the relationship (biosynthetic, degradative, recycling?) between the HSPGs detected in these different organelles.

The antibody raised against liver HSPGs appears to recognize plasma membrane–associated HSPGs from tissues other than liver because it stains plasma membranes of kidney epithelia and endothelia (34) and of cultured fibroblasts (35). However, it does not recognize HSPGs associated with basement membranes because it does not stain basement mem-

Published March 1, 1985



FIGURES 8 and 9 Localization of HSPG in endosomes and lysosomes by immunoperoxidase. In Fig. 8, reaction product is seen in an endosome (*en*)—with a tubular connection (arrow)—located near the sinusoidal cell surface. The sinusoidal domain of the plasmalemma (*cm*) is also stained. In Fig. 9, reaction product is seen in a presumptive endosome (*en*) and in a lysosome (*ly*). (Fig. 8) × 46,500; (Fig. 9) × 22,000.

branes in kidney and other tissues (34). Recently an antibody was raised against HSPGs purified from rat kidney glomeruli that stains basement membranes but does not stain epithelial or endothelial plasma membranes in the kidney (36) and in other tissues surveyed, including rat liver. These findings suggest that the plasma membrane HSPGs are antigenically distinct from the basement membrane–associated HSPGs. Further work in progress using anti-HSPG antibodies is aimed at determining the relationship between these two populations of HSPG.

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