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

JENNIFER LEA STOW, LENA KJELLEN,* ERIC UNGER,* MAGNUS HÖÖK,4 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) 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 (Kjellen, L., H. Pertoft, A. Oldberg, M. Höök, submitted for publication). The heparin-displaceable 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). Iodo beads were obtained from Pierce Chemical Co. (Rockford, IL), and Na[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).
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 50 μg proteins 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 [125I]-HSPGs and [125I]-PMs: Plasma membrane fractions (PMs) (19) and membrane HSPGs (16) were prepared from rat livers as detailed previously, and radioiodinated with the Chloramine T method using Iodobeads. [125I]-PMs were solubilized in 1% Triton X-100, in phosphate-buffered saline (PBS).

Preparation of [35S]Sulfate HSPG: Primary cultures of rat hepatocytes were radiolabeled with 100 μCi/ml of [35S]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 0.05 M Tris buffer (pH 8.0) for 30 min at 25°C to release membrane-intercalated HSPGs and HS polysaccharide. After centrifugation (to pellet unlsolubilized membrane HSPGs), PMs 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 next step.

Immunoprecipitation: [125I]-HSPGs (3 μg protein) or [125I]-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. [35S]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 pelletted by centrifugation, washed with 4 × 1 ml of PBS containing 0.5 M NaCl and 0.1% Triton X-100, and assayed for 125I- or 35S-radioactivity.

SDS PAGE: Immunoprecipitates obtained from [125I]-labeled samples were solubilized and analyzed on 7–15% polyacrylamide gradient gels with a 4.8% stacking gel (lanes 5, 7, and 8) is considered to be nonspecific since it is present with both normal and immune sera. Previous experiments have established that this is because no detectable amount of 125I-radioactivity is incorporated into HSPGs when intact PMs are iodinated (Kjellén, L., and M. Höök, unpublished observations). When 125I-PMs and 125I-HSPGs (constituting 40% of the total protein or 10% of the total 125I-radioactivity) were mixed, the precipitation of 125I-HSPGs was as efficient as with 125I-HSPGs alone, indicating that plasma membrane proteins do not inhibit antibody binding.

DIGESTION OF 125I-HSPGS

Digestion of the 125I-HSPG with a purified platelet heparinase (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 125I-HSPGs or a mixture of the latter and 125I-PMs with...
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 sinusoids (Fig. 3A). A similar distribution of reaction product was seen in 0.5-μm sections of plastic-embedded immunoperoxidase preparations (Fig. 3B). 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 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.
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 canicular 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 canicular (BC) and lateral (cm') domains of the plasmalemma. (Fig. 4) x 13,500; (Fig. 5) x 39,000; (Fig. 6) x 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-
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.

recognized 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 adenylyl 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-
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The authors gratefully acknowledge the expert assistance of Sue Ann Mentone (immunocytochemistry) and M. Lynne Wootton (word processing).

Received for publication 26 July 1984, and in revised form 29 October 1984.