Heterogeneous Distribution of a Basement Membrane Heparan Sulfate Proteoglycan in Rat Tissues

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Abstract. A heparan sulfate proteoglycan (HSPG) synthesized by murine parietal yolk sac (PYS-2) cells has been characterized and purified from culture supernatants. A monospecific polyclonal antiserum was raised against it which showed activity against the HSPG core protein and basement membrane specificity in immunohistochemical studies on frozen tissue sections from many rat organs. However, there was no reactivity with some basement membranes, notably those of several smooth muscle types and cardiac muscle. In addition, it was found that pancreatic acinar basement membranes also lacked the HSPG type recognized by this antiserum. Those basement membranes that lacked the HSPG strongly stained with antisera against laminin and type IV collagen. The striking distribution pattern is possibly indicative of multiple species of basement membrane HSPGs of which one type is recognized by this antiserum. Further evidence for multiple HSPGs was derived from the finding that skeletal neuromuscular junction and liver epithelia also did not contain this type of HSPG, though previous reports have indicated the presence of HSPGs at these sites.

The PYS-2 HSPG was shown to be antigenically related to the large, low buoyant density HSPG from the murine Engelbreth-Holm swarm tumor. It was, however, confirmed that only a single population of antibodies was present in the serum. Despite the presence of similar epitopes on these two proteoglycans of different hydrodynamic properties, it was apparent that the PYS-2 HSPG represents a basement membrane proteoglycan of distinct properties reflected in its restricted distribution in vivo.

BASEMENT membranes and a variety of cell surfaces have been shown to contain heparan sulfate proteoglycans (HSPGs)[1] (see reviews 13, 15, 17, 26). Those from cell surfaces appear to have hydrophobic characteristics which indicate that their core proteins may be distinct from the basement membrane HSPGs, these being extracellular matrix components. The cell-associated HSPGs have been strongly implicated with a role in cell adhesion processes (17, 21, 40), while the basement membrane forms, notably those found in the kidney glomerulus, are thought to control macromolecular permeability of their respective basement membranes (19). More recently, concentrations of HSPG in the neuromuscular junction basement membrane have been described which may have a role in the orientation or localization of acetylcholinesterase (1, 2). However, for most basement membranes, the role of HSPG is presently not understood.

There is a strong probability that basement membranes contain a family of proteoglycans, including some forms of chondroitin sulfate proteoglycan (CSPG) (5, 13, 18) and a potential candidate for one such proteoglycan from PYS-2 cell cultures has been identified (8). I aimed to characterize a basement membrane HSPG secreted by PYS-2 cells, and then, by immunochemical analysis, determine its relationship to other HSPGs and ubiquity in basement membranes. As an increasing number of extracellular matrix proteoglycans, including HSPGs, have now been described from cell cultures and in vivo sources (13, 15, 26, and references therein), it was felt to be necessary in this study to correlate structure with immunochemical and distribution data so that when protein core sequence data becomes available proteoglycan relationships can be more easily established. The situation is not straightforward in that both large, low density and small, high density HSPGs have been reported from PYS-2 cells (33) and the murine Engelbreth-Holm swarm (EHS) tumor, a rich source of basement membrane macromolecules (10, 14). The two HSPGs from the EHS tumor are immunochemically related (10, 14), which has given rise to the thesis that the smaller HSPG is a proteolytic product of the larger form, and that such processing may, to some extent, explain the wide variation in HSPG sizes from tissue sources (14, 15, 33). In addition, antibodies raised against EHS tumor HSPGs cross react with HSPGs from PYS-2 cells and a rat yolk sac tumor (10, 33, 38).

Previous studies with PYS-2 cells have shown the synthe-

1. *Abbreviations used in this paper*: CS, chondroitin sulfate; DS, dermatan sulfate; EHS, Engelbreth-Holm swarm; HS, heparan sulfate; PG, proteoglycan.

sis of HSPGs in addition to a dermatan sulfate proteoglycan (DSPG) (8, 10, 24, 33). In this report, a small, high density HSPG has been purified from bulk culture supernatants and a monospecific antiserum raised against it. Immunohistochemical analysis showed that not all basement membranes contain this HSPG, nor is there a concentration of it in the neuromuscular junction. The HSPG is also immunochemically unrelated to that found on cell surfaces and does not stain liver epithelial cells, consistent with the notion that HSPGs with hydrophobic properties have different core protein and functional capabilities. However, it remains highly likely that there are other HSPGs specific to basement membranes which may have distinct core proteins and fulfill different roles in basement membrane physiology. This is particularly borne out by the finding that not all basement membranes contain the type of HSPG reported here.

Materials and Methods

Materials

Cell culture reagents were from Flow Laboratories, Inc., McLean, VA. Tissue culture flasks and dishes were from Corning Glass Works, Corning, NY. Roller culture flasks were from Sterilin Ltd., Teddington, Middlesex, UK. $Na_2\ ^{35}SO_4$ was from Amersham Corp., Arlington Heights, IL (specific activities 1,440 Ci/mmol). DEAE-Sephacel, Sepharose CL-4B, Sephadex G-50 and G-200, and Mono Q column were all obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals were from Sigma Chemical Co., St. Louis, MO or Fisher Chemicals, Fairhaven, NJ. Chondroitinase ABC, heparitinase, and heparinase were from Miles Laboratories, Inc., Elkhart, IN, and leech hyaluronidase was from Biopharm (U.K.) Ltd., Swansea, Glamorgan, UK.

Cells: Culture and Experimental Conditions

PYS-2 cells, a teratocarcinoma-derived cell line related to the parietal endodermal cells of the mouse embryo (22) were grown at 37°C in DME containing 8% FBS, 25 μg/ml ascorbic acid, 1 mM serine, 2 mM proline, 1 mM sodium pyruvate, and 30 nM each of adenosine, cytidine, guanosine, and uridine. Some cultures in 100-mm petri dishes were radiolabeled with 20 μCi/ml [^{35}S]sulfate for 3 d. For bulk cultures, cells were grown for 3–6 d in 150-cm^2 flasks or roller bottles. Medium from large or small batches was decanted and centrifuged at 1,000 rpm in a TJ-6 centrifuge (Beckman Instruments, Inc., Palo Alto, CA) with TH-4 rotor and the supernatants were then further processed for the purification of proteoglycans. A similar murine cell line, PF-HR9, was grown in DME containing 10% FBS.

Purification and Analysis of HSPGs from PYS-2 Culture Supernatants

All buffers contained the protease inhibitors EDTA, phenylmethylsulfonyl fluoride (PMSF), and *N*-ethylmaleimide in addition to sodium azide at concentrations of 20, 0.2, and 10 mM, and 0.02% respectively. Radiolabeled medium from PYS-2 cultures was made 0.2 mM with PMSF, 10 mM with *N*-ethylmaleimide, and 4 M with urea before isolation of the proteoglycans on a column of DEAE-Sephacel as previously described (8). Labeled proteoglycans were then eluted with a gradient from 0.2–1.5 M NaCl in 0.05 M sodium acetate, pH 4.0, containing 4 M urea. Fifty 4-ml fractions were collected; 100 μl of each was analyzed for radiolabel, and the conductivity of every third fraction was also measured.

CS/DS was removed by overnight treatment at 37°C with 0.2 U/ml chondroitinase ABC in 0.05 M Tris acetate, pH 8.0, containing 10 mM EDTA, 5 mM *N*-ethylmaleimide, 0.2 mM PMSF, and 20 mM pepstatin followed by a repurification on a 3 × 1-cm column of DEAE-Sephacel as before. The digestion products of chondroitinase ABC action did not rebind DEAE-Sephacel under the conditions used. For analysis of CS/DS and HS content, gel chromatography was performed on a 30 × 2-cm column of Sephadex G-50 after chondroitinase ABC or nitrous acid (30) treatments. The column was equilibrated in 1 M NaCl and run at 15 ml/h. 2-ml fractions were collected and analyzed for radiolabel.

Size analysis of the proteoglycan was carried out on a 90 × 1.5-cm column of Sepharose CL-4B equilibrated with 0.1% SDS, 0.35 M NaCl, 0.05 M Tris-HCl, pH 8.0, or Dulbecco's PBS, both run at 6 ml/h. 1.5-ml fractions were collected for radiolabeled analysis. To examine whether the proteoglycans existed in disulfide-bonded aggregates, samples were boiled for 3 min in the SDS-containing buffer with 2 mM dithioerythreitol. Reduced samples were cooled, alkylated with excess 0.5 M iodoacetamide, and chromatographed on the column of Sepharose CL-4B.

Glycosaminoglycan chain size was determined after alkaline elimination (8) and chromatography on a column of Sepharose CL-4B as above or Sephadex G-200 equilibrated with 0.2 M NaCl (36). Columns were run at 6 ml/h and 1.5-ml fractions collected for radiolabel analysis. Samples for papain treatment were incubated for 16 h at 65°C in 0.05 M sodium acetate, pH 5.5, 2 M NaCl, 0.01 M cysteine HCl, and 0.01 M EDTA containing 2 mg/ml recrystallized papain. Chromatography was on a column of Sepharose CL-4B as above.

Bulk Preparations of Proteoglycans and Antiserum Production

To each 5–10 liter batch of medium, 3–5 ml of radiolabeled medium was added as a tracer for subsequent proteoglycan purification. The first stage was by anion-exchange chromatography essentially as before (8) except that a 3 × 5-cm column of DEAE-Sephacel was loaded at 50–100 ml/h.

After salt gradient elution, fractions from the first peak (Fig. 1) in 0.05 M sodium acetate, pH 4.0, 4 M urea, and protease inhibitors (8) were pooled and solid cesium chloride was added directly to a starting density of 1.4 g/ml. Centrifugation was at 100,000 *g* for 72 h at 4°C. 2-ml fractions were eluted from the bottom of the tubes, and an aliquot from each was analyzed for radiolabel content and density. Those fractions eluting at the bottom (density 1.45 g/ml) were pooled and dialyzed into PBS for ion exchange chromatography by HPLC. 5-ml aliquots were applied to a Mono Q column at 2 ml/min and eluted with a linear gradient of 0.2–1.6 M NaCl in 0.05 M sodium acetate, pH 4.0, at 2 ml/min. 1-ml fractions were collected and analyzed for radiolabel content, pooled (see below), and dialyzed into PBS for antiserum production. Samples were treated with chondroitinase ABC or nitrous acid and chromatographed on a column of Sephadex G-50 to confirm absence of CS/DSPGs.

As an approximate guide to protein content, it was estimated that protein was present to the equivalent of 10% of the glycosaminoglycan concentration and the HSPG in PBS was analyzed spectrophotometrically at 220 or 230 nm using heparan sulfate chains (a gift from Dr. M. Höök) as a standard. 1 mg HSPG by protein content in PBS was mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously in a New Zealand white rabbit which was pre-bled before injection to provide preimmune serum. The pre-bleed was used to check a lack of preexisting reactivity to rat tissue antigens. 2 and 4 wk later, the rabbit was boosted with HSPG at a similar concentration in Freund's incomplete adjuvant. Bleeds were taken from an ear vein at two-wk intervals. The data presented below are based on the eighth and ninth bleeds.

Bulk preparations of PYS-2 culture supernatants were also used to purify the CS/DSPG. The preparation was as above for HSPG, except that the second peak of material eluting from the DEAE-Sephacel was used (see below). This has previously been shown to be rich in CS/DSPG (8). To remove contaminating HSPG the material was dialyzed into 0.1 M sodium acetate, pH 7.0, containing 1 mM calcium acetate, 0.2 mM PMSF, and 1 mM benzamidine HCl. Before enzyme addition, 50 μg/ml leupeptin was added to the proteoglycan mixture. 0.25 U/ml each of heparitinase and heparinase were added for a 4–5-h incubation at 42°C. The CS/DSPG was then recovered in the void volume of a 30 × 2-cm column of Sephadex G-200 equilibrated and run at 10 ml/h in 0.2 M sodium chloride, 20 mM Tris-HCl, pH 7.5, with protease inhibitors as before. Under this regime, the HSPG core protein and heparan sulfate degradation products were included in the column. Hexosamine analysis confirmed the absence of HSPG in the CS/DSPG preparation (also confirmed by lack of activity of the anti-HSPG serum against this material; see below).

One bulk preparation of medium proteoglycans from PF-HR9 cells was used in the analyses of the anti-HSPG serum and was prepared by a single ion-exchange chromatography procedure on a 3 × 5-cm column of DEAE-Sephacel as described above.

ELISA

96-well plates were sensitized with 100 ng antigen/well in carbonate buffer, pH 9.8, for 2 h at 37°C, washed with PBST (PBS containing 0.15% Tween 20), and incubated with antisera in PBST for 1 h at 37°C. After repeated

washing, wells were then incubated with a 1:1,000 dilution of goat anti-rabbit IgG coupled to horseradish peroxidase (CooperBiomedical, Inc., Malvern, PA) in PBST for 1 h at 37°C. The plates were then washed five times with PBST, twice with distilled water, and color developed with DMAB/MBTH chromophore reagent, at 100 μl/well. The color was developed for 20 min at room temperature and absorbance read on a multiskan (Dynatech Laboratories, Inc., Alexandria, VA) at 540 nm. Control wells, not shown on the figures, include use of inappropriate peroxidase-conjugated second antibody, lack of antigen, first antibody or second antibody, and use of preimmune sera from the same rabbits that were subsequently used for raising antisera. All of these controls rendered absorbance readings at background levels.

Immunoblotting Analysis of the Antiserum

Electrophoresis was performed as previously (8) on 3–15% gradient SDS gels. Electrophoretic transfer onto cationized nylon membranes (Zetaprobe; Bio-Rad Laboratories, Richmond, CA) for 2 h at 0.2 A, and then 4 h at 0.6 A, was in 0.192 M glycine, 0.024 M Tris. In some cases, molecular mass standards or total protein were visualized on the membrane using a biotinylation technique, followed by avidin-peroxidase. Briefly, the method was as follows. The membrane was washed in PBS, then in 0.05 M sodium borate, 0.5 M NaCl, 5.0% 1-methyl 2-pyrollidinone, 0.2% Tween-20, pH 9.3, for 10 min. 100 ml fresh borate buffer containing 200 μl of 75 mM *N*-hydroxysuccinimide biotin (Boehringer Mannheim Biochemicals, Indianapolis, IN) in dimethylformamide was added for 15 min with constant agitation. The membranes were washed once in 100 ml borate buffer; then in 0.5 M NaCl, 0.02 M Tris-HCl, pH 7.5, 0.2% Tween-20, 5% 1-methyl 2-pyrollidinone before adding 100 μl horseradish peroxidase-conjugated avidin (Boehringer Mannheim Biochemicals) in 100 ml Tris-tween buffer for 1 h. Washes in Tris-tween buffer and Tris-saline were followed by color development with 4chloro-1-naphthol (Bio-Rad Laboratories) as per manufacturer's instructions.

For immunoblotting the membranes were blocked, following electrophoretic transfer with TBS/milk (0.5 M NaCl, 0.05 M Tris-HCl, pH 7.5, containing 0.5% dried milk powder) for at least 6 h. Primary antibody incubations were performed overnight at a 1:100 dilution in TBS/milk. A 1:3,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories) in TBS/milk was incubated for 1 h with the membranes as secondary antibody, followed by several washes and color development as per manufacturer's instructions.

Approximately 0.5 mg wet weight per lane of frozen EHS tumor (a gift from Dr. D. Abrahamson, University of Alabama at Birmingham) was dissolved directly in SDS sample buffer containing dithioerythreitol. All samples for electrophoresis, when reduced, were alkylated with 0.5 M iodoacetamide. Lyophilized large EHS tumor HSPG (a gift from Dr. S. Ledbetter, the Upjohn Company, Kalamazoo, MI) was hydrated in 2 M guanidinium hydrochloride in neutral buffer, and then dialyzed into heparitinase/heparinase buffer. Some samples were treated with 0.25 U/ml of both heparitinase and heparinase for 4 h at 42°C as above except that 0.5 mg/ml ovalbumin was added to further reduce proteolytic attack on the HSPG core protein.

Preparation of Total PYS-2 Culture Supernatant Proteins

Confluent PYS-2 cultures in eight 150-cm^2 flasks were washed with serum-free DME and then incubated with 30 ml/flask serum-free DME for 6 h at 37°C. The medium was harvested, spun at 1,000 rpm to remove cellular debris, and solid polyethylene glycol ($M_r \cong$ 8,000 D) added to 10% wt/vol. After stirring overnight at 4°C, the precipitated proteins were sedimented by centrifugation at 16,000 rpm for 30 min at 4°C in a Sorvall RC-5B centrifuge with SA600 rotor. The precipitates were thoroughly washed with 90% ethanol and solubilized directly with reducing buffer for SDS-PAGE. Samples were immunoblotted, viewed directly by silver staining of a fixed gel, or resolved by biotinylation followed by avidin peroxidase after transfer to cationized nylon membranes.

Dotblotting Technique

Proteoglycans were applied to hydrated cationized nylon membranes in a dotblotting apparatus (Bio-Rad Laboratories) at 100 μl/well and left to drain through the membrane for 2 h. Vacuum was then applied to draw the remainder of the liquid through the membrane. The membrane was then blocked with TBS/milk for at least 4 h at room temperature. Washes were with TTBS (TBS containing 0.2% purified Tween 20; Pierce Chemical Co., Rockford, IL). Primary antibodies (200 μl total volume per well) diluted in TTBS/milk were incubated for at least 2 h or occasionally overnight. After serial washing with TTBS, horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad Laboratories) at 1:3000 dilution in TTBS/milk (200 μl total volume per well) was incubated with the membrane for 1 h at room temperature. Color development was as above for immunoblotting.

Where anti-HSPG serum was preincubated with PYS-2 or EHS tumor HSPGs, the antiserum was diluted in TTBS/milk as above and allowed to incubate with 5 μg/ml HSPG isolated from PYS-2 culture supernatants or 5 μg/ml large, low density HSPG from the EHS tumor. Preincubation was for 1 h at 25°C before adding the antibody/antigen mixture to the wells for an additional 3 h at 37°C.

Immunohistochemical Staining

Fresh adult rat tissues were snap-frozen in isopentane chilled in a solid carbon dioxide/acetone bath and processed further as previously described (37). The primary rabbit antisera were anti-mouse laminin, anti-human type IV collagen, and anti-PYS-2 HSPG, used at 1:50, 1:40, and 1:30 dilutions in PBS, respectively. The antiserum against human type IV collagen has been characterized previously (37). That against EHS tumor-derived laminin was a gift from Dr. G. J. Harrap (Unilever Research, Colworth Laboratory, Bedford, U.K.) was monospecific, having no activity against murine type IV procollagen, fibronectin, entactin, or basement membrane proteoglycans. The anti-HSPG serum is characterized below.

After incubation with primary antibodies, the sections were washed with PBS, and then incubated with a 1:40 or 1:50 dilution of a FITC-conjugated $F(ab)_2$ fragment of goat anti-rabbit IgG [$F(ab)_2$ fragment specific (Cooper-Biomedical, Inc.)] for 45 min. at 37°C in a wet box. Finally the sections were washed in PBS and mounted for viewing on the microscope.

As with all the immunological techniques described here, controls included the use of preimmune sera from the rabbits which were subsequently used to raise antisera. A further control in the immunofluorescence studies was the use of fluorochrome-conjugated antibody alone, without primary antiserum incubation. In all cases, the controls gave low levels of fluorescence by comparison with positively stained sections.

Neuromuscular Junction Studies

Fresh rat thigh muscle was teased into single fibers under a dissecting microscope in PBS and stained unfixed with 100 μg/ml TRITC-conjugated α-bungarotoxin (Molecular Probes, Inc., Eugene, OR) to detect acetylcholine receptors, and anti-HSPG or anti-laminin sera under the same conditions as above. In double-stained preparations the α-bungarotoxin and primary antiserum were added simultaneously. After a 45-min incubation at room temperature and thorough washing in PBS, the preparations were treated with fluorescein-conjugated goat anti-rabbit IgG as before.

Microscopy

All sections were viewed on a Nikon Optiphot fitted with phase-contrast and epiillumination optics and appropriate filters to prevent fluorescence breakthrough. This was checked on single-stained specimens from the neuromuscular junction studies. Micrographs were taken on Ilford HP5 film. Where negative controls were photographed, the same exposure time as the appropriate positively stained specimen was used in each case. In double fluorescence experiments, the fluorescein image was photographed first.

Results

Characterization of the PYS-2 HSPG

[^{35}S]Sulfate-labeled proteoglycans released into the medium from PYS-2 cultures were harvested by anion-exchange chromatography, the column being eluted with an increasing salt gradient. The elution profile (Fig. 1) revealed two partly resolved peaks of radioactivity, and by subjecting each of the two pools to degradation by nitrous acid and chondroitinase ABC, it was found that the first peak contained on average 90% heparan sulfate. The second peak contained an average of ~70% chondroitin/dermatan sulfate. These results are similar to those reported previously (20). The material elut-

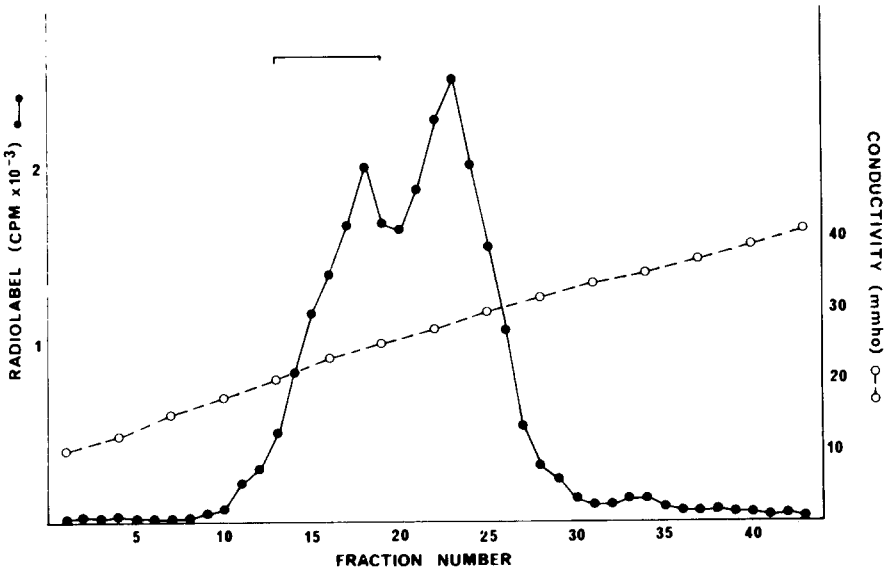

Figure 1. Anion exchange chromatography of [^{35}S]sulfate-labeled PYS-2 culture supernatants. A linear salt gradient of 0.2–1.5 M NaCl at pH 4.0 was used to elute the column. Fractions enriched in HSPG were pooled (*bar*) and taken for further analysis. Bulk culture supernatants were taken through their first purification stage by similar chromatography.

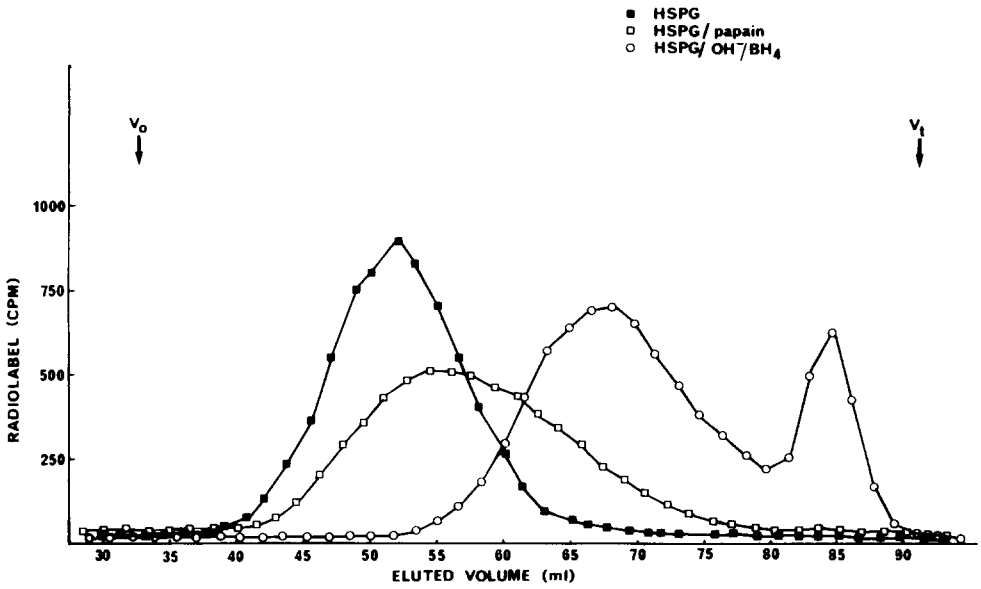

Figure 2. Gel chromatography on a column of Sepharose CL-4B of [^{35}S]sulfate-labeled HSPG from PYS-2 culture supernatants. Intact proteoglycans and papain or alkaline/borohydride-treated proteoglycans were chromatographed in the presence of 0.1% SDS, 0.35 M NaCl, 0.05 M Tris-HCl, pH 8.0, and protease inhibitors.

ing in the first pool was taken for further analysis after removal of contaminating chondroitin/dermatan sulfate by treatment with chondroitinase ABC followed by repurification on DEAE-Sephacel (not shown). The purified heparan sulfate eluted with a K_{av} of 0.30 on a column of Sepharose CL-4B in the presence or absence of SDS indicating an M_r of 200–300 kD. Further samples of proteoglycan were reduced and alkylated in the presence of SDS and chromatographed on a column of Sepharose CL-4B in the presence of SDS (not shown). There was no change in elution profile, indicating that this HSPG is not present as a disulfide-bonded dimer or oligomer. Furthermore, since the elution profile of the HSPG chromatographed in the presence of PBS or SDS were virtually identical, it would appear that there is little tendency of this proteoglycan to aggregate under physiological conditions.

The proteoglycan nature of this heparan sulfate material was confirmed by papain digestion followed by gel filtration chromatography. This caused a shift of the peak on a column of Sepharose CL-4B to K_{av} 0.38–0.43 and alkaline elimination produced an even larger shift to K_{av} 0.61 (Fig. 2). These data suggest that papain digestion leaves large protein remnants attached to each glycosaminoglycan chain, or, more likely, that papain releases peptides containing several glycosaminoglycan chains, these separated by small protease-resistant segments of protein core.

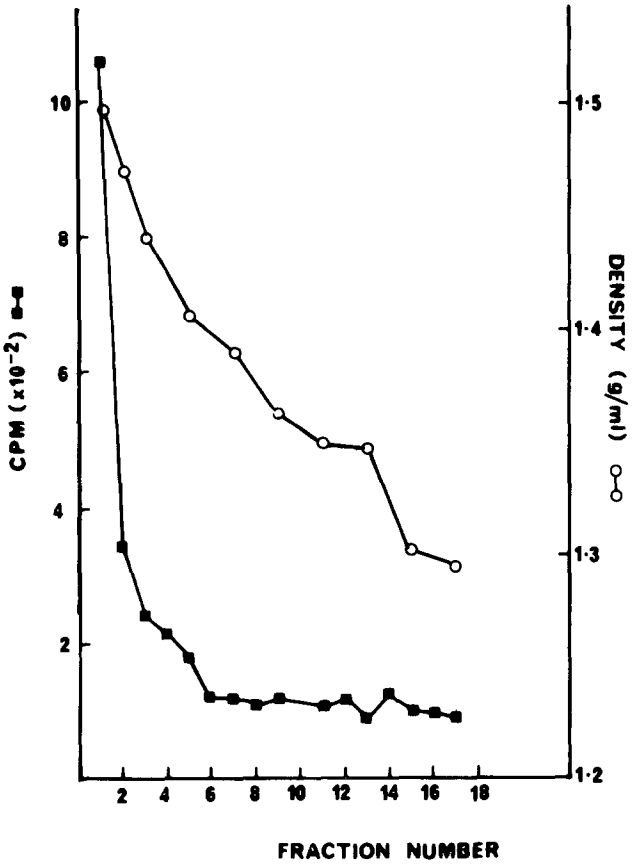

Figure 3. HSPG-containing fractions from bulk cultures to which a trace amount of [^{35}S]sulfate-labeled medium had been added and chromatographed as in Fig. 1 were subjected to cesium chloride density gradient centrifugation. Starting density was 1.4 g/ml. Note that virtually all the radiolabeled HSPG was measured from fractions having a density >1.4 g/ml.

The elution profile of isolated heparan sulfate chains released by alkaline elimination on a column of Sephadex G-200 gave a single peak with K_{av} 0.30 indicating an M_r of 17–18 kD (36). Further treatment of isolated chains showed them to be susceptible to nitrous acid degradation (not shown).

Amino acid and hexosamine analysis of the proteoglycan (Neame, P., and J. R. Couchman, unpublished data) verified that a large amount of glucosamine was present, consistent with an HSPG. These properties indicate that the proteoglycan described here is closely similar to that previously described from PYS-2 cells (24, 33).

Preparation of the Rabbit Antiserum against the PYS-2 HSPG

After addition of trace quantities of [^{35}S]sulfate-radiolabeled medium to 5–10 liter batches, the HSPG was purified in a three-stage procedure, starting with anion-exchange chromatography and elution with a salt gradient as shown previously in Fig. 1. Material eluting in the first peak was pooled, brought to a starting density of 1.4 g/ml with solid cesium chloride and subjected to density gradient centrifugation (Fig. 3). A very large proportion of the [^{35}S]sulfate-labeled HSPG eluted in the bottom fractions of density ≅1.5 g/ml (Fig. 3). Very little radiolabel was detected in the region of 1.3 g/ml density where a previously reported large HSPG from both PYS-2 cells and the EHS tumor has been isolated (10, 14, 33).

Final purification was by ion-exchange HPLC on a Mono Q column. Salt gradient elution revealed polydispersity in terms of charge characteristics and a conservative pooling excluded material of higher sulfation, as indicated in Fig. 4. Pooled proteoglycan fractions were then dialyzed into PBS for immunization. HSPG represented >95% of the total sulfated macromolecules as judged by chromatography of the treated radiolabeled material on a column of Sephadex G-50 after chondroitinase ABC digestion (not shown).

Characterization of the Anti-HSPG Serum

The antiserum was tested by a variety of techniques to show specificity for HSPG. By ELISA, no reactivity of the serum against murine laminin, human type IV collagen, or rat fibronectin could be detected (Fig. 5). Polyclonal antisera

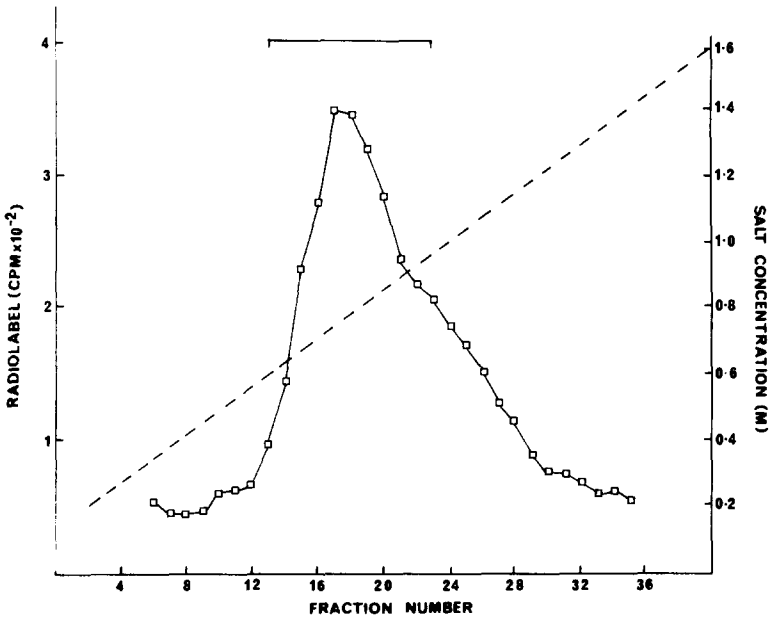

Figure 4. Final purification of PYS-2 bulk culture supernatants by HPLC anion-exchange chromatography. Up to 5-ml samples in PBS were loaded on to a Mono Q column and eluted with a 0.2–1.6 M NaCl gradient at pH 4.0. Eluted material was analyzed for tracer [^{35}S]sulfate-labeled HSPG. Fractions shown by the bar were pooled, concentrated, and injected into a rabbit. Further samples were subjected to amino acid and hexosamine analysis.

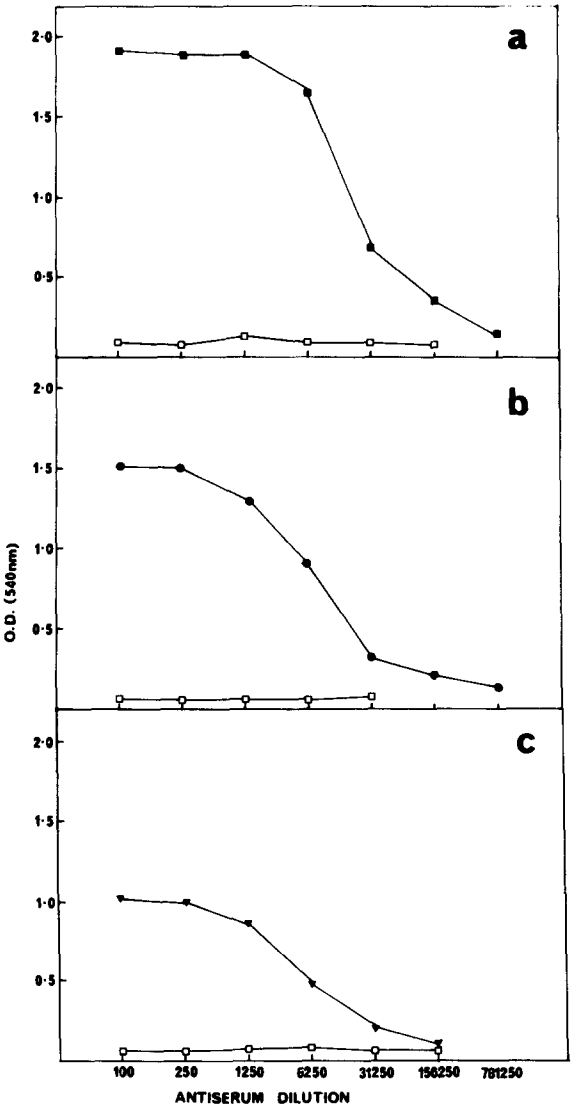

Figure 5. ELISAs with the anti-HSPG serum against 100 ng/well murine laminin (*a*), human type IV collagen (*b*), and rat plasma fibronectin (*c*). In each case, a fully characterized antiserum against the appropriate antigen was also used (*solid symbols*). Open squares denote the reaction of the anti-HSPG serum, and show no detectable reactivity with any antigen. Preimmune serum from the same rabbit that was used to raise the anti-HSPG was also used in this assay, and gave similar results to the immune serum (not shown).

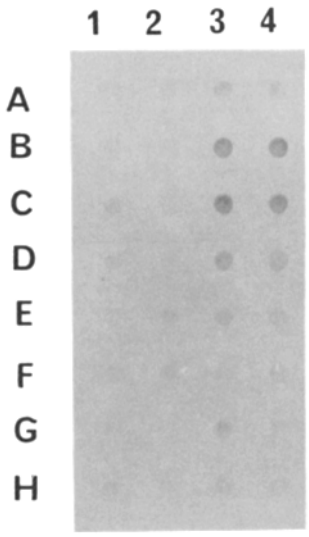

Figure 6. Dotblot analysis on cationized nylon membrane of the reactivity of the anti-HSPG serum to PYS-2-derived HSPG and CS/DSPG. Rows *B–D* were sensitized with 1 μg, 0.5 μg, and 0.1 μg HSPG, respectively; rows *E–G* were sensitized with the same quantities of CS/DSPG, respectively. Rows *A* and *H* were treated with 1% ovalbumin. Lanes *1* and *2* received preimmune serum from the rabbit that was used to raise the antibodies (lanes *3* and *4*), either at a 1:100 dilution (lanes *1* and *3*) or 1:200 dilution (lanes *2* and *4*). The antiserum while reacting with the HSPG does not appear to recognize the native CS/DSPG.

against each substrate molecule gave a strong response in each case. A lack of reactivity of the antiserum against the CS/DSPG synthesized by PYS-2 cells (8) was confirmed by dotblotting (Fig. 6) and immunoblotting (not shown). In immunoblotting, the antiserum gave a positive response to the HSPG (Fig. 7 *A*). One important feature of the immunoblotting was the failure of the HSPG to migrate substantially further into the SDS-PAGE gel after a combination of heparitinase and heparinase treatment; this feature is discussed below.

A further immunoblotting experiment on a preparation of chondroitinase ABC-digested proteoglycans from the closely related murine cell line, PF-HR9 (Fig. 7 *B*) showed that the anti-HSPG had no reactivity towards CS/DSPGs. In addition, there was evidence for a hybrid HS/CSPG released to the medium of this cell line. A polyclonal antiserum (R44) raised against chondroitinase ABC-treated bovine nasal cartilage proteoglycan, which has activity against the carbohydrate stubs generated by the enzyme (8) and hence is reactive with all CS/DSPGs after chondroitinase ABC treatment, revealed a very disperse band at high molecular mass consistent with a hybrid proteoglycan. In addition, a closely spaced doublet of CS/DSPG core proteins of ~30 kD was also present (lane *4*, *arrow*) which probably corresponds to that seen in PYS-2 cultures described previously (8). Importantly, neither of these species was detectable with the anti-HSPG serum (lane *2*). In contrast, a high molecular mass diffuse band of material was detected by the anti-HSPG serum (lane *2*), which is not CS/DSPG based on its lack of reactivity with R44 (lane *4*). These results confirm that the anti-HSPG has no activity against chondroitin sulfate- or dermatan sulfate-containing proteoglycans.

Immunoblotting of EHS tumor extracts separated by SDS-PAGE confirmed that the antiserum had no reactivity towards murine laminin, type IV collagen, or other tumor components (Fig. 8), and further showed the specificity of the anti-laminin and anti-type IV collagen sera (see also reference 20) used in this study. Although shown below that the anti-PYS-2 HSPG serum does cross react with the large EHS tumor HSPG, this could not be demonstrated by immunoblotting against total EHS tumor proteins, presumably as a result of insufficient proteoglycan content in the tumor matrix (see reference 10).

To check for reactivity of the antiserum with other proteins released by PYS-2 cells into the culture medium, polyethylene glycol precipitation of serum-free culture supernatants was analyzed by immunoblotting with anti-HSPG and antisera against laminin and type IV collagen. As shown in Fig. 9, a large number of polypeptides was detectable by silver staining of the gel and by a biotinylation/avidin peroxidase technique after electrophoretic transfer of these polypeptides to cationized nylon membrane. The anti-HSPG serum only reacted with a broad band of high molecular mass material, consistent with proteoglycan. It has been shown above (Fig.

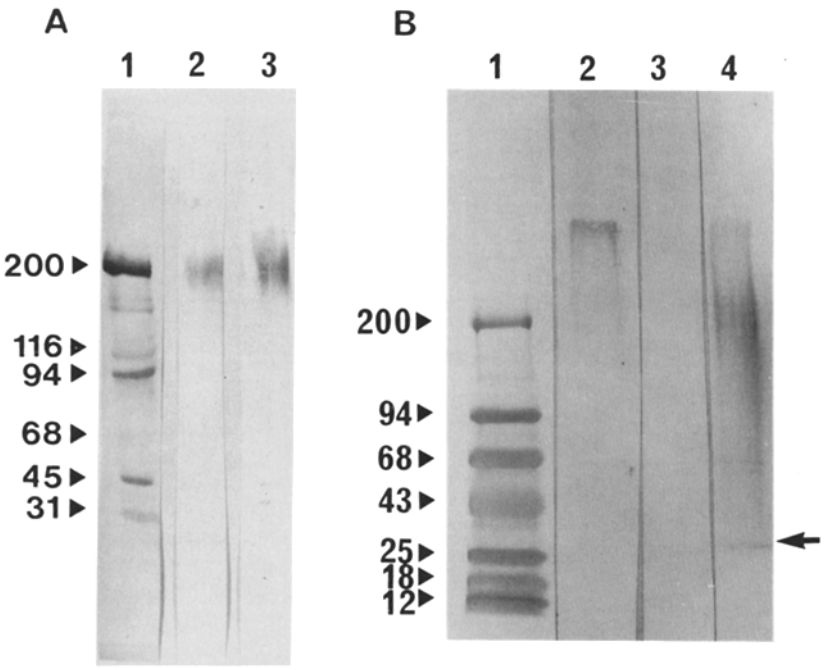

Figure 7. (*A*) Reactivity of the anti-HSPG serum with intact or heparitinase/heparinase digested PYS-2 HSPG. 2.8 μg HSPG before (lane *2*) or after (lane *3*) enzyme digestion was run on a 3–15% gradient SDS-PAGE and electrophoretically transferred to cationized nylon membrane and probed with the anti-HSPG serum. Although gel chromatography showed digestion products and hexosamine analysis confirmed the presence of glucosamine (see text), the only change in electrophoretic pattern was a broadening of the band. Lane *1* shows molecular mass markers revealed by biotin/avidin peroxidase on the membrane. Molecular masses (in kilodaltons) are assigned to the markers. (*B*) Lack of reactivity of the anti-HSPG serum with chondroitin sulfate- and dermatan sulfate-containing proteoglycans. Chondroitinase ABC-treated total proteoglycans from the murine endodermal cell line PF-HR9 were separated on a 3–15% SDS-PAGE minigel and transferred to cationized nylon membrane. Lane *1* shows prestained molecular mass markers (shown in kilodaltons). Lane *2* shows a single broad band staining with anti-HSPG serum at the top of the gel. Lane *3* is stained with preimmune serum and lane *4* is stained with R44, a polyclonal antiserum recognizing chondroitin/dermatan sulfate stubs remaining on core proteins after chondroitinase ABC treatment. A broad band of large size is detected along with a closely spaced doublet of peptides of ~30 kD (*arrow*). Neither of these CS/DSPGs is stained in lane *1*. Moreover, the diffuse nature of the large proteoglycan in lane *4* is potentially indicative of a hybrid molecule to which other glycosaminoglycan chains (e.g., heparan sulfate) are still attached.

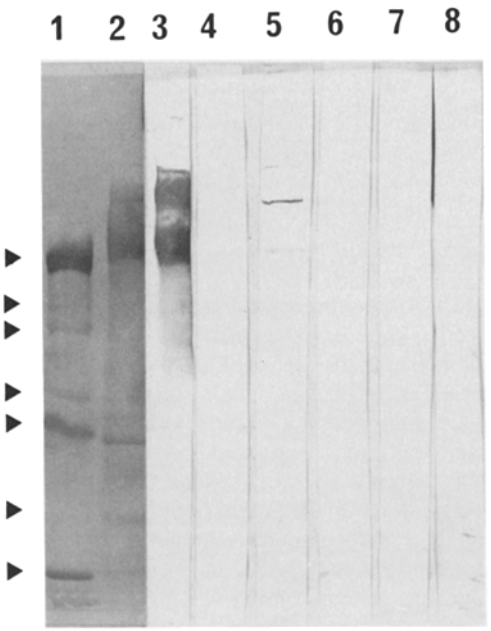

Figure 8. Immunoblot of EHS tumor probed with antibodies against laminin (lane *3*), type IV collagen (lane *5*), and HSPG (lane *7*). The preimmune sera appropriate to each of the three sera are shown in lanes *4*, *6*, and *8*, respectively. Lane *1* shows molecular mass markers (*arrows*): myosin (200 kD); β-galactosidase (116 kD); phosphorylase B (94 kD); BSA (68 kD); ovalbumin (45 kD); carbonic anhydrase (31 kD—not well resolved); and soybean trypsin inhibitor (21.5 kD). These and a lane showing total protein (lane *2*) were stained by biotin/avidin peroxidase. The proportion of HSPG in the tumor was presumably very low and not resolvable (cf. Fig. 10), but the figure shows a lack of reactivity of the anti-HSPG serum with other major components such as laminin and type IV collagen.

6) that there is no cross-reaction with a DSPG synthesized by PYS-2 and PF-HR9 cells (8). These results also confirm a lack of reactivity with laminin and type IV collagen of PYS-2 origin or any other polypeptides from PYS-2 culture supernatants.

Relationship of the PYS-2 HSPG to EHS Tumor HSPG

Two distinct HSPGs have been isolated from the EHS tumor (10, 14): one, with a large protein core and low, buoyant density when analyzed by cesium chloride density gradient centrifugation; the other, a small, high density HSPG, immunologically related to the larger form (10, 14) and possibly derived from it by proteolytic processing (14).

Fig. 10 shows a clear reaction by immunoblotting of both the native and heparitinase/heparinase digested core protein of the large HSPG with the antiserum raised against PYS-2 HSPG. Several polypeptides are, in fact, recognized by the antiserum after enzymatic digestion which may represent subspecies or degradation products of a single high molecular mass core protein. The strong response of the anti-HSPG serum to EHS tumor core protein indicated (*a*) the similarity of core protein epitopes and (*b*) that a major part of the polyclonal response is directed towards the proteoglycan core protein. The antiserum also recognized the intact and heparitinase/heparinase-treated EHS tumor proteoglycan by dot-

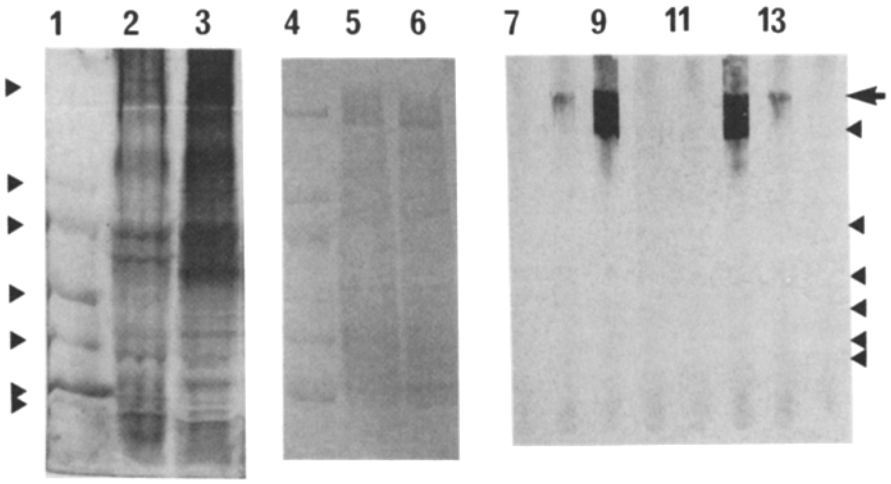

Figure 9. Reactivity of the anti-HSPG serum with macromolecules isolated form the culture supernatants of PYS-2 cells by polyethylene glycol precipitation. (Lanes *1–3*) Silver stained 3–15% SDS–polyacrylamide minigel showing molecular mass markers (lane *1*), myosin (200 kD); phosphorylase B (97 kD); BSA (68 kD); ovalbumin (43 kD); α-chymotrypsinogen (26 kD); β-lactoglobulin (18.5 kD); and cytochrome C (12.3 kD). Total precipitated proteins are shown lanes *2* and *3*. Lanes *4–14* were electrophoretically transferred to cationized nylon membrane. Transfer was confirmed by biotin/avidin peroxidase (lanes *5* and *6*). Twice as much protein was loaded in lanes *3* and *6* as in lanes *2* and *5*. Lane *4* shows the transfer of prestained molecular mass markers. (Lanes *7* and *14*) Probed with the appropriate preimmune serum for the anti-HSPG; (lanes *8* and *13*) anti-HSPG; (lanes *9* and *12*) anti-laminin; (lanes *10* and *11*) anti-type IV collagen. The anti-laminin lanes have been overdeveloped so that the small amount of type IV collagen synthesized by these PYS-2 cells is visible at 180–190 kD. Arrowheads to the right of lane *14* show the position of prestained molecular mass markers as they transferred to the membrane. (*Arrow*) The boundary of the stacking gel to resolving gel.

blotting (Fig. 11), indicating that cross-reactivity can occur with both SDS-denatured and native HSPG.

These results follow earlier findings that both high and low density PYS-2 HSPGs are recognized by antibodies raised against low density HSPGs from EHS tumor (33) and confirm their antigenic similarity.

Although under the culture conditions used here, no large, low density proteoglycan could be detected in [^{35}S]sulfate-labeled material either by gel filtration chromatography or cesium chloride density gradient centrifugation, a dotblotting experiment was performed to confirm that the antiserum was monospecific and did not separately recognize two populations of HSPG. The antiserum was preincubated with the small PYS-2 HSPG or large EHS tumor HSPG before exposure to the same or alternate substrate-adsorbed proteoglycans (Fig. 11). Preincubation of the antiserum with excess PYS-2 high density HSPG virtually eliminated all reactivity to the EHS tumor HSPG and PYS-2 HSPG. Preincubation of the antiserum with low density EHS tumor HSPG, on the other hand, removed all reactivity to that antigen, but some activity against the PYS-2 HSPG remained. Taken together, these results indicated that the antiserum was indeed monospecific, and confirmed an antigenic relationship between these HSPGs from PYS-2 and EHS tumor sources as reported previously (10, 33). In addition, these experiments indicated that the PYS-2 HSPG contained epitopes not represented on the EHS tumor HSPG.

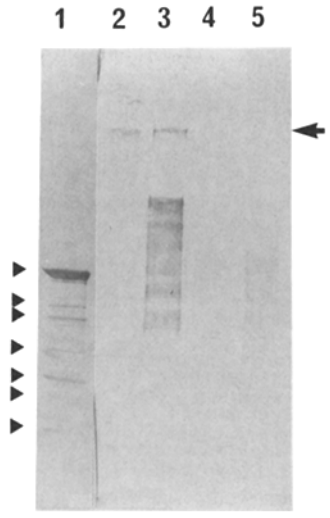

Figure 10. The anti-HSPG serum cross reacts with the large, low density HSPG from the EHS tumor. 5 μg HSPG was loaded in each of lanes *2–5*, either before (lanes *2* and *4*) or after (lanes *3* and *5*) heparitinase digestion. Electrophoretically separated products on a 3–15% SDS polyacrylamide gel were transferred to cationized nylon membrane and probed with anti-HSPG serum (lanes *2* and *3*) or the appropriate preimmune serum (lanes *4* and *5*). Several "core proteins" recognized by the antiserum appear to have been liberated by heparitinase treatment. The smaller subspecies were possibly proteolytically derived from a larger form. Molecular mass markers are denoted by arrowheads and correspond to those in Fig. 7. (*Arrow*) The boundary of the stacking gel to resolving gel.

HSPG Distribution in Adult Rat Tissues

A preliminary survey of a number of rat tissues by indirect immunofluorescence on frozen sections was carried out. Sections were stained with the anti-HSPG serum, anti-laminin, or anti-type IV collagen sera. The results are shown in Figs. 12–15 and are summarized in Table I. While a wide variety of basement membranes all stained positively for laminin and type IV collagen, only specific subsets stained with antiserum against the PYS-2 HSPG. Epithelial (Figs. 12–14), mesothelial (Fig. 12, *g–i*), and endothelial (Figs. 12–14) basement membranes from a number of rat tissues stained with all three antisera. The exception was liver, where the sinusoidal basement membranes were positive for type IV collagen (Fig. 14 *b*) and negative for HSPG (Fig. 14 *a*). Kidney tubular and glomerular basement membranes were particularly well stained with the anti-HSPG serum (Fig. 14, *c* and *d*). Basement membranes which were negative for the HSPG included those associated with some electrically excitable tissues, but in each case the same structures were clearly positive for laminin and type IV collagen. Most smooth muscle tested including that of esophagus, small intestine, uterus, bladder, and tracheole (Figs. 12 *d*, 13 *a*, 13

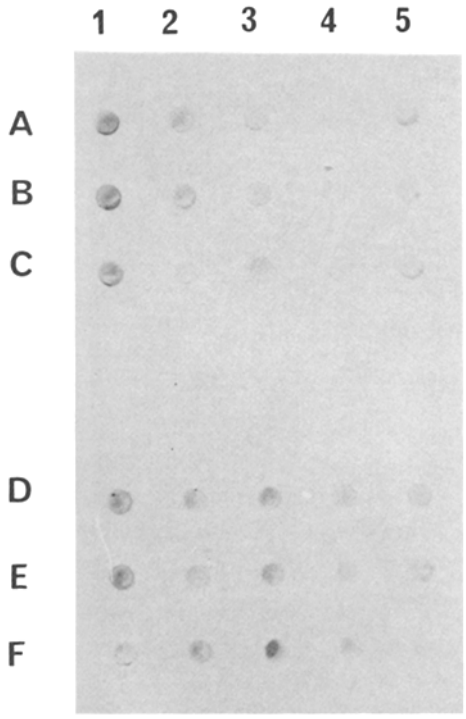

Figure 11. Absorption of the anti-HSPG serum with PYS-2 HSPG or EHS tumor large HSPG. EHS HSPG (rows *A–C*) or PYS-2 HSPG (rows *D–F*) were dotted on to cationized nylon membrane at 0.5 μg/well (rows *A* and *D*), 0.1 μg/well (rows *B* and *E*), or 0.02 μg/well (rows *C* and *F*). Lane *5* has no HSPG, but contained 1 mg/well ovalbumin. After blocking the membrane with ovalbumin, the wells had an equal amount of anti-HSPG serum or preimmune serum added. Lane *1* received the antiserum with no further additions and shows reactivity with both proteoglycans. The antisera for each well in lanes *2* and *3* were preadsorbed for 1 h with 1 μg PYS-2 HSPG (lane *2*) or EHS HSPG (lane *3*). While virtually all reactivity has been blocked in lane *2*, absorption with EHS HSPG only partially abrogates reactivity towards a PYS-2 HSPG substrate, but completely blocks reactivity with an EHS HSPG substrate. Lane *4* received preimmune serum while lane *5* received immune anti-HSPG serum.

d) were unlabeled for HSPG as was cardiac muscle (Fig. 14 *g*), and the neuromuscular junction of striated muscle (see below). Aortic and peripheral vascular smooth muscle (Figs. 13 *a*, 14 *a* and 14 *e*) and striated muscle (Fig. 16) were, however, positively labeled for HSPG and strongly stained for laminin and type IV collagen.

Attempts to uncover potentially masked HSPG epitopes by pretreatment of tissue sections with heparitinase/heparinase, chondroitinase ABC, or leech hyaluronidase before antibody treatment did not alter the staining pattern of any of the three antisera (not shown). Since the polyclonal anti-HSPG used in these studies stained a large number of basement membranes, and two other basement membrane components were consistently demonstrable, it seemed likely that the HSPG recognized by the antiserum to PYS-2 high density HSPG was absent or present to a minimal extent in a particular subset of muscle basement membranes.

In a further examination of the distribution of this type of HSPG with tissues where membrane depolarization is involved in functional activity, sections of pancreas and parotid gland were stained with antisera to basement membrane components (Fig. 15). It has been suggested that pancreatic acinar cells in particular undergo membrane depolarization as a result of neuronal and hormonal stimulation, resulting in the release of digestive enzymes from the luminal surface (27). Consistent with this, the basement membranes surrounding the pancreatic acini, but not those of the parotid gland (Fig. 15) were devoid of staining for this form of HSPG. The basement membranes of blood vessel endothelia, particularly those of pancreatic islet capillaries, were strongly stained with the anti-HSPG serum on the same tissue sections (Fig. 15 *a*). Not only were parotid gland acini positive, but all the ductular basement membranes in this organ contained this proteoglycan (Fig. 15 *c*). In contrast, laminin and type IV collagen were readily displayed in all basement membranes of these glands.

Liver epithelium is known to be a rich source of cell surface associated HSPG which has hydrophobic properties (20). The anti-HSPG serum failed to stain liver epithelium in frozen sections (Fig. 14 *a*) indicating that membrane-intercalated HSPG is probably antigenically unrelated to the basement membrane form discussed here. However, liver blood vessel basement membranes were strongly stained with the anti-HSPG serum in the same sections.

Absence of PYS-2 HSPG at the Neuromuscular Junction

Several previous reports have highlighted the presence of HSPG in *Xenopus* and chick embryo neuromuscular junction (1, 2, 4) and in vitro there is reported to be a considerable concentration of proteoglycan in this structure after experimental manipulation (1). To further characterize the PYS-2 HSPG, fresh adult rat striated muscle was teased into single fibers and stained, either with anti-HSPG serum, anti-laminin serum, or rhodamine-labeled α-bungarotoxin to detect acetylcholine receptors, either singly or in double staining. Although the neuromuscular junction and motor end plates could be easily stained with α-bungarotoxin (Fig. 16 *a*), no co-distribution with the antiserum against PYS-2 HSPG could be detected. General endomysial staining for HSPG could be seen in the same preparations (Fig. 16, *b* and *c*) even though this was consistently weak. Over 100 motor end plates were examined in double-stained preparations, all of which lacked any detectable staining with anti-HSPG serum, whereas co-distribution of laminin with acetylcholine receptors was detected (Fig. 16, *g–i*), indicating that steric hindrance did not hinder antibody penetration into the neuromuscular junction. It would appear, therefore, that concentrations of HSPG at adult rat neuromuscular junction, should they occur in a similar way to other systems (1, 2, 4), do not involve the HSPG characterized here.

Discussion

In this report, a small, high buoyant density HSPG released into culture supernatants of PYS-2 cells has been characterized and purified, and a monospecific antiserum has been raised against it. This proteoglycan, by virtue of its hydrodynamic properties and glycosaminoglycan chain size, is closely similar to that described previously from this cell

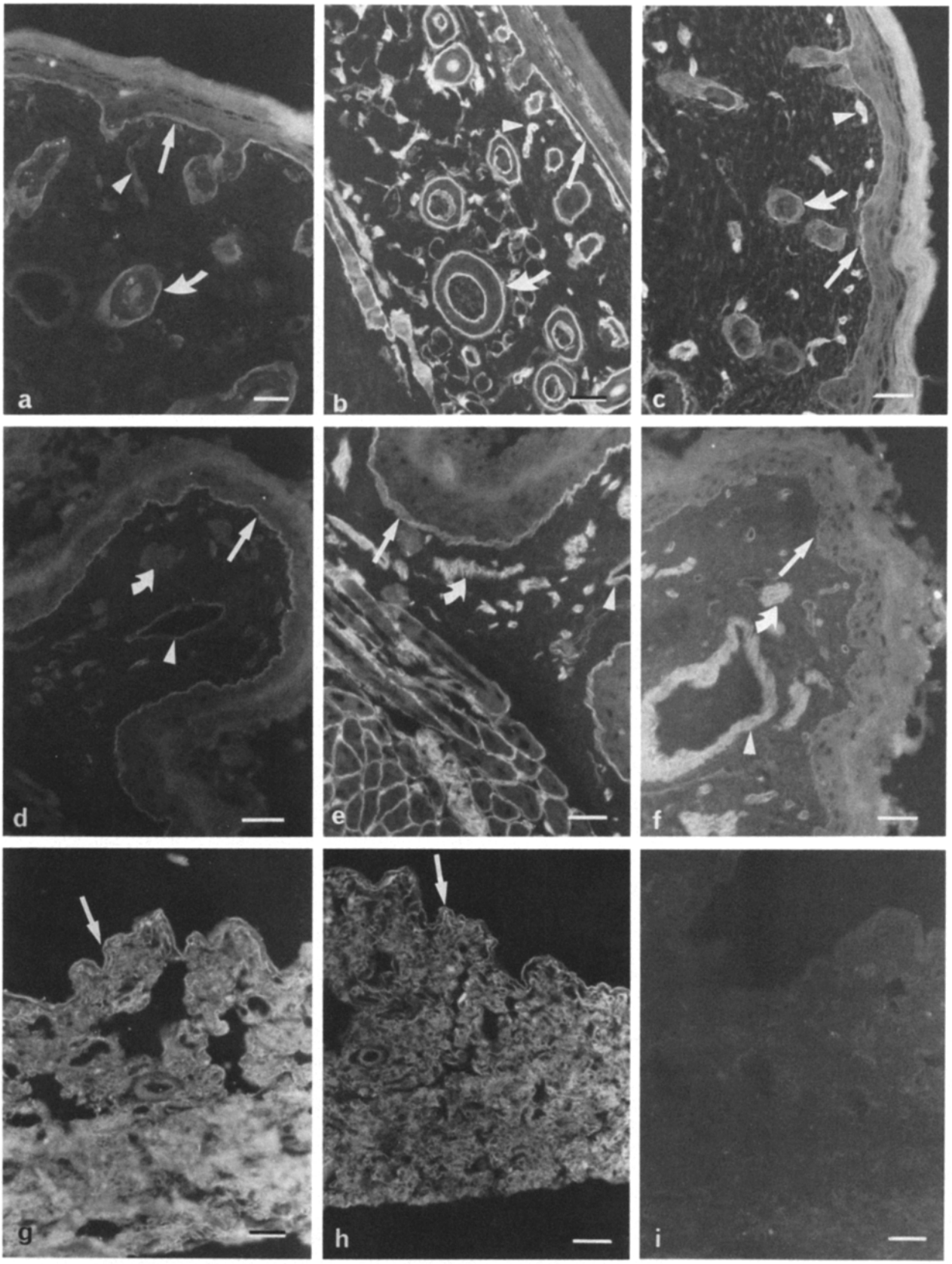

Figure 12. Immunohistochemical staining of frozen sections of adult rat skin (*a–c*), esophagus (*d–f*), and lung (*g–i*) with anti-HSPG serum (*a*, *d*, and *g*), anti-laminin serum (*b*, *e*, and *h*), anti-type IV collagen serum (*c* and *f*), or preimmune serum from the rabbit used to raise the anti-HSPG serum (*i*). Arrows in *a–f* indicate the basement membranes underlying the stratified epithelia; arrowheads denote upper dermal capillaries (*a–c*) or venules (*d–f*). Curved arrows in *a–c* mark follicular epithelial basement membrane. While esophageal smooth muscle was stained for laminin and type IV collagen (*curved arrows*, *e* and *f*), this was not stained with the anti-HSPG serum (*curved*

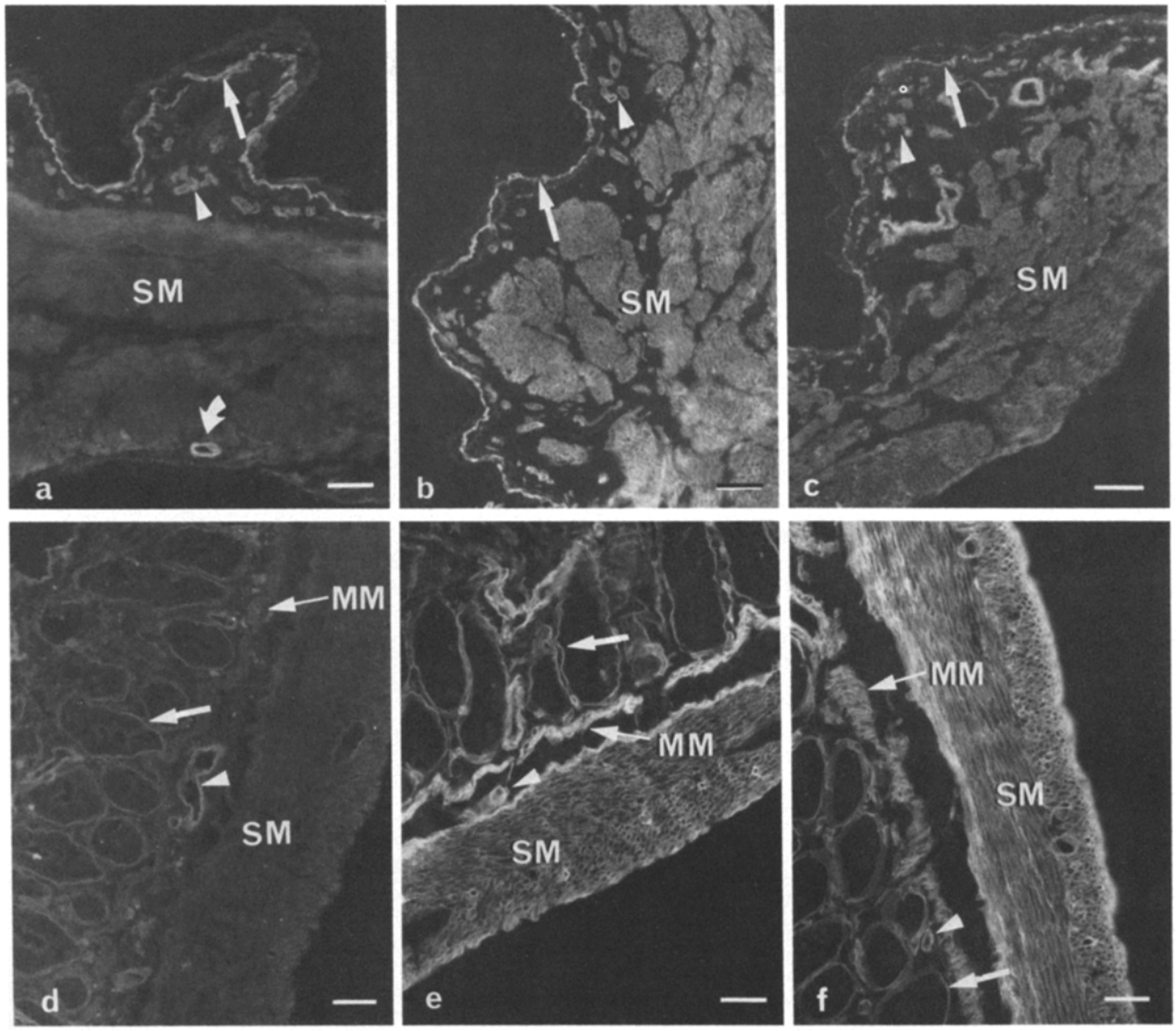

Figure 13. Frozen sections of rat bladder (*a–c*) and ileum (*d–f*) stained with antisera against PYS-2 HSPG (*a* and *d*), laminin (*b* and *e*), and type IV collagen (*c* and *f*). In all cases, epithelial (*arrows*) and blood vessel endothelial basement membranes (*arrowheads*) are stained. In contrast to laminin and type IV collagen, the inner longitudinal and outer circular bladder smooth muscles were not positive for the HSPG (*SM*; *a–c*). Similarly, the muscularis mucosa (*MM*) and inner circular and outer longitudinal smooth muscles of the ileum (*SM*) were unstained with anti-HSPG serum. A positively stained arteriole in the serosa of the bladder is marked in *a* (*curved arrow*). Bars: (*a–c*) 100 μm; (*d–f*) 50 μm.

type (24, 33). Results from immunoblotting assays and ELISAs confirmed that the antiserum had no reactivity with the DSPG synthesized by these cells (8), laminin, type IV collagen, fibronectin, or other polypeptides of PYS-2 or EHS tumor origin. In addition, no evidence was found to suggest that the antiserum has any reactivity with the glycosaminoglycan portion of PYS-2 HSPG and reactivity of the antiserum with heparitinase/heparinase-treated proteoglycan was demonstrated by immunoblotting and in vivo staining. It therefore appears likely that the antiserum recognizes epitopes on the core protein of the proteoglycan although it remains possible that the antiserum also has reactivity to N- or O-linked oligosaccharides.

The limited susceptibility of PYS-2 HSPG to heparitinase/heparinase (Fig. 7 *a*), in contrast to that from the EHS tumor, is of some interest. To rule out the possibility of a hybrid HS/CSPG being responsible for this result, supernatant proteoglycans from the murine endodermal cell line PF-HR9 were subjected to chondroitinase ABC and immunoblotting. All CSPGs or DSPGs were detected with an antiserum (R44) recognizing the carbohydrate stubs remaining on CS/DSPGs after enzyme treatment. There was no common recognition

arrows, *d*). Basement membranes of lung mesothelium were stained for HSPG and laminin (*arrows*) as were those of alveolar epithelia and capillary endothelia (*g* and *h*). No staining was seen with preimmune serum (*i*). Bars, 50 μm.

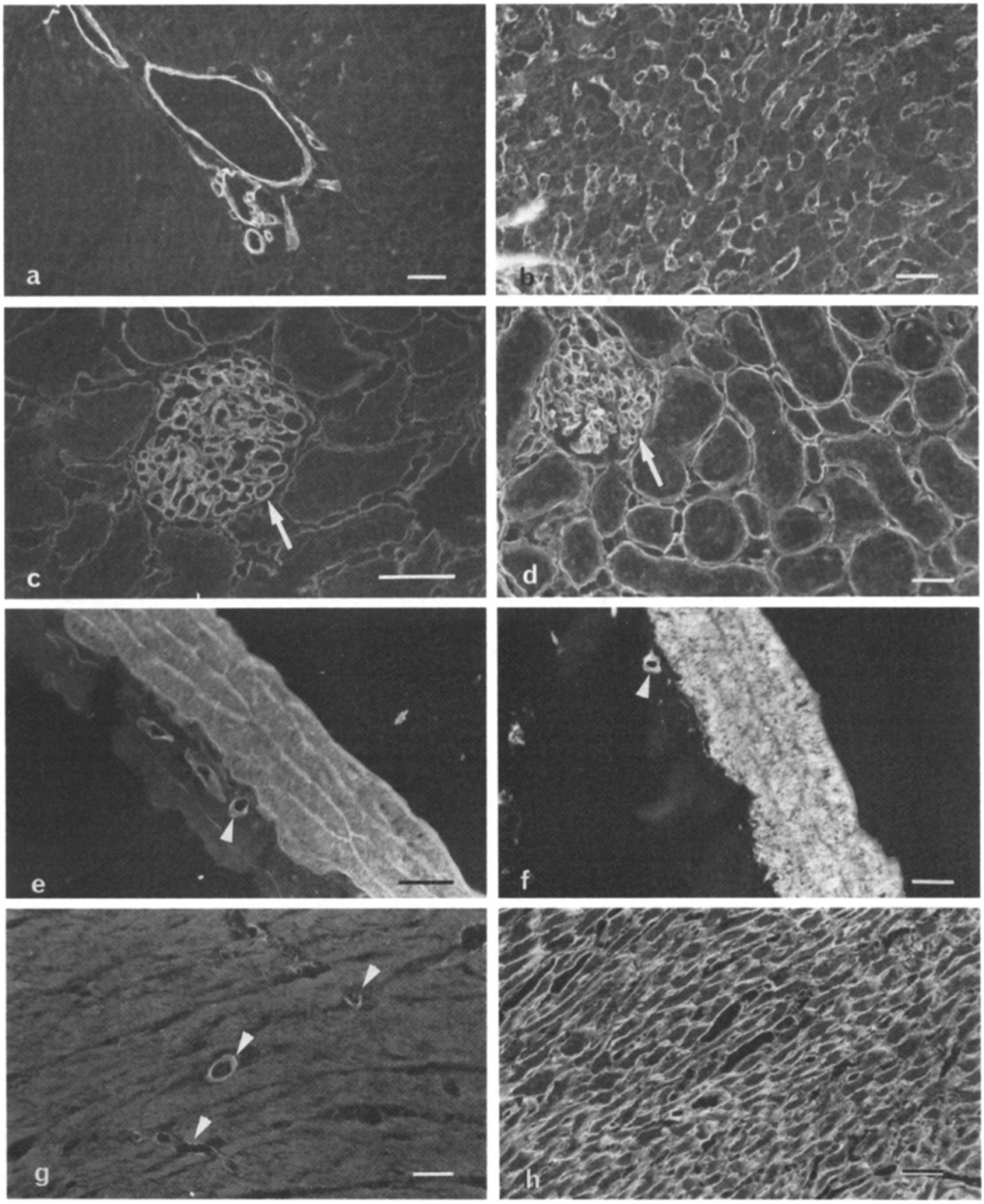

Figure 14. Frozen sections of rat liver (*a* and *b*), kidney (*c* and *d*), aorta (*e* and *f*), and heart ventricle (*g* and *h*) stained with anti-HSPG serum (*a*, *c*, *e*, and *g*), anti-type IV collagen serum (*b*), and anti-laminin serum (*d*, *f*, and *h*). While hepatic blood vessels were positive for HSPG, sinusoidal basement membranes were apparently unstained (but were stained with anti-type IV collagen serum; *b*). Kidney tubular and glomerular (*arrows*) basement membranes (*c* and *d*) were strongly stained with anti-HSPG and anti-laminin. The intima and media of aorta were also stained (*e* and *f*), as were vasa vasorum in the adventitia (*arrowheads*). Cardiac muscle (*g* and *h*) apart from blood vessels (*arrowheads*; *g*) was unstained with anti-HSPG serum, but the muscle basement membranes were strongly positive with anti-laminin serum (*h*). Bars, 50 μm.

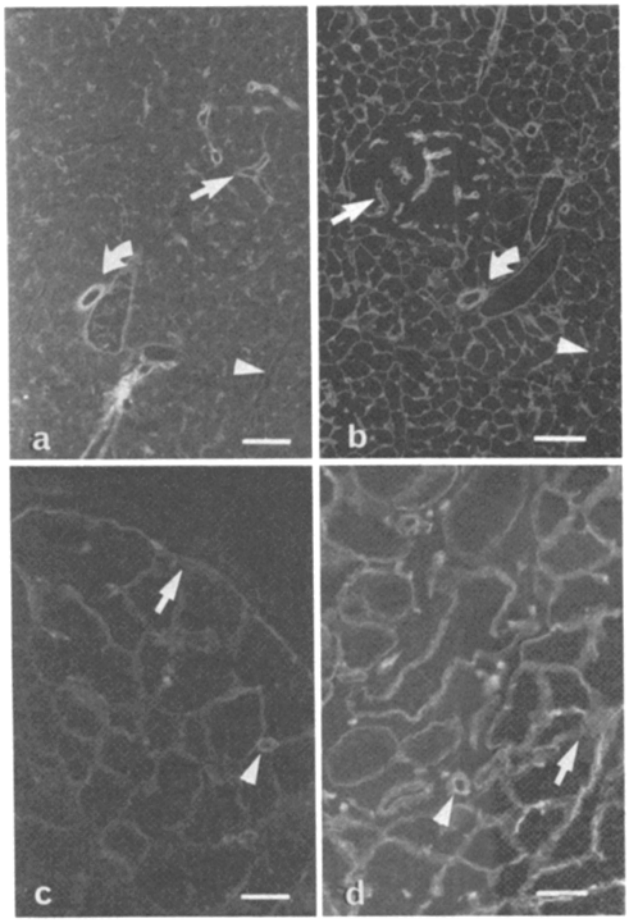

Figure 15. Sections of rat pancreas (*a* and *b*) and parotid gland (*c* and *d*) stained with anti-HSPG serum (*a* and *c*) and anti-laminin serum (*b* and *d*). While pancreatic islet capillaries (*arrows*) and other blood vessels (*curved arrows*) were stained for both basement membrane components, acinar basement membranes apparently lacked the HSPG (see *arrowhead* for example). In comparison, both acinar (*arrows*) and intralobular duct (*arrowheads*) basement membranes of the parotid gland were positive for both components. Bars: (*a* and *b*) 100 μm; (*c* and *d*) 50 μm.

of any proteoglycan species by R44 and the anti-HSPG serum further indicating that the latter was specific for HSPG. In addition, the experiment provided evidence for a hybrid proteoglycan in PF-HR9 supernatants. Such molecules have been described from other cell types before (9, 28), but whether the mouse endodermal variety is of cell surface origin or is secreted directly to the medium along with other basement membrane components is currently unknown.

The antigen, although somewhat resistant to heparitinase/heparinase treatment, was identified as an HSPG by hexosamine analysis (Neame, P., and J. R. Couchman, unpublished data) and sensitivity to nitrous acid deamination. A previous report (25) has shown that an HSPG from kidney glomerular basement membrane exhibited partial resistance to nitrous acid which was thought to result from reduced *N*-sulfation of glucosamine residents close to the core protein. Thus, a particular disposition of sulfate groups and/or iduronate residues may explain the relative resistance of the PYS-2 HSPG to bacterial enzyme degradation (see reference 12). Furthermore, it was also proposed that the heparan sulfate chains in a bovine glomerular HSPG were clustered in a small portion of the core protein (25) consistent with the fact that the PYS-2 HSPG was not totally papain sensitive, the enzyme releasing peptides bearing several glycosaminoglycan chains.

There are now many reports of HSPGs isolated from the medium or extracellular matrix of cultured cells, and from intact basement membranes of tissue origin. Two HSPGs have been isolated from the matrix of the EHS tumor: a large, low buoyant density HSPG and a smaller, high buoyant density HSPG (10, 14, 38). These have been shown to be immunologically related in that antisera raised against one form cross-react with the other (10, 14), which in turn has given rise to speculation that the larger form gives rise to the smaller by way of limited proteolysis (14). As shown here, the antiserum raised against the small, high density HSPG from PYS-2 cultures cross reacted with the large, low density HSPG from the EHS tumor. This raised the possibility that the polyclonal antiserum had activities against two different HSPGs or that there was antigenic similarity of the two proteoglycans. Absorption of the antiserum with the PYS-2 HSPG or with the large HSPG from the EHS tumor completely abrogated reaction with the large EHS HSPG, verifying the monospecificity of the antiserum and therefore, the close similarity of the two proteoglycans despite differences in core protein size. On the other hand, the absorption studies showed that the antiserum still reacted with the PYS-2 HSPG after preabsorption with the EHS tumor HSPG, thus implying that there are epitopes on the PYS-2 HSPG not present on the EHS tumor HSPG, even though the latter has a much larger core protein. This may result from different oligosaccharides, from conformational differences between the two core proteins, or from the fact that the EHS tumor HSPG may have some differences in protein sequence from that of the PYS-2 proteoglycan. The intriguing relationship between core proteins of HSPGs can only be resolved with protein sequence data, but studies on CSPGs (16) have shown that an antigenic relationship does not necessarily correlate with a closely related overall core protein structure.

The immunohistochemical study presented here demonstrated the unusual distribution of the HSPG antigen. By far the majority of basement membranes were positively stained with the anti-HSPG, anti-laminin, and anti-type IV collagen sera including those underlying most epithelia, mesothelia, and endothelia. However, the basement membranes of most smooth and cardiac muscle were negative when stained with the anti-HSPG serum although brightly labeled for the other two basement membrane components. In contrast, blood vessel and capillary endothelial basement membranes including those associated with muscle which were not positive for the HSPG were distinctly stained for all three components in the same tissue sections. Aortic and peripheral blood vessel smooth muscle basement membranes were stained with antisera against all three basement membrane components showing that not all smooth muscle was negative for this type of HSPG. Although staining of endomysial basement membranes in cross sections of striated muscle was not strong, it was clearly visible in *en face* staining of freshly dissected skeletal muscle fibers. Since a polyclonal antiserum was used in these experiments and treatment of tissue sections with heparitinase/heparinase, chondroitinase ABC, or hyaluronidase had no effect on staining for HSPG, it seems

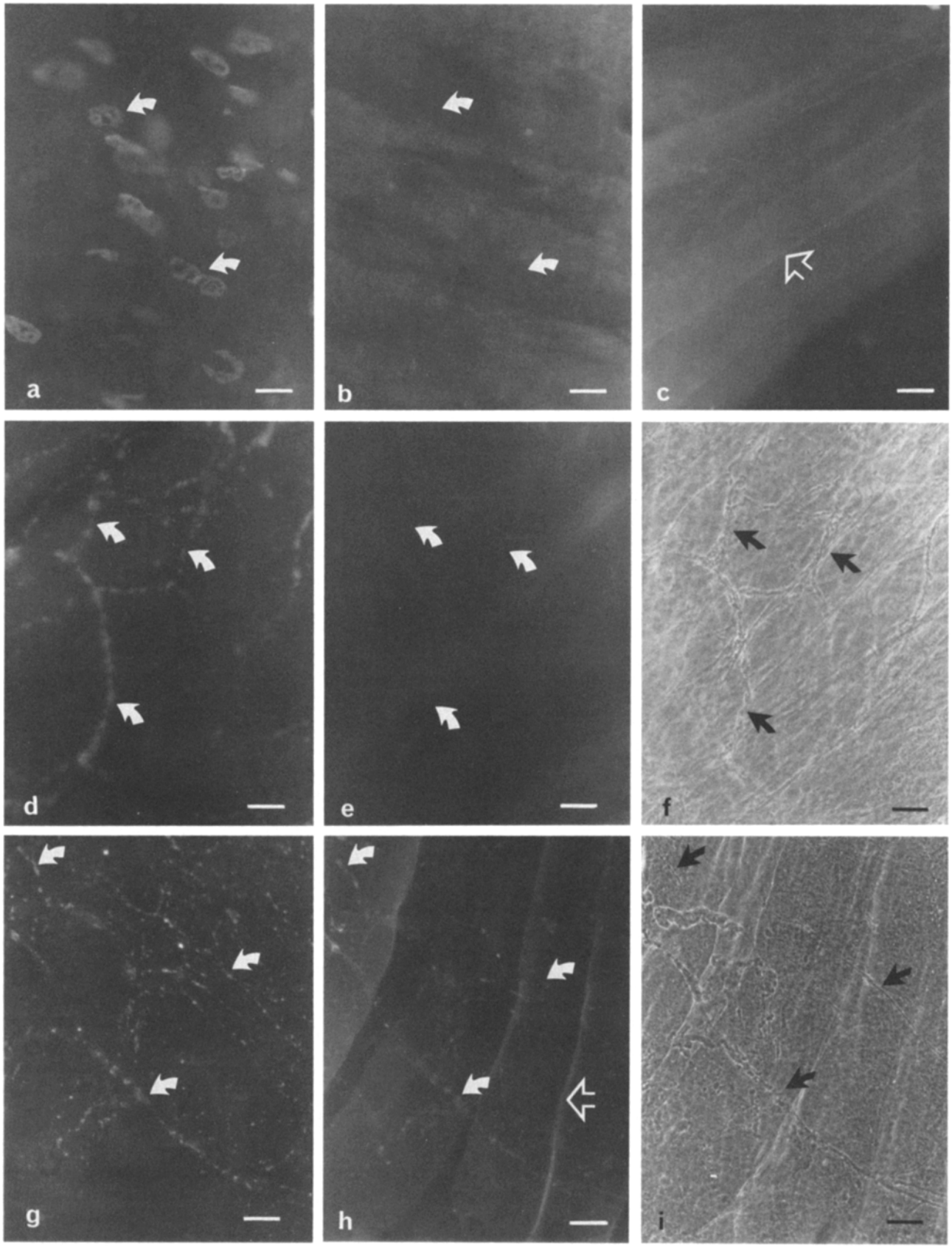

Figure 16. Absence of the PYS-2 form of HSPG at the neuromuscular junction of rat striated muscle. Freshly teased postaxial hindleg muscle was stained for neuromuscular junction by TRITC-α-bungarotoxin (*a*, *d*, and *g*). In double-stained preparations, end plates (*a* and *b*) and neuromuscular junction zones (*d–i*) could not be stained with anti-HSPG serum (*b* and *e*). Neuromuscular junction could, on the other hand, be stained with anti-laminin serum (*h*). *f* and *i* show matching phase-contrast micrographs. Curved arrows mark matched sites. Open arrows in *b*, *e*, and *h* show general endomysial staining for HSPG (*b* and *c*) and laminin (*h*). Bars, 50 μm.

Table I. Basement Membrane Staining with Antiserum against a PYS-2-derived HSPG

Cell type	Tissue	Staining	
Epithelia	Skin	+	
	Esophagus	+	
	Kidney tubule	+	
	Kidney glomerulus	+	
	Bronchiole	+	
	Bladder	+	
	Uterus	+	
	Ileum	+	
	Liver		−
	Pancreas acini		−
	Parotid acini	+	
	Parotid ducts (intra- and interlobular)	+	
Mesothelia	Lung	+	
Endothelia	Major and minor blood vessels (arterial and venous)	+	
	Capillaries	+	
Muscle	Striated	(+)	
	Striated-neuromuscular junction		−
	Smooth: Aortic	+	
	Peripheral blood vessels	(+)	
	Esophagus		−
	Bronchiole		−
	Bladder		−
	Uterus		−
	Ileum		−
	Cardiac		−

(+) denotes staining which was often weak.

highly probable that many smooth muscle types and cardiac muscle lack this form of HSPG in their basement membranes. Alternatively, the HSPG in these basement membranes may be in very much lower concentrations or be complexed in such a way to cause antigenic masking or steric hindrance of antibody penetration. Previous reports showing variability in tissue laminin and type IV collagen staining have concerned monoclonal antibodies where antigenic masking was a distinct possibility (11, 35).

One implication of these results is that for at least some smooth muscle and cardiac muscle, an alternative form of HSPG may be present. This form presumably has a core protein of sufficiently different characteristics that there is no antigenic relationship with the HSPG under study in this report. Additionally, it is also quite feasible that many basement membranes contain more than one HSPG. The fact that a group of muscle tissues are unstained implies that there is a correlation between their function and the nature of basement membrane composition.

The basement membranes of smooth and cardiac muscle abut the plasma membrane where the proteoglycans may exert their influence. Bearing in mind the requirement for membrane depolarization to be accompanied by calcium influx in these muscle types and the capacity of proteoglycans to bind with cations or perhaps control their diffusion (3, 23), it is tempting to speculate that electrostatic shielding or some function involving the availability of extracellular calcium for influx resulting from stimulation to contract involves certain proteoglycan species. On the other hand, striated muscle, with its abundant "T" tubule and sarcoplasmic reticulum network as calcium sources, may not use the same extracellular ion control mechanisms. In addition, vascular smooth muscle (which as striated muscle stained with the anti-HSPG serum) may also use calcium release from submembraneous sarcoplasmic reticulum in its contractile activity (34).

The idea of extracellular cation availability being associated with or controlled by basement membrane is supported by the finding that those of pancreatic acini were also not stained with the anti-HSPG serum. In this case, it has been proposed that membrane depolarization may be part of or a consequence of secretagogue stimulation (27, 39) and that basolateral extracellular calcium is important for enzyme and fluid release from the luminal surface of acinar cells under prolonged stimulus and for maintenance of a high resting membrane potential and resistance (27). Unlike the pancreas, however, the parotid gland acinar basement membranes contained the HSPG investigated here, perhaps indicative of different control mechanisms with regard to secretion (39).

Although there are now reports that one or more forms of basement membrane HSPG are located at the neuromuscular junction and may have a role to play in the orientation or localization of acetylcholinesterase (1, 2, 4), fluorescence studies showed that while motor end plates could be readily detected by rhodamine-conjugated α-bungarotoxin, there was no commensurate staining with the anti-HSPG serum. In the same experiment, double fluorescence studies resulted in endomysial staining of the muscle fibers with anti-HSPG serum, and antibodies against laminin stained the neuromuscular junction showing that antibody penetration into this zone was not sterically hindered. An antigenically distinct form of HSPG may thus be associated with these specialized zones. However, using a monoclonal antibody, others have shown that quite widely distributed HSPG species have some antigenic similarity to that located at the neuromuscular junction (4, 29). It is clear that further detailed characterization will be required to elucidate basement membrane proteoglycan relationships.

Major and minor blood vessel endothelia stained intensely with the anti-HSPG serum, but in contrast no staining of the thin, patchy basement membranes of rat liver sinusoids was detected. Type IV collagen was, however, detectable in this basement membrane. It remains possible, just as with the apparently negative muscle and acinar epithelial basement membranes, that the HSPG was present at levels below that which could be detected by indirect immunofluorescence microscopy.

One major class of HSPGs is the hydrophobic type at the cell surface of many cells including liver epithelia and fibroblasts (6, 9, 17, 20, 28, 40). In vitro studies indicate a role for hydrophobic HSPGs in cell adhesion to the extracellular matrix through an association with the microfilament-based cytoskeleton (17, 40, 41). An antiserum against cell surface HSPG from liver epithelial cells has been shown previously to cross react with fibroblast cell surfaces (41). The antiserum against PYS-2 HSPG did not stain liver epithelia or PYS-2 cell surfaces (Couchman, J. R., unpublished results), and studies in progress further indicate that fibroblast cell surfaces are also not stained (Woods, A., J. R. Couchman, and M. Höök, manuscript in preparation). This together

with previous evidence (31, 32) strongly suggests that the core proteins of cell surface and basement membranes HSPGs are distinct and immunologically unrelated. Furthermore, the fact that the antiserum only stained basement membranes indicates that it has no activity against cell surface forms of HSPG.

Finally, bearing in mind the present and previous data concerning the presence of CS/DSPGs in basement membranes (5, 7, 18), it is probable that a family of proteoglycans are present in these specialized extracellular matrices, each of which may have specific roles to play in the physiology of epithelial or parenchymal growth and differentiation.

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