Distinctive Populations of Basement Membrane and Cell Membrane Heparan Sulfate Proteoglycans Are Produced by Cultured Cell Lines

Jennifer L. Stow and Marilyn Gist Farquhar

Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510

Abstract. We have investigated the nature and distribution of different populations of heparan sulfate proteoglycans (HSPGs) in several cell lines in culture. Clone 9 hepatocytes and NRK and CHO cells were biosynthetically labeled with $^{35}SO_4$, and proteoglycans were isolated by DEAE-Sephacel chromatography. Heterogeneous populations of HSPGs and chondroitin/dermatan proteoglycans (CSPGs) were found in the media and cell layer extracts of all cultures. HSPGs were further purified from the media and cell layers and separated from CSPGs by ion exchange chromatography after chondroitinase ABC digestion. In all cell types, HSPGs were found both in the cell layers (20–70% of the total) as well as the medium. When the purified HSPG fractions were further separated by octyl-Sepharose chromatography, very little HSPG in the incubation media bound to the octyl-Sepharose, whereas 40–55% of that in the cell layers bound and could be eluted with 1% Triton X-100. This hydrophobic population most likely consists of membrane-intercalated HSPGs. Basement membrane-type HSPGs were identified by immunoprecipitation as a component (30–80%) of the unbound (nonhydrophobic) HSPG fraction. By immunofluorescence, basement membrane-type HSPGs were distributed in a reticular network in Clone 9 and NRK cell monolayers; by immunoelectron microscopy, these HSPGs were localized to irregular clumps of extracellular matrix located beneath and between cells. The cells did not produce a morphologically recognizable basement membrane layer under these culture conditions. When membrane-associated HSPGs were localized by immunoelectron microscopy, they were found in a continuous layer along the cell membrane of all cell types.

The results demonstrate that (*a*) two antigenically distinct populations of HSPG—an extracellular matrix and a membrane-intercalated population—are found at the surface of several different cultured cells lines; (*b*) these populations can be distinguished from one another by differences in their distribution in the monolayers by immunocytochemistry and can be separated by hydrophobic chromatography; and (*c*) basement membrane-type HSPGs are secreted and deposited in the extracellular matrix by cultured cells even though they do not produce a bona fide basement membrane-like layer.

HEPARAN sulfate proteoglycans (HSPGs)[1] of different molecular sizes and structures have been found at the surfaces of many different types of cells in tissues and in cultures (12). We have previously reported that two antigenically distinct populations of HSPG can be distinguished in a number of tissues based on their localization with specific antibodies: one type was associated exclusively with basement membranes (7, 24, 35, 37) and the other with the plasma membranes (7, 35, 36) of liver and kidney cells.

To determine whether or not similar populations of HSPGs are made by cultured cells, we have (*a*) analyzed HSPGs made by hepatocyte (Clone 9), normal rat kidney (NRK), and Chinese hamster ovary (CHO) cell lines; (*b*) partially characterized these HSPGs by ion-exchange and hydrophobic chromatography; and (*c*) determined their distribution by immunochemical and immunocytochemical procedures.

Materials and Methods

Materials

Guanidine hydrochloride (GuHCl), 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propane sulfonate (CHAPS), diaminobenzidine (DAB) hydrochloride, type II, phenylmethylsulfonyl fluoride (PMSF), benzamidine hydrochloride, 6-aminohexanoic acid, and Protein A-Sepharose 4B beads were obtained from Sigma Chemical Co. (St. Louis, MO). $Na^{35}[SO_4]$ (carrier free) was obtained from ICN (Chemical and Radioisotope Division, Irvine, CA). Chondroitinase ABC and heparitinase were from Miles Scientific Div. (Naperville, IL), and chromatographic resins were from Pharmacia Fine Chemicals (Piscataway, NJ).

1. *Abbreviations used in this paper*: CSPG, chondroitin/dermatan sulfate proteoglycan; DAB, diaminobenzidine; GBM, glomerular basement membrane; GuHCl, guanidine hydrochloride; HRP, horseradish peroxidase; HSPG, heparan sulfate proteoglycan; LM, liver microsomes.

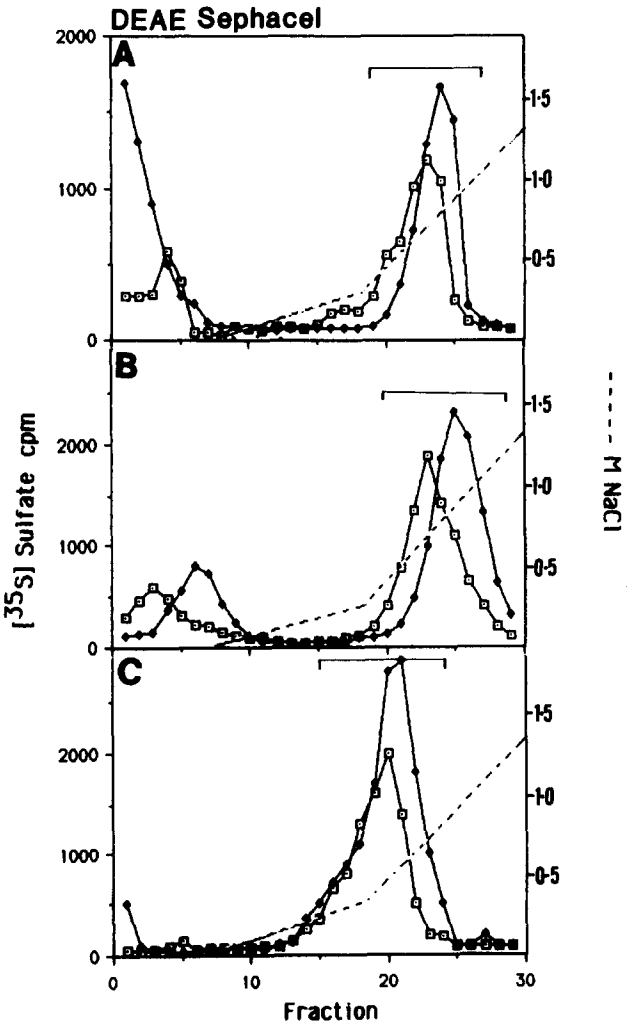

Figure 1. Ion exchange chromatography of $^{35}SO_4$-labeled extracts obtained from cultures of Clone 9 (*A*), NRK (*B*), and CHO (*C*) cells. Cultures were labeled for 24 h with $^{35}SO_4$, extracted with 4 M GuHCl, exchanged into 8 M urea buffer, applied to DEAE-Sephacel columns and eluted with a continuous NaCl gradient. In all cases, the proteoglycans extracted from the cell layers (○) and the media (●) consisted of broad peaks (indicated by the bars) which eluted at 0.4–1.0 M NaCl.

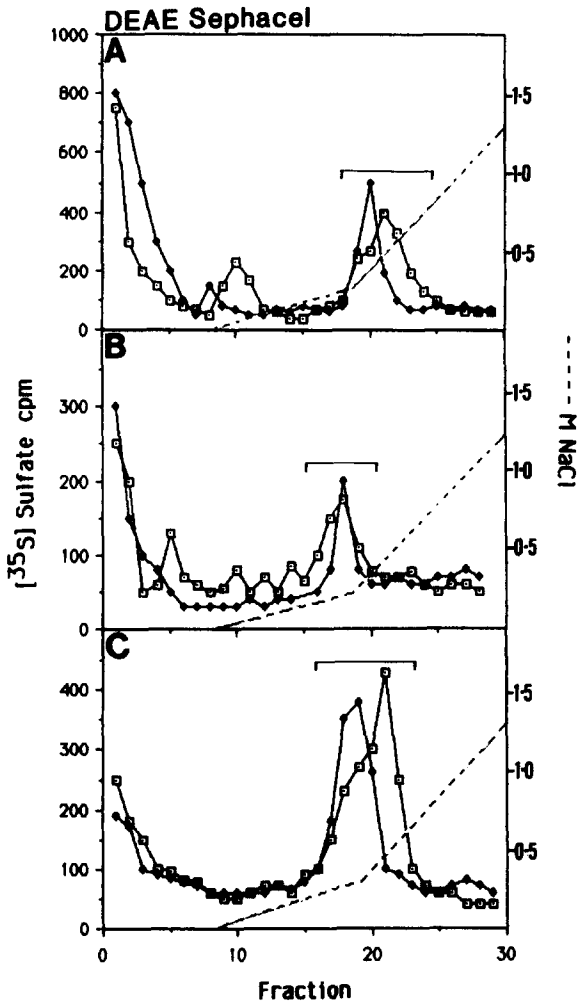

Figure 2. Ion exchange chromatography of chondroitinase ABC-digested, $^{35}SO_4$-labeled proteoglycans obtained from the media (●) and cell layers (○) of Clone 9 (*A*), NRK (*B*), and CHO (*C*) cells. Proteoglycan peaks eluted from DEAE-Sephacel columns (see Fig. 1) were concentrated, digested for 3 h at 45°C with chondroitinase ABC, and rechromatographed on DEAE-Sephacel. The digested CSPG fragments elute as one or two early peaks at low salt concentrations which could be clearly separated from the HSPG peak eluting at 0.4 M NaCl (indicated by the bars).

Antibodies

Polyclonal serum was prepared against purified glomerular proteoglycans and was shown to specifically recognize the core protein of the population of HSPGs (M_r 130,000) found in the rat glomerular basement membrane (GBM) by immunoprecipitation (37). By immunocytochemistry this antibody, referred to as anti-HSPG (GBM), stains basement membranes in the kidney and in other tissues. Polyclonal serum raised against HSPG purified from rat liver microsomes (LM) was kindly provided by Dr. Magnus Höök (University of Alabama). This antibody, referred to as anti-HSPG (LM) was previously shown to specifically recognize the core protein of the heparin-releasable and membrane-intercalated forms of membrane-associated HSPG (36) by immunoprecipitation. By immunocytochemistry it stains the basolateral or vascular domain of hepatocytes (36). Fab fragments of sheep anti-rabbit IgG conjugated to horseradish peroxidase (HRP) were from Biosys (Compiègne, France), and rhodamine-conjugated goat anti-rabbit IgG was from Cooper Biomedical Inc. (Malvern, PA).

Cell Culture

A rat hepatocyte cell line (Clone 9) was obtained from Dr. David Sabatini (New York University). Normal rat kidney (NRK) cells were provided by Dr. Soni Anderson (1) and were originally obtained from Dr. George Todaro, Oncogene (Seattle, WA). Chinese hamster ovary (CHO) cells were from the American Type Culture Collection (Rockville, MD). Cells were grown in MEM or in F12 nutrient mixture (Gibco, Grand Island, NY) supplemented with 10% FCS in an atmosphere of 95% air, 5% CO_2, and were passaged weekly. For immunocytochemical experiments they were plated onto 35-mm plastic dishes and grown to confluency, and for biosynthetic labeling experiments they were grown in 25-cm^2 flasks.

Biosynthetic Labeling of Proteoglycans

Confluent cultures were incubated for 24 h in 2 ml of MEM containing 5% FCS and $^{35}SO_4$ (200 μCi/ml). After labeling, the medium was collected and centrifuged (2,000 rpm, 10 min) to remove cell debris, two volumes of 6 M GuHCl, 0.075 M sodium acetate, 0.75% CHAPS, and 1 μM PMSF were added, and the medium was stored at −20°C. 2 ml of ice cold, 4 M GuHCl (containing 0.5% CHAPS, 50 mM sodium EDTA, 5 mM benzamidine HCl, 0.1 M 6-aminohexanoic acid, 1 μM PMSF, and 50 mM sodium acetate, pH 6.0) were added to the cell layer, and the cells were scraped off the dish and extracted in suspension for 16 h at 4°C with constant shaking. Unextracted residues were removed by centrifugation (13,000 rpm for 10

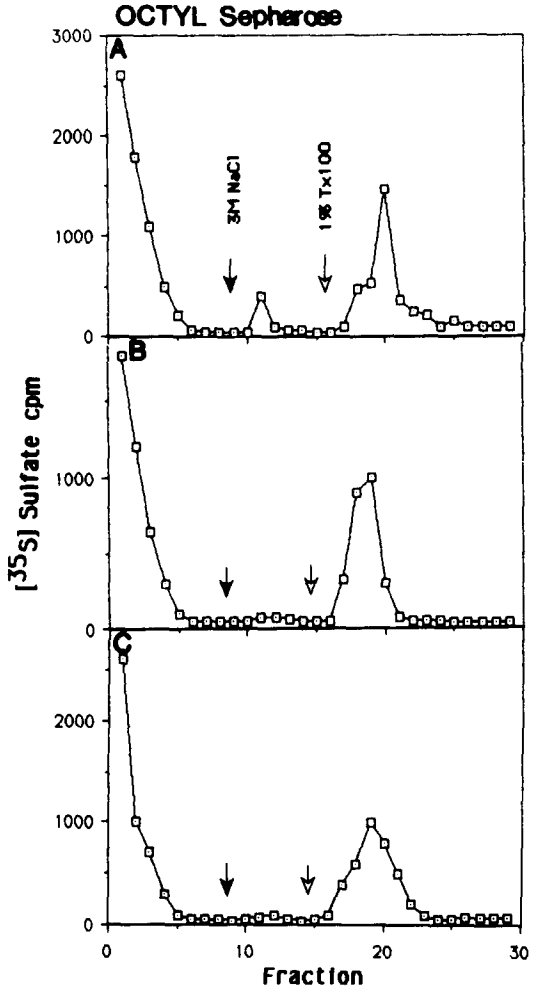

Figure 3. Octyl-Sepharose chromatography of HSPG fractions obtained as shown in Fig. 2 from Clone 9 (*A*), NRK (*B*), and CHO (*C*) cell layers. $^{35}SO_4$-HSPGs were incubated with octyl-Sepharose resin in the presence of 0.4% cholate and eluted step-wise with 3 M NaCl and 1% Triton X-100 at the points indicated. The peaks eluted with Triton X-100 represent the most hydrophobic populations of HSPG.

min), and the supernatants were stored at −20°C. This extraction procedure solubilizes 90–95% of the $^{35}SO_4$-labeled proteoglycans from the cell layer.

Isolation of Proteoglycans

Unincorporated $^{35}SO_4$, GuHCl, and other salts were removed from the medium and cell extracts by chromatography on Sephadex G-50 (fine) columns (0.5 × 16 cm) equilibrated and eluted with 8 M urea, 0.15 M NaCl, 0.05 M sodium acetate, pH 6.0 (Buffer A). The excluded fractions were then applied to a column of DEAE-Sephacel (5-ml bed volume) preequilibrated in Buffer A with 0.5% (wt/vol) CHAPS. After sample application, the columns were washed with ~5 ml of the same buffer and then eluted with a continuous NaCl gradient (0.15–1.5 M) in Buffer A using a total of 46 ml (40). Fractions (~1 ml) were collected at a flow rate of ~3 ml/h, and aliquots were counted for radioactivity.

Fractions containing the $^{35}SO_4$-labeled proteoglycans which eluted as a single, broad peak (see Fig. 1) were pooled and concentrated for use in the assays described below by diluting the pooled sample with 8 M urea to give a final concentration of <0.2 M NaCl. The sample was then reapplied to a DEAE-Sephacel column (200-μl bed volume) and eluted with 100-μl aliquots of 1 M NaCl in Buffer A. Recovery of radiolabeled proteoglycans was consistently ~90%.

Enzymatic Treatments

Proteoglycan fractions were digested either with chondroitinase ABC (0.05 U/ml) in 50 mM Tris-HCl, pH 7, with 1 μM PMSF for 3 h at 45°C (33) or with heparitinase (0.1 U/ml) in 50 mM Tris-HCl, 10 mM calcium acetate, pH 7, containing 1 μM PMSF for 3 h at 37°C (20).

Chromatography on Sepharose CL-6B

The relative proportion of heparan sulfate and chondroitin/dermatan sulfate proteoglycan (CSPG) in each extract was determined by Sepharose CL-6B chromatography before and after digestion with heparitinase or chondroitinase ABC. Sepharose CL-6B columns (0.7 × 100 cm) were equilibrated with 4 M GuHCl, 0.05 M Tris-HCl, 0.05 M sodium sulfate, 0.5% Triton X-100, pH 7.0. Fractions (0.6 ml) were collected, and aliquots were counted for radioactivity.

Isolation of HSPGs

$^{35}SO_4$-Labeled proteoglycan fractions that had been digested with chondroitinase ABC were diluted in Buffer A, reapplied to a DEAE-Sephacel column (2-ml bed volume), and eluted with a continuous NaCl gradient as described above. Chondroitin sulfate disaccharides eluted at low salt concentrations and were discarded. The remaining $^{35}SO_4$ radioactivity, representing intact HSPG, was collected and used for octyl-Sepharose chromatography.

Separation of HSPG by Hydrophobic Chromatography

Purified $^{35}SO_4$-HSPGs (~5–30 ml) were reapplied to DEAE-Sephacel columns (1 ml resin), and the bound HSPGs were washed with 20 ml 2% sodium cholate in 0.15 M NaCl, 20 mM Tris-HCl, pH 7.3, followed by 10 ml 0.4% cholate in the same buffer and then eluted with 0.4% cholate in 1 M NaCl, 20 mM Tris-HCl, pH 7.3. Fractions were diluted to 0.4% cholate, 0.6 M NaCl, 20 mM Tris, pH 7.3, and applied to an octyl-Sepharose column (2 ml resin); the column was incubated overnight at 4°C and then eluted sequentially with 10 ml of each of the following: (*a*) 0.15 M NaCl; (*b*) 3 M NaCl; and (*c*) 1% Triton X-100, 3 M NaCl, all in 20 mM Tris-HCl, pH 7.3 (31). 1-ml fractions were collected and counted for radioactivity.

Immunoprecipitation

Anti-HSPG (GBM) was bound to Protein A-Sepharose beads by incubating the washed beads in anti-HSPG serum (diluted 1:1 in PBS) for 1 h at 20°C. The beads were collected and washed twice in PBS and twice in RIPA buffer (5) (0.15 M NaCl, 100 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.1% deoxycholate, 0.1% SDS, 10 mM EDTA).

Isolated HSPG fractions (see Fig. 2) and subfractions eluted from the octyl-Sepharose column (see Fig. 3) were purified and concentrated over DEAE-Sephacel columns as described above. The fractions were then diluted to a volume of 1.5 ml in RIPA buffer containing 4 μl of normal rabbit serum, and after a 15-min incubation at 20°C, 40 μl Protein A-Sepharose was added and the samples were centrifuged (13,000 rpm for 2 min). The supernatant was mixed with 40 μl of Protein A-Sepharose beads with bound anti-HSPG (GBM) and incubated for 3 h at 20°C or 4°C overnight on a Nutator. The beads were washed three times in RIPA buffer, once in PBS, solubilized directly in scintillation fluid, and the amount of bound radioactivity was determined.

Immunofluorescence

Cultures grown on 35-mm petri dishes were prepared for immunofluorescence by one of two methods. Monolayers were either (*a*) fixed in formalin for 10 min and then permeabilized with 0.2% Triton X-100 in PBS for 10 min, or (*b*) they were treated with 2% Triton X-100 in PBS to extract the cells, and the matrix material remaining attached to the dishes was fixed in formalin. The cells and/or matrices were then reacted in the dishes sequentially with anti-HSPG (GBM) serum for 1 h, followed by goat anti-rabbit IgG conjugated to rhodamine, after which they were coverslipped and viewed in a Zeiss Photomicroscope III by epifluorescence illumination.

Immunoperoxidase Staining

Fixation and incubations were carried out as detailed previously (3). In brief, cells grown in 35-mM culture dishes were fixed for 3 h at 20°C by

*Table I. Percent HSPG and CSPG Found in the Media and Cell Layers of Cell Lines**

	Clone 9		NRK		CHO	
Cell types	Cell layer	Medium	Cell layer	Medium	Cell layer	Medium
Total $^{35}SO_4$ Proteoglycan (cpm per dish) $n = 6$	585,400	992,110	620,000	750,000	712,000	804,540
% HSPG	35	20	51	50	60	70
% CSPG	65	80	49	48	45	40

* Cultures were labeled for 24 h with 200 μCi/ml $^{35}SO_4$. The media and cell layers were extracted in 4 M GuHCl and 0.5% CHAPS and chromatographed over Sephadex G50 and DEAE-Sephacel columns as described in Materials and Methods. The total amount of $^{35}SO_4$-labeled proteoglycans in each extract was determined by counting the proteoglycan peak recovered off the DEAE-Sephacol column. The percentages of HSPG and CSPG were determined by counting the $^{35}SO_4$ radioactivity recovered in the proteoglycan peak by Sepharose CL6B chromatography before and after digestion of proteoglycans with chondroitinase ABC or heparitinase, respectively, as described in Materials and Methods.

replacing the growth medium with the fixative of McLean and Nakane (22) (2% paraformaldehyde, 0.75% lysine, 10 mM $NaIO_4$ in 35 mM phosphate buffer, pH 6.2). They were then (*a*) incubated in either anti-HSPG (GBM) (1/50), anti-HSPG (LM) (1/10), or normal rabbit (1/50) serum (in PBS); (*b*) incubated in F(ab) fragments of sheep anti-rabbit IgG conjugated to HRP (2 h); (*c*) fixed in 1.5% glutaraldehyde (1 h); (*d*) reacted with DAB and H_2O_2, followed by (*e*) postfixation in ferrocyanide-reduced OsO_4, and (*f*) dehydration and embedding in epoxy resin for electron microscopy. In some experiments, cells were scraped off the dish before postfixation in OsO_4, pelleted (in a Microfuge) and processed and embedded as pellets. In others the cell layers were processed in the dishes up to the propylene oxide step (when they detached) after which they were oriented during embedding so that the monolayers could be sectioned either vertically (top to bottom) or *en face*. Ultrathin sections were stained with lead citrate and photographed in a Philips 301 electron microscope.

Results

Characterization of Proteoglycans in the Culture Medium and Cell Layer

When Clone 9, NRK, and CHO cell cultures were labeled for 24 h with $^{35}SO_4$ and the extracted proteoglycans were analyzed by DEAE-Sephacel chromatography, the medium and cell extracts of all cell lines were found to contain heterogeneous populations of $^{35}SO_4$-labeled proteoglycans which eluted as broad peaks between 0.4–1.0 M NaCl (Fig. 1). Small peaks of $^{35}SO_4$ radioactivity, consisting of sulfated glycoproteins, eluted at low salt concentrations (0.1–0.3 M) (40) and were not analyzed further.

To determine the type of proteoglycans present, the proteoglycan fractions eluted from DEAE-Sephacel columns were analyzed by molecular sieve chromatography on Sepharose CL-6B columns before and after digestion with either chondroitinase ABC or heparitinase. The undigested proteoglycan fractions from all cell layer and medium extracts eluted from CL-6B columns as very broad peaks (not shown), indicating that the proteoglycans present are heterogenous in size. The percent of the total proteoglycans constituted by HSPG and CSPG is shown in Table I. HSPGs, identified by their susceptibility to digestion with heparitinase and not chondroitinase ABC, accounted for 20–50% of the total $^{35}SO_4$-labeled proteoglycans found in both the medium and cell extracts of Clone 9 and NRK cells and 60–70% of those made by CHO cells. The remaining proteoglycans were susceptible to chondroitinase ABC and thus consisted of CSPG.

When the $^{35}SO_4$-labeled proteoglycan fractions which had been treated with chondroitinase ABC were rechromatographed on DEAE-Sephacel (Fig. 2), purified HSPGs (which eluted at 0.5–0.9 M NaCl) could be separated from the digested CSPGs (which eluted earlier) and subjected to further analysis.

Separation of HSPG by Hydrophobic Chromatography

Proteoglycans of varying hydrophobicity can be separated by binding to octyl-Sepharose followed by their selective elution with detergent (16, 25, 31). When purified $^{35}SO_4$-labeled HSPGs obtained from GuHCl extracts of the medium and cell layers were separated according to their affinity for octyl-Sepharose, relatively little HSPG (~20%) in the medium obtained from any cell type bound to the column (Table II). However, a significant fraction (40–55%) of the HSPGs obtained from each of the cell layers did bind and was eluted with 1% Triton X-100 (Fig. 3, Table II). The HSPGs that bound and could be eluted with 1% Triton X-100 are assumed to represent membrane-intercalated HSPGs, and the unbound HSPGs are assumed to consist of other forms—i.e., not membrane-intercalated. These data suggest that among the HSPGs produced by all three cell types, a large proportion consists of HSPGs with core proteins containing more hydrophobic regions, presumably membrane-anchoring domains (16).

Immunoprecipitation of Basement Membrane HSPGs

The $^{35}SO_4$-labeled HSPG fractions prepared by hydrophobic chromatography were tested for their reactivity with an-

*Table II. Separation of Purified HSPG by Hydrophobic Chromatography**

	Clone 9		NRK		CHO	
Cell Line	Cell layer	Medium	Cell layer	Medium	Cell layer	Medium
% HSPG bound	40	15	40	9	55	18

*$^{35}SO_4$-labeled HSPG were isolated by chondroitinase-ABC digestion of the total proteoglycans, and the purified HSPG from each cell layer and medium extract was chromatographed on octyl-Sepharose columns eluted with Triton X-100 as described in Materials and Methods. These percentages reflect the total $^{35}SO_4$ radioactivity that bound to octyl-Sepharose in samples from six labeling experiments.

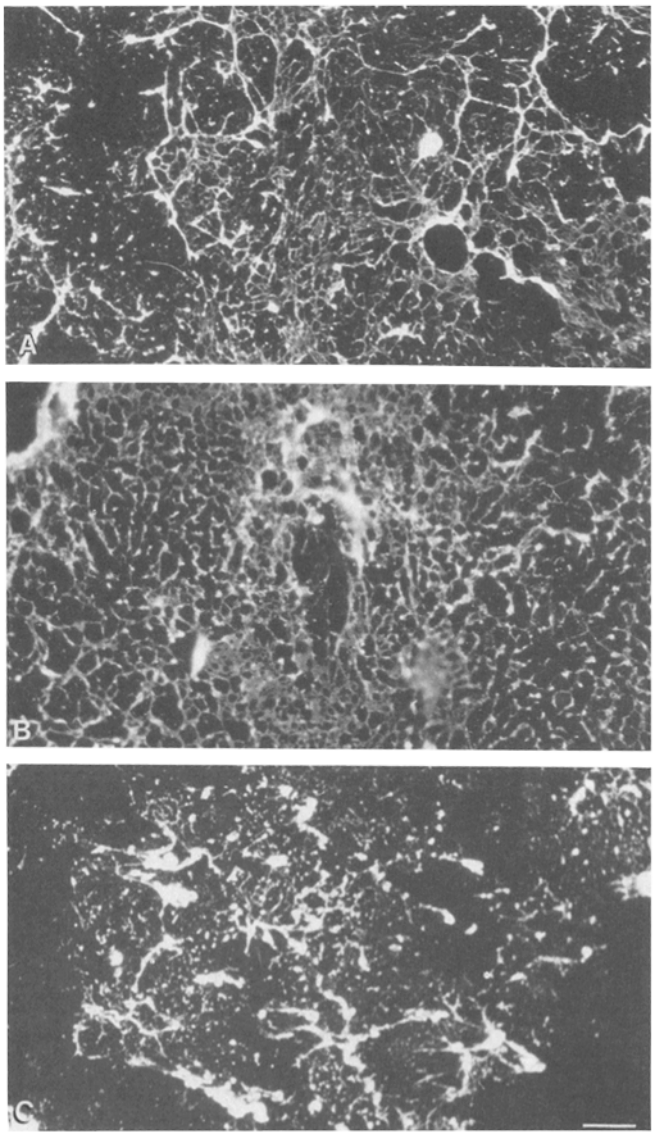

Figure 4. Immunofluorescence localization of extracellular matrix proteins in Clone 9 cell cultures following extraction of the cell layer. Monolayers were treated with 2% Triton X-100, and the material remaining in the dish was fixed in formalin and incubated with anti-HSPG (GBM) (*A*), anti-fibronectin (*B*), or anti-laminin (*C*) followed by rhodamine-conjugated goat anti-rabbit IgG. All three antibodies stain an irregular network of extracellular material adhering to the culture flask. Bar, 100 μm.

ti-HSPG (GBM) or normal rabbit serum by immunoprecipitation. HSPGs could be precipitated with anti-HSPG (GBM) serum from the fractions which did not bind to octyl-Sepharose, whereas they could not be precipitated from the bound fraction. The amount of HSPG precipitated from the unbound fractions obtained from the media and the cell layers varied considerably from cell type to cell type. The amount precipitated from the medium was very low in Clone 9 hepatocytes (2%) and NRK cells (7%), but was higher in CHO cells (22%). The amount precipitated from the cell layers was highest in Clone 9 (80%) and NRK cells (60%) and lowest (30%) in CHO cells. It can be concluded that (*a*) under these conditions basement membrane HSPGs do not bind to octyl-Sepharose; (*b*) they can be separated from more hydrophobic populations of HSPG by octyl-Sepharose chromatography; and (*c*) the amount of immunoprecipitated basement membrane HSPG present in the cell layers and released into the medium varies among different cell types. We were unable to carry out similar immunoprecipitations with anti-HSPG (LM) serum due to an insufficient supply of the antibody.

Distribution of Basement Membrane HSPG in the Monolayers

When Clone 9 cell monolayers were treated with 2% Triton X-100 (to extract the cells), and the residues were fixed and stained with anti-HSPG (GBM) by immunofluorescence, a web-like, reticular array of extracellular matrix-like material was seen adhering to the dish (Fig. 4 *A*). A similar web-like matrix was seen after staining NRK cells, but none was seen in CHO cell cultures. When the Clone 9 cell residues were stained for fibronectin (Fig. 4 *B*) and laminin (Fig. 4 *C*), a similar staining pattern was seen.

When the monolayers of Clone 9 hepatocytes were fixed, incubated with anti-HSPG (GBM), and reacted by immunoperoxidase for electron microscopy, clumps of DAB-containing extracellular material of varied sizes and shapes were seen adhering to the cell surface beneath and between cells (Fig. 5, *A–E*). These clumps are assumed to be cross sections through the web-like reticulum seen by immunofluorescence. Sometimes the stained extracellular material was located in deep pockets of the cell membrane whose connection to the cell surface was not always visible in the plane of the section (Fig. 5 *B*). In other cases, reaction product was concentrated in adhesion plaques located at the base of the cells where they attach to the culture dish (Fig. 5 *A*). Since the cells were not permeabilized, no intracellular staining was seen. Interestingly, there was no morphologically recognizable basement membrane-like layer layed down by this or any of the other cell types studied.

When cultured NRK cells were similarly reacted by immunoperoxidase, anti-HSPG (GBM)-reactive material was also found in extracellular locations (Fig. 6, *A–C*). The stained material was typically distributed in smaller patches which were closely adherent to the cell surface beneath and between the cells. It was often associated with bundles of smaller wispy fibers projecting from the cell surface (Fig. 6 *B*).

CHO cells possessed a relatively small amount of extracellular material that reacted with anti-HSPG (GBM) by immunoperoxidase when compared to Clone 9 and NRK cells. The reactive material was restricted to smaller patches located at points of adherence between adjacent cells (Fig. 7, *A* and *B*), and little or none was deposited beneath the cells.

It is concluded that (*a*) all three cell types studied produce basement membrane-type HSPG and deposit it extracellularly in irregular clumps of extracellular matrix material which are concentrated at sites of cell-cell or cell-substrate adhesion, and (*b*) these HSPGs are not incorporated into an organized basement membrane-like layer.

Distribution of Membrane-associated HSPG in the Monolayers

When cultures of Clone 9 cells were reacted with anti-HSPG

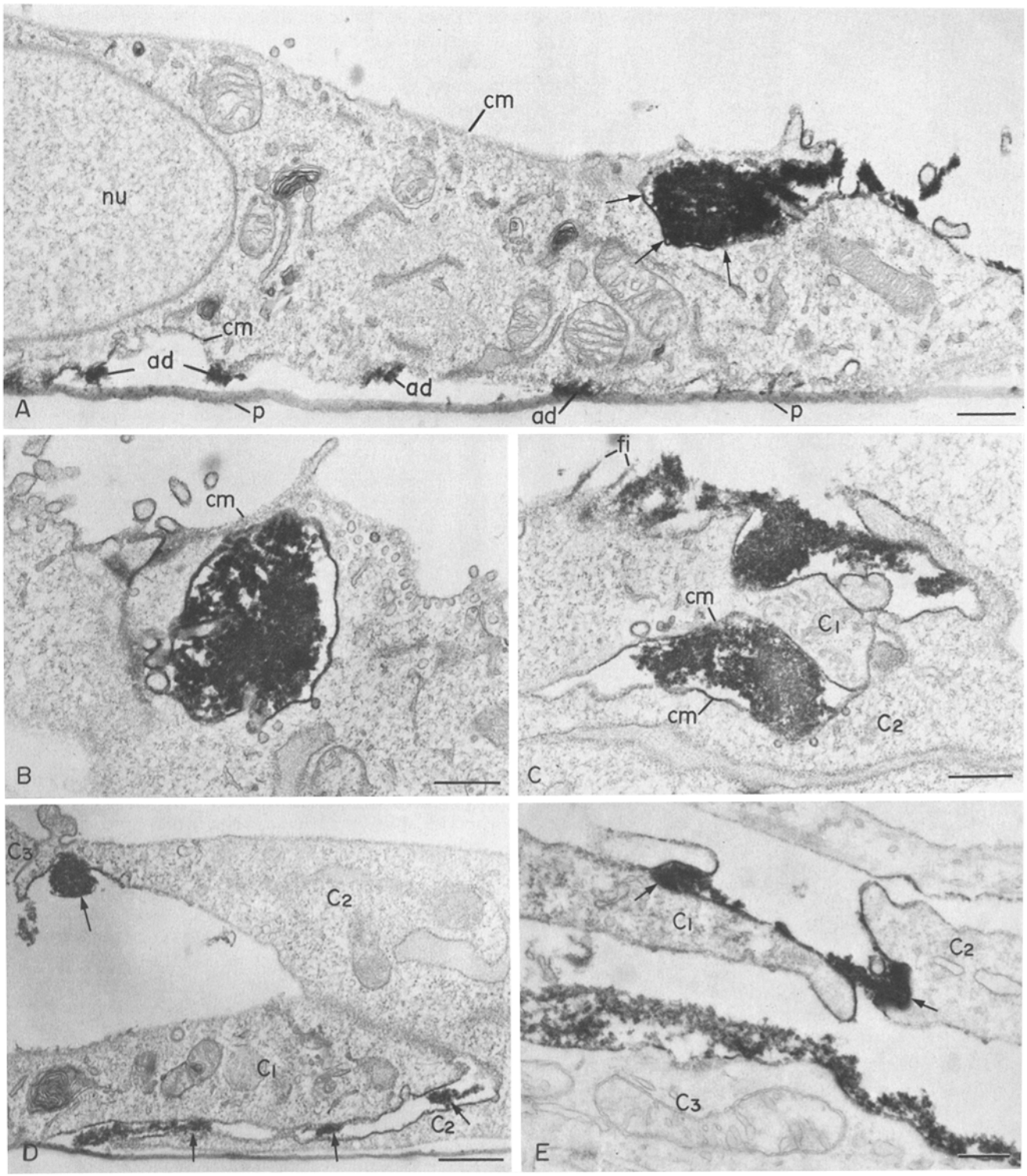

Figure 5. Distribution of basement membrane HSPGs in Clone 9 hepatocytes as seen by immunoperoxidase staining. Cells were incubated sequentially in anti-HSPG (GBM), sheep anti-rabbit Fab conjugated to HRP, and DAB medium. Deposits of extracellular material of variable size and organization which react with the antibody are found along the cells' surfaces. In *A*, these HSPGs are seen to be associated with adhesion plaques (*ad*) located at the base of the cell where it makes contact with the plastic substratum (*p*) and in a large deposit (*arrows*) of extracellular material located in a pocket of the cell membrane (*cm*). Note that the plasmalemma itself (*cm*) is not reactive; however, there appears to be some staining of the adjacent cell membrane due to diffusion of reaction product from its site of generation in the matrix. These cells do not make a morphologically recognizable basement membrane layer. *nu*, nucleus. *B* shows what is assumed to be a similar pocket containing reactive extracellular matrix material whose continuity with the plasma membrane is not evident in the plane of the section. *C* shows several such masses located in the intercellular spaces between the overlapping cell membranes (*cm*) of two adjacent cells (C_1 and C_2). Fibrillar strands (*fi*) are seen adjacent to the upper deposit. *D* and *E* demonstrate aggregates of matrix-like material that stains for basement membrane proteoglycans (*short arrows*) sandwiched between the overlapping edges of adjacent cells (C_1 and C_2). These HSPGs are also detected in a mass of material located near the junction between two cells (*long arrow* in *D*) and in a layer beneath the surface of another cell (C_3 in *E*). Bars: 0.5 μm.

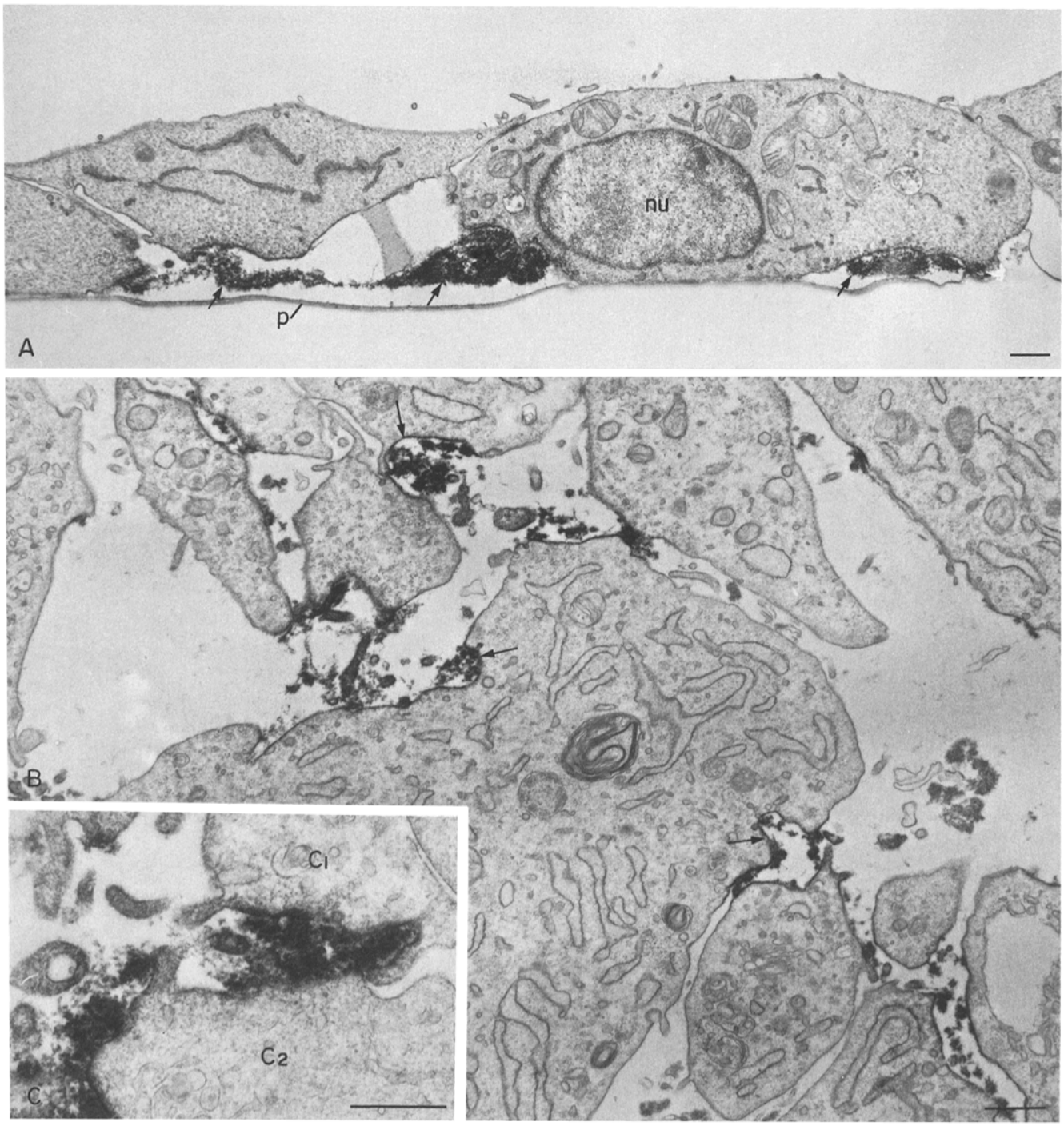

Figure 6. Distribution of basement membrane HSPGs in NRK cells incubated with anti-HSPG (GBM) as in Fig. 5. (*A*) Section cut through a monolayer (top to bottom) of NRK cells. Large clumps of stained material (*arrows*) are found between the basal surface of the cells and the plastic substratum (*p*). In contrast to Clone 9 cells, no clumps of extracellular matrix are seen on the upper surface of these cells which faces the culture medium. (*B*) Grazing section of cells cut *en face* showing clumps of reactive fibrillar material located in pockets of the cell membrane between adjacent cells (*arrows*). (*C*) The fibrillar extracellular material located in very close association with the surfaces of two adjacent cells (C_1 and C_2) is shown here at higher magnification. Bars: 0.5 μm.

(LM) by the immunoperoxidase procedure, the distribution of reaction product was quite different from that in specimens incubated with anti-HSPG (GBM): it was distributed in a continuous layer along the cell membrane (Fig. 8, *A* and *B*), and the extracellular material was not stained. Reaction product was not restricted to specific domains of the cell surface but occurred as a continuous layer outlining the entire exposed surface of the cells. It was also present in small vesicular profiles found in continuity with the cell membrane or located in the cytoplasm close to the cell surface. There was no intracellular staining under these conditions (no permeabilization).

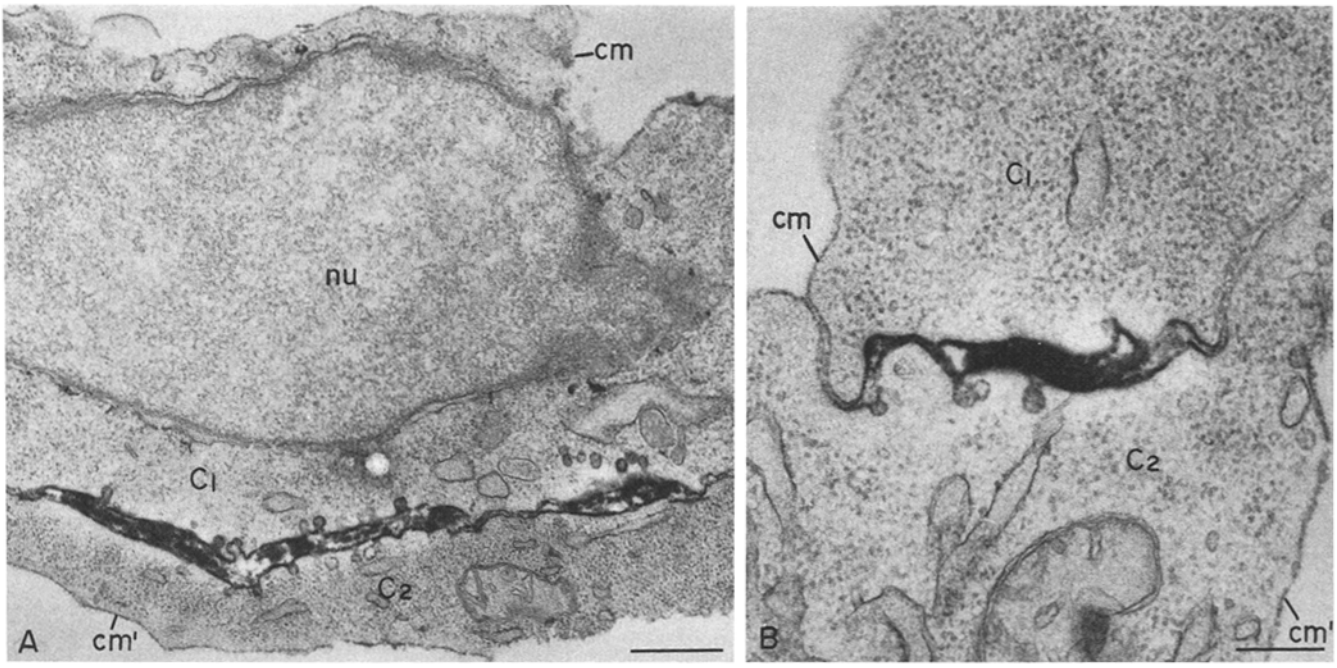

Figure 7. Distribution of basement membrane HSPGs in CHO cells reacted with anti-HSPG (GBM). The intercellular spaces between overlapping cells (C_1 and C_2) are filled with reactive extracellular matrix material, but no such material is seen on the free cell surfaces facing the medium (*cm*) or the substrate (*cm'*). Bars: (*A*) 0.5 μm; (*B*) 0.25 μm.

In NRK cells there was a similar distribution of staining with anti-HSPG (LM): reaction product was found along the entire surface of NRK cells, including vesicular profiles in continuity with the plasmalemma, but there was no staining of other structures (Fig. 9). In CHO cells, staining with anti-HSPG (LM) was similarly distributed but was somewhat weaker than in the other cell types.

Discussion

Previously we have identified two different populations of

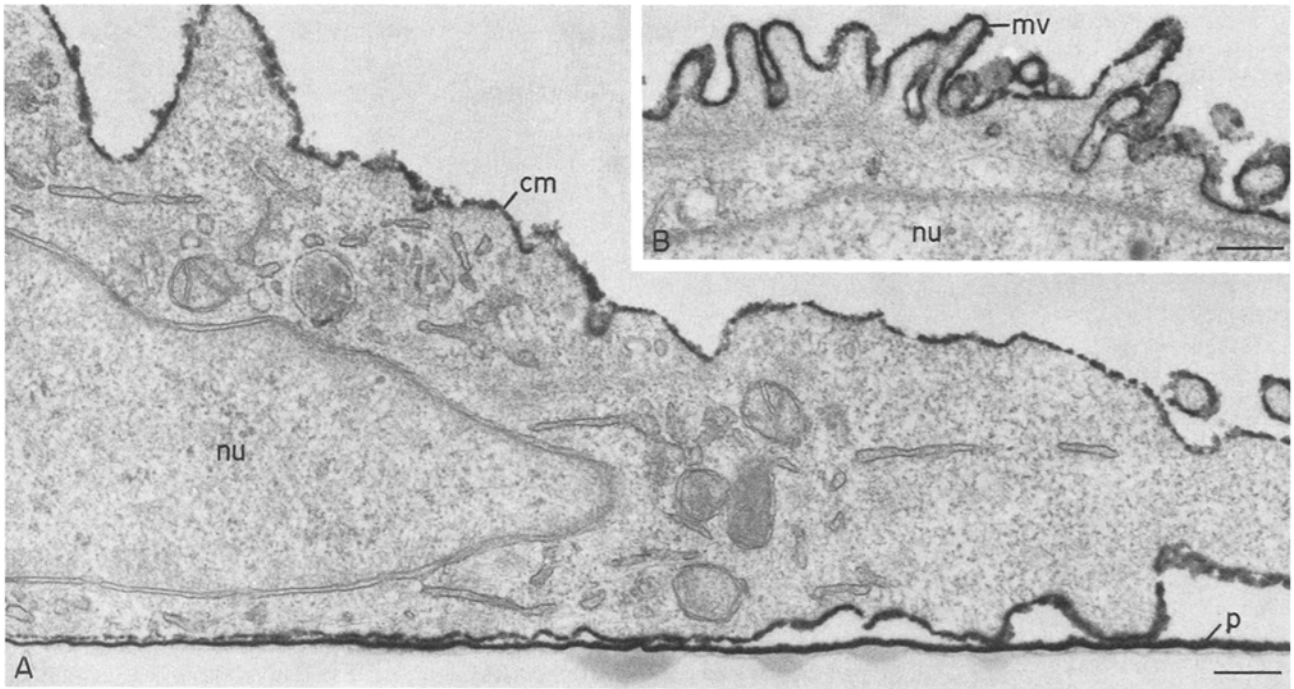

Figure 8. Localization of membrane HSPG in Clone 9 hepatocytes with anti-HSPG (LM). A continuous line of reaction product is distributed in a continuous layer on the outer surface of the entire plasmalemma (*cm*) on both the upper cell surface facing the medium and the basal surface facing the plastic substrate (*p*). In *B*, microvilli (*mv*) present on the cell surface facing the medium are continuously outlined by reaction product. Bar, 0.25 μm.

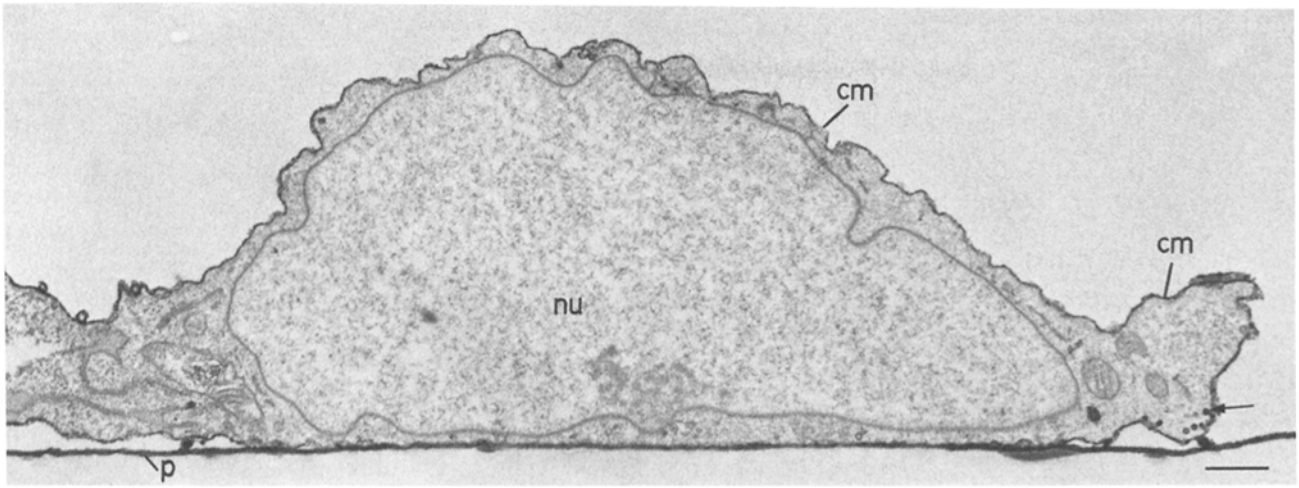

Figure 9. Localization of membrane HSPG in NRK cells after immunoperoxidase staining with anti-HSPG (LM). Only the cell membrane (*cm*) and small vesicular profiles close to the cell surface (*arrow*) are stained. *nu*, nucleus; *p*, plastic substratum. Bar, 0.5 μm.

HSPGs in rat tissues which can be distinguished by their characteristic distribution using specific antibodies. One type of HSPGs demonstrated by staining with anti-HSPG (GBM), was found to be associated with basement membranes in all tissues surveyed which included kidney, liver, ovary, pancreas, pituitary, and intestine (7, 24, 37; our unpublished observations). The other type of HSPG, demonstrated by staining with anti-HSPG (LM), was associated with cell membranes. These HSPGs proved to be preferentially concentrated on the basolateral (vascular) plasmalemmal domain of hepatocytes (36) and kidney tubule cells (35). In the present study we have obtained immunocytochemical data demonstrating that cell lines in culture also produce these two antigenically distinct populations of HSPG and that each of these populations has a characteristic and unique distribution in the cell monolayers. The HSPGs recognized by anti-HSPG (LM), raised against liver membrane HSPG, are distributed in a continuous layer along the entire cell membrane, whereas those stained by anti-HSPG (GBM) which recognizes basement membrane HSPGs, are concentrated in clumps of poorly organized extracellular matrix material of varying sizes and shapes located in pockets of the cell surface or in sites of cell-cell or cell-substrate attachment. By immunofluorescence these latter HSPGs colocalized in extracellular deposits with fibronectin and laminin, but none of these extracellular matrix components was incorporated into a morphologically recognizable basement membrane layer.

The main differences between the present findings in monolayers of cultured cell lines and those obtained previously in situ are as follows: (*a*) the matrix-type HSPGs are much more irregularly distributed and are not incorporated into a regular, organized basement membrane; and (*b* there is no preferential concentration of membrane-type HSPG on any particular domain of the plasmalemma. The latter observation is not surprising because, in contrast to liver and kidney in situ, these cells are not polarized—i.e., they do not make occluding junctions and do not deposit a basal basement membrane layer.

We also obtained corroborating biochemical evidence for the existence of at least two populations of HSPGs of varying hydrophobicity in the cell lines studied. When we isolated HSPGs (by chondroitinase ABC digestion of total proteoglycan fractions) and separated them on octyl-Sepharose columns, 40–50% (depending on the cell type) of the total HSPGs bound to the column and was eluted with 1% Triton X-100, whereas the remainder did not bind. Those that bound to octyl-Sepharose are assumed to correspond to membrane-intercalated HSPGs, based on their hydrophobic properties (16, 25) and on previous studies on rat liver (15, 16) and cultured cells (4, 25). The other type of HSPGs failed to bind to octyl-Sepharose and included basement membrane HSPGs, because ~40–50% of the total counts could be immunoprecipitated with anti-HSPG (GBM) serum. This fraction must also contain one or more additional, antigenically distinct populations of HSPGs (e.g., membrane-intercalated HSPGs which have lost their hydrophobic membrane tails [15, 28]), since 50–60% of the total was not recognized by anti-HSPG (GBM).

HSPGs with different properties have been found in association with the media and cell layers of a variety of cells in culture (2, 4, 11, 15, 18, 21, 23, 25, 27, 32, 38). In relatively few cases have HSPG populations been studied using specific antibodies (14, 23, 29, 38). Usually, they are characterized solely based on differences in their physical properties (hydrodynamic size, buoyant density). Their nature (membrane-intercalated vs. matrix-associated) and distribution in the monolayer has been deduced based on their ease of extraction. For example, those released by heparin treatment are assumed to represent either matrix-type HSPGs or membrane HSPGs that have lost their hydrophobic tails (13, 15); those released by detergent or trypsin are assumed to consist of membrane-intercalated HSPGs (40); and those not released by any of these treatments are assumed to consist of HSPGs associated with intracellular compartments (41). Our immunocytochemical results indicate that (*a*) both the membrane-type and the matrix- (basement membrane) type HSPGs are closely associated with the cell surface, and (*b*) the distribution of the latter HSPG in the monolayer can vary considerably, being found in some cases on one cell surface and in others on all cell surfaces (i.e., that facing the medium as

well as that between or beneath the cell layer). This situation can be expected to considerably complicate attempts to selectively extract either population of HSPG and to obtain purified populations of HSPGs for further analysis.

It is of interest that although Clone 9 hepatocytes and NRK cells, like many other cell lines, do not normally produce an organized basement membrane, they nevertheless produce basement membrane components, including HSPGs. Our immunofluorescence results have demonstrated that these HSPGs have a distribution which is similar to that of fibronectin and laminin. This colocalization of extracellular matrix components was described previously in NRK cell cultures by immunofluorescence (11). It was assumed that these elements were assembled into a basement membrane-like matrix; however, our immunoelectron microscopic results demonstrate that in both NRK and Clone 9 cells they are not deposited in a typical basally located basement membrane. Rather they are assembled into packets or aggregates of irregular sizes and shapes which in three dimensions form an irregular reticulum found between or underneath the cells in the monolayers. The factors instrumental in the assembly of basement membrane components into an organized membrane layer are unknown at present but are worthy of further study.

A further finding is that in Clone 9 cells matrix-type HSPGs are often localized in sites of cell–cell attachment as well as in adhesion plaques—i.e., points of contact between the basal cell membrane and the plastic dish which apparently function in cell–substrate attachment. It has been known for some time that HSPGs are an important component of adhesion sites in fibroblasts (18, 32, 38).

A well-known property of HSPGs is their ability to specifically interact with both cell membrane constituents (16) and matrix components including type I collagen (17), laminin (34), and fibronectin (39), which possess specific binding sites for heparan sulfate. One of the proposed functions of cell membrane HSPGs is that they form a link between the extracellular matrix, the cell surface, and the intracellular cytoskeleton (4, 27, 38).

At present it is not known how much homology or diversity exists between the protein cores of different populations of HSPG. It is clear from our studies (35–37) as well as those of others (14, 23) that there are antigenically distinct populations of basement membrane-associated and membrane-associated HSPGs. Within these two families further diversity in GAG composition and core protein size has been described. For example, cell membrane HSPGs with both chondroitin and heparan sulfate side chains have been isolated from mammary cells (6, 30). Also, two forms of basement membrane HSPGs have been isolated from the Englebreth-Holm swarm sarcoma (9, 10)—a large, low buoyant density HSPG and a smaller, high density HSPG—which are antigenically related. Ledbetter et al. (19) have identified a large precursor assumed to give rise by proteolysis to both the high and low density HSPG. A population of large HSPGs immunologically related to those of the Englebreth-Holm swarm sarcoma has been identified in the extracellular matrix produced by parietal yolk sac (PYS) cells in culture (26), and another large (750,000 mol wt) HSPG associated with basement membranes has also been isolated from L2 cell cultures (8). The relationship between the basement membrane HSPGs produced by tumor cell lines and those produced by normal cells in situ is not yet clear.

Further work is required to determine to what extent these different populations of basement membrane HSPGs resemble or differ from one another and from membrane-associated HSPGs. The availability in the future of the amino acid sequences of the core proteins of cell surface and basement membrane populations of HSPGs derived from different sources will be required to resolve these issues.

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