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Basement Membrane Heparan Sulfate Proteoglycan Is the Main Proteoglycan Synthesized by Glomerular Epithelial Cells in Culture

Jennifer L. Stow,* Carol J. Soroka,* Karen MacKay,† Liliane Striker,† Gary Striker,† and Marilyn Gist Farquhar*

From the Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut, and the Metabolic Disease Branch, National Institute of Diabetes, Digestive and Kidney Diseases, Bethesda, Maryland†*

The production and distribution of basement membrane-type heparan sulfate proteoglycans (BM HSPG) were investigated in a mouse glomerular epithelial cell line. Confluent cell monolayers were radiolabeled with [^{35}S]sulfate or [^{35}S]cysteine. Proteoglycans were isolated from the medium and cell layers by ion exchange chromatography and their nature determined by enzyme digestion (chondroitinase ABC) or degradative treatment (nitrous acid). It was found that more than 80% of the proteoglycans in both the cell layer and medium were heparan sulfate proteoglycans (HSPG) based on their susceptibility to nitrous acid degradation. More than half of the HSPG in the cell layer could be precipitated with an antiserum that specifically recognizes BM HSPG; only 10% of those released into the medium were precipitated with this antiserum. When immunoprecipitates of [^{35}S]sulfate-labeled proteoglycans were analyzed by SDS-PAGE, the mature proteoglycans ran as a broad band at the top of the gel. When immunoprecipitates of [^{35}S]cysteine-labeled proteoglycans were similarly analyzed, a 250 kd precursor core protein band was seen in addition to the mature proteoglycan. When BM HSPG were localized by immunofluorescence and immunoelectron microscopy (immunoperoxidase), they were found intracellularly in biosynthetic compartments (ER and Golgi cisternae) and extracellularly in deposits of basement membrane-like matrix located beneath and between the cells. These results indicate that 1) BM HSPG are the predominant type of proteoglycans made by glomerular epithelial cells in culture; 2) these HSPG are assembled into a loosely organized matrix that is deposited beneath and between the cells; and 3) this cell type produces a higher proportion of BM HSPG than other cultured epithelial cells studied previously. (Am J Pathol 1989, 135: 637–646)

Heparan sulfate proteoglycans (HSPG) are important structural and functional components of the glomerular basement membrane (GBM) where they are assumed to play a role in maintaining the size and charge selective properties of the GBM.[1–3] Changes in these HSPG or in the anionic sites where they are concentrated were reported in a number of glomerular diseases, including human[4] or experimental[5–7] diabetes, aminonucleoside nephrosis,[2,8–10] congenital nephrotic syndrome,[11] immune complex nephritis,[12] and lupus nephritis.[13]

In principle, all glomerular cell types, endothelial, mesangial, and epithelial, should be capable of making basement membrane-type HSPG (BM HSPG). Autoradiographic findings[2] and immunocytochemical[14] evidence suggest that the glomerular epithelium is the main source of BM HSPG that is incorporated into the GBM. However, it is difficult to study the contribution of the glomerular epithelial cell to the biosynthesis of GBM components *in vivo* or in isolated glomeruli due to the presence of the other cell types. The availability of glomerular epithelial cell lines in culture provides a useful system in which to study the biosynthesis of GBM components by these cells. Several earlier studies on epithelial and mesangial cells in culture[15–17] examined sulfate incorporation into the total pool of cellular and matrix proteoglycans, which would include

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Address reprint requests to Marilyn G. Farquhar, PhD, Department of Cell Biology, Yale University School of Medicine, 333 Cedar Street, P.O. Box 3333, New Haven, CT 06510.

those associated with the cell surface and intracellular compartments as well as those associated with the extracellular matrix. No study to date has specifically analyzed the synthesis of BM HSPG by these cells.

In this study we have taken advantage of the availability of specific antibodies against BM HSPG to investigate the production of this population of HSPG by normal mouse glomerular epithelial cells in culture.[18] We report here that these cells produce large amounts of BM HSPG that are secreted and assembled into an extracellular matrix deposited by the monolayer.

Materials and Methods

Materials

Diaminobenzidine (DAB) hydrochloride, type II, 3-[(3-chloramidopropyl) dimethyl-ammonio]-1-propane sulfonate (CHAPS), 6-aminohexanoic acid, phenylmethylsulfonyl fluoride (PMSF), and sodium borohydride were obtained from Sigma Chemical Co. (St. Louis, MO). Ultra pure guanidine hydrochloride (GuHCl) was purchased from Bethesda Research Laboratories (Gaithersburg, MD). Butyl nitrite was from Eastman-Kodak (Rochester, NY), and Triton X-100 (Surfact-Amps) was obtained from Pierce (Rockford, IL). Autofluor was from National Diagnostics (Manville, IL).

$Na_2{}^{35}SO_4$ (carrier free) and [^{35}S]cysteine were obtained from ICN (Radioisotope Division, Irvine, CA), and chondroitinase ABC was from Miles Scientific Division (Naperville, IL). The Superose 6 column, Q Sepharose, and Protein A-Sepharose were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Tissue culture medium, human fibronectin, and a rabbit antiserum to laminin were from Gibco (Grand Island, NY). Rhodamine-conjugated goat anti-rabbit IgG was from Tago (Burlingame, CA), and Fab fragments of sheep anti-rabbit IgG conjugated to peroxidase were from Biosys (Compiègne, France).

An antiserum to BM HSPG was prepared by immunizing rabbits with purified glomerular proteoglycans. This antiserum (anti-BM HSPG) was characterized previously[14] and shown to be specific for the core protein of the BM HSPG. It does not recognize cell membrane-associated HSPG.[19,20]

Cell Culture and Biosynthetic Labeling

Normal glomerular epithelial cells were isolated from outgrowths of mouse glomeruli by serial dilution cloning as described in detail previously.[18] Cells were grown on human fibronectin-coated plastic culture dishes, in DMEM/Hams F12 (3:1) medium supplemented with 1 mM glutamine, 100 μg/ml streptomycin and penicillin and 10% fetal bovine serum (FBS).[18] The cells were passaged weekly and used between passages 2 and 14 for the present studies.

Confluent cultures (day 7 after passage) were incubated for 1 to 24 hours in 2 ml culture medium containing 5% FBS and $^{35}SO_4$ (200 μCi/ml) or in 2 ml of cysteine-free medium containing [^{35}S]cysteine (200 μCi/ml). After radiolabeling, the medium was collected and centrifuged (10,000*g*, for 10 minutes) to remove cell debris and stored at −20 C.

Isolation and Analysis of Proteoglycans

Cells were scraped off the culture flask and extracted in 2 ml ice-cold GuHCl buffer (4 M GuHCl, 50 mM sodium acetate, pH 6, 4 mM sodium EDTA, 0.5% CHAPS) containing protease inhibitors (5 mM benzamidine HCl, 100 mM 6-aminohexanoic acid, 1 μM PMSF) by shaking at 4 C for 16 hours. The residue was removed by centrifugation at 13,000*g* for 10 minutes, and the soluble extract and residue monitored for radioactivity. Of the ^{35}S-labeled material in the cell layer, 97% was extracted with this procedure.

Cell layer extracts and medium were desalted over Sephadex G50 columns (0.5 × 16 cm) that were eluted with urea buffer (8 M urea, 50 mM sodium acetate, 0.15 M NaCl, pH 6). The excluded fractions were then chromatographed on a 2-ml column of Q Sepharose equilibrated in urea buffer containing 0.5% CHAPS. The bound glycoproteins were eluted in two peaks with a 46 ml gradient of 0.15 to 1.0 M NaCl in urea buffer. The eluate was collected in 1.0 ml fractions and aliquots were assayed for ^{35}S-radioactivity.

Fractions containing labeled proteoglycans were collected and concentrated for further analysis by diluting the eluate with 8 M urea to a concentration of 0.2 M NaCl and then rebinding the proteoglycans to a 200 μl column of Q Sepharose from which they were then eluted in 500 μl of 4 M GuHCl buffer or 50 mM Tris-HCl, pH 7.2, 1M NaCl. HSPG were prepared from the total proteoglycans by digestion with chondroitinase ABC (0.05 U/ml) in 50 mM Tris-HCl, pH 7.2, with 1 μM PMSF for 1 to 3 hours at 37 C and with subsequent ion exchange chromatography as described above.

Nitrous acid deamination was performed on total [^{35}S]-sulfate-labeled proteoglycans or immunoprecipitates according to the method of Cifonelli and King.[21] Alkaline β-elimination of O-linked GAG chains was conducted with 0.05 M NaOH and 1 M $NaBH_4$ according to Carlson.[22] Molecular sieve chromatography of intact [^{35}S]sulfate-labeled proteoglycans and nitrous acid- and alkaline borohydride-digested proteoglycans was carried out on a

Superose 6 FPLC column equilibrated in the GuHCl buffer containing 0.5% Triton X-100.

Immunoprecipitation

For characterization of HSPG, immunoprecipitation was carried out on chondroitinase ABC-digested proteoglycan fractions equilibrated in RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 0.15 M NaCl, and 10 mM EDTA in 25 mM Tris buffer, pH 7.2) with protease inhibitors. In biosynthetic studies immunoprecipitation was carried out on cell extracts or culture medium. For this purpose cells were extracted in 1 ml of RIPA buffer with protease inhibitors, and the culture medium was mixed with an equal volume of 2× RIPA buffer. The cell and medium extracts and proteoglycan fractions were centrifuged at 13,000*g* for 15 minutes (to remove unsolubilized material) and preincubated for 10 minutes at 20 C with Protein A-Sepharose beads in RIPA buffer. The beads were removed by centrifugation, and the cleared supernatants were incubated with 5 μl of anti-BM HSPG serum for 2 hours at 20 C on a nutator after which Protein A-Sepharose beads were added to the tubes for an additional 1 hour incubation. The beads were then washed (four changes of RIPA buffer over 6 hours and once with distilled water), solubilized in SDS PAGE sample buffer (0.15 M Tris, pH 6.8, 10% SDS, 25% glycerol, 5% β-mercaptoethanol, and bromophenol blue) for electrophoresis or in 4 M GuHCl for chromatography. Immunoprecipitates were analyzed by SDS PAGE on 4% to 15% Laemmli gels, followed by fluorography using Autofluor according to the manufacturer's specifications, or they were analyzed by ion exchange chromatography or molecular sieve chromatography as described above. In some instances immunoprecipitates were treated with nitrous acid before molecular sieve chromatography on a Superose 6 FPLC column.

Immunocytochemistry

Cell layers grown on 35 mm petri dishes were fixed in 4% paraformaldehyde in PBS for 10 minutes, extracted with 1% Triton X-100 in PBS, and incubated sequentially in anti-BM HSPG serum for 1 hour, followed by 1 hour in goat anti-rabbit IgG conjugated to rhodamine. The cultures were then mounted and viewed in a Zeiss Photomicroscope III by epifluorescence illumination.

Immunoperoxidase staining of the cultures was carried out as described previously.[19] Briefly, cell monolayers were fixed in PLP fixative (2% paraformaldehyde, 0.75% lysine, and 10 mM NaIO4 in 35 mM phosphate buffer, pH 6.2) for 4 hours at 20 C, permeabilized with 0.005% saponin in PBS, and then incubated in the presence of saponin with anti-BM HSPG serum for 12 hours at 4 C followed by Fab fragments of sheep anti-rabbit conjugated to HRP for 2 hours at 20 C. After incubation the cells were fixed in 1.5% glutaraldehyde, reacted with diaminobenzidine (DAB) and H_2O_2, postfixed in OsO_4, and embedded in Epox.

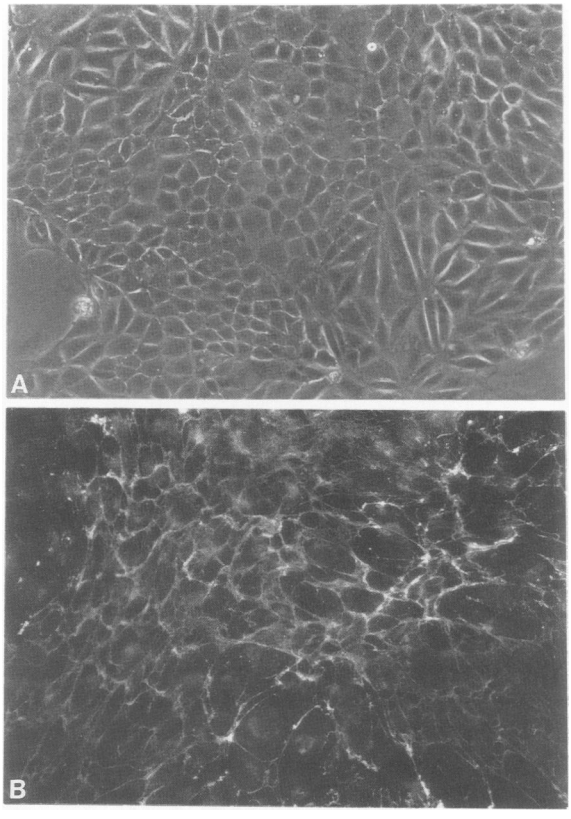

Figure 1. **A:** *Phase contrast micrograph of cultured mouse glomerular epithelial cells. The confluent monolayer (day 5) has the cobblestone appearance typical of epithelial cells in culture.* **B:** *Immunofluorescence localization of basement membrane HSPG in the extracellular matrix deposited by cultured mouse glomerular epithelial cells. Confluent monolayers were treated with 1% Triton X-100, and the matrix that remained attached to the dish was fixed with 4% paraformaldehyde and incubated with anti-BM HSPG serum followed by rhodamine-conjugated goat anti-rabbit IgG. An irregular web-like matrix adhering to the culture dish is brightly stained (×700).*

Results

Morphology of Epithelial Cells in Culture

At confluence the glomerular epithelial cells formed a monolayer with a typical cobblestone appearance (Figure 1A). The morphology and growth characteristics of these cultured mouse glomerular epithelial cells are similar to those described previously for cultures of human and rat glomerular epithelial cells.[15–18,23] The cultures retained

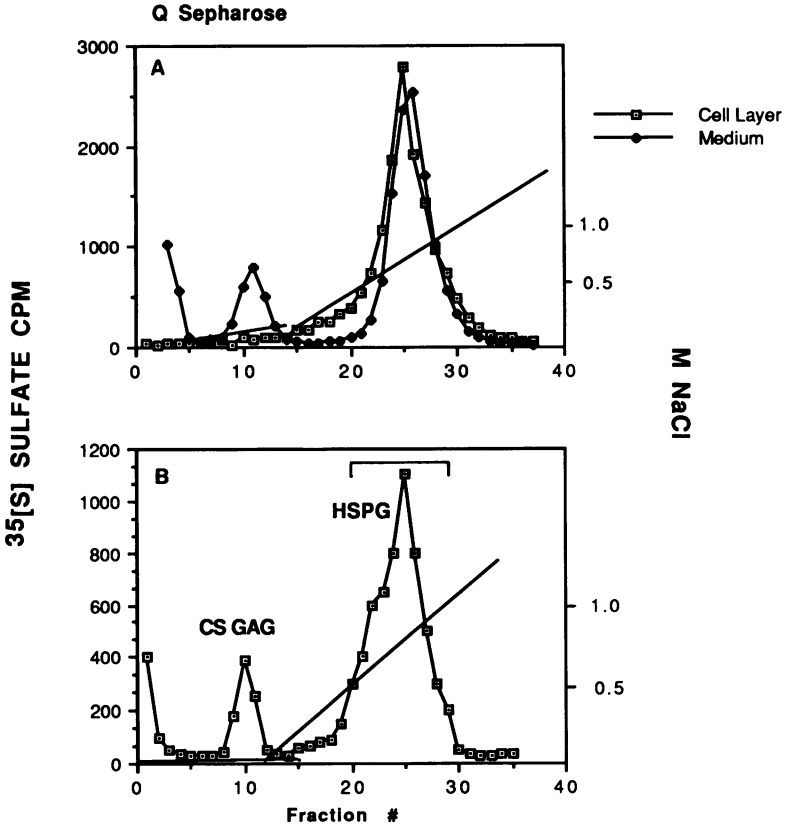

Figure 2. *Ion exchange chromatography of [^{35}S]-labeled extracts obtained from the cell layer (-□-) and the medium (-◆-) of cultured mouse glomerular epithelial cells. Cultures were labeled for 24 hours with [^{35}S]sulfate, extracted with 4 M GuHCl, exchanged into 8 M urea buffer, applied to a Q Sepharose column, and eluted with a continuous NaCl gradient (——). A: Most of the radioactivity in both the medium and cell layer extracts was found in proteoglycans that elute as a broad peak at 0.4 to 0.8 M NaCl. The small peak of radioactivity at the beginning of the elution profile in the medium extract represents sulfated glycoproteins. B: The proteoglycan peak from the cell layer was dialyzed, concentrated, digested for 3 hours at 37 C with chondroitinase ABC, and chromatographed on a Q Sepharose column. The digested [^{35}S]sulfate-labeled chondroitin and dermatan sulfate fragments eluted as an early peak at lower NaCl concentrations, whereas the undigested HSPG remained in the peak eluting at 0.4 to 0.8 M NaCl (indicated by the bar).*

their typical epithelial morphology and remained consistent with respect to proteoglycan synthesis and expression (Figure 1B) through 15 to 20 passages.

Glomerular Epithelial Cells Synthesize Predominantly HSPG

When glomerular epithelial cell cultures were labeled with [^{35}S]sulfate and extracted and analyzed by ion exchange chromatography on Q Sepharose, both the medium and the cell layers were found to contain ^{35}S-labeled proteoglycans that eluted as broad peaks at 0.4 to 0.8 M NaCl (Figure 2A). Approximately 49% of the total proteoglycans were recovered from the medium, whereas 51% were associated with the cell layer. When the proteoglycans associated with the cell layer and medium were digested with chondroitinase ABC, only 13% and 20% were digested, respectively, and therefore consisted of chondroitin/dermatan sulfate proteoglycans (CS/DSPG) (Figure 2B). Thus, most (>80%) of the proteoglycans in both the cell layer and medium were resistant to digestion with this enzyme, suggesting that they consist of HSPG.

To further characterize the proteoglycans in the cell layer and medium, the proteoglycan fractions obtained by ion exchange chromatography on Q Sepharose were further analyzed by molecular sieve chromatography on a Superose 6 FPLC column before (Figure 3A) and after nitrous acid and alkaline borohydride treatment (Figure 3B). The untreated proteoglycans from the medium eluted as a single peak (Kav 0.32) on the molecular sieve column, whereas those from the cell layer eluted in two peaks, one that coeluted with the medium proteoglycan (Kav 0.32) plus an additional peak (Kav 0.78) that eluted close to the total volume of the column. Nitrous acid treatment (which specifically degrades only heparan sulfate GAG chains) virtually eliminated both the peaks seen in the untreated sample from the cell layer confirming their identity as mainly HSPG (Figure 3B). Alkaline borohydride reduction (which releases intact GAG chains) resulted in the loss of the original proteoglycan peak (Kav 0.32), and a new peak representing GAG chains appeared at Kav 0.53 (Figure 3B). The peak at Kav 0.78 was unchanged by the treatment, suggesting that it consists of free heparan sulfate GAG chains associated with the cells. This pool of free GAG chains could also be extracted with Triton X-100 but was not released from the cell surface by trypsinization (data not shown), suggesting that it is located in intracellular compartments.

It was concluded that most of the proteoglycans in the medium and cells consist of HSPG, and that the medium contains mostly intact proteoglycans, whereas the cell

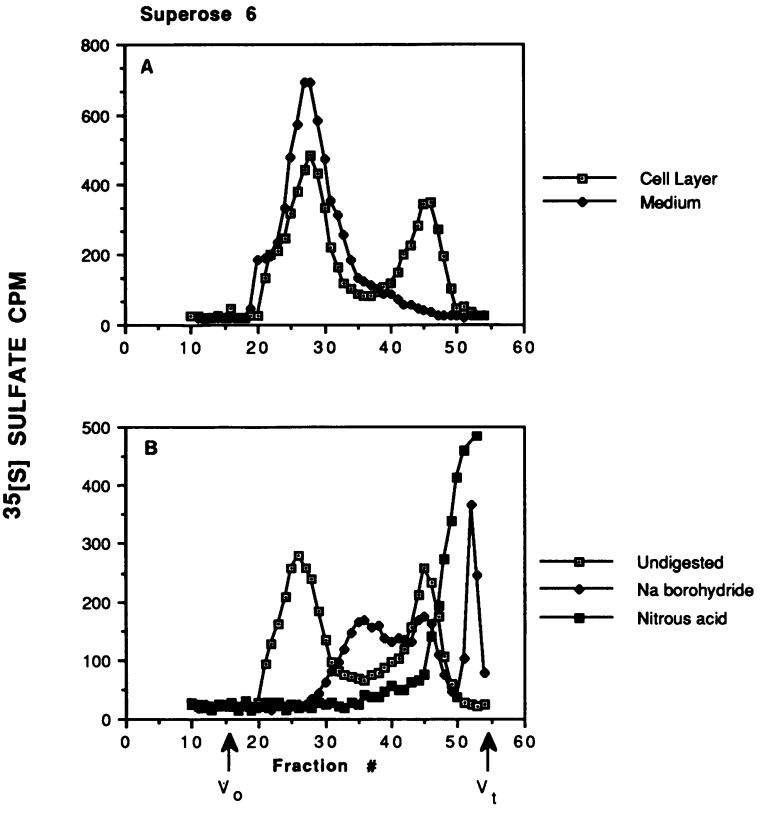

Figure 3. *Molecular sieve chromatography of [^{35}S]sulfate-labeled proteoglycans from the cell layer (-⊡-) and the culture medium (-♦-).* **A:** *Proteoglycan peaks eluted from Q Sepharose as described in Figure 2 were applied to a Superose 6 FPLC column and eluted in 4 M GuHCl buffer. The medium proteoglycans eluted as a single peak (Kav 0.32). Those associated with the cell layer eluted in two peaks, one of which coincided with medium proteoglycans and another, separate peak (Kav 0.78).* **B:** *Aliquots of the proteoglycans from the cell layer were subjected to treatment with either nitrous acid (-■-) or alkaline borohydride (-♦-) as described in Materials and Methods, and the digested samples were chromatographed on the Superose 6 FPLC column to compare the profiles of digested and undigested proteoglycans (-⊡-). Nitrous acid treatment, which specifically degrades heparan sulfate chains, reduced material in both peaks to small fragments that elute from the column in the V_t. Alkaline borohydride treatment, which releases GAG chains from proteoglycans, caused a shift in the larger peak (Kav 0.32 to Kav 0.53), and the appearance of a third peak in the V_t that presumably represents small (Mr, <6000) GAG fragments. This treatment did not shift the smaller peak (Kav 0.78).*

layer contains free GAG chain as well as intact proteoglycans.

BM HSPG is the Main Population of HSPG in the Cell Layer

When the [^{35}S]sulfate-labeled HSPG (prepared by digestion of total proteoglycans with chondroitinase ABC) were tested for their reactivity with anti-BM HSPG serum, 51% of the total HSPG from the cell layer was precipitated. Thus, basement membrane HSPG accounts for approximately half of the total HSPG associated with the glomerular epithelial cell layer in culture. By contrast, very little (10%) of the HSPG in the culture medium was immunoprecipitated by this antiserum.

When the immunoprecipitates were dissociated with 8 M urea or 4 M GuHCl and subjected to ion exchange and molecular sieve chromatography, the precipitated BM HSPG eluted as a single peak (Figure 4). When the precipitates were treated with nitrous acid before separation on the Superose 6 column, this peak of radioactivity disappeared. This indicates that the BM HSPG made by these cells carry largely or exclusively heparan sulfate GAG chains.

The Core Protein of the BM HSPG Is Synthesized as a 250 kd Precursor

After labeling glomerular epithelial cells with [^{35}S]cysteine for 12 hours, only mature BM HSPG, which comigrated with [^{35}S]sulfate-labeled immunoprecipitates on SDS PAGE (Figure 5A), was precipitated from the medium and cell extract with anti-BM HSPG (Figure 5B). However, in cultures pulse-labeled for only 30 minutes, an additional 250 kd band was precipitated from the cell layer (Figure 5C). This band was not seen in [^{35}S]sulfate-labeled precipitates of the cell layer and was not present in [^{35}S]-cysteine-labeled precipitates obtained from the medium. The fact that the 250 kd band was found in the cell layer but not in the medium, that it was not sulfated, and that it was most prominent after short labeling periods indicate that, it is most likely a precursor form of the BM HSPG core protein.

BM HSPG Is Localized in the Extracellular Matrix and in Biosynthetic Compartments of Glomerular Epithelial Cells

When monolayers of glomerular epithelial cells were permeabilized with Triton X-100 and stained by immunofluorescence with anti-BM HSPG, a web-like matrix was seen

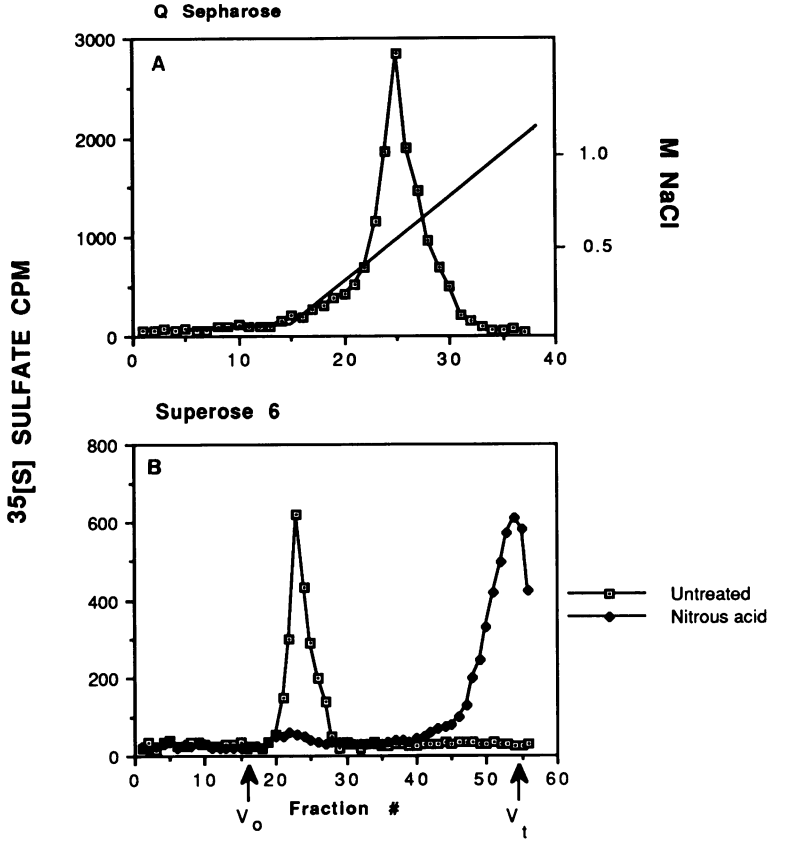

Figure 4. *Chromatography of BM HSPG immunoprecipitated from glomerular epithelial cells. [^{35}S]sulfate-labeled HSPG were prepared from cell extracts by ion exchange chromatography and digestion with chondroitinase ABC as described in Figure 2. The material was exchanged into RIPA buffer and subjected to immunoprecipitation with anti-BM HSPG.* **A**: *The immunoprecipitates were solubilized with 8 M urea and analyzed by ion exchange chromatography on a Q Sepharose column.* **B**: *Immunoprecipitates were solubilized with 4 M GuHCl before (-□-) or after (-◆-) digestion with nitrous acid and analyzed by molecular sieve chromatography on a Superose 6 column. The untreated immunoprecipitates eluted as a single peak off both columns. Those treated with nitrous acid were degraded to small fragments eluting in the V_t, confirming their identity as HSPG.*

between adjacent cells and beneath the cell layer (Figure 1B). A similar pattern was seen after staining with anti-laminin (data not shown).

When the BM HSPG was localized at the EM level by immunoperoxidase, it was found in irregular clumps of loosely organized extracellular matrix located between adjacent cells (Figures 6A, B) or between the cells and the culture dish (Figure 6C). These deposits of matrix were not assembled into a typical basement membrane-like layer, and they resembled those deposited by other epithelial (kidney and liver) cell lines studied previously.[20] However, the glomerular epithelial cells produce a more abundant HSPG-containing extracellular matrix than other epithelial cell lines we have studied.

BM HSPG was also detected in the rough ER and Golgi cisternae of the glomerular epithelial cells (Figure 6C) where it was seen more often than in the other cultured kidney and liver cell lines studied. The presence of these HSPG in biosynthetic compartments suggests that these cells are more actively synthesizing BM HSPG than the other cell lines studied to date.

Discussion

The purpose of this study was to determine the nature of the proteoglycans synthesized by mouse glomerular epithelial cells in culture with particular attention to basement membrane-type HSPG. We found that proteoglycans associated with the cell layer consisted predominantly (87%) of HSPG (based on their sensitivity to nitrous acid degradation) and that the major population (51%) of HSPG was BM HSPG based on its ability to be precipitated with a specific anti-BM HSPG serum. Similarly, 80% of the total proteoglycans in the culture medium consisted of HSPG, but very few of these (10%) could be precipitated by the anti-BM HSPG serum. By immunoelectron microscopy we found that the BM HSPG was localized in biosynthetic compartments (ER and Golgi) inside the cells or in clumps of extracellular matrix deposited beneath the cell layer or between adjacent cells. The cell layer also contained other populations of HSPG not recognized by the BM HSPG serum and a resident pool of free heparan sulfate GAG chains that could be separated from intact HSPG by FPLC molecular sieve chromatography.

It was important to establish the size of the fully glycosylated BM HSPG and the core protein synthesized by mouse glomerular epithelial cells because a number of different molecular sizes have been reported for BM HSPG isolated from other sources.[24-26] When BM HSPG were immunoprecipitated from the glomerular epithelial cell layer after labeling with radioactive sulfate, the mature sulfated BM HSPG eluted as a single peak from ion exchange and molecular sieve columns and appeared as a

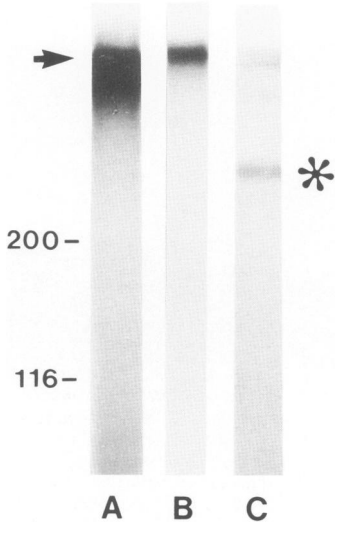

Figure 5. *Immunoprecipitates obtained with anti-BM HSPG. Glomerular epithelial cells were labeled with [^{35}S]sulfate (lane A) or [^{35}S]cysteine (lanes B and C), extracted with RIPA buffer, and the proteins immunoprecipitated from these extracts were analyzed by SDS-PAGE and fluorography. After a 12-hour labeling period the mature (approximately 400 kd) BM HSPG was the only protein band seen in the precipitates (lanes A and B). The fully glycosylated HSPG typically ran as a smeared band at the interface of the stacking and resolving gels (arrow). After a shorter (30-minute) labeling period with [^{35}S]-cysteine (lane C), another band migrating at approximately 250 kd was precipitated. This band (not seen in [^{35}S]sulfate-labeled precipitates) was assumed to be a nonsulfated, precursor of the mature BM HSPG.*

smeared band at the top of SDS gels. These results are consistent with those obtained previously for BM HSPG from rat GBM.[2,14] However, when immunoprecipitations were carried out after labeling the cell layer with [^{35}S]-cysteine and the precipitates were examined by SDS PAGE, an additional 250 kd band was seen, as well as the mature HSPG at the top of the gel. This 250 kd band predominated in immunoprecipitates after a short (20 to 30 minute) pulse, whereas at longer labeling times the mature BM HSPG predominated. The 250 kd band thus has the characteristics of a nonsulfated precursor of the core protein. The fact that it is not sulfated indicates that it has not yet reached the trans part of the Golgi complex where sulfation as well as terminal glycosylation take place,[27] and therefore it represents either an ER or an early (cis/middle) Golgi form of the HSPG. Further work is needed to determine the state of glycosylation of the 250 kd precursor band, as well as the size of the core protein of the BM HSPG. The range of sizes (Mr, 18,000 to 300,000) reported for BM HSPG core proteins could be due to proteolysis during their preparation or to differential processing of this molecule in different cell types. There may also be other precursor forms of the BM HSPG that we have not detected in these glomerular epithelial cells.

BM HSPG are the most abundant population of proteoglycans labeled in the glomerular epithelial cell cultures, but they are not the only type of proteoglycans these cells make. Like glomeruli *in vivo*[28] or *in situ*[29] or isolated glomeruli,[30–32] they make a small amount (approximately 15%) of CS/DSPG, and they also make one or more additional populations of HSPG not precipitated by our antibody. The identity of the remaining HSPG in the cell layer and medium is not known. They may represent, at least in part, HSPG associated with the cell membrane. We previously reported staining of the podocyte cell surface[2] as well as a variety of cells in culture[20] with an antibody generated against cell membrane-intercalated HSPG purified from rat liver.[19,33] These earlier immunocytochemical results suggest therefore that there is a separate, antigenically distinct population of HSPG on the podocyte and other cell surfaces. The presence of membrane-intercalated or more loosely associated forms of HSPG has been well documented on the surfaces of a wide variety of cells.[33] Their functions are for the most part unknown, although some have been shown to act as mediators of cell adhesion[34] or to link the extracellular matrix with the cytoskeleton.[35–37] The identification of additional populations of proteoglycans synthesized by glomerular epithelial cells will require the development and use of specific antibodies that recognize individual types of proteoglycans produced by these cells. No such antibodies are available at present.

By molecular sieve chromatography on an FPLC Superose column we detected a pool of [^{35}S]sulfate-labeled material in the cell layer that we determined consisted of free heparan sulfate GAG chains because it was not degraded by alkaline borohydride reduction, but was degraded by nitrous acid oxidation. Such pools of free GAG chains were found to be associated with various other cell types in culture.[38–40] In most of these cases, with one exception,[41] the free GAG were located intracellularly and were believed to arise by the degradation of endocytosed proteoglycans.[38,39] In the present study, we were not able to release the free heparan sulfate chains by trypsinization, suggesting that they are also located in intracellular compartments. Further work is needed to establish the source of this free GAG pool in glomerular epithelial cells.

The available evidence to date[2,14] suggests that the podocyte or glomerular epithelial cell synthesizes most of the BM HSPG that is incorporated into the GBM. Using our specific antibody to BM HSPG, we previously demonstrated by immunoperoxidase localization the presence of these BM HSPG in biosynthetic organelles (ER and Golgi) of the podocyte but not in endothelial or mesangial cells.[2,14] This is consistent with the findings in the present study of a similar pattern of intracellular staining in cultured mouse glomerular epithelial cells. In addition, our earlier autoradiographic studies demonstrated high concentrations of autoradiographic grains (67% of the total) over

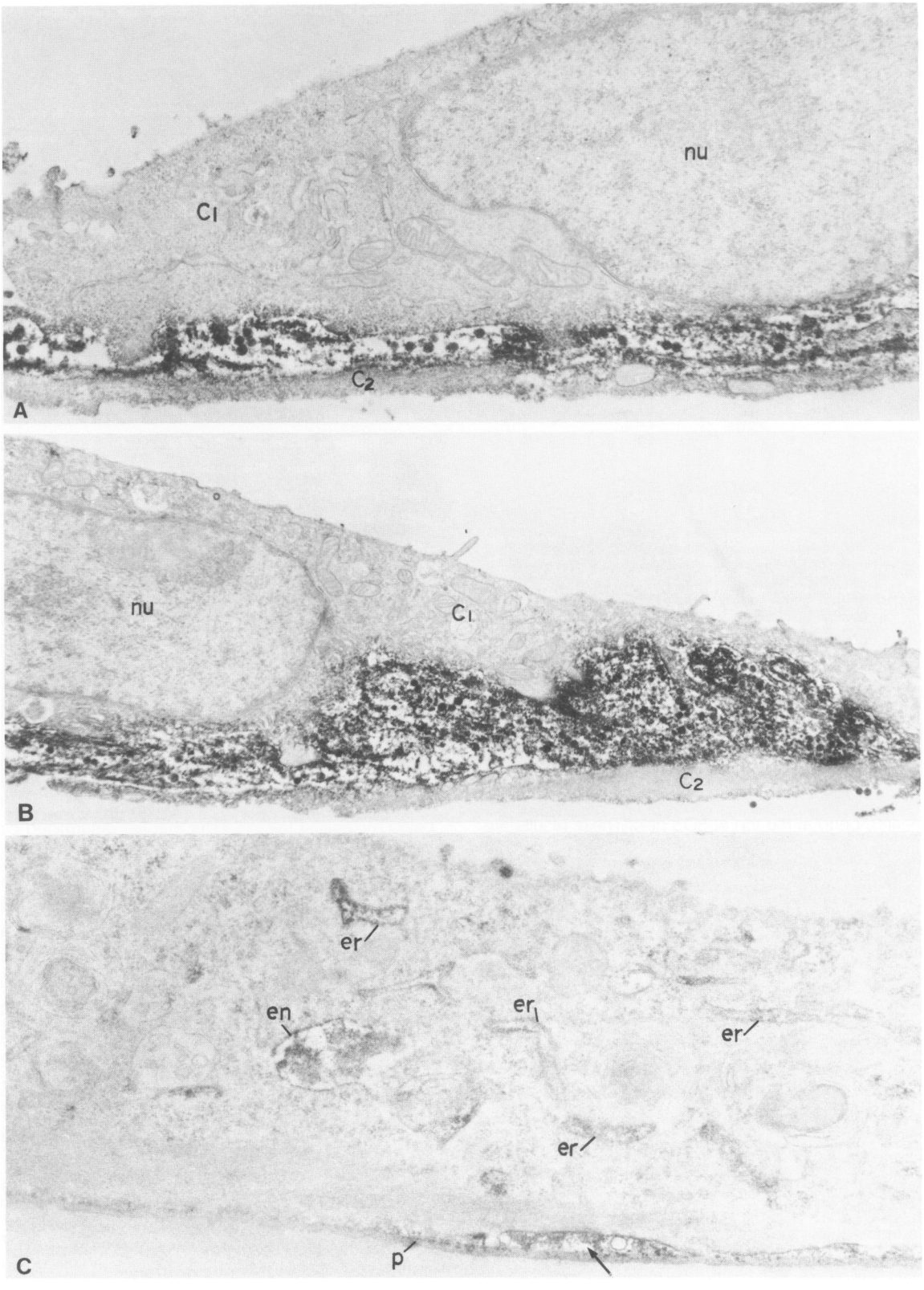

Figure 6. *Distribution of basement membrane HSPG in cultured glomerular epithelial cell monolayers as seen by immunoperoxidase staining. Cells were incubated sequentially with anti-BM HSPG, anti-rabbit Fab conjugated to HRP, and DAB medium. Large immunoreactive deposits of extracellular matrix material were found deposited between and beneath adjacent cells (C_1 and C_2). When the cells were permeabilized with 0.005% saponin (Figure 6C) before immunoperoxidase localization of the HSPG, intracellular staining could also be seen. Here reaction product is present in an endosome (en) and in a number of rough ER cisternae (er). Presumably the latter represents newly synthesized precursors of BM HSPG en route along the biosynthetic pathway. No staining of cell surfaces was seen, but deposits of loosely organized extracellular matrix were present beneath the cell (arrow). nu, nucleus; p, plastic substrate (A and B, ×19,000; C, ×33,000).*

the podocyte after radiolabeling of kidney slices with [^{35}S]-sulfate.[2] Endothelial cells may also contribute some, albeit a minor fraction, of BM HSPG to the GBM, as endothelial cells from various sources have been shown to produce HSPG, including BM HSPG, in culture.[24] Mesangial cells produce mainly CSPG[15] in culture, which is consistent with the finding in the glomerulus that these proteoglycans appear to be concentrated in the mesangial matrix.[42]

When BM HSPG were localized in glomerular epithelial cell cultures by immunofluorescence, they were found throughout the monolayer associated with an extracellular web-like matrix that colocalized with laminin. Their pattern of distribution was similar to that of BM HSPG in several other normal kidney and liver cell lines.[20,43] By immunoelectron microscopy after immunoperoxidase staining, BM HSPG were found in loosely organized deposits of extracellular matrix situated basally and between adjacent cells in the monolayer. Their size was generally larger than those found in the epithelial cell lines we studied previously,[20] although the glomerular epithelial cells, like the other cells lines,[20] did not make a morphologically recognizable basement membrane layer. Apparently additional unknown factors are required for the assembly of a bona fide, morphologically recognizable basement membrane layer by cells in culture.

Further work is now required to elucidate the factors controling the synthesis and secretion of BM HSPG and the laying down of a basement membrane by cultured glomerular epithelial cells to gain insights into how this cell regulates the synthesis and maintenance of the GBM components *in vivo*.

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