Biochemical and UltrastructuralStudies of Proteoheparan Sulfates Synthesized by PYS-2, a Basement Membrane–producingCell Line

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ABSTRACT The mouse teratocarcinoma-derived cell line, PYS-2, has been shown to produce laminin, a basement membrane–specific glycoprotein. In these studies we demonstrate that PYS-2 cells synthesize and secrete into the culture medium a proteoglycan which contains only heparan sulfate as its sulfated polysaccharide side chains, as well as type IV procollagen and laminin. The apparent molecular weights of the proteoglycan and its heparan sulfate side chain were estimated to be 400,000 and 25,000, respectively, by gel chromatography. A proteoheparan sulfate with properties closely similar, if not identical, to those of the proteoglycan in the medium, together with two heparan sulfate single chains of different molecular size, were extracted from the cell layer with 2% SDS in the presence of protease inhibitors. Ultrastructurally, a fine fibrillar intercellular matrix was recognized which contained discrete 100–200 Å diameter ruthenium red-positive granules interspersed throughout the filamentous meshwork. The PYS-2 cultures were shown by immunofluorescence to react with antibodies against the heparan sulfate-containing proteoglycan isolated from the mouse EHS sarcoma (Hassell, J. R., P. G. Robey, H. J. Barrach, J. Wilczek, S. I. Rennard, and G. R. Martin. 1980. Proc. Natl. Acad. Sci. U. S. A. 77:4494-4498). Immunoelectron microscopic examination, using the same antibodies, revealed that the proteoheparan sulfate was located not only at the edges but also within the interstices of the matrix. These findings indicate that PYS-2 cells synthesize and secrete a proteoglycan with properties similar to those of basement membrane proteoglycan. These cells may therefore serve as a useful model system for the study of the biosynthesis and structure of basement membranes.

The basement membrane is a felt-like complex of extracellular macromolecules that separates epithelial and endothelial cells from the adjacent matrix. Recent studies have elucidated the biochemical properties of some of the constituents of basement membranes. For example, the major collagenous component, known as type IV collagen (1), is found only in basement membranes (2, 3), and two large noncollagenous glycoproteins, fibronectin (4, 5) and laminin (6), are also thought to contribute to their structure. Fibronectin is broadly distributed in tissues (3) but laminin may be uniquely located in basement membranes (7). By ultrastructural criteria, the basement membrane is composed of a layer of low electron density adjacent to the cells (the lamina lucida) and a layer of greater electron density (the lamina densa). Recent immunoelectron microscopic studies suggest that the above constituents are predominantly located in one or another of these layers of the basement membrane (5, 7).

Glycosaminoglycans have also been shown to be integral constituents of basement membranes by biochemical and ultrastructural criteria. Heparan sulfate is a common sulfated glycosaminoglycan in basement membranes but both hyaluronic acid and chondroitin sulfates also occur (8–11). Hassell et al. (12) isolated a heparan sulfate-containing proteoglycan from a murine tumor, the EHS sarcoma, and demonstrated that antibodies against this proteoglycan reacted with several authentic basement membranes.

The mouse teratocarcinoma-derived cell line, PYS-2 (13), is related to the parietal endoderm cells of the developing embryo,
which are known to produce Reichert’s membrane (14). This cell line has been shown to produce a large amount of laminin (15) and could therefore be an in vitro experimental model for the production of basement membranes. However, neither the collagen nor proteoglycans synthesized by PYS cells have been characterized. In the present work, we have examined proteoglycan synthesis by PYS cells biochemically and analyzed the extracellular matrix using histochemical and immunocytochemical methods to determine whether these cells synthesize a type of proteoglycan that may be specific for basement membranes. We have also studied the synthesis of laminin and of type IV procollagen by these cells. We conclude that PYS cells may serve as a good model for the production of basement membranes.

MATERIALS AND METHODS

Materials

The following materials were purchased commercially: acid mucopoly saccharide kit as standards for electrophoresis and heparan sulfate (bovine kidney) from Serva, Heidelberg, Germany; cellulose acetate (Sepharose CL-4B) from Pharmacia Fine Chemicals, Piscataway, N.J.; carrier-free Na\(^{35}\)SO\(_4\) and D-[\(^{3}H\)]-glucosamine (34 Ci/mmol) from Amersham Corp., Arlington Heights, Ill.; 1-[\(^{3}H\)]proline (22.7 Ci/mmol) from New England Nuclear, Boston, MA; ruthenium red from Ted Pella, Inc., Tustin, CA; Iodofor I-4 emulsion from Iodofor, Ltd., Iford, fluorescein-conjugated goat anti-rabbit IgG antibody and fluorescein-conjugated rabbit anti-goat IgG antibody from Calbiochem-Behring Corp., La Jolla, CA. Purified hemocyanin was kindly supplied by Dr. James Koehler (University of Washington, Seattle).

Cell Culture and Metabolic Labeling

PYS-2 cells were obtained as a gift from Dr. John Lehman, University of Colorado, Denver. The cells were cultured in Dulbecco’s modiﬁed Eagle’s medium (DMEM) supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 16% VSP neonatal calf serum (Biocell Laboratories, Carson). Labeling was performed using confluent cultures. Cultures were preincubated for 1 h in DMEM supplemented with sodium ascorbate (50 μg/ml), β-amino proline (80 μg/ml), and penicillin/streptomycin, and then labeled with 25 μCi/ml of Na\(^{35}\)SO\(_4\) and 5 μCi/ml of [\(^{3}H\)]-glucosamine, with 25 μCi/ml of Na\(^{35}\)SO\(_4\) or, with 50 μCi/ml of [\(^{3}H\)]proline, in the same medium. Under these conditions, the incorporation of \(^{35}\)S was linear with time up to 24 h. Because the incorporation of \(^{35}\)S increased rapidly with increasing concentrations of serum in the labeling medium, and reached a plateau at a concentration of 4% (116% of the serum-free sample), the cultures were labeled in the presence of 4% serum in some experiments.

Processing of Culture Medium and Cell Layers

After labeling, the medium was harvested in the presence of protease inhibitors (0.2 mM phenylmethyl-sulphonyl fluoride (PMSF), 10 mM N-ethylmaleimide (NEM) and 20 mM EDTA) at 0°C. The cell layer was washed at 4°C with PBS containing the protease inhibitors. The wash was combined with the medium. After centrifugation at 400 g for 10 min to remove cellular debris, labeled macromolecules were precipitated from the supernatant by adding one part of (NH\(_4\))\(_2\)SO\(_4\) to two parts of supernatant (wt/vol). The precipitate obtained from centrifugation at 4°C was dissolved in 8 ml of 4 M deionized urea containing 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 1 mM NEM, and then chromatographed on a column (1.3 x 78 cm) of Sepharose CL-4B under the same conditions.

Immune Reagents

Goat anti-laminin antibodies and rabbit anti-basement membrane-proteoglycan antibodies were kindly donated by Dr. George R. Martin (National Institute of Dental Research, Bethesda, Md.). Goat anti-laminin antibodies were purified essentially by the same method described previously (18). Preparation of goat anti-basement membrane-proteoglycan antibodies (SAGE) (19) using a 4% stacking gel with a 5% separating gel. Fluorescence autoradiography was carried out as described previously (18). Protein was estimated by the method of Lowry et al. (20) using bovine serum albumin (BSA) as a standard. Hexuronic acid was estimated by the method of Bitter and Muir (21) using glucuronolactone as a standard. Glycosaminoglycans were identified by specific degradation, either with chondroitinases-AC II (22) or with nitrous acid (23). Immune reagents were used in some experiments.

Indirect Immunofluorescence

Cells were grown on coverslips and treated as described previously (25), with minor modifications. After the medium was removed, the cover slips were washed with DMEM containing 1.0 mg/ml of BSA at room temperature for 30 min.

Purification of Proteoglycans in the Medium

The sulfated proteoglycans, obtained by gel filtration of the medium proteins on Sepharose CL-4B in urea buffer, were pooled and concentrated on a Diaflo XM-100A membrane. The solution was dialyzed at 4°C against 4 M urea containing 50 mM Tris-HCl, pH 7.5, 0.2 mM PMSF, 2 mM EDTA, and 1 mM NEM, and then chromatographed on a column (0.7 x 11 cm) of DEAE cellulose with a NaCl gradient ranging from 0 to 0.7 M at 4°C. The sulfated proteoglycan fractions were pooled and again chromatographed on Diaflo XM-100A membrane. The proteoglycans were precipitated from the concentrated solution by adding 3 vol of 95% ethanol-1.3% potassium acetate at 0°C. After centrifugation, the precipitate was dissolved in 4 M guanidine-HCl containing 50 mM Tris-HCl, pH 7.5, 0.2 mM PMSF, 2 mM EDTA, and 1 mM NEM. Ultracentrifugation was carried out in a CsCl density gradient at an initial density of 1.45 g/ml in guanidine-HCl buffer at 10°C using a Beckman SW 50.1 rotor at 36,000 rpm for 50 h.
cell cultures, representative monolayers were fixed in situ with 0.1 M cacodylate buffered 3% glutaraldehyde, pH 7.3, containing 0.2% ruthenium red for 1 h at room temperature (26). The monolayers were then rinsed with 0.1 M cacodylate buffer containing 7.5% sucrose and 0.1% ruthenium red and postfixed with 0.1 M cacodylate-buffered 1% osmium tetroxide pH 7.3 containing 0.05% ruthenium red for 30 min. After a brief rinse with distilled water, the monolayers were dehydrated and embedded in epoxy resin as previously described (26). Thin sections were cut with a diamond knife on a LKB Ultratome III, double-stained with uranyl acetate and lead citrate (27), and examined in a JEOL 100-B electron microscope. Parallel dishes were incubated for 1 h with nitrous acid to specifically identify heparan sulfate-staining sites in the monolayers (23). Controls for this degradation consisted of similar dishes incubated with acetate buffer at pH 3.4 for the same time period. After these treatments, the dishes were rinsed with acetate buffer and the monolayers were fixed in the presence of ruthenium red as described above.

**Immunoelectron Microscopy**

Direct and indirect methods were used to localize proteoheparan sulfate. The antibodies against proteoheparan sulfate were the same antibodies used for the immunofluorescence studies. In the direct method, the purified antibodies were labeled with 125I-site by the chloramine T method (28, 29). The specificity of labeled antibodies was 18 µCi/µg. The final dilution of the antibodies was 1:100. As control, rabbit IgG (33 µg in 10 µl PBS) was labeled with Na225I in an identical fashion. The indirect method involves the conjugation of the electron-opaque molecule, hemocyanin, to goat anti-rabbit IgG using 5% glutaraldehyde as previously described (29).

Both the direct and indirect methods for the immunoelectron microscopic localization of proteoheparan sulfate involve fixing cells initially for 3 h in 3% of paraformaldehyde in PBS, anti-laminin (or anti-basement membrane proteoglycan) antibodies, and fluorescein isothiocyanate-conjugated rabbit anti-goat IgG or goat anti-rabbit IgG antibodies. Slides were examined with a Zeiss photomicroscope III equipped with epillumination.

**Electron Microscopic Histochemistry**

To demonstrate proteoglycans within the intercellular matrix of the PYS-2 cells, representative monolayers were fixed in situ with 0.1 M cacodylate-buffered 3% glutaraldehyde, pH 7.3, containing 0.2% ruthenium red for 1 h at room temperature (28). The monolayers were then rinsed with 0.1 M cacodylate buffer containing 7.5% sucrose and 0.1% ruthenium red and postfixed with 0.1 M cacodylate-buffered 1% osmium tetroxide pH 7.3 containing 0.05% ruthenium red for 30 min. After a brief rinse with distilled water, the monolayers were dehydrated and embedded in epoxy resin as previously described (26). Thin sections were cut with a diamond knife on an LKB Ultratome III, double-stained with uranyl acetate and lead citrate (27), and examined in a JEOL 100-B electron microscope. Parallel dishes were incubated for 1 h with nitrous acid to specifically identify heparan sulfate-staining sites in the monolayers (23). Controls for this degradation consisted of similar dishes incubated with acetate buffer at pH 3.4 for the same time period. After these treatments, the dishes were rinsed with acetate buffer and the monolayers were fixed in the presence of ruthenium red as described above.

**Components in the Culture Medium**

The material that precipitated from the culture medium of PYS-2 cells with (NH4)2SO4 was dissolved and chromatographed on Sepharose CL-4B (Fig. 1). The [35S] sulfate-labeled material was eluted from the column as a single peak with the Kav value of 0.31, whereas the [3H]glucosamine-labeled material was separated into two broad peaks by the gel filtration. The main [3H]-labeled peak (Kav = 0.15) was shown by SDS-PAGE under reducing conditions to be composed mainly of two high molecular weight, collagenase-resistant polypeptides with mol wt of ~400,000 and 200,000 (Fig. 2). Because these polypeptides reacted with anti-laminin antibodies (data not shown, but see the immunofluorescence in Fig. 9b), they were identified as subunits of laminin. When the culture was labeled with [3H]proline, there appeared a new small peak in the V0 fraction, in addition to the laminin peak and the broad, low molecular weight peak (Fig. 1). This [3H]proline-labeled peak was shown, by SDS-PAGE under reducing conditions, to contain a doublet with a mobility slightly faster than that of the small subunit of laminin (Fig. 2). This doublet was susceptible to digestion with collagenase (Fig. 2, lane 2) and reacted with antibodies against type IV collagen prepared from human placenta (24, data not shown). Because type IV procollagen is known to be composed of two subunits with apparent mol wt

**FIGURE 1** Gel chromatography on Sepharose CL-4B of the labeled material obtained from the culture medium of the PYS-2 cells labeled with both [3H]glucosamine and [35S] sulfate or with [3H] proline for 24 h. The column was run in 4M urea-Tris buffer containing protease inhibitors. Fractions of 3.6 ml were collected. The solid bar indicates the fractions pooled for further purification of the sulfated material. V0, void volume; Vp, total volume.

**FIGURE 2** SDS-PAGE of [3H] proline-labeled medium proteins resolved by Sepharose CL-4B chromatography (see Fig. 1). Proteins were incubated with (+) or without (−) bacterial collagenase and then analyzed in the presence of 50 mM dithiothreitol. Lane 1, proteins in fraction 29 of Sepharose CL-4B chromatogram (see Fig. 1); lane 2, collagenase digest of these proteins; lane 3, proteins in fraction 37; lane 4, collagenase digest of these proteins. FN indicates the migration position of fibronectin isolated from bovine plasma. PIV indicates the proα1(IV) and proα2(IV) chains of type IV procollagen.

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of 180,000 and 165,000 (2, 3), this doublet can be identified as type IV procollagen.

**Sulfated Proteoglycans in the Medium**

The peak containing sulfated material, pooled as shown in Fig. 1, was chromatographed on a DEAE cellulose column (Fig. 3). The $[^{3}H]$ glucosamine-labeled material was separated into two peaks. The main peak which eluted first was shown to contain laminin by SDS-PAGE (data not shown). The minor peak eluted at the same position as the sulfated peak. This sulfated peak was further purified by ultracentrifugation in a CsCl density gradient (Fig. 4). The bottom fraction with the density of over 1.50 g/ml contained most of the sulfated material, while the $[^{3}H]$-labeled material was recovered both in the bottom fraction and in the top fraction. This showed that contaminating glycoproteins were largely separated from the sulfated material by CsCl density gradient ultracentrifugation. The bottom fraction thus prepared from 40 150-mm plates contained 30 μg of protein and 22 μg of hexuronate.

An aliquot of the purified, sulfated material was precipitated by adding 3 vol of 95% ethanol-1.3% potassium acetate at 0°C and the precipitate was dissolved in 0.2 N NaOH. After incubation for 16 h at room temperature (which cleaves glycosaminoglycans from proteoglycans), the solution was neutralized by adding 0.2 N HCl at 0°C and then subjected to gel filtration on Sephadex G-200 (Fig. 5). The sulfated glycosaminoglycan was eluted from the column as a single peak with $K_{av}$ of 0.19. Because the sulfated material, before alkali treatment, was excluded from the column, this sulfated glycosaminoglycan was linked to a protein core. The apparent mol wt of the glycosaminoglycan was 25,000, based on the elution position of chondroitin sulfate side chains of known mol wt (34). The elution position of the glycosaminoglycan was not changed by digestion with chondroitinase-AC followed by gel filtration on the column (data not shown), but treatment with nitrous acid degraded the glycosaminoglycan completely to small molecules (Fig. 5). These findings suggest that this proteoglycan contains only heparan sulfate as sulfated polysaccharide side chains.

**Extraction of Sulfate Material from the Cell Layer**

After labeling with 25 μCi/ml of $[^{35}S]$ sulfate for 24 h in the presence of 4% serum, the cell layer was treated with 2% SDS containing protease inhibitors. The extract was separated into three radioactive peaks by gel chromatography on Sepharose CL-4B in SDS-buffer: $L$ ($K_{av} = 0.29$), $M$ ($K_{av} = 0.65$), and $S$ ($K_{av} = 0.88$) (Fig. 6). When the medium, treated with SDS, was chromatographed on the same column, there appeared only one sulfate-containing peak ($K_{av} = 0.30$) near the elution position of component $L$ isolated from the cell layer (Fig. 6). This elution pattern of the sulfated material in the medium is consistent with that obtained from the Sepharose CL-4B column equilibrated with the urea-buffer (shown in Fig. 1).

The three sulfate-containing peaks from the cell layer were pooled separately and lyophilized. The dried material was dissolved in water and the solution was dialyzed against 8 M urea containing 50 mM Tris·HCl, pH 7.5, 0.2 mM PMSF, 2 mM EDTA and 1 mM NEM at 4°C and then against the 4 M urea buffer for DEAE cellulose chromatography. The elution pattern from the DEAE cellulose column of the sulfated material from each sample was closely similar to that shown in Fig. 3; there occurred only one sulfate-containing peak in each sample (data not shown). The sulfated glycosaminoglycans were analyzed in the same way as those described in relation to Fig. 5. Mild alkali treatment of component $L$ produced a sulfated glycosaminoglycan that was eluted from the Sephadex G-200 column at the same position ($K_{av} = 0.19$) as the heparan sulfate side chain of the proteoglycan in the medium (see Fig. 5), while the nontreated component $L$ was excluded from the column (Fig. 7). The elution positions of both component $M$ ($K_{av} = 0.19$) and component $S$ ($K_{av} = 0.46$) were not changed by alkali treatment, suggesting that they were not linked to a protein core, or to only a small peptide. The apparent mol wt of component $M$ was estimated to be 25,000, which was the same value as that of the heparan sulfate side chain of the proteoglycan. Component $S$ had an average mol wt of 12,000, based on the elution position of chondroitin sulfates of known mol wt (34).

The alkali-treated glycosaminoglycans were digested either with chondroitinase-AC or with nitrous acid to investigate their chemical nature. None of the samples produced the specific disaccharide products of chondroitinase-AC digestion (data not shown), whereas incubation with nitrous acid degraded all of the glycosaminoglycans to small molecules (Fig. 7). These findings show that component $L$, extracted from the cell layer with the SDS solution, has properties similar, if not identical, to those of the proteoheparan sulfate in the culture medium.

**Glycosaminoglycans**

To confirm the finding that the predominant sulfated glycosaminoglycan synthesized by these cells is heparan sulfate,
Sephadex G-200 column chromatography of the purified, sulfated component (see Fig. 4) treated either with 0.2 N NaOH (●—●) or with nitrous acid after treatment with 0.2 N NaOH (○—○). The column (0.7 x 28 cm) was equilibrated with 0.2 M NaCl containing 20 mM Tris-HCl, pH 7.5, and 1 mM sodium azide at 4°C. The solid bar indicates the elution position of the non-alkali-treated sample. Fractions of 0.22 ml were collected. V0, void volume. Vt, total volume.

Indirect Immunofluorescence

Sephagrose CL-4B gel chromatography in SIDS buffer of SIDS extracts of the cell layer (●—●) and the culture medium (○—○). PYS-2 cultures were labeled with [35S]sulfate for 24 h. Horizontal lines indicate fractions of components L, M, and S which were pooled for further analyses. Fractions of 1.8 ml were collected. V0, void volume. Vt, total volume.

glycosaminoglycans were isolated directly by pronase digestion either from the culture medium or from the cell layer and analyzed by two-dimensional electrophoresis on cellulose acetate. There appeared in each case only one Alcian Blue-stained spot with a mobility corresponding to that of authentic heparan sulfate (Fig. 8 a and c). The autoradiogram of the sample from the medium also exhibited only one radioactive spot in exactly the same position as that of the Alcian Blue-stained spot (Fig. 8 b). The main radioactive spot in the sample from the cell layer was also found at the position of the Alcian Blue-stained spot. Additionally, there was a very faint radioactive spot with mobility corresponding to that of authentic chondroitin sulfates (Fig. 8 d).

The Alcian Blue-stained spots both from the medium and from the cell layer were shown to be resistant to digestion with chondroitinase-AC but susceptible to degradation with nitrous acid (data not shown, but see Fig. 5), indicating that the material contained heparan sulfates.

Indirect Immunofluorescence

The PYS-2 cultures, especially the extracellular matrices, reacted both with anti-laminin antibodies and with anti-base-

Chondroitin 4- and 6-sulfates, dermatan sulfate, and heparan sulfate (50 μg of each glycosaminoglycan) were added as carriers. Solid bars indicate the elution position of the sample before NaOH treatment. V0, void volume. Vt, total volume.

Two-dimensional electrophoresis of glycosaminoglycan preparations on cellulose acetate. The electrophoretograms shown are preparations from the culture medium of PYS-2 cells labeled with [35S] sulfate for 24 h (a) and the corresponding radioautogram (b); preparation from the cell layer (c) and the corresponding radioautogram (d). Note a faint spot (arrow) at the position of authentic chondroitin sulfates. A reference glycosaminoglycan mixture which consists of hyaluronic acid (HA), heparan sulfate (HS), keratan sulfate (KS), dermatan sulfate (DS), chondroitin 4-sulfate (CS), and heparin (HP) was used.
the extracellular matrix in the form of fibrils (Fig. 9f). The proteoheparan sulfate synthesized by PYS cells is thus shown to be immunologically related to the proteoglycan synthesized by EHS sarcoma and to be deposited extracellularly.

**Ultrastructural Examination**

Confluent monolayers consisted of cells arranged in a contiguous pattern separated by pockets of interstitial space filled with a finely granular matrix (Fig. 10). The majority of the cells contained abundant rosettes of polyribosomes and distended cisternae of rough endoplasmic reticulum which contained material resembling that present in the intercellular space. Cross sections of the monolayers revealed that the matrix did not form a continuous sheet beneath the cultured cells but rather was present in pockets formed either where cells came into close contact (Fig. 11) or by long, thin extension of cytoplasm (Fig. 10). High magnification of the intercellular matrix revealed that it was composed of a disorganized array of fine filaments measuring 20 to 50 Å in diameter and of 90 to 150 Å Diam ruthenium red-positive granules (Fig. 12). The fine filaments were frequently observed attached to the plasma membrane of the cells and in communication with the filamentous material of the intercellular space (Fig. 12). Some of the membrane filaments frequently exhibited a granular substructure and demonstrated an affinity for ruthenium red.

Discrete ruthenium red-positive granules were present interspersed among the fine filaments in the intercellular matrix, and in some areas they appeared to be regularly spaced with center-to-center distances measuring ~600 to 900 Å (Fig. 12). Treatment of the monolayers with nitrous acid completely removed the small ruthenium red-positive granules but left the bulk of filamentous meshwork of the intercellular matrix intact (data not shown).

Autoradiographs of cells incubated with 125I-labeled anti-proteoglycan antibodies revealed that the antigen was distributed throughout the matrix between the cells and not confined to any particular region of the intercellular matrix (Fig. 13). Control cells incubated with 125I-labeled IgG were completely
negative. A similar distribution of antibodies was observed using the electron-dense marker hemocyanin coupled to goat anti-rabbit IgG antibodies (Fig. 14). The label was prominent both at the edges and within the interstices of the matrix. In addition, the markers were infrequently observed attached to the surface of the cells in small clumps (Fig. 14, inset). Control
monolayers which were incubated with nonimmune rabbit IgG and then labeled with hemocyanin conjugated to goat antirabbit IgG antibodies were negative.

DISCUSSION

Although basement membranes are believed to be involved in a variety of cellular functions (for review, see reference 35), a biochemical study using normal tissues is difficult due to the low content of basement membrane in tissues and to the poor solubility of its components. A transplantable mouse tumor, the EHS sarcoma, has been shown to be a useful experimental model for basement membrane production (3, 6, 12, 36). Additionally, several cell culture systems have been suggested as in vitro experimental models (15, 24, 37-40). However, proteoglycan synthesis has been examined in none of these in vitro systems. The present biochemical and histological studies re-
reveal that the PYS-2 cells synthesized, secreted, and accumulated a particular type of proteoheparan sulfate as well as laminin and type IV procollagen. This proteoheparan sulfate was virtually the only sulfated proteoglycan synthesized and secreted by the cell line, and was shown to be immunologically related to the basement membrane proteoglycan isolated from the EHS sarcoma (12).

The overall size of this proteoheparan sulfate was roughly estimated to be 400,000 based on its elution position from Sepharose CL-4B (12). The macromolecule has a ratio of hexuronate to protein of 2:3. Assuming that the heparan sulfate side chain is composed of a number of repeating units of hexuronyl-hexosamine bearing one sulfate residue, the heparan sulfate to protein ratio would be 5:3. Because the average mol wt of the heparan sulfate side chains is 25,000, this proteoglycan contains, on the average, 10 heparan sulfate side chains per molecule. This proteoglycan is considerably larger than the heparan sulfate proteoglycans isolated from hepatocyte plasma membranes (41, 42) and from kidney glomerular basement membrane (43). However, the molecule is smaller, both in overall size and in the size of its heparan sulfate side chains, than the proteoglycan isolated from the EHS sarcoma (12).

By histochemical and immunocytochemical methods, the proteoheparan sulfate synthesized and deposited as an insoluble matrix by PYS cells could be localized throughout the fine fibrillar material between the cells of the monolayers. The size and staining characteristics of the granules observed in the matrix after fixing the monolayers in the presence of ruthenium red were similar to those of the granules identified in basement membranes of various organs in vivo (8, 44-46). In the glomerular basement membrane, these small granules have been shown to contain heparan sulfate (9, 11), and similar granules in basement membranes below the endothelium of small and large blood vessels resist digestion with chondroitinase ABC, suggestive of their heparan sulfate nature (46). The heparan sulfate ruthenium red-positive granules associated with basement membranes are clearly smaller (100–200 Å in Diam) than the larger ruthenium red-positive proteoglycan granules (200–500 Å in Diam) present in the intercellular matrix of various tissues, including cartilage (47) and blood vessels (46). These larger granules have been shown to contain principally chondroitin sulfate or a hybrid chondroitin-dermatan sulfate mixture.

The granular nature of the proteoglycan identified by ruthenium red staining undoubtedly represents an artifact incurred during tissue processing for electron microscopy, because purified preparations of proteoglycans appear as extended bottlebrush structures when examined in the electron microscope (47, 48). Because glycosaminoglycans are highly charged anionic polymers and occupy very large hydrodynamic domains, displacement of water by interaction with ruthenium red could alter their native configuration causing them to occupy a smaller, less hydrated volume. Removal of water during processing could then lead to further collapse of the once extended macromolecules.

Recently, Hascal demonstrated that the size of ruthenium red-stained granules present in cartilage matrix corresponded to the width of purified spread proteoglycan monomers (47). More recently, Iozzo et al. (49) have demonstrated a direct correlation between the size of the ruthenium red granules and the molecular weight of the major populations of proteoglycans present in normal and malignant human colon. It is interesting to note in this regard that tissues which contain predominately large ruthenium red granules such as cartilage and blood vessels possess proteoglycans with mol wt in excess of $1 \times 10^6$. On the other hand, the proteoglycans present in basement membranes which contain the smaller ruthenium red granules possess mol wt in the range of $75 \times 10^3$ to $750 \times 10^3$ (12, 41–43). The proteoheparan sulfate produced by the PYS cells and deposited in the matrix of these cultures falls within this range.

The finding that heparan sulfate is virtually the only glycosaminoglycan synthesized, secreted, and accumulated by PYS cells is unusual, because various tissues that produce basement membranes have been shown to synthesize not only heparan sulfate but also chondroitin sulfate and hyaluronic acid (9-11, 44, 45). For example, the labeled glycosaminoglycans produced by mouse mammary epithelium are hyaluronic acid (~55%), chondroitin sulfates (~5%), and heparan sulfate (~40%) (10). Rat glomeruli synthesize predominantly heparan sulfate (~50%), in addition to hyaluronic acid (~10%) and chondroitin sulfates (~20%) (11). The biosynthesis of proteoheparan sulfate may be more complicated than that of other sulfated proteoglycans, because this proteoglycan contains both iduronic acid and glucuronic acid, and both N- and O-sulfated groups in its polysaccharide moiety (50). PYS-2 cells may therefore be useful in elucidating the mechanism and regulation of synthesis of proteoheparan sulfate, a macromolecule which may be involved in cell surface-extracellular matrix interactions (51, 52).

The occurrence of two heparan sulfate single chains with different molecular weights in the extract of the cell layer (component M and component S in Fig. 6) is puzzling. One explanation is that these chains are artificial degradation products produced during the extraction procedure, even though the SDS solution contained enzyme inhibitors. However, under the same extraction conditions endothelial cells from bovine aorta, which synthesize at least two distinct types of proteoheparan sulfate, yielded, if any, only a very small amount of single chain heparan sulfates, whereas a human epidermoid carcinoma cell line, A-431, yielded a single-chain heparan sulfate similar to component S, as well as a proteoheparan sulfate (Oohira, Wight, and Bornstein. Unpublished observation). Furthermore, pulse-chase experiments revealed that the predominant sulfated material in the SDS extract of the 4-h-pulse-labeled culture was component L. Small amounts of components M and S were found. Prolonged chase incubation (up to 20 h) resulted in an increase in the radioactivity of component S and a decrease in the radioactivity of both components L and M (data not shown). These observations suggest that some cell lines, perhaps those derived from tumors, rapidly process endogenous proteoheparan sulfates into single-chain glycosaminoglycans. Alternatively or additionally, there is the possibility that cells synthesize protein-free glycosaminoglycans under certain conditions. For example, Culp et al. (51) propose that heparan sulfate chains as well as proteoheparan sulfate exist in cell-substrate adhesion sides and may play a role in cell attachment-detachment. Newly synthesized single-chain chondroitin sulfates were also found in aged costal cartilages from the rat, although the amount was very small compared to total glycosaminoglycans (53).

In addition to proteoheparan sulfate, PYS-2 cells synthesize and secrete a large amount of laminin into the culture medium. As shown in Figs. 1 and 2, laminin could be separated from type IV procollagen, by chromatography of medium proteins on Sepharose CL-4B. Laminin was further purified by DEAE cellulose chromatography (Fig. 3). Thus, three unique constituents of basement membranes could easily be separated from each other, demonstrating that PYS cells can serve as a good source of basement membrane components. The yield of pure
laminin from the 24-h culture medium of 30 150-mm plates is >1 mg. Using this preparation, we have prepared specific rabbit anti-laminin antisera which, by the ELISA method, do not crossreact with type IV collagen from human placenta, with fibronectin from bovine plasma, or with proteoglycan from PYS-2 culture medium (unpublished observations).

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REFERENCES

1. Kefalides, N. A. R., A. Alper, and C. C. Clark. 1979. Biochemistry and metabolism of basement membranes. Int. Rev. Cytol. 61:167-228.

2. Bornstein, P., and H. Sage. 1980. Structurally distinct collagen types. Annu. Rev. Biochem. 49:957-1003.

3. Timpl, R., and G. R. Martin. 1981. Components of basement membranes. In: Immunocytochemistry of the Extracellular Matrix. H. Furchtmy, editor. CRC Press, Boca Raton, in press.

4. Madri, J. A., J. R. Holli, and J. M. Foidart. 1980. Ultrastructural localization of fibronectin and laminin in the basement membranes of the murine kidney. J. Cell Biol. 86:682-687.

5. Timpl, R., H. Rohde, P. G. Robey, S. I. Renard, J. M. Foidart, and G. R. Martin. 1979. Laminin - a glycoprotein from basement membranes. J. Biol. Chem. 254:9933-9937.

6. Foidart, J. M., E. W. Bere, Jr., M. Yaar, S. J. Rennard, M. Gullino, G. R. Martin, and S. I. Katz. 1980. Distribution and immunoelectron microscopic localization of laminin, a noncollagenous basement membrane glycoprotein. Lab. Invest. 42:336-342.

7. Kanwar, Y. S., and M. G. Fargnudi. 1979. Anionic sites in the glomerular basement membrane. In vivo and in vitro localization to the lamina rara by cationic probes. J. Cell Biol. 181:177-153.

8. Kefalides, N. A., ed. 1978. Biology and chemistry of basement membranes. Academic Press, New York, 615 p.

9. Timpl, R., G. R. Martin, P. Bruckner, G. Wick, and H. Wiedemann. 1978. Nature of the collagenous protein in a tumor basement membrane. J. Cell Biol. 80:43-52.

10. Oldberg, A., L. Kjebbin, and M. Hook. 1977. Cell-surface associated structural proteins in collagenous basement membranes. Dev. Biol. 57:327-327.

11. Oldberg, A., L. Kjebbin, and M. Hook. 1977. Cell-surface associated structural proteins in collagenous basement membranes. Dev. Biol. 57:327-327.

12. Oldberg, A., L. Kjebbin, and M. Hook. 1977. Cell-surface associated structural proteins in collagenous basement membranes. Dev. Biol. 57:327-327.