Multiple Heparan Sulfate Proteoglycans Synthesized by a Basement Membrane Producing Murine Embryonal Carcinoma Cell Line†

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ABSTRACT: The murine embryonal carcinoma derived cell line M1536-B3 secretes the basement membrane components laminin and entactin and, when grown in bacteriological dishes, produces and adheres to sacs of basement membrane components. Heparan sulfate proteoglycans have been isolated from these sacs, the cells, and the medium. At least three different heparan sulfate proteoglycans are produced by these cells as determined by proteoglycan size, glycosaminoglycan chain length, and charge density. The positions of the *N*- and *O*-sulfate groups in the glycosaminoglycan chains from each proteoglycan appear to be essentially the same despite differences in the size and culture compartment locations of the heparan sulfate proteoglycan. Additionally, small quantities of chondroitin sulfate proteoglycans are found in each fraction and copurify with each heparan sulfate proteoglycan. Because this cell line appears to synthesize at least three different heparan sulfate proteolyglycans which are targeted to different final locations (basement membrane, cell surface, and medium), this will be a useful system in which to study the factors which determine final heparan sulfate proteoglycan structures and culture compartment targeting and the possible effects of the protein core(s) on heparan sulfate carbohydrate chain synthesis and secretion.

Basement membranes are specialized extracellular structures which separate epithelial or endothelial cell layers from the underlying stroma (Kefalides et al., 1979). Biochemical and immunological analyses of basement membranes have shown the presence of collagen type IV (Bornstein & Sage, 1980; Kefalides et al., 1979), which is chemically distinct from interstitial collagens, and the glycoproteins laminin (Chung et al., 1979; Foidart et al., 1980; Madri et al., 1980; Timpl et al., 1979), entactin (Carlin et al., 1981), and heparan sulfate proteoglycan (Gordon & Bernfield, 1980; Hassell et al., 1980; Kanwar & Farquhar, 1979; Kanwar et al., 1981; Oohira et al., 1982, 1983; Parthasarthy & Spiro, 1982). These same molecules are produced by a variety of cell types which can be used as model systems for the study of the synthesis of basement membrane components. In particular, these include the EHS sarcoma (Hassell et al., 1980), various cultured endothelial cell types (Oohira et al., 1983), and the murine PYS (Oohira et al., 1982) and M1536-B3 (Chung et al., 1979) cell lines. However, to date, the study of basement membrane components produced by most of these cells has been performed with extracts of excised tumors (Hassell et al., 1980), the culture media (Oohira et al., 1982), or the material present in the cell layer (Oohira et al., 1983).

The murine M1536-B3 cell line derived from an embryonal carcinoma and developed by Chung and co-workers is a unique exception to other systems under study. These cells have been shown to synthesize, adhere to, and proliferate on sacs of the basement membrane components laminin and entactin (Carlin et al., 1981; Chung et al., 1977a,b, 1979), which can be separated from the cells for analyses (Chung et al., 1977b). These observations suggest that the M1536-B3 cell is a useful model system to study basement membranes in the absence of the influence of other cell types. Furthermore, since these sacs lack detectable collagen, these studies indicate that collagen is not required for the formation, from known basement membrane components such as laminin and entactin, of an organized structure capable of supporting cell growth. We now report that these sacs of basement membrane molecules also contain a heparan sulfate proteoglycan (HSPG),[1] as has been found in other basement membranes (Kanwar & Farquhar, 1979). In addition, we have found that the M1536-B3 cells produce at least two other unique heparan sulfate proteoglycans which are localized in different culture compartments.

Materials and Methods

Materials. Trypsin (1× crystallized) was obtained from Worthington Biochemical Corp. Cytochalasin B was obtained from Sigma Chemical Co. and chondroitin ABC lyase from Miles Laboratories. The radioisotopes $H_2{}^{35}SO_4$ (carrier free), D-[6-^{3}H(N)]glucosamine (20 Ci/mmol), D-[1-^{14}C]glucosamine (55 Ci/mol), D-[1-^{3}H(N)]galactose (14.2 Ci/mmol), and L-[G-^{3}H]serine (5 Ci/mmol) were purchased from New England Nuclear. Sephadex G-50 and G-100, Sephacryl S-200 and S-300, and DEAE-Sephadex A-25 were obtained from Pharmacia Fine Chemicals, Inc. Dowex AG 1-X8 (200–400 mesh) and AG 50W-X8 (200–400 mesh) and Bio-Gel A-15m, Bio-Gel P10, and Bio-Gel P2 were obtained from Bio-Rad Laboratories. The DE-52 DEAE-cellulose was obtained from Whatman. The nonsulfated disaccharide 2,5-anhydro-*O*-(α-L-idopyranosyluronic acid)-D-mannitol (IM) was a gift from Dr. B. Weissmann of the University of Illinois, Chicago.

Cell Culture. The M1536-B3 cells were obtained from Dr. A. E. Chung in whose laboratory they were derived (Chung et al., 1977a). The cells were maintained in our laboratory as previously described for other murine cell lines (Underhill & Keller, 1975). For the isolation of basement membrane sacs, 5×10^5 cells were added to 100-mm bacteriology dishes containing 8 mL of Dulbecco's modified Eagle's minimal

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[1] Abbreviations: HSPG, heparan sulfate proteoglycan; GAG, glycosaminoglycan; CB HSPG, cytochalasin B HSPG; low M_r HSPG, low molecular weight medium HSPG; high M_r HSPG, high molecular weight medium HSPG; BM HSPG, basement membrane HSPG; Tris, tris(hydroxymethyl)aminomethane; Cl_3CCOOH, trichloroacetic acid; Gdn·HCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate.

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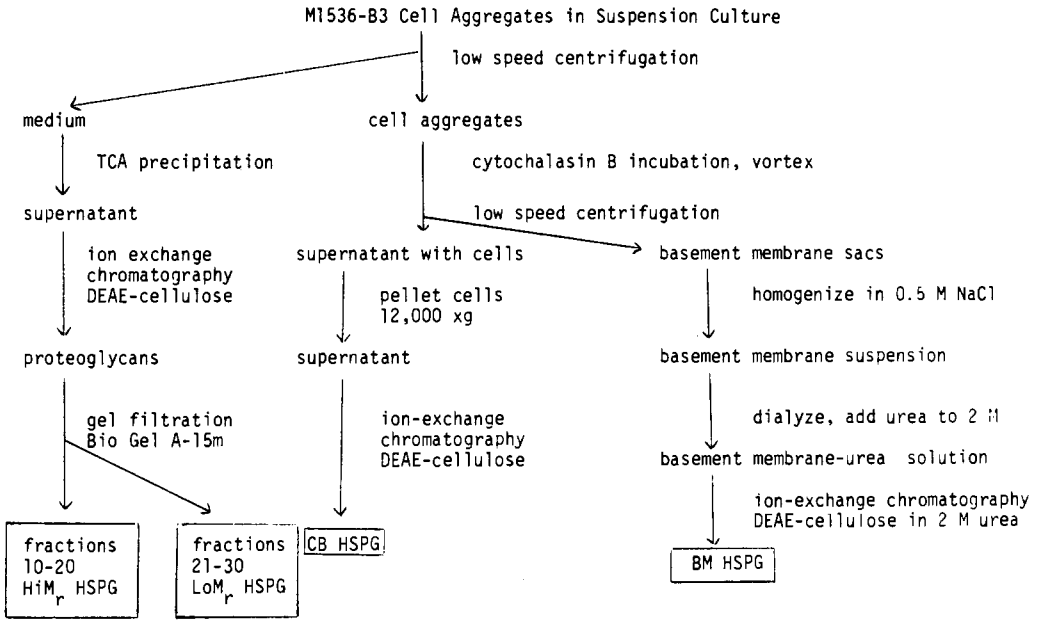

FIGURE 1: Preparation of M1536-B3 heparan sulfate proteoglycans.

essential medium containing 10% fetal bovine serum and grown for 10 days (Chung et al., 1977b). In these cultures, the cells do not attach to the dishes but make and adhere to sacs of basement membrane components. Incorporation of radioisotopically labeled metabolites was performed with modified media in order to maximize incorporation of radiolabels: low sulfate medium (Underhill & Keller, 1975) with $H_2{}^{35}SO_4$ (5 μCi/mL) for 10 days or low glucose medium (Underhill & Keller, 1975) for 6 days in the presence of either D-[6-^{3}H]glucosamine (1 μCi/mL), D-[1-^{14}C]glucosamine (0.5 μCi/mL), or D-[1-^{3}H]galactose (1 μCi/mL).[2] As reported earlier for other cell types (Underhill & Keller, 1975), the heparan sulfate proteoglycans obtained from M1536-B3 cells grown in low glucose or low sulfate medium are identical by size and charge density with [^{3}H]glucosamine-labeled proteoglycans obtained from cultures labeled in normal medium. Cell cultures to which [^{35}S]sulfate was added at days 0, 2, 4, 6, and 8 showed that all HSPGs after 2-day lag are continuously synthesized during the entire period of growth (see Table I). [G-^{3}H]Serine (1 μCi/mL) was added to $H_2{}^{35}SO_4$-labeled cells 2 days prior to harvesting because our work with Swiss mouse 3T3 cells has suggested that some radiolabel from the [^{3}H]serine is metabolically converted to carbohydrate after 48 h.[3] We have recently labeled the M1536-B3 cells with a mixture of [^{3}H]leucine, [^{3}H]valine, and [^{3}H]isoleucine (which are not glucogenic) by using the same protocol as for [^{3}H]serine, and the same labeling pattern observed with [^{3}H]serine label is found.

Isolation of Proteoglycans. The cell–basement membrane complexes were pelleted out of the medium and washed with buffered saline (0.137 M NaCl, 1 mM KH_2PO_4, 2 mM KCl, 8 mM Na_2HPO_4, 1 mM $MgCl_2$, and 20 mM Tris-HCl, pH 7.4) containing protease inhibitors (1 mM *o*-phenanthroline, 0.1 mM phenylmethanesulfonyl fluoride, 1 mM *N*-ethylmaleimide or 1 mM iodoacetic acid, and 1 μg/mL soybean trypsin inhibitor) by low-speed centrifugation in a clinical centrifuge. Cells were subsequently removed from the basement membrane sacs by using essentially the method reported previously (Chung et al., 1977b). This methodology is summarized in Figure 1. Briefly, washed cell–basement membrane complexes were incubated in buffered saline with protease inhibitors containing 20 μg/mL cytochalasin B for 6 h at 37 °C. The suspensions were then vortexed and the basement membranes pelleted by low-speed centrifugation in a clinical centrifuge (about 50*g* for 2 min), leaving the detached cells in suspension. The basement membranes were washed with the same buffer until inspection with phase-contrast microscopy revealed clean membranes devoid of attached cells. The purified basement membranes were then homogenized in 0.5 M NaCl with protease inhibitors (as above), stirred at 4 °C for 48 h, and then dialyzed overnight against distilled water. The flocculent precipitate which formed in the dialysis bag was dissolved by adding solid urea to a final concentration of 2 M. The cytochalasin B buffered saline supernatant, which contains the detached cells, was spun at 12000*g* for 10 min in order to remove the cells and cell debris, which were discarded. The bulk serum proteins in the medium were precipitated with 7% Cl_3CCOOH at 4 °C [it has been shown that no heparan sulfate is precipitated under these conditions (Johnston et al., 1979; Kraemer, 1971)], and the precipitate was removed by centrifugation at 16000*g* for 10 min. More recently, the medium heparan sulfate proteoglycans have been purified in the absence of the Cl_3CCOOH precipitation step in order to obtain the laminin and entactin in the medium. With this latter methodology, the same proteoglycans were isolated in about the same amounts, indicating

Table I: Sulfate Incorporation into Heparan Sulfate Proteoglycans

fraction	day 2[a]	4	6	8	10
basement membrane	22 950[b]	23 870	27 240[c]	56 570	49 140
cytochalasin B	44 220	43 070	ND[d]	101 330	77 610
high M_r medium	4 400	7 783	4 965[c]	20 419	24 817
low M_r medium	4 400	9 758	16 609[c]	52 505	49 043

[a] Days grown in the presence of labeled substrate. All cultures were initiated on the same day and harvested on day 10. Isotope was added to two dishes on day 0, and thereafter on every second day two more dishes were labeled. [b] Total counts per minute obtained in each fraction after DEAE ion-exchange chromatography. [c] These are minimal values as some material (<50%) was lost during analysis. [d] ND, not determined.

[2] The 6-day growth period in low glucose medium was chosen because the cells grew poorly after 6 days in this medium.

[3] L. J. Lowe-Krentz and J. M. Keller, unpublished results.

that the use of Cl_3CCOOH had no effect on the proteoglycan structure or yield. In both cases, the resultant supernatant, which contains the proteoglycans, was dialyzed extensively against distilled water. The medium and cytochalasin B supernatant fractions containing HSPGs were applied to ion-exchange columns of DEAE-cellulose (in 10 mM NaCl–20 mM Tris-HCl, pH 7.0) and eluted with a linear gradient of 10 mM–1 M NaCl (Underhill & Keller, 1975). The 2 M urea basement membrane extract containing HSPGs was treated similarly except that all solutions contained 2 M urea.

Two alternative methods for isolating the proteoglycans from the cell–basement membrane complexes were employed in order to demonstrate that no degradation of the proteoglycan glycosaminoglycan chains was occurring during the 6-h cytochalasin B incubation. In the first method, the [^{35}S]-sulfate-labeled cell–basement membrane complexes were suspended in buffered saline (without $MgCl_2$ or protease inhibitors) containing 1 mg/mL trypsin for 30 min at 37 °C. The trypsinized cell–basement complexes were pelleted at low speed in a clinical centrifuge. [^{35}S]Sulfate was associated with both the released proteoglycan material present in the supernatant fraction and the pelleted cell–basement membrane complexes. The pellet was washed with buffered saline, treated with cytochalasin B, and processed as described above. Both the trypsin supernatant and the cytochalasin B extract were analyzed as described below. Alternatively, the proteoglycans were extracted from [^{35}S]sulfate-labeled cell–basement membrane complexes, with buffered saline containing 4 M Gdn·HCl, and debris removed by centrifugation at 12000g for 10 min. In both the cytochalasin B preparation and the guanidine extraction, greater than 90% of the [^{35}S]sulfate-labeled materials were extracted. The guanidine-extracted materials were subjected to dissociative CsCl gradient centrifugation as described below. The guanidine-extracted proteoglycans from the three densest gradient fractions were analyzed by DEAE-cellulose ion-exchange chromatography and gel filtration as described below.

Analytical Methods. Analysis of radioactivity was performed as described previously (Underhill & Keller, 1975; Keller et al., 1978, 1980). Ion-exchange chromatography was performed as above or on DEAE-Sephadex A-25 eluted with a linear gradient from 50 mM to 1.5 M NaCl as described previously (Keller et al., 1980). Gel filtration was performed on a 1 × 60 cm column of Bio-Gel A-15m, Sephadex G-100, or Sephacryl S-200, a 1 × 45 cm column of Sephadex G-50, or a 1 × 200 cm column of Sephacryl S-300, Bio-Gel P2, or Bio-Gel P10 in 20 mM Tris-HCl, pH 7.0, containing 1.0 M NaCl. Two-milliliter fractions were collected from all columns except Sephadex G-50 (where 1.2-mL fractions were collected). Blue dextran or azoprotein was used to determine V_0, and phenol red or ^{35}S-labeled inorganic sulfate was used to determine V_t. Columns were calibrated with defined dextran fractions as described previously (Johnston et al., 1979). The molecular weight values obtained are only estimates since the physical parameters of the molecules which determine their behavior on these columns (e.g., frictional coefficients and protein–carbohydrate contents) are unknown and thus a definitive set of standards cannot be selected. The values obtained, however, do give relative size estimates and are consistent with values obtained by others (see Discussion). Recoveries of radiolabeled material from these columns were typically greater than 95%.

Analysis of radiolabeled (^{3}H or ^{14}C) hexosamines was as previously described (Underhill & Keller, 1975). Uronic acid analysis of HSPGs prepared from cells grown in the presence of [^{3}H]galactose was as described previously (Keller et al., 1980). A minimum of 2500 cpm of uronic acid or hexosamine was used in these determinations.

Density gradient centrifugation was performed on CsCl gradients containing 4 M Gdn·HCl in 0.15 M NaCl–20 mM Tris-HCl, pH 8.0. The sample and 1.2 g of CsCl were dissolved in 3.6 mL of buffer, and the centrifugation was carried out for 72 h by using a Beckman SW 50.1 rotor at 100000g and 20 °C (Hassell et al., 1980). The gradients were collected in 10 equal fractions through a needle inserted into the bottom of the tube. Aliquots (100 μL of each fraction) were weighed to determine the density gradient or assayed for radioactivity. In all cases, essentially 100% of the applied radioactive sample was recovered from the gradient.

Proteoglycans or released glycosaminoglycan (GAG) chains were treated with chondroitin ABC lyase as previously described (Johnston et al., 1979). Degradation of the heparan sulfate GAGs by nitrous acid employed low pH conditions (Underhill & Keller, 1975; Keller et al., 1980). Alkaline elimination of the O-linked carbohydrate chains was performed in 0.5 M NaOH containing 1 M $NaBH_4$ for 24 h at room temperature (Kraemer & Smith, 1974). The reaction products were subsequently dialyzed and analyzed as described under Results.

The methods used for the oligosaccharide "fingerprinting" technique have been reported previously (Keller et al., 1980). This procedure involves separation of the inorganic sulfate released by nitrous acid treatment of HSPGs from the bulk of the O-sulfated oligosaccharides by gel filtration on Bio-Gel P2. In the fingerprinting procedure, one [^{14}C]glucosamine-labeled and one [^{3}H]glucosamine-labeled HSPG were mixed and degraded with nitrous acid, and the bulk of the oligosaccharides were separated from free sulfate by gel filtration on Bio-Gel P2. These oligosaccharides were then analyzed by gel filtration on Bio-Gel P10. The individual peaks from the P10 column were subsequently analyzed by ion-exchange chromatography on DEAE-Sephadex A-25.

Results

Isolation of Heparan Sulfate Proteoglycans. HSPGs were isolated from M1536-B3 cells by using the techniques described under Materials and Methods and outlined in Figure 1. The [^{35}S]sulfate-labeled material solubilized from the basement membrane (BM) sacs was separated by ion-exchange chromatography on DEAE-cellulose in the presence of 2 M urea, and the [^{35}S]sulfate-containing fractions were pooled as indicated (Figure 2). Ion-exchange chromatography of BM samples from preparations radiolabeled with glucosamine or galactose gave an additional peak before the [^{35}S]sulfate peak between fractions 25 and 50 which was well separated from the apparent proteoglycans and, when analyzed by SDS–polyacrylamide gel electrophoresis, was found to contain laminin and entactin. The [^{35}S]sulfate-labeled material from the cytochalasin B supernatant (CB extract) and from the growth medium produced very similar peaks when subjected to ion-exchange chromatography on DEAE-cellulose. As described above, similar analyses of these fractions from cells radiolabeled with [^{3}H]- or [^{14}C]glucosamine or [^{3}H]galactose demonstrated the presence of a labeled glycoprotein fraction which eluted significantly before the [^{35}S]sulfate-labeled material. The [^{35}S]sulfate-labeled material isolated by ion-exchange chromatography from the medium, the CB extract, and the basement membrane sacs was further purified by gel filtration on Bio-Gel A-15m (Figure 3). The [^{35}S]sulfate-labeled CB extract yielded only a single peak near the total volume of the column (Figure 3A). If the cells were radio-

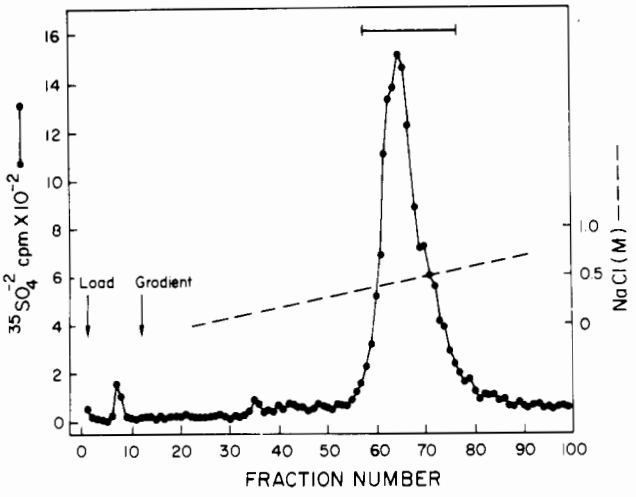

FIGURE 2: Ion-exchange chromatography of basement membrane components. [^{35}S]Sulfate-labeled basement membrane was solubilized in 2 M urea and subjected to ion-exchange chromatography on DEAE-cellulose in the presence of 2 M urea as described under Materials and Methods. The column was loaded starting at fraction 1, and the gradient was begun at fraction 12. (●) [^{35}S]Sulfate; (---) NaCl (molar concentrations).

labeled with [^{14}C]- or [^{3}H]glucosamine, [^{3}H]galactose, or [^{3}H]serine, the isolated proteoglycans also yielded only a single peak at this same position. The [^{35}S]sulfate-labeled medium proteoglycans were separated into two fractions by gel filtration on Bio-Gel A-15m as shown in Figure 3B, an early eluting fraction (high molecular weight proteoglycan) and a late eluting fraction (low molecular weight proteoglycan). Identical A-15m gel filtration profiles were observed with the pooled ion-exchange column proteoglycan fractions derived from cells which had been radiolabeled with [^{14}C]- or [^{3}H]glucosamine, [^{3}H]galactose, or [^{3}H]serine. The [^{35}S]sulfate-labeled basement membrane proteoglycan fractions yielded a single major component by gel filtration on Bio-Gel A-15m (Figure 3C). The small peak at V_0 was not reproducible. Radiolabeled fractions were pooled as indicated for further study. As with the other proteoglycans, analyses of [^{3}H]galactose-, [^{14}C]- and [^{3}H]glucosamine-, and [^{3}H]serine-radiolabeled proteoglycans gave the same profiles when chromatographed in the Bio-Gel A-15m column.

The homogeneity of all radiolabeled proteoglycans was determined by dissociative density gradient centrifugation as described under Materials and Methods. All four proteoglycans sedimented in the most dense fractions (Figure 4). The dissociative density profiles of the proteoglycans radiolabeled with [^{35}S]sulfate, [^{3}H]serine, or [^{3}H]glucosamine (data not shown) were essentially identical. These data suggest that the A-15m pooled fractions are free of associating proteins or glycoproteins. Indeed, after CsCl gradient centrifugation, the proteoglycans had identical profiles on Bio-Gel A-15m columns.

The possible presence of chondroitin sulfate and heparan sulfate chains in the proteoglycan preparations was examined by treatment of the various proteoglycans with chondroitin ABC lyase and nitrous acid as described under Materials and Methods. Following digestion, the [^{35}S]sulfate-labeled reaction products were compared with those from untreated controls by gel filtration on Sephadex G-50 or G-100. No peak was obtained eluting close to V_t after chondroitin ABC lyase digeston, showing that no degradation was obtained with BM proteoglycan, CB proteoglycan, and low molecular weight proteoglycan. A small release (about 5%) of oligosaccharide from high molecular weight proteoglycan suggested the

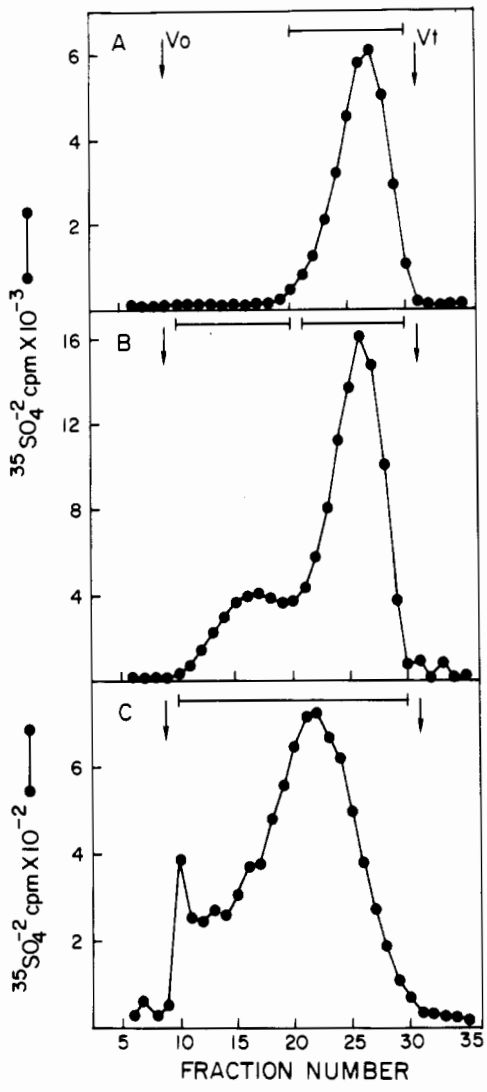

FIGURE 3: Fractionation of HSPGs on Bio-Gel A-15m. The [^{35}S]-sulfate-labeled HSPGs, isolated by ion-exchange chromatography (see Figure 2), were chromatographed on Bio-Gel A-15m and pooled as indicated by the bars. CB HSPG (A); medium HSPGs (B); BM HSPG (C).

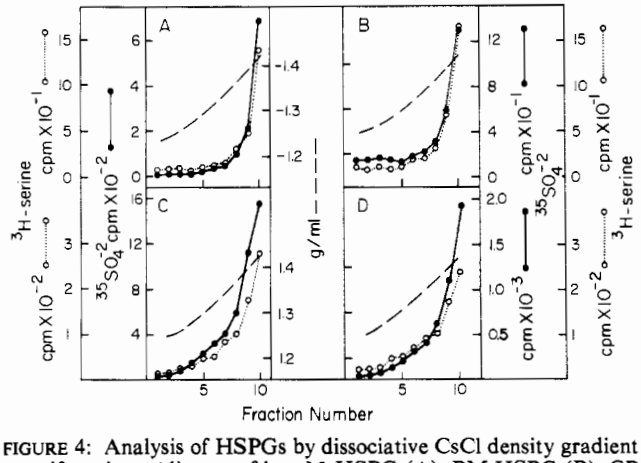

FIGURE 4: Analysis of HSPGs by dissociative CsCl density gradient centrifugation. Aliquots of low M_r HSPG (A), BM HSPG (B), CB HSPG (C), and high M_r HSPG (D) were analyzed by CsCl density gradient centrifugation in 4 M Gdn·HCl. (●) [^{35}S]Sulfate; (○) [^{3}H]serine; (---) grams per milliliter.

presence of traces of chondroitin sulfate proteoglycan. Treatment of all four [^{35}S]sulfate-labeled HSPGs with nitrous acid, as described under Materials and Methods, appeared to result in 90–95% conversion to low molecular weight products

Table II: M1536-B3 Heparan Sulfate Molecules

fraction	proteoglycan size[a] (daltons)	glycosaminoglycan chain size (daltons)	% uronic acid composition: iduronate[b]	% uronic acid composition: glucuronate[b]
BM HSPG (basement membrane)	100 000	60 000	44	56
CB HSPG (cell)	20 000	10 000	47	53
low M_r HSPG (medium)	20 000	10 000	41	59
high M_r HSPG (medium)	700 000	29 000	41–49	51–56

[a] Determined by gel filtration on calibrated columns of Bio-Gel A-15m, Sephacryl S-200 (CB, low M_r and high M_r HSPGs), or Sephacryl S-300 (BM HSPG). [b] Values are expressed as the percent of total [^{3}H]uronic acid in the sample except as indicated; all numbers are the average of two experiments with ±2% variance. All samples were treated with alkali and chondroitin ABC lyase before uronic acid analysis.

which appeared in the low molecular weight region of a G-100 column (not shown). This indicates the heparan sulfate nature of the proteoglycans.

Characterization of the HSPGs. The sizes of the [^{35}S]-sulfate-labeled or [^{3}H]- or [^{14}C]glucosamine-labeled HSPGs were determined by gel filtration on calibrated Bio-Gel A-15m or Sephacryl S-200 columns (Table II). Sharp single peaks were obtained for CB HSPG (M_r 20 000), low M_r HSPG (M_r 20 000), and high M_r HSPG (M_r 700 000). The K_{av} values for BM HSPG on Sepacryl S-300 and Bio-Gel A-15m suggested a M_r of 100 000.

The heparan sulfate GAG chains were released from the intact proteoglycans with alkaline borohydride as described under Materials and Methods. The molecular weight values for these chains were determined by gel filtration on calibrated columns of Sephacryl S-200 or S-300 (Table II). The CB HSPG and low M_r HSPG have identical M_r 10 000 GAG chains (in addition to identical proteoglycan sizes), while the GAG chains of the BM HSPG and high M_r HSPG have unique sizes (M_r 60 000 and 29 000, respectively). The relative elution behavior of these GAG chains on gel filtration was not altered in the presence of 4 M guanidine which indicates that the size differences are not the result of aggregation or self-association (Fransson, 1981). Proteoglycans isolated from cell–basement membrane complexes by trypsin treatment or extraction with 4 M guanidine also had GAG chain sizes of M_r 60 000 and 10 000 (identical with the sizes of the BM HSPG and CB HSPG GAG chains), showing that the heparan sulfate GAG chains are not significantly degraded during the 6-h cytochalasin B incubation although low levels of degradation might be responsible for the broad peak observed in Figure 3C (see Materials and Methods for a discussion of these experiments). In addition, incubation of high M_r HSPG in the cytochalasin B extraction solution for 6 h did not alter the elution behavior of this species on subsequent gel filtration.

Chemical Composition. The hexosamine content of each HSPG was determined as described under Materials and Methods. All four HSPGs had 80–85% glucosamine with 15–20% galactosamine. The galactosamine content of the HSPGs was not altered by ion-exchange chromatography, gel filtration, or dissociative CsCl gradient centrifugation.

The amounts of iduronic and glucuronic acids in each HSPG were determined and are shown in Table II. Before analysis, all samples were alkali treated and digested with chondroitin ABC lyase. All four HSPGs contained between 41 and 49% iduronic acid and between 51 and 59% glucuronic acid.

Cochromatographic Comparisons of HSPGs. The four HSPG fractions were compared by ion-exchange cochromatography. These data showed that CB HSPG and low M_r HSPG have similar charge densities while high M_r HSPG and BM HSPG have greater charge densities when the relative rates of migration are calculated (an average of two experiments). Relative to an arbitrary charge density of 1 for the low M_r HSPG, the charge densities of CB, high M_r, and BM HSPGs are 0.98, 1.10, and 1.14, respectively.

Fingerprinting of HSPGs. The GAG structures of the HSPGs were compared by cochromatographic fingerprinting of nitrous acid produced fragments. Two HSPGs (one [^{14}C]glucosamine labeled and one [^{3}H]glucosamine labeled) were treated simultaneously with nitrous acid, and the degradation products were chromatographed on Bio-Gel P10 (Figure 5) before (A) or after (B–D) separation of inorganic sulfate on Bio-Gel P2 (see Materials and Methods). All combinations of HSPGs were analyzed, but because the low M_r HSPG and CB HSPG appeared identical (Figure 5D), only four pairs are shown. As is evident, the only major differences are in the earlier eluting column fractions shown as pools I and II in Figure 5A. All of the galactosamine is in these fractions (see below) along with some glucosamine and most likely the protein core(s). Comparisons between column runs cannot be made because three different Bio-Gel P10 columns were used. It should be noted that pool IX in Figure 5A is not present in Figure 5B–D because it was removed with inorganic sulfate on Bio-Gel P2 (Keller et al., 1980). The similar distribution profile for the released oligosaccharides indicates that the arrangement of *N*-sulfates is the same for all the heparan sulfate GAG chains from the different HSPGs. GAG chains released from high M_r HSPG and low M_r HSPG by alkaline elimination were also degraded with nitrous acid and analzyed by gel filtration on Bio-Gel P10. Although not shown, the profiles were similar to if not identical with that produced by the intact HSPGs (Figure 5C).

The individual peaks resolved by gel filtration of the nitrous acid generated HSPG fragments (Figure 5A) were further fractionated by ion-exchange chromatography on DEAE-Sephadex A-25. The charge heterogeneity of the components in peak I from Figure 5A–C is shown in Figure 6. Within a single-column elution profile, the double-labeled peaks align quite well, which suggests that they are the same components but exist in different relative amounts in the different proteoglycans. Again, it must be noted that, without identification of individual peaks, comparisons between column runs should not be made as a new column is packed for each analysis and slight differences in elution occur. The possible presence of chondroitin sulfate chains in the pool I fractions used in these comparisons does not affect the appearance of the individual peaks as chondroitin sulfate is not fragmented by nitrous acid and elutes as a single broad peak (see below). Each pool from each Bio-Gel P10 column was analyzed by this same method of DEAE-Sephadex ion-exchange chromatography. Slight differences in the proportional distribution of isotope among the fractions resolved from pool II were apparent (Figure 7), but later pools did not demonstrate such obvious differences. Similar results were obtained with Bio-Gel P10 pools from the low M_r HSPG–BM HSPG and high M_r HSPG–BM HSPG comparisons. The ion-exchange analyses of pools I–VIII of low M_r HSPG–CB HSPG, as in the Bio-Gel P10 analysis, showed no differences (data not shown).

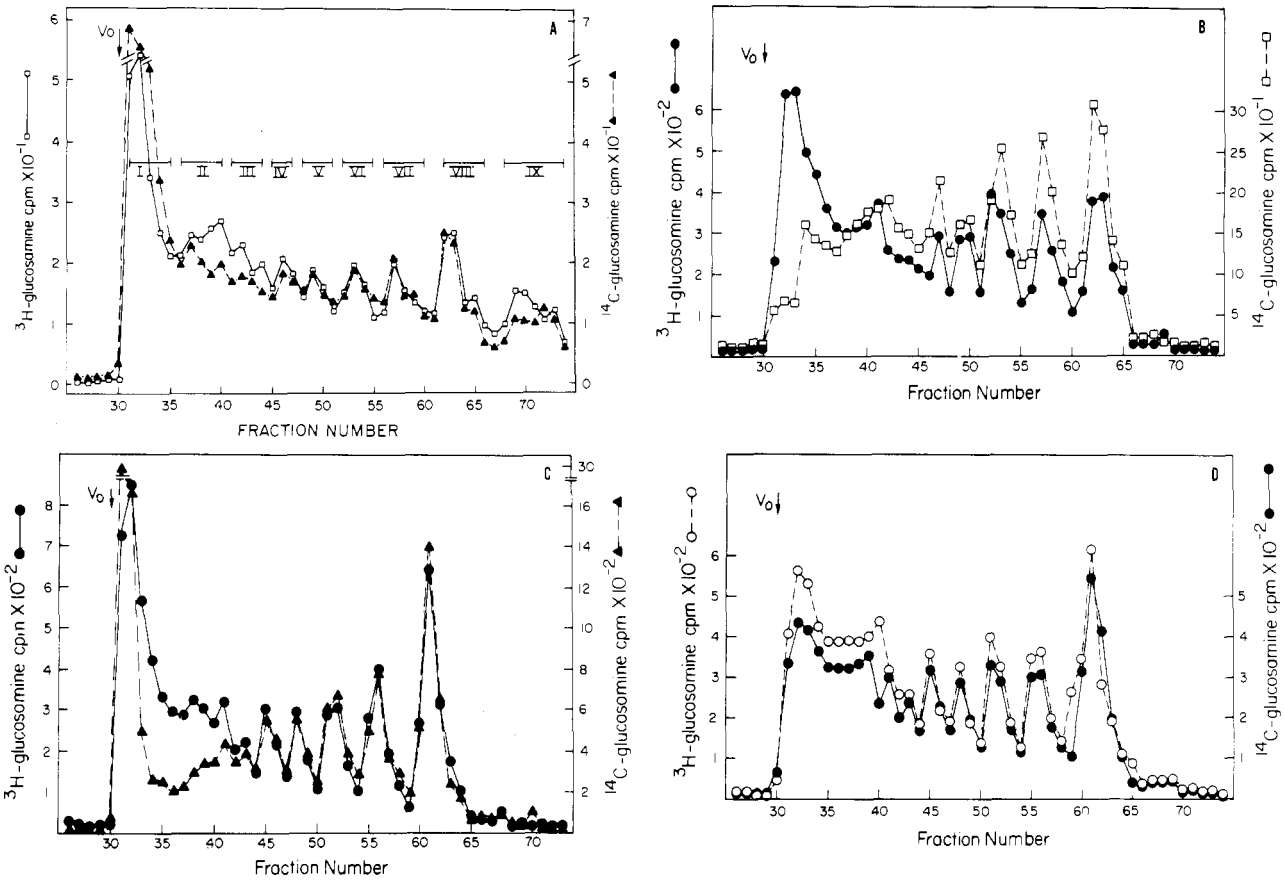

FIGURE 5: Bio-Gel fingerprint analysis of HSPGs. Nitrous acid generated fragments [before (A) or after (B–D) separation of inorganic sulfate on Bio-Gel P2]. (A) High M_r HSPG [[^{14}C]glucosamine (▲)] and BM HSPG [[^{3}H]glucosamine (□)]; (B) low M_r HSPG [[^{3}H]glucosamine (●)] and BM HSPG [[^{14}C]glucosamine (□)]; (C) low M_r HSPG [[^{3}H]glucosamine (●)] and high M_r HSPG [[^{14}C]glucosamine (▲)]; (D) low M_r HSPG [[^{14}C]glucosamine (●)] and CB HSPG [[^{3}H]glucosamine (○)]. Fraction 64 is the elution position of a nonsulfated dissaccharide standard (IM) as described (Weissman & Chao, 1981).

Characterization of the Galactosamine Component. [^{3}H]- or [^{14}C]glucosamine-labeled low molecular weight, high molecular weight, and CB proteoglycans were treated with nitrous acid as described under Materials and Methods, and the digestion products were fractionated by gel filtration on Bio-Gel P10. The fractions were pooled as shown in Figure 5A, and the galactosamine and glucosamine contents (as percent of radiolabel) of each pool were determined as described under Materials and Methods. Essentially all of the galactosamine present in high molecular weight proteoglycan was found in pools I and II (fractions 31–40). The galactosamine present in the CB and the low molecular weight proteoglycans was distributed similarly with 75% present in pool I (fractions 31–35), 15% in pool II (fractions 36–40), and 8% present in pool III (fractions 41–44). The galactosamine-containing fractions were pooled and treated with alkaline borohydride, as described under Materials and Methods, to release the carbohydrate chains from the proteoglycan core.

The radiolabeled alkali-released chains were then fractionated on DEAE-Sephadex A-25 as described under Materials and Methods (Figure 8). A relatively sharp peak eluting between fractions 15 and 35 contained essentially all of the radiolabeled glucosamine (greater than 95%). A broad highly charged peak eluted between fractions 35 and 70 and contained all of the radiolabeled galactosamine. This same pattern held regardless of which proteoglycan was used for the isolation of radiolabeled galactosamine carbohydrate.

Despite the insensitivity of the intact proteoglycans to chondroitin ABC lyase (see above), the elution positions from the DEAE Sephadex A-25 columns indicated that the galactosamine-containing material was a glycosaminoglycan, and so the isolated chains were tested for susceptibility to chondroitin ABC lyase (as described under Materials and Methods). The galactosamine-containing carbohydrates from the high molecular weight, low molecular weight, and CB proteoglycans were all degraded by chondroitin ABC lyase. Insufficient quantities of radiolabeled BM proteoglycan were available to determine chondroitin ABC lyase susceptibility of the galactosamine-containing carbohydrate, but it is probably also chondroitin sulfate. We thus conclude that the galactosamine in these HSPG preparations is in chondroitin sulfate which is not susceptible to degradation in the intact proteoglycans.

The sizes of the [^{3}H]- or [^{14}C]glucosamine-labeled chondroitin sulfate GAGs were analyzed by gel filtration on Sephacryl S-200 (as described under Materials and Methods). Despite the fact that they each represented 15–20% of the hexosamine in very different HSPG preparations (see above), all chondroitin sulfate chains had similar molecular weights of about 16 000.

Discussion

The M1536-B3 cells provide an excellent system in which to study basement membrane HSPGs because they produce readily isolatable sacs of basement membranes which contain little if any collagen (Chung et al., 1977b). Because of their high charge density, the proteoglycans present can easily be separated from the other basement membrane glycoproteins. We have isolated and purified the BM proteoglycans as well as cell-associated proteoglycans released when the cells are

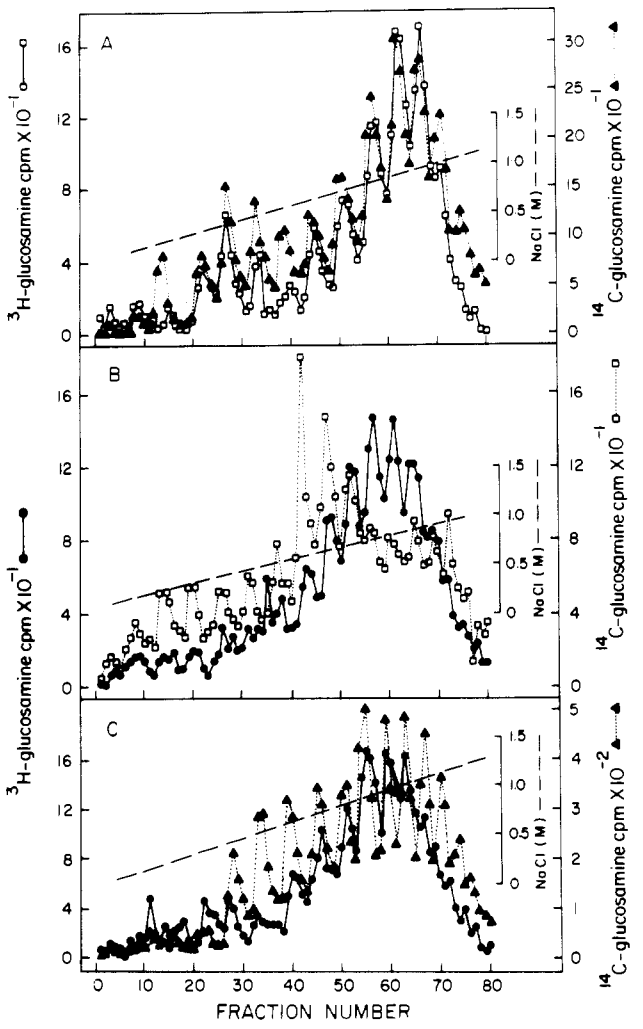

FIGURE 6: DEAE-Sephadex A-25 fingerprint analysis of Bio-Gel P10 pool I HSPG fractions. Pool I fractions (as designated in Figure 5A) were compared by using ion-exchange chromatography on DEAE-Sephadex A-25. (A) [^{3}H]Glucosamine-labeled BM HSPG (□) and [^{14}C]glucosamine-labeled high M_r HSPG (▲) (from fractions 31–35 of Figure 5A); (B) [^{3}H]glucosamine-labeled low M_r HSPG (●) and [^{14}C]glucosamine-labeled BM HSPG (□) (fractions 31–34 from Figure 5B); (C) [^{3}H]glucosamine-labeled low M_r HSPG (●) and [^{14}C]glucosamine-labeled high M_r HSPG (▲) (fractions 31–34 from Figure 5C); (---) NaCl (molar concentrations).

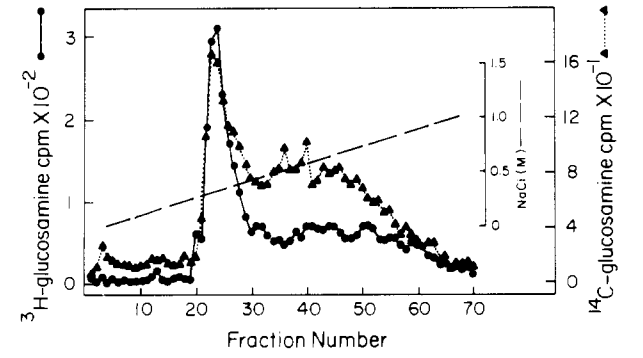

FIGURE 7: DEAE-Sephadex A-25 fingerprint analysis of Bio-Gel P10 pool II. [^{3}H]Glucosamine-labeled low M_r HSPG (●) and [^{14}C]-glucosamine-labeled high M_r HSPG (▲) pool II (fractions 35–39, Bio-Gel P10 analysis, Figure 5C) was analyzed by ion-exchange chromatography using DEAE-Sephadex A-25.

removed from the basement membranes and two proteoglycans, which differ in size, from the growth medium of these cells. Following purification by ion-exchange chromatography, no protein or carbohydrate moieties can be separated from any of the four HSPGs by size, charge, or dissociative CsCl density gradient centrifugation. The BM and high M_r HSPGs are distinguished from each other on the basis of different proteoglycan size and GAG chain length. The CB and low M_r HSPGs are essentially identical with each other as judged by proteoglycan size, GAG chain length, and charge density, but they differ from the BM HSPG and high M_r HSPG in all of these characteristics. It is clear from the analyses (fingerprints) of the products produced by treatment with nitrous acid that the HSPG GAG chains are very similar in their N-sulfated regions, since for all of the HSPGs, the small products released by nitrous acid appear similar in structure and percent of total carbohydrate. Not only are the relative amounts of the various sized small oligosaccharides identical but also the ion-exchange profiles of these pieces are essentially identical, showing that the *O*-sulfate patterns in the N-sulfated regions are the same for all of the heparan sulfate GAGs. Furthermore, the iduronic acid to glucuronic acid ratios differ only slightly between the heparan sulfate GAG chains in all four HSPGs, falling in a range similar to previously published values for heparan sulfate (Keller et al., 1980; Oldberg et al., 1977; Riesenfeld at al., 1982). The lack of differences in the N- and O-sulfation patterns suggests that the differences in the HSPGs are not due to selective actions of polymer-modification enzymes. Rather, these cells apparently are able to produce multiple heparan sulfate chains of varying lengths by anabolic or specific catabolic (e.g., endoglycosidases; Oldberg et al., 1980) mechanisms.

All four proteoglycans contain 15–20% galactosamine which becomes sensitive to chondroitin ABC lyase only after the chains are released with alkali. Chondroitin sulfate proteoglycan standards added to the reaction mixture as controls are degraded in the presence of the intact HSPGs, eliminating the possibility of an enzyme inhibitor in the HSPG preparations. This observation suggests that there is chondroitin sulfate present in each proteoglycan fraction. However, it is not clear whether these chondroitin sulfate chains are present on a contaminating proteoglycan in each HSPG fraction or actually somehow linked to the HSPGs. We have been unable to separate chondroitin and heparan sulfate proteoglycans in these fractions by using ion-exchange chromatography, CsCl density gradients, and gel filtration in the presence of urea or guanidine, methods which have proven satisfactory in the separation of chondroitin and heparan sulfate proteoglycans isolated from fibroblasts.[3] Additionally, the isolated chondroitin sulfate GAG chains are similar to (if not identical with) each other in size. The reasons for the copurification of the chondroitin sulfate molecules with the HSPGs in the same ratios and their lack of degradation by chondroitin ABC lyase in the intact proteoglycans are unclear. We have not observed a lack of degradation of chondroitin sulfate chains in the proteoglycans from other cell lines, nor are we aware of similar results obtained by other investigators. These matters are currently under investigation.

Comparisons of the M1536-B3 HSPGs with basement membrane proteoglycans isolated by others reveal many similarities. The HSPG (BM-1) isolated from the EHS sarcoma, M_r 750000 (Hassell et al., 1980), is similar in size to the M1536-B3 high M_r HSPG and larger than the M1536-B3 BM HSPG. The PYS-2 HSPG, from the medium or cell layer (Oohira et al., 1982), was smaller (M_r 400000) than the high M_r HSPG and larger than the BM HSPG, and its GAG chains (M_r 25000) were similar in size to those of the high M_r HSPG. The glomerular basement membrane HSPG was found to have an M_r of 130000 (Kanwar et al., 1981), similar to the

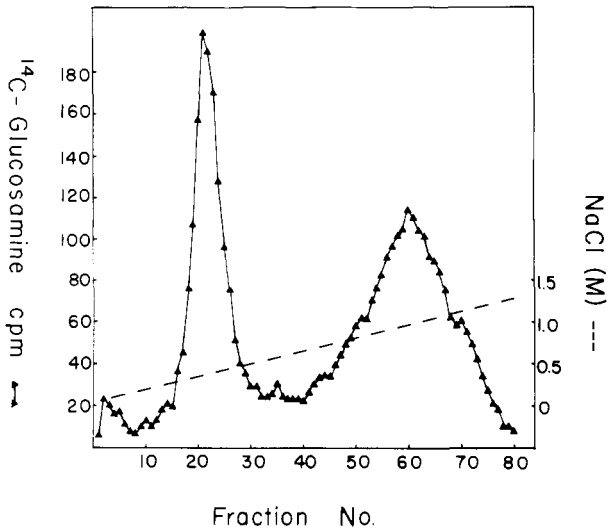

FIGURE 8: DEAE-Sephadex A-25 ion-exchange chromatography of alkali-released galactosamine-containing carbohydrate. [^{14}C]-Glucosamine-labeled high molecular weight pools I, II, and III from Bio-Gel P-10 gel filtration of nitrous acid degradation products were alkali eliminated as described under Materials and Methods. The alkali-released carbohydrate was subjected to ion-exchange chromatography on DEAE-Sephadex A-25. (---) NaCl (molar concentrations).

M1536-B3 BM HSPG and much smaller than the M1536-B3 high M_r HSPG. However, the GAG chains of the glomerular basement membrane HSPG are similar to those from the M1536-B3 high M_r HSPG. HSPGs isolated from bovine aortic endothelial cell layers and medium are of two varieties. Both the medium and cell layer (which includes extracellular matrix material) contain HSPGs of about M_r 1 000 000 with chains of about M_r 36 000 while the cell layer also contains a HSPG of M_r 130 000 with GAG chains of about M_r 20 000 (Oohira et al., 1983). These proteoglycans are similar in size to the high M_r HSPG and BM HSPG, respectively; however, the chain sizes differ in both cases. Like the M1536-B3 HSPGs, the EHS sarcoma BM-1 proteoglycan was insensitive to chondroitin ABC lyase although galactosamine was listed as a minor component (Hassell et al., 1980). About 15% of the proteoglycans isolated from kidney glomerular basement membrane were degraded by chondroitin ABC lyase, but no galactosamine analysis was reported (Kanwar et al., 1981). Finally, the PYS-2 HSPG was also insensitive to chondroitin AC lyase, but no analysis of glucosamine and galactosamine was reported (Oohira et al., 1982). Clearly, the basement membrane HSPGs are large proteoglycans and may well vary between different basement membranes as cell-surface heparan sulfates have been shown to vary between cell types (Keller et al., 1982). Therefore, it will be important to characterize the HSPGs of different basement membrane systems in order to appreciate the functional relationships between the HSPGs from various basement membranes.

Our identification of three and possibly four distinct HSPGs produced by the M1536-B3 cells, only one of which appears to be incorporated into their basement membranes, suggests that there must be a mechanism that either directs the synthesis of each form of HSPG or organizes the modification of some HSPGs after synthesis. It is tempting to speculate that a precursor–product type of system, possibly involving heparan sulfate endoglycosidases (Oldberg et al., 1980) and proteases, is responsible for the occurrence of the three to four varieties of HSPG; however, we have no convincing evidence to support such a model at the present time. We are currently using this cell system to study the factors which determine the final HSPG structure and the role of the protein core(s) in HSPG synthesis, secretion, and culture compartment localization.

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