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Heparan Sulfate Proteoglycans of Human Neuroblastoma Cells: Affinity Fractionation on Columns of Platelet Factor-4[+]

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Human neuroblastoma cells (Platt) were detached from tissue culture substrata with a Ca^{2+} chelating agent, and then the suspended cells were extracted with a sodium dodecyl sulfate (SDS)-containing buffer to maximally solubilize their sulfate-radiolabeled proteoglycans. The majority of the high-molecular-weight material in these dissociative extracts was heparan sulfate proteoglycan, which resolves into two heterodisperse size classes upon gel filtration on columns of Sepharose CL4B. After removal of SDS from these extracts by hydrophobic chromatography on Sep-Pak C_{18} cartridges, extracts were further fractionated on various affinity matrices. All of the sulfate-radiolabeled material eluted as one peak from DEAE-Sephadex ion-exchange columns. In contrast, affinity fractionation on Sepharose columns derivatized with the heparan sulfate-binding protein, platelet factor-4, resolved three major and one minor subsets of these components. The nonbinding fraction contained some heparan sulfate proteoglycan and some chondroitin sulfate. The weak-binding fraction contained principally heparan sulfate proteoglycan, as well as a small amount of chondroitin sulfate proteoglycan; the gel-filtration properties of these proteoglycans before or after alkaline borohydride treatment indicated that they were small in size, containing perhaps 2 to 4 glycosaminoglycan chains. The high-affinity fraction eluted from platelet factor 4–Sepharose was composed entirely of "single-chain" heparan sulfate. A portion of the heparan sulfate proteoglycan of the original extract bound to the hydrophobic affinity matrix, octyl–Sepharose, and this hydrophobic proteoglycan partitioned into the nonbinding and weak-binding fractions of the platelet factor 4–Sepharose affinity columns. These studies reveal that the majority of the proteoglycan made by these neuronal cells in culture is of the heparan sulfate class, is small in size when compared to other characterized proteoglycans, and can be resolved into several overlapping subsets when fractionated on affinity matrices.

The mechanisms by which neuronal cells adhere to various extracellular matrices are critically important in the development and eventual function of the nervous system in animals. In order to study these processes at the molecular level more easily, a number of laboratories have resorted to tissue culture model systems. In some cases for appropriate biochemical analyses, it is necessary to use neuroblastoma tumor cells which can be cloned and grown in large quantities but which remain inducible to form differentiated neurites under appropriate culture conditions (1, 2). Letourneau (3, 4) initiated studies on the chemical requirements of the tissue culture substratum as it effects neurite outgrowth. Recent studies have reported that conditioned media from a variety of sources, when adsorbed to substrata, promote neurite outgrowth (5–7) with some indication

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that a heparan sulfate-containing moiety may be an active factor (8).

Another approach has been taken to identify the cell-synthesized macromolecules enriched in the adhesion sites under the cell bodies and growth cones of neuroblastoma cells in tissue culture. EGTA[3]-mediated detachment of differentiated rat or human neuroblastoma cells leaves small focal adhesion sites as substratum-attached material (9, 10). Scanning electron microscopic studies reveal that the structural organization of these focal adhesion sites is significantly different between the cell body and the growth cone (11). Biochemical analyses indicate that these sites from either differentiated—that is, neurite-producing—or undifferentiated cells are greatly enriched in heparan sulfate proteoglycan [(9, 10); Vallen *et al.*, manuscript in preparation] and, in contrast to fibroblast adhesion sites (12), depleted of hyaluronic acid and chondroitin sulfate proteoglycan (9). This adhesive material is also enriched in a fibronectin-like glycoprotein which is cell-synthesized (9). The significance of this fibronectin-like glycoprotein in the adherence of these cells is supported by experiments showing that neuroblastoma cells attach and spread very well on substrata coated with polyclonal or monoclonal antibodies to plasma fibronectin (13). Furthermore, neuroblastoma cells (9), chick neural retina cells (14), and chick dorsal root ganglion cells (15, 16) have been shown to extend neurites on fibronectin-coated substrata. This evidence, taken together with additional evidence that fibronectin is a potent, multivalent heparan sulfate-binding molecule (17, 18, 19) and that substratum-attached material of neuroblastoma cells contains a very high proportion of heparan sulfate which binds to fibronectin (20), provides strong indication that heparan sulfate proteoglycans on neuronal cells are critically important in the adhesion of growth cones and/or cell bodies to certain extracellular matrices.

For these reasons, we have initiated a series of studies to biochemically analyze the heparan sulfate proteoglycans of neuroblastoma cells. This report focuses on the properties of these proteoglycans in the cell fraction, while another study (Vallen *et al.*, manuscript in preparation) will deal with the proteoglycans in the substratum adhesion sites. Recently, fibroblasts have been shown to attach, spread, reform microfilament bundles, and form close adhesive contacts on substrata coated with the heparan sulfate-binding protein, platelet factor-4 (PF4) (21, 22). Neuroblastoma cells respond similarly on PF4-coated substrata (Tobey, Chernoff, and Culp, manuscripts in preparation). Therefore, we have taken advantage of these special properties of PF4 to use it as an affinity ligand in the purification and separation of subsets of heparan sulfate proteoglycans from Platt human neuroblastoma cells (10, 23).

MATERIALS AND METHODS

Cell growth and fractionation. *Mycoplasma*-free Platt human neuroblastoma cells were grown as described previously (10) in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 10 mM Hepes buffer, 250 U/ml penicillin, and 250 μg/ml streptomycin sulfate. For radiolabeling, 15×10^6 cells were seeded into 1750-cm^2 plastic roller bottles for 72 h of exponential growth in the same medium without streptomycin sulfate and penicillin but with 25 μCi/ml $Na_2{}^{35}SO_4$ until 70–80% confluence of the cultures had been achieved. The cells were detached with 0.5 mM EGTA in phosphate-buffered saline (PBS) after extensive washing with PBS as described previously (10). The cells were then pelleted by centrifugation out of PBS and resuspended in 10 ml PBS containing the protease inhibitors phenylmethylsulfonyl fluoride (1 mM), *N*-ethylmaleimide (1 mM), pepstatin (0.1 mM), and EDTA (1 mM). The cell suspension was made 0.2% in SDS to dissolve cell membranes and organelles, and stirred for 1 h at room temperature. The extract was centrifuged at 16,000 rpm for 30 min at 4°C in a Sorvall centrifuge to pellet insoluble chromatin and other materials, and the supernatant was passed through a 0.2-μm Millipore membrane filter to obtain the solubilized extracts. Greater than 95% of ^{35}S-radiolabeled macromolecules could be recovered in the SDS extract.

[3] Abbreviations used: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; GdnHCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; PF4, platelet factor-4; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; HMW, high molecular weight.

Fractionation of extracts. Cell extracts were chromatographed over Sepharose CL6B or CL4B columns (1.4 × 110 cm) eluted with 0.2% SDS and 150 mM Tris-HCl (pH 7.4), with collection of 1.5-ml fractions and with recoveries in the range 80–90%. Small aliquots of each fraction were assayed for radioactivity by scintillation counting. Indicated regions of the profile were pooled for further analysis.

Proteoglycan and heparan sulfate contents of fractions. Alkaline sodium borohydride reduction was used to determine the proteoglycan contents in various chromatographic fractions by determining the shift of sulfate-radiolabeled material to more-included regions of Sepharose chromatography profiles (24–26). To half of each sample was added an equal volume of 7.5% (w/v) $NaBH_4$ in 0.1 N NaOH. The control half received nothing. Both were incubated at 45°C for 19 h (26). The experimental sample was neutralized with concentrated acetic acid. Both samples were brought to 0.2% in SDS and chromatographed on Sepharose CL6B columns as described above.

Nitrous acid deamination was performed on samples to determine the amount of *N*-sulfated heparan sulfate (27). Sodium nitrite in 1.8 M acetic acid was added to a concentration of 1.8% to one-half of the samples. The control halves received acetic acid only. All samples were incubated at room temperature for 80 min, at which time an equal volume of 2 M ammonium sulfamate was added to end the reaction. Samples were made 0.2% in SDS, and chromatographed on Sepharose CL6B columns as described above. Previous evidence (26) demonstrates that these nitrous acid treatment conditions do not lead to degradation of the glycoproteins in cell extracts.

Affinity chromatography. Heparan sulfate moieties in these cell extracts were fractionated based on their binding affinity for PF4 (22); the only other neuronal glycosaminoglycan to bind to PF4 with weak affinity was chondroitin sulfate (See RESULTS). PF4 was isolated from outdated human platelet pack plasma obtained from the Cleveland Red Cross by affinity chromatography (A. Nicol and L. A. Culp, unpublished data). In brief, 100–150 packs of outdated human platelets were frozen and thawed three times, and centrifuged in a Sorvall RC-5 centrifuge at 4000 rpm for 20 min at 4°C. The supernatant fraction was filtered through glass wool and Whatman No. 1 filter paper, made 0.05% in sodium azide, and passed at room temperature over a 100-ml Sepharose 4B column conjugated with intestinal mucosa heparin (5 mg/ml) using cyanogen bromide (28). After adsorption, the column was rinsed with PBS and eluted successively with 0.5 M NaCl, 1.0 M NaCl, and 2 M NaCl (all in 50 mM Tris-HCl, pH 7.2). Most of the PF4 was found in the 1.0 M NaCl fraction, but was contaminated with some higher-molecular-weight protein. This fraction was therefore concentrated by vacuum dialysis at 4°C to a volume of 2–3 ml and further purified by passage through a Sephadex G-75 column (1.5 × 90 cm) eluted with 1.0 M NaCl in 5 mM Tris-HCl (pH 7.2). The most-included material was collected and its concentration was assayed for protein content; it was verified as PF4 by polyacrylamide gel electrophoresis and amino acid analysis. The PF4 was stored at concentrations of less than 1 mg/ml in PBS supplemented with 0.2 M NaCl (22).

To crosslink PF4 to Sepharose 4B, special precautions were taken to avoid inactivating the lysine residues in its active site (42). Sepharose 4B was activated with cyanogen bromide by the method of March *et al.* (28) with some modifications. The amount of cyanogen bromide in acetonitrile (2 mg/ml) added to activate the Sepharose was reduced by fivefold from the usual concentration to 0.01 vol of the resin in water. The PF4 was dialyzed against 0.2 M sodium bicarbonate in water (pH 9.5) overnight with two changes, and was allowed to react with the activated Sepharose for only 4 h at 4°C. More stringent conditions were found to inactivate the PF4.

Cell extracts from Sepharose CL6B columns containing the majority of the nitrous acid-sensitive material were pooled, supplemented with 1 mM phenylmethylsulfonyl fluoride and 1.5 mM pepstatin, concentrated by vacuum dialysis, and dialyzed against PBS overnight at 4°C. Sep-Pak C_{18} cartridges were used to rid samples of their SDS. These cartridges were activated by sequentially eluting glass-distilled water, reagent-grade methanol, and then water through them. No more than 1 ml cell extract was passed over the activated Sep-Pak C_{18} columns, followed by 1 ml glass-distilled water. Recovery of samples was generally greater than 90% of the radioactivity loaded, and no single component of the samples seemed to bind irreversibly to the Sep-Pak resin (as determined by rechromatographing the samples over Sepharose CL6B columns). Samples were made 50 mM in Tris-HCl, 1 mM in $MgCl_2$, and 1 mM in $CaCl_2$ (pH 7.2) (referred to as Tris buffer) by the addition of 10× Tris buffer. These samples were passed through 2- to 3-ml minicolumns of PF4–Sepharose (1 mg/ml). The columns were rinsed with Tris buffer, eluted with a linear gradient from 0 to 0.6 M NaCl in Tris, then 0.6 M NaCl in Tris, and finally rinsed with 2.0 M NaCl in Tris buffer to elute any tightly bound material. Refractive indices to establish ionic strengths were measured on a Bausch and Lomb refractometer.

Hydrophobic affinity chromatography. Various fractions, after ridding them of SDS on Sep-Pak C_{18} cartridges, were allowed to adsorb to the hydrophobic matrix, octyl-Sepharose 4B, to determine if this proteoglycan material has the potential to be membrane associated (29, 30). Samples were dialyzed overnight at 4°C with three changes of 3 M NaCl in buffer A (0.5 M GdnHCl, 0.2 M Tris-HCl, pH 7.3). This sample and octyl-Sepharose in the same buffer (1:3, v/v) were rotated overnight at room temperature. The matrix

was then poured into a minicolumn of 2-3 ml and successively eluted as modified from Kjellen *et al.* (29) with (i) 3 M NaCl in 0.2 M Tris-HCl (pH 7.3); (ii) 0.1 M NaCl in 0.2 M Tris-HCl (pH 7.3); (iii) 3 M NaCl in 0.2 M Tris-HCl (pH 7.3); (iv) 1% (w/v) octylglucoside, 3 M NaCl, 0.2 M Tris-HCl (pH 7.3); and, finally (v) 0.2% (w/v) SDS. Both high and low ionic strength buffers were used to elute the octyl-Sepharose columns (prior to octylglucoside elution), because some proteoglycan material invariably eluted in both buffers. Fractions were assayed for radioactivity by scintillation counting.

Materials were purchased from the following sources: Sepharose CL6B, Sepharose CL4B, Sepharose 4B, octyl-Sepharose 4B, Sephadex G-75, and DEAE-Sephadex from Pharmacia Fine Chemicals; 1750-cm^2 tissue culture plastic roller bottles from Falcon Labware; $Na_2{}^{35}SO_4$ from Amersham Corporation; porcine mucosal [^{3}H]heparin and NEF-963 aqueous scintillation mixture from New England Nuclear Corporation; phenylmethylsulfonyl fluoride, pepstatin A, *N*-ethylmaleimide, and EDTA from Sigma Chemical Company; EGTA from Eastman Organic Chemicals; Dulbecco's modified Eagle's medium from Grand Island Biological Company; Sep-Pak C_{18} cartridges from Waters Associates; and fetal calf serum from K. C. Biologicals Company. A sample of $^{35}SO_4^{2-}$-radiolabeled chick embryonic limb cartilage chondroitin-keratin sulfate proteoglycan was kindly provided by Dr. Arnold Caplan of the Biology Department of Case Western Reserve University.

RESULTS

Gel filtration properties. Platt human neuroblastoma cells were grown for 72 h in medium containing $^{35}SO_4^{2-}$ to uniformly radiolabel the sulfated glycosaminoglycans of these cells. After EGTA-mediated detachment of the cells, SDS was used to quantitatively extract the glycosaminoglycan and proteoglycan moieties from the suspended cells, as described under Materials and Methods, using several protease inhibitors to protect against breakdown. The extract was then passed over a Sepharose CL6B column eluted with a SDS-containing buffer. As shown in Fig. 1, a sizable fraction of the ^{35}S-radiolabeled material elutes in the V_0 region of the column where proteoglycan would be expected to elute [since the glycosaminoglycan chains produced by these cells are well-included on a Sepharose CL6B column (10)]. Fractions 30-70 were pooled as the "high-molecular-weight" (HMW) fraction and will provide the material analyzed below.

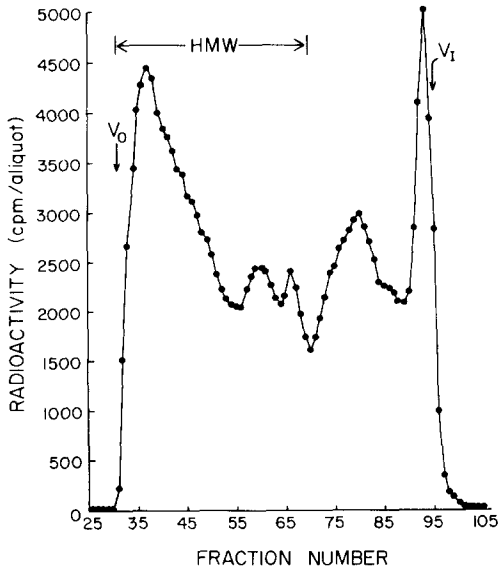

FIG. 1. Gel filtration chromatography of SDS-solubilized cell extracts. $^{35}SO_4$-Radiolabeled and EGTA-detached Platt neuroblastoma cells were extracted with a SDS-containing buffer as described under Materials and Methods. The extract was then eluted through a Sepharose CL6B column with 0.2% SDS in 150 mM Tris-HCl, (pH 7.4). An aliquot of each fraction was assayed for radioactivity, and V_0 and V_I were determined with Blue Dextran and dinitrophenylated glycine, respectively. The high-molecular-weight (HMW) fraction was pooled and concentrated for further analysis.

The HMW fraction of Fig. 1 was then chromatographed on a Sepharose CL4B column to get a more accurate determination of the size distribution of these moieties (Fig. 2). There was a heterodisperse class of ^{35}S-radiolabeled material that extended from fraction 35(V_0) through fraction 70, and a more homogeneous class of material at fractions 75-90 that appears more monodisperse.

The biochemical nature of the HMW fraction was then tested by chemical or enzymatic treatment (Fig. 3). Nitrous acid deamination shifted 80-85% of the radioactivity toward the V_I region of the profile of an SDS-eluted Sepharose CL6B column (Fig. 3A), indicating that the major portion of the radioactive material is heparan sulfate. This was confirmed by taking a portion of HMW, ridding it of SDS as described under Materials and Methods, and

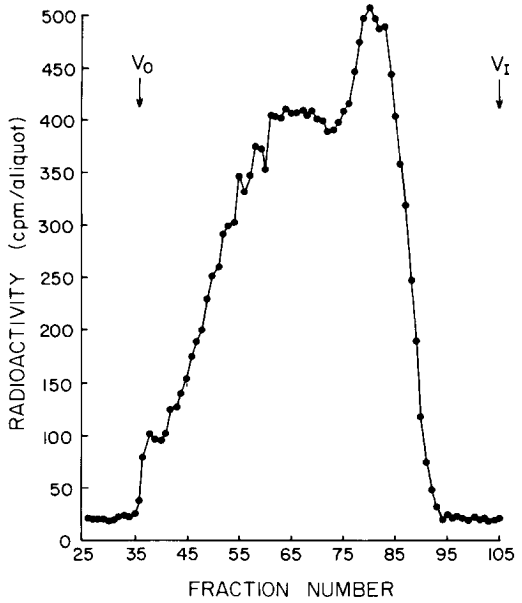

FIG. 2. Gel filtration chromatography of the HMW fraction. The high-molecular-weight region (HMW) of the Sepharose CL6B column described in Fig. 1 was concentrated and passed through a Sepharose CL4B column eluted with 0.2% SDS in 150 mM Tris-HCl (pH 7.4). Fractions were analyzed and the column was calibrated as described in Fig. 1.

digesting it with *Flavobacter heparinum* heparanase kindly donated by Dr. Jeremiah Silbert of Boston's Veterans Administration Outpatient Clinic (data not shown). The nitrous acid-resistant material in Fig. 3A is chondroitin sulfate (susceptible to chondroitinase AC treatment). Analysis of the total cell glycosaminoglycan from [^{3}H]glucosamine-radiolabeled cells showed little detectable dermatan sulfate or hyaluronate. The predominant chondroitin isomer was chondroitin-4-sulfate (E. A. G. Chernoff, G. A. Maresh, and L. A. Culp, unpublished data).

The proteoglycan nature of the extract was tested by treating the HMW fraction with alkaline borohydride. As shown in Fig. 3B, most of the ^{35}S-radiolabeled material between fractions 30 and 45 shifted to a lower-molecular-weight region of the profile, indicating that the higher-molecular-weight material is proteoglycan. However, the ^{35}S-radiolabeled material between fractions 50 and 70 failed to shift, indicating that these are free glycosaminoglycan chains with either a small peptide linked to it or no peptide (this assay could not distinguish between these two possibilities); that the majority of these chains are heparan sulfate was then shown by their sensitivity to nitrous acid (see Fig. 3A). We can conclude from these data that heparan sulfate proteoglycan and "single-chain" heparan sulfate comprise the major portion of the ^{35}S-radiolabeled HMW fraction.

Ion-exchange chromatography. Since success has been achieved in fractionating proteoglycans on ion-exchange columns (31, 32), we made a similar attempt in this system. It was first necessary to rid the

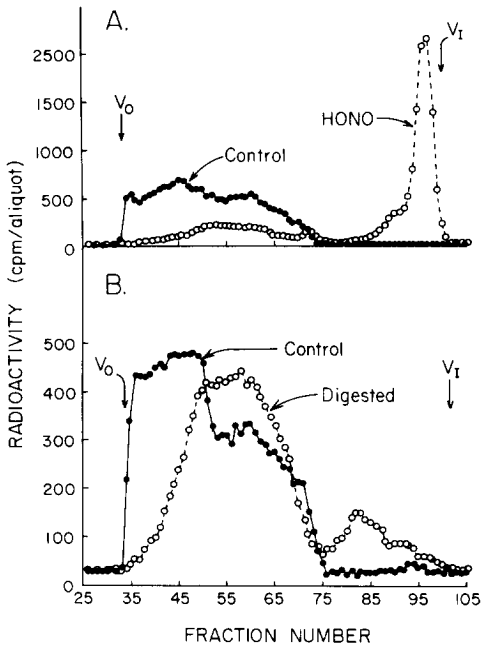

FIG. 3. Sensitivity to digestions of the HMW fraction. The HMW fraction from Fig. 1 was pooled and concentrated. The material was then divided into four portions. Two portions (A) were treated as a control (●) or with nitrous acid (○) to determine the heparan sulfate content of the fraction, as described under Materials and Methods. The other portions (B) were treated as a control (●) or with alkaline borohydride (○) to determine proteoglycan content, as described under Materials and Methods and by Lark and Culp (25, 26). The digests were then eluted through the same Sepharose CL6B column and analyzed for radioactivity as described under Materials and Methods.

HMW fraction (Fig. 1) of its SDS as described under Materials and Methods. The yield of ^{35}S-radiolabeled material from Sep-Pak C_{18} cartridges was routinely 88–96% of the material loaded, and there was so little detergent in the eluate that they could be digested for several hours with *Streptomyces* hyaluronidase, testicular hyaluronidase, or *Flavobacter* heparanase with minimal perturbation of enzyme activity (data not shown). When the HMW fraction was eluted through a DEAE-Sephadex column, the ^{35}S-radiolabeled material eluted as a rather homogeneous class of material at 0.5–0.7 M NaCl without any resolution of distinct entities (probably because the majority of this material is the heparan sulfate class) (data not shown). DEAE-Sephadex columns were therefore of limited usefulness in fractionating the HMW fraction, but do demonstrate that there is very little sulfate-radiolabeled glycoprotein in these HMW extracts which would bind more weakly to this ion-exchange resin.

Affinity chromatography on columns of platelet factor-4. Since the majority of the polysaccharide radiolabeled with $^{35}SO_4^{2-}$ in HMW was shown to be heparan sulfate, and since the heparan sulfate-binding protein PF4 was shown to promote physiologically compatible adherence and spreading of fibroblast cells when adsorbed to the tissue culture substratum (21, 22), we attempted to use affinity columns of PF4-Sepharose to further fractionate the various moieties in HMW. Special care was taken in preparation of the affinity columns to prevent inactivation of the lysine residues in the glycosaminoglycan-binding site of PF4 (see Materials and Methods). As demonstrated in Fig. 4A, this affinity matrix readily resolved the chick cartilage chondroitin sulfate proteoglycan with weak binding affinity (0.1 M NaCl) and the high-affinity-binding heparin (0.55 M NaCl). When the HMW fraction was eluted through PF4-Sepharose, three major and one minor classes of components could be resolved. Each of the major classes (A, B, and C), when pooled and rerun on a second PF4-Sepharose column (Fig. 4B), chromatographed identically, indicating that

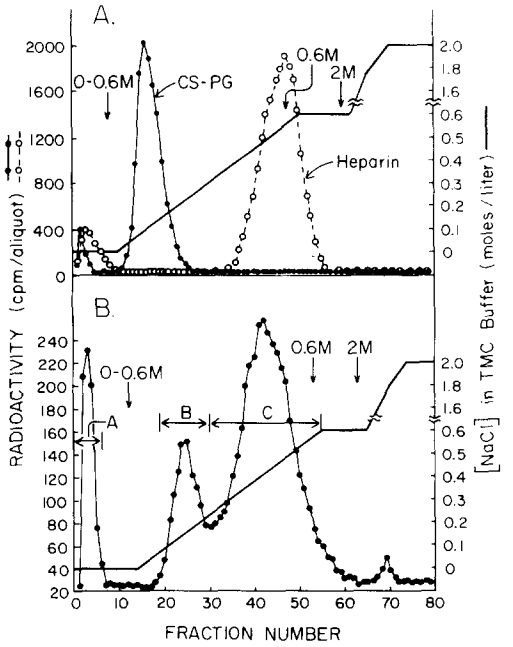

FIG. 4. Platelet factor-4 affinity chromatography. The HMW fraction from Fig. 1 was depleted of SDS with Sep-Pak C_{18} hydrophobic cartridges, and then passed onto a PF4-Sepharose affinity column as described under Materials and Methods. The column was calibrated (A) with ^{35}S-radiolabeled chick cartilage chondroitin sulfate proteoglycan (●) and [^{3}H]heparin (○). Chromatography of the HMW fraction is shown in (B). The column was first eluted with Tris buffer (referred to as TMC), then with a linear gradient of 0–0.6 M NaCl in Tris [0–0.6 M], then with 0.6 M NaCl in Tris [0.6 M], and finally with 2 M NaCl in Tris [2 M]. Aliquots of fractions were assayed for radioactivity with correction for quenching and for salt concentration by refractometry. Peaks A, B, and C of radiolabeled material in (B) were pooled for further analysis.

the column was not saturated with glycosaminoglycan (data not shown). Time was not a factor in this affinity system; batch adsorption of HMW to the PF4-linked beads for as long as 4 h gave exactly the same profile of elution as material eluted straight through the column.

The PF4-resolved classes were then analyzed biochemically. Approximately 60% of the weak-affinity material in peak B (from Fig. 4B) was sensitive to nitrous acid digestion (Fig. 5A), indicating a slighty higher proportion of heparan sulfate than chondroitin sulfate. The majority of the

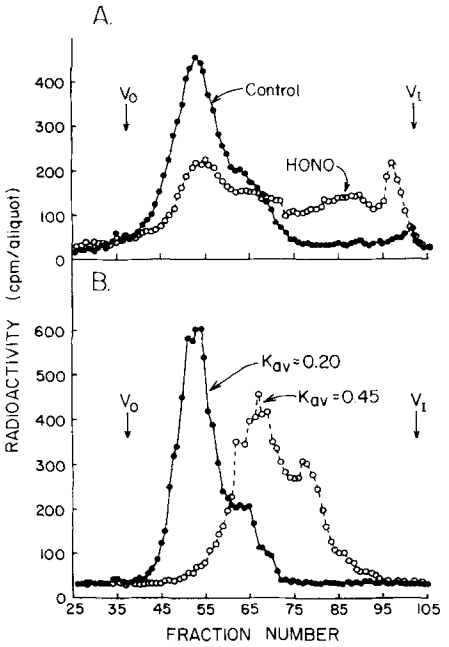

FIG. 5. Sensitivity to digestion of peak B material. Peak B from the PF4-Sepharose affinity column (Fig. 4B) was split into four portions and treated with nitrous acid (A) to identify heparan sulfate sequences, or with alkaline borohydride (B) to identify proteoglycan species. Control samples (●) were chromatographed on the same Sepharose CL6B column as the digests (○) using 0.2% SDS in 150 mM Tris-HCl (pH 7.4) to elute the column.

material shifted upon alkaline borohydride treatment (Fig. 5B) from K_{av} of 0.20 to a K_{av} of 0.45. This would indicate that these are relatively small heparan sulfate *and* chondroitin sulfate proteoglycans with no more than 2-4 glycosaminoglycan chains per core protein; it is also likely that there are two separate proteoglycans in this material, one containing heparan sulfate and a second chondroitin sulfate, since they can be separated on Fibronectin-Sepharose affinity columns (data not shown).

The high-affinity-binding material in peak C of Fig. 4B was similarly analyzed (Fig. 6). This fraction elutes from the PF4-Sepharose column (see Fig. 4B) at 0.45 M NaCl, just slightly below the affinity of heparin chains (see Fig. 4A). Peak C is composed entirely of heparan sulfate which is nitrous acid sensitive (Fig. 6A), and is "single-chain" either with or without a small peptide linked to it (not proteoglycan in the conventional sense), since it is resistant to alkaline borohydride treatment as determined by this assay (Fig. 6B). It therefore appears that this affinity matrix binds free heparan sulfate chains more effectively than it does the multivalent proteoglycan from these neuronal tumor cells. The flow-through material in peak A of Fig. 4B was also analyzed (data not shown). It contains principally heparan sulfate proteoglycan (approx 70–75%), with the remainder being chondroitin sulfate proteoglycan.

Hydrophobic affinity chromatography. A number of laboratories have identified a membrane-associated form of heparan sulfate proteoglycan in a variety of cell systems which has a hydrophobic core protein (29, 30, 33). To test for such a proteoglycan in this neuronal system in a preliminary way, the assay of Kjellen *et al.* (29) was used with octyl-Sepharose affinity

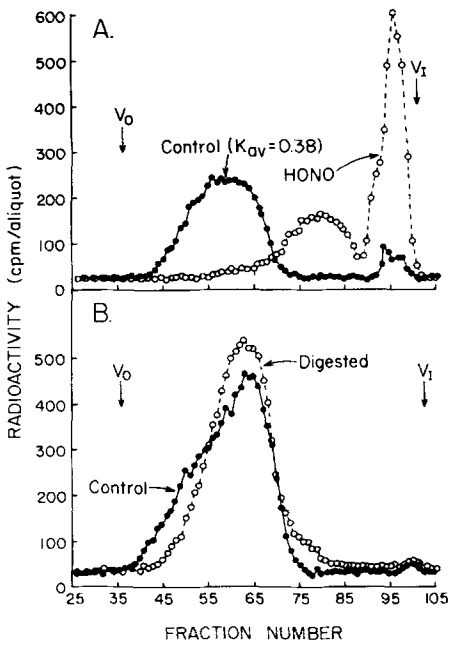

FIG. 6. Sensitivity to digestion of peak C material. Peak C from the PF4-Sepharose affinity column (Fig. 4B) was split into four portions and treated with nitrous acid (A) to identify heparan sulfate sequences, or with alkaline borohydride (B) to identify proteoglycan species. Control samples (●) were chromatographed on the same Sepharose CL6B column as the digests (○) using 0.2% SDS in 150 mM Tris-HCl (pH 7.4) to elute the column.

chromatography and sensitivity to elution with the detergent octylglucoside. When the HMW fraction was batch-adsorbed to octyl-Sepharose beads, a variety of components bound to this matrix (Fig. 7A). Two classes of material could be eluted with low (0.1 M NaCl) and high (3 M NaCl) salt. Approximately 20–22% of the ^{35}S-radiolabeled material could then be eluted from this affinity matrix with octylglucoside, and this particular fraction was completely sensitive to nitrous acid treatment (data not shown). This indicates that there is a subset of heparan sulfate proteoglycan which probably has a hydrophobic sequence in its core protein.

The HMW material was then fractionated on PF4-Sepharose (as in Fig. 4B), and the three major pools (A, B, and C) were tested for their binding to octyl-Sepharose

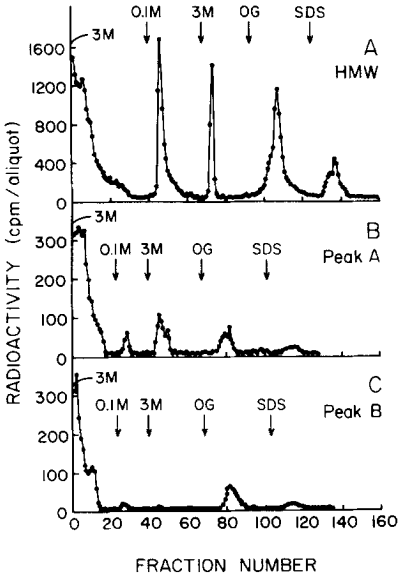

FIG. 7. Octyl-Sepharose affinity chromatography. The HMW fraction (A) (after ridding it of SDS), peak A of the PF-4 affinity column (B), and peak B of the PF-4 affinity column (C) were dialyzed against 3 M NaCl in buffer A, and then batch-adsorbed to octyl-Sepharose as described under Materials and Methods. A column was then poured and eluted successively with 3 M NaCl in 0.2 M Tris-HCl (pH 7.3) [3 M]; 0.1 M NaCl in 0.2 M Tris-HCl (pH 7.3) [0.1 M]; 3 M NaCl in 0.2 M Tris-HCl (pH 7.3) [3 M]; 1% octylglucoside in 3 M NaCl plus 0.2 M Tris (pH 7.3) [OG]; and finally 0.2% SDS in water. Fractions were collected and an aliquot was assayed for radioactivity.

columns. None of the single-chain heparan sulfate of peak C (from Fig. 4B) bound to octyl-Sepharose, as would be expected (data not shown). However, a small fraction of peak A (Fig. 7B) and peak B (Fig. 7C) did bind to octyl-Sepharose. These data indicate that these human neuroblastoma cells generate, by unknown mechanisms, multiple heparan sulfate proteoglycans with overlapping properties for binding to platelet factor-4 and a hydrophobic lipid-like moiety.

DISCUSSION

This study initiates the characterization of heparan sulfate proteoglycans from a neuronal cell derivative of the human central nervous system (10, 23), Platt neuroblastoma cells. This is far-and-away the predominant proteoglycan made by these cells, along with small amounts of chondroitin sulfate proteoglycan. There is mounting evidence for the importance of this proteoglycan in adherence of neuronal cells to various extracellular matrices. Heparan sulfate is the predominant glycosaminoglycan moiety enriched in the tissue culture substratum adhesion sites of rat and human neuroblastoma cells [(9, 10); Vallen *et al.*, manuscript in preparation]. Neuronal cells from various sources attach and spread on the heparan sulfate-binding proteins fibronectin (9, 14–16) or platelet factor-4 (Tobey, Chernoff, and Culp, manuscripts in preparation). And, Lander *et al.* (8) generated evidence that heparan sulfate proteoglycan from conditioned media was an active factor in promoting neurite outgrowth of rat sympathetic neurons. It is therefore important to biochemically characterize the proteoglycans of neuronal cells.

The evidence reported herein indicates that there are multiple heparan sulfate moieties associated with the Platt human neuroblastoma *cell fraction* [excluding the substratum adhesion sites which will be reported separately (Vallen *et al.*, manuscript in preparation)]. Fractionation based on size and on binding to two different affinity matrices has resolved several subsets of heparan sulfate proteoglycan. The SDS-solubilized extracts contain a sizable

amount of "single-chain" heparan sulfate (either with or without a small peptide linked to it) which binds to PF4-Sepharose columns with the highest affinity. The source of these chains and their location in the cell must be resolved with other experiments. In this regard, it has recently been shown (26, 41) that fibroblast substratum adhesion sites contain a subset of heparan sulfate proteoglycan which is catabolized to single-chain glycosaminoglycan, perhaps by an endoglycosidase activity as found in other systems (34-36). The chemical nature of the substratum can also affect the turnover properties of proteoglycan (37, 38).

The PF4-Sepharose affinity matrix distinguishes two different heparan sulfate proteoglycans in human neuroblastoma cells. One class fails to bind at all, even after repeated exposure to a PF4 column. A second proteoglycan does bind to the column, is relatively small in size and included on Sepharose CL6B gel filtering columns, and contains no more than 2-4 glycosaminoglycan chains, as evidenced by the shifting of ^{35}S radioactivity after alkaline borohydride treatment. Whether this small proteoglycan is an intermediate in the catabolism of a larger proteoglycan initially made by these cells or is, in fact, a distinct species synthesized by these cells remains to be determined. It is also interesting to note that the substratum adhesion sites of murine fibroblasts contain two distinguishable heparan sulfate proteoglycans—one which binds to PF4 and a second which does not (42). The molecular basis for the resolving properties of the PF4 affinity columns remains to be elucidated.

The subsets of the PF4-binding and PF4-nonbinding proteoglycans are multiplied when they are tested on the hydrophobic affinity matrix, octyl-Sepharose. For both PF4 classes, there is a small subset which binds to octyl-Sepharose, requiring a detergent to elute it (29, 30), and therefore may have a hydrophobic sequence in its core protein which is membrane associated. Further experiments with a liposome system (29, 33) should resolve this important issue. A sizable amount of the heparan sulfate proteoglycan, either in the HMW fraction or in both of the PF4 classes, fails to bind to octyl-Sepharose and therefore would be associated with the surface of these neuronal cells by a binding receptor-dependent process as described previously (29). In any case, these affinity chromatography studies have resolved four distinctly different classes of heparan sulfate proteoglycan made by Platt human neuroblastoma cells. Furthermore, the physiological signficance of the PF4-binding class has been demonstrated with experiments on the response of Platt cells to PF4-coated substrata (Tobey and Culp, manuscript in preparation)—they attach, spread, and reorganize their cytoskeletal networks similarly to the recent studies of Laterra *et al.* (21, 22) with murine fibroblasts.

Proteoglycans are now being isolated and characterized from a number of noncartilage cell lines (39, 40, 31-33). Two subsets of heparan sulfate proteoglycan have been resolved in a number of systems. Kjellen *et al.* (29) and Norling *et al.* (30) originally described octyl-Sepharose binding and nonbinding proteoglycans from several sources. Oohira *et al.* (32) have reported small-sized high-density and larger low-density heparan sulfate proteoglycans isolated from the culture medium or extracted directly from bovine aortic endothelial cells with SDS-containing buffers. It is therefore becoming increasingly clear that there are multiple subsets of heparan sulfate proteoglycans in many, if not all, cell systems being studied. The molecular bases and the functional significance of these differing subsets have become a high-priority item for future investigation.

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