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Immunopurification and Characterization of A Neuronal Heparan Sulfate Proteoglycan[1]

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Abstract

We have identified a unique heparan sulfate (HeS) proteoglycan synthesized by the neuronal-like cell line PC12. The proteoglycan, purified with monoclonal antibodies from medium conditioned by PC12 cells, has an apparent molecular weight of 350,000, and it contains a M_r 80,000 core protein and HeS side chains of M_r 15,000 each. The purified molecule has the same apparent size and density as it has in conditioned medium.

HeS proteoglycans that are indistinguishable antigenically but very difficult to solubilize are found on the external surface and in the interior of PC12 cells and neurons. Mild proteolysis converts the surface proteoglycan into a molecule closely resembling that found in the medium. The same surface antigens are also present on a subpopulation of T-cells and on a non-neuronal accessory cell found in dorsal root ganglion cultures.

The PC12 cell line and the non-neuronal dorsal root ganglion cells secrete a factor into medium that, after adsorption to polylysine-coated surfaces, induces rapid neurite outgrowth by primary sympathetic neurons. The monoclonal antibodies used to purify the neuronal HeS proteoglycan from PC12 cells are capable of depleting this conditioned medium of its neurite-promoting activity. These studies suggest that a HeS proteoglycan synthesized and secreted by neurons and certain accessory cells plays a role in regulating neurite outgrowth.

Proteoglycans have been found in extracellular matrices (Hassell et al., 1980), on the cell surface (Oldberg et al., 1979), and inside of the cell (Pacifici et al., 1983), often associated with particular organelles. Specific proteoglycans have been found in cell adhesion plaques and thereby have been implicated in cell adhesion and motility (Lark and Culp, 1983). Others appear to play important roles in organizing basement membranes (Schubert et al., 1983) and regulating the permeability of these membranes to small molecules (Van Harreveld et al., 1971). Proteoglycans have been found in transmitter-containing vesicles and have been postulated to play an important role in transmitter storage (Bennett and Leblond, 1977; Cook, 1977) or in alignment of synaptic structures, by dint of their deposition in the synaptic cleft (Buckley et al., 1983).

Many different cells secrete factors into medium that, when attached to the substrate, induce neurite outgrowth (reviewed by Berg, 1984). One such factor, secreted by corneal endothelial cells, has been partially purified and shown to contain a HeS proteoglycan associated with the biological activity (Lander et al., 1982, 1983). In this report, we describe the biochemical properties of a purified neuronal HeS proteoglycan which is associated with a similar neurite outgrowth activity.

Materials and Methods

Materials. Chondroitinase ABC, chondroitinase AC, hyaluronidase, hyaluronic acid (grade IV), trypsin (type III), soybean trypsin inhibitor, and neuraminidase (type IX) were obtained from Sigma Chemical Co.; Pronase was from Calbiochem-Behring Corp.; CsCl was from Schwartz-Mann; and bovine lung heparan sulfate was from Upjohn Laboratories. Antiserum to NGF was the generous gift of Dr. Eric M. Shooter; crude heparinase was the generous gift of Dr. Alfred Linker.

PC12 cells. The rat pheochromocytoma cell line, PC12 (Greene and Tischler, 1976) was obtained from Dr. D. Schubert (Salk Institute). For preparation of conditioned medium (CM), cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal calf serum and 10% horse serum. CM was harvested from cultures that were at least 2 days old with final densities of 10^6 cells/ml. Serum-free CM (CM_{SF}) was prepared by incubating confluent monolayers for 2 days in DMEM alone or in DMEM supplemented with transferrin (50 μg/ml), insulin (5 μg/ml), SeO_2 (4 ng/ml) (Block and Bothwell, 1983), glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). Both CM and CM_{SF} were centrifuged and filtered through a 0.45-μm filter before storage at 4°C.

PC12 membranes were prepared from 5×10^7 cells and rinsed twice with DMEM and once with phosphate-buffered saline (PBS) containing 0.5 mM *phenylmethylsulfonyl fluoride* (PMSF). Cells were scraped from the dishes with a rubber policeman in 30 ml of cold PBS/PMSF, homogenized in a glass-Teflon homogenizer and centrifuged at 20,000 × *g* for 20 min. Then followed four sequential cycles of homogenization and centrifugation at 4°C in H_2O/PMSF, 1 M NaCl/PMSF, PBS/PMSF, and PBS. THe final pellet was resuspended in 1 ml of PBS and stored at −80°C.

Radiolabeling of PC12 cells was carried out by growth in DMEM with no inorganic sulfate, no penicillin or streptomycin sulfate, and only 10% of normal amino acid concentration, supplemented with 20 μCi/ml of [^{35}S] sulfate (Amersham/Searle Corp.) and 50 μCi/ml ^{3}H-amino acid mixture (Amersham). Cells were plated at 5×10^5/ml, grown for 4 days, and then harvested by low-speed centrifugation. CM was millipore-filtered, adjusted to 1% calf serum, and stored at −20°C.

Immunopurification of the proteoglycan. Isolation of hybridoma-secreting cell lines and preparation of antibodies was carried out as described in Matthew et al. (1981). Immunoaffinity columns were prepared by linking each of five monoclonal antibodies, purified from ascites fluid, and normal mouse serum to Sepharose 4B (Hudson and Hay, 1976). To purify the antigen, 4 ml of PC12 CM, labeled with [^{35}S]sulfate and ^{3}H-amino acids, were incubated

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sequentially with 2 ml of each of the Sepharose-antibodies (aPG3, aPG22, aPG42, and normal mouse serum) for 1 hr at 25°C with gentle rocking, followed by washing with 100 ml of PBS, 5% calf serum, and 10 ml of 0.9% NaCl. In other words, the immunopurified antigen from the aPG3 column was next purified on a column of aPG22 followed by aPG42. The antigen was eluted from the first two antibodies with 0.1 M Na-glycine, pH 3.0. The third immunoaffinity column was eluted with 4 M GuCl/50 mM Tris, pH 7.0. The nonadhering fraction was collected from the final column of normal mouse serum.

Density and molecular weight determinations. Samples of radiolabeled PC12 medium were prepared for ultracentrifugation by layering 1-ml aliquots onto Bio-Gel P-2 columns (exclusion limit, M_r 1800) equilibrated with 4 M GuCl/20 mM Tris, pH 7.0, collecting void volumes, adjusting the total volume to 4.89 ml with 4 M GuCl, and dissolving 2.87 g of CsCl in it. Samples of purified proteoglycan were treated similarly, with the omission of the Bio-Gel P-2 column. Samples were centrifuged to equilibrium in a Beckman SW 50.1 rotor at 40,000 rpm for 70 hr. 280-μl fractions were collected, of which 50 μl were weighed and then counted in Aquasol.

For gel filtration, 50-μl aliquots of radiolabeled PC12 CM or immunopurified proteoglycan, adjusted to 0.9% NaCl or 4 M GuCl, were layered onto Sepharose CL-4B columns equilibrated with 0.9% NaCl or 4 M GuCl and 50 mM Tris, pH 7.0 at 4°C. One-ml fractions were collected, and an aliquot from each fraction was counted in Aquasol. For those fractions containing 4 M GuCl, a 50-μl sample was layered onto Bio-Gel P-2 columns equilibrated with PBS. The void material was collected and incubated in multiwells or polylysine-coated multiwells for radioimmunoassays. Each of the five antibodies was used to monitor the elution of antigen and gave identical results. Columns were calibrated with IgG (M_r 150,000), ferritin (M_r 440,000), and IgM (M_r 1,000,000).

Gel filtration of the proteoglycan core protein after β-elimination was carried out on a 100-μl sample of immunopurified proteoglycan by layering it onto a Bio-Gel P-2 column equilibrated with 0.2 M sodium sulfite, pH 11.5 at 25°C, and incubating the void material at 25°C for 24 hr (Simpson et al., 1972). After adjustment to 4 M GuCl, the sample was concentrated by dialysis against solid polyethylene glycol and chromatographed on a Bio-Gel P-150 column equilibrated with 4 M GuCl/50 mM Tris, pH 7.0. The column was calibrated with chymotrypsinogen A, ovalbumin, and bovine albumin.

Carbohydrate chains of the proteoglycan were prepared from a 100-μl sample of immunopurifed proteoglycan layered onto a Bio-Gel P-2 column equilibrated with PBS, collected in the void volume, and digested with 1 mg/ml Pronase for 4 hr at 25°C. The sample was concentrated to 50 μl by dialysis against solid polyethylene glycol at 25°c for 2 hr, adjusted to 4 M GuCl, and subjected to gel filtration on a Sephadex G-150 column equilibrated with 4 M GuCl/50 mM Tris, pH 7.0. The eluted fractions were counted in Aquasol. The column was calibrated with samples of dextrans and dextran sulfates of molecular weights 5,000, 8,000, 10,000, 15,000, and 50,000.

TABLE I

Competition between different antibodies

Five aPG antibodies were tested for steric hindrance of the binding of one of them, ^{125}I-aPG3, to synaptic membranes (SM) and PC12 membranes (PC12M). Initially, a serial dilution of each membrane preparation was assayed to ensure that each antigen would be saturated by the concentration of antibody in culture supernatants. Antibody concentrations in the hybridoma supernatants were 5 to 10 μg/ml. Limiting amounts of membrane were adsorbed to microwells; then hybridoma supernatants of the different aPG culture supernatants were incubated in the microwells overnight at 4°C. A trace amount (approximately 10 μg/ml) of ^{125}I-aPG3 antibody was added to each microwell, washed, dried and counted. As controls, two antibodies against different determinants were included: aGA9 recognizing galactose moieties on myelin membranes (Matthew, 1981) and aSV48 recognizing a synaptic vesicle protein (Matthew et al., 1981).

Competing Antibody	Percentage of ^{125}I-aPG3 Binding	
	SM	PC12M
aPG3	0	0
aPG15	38	47
aPG22	1	1
aPG31	88	69
aPG42	32	11
aGA9	97	100
aSV48	98	99

TABLE II

Distribution of HeS proteoglycan in PC12 cells

To measure internal antigen, 10^7 PC12 cells were washed and trypsinized with 2 mg/ml trypsin for 30 min at 25°C and then washed twice by pelleting in DMEM at 1000 × *g* for 5 min. Cells were retrypsinized for 10 min at 4°C and then washed once in DMEM with 5% fetal calf serum, once in DMEM with 5% fetal calf serum and 5 mg/ml soybean trypsin inhibitor, and three times in DMEM. 80% of the trypsinized cells were frozen immediately after the washes, thawed and homogenized, and then assayed by radioimmunoassay on polylysine-coated multiwell plates. As controls, the 20% of the cells kept unfrozen were maintained at 4°C and assayed for residual surface antigen. These control cells were incubated in suspension with aPG antibody and then ^{125}I-second antibody and were washed three times between each step. They bound none of the tested antibodies, verifying the complete removal of all surface antigen.

To measure surface antigen, 50-ml cultures of PC12 cells were grown to 2×10^5 cells/ml, collected, centrifuged three times in 10 volumes of medium at 500 × *g* for 10 min, and resuspended at 10^5 cells/ml. One-milliliter aliquots were plated into each of the 24 wells in a Falcon tissue culture dish and allowed to adhere for 90 min. All subsequent incubations and washes were done with ice-cold PC12 medium. Each well was rinsed three times, and 1 ml of hybridoma culture fluid was applied for 1 hr on ice. Cultures were rinsed four times, and 1 ml containing 1 μCi of ^{125}I-goat anti-mouse Ig serum (20 μCi/μg) was added in PC12 medium. After 1 hr on ice, cultures were washed five times and digested with 100 μl of 1 mg/ml Pronase for 1 hr at 25°C. After addition of 0.4 ml of 10% SDS, the contents of each well were removed and counted.

To measure secreted antigen, washed cells were grown for 24 hr in fresh growth medium at 2×10^5 cells/ml and removed by centrifugation at 500 × *g* for 10 min. The CM was filtered through a Millipore filter (0.22 μm) and aliquoted into polylysine-coated multiwell plates for radioimmunoassay. Results are presented for two of the aPG antibodies, but all five gave similar results. Controls consisted of wells incubated with medium minus mouse antibodies (100 cpm) or with indifferent antibodies aGA9 and aSV48 (see legend to Table I). All assays were done in triplicate and did not vary by more than 4%.

Antibody	^{125}I cpm		
	Internal	Surface	Secreted
aPG22	3485	2036	1416
aPG3	5216	2503	2300
aSV48	3357	125	82
aGA9	76	106	62

TABLE III

Effects of enzyme digestion on antibody binding

CM_{SF} was prepared from PC12 cells as described in "Materials and Methods" and adjusted to 10 mg/ml bovine serum albumin. Aliquots were incubated for 12 hr at 25°C with each enzyme. The digested samples were adsorbed to polylysine-coated assay wells and tested in a radioimmunoassay.

Antibody	Control	Percentage Remaining			
		Hyaluronidase	Chondroitinase ABC	Chondroitinase AC	Heparinase
aPG3	100	92	102	98	9
aPG15	100	83	99	96	21
aPG22	100	87	95	101	25
aPG31	100	97	96	94	12
aPG42	100	86	86	105	18

Solid-phase radioimmunoassay. Radioimmunoassays were done on soluble antigens and membrane preparations after adsorption to polyvinyl chloride 96-well plates (Klinman, 1972). After thorough washing, antibodies were applied to the wells for 6 hr at 25°C, rinsed well, and then incubated with ^{125}I-labeled second antibody, washed, and counted. For competition experiments, the blocking antibody was incubated together with antigen overnight. For radioimmunoassays on soluble glycosaminoglycan-containing material, plates were first coated with 0.1 ml of poly-D-lysine at 1 mg/ml for

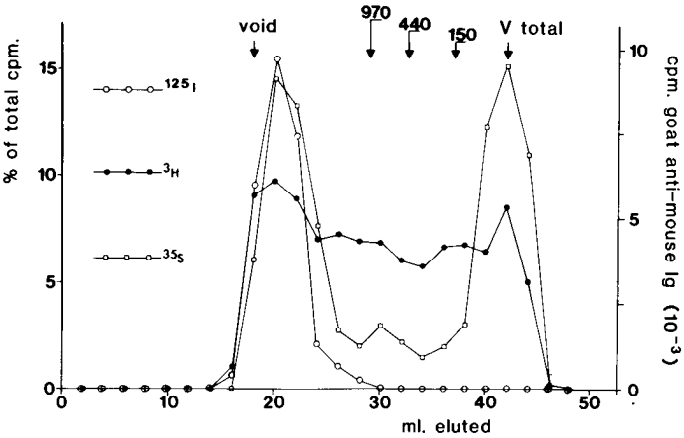

Figure 1. Permeation chromatography of PC12 CM in associative conditions. PC12 CM, metabolically labeled with ^{3}H-amino acids and inorganic [^{35}S]sulfate, was chromatographed on Sepharose CL-4B equilibrated in PBS. The elution profiles of ^{35}S (□), ^{3}H (●), and antibody aPG3 binding (O) are shown.

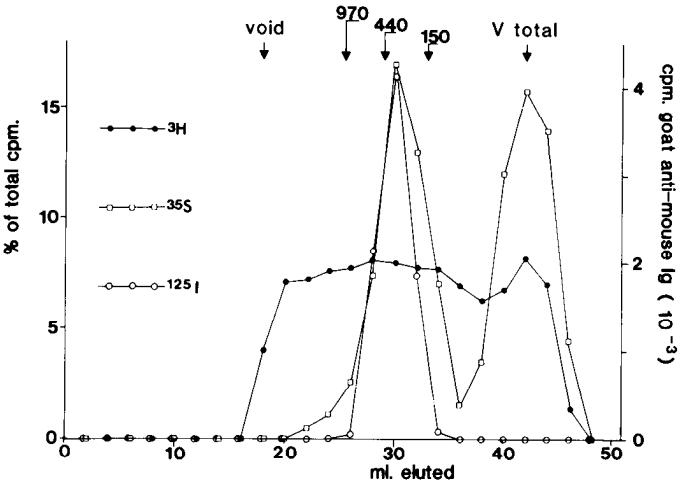

Figure 2. Permeation chromatography of PC12 CM in dissociative conditions. PC12 CM, metabolically labeled with ^{3}H-amino acids and inorganic [^{35}S]sulfate, was chromatographed on Sepharose CL-4B equilibrated in 4 M GuCl/50 mM Tris-Cl, pH 7.0. The elution profiles of ^{35}S (□), ^{3}H (●), and antibody aPG3 binding (O) are shown.

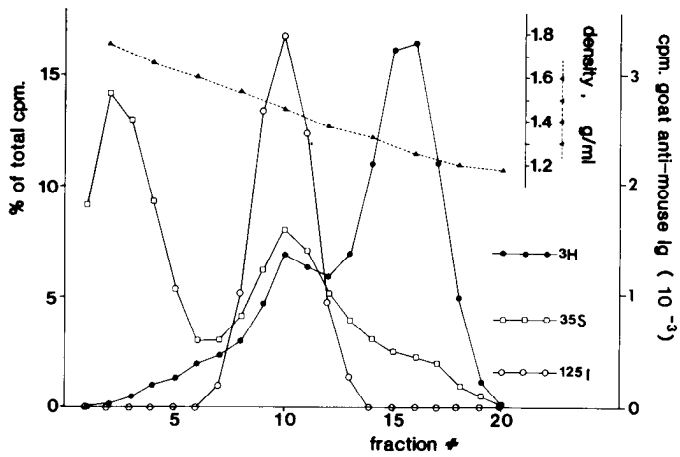

Figure 3. CsCl density gradient ultracentrifugation of CM. PC12 CM, metabolically labeled with ^{3}H and ^{35}S, was desalted and centrifuged to equilibrium in a CsCl gradient containing 4 M GuCl. The density (▲), ^{3}H distribution (●), ^{35}S distribution (□), and antibody aPG22 binding (O) in each fraction are shown.

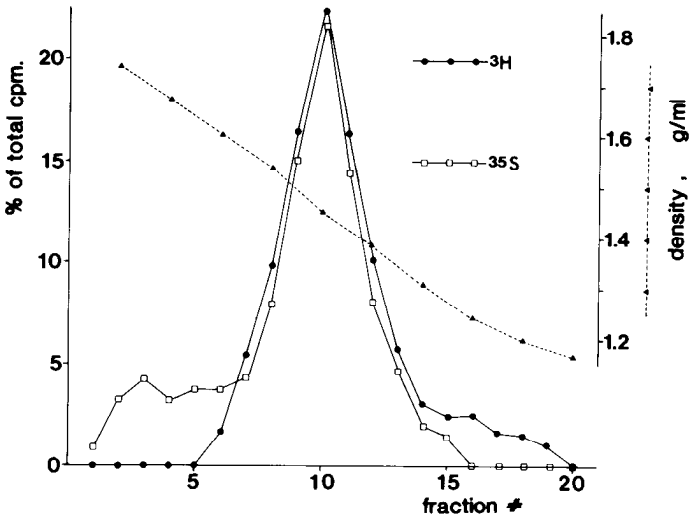

Figure 4. CsCl density centrifugation of immunopurified antigen. The purified antigen from metabolically labeled PC12 CM was centrifuged as described in Figure 3. The density (▲), ^{3}H distribution (●), and ^{35}S distribution (□) in each fraction are shown.

1 hr and then emptied, air dried, and rinsed thoroughly with distilled water. Iodination of immunoglobulins was carried out with 1 mCi carrier-free ^{125}I (Amersham) according to the method of Hunter and Greenwood (1962), to a specific activity of 10 to 30 μCi/μg.

As controls in the various antibody experiments, monoclonal antibodies against a synaptic vesicle protein, aSV30 and aSV48 (Matthew et al., 1981), or against galactose moieties on myelin membranes and desmin intermediate filaments, aGA9 (Matthew, 1981), were used.

Enzymatic treatments. Trypsin was used at 100 μg/ml for 2 hr at 37°C, after which soybean trypsin inhibitor was added to a final concentration of 500 μg/ml. As a control, trypsin inhibitor was used at 100 μg/ml for 2 hr at 37°C. Neuraminidase was used at 2.5 U/ml for 5 hr at 37°C. Chondroitinase ABC and AC were used at 2 U/ml for 6 hr at 37°C. Heparinase was used at 30°C as indicated.

Primary cell cultures. Sympathetic, sensory, cerebellar, cerebral cortical, and olfactory bulb neurons from neonatal Sprague-Dawley rats were dissociated and cultured as described in Lander et al. (1982); leptomeningeal cells were cultured as described in Raff et al. (1979). Thymocytes and spleen cells were prepared by mechanical shredding of the appropriate adult rat tissues. Fibroblasts and a non-neuronal accessory cell, henceforth referred to as "flat cells," were cultured from dorsal root ganglia prepared according to Fields et al. (1978). Flat cells were obtained by allowing DRG coverslip cultures to grow for 10 to 12 days, by which time they had expanded from 1 to 50% of the population and formed a very loosely adherent layer on top of all other cell types. An enriched culture was then produced by gently washing the loosely adherent flat cells from the coverslips and plating them in DMEM with 10% calf serum. An immortal cell line was derived from the enriched culture by maintaining cells in this way for 3 months. CM from flat cells was prepared in the same way as for PC12 cells (see above).

Neurite outgrowth assays. Assays of neurite extension by neonatal cells from rat superior cervical ganglion, preparation of culture substrata from CM, enzymatic treatments of these substrata, and the evaluation of neurite outgrowth were carried out as described in Lander et al. (1982). All values of neurite outgrowth are the averages of two to four assays, each representing counts of 75 to 100 cells.

Immunofluorescence on dissociated cells. Cells were plated on collagen-coated glass coverslips. Primary cells were grown for at least 4 days before staining. Coverslips were removed from the culture dish, rinsed in 5% calf serum, incubated with undiluted hybridoma culture supernatant for 40 min at 25°C, rinsed, incubated with a 40-fold dilution of fluorescein-coupled goat-anti-mouse Ig serum for 40 min at 25°C, mounted in elvanol, and viewed with a Zeiss IM fluorescence microscope. Rabbit sera were generally used at a 100-fold dilution.

Cell types were identified in these cultures by means of cell-type specific antisera in double-label experiments with monoclonal antibodies. Tetanus toxin (a gift of Lederle Laboratories) identified neurons (Fields et al., 1978). In peripheral nervous system cultures, anti-RAN-1 (a gift of Dr. K. Fields)

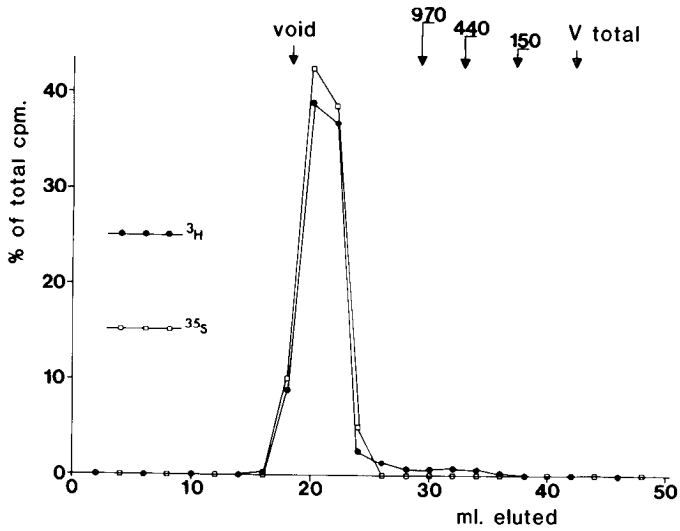

Figure 5. Gel chromatography of immunopurified antigen under associative conditions. The metabolically labeled, purified antigen was chromatographed on Sepharose CL-4B in PBS. The elution profiles of ^{3}H-protein (●) and ^{35}S-carbohydrate (□) are shown.

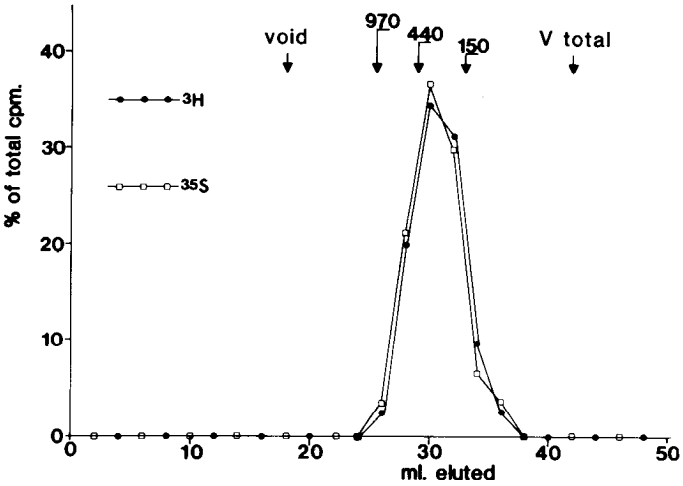

Figure 6. Gel chromatography of immunopurified antigen under dissociative conditions. The metabolically labeled, purified antigen was chromatographed on Sepharose CL-4B in 4 M GuCl/50 mM Tris, pH 7.0. The elution profiles of ^{3}H-protein (●) and ^{35}S-carbohydrate (□) are shown.

identified Schwann cells. In central nervous system cultures, antigalactocerebroside (a gift of D. Shelton) identified oligodendrocytes and antiglial fibrillary acidic protein (a gift of Dr. L. Eng) identified astrocytes (Raff et al., 1979). Fibroblasts fail to bind any of the toxins or sera cited above, and they also have a distinctive appearance. Anti-Thy-1.1 (a gift of Dr. J. Goodman) and antifibronectin (a gift of Dr. K. Fields) bind most of these cells (Raff et al., 1979).

Immunoprecipitations of CM neurite-promoting factor from flat cells and PC12 cells. For immunoprecipitation of flat cell CM, aPG antibodies 15, 22, 31, and 42 were immobilized by attachment to Sepharose 4B (see above) and, in a separate experiment, by adsorption to formaldehyde-fixed Staph A bacteria. For each antibody, 1 ml of CM was incubated overnight, gently rocking, with a quantity of packed beads (1 ml) or fixed bacteria (0.3 ml) estimated to carry approximately 1 to 2 mg of bound antibody. After centrifugation and millipore filtering, the CM supernatant was diluted to a concentration at which unadsorbed CM gave an approximately half-maximal response (25% of the cells extending neurites), and it was then applied to the polylysine-coated substrate for the outgrowth assay. Controls consisted of CM adsorptions with Sepharose 4B and Staph A, neither of which had any bound antibody.

In the case of PC12 CM, immunoprecipitations were done with aPG 31 and with an indifferent antibody aSV30, a monoclonal antibody specific for a synaptic vesicle antigen (Matthew et al., 1981). For each antibody, 1 ml of

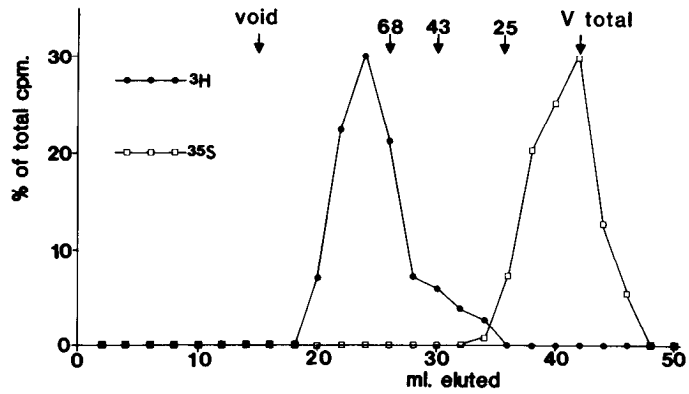

Figure 7. Estimation of core protein molecular weight by chromatography. The metabolically labeled and purified proteoglycan was incubated with 0.2 M sodium sulfite, pH 11.5, for 24 hr at 25°C; the solution was adjusted to 4 M GuCl and chromatographed on Bio-Gel P-150 in 4 M GuCl/50 mM Tris, pH 7.0. The elution profiles of ^{3}H-protein (●) and ^{35}S-carbohydrate (□) are shown.

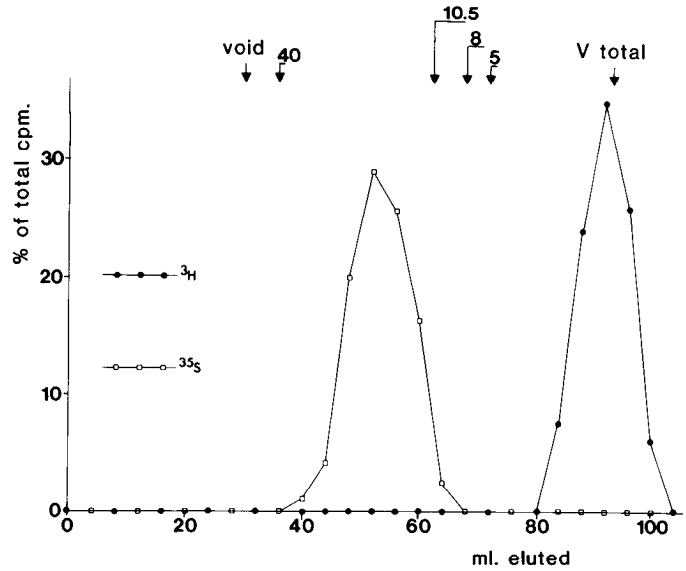

Figure 8. Estimation of GAG molecular weight by chromatography. Metabolically labeled and purified proteoglycan was digested with 1 mg/ml Pronase and chromatographed on Sephadex G-75 in 4 M GuCl/50 mM Tris, pH 7.0. The elution profiles of ^{35}S-carbohydrate (□) and ^{3}H-protein (●) are shown.

CM was incubated overnight at 4°C with a volume of ascites fluid containing 1 mg of antibody. Excess staph A was added to the mixtures, incubated for 1 hr, and then removed by centrifugation. The supernatant was millipore-filtered and then assayed as described above.

Results

Monoclonal antibodies to a neuronal HeS proteoglycan. Five different monoclonal antibodies, made against synaptic junctional protein from rat brain, bind a set of closely neighboring determinants found in synaptic membranes. A competition assay demonstrates this, showing that when one of the antibodies, aPG3, is radioactively labeled, its binding to these membranes is partially inhibited by each of the other four (Table I).

The neuron-like cell line PC12 (Greene and Tischler, 1976) bears an antigenically indistinguishable complex in its membranes (Table I). Furthermore, these cells secrete the same antigens into the medium, as well as harboring them internally (Table II; cf. Greif and Reichardt, 1982).

Given that all five antibodies recognized the same (presumably major) component and that glycosaminoglycans and proteoglycans

TABLE IV

Size and antibody binding of purified antigen after digestion

^{35}S-labeled proteoglycan was immunopurified from PC12 CM_{SF} as described in "Materials and Methods," adjusted to 10 mg/ml bovine serum albumin, then aliquoted and digested with 1 mg/ml of each enzyme for 4 hr at 25°C. Nitrous acid degradation has been described in Lander et al. (1982). Bio-Gel P-6 columns were used to determine the approximate size of ^{35}S-labeled material. Void materials have molecular weights greater than 5,000, and V_{Total} materials have molecular weights less than 1,500. The immunoprecipitability of the treated material was assayed in multiwell plates coated with ascites aPG3. Each well received 2000 cpm of ^{35}S and was incubated for 4 hr at 25°C. The nitrous acid treated antigen was not tested in this assay. Values cited are the average of triplicate wells which did not differ by more than 5%.

	Percentage of cpm		Percentage Immunoprecipitable
	Void	V_{Total}	
Control	100	0	90
Hyaluronidase	90	6	72
Heparinase	7	91	6
Chondroitinase AC	97	1	85
Chondroitinase ABC	90	7	79
Nitrous acid	29	65	

TABLE V

Binding of aPG antibodies to the HeS proteoglycan, Its component core protein, and GAG fragments

The immunopurified HeS proteoglycan antigen, core protein, and GAG chains were assayed in the standard radioimmunoassay (see "Materials and Methods"). Core protein was adsorbed to untreated assay wells, whereas GAG chains were adsorbed to polylysine-coated wells. Assays were done in triplicate and did not vary by more than 3%. Controls included no mouse antibody (100 cpm) and aGA9 and aSV48 (see legend to Table I). Values have had background (approximately 100 cpm) subtracted.

Antibody	cpm ^{125}I-Second Antibody		
	Immunopurified Antigen	Core Protein	GAG Chain
aPG3	9764	7	1998
aPG15	4264	370	76
aPG22	8165	650	90
aPG31	6090	15	1689
aPG42	7442	287	56
aGA9	9	0	0
aSV48	35	1	7

are abundant in brain (Margolis, 1967; Margolis et al., 1972), it seemed reasonable to test for linkage between the antigens and glycosaminoglycan. Enzyme treatments of CM containing the antigens from PC12 cells demonstrate that all five determinants are closely associated with a heparan sufate-containing molecule (Table III), inasmuch as heparinase destroys their binding to polylysine.

Further evidence for the proteoglycan nature of the molecule comes from permeation chromatography under associating and dissociating conditions. Proteins in CM from PC12 cells were metabolically labeled with ^{3}H-amino acids, while sulfated carbohydrates, including glycosaminoglycans, were labeled with [^{35}S]sulfate. Permeation chromatography on Sepharose 4B, followed by an antibody binding assay to the different fractions, shows the antigens eluting in a unimodal peak centered under the major macromolecular peak of sulfate label and one of the main peaks of amino acid incorporation (Fig. 1), corresponding to a molecular weight of approximately 10,000,000 for a compact protein. Fractionation of similarly radiolabeled CM in the presence of 4 M guanidine chloride (GuCl), conditions that dissociate proteoglycan complexes, produces a coincident shift in the peaks of sulfate labeling and of antibody binding (Fig. 2), down to a position corresponding to a molecular weight of approximately 350,000.

TABLE VI

Solubilization of antigen from synaptic membranes

Column headings refer to solubilization treatments; row titles refer to the subcellular fraction assayed. A 100-μl aliquot of synaptic membranes in PBS (protein = 100 μg/ml) was adjusted to 0.1% Triton X-100 (TX-100) and incubated for 20 min at 25°C. An identical sample was maintained in PBS. Both samples were centrifuged at 100,000 × *g* for 1 hr in an airfuge (Beckman). The pellets were washed two additional times in PBS. Supernatants from the three centrifugations were pooled for assay. The final pellets were resuspended in PBS. The total number of antigen units in supernatants and pellets was determined for each sample by radioimmunoassay (see "Materials and Methods"). The samples containing Triton were diluted 50-fold in PBS containing 5% calf serum in order to permit binding to the plate.

Trypsin treatment consisted of incubating 4-ml synaptic membranes in PBS (protein = 10 μg/ml) with 0.025% trypsin/5 mM EDTA/5 mM EGTA for 20 min at 37°C. The reaction was terminated with 0.03% soybean trypsin inhibitor, and the membranes were pelleted at 10,000 × *g* for 15 min. The supernatant was recovered, and 100-μl aliquots were adsorbed to multiwell plates for 2 hr at 25°C and 10 hr at 4°C. The membrane pellet was washed three times and separated into 40 microfuge tubes. Forty equivalent tubes of untrypsinized synaptic membrane were aliquoted in parallel. The amount of antigen was quantified by binding aPG 22 or aPG31 and ^{125}I-goat anti-mouse Ig serum in solution. The membranes were washed three times between each incubation by pelleting at 10,000 × *g* for 10 min. In both assays, the monoclonal antibody and goat anti-mouse Ig serum were used at saturating concentrations. Controls included normal mouse serum or the absence of mouse antibody. Each assay was done in triplicate, and values did not vary by more than 4%. Values are expressed as a percentage of total binding in the control.

aPG Antibody	PBS		TX-100		Trypsin	
	22	31	22	31	22	31
Membrane	98	99	97	98	67	76
Soluble	2	1	3	2	56	76
Total	100	100	100	100	123	152

Since most proteoglycans have buoyant densities intermediate between protein and carbohydrate, they can be separated from proteins and glycoproteins in density gradients. CsCl density gradient centrifugation of radiolabeled CM from PC12 cells in the presence of 4 M GuCl gives results consistent with a proteoglycan identity for the antigens (Fig. 3). The major peak of ^{3}H-labeled protein equilibrates at 1.30 g/ml, while a smaller protein peak coincient with a peak of ^{35}S-labeled carbohydrate equilibrates at 1.45 g/ml. The remainder of radioactive sulfate is concentrated at the bottom of the gradient (1.60 g/ml). Antibody binding activity equilibrates as a single molecular species at 1.45 g/ml, coinciding exactly with the superimposed peaks of ^{3}H and ^{35}S.

Purification and characterization of the antigen. A conclusive demonstration that these antigens are part of a unique proteoglycan requires that a purified molecule retain all of the properties found associated in CM. To this end, radiolabeled CM from PC12 cells was subjected to sequential immunopurification with monoclonal antibodies aPG3, aPG22, and aPG42 as described in "Materials and Methods," and the purified antigen was compared to whole CM.

The purified antigen equilibrates in a dissociating CsCl gradient at a density identical to that found with CM (1.45 g/ml), with over 90% of the ^{35}S- and ^{3}H-label forming a monotonic peak (Fig. 4). The residual protein and sulfated carbohydrate may have been specifically associated with the proteoglycan under the nondenaturing conditions of purification, only to be dissociated by GuCl in the gradient.

Permeation chromatography also indicates nearly homogeneous purity of the antigen and once again matches the findings with whole CM. Under associative conditions, the purified proteoglycan elutes as a single peak of molecular weight of approximately

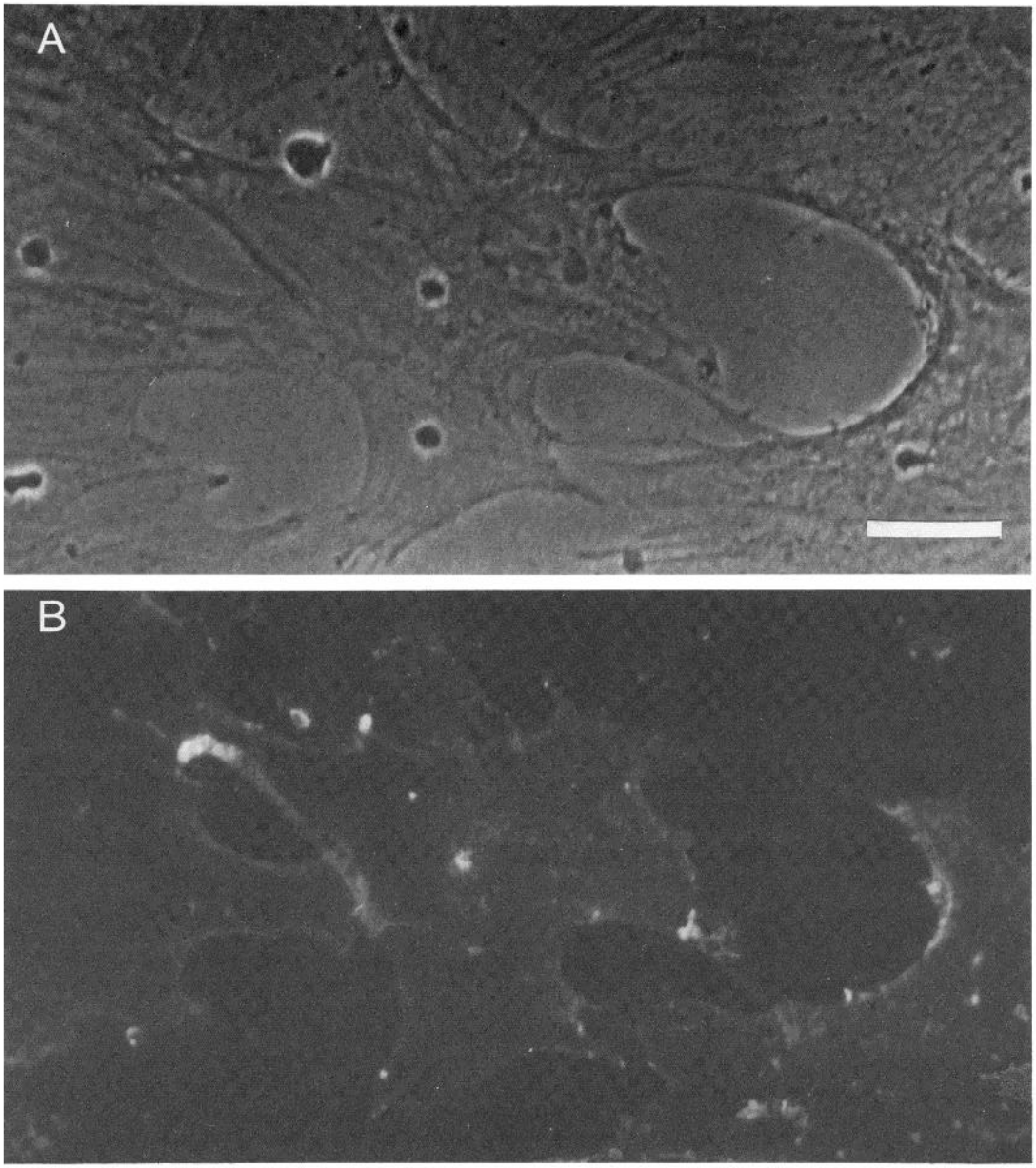

Figure 9. Flat cells derived from DRG cultures. (*A*) Phase-contrast micrograph. (*B*) Indirect immunofluorescence with aPG3. *Bar* = 50 μm.

10,000,000 (Fig. 5), as if associated in a large complex. In GuCl, it elutes as a single peak of apparent molecular weight of 350,000 (Fig. 6). Even though the standards used were proteins, not proteoglycans, this is probably a reasonable estimate of molecular weight, since the standards are extended in 4 M GuCl (Tanford, 1968). The coincidence of ^{3}H and ^{35}S in a single peak indicates that the proteoglycan is considerably pure.

Characterization of protein and cabohydrate moieties. Treatment of proteoglycans with mild alkali destroys protein-*O*-glycosaminoglycan linkages by β-elimination (Simpson et al., 1972). A sample of purified proteoglycan so treated was chromatographed in dissociative conditions on Bio-Gel P-150, producing a single protein peak with apparent molecular weight of 80,000 (Fig. 7). Since the protein core may still contain *N*-linked, unsulfated carbohydrate side chains, this may be an overestimate. The ^{35}S-GAG molecules migrate very near the end of this chromatography, as expected for molecules of molecular weight 10,000 to 15,000.

A more accurate size measurement of the GAG side chains was obtained after Pronase digestion of the purified proteoglycan and chromatography on Sephadex G-75 (Fig. 8). Now the ^{35}S-label elutes as a single peak of molecular weight 15,000.

Enzyme treatments verified that the GAG side chains of this proteoglycan are indeed HeS. Digestion of purified proteoglycan with hyaluronidase, chondroitinase ABC, or chondroitinase AC had no effect on its apparent molecular weight in a Bio-Gel column, while both heparinase and nitrous acid reduced its apparent size (Table IV). Nitrous acid specifically hydrolyzes HeS (Cifonelli, 1968). Coincident with a reduction in molecular weight, heparinase treatment also specifically eliminated binding of aPG3 (Table IV). Taken altogether, these data describe a proteoglycan consisting of approximately 18 HeS side chains linked to a protein core.

The separability of the proteoglycan into protein core and HeS side chains made possible a determination of which antibodies specifically bound one moiety as opposed to the other. Pronase digestion provided isolated heparan sulfate chains, while mild alkali treatment followed by gel filtration yielded protein cores, and these were assayed for antigenic activity in solid-phase radioimmunoassays. Each antibody binds protein or carbohydrate but not both

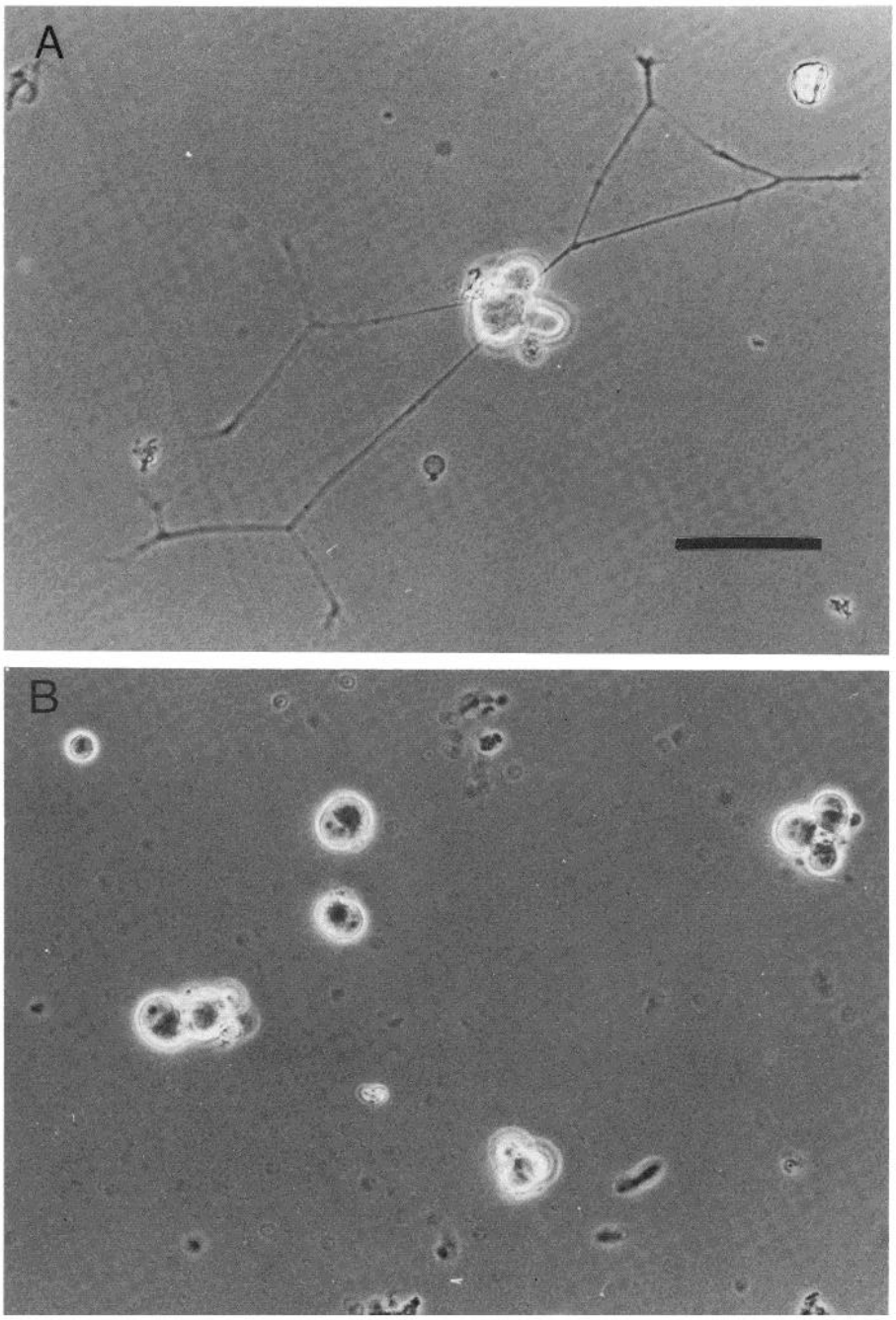

Figure 10. Neurite outgrowth response to flat cell CM. Primary rat superior cervical ganglion neurons were plated onto polylysine substrata which had been incubated with (*A*) flat cell CM or (*B*) fresh medium and photographed 8 hr later. *Bar* = 50 μm.

(Table V), with antibodies aPG15, aPG22, and aPG42 specific for the former and aPG3 and aPG31 specific for the latter. None of the antibodies shows signficant binding to purified HeS from bovine lung (data not shown).

Distribution of the antigen. Having purified a HeS proteoglycan from pheochromocytoma cells with antibodies made against synaptic junctional protein from brain, it was of interest to know where else this molecule resided. As described earlier, it is present in synaptic membranes from rat brain. This form turns out to be insoluble in non-ionic detergents (Table VI), requiring sodium dodecyl sulfate for full solubilization. Mild trypsinization of these membranes releases a soluble form of the molecule, as measured by antibody binding to supernatant as opposed to the treated membranes (Table VI); it also apparently exposes more antigenic sites. These results suggest that the antigenic determinants are linked to the cell membrane by means of a trypsin-sensitive protein.

The cell types displaying these antigens, as assayed by immunofluorescence on dissociated cells, are neurons, thymocytes, PC12 cells, and a previously unidentified non-neuronal accessory cell found in DRG cultures, "flat cells" (Fig. 9; also see "Materials and Methods"). In all cases except thymocytes, the cells stained positively and with a similar pattern for all five aPG antibodies. Subpopulations of thymocytes showed differential staining for the antigens defined by aPG31 or aPG42, representing the only instance encountered so far in which the five determinants do not colocalize to the same molecule. The neurons tested came from cerebellum, cerebral cortex, superior cervical ganglion, and dorsal root ganglion. Oligodendrocytes, leptomeningeal cells, and Schwann cells failed to stain for the antigens, as did astrocytes (except for a subpopulation found in optic nerve). Fibroblasts and spleen cells did not display the antigens either.

Flat cells distinguish themselves by their constellation of surface antigens. While resembling fibroblasts in many respects—morphology in phase-contrast, positive staining for anti-Thy-1.1, failure to

TABLE VII

Immunoprecipitation of neurite-inducing factor by aPG antibodies

Flat cell CM was incubated with aPG antibodies bound previously to either Sepharose 4B or Staph A and then centrifuged, and the supernatant filtered and assayed as described in "Materials and Methods." In the controls, CM was incubated with either Sepharose or Staph A containing no bound antibody or else was not adsorbed at all.

PC12 CM_{SF} was incubated with 1 mg ascites and then precipitated with excess Staph A and assayed as described in "Materials and Methods." Controls were done with the an antibody (aSV30) that recognizes a synaptic vesicle protein (Matthew et al., 1981) or with no CM.

	Percentage of Responding Neurons
A. "Flat cell" CM	
Antibody	
Sepharose or Staph A alone	21.0 ± 7.3[a]
aPG15	9.3 ± 1.3
aPG22	5.5 ± 3.4
aPG31	8.5 ± 2.6
aPG42	10.0 ± 6.1
B. PC12 CM	
aSV30	37.6[b]
aPG31	5.0
Without CM	4.2

[a] Mean ± SD for four assays.

[b] Average of duplicate assays.

stain with anti-RAN-1, anti-glial fibrillary acidic protein, or tetanus toxin—they differ in their failure to stain with anti-fibronectin. Moreover, the five aPG antibodies stain flat cells in a similar speckled pattern (Fig. 9) while failing to stain fibroblasts at all. Flat cells comprise a very small fraction (approximately 1%) of cells in freshly dissociated cultures of DRG, as well as in other tissues tested, superior cervical ganglion, cerebral cortex, and rat heart.

Antibodies precipitate neurite promoting activity from PC12 and flat cell CM. Given that the neurite outgrowth activity described by Lander et al. (1982) is associated with a heparan sulfate proteoglycan, that PC12 cells synthesize and secrete a heparan sulfate proteoglycan, and that flat cells appear to make the same molecule, we reasoned that these two cell types were likely to produce a similar activity. CM and CM_{SF} from cultures of either of these cell types, when first attached to a polylysine substrate, will induce vigorous neurite extension of neonatal superior cervical ganglion neurons within 16 hr (Fig. 10). The polylysine substrate serves to attach and presumably concentrate the anionic proteoglycan material (cf. Lander et al., 1982). Under these conditions, neurite growth requires neither NGF nor serum.

Moreover, the activity obtained from PC12 cells is sensitive, in this assay, to the enzymes trypsin and heparinase, but not to hyaluronidase, chondroitinase ABC, or chondroitinase AC (flat cell CM was not tested in this experiment). Trypsin and heparinase reduced the neurite outgrowth response to background levels (5%), while the other enzyme treatments gave results comparable to controls with no enzyme or with trypsin plus soybean trypsin inhibitor (range, 18 to 28%).

With the demonstration that these cells do make the neurite activity and that it behaves as if associated with a HeS proteoglycan, we reasoned further that the aPG antibodies ought to be capable of removing this activity from CM. Immunoprecipitation experiments of flat cell or PC12 CM with aPG antibodies confirmed our expectation (Table VII). With flat cell CM, several antibodies were tried and, not surprisingly, they differed in the efficiency with which they removed the activity. The results indicate association of this unique complex of antigens with neurite-promoting activity and therefore, as defined by the PC12 cells, with the HeS proteoglycan the biochemical identity of which we have established.

Attempts to induce neurite outgrowth with the immunopurified HeS proteoglycan failed. Two different preparations were tried, one having been eluted in the final step with 4 M GuCl, the other with 0.1 M glycine, pH 3. Under either set of conditions, dissociation of other proteins or denaturation of the proteoglycan would be likely (cf. Lander et al., 1983).

Discussion

Monoclonal antibodies prepared against synaptic membranes have identified a unique HeS proteoglycan. Molecular weights for each component of this molecule have been estimated by gel filtration. The core protein appears to have a molecular weight of 80,000, the HeS side chains have a molecular weight of 15,000, and the whole molecule has a molecular weight of 350,000. While we have purified only the PC12 molecule, we have identified a protein in brainsynaptic membranes that shares important biochemical and antigenic characteristics (cf. Rapraeger and Bernfield, 1983). Likewise, the flat cell molecule, in addition to sharing antigenic identity, displays an analogous biological property: association with a neurite-promoting activity. These correlations suggest that the aPG antibodies recognize and define a HeS proteoglycan which is common to central and peripheral neurons as well as present on a previously unidentified non-neuronal accessory cell, which we have named flat cells.

Functions for proteoglycans are unknown, though as many have been proffered as there are putative locations for the molecules. Their postulated roles include control of tissue hydration (Polansky et al., 1974), maintenance of structural integrity (Mustafa and Kamat, 1973), control of neuronal migration and differentiation (Margolis et al., 1975), regulation of segregation and storage of other molecules (Farquhar and Palade, 1981), and mediation of adhesion by fibroblasts (Lark and Culp, 1983), myoblasts (Schubert et al., 1983), and synaptic terminals (Buckley et al., 1983).

Experiments with CM from corneal endothelial cells by Lander et al. (1982) first established a link between an activity-promoting neurite outgrowth and HeS proteoglycan. Two hypotheses arose from the work, indistinguishable at the time, regarding the relationship of the proteoglycan to the activity. Either the proteoglycan *is* the activity, or else it is part of a complex containing the activity. Subsequent work has made it clear that another molecule is necessary for this activity. A monoclonal antibody raised against PC12 cell CM and capable of blocking neurite extension (Matthew and Patterson, 1983) recognizes the M_r 350,000 proteoglycan only when it is associated with a nonsulfated molecule the molecular weight of which is approximately 1 million in 4 M GuCl. Moreover, this blocking antibody fails to stain neurons at all, and it does stain peripheral glial cells. Independent evidence comes from further purification of the activity from bovine corneal endothelial cells, demonstrating a separable, nonproteoglycan protein factor capable of eliciting the response (Lander et al., 1983; A. L. Lander, unpublished data).

The association of a neurite-promoting factor with HeS proteoglycan, however, remains a consistent observation, bolstered by the results reported here. Our findings demonstrate that two different cell types synthesize and secrete the same antigenically distinct HeS proteoglycan and that, in both cases, it is part of an apparent complex with neurite-promoting activity. Plausibly, such an association could be a vehicle for ensuring proper deposition of the factor in the extracellular matrix. Given the apparent involvement of proteoglycans in cellular adhesion complexes (e.g., Lark and Culp, 1983; Schubert et al., 1983), and the presence of our HeS proteoglycan on neuronal surface membranes, its propensity for association with neurite outgrowth factor could also indicate an adhesive role for the proteoglycan in binding the neuron to its extracellular substratum.

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