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The neuronal cell-surface molecule mitogenic for Schwann cells is a heparin-binding protein

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ABSTRACT **The cell surface of embryonic peripheral neurons provides a mitogenic stimulus for Schwann cells. We report (*i*) the solubilization of this mitogenic activity from rat dorsal root ganglion neurons grown in tissue culture and (*ii*) the solubilization and partial purification of mitogenic activity from neonatal rat brains. Extracted mitogenic activity is peripheral rather than intrinsic to the membrane, stable after extraction, and active as a mitogen in the absence of serum (the most stringent criterion defining the neuronal mitogen). We have previously provided evidence suggesting that a neuronal cell-surface heparan sulfate proteoglycan is required for expression of the neurons' mitogenic activity. We now show that mitogenic activity can be extracted from the membrane dissociated from proteoglycan as assayed by its ability to bind to immobilized heparin. After dissociation, low concentrations of heparin (1 μg/ml) inhibit the ability of the mitogen to stimulate Schwann cell division. Basic fibroblast growth factor (FGF) is weakly mitogenic for Schwann cells, but it is not present in mitogenic brain extracts (based on immunoblotting). Immunodepletion experiments with specific antibodies to FGF indicate that the mitogenic activity extracted from neurons is not a form of this heparin-binding mitogen. Acidic FGF is not mitogenic for Schwann cells and is not present in mitogenic brain extracts. We suggest that these and previous data indicate the neurite mitogen is a proteoglycan–growth factor complex that limits mitogenic activity to the axonal surface, protects mitogen against inactivation by other proteoglycans, and provides for effective presentation of mitogen to the Schwann cell.**

A complex series of cell–cell interactions among neurons, Schwann cells, and fibroblasts lead to the formation of the peripheral nerve. One of these interactions is the neuron-stimulated proliferation of Schwann cells, first suggested by experiments in which the ratio of Schwann cells to neurons remained constant when neuronal numbers were altered (1). These *in vivo* observations have been extended in an *in vitro* system is which neurons and Schwann cells can be separated and recombined; *in vitro* studies demonstrate that contact with the neuronal surface causes Schwann cells to undergo a dramatic increase in both DNA synthesis and cell division (2–4). Several lines of evidence indicate that the cell surface of the neuron (rather than a released factor) carries the mitogenic stimulus. Membrane fragments from dorsal root ganglion neurons grown in tissue culture mimic the mitogenicity of whole cells, and trypsin treatment of intact neurons in culture prevents the mitogenic response of Schwann cells added to these neurons (5). Furthermore, a semipermeable collagen membrane interposed between Schwann cells and neurons in the culture dish inhibits the mitogenic response (6), and conditioned medium from neurons is not mitogenic for Schwann cells (2, 4).

Recent tissue culture studies indicate that the neuronal cell-surface mitogen is associated covalently or noncovalently with a cell-surface heparan sulfate proteoglycan. An inhibitor of proteoglycan biosynthesis, 4-methylumbelliferyl β-D-xyloside, decreases the mitogenicity of embryonic dorsal root ganglion neurons and of neuronal plasma membranes derived from neurons grown in the presence of the inhibitor (7); inhibitors of glycoprotein processing do not have a similar effect (8). Heparitinase, an enzyme that degrades heparan sulfate proteoglycans, also diminishes the mitogenicity of neurons and membranes derived from the neurons for Schwann cells (7).

To further characterize the cell–cell interaction that results in Schwann cell proliferation, we have begun to purify the neuronal cell-surface mitogen. We demonstrate the solubilization of the mitogen from dorsal root ganglion neurons in culture, which can only be obtained in limited quantity. A much more abundant source of the mitogen has been identified in extracts of neonatal rat brain. The solubilized mitogenic activity can be separated from the neuronal cell-surface proteoglycan, as assayed by its ability to bind to immobilized heparin. These results have been reported in abstract form (9, 10).

MATERIALS AND METHODS

Schwann Cell Culture. Primary Schwann cells were prepared from 1- to 3-day-old rat sciatic nerves by a slight modification (8, 10) of the method of Brockes *et al.* (11). After a single treatment with anti-thy1.1, cells were plated onto uncoated glass Lab-Tek slides at 1.5–2 × 10^4 cells per well in Dulbecco's modified Eagle's medium containing 10% fetal calf serum at 35°C.

Mitogenicity Assays. Forty-eight fours after plating, the Schwann cells were mitotically quiescent. Mitogen was added to cells in defined N2 medium (8) without nerve growth factor and with the addition of garamycin (2 μg/ml) and fungizone (2.5 μg/ml) in a total vol of 300 μl. After 16–18 hr, 10 μl of [^{3}H]thymidine (6.7 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) was added to each well to a final concentration of 1.3 μCi/ml, and the incubation continued for an additional 24 hr. Fixation with acetic acid/ethanol, processing for autoradiography, toluidine blue staining, and cell counting have been described (9).

Membrane Preparation. Growth of mass cultures of embryonic day 15 dorsal root ganglion neurons and preparation of membranes from these primary cells has been described (7). For membrane preparation from neonatal rats, 1- to 24-hr-old rats were anesthetized by cold exposure, decapitated, and brains were removed. Brains were homogenized at a ratio of 10 brains to 40 ml of homogenization buffer (HB) (0.25 M sucrose/0.1 mM $MgCl_2$/10 mM Tris·HCl/0.63 mM

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Abbreviations: FGF, fibroblast growth factor; bFGF, basic FGF; aFGF, acidic FGF.
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phenylmethylsulfonyl fluoride/17 milliunits of aprotinin per ml) with 50 strokes in a Dounce homogenizer. Nuclei were removed by centrifugation (10 min at 2000 × *g*) and crude membranes were isolated by centrifugation from the resultant supernatant (130,000 × *g* for 1 hr).

Solubilization of Mitogenic Activity. Membranes were solubilized under various conditions (see Fig. 1) in HB plus 2 mM EGTA at a ratio of 10 brains to 35 ml of buffer. After 60–90 min of incubation on ice, insoluble material was removed by centrifugation (130,000 × *g* for 1 hr).

Partial Purification of Mitogen. Solubilized extract from 10 rat brains was incubated with 2 ml of Affi-Gel-heparin (Bio-Rad) per 10 brains for 60–90 min at 4°C with rotation. The gel was then poured into a column and washed with 10 column vol of loading buffer. Mitogenic activity was eluted with either 4 ml of 1.2 M NaCl in HB or a 20-ml salt gradient of 0.5–1.5 M NaCl in HB.

Fibroblast Growth Factor (FGF). Basic FGF (bFGF) was prepared from bovine pituitaries as described (12) and was a gift of B. Wice (Washington University, St. Louis). Samples were also obtained from Collaborative Research. Acidic FGF (aFGF) was prepared from bovine brains according to published procedures (13). Recombinant endothelial cell growth factor was the gift of Michael Jaye (Meloy Laboratories, Horsham, PA).

Anti-FGF. IgG from polyclonal antisera against amino acids 1–24 of bFGF and intact aFGF were the gifts of Terry Riss (University of Texas at Houston). IgG fractions from each antiserum were obtained by chromatography on protein A-Sepharose and elution with 0.1 M acetic acid, followed by immediate neutralization. IgG fractions were dialyzed against phosphate-buffered saline (pH 7.4) (PBS) and stored at −20°C.

Electrophoretic Protein Transfer Blot. Protein (25 ng of FGF or 75 μg of heparin purified mitogenic brain extract) was electrophoresed on 12% $NaDodSO_4$/polyacrylamide gels and transferred to nitrocellulose paper according to standard procedures. The nitrocellulose paper was then blocked with gelatin overnight and incubated with either IgG fraction overnight (aFGF) or 1 hr (bFGF). Bound IgG was then visualized by 1-hr incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG, followed by color development.

DNA Synthesis in Serum-Deprived NR6 Cells. NR6 cells, an epidermal growth factor receptor-negative variant of Swiss 3T3 cells (14), were used to assay mitogenicity of FGF as described (15).

RESULTS

We have found that a combination of 30 mM octylglucoside and 0.5 M NaCl solubilizes mitogenic activity from membranes derived from dorsal root ganglion neurons grown in tissue culture (Fig. 1). The limiting amounts of dorsal root ganglion neuronal membrane available for analysis led us to search for a more abundant source of mitogen for further purification that shared with the dorsal root ganglion mitogen activity in serum-free chemically defined medium. We found that neonatal rat brain membranes are mitogenic for Schwann cells under these conditions; the specific activity is approximately one-half that of dorsal root ganglion neuron membranes (data not shown; see ref. 4).

Large amounts of neonatal brain membranes enabled us to carry out controlled experiments to test conditions that remove peripheral or intrinsic proteins from brain membranes for their ability to extract mitogenic activity. In three independent experiments, activity was recovered after extraction in 0.13 M sodium pyrophosphate or 0.5 M NaCl, conditions that extract molecules peripherally associated with membranes. Detergent alone extracted mitogen of low specific activity. Detergent plus 0.5 M NaCl, a condition that extracts intrinsic proteins from membranes, extracted maximum mitogenic activity from brain membranes (Table 1). By this criterion, 61% of the brain membrane-derived mitogenic activity is peripherally associated with the membrane.

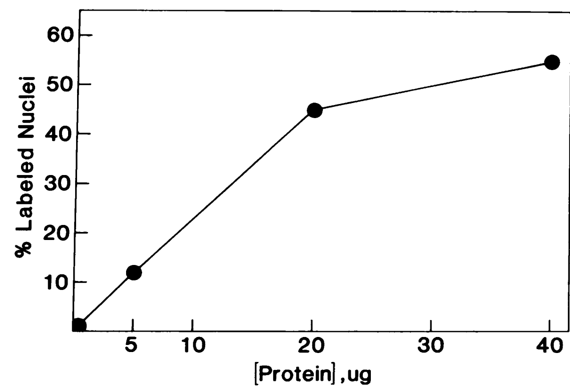

FIG. 1. Extraction of mitogenic activity from dorsal root ganglion neurons. Dorsal root ganglion neurons from 800 embryonic day 15 dorsal root ganglia were grown for 3 weeks *in vitro*. Membranes were prepared from neurons, free of nonneuronal cells, and solubilized in 30 mM octyl β-D-glucopyranoside/0.5 M NaCl/0.2 mM EGTA in homogenization buffer. After dialysis against Dulbecco's modified Eagle's medium, samples were added to Schwann cells in defined serum-free medium for mitogenicity assay. Protein concentration was based on the A_{280} of the extract prior to dialysis.

Since we have previously shown that the neuronal cell-surface mitogen is either itself a heparan sulfate proteoglycan or is associated with a heparan sulfate proteoglycan (7), the ability of mitogenic extracts to bind to a heparin affinity matrix was tested. NaCl (0.5 M) extracts of brain membranes were incubated with Affi-Gel-heparin. The recovery of mitogenic activity from Affi-Gel-heparin after elution with 1.2 M NaCl is shown in Table 2. These data, taken together with previous observations (7), suggest that the neuronal mitogen can be separated from a membrane-associated proteoglycan, which therefore is not required for activity of the solubilized mitogen, and show that mitogenic activity can be partially purified by using immobilized heparin.

To determine the affinity of the neuronal mitogen for heparin, activity was eluted from Affi-Gel-heparin using a salt gradient (Fig. 2). In four separate experiments, activity eluted from Affi-Gel-heparin at 0.55–0.76 M NaCl. Gradient elution from Affi-Gel-heparin resulted in 2-fold higher specific activity than step elution.

Heparin at concentrations up to 1 mg/ml does not inhibit the proliferation of Schwann cells stimulated by intact dorsal

Table 1. Extraction of mitogenic activity from neonatal rat brain membranes

Extraction condition	Units recovered	Specific activity
Sodium pyrophosphate (0.13 M)	18.5	38.5
NaCl (0.5 M)	92.4	30.3
Octylglucoside (30 mM)	50	5
NaCl (0.5 M) + octylglucoside (30 mM)	150	16.7

Crude brain membranes derived from neonatal rat brains were aliquoted into four samples and extracted in homogenization buffer including salt and/or detergent as shown for 1 hr on ice. Samples were clarified by centrifugation (1 hr at 130,000 × *g*) and extracts were dialyzed against 2–100 vol changes of PBS. Samples were added to Schwann cells at several dilutions and percentage labeled Schwann cell nuclei (LI) were determined after autoradiography. One unit of activity was arbitrarily defined as the amount of mitogen required to stimulate one-half of the maximum mitogenic response (usually LI ≈ 50%). Concentration of protein was determined by a modified Lowry assay (16). Specific activity is expressed as units per mg of protein.

Table 2. Partial purification of neonatal brain extract on Affi-Gel-heparin

Condition	Units/mg	% recovery	Enrichment
NaCl extract (0.5 M)	47.6	100	
Eluate of NaCl extract (1.2 M)	1668	82	35-fold

Control experiments determined that binding of mitogen to Affi-Gel-heparin was maximal after 60 min on ice; longer incubations led to decreased specific activity. Resin was titrated with mitogenic extract and equivalent results were obtained by using 2–10 ml of Affi-Gel-heparin per extract per 10 brains. Samples were eluted with 2 column vol of 1.2 M NaCl in HB and dialyzed against 1000 vol of PBS before addition to Schwann cell mitogenicity assays as described for Table 1. When data for five independent experiments were pooled, the average recovery of activity in the mitogen eluted from heparin was 52.2% ± 21.8%, with an enrichment of 21.8 ± 8.3-fold.

root ganglion neurons in tissue culture (Table 3). However, 90% of the mitogenic response of Schwann cells to solubilized mitogen is blocked by heparin (1 μg/ml). The inhibition is dose dependent and is half maximal at 0.5–0.7 μg/ml. Heparan sulfate is less effective in blocking mitogenic activity. Chondroitin sulfate, a glycosaminoglycan based on repeating heterodimers of *N*-acetyl-D-galactosamine and D-glucuronic acid is not inhibitory to the extract.

Since the neuronal Schwann cell mitogen is a heparin-binding protein and because heparin has been shown to inhibit proliferation of endothelial cells stimulated by FGF (17), we assessed the possibility that the neuronal mitogen is aFGF or bFGF, heparin-binding mitogens present in brain (16, 18). The affinity of the neuronal mitogen for heparin differs markedly from that of aFGF or bFGF, which elute from heparin columns at 1 M and 1.5–2 M NaCl, respectively (16, 18). The neuronal mitogen and FGF were further compared on the basis of their ability to stimulate Schwann cell proliferation and the ability of antisera specific for aFGF or bFGF to recognize proteins present in neuronal mitogen preparations. We found that bFGF causes dose-dependent Schwann cell proliferation at concentrations identical to those that maximally stimulate the proliferation of serum-starved NR6 cells (Fig. 3). However, while the neuronal mitogen stimulates 60–80% of Schwann cells in a preparation to incorporate [^{3}H]thymidine in a 40-hr bioassay, bFGF is only weakly mitogenic, stimulating 5–10% of Schwann cells to incorporate label (Fig. 3). Neither aFGF nor endothelial

Table 3. Effect of heparin on Schwann cell proliferation

Heparin, μg/ml	% labeled nuclei: Intact neurons*	% labeled nuclei: Solubilized mitogen†
0	71.6	19.7
1	ND	1.4
10	69.1	0.6
100	72.3	ND
1000	71.5	ND

ND, not determined.

*Dorsal root ganglion neurons free of nonneuronal cells were grown on collagen-coated coverslips (8). Purified Schwann cells, scraped off plastic Petri dishes, were added for 1.5 hr in serum-containing medium. Medium was changed to defined N2 medium including [^{3}H]thymidine (2 μCi/ml) ± heparin for 24 hr before fixation and autoradiography. At the end of the incubation, all Schwann cells were tightly associated with neurons; there was no indication of cell loss even in the presence of heparin (1 mg/ml). The data are representative of those obtained in three separate experiments.

†Heparin-purified brain mitogen was added to purified Schwann cells in the presence or absence of glycosaminoglycans in the standard proliferation assay. Background was 0.4%. In the same experiment, chondroitin sulfate (100 μg/ml) did not inhibit Schwann cell proliferation (LI, 22.3%). Heparin sulfate (10 μg/ml) partially inhibited proliferation (LI, 6.4%). The data are representative of those obtained in 10 separate experiments.

cell growth factor was mitogenic for neonatal rat Schwann cells at concentrations up to 250 ng/ml; these preparations were active on serum-starved NR6 cells at a half-maximal concentration of 1 ng/ml (data not shown).

To ensure that the responsiveness of Schwann cells to brain extracts was not due to FGF associated with an activating molecule, we transferred FGF (both acidic and basic) and neuronal mitogen, partially purified on Affi-Gel-heparin, to nitrocellulose paper after $NaDodSO_4$ gel electrophoresis and probed the paper with polyclonal antisera to aFGF or bFGF. The monospecific antisera detected 25 ng of the corresponding FGF but did not detect any immunoreactivity in the neuronal extracts (data not shown). Based on the stimulation of Schwann cell proliferation observed with these neuronal extracts, and assuming that all of that activity was due to bFGF, there should have been at least 170 ng of FGF in those lanes, yet none was detected.

To corroborate these results, IgG fractions of antisera against bFGF were tested for their ability to deplete activity

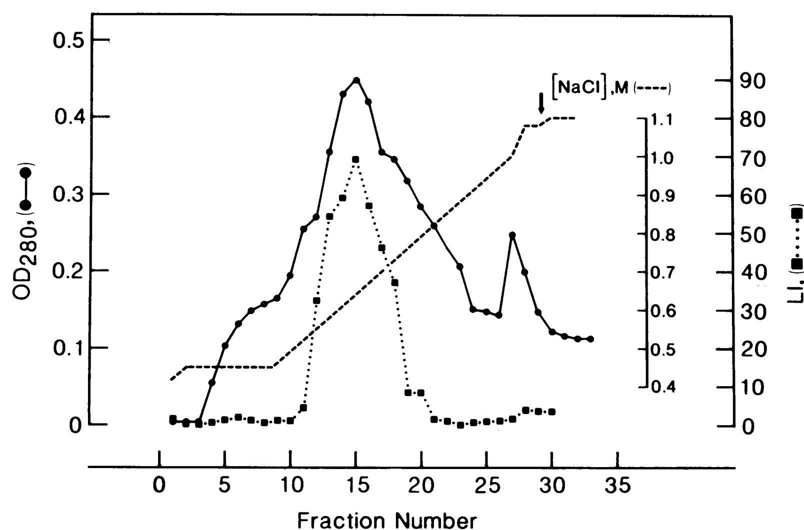

FIG. 2. Elution of mitogenic activity from Affi-Gel-heparin. Extract from 20 neonatal brains was bound in batch to Affi-Gel-heparin and eluted with a 0.5–1.5 M NaCl gradient. The A_{280} of the unbound fraction was 1.1. Fractions (0.8 ml) were collected at a flow rate of 12 ml/hr. Thirty microliters of each column fraction was added to Schwann cells for mitogenicity assay after dialysis against PBS.

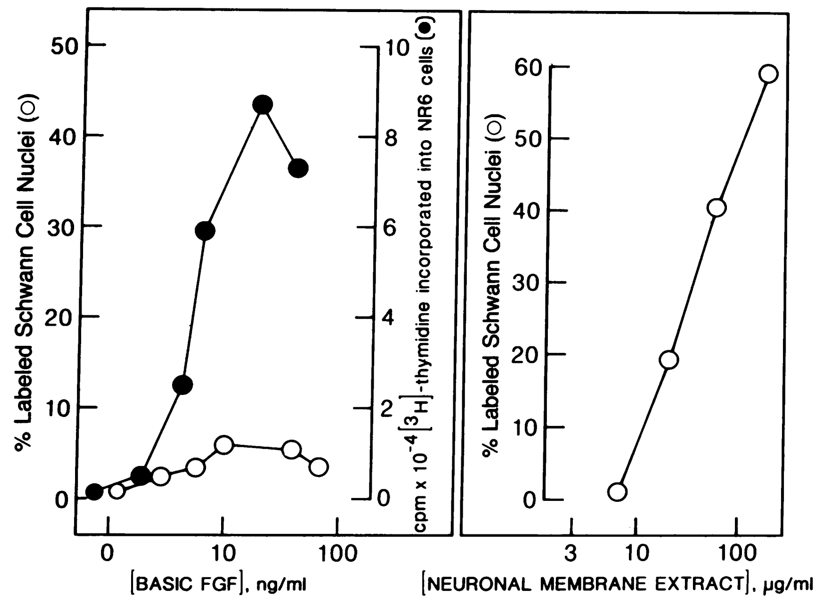

FIG. 3. bFGF is weakly mitogenic for rat Schwann cells. bFGF was added in parallel to serum-starved NR6 cells and neonatal rat Schwann cells (*Left*) in serum-free N2 medium. Crude 0.5 M NaCl extract was added to Schwann cells (*Right*) in serum-free N2 medium. NR6 cell assays were quantitated after trichloroacetic acid precipitation; Schwann cell assays by autoradiography.

from neuronal extracts. Fig. 3 shows the results of two such experiments. Although 6 μg of anti-bFGF bound to protein A-Sepharose beads was able to deplete 80% of the bFGF mitogenic activity for NR6 cells, the antibody at the same concentrations did not deplete mitogenic activity for Schwann cells from a neuronal extract. Anti-bFGF (42 μg) depleted 90% of the mitogenic activity of bFGF for Schwann cells, while 73 μg did not deplete mitogenic activity from neuronal extracts. These results indicate that the neuronal mitogen is immunologically unrelated to bFGF.

DISCUSSION

Membrane fractions and extracts derived from both embryonic peripheral and neonatal central nervous system vigorously stimulate Schwann cell proliferation in a defined serum-free medium. We have not formally shown that the peripheral and central nervous system mitogens are identical; purification to homogeneity must first be achieved. We have, however, demonstrated the similarity of these mitogens by using the most restrictive assay conditions for the study of the

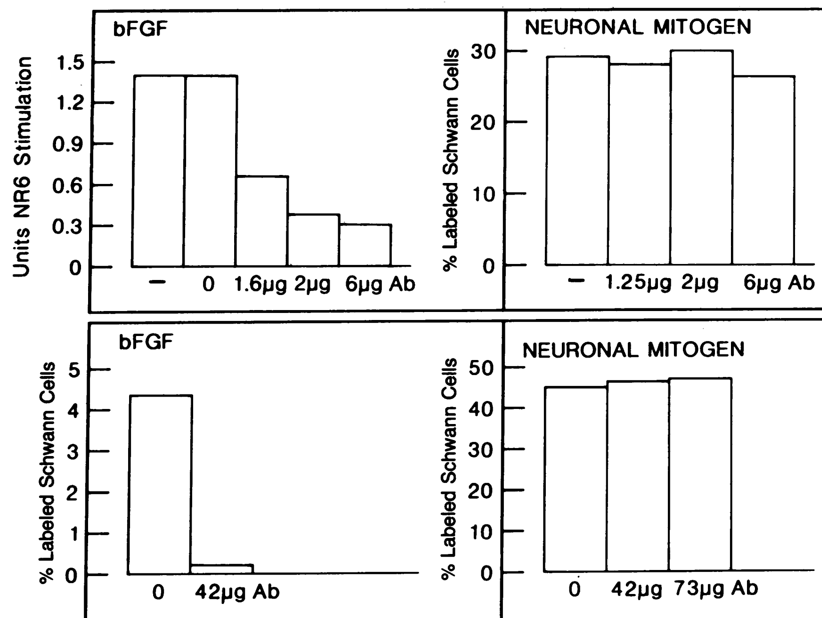

FIG. 4. Antibodies that recognize bFGF do not deplete activity from neuronal mitogenic extracts. (*Upper*) bFGF (7.5 ng = 3 units) or heparin-purified neuronal mitogen (30 μl = 5 units) was mixed with antibody for 2 hr at room temperature in a final vol of 170 μl of Dulbecco's modified Eagle's medium containing 0.1% gelatin. Protein A-Sepharose beads were added for an additional 1 hr at room temperature with occasional mixing. Beads were removed by centrifugation and 50 μl of supernatant was added in duplicate to NR6 cells (for bFGF) and 30 μl was added to Schwann cells (for neuronal mitogen). (*Lower*) Protein A-Sepharose was coated with anti-bFGF IgG and washed four times with PBS. Fifty or 87.5 μl of beads (with or without antibody) was added to either bFGF (4 units) or neuronal extract (4 units) in a final vol of 200 μl containing Dulbecco's modified Eagle's medium with 0.1% fetal calf serum. After a 1-hr incubation at room temperature, beads were removed by centrifugation and 50-μl aliquots were assayed for mitogenicity on Schwann cells. –, No antibody or protein A-Sepharose; 0, no antibody with protein A-Sepharose; Ab, antibody and protein A-Sepharose.

mitogenic response. In contrast, membrane fractions from a pheochromocytoma cell line, PC12, and from adult brain are mitogenic for Schwann cells only in the presence of 10% fetal calf serum (19–22).

The usefulness of the neonatal brain preparation is the essentially unlimited supply of material available for purification. Neonatal rat brains were selected as starting material for mitogen purification because they represent a population of cells highly enriched for neurons. By extrapolation from data on optic nerve and olfactory bulb, neonatal rat brains contain no oligodendrocytes and 25% of the adult complement of astrocytes (25, 23, 24). The major contaminant of this preparation is endothelial cells and their adherent basal laminae; we have not made any effort to purify neuron-specific membranes.

It is interesting that substantial mitogenic activity for peripheral nervous system glia can be extracted from central nervous system tissue. *In vitro* central neurons are mitogenic for Schwann cells (4), and peripheral neurons are mitogenic for embryonic and adult oligodendrocytes (25, 26). The function of the neuronal mitogen in neonatal rat central nervous systems may be to support the burst of oligodendrocyte proliferation that begins shortly after birth in the rat (23, 24).

We have identified an additional diagnostic tool defining the neuronal mitogen in the ability of low concentrations of heparin to inhibit Schwann cell proliferation stimulated by this mitogen.

The neuronal mitogen has been compared to FGF, since aFGF and bFGF are heparin-binding mitogens known to be present in adult brain (19, 20) and thus may also be present in the neonatal brain. The neuronal mitogen differs from FGF in its ability to stimulate Schwann cell proliferation, its affinity for heparin, and by immunological criteria. These data argue strongly that the neuronal mitogen is not aFGF or bFGF, either associated with membranes *in situ* or nonspecifically adsorbed to membranes during purification, and substantiate the argument that the neuronal cell surface contains a unique mitogen for Schwann cells. We have not addressed the similarity of the neuronal mitogen to glial growth factor (27). The possibility that these Schwann cell mitogens are related or identical is an important issue remaining to be resolved.

Mitogenic activity can be removed from membranes under conditions that extract peripheral membrane proteins, suggesting that the mitogen is peripherally associated with the lipid bilayer. Because mitogenic extracts are significantly purified by heparin affinity chromatography and heparin inhibits the proliferation of Schwann cells stimulated by mitogenic extracts, we suggest that solubilization from the membrane separates the neuronal membrane mitogen from the heparan sulfate proteoglycan with which it is associated on the neuronal cell surface (7). In our working model of the mitogen, association of the neuronal mitogen with a proteoglycan on the cell surface restricts the mobility of the mitogen, allowing precise localization of activity in space and during different developmental stages. Furthermore, the association of mitogen and proteoglycan on the neuronal surface allows the mitogen to be specifically presented to the Schwann cell, at the same time preventing association with and inactivation by other proteoglycans, such as heparin.

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