Schwannoma Cell-derived Inhibitor of the Neurite-promoting Activity of Laminin

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Abstract. During the purification of laminin-proteoglycan complexes from rat RN22 Schwannoma cell-conditioned medium, a laminin-rich fraction was obtained which lacked neurite-promoting activity. Since laminin from several sources is known to have potent neurite-promoting activity, this result suggested that either this laminin was inactive or its activity was somehow masked by associated molecule(s). The latter possibility was supported by the demonstration that the inactive laminin-containing fraction inhibited active laminin-containing fractions. This inhibitory activity was partially purified by using ion exchange chromatography and isopycnic centrifugation. The purified material contained proteoglycan based on its high affinity for cationic resin, high buoyant density, large heterodisperse appearance on electrophoretic gels, ability to label with inorganic sulfate, sensitivity to trypsin and glycosaminoglycan lyases, and heat stability.

A quantitative in vitro bioassay was used to monitor the inhibitor after treatments aimed at defining its activity. The isolated Schwannoma-derived inhibitor (*a*) inhibits the neurite-promoting activity of purified rat, mouse, and human laminin; (*b*) is active whether presented to laminin in solution or after either the inhibitor or laminin is first bound to the culture substratum; (*c*) does not act by displacing laminin from the substratum; (*d*) can be prevented from binding to neurite-promoting laminin substrates by polyclonal and monoclonal anti-laminin or polyclonal anti-entactin antibodies; and (*e*) is abolished by proteases or glycosaminoglycan lyases but not by heat. The above results suggest that the neurite-promoting activity of laminin is subject to regulation through association with a proteoglycan and entactin.

Components of the basal lamina promote neuritic outgrowth from several types of embryonic neurons in vitro (Baron von Evercooren et al., 1982; Davis et al., 1985*a*; Edgar et al., 1984; Lander et al., 1982, 1985; Manthorpe et al., 1983; Rogers et al., 1983). Several extracellular matrix macromolecules including fibronectin (Akers et al., 1981), laminin (Baron von Evercooren et al., 1982; Davis et al., 1985*a*), collagens type I and IV (Carbonetto et al., 1983), and a heparan sulfate proteoglycan (Hantez-Ambroise et al., 1987) have been reported to possess neurite-promoting activity. In vitro, cells release into their culture media neurite-promoting factors that express their activity when bound to polycationic substrata (Collins, 1978; Davis et al., 1985*a*). Of these, laminin appears to be the most potent factor and it is often found complexed with other extracellular matrix constituents such as proteoglycans and entactin/nidogen (Carlin et al., 1981; Davis et al., 1985*a*; Dziadek et al., 1986; Lander et al., 1985; Paulsson et al., 1987). These associated molecules affect the behavior of laminin during purification and may alter its character and interaction with extracellular matrix and cell surface components. Also, preparations of laminin have been described that have different molecular compositions, immunological properties, heparin binding properties, and proteoglycan affinities (Davis et al., 1985*a*,*b*, Edgar et al., 1988; Lander et al., 1983, 1985; Sakashita et al., 1980).

The significance of laminin's association with entactin and proteoglycans remains unclear. In one report epidermal cell attachment to laminin was significantly improved due to entactin in a laminin-entactin matrix (Alstadt et al., 1987). Proteoglycans and their component glycosaminoglycan chains have generally not been found to promote neurite outgrowth, and may even inhibit outgrowth on other substrates (Akeson and Warren, 1986). Also, inhibition of cell attachment to extracellular matrix proteins by extracellular proteoglycans has been demonstrated (see Ruoslahti, 1988).

Analysis of conditioned medium from rat RN22 Schwannoma cells showed that low buoyant density proteoglycans and entactin copurified with laminin neurite-promoting activity (Davis et al., 1987). Laminin has been shown to be essential for the neurite-promoting activity of these complexes (Davis et al., 1985*a*; Lander et al., 1985). In the present study we found that in vitro some Schwannoma cell laminin was released in a complexed form that did not express any neurite-promoting activity. From these complexes we have isolated a material which inhibits the neurite-promoting activity of purified laminin. Pretreating neurite-promoting laminin-entactin substrates with anti-laminin or anti-entac-

tin antibodies protected against subsequent inhibition. The inhibitory material has high bouyant density and contains heparan/chondroitin sulfate proteoglycan and its inhibitory activity is sensitive to glycosaminoglycan lyases.

Materials and Methods

Isolation of ^{35}S-labeled Schwannoma Neurite-promoting and Inhibitory Activities

Growth conditions for the rat RN22 Schwannoma cells and the methods for labeling with $^{35}SO_4$ have been described (Davis et al., 1987). 8 liters of RN22 conditioned medium containing 80 ml ^{35}S-labeled RN22 medium was first applied to a 100-ml bed volume DEAE (DE52; Whatman, Inc., Clifton, NJ) ion-exchange column (2.5-cm-diam) and eluted with a 600-ml linear salt gradient from 0.15–0.6 M NaCl in 10 mM phosphate buffer, pH 8.0. Laminin immunoreactivity began to elute at 0.3 M NaCl in the gradient and overlapped with the elution of radiosulfated material (see Results). Fractions eluted between 300 and 420 ml of the gradient were pooled and diluted to 0.15 M NaCl with phosphate buffer (10 mM, pH 8.0). This diluted pool was applied to a 10-ml bed volume DEAE column and bound material was eluted using a 60-ml NaCl gradient (0.15–0.6 M). The laminin immunoreactivity which eluted at 0.2-0.3 M NaCl was collected and pooled (POOL I). Also, the inhibitory activity (see below) began to elute at 0.3 M NaCl and was also collected and pooled (POOL II). Pool II was concentrated by ultrafiltration using an XM100 membrane (Amicon Corp., Danvers, MA) and dialyzed against 0.4 M guanidine-HCl in 50 mM Tris-HCl, pH 7.5. Solid CsCl was added to give density of 1.35 g/ml and samples were subjected to isopycnic centrifugation at 180,000 *g* for 48 h. High density fractions (>1.5 g/ml) were pooled (Pool A) and dialyzed against PBS, pH 7.2. Laminin antigen was measured by an ELISA (Engvall, 1980) performed in polystyrene, 96-well plates using polyclonal and monoclonal antibodies as previously described (Davis et al., 1985*a*). Fractions were assayed for neurite-promoting activity using 8-d ciliary ganglion neurons (Manthorpe et al., 1981, 1983). Radioactivity was monitored by liquid scintillation. Protein was measured by the method of Lowry et al. (1951) using BSA as a standard. Glycosaminoglycan content was measured by Alcian blue binding using heparan and chondroitin sulfates (Sigma Chemical Co., St. Louis, MO) as standards according to Bartold and Page (1985).

To address whether proteoglycan is involved in the inhibitory activity, we cultured the RN22 cells in the presence of an inhibitor of proteoglycan assembly, 4-methyl umbelliferyl-β-D-xyloside. Conditioned medium from RN22 cultures grown in the presence of 1 mM β-D-xyloside was collected, heat-treated, and assayed for activity.

Gel Electrophoresis, Immunoblotting, and Autoradiography

SDS-PAGE (4-12%) was run under reducing conditions using the buffer conditions of Laemmli (1970). Unstained gels were electroblotted to nitrocellulose sheets by the method of Towbin et al. (1979) and the blots were immunostained for laminin as described by Davis et al. (1985*a*) or stained by colloidal gold using Aurodye (Janssen Life Science Products, Piscataway, NJ). Autoradiography was performed on the immunoblots by exposing them to XAR-5 x-ray film (Eastman Kodak Co., Rochester, NY) for 5 d at −70°C.

Enzyme Digestion

Some test samples were treated with proteoglycan lyases or proteolytic enzyme before assay for activity or analysis by SDS-PAGE. For analysis of proteoglycans, samples were incubated for 3 h at 37°C with heparitinase (heparin lyase II; Sigma Chemical Co.) at 1 U/ml in 50 mM Tris-HCl, pH 7.5, containing 25 mM sodium acetate and 5 mM calcium acetate and/or chondroitinase ABC (Miles Laboratories, Inc., Naperville, IL) at 0.2 U/ml, under the same conditions except that calcium acetate was excluded. Digestions were terminated by boiling. Protease digestion was performed using 100 μg/ml trypsin (Irvine Scientific, Santa Ana, CA) in PBS, followed by the addition of 200 μg/ml trypsin inhibitor (Sigma Chemical Co.).

Induction and Inhibition of Neurite Outgrowth

Neurite promotion and inhibition were assayed as substratum-bound activities on polyornithine-coated tissue culture wells (Manthorpe et al., 1983). For neurite promotion assays, polyornithine wells were treated for 2 h with purified rat yolk sac laminin purified according to Engvall et al. (1983) or rat RN22 Schwannoma cell conditioned medium fractions in PBS (50 μl) and then washed with sterile PBS. Embryonic day 8 chick ciliary ganglion neurons were seeded (10^3 neurons per well) in serum-free N1 medium (Bottenstein and Sato, 1979) containing 1% albumin and ciliary neuronotrophic factor (40 trophic units/ml) (Barbin et al., 1984) and incubated in 5% CO_2 in humidified air. Neurite outgrowth was scored by phase-contrast microscopy after 4 h by counting the percentage of neurons bearing processes greater than four cell body diameters. When serial dilutions of purified rat laminin were presented to polyornithine-coated tissue culture wells and ciliary neurons cultured on the resulting substratum, the maximal neurite outgrowth occurred when ~25 ng laminin antigen was applied (i.e., 500 ng/ml × 50 μl/well). Over 95% of the laminin antigen had adsorbed to the polyornithine-coated wells (determined by ELISA of the unadsorbed solution, data not shown).

Neurite-inhibitory activity was assayed by mixing rat laminin and samples to be tested for inhibitory activity (50 μl total volume) and incubating the mixture in polyornithine wells for 2 h, followed by three substrate washes with sterile PBS. In some experiments samples were added as follows: (*a*) laminin was first bound to polyornithine wells for 2 h followed by washing (the wells were blocked with 1% serum albumin solution in some cases) and then inhibitory sample was added; (*b*) the inhibitory sample was first incubated in polyornithine wells for 2 h followed by washes and addition of laminin; (*c*) the inhibitory sample and laminin were preincubated for 1 h in polypropylene tubes before being applied to and incubated in polyornithine wells for 2 h. Ciliary test neurons were seeded, cultured, and scored as described above. We define the half maximal inhibition as 1 neurite inhibitory unit (NIU)[1] and the titer in NIU/ml as that dilution of test sample (total volume = 50 μl) added to the well during substratum treatment eliciting 1 NIU.

Results

Fractionation of RN22-derived Neurite-promoting and Inhibitory Activities

Fractionation of RN22-conditioned medium on DEAE cellulose is shown in Fig. 1. Over 95% of the recovered protein eluted as one early peak before 0.25 M NaCl while two peaks of laminin antigen eluted with ~0.3 and 0.4 M salt. The first and second laminin peaks represented about one-third and two-thirds, respectively, of the total recovered laminin antigen measured by an ELISA using rabbit polyclonal anti-rat laminin antiserum. Over 95% of the recovered radioactivity eluted in one peak immediately after, and overlapping with, a second laminin peak. Essentially these same elution profiles were obtained using the previous smaller scale fractionation (Davis et al., 1987).

The previous study (Davis et al., 1987) indicated that the overlapping laminin and $^{35}SO_4$ peaks from the DEAE column contained laminin–proteoglycan complexes possessing neurite-promoting activity. In an attempt to resolve free laminin, free proteoglycan, and laminin–proteoglycan complexes, the overlapping laminin and $^{35}SO_4$ fractions were pooled as indicated in Fig. 1, diluted and fractionated on a second, smaller DEAE column (Fig. 2). The radioactivity eluted in a slightly broader and earlier peak with 0.3–0.5 M NaCl (Fig. 2 *A*) as compared with the first column (cf. Fig. 1). The laminin antigen (measured by ELISA using polyclonal anti-rat laminin) eluted as two broad and slightly overlapping peaks with ~0.2–0.3 M and 0.3–0.4 M NaCl and these peaks contained about one- and two-thirds, respectively, of the recovered antigen. Thus, the single laminin–$^{35}SO_4$ pool from the first column resolved in the second column into two laminin peaks,

1. *Abbreviations used in this paper*: NIU, neurite inhibitory unit; NPU, neurite-promoting unit.

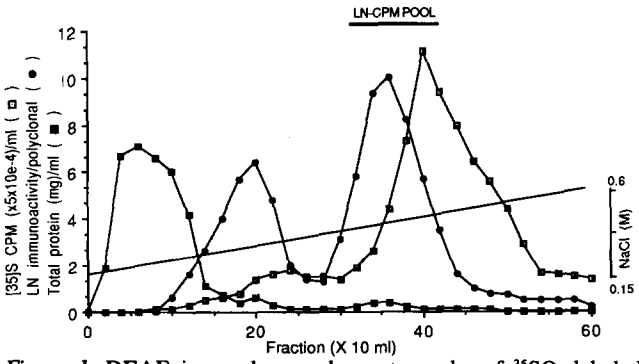

Figure 1. DEAE ion-exchange chromatography of $^{35}SO_4$-labeled RN22 Schwannoma conditioned medium. 8 liters of unlabeled plus 80 ml of ^{35}S-labeled conditioned media were applied to a 100-ml DEAE column from which bound material was eluted by a 600-ml 0.15–0.6 M-linear NaCl gradient (represented by a line connecting the onset and completion of the elution). Fractions (60 × 10 ml) were assayed for total protein (■), radioactivity (□), and laminin immunoactivity in an ELISA using polyclonal rat laminin antiserum (●). The late-running peak of laminin immunoactivity (fractions 30–42, indicated by bar) which overlapped with the $^{35}SO_4$ peak was collected and pooled for further fractionation.

one of which was associated with nearly all the recovered ^{35}S-radioactivity. The elution of two laminin peaks from the second DEAE column occurred with a little less salt concentration as that of the laminin from the first column. This result suggests that the laminin–$^{35}SO_4$ peak from the first column contained laminin–proteoglycan complexes from which the laminin component can be released with additional fractionation.

When these same fractions from the second DEAE column were examined for laminin antigen using the mouse monoclonal antibody, termed 2E8, the majority of immunoactivity was found in one peak which eluted before the peak of radioactivity. In fractions containing most of the ^{35}S-radiosulfated material, laminin antigen could only be detected by the polyclonal antibodies. Since the 2E8 antibody is known to bind to an epitope located in the cross region of laminin on the 200-kD B subunit (Engvall et al., 1986), these results suggest that in the radioactive peak fractions the 2E8 epitope of the laminin B chain is either missing or masked. However, 2E8 immunoactivity was demonstrated on western blots of the same column fractions (Fig. 2 *B*) showing that the 2E8 epitope was present in fractions containing the peak of ^{35}S-radioactivity but apparently was prevented from interacting with the 2E8 antibody in the ELISA.

Autoradiography on the immunoblots revealed two bands of ^{35}S-radioactivity, neither of which comigrated with laminin polypeptides (Fig. 2 *C*). One radioactive band was broad, diffuse, and in a region corresponding to a relative molecular mass of 300–900 kD and is likely to contain radiosulfated proteoglycan. The other band was focussed at 150 kD and comigrated with the sulfated, laminin-binding protein, entactin (Paulsson et al., 1987). Immunoblots of these same DEAE fractions using anti–entactin antibodies showed staining of this 150-kD radiosulfated band, thus supporting its identification as entactin (data not shown).

Each fraction of the second DEAE column was examined and scored as containing (+) or not containing (−) neurite-promoting activity using the CG_8 test neurons. The results are depicted in Fig. 2 *D*. At the concentration tested (20 μl of sample plus 30 μl buffer per well) all fractions expressed neurite-promoting activity except in those associated with the peak of ^{35}S-radioactivity. Thus, the lack of neurite-promoting activity correlated with the presence of proteoglycan and entactin and the absence of monoclonal antibody 2E8 binding. However, significant amounts of laminin antigen are detected by immunoblotting in these same peak radioactive fractions (cf. Fig. 2), suggesting that the neurite-promoting activity, like the 2E8 epitope, was masked by material within the peak ^{35}S-radiolabeled fractions, e.g., by proteoglycan and/or entactin.

Inhibition of Laminin Neurite-promoting Activity

Next, we explored more directly whether the peak of ^{35}S-radioactivity in POOL II contained a material that could inhibit the neurite-promoting activity of the laminin in POOL I. Pools I and II were tested for neurite-promoting activity separately and after combination. Results are shown in Fig. 3. When POOL II was combined with the neurite-promoting POOL I, this activity was no longer detected (Fig. 3 *C*). Thus, POOL II contains an activity capable of eliminating the neurite-promoting activity in POOL I. Purified mouse, rat, and human laminin, all previously shown to possess potent neurite-promoting activity (Engvall et al., 1986; Manthorpe et al., 1983; Wewer et al., 1983), were similarly inhibited by POOL II (data not shown).

To monitor and characterize the inhibitor in more detail, we used a quantitative bioassay as described in Materials and Methods. When serial dilutions of POOL II were incubated in wells treated with 500 ng/ml × 50 μl/well (25 ng/well) of laminin, a concentration-dependent inhibition of the laminin activity was observed. The results are shown in Fig. 3 *D*.

Characterization of the Schwannoma-derived Neurite Inhibitor

The inhibitory material eluted relatively late from the DEAE column (Fig. 2) and was associated with $^{35}SO_4$-labeled material, suggesting that it may contain proteoglycan. To isolate this component, POOL II was submitted to fractionation by isopycnic centrifugation on a CsCl gradient. The CsCl density gradient fractions were examined for radioactivity, laminin immunoreactivity, neurite-promoting, and inhibitory activities. The results are shown in Fig. 4. Most of the radiosulfated material had a high buoyant density (>1.5 g/ml) although a small proportion of radioactivity appeared at a lower density (1.35 g/ml). The high density fractions lacked laminin immunoreactivity (using polyclonal antibodies in ELISA) and contained inhibitory activity (Pool A). Laminin antigen was present as a distinct peak (1.35 g/ml) which contained neurite-promoting activity (Pool B). Apparently the high ionic strength of this gradient (0.4 M guanidine-HCl and 8 M CsCl) effectively separated neurite-promoting activity from inhibitory activity. Thus, when POOL II samples containing laminin but no neurite-promoting activity were subjected to isopycnic centrifugation, neurite-promoting activity was recovered.

The neurite-promoting and inhibitory activities of fractions obtained during various fractionation steps are shown in Table I. The inhibitory activity recovered in the high den-

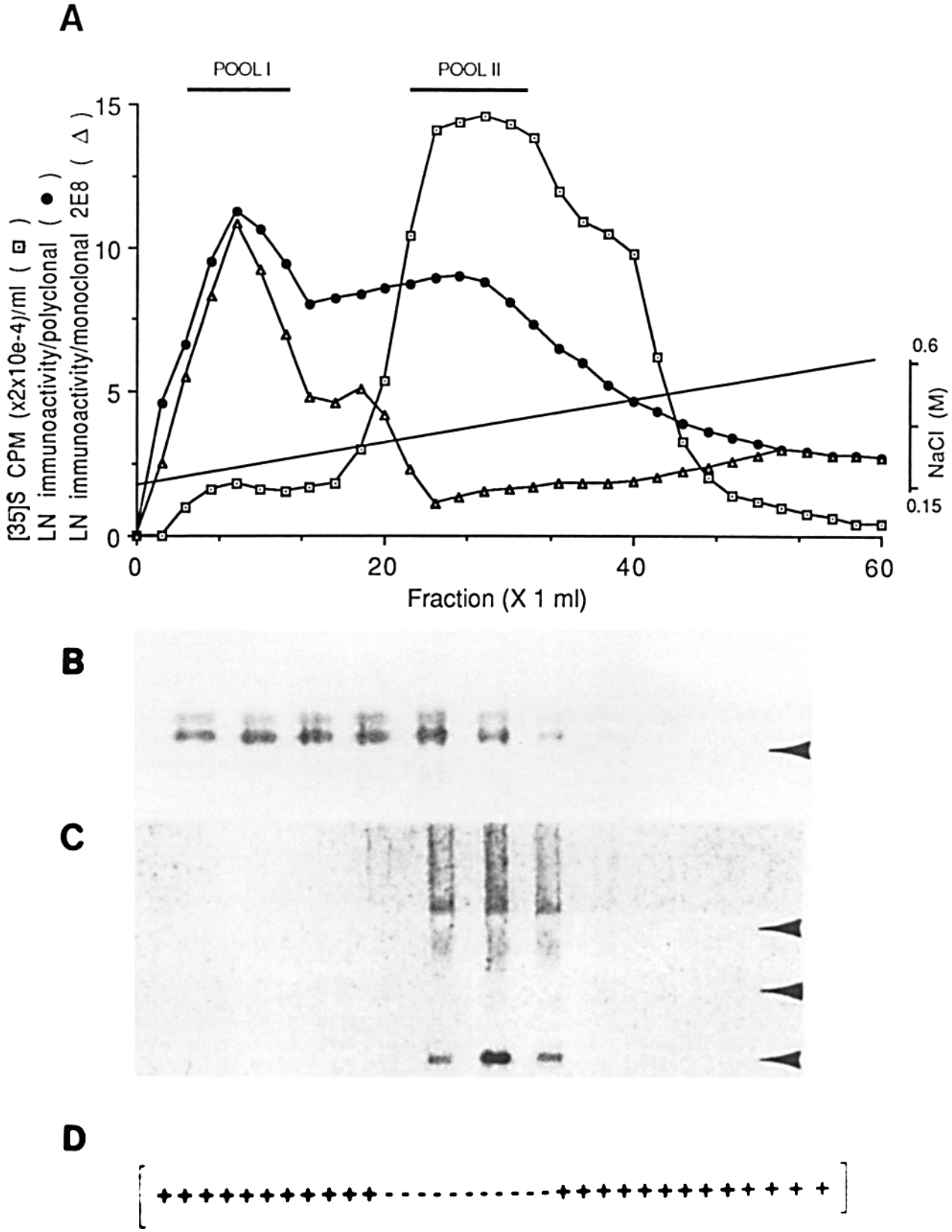

Figure 2. Further fractionation of the laminin and ^{35}S-labeled DEAE fraction by a second DEAE ion-exchange step. Fractions 30–42 from the first DEAE step (see Fig. 1) were diluted to 0.15 M NaCl and applied to a 6-ml DEAE column from which bound material was eluted in 1-ml fractions by a 60-ml 0.15–0.6 M linear NaCl gradient (represented by a line connecting the onset and completion of the elution). (*A*) Fractions were assayed for radioactivity (⊡) and laminin immunoactivity in an ELISA using a polyclonal antiserum (●) and monoclonal antibody No. 2E8 (Δ). Bars indicate POOL I (fractions 4–12) and POOL II (fractions 20–32). (*B*) Western blots from reducing SDS gel profiles of every fifth fraction (20 μl) were immunostained for laminin with monoclonal No. 2E8. Arrow indicates a relative molecular

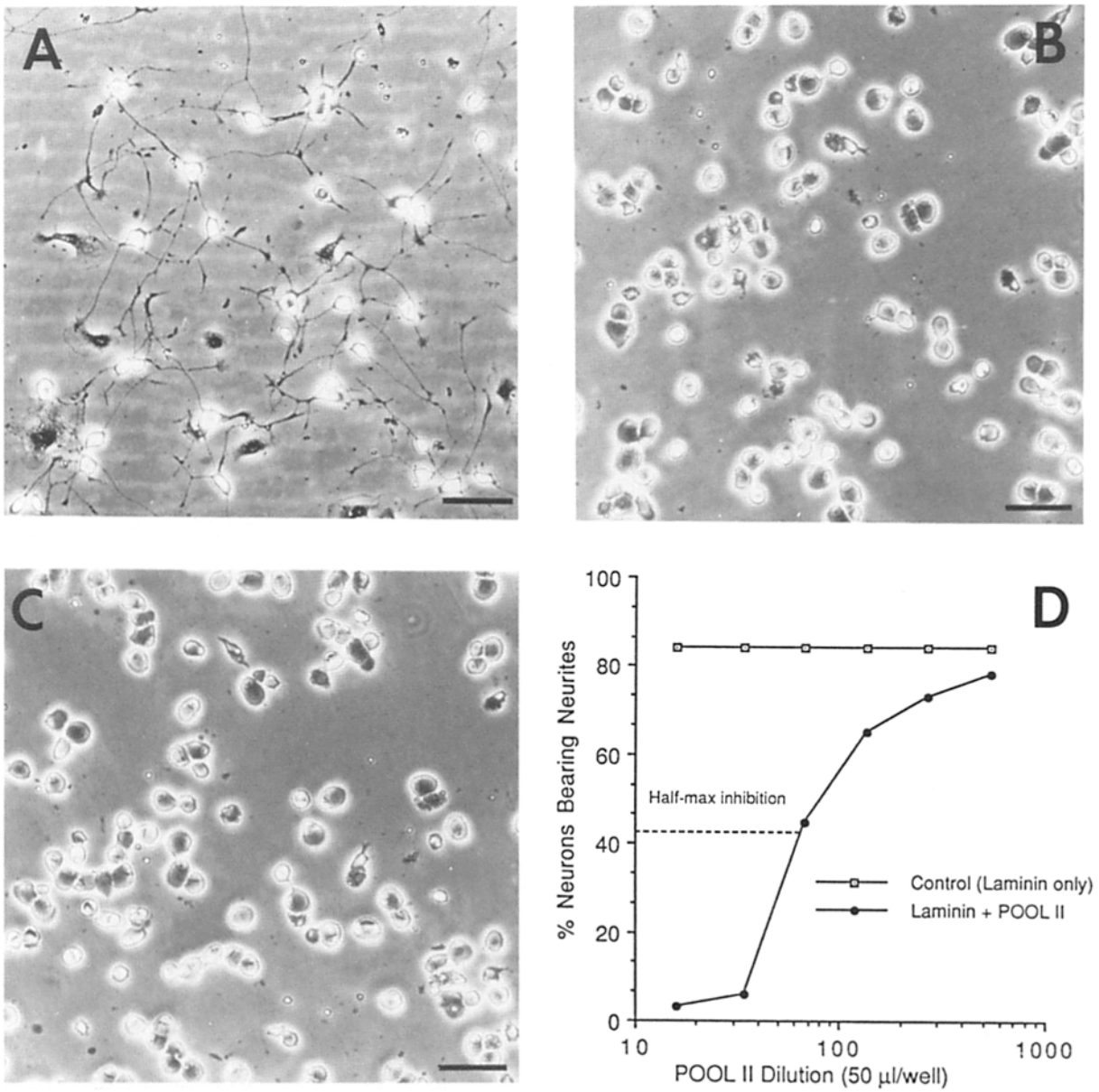

Figure 3. Bioassay of substratum-bound neurite-promoting and neurite-inhibiting activities derived from Schwannoma-conditioned medium. Purified embryonic day 8 ciliary ganglion neurons were cultured according to Material and Methods on polyornithine-coated tissue culture wells pretreated with samples from DEAE ion-exchange (*A*) POOL I, (*B*) POOL II, and (*C*) POOL I and POOL II combined, each diluted 1:10 in a volume of 50 µl. Phase-contrast photomicrographs were taken after 4 h in culture. (*D*) A maximal neurite-promoting response by CG_8 neurons was achieved by incubating the polyornithine-wells with 50 µl of 500 ng/ml (25 ng/well) of purified rat laminin (⊡). A titration curve for the inhibitory activity of POOL II sample (●) indicates a titer of 65 NIU/ml. CG_8 neurons were seeded and maintained for 4 h before assessing the percentage of cells bearing neurites greater than four cell diameters. Assays counting 50–100 neurons per well were performed in duplicate and the data points represent the mean from four experiments. Standard deviations were <5%. Bars, 50 µm.

sity CsCl fractions (POOL A) represented approximately four times (i.e., 3,800 vs. 1,000 NIUs loaded) the activity found in the native second DEAE POOL II starting material, apparently because laminin (with its neurite-promoting activity) was separated away from the higher density inhibitor. This result indicates that the interaction between laminin and inhibitor is reversible.

Given that laminin is heat sensitive (Ott et al., 1982) while proteoglycans are often heat stable, we attempted to destroy selectively the laminin activity by heat treatment and thereby possibly release inhibitor from laminin–inhibitor complexes. The results are shown in Table I. Heat treatment (90°C, 15 min) of conditioned medium from RN22 cells and of the laminin-radiosulfated pool from the first DEAE step resulted in

mass of 200 kD. (*C*) The western blot shown in *B* was exposed to x-ray film for 3 d and developed. The arrows indicate molecular mass markers: rat yolk sac laminin (400 and 200 kD) and entactin (150 kD). (*D*) An aliquot (20 µl) of each of the fractions from the second DEAE column elution was examined for neurite-promoting activity using ciliary ganglion test neurons and scored as containing (+) or not containing (−) such activity.

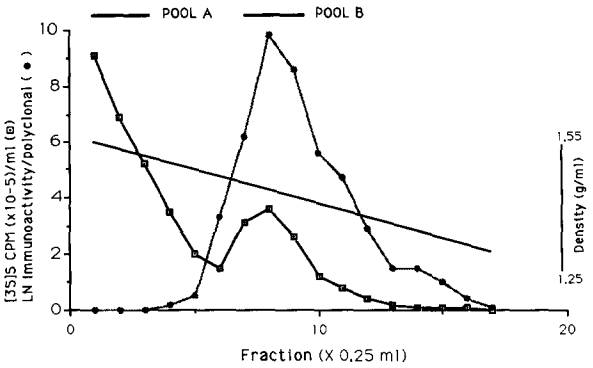

Figure 4. Isopycnic centrifugation of the neurite inhibitor. For buoyant density analysis, POOL II (from the DEAE fractionation step shown in Fig. 3) was made 0.4 M in guanidine-HCl, adjusted to 1.35 g/ml with crystalline CsCl, and then centrifuged. Gradient fractions were assayed for ^{35}S-radioactivity (⊡), laminin immunoactivity in an ELISA using polyclonal antibodies (●), and for the presence of neurite-promoting activity (POOL B), and inhibitory activity (POOL A) (top horizontal bars).

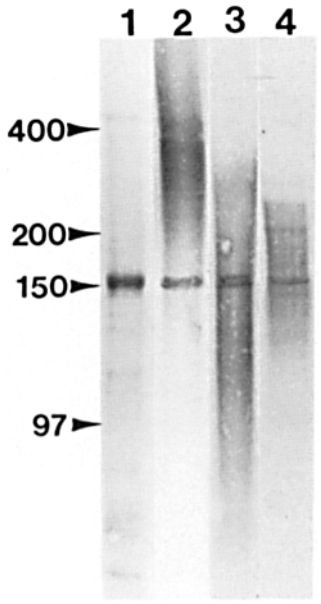

Figure 5. SDS-PAGE of CsCl inhibitory fraction (POOL A). (Lane *1*) Aurodye protein staining of the inhibitory material electrophoresed on 4–12% acrylamide gels under reducing conditions and then transferred to nitrocellulose. The inhibitory material was examined by autoradiography before (lane *2*) and after digestion with heparitinase (lane *3*) or chondroitinase ABC (lane *4*). The arrows indicate molecular mass markers: rat yolk sac laminin (400 and 200 kD) and entactin (150 kD) and phosphorylase *b* (97 kD).

the entire loss of neurite-promoting activity and the appearance of inhibitory activity. Heat treatment of POOL I samples from the second DEAE step eliminated its neurite-promoting activity but generated no inhibitory activity in accordance with the view that this material contains laminin but no inhibitor. When Pool II samples were heated, the titer of the inhibitory activity increased approximately fourfold. Thus, exposure of POOL II to heat (to inactivate laminin neurite-promoting activity) increased its inhibitory activity to about the same extent as does the isopycnic centrifugation (which separated inhibitor from laminin neurite-promoting activity). The specific activity of the inhibitor from the CsCl gradient pool (Fig. 4) was ~17,000 inhibitory units/mg protein, representing a 90,000-fold increase over the serum-containing conditioned medium starting material (after heating).

The inhibitory fractions from the CsCl gradient were pooled (POOL A) and analyzed by SDS-PAGE and electroblotting. The results are shown in Fig. 5. Sensitive protein staining revealed a major band with an apparent molecular mass of 150 kD and a few minor bands (lane *1*). The 150-kD band reacted with anti-entactin antibodies but no laminin immunoreactivity was detected (results not shown). Autoradiography of the radiosulfate labeled material showed a broad band, that was most dense in a region corresponding to a relative molecular mass of 300–400 kD, and a narrow 150-kD band (lane 2). The electrophoretic mobility of the high molecular weight substance was increased after digestion by heparitinase (lane *3*) and by chondroitinase ABC (lane *4*). Lyases that digest different glycoaminoglycans had no effect (not shown). These results indicated that the components in the inhibitory CsCl sample are heparan/chondroitin sulfate proteoglycan and entactin.

Table I. Neurite-promoting and Inhibiting Activity Levels at Various Fractionation Steps or after Heat Treatment

	Native		Heat-treated	
Fraction	NPU	NIU	NPU	NIU
RN22 medium	64,000	0	0	13,800
First DEAE Pool (Fig. 1)	33,000	0	0	11,000
Second DEAE Pool I (Fig. 2)	5,000	0	0	0
Second DEAE POOL II (Fig. 2)	0	1,000	0	4,100
CsCl Pool A (Fig. 4)	0	3,800	0	4,000
CsCl Pool B (Fig. 4)	1,400	0	0	0

Characterization of the Inhibitory Activity

The above fractionation studies suggested that the inhibitory activity may involve proteoglycan and entactin. To analyze further the properties of the inhibitory activity, we submitted the CsCl preparation to several treatments followed by bioassay. The results are shown in Table II. The inhibitor appears to be heat stable but trypsin sensitive suggesting that protein is important in expression of the inhibitory activity. The ability of heparitinase or chondroitinase ABC to eliminate the inhibitory activity suggests the involvement of both heparan and chondroitin sulfate glycosaminoglycans in the inhibitory activity. The products of lyase digestion (i.e., glycosaminoglycan fragments plus core protein) were not active. Also, purified heparin, heparan sulfate, chondroitin sulfate (up to

Table II. Effects of Selected Treatments on the Inhibitory Activity

Treatment	Percent of remaining inhibitory activity*
None	100 ± 2
Heat: 95°C, 15 min	100 ± 2
Trypsin	0 ± 2
Chondroitinase ABC	5 ± 5
Heparitinase	10 ± 5

Inhibitory sample of CsCl preparation (POOL A, Fig. 4) was treated with heat or with either trypsin (100 μg/ml), chondroitinase ABC (0.2 U/ml), or heparitinase (1 U/ml) for 3 h at 37°C. Inhibitory titer (cf. Fig. 3) was determined after treatments. Data are averages of quadruplicate assays in three separate experiments, presented ± SD of the means.
* Starting activity = 160 NIU/ml.

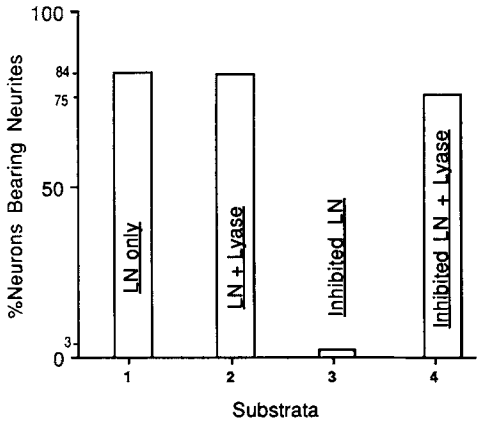

Figure 6. Inhibition of substratum-bound laminin neurite-promoting activity and recovery of activity after inactivation of inhibitor with heparitinase. (*1*) Polyornithine wells were coated with laminin (50 ng/well) which resulted in maximal neuritic outgrowth by ciliary test neurons. (*2*) Treatment of the laminin substratum with heparitinase (heparin lyase II) did not alter the activity of laminin. (*3*) The neurite-promoting activity of laminin was inhibited by subsequent treatment with the Schwannoma-derived inhibitor (16 NIU/well). (*4*) The neurite-promoting activity of laminin was recovered when the inhibited laminin substratum was treated with heparitinase (0.1 U/well for 2 h) before initiating the neurite outgrowth assay. The percentage of test neurons (CG_8) bearing neurites was determined as previously described. Data represent the mean of quadruplicate assays from two separate experiments.

20 μg/ml), or the chondroitin sulfate proteoglycan decorin, which inhibits cell attachment to fibronectin (Brennan et al., 1983), did not decrease the percentage of neurons bearing neurites in response to a laminin substratum.

To address further whether proteoglycan is involved in the inhibitory activity, conditioned medium from RN22 cultures grown in the presence of methyl-umbelliferyl β-D-xyloside was collected, heat treated, and assayed for activity. Compared to heat-treated medium from control cultures, medium from β-D-xyloside–treated cultures (having the same number of cells) contained <20% of the inhibitory activity (1.7 and 0.3 NIU/ml, respectively), suggesting that this activity involved an intact proteoglycan. Although we were unable to assay directly the expression of proteoglycan core protein, assays of these conditioned media for glycosaminoglycan content suggested that xyloside treatment did not alter the synthesis (or release) of this major proteoglycan component.

Several experiments were undertaken to test whether the inhibitory activity in our assays was due to displacement of laminin from the polyornithine-coated substratum. In one experiment, an inhibited substratum, created by adding the inhibitor to a defined amount of laminin, was treated with heparitinase and the treated substratum then seeded with CG_8 neurons to test for neurite-promoting activity. The results are shown in Fig. 6. The enzyme treatment eliminated the inhibitory activity and restored the neurite-promoting activity of laminin. This shows that the inhibitor does not displace laminin from the substratum but binds to and blocks the substratum-bound laminin. In addition, when a completely inhibited substratum (4 neurite-promoting units [NPU]/well of laminin plus 8 NIU/well inhibitor) was then treated with a high amount of laminin (16 NPU/well), a neurite-promoting substratum was created (64% ± 14 neurons bearing neurites). These results suggest that the inhibitor can be saturated with additional laminin and that the presence of inhibitor is not toxic to neurons.

The inactivation of substratum-bound laminin can be localized and will persist for many days in vitro. When a laminin-substratum was treated with localized inhibitor, neurons seeded onto the inhibited area did not extend neurites within 24 h and exhibited nominal, if any, neurite outgrowth even after 5 d (Fig. 7). Neurons that had attached adjacent and immediately proximal to the inhibited area of the laminin-substratum showed extensive outgrowth of neurites which, however, generally did not enter the inhibited region.

To test whether a particular sequence of substratum additions was critical for inhibitory activity, dilutions of the inhibitor were presented to wells before addition of the laminin, simultaneously with the laminin, or after preincubation with laminin in tubes. The results are shown in Fig. 8. The inhibitor retained its inhibitory effects regardless of the presentation sequence. However, the inhibitor exhibited the greatest potency (i.e., had the highest titer) when allowed to bind to the substratum before the addition of laminin and when preincubated with the laminin in solution before addition to the polycationic substratum.

Antibodies Bound to Laminin Protect Its Neurite-promoting Activity from Inhibition

The inhibitory pool from the CsCl centrifugation (POOL A, Fig. 4) was shown to be free of laminin but yet retained the capacity to interact with laminin and inactivate its neurite-promoting activity. To define further the nature of this association, we examined whether antibodies which bind to laminin would prevent its inactivation by the inhibitor. This approach used purified RN22 laminin and antibodies that bind to laminin but do not block its neurite-promoting activity. The results are shown in Table III. When RN22 laminin was preincubated with polyclonal antibodies raised against rat yolk sac tumor laminin, which do not block RN22 laminin activity, its neurite-promoting activity was minimally decreased by the inhibitor. Similarly, pretreatment of the RN22 laminin with 2E8 anti–human laminin monoclonal antibody also prevented inhibition. Since the 2E8 epitope on laminin is located at the cross-region of the laminin molecule (Engvall et al., 1986), this region of laminin might participate in the interaction between the inhibitor and laminin resulting in inhibition.

The partially purified inhibitor contains relatively high levels of the laminin-binding protein, entactin, which copurified with the inhibitory proteoglycan even under conditions which separated inhibitory activity from laminin neurite-promoting activity (i.e., isopycnic centrifugation). Like the 2E8 antibody, entactin is known to bind to laminin near the cross-region of laminin (Paulsson et al., 1987). Thus, we examined whether anti–entactin antibodies could interfere with the ability of the inhibitor to interact with laminin. The results are presented in Table III. Entactin antibodies, which do not by themselves interfere with the neurite-promoting activity of RN22 laminin (a preparation containing entactin), did prevent the inhibition from occurring.

In related experiments, anti–entactin antibodies were shown to be immunoreactive with the inhibitory material as well as with various tested laminin preparations. To further

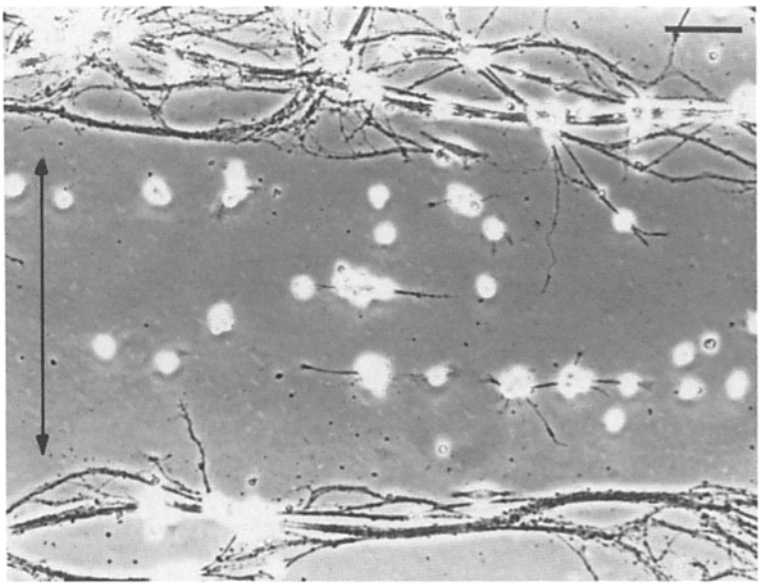

Figure 7. Inhibition of a laminin-substratum is localized and persistent. The substratum was prepared by treating a polyornithine-coated tissue culture dish with RN22 laminin (500 ng/ml). A narrow strip of inhibitor (CsCl POOL A, 1:100) (*arrows*) was applied by allowing the inhibitor solution to be drawn across the substratum by capillary action under a fine glass fiber. All incubations were performed in water saturated air to prevent drying. The substratum was washed and CG_8 neurons were seeded uniformly and cultured in NI medium containing albumin and ciliary neuronotrophic factor. Phase-contrast photomicrograph was taken after seven days. Bar, 50 μm.

examine the role of entactin in the inhibitory activity, the isolated inhibitory fraction (CsCl Pool A, Fig. 4) was fractionated by isopycnic centrifugation run under dissociating conditions (in the presence of 2 M guanidine-HCl). A high buoyant density fraction containing heparan/chondroitin sulfate proteoglycan, but no entactin, was obtained that contained ~60% of the original inhibitory activity. However, the laminin preparation used in these assays contained entactin immunoreactivity. All preparations of laminin tested contained substantial entactin immunoreactivity and conditions required to dissociate entactin from laminin (see Paulsson et al., 1987) compromised the neurite-promoting activity of isolated laminin. Further study using carefully characterized preparations of laminin and entactin and neurite-promoting laminin fragments which do not associate with entactin will be required to assess whether entactin is required for the inhibitory activity.

Discussion

Here we report a first characterization of a material that can bind to laminin and interfere with its ability to promote neurite outgrowth from cultured neurons. This material has been isolated from medium conditioned by RN22 Schwannoma cells by ion-exchange chromatography followed by isopycnic centrifugation. The inhibitory material contains proteoglycan based upon its high affinity for cationic resin, high bouyant density, large heterodisperse appearance on SDS gels, ability to become labeled with inorganic radiosulfate, sensitivity to trypsin and certain glycosaminoglycan lyases, and heat stability. The proteoglycan-containing material obtained from the CsCl density gradient also contained a substantial amount of the sulfated, 150-kD laminin-binding protein, entactin. The fact that the entactin sediments in the high density fraction, which is devoid of laminin, suggests that entactin retains an association with the inhibitory proteoglycan-containing material.

The proteoglycan associated with the inhibitory activity is apparently different from another laminin-binding proteoglycan in RN22 medium described by Davis et al. (1987) which has a lower density, higher molecular weight, and does not inhibit laminin neurite-promoting activity. In ion-exchange chromatography, much of the total RN22-conditioned medium laminin co-eluted with proteoglycans under high

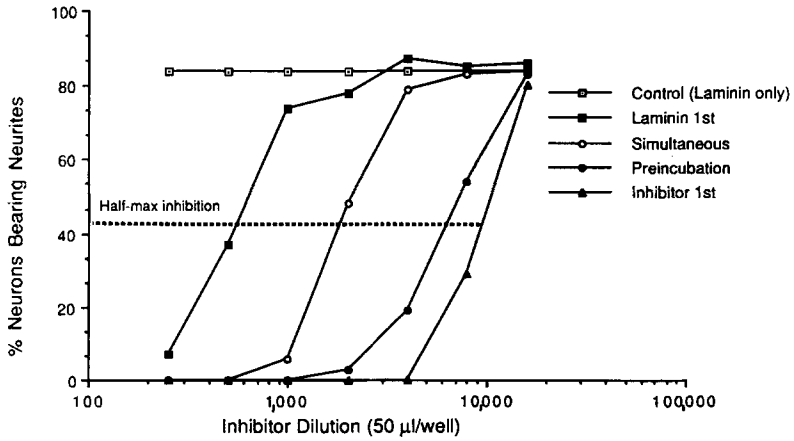

Figure 8. Effect of application sequences on inhibitory titer. Serial dilutions of inhibitor (CsCl, POOL A) and 25 ng of laminin were presented to polyornithine-coated wells in various sequences. Inhibitor was presented (*a*) after laminin, (*b*) simultaneously with the laminin, (*c*) preincubated in tubes before their combined presentation to the wells, or (*d*) before addition of laminin. For each step the applied samples were allowed to bind the polyornithine wells for 2 h at 25°C, the wells were washed, the CG_8 neurons seeded and after 4 h the percentage of neurons with neurites greater than four cell body diameters in length was determined. 50–100 neurons were scored per well and two wells were counted per experiment; the data points represent the mean from four such experiments. Standard deviations were <5%.

Table III. Antibody Interference of the Inhibitors Action on the Neurite-promoting Activity of RN22 Laminin

Antibody treatment	Substratum (neurons bearing neurites)	
	Laminin	Plus inhibitor
	%	%
None	84	0
Controls (preimmune anti-NF, anti-FN)	75–85	0
Anti-rat yolk sac laminin	75	74
Anti-human laminin No. 2E8	83	70
Anti-entactin	85	76

RN22 laminin (25 ng) was incubated in 6-mm-diam polyornithine-wells for 2 h. The laminin-polyornithine substratum was treated with PBS containing 1% BSA followed by a 30-min incubation with PBS alone or PBS containing 1:50 dilutions of the following antibodies: polyclonal anti-human fibronectin (*FN*), monoclonal anti-neurofilament (*NF*), polyclonal anti-rat laminin, monoclonal anti-rat laminin No. 2E8, or polyclonal anti-mouse entactin. Without removing the antibodies, inhibitor (160 NIU/ml; CsCl inhibitory fraction) was added for 2 h and then the wells were washed. CG_8 neurons (10^3/well) were seeded and the percentage of neurons bearing neurites was determined 4 h later by scoring 50–100 neurons per well. Conditions were replicated eight times in four separate experiments. Standard deviations were <10%.

salt from DEAE and was active in promoting neurites (cf. Fig. 1). When this laminin-proteoglycan pool was refractionated on a second DEAE column, only half of the loaded laminin antigen eluted with the proteoglycans and a substantial amount of laminin now eluted with less salt independent of proteoglycan (cf. Fig. 2). Only the proteoglycan-associated laminin lacked neurite-promoting activity. Subsequent analysis of this material (POOL II, Fig. 2) indicated the presence of an inhibitor of the associated laminin. The inhibitory activity could be destroyed by glycosaminoglycan lyase, which resulted in the reexpression of laminin neurite-promoting activity. Conversely, the laminin could be selectively denatured by heat, which apparently caused release of the inhibitor. Thus, by fractionation or treatments that selectively destroys one activity, neurite-promotion or inhibition can be expressed independently. In summary, unfractionated RN22 medium contains antagonistic neurite-promoting and inhibiting activities, with neurite-promoting activity dominating.

Chromatography and enzyme digestions indicate that the proteoglycan-associated inhibitor is degraded and inactivated by heparitinase or chondroitinase (Fig. 5 and Table II), suggesting the involvement of both heparan and chondroitin sulfate glycosaminoglycans in the inhibitory activity. The inhibitor is heat stable but trypsin sensitive, suggesting an intact protein to be important in the manifestation of the inhibitory activity. Additional evidence for the role of an intact proteoglycan in the inhibitory activity was obtained by growing RN22 cultures in the presence of 4-methylumbelliferyl-β-D-xyloside, a competitive inhibitor of glycosaminoglycan addition to proteoglycan core protein (Ratner et al., 1985; Galligani et al., 1975). Conditioned medium from xyloside treated RN22 cells contained only a small fraction of the inhibitory activity of that of untreated cells.

Proteoglycans found in culture conditioned media are thought to be derived from membrane-associated proteoglycan (Jalkanen et al., 1987) and a membrane fraction from RN22 cells contains a proteoglycan with properties very similar to inhibitor isolated from the conditioned medium (i.e., similar fractionation, appearance by autoradiography, and lyase inactivations). The regulation of proteoglycan synthesis and deposition may dictate whether laminin expresses its inherent neurite-promoting activity. The degradation of proteoglycan by specific lyases may remove the inhibition and restore neurite-promoting activity to a previously suppressed substratum. Thus, laminin neurite-promoting activity may depend as much on a fine tuning of proteoglycan synthesis and degradation as on laminin production and its cell surface or extracellular deposition.

Laminin preparations contain variable amounts of entactin, a sulfated protein that binds to a region near the cross region of laminin (Paulsson et al., 1987). Since entactin itself can bind laminin, but also copurifies with the inhibitor even through high ionic strength conditions, entactin may have the ability to interact with both laminin and the inhibitor. Anti-entactin antibodies prevent the inhibitor from blocking laminin neurite-promoting activity, Also, the monoclonal antibody 2E8 is known to bind to the cross region of rat laminin and, without diminishing neurite-promoting activity (Davis et al., 1987; Engvall et al., 1986), will protect the neurite-promoting activity of laminin. Thus, the ability of the inhibitor to interact with laminin may depend on its association with the cross region of the laminin tertiary structure, a region different from the postulated neurite-promoting domain at the end of the 400-kD long arm (Edgar et al., 1984; Engvall et al., 1986). Through interaction with the cross region of the laminin molecule, the inhibitor may influence the distant neurite-promoting site either directly, by additional binding to another portion of the laminin molecule, or indirectly, by inducing laminin conformational changes. The ability of the 2E8 and anti-entactin antibodies to protect the neurite-promoting activity of laminin raises the possibility that entactin or other components may mediate the formation of laminin complexes which modulate the potential neurite-promoting activity of laminin in basement membranes.

Schwannoma cells have been used in this study as a model for Schwann cells in vitro. Recently we have established purified populations of rat sciatic nerve Schwann cells and have found that their conditioned media also contain activity which inhibits neuritic outgrowth in response to laminin. The Schwann cell inhibitory activity had properties identical to the RN22 inhibitor (unpublished results). Future studies will need to address the inhibitors presence and function in vivo.

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