# Purification of a Factor That Promotes Neurite Outgrowth: Isolation of Laminin and Associated Molecules

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ABSTRACT When culture medium, conditioned by any of several cell types, is applied to a polycationic substratum, a substance is adsorbed that causes neurons cultured on that substratum to extend processes (neurites) rapidly and profusely. We have purified the factor responsible for this effect from medium conditioned by bovine corneal endothelial cells, and have shown that it is composed of the glycoprotein laminin and two associated laminin-binding molecules: a sulfated protein known as entactin, and a large heparan sulfate proteoglycan. Of these molecules, only laminin was found to be present throughout the purification in all fractions possessing neurite outgrowth-promoting activity and absent from all fractions lacking activity. Laminin, purified from other sources, has been shown previously to promote extensive outgrowth by cultured neurons. These and other data presented here support the conclusion that laminin is responsible for the neurite outgrowth-promoting activity of the conditioned medium factor. Evidence is also presented that the association of a proteoglycan with laminin promotes efficient attachment of laminin to polycationic substrata, particularly in the presence of competing molecules.

Little is known about the cellular mechanisms that generate the complex patterns of axon growth seen in nervous system development. The search for factors that may control axonal growth and guidance in vivo has relied heavily on the study of neurite growth in vitro (1). While it has long been appreciated that cell–substratum interactions influence the growth of neurites (2, 3), only recently have candidates emerged for molecules that might serve such a function in vivo. Helfand et al. (4) and Collins (5) were the first to report that cultured heart cells produce a substance that, when adsorbed to a substratum, dramatically enhances neurite outgrowth by neurons plated on that substratum. Since then, many other cell types have been shown to release into conditioned medium (CM)[1] a substance with a similar effect on neurons (6–9). It is characteristic of these "neurite outgrowth-promoting factors" found in CM to adsorb preferentially to polycationic substrata (i.e., substrata coated with polylysine or polyornithine) and cause many types of neurons to extend neurites both sooner and more rapidly than they would on untreated polycationic substrata or other conventional culture substrata (e.g., plastic, collagen). Interestingly, neurite outgrowth-promoting factors do not replace trophic factors in supporting the long-term survival of neurons, and are unrelated to nerve growth factor (7, 8).

Recently, considerable efforts have been made to characterize the neurite outgrowth-promoting factors found in CM (10). They are produced by a large number of cell types (6, 8), are protease sensitive (5, 7–9, 11), and are of a very large apparent molecular size (8, 9, 12). Their activity can be detected in the substratum-attached material deposited by cultured cells (6, 8, 13–15), an observation that suggests that these factors consist of, or are related to, components of the extracellular matrix. Consistent with this possibility, evidence has been presented that neurite outgrowth-promoting factors are associated with heparan sulfate (HeS) proteoglycans, molecules that are major extracellular matrix constituents (8, 9, 16). To investigate this possibility more fully, several of the extracellular matrix molecules that have recently been purified have been applied to culture substrata and screened for

[1] *Abbreviations used in this paper:* BCE, bovine corneal endothelial; CM, conditioned medium (media); DME-0.1, Dulbecco's modified Eagle's medium containing 0.1% glucose; EIA, enzyme-linked immunoassay; HeS, heparan sulfate; TBS, Tris-buffered saline.



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neurite outgrowth-promoting activity comparable to that found in CM. Proteoglycans, glycosaminoglycans, and collagens that have thus been tested have no such activity (8, 17). Fibronectin modestly enhances neurite outgrowth, but this has been observed only for certain types of neurons (16–19). In contrast, laminin dramatically promotes rapid profuse neuritic growth by many types of neurons (17, 20). In fact, the effects of substratum-bound laminin on neurite outgrowth are indistinguishable from those of CM-treated substrata (21).

The ability of purified laminin to mimic the biological effects of CM-treated substrata raises the possibility that the neurite outgrowth-promoting factors in CM consist of laminin. Indeed, some of the properties of laminin are strikingly similar to those of neurite outgrowth-promoting factors. For example, laminin is a very large (850–1,000 kD) glycoprotein (22) that can associate with HeS proteoglycans (23, 24). Nevertheless, there appear to be immunochemical differences between laminin and neurite outgrowth-promoting factors. Antibodies raised against purified laminin will block the ability of laminin-treated substrata to promote neurite outgrowth, but these same antibodies, when applied to most CM-treated substrata, fail to block this activity (9, 16, 17, 25). These results have been taken to imply that laminin cannot be responsible for the neurite outgrowth-promoting activity of CM (17, 25). Recently, we have challenged this interpretation by showing that laminin is present in CM that promote neurite outgrowth, and demonstrating that removal of laminin by immunoprecipitation results in the loss of neurite outgrowth-promoting activity (26). We have offered several explanations for the inability of laminin-blocking antibodies to block the neurite outgrowth-promoting activity of CM-treated substrata. These include the possibility that molecules bound to laminin in CM interfere with the binding of blocking antibodies, or that there exist multiple forms of laminin, differing in amino acid sequence or carbohydrate composition, that are immunochemically distinct from one another (26).

In the present study, we describe the purification of the neurite outgrowth-promoting factor in bovine corneal endothelial (BCE) CM. The purification yields laminin, which is found in association with two laminin-binding molecules: the sulfated 150-kD protein entactin (27), and a large HeS proteoglycan. These associated molecules are not essential for neurite outgrowth-promoting activity, but they do affect laminin's behavior during purification. The proteoglycan also appears to play a role in the attachment of laminin to polycationic substrata. A summary of this work has been published in abstract form (28).

## MATERIALS AND METHODS

*Materials:* Swine skin gelatin was coupled to CNBr-activated (29) Sepharose 4B (Pharmacia Inc., Piscataway, NJ) at 1 mg gelatin/ml resin. Laminin was purified from the EHS sarcoma (22, 30), and a rabbit antiserum to it was prepared (31). Anti-laminin antibodies were affinity-purified from this antiserum by chromatography on a column of laminin (coupled to Affigel 10 [Bio-Rad Laboratories, Richmond, CA] at 0.8 mg/ml of gel). The coupled laminin had been previously chromatographed on Biogel A 1.5M (Bio-Rad Laboratories) to free it of low molecular weight contaminants (32). Heparitinase was purified from *Flavobacterium heparinum* by hydroxylapatite chromatography and was free of detectable chondroitinase activity (33). Cell culture media were prepared by the University of California at San Francisco cell culture facility. Radiochemicals were obtained from Amersham Corp. (Arlington Heights, IL). Nonimmune rabbit immunoglobulin was prepared by batch adsorption and elution from DEAE-cellulose (34). Goat anti-rabbit immunoglobulin was from Cappel Laboratories (Cochranville, PA). Phosphate-buffered saline (PBS) consisted of 137 mM NaCl, 2.7 mM KCl, 7.8 mM $Na_2HPO_4$, and 1.5 mM $KH_2PO_4$ adjusted to pH 7.4. Antiserum against entactin, which did not cross-react with laminin, was the generous gift of Dr. Hynda Kleinman (National Institutes of Health).

*Conditioned Media:* BCE cells were established and maintained as described (35, 36), in a growth medium of Dulbecco's modified Eagle's medium containing 0.1% glucose (DME-0.1) and supplemented with glutamine (2 mM), gentamycin (50 μg/ml), Fungizone (0.25 μg/ml), and fetal bovine serum (15%). Metabolic labeling of cells with [$^{35}$S]methionine was accomplished by rinsing cultures twice with methionine-free DME-0.1, then adding DME-0.1 containing [$^{35}$S]methionine (40 μCi/ml, 1,110 Ci/mmol) and unlabeled methionine to a final methionine concentration of 21 μM, a reduced amount of serum (0.5%), and glutamine and antibiotics as above. After 3 d of culture, an additional 20 μM methionine was supplied by adding 1 ml DME-0.1 per 10-ml culture fluid. After 24 h, the CM was harvested, filtered (0.2 μm), treated with the protease inhibitors phenylmethylsulfonyl fluoride (1 mM), EDTA (3 mM), pepstatin ($10^{-7}$ M), and *N*-ethyl maleimide (2 mM), and either used at once or stored at −20°C.

Metabolic labeling with [$^{35}$S]sulfate was accomplished by rinsing cultures twice in sulfate-free DME-0.1, then adding DME-0.1 containing carrier-free [$^{35}$S]sulfate (40 μCi/ml) and unlabeled sulfate ($MgSO_4$) to a final sulfate concentration of 4 μM, a reduced amount of serum (0.5%), glutamine, and penicillin. After 4 d of culture, the CM was harvested, treated, and stored as described above.

*Purification of the Neurite Outgrowth-promoting Factor:* Freshly prepared labeled CM was mixed with gelatin-Sepharose in PBS (6 ml resin/100 ml of CM), stirred slowly at room temperature for 2 h, then filtered. The resin was washed with 1 bed vol of PBS, and this was pooled with the filtrate. CM absorbed in this way was cooled to 4°C and stirred, as saturated $(NH_4)_2SO_4$ was added, dropwise, to 48% of saturation. All subsequent steps were done at 4°C. The solution was stirred overnight, then centrifuged at 20,000 *g* for 1 h. The pellet was resuspended in 0.05 M Tris-HCl, 0.1 M NaCl, pH 7.4 (Tris-buffered saline, TBS) containing 0.1% Triton X-100 and protease inhibitors (all four inhibitors described above, at the indicated concentrations except for EDTA, which was used at 2 mM). This material was dialyzed overnight against 400 vol of TBS containing protease inhibitors (EDTA was reduced in this instance to 0.2 mM). The retained material was centrifuged at 25,000 *g* for 1 h to remove the slight precipitate that remained. The supernatant was mixed with DEAE-cellulose (DE52, Whatman Inc., Clifton, NJ) equilibrated in TBS containing 0.1% Triton X-100. 1 ml of resin was added for each 100 ml of labeled CM used at the start of purification. The suspension, sealed within a disposable polypropylene column (Bio-Rad Laboratories), was mechanically rocked for 2 h. The unbound material was eluted from the column bottom, and the ion-exchange matrix washed with 2 bed vol of TBS containing 0.1% Triton X-100. Bound material was eluted with 1 M NaCl, 0.05 M Tris-HCl, 0.1% Triton X-100, pH 7.4. Fractions were collected manually, and the appearance of eluted radioactivity was monitored by liquid scintillation counting using Aquasol-II (New England Nuclear, Boston, MA). Greater than 90% of the bound radioactivity eluted within the first 1.3 ml, and this material was pooled. To this sample was added solid sucrose (ultra-pure, Schwarz-Mann, Spring Valley, NY) to 5% (wt/vol), in order to increase its density, and sufficient phenol red (concentrated aqueous solution) to color it lightly. The sample was then applied to a column (1.5 × 28.5 cm) of Sepharose CL4B (Pharmacia Inc.) equilibrated in 0.5 M NaCl, 0.05 M Tris-HCl, 0.1% Triton X-100, 0.2 mg/ml hemoglobin (human, 2× recrystallized, Sigma Chemical Co., St. Louis, MO), pH 7.4, containing the aforementioned four protease inhibitors (EDTA at 2 mM). The column was run at 3.5 ml/h, and 1-ml fractions were collected. Calibration of the column was performed separately using three markers: β-galactosidase was detected enzymatically (37), and high molecular weight aggregates of the enzyme served as a convenient void volume ($V_0$) marker (38). Laminin was iodinated using chloramine-T (34) and detected by gamma counting. [$^{35}$S]Sulfate was used to determine the total volume ($V_t$) and was detected by liquid scintillation counting.

After gel filtration, fractions with neurite outgrowth-promoting activity (see text) were pooled, placed into dialysis tubing, and embedded in dry Sephadex (Pharmacia Inc.). Once sufficiently concentrated, the sample was dialyzed against TBS containing protease inhibitors (EDTA was 2 mM). To 0.4 ml of recovered material were added the sedimentation velocity standards β-galactosidase (2.5 U, Worthington Biochemical Corp., Freehold, NJ) and intestinal alkaline phosphatase (3.4 U, Sigma Chemical Co.). The solution was layered onto a preformed density gradient (10.7 ml of a 5–20% sucrose gradient in TBS containing 0.1% Triton X-100, resting atop 0.4 ml of 60% sucrose in the same buffer) in a polyallomar ultracentrifuge tube (Beckman Instruments, Inc., Palo Alto, CA), and centrifuged in an SW41 Ti rotor at 40,000 rpm for 11.7 h

at 4°C. Fractions were eluted by puncture of the tube bottom. Sedimentation standards were located by enzymatic assay (37, 39).

*Determination of Neurite Outgrowth-promoting Activity:* Neurite outgrowth-promoting activity was assayed as described previously (8, 26). Briefly, polylysine-coated tissue culture wells were treated overnight with 50 μl of solutions to be assayed and were washed with neuronal culture medium. Dissociated neonatal rat superior cervical ganglion cells were added to treated wells and incubated at 37°C (in a 8% $CO_2$ atmosphere) for 11 h, then fixed with 2.5% glutaraldehyde in PBS containing 5% sucrose. Wells were washed and scored by microscopic evaluation of random fields. The percentage of cell bodies with neuronal morphologies that possessed neurites >2 cell diameters in length was determined for 100 or more cells in each well. Data are presented as means of duplicate wells ± the deviation from the mean. In certain experiments, the quantity of biological activity in each sample is expressed in neurite outgrowth-promoting units. This represents the inverse of the dilution that gave a half-maximal response multiplied by the sample volume in milliliters. In a typical maximal response, 75–85% of the neurons possessed neurites.

*Immunochemical Methods:* Enzyme-linked immunoassays (EIAs) were performed on samples by adsorbing them to polylysine-coated tissue culture plastic wells (as described above) overnight, washing, blocking the wells with EIA buffer (5% newborn calf serum in PBS), and applying affinity-purified anti-laminin at 1 μg/ml overnight. After washing, peroxidase-conjugated goat anti-rabbit immunoglobulin G (Cappel Laboratories) was added at 1:2,500 for 2 h. After further washing, bound peroxidase activity was determined by colorimetric assay monitored at 415 nm (40).

Immunoprecipitation was performed as described (26). Briefly, samples of CM (0.3 ml) were mixed with anti-laminin (2 μg) and carrier nonimmune rabbit immunoglobulin (8 μg) and incubated overnight at 4°C. Precipitating antiserum (goat anti-rabbit immunoglobulin) was added to the equivalence point (determined to be 20 μl), and the sample was incubated overnight at 4°C. The precipitate was collected by centrifugation, the supernatant decanted, and the pellet washed once with TBS. The pellet was redissolved in 9 M urea overnight before electrophoretic analysis.

Immunoblotting (41) was carried out on a sample of fibronectin-depleted BCE-CM that was concentrated by ultracentrifugation (26), subjected to nonreducing SDS PAGE, electrophoretically transferred to nitrocellulose, and probed with affinity-purified rabbit anti-laminin (1 μg/ml) or rabbit anti-entactin serum at 1:100. Bound antibody was detected autoradiographically after exposure of the nitrocellulose to $^{125}$I-goat anti-rabbit immunoglobulin G.

*Electrophoresis:* The SDS PAGE system of Laemmli (42) was used. Samples were prepared in buffer without 2-mercaptoethanol, except where indicated. Gels were cast on GelBond PAG supports (Marine Colloids Inc., Springfield, NJ) and were 2.8% acrylamide in the stacking portion and 2.9–15% exponential gradients of acrylamide in the separating portion. Nonradioactive marker proteins were stained with Coomassie Brilliant Blue. Radioactively labeled material was visualized by fluorography using 2, 5-diphenyloxazole in dimethylsulfoxide (43). Gels were exposed to preflashed Kodak XAR-2 film at −70°C (44). Fluorographic exposure over labeled bands was quantified by elution and turbidimetric detection of silver grains at 500 nm (45).

*Heparitinase Digestion:* Samples to be digested were made 2.5 mM in $CaCl_2$, and 35 μg/ml of purified heparitinase in TBS was added. Controls received TBS in place of enzyme. After incubation at 43°C for 2 h (33), the reaction was stopped by cooling to 4°C, or if appropriate, by the addition of SDS-containing electrophoresis buffer.

## RESULTS

### Purification of the Neurite Outgrowth-promoting Factor from BCE-conditioned Medium

To aid in the detection of proteins present at low concentrations, purification was carried out using BCE-CM that had been metabolically labeled with [$^{35}$S]methionine. In the first step of purification (Table I), BCE-CM was depleted of fibronectin by absorption with gelatin-Sepharose (46). Neurite outgrowth-promoting activity was detected only in the fraction not bound to the affnity matrix (Table I, line *B*). To this fraction was added ammonium sulfate to 48% of saturation, the minimum concentration required to precipitate all outgrowth-promoting activity (Table I, line *C*). The ammonium sulfate precipitate was collected, redissolved, dialyzed, and applied to DEAE-cellulose. Nearly all of the neurite outgrowth-promoting material bound to the matrix, whereas > 85% of the applied [$^{35}$S]methionine did not (Table I, line *D*). The active material was eluted from the DEAE-cellulose with a step of 1.0 M NaCl; salt gradient elution was not used because the active material was found to elute in a very broad peak between 0.1 and 0.8 M NaCl. The DEAE eluate was further fractionated by gel filtration on a column of Sepharose CL4B (Table I, line *E*). As shown in Fig. 1, neurite outgrowth-promoting activity appeared in a single peak just after the void volume, with a shoulder trailing off into more included fractions. The majority of applied [$^{35}$S]methionine was found in later fractions not associated with outgrowth-promoting activity. This pattern of fractionation was observed in all preparations, although the relative proportions of the peak and shoulder varied. As a final step of purification, pooled fractions from gel filtration were concentrated, dialyzed, and sedimented through a 5–20% sucrose gradient. The labeled material fractionated into four peaks (Fig. 2). The most mobile peak (~19S) was associated with neurite outgrowth-promoting activity, as was the leading edge of the second most

TABLE I. *Purification of Neurite Outgrowth-promoting Activity from BCE-CM*

| | Neurite outgrowth-promoting activity | Incorporated [$^{35}$S]methionine | Specific activity | Apparent yield |
|---|---|---|---|---|
| | *U* | *cpm × 10$^{-6}$* | *U/(cpm × 10$^{-6}$)* | *%* |
| A. BCE-CM | 155 | 1480 | 0.105 | 100 |
| B. Gelatin-Sepharose, not bound | 170 | 1224 | 0.139 | 109 |
| bound, eluted with 4 M urea | <20 | 139 | | |
| C. Ammonium sulfate, pellet | 213 | 586 | 0.363 | 137 |
| supernatant | <50 | 651 | | |
| D. DEAE-cellulose, bound, eluted | 200 | 71.1 | 2.81 | 129 |
| not bound | <20 | 520 | | |
| E. Sepharose CL4B, fractions 19–28 | 166 | 9.13 | 18.2 | 107 |
| F. Velocity sedimentation, fractions 5–14 | 140 | 4.14 | 33.8 | 90 |

Methionine-labeled BCE-CM was fractionated as described (Materials and Methods). For fractions containing unincorporated [$^{35}$S]methionine (i.e., before dialysis of ammonium sulfate pellet), the data shown refer to trichloroacetic acid-precipitable material (removal of free methionine by dialysis gave similar results). For subsequent fractions, total $^{35}$S content is shown. Neurite outgrowth-promoting units are defined in Materials and Methods. Where neurite outgrowth-promoting activity was not detectable, activity is expressed as less than the greatest number of units that, given the sample volume, would have just reached the limit of detection (~0.25 U/ml). Explanation for apparent increases in the total number of units during the purification are discussed in the text. Given this behavior, an accurate assessment of the purification factor is not possible, and yields must be expressed as apparent. The fraction numbers shown for the gel filtration and velocity sedimentation steps refer to Figs. 1 and 2, respectively.

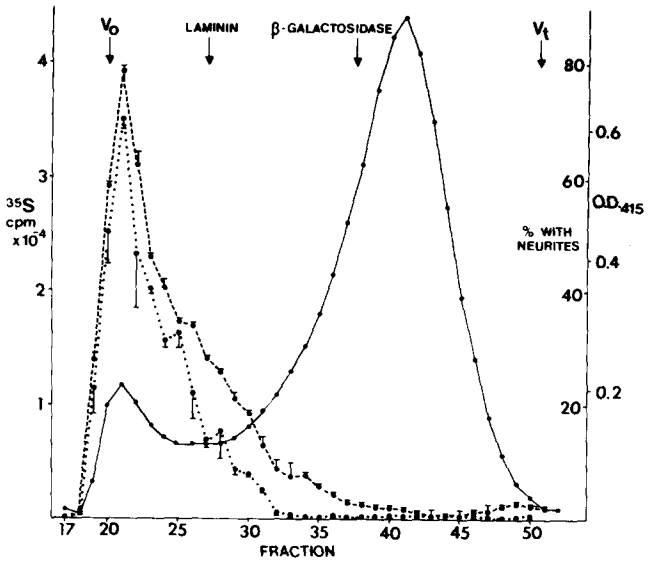


FIGURE 1 Gel filtration on Sepharose CL4B. The presence of [$^{35}$S]methionine label was assayed in 10-μl samples of each fraction (——). Dilutions of each fraction (1:12 in TBS) were applied to polylysine-treated wells for determination of neurite outgrowth-promoting activity (· · ·) and laminin immunoreactivity (– – –) as described. Chromatographic positions of standards (see Materials and Methods) are shown (arrows).

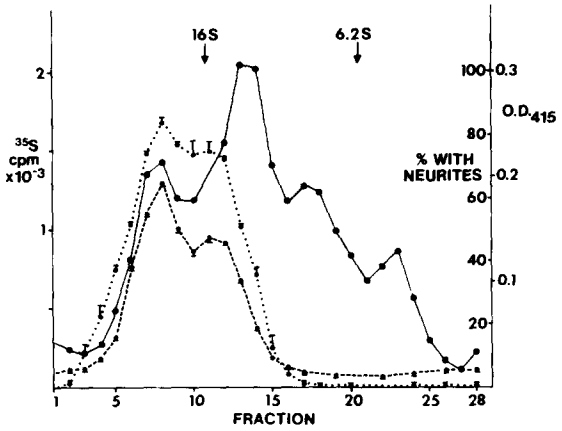


FIGURE 2 Velocity sedimentation through 5–20% sucrose. The presence of [$^{35}$S]methionine label was assayed in 10-μl samples of each fraction (——). Dilutions of each fraction (1:8 in TBS) were applied to polylysine-treated wells for determination of neurite outgrowth-promoting activity (· · ·) and laminin immunoreactivity (– – –) as described. Positions of sedimentation standards (β-galactosidase, *16S*, and intestinal alkaline phosphatase, *6.2S*) are shown (arrows).

mobile peak (~13S). The two peaks of lowest mobility (~9S and ~4S) lacked biological activity. This pattern was highly reproducible. The immunoassay results shown in Figs. 1 and 2 will be discussed below.

As shown in Table I, each purification step resulted in an increase in specific activity. This increase was ~2.6-fold after ammonium sulfate precipitation, a further 7.8-fold after ion-exchange chromatography, 6.5-fold after gel filtration, and 1.9-fold after velocity sedimentation. The net increase in specific activity was 322-fold. This number cannot be considered an accurate estimate of fold purification because, as Table I shows, the total quantity of neurite outgrowth-promoting activity markedly increased after each of the first two purification steps. Results such as these usually indicate that substances present in the starting material inhibit activity or interfere with the assay, artificially depressing the quantity of activity measured. For this reason, the actual amount of neurite outgrowth-promoting activity initially present is not known, but must be at least as great as the amount measured after the second purification step, and most likely even greater (since some loss probably occurred during the first two purification steps). Thus, the purification factor can be estimated as <235-fold.

## Composition of the Neurite Outgrowth-promoting Factor

To identify the molecules purified by the above procedure, fractions from different stages of purification were analyzed by denaturing SDS PAGE in 2.9–15% exponential gradient gels under nonreducing conditions, and labeled molecules were visualized by fluorography (Fig. 3). Comparison of lanes *1* and *2* illustrates that absorption with gelatin-Sepharose removes a major protein band of 440 kD, representing fibronectin, from the starting material. Subsequent purification by ammonium sulfate precipitation (lane *3*) and ion-exchange

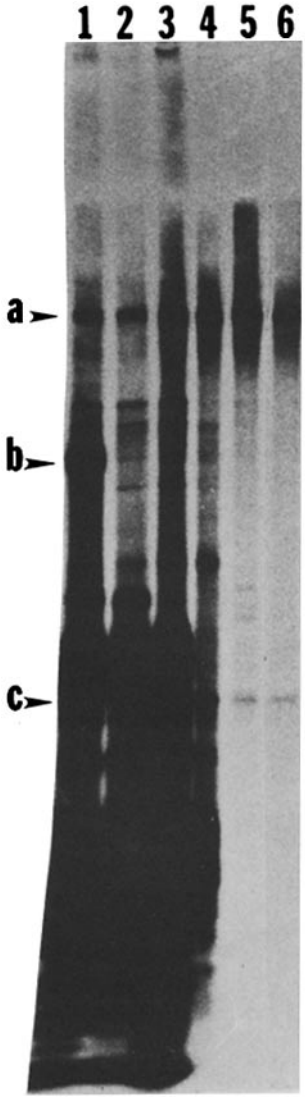


FIGURE 3 Analysis of purification by SDS PAGE. Samples at different stages of purification were analyzed by SDS PAGE under nonreducing conditions (see Materials and Methods). The samples in each lane and the amount (cpm × $10^{-3}$) applied were: Lane *1*, BCE-CM (191); lane *2*, material not adsorbed to gelatin-Sepharose (142); lane *3*, ammonium sulfate pellet (309); lane *4*, material bound to DEAE-cellulose and eluted with 1 M NaCl (33), lane *5*, gel filtration, fraction 21 (see Fig. 1) (5.9); lane 6, gel filtration, fraction 25 (see Fig. 1) (3.3). Standards: (*a*) laminin (850–1,000 kD), (*b*) cellular fibronectin (440 kD), and (c) mouse immunoglobulin (150 kD).

chromatography (lane *4*) removes many additional protein bands. Lane *5* shows the labeled molecules present in the active peak of the gel filtration profile (fraction 21 of Fig. 1), and lane *6* is from the "shoulder" (fraction 25 of Fig. 1). Whereas lane *5* contains some bands not present in lane *6*, only two bands are present in both lanes, one that co-migrates with a laminin standard (850–1,000 kD), marker *a*, and one that co-migrates with a mouse immunoglobulin G standard (150 kD), marker *c*. The unusually high molecular weight of the first band allows it to be visualized even in crude CM (lane *1*). By following it through the steps of purification, examining side fractions as well (not shown), it can be seen that each purification step enriches for this protein. Also present in lanes *4*, *5*, and *6* is labeled material that does not band sharply, but appears as a diffuse blur beginning at the top of the separating gel and extending beyond the location of the laminin standard. It is particularly apparent in lane *5*.

Fig. 4 illustrates in greater detail the fractionation of labeled molecules during gel filtration. Alternate fractions from the experiment shown in Fig. 1 were analyzed by SDS PAGE under nonreducing conditions. The distribution and amount

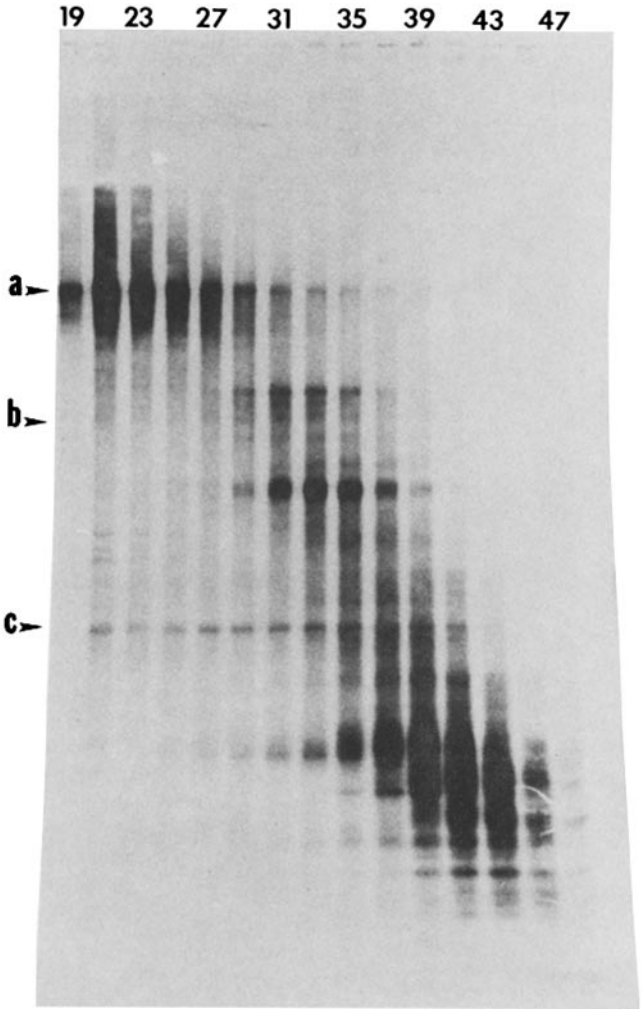


FIGURE 4 Gel filtration on Sepharose CL4B. Analysis by SDS PAGE. Equal volumes of odd-numbered fractions from the material shown in Fig. 1 were analyzed by nonreducing SDS PAGE. Fractions are identified by numbers above lanes. Standards: (*a*) laminin (850–1,000 kD), (*b*) fibronectin (440 kD), and (*c*) mouse immunoglobin (150 kD).

of fluorographic exposure of the band that co-migrates with a laminin standard (marker *a*) correlates well with the distribution and amount of neurite outgrowth-promoting activity (Fig. 1, fractions 19–31). Also, broadly distributed over these same fractions is the "high molecular weight blur" described in lanes *4–6* of Fig. 3. A few other molecules are present in neurite outgrowth-promoting fractions; however, one of these, represented by a 150-kD band (marker *c*), is also distributed among fractions that lack neurite outgrowth-promoting activity (i.e., fractions 33–41), and the others, represented by a group of bands between markers *b* and *c*, are present in the void volume peak (fractions 19–23) of outgrowth-promoting activity, but absent from the adjacent shoulder (fractions 25–31) of activity. Thus, the molecules that best correlate with neurite outgrowth-promoting activity are those represented by the band that co-migrates with laminin (marker *a*) and the high molecular weight blur.

To determine how the labeled molecules purified by gel filtration behaved when subjected to velocity sedimentation, fractions from the sucrose gradient shown in Fig. 2 were analyzed by SDS PAGE under nonreducing conditions. As shown in Fig. 5, the labeled molecules applied to the sucrose gradient—i.e., the band that co-migrates with laminin (marker *a*), the 150-kD band (marker *c*), the minor bands present in the peak but not the shoulder of the gel filtration profile, and the high molecular weight blur—can all be accounted for. The most mobile peak (~19S; fractions 5–10) contains the band that co-migrates with laminin, the 150-kD band, and the high molecular weight blur. This peak of radioactivity is associated with a peak of neurite outgrowth-promoting activity (Fig. 2). The second peak (~13S; fractions 11–16) contains the same three molecules in its leading fractions (fractions 11–13), but contains only the high molecular weight blur in its trailing fractions (fractions 14–16). Only the leading fractions of this peak are associated with neurite outgrowth-promoting activity (Fig. 2). The third peak (~9S; fractions 17–20) contains only the high molecular weight blur, and the fourth (~4S; fractions 21–24) contains much of the 150-kD band, as well as the other minor bands noted in Fig. 4, fractions 19–23. Neither of these peaks is associated with outgrowth-promoting activity.

The data in Figs. 4 and 5 demonstrate that all neurite outgrowth-promoting fractions contain three different labeled molecules: the band that co-migrates with laminin, the 150-kD band, and the high molecular weight blur. The identity of these molecules, and evidence that they physically associate with one another, are presented below. Figs. 4 and 5 also demonstrate that, whereas the 150-kD band and the high molecular weight blur may be found in fractions that lack neurite outgrowth-promoting activity, the band that co-migrates with laminin is only found in fractions possessing this activity. In fact, the profile of neurite outgrowth-promoting activity in Figs. 1 and 2 correlates well with the distribution of the molecular represented by this band, as judged by the intensity of fluorographic exposure in Figs. 4 and 5, respectively.

## Laminin and Entactin

It seemed likely that the labeled molecule in Figs. 4 and 5 that correlates with the presence of neurite outgrowth-promoting activity and co-migrates with a laminin standard was, in fact, laminin, a glycoprotein known to promote neurite

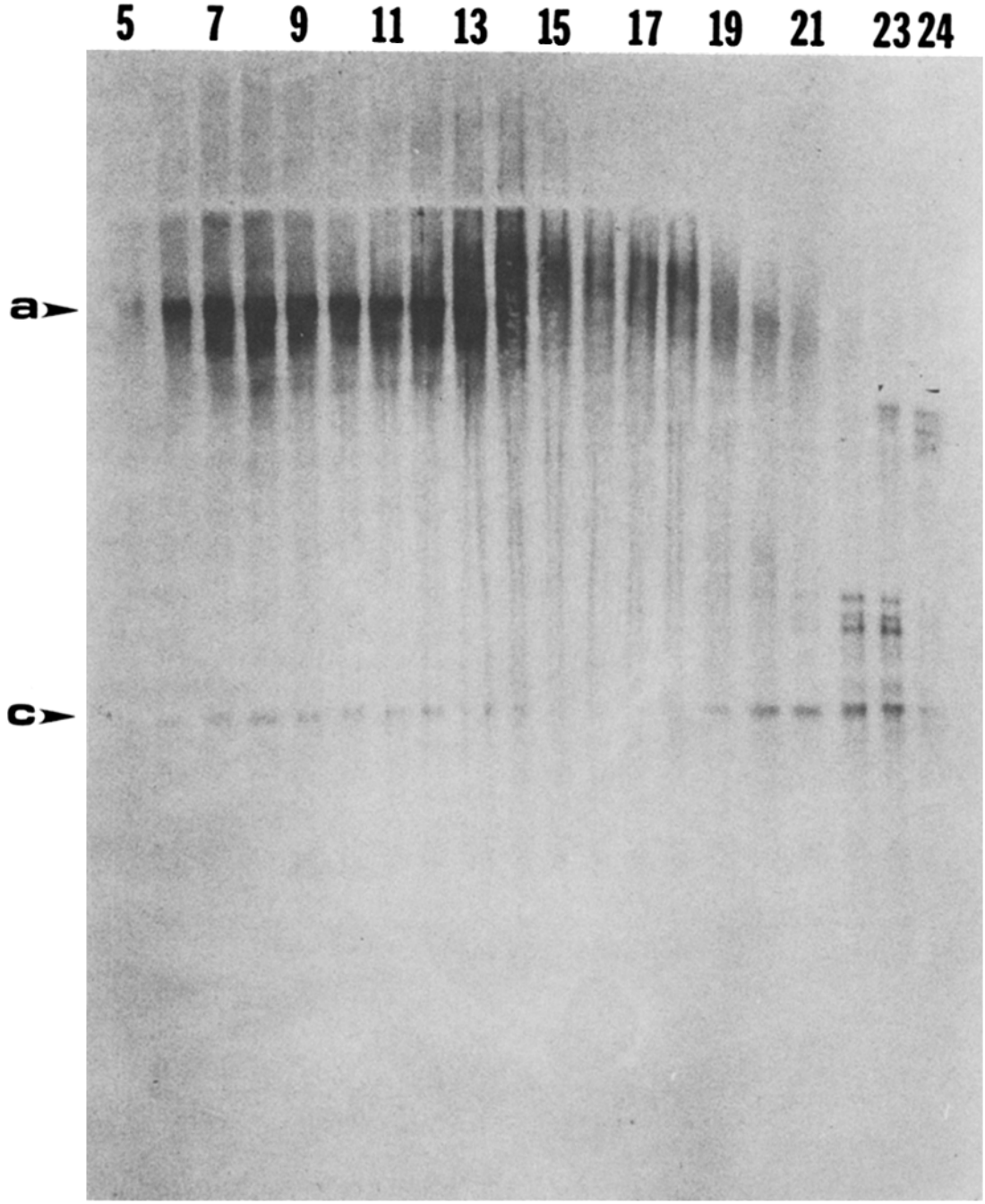


FIGURE 5 Velocity sedimentation through 5–20% sucrose. Analysis by SDS PAGE. Equal volumes of fractions 5–24 from the material shown in Fig. 2 were analyzed by nonreducing SDS PAGE. Fractions are identified by numbers above lanes. Standards: (a) laminin (850–1,000 kD) and (c) mouse immunoglobulin (150 kD).

outgrowth in this assay system (16, 17, 20, 26). To test this hypothesis, fractions from gel filtration were assayed for laminin by enzyme-linked immunoassay (EIA) using affinity-purified antibodies directed against laminin from the (murine) EHS sarcoma. The results, shown in Fig. 1, indicate a very good correlation between laminin immunoreactivity, neurite outgrowth-promoting activity, and the band that co-migrates with a laminin standard (Fig. 4, marker *a*). Similar analysis was carried out on fractions that had been subjected to velocity sedimentation. Fig. 2 shows that the profile of anti-laminin binding again matches the profile of neurite outgrowth-promoting activity, as well as that of the band that co-migrates with the laminin standard (Fig. 5, marker a).

To establish that this band indeed represents laminin, immunoprecipitates of [$^{35}$S]methionine-labeled BCE–CM were prepared using affinity-purified anti-laminin and analyzed by nonreducing SDS PAGE (Fig. 6). In order that all immunoprecipitated molecules be detected, precipitates were not extensively washed, and therefore contained some nonspecifically precipitated molecules. These nonspecifically precipitated molecules could be distinguished as bands present in precipitates prepared both with (Fig. 6, lane *1*) and without (Fig. 6, lane *2*) anti-laminin. The major molecule that was specifically precipitated (i.e., present in lane *1* but not lane *2*)

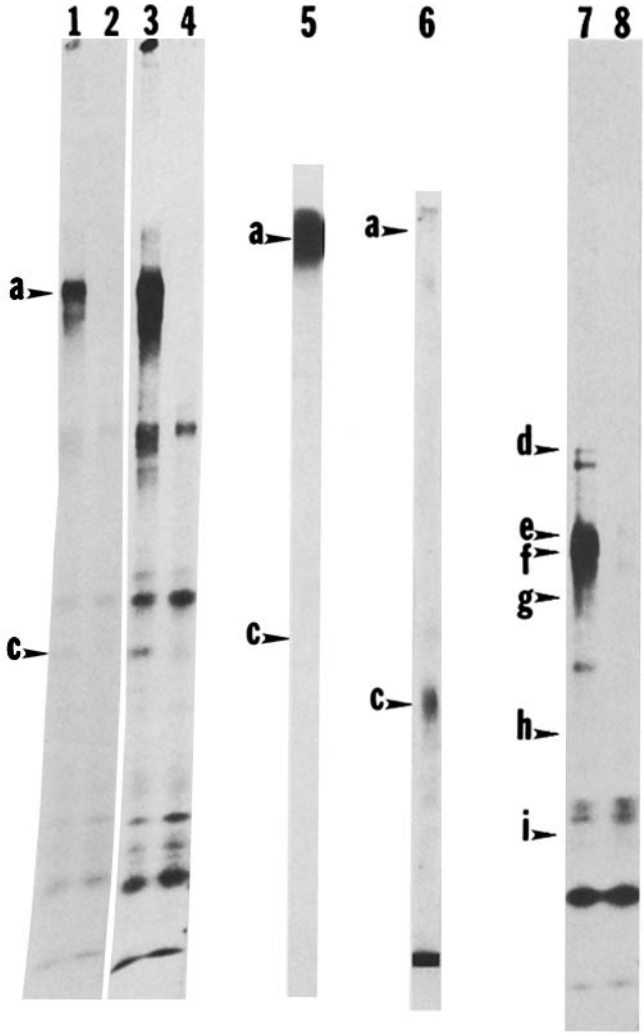


FIGURE 6 Analysis of BCE-CM by immunoprecipitation and immunoblotting. [$^{35}$S]Methionine-labeled BCE-CM was subjected to immunoprecipitation with affinity-purified anti-laminin, as described, and the pellet was analyzed by nonreducing SDS PAGE. Lanes *1* and *3*, experimental; lanes *2* and *4*, control (no anti-laminin used). Lanes *3* and *4* were exposed four times longer than were lanes *1* and *2*. Note the protein band at 150 kD that specifically coprecipitates (i.e., present in lanes *1* and *3*, but not *2* and *4*) with laminin. Lanes 5 and 6 represent immunoblots of unlabeled BCE-CM with affinity-purified rabbit anti-laminin (lane 5) and rabbit anti-entactin serum (lane 6) (see Materials and Methods). Standards: (*a*) laminin (850–1,000 kD) and (*c*) mouse immunoglobin (150 kD). Anti-laminin immunoprecipitates analyzed in lanes *1–4* were also prepared in sample buffer containing 5% 2-mercaptoethanol for analysis by SDS PAGE under reducing conditions. Lane *7*, experimental (same sample as lanes *1* and *3*); lane 8, control (same sample as lanes *2* and *4*). Standards: (*d*) laminin, heavy chain (400 kD), (e) cellular fibronectin (220 kD), (f) laminin, light chain (200–220 kD), (g) myosin (200 kD), (*h*) β-galactosidase (116 kD), and (*i*) bovine serum albumin (68 kD). Note the 150-kD band found between standards *g* and *h* in lane *7*.

appeared as a band that co-migrated with a laminin standard (marker *a*), supporting the conclusion that this band represents laminin. Interestingly, another band at 150 kD (marker *c*) was also immunoprecipitated by anti-laminin antibodies. It is especially apparent in lane *3* (a longer exposure of lane *1*), and its absence in lane *4* (a longer exposure of lane *2*) establishes that it was specifically immunoprecipitated. This result implies either that the 150-kD band represents a molecule recognized by anti-laminin antibodies, or it represents a molecule that co-immunoprecipitates with laminin because it associates with laminin. To distinguish between these possibilities, BCE-CM was electrophoretically blotted onto nitrocellulose and probed with anti-laminin antibodies (Fig. 6, lane *5*). These antibodies bound to a band co-migrating with a laminin standard (marker *a*), but did not recognize any band at or near 150 kD. Thus, there appears to be a 150-kD protein in BCE-CM that is associated with laminin. This observation explains why, during purification of the neurite outgrowth-promoting factor from BCE-CM, a 150-kD band was consistently seen in all fractions that contained the laminin band (cf. Figs. 3–5).

The identity of this 150-kD band was strongly suggested by the literature. Several groups have reported that a laminin-associated protein of 150 kD is present in a number of rat and mouse CM and tissues (47–49), and this protein has been purified and named entactin (50) (also known as glycoprotein C [51]). To confirm that the laminin-associated 150-kD protein present in BCE-CM is (bovine) entactin, immunoblots were probed with an antiserum specific for entactin (generously provided by Dr. Hynda Kleinman). As shown in lane *6* of Fig. 6, this antiserum recognized the 150-kD band in BCE-CM.

As further proof that the molecules immunoprecipitated from BCE-CM by anti-laminin antibodies were indeed laminin and entactin, the immunoprecipitates analyzed by nonreducing SDS PAGE in lanes *1–4* of Fig. 6 were further analyzed under reducing conditions (lanes *7–8*). As expected, the band that co-migrated with nonreduced laminin was replaced by bands co-migrating with the light and heavy chains of reduced laminin. The light chain appeared as a diffuse band, and instead of a single heavy chain, a doublet was observed, although the doublet was not a consistent finding in all batches of CM (splitting of the chains of laminin into doublets has been reported by others, e.g., reference 22). Under reducing conditions, there appeared to be no significant change in the apparent molecular weight of the 150-kD band, entirely consistent with the fact that entactin consists of a single polypeptide chain (50). Additional evidence that this protein is entactin comes from [$^{35}$S]sulfate labeling, described below.

## Proteoglycan

The only other labeled material detected in all neurite outgrowth-promoting fractions in Figs. 4 and 5 is the high molecular weight blur that, like the 150-kD molecule identified as entactin, is also found in some fractions that do not promote outgrowth (e.g., Fig. 5, fractions 15–20). For several reasons, it was suspected that this material represents a heparan sulfate proteoglycan. First, there is some evidence suggesting that neurite outgrowth-promoting factors in BCE-CM and other CM are associated with proteoglycans of the HeS class (8, 9, 16, 52). Second, laminin is known to bind HeS (23, 24), which in CM is found as part of HeS proteoglycans (e.g., 52–54); it would not, therefore, be unexpected to find an HeS proteoglycan co-purifying with the laminin in BCE-CM (e.g., in Figs. 3–5). Third, when proteoglycans are subjected to SDS PAGE, they often migrate as diffuse blurs (e.g., 55). To establish that the high molecular weight blur in Fig. 3–5 represents an HeS proteoglycan, several experiments were done.

First, BCE-CM was prepared from cells grown in [$^{35}$S]-

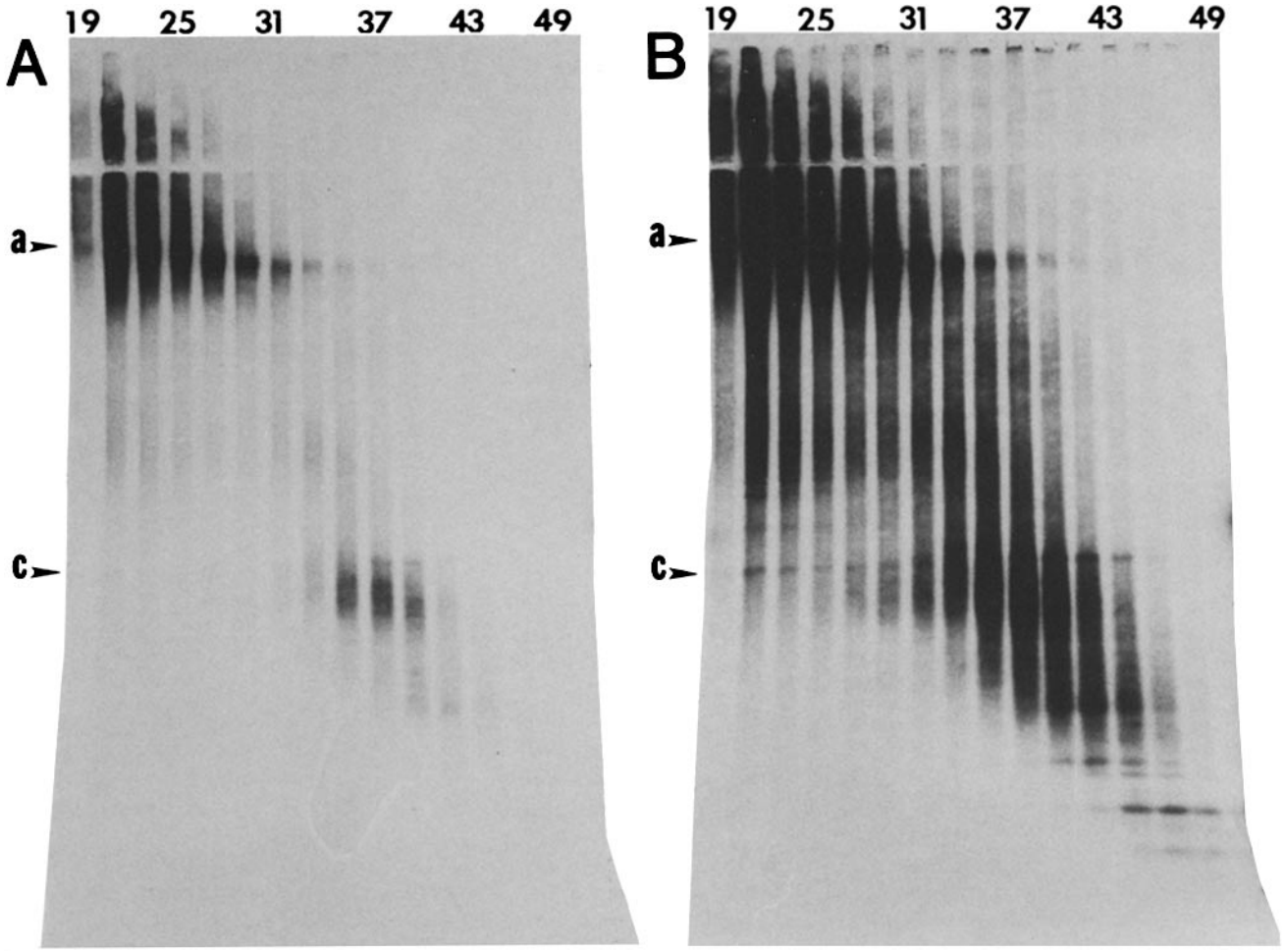


FIGURE 7 (*A*) Gel filtration of [$^{35}$S]sulfate-labeled BCE-CM. Samples were purified and analyzed as in Fig. 4, except [$^{35}$S]sulfate-labeled BCE-CM was used. (*B*) The same gel, exposed seven times longer. Fractions are identified above lanes and are analogous to those in Fig. 4. Standards: (a) laminin (850–1,000 kD) and (c) mouse immunoglobulin (150 kD). Note the presence of a sulfated band at 150 kD.

sulfate, which labels the glycosaminoglycan side chains of most classes of proteoglycans. Sulfate-labeled material was taken through all purification steps described in Table I, except for the final step of velocity sedimentation. Fractions from gel filtration were then analyzed by SDS PAGE (Fig. 7*A*). The major sulfated molecular species appears as a blur in the very high molecular weight region of the gel. This material is present in the completely excluded fractions and trails off into included fractions. When Fig. 7*A* is compared with Fig. 4, it can be seen that methionine (Fig. 4) and sulfate (Fig. 7*A*) are both incorporated into high molecular weight blurs with the same electrophoretic and gel filtration behavior. This result suggests that methionine and sulfate are incorporated into the same molecule, as would be expected for a proteoglycan.

Very few protein bands seen in Fig. 4 are also seen in Fig. 7, *A* or *B* (a longer exposure of 7*A*), reflecting the fact that sulfated (glyco)proteins are relatively rare. One band, however, that clearly appears in Fig. 7*B* is a band corresponding to the 150-kD protein described above. Since entactin has been shown to be sulfated (27, 51) this result furthers corroborates the identification of the 150-kD band as bovine entactin.

The sulfate and methionine labeling of the high molecular weight blur in Figs. 4 and 7 provides suggestive evidence that this blur represents a proteoglycan. To obtain conclusive evidence, [$^{35}$S]methionine-labeled samples that had been purified and subjected to velocity sedimentation (i.e., the fractions from Fig. 2) were digested with purified heparitinase, an enzyme that specifically degrades HeS side chains (33). Digested samples were analyzed by SDS PAGE, as shown in Fig. 8. Comparison of this figure with Fig. 5—SDS PAGE of the same samples before heparitinase digestion—shows clearly that the effect of digestion with heparitinase was to convert all of the high molecular weight blur to a single band of ~400 kD. This indicates that the original blur represented an HeS proteoglycan, whose protein core has a molecular weight of ~400 kD.[2] When subjected to SDS PAGE under reducing

[2] The appearance, after heparitinase digestion, of apparently the same 400-kD core protein in every fraction that had previously contained a high molecular weight blur justifies the conclusion that a single species of HeS proteoglycan is present in all of these fractions. This proteoglycan may, of course, exhibit heterogeneity in length or number of carbohydrate chains. Although this was not assessed directly, it would be reasonable to expect that carbohydrate heterogeneity would give rise to a certain amount of heterogeneity in sedimentation behavior and electrophoretic mobility. Indeed, behavior consistent with this expectation may be noted in Fig. 5: proteoglycan molecules that have sedimented to different positions in the sucrose gradient appear to give rise to high molecular weight blurs of slightly different electrophoretic mobility (e.g., compare fractions 9, 16, and 19).

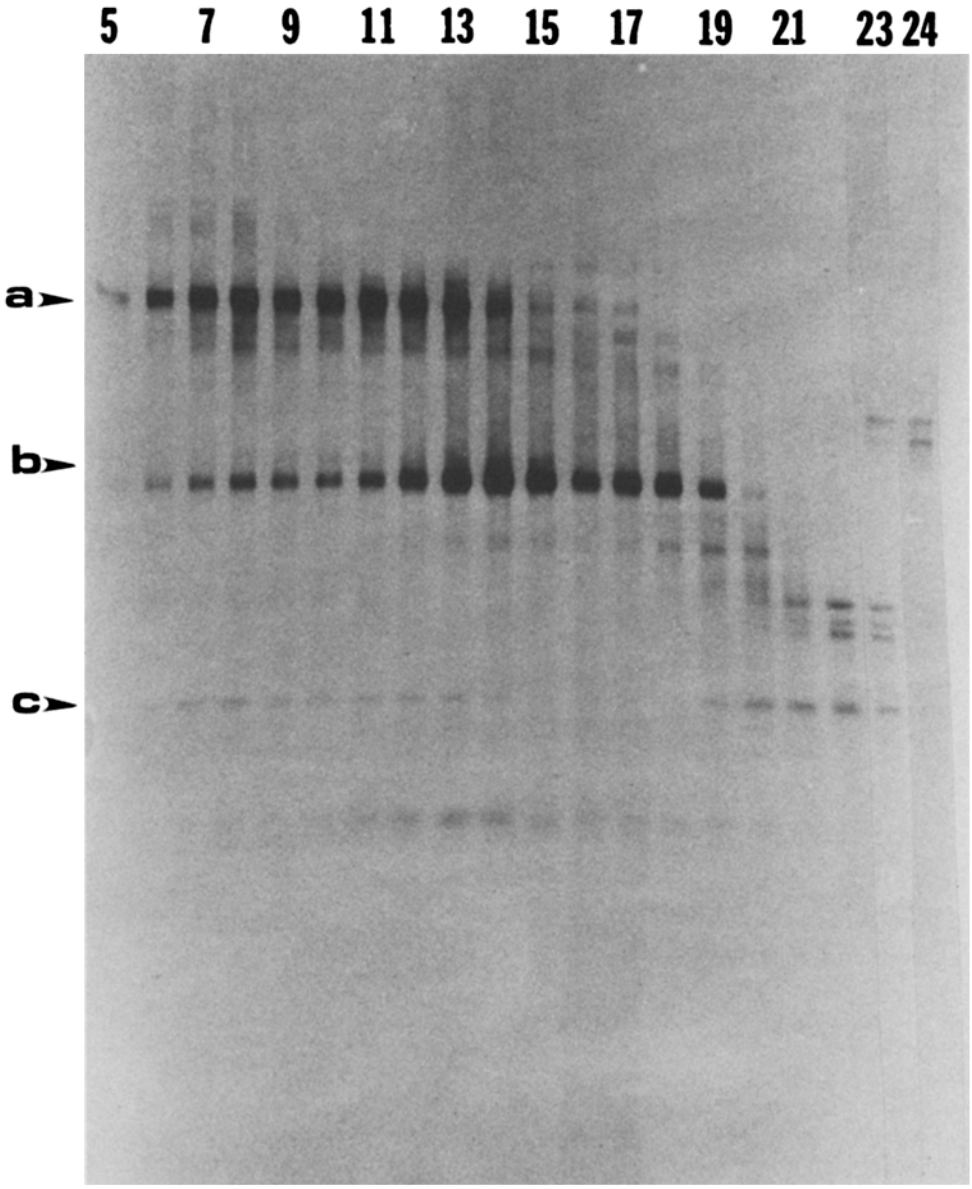


FIGURE 8 Heparitinase digestion of samples from velocity sedimentation. Those samples that were analyzed by SDS PAGE in Fig. 5 were digested with heparitinase (see Materials and Methods) and then analyzed by nonreducing SDS PAGE. Fractions are identified above lanes. Standards: (*a*) laminin (850–1,000 kD), (*b*) cellular fibronectin (440 kD), and (c) mouse immunoglobin (150 kD). Note the disappearance of the high molecular weight blur present in Fig. 5 and the appearance of a core protein band at 400 kD.

conditions, the core protein again migrated as a single band of ~400 kD, indicating that it consists of a single polypeptide chain. These data are shown in Fig. 9. The other smaller bands in Fig. 9 represent the two chains of laminin and entactin.

With the aid of Fig. 8, it is possible to identify those molecules contributing to the different peaks of total labeled protein at the last step of purification (Fig. 2). The peak at ~19S (fractions 5–10), which possesses neurite outgrowth-promoting activity, contains laminin, proteoglycan, and entactin. The peak at ~13S (fractions 11–16), which possesses neurite outgrowth-promoting activity in its leading fractions (fractions 11–13), contains laminin, proteoglycan, and entactin in its leading fractions, but only proteoglycan in its trailing fractions. The peak at ~9S (fractions 17–20) contains proteoglycan only, and the peak at ~4S (fractions 21–24) contains entactin and other molecules not yet identified.

The appearance of entactin, both in the laminin-containing fractions and in the 4S peak, presumably reflects the fact that a portion of the entactin is bound to laminin and a portion is free. Likewise, the appearance of the proteoglycan in all laminin-containing fractions, as well as in the 9S peak and trailing edge of the 13S peak, suggests that a portion of the proteoglycan is bound to laminin and a portion is free. Evidence that some of the proteoglycan is indeed physically associated with laminin was obtained by showing that immunoprecipitation of one molecule brings down the other, the same approach that was taken to demonstrate the association of entactin with laminin (cf. Fig. 6). It was necessary to modify the procedure slightly over that used in Fig. 6, in order to enhance the visibility of the proteoglycan in SDS gels. This was done by performing anti-laminin immunoprecipitations with [$^{35}$S]sulfate–labeled BCE–CM, rather than [$^{35}$S]methionine-labeled BCE–CM. As shown earlier (Fig. 7), [$^{35}$S]sulfate strongly labels the proteoglycan and does not label laminin; this was helpful, given that laminin and the proteoglycan normally migrate to somewhat overlapping positions in SDS gels (e.g., Figs. 3–5). The results of these immunoprecipitations are shown in Fig. 10. The characteristic blur of the proteoglycan is clearly present in the anti-laminin immunoprecipitate (lane *1*) but not the control (lane *2*). Precipitation of the proteoglycan with anti-laminin antibodies could not

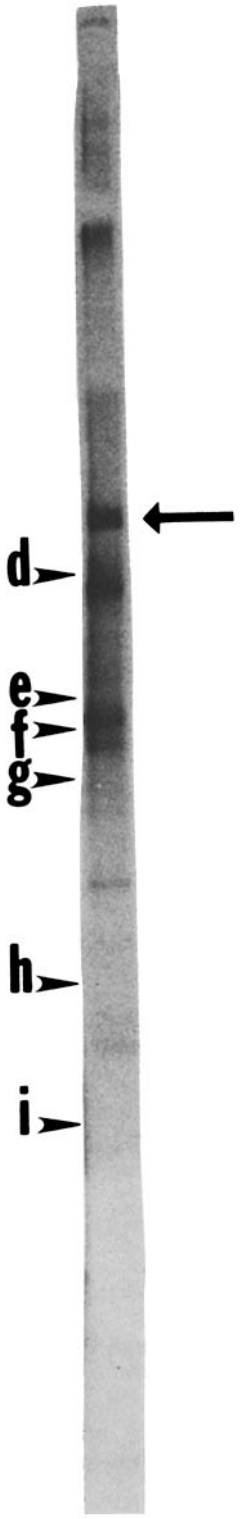


FIGURE 9 Analysis of heparitinase-digested neurite outgrowth-promoting factor by reducing SDS PAGE. Material purified from [$^{35}$S]methionine-labeled BCE-CM through the gel filtration step (i.e., analogous to pooled fractions 19-25 in Fig. 1) was digested with heparitinase as described, mixed with sample buffer containing 5% 2-mercaptoethanol, and analyzed by SDS PAGE. Standards: (*d*) laminin, heavy chain (400 kD), (*e*) cellular fibronectin (220 kD), (*f*) laminin, light chain (200–220 kD), (*g*) myosin (200 kD), (*h*) β-galactosidase (116 kD), and (*i*) bovine serum albumin (68 kD). Arrow: proteoglycan core protein. Entactin is found between standards *g* and *h*.

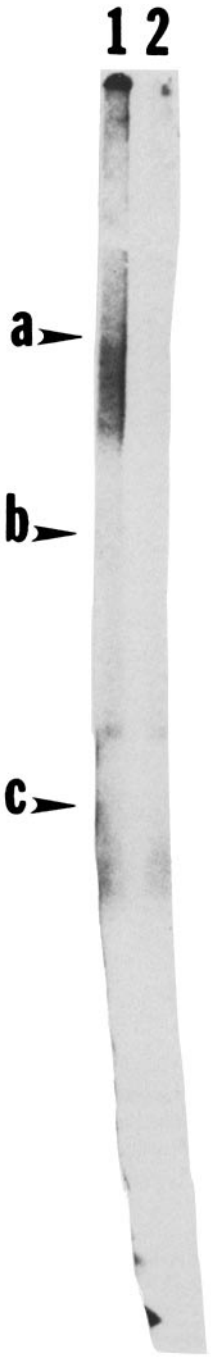


FIGURE 10 Immunoprecipitation of [$^{35}$S]sulfate-labeled BCE-CM with anti-laminin. Sulfate-labeled CM was subjected to immunoprecipitation as described, and as in Fig. 6. The pellet was analyzed by nonreducing SDS PAGE. Lane *1*, experimental; lane *2*, control (no anti-laminin used). Standards: (*a*) laminin (850-1,000 kD), (*b*) cellular fibronectin (440 kD), and (*c*) mouse immunoglobulin (150 kD).

have been due to reactivity of the antibodies towards the proteoglycan, because examination of Figs. 2, 5, and 8 reveals that anti-laminin antibodies show no binding to sucrose gradient fractions that contain the proteoglycan but lack laminin (e.g., fractions 17–19). In addition, the proteoglycan blur was not recognized by anti-laminin on immunoblots (Fig. 6, lane *5*). Thus, some of the HeS proteoglycan in BCE-CM must be physically associated with laminin.

It is known that purified laminin (from the EHS sarcoma) will bind glycosaminoglycans, particularly heparin and HeS, and that the binding of heparin and HeS to EHS-sarcoma laminin can be blocked by high (0.5 M) salt (23, 24, 56). To see whether this interaction accounted for binding of the HeS-proteoglycan to laminin in BCE-CM, [$^{35}$S]methionine-labeled BCE-CM was purified, as described above, through the gel filtration step (cf. Figs. 1 and 4), and fractions with neurite outgrowth-promoting activity were pooled and divided in equal aliquots, one of which was sedimented through a sucrose gradient in 0.1 M NaCl, the other through a sucrose gradient in 0.5 M NaCl. The profile of total labeled protein in the 0.1 M NaCl gradient (Fig. 11*A*) was nearly identical to that in the 0.5 M NaCl gradient (Fig. 11*B*). The distribution of laminin, assessed by EIA, also appeared the same in the two gradients. The relative amounts of proteoglycan in alter-

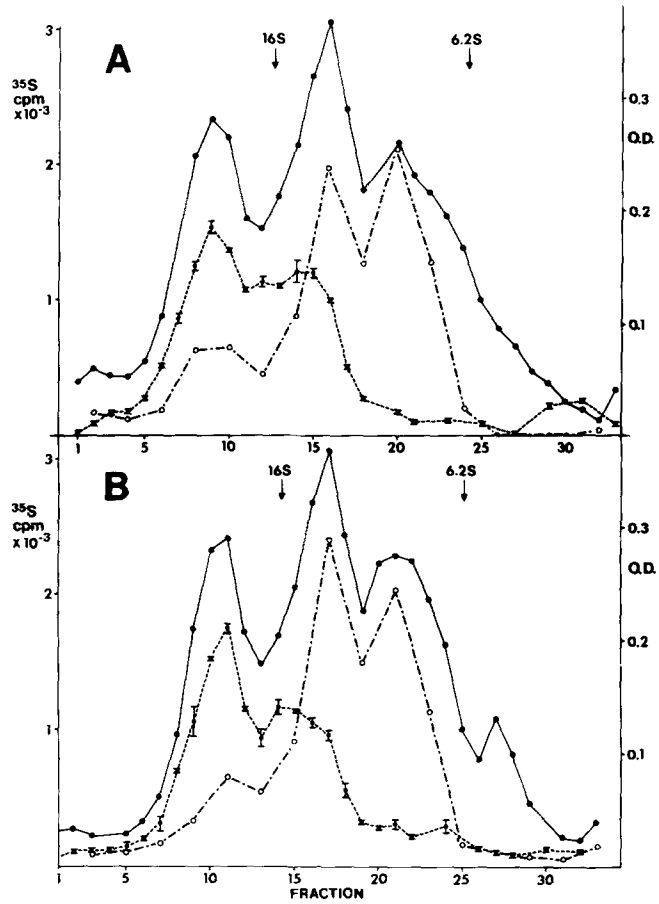


FIGURE 11 Effect of increased salt concentration on laminin-proteoglycan association. [$^{35}$S]Methionine-labeled BCE-CM was purified through the gel filtration step, then divided into two aliquots. One, shown in *A*, was sedimented through 5–20% sucrose in TBS, as described (see Fig. 2). The other, shown in *B*, was adjusted to 0.5 M in NaCl by the addition of 4 M NaCl, and sedimented in the same manner through 5–20% sucrose in 0.5 M NaCl, 0.05 M Tris HCl, 0.1% Triton X-100, pH 7.4. [$^{35}$S]Methionine label was assayed in 10-μl aliquots of each fraction (——), and dilutions of each fraction (1:9 in TBS) were applied to polylysine-treated wells for determination of laminin immunoreactivity by EIA (– – –), as described. Aliquots of selected fractions were also digested with heparitinase and analyzed by nonreducing SDS PAGE, in order to produce fluorographs, such as the one shown in Fig. 8, in which the [$^{35}$S]methionine incorporated into proteoglycan runs as an easily identifiable core protein band. The density of exposure of this band in each lane, measured as described in Materials and Methods, was plotted (— · — · —) and indicates the proteoglycan content of different fractions. Sedimentation standards are shown as in Fig. 2. As a rough measurement of the amount of proteoglycan that was associated with laminin in each gradient, the measured amount of core protein sedimenting between 13–23S in each gradient was divided by the total amount of core protein in that gradient. The 13–23S region corresponded to fractions 4–16 in *A* and 7–17 in *B* and was chosen because within it was found nearly all of the laminin in each gradient. The ratio thus obtained was 0.49 for *A* and 0.48 for *B*.

nate fractions were assessed by heparitinase digestion, SDS PAGE of the digests, and quantification of core protein by measurement of the density of fluorographic exposure. Again, the profiles in the two gradients were not significantly different. In particular, the proportion of the total quantity of applied proteoglycan that was found in laminin-containing fractions was the same for the two gradients (see legend to Fig. 11). It appears, therefore, that the interaction between the HeS proteoglycan and laminin from BCE-CM is less salt-labile than has been reported for HeS and purified mouse laminin. These findings are consistent with previous observations on BCE-CM, in which neurite outgrowth-promoting activity remained associated with [$^{35}$S]sulfate-labeled material in the presence of 3 M CsCl (8).

## Contribution of the HeS Proteoglycan to Neurite Outgrowth-promoting Activity

The experiments described above demonstrate that a HeS proteoglycan and the 150-kD glycoprotein entactin associate with laminin in BCE-CM. All three molecules co-immunoprecipitate (Figs. 6 and 10), and significant amounts of HeS proteoglycan and entactin co-purify with the laminin in BCE-CM (Figs. 3–5). Removal of all three from BCE-CM by immunoprecipitation with anti-laminin results in the disappearance of neurite outgrowth-promoting activity (26). While all three molecules may be considered components of the neurite outgrowth-promoting factor, there is ample evidence that laminin is most directly involved in promoting neurite outgrowth; this will be reviewed in the discussion. There is some evidence, however, from previous work with unfractionated BCE-CM that suggests that the proteoglycan also contributes to neurite outgrowth-promoting activity (8). To determine how this might occur, the neurite outgrowth-promoting factor, purified as described above, was digested with heparitinase to completion, as judged by SDS PAGE, and neurite outgrowth-promoting activity was assayed under three different conditions (Table II).

First, the digested and control (undigested) samples were diluted in an equal volume of buffer (TBS) and assayed. Both samples promoted neurite outgrowth equally well (Table II, line *A*). In fact, assays of dilutions of these samples failed to reveal a significant difference in the quantity of neurite outgrowth-promoting activity they contained. Thus, the proteoglycan, or at least its HeS side chains, is not essential for neurite outgrowth-promoting activity. In the second part of the experiment, the digested and control samples were diluted not with TBS, but with 20% fetal bovine serum in TBS (Table

TABLE II. *Role of HeS Proteoglycan in Neurite Outgrowth-promoting Activity of Material Purified from BCE-CM: Effect of Heparitinase Digestion on Ability to Withstand Addition of Other Substances*

| Sample mixed with | Neurite outgrowth: Heparitinase-digested sample | Neurite outgrowth: Control (undigested) sample |
|---|---|---|
| | % | |
| A. TBS | 71 ± 1.7 | 67 ± 0.8 |
| B. 20% Fetal bovine serum in TBS | 0 | 70 ± 2.9 |
| C. Laminin-depleted CM | 27 ± 6.3 | 75 ± 5.3 |

Neurite outgrowth-promoting material from BCE-CM was purified through the gel filtration step (see text), and an aliquot was digested with heparitinase as described (Materials and Methods). Aliquots of digested and undigested (control) material were mixed with equal volumes of the indicated solutions, and applied to polylysine-treated substrata for determination of neurite outgrowth-promoting activity, which is expressed as the percentage of plated neurons bearing neurites. Laminin-depleted CM refers to serum-free BCE-CM that was depleted of laminin by immunoprecipitation with anti-laminin antibodies (see Materials and Methods). Neurite outgrowth on wells treated with laminin-depleted CM alone was 9%, as compared with 75% before depletion.

II, line *B*). When mixed with this amount of serum, the digested sample showed no neurite outgrowth-promoting activity at all, while the control sample remained as active as before. In the third part of the experiment, the digested and control samples were diluted with laminin-depleted CM, which was serum-free BCE–CM that had been depleted of laminin, and neurite outgrowth-promoting activity, by immunoprecipitation with anti-laminin antibodies. (Table II, line *C*). Dilution with this laminin-depleted CM also markedly inhibited the activity of the digested sample, but not the control sample.

This experiment demonstrates a clear effect of digestion of HeS chains on neurite outgrowth-promoting activity under some, but not all, assay conditions. Thus, compared with the undigested neurite outgrowth-promoting factor, the heparitinase-digested factor, though fully capable of promoting neurite outgrowth, is much more easily inhibited by the addition of molecules ordinarily encountered in CM (i.e., serum [line *B*] and molecules secreted by BCE cells [line C]). It seems most likely that this inhibition represents interference with attachment of the neurite outgrowth-promoting factor to the substratum, against which the intact proteoglycan provides protection. Observations supporting this interpretation are discussed below.

## DISCUSSION

### *Purification of the Neurite Outgrowth-promoting Factor*

The results presented above demonstrate that purification of the neurite outgrowth-promoting factor from BCE–CM yields laminin, entactin, and a HeS proteoglycan. Because only [$^{35}$S]methionine-labeled protein—not total protein—was monitored during the purification, it is theoretically possible that other proteins also contribute to the composition of the neurite outgrowth-promoting factor, but escape detection because they are unlabeled. This seems very unlikely for two reasons: (*a*) BCE cells were labeled for a very long time (4 d) so that all methionine-containing proteins they secrete should be labeled, and (*b*) the only other source of unlabeled proteins in BCE–CM is the 0.5% fetal bovine serum in the labeling medium. Since the neurite outgrowth-promoting factor is produced by BCE cells in normal amounts in the absence of serum and can promote outgrowth in the absence of serum (8), it is very unlikely that serum molecules are associated in any functionally significant way with the factor.

The scheme devised for purification of the neurite outgrowth-promoting factor consists largely of common fractionation methods such as ammonium sulfate precipitation, ion-exchange chromatography, and gel filtration. Certain points, however, merit discussion. For example, the purification begins with absorption of CM with gelatin–Sepharose to remove fibronectin. Very poor yields were consistently obtained when BCE–CM was not thus treated, because in the presence of fibronectin, concentration of the activity at any step, e.g., by ammonium sulfate precipitation, absorption to DEAE, or ultracentrifugation (16), resulted in the appearance of insoluble material in the concentrate and losses of 40–90% of the neurite outgrowth-promoting factor, apparently due to trapping in the insoluble fraction. Analysis of the insoluble material by SDS PAGE indicated that its major component was fibronectin (not shown). BCE cells produce a large amount of fibronectin (57), and cellular fibronectin, when concentrated, is capable of spontaneous covalent polymerization (58).

Several precautions were taken during purification to minimize nonspecific losses. Triton X-100 was used in all buffers after ammonium sulfate precipitation. To minimize adsorption to Sepharose during gel filtration, the column buffer also contained high salt (0.5 M) and a pure protein carrier (hemoglobin). Inclusion of hemoglobin also made it possible to concentrate active fractions from gel filtration without major losses, by loading them into dialysis bags and dehydrating against dry Sephadex. Hemoglobin was later separated from the neurite outgrowth-promoting factor during velocity sedimentation (in Fig. 2, the hemoglobin peak is fraction 25). Precautions were also taken during purification to minimize proteolysis. Protease inhibitors were added to the CM just after collection and included in all buffers. Despite this, one fractionation step, precipitation with polyethylene glycol, that had been used in early attempts at purification (16) was found to greatly accelerate the degradation of many proteins in BCE–CM, including laminin; this step was abandoned.

Table I summarizes the results of a typical purification. As discussed above, the fact that the total amount of neurite outgrowth-promoting activity increased 37% after the first two steps of purification suggests that there steps removed inhibitors from the CM. These inhibitors need not be molecules that block neurite outgrowth-promoting activity per se, but may simply be molecules in BCE–CM that bind polylysine, and can thereby compete with the neurite outgrowth-promoting factor for attachment to the culture substratum. The presence of inhibitors in BCE–CM causes fold purification to be overestimated when calculated from specific activities. As already stated, the degree of purification of the neurite outgrowth-promoting factor is therefore ≤235-fold. Consistent with this estimate, when the laminin band seen on SDS PAGE at each step of purification was quantified (using Fig. 3 and results from other batches of BCE–CM), it was found to be enriched 75–200-fold, depending on the batch of CM (calculations not shown).

### *Composition of the Neurite Outgrowth-promoting Factor*

In the most purified fractions of the neurite outgrowth-promoting factor, three molecules—laminin, entactin, and an HeS proteoglycan—have been identified. These three molecules appear to associate physically with each other (illustrated in Fig. 12), although no claim is made that they exist in a stoichiometric complex.

Laminin is identifiable as an [$^{35}$S]methionine-labeled band that co-migrates by nonreducing SDS PAGE with a laminin standard (Figs. 3–5), is specifically precipitated from BCE–CM by antibodies to mouse laminin, and is recognized by these antibodies on immunoblots of BCE–CM (Fig. 6). During gel filtration and velocity sedimentation, the profile of anti-laminin immunoreactivity correlates with the amounts of the laminin band present in each fraction (compare Figs. 1 and 4, 2 and 5). The polypeptide composition of the laminin in BCE–CM indicates that heavy and light chains are present and similar in molecular weight to the heavy and light chains of mouse laminin (Figs. 6 and 9). The stoichiometry of these chains cannot be determined from [$^{35}$S]methionine-labeled material without information about methionine content. However, assuming that the proportion of methionine in the

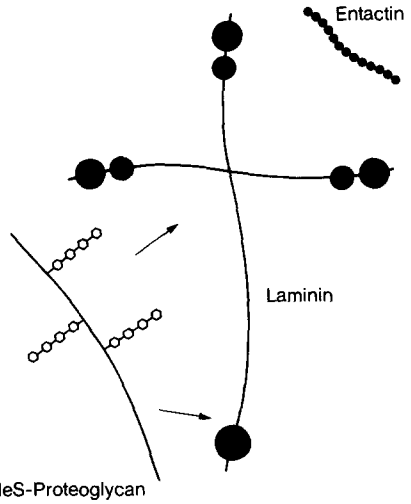


FIGURE 12 Components of the neurite outgrowth-promoting factor. The drawing schematically depicts the molecules that associate with laminin in BCE-CM. The structure of laminin is based on electron microscopic data (59). The structures of the HeS proteoglycan and entactin are speculative. The interaction of the HeS proteoglycan with heparin-binding domains of laminin (73) is indicated by arrows. The binding site for entactin is not known.

two chains is not too different, the relative amount of heavy chain in Fig. 6 appears to be lower than expected, given the 1:1.6 heavy/light chain weight ratio reported for mouse laminin from the EHS sarcoma (59). Furthermore, the relative amount of heavy chain seen by SDS PAGE varied among preparations (compare Figs. 6 and 9), and often decreased during the course of purification. In certain instances, the purified neurite outgrowth-promoting factor, though fully active, appeared by reducing electrophoresis to lack the heavy chain altogether (Lander, A. D., unpublished observations). The fact that decreases occurred during purification suggests that proteolysis of the heavy chain was taking place and is consistent with the observation that the heavy chain of mouse laminin is very sensitive to proteolysis, whereas the light chain is relatively stable (56). Because losses of heavy chain seen by reducing electrophoresis were never accompanied by a change in the nonreduced molecular weight of the laminin, it seems likely that proteolysis of the heavy chain was limited to regions of the polypeptide cross-linked by intrachain disulfide bonds, so that fragmentation could occur only after reduction.

Entactin, a sulfated laminin-binding protein (27, 50, 51), is also a component of the purified neurite outgrowth-promoting factor. The 150-kD protein present in Figs. 3–6 can be identified as entactin because it is of the correct molecular weight before (Figs. 4–6) and after (Figs. 6 and 9) reduction, is sulfated (Fig. 7), and is recognized by anti-entactin antibodies (Fig. 6). It appears to be physically associated with the neurite outgrowth-promoting factor because it is precipitated with laminin by anti-laminin antibodies (Fig. 6). Entactin is known to be a laminin-binding protein, which probably accounts for this association. While entactin is present in every fraction with neurite outgrowth-promoting activity (Figs. 1, 2, 4, and 5), it is not possible to calculate its stoichiometry without knowing its methionine content.

The third constituent of the purified neurite outgrowth-promoting factor is an HeS proteoglycan. When analyzed by SDS PAGE, it appears as a high molecular weight blur, which is characteristic of proteoglycans (e.g., reference 55). As expected for an HeS proteoglycan, it can be labeled with [$^{35}$S] sulfate (Fig. 7). Purified heparitinase, which digests HeS but not other classes of glycosaminoglycans, changes the mobility of this molecule to that of a single polypeptide of ~400 kD (Figs. 8 and 9). This proteoglycan appears to be physically associated with the neurite outgrowth-promoting factor because it is precipitated, along with laminin, by anti-laminin antibodies (Fig. 9). The ability of laminin to bind to HeS (23, 24) could account for such an association. Interestingly, an HeS proteoglycan with a 400-kD core protein has been reported by others to be a component of murine basement membranes (60). The proteoglycan identified in this report may be the bovine form of this molecule.

## *Physico-chemical Properties of the Neurite Outgrowth-promoting Factor*

Most of the behavior of the neurite outgrowth-promoting factor in BCE-CM during purification can be understood in terms of the known physico-chemical properties of laminin and the effects that bound proteoglycan and entactin might have on these properties. For example, the strong binding of the neurite outgrowth-promoting factor to DEAE-cellulose (requiring 1 M NaCl for complete elution) likely reflects the presence of the HeS proteoglycan, a polyanion. Fractionation of the neurite outgrowth-promoting factor on Sepharose CL4B (Fig. 1) indicates that most of it behaves as a larger entity than purified murine laminin (note laminin standard in Fig. 1). This could reflect bound proteoglycan and entactin, or it could reflect self-aggregation of laminin (59). Some of the active factor in Fig. 1 does, however, migrate appropriately for pure laminin (fractions 25–31) forming a shoulder off the main peak (fractions 19–23). Significantly, these shoulder fractions appear to contain less of the proteoglycan than the peak fractions, as judged by the amount of high molecular weight blur seen in Fig. 4.

In sucrose gradients, the neurite outgrowth-promoting factor sediments more rapidly than does purified laminin. In Fig. 2, neurite outgrowth-promoting activity is associated with peaks at ~19S and ~13S, whereas purified laminin sediments at 11.5S (59). These results also suggest that molecules are bound to laminin, but self-aggregation of laminin could account for some of this behavior.

One unexpected result, shown in Fig. 11, is that the purified neurite outgrowth-promoting factor is stable to a salt concentration that should disrupt the binding of HeS or heparin to laminin (23, 24, 56). It is possible that binding of the HeS proteoglycan to laminin is stabilized by entactin, or by the protein core of the proteoglycan. There is some evidence that the core proteins of proteoglycans influence their binding to laminin. For example, a fragment of the HeS proteoglycan present in the EHS sarcoma, which contains all of the HeS chains of the intact molecule but lacks much of the core protein, shows reduced binding to laminin (61). It is also possible that there are modifications of the HeS chains of the proteoglycan in BCE-CM that increase its effectiveness as a ligand. Finally, if there is more than one HeS-binding site on laminin (cf. reference 56), a very large HeS proteoglycan such as the one in BCE-CM may be able to bind to both sites simultaneously, giving rise to an interaction of high avidity. Although it is not yet clear why laminin's interaction with the proteoglycan appears more stable than its interaction with glycosaminoglycans, it is interesting to note that similar ob-

servations have been made regarding the interaction of another extracellular matrix glycoprotein, fibronectin, with HeS proteoglycans (55).

## Functional Roles of the Components of the Neurite Outgrowth-promoting Factor

Because purification of the neurite outgrowth-promoting factor yields three molecules—laminin, entactin, and an HeS proteoglycan—it is important to determine which of the three are involved in the promotion of neurite outgrowth. We believe that laminin is primarily responsible for this activity. Laminin was purified from BCE-CM to approximately the same extent as was the neurite outgrowth-promoting factor (75–200-fold, as discussed above). During the purification, all fractions containing laminin promoted neurite outgrowth; all fractions lacking laminin did not. Furthermore, the profiles of neurite outgrowth-promoting activity after gel filtration (Fig. 1) and velocity sedimentation (Fig. 2) are closely paralleled by the profiles of laminin immunoreactivity (Figs. 1 and 2) and by the electrophoretic band representing laminin (Figs. 4 and 5). Importantly, when laminin is specifically removed from BCE-CM by immunoprecipitation, laminin and neurite outgrowth-promoting activity disappear concomitantly (26).

The hypothesis that laminin is responsible for the activity of the neurite outgrowth-promoting factor in BCE-CM is consistent with the observation that pure murine laminin derived from the EHS sarcoma can promote neurite outgrowth (16, 17, 20, 26). The specific activity of EHS sarcoma laminin is 18–38 neurite outgrowth-promoting units per microgram (17). By silver staining SDS polyacrylamide gels on which anti-laminin immunoprecipites of BCE-CM were run alongside known amounts of EHS sarcoma laminin, it has been possible to estimate the amount of laminin in BCE-CM (~0.2–0.7 $\mu$g/ml in different batches of CM, data not shown). Assuming the same specific activity as EHS sarcoma laminin, this quantity is sufficient to account for the approximately 12 neurite outgrowth-promoting units found in each milliliter of BCE-CM (8).

Neither of the two other components of the neurite outgrowth-promoting factor, entactin and the HeS proteoglycan, appear able to promote neurite outgrowth, since both are frequently found in fractions that lack this activity (cf. Figs. 1, 2, 4, and 5). We also do not believe that these molecules must necessarily be present along with laminin for neurite outgrowth-promoting activity to be seen, for the following reasons: (*a*) Much of the entactin associated with the neurite outgrowth-promoting factor in BCE-CM can be removed by velocity sedimentation in 2 M urea, without reducing the factor's activity (data not shown). Furthermore, the neurite outgrowth-promoting factor in some other CM lacks entactin entirely, yet are fully active (26). (*b*) The HeS proteoglycan associated with the neurite outgrowth-promoting factor in BCE-CM can be digested with heparitinase (which removes HeS) without a reduction in activity (Table II).

Although the HeS proteoglycan is not absolutely required for the activity of the neurite outgrowth-promoting factor in BCE-CM, the data in Table II suggest that it plays an important role. We propose that this role is to mediate the attachment of laminin to polylysine substrata, and is achieved through the interaction of polyanionic HeS chains with polylysine, a polycation. We believe that such a role is important, despite the fact that laminin by itself can adsorb to polycationic substrata (e.g., reference 17), because laminin is only a minor constituent of CM, being outnumbered > 100-fold by other secreted proteins in BCE-CM and up to 10,000-fold by serum proteins, depending on the serum content of the CM. The role of HeS proteoglycan in making attachment of laminin favorable under these circumstances is clearly reflected in the marked ability of serum, and molecules secreted by BCE cells, to inhibit the heparitinase-digested neurite outgrowth-promoting factor. The fact that serum is routinely present throughout the neurite outgrowth assay (as part of the neuronal culture medium), yet only inhibits the digested factor when it is present at the time of application of the factor to the substratum, confirms that the inhibitory effect of serum on the digested factor is the result of interference with attachment to the substratum, not interference with neurite outgrowth-promoting activity per se.[3] Interestingly, these results suggest that, were it not for the HeS proteoglycan, the amount of neurite outgrowth-promoting activity detectable in serum-free BCE-CM would be low, and in BCE-CM with ≥10% serum (see reference 8), none would be detected at all.

## Neurite Outgrowth-promoting Factors in Other CM

Neurite outgrowth-promoting activity, analogous to that found in BCE-CM, has been observed in media conditioned by a great variety of cell types (5–8). Partial purification of the active material from many of these CM has revealed gel filtration and ion exchange behavior similar to that seen in this study (7, 9, 11, 12, 62). Recently, we have surveyed CM from six different cell types, shown that laminin is present in each, and that removal of this laminin eliminates neurite outgrowth-promoting activity (26). Some of these CM have also been fractionated by the steps of purification listed in Table I. In each case, neurite outgrowth-promoting activity was always restricted to fractions containing a protein that had the electrophoretic mobility of laminin, and reacted with anti-laminin antibodies (63, 64). In a recent abstract, a purified neurite outgrowth-promoting factor from rat Schwannoma CM was reported to be associated with a polypeptide of ~200 kD (under reducing conditions) that is recognized by anti-laminin antibodies (65). It seems likely that this polypeptide represents a light chain of laminin. The absence of a 400-kD band in this preparation does not necessarily mean the laminin heavy chain is not present. As discussed above for BCE-CM, limited proteolysis may cause the heavy chain, while actually present and intact, to appear absent or in very low quantity after reduction.

We have emphasized above that in order for neurite outgrowth-promoting activity in BCE-CM to be detectable, laminin must successfully compete with other molecules for substratum attachment. The results of Table II suggest that the HeS proteoglycan associated with laminin is critical for

[3] In an earlier study (8), we reported that treatment of BCE-CM with crude heparinase (which contains heparitinase) resulted in complete inactivation of the neurite outgrowth-promoting factor, and we suggested that an HeS proteoglycan was absolutely required for neurite outgrowth-promoting activity. It is now clear that treatment with crude heparinase did not actually inactivate the factor, but rather left it unable to attach efficiently to the substratum in the presence of the other molecules in BCE-CM, which included an additional 1 mg/ml of protein contributed by the crude enzyme.

this process, especially when serum (at 10%) is present. Since the neurite outgrowth-promoting factors in most CM appear quite active in the presence of similar amounts of serum (see reference 6), it would be reasonable to suppose that an HeS proteoglycan is also associated with laminin in these CM. Indeed, we have obtained evidence from isopycnic centrifugation (16) and enzymatic digestion (9, 16) suggesting that an HeS proteoglycan is associated with the neurite outgrowth-promoting factors in several, possibly all, CM. Recently, we have shown that monoclonal antibodies that recognize an HeS proteoglycan in PC12 pheochromocytoma CM immunoprecipitate neurite outgrowth-promoting activity from that CM (52). Interestingly, these antibodies recognize a proteoglycan quite distinct, in core protein size and total molecular weight, from the HeS proteoglycan found in BCE-CM. Which species of HeS proteoglycan associate with laminin in different CM may reflect the synthesis of different HeS proteoglycans by different cell types as well as differences in the affinity of different HeS proteoglycans for laminin (see reference 61).

Entactin, although a component of the neurite outgrowth-promoting factor in BCE-CM, does not appear to be an invariant constitutent of such factors. In at least two of six CM surveyed, no polypeptide with the molecular weight of entactin co-immunoprecipitated with laminin (26). Indeed, some cell types that synthesize laminin may fail to make entactin (66).

We believe that the conclusion that the neurite outgrowth-promoting factors in CM consist of laminin in association with certain laminin-binding molecules is well supported by the available data. One observation that remains to be explained, however, is why antibodies that block the outgrowth-promoting activity of laminin fail to block the activity of the neurite outgrowth-promoting factors in most CM (16, 17, 25). If the neurite outgrowth-promoting factors in most CM consist of laminin with bound proteoglycan (and possibly entactin), it is possible that sites on laminin recognized by blocking antibodies are made inaccessible, or structurally altered, by bound molecules (26). Consistent with this possibility, blocking antibodies appear to recognize a region of the laminin molecule that is associated with a heparin-binding site (25). Limited proteolysis of laminin in CM could also alter sites recognized by blocking antibodies. A third, intriguing possibility is that most cells in culture synthesize a form of laminin that is immunochemically different from the laminin purified from the EHS sarcoma. Polyclonal and monoclonal antibodies that do block the activity of CM-derived neurite outgrowth-promoting factors have recently been described and should be useful in distinguishing among these possibilities (25, 67).

## Role of Laminin in Neuronal Development In Vivo

The results discussed above suggest that the activity of the neurite outgrowth-promoting factors found in numerous CM can be attributed to a single molecule—laminin. This conclusion supports the view that laminin elicits a highly specific neuronal response, and is not merely one of several agents that increase substratum adhesiveness (see reference 3). Furthermore, the effects of laminin on neurons are not limited to the promotion of neurite outgrowth. Some types of neurons, when cultured on laminin substrata, can be kept alive by considerably lower concentrations of trophic factors such as nerve growth factor; other neurons do not respond to nerve growth factor at all unless provided a laminin substratum (25). For spinal motoneurons, a type of neuron whose trophic factor requirements have not yet been defined, laminin substrata permit at least several days of survival in culture (9, 64). As might be expected, substrata treated with CM, or with neurite outgrowth-promoting factors purified from CM, also possess these activities (9, 64, 68, 69).

These observations suggest that laminin is a good candidate for an extracellular matrix molecule that plays a role in neuronal development in vivo. Indeed, immunohistochemical studies have detected laminin in sites where growing axons would be in contact with it either during initial outgrowth or during regeneration. These locations include the endoneurium of peripheral nerves (70), the basal lamina surrounding muscle fibers (71), the ventral root pathways through which early spinal cord axons grow into the periphery (72), and the sites of forming sensory ganglia (72). Whether laminin in these locations actually influences the growth or guidance of axons remains to be demonstrated.

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