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# Laminin is associated with the "neurite outgrowth-promoting factors" found in conditioned media

(extracellular matrix/cell–substratum interaction/axonal growth and guidance)

ARTHUR D. LANDER*, DENNIS K. FUJII†, AND LOUIS F. REICHARDT*

*Division of Neuroscience, Department of Physiology, and †Department of Obstetrics, Gynecology and Reproductive Sciences, University of California School of Medicine, San Francisco, CA 94143

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ABSTRACT **Conditioned media (CMs) from many cell types contain a factor that can adsorb to a polycationic substratum and cause neurons plated on that substratum to extend neurites rapidly and profusely. The extracellular matrix glycoprotein laminin, when bound to a substratum, elicits a similar response by neurons. In this report, six CMs that contain a "neurite outgrowth-promoting factor" were studied. Immunoprecipitation with affinity-purified anti-laminin antibodies demonstrated that laminin is present in all of them, and when it was selectively removed, there was a corresponding loss of neurite outgrowth-promoting activity in each CM. Antibodies to purified laminin failed, however, to block the outgrowth-promoting activity of five of the CMs tested, even though these antibodies blocked the outgrowth-promoting activity of purified laminin in the same assay. This result could reflect differences in amino acid sequence or protein modification between CM-derived laminin and the purified laminin used in generating antibodies. Alternatively, it could reflect the fact that other molecules bind to laminin in CMs and could interfere with the binding of antibodies to sites on laminin that are important for biological activity.**

The role of the extracellular matrix in influencing axon outgrowth has been the subject of several recent *in vitro* studies (1–6). Neurons of various types, when plated on extracellular matrix deposited by cultured cells, respond dramatically with neurite growth that commences quickly and progresses rapidly and extensively (4). This response occurs even in the absence of nerve growth factor or other appropriate survival factors (4, 7). A similar response is seen when neurons are plated on polycationic substrata that have been exposed to media conditioned by almost any cell type (1–4). This observation suggests that the factor responsible for the neurite outgrowth-promoting activity of extracellular matrix is also present in conditioned medium (CM). Several groups have partially purified and characterized the neurite outgrowth-promoting activity found in several CMs (1, 2, 4, 8, 9). Similarities in results suggest that a single substance may mediate activity in each case.

Neurons also have been cultured on substrata treated with purified extracellular matrix molecules (5, 6, 10). Of these, only laminin-treated substrata promote rapid, extensive neurite outgrowth that is qualitatively and quantitatively similar to that seen on CM-treated substrata (6, 10). Laminin-treated substrata also mimic other effects of CM-treated substrata on neurons: they potentiate neuronal responses to nerve growth factor (11), and they have permitted survival in culture of certain neurons that have not survived on other substrata (e.g., ref. 12).

To determine whether the effects of CM-treated substrata on neurons are the result of laminin in CMs, several investigators have prepared antisera directed against laminin that completely block neuronal responses to laminin-treated substrata (6, 10, 11). These sera have failed to affect the activities of most CM-treated substrata, suggesting that the active material in most CMs is not laminin. Recently, however, we have completed the purification of the neurite outgrowth-promoting factor present in bovine corneal endothelial (BCE) CM. In agreement with earlier results (4, 10, 13), we found that the active factor is an aggregate involving a heparan sulfate proteoglycan and two large proteins. One of these proteins has been identified as laminin. The other components can be separated from laminin and do not promote neurite outgrowth on their own. Details of the purification will be presented elsewhere.

In this report, we present evidence that laminin is a component not only of the active factor in BCE-CM but also of the active factors in CMs produced by several other cell types. We suggest that laminin is responsible for the neurite outgrowth-promoting activity of all CMs with this activity. Reasons for the failure of anti-laminin antibodies to block the activity of many CM-treated substrata are discussed.

## MATERIALS AND METHODS

**Materials.** Laminin was purified from the Engelbreth–Holm–Swarm (EHS) sarcoma (14). Gelatin-Sepharose 4B was prepared at 1 mg of gelatin per ml of resin (15). Goat anti-rabbit immunoglobulin was from Cappel Laboratories (Cochranville, PA). Phosphate-buffered saline (pH 7.4) was prepared as described (16). Heparitinase was purified from *Flavobacterium heparinum* (17).

**Conditioned Media.** BCE cells were maintained and serum-free CM was produced as described (4). Other cells were maintained in Dulbecco's modified Eagle's medium containing 0.1% glucose (DME-1 medium) or 0.4% glucose (DME-4 medium) as indicated and 2 mM glutamine, 100 units of penicillin per ml, 100 μg of streptomycin per ml, and serum. Specifically, RN22 cells and PC12 cells were maintained in DME-4 medium with 10% horse serum and 5% fetal calf serum, and PFHR9 cells were maintained in DME-1 medium with 10% fetal calf serum. CM was medium that was incubated 2–3 days over dense cultures. $C_2$ cells (18) were maintained in DME-4 medium with 20% fetal calf serum, grown to near confluence, switched to DME-4 medium with 10% horse serum, and allowed 2–3 days to fuse into myotubes. Medium was conditioned 2 days over resting myotube cultures. Primary embryonic chicken muscle cultures were prepared, fused, and used to prepare serum-free CM as in ref. 12. CMs metabolically labeled with [$^{35}$S]methionine were prepared by rinsing cultures with methionine-free DME-1 medium and labeling in DME-1 containing glutamine and antibiotics as above, [$^{35}$S]methionine (40 μCi/ml, 1110

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Abbreviations: CM, conditioned medium; BCE, bovine corneal endothelial; EHS sarcoma, Engelbreth–Holm–Swarm sarcoma.

Ci/mmol, Amersham; 1 Ci = 37 GBq), unlabeled methionine (final concentration, 21 μM), and reduced levels of serum (for BCE cells, 0.5% fetal calf serum; for PC12 and RN22, 0.5% fetal calf serum and 1% horse serum; for PFHR9, 1% fetal calf serum; and for $C_2$ and chicken muscle cells, 1% horse serum). Cultures were maintained 3 days (BCE) or 2 days (all others) and then fed with 1 ml of DME-1 medium per 10 ml of culture fluid, supplying an additional 20 μM methionine. After 24 hr, the labeled CMs were harvested, filtered (0.2-μm pores), and treated with the protease inhibitors phenylmethylsulfonyl fluoride (1 mM), EDTA (3 mM), pepstatin (0.1 μM), and *N*-ethylmaleimide (2 mM).

**Assay for Neurite Outgrowth-Promoting Activity.** Polylysine-coated multiwell tissue culture plates (Costar, Cambridge, MA) were treated with CM samples overnight and washed extensively (4). Antisera, if applied, were diluted 1:100 in sympathetic neuron culture medium (see below) and added for 5 hr at room temperature. Antisera were either washed off extensively or were left in the wells during the culture period (as indicated in the text). Neonatal rat sympathetic neurons (4) were cultured in DME-4 medium supplemented with 5% fetal calf serum, glutamine, and antibiotics, as above, for 11 hr at 37°C in an 8% $CO_2$ atmosphere. Cells were fixed (4), and neurite outgrowth was quantified as the percentage of neuronal cell bodies bearing neurites >2-cell diameters long (4). Data are averages of duplicate wells (± the deviation). Each experiment was repeated at least three times.

**Immunochemical Techniques.** A rabbit antiserum to mouse laminin was prepared (19). Serum antibodies were purified by affinity chromatography on laminin coupled to Affi-Gel 10 (Bio-Rad) at 0.8 mg/ml of gel. The laminin used for coupling had been further purified on Bio-Gel A-1.5m (Bio-Rad) (20) and was free of detectable low molecular weight contaminants. For immunoprecipitations, samples of CMs (0.3 ml) mixed with affinity-purified anti-laminin (amounts as indicated) and carrier nonimmune rabbit Ig (5–25 μg) were incubated overnight at 4°C. Precipitating antiserum (goat anti-rabbit Ig) was added to the equivalence point (2.5 μl/μg of carrier Ig). After 12–24 hr at 4°C, the precipitate was removed by centrifugation. Enzyme-linked immunoassays (21) were performed on samples applied to polylysine-coated tissue culture wells by using affinity-purified anti-laminin at 0.5 μg/ml.

**Velocity Sedimentation Analysis.** BCE-CM labeled with [$^{35}$S]methionine (54 ml) was stirred with gelatin-Sepharose (3 ml) for 2 hr, separated from the resin by filtration, and centrifuged in a 45 Ti rotor at 42,000 rpm for 11 hr. The pellet was resuspended in Tris-buffered saline (0.1 M NaCl/0.05 M Tris·HCl, pH 7.4) with 0.1% Triton X-100 and 2 mM EDTA and was centrifuged at 44,000 rpm for 10 hr. The pellet was resuspended in 0.53 ml of the same buffer, and insoluble material was removed by brief centrifugation. The supernatant material was mixed with the sedimentation standards alkaline phosphatase (3.4 units) and β-galactosidase (2.5 units) (assayed as in refs. 22 and 23) and was layered onto a gradient of sucrose (5–20%) in Tris-buffered saline/Triton X-100 and centrifuged in an SW 41 Ti rotor at 38,000 rpm for 12 hr.

**Electrophoresis.** The $NaDodSO_4$/polyacrylamide gel system of Laemmli was used (24). Separating gels were 3–15% exponential acrylamide gradients. Stacking gels were 2.8% acrylamide. Gels were cast on GelBond solid supports (Marine Colloids, Rockland, ME). Labeled material was detected by fluorography (25) with preflashed Kodak XAR-2 film at −70°C (26).

## RESULTS

CMs were prepared from six cell types: BCE cells, a rat Schwannoma line (RN22), a rat pheochromocytoma line (PC12), a mouse endodermal cell line (PFHR9), a mouse myoblast line ($C_2$), and primary embryonic chicken muscle cells. Polylysine substrata were treated with each CM, and rat sympathetic neurons were cultured on them, in the absence of nerve growth factor, for 11 hr. Neurite growth was evident on all of the CM-treated substrata and was absent on an untreated polylysine substratum (Fig. 1; Table 1). Extensive neurite outgrowth was also evident on polylysine substrata treated with purified laminin (Table 1). When laminin-treated substrata were exposed to anti-laminin antiserum, neurons plated on these substrata failed to extend neurites. However, when six CM-treated substrata were exposed to sufficient anti-laminin antiserum to block saturating amounts of laminin, neurite outgrowth-promoting activity was unaffected on five. The sixth (PFHR9-CM) was partially blocked (Table 1). Similar findings have been reported by others (e.g., refs. 6 and 10).

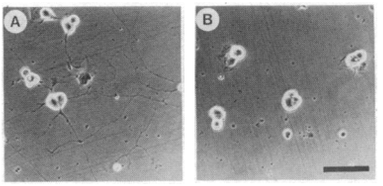


FIG. 1. Neurite outgrowth-promoting effect of CM-treated substrata. Rat sympathetic neurons were prepared and cultured for 11 hr on a polylysine substratum treated with BCE-CM (*A*) or untreated (*B*). (Bar = 50 μm.)

These results seem to suggest that laminin is not the active factor in many CMs. However, when CMs were subjected to immunoprecipitation with affinity-purified anti-laminin antibodies (Table 2), neurite outgrowth-promoting activity was specifically removed from all CMs tested, implying that laminin immunoreactivity is associated with the active factor in these CMs. To determine whether the immunoreactive molecule in each CM is indeed laminin, identical immunoprecipitations were performed with [$^{35}$S]methionine-labeled CMs. Precipitates, washed only once in 0.1 M NaCl/0.05 M Tris, pH 7.4, were subjected to electrophoresis under nonreducing conditions (Fig. 2*A*). A band that comigrates with a laminin standard was found to be specifically immunoprecipitated from each CM (in chicken muscle CM, this band has a slightly lower apparent molecular weight). Upon reduction (Fig. 2*B*), this band was replaced in each case by bands comigrating with the heavy and light chains of a laminin standard (the chicken muscle CM bands are of slightly lower apparent molecular weight). In some CMs, a doublet, rather than a single band, corresponded to the high molecular weight chain of laminin (also described in ref. 19). The rela-

Table 1. Effect of anti-laminin antiserum on the neurite outgrowth-promoting activity of CM-treated and laminin-treated substrata

| | Neurite outgrowth, % with neurites | | |
|---|---|---|---|
| | | After antibody treatment | |
| Substratum | No antibody | Anti-laminin applied and washed off before culture | Anti-laminin present in culture |
| BCE-CM | 65 ± 3.8 | 72 ± 5.4 | 64 ± 6.5 |
| PC12-CM | 53 ± 0.4 | 50 ± 2.4 | 51 ± 2.6 |
| RN22-CM | 49 ± 1.0 | 52 ± 0.5 | 47 ± 1.4 |
| $C_2$-CM | 31 ± 2.9 | 35 ± 1.7 | 32 ± 1.6 |
| Chicken muscle-CM | 73 ± 3.4 | 75 ± 7.9 | 76 ± 2.4 |
| PFHR9-CM | 59 ± 0.6 | 32 ± 1.0 | 14 ± 1.9 |
| Laminin (5 μg/ml) | 77 ± 1.2 | 6 ± 0.7 | 2 ± 0.5 |
| Control | <2 | <2 | <2 |

Table 2. Removal of neurite outgrowth-promoting activity from CMs by immunoprecipitation

| CM | Affinity-purified anti-laminin, μg per ml of CM | Neurite outgrowth, % |
|---|---|---|
| BCE | 0 | 85 ± 1.6 |
| | 9 | 5 ± 1.4 |
| PC12 | 0 | 60 ± 2.1 |
| | 9 | 2 ± 1.2 |
| RN22 | 0 | 28 ± 2.1 |
| | 9 | 1 ± 0.6 |
| $C_2$ | 0 | 35 ± 4.1 |
| | 9 | 2 ± 1.5 |
| Chicken muscle | 0 | 82 ± 1.4 |
| | 9 | 49 ± 0.9 |
| | 27 | 14 ± 1.4 |
| PFHR9 | 0 | 60 ± 2.3 |
| | 9 | 21 ± 3.8 |
| | 27 | 4 ± 1.5 |

tive intensity of labeling of heavy and light chains was considerably different among CMs (Fig. 2*B*). The true stoichiometry of the chains cannot be deduced without information about methionine content. However, the appearance that some CMs have a lower-than-expected amount of heavy chain for the amount of light chain seen (cf. ref. 14) is consistent with the occurrence of limited proteolysis within disulfide-bridged regions of the heavy chain, which would not alter the nonreduced molecular weight of laminin (Fig. 2*A*).

Nearly all of the other bands in Fig. 2 represent proteins that were nonspecifically precipitated (i.e., are present in control lanes 2, 4, 6, 8, 10, and 12). The one exception is a band of ≈ 150,000 daltons (unchanged by reduction) that was specifically immunoprecipitated from BCE-CM, RN22-CM, and $C_2$-CM. Analysis of electrophoretic immunoblots (not shown) indicated that this band is not recognized by anti-laminin antibodies. A band of similar molecular weight has been reported by many investigators to coimmunoprecipitate with laminin (27–29) and probably represents entactin, a sulfated glycoprotein that binds laminin tightly (30, 31).

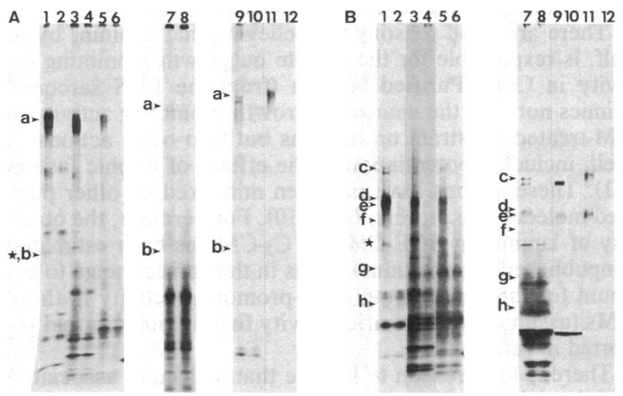


FIG. 2. Immunoprecipitation of [$^{35}$S]methionine-labeled CMs with affinity-purified anti-laminin. Immunoprecipitates, prepared as described, were washed once in Tris-buffered saline and resuspended in 9 M urea. After 1–12 hr, samples were diluted in electrophoresis buffer (24) prepared in 9 M urea and lacking 2-mercaptoethanol. Samples were heated (100°C) for 10 min. Aliquots were subjected to electrophoresis under nonreducing conditions (*A*) or were made 5% in 2-mercaptoethanol for electrophoresis under reducing conditions (*B*). The CMs and the amount of anti-laminin used per ml of CM were as follows (for both *A* and *B*): BCE-CM, 9 μg (lane 1) and 0 μg (lane 2); RN22-CM, 9 μg (lane 3) and 0 μg (lane 4); $C_2$-CM, 9 μg (lane 5) and 0 μg (lane 6); PC12-CM, 9 μg (lane 7) and 0 μg (lane 8); embryonic chicken muscle CM, 27 μg (lane 9) and 0 μg (lane 10); and PFHR9-CM, 27 μg (lane 11) and 0 μg (lane 12). Fluorographic exposure was 4 days for lanes 1–6, 9, and 10; 7 days for lanes 7 and 8; and 2.5 hr for lanes 11 and 12. (*A*) Unreduced molecular weight standards marked a and b were purified laminin and mouse IgG, respectively. (*B*) Reduced molecular weight standards marked c–h were: c, laminin, large subunit; d, cellular fibronectin; e, laminin, small subunit; f, myosin; g, β-galactosidase; and h, bovine serum albumin. Note the 150,000-dalton polypeptide (*) in lanes 1, 3, and 5 of *A* and *B*.

To further substantiate the claim that elimination of outgrowth-promoting activity from the CMs reflects removal of laminin, labeled BCE-CM was subjected to immunoprecipitation with various amounts of affinity-purified anti-laminin. The supernatants were assayed for neurite outgrowth-promoting activity and were analyzed by electrophoresis under nonreducing conditions (Fig. 3). The loss of activity seen with increasing amounts of antibody correlated well with the removal of the band that comigrates with laminin. Such a good correlation would not have been expected if loss of activity depended on antibodies that recognized and precipitated a molecule distinct from laminin.

If laminin is indeed associated with the active factor in various CMs, it should be possible to purify this factor on the basis of its activity and to demonstrate that neurite outgrowth-promoting activity is always found associated with laminin. In fact, fractionation of BCE-CM yielded this result. An illustration of this is presented in Fig. 4, and described below:

BCE-CM labeled with [$^{35}$S]methionine was mixed with gelatin-Sepharose to remove cellular fibronectin (10, 15), ultracentrifuged to pellet large molecules (S > 14), resuspended, and ultracentrifuged again, resulting in an increase in specific activity of about 30-fold. The partially purified material was then analyzed by velocity sedimentation (Fig. 4). The sedimentation profiles of neurite outgrowth-promoting activity, [$^{35}$S]methionine, and laminin immunoreactivity are shown in Fig. 4*A*. Biological activity was broadly distrib-

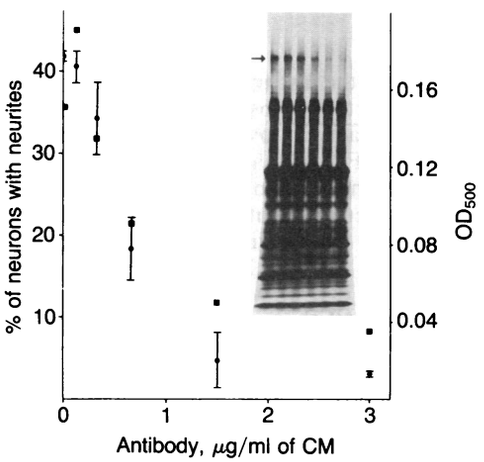


FIG. 3. Concomitant removal of laminin and outgrowth-promoting activity from BCE-CM by immunoprecipitation. [$^{35}$S]Methionine-labeled BCE-CM was subjected to immunoprecipitation with various amounts of affinity-purified anti-laminin. After removal of the precipitate, the supernatants were assayed for neurite outgrowth-promoting activity at a 1:16 dilution (chosen within a range where the neuronal response would be roughly linear with concentration of the active factor). The same samples were also analyzed by electrophoresis under nonreducing conditions. (*Inset*) Supernatants of precipitates that were performed with (from left to right) 0, 0.12, 0.33, 0.67, 1.5, and 3.0 μg of anti-laminin per ml of CM. In each lane, the density of fluorographic exposure of the band that comigrates with a laminin standard (arrow) was measured (32). These data are plotted (■) together with the neurite outgrowth determinations (percent of neurons with neurites) (●). Loss of neurite outgrowth-promoting activity coincides with depletion of laminin.

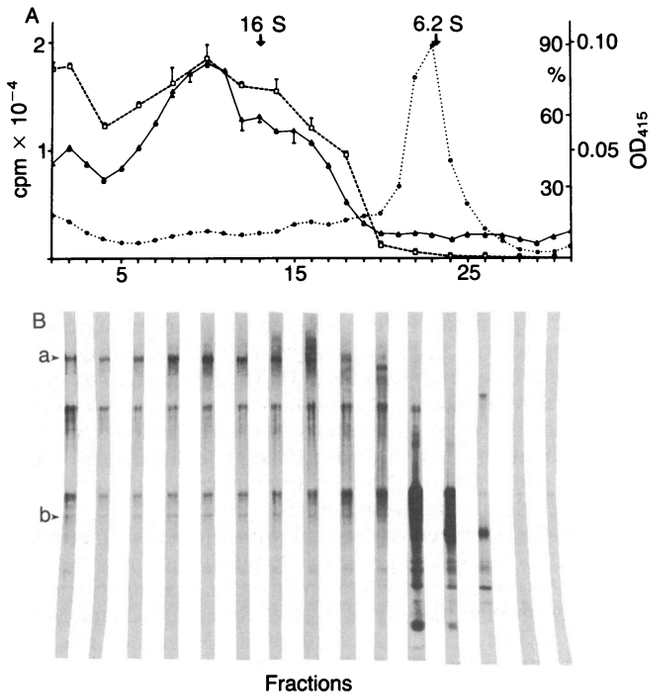


FIG. 4. Velocity sedimentation of [$^{35}$S]methionine-labeled BCE-CM. Labeled BCE-CM, partially purified as described, was sedimented through a 5–20% sucrose gradient. (*A*) Eluted fractions were assayed for radioactivity (·····), neurite outgrowth-promoting activity (—), and laminin-immunoreactivity (----). The positions of sedimentation standards ($\beta$-galactosidase, 16 S; and alkaline phosphatase, 6.2 S) are marked (arrows). (*B*) Aliquots of alternate fractions were electrophoretically analyzed under nonreducing conditions. Lanes have been aligned under appropriate fractions in *A*. Molecular weight markers were: a, laminin; and b, mouse IgG. The 150,000-dalton protein that associates with laminin comigrates with marker b.

uted over many of the fastest-sedimenting fractions. The distribution of this activity closely matched the distribution of laminin immunoreactivity over the same fractions. Electrophoretic analysis (Fig. 4*B*) shows that a band that comigrated with a laminin standard also followed the same profile.

The broad uneven distribution of laminin in this gradient is similar to that seen upon sedimentation of other laminin-containing CMs (e.g., see ref. 33) and reflects aggregation of laminin with itself (34) or with other molecules in the CM (33). Indeed, Fig. 4*B* shows that several labeled molecules cosedimented with laminin, although most of these were also found in equal or greater concentration in slower-sedimenting fractions that possessed no neurite outgrowth-promoting activity. Among the molecules that cosedimented with laminin was a 150,000-dalton band. It comigrated with the 150,000-dalton band pointed out in Fig. 2. Also visible in the high molecular weight region of the gel is weakly labeled material that migrated as a diffuse blur (see especially fractions 14–20). Its identify is discussed below.

The association of laminin with the active factor in BCE-CM is further supported by a more extensive purification to be presented elsewhere. At the end of this purification, electrophoretic analysis (Fig. 5, lane 1) revealed only the laminin band, a 150,000-dalton band, and a diffuse blur in the high molecular weight region of the gel (surrounding the laminin band). The identity of the diffuse high molecular weight blur was established by digestion with the enzyme heparitinase, which converted this labeled material into a single band representing a polypeptide of ≈400,000 daltons (Fig. 5, lane 2). This indicates that the high molecular weight blur in lane 1

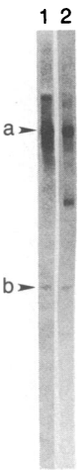


FIG. 5. Purified "neurite outgrowth-promoting factor" from BCE-CM. Lanes: 1, [$^{35}$S]methionine-labeled BCE-CM was fractionated by gelatin-Sepharose absorption, ammonium sulfate precipitation, DEAE-cellulose chromatography, gel filtration, and velocity sedimentation (cf. ref. 10) and was analyzed by electrophoresis under nonreducing conditions; 2, the sample analyzed in lane 1 was digested before electrophoresis, with heparitinase (35 $\mu$g/ml) for 2 hr at 43°C (17). Molecular weight markers were: a, laminin; and b, mouse IgG. The 150,000-dalton protein comigrates with marker b. The band that appears after heparitinase digestion (lane 2) represents the core protein of a heparan sulfate proteoglycan (see text).

represents a heparan sulfate proteoglycan (cf. refs. 4, 10, and 16). Like the 150,000-dalton band, this proteoglycan will coimmunoprecipitate with the laminin in BCE-CM, although [$^{35}$S]methionine labels it too faintly to make it apparent in Fig. 2 (data not shown). Thus, the laminin in BCE-CM is associated with a 150,000-dalton protein (cf. refs. 30 and 31) and a heparan sulfate proteoglycan.

## DISCUSSION

The data presented here demonstrate that laminin is associated with the neurite outgrowth-promoting activity present in a variety of CMs. Thus, either laminin is responsible for neurite outgrowth-promoting activity, or molecules associated with laminin are responsible for activity, or both laminin and associated molecules are required for activity.

There are good reasons for believing that laminin, by itself, is responsible for the neurite outgrowth-promoting activity in CMs. Purified laminin (from the EHS sarcoma) mimics not only the neurite outgrowth-promoting activity of CM-treated substrata on neurons but also other actions as well, including potentiation of the effects of trophic factors (11). These actions have not been mimicked by other purified molecules (e.g., refs. 6 and 10). Furthermore, the quantity of laminin in BCE-CM and $C_2$-CM has been estimated (unpublished observations) and is in the correct range to account for the neurite outgrowth-promoting activity in these CMs (assuming the specific activity for laminin was that reported in ref. 6).

There is little reason to believe that molecules associated with laminin are themselves responsible for promoting neurite outgrowth. Both the proteoglycan and the 150-kDa protein in BCE-CM can be isolated free of laminin—e.g., in the slower sedimenting fractions of sucrose gradients similar to the one shown in Fig. 4—and do not have this activity (not shown). There is also little reason to believe that the presence in CMs of both laminin and associated molecules is necessary for the promotion of neurite outgrowth. The 150-kDa protein is present only in some CMs (Fig. 2). In BCE-CM and $C_2$-CM, most of it can be separated from laminin without any loss of outgrowth-promoting activity. The proteoglycan in BCE-CM also can be separated in large part from laminin without diminishing activity (unpublished observations).

There are at least two ways to explain the failure of anti-laminin antibodies to block the activity of CM-treated substrata. First, there may be structural differences between the purified laminin used in this study (derived from a mouse tumor, the EHS sarcoma) and the laminin in CMs, so that differences exist in the epitopes present on biologically important regions of the molecule. Antigenic variation among species may explain why the activity of BCE-CM (bovine)

and chicken muscle CM is insensitive to anti-mouse laminin. However, anti-mouse laminin antibodies also failed to block the activity of two CMs produced by rat cells (RN22 and PC12), although purified rat laminin has been reported to be antigenically very similar to mouse laminin (35) and antibodies to purified rat laminin block the neurite outgrowth-promoting activity of purified mouse laminin (6). Furthermore, anti-mouse laminin antibodies failed to block the activity of a CM produced by mouse cells ($C_2$). These results raise the possibility that different cells, even within a species, may produce isoforms of laminin (cf. cellular vs. plasma fibronectin) that differ in protein sequence or pattern of glycosylation.

A second possibility is that the laminin in CMs is sufficiently like EHS sarcoma-derived laminin that it should be blocked by the same antibodies, but molecules bound to laminin in CMs restrict antibody access to certain epitopes or cause conformational changes that eliminate epitopes. It is noteworthy that the neurite outgrowth-promoting activity of EHS sarcoma-derived laminin can be blocked by antibodies that bind at or near a heparin binding site (11). If this region of the molecule is responsible for promoting neurite outgrowth and laminin in CMs has a heparan sulfate proteoglycan bound to this site, then antibody binding to this region might be altered. If this were the case, removal of bound proteoglycan from the laminin in $C_2$-CM, or other murine CMs, would restore sensitivity to blockade by anti-laminin.

Insight into how laminin in CMs resists blockade by anti-laminin may come from examination of PFHR9-CM, the one CM partially blocked by anti-laminin (Table 1; ref. 6). If laminin isoforms exist, one might expect to find that PFHR9-derived laminin more closely resembles EHS sarcoma-derived laminin than do the laminins in other CMs. Alternatively, it may be that most of the laminin in PFHR9-CM remains free of laminin-binding molecules and, therefore, is sensitive to blockade by anti-laminin antibodies. PFHR9-CM contains much more laminin than the other cell types examined in this study, and synthesis of laminin appears to exceed that of the laminin-binding molecule entactin (unpublished observations), which PFHR9 cells also secrete (31). Therefore, it seems possible that much of the laminin in PFHR9-CM lacks bound molecules.

In the present study, six CMs, produced by various cell types, are shown to contain laminin that is associated with neurite outgrowth-promoting activity. CMs from a great number of other cell types have similar outgrowth-promoting activity. It would seem, therefore, that a wide variety of cells synthesize laminin *in vitro*. Whether as many cells do so *in vivo*—where laminin appears restricted to basal laminae—is not known (cf. ref. 36).

Since laminin's effects on neurons *in vitro* are so striking and appear unique to laminin among extracellular matrix molecules, it is tempting to speculate that laminin plays a role in nervous system development *in vivo*, influencing initial growth or regeneration of peripheral axons. One of the ways in which this role might be assessed is to observe the effects of anti-laminin antibodies *in vivo*. Although this approach is attractive, the results of this study suggest that currently available antibodies may be ineffective in some of these experiments, not because laminin is unimportant, but because the antibodies are directed against inappropriate isoforms of laminin or against laminin epitopes that are shielded or altered by associated molecules.

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