Studies on Extracellular Matrix Components That Promote Neurite Outgrowth

A.D. LANDER, K. TOMASELLI, A.L. CALOF, AND L.F. REICHARDT

Department of Physiology, Division of Neurobiology, University of California, San Francisco, San Francisco, California 94143

An important determinant in the development of multicellular organisms is the extracellular matrix (ECM) upon which cells attach, migrate, and differentiate. It is likely that a class of substances that affect neuronal development will be found to be associated with the ECM. During early stages of neuronal development, strong spatial and temporal correlations are seen between the appearance of fibronectin and migration of granule cells to form the external granule cell layer in the cerebellum (Hatten et al. 1982) and migration of neural crest cells to form the variety of tissues derived from the crest (Thiery et al. 1982). When cerebellar granule cells or neural crest cells are cultured in vitro, their abilities to adhere to and migrate on fibronectin substrata correlate temporally with their migratory behaviors in vivo (Hatten et al. 1982; Rovasio et al. 1983).

The ECM is also important in later stages of neuronal development. The interaction between a growth cone and its substratum can determine the rate at which it grows and the paths it follows in vitro (Letourneau 1975). In vivo, axons may also follow routes determined by the substratum (Katz and Lasek 1979). In some cases, this reflects the association of axons with already oriented cells, e.g., radial glia in the cerebral (Rakic 1974) and pioneer fibers in Daphnia (Levintal et al. 1976).

There are several factors that, when bound to culture substrata, stimulate outgrowth from particular classes of neurons. Of these, fibronectin and laminin (Akers et al. 1981; Baron-Van Evercooren et al. 1982) are known components of the ECM in vivo. One group of factors is derived from cultured cells (Collins 1978; Adler et al. 1981; Coughlin et al. 1981; Lander et al. 1982), and, when attached to a substratum, these factors promote profuse and rapid neurite outgrowth. When tested on sympathetic neurons, neurite outgrowth is seen even in the absence of nerve growth factor (NGF). The properties of these factors are discussed in this paper.

RESULTS

Extracellular Matrix Promotes Neurite Outgrowth

Our attention was originally drawn to the ECM by observations that PC12 (pheochromocytoma) cells, rat sympathetic neurons, and other neuronal cell types exhibit profuse and rapid process outgrowth on the matrix secreted by corneal endothelial cells in vitro (Fujii et al. 1982; Lander et al. 1982). PC12 cells and rat sympathetic neurons extend neurites on the ECM even in the absence of NGF, and process outgrowth is not prevented by preincubation of ECM with antiserum to NGF or inclusion of NGF antiserum in the culture medium.

The behavior of neurons on ECM was unusual in other ways (Table 1). Neurites appeared earlier and grew more rapidly on ECM than on polylysine-coated plastic. Within 6 hours after plating onto ECM, more than 80% of the neurons had neurites, many of them several cell diameters in length. In contrast, neurons plated on polylysine and cultured with NGF had very few processes by 6 hours or even 12 hours, although most cells extended neurites by 24 hours.

Although neurites appeared early and grew rapidly on ECM in the absence of NGF, their rate of growth slowed dramatically after 24 hours and cell viability (estimated by morphological criteria) fell steadily thereafter. By 72 hours, less than 20% of the neurons appeared alive; none survived over 5 days. Massive cell death could be avoided only if NGF was present in the culture medium. Then, good viability was maintained for over a week, the longest time assayed. Thus, ECM can substitute for NGF in inducing short-term process outgrowth, but not in maintaining long-term neuronal viability.

Table 1. Comparison of the Response of Neurons to NGF and ECM

| Substratum | Medium | 6 | 12 | 24 | 48 | 72 |
|------------|--------|---|----|----|----|----|
| Polylsine  | + NGF  | 2 | 6  | 75 | 86 | 84 |
| ECM        | - NGF  | 86| 78 | 73 | 40 | 2 |
| Polylsine  | + NGF  | 94| 94 | 86 | 87 | 85 |
| ECM        | - NGF  | 99| 93 | 78 | 49 | 17 |

Rat sympathetic neurons cultured with NGF on polylysine-coated tissue culture plastic and without NGF on ECM were fixed at various times after plating. Neurite outgrowth was measured by counting random fields and determining the percentage of presumptive neurons with neurites. Survival was estimated crudely as the percentage of presumptive neurons lacking morphological signs of cell death or injury, including cell swelling, loss of adhesiveness, retraction of neurites, and accumulation of intracytoplasmic granules. Over 100 neurons were counted for each point shown above.
Table 2. Neuronal Response to CM_{SF} in the Presence and Absence of Serum

| Substratum                  | Medium | 5  | 13 | 24 | 48 |
|-----------------------------|--------|----|----|----|----|
| (a) Percent neurite outgrowth |        |    |    |    |    |
| CM_{SF}-treated polylysine  | + serum | 18 | 56 | 63 | 35 |
|                             | − serum | 57 | 58 | 48 | 21 |
| (b) Percent neuronal survival|        |    |    |    |    |
| CM_{SF}-treated polylysine  | + serum | 95 | 96 | 91 | 48 |
|                             | − serum | 89 | 89 | 81 | 25 |

Rat sympathetic neurons were plated on polylysine-coated tissue culture plastic that had been treated with CM_{SF}. Neurons were cultured without NGF and with or without serum. Neurite outgrowth and survival were determined as in Table 1.

Identification of Active Factors Associated with the ECM

Two approaches have been used to characterize factors in the ECM that promote neurite outgrowth. Major purified components, such as fibronectin and laminin, have been directly tested for effects on neurite outgrowth, and factors secreted by cells into serum-free conditioned media have been identified, purified, and characterized.

Polycationic Surfaces Treated with Conditioned Medium Can Substitute for ECM

One class of neurite outgrowth-promoting factors can be identified in medium conditioned by growth with corneal endothelial cells. Polysine-coated tissue culture dishes were exposed to this conditioned medium (CM) and washed thoroughly. Sympathetic neurons plated onto this substratum responded as they did on ECM, by rapidly extending neurites. Serum-free conditioned medium (CM_{SF}) applied in this way also produced an active substratum (Table 2). Therefore, the active substance(s) is synthesized and secreted by corneal endothelial cells and is not a modified or concentrated component of serum. Table 2 also shows the results obtained when neurons plated on CM_{SF}-treated polysine coated surfaces were cultured without serum. Since rapid neurite outgrowth was also observed under these conditions, the CM_{SF}-coated surface was not acting merely by adsorbing and concentrating some serum component onto the substratum. Instead, it appears to act directly on sympathetic neurons. Figure 1 shows the appearance of cells on CM_{SF}-treated polysine-coated surfaces when grown in the presence and absence of serum. Surfaces not treated with polysine did not promote measurable neurite outgrowth after incubation with CM_{SF}.

CM_{SF}-treated Substrata Promote Neurite Outgrowth by Many Different Types of Neurons

In addition to rat sympathetic neurons, other neuronal cultures were plated onto CM_{SF}-treated, polysine-coated substrata. Rat and chick sensory neurons and sympathetic neurons all responded in the characteristic manner already described, i.e., with early, rapid, extensive neurite outgrowth (Fig. 2). NGF, which these cells normally require for outgrowth and survival, was not required for the response. Thus, two classes of peripheral NGF-dependent neurons from two different species respond to the factor contained in CM_{SF}. Others have reported that parasympathetic neurons also respond to similar factors (Adler et al. 1981).

Figure 1. Response of neurons to CM_{SF}-treated substrata. Rat sympathetic neurons plated on BCE CM_{SF}-treated polylysine substrata, cultured without NGF for 13 hr in serum-containing (A) and serum-free (B) medium. Bar, 50 µm.
At least one class of central neurons responds to CMsf-treated substrata. Embryonic chick motor neurons were purified in a fluorescence-activated cell sorter from embryos whose limbs had been injected previously with a purified conjugate of Lucifer Yellow-wheat germ agglutinin (modified from the procedure of McPheeters and Okun 1980). This conjugate is transported retrogradely and labels the motor neurons but no other cell types in the spinal cord. When these fluorescent neurons were purified on the cell sorter and plated, neurite outgrowth in culture was observed in the first 24 hours on CMsf-treated, but not untreated, polylysine-coated substrata (not shown). Short-term survival did not require other factors, but long-term survival was promoted by addition of chick muscle CM. In contrast, when dissociated cells from cerebellum and olfactory bulb of neonatal rats were plated on CMsf-treated substrata, no positive or negative effects on growth were observed (not shown). When examining cultures not requiring NGF, such as these, negative results may be equivocal, since neurite outgrowth may normally be quite rapid and growth-promoting factors may effect only a minimal increase. With this in mind, however, it is interesting to note that of the cell types tested, all of those with peripheral axons in vivo responded to CMsf; those with processes only in the CNS were not noticeably affected.

Many Cell Types Produce Factors with Similar Properties

Serum-free media conditioned by confluent cultures of various cell types were prepared as described (Lander et al. 1982) and assayed for neurite outgrowth-promoting activity.
promoting activity in the standard manner, using rat sympathetic neurons. Media conditioned by bovine vascular endothelial cells, bovine vascular smooth muscle cells, bovine adrenal cortical cells, human skin fibroblasts, embryonic chick myotubes, rat primary cells that appear to be derived from pericytes (N. Dekker et al., unpubl.), and the cell lines C2 (mouse skeletal muscle), PTK-1 (kangaroo rat epithelium), A-431 (human vulva carcinoma), RN-22 (rat Schwannoma), N-18 (mouse neuroblastoma), and PC12 (rat pheochromocytoma) all possessed activity indistinguishable from that of CM\textsubscript{SF}, and these factors appear to have similar biochemical properties. First, the factors that have been examined appear to be large molecules. As exemplified in Figure 3, the neurite outgrowth-promoting factors that have been examined eluted near the void volume in permeation chromatography on Sepharose 6B (exclusion limit $4 \times 10^6$ daltons for globular proteins; $1 \times 10^6$ daltons for polysaccharides).

CM\textsubscript{SF} from several sources has also been fractionated by isopycnic sedimentation in CsCl density gradients under nondissociating conditions. The results in Figure 4 show that the factors from a variety of sources have densities in CsCl between 1.30 and 1.40. The activities in CM from bovine corneal endothelial (BCE), chick mesenchymal, and putative pericyte cultures ap-

![Figure 4. Isopycnic sedimentation in associative CsCl gradients. BCE CM\textsubscript{SF} from different cell types was centrifuged to equilibrium in CsCl containing 0.4 M GSH, as described in Lander et al. (1982). Fractions were collected and the density of each was measured (---). After dialysis, fractions were assayed for neurite outgrowth-promoting activity. Neurite outgrowth was assayed in CM\textsubscript{SF} from several sources: BCE cells (•-----•, bottom); RN22 Schwannoma cells (•-----•, top); embryonic chick myotubes (•-----•, bottom); PC12 pheochromocytoma cells (•-----•, top); and P cells, an unknown primary cell line whose antigenic properties and morphology suggest that they are derived from pericytes (•-----•, bottom) (N. Dekker et al., unpubl.). Embryonic chick muscle culture CM\textsubscript{SF} was assayed using chick motor neurons purified on the cell sorter (McPheeters and Okun 1980). CM\textsubscript{SF} from other sources were assayed with rat sympathetic neurons (Lander et al. 1982).](image-url)
pear to be single peaks of activity with average densities of 1.35. The activities in CM grown with RN-22 and PC12 cells appear to be bimodal with density peaks at 1.37 and 1.32. The presence of two peaks may reflect molecular heterogeneity. Alternatively, this appearance may be an artifact: If a molecule roughly co-sedimenting with the factor also bound polysine, it might outcompete the factor for binding to the substra-
tum, thus artificially depressing the level of activity observed in certain fractions. It is unclear at this point which alternative is correct.

Since these activities have densities between those of pure proteins and carbohydrates, it appeared that these factors might contain carbohydrate, as well as protein. To obtain more detailed information, corneal endothe-
lial CMsF, metabolically labeled with [3H]amino acids and [35S]sulfate, was prepared and centrifuged in CsCl. With some qualifications (Branford-White 1980), the sulfate label is specific for glycosaminoglycans and, therefore, marks the position of proteoglycans in these gradients. The leucine label marks proteins and, therefore, also glycoproteins and proteoglycans. When con-
centrated CMsF, to which aliquots of leucine-labeled and sulfate-labeled CMsF were added, was centrifuged in CsCl, the neurite outgrowth-promoting activity banded with a peak of [35S]sulfate and [3H]amino acid-
labeled material, suggesting that the neurite outgrowth-
promoting factor is or is associated with a sulfated gly-
coprotein, mucin, or proteoglycan (Lander et al. 1982).

To help distinguish between these possibilities, CM was prepared in the presence of drugs that interfered selectively with the synthesis of individual classes of carbohydrate. The results in Figure 5 show that neurite outgrowth-promoting activity could be detected in CMsF prepared by growth in the presence of 2.5 mm p-nitrophenyl-β-d-xyloside, but the density of this activity was shifted to 1.30 g/ml. Because β-d-xylosides compete with xylene-primed core proteins as templates for glycosaminoglycan synthesis, cells exposed to these drugs secrete proteoglycans that contain fewer and/or shorter glycosaminoglycan chains (Stevens and Austen 1982) and are therefore less dense. This result indicates that the biologically active factor includes a proteogly-
can, since synthesis of mucins and N-linked carbohydrates is not sensitive to the presence of β-d-xylosides.

To identify the properties of the molecules essential for activity of the proteoglycan-associated factors, CMsF from BCE cells and PC12 cells and active frac-
tions from CsCl gradients were subjected to various treatments prior to assay. The corneal endothelial fac-
tor is inactivated by low or high pH or by heating to 80% (Lander et al. 1982). The results in Table 3 show that the activities of the corneal endothelial and PC12-derived factors are destroyed by trypsin. No decrease in the activity of BCE CMsF was seen after

Figure 5. Sensitivity of density of neurite outgrowth-promoting activity to growth with β-xylosides. CMsF was prepared from BCE cells grown in standard conditions (-----O) (Lander et al. 1982) or in the presence of 2.5 mm p-nitrophenyl-β-d-xyloside for 4 days (-----O). After centrifugation to equilibrium in 0.4 M GuHCl, density and neurite outgrowth-promoting activity were measured as described in the legend to Fig. 4. (-----O) The densities of the fractions.
exposure to collagenase or neuraminidase. To identify the class of proteoglycan associated with neurite outgrowth-promoting activity, active fractions were pooled and digested with chondroitinase ABC or heparinase. The results in Table 3 show that the neurite outgrowth-promoting activity is not sensitive to chondroitinase ABC, which degrades the chondroitin and dermatan sulfates, but is sensitive to heparinase, which degrades the remaining class of xylose-initiated glycosaminoglycans, the heparan sulfates. If aliquots of the heparinase were first passed over glycosaminoglycan-Sepharose columns, the eluate that was not retained on chondroitin sulfate-Sepharose retained ability to inactivate the factor, whereas the eluate that was not retained on heparan sulfate-Sepharose no longer possessed the ability to inactivate the neurite outgrowth-inducing factor. Therefore, the degradative enzyme binds heparan sulfate, but not chondroitin sulfate, and the factors appear to be heparan sulfate-proteoglycans or heparan sulfate-proteoglycans complexed with other molecules.

### Purification and Characterization of One Factor

The neurite outgrowth-promoting activity from corneal endothelial CSMF labeled with [3H]leucine and [35S]sulfate has been purified by fractionation with ammonium sulfate, polyethylene glycol, DEAE-cellulose chromatography, and sucrose gradient velocity sedimentation. Results are summarized in Table 4. When the entire peak of active material from the velocity sedimentation was pooled, the factor was found to be purified approximately 40-fold over 3H-labeled material and 30-fold over 35S-labeled material. These numbers must be considered low estimates, since some of the flanking fractions pooled in with the peak clearly contained contaminating material. The sedimentation

### Table 3. Sensitivity to Enzymatic Digestion

| Treatment of enzyme | Control | Heparinase | ABC | Trypsin | Collagenase | Neuraminidase |
|---------------------|---------|------------|-----|---------|-------------|---------------|
| BCE CSMF            | 38      | 39         | 43  | 0       | 0           | 0             |
| PC12 CSMF           | 53      | 1          | 44  | 0       | 0           | 0             |
|                     | 21      | 5          | 18  | 0       | 0           | 0             |

In a, CSMF was exposed to various treatments and assayed. In b, 50-µm aliquots of heparinase (6.0 mg/ml in 0.1 sodium acetate [pH 7.0]) were applied to columns containing 100 µl of glycosaminoglycan-conjugated Sepharose and eluted, at 4°C, in 200 µl of buffer. Controls were not chromatographed, but were simply diluted with buffer to 1.5 mg/ml and 0.15 mg/ml. Samples were added to 4 volumes of partially purified factor and the mixture was incubated for 4 hr at 30°C and assayed for neurite outgrowth-promoting activity. A dilution of the factor affording maximal sensitivity was used. Data are averages of duplicate assays ± deviation from the mean.

### Table 4. Purification of the BCE-derived CSMF Factor

| Specific activity | Neurite outgrowth-promoting activity (arbitrary units) | 3H dpm (×10⁻⁶) | 35S dpm (×10⁻⁶) | Outgrowth activity (+ 3H) | Outgrowth activity (+ 35S) | Yield (%) |
|-------------------|-----------------------------------------------|-----------------|-----------------|-------------------------|---------------------------|-----------|
| CSMF              | 245                                            | 39.5            | 4.4             | 6.2                     | 56                        | (100)     |
| Ammonium sulfate pellet | 192                                              | 14.6            | 1.0             | 13.0                   | 192                       | 78        |
| Polyethylene glycol pellet | 112                                             | 5.90            | 0.298           | 19.0                   | 376                       | 46        |
| DEAE eluted with 1 M NaCl | 94                                             | 1.44            | 0.127           | 67.0                   | 740                       | 38        |
| Sucrose gradient: pooled active fractions (#8–19) | 34                          | 0.128           | 0.0223          | 266.0                   | 1525                      | 14        |

Pooled labeled and unlabeled CSMF was treated with ammonium sulfate (48% saturation), and the precipitate was recovered by centrifugation. The pellets were redissolved in 50 mM Tris-HCl (pH 7.4), and polyethylene glycol 6000 was added to a final concentration of 14% (w/v). The precipitate was recovered by centrifugation, and the pellet was resuspended in Tris-saline buffer (0.1 M NaCl/50 mM Tris-HCl [pH 7.4]). Material that failed to redissolve was removed by centrifugation. The solution was then mixed with 0.5 ml of a slurry of DEAE-cellulose equilibrated in Tris-saline buffer and shaken overnight. After the unbound material was eluted, the DEAE-cellulose was washed with 4 volumes of Tris-saline buffer and eluted with 1 M NaCl/50 mM Tris-CI (pH 7.4). The material thus eluted was mixed with 2 volumes of 50 mM Tris-CI (pH 7.4) and sedimented on a linear 5–20% sucrose gradient in Tris-saline buffer.

*Neurite outgrowth-promoting activity: One unit per milliliter gives a half-maximal response.
behavior of the factor is noteworthy: At very low concentration, most of the neurite outgrowth-promoting activity is found at about 14S–15S, with a smaller peak at about 17S. At higher concentration, the factor sediments farther, with two peaks often seen at 17S and 19S. At the highest concentrations tested, a very broad peak at 21S was seen. In every case examined, regardless of where the peak of outgrowth-promoting activity appeared, the major peak(s) of fast-sedimenting \(^{35}S\) and \(^{3}H\) cosedimented with it. This unusual sedimentation behavior, probably reflecting aggregation, has therefore proved useful in firmly identifying the labeled peaks with the active factor.

**Characterization of the Factor**

Significant amounts of reasonably purified factor could be obtained quickly, taking advantage of its unusual sedimentation behavior, by centrifuging it to concentrate it, sedimenting it at very high concentration on a sucrose gradient (where it runs near 21S), pooling the active material, and resedimenting it on a sucrose gradient at low concentration. As shown in Figure 6a, material from the first sucrose gradient, rerun at low concentration, behaves as a peak at 14S with a small shoulder at 17S. Because peaks are not sharply separated on sucrose gradients, the pooled 21S material from the first gradient was contaminated with a small fraction of the peaks found in other regions; for this reason, a shoulder at 6S–7S is seen, representing residual contamination with the major high-molecular-weight protein secreted by the corneal endothelial cells. Little other contamination is readily apparent.

The results in Figure 6b–d indicate the effects of digestion with various enzymes on the material shown in Figure 6a. Incubation with chondroitinase ABC did not reduce measurably the size of the labeled material (Fig. 6c). In contrast, incubation with highly purified heparitinase (selective for low-sulfate-substituted heparan sulfate-proteoglycans) reduced the rate at which \(^{3}H\) and \(^{35}S\) sediment in sucrose (Fig. 6d). These results support previous evidence that a heparan sulfate-proteoglycan is an integral part of the neurite outgrowth factor. The size of the factor also appeared to be reduced.

---

**Figure 6.** Enzymatic digestion of sucrose gradient-purified CMAsF factor. Peak fractions from concentrated, sucrose gradient-purified CMsF were pooled, and four 0.15-ml aliquots were removed. To one aliquot was added 0.15 ml purified collagenase (Sigma type VII, 150 units/ml in 0.1 M sodium phosphate, 0.05 M NaCl, 0.33 mM CaCl\(_2\) [pH 7.4]). Another aliquot received 0.15 ml purified Flavobacterium heparinum heparitinase (35 µg protein/ml in 0.1 M sodium phosphate, 0.05 M NaCl [pH 7.4]). The third received 0.15 ml of human hemoglobin (Sigma, 4 x recrystallized, 2 mg/ml) was added to each aliquot to reduce adsorptive loss of proteins and minimize the effects of possible contaminating proteases. The four samples were incubated for 6 hr: the heparitinase digest at 43°C, the others at 37°C. After removing aliquots for SDS-acrylamide gels, the samples were fractionated on 5–20% sucrose gradients as described.
by incubation with collagenase (Fig. 6b), but the possibility that the collagenase contained small amounts of other proteases has not been completely ruled out.

The samples analyzed by sedimentation (Fig. 6a–d) were also subjected to SDS-acrylamide gel electrophoresis and analyzed by fluorography (Fig. 7). The results show more than one labeled component. A very broad band of high-molecular-weight labeled material is seen on these gels that is insensitive to collagenase or chondroitinase ABC, but is eliminated by heparitinase. This material is clearly the heparan sulfate-proteoglycan. Other bands are visible that are not seen in gels of material from sucrose gradient fractions that do not contain neurite outgrowth-promoting activity, and these appear to be proteins or glycoproteins that purify with the heparan sulfate-proteoglycan. A sharp band is visible in samples digested with heparitinase. This could be the core protein of the heparan sulfate-proteoglycan or another protein (glycoprotein) whose presence was masked in undigested fractions by the broad heparan sulfate-proteoglycan band.

Possible models for this class of factors are shown in Figure 8. Figure 8A is a model of a heparan sulfate-proteoglycan that exists free in solution and is not complexed with other molecules. The data at this time do not support this model. Figure 8B is a model of the same proteoglycan associated with other molecules that are not required for activity, which resides solely in the heparan sulfate-proteoglycan. Alternatively (Fig. 8C), the factor could consist of a complex formed by a heparan sulfate-proteoglycan and other molecules, with the integrity of the complex being required for activity. Such other molecules probably would not include hyaluronic acid or other proteoglycans, since the factor is resistant to chondroitinase ABC. A protein(s) or glycoprotein(s) would be more likely. To explain why only an intact complex would be active, it is not necessary to assume that neurons must recognize more than one element of the complex. One possibility is that neurons recognize only the protein or glycoprotein component, but that the presence of a proteoglycan is necessary to anchor the complex to an appropriate substratum (e.g., polylysine, ECM). Certain molecules known to be secreted by corneal endothelial cells, such as fibronectin and laminin (Gospodarowicz et al. 1981), are plausible candidates for the nonproteoglycan portion of such a complex, but proteins with appropriate $M$,s have not been identified in the SDS-acrylamide gels of the partially purified factor.

If, as in Figure 8, A and B, a proteoglycan is the only molecule essential for promoting neurite outgrowth, the protein core of the proteoglycan must be necessary for the molecule’s function, since activity is eliminated by trypsin. Compatible with this is the observation that
suggests that the proteoglycan may be subject to denaturation that is not readily reversible; this would almost certainly involve the core protein.

cause a component of the complex is denatured by these conditions or because the complex is unable to reassemble efficiently after being dissociated.

Purified heparan sulfate would be expected to lack reactivity, since it lacks components of the complex. Exposure to $4 \text{ M} \text{ GuHCl}$ could destroy activity either because a component of the complex is denatured by these conditions or because the complex is unable to reassemble efficiently after being dissociated.

Effect of Other ECM Components on Neurite Outgrowth

Fibronectin, a glycoprotein important in cellular adhesion (Culp et al. 1979), has been reported to promote the outgrowth of neurites from fetal human sensory neurons (Baron-Van Evercooren et al. 1982) and embryonic chick retinal neurons (Akers et al. 1981), but fibronectin was not an effective promoter of neurite outgrowth by rat sympathetic neurons in our assay system (Table 5). To examine the possibility that fibronectin might be a functionally important part of the heparan sulfate-proteoglycan-associated neurite outgrowth-promoting factors, BCE CMsF-coated substrata. The antiserum does not prevent outgrowth on CMsF-coated substrata even when included in the neuronal growth medium, so laminin is not likely to be a functionally important part either of the heparan sulfate-proteoglycan-associated factors or of the surfaces of the growth cones of sympathetic neurons.

Although laminin and fibronectin do not appear to be functionally essential components of the heparan sulfate-proteoglycan-associated neurite-promoting factors, both are synthesized by many of the cell types that secrete neurite outgrowth-promoting factors, including BCE cells, RN22 cells (Palm and Furcht 1983), and PC12 cells (K. Tomaselli, unpubl.). Furthermore, both glycoproteins bind glycosaminoglycans, especially heparan sulfate (e.g., Hay 1981). Preliminary experiments using radioimmunoassays indicate that some laminin

| Concentration of laminin or fibronectin solution applied (µg/ml) | Laminin applied to PLYS-coated tissue culture plastic | Laminin applied to untreated tissue culture plastic | Fibronectin applied to PLYS-coated tissue culture plastic |
|---------------------------------------------------------------|------------------------------------------------------|---------------------------------------------------|------------------------------------------------------|
| 450                                                          | 25 ± 3.7                                             | 6 ± 0.9                                           | 1                                                    |
| 113                                                          | 25 ± 3.3                                             | 9 ± 2.1                                           |                                                      |
| 28                                                           | 26 ± 4.0                                             | 8 ± 2.0                                           |                                                      |
| 0                                                             | 2                                                   | 2                                                 |                                                      |

Solutions of purified laminin or fibronectin (0.05 ml) in Dulbecco’s PBS were applied to wells for 12 hr at 4°C. After washing of the substrata, rat sympathetic neurons were cultured upon them for 13 hr.

Data represent the mean of duplicate assays and are the percentage of neurons with neurites.
may be associated with the purified factor from BCE CMsF and may be deposited on PC12 producer of neurite outgrowth or as identifiable protein bands in SDS-acrylamide gels.

**DISCUSSION**

Evidence exists that components of the ECM have strong stimulatory effects on neurite outgrowth by rat sympathetic neurons in vitro. The properties of the neurite outgrowth-promoting factors detected in CM suggest that they are very closely related to each other and to factors described by others (Collins 1978; Dribin and Barrett 1980; Adler et al. 1981; Coughlin et al. 1981; Henderson et al. 1981). In particular, the factors investigated in this report have densities in CsCl gradients intermediate between those of carbohydrate and protein and are sensitive to digestion by heparinase and heparitinase, but not chondroitinase ABC. This suggests that each of these factors contains a heparan sulfate-proteoglycan as an element essential for activity.

Heparan sulfate-proteoglycans are ubiquitous molecules that may have a great variety of biological functions. They are widely distributed in tissues, including the brain (Toledo and Dietrich 1977), and are found on the surfaces of many cell types (Keller et al. 1978), and are present in basement membranes (Hassel et al. 1980; Kanwar and Farquhar 1979). The polysaccharide portions of heparan sulfate-proteoglycans are diverse in length, degree of sulfation, and uronic acid composition (Lindahl et al. 1977; Oldberg et al. 1979; Radha-krishnamurthy et al. 1980). Some appear to be integrally associated with cell membranes; others may bind cell-surface receptors (Kjellen et al. 1980; Hurst et al. 1981). Heparan sulfates may be a negative regulator of cell proliferation (Kraemer and Tobey 1972; Chiarugi et al. 1976; Cohn et al. 1976). They may play a role in neuronal development, since levels are much higher in developing animals than in adults (Margolis et al. 1975).

Heparan sulfate-proteoglycans may also function in the control of cell motility and adhesion, since heparan sulfate is present in the newly formed adhesion sites of fibroblasts, glioma cells, and neuroblastoma cells (e.g., Culp et al. 1979, 1980). Heparinase digestion of the cell surface prevents the normal spreading of fibroblasts on fibronectin-coated surfaces and indicates that heparan sulfate-proteoglycans are critical mediators of cell adhesion (Laterra et al. 1983). The factors described in this report may, in fact, function by increasing adhesion between the neuronal plasmalemma and the substratum.

The one neurite outgrowth-promoting factor that has been purified and studied in detail in this report appears to be a complex containing a heparan sulfate-proteoglycan and other proteins (or glycoproteins), and sediments with a high sedimentation coefficient. The properties of this factor, therefore, resemble those of the adhesion-mediating particles described by Schubert and LaCorbiere (1980a,b, 1982), which contain a mixture of glycosaminoglycans, collagen, and glycoproteins. The factor from BCE CMsF, though, appears to have a simpler composition because it contains no sulfated glycosaminoglycan that is sensitive to chondroitinase ABC. The BCE-derived factor also does not aggregate in the presence of Ca++, unlike the particles described by Schubert and LaCorbiere (1982). The other similarities between these particles, though, suggest that both may promote cell aggregation and adhesion to substrata.

The results also make it clear that not all factors in CM that promote neurite outgrowth attach efficiently enough to a polyionic substrata to be detected in the neurite outgrowth assay. Both fibronectin and laminin promote neurite outgrowth by some neuronal cell types (Akers et al. 1981; Baron-Van Evercooren et al. 1982; M. Manthorpe et al., in prep.) and can be detected immunologically in media conditioned by many of the cells used in these investigations (e.g., Palm and Furcht 1983). Yet antibodies to fibronectin (Collins 1980) or laminin (Table 7) do not diminish noticeably the outgrowth of neurites induced by CMsF-derived CM, and neurite-promoting activity has not been seen at lower densities in CsCl gradients. Thus, the standard assay for neurite outgrowth detects only a subset of those

**Table 7. Effect of Anti-laminin Antiserum on Neurite Outgrowth**

| Percent of cells with neurites | Laminin on PLYS | PC12 CMsF | BCE CMsF on PLYS |
|-------------------------------|-----------------|-----------|-----------------|
| Control (no antibody)         | 23 ± 3.5        | 22        | 85 ± 3.9        |
| Substratum treated with       |                 |           |                 |
| anti-laminin at               |                 |           |                 |
| 1:100                         | 1               | 20        | 88 ± 0.2        |
| 1:500                         | 2 ± 0.3         | —         | —               |
| 1:2500                        | 19 ± 2.5        | —         |                 |
| Substratum treated with,      |                 |           |                 |
| and neurons grown in,         |                 |           |                 |
| anti-laminin at               |                 |           |                 |
| 1:100                         | 1               | 19        | 87 ± 1.3        |
| 1:500                         | 1 ± 0.5         | —         | —               |
| 1:2500                        | 12 ± 3.9        | 23        |                 |

Laminin was applied at 100 μg/ml in PBS. CMsF was applied undiluted. Data represent the mean of duplicate assays. Assay time: 13 hr.
molecules that may play a role in vivo. Only polyanionic molecules can be expected to attach efficiently enough in the presence of competing molecules to be detected as inducers of neurite outgrowth in unfractionated CM. Fibronectin and laminin can be studied in isolation only because they are major, well-characterized glycoproteins of the ECM. Clearly, other molecules that are important may exist and require more sophisticated assays for detection.

There is some evidence for selectivity in the response of different classes of neurons to the factors discussed in this report. Laminin markedly induces neurite outgrowth by both central and peripheral neurons (M. Manthorpe et al., in prep.), but the heparan sulfate-proteoglycan-associated factors have measurable effects in our assay only on neurons that extend processes into the periphery (Lander et al. 1982). Fibronectin appears to have measurable effects on an even smaller subset of neurons. These results are tentative because the heparan sulfate-proteoglycan-associated factors, laminin, and fibronectin differ in potency in this assay.

Possible Role of Factors In Vivo

Antibodies to neurite outgrowth-promoting factors derived from mouse heart, rat pheochromocytoma, and rat Schwannoma have been made that block the response of neurons to these factors and should be useful in future studies in vivo (Coughlin and Kessler 1982; M. Manthorpe, pers. comm.; Matthew and Patterson, this volume). Laminin is produced by both Schwannoma cells and astrocytes in vitro (Liesi et al. 1983; Palm and Furcht 1983). Laminin is a prominent constituent of the endoneurium of peripheral nerves in vivo (Palm and Furcht 1983) and hence is appropriately located to be important in the regeneration and myelination of peripheral nerves. Laminin is not visible at early times during regeneration of newt limbs and hence seems unlikely to guide the pioneer fibers into the blastema of these limbs (Gulati et al. 1983). Fibronectin is present at early times in regenerating blastema and hence could be an important component of the substratum on which pioneer fibers enter peripheral tissues (Gulati et al. 1983). Although fibronectin is not a prominent constituent of the endoneurium of peripheral nerves, enough is present for it also to be important in promoting axon growth during regeneration. The role of laminin in CNS development is not clear. Even though laminin is produced by embryonic astrocytes in culture (Liesi et al. 1983), it has been detected in embryonic rat brain only in association with capillaries (K. Valentino, unpubl.).

The synthesis of neurite outgrowth-promoting factors may be regulated in targets by innervating neurons. Denervation of the chick limb results in a striking increase in neurite outgrowth-promoting activity detected in extracts of the limb (Henderson et al. 1983). The growth state of neurons also may correlate with the level of these factors. Denervation of the sympathetic ganglion results in increased levels of antigenic substances that are cross-reactive with the neurite outgrowth factor in the sympathetic ganglion (Matthew and Patterson, this volume). The responsiveness of neurons to neurite outgrowth-promoting factors also appears to be regulated by their growth state. Chick ciliary neurons have been shown to lose responsiveness to these factors at approximately the same time as they establish peripheral connections (Collins and Lee 1982). Responsiveness is not regained at later times in vivo in normal animals, but can be regained by culture in vitro. Similar experiments with retinal ganglion cells have shown that prior denervation increases the ability of neurons to extend processes rapidly in vitro (Landretti and Agranoff 1979).

Adhesive factors that promote neurite outgrowth may have additional roles in vivo. Polycationic substrate-adherent factors are required for the survival of chick motor neurons and a class of chick sensory neurons in vitro (Bennett et al. 1980; Edgar and Thoenen 1982). Sensory neurons are able to survive in the presence of much lower concentrations of NGF on a CM-treated substratum than on more conventional substrata (Edgar and Thoenen 1982). Thus, adhesive factors may be important in both modulating neuronal survival and responsiveness to trophic factors during development.

Finally, it is clear that the major components of the ECM described in this report may be important permissive constituents of the substratum that neurites follow in vivo, but their distributions do not explain adequately the precision with which early pathways are established by pioneer fibers or the specificity for different pathways exhibited by nerve fibers during later development. There may be molecules, precisely placed in the extracellular environment, which guide growth cones along the many stereotyped pathways they follow, but if such molecules are there, we must keep looking for them.

Note Added in Proof

We have recently completed the purification of the neurite outgrowth-promoting factor from BCE cells. Our results confirm that the factor consists of several proteins found associated with a heparan sulfate proteoglycan. Preliminary evidence suggests that the protein portion of the factor interacts with neurons, and the proteoglycan portion mediates the binding of the factor to the substratum.

ACKNOWLEDGMENTS

We thank Denis Gospodarowicz and Dennis Fujii for help with this work. This research was supported by grants to A.D.L. from the National Institutes of Health, and to L.F.R. from the National Science Foundation, National Institutes of Health, March of Dimes-Birth Defects Foundation, Muscular Dystrophy Association, and Wills Foundation.
REFERENCES

ADLER, R., M. MANTHORPE, S. SKAPER, and S. VARON. 1981. Polyornithine-attached neurite-promoting factors (PNPFs). Culture sources and responsive neurons. Brain Res. 206: 129.

AKERS, R.M., D.R. MOSHER, and J. LILLEN, 1981. Promotion of motoneuronal outgrowth by substrate-bound fibronectin. Dev. Biol. 86: 179.

BARON-VAN EVERCOOREN, A., H.K. LEIKIN, S. OHNO, P. MARANGOS, J.P. SCHWARTZ, and M.E. DUBOIS-DALCQ, 1982. Nerve growth factor, laminin and fibronectin promote nerve growth in human fetal sensory ganglia cultures. J. Neurosci. Res. 8: 179.

BENNETT, M.R., K. LAI, and V. NURCOME. 1980. Characterization of embryonic motoneurons in vitro: Their survival is dependent on skeletal muscle. Brain Res. 20: 537.

BONNER, W.M. and R.A. LASKEY. 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46: 83.

BRANFORD-WHITE, C.J. 1980. Sulfated glycoproteins in synaptosomes. Neurosci. Lett. 16: 307.

CIBARUCHI, V.P. and S. VANNUCCI. 1976. Surface heparan sulfate as a control element in eukaryotic cells: A working model. J. Theor. Biol. 61: 459.

COHN, R.H., J.-J. CASSIMAN, and M.H. BERNFIELD. 1976. Relationship of transformation, cell density and growth control to the cellular distribution of newly synthesized glycoaminoglycan. J. Cell Biol. 71: 280.

COWINS, F. 1978. Induction of neurite outgrowth by a conditioned-medium factor bound to the culture substrate. Proc. Natl. Acad. Sci. 75: 5210. 1980. Neurite outgrowth induced by the substrate attached material from nonneuronal cells. Dev. Biol. 79: 247.

DIETZ, K., M. LEE. 1982. A reversible developmental change in the ability of clonal ganglion neurons to extend neurites. J. Neurosci. 2: 424.

DOUGHERTY, M.D. and J.A. KESSLER. 1982. Antiserum to a new neuronal growth factor: Effects on neurite outgrowth. J. Neurosci. Res. 8: 298.

DOUGHERTY, M.D., E.M. BLOOM, and I.B. BLACK. 1981. Characterization of a neuronal growth factor from mouse heart-cell-conditioned medium. Dev. Biol. 82: 36.

DU PONT., L.A. 1983. Adhesion sites of spinal cord cells: Biochemical composition. Biochemistry 19: 5899.

EDGAR, D. and H. THOENEN. 1982. Modulation of NGF-induced survival of chick sympathetic neurons by contact with a conditioned medium factor bound to the culture substrate. Dev. Brain Res. 5: 89.

FUJII, D.K., S.L. MASSOGGLIA, N. SAVION, and D. GOSPODAROWICZ. 1982. Neurite outgrowth and protein synthesis by PC12 cells of a function of substrate and nerve growth factor. J. Neurosci. 2: 1157.

GOSPODAROWICZ, D., G. GREENBURG, J.M. FORDART, and N. SAVION. 1981. The production and localization of laminin in cultured vascular and corneal endothelial cells. J. Cell. Physiol. 107: 173.

GULATI, A.K., A.A. ZALEWSKI, and A.H. REDD. 1983. An immunofluorescent study of the distribution of fibronectin and laminin during unit regeneration in the adult newt. Dev. Biol. 96: 355.

HASSEL, J.R., P.G. ROBEY, H.-J. BARRACH, J. WILCZEK, S.I. RENNARD, and G.R. MARTIN. 1980. Isolation of a heparan sulfate-containing proteoglycan from basement membrane. Proc. Natl. Acad. Sci. 77: 4494.

HATTEN, M.E., M.B. FURIE, and D.B. RIFKIND. 1982. Binding of developing mouse cerebellar cells to fibronectin: A possible mechanism for the formation of the external granular layer. J. Neurosci. 2: 1195.

HAY, E. 1981. Extracellular matrix. J. Cell Biol. 91: 205S.

HENDERSON, C.E., M. HUCHET, and J.-P. CHANGHEUX. 1981. Neurite outgrowth from embryonic chicken spinal neurons is promoted by media conditioned by muscle cells. Proc. Natl. Acad. Sci. 78: 2625.

HURST, R.E., R.T. PARMLEY, S. NAKAMURA, S. WEST, and F.R. DENYS. 1981. Heparan sulfate of AH-130 ascites hepatoma cells: A cell-surface glycosaminoglycan not displaced by heparin. J. Histochem. Cytochem. 29: 731.

KANWAR, Y.S. and M.G. FARQUHAR. 1979. Isolation of glycosaminoglycans (heparan sulfate) from glomerular basement membranes. Proc. Natl. Acad. Sci. 76: 4493.

KATZ, M.J. and J.M. LASKER. 1979. Substrate pathways which guide growing axons in Xenopus embryos. J. Comp. Neurol. 183: 817.

KELLER, K.L., C.B. UNDERHILL, and J.M. KELLER. 1978. Multiple types of cell surface heparan sulfate are produced by primary cultures of embryonic mouse cells. Biochem. Biophys. Acta 540: 431.

KELLEN, L.A. OLDEN, and M. HÖÖK. 1980. Cell surface heparan sulfate. Mechanisms of proteoglycan-cell association. J. Biol. Chem. 255: 10407.

KRAEMER, P.M. and R.A. TOREY. 1972. Cell cycle dependent desquamation of heparan sulfate from the cell surface. J. Cell Biol. 55: 713.

LAEMMLI, U.K. 1970. Cleavage of structural proteins during the assembly of bacteriophage T4. Nature 227: 680.

LANDER, A.D., D.K. FUJII, D. GOSPODAROWICZ, and L.F. REICHARDT. 1982. Characterization of a factor that promotes neurite outgrowth: Evidence linking activity to a heparan sulfate proteoglycan. J. Cell Biol. 94: 574.

LANDRETTI, G.E. and B.W. AGRAFONOFF. 1979. Explant culture of adult goldfish retina: A model for the study of CNS regeneration. Brain Res. 161: 39.

LATTARA, J., J.E. GILBERT, and E. L. CULP. 1983. Cell surface heparan sulfate mediates some adhesive responses to glycosaminoglycan-binding matrices, including fibronectin. J. Cell Biol. 96: 112.

LETORNEAU, P.C. 1975. Cell to substratum adhesion and guidance of axonal elongation. Dev. Biol. 44: 92.

LEVINTHAL, F., E. MACAGNO, and C. LEVINTHAL. 1976. Anatomy and development of identified cells in isogenic organisms. Cold Spring Harbor Symp. Quant. Biol. 40: 321.

LIES, P., D. DAHL, and A. VAHERI. 1983. Laminin is produced by early rat astrocytes in primary culture. J. Cell Biol. 96: 920.

LINDHALL, U., M. HÖÖK, G. BACKSTRÖM, I. JACOBSSON, J. RISENFELT, A. MALMSTROM, L. RODEN, and D.S. FEINGOLD. 1977. Structure and biosynthesis of heparin-like polysaccharides. Fed. Proc. 36: 19.

MARGOLIS, R.U., R.K. MARGOLIS, L.B. CHANGE, and C. PRETI. 1975. Glycosaminoglycans of brain during development. Biochemistry 14: 85.

MCPEETERS, M. and L.M. OUKEN. 1980. Identification and isolation in vitro of presumptive motoneurons marked by retrograde transport of a new fluorescent tracer. Soc. Neurosci. Abstr. 6: 733.

OLDEN, A., L. KELLEN, and M. HÖÖK. 1979. Cell surface heparan sulfate. Isolation and characterization of a proteoglycan from rat liver membranes. J. Biol. Chem. 254: 8505.

PALM, S.L. and L.T. FURCHT. 1983. Production of laminin...
and fibronectin by Schwannoma cells: Cell-protein interactions in vitro and protein localization in peripheral nerve in vivo. J. Cell Biol. 96: 1218.

Radhakrishnamurthy, B., F. Smart, E.R. Dalferes, Jr., and G.S. Berenson. 1980. Isolation and characterization of proteoglycans from bovine lung. J. Biol. Chem. 255: 7575.

Rakic, P. 1974. Intrinsic and extrinsic factors influencing the shape of neurons and their assembly into neuronal circuits. In Frontiers in neurology and neuroscience research (ed. P. Seeman and G.M. Brown), p. 112. Toronto University Press, Toronto, Canada.

Rovasio, J.B., A. Delouvee, K.M. Yamada, R. Timpl, and J.P. Thiery. 1983. Neural crest migration: Requirements for exogenous fibronectin and high cell density. J. Cell Biol. 96: 462.

Schubert, D. and M. Lacorbiere. 1980a. Altered collagen and glycoaminoglycan secretion by a skeletal muscle myoblast variant. J. Biol. Chem. 255: 11567.

—. 1980b. A role of secreted glycoaminoglycans in cell-substratum adhesion. J. Biol. Chem. 255: 11564.

—. 1982. Properties of extracellular adhesion-mediating particles in myoblast clone and its adhesion-deficient variant. J. Cell Biol. 94: 108.

Stevens, R.L. and K.F. Austen. 1982. Effect of p-nitrophenyl-beta-D-xyloside on proteoglycan and glycosaminoglycan biosynthesis in rat serosal mast cell cultures. J. Biol. Chem. 257: 253.

Thiery, J.P., J.L. Duband, and A. Delouvee. 1982. Pathways and mechanisms of avian trunk neural crest cell migration and localization. Dev. Biol. 93: 324.

Toledo, O.M.S. and C.P. Dietrich. 1977. The specific distribution of sulfated mucopolysaccharides in mammals. Biochim. Biophys. Acta 498: 114.