Attachment to an Endogenous Laminin-like Protein Initiates Sprouting by Leech Neurons

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Abstract. Leech neurons in culture sprout rapidly when attached to extracts from connective tissue surrounding the nervous system. Laminin-like molecules that promote sprouting have now been isolated from this extracellular matrix. Two mAbs have been prepared that react on immunoblots with a ≈220- and a ≈340-kD polypeptide, respectively. These antibodies have been used to purify molecules with cross-shaped structures in the electron microscope. The molecules, of $\approx 10^3$ kD on nonreducing SDS gels, have subunits of ≈340, 220, and 160–180 kD. Attachment to the laminin-like molecules was sufficient to initiate sprouting by single isolated leech neurons in defined medium. This demonstrates directly a function for a laminin-related invertebrate protein. The mAbs directed against the ≈220-kD chains of the laminin-like leech molecule labeled basement membrane extracellular matrix in leech ganglia and nerves. A polyclonal antiserum against the ≈220-kD polypeptide inhibited neurite outgrowth. Vertebrate laminin did not mediate the sprouting of leech neurons; similarly, the leech molecule was an inert substrate for vertebrate neurons. Although some traits of structure, function, and distribution are conserved between vertebrate laminin and the invertebrate molecule, our results suggest that the functional domains differ.

NEURONS from the central nervous system of the leech, *Hirudo medicinalis*, grow to regenerate after injury and form synapses in the animal as well as in tissue culture (for reviews see references 26, 28). From this simple nervous system, single cells can be isolated and cultured for several weeks (7). Leech neurons are especially useful because they can grow neurites on certain substrates and form synapses in defined media (1, 3, 7). This allows us to dissect the mechanism of regeneration into distinct steps that can be studied separately (5).

To isolate physiologically relevant molecules from leech tissues, functional assays were established for single cultured neurons. Focusing on the mechanism of neurite outgrowth, it was found that only a few substrates, including leech extracellular matrix (ECM),[1] mediate sprouting by identified leech neurons in culture; no other cells or soluble growth factors are needed (3). We have recently demonstrated that neurite-promoting activity is concentrated in high molecular mass fractions isolated from leech ECM (3).

Substrates coated with the vertebrate basement membrane protein, laminin, are known to promote neurite formation by vertebrate neurons (8). Recently, molecules that are related to laminin have been described in some invertebrates (14, 25). We therefore suspected that a laminin-like component from our leech ECM extracts (5) could be at least in part responsible for the observed activity. In this paper, we report the isolation of laminin-like leech molecules using mAbs. This enabled us to determine their neurite-promoting activity. We asked whether attachment to these molecules was sufficient to initiate the sprouting of isolated leech neurons, and whether they were the major or merely one of several neurite-promoting components in leech ECM. We also tested whether vertebrate laminin and the leech molecules would promote the sprouting of neurons across phyla. Finally, the mAb allowed us to study the distribution of laminin-like molecules within the leech central nervous system.

Materials and Methods

Preparation of ECM Extracts

Ganglion capsule ECM was prepared from leeches (*H. medicinalis*; Ricarimpex, Audenge, France) as described (3). Briefly, chains of ganglia that make up the central nervous system of adult leeches were dissected in Ringer solution (26) and transferred to 10 mM Tris-HCl buffer (pH 7.4) containing 2% Triton X-100 (E. Merck GmbH, Darmstadt, Federal Republic of Germany) and protease inhibitors (3). Ganglia were crushed first with forceps and then with a Dounce homogenizer and extracted overnight at 4°C in an excess of buffer. The insoluble material was sedimented at 10,000 *g* (5 min) and washed three times with distilled water. The pellet was extracted for 24 h at 4°C with 4 M urea (100 μl per ganglion chain) as described (3), or alternatively with 150 mM NaCl, 10 mM EDTA, 10 mM Tris-HCl, pH 7.4 (TES buffer; 50 μl per ganglion chain). No protease inhibitors were included in the extraction buffers because of toxic effects in subsequent bioassays. The amount of protein extracted from one ganglion chain

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1. *Abbreviations used in this paper*: ECM, extracellular matrix; EHS, Englebreth-Holm swarm; NGF, nerve growth factor; TES buffer, Tris-HCl/EDTA/saline.

was 15–25 μg (EDTA extract) or ≈50 μg (urea extract) as estimated from A_{280} assuming an average absorption coefficient of A_{280} = 1,000 cm^2 g^{-1}.

Isolation of mAbs 203 and 206

Urea extracts from leech ganglion capsules (3) were dialyzed against 150 mM NaCl, 20 mM sodium phosphate, pH 7.3 (PBS). BALB/c mice (Madörin AG Kleintierfarm, Füllinsdorf, Switzerland) were injected intraperitoneally with 100 μl extract (50 μg antigen) which was suspended in 100 μl complete Freund's adjuvant (Gibco Laboratories, Grand Island, NY). Mice were boosted after 1 mo with 100 μl dialyzed extract injected into the tail vein. This was repeated 2 d later, and after two more days the spleens were removed. Lymphocytes isolated from each spleen were fused (13) with 5×10^7 PAI myeloma cells (33). Resulting hybridoma lines were grown as described (16). Culture supernatants were screened by an ELISA (12) on microtiter wells (No. 3911; Falcon Labware, Oxnard, CA) coated with 200-fold diluted antigen extract. 150 hybridoma lines from two fusions yielded 0.5–>2.0 OD (488 nm) above background in this assay; these were rescreened on whole mounts of fixed and permeabilized leech ganglia (1). The supernatants of 70 lines clearly stained capsule ECM. These were tested by immunoblotting for their reactivity with specific polypeptides found in neurite-promoting ECM fractions (3). Selected hybridoma lines were cloned by serial dilution (16) and after rescreening, the clones were injected into pristane-primed (Aldrich Chemical Co., Milwaukee, WI) BALB/c mice to generate ascites tumors. Mice were killed and ascites fluid was collected 10 d later. Immunoglobulins were precipitated with 50% saturated ammonium sulfate, dissolved in the original volume, dialyzed against PBS, Millipore filtered, and stored at −70°C. Single immunoglobulin batches obtained from clones 203-1B5 and 206-1H1 were used. On SDS-polyacrylamide gels, mAbs 203 and 206 revealed one heavy chain (≈50 kD) and one light chain; they are IgGs (not shown). Culture supernatants from all screened subclones of hybridomas 203 and 206 gave identical staining patterns by immunofluorescence and immunoblotting when compared to the original lines, thus confirming their monoclonal origin.

Other Immunoreagents and Specificity Controls

A polyclonal antiserum (No. 99) against a ≈220-kD polypeptide from neurite-promoting extracts was generated as follows. Ganglion capsule urea extract (3) was run on a preparative SDS-polyacrylamide gel under reducing conditions. The ≈220-kD region was cut out, washed briefly with distilled water, and minced in a Dounce homogenizer. Two rabbits were injected subcutaneously, each with material obtained from 10 ganglion chains suspended in complete Freund's adjuvant. A boost was made with the same amount of antigen in incomplete adjuvant after 1 mo, and the rabbits were killed and the serum was collected 1 wk later.

Several control experiments were done to ascertain the target specificity of mAbs 203 and 206 and antiserum No. 99. To account for effects of nonspecific IgG binding, the unrelated mAb M1 (against chick tenascin [4]) and rabbit preimmune serum were used. These gave very low or no background in all assays described. We also used other immunoreagents as positive controls. mAb 65 reacted broadly with many polypeptides present in leech ECM extract. Antiserum No. 101 was prepared against and strongly bound to the ≈70-kD bands present in ECM extracts, but did not inhibit sprouting (data not shown).

SDS Gel Electrophoresis and Immunoblotting

Extract samples and column fractions were analyzed by SDS-PAGE according to Laemmli (20) with reagents from Bio-Rad Laboratories (Richmond, CA). Samples were run with or without reduction by 0.1 M dithiothreitol (DTT; Sigma Chemical Co., St. Louis, MO) on 7.5% straight or 3–15% gradient acrylamide gels. Two-dimensional gels (nonreduced vs. reduced) were run by cutting gel lanes from a gradient gel run under nonreducing conditions, turning them by 90°, and placing them on top of a second gel. Lanes were overlaid with sample buffer containing DTT and run in the second dimension. All gels were stained with 0.2% Coomassie Brilliant Blue (Bio-Rad Laboratories). Molecular mass standards were obtained from Sigma Chemical Co. (6-H); calibrations ($\times 10^{-3}$ kD) are indicated at left of each figure.

Immunoblotting was done as described (36). Briefly, polypeptides were transferred from unfixed SDS gels onto nitrocellulose paper (BA-85; Schleicher & Schuell, Inc., Dassel, FRG). Free protein binding sites were blocked with PBS containing 10% horse serum (North American Biologicals, Miami, FL) and the sheets were incubated serially (each step for 1 h at 37°C) with mAb or antiserum (diluted 1:200), goat anti-mouse or anti-rabbit Ig (diluted 1:100, Cappel Laboratories, Cochranville, PA), and mouse or rabbit peroxidase-antiperoxidase complex (diluted 1;3,000; Jackson Immunoresearch Laboratories, Avondale, PA). PBS-horse serum was used to dilute the reagents and to wash between the incubations. The blots were developed with 4-chloro-1-naphthol (E. Merck GmbH) as published (18).

Immunoaffinity Purification

About 3 mg mAb 203 or 206, respectively (1 ml of the ammonium sulfate cut of ascites fluid), were coupled to 1 g CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) as recommended by the manufacturer. Typically, 0.5 ml EDTA extract was applied to a column containing 0.5 ml Sepharose coupled with antibody 203. The column was washed with TES buffer, then with a mixed detergent buffer (0.3 M NaCl, 0.1% SDS, 0.05% Triton X-100, 10 mM Tris-HCl, pH 8.4) followed by TES buffer. Bound antigen was eluted either with 0.1 M triethylamine, pH 11.0, or with 4 M urea containing 10 mM EDTA and 10 mM Tris-HCl, pH 7.4. Both types of eluates looked identical upon SDS gel electrophoresis. Neurite-promoting activity could only be restored from the urea eluates after dialysis against TES buffer. Material which did not bind to the antibody 203 column was reapplied to a 0.5-ml column of antibody 206-Sepharose. In this case, adsorbed antigen was released by the mixed detergent buffer (see above). Protein concentration in the column eluates was estimated by comparing Coomassie Blue-stained gel bands to a mouse laminin standard (reference 31; gift of Dr. M. Paulsson, Biocenter, University of Basel, Basel, Switzerland).

Electron Microscopy of ECM Fractions

Fractions from antibody affinity columns were examined by EM using the rotary shadowing technique as described (32). Samples were dialyzed against 0.2 M ammonium bicarbonate ('AnalaR'-grade; BDH Chemicals Ltd., Poole, England), pH 7.9, mixed with an equal volume of 87% glycerol (E. Merck GmbH) and sprayed onto freshly cleaved mica sheets (11). Samples were dried in vacuo and rotary shadowed (120 rpm) using an electron beam evaporator in a Balzers BAF 300 or BAE 307 D, respectively, with platinum-carbon at 5-7° to a thickness of 0.6–0.7 nm followed by carbon at 90°. The replicas were floated on distilled water, picked up on 400-mesh copper grids, and examined with an electron microscope (model 109; Carl Zeiss GmbH, Oberkochen, FRG) (80 kV) at a magnification of ~50,000. Calibration was performed regularly with negatively stained catalase. The dimensions of all structures were measured at a total magnification of ~500,000 using a graphics tablet.

Immunohistochemistry

For whole-mount preparations, leech ganglia were fixed with 4% paraformaldehyde and permeabilized with 0.4% Triton X-100 as described (1). For sectioning, unfixed ganglia were embedded in Tissue Tek (Miles Laboratories, Inc., Elkhart, IN) and frozen on dry ice. 10-μm sections prepared on a cryostat microtome (model 2,000; Reichert Jung, Vienna, Austria) were mounted on gelatin-coated slides and routinely stained with ascites or antiserum diluted 1:200 with PBS containing 10% horse serum (KC Biological Inc., Lenexa, KS), followed by rhodamine-conjugated second antibodies (Cappel Laboratories) (4).

Neuronal Cell Cultures

Retzius and Anterior Pagoda neurons were isolated from leech (*H. medicinalis*; Ricarimpex) ganglia and cultured at 20°C as described (3, 7) in either standard medium containing 2% FCS (KC Biological Inc.) (7) or in protein-free Leibovitz-15 medium (Gibco Laboratories) supplemented with 30 mM glucose and 2 mM L-glutamine. For antibody inhibition experiments, serum-containing media were supplemented with heat-inactivated (56°C, 30 min) antiserum No. 99 or preimmune serum at a dilution of 1:50. Dorsal root ganglion neurons were isolated by Dr. H. Vedder (Biocenter, University of Basel) from newborn rats (Madörin AG Kleintierfarm) as described (2) and cultured at 37°C and 5% CO_2 in Ham's F12/DME (1:1) containing N2 supplement (2) with 20 ng/ml β-nerve growth factor (NGF) (8). This NGF concentration is high enough to ensure neuronal survival for at least 2 d, but induces very little neurite outgrowth on polylysine-coated control dishes (8), thus allowing detection of substrate-mediated neurite outgrowth by the vertebrate neurons. Cell culture microwell dishes (No. 3034;

Falcon Labware) were coated with leech ECM fractions or 100 μg/ml mouse Englebreth-Holm swarm (EHS) tumor laminin-nidogen complex (reference 29; gift of Dr. M. Paulsson, Biocenter, University of Basel) diluted in TES buffer for 2 h at room temperature (5 μl per well). After washing the dishes three times with sterile water, dishes were ready for use.

Results

Leech ECM Extract Contains Molecules with a Subunit Composition Similar to Mouse Laminin

When samples of EDTA extract from leech ganglion capsules were run on SDS-acrylamide gradient gels under nonreducing conditions, two separate components of ≈10^3 kD were detected that roughly comigrated with nonreduced mouse laminin (Fig. 1, *a* and *b*). Bands usually seen after reduction (i.e., ≈350- and 220-kD polypeptides as well as minor components of ≈180 and 160 kD (Fig. 1 *d*), were lacking. The region from a nonreducing gel including the two ≈10^3 kD components was run in the second dimension on a reducing gel (Fig. 1 *e*). The more slowly migrating band of ≈10^3 kD dissolved into two spots of ≈340 and 220 kD, whereas the more rapidly migrating band was split into polypeptides of ≈340, 180, and 160 kD; a trace at ≈220 kD was visible as well. Thus, the electrophoretic components of ≈10^3 kD consist of disulfide-linked subunits similar in size as those of vertebrate laminin (Fig. 1 *c*).

mAbs 203 and 206 Specifically Bind to Laminin-like Molecules from Leech ECM

Polyclonal antisera against vertebrate laminin did not cross-react with leech ECM (3, 5). To purify and characterize leech components that resemble vertebrate laminin in their subunit composition, we generated a hybridoma library against urea extract of detergent-washed leech ganglion capsules. From this library we selected cell lines 203 and 206, which secrete IgGs reacting on immunoblots solely with a ≈220- and a ≈340-kD polypeptide, respectively, found in ECM extracts (Fig. 2. *b* and *c*).

Antibodies 203 and 206 were bound to cyanogen bromide-activated Sepharose and used as an affinity matrix. When EDTA extracts of leech ganglion capsules (Fig. 3, *a* and *e*) were applied to an antibody 203 column, most of the ≈220-kD and part of the ≈340-kD polypeptide was specifically retained. The two bands were eluted together at high pH or with 4 M urea (Fig. 3 *f*). Upon electrophoresis of these eluates under nonreducing conditions, an ≈10^3 kD component could be detected, indicating that the two polypeptides retained on the column are disulfide linked (Fig. 3 *b*). Comparing the eluate with the applied material, it was clear that, of the two ≈10^3 kD components visible in EDTA extract, the more slowly migrating was preferentially retained on the antibody 203 column (cf. Fig. 3, *a* and *b*). This fits well with the result that the "slow" ≈10^3 kD molecule contains most of the ≈220-kD subunits (Fig. 1 *e*) carrying the epitope of antibody 203 (Fig. 2 *b*). Eluates from the antibody 203 column were also analyzed in the electron microscope after

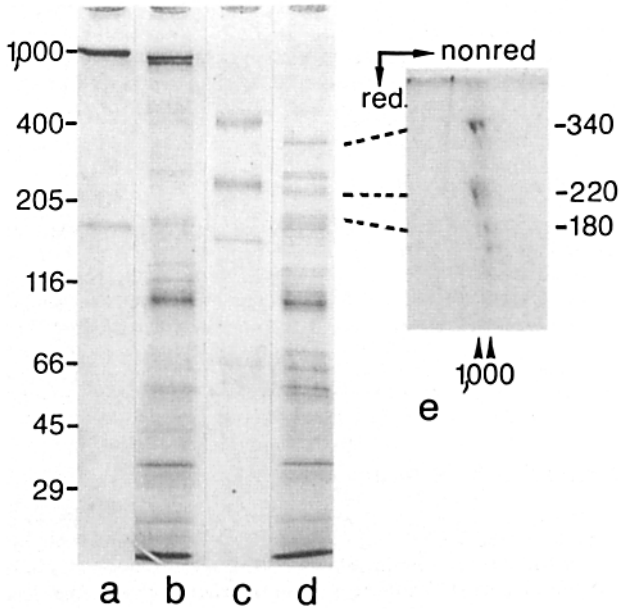

Figure 1. Analysis of neurite-promoting leech ECM extract on an SDS-polyacrylamide (3-15% gradient) gel with and without reduction; comparison to mouse EHS laminin. Mouse laminin/nidogen complex (3 μg; lanes *a* and *c*) was run next to leech ganglion capsule EDTA extract (30 μl; lanes *b* and *d*) under nonreducing (lanes *a* and *b*) and reducing (lanes *c* and *d*) conditions, respectively. The two nonreduced bands of ≈10^3 kD in EDTA extract (*b*) that comigrate with intact mouse laminin (*a*) were run on a 7.5% gel in the second dimension after reduction (*e*); the more slowly migrating band is at left.

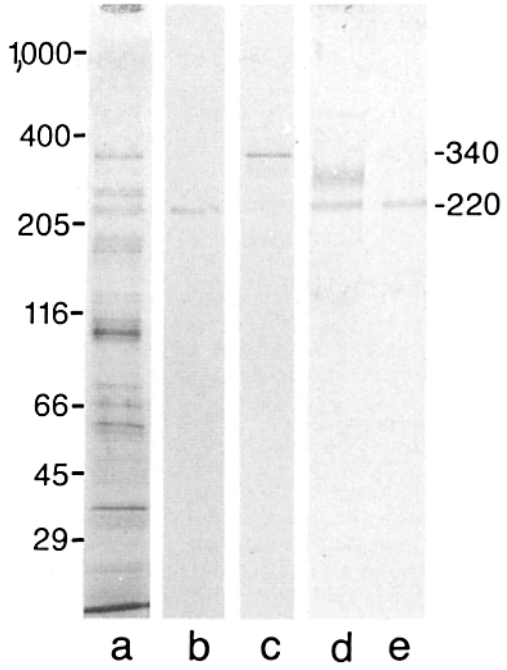

Figure 2. Immunoblotting of neurite-promoting leech ECM fractions with mAbs 203 (lane *b*) and 206 (lanes *c*) and polyclonal antiserum No. 99 (lanes *d* and *e*). (Lane *a*) Coomassie Blue-stained SDS-polyacrylamide (3-15%) gel of ganglion capsule EDTA extract that was transferred to nitrocellulose to be developed with antibody 203 (lane *b*), 206 (lane *c*), and antiserum No. 99 (lane *d*) as described in Materials and Methods. (*e*) Antiserum No. 99 bound to a blot of partially purified (on DEAE-cellulose [3]) neurite-promoting fraction.

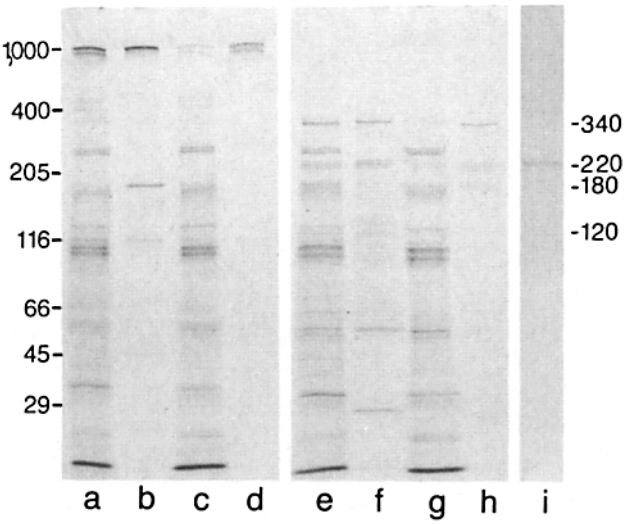

Figure 3. Immunoaffinity purification of antigen reacting with mAb 203 and 206. Ganglion capsule EDTA extract (lanes *a* and *e*) was applied to Sepharose coupled with antibody 203, and bound protein (lanes *b* and *f*) was eluted by 0.1 M triethylamine. Unbound material was reapplied to an antibody 206–Sepharose column, the flow-through was collected (lanes *c* and *g*), and bound antigen (lanes *d* and *h*) was released with mixed detergent buffer as described in Materials and Methods. Samples were run on a 3–15% gradient polyacrylamide-SDS gel stained with Coomassie Blue. Samples were applied before (lanes *a–d*) or after (lanes *e–h*) reduction with DTT. 30 μl (lanes *a* and *e*) or 60 μl (lanes *b–d* and *f–h*) were loaded per lane. The bands at ≈150 kD in *b* and at ≈50 and 25 kD in *f* are not present in EDTA extract; they are a contaminant (IgG) originating from the antibody 203 column. Other bands seen in lanes *b*, *d*, *f*, and *h* are components specifically retained by antibody 203– and 206–Sepharose, respectively. Lane *i* shows an immunoblot of material eluted from antibody 206–Sepharose, electrophoresed after reduction, and developed with antibody 203.

rotary shadowing. The only large molecules found in such fractions had the typical cross-shaped, laminin-like appearance (Fig. 4) described below; six-armed structures typical for tenascin (37) or rod-shaped molecules resembling vertebrate collagens (35), which were found in crude extract (24a), could not be detected in the electron microscope after extensive searching. From these results, we conclude that antibody 203 recognizes a small subunit of a leech molecule which has a subunit composition and domain structure similar to that of vertebrate laminin.

Material not retained by the antibody 203 column was reapplied to Sepharose coupled with antibody 206. In this case, bound antigen consisted of a mixture of both ≈10^3 kD components (Fig. 3 *d*), which upon reduction was dissolved into polypeptides of ≈340, 220, and 160–180 kD (Fig. 3 *h*). Again, this finding is in accordance with the fact that both ≈10^3 kD components contain ≈340-kD subunits (Fig. 1 *e*) which carry the epitope of antibody 206 (Fig. 2 *c*). The mixture of ≈10^3 kD components eluted from the antibody 206 column was analyzed in the electron microscope, and again only cross-shaped, laminin-like molecules were found which could not be distinguished from antibody 203 affinity-purified material (not shown). Taken together, these results strongly suggest that both ≈10^3 kD components are laminin-like molecules with a common large subunit to which antibody 206 binds.

Direct proof that the epitopes of antibodies 203 and 206 can indeed reside on different subunits of the same molecule was obtained as follows: EDTA extract was passed over antibody 206–Sepharose, and bound material was eluted, electrophoresed after reduction, and blotted to nitrocellulose. Staining of the blot with antibody 203 (Fig. 3 *i*) revealed the presence of a ≈220-kD subunit in molecules which had bound to antibody 206 via the ≈340-kD polypeptide.

Whereas the flow-through from an antibody 203 column still contained most or all of the rapidly migrating ≈10^3 kD component (not shown), ECM extract passed over both antibody columns was very much depleted from the two ≈10^3 kD bands; otherwise, the electrophoretic pattern looked identical to the applied material (cf. Fig. 3, *a*, *c*, *e*, and *g*).

Structure of the Laminin-like Leech Molecules

The eluates from the antibody 203 (Fig. 3, *b* and *f*) and 206 (Fig. 3, *d* and *h*) columns contained almost exclusively cross-shaped molecules when analyzed in the electron microscope (Fig. 4, *top*). These have all characteristic features reported for laminin from a mouse tumor (10, 11). They consist of one long arm and three shorter arms each bearing terminal globules, and additional globules near the middle of the short arms (Fig. 4, *bottom*). The globule of the long arm is significantly thicker than those of the short arms (Fig. 4). The length of the long arm measured from the center of the cross up to the middle of the terminal globule is 94 nm (SEM 5 nm, n = 102); i.e., it is ~15 nm longer than reported for mouse tumor laminin (11). For the majority of molecules, the length of each short arm is 36 nm (SEM 4 nm, n = 102), which is identical to the corresponding value of mouse tumor laminin (10). 40% of the leech molecules have one elongated short arm of 53 nm (SEM 4 nm, n = 44). About 20% of the laminin-like structures have an additional extension with a globule attached to one short arm. This might be a nidogen-like molecule (29) and might correspond to the minor ≈120-kD band specifically retained by the antibody columns (Fig. 3, *b* and *f*).

Distribution of Laminin-like Molecules in the Leech Central Nervous System

Cryosections through leech segmental ganglia were labeled by immunofluorescence with antibodies 203 and 206. The staining patterns coincided very well with the distribution of basement membrane–like material within the leech central nervous system (6). An intensely stained sharp layer on the outer surface of the outer capsule marked the location of endothelial basement membrane (Fig. 5 *a* and *b*). Parallel-labeled layers were seen within the capsule matrix, giving it a laminated appearance. The interface between the capsule matrix and the neuronal giant packets (i.e., the surface of the giant package glial cell) was more fuzzily stained. Labeled granular material partially outlined the surfaces of the neuronal cell bodies. The periphery of the inner capsule (which is located between the neuronal packets and the neuropil) was labeled as well, whereas no staining was found within the neuropil (Fig. 5, *a* and *b*). In connective nerves, staining was restricted to the capsule matrix and to basal surfaces of endothelial and glial cells (Fig. 5, *c* and *d*). Thus, the laminin-like molecule recognized by antibodies 203 and 206 appears to be a component of leech ECM comparable to vertebrate basement membranes.

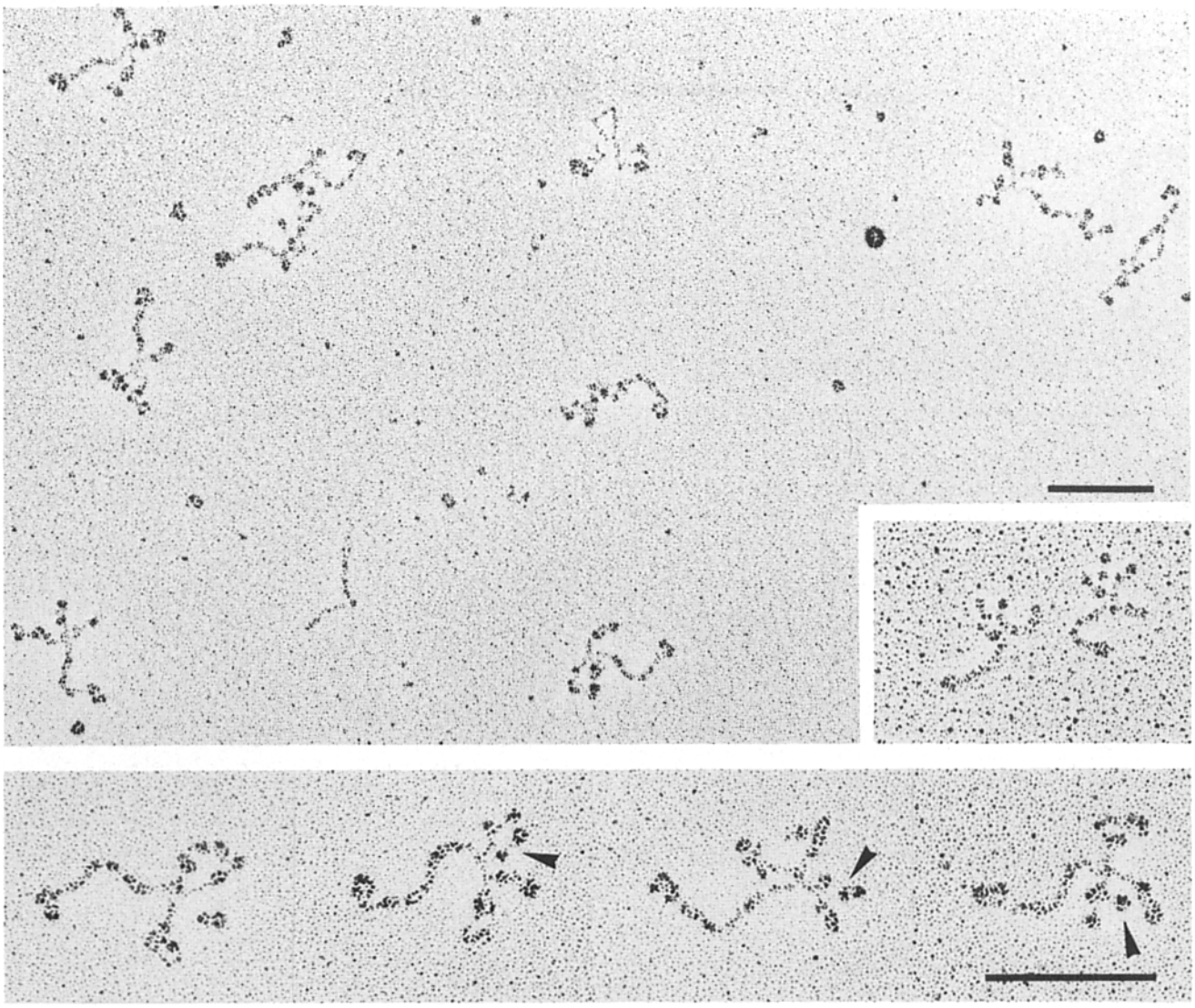

Figure 4. Electron micrographs of laminin-like leech molecules after rotary shadowing. The overview shows a 0.1-M triethylamine eluate from an antibody 203 affinity column loaded with crude EDTA extract. Nine laminin-like molecules are clearly recognizable in this field. They are characterized by one long and three shorter arms each bearing one or two globules, respectively. The inset shows two laminin–nidogen complexes from the EHS mouse tumor at the same magnification. Selected leech molecules are shown in the lower row at higher magnification. *Arrowheads*, extensions on short arms which might correspond to nidogen-like molecules. Bars, 100 nm.

The staining patterns of antibodies 203 and 206 appeared identical except that antibody 203 labeling was somewhat more fuzzy. Antiserum No. 99 directed against the ≈220-kD polypeptide produced a higher background staining of axons and glia (Fig. 5 *e*) which was also seen with preimmune serum at the same concentration (not shown).

Attachment to the Laminin-like Component Is Sufficient to Initiate Sprouting by Leech Neurons

In dishes coated with EDTA extract from ganglion capsules, the extent of neuronal sprouting depended on the protein concentration (Fig. 6). The half-maximal rate of neurite growth was reached at ~20–30 μg protein per ml coating solution (Fig. 6 *b*). When laminin-like protein that had eluted from the antibody 203 and 206 affinity columns was incubated with culture dishes (Fig. 6, *d–f*), leech neurons sprouted extensively. The protein concentration of the column eluates was estimated (from Coomassie Blue–stained gels using mouse laminin as a standard) to be ~10 μg/ml; purified material was still active at 3 μg/ml (Fig. 6 *f*). These results suggest that specific neurite-promoting activity of the purified material is ~10-fold higher than that of crude extract.

EDTA extract passed twice over an antibody 203 affinity column was still active at concentrations comparable to original EDTA extract (not shown). Extract passed over both antibody 203 and 206 columns, however, failed to promote extensive neurite outgrowth at all concentrations tested (Fig. 6, *g–i*). Thus, depletion of EDTA extract from both the "slow" and the "fast" ≈10^3 kD component (cf. Fig. 3 *c*) abolishes most of the activity, suggesting that laminin-like molecules are major neurite-promoting components in leech ganglion capsule ECM. Fig. 6 *e* also shows that leech neurons grew

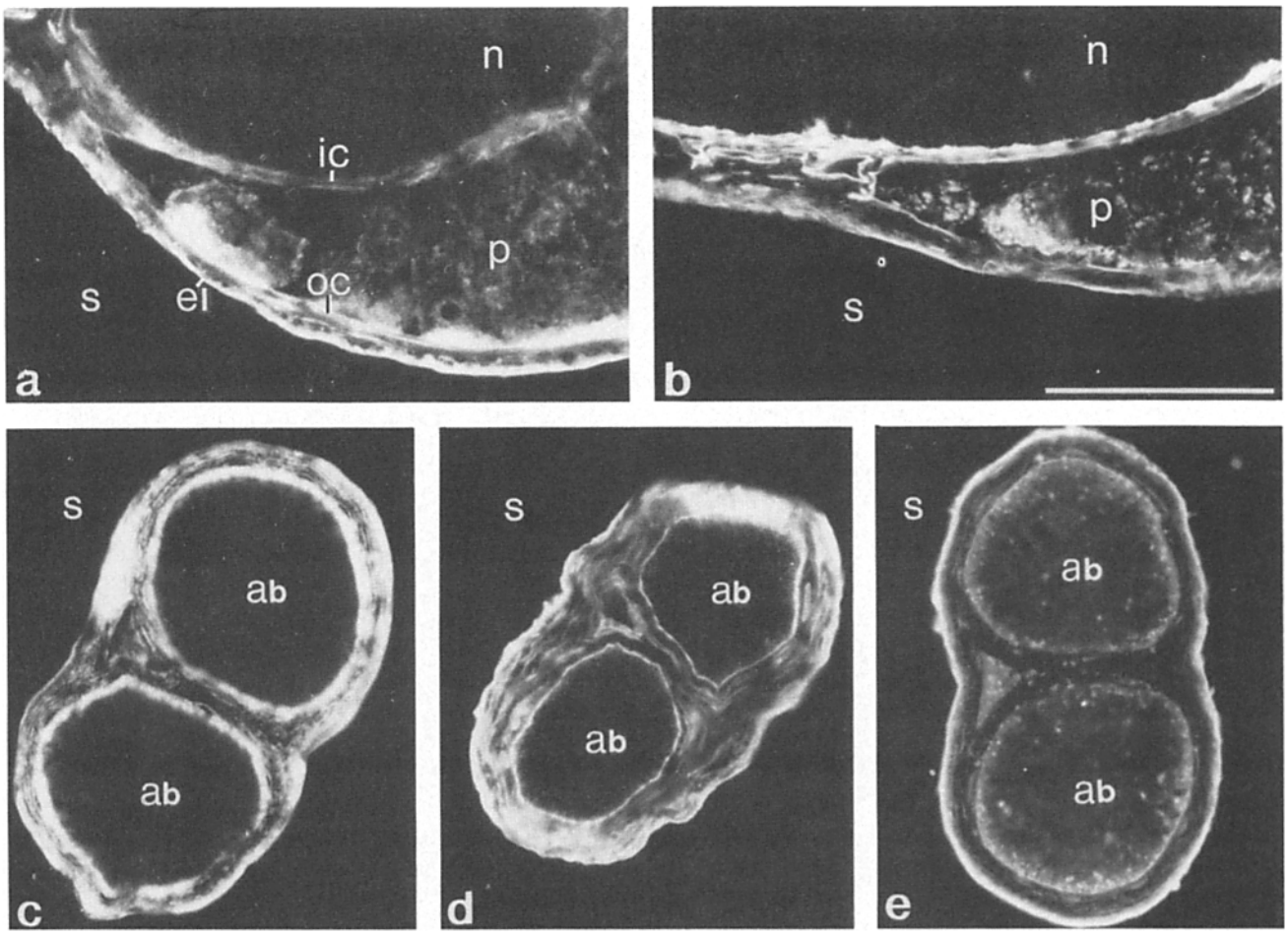

Figure 5. Distribution of laminin-like molecules within the leech central nervous system as revealed by staining with antibodies. Cryosections through a segmental ganglion (*a* and *b*) and through a connective nerve (*c–e*) were incubated with antibody 203 (*a* and *c*), 206 (*b* and *d*), or antiserum No. 99 (*e*) followed by rhodamine-labeled second antibody. The structural details are described in the text. *oc*, outer capsule; *ic*, inner capsule; *el*, endothelial layer; *p*, neuronal package; *n*, neuropil; *ab*, axon bundles; *s*, blood sinus. Bar, 100 μm.

well in serum-free L-15 medium on dishes coated with the purified material. Neuronal survival after ≥2 d was better in the presence of serum. We conclude that neurite growth by isolated leech neurons can be induced by the laminin-like substrate; soluble growth factors or the presence of other cells are not needed.

An Antiserum against the ≈220-kD Polypeptide Inhibits Sprouting of Leech Neurons on ECM Extract

mAbs 203 and 206 had no effect on the sprouting of isolated leech neurons when added to culture medium (not shown). Additional evidence that activity resides on the laminin-like molecules was provided by an inhibitory polyclonal antiserum against SDS-denatured subunits (see Materials and Methods). One of the antisera, No. 99, reacted with the ≈220-kD polypeptide on immunoblots of ECM fractions enriched for laminin-like molecules (Fig. 2 *e*).

Isolated leech neurons were cultured on dishes coated with EDTA extracts from ganglion capsule matrix. To standard culture medium, antiserum No. 99 or preimmune serum was added at a dilution of 1:50. Cells were photographed at daily intervals and the growth rate of neurites was determined. In medium containing preimmune serum, the total length of neurites formed per cell/day was 650 μm (SEM 120 μm, n = 13), compared to 120 μm (SEM 60 μm, n = 10) in the presence of antiserum No. 99. Thus, compared to the control, antiserum No. 99 inhibited neurite outgrowth by 80%. Not only was the growth of neurites delayed but extensions were stubbier, more branched and fasciculated more frequently (Fig. 7, *a* and *b*).

In addition to binding to the ≈220-kD subunits of laminin-like molecules, antiserum No. 99 reacted with a second band of ≈300 kD in EDTA extract which may or may not be related to laminin-like components (Fig. 2 *d*). We therefore tested whether antiserum No. 99 also inhibited growth of neurons cultured on affinity-purified laminin-like material. In such an experiment, total neurite length per cell/day was 1,030 μm (SEM 170 μm, n = 14) in 1:50 diluted preimmune serum and 220 μm (SEM 40 μm, n = 16) in the same concentration of antiserum No. 99, which again amounts to an inhibition of ~80%. Representative cells are shown in Fig. 7, *c* and *d*.

Neurite growth in the absence of any rabbit serum was not significantly different from the values found with preimmune

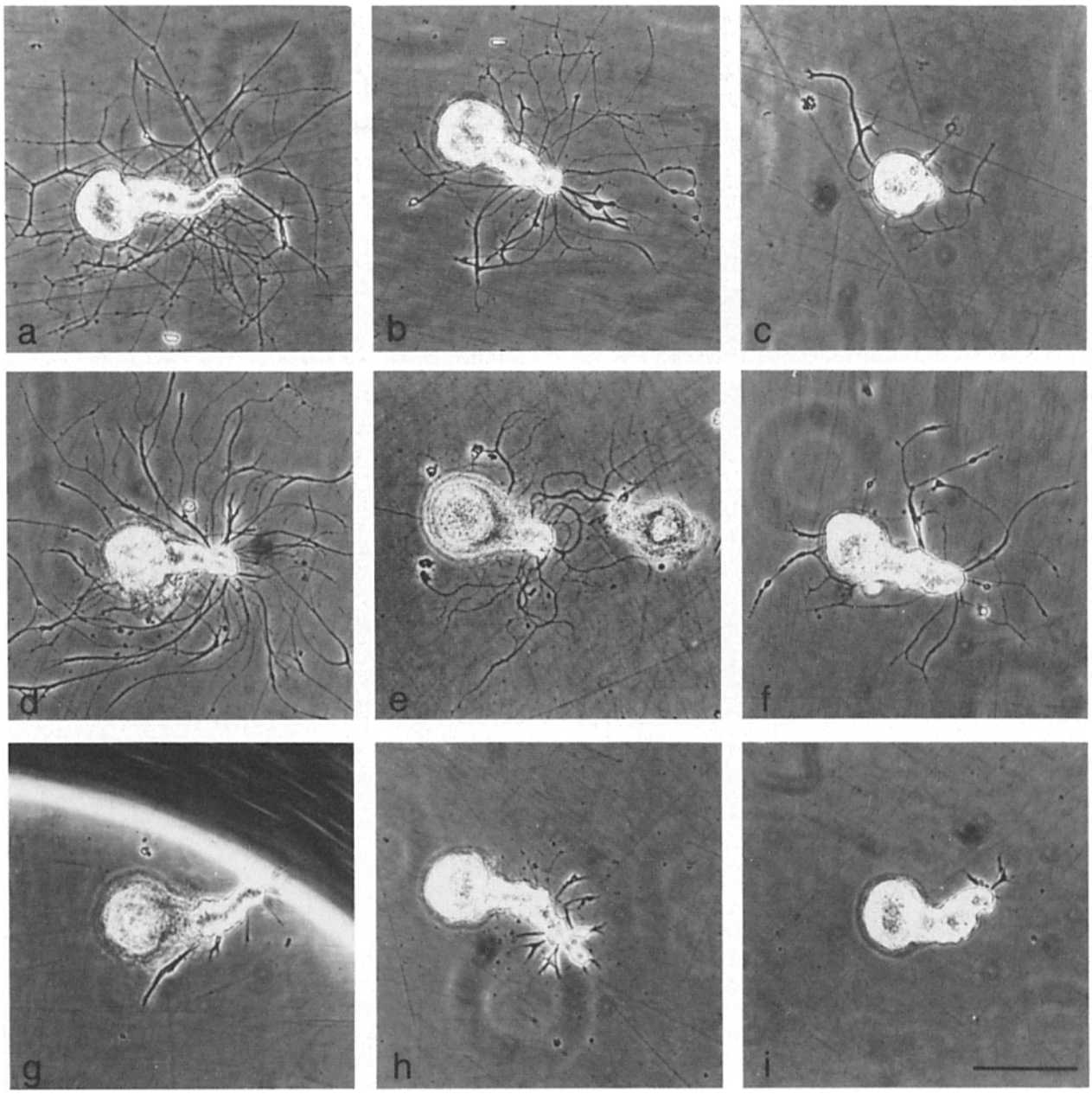

Figure 6. Concentration-dependent neurite-promoting activity of leech ECM extract and of the affinity-purified laminin-like molecules. Culture dishes were coated with ganglion capsule EDTA extract (*a–c*), eluates from antibody 203 (*d* and *e*) and 206 (*f*) columns, and material not retained by antibody 203 and 206 affinity columns (*g–i*). Estimated protein concentrations of the coating solutions were 72, 22, and 7 μg/ml (*a–c*) for EDTA extract; 10 (*d* and *e*) or 3 μg/ml (*f*) for the purified material; and 80, 27, and 8 μg/ml (*g–i*) for the flow-through, respectively. Isolated Retzius neurons were plated in medium containing 2% serum (*a–d* and *f–i*) or serum-free medium (*e*), and photographed after 2 d. For each substrate and concentration, a neuron showing optimal growth (from a sample of five) is shown. Bar, 100 μm.

serum, and an antiserum reacting with unrelated bands in EDTA extract (No. 101; see Materials and Methods) did not inhibit sprouting (not shown). Thus, inhibition is specific for antiserum No. 99.

The Laminin-like Molecule from Leech ECM Promotes the Sprouting of Leech, but Not Vertebrate, Neurons and Vice Versa

We tested whether cultured leech neurons grew neurites on substrates coated with vertebrate ECM components. Gelatin, rat tail collagen, bovine serum fibronectin, chick tenascin (4), mouse tumor laminin and its fragments E1 and E8 (29), as well as intact chick retinal basement membranes (15) were inactive in promoting the sprouting of leech neurons (3) (not shown). Moreover, neither of the antisera against the vertebrate molecules reacted specifically with a related leech component on immunoblots (not shown). We therefore asked whether the laminin-like leech molecules promoted the growth of neurons from a different phylum.

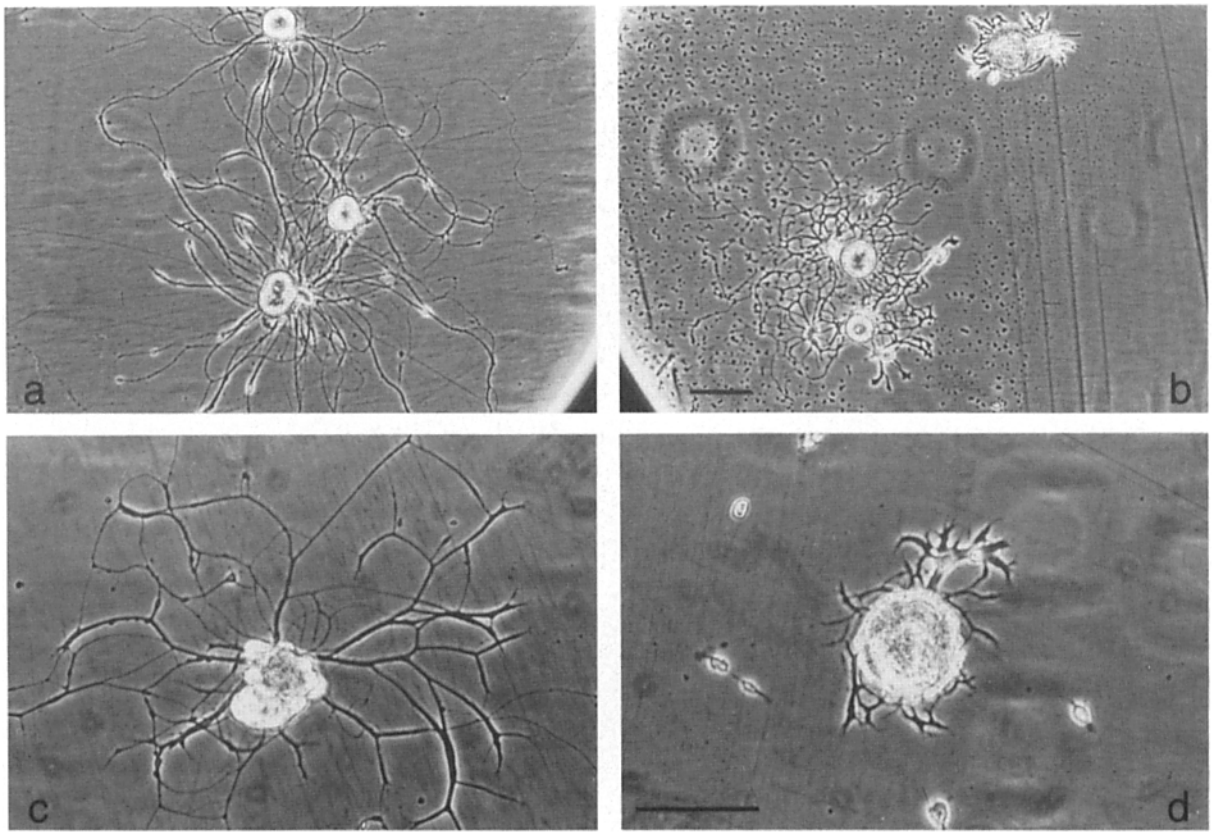

Figure 7. Inhibition of neurite outgrowth by antiserum against the ≈220-kD polypeptide. Retzius neurons were grown for 5 d on dishes coated with ganglion capsule EDTA extract (*a* and *b*) or for 2 d on affinity-purified laminin-like molecule (*c* and *d*) in the presence of preimmune serum (*a* and *c*) or antiserum against the ≈220-kD subunit of laminin-like molecules (*b* and *d*). Representative cells are shown; quantitative data are given in Results. Bars, 100 μm.

Embryonic rat dorsal root ganglion neurons were grown in defined medium in the presence of enough β-NGF (20 ng/ml) to allow survival but only marginal neurite outgrowth on polylysine-coated dishes (not shown, cf. reference 8). Under identical conditions, the rat neurons grew an extensive neurite network within 24 h on a substrate coated with mouse EHS tumor laminin (Fig. 8 *b*). In contrast, on dishes coated with leech ECM fractions enriched for the laminin-like molecule, neurite formation by the rat cells was as poor as on polylysine-coated control substrates (Fig. 8 *d*). In the same experiment, isolated leech neurons were plated onto identically treated substrates, and the opposite result was obtained: leech neurons grew well on the leech molecule but not on mouse laminin (Fig. 8, *a* and *c*). Despite the similarity between the two molecules, they do not interact functionally with cell surfaces from neurons of another phylum.

Discussion

We report here the isolation of laminin-like molecules from leech ECM and their localization in basement membrane-like structures within the central nervous system. These molecules act as a neurite-promoting substrate for cultured leech neurons. The structural features as seen in the electron microscope are shown to be very similar to vertebrate laminin.

Leech ganglion capsule extract contains two electrophoretic components of $\approx 10^3$ kD. The slow variant with subunits of ≈340 and 220 kD is unambiguously identified as a laminin-like component by its purification with the aid of antibody 203 and by its appearance in the electron microscope. This molecule promotes neurite growth. By analogy with vertebrate laminin (10, 11, 24), it is probably a trimeric molecule with one large and two smaller subunits. The fast $\approx 10^3$ kD variant with subunits of ≈340 and 160–220 kD is in all likelihood a laminin-like molecule as well, since antibody 206 cross-reacts with both $\approx 10^3$ kD components and since mixtures of both reveal only laminin-like structures in the electron microscope. Although the fast variant has not been obtained in pure form, it is presumably active in neurite promotion as well, since only removal of both $\approx 10^3$ kD components abolishes the activity of ECM extract. It is not clear at present whether the fast variant arises from the slow by proteolytic cleavage, or whether the two components are isoforms of laminin-like leech molecules. It is noteworthy that both mAb 203 and polyclonal antiserum No. 99 react with the ≈220-kD, but not with the ≈160–180-kD polypeptides, arguing against them being derived proteolytically from one another. Laminin isoforms with different subunit compositions have been described in vertebrates (9, 39).

Molecules with a typical cross-shaped appearance in the electron microscope and with a subunit composition resembling mouse EHS tumor laminin have been identified in other invertebrates, namely in *Drosophila* (14) and in sea urchin (25). For laminin-like molecules from leech ECM, we

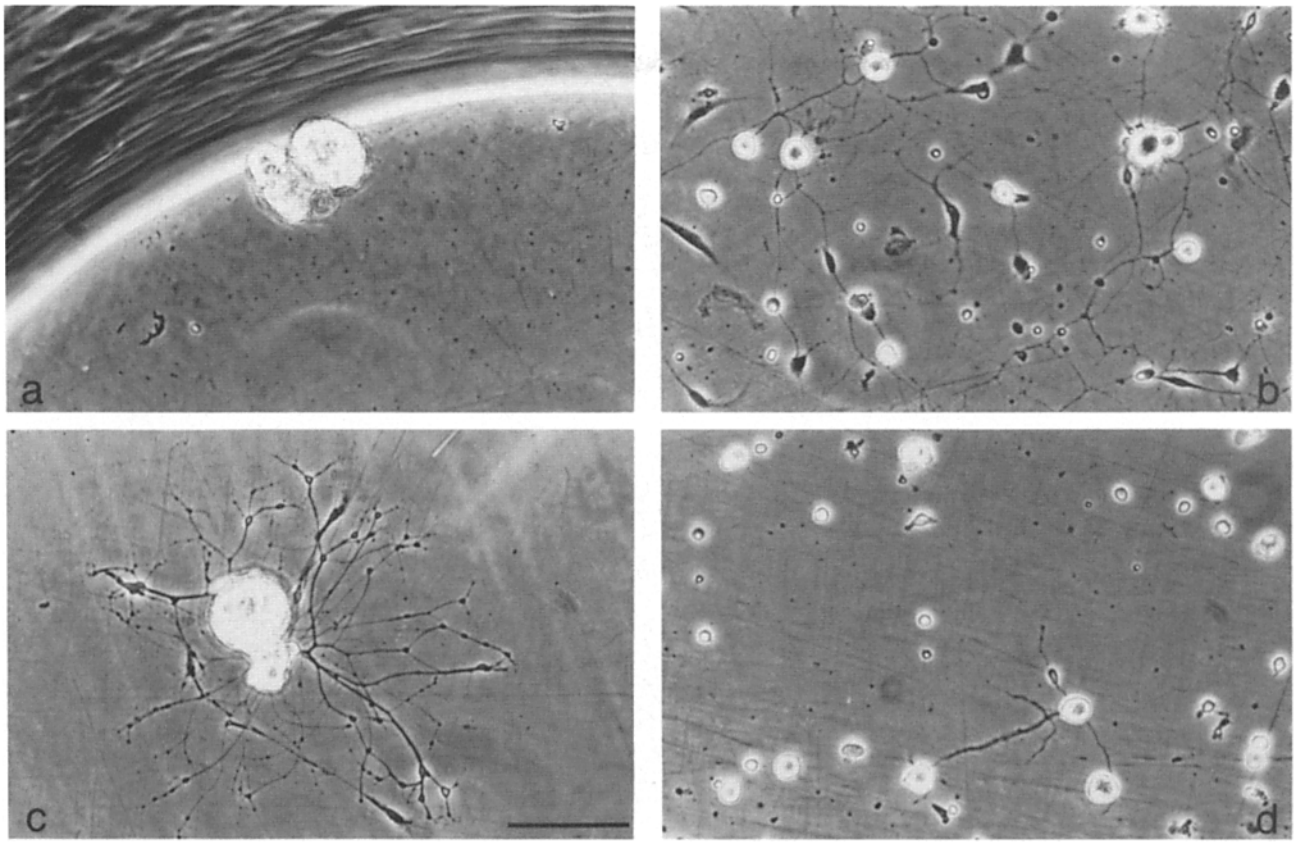

Figure 8. Neurite outgrowth of leech and vertebrate neurons on laminin from different species. Culture dishes were coated with mouse EHS tumor laminin (*a* and *b*) or with laminin-like leech molecule (*c* and *d*). Leech Retzius neurons (*a* and *c*) or rat dorsal root ganglion neurons (*b* and *d*) were plated on both substrates and photographed after 1 d. Culture conditions are described in Materials and Methods. Bar, 100 μm.

were able to demonstrate a biological function by a direct assay in vitro. This function, promotion of neurite outgrowth, is also one of the well-characterized activities attributed to vertebrate laminin (8). It has been shown that laminin-containing substrates can act synergistically with soluble growth factors such as NGF to induce the sprouting of vertebrate neurons (8). For example, low NGF concentrations will ensure the survival of vertebrate dorsal root ganglion neurons, but not promote their growth on a nonphysiological substrate. Under these conditions, however, the neurons will sprout extensively on a laminin substrate (8). We show here that attachment to the affinity-purified preparation of the laminin-like molecule is sufficient to initiate the sprouting of leech neurons without soluble growth factors.

It is difficult to rule out that a low molecular mass growth factor is tightly bound to the laminin-like protein. However, this seems very unlikely, since activity copurifies with the laminin-like molecule even after its treatment with 4 M urea, and since an antiserum against its ≈220-kD subunit inhibits sprouting. For vertebrate laminin, the neurite-promoting activity has been localized to a specific domain on the long arm of the protein which can be obtained by limited elastase digestion (8, 24, 29). We are currently fragmenting the leech molecule in an attempt to localize the active domain, and to find out whether this function is reflected by a conserved structure as well.

Vertebrate laminin does not promote neurite outgrowth by leech neurons, and vice versa. The neurite-promoting domains of laminin-like molecules might differ in this respect from the best known cellular recognition signal, the short peptide sequence RGD, found within cell attachment domains of several vertebrate ECM molecules (31). A family of cell surface receptors termed integrins which recognize this sequence has been characterized (17). The RGD recognition system seems to be conserved during evolution (27, 40). The neurite-promoting domain of laminin, on the other hand, might depend on the tertiary structure of a segment of the long arm involving all three subunits (8, 29, 30). In the case of laminin and related invertebrate components, structural and functional similarities point to a common ancestor protein. However, it appears that the recognition sites on the molecules that are responsible for neurite promotion, and with them the corresponding cell surface receptor domains, are quite different between phyla.

In the leech, the development of single neurons can in some cases be traced back to the zygote: the complete cell lineage (34, 38), migration of neuroblasts (34), pattern of neurite outgrowth (19), and synapse formation (38) have been studied in detail. Moreover, adult leech neurons regenerate functional connections with a high degree of precision (23). Laminin is believed to be important for the development and regeneration of the vertebrate nervous systems (21,

22). The specific antibodies will allow us to study the role of a laminin-like molecule in a simple animal in which analysis at the level of single neurons of known origin and function is possible.

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