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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 ∼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...
was 15–25 μg (EDTA extract) or =50 μg (urea extract) as estimated from A280 assuming an average absorption coefficient of A280 = 1,000 cm−1 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 (Madison, Miss.; Fellinl World, 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 × 10⁴ PAI myeloma cells (33). Resulting hybridoma lines were grown as described (16) using supernatants 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 the original lines, thus confirming their monoclonal origin.

**SDS Gel Electrophoresis and Immunoblotting**

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.

**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 replicates were fixed 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 ~500,000. 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.

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**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 2000; Reichert Jung, Vienna, Austria) were mounted on gelatin-coated slides and routinely stained with ascites or antisera diluted 1:200 with PBS containing 10% horse serum (KC Biological Inc., Lenexa, KS), followed by rhodamine-conjugated secondary antibodies (Cappel Laboratories) (4).
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 non-reducing conditions, two separate components of \( \approx 10^3 \) kD were detected that roughly comigrated with nonreduced mouse laminin (Fig. 1, a and b). Bands usually seen after reduction (i.e., \( \approx 350 \) - and \( 220 \)-kD polypeptides as well as minor components of \( \approx 180 \) and \( 160 \) kD (Fig. 1 d), were lacking. The region from a nonreducing gel including the two \( \approx 10^3 \) kD components was run in the second dimension on a reducing gel (Fig. 1 e). The more slowly migrating band of \( \approx 10^3 \) kD dissolved into two spots of \( \approx 340 \) and \( 220 \) kD, whereas the more rapidly migrating band was split into polypeptides of \( \approx 340, 180, \) and \( 160 \) kD; a trace at \( \approx 220 \) kD was visible as well. Thus, the electrophoretic components of \( \approx 10^3 \) 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 \( \approx 220- \) and a \( \approx 340 \)-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 \( \approx 220 \)-kD and part of the \( \approx 340 \)-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 \( \approx 10^3 \) 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 \( \approx 10^3 \) 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" \( \approx 10^3 \) kD molecule contains most of the \( \approx 220 \)-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
polyacrylamide-SDS gel stained with Coomassie Blue. Samples through was collected (lanes c and g), and bound antigen (lanes d 203 and 206. Ganglion capsule EDTA extract (lanes a and e) was
Figure 3. rotary shadowing. The only large molecules found in such
body 203 recognizes a small subunit of a leech molecule
extensive searching. From these results, we conclude that an-
tegrates (35), which were found in crude extract
per lane. The bands at =150 kD in b and at =50 and 25 kD in f
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
by immunofluorescence with antibodies 203 and 206. The
Cryosections through leech segmental ganglia were labeled
Distribution of Laminin-like Molecules in the Leech
Structure of the Laminin-like Leech Molecules

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 anti-
body 206–Sepharose, and bound material was eluted, elec-
trophoresed 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 ≈103 kD
component (not shown), ECM extract passed over both anti-
body columns was very much depleted from the two ≈103
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 globu-
les, 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 fuzzy stained. Labeled
granular material partially outlined the surfaces of the neu-
ronal 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 lami-
nin-like molecule recognized by antibodies 203 and 206 ap-
ppears to be a component of leech ECM comparable to ver-
tebrate basement membranes.
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” ~101 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 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.
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.

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.
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 ≈103 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 ≈103 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 ≈103 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 ≈103 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
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 $\approx 220$-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.

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 $\mu$m.

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,
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References

1. Aréchiga, H., M. Chiquet, D. P. Kuffer, and J. G. Nicholls. 1986. Formation of specific connections in culture by identified leech neurones containing serotonin, acetylcholine and peptide transmitters. J. Exp. Biol. 126:15-31.

2. Bottenstein, J., E. and C. Sato. 1985. Cell Culture in the Neurosciences. Plenum Press, New York. 1-21.

3. Chiquet, M., and S. E. Acklin. 1986. Attachment to Con A or extracellular matrix initiates rapid sprouting by cultured leech neurons. Proc. Natl. Acad. Sci. USA. 83:6188-6192.

4. Chiquet, M., and D. F. Fambrough. 1984. Chick myotendinous antigen. I. A monoclonal antibody as a marker for tendon and muscle morphogenesis. J. Cell Biol. 98:1926-1936.

5. Chiquet, M., and J. G. Nicholls. 1987. Neurite outgrowth and synapse formation by identified leech neurones in culture. J. Exp. Biol. 152:191-206.

6. Coggeshall, R. E., and D. W. Fawcett. 1964. The fine structure of the central nervous system of the leech Hirudo medicinalis. J. Neurophysiol. (Bethesda). 27:229-239.

7. Dietzel, I. D., P. Drapeau, and J. G. Nicholls. 1986. Voltage dependence of 5-hydroxytryptamine release at a synapse between identified leech neurones in culture. J. Physiol. (Lond.). 372:191-205.

8. Edgar, D., R. Timpl, and H. Thoenen. 1984. The heparin-binding domain of laminin is responsible for its effects on neurite outgrowth and neuronal survival. EMBO (Eur. Mol. Biol. Organ.) J. 3:1463-1468.

9. Edgar, D., R. Timpl, and H. Thoenen. 1988. Structural requirements for the stimulation of neurite outgrowth by two variants of laminin and their inhibition by antibodies. J. Cell Biol. 106:1299-1306.

10. Engel, J., and H. Furthmayr. 1987. Electron microscopy and other physical methods for the characterization of extracellular matrix components: laminin, fibronectin, collagen IV, collagens VI, and proteoglycans. Methods Enzymol. 145:3-78.

11. Engel, J., E. Odermatt, A. Engel, J. A. Madri, H. Furthmayr, H. Rohde, and R. Timpl. 1981. Shapes, domain organization and flexibility of laminin and fibronectin, two multifunctional proteins of the extracellular matrix. J. Mol. Biol. 150:97-120.

12. Engvall, E. 1980. Enzyme immunoassay ELISA and EMIT. Methods Enzymol. 70:419-439.

13. Fazekas de St. Groth, S., and D. Scheidegger. 1980. Production of monoclonal antibodies: strategy and tactics. J. Immunol. Methods. 35:1-21.

14. Fessler, L., G. A. Campbell, G. G. Duncan, and J. H. Fessler. 1987. Drosophila laminin: characterization and localization. J. Cell Biol. 105:2383-2391.

15. Halfwer, W., and S. Deiss. 1986. Axonal pathfinding in organ cultured embryonic avian retinae. Dev. Biol. 114:296-310.

16. Hauri, H. P., E. Sterchi, D. Bientz, J. M. Fransen, and A. Marxer. 1985. Expression and intracellular transport of microvillus membrane hydrodases in human intestinal epithelial cells. J. Cell Biol. 101:838-851.

17. Hynes, R. O. 1987. Integrins: a family of cell surface receptors. Cell, 48:549-554.

18. Imhof, B. A., U. Marti, K. Boller, H. Frank, and W. Birchmeier. 1983. Association between coated vesicles and microtubules. Exp. Cell Res. 145:199-207.

19. Kramer, A. P., and G. S. Stent. 1985. Developmental arborization of sensory neurones in the leech Haemanteria ghiliani. II. Experimentally induced variations in the branching pattern. J. Neurosci. 5:768-775.

20. Lammli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.). 227:680-685.

21. Liesi, P. 1985. Do neurons in the vertebrate CNS migrate on laminin? EMBO (Eur. Mol. Biol. Organ.) J. 4:1163-1170.

22. Liesi, P. 1985. Laminin-immunoactive glia distinguish regenerative adult CNS systems from non-regenerative ones. EMBO (Eur. Mol. Biol. Organ.) J. 4:2505-2511.

23. Macagno, E. R., K. J. Muller, and S. A. DeRiemer. 1985. Regeneration of axons and synaptic connections by touch sensory neurones in the leech central nervous system. J. Exp. Biol. 116:151-173.

24. Martin, G. R., and R. Timpl. 1987. Laminin and other basement membrane components. Annu. Rev. Cell Biol. 3:57-85.

25. Masuda-Nakagawa, L., K. Beck, and M. Chiquet. 1986. Identification of molecules in leech extracellular matrix that promote neurite outgrowth. Proc. R. Soc. Lond. B. Biol. Sci. In press.

26. McCarthy, R. A., K. Beck, and M. M. Burger. 1987. Laminin is structurally conserved in sea urchin basal lamina. EMBO (Eur. Mol. Biol. Organ.) J. 6:1587-1593.

27. Muller, K. J., J. G. Nicholls, and G. Stent. 1981. Neurobiology of the Leech. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 249-275.

28. Nieder, C., M. Sémérevra, K. M. Yamada, and J.-P. Thiery. 1987. Peptides containing the cell-attachment recognition signal Arg-Gly-Asp prevent gasterulation in Drosophila embryos. Nature (Lond.). 325:348-350.

29. Nicholls, J. G. 1987. The search for connections: studies of regeneration in the nervous system of the leech. Magnes Lecture Ser. 2:1-96.

30. Paulsson, M., M. Auerbacher, R. Deutzmann, R. Timpl, K. Beck, and J. Engel. 1987. Laminin-nidogen complex. Extraction with chelating agents and structural characterization. Eur. J. Biochem. 166:11-19.

31. Paulsson, M., R. Deutzmann, R. Timpl, D. Dalzoppo, E. Odermatt, and J. Engel. 1985. Evidence for coiled-coil a-helical regions in the long arm of laminin. EMBO (Eur. Mol. Biol. Organ.) J. 4:309-316.

32. Ruoslabhi, E., and M. D. Pierschbacher. 1987. New perspectives in cell adhesion: RGD and integrins. Science (Wash. DC). 238:491-497.

33. Shotton, D. M., B. Burke, and D. Branton. 1979. The molecular structure of human erythrocyte spectrin, biophysical and electron microscopic studies. J. Mol. Biol. 131:303-329.

34. Stocker, J. W., H. K. Forster, V. Miggiano, C. Staeblin, G. Staiger, B. Takacs, and T. Staehelin. 1982. Generation of 2 new mouse myeloma cell lines "PAI" and "PAI-0" for hybridoma production. Res. Disclosure. 217:155-157.

35. Stuart, D. K., S. S. Blair, and D. A. Weisblat. 1987. Cell lineage, cell death, and the developmental origin of identified serotonin- and dopamine-containing neurones in the leech. J. Neurosci. 7:1107-1122.

36. Timpl, R., H. Wiedemann, V. van Deldcn, H. Furthmayr, and K. Kühn. 1981. A network model for the organization of type IV collagen molecules in basement membranes. Eur. J. Biochem. 121:203-211.

37. Towbin, H., T. Staeblin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA. 76:4350-4354.

38. Vaughan, L., S. Huber, M. Chiquet, and K. H. Winterhalter. 1987. A major six-armed glycoprotein from embryonic cartilage. EMBO (Eur. Mol. Biol. Organ.) J. 6:349-353.

39. Weisblat, D. A. 1081. Development of the nervous system. In Neurobioloy of the Leech. K. J. Muller, J. G. Nicholls, and G. S. Stent, editors. Cold Spring Harbor Laboratory. Cold Spring Harbor, New York. 173-195.

40. Wexer, U. M., D. Tichy, A. Damjanov, M. Paulsson, and I. Damjanov. 1987. Distinct antigenic characteristics of murine parietal yolk sac laminin. Dev. Biol. 121:397-407.

41. Wilcox, M., and M. Leptin. 1985. Tissue specific modulation of a set of cell surface antigens in Drosophila. Nature (Lond.). 316:351-354.