Structural Requirements for the Stimulation of Neurite Outgrowth by Two Variants of Laminin and Their Inhibition by Antibodies

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Abstract. Laminin derived from the Engelbreth-Holm-Swarm (EHS) tumor and a lamininlike molecule synthesized by RN22 Schwannoma cells both stimulate rapid neurite outgrowth, consistent with a common neurite-promoting site. However, antilaminin antisera can only inhibit the activity of the EHS laminin. The blocking antibodies in such sera are directed against the terminal heparin-binding domain of the laminin long arm (Edgar, D., R. Timpl, and H. Thoenen. 1984. *EMBO [Eur. Mol. Biol. Organ.] J.* 3:1463–1468). These epitopes are demonstrated by immunoblotting to be part of the A chain and to be absent in RN22 laminin, showing (through metabolic labeling) that the cells synthesized little if any 440-kD A chain. This indicates that the antibody inhibition was probably due to steric hindrance, a common neurite-promoting site, apparently not being antigenic in native molecules. Antibodies raised against a 25-kD proteolytic fragment derived from the long arm of laminin were then used as probes to identify other potential neurite-promoting structures. Although these antibodies do not cross-react with native laminin, they recognized the B chains of denatured EHS and RN22 molecules on immunoblots. The antibodies also bound to the large proteolytic fragment, derived from the long arm of laminin that contains the neurite-promoting site, thus inhibiting its activity. Taken together, these results point to the localization of normally nonantigenic, defined, B chain sequences within or close to the neurite-promoting site of laminin.

NEURONS can be stimulated to extend neurites rapidly when cultured on substrates consisting either of specific cell types (notably glia [19, 29]), or alternatively if the substrate contains molecules secreted or "shedded" by many types of cells (2, 8). Although the cell surface molecules responsible for stimulating neurite outgrowth are currently only starting to be identified (see references 4, 5, 35), the well-characterized basement membrane protein laminin (26) has been shown to evoke many effects of extracellular matrices on neural cells: when used as a tissue culture substrate, laminin stimulates rapid neurite outgrowth (3), potentiates the survival-promoting activities of soluble neurotrophic factors (16), and can both induce an activation and increase the amount of the enzyme tyrosine hydroxylase in adrenal chromaffin cells (1). Laminin has also been shown to stimulate the division of Schwann cells (27).

Antibody inhibition studies have been used to analyze the molecular structures mediating these effects of laminin; the ability of domain-specific antibodies to block the effect of laminin on neural cells indicates that a site at or near the end of the long arm of laminin (see Fig. 1) is responsible for the stimulation of neurite outgrowth (16, 17), potentiation of neuronal survival (16), and induction of tyrosine hydroxylase (1). The blocking antibodies detected in the original study were all directed against a 50-kD heparin-binding fragment (E3) derived from the terminal globular domain from the long arm of mouse Engelbreth-Holm-Swarm (EHS)[1] tumor laminin (16). Direct evidence for the involvement of this domain could not be provided, however, because the heparin-binding fragment itself had no stimulatory effect on neurons. Indeed, the smallest proteolytic fragment of laminin (E8; M_r ~280 kD) able to promote neurite outgrowth consisted not only of the whole terminal globular domain, but also contained a 30-nm rodlike segment of the long arm (16; see Fig. 1). More recently, it has been shown that monoclonal antibodies directed against epitopes on or near to the terminal globular domain from the long arm of human placental laminin can also inhibit neurite outgrowth to some extent (17). Significantly, the degree of antibody inhibition was increased by the use of secondary antibodies in a sandwich, consistent with inhibition caused by steric hindrance, although other possibilities (e.g., changes in antibody affinity) might also account for such findings (17).

In addition to purified EHS laminin, a considerable number of other neurite-promoting factors have been described, the activity of which appears to be due to laminin that is often complexed noncovalently with proteoglycan and with the protein entactin/nidogen (12, 13, 24, 25). Antisera against

1. *Abbreviation used in this paper*: EHS, Engelbreth-Holm-Swarm.

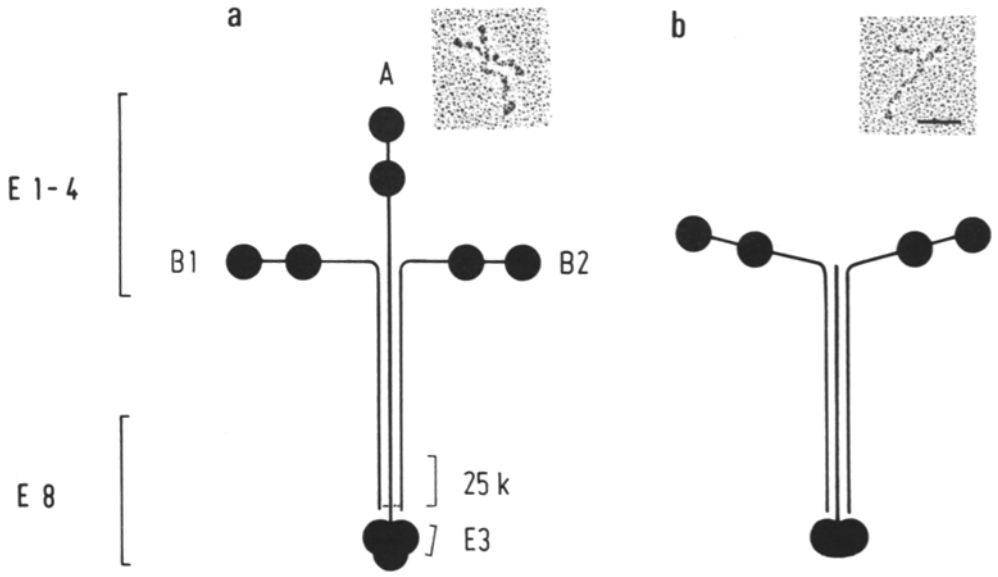

Figure 1. Rotary-shadowing images (*inserts*) and schematic drawings of EHS laminin (*a*) and RN22 cell laminin (*b*). For rotary shadowing, EHS laminin was prepared as described previously (39), and RN22 laminin was isolated essentially as described (12; Edgar, D., and M. Paulsson, unpublished observations). The location of the polypeptide chains in the illustration of EHS laminin (*a*) is based on recently proposed models (26, 41). The origins within the native laminin molecule of the proteolytic fragments used in this study are indicated by brackets. Contributions of the three laminin polypeptide chains to each fragment are shown in the illustration, although it should be noted that the 25-kD fragment consists only of two disulfide-bonded polypeptides derived from the B chains (33), and fragment E8 is derived exclusively from the laminin A chain (41). The molecular masses of the fragments have previously been determined to be 50 kD for E3 (30); 280 kD for E8 (33); 700 kD for E1–4 (30, 38); and 25 kD for 25K (33). Fragment E3 comprises part of the terminal globular domain of the long arm, and has been shown both to bind heparin (30) and to provide the epitopes necessary for antibody inhibition of neurite outgrowth on native laminin (16). The locations of the polypeptide chains of RN22 laminin (*b*) are tentative and their identities open to speculation; the drawing is a hypothetical working model based on the rotary shadowing image (12). Drawings are not to scale. Bar, 50 nm.

EHS laminin cannot block the neurite-promoting activity of such factors, although some antibody recognition evidently occurs as the antisera can immunoprecipitate the activities (12–14, 24, 25). To explain these findings, it has been proposed that the proteoglycan of the neurite-promoting complex could mask those epitopes necessary for antibody inhibition of neurite outgrowth (24). It has, however, been demonstrated that the complex of proteoglycan and laminin can create an epitope that is absent on either component alone, and that will bind antibodies to block neurite outgrowth on the complex (7).

One particularly well-studied neurite-promoting factor is that produced by RN22 rat Schwannoma cells, the activity of which is also not inhibited by antisera against laminin (12, 13). This factor has been shown to consist of laminin molecules that lack the 440-kD A chain and, according to current models (39), might consequently be expected also to lack the heparin-binding fragment, E3, previously implicated in neurite outgrowth (16). A comparison between the laminins of the EHS tumor and RN22 cells, with respect to their structure and interactions with antibodies, therefore affords an excellent opportunity to define the neurite-promoting site more precisely: it is to be expected that the laminin molecular structures involved in neurite stimulation will be common to both molecules. Taken together, the results of this comparison point for the first time to the involvement of laminin long arm B chain sequences in its neurite-promoting site.

Materials and Methods

Preparation of Laminin, Proteolytic Fragments, and Antibodies

Laminin was purified from the mouse EHS tumor as previously described (39). The laminin-nidogen complex was isolated by a more recent procedure from which the E8 fragment could be prepared by mild proteolytic digestion with elastase (32). The E1–4 and 25-kD proteolytic fragments were prepared as described (30, 33, 38) and laminin and its fragments used for the immunization of rabbits by standard procedures (37). Antibodies were then purified from the antisera by affinity chromatography (37). Antibody cross-reactivities and antigen-binding capacities (see reference 28) were determined by standard radioimmunobinding assays using solutions of laminin and its fragments (37).

Neurite Outgrowth Assay

Tissue culture multiwell plates (24-well cluster; Costar, Cambridge, MA) were first coated with poly-DL-ornithine (Sigma Chemical Co., St. Louis, MO) and then incubated overnight at 4°C with samples to be tested for neurite-promoting activity (15, 16). The amount of laminin and fragment E8 adsorbed to the culture substrate was determined by including small amounts of ligand that had been labeled with ^{125}I (Amersham Buchler GmBH, Braunschweig, FRG) by the chloramine T method (37). At the low ligand concentrations necessary for maximal neurite outgrowth it was found that some 35–45% of laminin or fragment E8 bound to the substrate. The neurons used were dissociated from the paravertebral sympathetic ganglia of 12-d-old chick embryos, and separated from nonneuronal cells by a preplating technique (15, 16). The culture medium was F14 (Gibco, Karlsruhe, FRG) containing 50 μg/ml streptomycin sulfate and 50 U/ml penicillin sulfate (Sigma Chemical Co.), together with 10% (vol/vol) heat-inactivated horse serum (Gibco) and 20 ng/ml 2.5S nerve growth factor, prepared in our laboratory as described (36). Neurons (2,000/well) were cultivated in 400 μl of medium, and essentially all of them attached to the substrate within 30 min. Neurite outgrowth was evaluated by phase-contrast microscopy after 2 h of culture, and quantified by counting the percentage of neurons with neurites in randomly chosen fields of view. Variation between different fields of view in the same dish and between duplicate dishes was always <10%. Neurites were defined as processes of length equivalent to at least five cell diameters (~50 μm), and under the conditions of the assay no neurite development was apparent unless laminin or an equivalent substrate was used (see reference 16).

Preparation of Conditioned Media and Metabolic Labeling

The mouse PYS-2 teratocarcinoma cell line (provided by J. M. Lehman, University of Colorado) and the rat RN22 Schwannoma cell line (provided

by S. Pfeiffer, University of Connecticut) were cultured in DME with the antibiotics described for the neurite assay, and supplemented with 10% (vol/vol) FCS (Gibco). Preliminary experiments showed that the maximal neurite-promoting activity of RN22-conditioned medium was found after 3 d culture of confluent cells, and it was this medium used in the experiments shown.

For long-term metabolic labeling, confluent PYS-2 and RN22 cells in 35-mm tissue culture dishes were incubated overnight with 1.5 ml DME supplemented with 1% (vol/vol) FCS and 100 μCi/ml [^{35}S]methionine (800 Ci/mmol; New England Nuclear Chemicals, Dreieich, FRG). After collection the media were centrifuged to remove cell debris, supplemented with 2 mM phenylmethylsulfonyl fluoride (PMSF; Sigma Chemical Co., and stored at −70°C until required.

For short-term pulse-chase experiments, confluent PYS-2 and RN22 cells in multiwell culture plates were incubated for 1 h in 0.5 ml methionine-free DME containing 10% (vol/vol) dialyzed FCS. The cells were then pulsed for 10 min with the same medium containing 100 μCi/ml [^{35}S]methionine, after which time the labeled medium was replaced by normal DME plus 10% (vol/vol) FCS. The cells were extracted at the chase times shown using 50 mM Tris-HCl, pH 7.8, containing 150 mM NaCl, 1% Triton X-100, 2 mM *N*-ethylmaleimide, 2 mM EDTA, and 2 mM PMSF. The extracts (500 μl/culture well) were centrifuged to remove debris and stored at −70°C until required.

Immunoprecipitation

Aliquots of culture medium (20–100 μl) were added to 800 μl of immunoprecipitation buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1% Triton X-100, 2 mM *N*-ethylmaleimide, 2 mM EDTA, 2 mM PMSF) containing 100 μl of *Staphylococcus aureus* suspension (10% in the immunoprecipitation buffer; pansorbin; Calbiochem, Frankfurt, FRG). After preadsorption for 1 h at room temperature, the Pansorbin was removed by centrifugation and 10 μg of antibodies added to the supernatant. The mixture was shaken for 45 min at room temperature, 50 μl of the Pansorbin suspension was added, and adsorptions were carried out for a further 45 min. The Pansorbin pellets were then washed twice with immunoprecipitation buffer, and twice with 50 mM Tris-HCl, pH 7.8, containing 1 M NaCl, 1% NP-40, and the protease inhibitors. Pellets were then boiled for 5 min in 60 μl of Laemmli sample buffer (23), with or without 2-mercaptoethanol, and subjected to SDS-PAGE on 3–6% exponential gradient gels (23). The gels were calibrated with a standard of the laminin-nidogen complex (32) and ^{35}S-labeled proteins were detected by fluorography at −70°C using Fuji x-ray film, after impregnating the gels with 1 M sodium salicylate.

Immunoblots

Aliquots of conditioned medium (250–1,000 μl) were treated with 12% (wt/vol) polyethylene glycol 6,000 (Serva, Heidelberg, FRG) to precipitate high molecular weight components. The precipitates were rinsed with 70% ethanol and dissolved in Laemmli sample buffer for SDS-PAGE on 3–6% gels (see above). After electrophoresis, proteins were electrotransferred onto nitrocellulose (40), and the blots calibrated by staining transferred standards reversibly with 0.1% Ponceau S 5% TCA (Sigma Chemical Co.). After destaining with water, the nitrocellulose was blocked with 50 mM Tris-HCl, pH 7.8, plus 150 mM NaCl (TBS) containing 2.5% (wt/vol) BSA (Sigma Chemical Co.), 0.2% Tween 20 (Sigma Chemical Co.), and 0.02% (wt/vol) sodium azide. Blocking was for 30 min at 40°C, followed by 1 h at room temperature. Blots were then incubated with the antibodies shown (10 μg/ml) for 90 min, and then washed extensively in TBS. Detection of the bound antibodies was by ^{35}S-labeled protein A (1 μCi/ml, 700 Ci/mmol; Amersham Buchler GmBH, Braunschweig, FRG) dissolved in the above blocking buffer. After 90 min at room temperature the nitrocellulose was extensively washed in TBS and blotted dry on filter paper (3 MM; Whatman Inc., Clifton, NJ). Exposure of Fuji x-ray film to the blot for autoradiography was generally 1–2 d.

Results

Stimulation of Neurite Outgrowth

Sympathetic neurons rapidly extended neurites when grown on substrates of laminin, on the E8 long arm fragment of laminin (Fig. 1), or alternatively on substrate-attached molecules from the conditioned medium of RN22 Schwannoma cells (Fig. 2). The assay method used here involved precoating the tissue culture plastic with poly-DL-ornithine, before

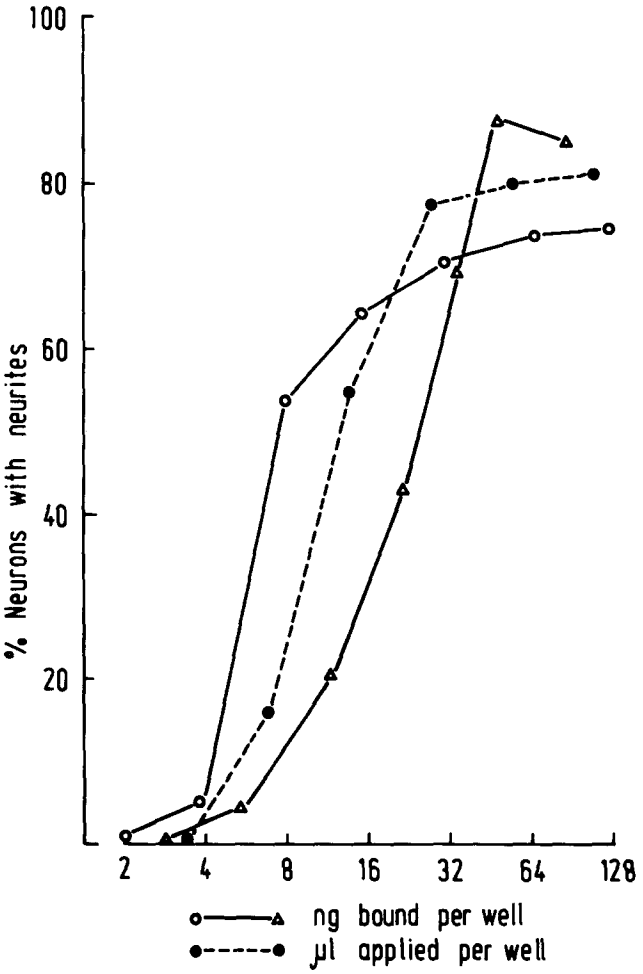

Figure 2. Dose-response curves for the stimulation of neurite outgrowth by different laminin substrates. Poly-DL-ornithine-coated tissue culture wells were incubated overnight with various amounts of EHS tumor laminin (△), fragment E8 (○), and RN22 Schwannoma-conditioned medium (●). The amounts of laminin and E8 adhering to the wells was determined by including trace amounts of ^{125}I-labeled protein. 2,000 sympathetic neurons were then cultured in the wells for 2 h and the percentage of neurons displaying neurites of length greater than 5 cell diam (>60 μm) was determined. The values shown are the means of duplicate cultures (variation between individual values <5%) and are typical of at least five independent experiments.

coating with laminin or other neurite-promoting factors. Thus the same numbers of neurons attached to the culture substrates irrespective of the presence or absence of laminin (see reference 16), and so the percentages of those neurons with neurites after 2 h culture are directly comparable from substrate to substrate. It should be noted that the maximal levels of neurite outgrowth were broadly similar for the three substrates, the apparently higher potency of E8 (with respect to that of laminin) reflecting its lower molecular weight; half maximal stimulation of neurite outgrowth occurred when the density of laminin or E8 substrate was ~10 fmol/cm^2. Estimates of the speed of neurite outgrowth generated similar dose-response curves, with maximal rates of ~1 μm/min. A substrate coating density of 100 ng per multiwell was therefore chosen for subsequent antibody inhibition studies. Similarly, 100 μl of RN22-conditioned medium was used to coat the wells for the inhibition studies, this amount giving maxi-

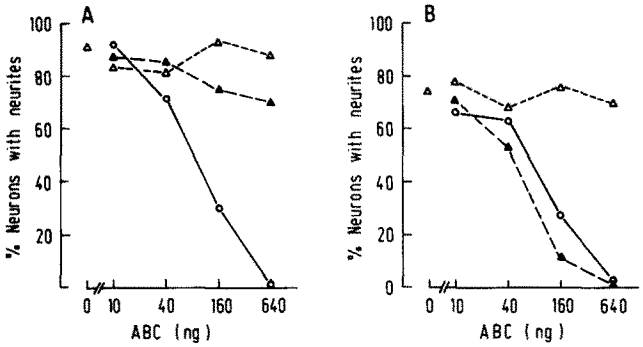

Figure 3. Antibody inhibition of neurite outgrowth on laminin (*A*) and on fragment E8 (*B*). Conditions of the assay are those described in Fig. 2, 100 ng of laminin and fragment E8 being bound to the wells. The antigen-binding capacities (*ABC*) of antibodies included throughout the culture period are given: (○) anti-E3; (△) anti-El-4; (▲) anti-25-kD. Means of duplicate cultures are shown, and are typical of three independent experiments.

mal neurite outgrowth, equivalent to that seen with 100 ng bound laminin or E8 (Fig. 2).

Antibody Inhibition of Neurite Outgrowth

As previously described (16), antibodies against fragment E3, derived from the terminal globular domain of the long arm of laminin (see Fig. 1), inhibited neurite outgrowth both on laminin (Fig. 3 *A*) and E8 (Fig. 3 *B*). In contrast, equivalent amounts of antibodies directed against the short arm fragment El–4 failed to inhibit neurite outgrowth on either substrate (Fig. 3). This pattern of antibody inhibition was also seen for PYS-2 cell laminin (data not shown, see also reference 24). Significantly, while antibodies against the 25-kD long arm fragment (see Fig. 1) displayed only slight (maximally 20%) inhibition of neurite outgrowth on laminin (Fig. 3 *A*) these antibodies were able to block neurite outgrowth on E8 totally, being as effective as the anti-E3 antibodies (Fig. 3 *B*). None of these antibodies—or antibodies against whole laminin—inhibited neurite outgrowth on substrates of RN22 conditioned media molecules (data not shown; see also references 12, 13, 24).

Table I. Immunoprecipitation of Neurite-promoting Activities from Solution by Antibodies to Laminin and Its Fragments

	Percentage of neurite-promoting activity remaining after immunoprecipitation of		
Antibody against	EHS laminin	Fragment E8	RN22 medium
Control	86	75	81
EHS laminin	2	5	5
E1-4	6	78	6
E3	5	2	76
25-kD fragment	68	12	73

1-ml aliquots of EHS laminin and fragment E8 (250 ng/ml in DME + 10% FCS) and RN22-conditioned medium (250 μl diluted in 1 ml DME + 10% FCS) were mixed with the antibodies shown (antigen-binding capacities 1.25 μg) for 1 h at room temperature, and for 1 h further after addition of 100 μl Pansorbin (10% wt/vol suspended in DME + 10% FCS). The supernatants were used to coat polyornithine culture substrates. The results given are the means of duplicates (determined after 2 h culture), that differed by <5%, and are typical of three independent experiments. Control precipitation was with nonimmune IgG.

Antibody Recognition of Neurite-promoting Molecules

As previously reported (12), antilaminin antibodies precipitated the neurite-promoting activities of laminin solutions and RN22-conditioned media (Table I), consistent with a molecule antigenically related to laminin being responsible for the latter activity. Similarly, antibodies to the laminin fragment El–4 recognized both laminin and the RN22 molecules, indicating the presence of short-arm epitopes on the RN22 neurite-promoting molecule. The latter antibodies did not, of course, recognize the long-arm fragment E8, so that its neurite-promoting activity was left in solution. Significantly however, while recognizing both laminin and fragment E8, the blocking antibodies against fragment E3 failed to precipitate the neurite-promoting activity of the RN22 cells (Table I). Antibodies to the 25-kD fragment were found to be able to precipitate only E8 activity and not that of the RN22 cells or laminin. The last result is consistent with the previously noted low cross-reactivity between these antibodies and lamin (33). Immunoprecipitation of PYS-2 cell laminin gave essentially the same results as those for EHS laminin solutions in that anti–fragment E3 antibodies precipitated >80% of the activity of PYS-2–conditioned medium.

Immunoblotting experiments were then performed to determine the reason for the specific lack of recognition of RN22 neurite-promoting activity by anti–fragment E3 blocking antibodies. While antilaminin antibodies recognized both the A (440 kD) and B (220 kD) chains of EHS and PYS laminin, the same antibodies only detected two well resolved polypeptides of ~220 kD on blots of RN22-conditioned media molecules (Fig. 4 *b*). The blocking antibodies against fragment E3 recognized only the A chain of mouse laminin (Fig. 4 *c*), consistent with the recent assignation of the heparin-binding domain of laminin to the A chain (see references 6, 26, 41). Significantly, anti–fragment E3 antibodies failed to cross-react on blots of RN22 molecules, indicating the absence (rather than masking) of E3 epitopes (Fig. 4 *c*). The anti–fragment 25-kD antibodies cross-reacted with the B chains of mouse laminin and with the 220-kD doublet of RN22 cell laminin on blots (Fig. 4 *d*). As previous work has shown fragment 25 kD to comprise the carboxyl-terminal segments of the two B chains of laminin (33), then the 220-kD doublet of RN22 cell laminin can also be regarded as B chains.

Metabolic Labeling Studies

To compare the structures of RN22 and PYS-2 cell laminins in more detail, the cells were metabolically labeled with [^{35}S]methionine, and the proteins released into their culture media analyzed by immunoprecipitation. As previously reported (12), antilaminin antibodies precipitated a relatively broad band of ^{35}S-protein from the RN22 medium (Fig. 5 *a*). On SDS-PAGE the mobility of the slowest migrating components of this band corresponded to that of PYS-2 laminin, whereas the bulk of the labeling migrated significantly faster (Fig. 5 *a*). In addition, a noncovalently bound band was seen with a mobility equivalent to 150 kD. This band was previously identified as nidogen (13). On reduction, the RN22 laminin was seen to comprise two well-defined chains of ~220 kD that comigrated with the B chains of PYS laminin, together with the 150-kD band. As previously noted, however (24), a small amount of labeled material could also

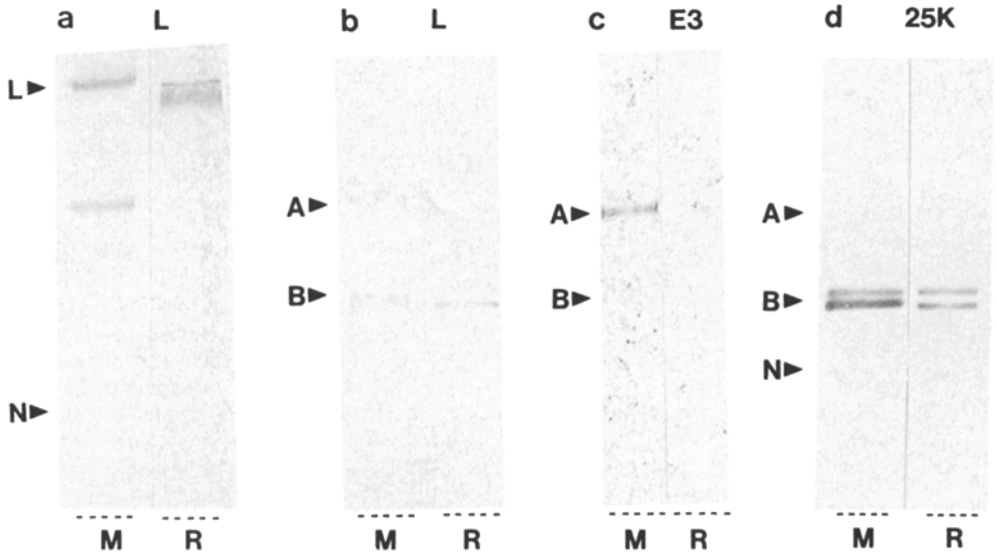

Figure 4. Immunoblots of mouse PYS-2 cell laminin (*M*) and rat RN22 cell laminin (*R*). Proteins from the culture media of these cells were subjected to SDS-PAGE under nonreducing (*a*) and reducing (*b–d*) conditions. Antibodies used for immunostaining were (*a* and *b*) antilaminin, (*c*) anti-E3, (*d*) anti-25-kD. Detection of bound antibodies was with ^{35}S-labeled protein A, followed by autoradiography. The laminin–nidogen complex (32) was included as molecular mass standard in a separate lane, and detected after electrotransfer by staining the blots with Ponceau S. *L*, laminin (800 kD); *N*, nidogen/entactin (150 kD); *A*, laminin A chain (440 kD); *B*, laminin B chains (220 kD).

be seen migrating as a doublet slightly faster than the 440-kD laminin A chain (Fig. 5 *b*). The amounts of this doublet varied from experiment to experiment, always being very small relative to the B chains, and often being undetectable – which perhaps might explain why its existence was not noted in several previous studies (12, 31).

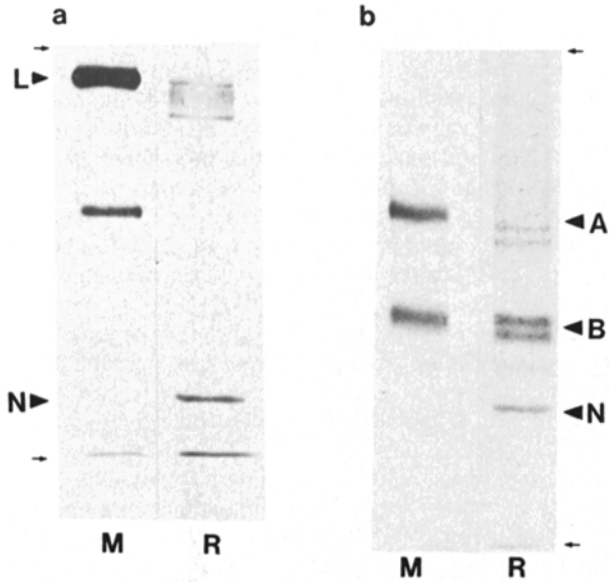

Figure 5. Immunoprecipitation of [^{35}S]methionine-labeled proteins from mouse PYS-2 cell (*M*) and rat RN22 cell (*R*) conditioned media. Proteins were precipitated with antilaminin antibodies and Pansorbin, and subjected to SDS-PAGE under nonreducing (*a*) and reducing (*b*) conditions. Arrows show the origin of the separating gel and the buffer front, and arrowheads show the positions of molecular mass standards: *L* laminin (800 kD); *N*, nidogen (150 kD); *A*, laminin A chain (440 kD); *B*, laminin B chains (220 kD). Standards were detected by staining the gel with Coomassie Blue. ^{35}S-labeled proteins were detected by fluorography.

To determine the reasons for the substoichiometric labeling of this doublet and for the broad banding of the unreduced RN22 cell laminin, the unreduced band was excised in three parts from the nonreducing gel and again subjected to SDS-PAGE, this time under reducing conditions. The results (Fig. 6) indicate that the broad banding of unreduced RN22 cell laminin is due to heterogeneity: only that part of the band comigrating relatively slowly with PYS-2 laminin contains the high molecular weight doublet. The faster moving components, corresponding to the bulk of the unreduced RN22 cell laminin, only contained the B chains and did not have any detectable high molecular weight doublet: indeed on reduction, the fastest-migrating portion displayed the B chains together with an even smaller labeled band of ~180 kD.

To exclude that the heterogeneity of RN22 cell laminin was due to degradation of any 440-kD A chain initially produced by these cells, preliminary experiments were performed to show that no selective degradation of ^{125}I-labeled EHS tumor laminin chains could be detected when exposed to RN22 cells (data not shown). To exclude both external and intracellular degradation of laminin components, a pulse–chase experiment was performed, labeling the laminin with a 10-min pulse of [^{35}S]methionine. While equivalent labeling of both A and B chains of PYS-2 intracellular laminin was seen throughout the chase period (Fig. 7 *b*), little if any detectable A chain could be detected in the RN22 cells, even immediately after the pulse (Fig. 7 *a*). A similar labeling pattern was seen in RN22 cells that had been continuously exposed to 10^{-4} M leupeptin (Sigma Chemical Co.) for 18 h before and during the pulse to inhibit lysosomal degradation (data not shown; see reference 20). The lack of detectable 440-kD A chain from the RN22 cells is therefore most probably due to a lack of synthesis, rather than specific degradation.

Discussion

The results presented here show that RN22 Schwannoma cell

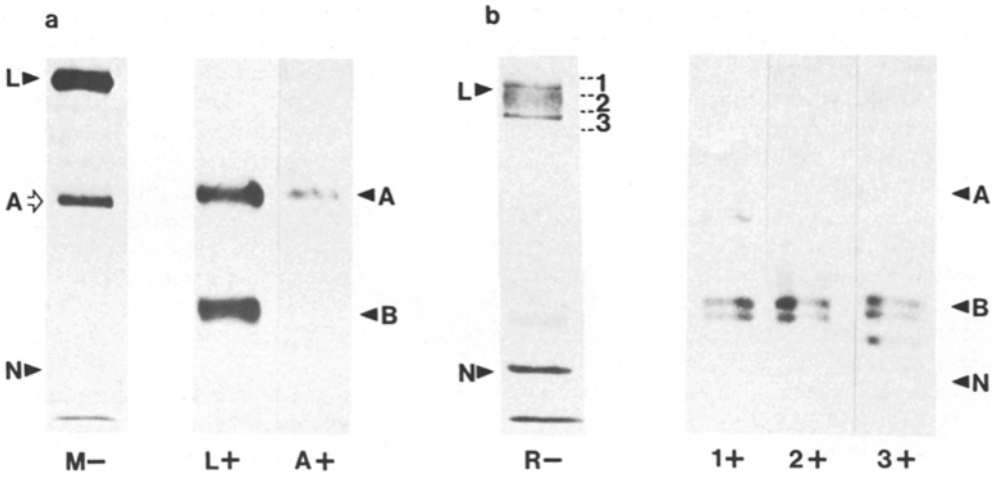

Figure 6. Two-dimensional SDS-PAGE of (*a*) PYS-2 cell and (*b*) RN22 cell immunoprecipitates. ^{35}S-labeled immunoprecipitates from conditioned media (see Fig. 4) were first subjected to electrophoresis under nonreducing conditions (−). Pieces of gel corresponding to *L* and *A* (*a*) and *1*, *2*, and *3* (*b*) were excised and their proteins then subjected to SDS-PAGE under reducing conditions (+). Arrowheads show the positions of molecular mass standards: *L*, laminin (800 kD); *N*, nidogen (150 kD); *A*, laminin A chain (440 kD); *B*, laminin B chains (220 kD). ^{35}S-labeled proteins were detected by fluorography.

laminin is not recognized by specifically those antibodies that can inhibit the neurite-promoting activity of EHS laminin. This is due to the lack of the 440-kD A chain in the majority of the RN22 laminin molecules, part of this chain constituting the heparin-binding fragment which contains the blocking epitopes. These observations indicate that this fragment is most likely not the actual neurite-promoting site of laminin, the most parsimonious hypothesis being that another (nonantigenic) site in the vicinity of the heparin-binding fragment, and common to EHS and RN22 laminins, is responsible for both their activities. A good candidate fulfilling these criteria appears to be the sequence of the B chains of laminin, corresponding to the defined proteolytic 25-kD fragment. This fragment originates from a region close to the carboxyl terminus of the B chains, which is located near the heparin-binding domain of EHS laminin, and is not antigenic within the native molecule (see reference 33). However, on partial proteolysis of laminin to produce the long arm fragment containing its neurite-promoting site, E8, these epitopes become exposed so that they can now bind antibodies. The resulting inhibition of neurite outgrowth provides direct evidence for the involvement of the B chains at or near to the neurite-promoting site of laminin from the EHS tumor, and also, by implication, of RN22 cell laminin and other neurite-promoting factors.

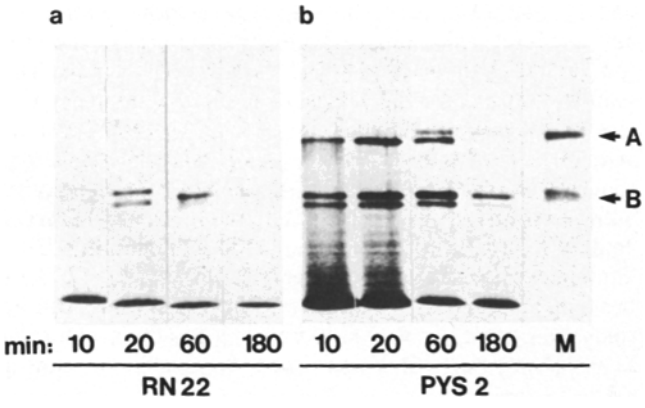

Figure 7. Pulse-chase metabolic labeling of laminin in (*a*) RN22 and (*b*) PYS-2 cells. The cells were pulsed with [^{35}S]methionine for 10 min followed by a chase of unlabeled methionine for the times shown in minutes (*min*). The cells were extracted with detergent and the laminin in the extracts subjected to immunoprecipitation followed by SDS-PAGE under reducing conditions and fluorography (see Fig. 5). Lane *M* shows the position of the laminin A (440 kD) and B (220 kD) chain molecular mass markers obtained from the culture medium of PYS-2 cells labeled for 18 h with [^{35}S]methionine.

Comparison of EHS and RN22 Laminins

The purpose of this comparison is not to present a comprehensive structural analysis of the two laminin molecules (the complete molecular structure of EHS tumor laminin has still not been established; see references 26, 41); the point is to define molecular structures that are either unique to one variant of laminin, or alternatively that are shared by the two molecules, and which could therefore account for a common neurite-promoting site. A further aim of this analysis was to try to provide a precise explanation in molecular terms for the lacking antibody inhibition of neurite outgrowth on RN22 cell laminin.

Initial immunoprecipitation and immunoblotting experiments showed that antibodies directed against the heparin-binding fragment of EHS laminin failed to recognize RN22 cell laminin. Although this could be due to species differences, we think it unlikely because it has been shown previously that mouse and rat laminins are antigenically very similar (18). Furthermore, the immunoblots presented here and elsewhere (6) indicated that the heparin-binding fragment of laminin is found on the A chain so that it seems the lack of the epitopes is a direct consequence of the absence of the 440-kD A chain.

The presence of B chains without the 440-kD A chain has previously been reported for laminin from a variety of tissues and species, including mouse (9, 11–13, 22, 24). We show here by metabolic labeling that a minor proportion of the laminin molecules synthesized by RN22 Schwannoma cells (estimated to be <20% from the data presented in Fig. 6) does indeed contain relatively high molecular mass components that migrate as a doublet, somewhat faster than the A chain

of laminin synthesized by PYS-2 teratocarcinoma cells or EHS tumor laminin (see also references 24, 34). The majority of the laminin molecules from the RN22 cells, however, consists of covalent oligomers of bands that, when reduced, migrate as 220-kD B chains with no detectable polypeptide migrating in the vicinity of the A chain. A third (minor) subpopulation of molecules was found to also contain a component migrating somewhat faster than the B chains (M_r ~180 kD). The relationship of this polypeptide to the others remains unknown; it was not possible to obtain any evidence that it is a breakdown product of one of the larger chains, the occasional appearance of the 180-kD band not being correlated with the decrease in the amount of any of the other bands.

Since the mobility of RN22 cell laminin is only marginally faster than that of those molecules containing both A and B chains (see also reference 12), then RN22 laminin most probably comprises more than just two B-chains held together by disulfide bonds. This is indicated by rotary shadowing (Fig. 1) that reveals a structure larger and more complex than could be accounted for by two B chains alone (12, 26). Furthermore, B chain dimers have been shown to have a mobility similar to that of the 440-kD A chain on SDS-PAGE (34; and own unpublished observations), and no such species was seen in nonreduced RN22 cell laminin (Fig. 6 *b*). It is interesting to note that pulse–chase experiments with human choriocarcinoma cells have shown that in order to be released from the cells, it is necessary for the A chain to be present in the laminin molecule (34), whereas A chains alone can be released from both these and teratocarcinoma cells (10, 34; see also Fig. 6 *a*). Such observations have led to the suggestion that the A chain functions as a "secretory peptide" (34). If this should be the case, then RN22 laminin might contain a smaller A chain variant that comigrates with the B chains, and one that does not have those A chain epitopes recognized by blocking antibodies. The speculative model presented in Fig. 1 is compatible with the Y-shaped (rather than cross-shaped) structure observed after rotary shadowing of purified RN22 cell laminin (12; Edgar, D., and M. Paulsson, unpublished observations).

The absence of a 440-kD A chain in RN22 Schwannoma laminin and elsewhere (12, 22, 24, 31) may be a consequence of degradation. However, the pulse–chase experiments shown here do not provide evidence for the rapid degradation of A chain by RN22 cells. More definitive evidence for or against the possibility of specific A chain degradation awaits measurement of the relative levels of mRNAs for the individual laminin chains. It should be noted however that low levels of A chain mRNA relative to that of the B chains have been reported recently in a variety of tissues (21). Thus, low levels of laminin 440-kD A chains, or the presence of A chain variants that do not coss-react with the probes available, seems not to be restricted to RN22 Schwannoma cells. Whether such variations in laminin result in differing biological properties remains to be seen.

Antibody Inhibition of Neurite Outgrowth

The analysis of the structure of RN22 cell laminin and its interaction with antibodies shows that the epitopes provided by the heparin-binding fragment are absent. This observation provides a direct explanation why no antibody inhibition of RN22 laminin is seen: these epitopes are required if antisera against EHS laminin are to block neurite outgrowth. It has recently been reported that human placental laminin is unable to bind heparin although it stimulates neurite outgrowth (17). Taken together, these results indicate that the heparin-binding fragment of EHS laminin does not constitute the actual neurite-promoting site, and that the antibody inhibition observed is due to steric hindrance. One corollary of these observations is that the neurite-promoting site of native laminin molecules does not seem to be significantly antigenic, antibody inhibition relying either on epitopes provided by the 440-kD A chain close to the site (as discussed for EHS laminin), or by more distant sites when the range of the blockade is increased by the use of secondary antibodies (17). Alternatively, it has been shown that a blocking epitope can be created by the association of laminin with other molecules (7).

The accessibility of epitopes to blocking antibodies is clearly a prerequisite for inhibition of neurite outgrowth. This is well illustrated by differences in the susceptibilities to antibody inhibition of neurite outgrowth on laminin and its long arm fragment E8 (see model presented in Fig. 1). Antibodies raised against the 25-kD laminin B chain fragment from the end of the rodlike segment of the long arm show little inhibition of neurite outgrowth on laminin, as they fail to recognize this epitope efficiently within the native molecule (33). Such antibodies are however able to recognize the long arm fragment E8 (see also reference 33), and so have the potential to block neurite outgrowth on this fragment. Thus, the rest of the intact laminin molecule is either hindering access of these antibodies to their binding sites within the long arm, or alternatively the structure of the 25-kD fragment epitopes is constrained within the intact molecule, rendering them unrecognizable.

As with all antibody inhibition studies, however, it cannot be concluded whether the anti–25 kD antibodies bind to the actual neurite-promoting site of fragment E8 (and therefore of laminin; see reference 16), or if their inhibitory activity, like that of antibodies to the heparin-binding fragment, is a consequence of steric hindrance. However, as the epitopes of the 25-kD fragment are also present on the (denatured) B chains of RN22 laminin, this fragment can now be regarded as a candidate to be either whole or part of the neurite-promoting site common to both EHS and RN22 laminins. The inability of anti–25-kD fragment antibodies to recognize their epitopes when present in either EHS or RN22 laminins adds weight to this hypothesis, as it is consistent with the notion that the neurite-promoting site cannot be antigenically active in the native molecules (see above). This 25-kD fragment is well defined, its sequence and location within EHS laminin being characterized (see references 26, 33). This presents a possibility to construct oligopeptides that can be tested in competition with laminin for interactions with cells to establish directly if it constitutes the neurite-promoting site.

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