Mapping of Domains in Human Laminin Using Monoclonal Antibodies: Localization of the Neurite-promoting Site

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Abstract. Monoclonal antibodies were made against a truncated form of human laminin isolated from placenta. 12 antibodies were isolated and characterized. All antibodies stained basement membranes in placenta and immunoprecipitated laminin from media of cultured choriocarcinoma cells. Three antibodies, 3E5, 4C7, and 4E10, partially blocked the neurite-promoting activity of laminin. Addition of a second antibody, goat anti-mouse IgG, caused more complete blocking of the activity. Two of the blocking antibodies, 4C7 and 4E10, reacted with epitopes within the globular domain at the end of the long arm of laminin, and the third one, 3E5, reacted at the end of the rod-like portion of the long arm adjacent to the globular domain, as shown by electron microscopy after rotary shadowing. Five nonblocking antibodies used in the same test reacted with epitopes in other domains of the molecule. Blocking antibodies 3E5 and 4E10 could be used in immunoblotting and both antibodies reacted with the same polypeptides in pepsin fragments of human laminin, the predominant polypeptides being ~400 kD. When a crude extract of human amnion was used as a source of intact laminin, the 4E10 antibody detected a single polypeptide of ~400 kD. A nonblocking antibody, 2E8, which reacted at the center of the laminin cross, reacted predominantly with a 200-kD polypeptide in human laminin fragments and exclusively with a 200-kD polypeptide in amnion extract and in rat laminin. Our results with human laminin match the results by Edgar, D., R. Timpl, and H. Thoenen, 1984, *EMBO (Eur. Mol. Biol. Organ.) J.*, 3:1463–1468, in which the neurite-promoting activity of mouse laminin resides at the end of the long arm, which is also the site for heparin binding. However, since the active fragments of human laminin did not bind to heparin, the neurite-promoting site should be different from the heparin-binding site. Our results further suggest that the neurite-promoting site may be contained in or close to the 400-kD component of laminin.

LAMININ is a large glycoprotein present in all basement membranes (Chung et al., 1977; Timpl et al., 1979; Hogan, 1980; Sakashita and Ruoslahti, 1980). It has a molecular mass of ~900 kD and is composed of disulfide-cross-linked polypeptides with M_rs of ~200 and 400 kD, as determined by SDS PAGE. In electron microscopy after rotary shadowing, purified laminin has the shape of a cross with one long and three short arms (Engel et al., 1981). The long arm ends with one large globule and the short arms each end with two smaller globules.

Numerous biological activities have been assigned to laminin (Timpl et al., 1983; Kleinman et al., 1985; von der Mark and Kuhl, 1985). Laminin has been reported to affect cell adhesion and migration (Terranova et al., 1980; Carlsson et al., 1981; Johansson et al., 1981; Darmon, 1982; Terranova et al., 1982; McCarthy et al., 1983; Couchman et al., 1983; Bronner-Fraser, 1984; Goodman and Newgreen, 1985) and growth and differentiation of nucleated cells in vitro (Rizzino et al., 1980; Grover et al., 1983), to promote adhesion of platelets (Ill et al., 1984), and to bind to collagen (Woodley et al., 1983; Kleinman et al., 1983; Charonis et al., 1985), heparin, and other glycosaminoglycans (Sakashita et al., 1980; Del Rosso et al., 1981), lectins (Shibata et al., 1982), plasminogen and its tissue-type activator (Salonen et al., 1984), sulfated glycolipids (Roberts et al., 1985), bacteria (Switalski et al., 1984; Speziale et al., 1982; Lopes et al., 1985), and the complement components Clq (Bohnsack et al., 1985) and C3 (Leivo and Engvall, 1986). Some of these activities have been assigned to different molecular domains of laminin. Thus, the end of the long arm of laminin may bind to heparin (Ott et al., 1982), type IV collagen (Charonis et al., 1985), and other laminin molecules (Yurchenco et al., 1985), and the ends of the short arms may bind to type IV collagen (Charonis et al., 1985), the collagenous domains of Clq (Bohnsack et al., 1985), and to other laminin molecules (Yurchenco et al., 1985).

Perhaps the most potent activity of laminin described so far is its capacity to induce and promote neurite outgrowth

in cultured neurons from both the central and peripheral nervous systems (Baron-Van Evercooren et al., 1982; Manthorpe et al., 1983; Rogers et al., 1983; Wewer et al., 1983; Edgar et al., 1984; Smalheiser et al., 1984; Adler et al., 1985). Other extracellular matrix components, including fibronectin and different types of collagens, may also show neurite-promoting activity (Akers et al., 1981; Vlodavsky et al., 1982; Rogers et al., 1983; Manthorpe et al., 1983; Adler et al., 1985), but their specific neurite-promoting activities vary with different cell types and tend to be lower than that of laminin (Davis et al., 1985b).

Further evidence for the high neurite-promoting potency of laminin comes from experiments in which factors capable of promoting neurite outgrowth from cultured neurons have been demonstrated in and purified from conditioned media from different cell types (Davis et al., 1985a; Lander et al., 1985). These purified factors have subsequently been shown to contain laminin, often in complex with proteoglycans, but not to contain fibronectin or collagen. It seems likely that laminin is the active species in these factors.

Relationships between structure and function of laminin are incompletely known. In our hands it has been difficult to prepare defined domains of laminin by proteolytic digestion for structural and functional studies. Furthermore, at least the neurite-promoting activity of laminin is dependent on an intact secondary or tertiary structure of laminin at the site that interacts with neurons. Conformational changes in laminin induced by urea or low pH lead to loss of neurite-promoting activity (our unpublished observation). We therefore decided to prepare monoclonal antibodies against laminin and to use these to probe for structure and function. In this report we describe the production and characterization of such antibodies and the use of these antibodies to localize the neurite-promoting site of laminin. Our findings with human laminin are consistent with the results of Edgar et al. (1984) who reported that the neurite-promoting activity of mouse laminin is located close to the previously described heparin-binding domain at the end of the long arm. However, since the truncated human laminin used in the present work does not bind to heparin but does promote neurite outgrowth, the neurite-promoting site appears to be different from the heparin-binding site. In addition, we show here that epitopes in the 400-kD component of laminin are present at the end of the long arm and suggest that the neurite-promoting site of laminin may be contained within its 400-kD component.

Materials and Methods

Isolation of Laminin

Human laminin was isolated in a truncated form as a set of large fragments from a mild pepsin digest of human placenta by affinity chromatography on monoclonal antibody Sepharose essentially as previously described (Wewer et al., 1983). The modifications used included the use of a lower concentration of pepsin (0.1 mg/ml) for digestion and the use of 4 M KSCN for elution of laminin from the antibody column. The eluted material was dialyzed against 50 mM Tris-HCl, pH 8.2, and further purified by fast protein liquid chromatography (Pharmacia Fine Chemicals, Piscataway, NJ) using a Mono Q column (Pharmacia Fine Chemicals). The column was eluted using stepwise increases in NaCl concentration. A major protein peak with laminin immunoreactivity eluted at salt concentrations between 0.30 and 0.35 M. This material was used in the present study. Rat laminin was purified from rat yolk sac tumors (Engvall et al., 1983). Mouse laminin was purchased from Bethesda Research Laboratories, Gaithersburg, MD.

Analysis of Heparin-binding Capacity of Human Laminin Fragments

3 mg of purified human laminin fragments in PBS, pH 7.2 (0.05 M phosphate, 0.10 M NaCl), were fractionated on a 1.5 × 16-cm column of heparin-Sepharose (Pharmacia Fine Chemicals) in PBS. The column was washed with PBS and eluted with 0.5 M NaCl in PBS. Laminin in the bound and unbound fractions was quantitated by absorbance at 280 nm and by reactivity with antibodies in ELISA (Engvall and Perlmann, 1972).

Production of Monoclonal Antibodies

BALB/c mice (Bantin and Kingman, Fremont, CA) were immunized with human laminin fragments and the spleen cells from immunized mice were fused with cells of the myeloma line X63-Ag8.563, using polyethylene glycol according to techniques described previously (Ruoslahti et al., 1982; Hessle and Engvall, 1984). Hybridomas were selected based on production of antibodies reacting with human laminin in ELISA (Engvall and Perlmann, 1972), and on capacity to stain basement membranes in indirect immunofluorescence of sections of placenta (Hessle et al., 1984). Quantities of monoclonal antibodies were produced from cloned hybridomas by cell culture or by ascites production in syngeneic mice. The antibodies were purified by ammonium sulfate precipitation and protein A Sepharose (Pharmacia Fine Chemicals) affinity chromatography (Ey et al., 1978).

Indirect Immunofluorescence and Immunoprecipitation of Laminin from Cultured Cells

The procedures used were those described earlier (Hessle et al., 1984; Hessle and Engvall, 1984). Frozen sections of placenta were air dried and fixed in acetone. The sections were incubated with hybridoma culture medium, washed, and incubated with fluorescein-labeled goat anti-mouse IgG diluted in PBS. Choriocarcinoma cells (JAR) were labeled metabolically with [^{35}S]methionine (New England Nuclear, Boston, MA). Culture media were collected and made 0.5% Triton X-100, 0.5 M NaCl, and 0.2 mM phenylmethylsulfonylfluoride. Monoclonal antibodies were adsorbed to protein A Sepharose beads, presaturated with rabbit anti-mouse IgG, and the antibody beads were then incubated with labeled culture media for 3 h at room temperature. The beads were washed with PBS-Tween 20 and bound protein was eluted by boiling in 4% SDS. The samples were analyzed by SDS PAGE. The gels were fixed and treated with Enlightning (New England Nuclear) before drying and autoradiography.

Ranking of Affinities of Monoclonal Antibodies

The relative affinities of the antibodies were ranked by diluting purified antibodies in microtiter wells coated with 100 μl of a solution of 1 μg/ml human laminin and by detecting bound antibodies with ELISA (Engvall and Perlmann, 1972). The lower the antibody concentration required to achieve 50% of maximum binding, the higher the affinity of that antibody. Therefore, the concentration of antibody at half of its maximal absorbance in ELISA was taken as a relative measure of its affinity (Van Heyningen et al., 1983). Assays were also done using microtiter wells pretreated with polyornithine (see below).

Electron Microscopy of Laminin-Antibody Complexes

100 μg of laminin and 30 μg of monoclonal antibody in 1 ml 0.1 M ammonium acetate, pH 7, were incubated at 37°C for 1 h. The solution was then sprayed on freshly cleaved mica discs and evaporated. The samples were processed for transmission electron microscopy after rotary shadowing as described (Engvall et al., 1983; 1986).

SDS PAGE and Immunoblotting

SDS PAGE was performed either on gels of 5% acrylamide using a discontinuous buffer system according to Laemmli (1970) or on 2–16% gradient gels (Pharmacia Fine Chemicals) at pH 7.2. After electrophoresis, proteins on the gels were electrophoretically transferred to nitrocellulose in a Tris-glycine buffer, pH 7.4, at 20 V for 16 h. Under these conditions, laminin polypeptides transferred completely and only traces of myosin used as molecular mass marker remained in the gels. Polypeptides on the nitrocellulose were stained with Amido black or detected immunologically by ELISA

after saturation of the nitrocellulose with 2% BSA in PBS. Hybridoma culture medium diluted 1:5 or rabbit antiserum diluted 1:1000 were incubated with the filters for 3 h. Peroxidase-labeled antiimmunoglobulin (Bio-Rad Laboratories, Richmond, CA) was used to detect bound antibodies and diamino benzidine (Litton Bionetics, Inc., Charleston, SC) was used for the color reaction.

Neurite Promotion and Inhibition Assays

The assays were performed using embryonic day 8 chick ciliary ganglion neuronal cultures essentially as previously described (Manthorpe et al., 1983; Davis et al., 1985a). Briefly, polyornithine-coated tissue culture plastic wells (6-mm diameter, 96-well microplates) were treated with 5 μg/ml of human laminin in PBS for 2–3 h at 37°C. The wells were washed once with 100 μl PBS containing 1% BSA and were either left untreated or incubated for 3–4 h at 37°C with a 1:5 dilution of hybridoma supernate or 1 μg/ml purified monoclonal antibodies. After washing two times 100 μl with PBS, the wells were incubated 2 h at 37°C with a 1:50 dilution of rabbit anti-mouse IgG or control (rabbit anti-human alpha-fetoprotein) antisera. The wells were again washed once with 100 μl PBS and 100 μl culture medium (Dulbecco's modified Eagle's basal medium supplemented with 0.5% BSA, 8×10^{-7} M insulin, 3.3×10^{-2} M glucose, 2.6×10^{-2} M $NaHCO_3$, 2×10^{-3} M L-glutamine, 100 μm/ml penicillin, and 100 trophic units/ml ciliary neuronotrophic factor) containing 1,000 neurons was added. Cultures were fixed after 3 h by the addition of 200 μl 2% glutaraldehyde for 20 min, washed with water, and stained with 0.1% toluidine blue in water. About 150 neurons were observed microscopically for each culture condition. Neurons were recorded as neurite-bearing if they possessed at least 50 μm of total neurite length (Davis et al., 1985c).

Results

Characterization of Human Laminin Pepsin Fragment

The material purified by fast protein liquid chromatography was characterized by SDS PAGE (Fig. 1). Unreduced, the truncated human laminin ran as a major band of slightly smaller molecular size than unreduced mouse laminin used for comparison. After reduction, the mouse laminin showed the characteristic 200- and 400-kD polypeptides, while the human laminin showed several bands ranging from <100 kD to >400 kD. Because of the complex pattern of polypeptides in human laminin, it was not possible in this test to establish a relationship of any particular polypeptide in human laminin with either the 400- or the 200-kD polypeptide in mouse or rat laminin. Furthermore, the presence of polypeptides larger than 400 kD in the reduced human laminin suggests that laminin in normal tissues, such as placenta, may be cross-linked by other than only disulfide bonds. The isolated laminin fragments did not bind to heparin in affinity chromatography under the conditions used (not shown). Most mouse and rat laminins bind to heparin under similar conditions (Sakashita et al., 1980; Engvall et al., 1983).

Isolation and Characterization of Monoclonal Antibodies to Human Laminin

12 monoclonal antibodies that reacted with human laminin in ELISA were raised and characterized. All the antibodies were of the IgG_1 subclass and had κ light chains. When tested in ELISA against rat and mouse laminins, only antibody 2E8 reacted with rat laminin, and, as expected, none of the antibodies reacted against mouse laminin. In indirect immunofluorescence, all the antibodies gave similar staining of both the trophoblast and the blood vessel basement membranes in human placenta and all the antibodies immunoprecipitated laminin from culture medium of JAR choriocarcinoma cells (not shown).

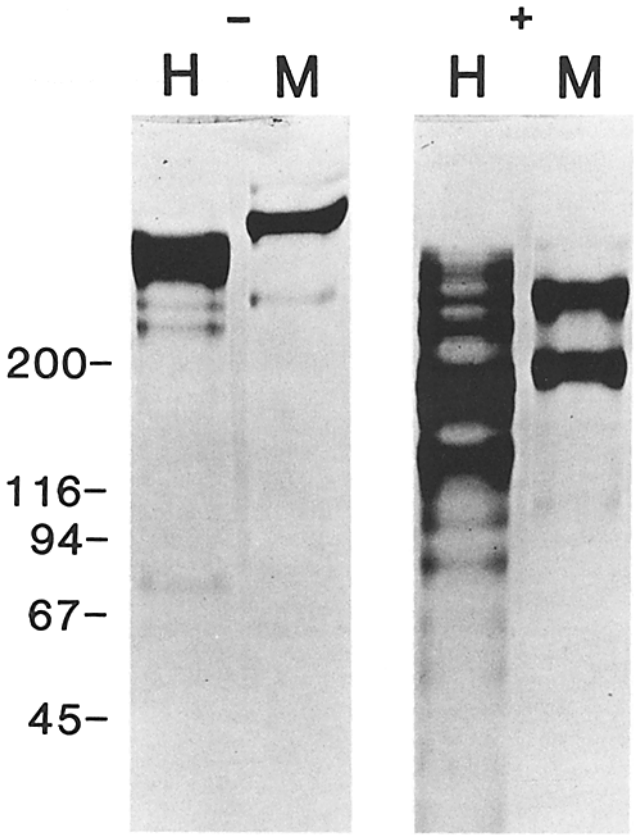

Figure 1. SDS PAGE analysis of purified laminins on a 2–16% acrylamide gradient gel. Pepsin-fragmented human laminin after purification by antibody affinity chromatography and fast protein liquid chromatography (H) and mouse laminin (M) were electrophoresed without (−) or with (+) reduction with 0.1 M 2-mercaptoethanol. The mobility on the same gel of proteins of known molecular masses are indicated.

Effect of Monoclonal Antibodies on Outgrowth of Neurites from Neurons Cultured on Human Laminin

Neurons from chicken embryo ciliary ganglia show extensive outgrowth of neurites after 3 h of culture on a substrate of polyornithine and laminin (Davis et al., 1985c). Treatment of the polyornithine–human laminin substrate with culture medium from 9 out of 12 hybridomas had no apparent effect on neurite outgrowth (Fig. 2, Table I). However, three hybridomas, 3E5, 4C7, and 4E10, appeared to produce antibodies capable of reducing the incidence of neurite-bearing cells and the length of individual neurites (Fig. 2).

Amplification of Monoclonal Antibody Blocking by Second Antibody

To test possible mechanisms by which the three antibodies, 3E5, 4C7, and 4E10, interfered with the neurite-promoting activity of laminin, we added a second antibody, an antibody to mouse IgG, to the neurite assay. The second antibody enhanced the blocking activity of antibodies 3E5, 4C7, and 4E10, suggesting that the blocking is the effect of steric hindrance (Fig. 2, Table II). The second antibody had no effect on the neurite-promoting activity of laminin when used alone or in combination with other laminin antibodies. Rab-

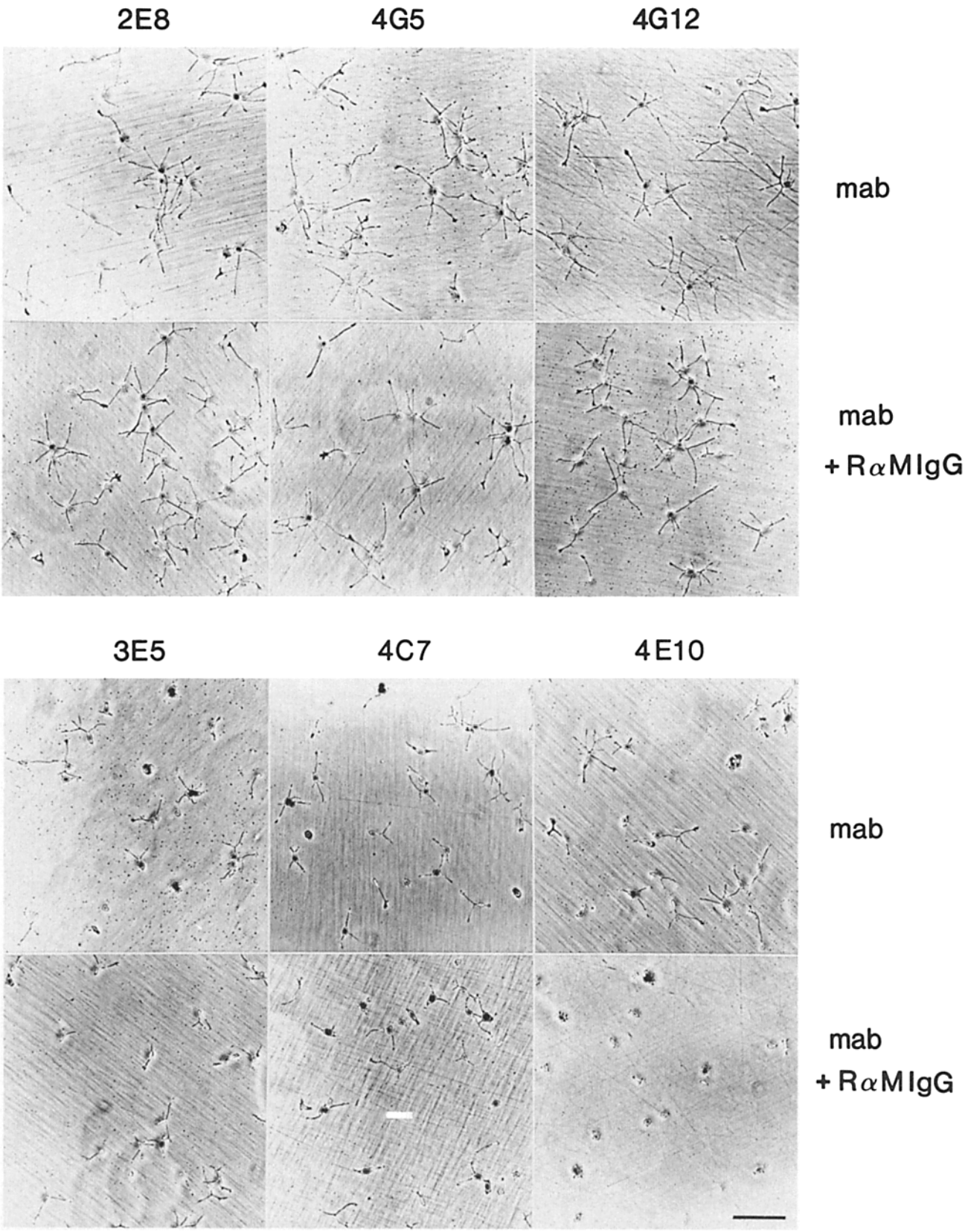

Figure 2. Effect of monoclonal antibodies to human laminin on extension of neurites from neurons cultured on human laminin-coated substrata. Neurons were plated in micro wells treated sequentially with polyornithine, human laminin, hybridoma culture medium (mAb: 2E8, 4G5, 4G12, 3E5, 4C7, and 4E10), and second antibody (rabbit anti-mouse IgG, RαMIgG, or rabbit anti-human alpha-fetoprotein as control). The cells were fixed after 3 h and stained with toluidine blue. Bar, 98 μm.

Table I. Influence of Monoclonal Antibodies on the Neurite-promoting Activity of Human Laminin

Monoclonal antibody	Percent of neurons with neurites			
	Experiment I*		Experiment II‡	
	− 2nd Ab§	+ 2nd Ab	− 2nd Ab	+ 2nd Ab
−	85	82	85	85
2D9	86	81	NT‖	NT
2D10	85	74	NT	NT
2E8	85	79	86	88
2G4	82	79	NT	NT
2G6	82	76	90	91
3C7	81	77	NT	NT
3E5	46	33	61	42
4C7	82	51	66	39
4C9	77	61	86	75
4E10	16	1	44	4
4G5	86	76	NT	NT
4G12	79	70	89	75

* Performed using hybridoma culture medium at a 1:5 dilution.
‡ Performed using purified antibody at 1 μg/ml.
§ 2nd Ab, rabbit anti-mouse IgG.
‖ NT, not tested.

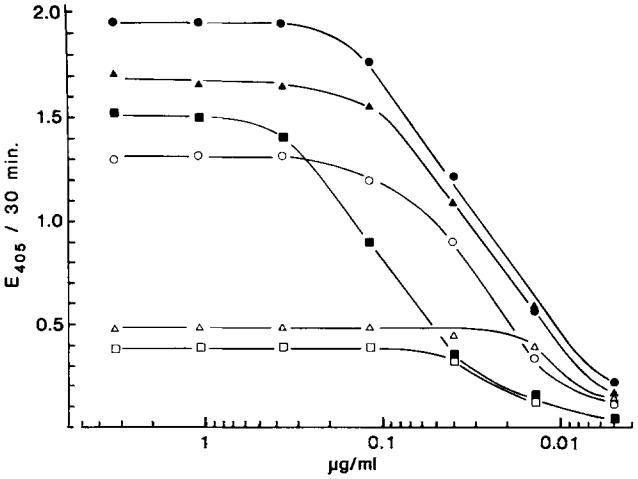

Figure 3. Titration of monoclonal antibodies in ELISA. Purified antibodies were serially diluted in microtiter wells coated with laminin. Bound antibodies were detected using goat anti-mouse IgG coupled to alkaline phosphatase and the enzyme activity was measured using *p*-nitrophenyl phosphate. The antibodies shown are 2E8 (*solid circle*), 4E10 (*solid triangle*), 2G6 (*solid square*), 3E5 (*open circle*), 4C7 (*open triangle*), and 4G5 (*open square*).

bit antibodies of irrelevant specificities had no effect on blocking or nonblocking monoclonal antibodies.

Lack of Correlation between Neurite Blocking Activity and Antibody Affinity

To exclude the possibility that the differences in the capacities of the antibodies to block the neurite-promoting activity of laminin would be due to differences in antibody affinity or level of antibody binding to laminin, the antibodies were titrated in ELISA (Fig. 3). The highest level of binding of antibody to laminin was obtained with antibodies 2E8, 2G4, and 4E10 (Fig. 3, Table II). Since the laminin was isolated with the 4E10 antibody, the 4E10 binding level was arbitrarily set at 100%. Other antibodies reached plateau levels of 75–100%, suggesting that most, but not all, molecules of laminin on the solid phase contained the epitopes for these antibodies. A few antibodies, including 4C7 and 4G5 (Fig. 3), reached lower plateau levels, indicating that these antibodies reacted with epitopes not present in all the laminin fragments or with epitopes unavailable in some laminin molecules coated on polystyrene. Similar results were obtained with assay plates pretreated with polyornithine.

The highest relative affinities were measured for antibodies 4C7 and 4C9 (Table II). 4C7 showed significant inhibition of neurite-promoting activity of laminin while 4C9 did not. Out of four antibodies with relative affinities between 1.4 and 1.8×10^{-8} M, two, 3E5 and 4E10, showed inhibition, while the other two, 2E8 and 4G5, did not. Thus, there was no direct correlation between affinity and blocking capacity.

Table II. Relative Affinities of Monoclonal Antibodies to Human Laminin Measured as the Concentration of Antibody Required to Attain 50% of Maximal Binding in ELISA

Antibody	Relative affinity	Level of maximum binding*
	$M \times 10^{-8}$	%
2D9	1.3	75
2D10	7.3	57
2E8	1.8	115
2G4	9.0	103
2G6	5.7	89
3C7	NM‡	>61
3E5	1.7	76
4C7	0.63	25
4C9	0.83	58
4E10	1.7	100
4G5	1.5	23
4G12	2.1	86

* Measured as percent of binding of antibody 4E10.
‡ NM, not measurable.

Localization of Epitopes in Laminin by Electron Microscopy

To localize the epitopes for the monoclonal antibodies in laminin, complexes of human laminin and individual monoclonal antibodies were prepared and examined by electron microscopy after rotary shadowing. Fig. 4 shows a gallery of images of complexes prepared with different monoclonal antibodies. The blocking antibodies, 3E5, 4C7, and 4E10, all reacted with epitopes located near the end of the long arm. The 4E10 and 4C7 antibodies bound to the large globular domain at the end of the long arm and the 3E5 antibody bound on the long arm of laminin just above this domain. The nonblocking antibodies, 2E8, 2G6, 4C9 (not shown), 4G5, and 4G12, bound to sites elsewhere in the molecule (Fig. 4). For most antibodies tested, a high incidence of laminin–antibody interactions was observed with the rotary shadowing technique. The majority of structures that could be identified as laminin had one antibody molecule bound per laminin molecule and the antibody was always bound in the same place. A high incidence of laminin–antibody interaction was also seen with antibody 4C7, in contrast to the relatively low level

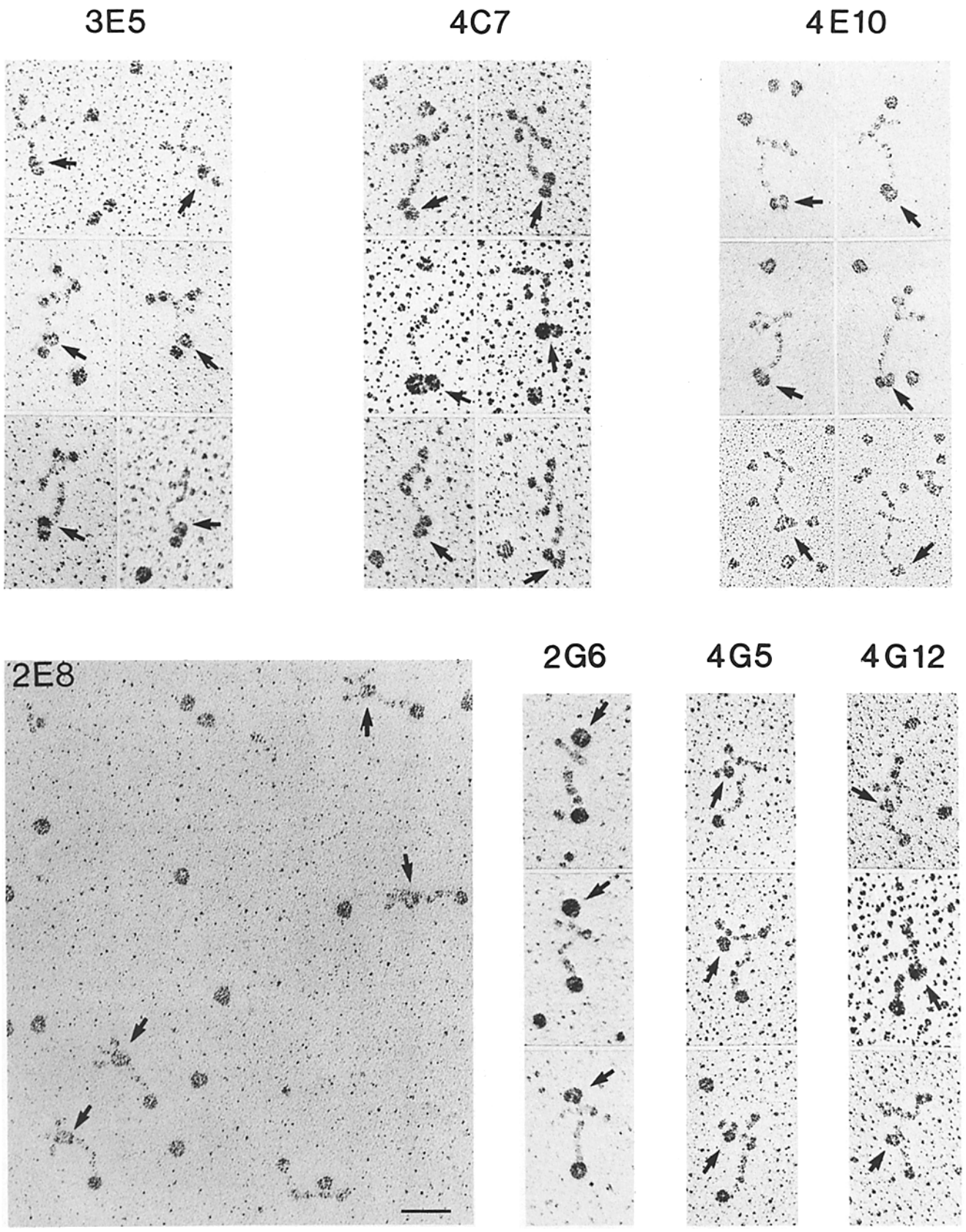

Figure 4. Electron microscopy after rotary shadowing of laminin-antibody complexes prepared using antibodies 4C7, 4E10, 3E5, 2E8, 2G6, 4G5, and 4G12. Arrows indicate antibodies bound to laminin. Bar, 68 μm.

of reactivity of this antibody with laminin in ELISA (Fig. 3). For antibodies 4G5 and 2G6, a lower incidence of laminin interaction was apparent, possibly because these antibodies reacted with sites on the short arms that were often missing from the truncated form of laminin. When laminin was incubated with an unrelated antibody, no laminin–antibody complexes were found.

These results suggested that the neurite-promoting site of laminin is located at the end of the long arm in the vicinity of the 4E10, 4C7, and 3E5 epitopes.

Reactivities of Monoclonal Antibodies with Laminin Subunits

Immunoblotting was used to define the reactivity of the monoclonal antibodies with laminin subunits. 3 out of the 12 antibodies, 2E8, 3E5, and 4E10, reacted with human laminin polypeptides transferred to nitrocellulose after SDS PAGE (Fig. 5). All three reacted with multiple bands, reflecting the heterogeneous nature of the truncated human laminin and the presence of proteolytic cleavages at multiple sites in each polypeptide component. 3E5 and 4E10 gave identical patterns of reactivity, which were different from the pattern given by 2E8. The bands stained most intensely with antibodies 3E5 and 4E10 were two closely spaced bands at the level of the 400-kD component of rat laminin. Other strongly stained bands were located below the level of the 200-kD component of rat laminin. The band that stained most strongly with antibody 2E8 had a mobility equal to the 200-kD component. When 2E8 was used in immunoblotting of rat laminin, only the 200-kD component of rat laminin was stained (Fig. 6).

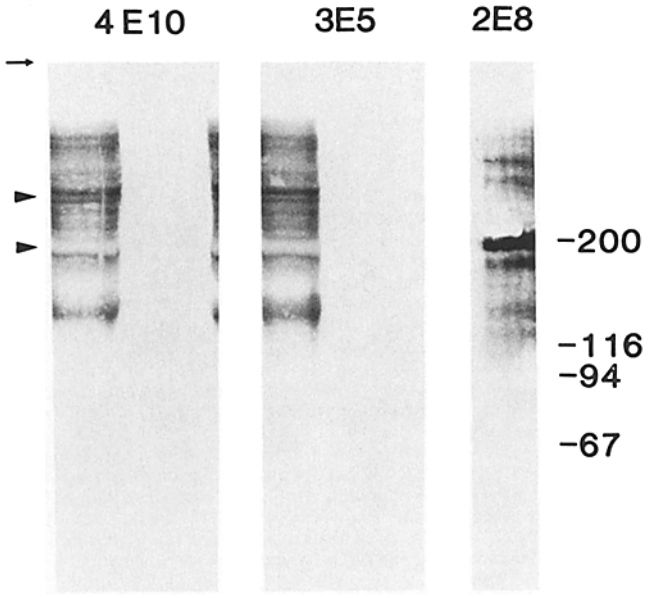

Figure 5. Immunoblotting of pepsin-fragmented human laminin using monoclonal antibodies. Purified human laminin and a mixture of marker proteins were reduced and submitted to electrophoresis in alternating lanes of a 2–16% gradient gel, transferred to nitrocellulose, and stained using monoclonal antibodies 2E8, 3E5, and 4E10 and peroxidase-labeled goat anti-mouse IgG. Mobilities of marker polypeptides are shown on the right and those of reduced mouse laminin are indicated by arrowheads on the left. The arrow indicates the top of the gel.

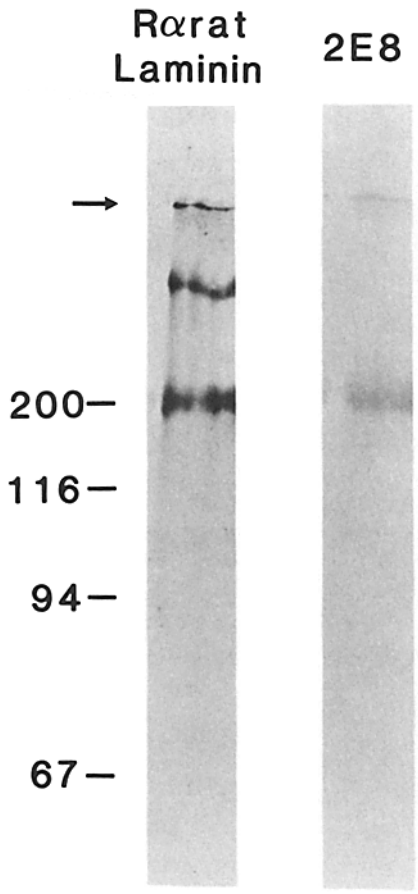

Figure 6. Immunoblotting of rat laminin, electrophoresed on a 5% acrylamide gel, using monoclonal antibody 2E8 and rabbit anti-rat (Rαrat) laminin. The arrow indicates the top of the gel.

Since we did not have sufficient amounts of intact human laminin available, a 2% SDS, 1% mercaptoethanol extract of human amnion was used as a source of intact laminin, this material was subjected to SDS PAGE, and the protein bands were transferred to nitrocellulose. In this complex mixture of amnion proteins, the 4E10 antibody could be shown to react only with a band at 400 kD, whereas the 2E8 antibody reacted only with a band at 200 kD (Fig. 7).

Discussion

We have produced a panel of monoclonal antibodies to human laminin and used these to localize the neurite-promoting site. 3 out of the 12 monoclonal antibodies characterized partially blocked the neurite-promoting activity of laminin. More substantial blocking of this activity could be obtained by adding an antibody to mouse IgG. These three blocking antibodies reacted at or close to the globular domain at the end of the long arm of laminin. Monoclonal antibodies reacting with domains in the laminin molecule distant from the end of the long arm had no effect on the neurite promotion either by themselves or in combination with antimouse IgG. We conclude that the three blocking antibodies react close to the neurite-promoting site of laminin. The fact that antibody affinity was not correlated with blocking activity and that an antibody to mouse IgG enhances the blocking by the

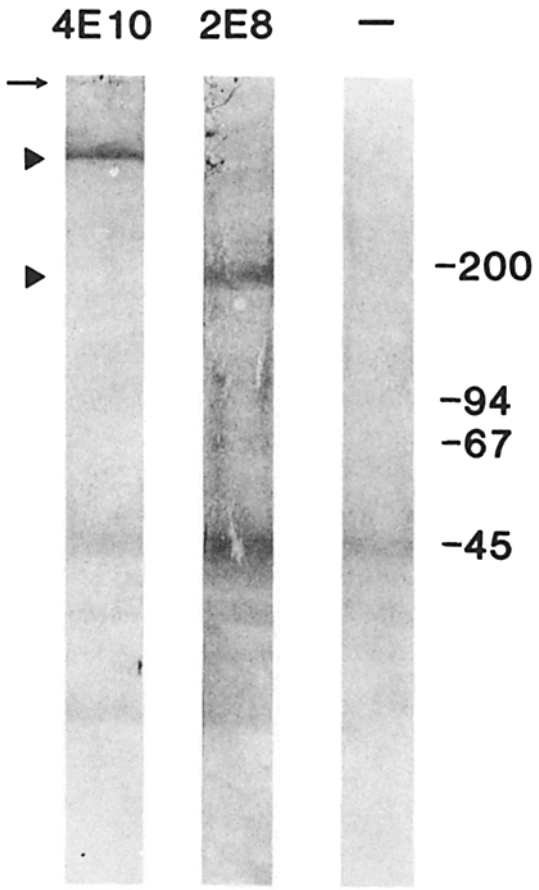

Figure 7. Immunoblotting of proteins from amnion. Proteins were extracted using 2% SDS and 1% mercaptoethanol, electrophoresed on a 2–16% gradient gel, transferred to nitrocellulose, and reacted with antibodies 4E10 and 2E8. Mobilities of marker polypeptides are shown on the right and those of reduced mouse laminin on the left (arrowheads). The arrow indicates the top of the gel.

primary antibody suggests that the mechanism of the blocking is by steric hindrance, the double layer of antibodies being more efficient in interfering with the neurite-promoting site than a single antibody.

Our results on the location of the neurite-promoting domain in laminin at the end of the long arm are similar to those of Edgar et al. (1984). These workers showed that a large fragment from mouse laminin consisting of the globular domain at the end of the long arm and a portion of the rod-like domain of the long arm contained neurite-promoting activity. Furthermore, rabbit antibodies against only the globular end domain blocked the neurite-promoting activity of intact laminin. Antisera against other fragments of laminin had no effect. That the end of the long arm of mouse laminin binds to heparin (Ott et al., 1982) in addition to promoting neurite outgrowth may be fortuitous. The two activities appear not to be mediated by the same site since the human laminin fragments that had full neurite-promoting activity did not bind to heparin.

The molecular architecture of laminin is not yet known in detail. A 400-kD polypeptide has been proposed to make up all (Rao et al., 1982; Liotta et al., 1981) or a major part (Palm et al., 1985) of the long arm of laminin and the 200-kD polypeptides are thought to make up the short arms. However, results from physical studies (Engel et al., 1981; Ott et al., 1982) and sequencing of cDNA (Barlow et al., 1984), as well as sequencing of proteolytic fragments (Paulson et al., 1985), have indicated that the COOH-terminal portions of the 200-kD polypeptides may form the rod-like portion of the long arm of laminin. Monoclonal antibodies could be useful in locating the individual polypeptides to different portions of the laminin cross. Three of our monoclonal antibodies reacted with conformation-independent determinants and could therefore be used to identify which laminin subunit contained the epitope by immunoblotting. One antibody, 2E8, reacted with a 200-kD polypeptide in both human and rat laminin. Its reactivity with the center of the laminin cross in electron microscopy thus establishes the presence of a 200-kD polypeptide in that area. The other two antibodies, 3E5 and 4E10, bound to sites a short distance apart in the laminin molecule, as evidenced by electron microscopy, and bound to the same peptides in human laminin fragments and with the 400-kD component in intact laminin. 3E5 and 4E10 therefore appear to bind to relatively closely spaced epitopes within the 400-kD polypeptide of laminin. Since both 3E5 and 4E10 were capable of blocking the neurite-promoting activity, the neurite-promoting site should be located close to the binding sites of these antibodies and would therefore be likely to reside within the 400-kD component. However, the assignment of the neurite-promoting site to a particular polypeptide domain will require further experimentation.

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