

## Laminin receptors for neurite formation

(neural outgrowth/cell surface/basement membrane/cell adhesion)

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**ABSTRACT** Laminin, a basement membrane glycoprotein promotes both cell attachment and neurite outgrowth. Separate domains on laminin elicit these responses, suggesting that distinct receptors occur on the surface of cells. NG108-15 neuroblastoma-glioma cells rapidly extend long processes in the presence of laminin. We report here that  $^{125}\text{I}$ -labeled laminin specifically binds to these cells and to three membrane proteins of 67, 110, and 180 kDa. These proteins were isolated by affinity chromatography on laminin-Sepharose. The 67-kDa protein reacted with antibody to the previously characterized receptor for cell attachment to laminin. Antibodies to the 110-kDa and 180-kDa bands demonstrated that the 110-kDa protein was found in a variety of epithelial cell lines and in brain, whereas the 180-kDa protein was neural specific. Antibodies prepared against the 110-kDa and 180-kDa proteins inhibited neurite outgrowth induced by the neurite-promoting domain of laminin, whereas antibodies to the 67-kDa laminin receptor had no effect on neurite outgrowth. We conclude that neuronal cells have multiple cell-surface laminin receptors and that the 110-kDa and 180-kDa proteins are involved in neurite formation.

Laminin, a major glycoprotein of basement membranes, induces a variety of cellular responses, including cell attachment (1–3) and neurite outgrowth (4–9). Laminin is composed of three chains, including the A chain (400 kDa), B1 chain (210 kDa), and B2 chain (200 kDa), which are arranged in a cross-shaped structure. A large fragment of laminin with cell-attachment activity has been produced by proteolysis and localized to the central portion of the cross where the three chains intersect (10). Subsequently, a synthetic pentapeptide, Tyr-Ile-Gly-Ser-Arg, was found to exhibit cell attachment activity for a variety of epithelial cells (11). However, this is not the only biologically active domain in laminin, since a separate site near the globule at the end of the long arm of the cross has neurite-promoting activity and cell attachment activity for certain cells (12, 13). Since these separate domains elicit distinct activities, it is likely that different receptors mediate these activities.

Previous studies have shown that there is a specific laminin receptor on cells (67 kDa) that recognizes the synthetic peptide Tyr-Ile-Gly-Ser-Arg from a sequence in the B1 chain (11, 14–16). Evidence that this receptor mediates cell attachment has been shown with antibody to the receptor, the Tyr-Ile-Gly-Ser-Arg peptide, and proteolytic fragments of laminin, all of which inhibit the attachment of a variety of cells to laminin (11). However, Tyr-Ile-Gly-Ser-Arg peptide and synthetic fragments from the intersection of the chains lack neurite-promoting activity. The cell-surface ligand responsible for laminin-mediated neurite outgrowth

has not been identified, but several candidates exist, including integrin, sulfatides, and gangliosides (17–20).

Here we have used laminin affinity chromatography to identify receptors on neuronal cells that might mediate neurite outgrowth. The NG108-15 neuroblastoma-glioma cells respond to laminin and extend processes within 1 hr (21). We find that these cells have three major cell-surface ligands that bind laminin, including the previously identified 67-kDa laminin adhesion receptor and two additional proteins at 110 kDa and 180 kDa.

### METHODS

**Cells.** NG108-15 neuroblastoma-glioma cells (from M. Nirenberg, National Institutes of Health) exhibit many neuronal characteristics (22) and extend processes in the presence of laminin (21).

Undifferentiated stem cells of the PCC4 teratocarcinoma (23) were grown in high glucose Dulbecco's modified Eagle's medium (DMEM) without sodium pyruvate (GIBCO) supplemented with 10% fetal bovine serum and antibiotics. To obtain differentiated cells, cells were plated in 35-mm dishes at  $1.5 \times 10^5$  cells per ml and were deprived of fresh medium for 6 days. The medium was replaced every 3 days until day 23.

**Laminin Preparation and Binding to Cells.** Laminin was prepared from the EHS (Englebreth-Holm-Swarm) tumor (1) and coupled to CNBr-activated Sepharose 4B. Laminin was iodinated (specific activity,  $1 \mu\text{Ci}/\mu\text{g}$ ;  $1 \text{ Ci} = 37 \text{ GBq}$ ) using the chloramine-T procedure (24).

Binding was carried out with cells in suspension according to Huard *et al.* (25). Duplicate samples of 200  $\mu\text{l}$  of cells ( $1 \times 10^6$ ) were incubated for 1 hr at room temperature in DMEM/hypoxanthine/aminopterin/thymidine medium with 1% bovine serum albumin and 20 mM Hepes containing various concentrations of  $^{125}\text{I}$ -labeled laminin ( $^{125}\text{I}$ -laminin) with or without a 10-fold excess of unlabeled laminin. After the cells were centrifuged and washed, the pellet and washes were counted to determine "bound" and "unbound" laminin.

**Affinity Chromatography of Detergent Extracts of Cell Membranes.** Twenty (150-mm diameter) confluent plates of cells were harvested at 4°C in 0.15 M NaCl/50 mM phosphate, pH 7.5, containing 1 mM phenylmethylsulfonyl fluoride, and collected by centrifugation. After homogenization in a Dounce homogenizer, cell membranes were prepared by discontinuous sucrose-density cushion centrifugation (16). The final cell-membrane pellet was extracted with 1.0% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) in 0.01 M Tris-HCl (pH 7.4) for 2 hr and then

Abbreviations:  $^{125}\text{I}$ -laminin,  $^{125}\text{I}$ -labeled laminin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; LAM-P, pepsin-digested laminin.

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circulated after dialysis in column buffer on a laminin-Sepharose column equilibrated in 0.1% CHAPS/0.01 M Tris-HCl, pH 7.4. The 67-kDa laminin receptor was eluted with a salt gradient [0.01–1 M NaCl in 0.01 M Tris-HCl (pH 7.4) containing 0.1% CHAPS] and then the column was washed with 0.1 M glycine (pH 2.0). The glycine eluate was dialyzed against water, lyophilized, and resuspended in 4.0 M guanidine hydrochloride/0.05 M Tris, pH 7.4, containing 0.1% CHAPS. The sample was chromatographed on molecular sieve fast protein liquid chromatography in the same buffer.

**Laminin and Antibody Binding to Cell Homogenates on Nitrocellulose.** Confluent cells were scraped from three flasks (75 cm<sup>2</sup>) and were collected by centrifugation. The cell pellet was extracted for 1 hr at 4°C with 0.1% CHAPS in 0.01 M Tris-HCl (pH 7.4) and then centrifuged. Aliquots of the extract and of a suspension of the pellet were placed in sample buffer, sonicated, and electrophoresed on a 7.5% polyacrylamide gel and either stained with Coomassie brilliant blue R250 or transferred to nitrocellulose (26). After blocking the nitrocellulose with 1% bovine serum albumin, it was directly incubated with <sup>125</sup>I-laminin in 0.05 M Tris-HCl (pH 7.4) containing 1% bovine serum albumin and 0.05 M NaCl for 2–18 hr. The filters were then washed seven times for 5 min each in buffer lacking the <sup>125</sup>I-laminin. The nitrocellulose was air-dried and exposed to film.

For immunoblots, tissues or cells solubilized directly in sample buffer were sonicated and the proteins in the extract were electrophoresed and transferred to nitrocellulose as described above (26).

**Antibody Preparation and Immunofluorescence.** Antibodies to the 67-kDa laminin receptor were obtained from K. von der Mark (Max-Planck-Institut, Martinsried, F.R.G.) and those to the CSAT (fibronectin-laminin) receptor were from C. Buck (Wistar Institute) and G. Tarone (Univ. Turin). Antibodies to the 110-kDa and 180-kDa proteins were prepared in rabbits from material that had been chromatographed on a laminin affinity column prior to electrophoresis. Several lanes from the gel were pooled, mixed with RIBI adjuvant (RIBI Immunochem, Hamilton, MT), and injected into rabbits. The animals received a total of three injections over 8 weeks.

NG108-15 cells were grown on glass coverslips for 2–3 days, fixed in 3% paraformaldehyde in 0.1 M phosphate buffer, and incubated with primary antibodies absorbed with rat and mouse liver powder in solution and diluted 1:10. Cells were incubated in secondary antibody (goat anti-rabbit IgG, coupled to fluorescein isothiocyanate; Cappel Laboratories, Cochranville, PA) diluted 1:500 in phosphate-buffered saline (PBS), and mounted in 90% (vol/vol) glycerol in PBS containing 4% *n*-propylgallate (27). PCC4 cells were stained with primary antibody diluted 1:20 followed by goat anti-rabbit antibody coupled with rhodamine (prepared by C.D.L. and T.M.S.). Stained cultures were examined with a Leitz microscope with Ploemak filters and photographed with Tri-X Kodak film.

**Antibody Inhibition of Neurite Outgrowth.** Cells were incubated for 45 min at room temperature in DMEM containing 0.02% bovine serum albumin with various amounts of each antibody and of preimmune sera (all heat-inactivated at 58°C for 30 min). The cells were then centrifuged and washed in 3 ml of the incubation buffer lacking the antibody before use. Plates were preincubated with 30 μg of pepsin-digested laminin (LAM-P), which had been chromatographed on heparin Sepharose followed by DEAE-cellulose chromatography (G. Sephel, personal communication). This material is active in neurite outgrowth but not in cell adhesion. After a 2-hr incubation, the cells were photographed. The number of processes was determined by direct counting. The number of attached cells in parallel plates was determined.

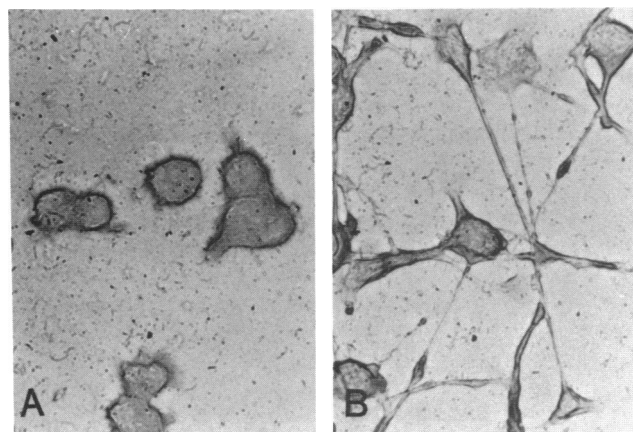


FIG. 1. Ability of laminin to promote process formation. (A) On plastic, no processes were observed after 1 hr. (B) On a laminin substrate, processes were observed after 1 hr. (Fixed in 5% formalin; stained with 1% methylene blue.)

## RESULTS

Laminin induced the formation of processes in NG108-15 within 1 hr of plating (Fig. 1). Since it was likely that this effect was due to the interaction of laminin with surface receptors, we measured the specific binding of <sup>125</sup>I-laminin to whole cells. <sup>125</sup>I-laminin bound to the cells and part was displaced by an excess of unlabeled laminin (Table 1).

To identify laminin-binding cell-surface proteins, we prepared cell membranes and extracted them with detergent. These extracts were chromatographed on a laminin affinity column. Three major proteins (67, 110, and 180 kDa) bound to the laminin affinity column (Fig. 2 and 3A). The 67-kDa laminin receptor bound to laminin and was eluted by NaCl. It was identified as the previously described laminin receptor, since it stained with authentic anti-receptor antibody (data not shown) and had a characteristic migration pattern before and after reduction (Fig. 3B). Proteins at 110 and 180 kDa required stronger eluent—i.e., 0.1 M glycine (pH 2.0) (Fig. 3A). Thus, the 67-kDa laminin receptor appears to bind to laminin with a lower avidity than the 110- and 180-kDa laminin-binding proteins. The 67-kDa laminin receptor without reduction migrated at 55 kDa as reported previously, while the migration of the 110-kDa laminin-binding protein was slightly slower and the 180-kDa laminin-binding proteins migrated slightly faster in the absence of reducing agents (Fig. 3B). These latter two proteins were further purified by fast protein liquid chromatography (Fig. 3C).

These three proteins (67, 110, and 180 kDa) bound to laminin, using a nitrocellulose filter incubated with <sup>125</sup>I-laminin. Cells were extracted with detergent and the solubilized material, as well as an aliquot of the residue (suspended in sample buffer with sonication), were electrophoresed on a NaDodSO<sub>4</sub>/polyacrylamide gel, transferred to nitrocellulose, and incubated with <sup>125</sup>I-laminin. Labeled laminin bound the three major components (67, 110, and 180 kDa) (Fig. 4). None of these components reacted with anti-CSAT antibody (data not shown). The 180-kDa band was present in the soluble fraction of the detergent extract.

Table 1. Binding of <sup>125</sup>I-laminin to cells

<sup>125</sup> I-laminin added, ng	<sup>125</sup> I-laminin bound, cpm × 10 <sup>-3</sup>	<sup>125</sup> I-laminin bound in presence of unlabeled laminin	Specific binding, cpm × 10 <sup>-3</sup>
67.5	3.2 ± 0.3	2.0 ± 0.4	1.2
135.0	4.5 ± 0.1	0.9 ± 0.2	3.5

Binding is expressed as mean ± SEM.

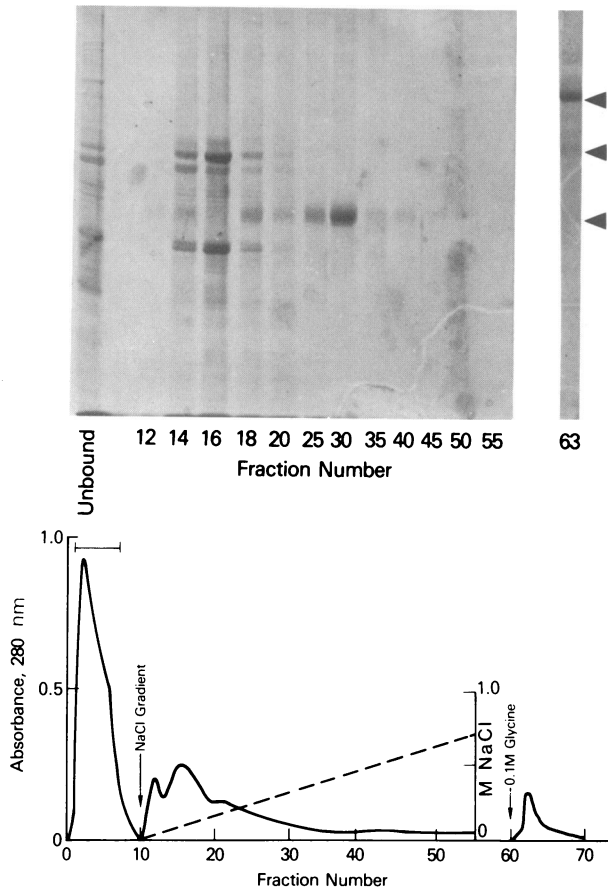


FIG. 2. Laminin affinity chromatography of detergent extracts of NG108-15 cell membranes. The 67-kDa laminin adhesion receptor eluted principally in tube 25, whereas the 110- and 180-kDa proteins were present in the low pH glycine wash. Variable amounts of other proteins at 90 and 55 kDa were present in some but not all of the preparations and may be degradation products.

We next prepared antibodies to the laminin-binding proteins. The proteins were isolated on a laminin affinity column, separated by electrophoresis, excised from the gel, and used to immunize rabbits. The resulting antibodies were used to assess the occurrence of these proteins in extracts of various cells and tissues. The 110-kDa laminin-binding protein was present in a variety of epithelial tissues (Fig. 5A;

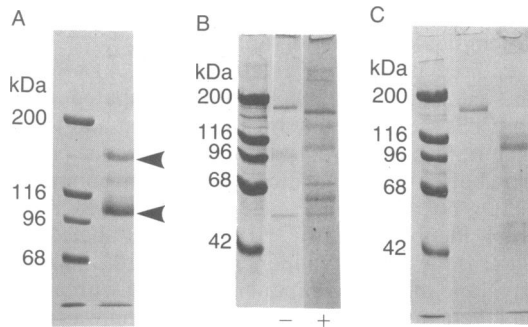


FIG. 3. (A) NaDodSO<sub>4</sub>/polyacrylamide gel of 0.1 M glycine (pH 2.0) eluate from laminin affinity column. (B) NaDodSO<sub>4</sub>/polyacrylamide gel of 68-, 110-, and 180-kDa laminin-binding proteins. Samples were electrophoresed on a 5% polyacrylamide gel in the presence (+) or absence (-) of reducing agent (dithiothreitol). The 65- and 55-kDa bands are likely degradation products of the 67-kDa molecule. (C) Purification of the 110- and 180-kDa proteins from the glycine eluate by fast protein liquid chromatography (FPLC). The sample from A was chromatographed on a FPLC molecular sieve column (Sepharose 6) at a flow rate of 0.5 ml/min.

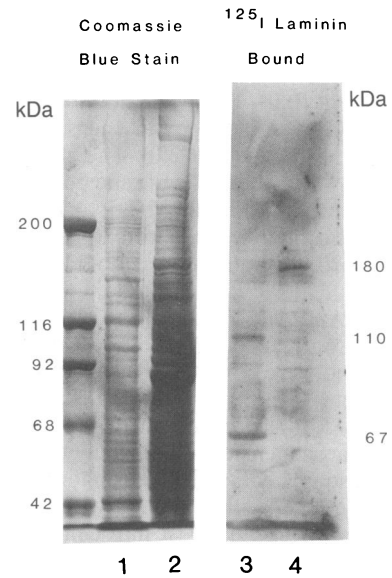


FIG. 4. Binding of <sup>125</sup>I-laminin to NG108-15 cell homogenates after NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis and transfer to nitrocellulose. Cells were extracted with 0.1% CHAPS for 1 hr and aliquots of a suspension of the pellet (lanes 1 and 3) and of the extract (lanes 2 and 4) were electrophoresed on a 5% polyacrylamide gel. The pellet (lane 1) and extract (lane 2) were stained with Coomassie blue and parallel lanes were transferred to nitrocellulose and incubated with <sup>125</sup>I-laminin. Three major bands (67, 110, and 180 kDa) bound labeled laminin.

Table 2), including brain and EHS tumor tissue, as well as in cultured cells including F9 teratocarcinoma, HT-1080, NG108-15 neuroblastoma, and B16 melanoma cells. No significant reactivity was seen in muscle, cartilage, fat, and human skin and 3T3 fibroblasts. Thus, this protein is broadly

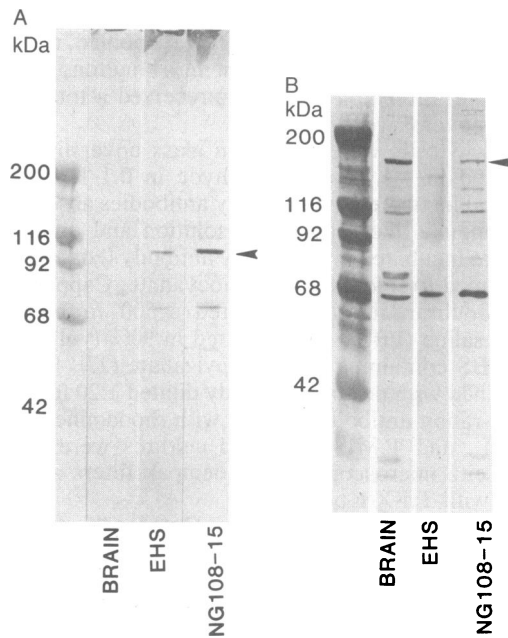


FIG. 5. (A) Immunoblot with anti-110-kDa antibody. Brain, EHS tumor, and NG108-15 cells were placed in sample buffer, sonicated, and directly electrophoresed on a 7.5% polyacrylamide gel. Arrow indicates the 110-kDa reactive band. The other lower molecular mass bands observed, particularly the band at 67 kDa, also stained with preimmune sera. (B) Immunoblot with anti-180-kDa antibody. All tissues and procedures were the same as described in A. The lower molecular mass bands were also observed using the preimmune sera from this animal.

Table 2. Distribution of 110- and 180-kDa laminin-binding proteins in tissues and in cells

	Cells						Tissue				
	F9 terato- carcinoma	HT-1080 fibrosarcoma	NG108-15 neuroblastoma- glioma	B16 melanoma	3T3 fibroblasts	Skin fibroblasts	Muscle	Cartilage	Brain	Fat	EHS
110 kDa	+	+	+	+	+	0	0	0	+	0	+
180 kDa	0	0	+	+	0	0	0	0	+	0	0

Homogenates of cell pellets and tissues were evaluated by immunoblot analyses. 0, No reaction; +, stained band.

distributed among epithelial and neural cells. The 180-kDa laminin-binding protein had a more limited distribution (Fig. 5B; Table 2) and was found only in brain and in cell lines derived from neuroectoderm, such as NG108-15 and B16 melanoma cells. Thus, the 180-kDa laminin-binding protein in the cells and tissues that we have examined appears to be restricted to cells of neural lineage.

Antibody to the 180-kDa protein stained the surfaces of the cell bodies and processes of NG108-15 cells (Fig. 6A). Antibody to the 110-kDa protein (Fig. 6B) showed a weaker pattern of staining. Staining with both antibodies was greater than that of serum from the preimmune control rabbits (Fig. 6C). The specificity of the 180-kDa protein antibody for neural cells was confirmed with staining of teratocarcinoma cells, which contain only 5% neural cells. Only cells with long neural processes stained with this antibody (Fig. 7A). These same cells also stained with the neural-specific antibody TuJi (28) (data not shown).

A heparin-binding fragment from a pepsin digest of laminin (LAM-P) was isolated. As shown previously, this fragment is the principal neurite-promoting site in laminin (12) (Fig. 8). Addition of antibody directed toward either the 110- or the 180-kDa receptor almost completely blocked the formation of neural processes (Fig. 8). On the other hand, cells preincubated either with antibody to the 67-kDa adhesion receptor or with preimmune serum formed the same number of processes on LAM-P substrates as cells without preincubation (Fig. 8). None of the antibodies reduced cell attachment to the LAM-P substrate (data not shown). These data suggest that the 110- and 180-kDa proteins are laminin receptors that mediate neurite outgrowth.

## DISCUSSION

Laminin is a large multifunctional molecule that affects the behavior of many cells, including the attachment of cells to a substrate and induction of morphological changes and cell differentiation (3). Separate domains have been demonstrated for cell adhesion and for neurite outgrowth (12); therefore, it is not unexpected that there are multiple cellular

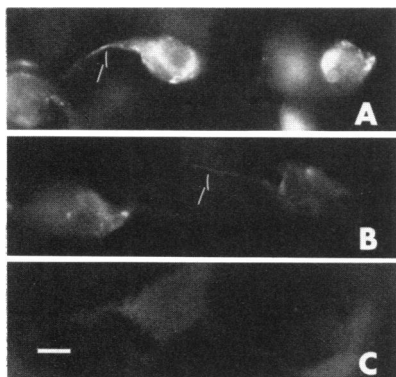


FIG. 6. Immunofluorescence of NG108-15 cells showing surface staining with antibody to 180-kDa protein (A), 110-kDa protein (B) on cell bodies and processes (arrows). No staining is seen with preimmune serum (C). (Bar = 10  $\mu$ m.)

receptors for laminin. We demonstrate here that neuroblastoma-glioma cells contain two additional laminin-binding proteins (110 and 180 kDa). We have also isolated these proteins from a human neuroblastoma cell line, LAN (unpublished data). These proteins are distinct from the previously described laminin receptor (67 kDa), which is present on many cells (14-16) and is involved in cell adhesion to laminin through the Tyr-Ile-Gly-Ser-Arg site on the B1 chain of laminin (11). The two proteins described here have a greater affinity for laminin because they require stronger elution conditions from the laminin affinity column than the 67-kDa receptor. Recently a 120-kDa laminin-binding protein from NG108-15 cells has been identified and is likely related to our 110-kDa protein (29).

The 67, 110, and 180-kDa laminin receptors all have a stronger affinity for laminin than integrin (CSAT). Binding of laminin to integrin cannot be readily demonstrated by affinity chromatography or by immunoblotting (17, 30). In addition, antibody to the  $\beta$  component of integrin does not react with either the 110- or 180-kDa band (unpublished data). We also do not find the 110- or 180-kDa protein on fibroblasts. Thus, the laminin-binding proteins described here are not likely to be directly related to integrin, although antibodies to integrin can block laminin-mediated neurite outgrowth (31). Possibly the anti-integrin antibody inhibits neural process formation for steric reasons by reacting with other components of the receptor coupled in the membrane. Also, RGDS has been shown to be the interactive site on fibro-

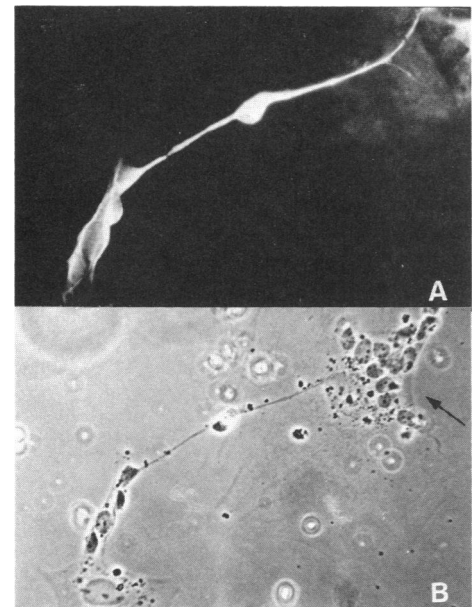


FIG. 7. Immunofluorescence of neuron-like cells in differentiated PCC4 teratocarcinoma cells stained with antibody to 180-kDa protein. (A) Antibody to the 180-kDa protein. (B) Cells viewed in phase contrast. Cells with long thin processes stain with this antibody, whereas flat epithelial-like cells are unstained (arrows). The neuronal-like cells also stained with TuJi antibody, a neural-specific  $\beta$ -tubulin antibody.

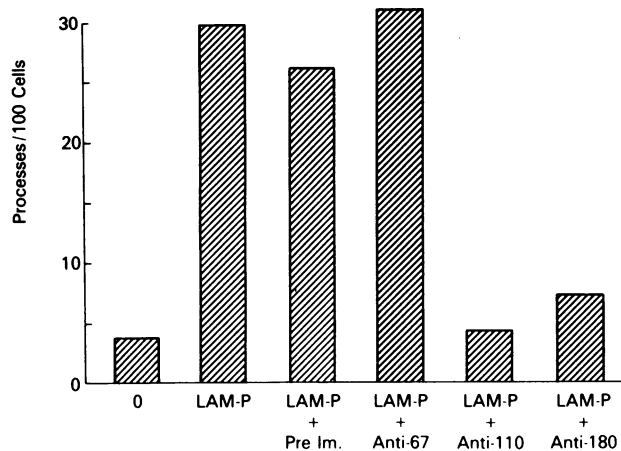


FIG. 8. Effect of antibodies on inhibiting NG108-15 process outgrowth mediated by a laminin fragment. Preimmune serum, anti-67-kDa, 110-kDa, and 180-kDa antibodies, each at a final concentration of 30%, were preincubated with NG108-15 cells as described.

nectin that binds to integrin (32), yet RGDS does not promote neurite outgrowth and does not inhibit laminin-mediated neurite outgrowth (L.L.-E., unpublished data). Thus, this site (RGDS) on fibronectin may not have a direct effect on neurite formation.

Our evidence suggests that the 180-kDa receptor is specific to the nervous system and that the 110-kDa receptor is common to many epithelial and neural cells. Both molecules are involved in neurite outgrowth since their respective antibodies can block process formation. Based on their different cellular distribution, it is likely that the 110- and 180-kDa proteins are not part of a receptor complex similar to that observed for integrin (17, 18, 30), but rather exist as separate receptor(s). It is not clear whether the receptors both bind to the same site on laminin or if two separate sites exist that "cooperate" to form processes.

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