

# Laminin Fragment E8 Mediates PC12 Cell Neurite Outgrowth by Binding to Cell Surface $\beta$ 1,4 Galactosyltransferase

Paul C. Begovac,\* Deborah E. Hall,† and Barry D. Shur\*

\*Department of Biochemistry and Molecular Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030; and †Athena Neurosciences, South San Francisco, California 94080

**Abstract.** A number of cell surface receptors bind to distinct laminin domains, thereby mediating laminin's diverse biological activities. Cell surface  $\beta$ 1,4-galactosyltransferase (GalTase) functions as one of these laminin receptors, facilitating mesenchymal cell migration and PC12 cell neurite outgrowth on laminin. In this study, the GalTase binding site within laminin was identified as the E8 fragment by assaying purified fragments and by immunoprecipitating and immunoblotting galactosylated laminin using E8-reactive antibodies. Compared with intact laminin and other laminin fragments, E8 possessed the highest GalTase binding activity, using both membrane-bound and solubilized

GalTase. More significantly, the neurite-promoting activity of fragment E8 was shown to be dependent upon its interaction with GalTase. Pregalactosylating purified E8 eliminated subsequent GalTase binding and consequently inhibited neurite initiation; parallel studies on laminin fragments E1-4 or E1 failed to affect neurite outgrowth. Furthermore, anti-GalTase IgG inhibited neurite initiation on purified E8 substrates; control IgG had no effect. These results localize the predominant GalTase binding domain in laminin to fragment E8 and demonstrate that the neurite-promoting activity of E8 is dependent upon its interaction with GalTase.

LAMININ is a major constituent of basement membranes. It is a large ( $\sim$ 900 kD) multidomain glycoprotein composed of three distinct chains, A (440 kD), B1 (225 kD), and B2 (205 kD) for which each of the murine cDNAs have been cloned (Sasaki and Yamada, 1987; Sasaki et al., 1987, 1988). A number of biological properties have been attributed to laminin, including the ability to modulate growth and differentiation of epithelial and tumor cells, to support cell attachment and migration, and to function during extracellular matrix assembly (reviewed in Kleinman et al., 1985; Martin and Timpl, 1987). Laminin is also a potent inducer of neurite formation in a variety of central and peripheral nervous system neurons and neuronal cell lines (reviewed in Sephel et al., 1989a). The diverse biological activities of laminin are believed to be localized to distinct laminin domains, each of which is recognized by appropriate cell surface receptors.

The three laminin chains (i.e., A, B<sub>1</sub>, B<sub>2</sub>) generate a cruciform molecule that possesses distinct binding domains (see Fig. 1). Collagen binds to the ends of the short arms (Rao et al., 1982; Terranova et al., 1983; Charonis et al., 1985, 1986), whereas heparin binds to the terminal globule of the long arm, the inner globule of the short arm, and the outer globule of the short arm (Sakashita et al., 1980; Ott et al., 1982; Charonis et al., 1988; Kouzi-Koliakos et al., 1989).

Peptide sequences having cell binding activity include a YIGSR sequence in the B1 chain (Graf et al., 1987) and an RGD sequence in the A chain short arm region (Sasaki et al., 1988; Aumailley et al., 1990). The major neurite-promoting activity of laminin has been localized to the E8 elastase fragment of laminin (Edgar et al., 1984, 1988; Engvall et al., 1986). Within E8, several putative neurite-promoting sites have been reported (Edgar et al., 1988; Liesi et al., 1989; Tashiro et al., 1989). A less potent neurite-promoting site near the upper cross-region of the laminin molecule has also been reported (Edgar et al., 1984; Sephel et al., 1989b).

Cell surface receptors identified thus far for laminin include a 67-kD laminin receptor, which recognizes the YIGSR sequence within the B1 chain (Malinoff and Wicha, 1983; Douville et al., 1988; Kleinman et al., 1988), 110-kD and 180-kD proteins present on neuronal cells (Kleinman et al., 1988), the integrin class of heterodimeric receptors, which binds to two distinct laminin domains (Horwitz et al., 1985; Tomaselli et al., 1987, 1988; Hall et al., 1990), and  $\beta$ 1,4-galactosyltransferase (GalTase) which binds to terminal *N*-acetylglucosamine residues on *N*-linked oligosaccharides on laminin (Runyan et al., 1988).

GalTase is present both within the Golgi complex, where it functions during glycoconjugate biosynthesis, as well as on the plasma membrane, where it functions as a cell surface receptor binding appropriate substrates on adjacent cell surfaces and in the extracellular matrix. Cell surface GalTase functions as a receptor in a number of cellular interactions

Dr. Begovac's present address is Department of Zoology, University of Oklahoma, Norman, OK 73019.

including mouse sperm-egg binding (Shur and Hall, 1982; Lopez et al., 1985), mouse preimplantation development (Bayna et al., 1988), ectoplacental cone intercellular adhesion (Hathaway et al., 1989), trophectoderm outgrowth (Romagnano, L., and B. Babiartz. 1989. *J. Cell Biol.* 109:62a), melanoma cell spreading (Runyan et al., 1988), mesenchymal cell migration (Eckstein and Shur, 1989), and PC12 cell neurite outgrowth (Begovac and Shur, 1990).

Laminin serves as the ligand, or substrate, for surface GalTase during selected cell-matrix interactions. Melanoma cell spreading and PC12 neurite outgrowth on laminin can be inhibited or stimulated by reagents that selectively inhibit or stimulate, respectively, cell surface GalTase (Runyan et al., 1988; Begovac, and Shur, 1990). Alternatively, blocking or removing GalTase binding sites within laminin inhibits cell spreading and neurite outgrowth. Laminin induces a threefold increase in the levels of surface GalTase on migrating mesenchymal cells (Eckstein and Shur, 1989), which is preferentially localized to the leading lamellipodia (Eckstein and Shur, 1989) and to the growth cones of developing neurites (Begovac and Shur, 1990). Collectively, these studies show that GalTase mediates lamellipodial and growth cone formation and extension on laminin. Furthermore, GalTase has a secondary role during the continued outgrowth of pre-existing neuronal processes (Begovac and Shur, 1990).

A critical issue that remains unresolved is where on the laminin molecule does GalTase bind and how do these binding site(s) compare with previously identified domains in laminin that have defined biological activities. Evidence presented in this study shows that the principle GalTase binding domain is associated with fragment E8 and, more significantly, that the biological activity of E8 is dependent upon its interaction with cell surface GalTase.

## Materials and Methods

### Reagents

Laminin was purified from an Engelbreth-Holm-Swarm (EHS) tumor (Timpl et al., 1979; Kleinman et al., 1982) or obtained from Sigma Chemical Co. (St. Louis, MO) and diluted in PBS or Medium B (127 mM NaCl, 5.3 mM KCl, 18.2 mM Hepes, pH 7.2). All other reagents were obtained from Sigma Chemical Co. unless otherwise indicated.

Laminin fragments E1-4 and E8 were prepared by elastase digestion (E0258; Sigma Chemical Co.; 1:100 enzyme/substrate ratio) for 3-4 h at 37°C and isolated according to previously published methods (Ott et al., 1982; Paulsson et al., 1985; Aumailley et al., 1987; Goodman et al., 1987; Timpl et al., 1983). Laminin fragments E1 and E3 were purified after a 14-h elastase digest (1:100 enzyme/substrate ratio) according to Ott et al. (1982). Purification of laminin fragments was verified by SDS-PAGE, rotary shadowing, and biological activity (Hall et al., 1990). Protein concentrations were determined using the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL) using BSA as standard.

Rabbit anti-GalTase IgG antibodies used for this study have been previously characterized (Bayna et al., 1988; Lopez et al., 1985) and are cross-reactive to PC12 GalTase (Begovac and Shur, 1990). The anti-E3 rabbit polyclonal antibody used has also been previously described (Hall et al., 1990).

### Cells

Rat pheochromocytoma (PC12) cells (Greene and Tischler, 1976) were grown in DME supplemented with 10% heat-inactivated horse serum, 5% heat-inactivated FBS, 2 mM fresh glutamine, and penicillin/streptomycin in a 10% CO<sub>2</sub> humidified incubator at 37°C, and passaged weekly. PC12 cells were maintained and primed with nerve growth factor as previously described (Begovac and Shur, 1990).

## GalTase Activity toward Laminin Fragments

To quantitate the number of binding sites present on laminin fragments, two different enzyme sources were used to galactosylate various laminin fragment preparations. The first method used PC12 cell membranes isolated according to Lopez et al. (1989) as the GalTase source. PC12 cells were harvested in Ca<sup>2+</sup>-Mg<sup>2+</sup>-free PBS, and washed three times in the same buffer. Cells were placed in homogenization buffer containing 1 mM triethanolamine, 0.25% sucrose, and protease inhibitor cocktail (PIC; 2 µg/ml anti-pain, 0.1% aprotinin, 10 µg/ml benzamide, 1 µg/ml chymostatin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin), and transferred into centrifuge tubes. Cells were homogenized using a Polytron (Brinkmann Instruments, Westbury, NY) for 40 s (setting 4) and centrifuged for 10 min at 500 g to remove nuclei and organelles. The supernatant was transferred to a fresh tube and membranes were pelleted by centrifugation at 33,000 g for 60 min at 4°C. The membrane pellet was washed three times with medium B/PIC and resuspended in the same buffer by light sonication. This intact membrane preparation was used directly for galactosylation of fragments or subsequently solubilized with 30 mM *n*-octylglucoside prior to use in galactosylation assays. For each assay, 500-750 ng of laminin or laminin fragment was lyophilized to remove residual ammonium bicarbonate from the preparation. To each protein sample ~1.5 × 10<sup>6</sup> cell membrane equivalents were added in a total volume of 50 µl in Medium B containing 10 mM MnCl<sub>2</sub>, 0.25 M NaCl, 100 µM UDP[<sup>3</sup>H]galactose (574 dpm/pmol; Dupont Co., Wilmington, DE; New England Nuclear, Boston, MA) and incubated for 90-120 min at 37°C. The reaction was terminated by adding 10 µl of 0.2 M EDTA, 0.05 M Tris buffer, pH 7.2. Individual samples were spotted onto chromatography paper and subjected to high-voltage electrophoresis (Shur, 1982). The origins were cut from the paper and placed in BudgetSolve (Research Products International, Mount Prospect, IL) scintillation cocktail. Incorporation was quantitated by liquid scintillation counting.

Affinity-purified bovine milk GalTase was also used to assess activity toward laminin and laminin fragments. Lyophilized laminin or laminin fragments (500-750 ng/assay) were resuspended in 50 µl Medium B containing 0.25 M NaCl, 10 mM MnCl<sub>2</sub>, 100 µM UDP[<sup>3</sup>H]galactose, and 1 µg GalTase. This mixture was incubated for 45 min at 37°C, and the reaction was stopped and subjected to high-voltage borate electrophoresis as described above.

### Blot Assay

Laminin was digested with elastase for 4 h at 4°C as described above, the digests were boiled in nonreducing SDS sample buffer, and subjected to SDS-PAGE on 6-15% gradient gels (Wallace and Selman, 1985). The gels were divided with one half being fixed and double stained with silver and Coomassie blue R-250. The other half of the gel was electroblotted onto nitrocellulose (Bio-Rad Laboratories, Richmond, CA) according to the methods of Towbin et al. (1979). The blots were cut into strips corresponding to the gel lanes, briefly rinsed in medium B for 5 min, and blocked with 2.5% BSA in Medium B for 2 h at 37°C. The blots were then incubated in a total volume of 4 ml Medium B containing 100 µg of purified GalTase, 10 mM MnCl<sub>2</sub>, and 87.5 µM UDP[<sup>3</sup>H]galactose for 2-3 h at 37°C with gentle agitation. Finally, the blots were rinsed with three changes of Medium B over 90 min and allowed to dry. The nitrocellulose strips were cut into 5-mm segments and placed into scintillation vials with BudgetSolve cocktail, and the samples were counted.

### Immunoblots and Immunoprecipitation

Western transfers of laminin digests prepared as described above were blocked with 2.5% BSA for 60 min in Tris-buffered saline containing 0.2% polysorbate-20 (TTBS; 10 mM Tris-Cl, 0.9% NaCl, pH 7.5). The blots were incubated in TTBS containing 2% normal goat serum and primary antisera (anti-E3 or normal rabbit; 1:50 dilution) for 3 h with gentle agitation. Blots were rinsed in three changes of TTBS and incubated in a commercial secondary goat anti-rabbit biotin antibody conjugate (Vector Laboratories, Inc., Burlingame, CA) that was later tagged with an avidin-horseradish peroxidase conjugate. All steps subsequent to the primary antibody incubation were carried out according to the manufacturer's recommendations.

Immunoprecipitation of fragment E8 was carried out using an anti-E3 rabbit polyclonal antibody (Hall et al., 1990). 150 µg of laminin was galactosylated in a total volume of 300 µl for 2 h at 37°C using 40 µg of bovine milk GalTase, 10 mM MnCl<sub>2</sub>, and 100 µM UDP[<sup>3</sup>H]galactose. The galactosylated laminin was dialyzed against 0.2 M ammonium bicarbonate, pH 7.9, digested with elastase (1:100 enzyme/substrate ratio) for 4 h at 4°C, and

the reaction was stopped with 1 mM PMSF. Half the digest was incubated with rabbit anti-E3 antiserum (1:50 dilution) and the other portion was incubated in control normal rabbit serum (1:50 dilution). The primary antibody incubation was carried out overnight at 4°C followed by immunoprecipitation with 75  $\mu$ l of packed volume of protein A-Sepharose (Pierce Chemical Co.) in 0.2 M ammonium bicarbonate buffer for 8 h at 4°C. The protein A-Sepharose was pelleted and washed three times with the same buffer; the final pellet boiled in nonreducing SDS sample buffer. The samples were electrophoresed on 6–15% gradient gels, treated with EN<sup>3</sup>HANCE fluor (New England Nuclear, Boston, MA), dried, and exposed to Kodak X-OMAT x-ray film for fluorography.

### Neurite Outgrowth Assays

PC12 cell neurite outgrowth assays were performed as detailed in Begovac and Shur (1990). Briefly, primed PC12 cells were plated in DME onto 48-well Costar dishes (Cambridge, MA) that had been prepared as follows. Wells were coated with purified fragment E1-4 (17  $\mu$ g/ml), fragment E1 (6  $\mu$ g/ml), or fragment E8 (6  $\mu$ g/ml) in PBS for 2 h at 37°C. Wells were washed three times with Medium B and blocked with 2 mg/ml BSA in Medium B for 60 min at 37°C. Wells were then washed three times with Medium B and used directly for antibody experiments or treated further to block GalTase substrates in the matrices. GalTase binding sites were consumed by incubating wells with 10  $\mu$ g of purified GalTase, 10 mM MnCl<sub>2</sub>, and 1 mM UDP-galactose in a total volume of 200  $\mu$ l Medium B. This incubation cocktail was changed three times during an overnight incubation. The wells were again washed four times with Medium B before adding cells. Previous assays determined that >99% of the GalTase binding sites are consumed after these incubations (Begovac and Shur, 1990).

Neurite outgrowth on specific fragment substrates was assessed by two separate assays described in Begovac and Shur (1990). The first assay quantitated neurite initiation as the percentage of cells that deviated from a round morphology. Initiation was assayed at 30, 60, and 90 min after plating cells. The second assay quantitated neurite formation as the percentage of cells that possessed neurites greater than one cell body in length. Neurite formation was determined after 4 h in culture. The data was plotted as mean percentage ( $\pm$  SEM) of outgrowth relative to control values. Cells were photographed using Hoffman interference microscopy with Kodak Tek-Pan film.

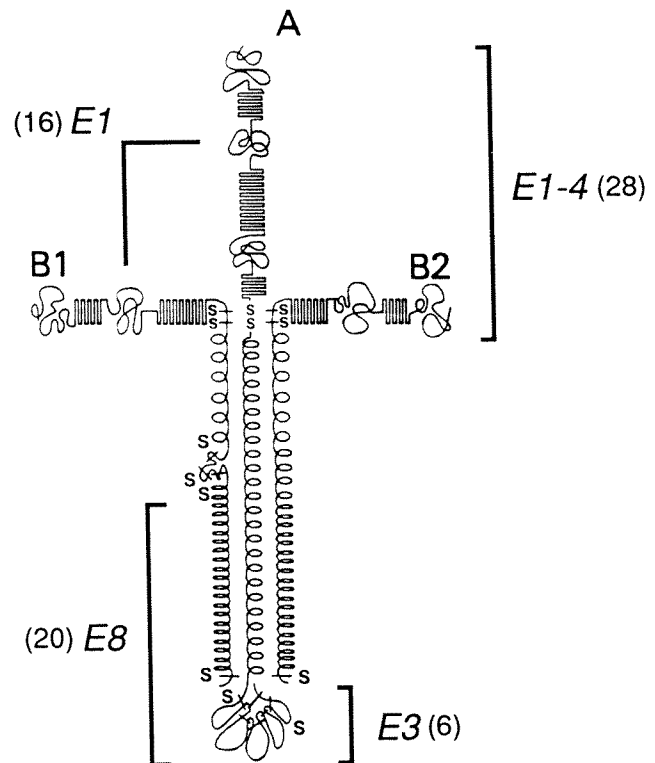
## Results

### Experimental Approach

Biochemical and biological assays were used to evaluate GalTase interactions with laminin fragments. Initially,  $\beta$ 1,4-GalTase from both intact and solubilized PC12 membranes, as well as affinity-purified  $\beta$ 1,4-GalTase from bovine milk, was used to define the distribution of GalTase binding sites within laminin and the binding specificity of GalTase toward purified laminin fragments (Fig. 1). The binding sites were identified by virtue of GalTase catalytic activity, which radiolabels substrates with [<sup>3</sup>H]galactose transferred from UDP[<sup>3</sup>H]galactose. Laminin fragment identity was determined by molecular weight, biological activity, and reactivity with specific antibodies. The biological significance of the GalTase-laminin fragment interaction was investigated by assaying neurite outgrowth on individual fragments in the presence of anti-GalTase antibodies or on laminin substrates in which GalTase binding sites had been blocked by pregalactosylation. The effects of these perturbations on both neurite initiation and neurite formation were quantitated.

### GalTase Activity toward Purified Laminin Fragments

Intact PC12 membranes were used as a source of GalTase, since this is the most biologically relevant form of the enzyme and most closely resembles the native interaction between cell surface GalTase and laminin. Intact PC12 mem-



**Figure 1.** Model of laminin illustrating various laminin fragments and potential N-glycosylation sites deduced from sequence data. Laminin chains A, B<sub>1</sub>, and B<sub>2</sub> are indicated as are the putative sulfhydryl group(s). Elastase fragments E1-4, E1, E8, and E3 are shown. The numbers in parentheses represent potential N-linked sites on individual fragments. Modified from Sasaki et al. (1988) with permission.

branes galactosylated fragment E8 at 8–10 times the specific activity of intact laminin or fragment E1, and >47 or 28 times the specific activity of fragments E1-4 or E3, respectively (Table I). Comparison of specific activities on a molar basis revealed that fragment E8 is galactosylated at nearly 1.5 times that of intact laminin, 4 times that of E1, 10 times that of E1-4, and 80 times that of E3.

Although these results clearly demonstrate that PC12 GalTase preferentially recognizes the E8 domain within laminin, it does not address whether the biological activities of E8 are dependent upon GalTase binding. This can be assessed by pregalactosylating the GalTase binding sites in E8 and examining the resultant effects on E8 biological activity. However, to exhaustively galactosylate laminin or its fragments, GalTase must be present in excess, and the amount of enzyme available from PC12 membranes is limiting making this approach unfeasible. Therefore, we took advantage of a commercially available form of soluble  $\beta$ 1,4-GalTase, affinity-purified from bovine milk, that can be added in excess to completely galactosylate all GalTase binding sites in laminin. As shown in Table I, affinity-purified GalTase from bovine milk labeled E8 to high specific activity, although activity towards all laminin preparations was higher than using PC12 membrane GalTase. Furthermore, the soluble milk enzyme had a broader substrate specificity than did the membrane-bound enzyme, since the relative difference in

**Table I. Membrane-bound GalTase Preferentially Recognizes Laminin Fragment E8**

| GalTase source   | n | Intact laminin | Fragment |         |         |       |
|--|---|----------------|----------|---------|---------|-------|
|  |   |                | E1-4     | E1      | E8      | E3    |
| <i>Specific activity (pmol galactose transferred/μg protein/h)</i> |   |                |          |         |         |       |
| Intact PC12 membrane   | 4 | 1.19           | 0.24     | 1.41    | 11.25*  | 0.40  |
| Soluble PC12 membrane  | 4 | 59.27          | 15.33    | 53.51   | 71.68   | 2.63  |
| Affinity purified from bovine milk                                 | 3 | 40.91          | 51.35    | 108.75‡ | 106.33§ | 37.69 |

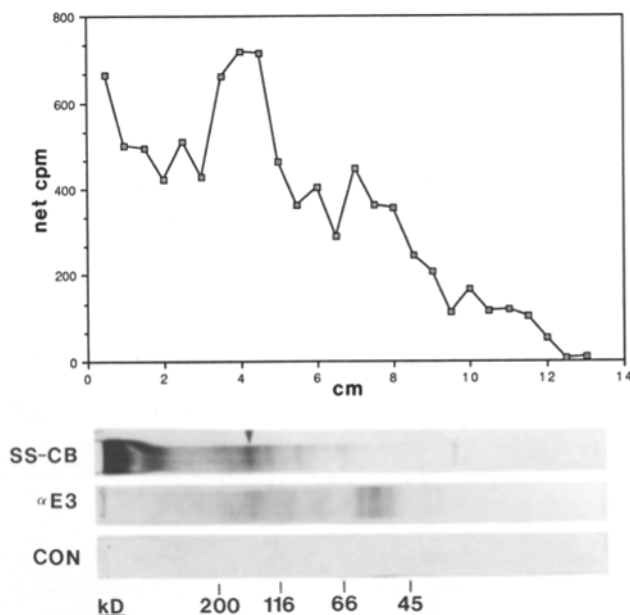
n, number of experiments. Data are expressed as mean values. \*  $p = 0.008$ , ‡  $p = 0.009$ , §  $p = 0.011$  relative to intact laminin based upon  $t$  test. All other values,  $p \geq 0.05$ .

galactosylation between E8 and other laminin substrates was decreased using the soluble enzyme. This higher labeling efficiency and reduced substrate specificity was a result of using a soluble form of the enzyme, since GalTase from detergent-solubilized PC12 membranes galactosylated laminin substrates similar to bovine milk GalTase (Table I). Interestingly, using all these GalTase sources, fragment E1 had higher substrate activity than its parent fragment E1-4, which contains the terminal globule domains on the laminin short arms (Paulsson et al., 1985). Thus, further proteolysis of E1-4 to obtain E1 apparently reveals cryptic GalTase binding sites. This observation is similar to other studies in which an RGD cell attachment site in laminin is exposed as a conse-

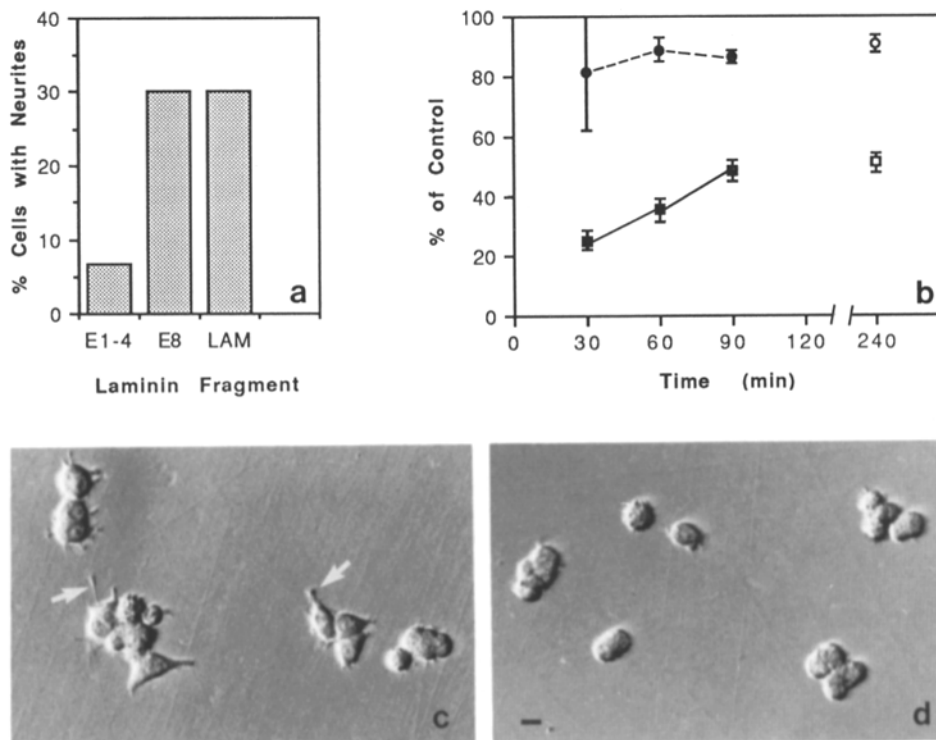
quence of limited proteolysis (Aumailley et al., 1990). Collectively, the results of these studies show that intact PC12 membranes preferentially galactosylate fragment E8 relative to other laminin domains. Furthermore, soluble forms of GalTase can also be used to exhaustively galactosylate individual laminin fragments to assess the biological consequences of pregalactosylation on neurite initiation and outgrowth. For these purposes, it is unimportant that soluble GalTase has a broader substrate specificity than membrane-bound GalTase. It is only necessary that soluble GalTase galactosylate those substrates recognized by PC12 membrane bound GalTase, any additional galactosylation is irrelevant.

#### GalTase Activity toward Total Laminin Digests

The experiments described above used purified laminin fragments to determine the relative GalTase substrate specificity. To independently assess the GalTase binding domains in laminin, intact laminin was digested with elastase and GalTase activity toward the total digest was determined using a blot assay. The digestion products were separated by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose strips were then galactosylated with affinity-purified GalTase and UDP[<sup>3</sup>H]galactose. Peaks of GalTase substrate activity were observed corresponding to partially digested laminin, fragment E1-4, and a minor peak of activity was associated with a protein of ~50 kD (Fig. 2, graph and lane SS-CB). However, the major peak of activity was associated with a digestion product of 140 kD (Fig. 2, lane SS-CB, arrowhead), the molecular weight of E8 on nonreducing gels (Paulsson et al., 1985; Deutzmann et al., 1988). An anti-E3 rabbit polyclonal antibody was used to further characterize the laminin digestion products by western blot analysis. The anti-E3 antibody recognizes E3 epitopes contained within undigested laminin, the isolated laminin A chain, fragment E8, and fragment E3 (see Fig. 1). The anti-E3 antibody recognized a 140-kD protein of the laminin digest that corresponded to the major peak of GalTase activity, confirming it as the E8 fragment (Fig. 2, lane αE3). Fragment E3 is also present in the digest and corresponded to a minor peak of GalTase activity (Fig. 2, lane αE3). Control rabbit serum showed no immunoreactivity (Fig. 2, lane CON). Similarly, when intact laminin was galactosylated with affinity-purified GalTase and UDP[<sup>3</sup>H]galactose, and elastase-digested, anti-E3 antibody immunoprecipitated [<sup>3</sup>H]galactosylated bands corresponding to fragment E3, fragment E8, and higher molecular weight components believed to be laminin A chain and partially digested laminin (data not shown).



**Figure 2.** Fragment E8 contains the principle GalTase binding sites within laminin. Elastase-digested laminin was subjected to SDS-PAGE on a 6–15% nonreducing gel and transferred to nitrocellulose. One lane was galactosylated with purified GalTase and UDP-<sup>3</sup>H]galactose as described in Materials and Methods. Net counts per minute of [<sup>3</sup>H]galactose incorporation is plotted as a function of position in centimeters (cm) on the nitrocellulose blot. The corresponding silver- and Coomassie blue-stained lane (SS-CB) of the same laminin digestion is shown. Anti-E3 antisera immunoreactivity (αE3) towards the same protein preparation identifies fragment E8 (140 kD, arrowhead) and fragment E3 (50 kD). Control antisera (CON) immunoblot of the same laminin digest depicted in the SS-CB lane above. Molecular weight standards are shown.



**Figure 3.** Pregalactosylating laminin fragment E8 inhibits its neurite-promoting activity. (a) Comparison plot of neurite formation on fragments E1-4, E8, and intact laminin (LAM). Values represent mean percent of cells possessing neurites after 4 h of culture. (b) Blocking GalTase binding sites by pregalactosylation inhibits neurite initiation (■) and neurite formation (□) on fragment E8 ( $n = 3$ ). Minimal inhibition of neurite initiation (●) or neurite formation (○) is observed on fragment E1-4 ( $n = 4$ ). Values represent the mean percent outgrowth on galactosylated E8 versus unmodified E8 or galactosylated E1-4 versus unmodified E1-4. Error bars, SEM. Neurite initiation on (c) unmodified E8 (control) or (d) galactosylated E8 after 50 min in culture. Note the increased cell flattening and initiating neurites (arrows) on control E8 substrate, whereas cells on galactosylated E8 remain round. Bar, 10  $\mu$ m.

Thus, on the basis of these biochemical analyses, fragment E8 appears to be the principle binding site for membrane bound GalTase and contains significant binding sites for solubilized GalTase, compared with the other domains of the laminin molecule.

#### Neurite Outgrowth on Pregalactosylated Laminin Fragments

The biological significance of the preferential binding between GalTase and E8 was determined by pregalactosylation of the substrate with purified GalTase and UDP-galactose. As a consequence, all binding sites for cell surface GalTase are selectively consumed. The effects of pregalactosylating GalTase substrates in fragments E1-4 and E8 were determined on PC12 cell neurite outgrowth. As previously reported, the greatest neurite outgrowth-promoting activity is associated with E8, but fragment E1-4 is also capable of supporting neurite outgrowth to a lesser degree (Fig. 3 a; Edgar et al., 1984). Blocking GalTase substrates in fragment E1-4 resulted in only a minor reduction ( $18 \pm 1\%$ ) in neurite initiation at 30–90 min of incubation compared with control E1-4, and insignificant differences ( $10 \pm 3\%$  inhibition compared with control E1-4) were observed in neurite formation after 4 h of culture (Fig. 3 b). Similar results were obtained when fragment E1 was used as a substrate (data not shown). In contrast, pregalactosylation of fragment E8 substrates inhibited neurite initiation by 75% ( $\pm 3\%$ ) relative to unmodified E8 control after 30 min of incubation and recovered to 51% ( $\pm 3\%$ ) inhibition by 90 min (Fig. 3 b). Neurite formation on pregalactosylated E8 was inhibited by 49% ( $\pm 3\%$ ) compared to control E8 after 4 h of culture. The morphology of cells during neurite initiation is illustrated for incubations on control mock-incubated E8 (Fig. 3 c), compared to cells on galactosylated E8 (Fig. 3 d).

Thus, both neurite initiation and neurite formation on fragment E8 are dependent upon interaction with GalTase, although GalTase effects are more significant during neurite initiation. In contrast, the low level of neurite outgrowth on fragment E1-4 is independent of GalTase.

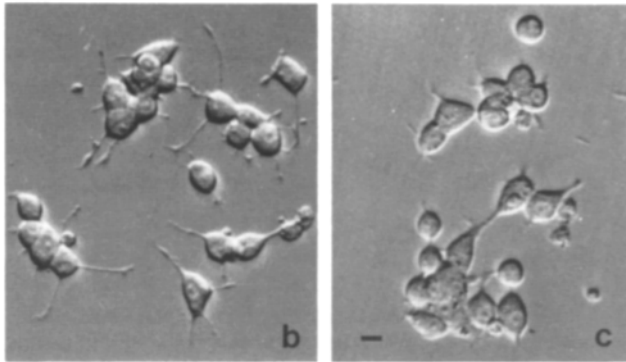
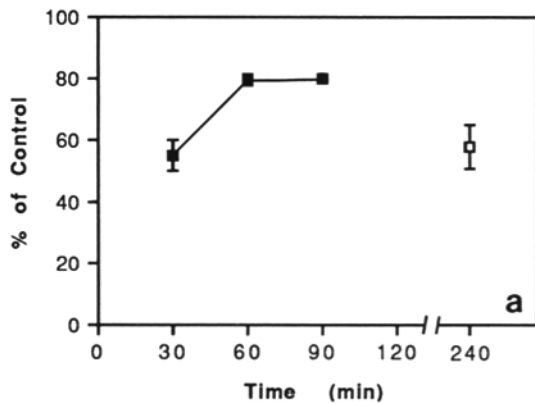
#### Neurite Outgrowth on Laminin Fragments in the Presence of Anti-GalTase IgG

Neurite outgrowth on laminin fragments was also examined in the presence of anti-GalTase IgG, which blocks the interaction of cell surface GalTase with its matrix substrate. A concentration of 500  $\mu$ g/ml of IgG was used, since this concentration was found to be optimal in previous dose-response studies (Begovac and Shur, 1990). Anti-GalTase IgG inhibited neurite initiation on fragment E8 by 46% ( $\pm 6\%$ ) of control IgG at 30 min and recovered to 22% ( $\pm 2\%$ ) inhibition relative to control at 90 min (Fig. 4 a). Neurite formation was inhibited by anti-GalTase IgG by 42% ( $\pm 7\%$ ) of control IgG after 4 h of incubation. In contrast, anti-GalTase IgG had little effect on neurite initiation or neurite formation on cells plated on either fragment E1-4 or E1 (data not shown). The inhibition of neurite formation in the presence of anti-GalTase IgG relative to control IgG is illustrated in Fig. 4, c and b, respectively.

In summary, either selectively consuming the GalTase binding sites within E8 by pregalactosylation, or inhibiting surface GalTase function with anti-GalTase IgG inhibits neurite initiation and neurite formation.

#### Discussion

This study establishes that fragment E8 is the principle GalTase binding domain within laminin and, more significantly, that the neurite-promoting activity of E8 is dependent upon



**Figure 4.** (a) Anti-GalTase IgG ( $n = 4$ ) inhibits neurite initiation at early times (■) and neurite formation at 4 h (□). Values represent the mean percent outgrowth on fragment E8 in anti-GalTase IgG versus control normal rabbit IgG. Error bars, SEM. The concentration of IgG used was 500  $\mu\text{g}/\text{ml}$ . Neurite formation on fragment E8 after 4 h of culture in the presence of normal rabbit IgG control (b) or in anti-GalTase IgG (c). Note the presence of well defined neurites on control cells (b) and the inhibition of neurite formation in the presence of anti-GalTase IgG (c). Bar, 10  $\mu\text{m}$ .

interaction with GalTase. The remaining laminin fragments also possess GalTase binding activity but the specific activity is less than that observed for fragment E8. The most striking illustration of this specificity comes from using membrane-bound GalTase, which mimics the surface GalTase-laminin interaction in situ. Solubilized GalTase shows reduced substrate specificity, although E8 is still galactosylated at higher efficiencies than other laminin fragments. The differences in substrate specificity between membrane-bound and soluble GalTase most likely reflect differences in enzyme kinetics, since the soluble enzyme has much greater access to its glycoprotein substrates than does the membrane bound enzyme whose particulate nature would produce fewer enzyme/substrate collisions resulting in lower glycosylation. Consequently, galactosylation towards all laminin fragments was higher using soluble GalTase compared with membrane-bound GalTase. However, the membrane environment also modulates the substrate specificity of GalTase, since the relative galactosylation of the laminin fragments differs between the soluble and membrane-bound enzymes. The fact that the membrane-bound and soluble GalTase show distinct kinetics and substrate specificity is consistent with recent studies of sperm surface GalTase, which selectively glycosylates only

one of three zona pellucida glycoproteins although all three glycoproteins are substrates for soluble GalTase (Miller, D. J., M. B. Macek, and B. D. Shur. 1990. *J. Cell Biol.* 111: 489a). Similarly, the behavior of other glycosyltransferases is modulated by the membrane environment; properties that can not be recovered by simply reconstituting the purified enzyme into liposome vesicles (Dannenberget al., 1989). Collectively, these results emphasize that the membrane environment can dramatically affect the enzyme's substrate specificity.

The biological significance of GalTase interaction with the E8 domain was assessed by assaying neurite initiation and outgrowth on fragments E1-4, E1, and E8 in the presence of anti-GalTase IgG antibodies or on fragment substrates in which GalTase binding sites had been blocked by pregalactosylation. With both of these perturbations, PC12 neurite initiation on individual fragments was inhibited only on fragment E8. On either E1-4 or E1, only a small inhibitory or no effect was observed. It is of interest that pregalactosylation of E8 produced a biphasic response with respect to neurite outgrowth, since neurite initiation was inhibited by 75% at early times, whereas formation of mature neurites was inhibited by only 50% after 4 h of incubation. These results mimic those reported previously for the role of surface GalTase in neurite initiation on intact laminin, possibly reflecting growth cone adherence to laminin, and a secondary role during neurite elongation (Begovac and Shur, 1990).

The mapping of GalTase substrates primarily to fragment E8 with lesser activity toward E1-4 and E1 is consistent with the carbohydrate composition of laminin. The carbohydrate content of laminin is reported to be ~13-15% (Chung et al., 1979; Engvall et al., 1983) to 25% (Knibbs et al., 1989). Primary sequence data from the cloning of the murine laminin chains (Sasaki and Yamada, 1987; Sasaki et al., 1987, 1988) predict that 73 potential *N*-linked oligosaccharide attachment sites are present. These linkage sites (Fig. 1) are distributed to the A chain (46 sites), B1 chain (13 sites), and the B2 chain (14 sites). Structural studies of the carbohydrate content of laminin suggests that the majority of oligosaccharide chains are *N*-linked and that approximately 40 asparagine linkage sites are utilized for glycosylation (Arumugham et al., 1986; Fujiwara et al., 1988). Characterization of terminal oligosaccharide residues indicate that  $\alpha$ -galactose,  $\beta$ -galactose, sialic acid, and  $\beta$ -*N*-acetylglucosamine are major sugar residues at the non-reducing terminus (Arumugham et al., 1986). The distribution of the sugar chains to various fragments has also been partially established. Fragment E1 contains 8-10 chains, fragment E4 contains 2-3 chains, and fragment E3 contains 1-2 chains (Fujiwara et al., 1988). Although there are no structural data for the actual number of fragment E8 glycosylation sites utilized, E8 has 20 potential *N*-linkage sites (Sasaki and Yamada, 1987; Sasaki et al., 1987, 1988). Previously, GalTase binding sites in laminin have been shown to be preferentially associated with *N*-linked oligosaccharide chains distributed between the A chain and the B chains at a 3:1 ratio (Runyan et al., 1988). Thus, our results indicating that GalTase preferentially binds fragment E8 are consistent with available structural data regarding both carbohydrate composition and distribution within laminin.

The importance of sugar residues on laminin during cell-matrix interactions has become apparent only recently. Lectin inhibition studies have suggested that wheat germ ag-

glutinin interferes with cell attachment on laminin, while con A inhibits cell spreading (Dean et al., 1988). Runyan et al. (1988) presented evidence that  $\beta$ -N-acetylglucosamine residues in laminin are important in melanoma cell spreading but not during cell attachment. Consistent with this, unglycosylated laminin supports melanoma cell and PC12 cell attachment but inhibits melanoma spreading and PC12 cell neurite outgrowth (Dean et al., 1990). These studies emphasize the role of oligosaccharides in cell interactions with laminin.

The mechanisms by which laminin promotes neurite outgrowth have become increasingly complex. In addition to binding sites for integrins (Bozyczko and Horwitz, 1986; Tomaselli et al., 1987, 1988) and GalTase (Begovac and Shur, 1990; present study), peptide sequences within E8 that support cell attachment and neurite growth include an Arg-Asn-Ile-Ala-Glu-Ile-Ile-Lys-Asp-Ile (p20) peptide in the B2 chain (Liesi et al., 1989) and an Ile-Lys-Val-Ala-Val (IKVAV) peptide in the A chain (Sephel et al., 1989c; Tashiro et al., 1989). It is unclear at this time how this multiplicity of surface receptors and laminin binding domains function collectively to mediate neurite outgrowth. Some evidence suggests that they may have temporally distinct roles, since initial attachment to laminin appears to be mediated by integrin binding sites (Tomaselli et al., 1987) as well as the p20 and IKVAV peptide domains (Liesi et al., 1989; Tashiro et al., 1989), whereas neurite initiation is mediated by GalTase binding to oligosaccharide residues within E8 (Begovac and Shur, 1990; present study). Once the neurite is established, additional outgrowth and elongation appears to require all of these receptors and binding domains, since blocking any of them individually results in only partial inhibition of neurite formation. Thus, while no single receptor or binding site is solely responsible for laminin's neurite outgrowth-promoting activity, there is likely to be a hierarchy of importance as well as cooperativity among the individual mechanisms.

In summary, two critical conclusions result from this study. First, cell surface GalTase, which has been shown to function as a laminin receptor during mesenchymal cell spreading, migration, and PC12 neurite outgrowth, preferentially binds to the E8 domain of laminin. Second, and more significantly, these studies show that the neurite-promoting activity of E8 is dependent upon its interaction with GalTase, since interfering with GalTase-E8 interactions by pregalactosylation of E8 substrates or incubation with anti-GalTase IgG inhibits neurite initiation and outgrowth. The significance of these conclusions is strengthened by the fact that the preferential binding of GalTase to fragment E8 is directly correlated with the biological activity of E8 during neurite outgrowth. Therefore, the neurite-promoting activity of fragment E8 is dependent upon the interaction between growth cone associated GalTase binding to N-linked oligosaccharides within the E8 domain. Presumably this binding stabilizes the interaction between the growth cone and laminin, allowing for continued growth cone extension. Unlike a migrating cell, the developing neurite does not need to dissociate from the underlying matrix for continued elongation. Consequently, it is unclear whether the GalTase:GlcNAc complex remains intact or is dissociated, a process that could occur catalytically by making available an appropriate galactose donor. However, it is still unknown whether such sugar donors are available extracellularly, although there is evi-

dence to suggest that glycosyltransferases can function catalytically on the cell surface in some situations (Turley and Roth, 1979). Further studies are needed to resolve this issue as well as the precise functional relationship between surface GalTase and other laminin receptors involved in neurite formation.

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