Cell Surface Galactosyltransferase Mediates the Initiation of Neurite Outgrowth from PC12 Cells on Laminin

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Abstract. Neurite outgrowth from PC12 pheochromocytoma cells, as well as from peripheral and central nervous system neurons in vitro, is mediated by the extracellular matrix molecule, laminin. We have recently shown that mesenchymal cell spreading and migration on laminin is mediated, in part, by the cell surface enzyme, β1,4 galactosyltransferase (GalTase). GalTase is localized on lamellipodia of migrating cells where it functions as a laminin receptor by binding to specific *N*-linked oligosaccharides in laminin (Runyan et al., 1988; Eckstein and Shur, 1989). In the present study, we examined whether GalTase functions similarly during neurite outgrowth on laminin using biochemical and immunological analyses. PC12 neurite outgrowth was inhibited by reagents that perturb cell surface GalTase activity, including anti-GalTase IgG and Fab fragments, as well as the GalTase modifier protein α-lactalbumin. Control reagents had no effect on neurite outgrowth. Furthermore, blocking GalTase substrates on laminin matrices by earlier galactosylation or enzymatic removal of GalTase substrates also inhibited neurite outgrowth. Conversely, neurite outgrowth was enhanced by the addition of UDP-galactose, which completes the GalTase enzymatic reaction, while inappropriate sugar nucleotides had no effect. The effects of all these treatments were dose and/or time dependent. Surface GalTase was shown to function during both neurite initiation and elongation, although the effects of GalTase perturbation were most striking during the initiation stages of neurite formation. Consistent with this, surface GalTase was localized by indirect immunofluorescence to the growth cone and developing neurite. Collectively, these results demonstrate that GalTase mediates the initiation of neurite outgrowth on laminin, and to a lesser extent, neurite elongation. Furthermore, this study demonstrates that process extension from both mesenchymal cells and neuronal cells is partly dependent upon specific oligosaccharide residues in laminin.

NEURITE outgrowth is influenced by soluble growth factors, such as nerve growth factor, (NGF[1]; reviewed in Levi-Montalcini, 1987) as well as by the extracellular matrix component laminin (Baron-Van Evercooren et al., 1982; Lander et al., 1983, 1985; Manthorpe et al., 1983; Edgar et al., 1984; Engvall et al., 1986; Tomaselli et al., 1987; Kleinman et al., 1988). Other extracellular matrix molecules, such as fibronectin (Baron-Van Evercooren et al., 1982; Rogers et al., 1983; Akeson and Warren, 1986; Schwarz et al., 1989) and collagen Type IV (Carbonetto et al., 1983; Turner et al., 1987) also appear to facilitate neurite outgrowth, but to a lesser degree than laminin. A recent study has suggested that a 25-kD fragment within the E8 fragment of laminin contains the neurite-promoting domain (Edgar et al., 1984, 1988; Engvall et al., 1986). The mechanism by which this portion of the laminin molecule interacts with the neuronal cell surface to promote neurite outgrowth remains unclear.

A number of studies have identified several cell surface molecules that interact with laminin during neurite outgrowth. Among these are the high-affinity 67-kD laminin receptor (Kleinman et al., 1988; Douville et al., 1988) as well as the integrin class of molecules (Tomaselli et al., 1987, 1988), which in particular have been shown to block neurite outgrowth from PC12 cells (Tomaselli et al., 1987). Recently, the cell surface enzyme β1,4 galactosyltransferase (GalTase) has also been shown to function as a laminin receptor during mesenchymal cell spreading and migration by binding to *N*-linked oligosaccharides in laminin (Runyan et al., 1988).

GalTase is a member of the glycosyltransferase super family of enzymes that is responsible for the biosynthesis of oligosaccharide chains on complex glycoconjugates. Specifically, GalTase transfers galactose from the nucleotide donor UDP-galactose (UDPGal) to its *N*-acetylglucosamine (GlcNAc) acceptor on various cellular and extracellular substrates (Pierce et al., 1980). GalTase is usually associated with the Golgi complex where it functions synthetically. However, GalTase is also found on the surface of many cells where it functions as a recognition molecule in a variety of

1. *Abbreviations used in this paper*: α-LA, α-lactalbumin; GalTase, Galactosyltransferase; GlcNAc, *N*-acetylglucosamine; NGF, nerve growth factor; UDPGal, UDP-galactose.

cellular interactions including fertilization, embryonic cell adhesion and migration, and growth control (reviewed in Pierce et al., 1980; Shur, 1988).

The function of surface GalTase during cell migration has been defined using a variety of cell biological, biochemical, and immunological approaches. For example, avian neural crest cell migration on basal lamina-like matrices can be both negatively and positively influenced by reagents that block or enhance GalTase activity, respectively (Runyan et al., 1986). GalTase has been localized to lamellipodia and filopodia of mouse 3T3 fibroblasts migrating on laminin, but GalTase is not detectable on lamellipodia of cells migrating on fibronectin, indicating that GalTase functions specifically during cell-laminin interactions (Eckstein and Shur, 1989). GalTase does not appear to function during the initial adhesion of cells to laminin, since reagents that perturb surface GalTase activity selectively inhibit cell spreading and migration on laminin; initial cell attachment is not affected (Runyan et al., 1988). Together, these observations indicate that GalTase is present on the surface of migratory cells at the leading edges of lamellipodia and filopodia where it binds to *N*-linked oligosaccharides in laminin. Since neurite outgrowth also occurs on laminin, these studies raise the possibility that filopodial extension and neurite outgrowth on laminin are mediated by analogous cell-matrix interactions.

In the present study, we examined the role of surface GalTase during neurite outgrowth on laminin using reagents that specifically interfere with cell surface GalTase activity or that modify GalTase substrates in laminin. The PC12 neuronal cell line was chosen as the experimental system for this study due to their widespread use as an in vitro model for neurite outgrowth (Akeson and Warren, 1986; Turner et al., 1987; Tomaselli et al., 1987; Schwartz et al., 1989; Sephal et al., 1989). Treatment of PC12 cells with NGF generates a differentiated population of cells possessing many properties of sympathetic neurons (Greene and Tischler, 1976). The primary advantage in using these cells is that neurite outgrowth can be examined without contamination of non-neuronal cells. This investigation specifically examined the potential function of GalTase during neurite outgrowth on laminin, since laminin is the extracellular matrix molecule that possesses the greatest neurite promoting activity in a variety of isolated neurons as well as neuronal cell lines. Furthermore, neurite outgrowth of PC12 cells occurs to a greater degree on laminin than on other extracellular matrix molecules and this substrate preference is similar to that of sympathetic neurons (Tomaselli et al., 1987). We present evidence to suggest that surface GalTase is localized to the developing PC12 neurite where it mediates the initiation of neurite outgrowth on laminin, and to a lesser extent neurite elongation.

Materials and Methods

Reagents

Laminin was purified from EHS tumor (gift of Dr. Raymond Runyan, University of Iowa, Iowa City, IA) or obtained from Sigma Chemical Co. (St. Louis, MO) and diluted in Medium B (127 mM NaCl, 5.3 mM KCl, 18.2 mM Hepes, pH 7.2). Specific reagents used to perturb cell surface GalTase included α-lactalbumin (α-LA) (control reagents: carboxymethyl α-LA or lysozyme); UDPGal (control reagent: UDP-glucose); and rabbit anti-bovine milk galactosyltransferase (anti-GalTase) IgG antibody (control reagent: normal rabbit IgG). Anti-GalTase Fab fragments were prepared using immobilized papain (Pierce Chemical Co., Rockford, IL) following the manufacturer's protocol. The purity of the Fab fragment preparation was verified by SDS-PAGE.

Anti-GalTase IgG was prepared as previously described and has been shown to specifically recognize murine GalTase by a variety of criteria, including immunoprecipitation and inhibition of GalTase enzymatic activity, and by immunoprecipitation of a single metabolically labeled membrane protein (Lopez et al., 1985; Bayna et al., 1988). For the present study, the anti-GalTase IgG was shown to recognize PC12 GalTase by immunoprecipitation of GalTase activity from solubilized membranes as described by Eckstein and Shur (1989). All reagents were obtained from Sigma Chemical Co. unless otherwise indicated.

For each experiment, reagents were reconstituted directly into DME (Gibco Laboratories, Grand Island, NY) at given concentrations except for anti-GalTase antibodies, which were dialyzed directly against DME. Reagents in media were equilibrated in 10% CO_2 before addition to cells.

Cells

Rat pheochromocytoma (PC12) cells (Greene and Tischler, 1976) were obtained as a gift from Dr. Kim Angelides (Baylor College of Medicine, Houston, TX). Cells were grown in DME supplemented with 10% heat-inactivated horse serum, 5% FBS, 2 mM fresh glutamine, and penicillin/streptomycin in a 10% CO_2 humidified incubator at 37°C and passaged weekly. PC12 cells were primed by plating cells on dishes coated with laminin (1 μg/ml) and treating cells with 2.5 S NGF (50 μg/ml) for 7–10 d (Greene, 1977). For individual experiments, primed cells were harvested by trituration in calcium-magnesium-free Dulbecco's PBS for 5–10 min and collected by centrifugation for 4 min at 500 *g*. Cells were washed three times in unsupplemented DME and then resuspended in DME containing the appropriate experimental or control reagent. Cells were plated at a density of 2–5 × 10^4 cells per well. Outgrowth assays for each perturbation were performed as described below.

Substrate Preparation

Substrates for all experiments examining cell surface GalTase perturbation were prepared by coating four-well (2.1 cm^2 wells) tissue culture dishes (Nunc, Inc., Naperville, IL) with 0.5 ml of laminin (10 μg/ml) for 60–90 min at 37°C. Wells were washed three times in Medium B before plating cells in reagent-containing medium.

Substrates for experiments examining laminin matrix modifications were prepared as follows. Four-well dishes (Nunc, Inc.) were coated with laminin (10 μg/ml) for 2 h at 37°C. Wells were washed three times with Medium B and incubated with 2 mg/ml BSA in Medium B for 60 min at 37°C. Wells were again washed three times with Medium B and incubated with one of three treatments. In the first modification, laminin was galactosylated to consume GalTase binding sites by incubation overnight at 37°C with purified GalTase (20 μg/well) in Medium B containing 2 mM UDPGal and 10 mM $MnCl_2$. The reaction mixture was replaced with fresh reagents three times during the overnight incubation. Control wells were treated in parallel with Medium B. Wells were then washed four times with Medium B before plating cells. Outgrowth assays were performed as detailed below.

The second modification of the laminin matrix was performed using β-*N*-acetylglucosaminidase to remove terminal GlcNAc residues. Wells were incubated with three changes of either Medium B, 0.1 M sodium citrate buffer, pH 5.25, or 0.1 M sodium citrate buffer, pH 5.25, containing 2 U of jack bean β-*N*-acetylglucosaminidase overnight at 37°C. Wells were then washed four times with Medium B before plating cells.

The third matrix modification involved the enzymatic removal of *N*-linked oligosaccharide chains in laminin with *N*-glycanase (Genzyme Corp., Boston, MA). After coating 48-well tissue culture dishes with laminin, wells were washed and incubated with 60 U/ml of *N*-glycanase in 0.2 M sodium phosphate buffer, pH 8.6, overnight at 37°C. The enzyme treatment was not preceded by a BSA block in an attempt to maximize the effectiveness of the *N*-glycanase. Wells were then washed four times with Medium B, blocked with BSA, and washed as described above before plating cells.

The effectiveness of these enzymatic treatments at either blocking or removing GalTase substrates in laminin was evaluated as described in Runyan et al. (1988). Briefly, after final washing, wells were incubated with purified GalTase (20 μg/well) in Medium B containing 87.5 μM UDP[^{3}H]-Gal (574 dpm/pmol; DuPont Co., Wilmington, DE; New England Nuclear, Boston, MA) and 10 mM $MnCl_2$ at 37°C for 2 h. Wells were then washed four times with Medium B and bound radioactivity was quantitated by solubilizing the matrix in 1 N NaOH followed by liquid scintillation counting.

Outgrowth Assays

Two different assays were used to determine the effects of each particular reagent on neurite outgrowth. Both assays used PC12 cells that had been primed with NGF. However, no additional NGF was added during the neurite outgrowth assay (Fig. 1), since NGF accelerates the rate of PC12 cell adhesion and neurite formation (Schubert and Whitlock, 1977; Chandler and Herschman, 1980). Perturbations of surface GalTase in the absence of NGF produced more striking effects than in the presence of NGF, which required an increased concentration of GalTase perturbants to produce a similar inhibition of neurite outgrowth.

The first assay quantitated neurite initiation by scoring the number of cells that deviate from a round morphology. Round morphology was defined as phase bright refractive cells lacking any process extensions from the cell body. Observations were carried out on live cells at 30, 60, and 90 min after plating using an inverted phase microscope, with the stage maintained at 37°C and 10% CO_2 in a plexiglass incubator. At least 200 cells were counted for each experimental group at each timepoint. The data are expressed as the percentage of cells that deviate from a round morphology. Representative cultures incubated in control medium for 0, 60, and 90 min are shown in Fig. 1, *a–c*.

The second assay quantitated neurite formation by scoring the number of cells possessing neurites. Neurites were defined as process extensions greater than one cell body in length possessing a broadened growth cone (Heidemann et al., 1985; Turner et al., 1987). These assays were carried out on longer term cultures (3–6 h). At the end of the culture period, cells were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.2, for 30 min at 23°C and washed twice in 0.1 M sodium phosphate buffer, pH 7.2. Cells were then examined on an inverted phase microscope and scored for the presence or absence of neurites as defined above. The data are expressed as the percentage of cells possessing neurites. At least 200 cells were counted for each treatment group. A representative culture in control medium is shown in Fig. 1 *d*.

For both assays, cell clumps containing more than five cells were not counted in the results. All values are expressed as the mean ± SEM.

In addition to these assays, cells were photographed with either phase-contrast or Hoffman modulation interference microscopy to document cell morphology with each of the experimental manipulations.

Rate of Neurite Elongation

Experiments to determine the rate of neurite elongation were performed using one cell surface perturbation, anti-GalTase IgG, and one matrix modification, before galactosylation. For each perturbation, cells were harvested and treated as detailed above. Cultures were incubated for 2 h to allow processes to form, at which time random fields were photographed. 2 h later, the same fields were rephotographed and these micrographs were analyzed using an X-Y digitizing board to measure neurite lengths (VIAS software; Ted Pella, Inc., Tustin, CA). Neurite lengths were measured only on those cells for which preexisting process extensions were observed at the 2 h timepoint. The difference in process lengths between 2 and 4 h were used to generate the mean rate (micrometers per hour) of neurite elongation. Data analysis using the Student's *t*-test for statistical comparison was carried out using VSTAT software (Ted Pella, Inc.).

Immunofluorescence

Chambered microscope slides were coated with laminin (5–10 μg/ml) overnight at 4°C and then washed three times with Medium B. Primed PC12 cells were plated into wells and allowed to extend neurites for various periods of time (30–90 min). The cells were then incubated with anti-GalTase IgG antibody (500 μg/ml) in DME containing 5% chicken serum as blocking protein for 45 min at 23°C. Wells were rinsed three times over 15 min with PBS followed by incubation in fluorescein conjugated goat anti-rabbit IgG (1:50 dilution; Boehringer Mannheim Biochemicals, Indianapolis, IN) for 40 min at 23°C. Wells were then washed three times with PBS for 15 min and fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 30 min at 23°C. Wells were washed an additional 30 min in several changes of 0.1 M sodium phosphate buffer and coverslipped. Localization of GalTase was performed using a Leitz epifluorescence microscope equipped with a fluorescein filter cube. Images were photographed using Kodak Tri-X film (400 ASA).

Results

Perturbation of Cell Surface GalTase

Cell surface GalTase was perturbed with specific reagents that either negatively or positively affect GalTase activity. The effect of each perturbation on neurite outgrowth was determined by assaying the extent of neurite initiation and neurite formation in experimental and control groups. In all instances, perturbation of surface GalTase did not appreciably affect initial cell attachment to laminin, although neurite outgrowth was significantly affected.

Anti-GalTase IgG. The addition of anti-GalTase IgG reduced neurite initiation to 41% (±4%) of control IgG at 30 min and recovered to 57% (±3%) of control at 90 min (Fig. 2 *a*). In longer term assays, anti-GalTase IgG reduced the number of cells possessing neurites to 57% (±7%) of control normal rabbit IgG. Lower concentrations of anti-GalTase IgG (50–150 μg/ml) were less effective at inhibiting neurite outgrowth, whereas higher concentrations (1 mg/ml) did not result in greater inhibition. Anti-GalTase Fab fragments (300–500 μg/ml) also reduced neurite formation to 70% (±8%) of media control in long term cultures (4–6 h) similar

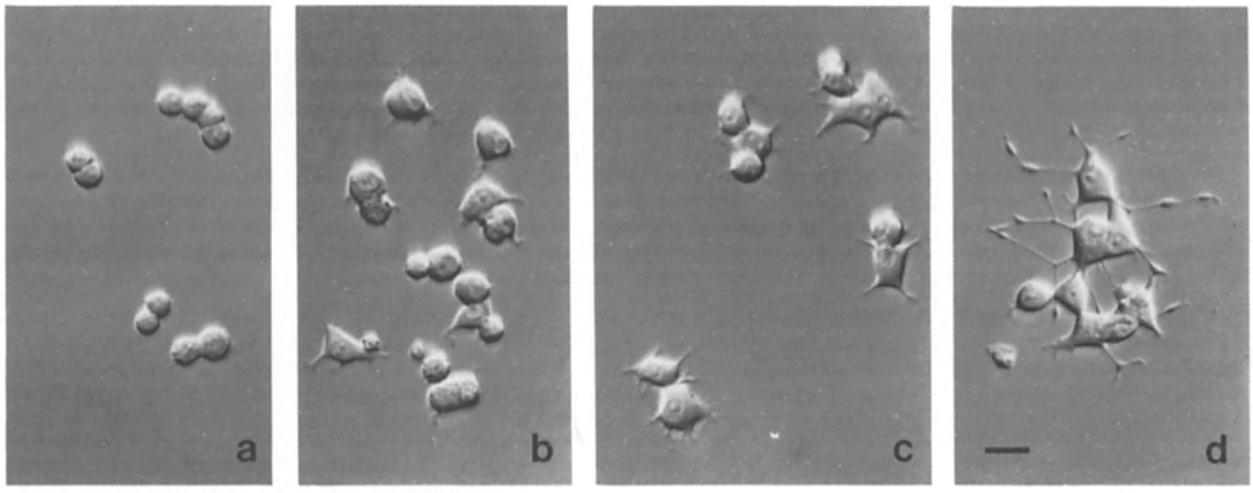
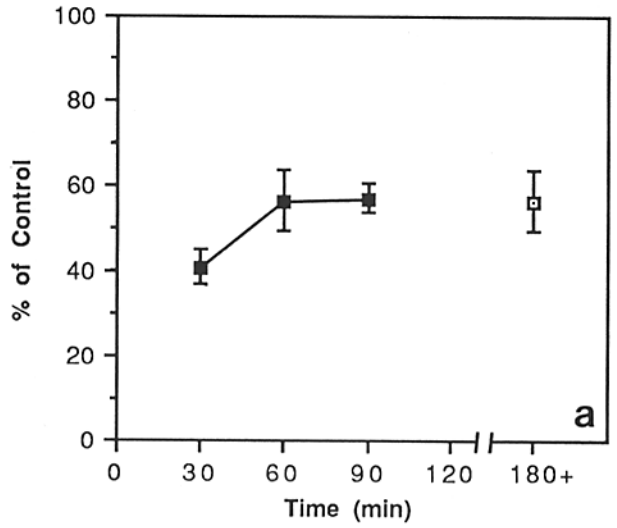


Figure 1. Representative timecourse of neurite outgrowth from NGF-primed PC12 cells on laminin in the absence of additional NGF. *a*, immediately after plating (0 time); *b*, 60 min; *c*, 90 min; *d*, 4 h. Bar, 20 μm.



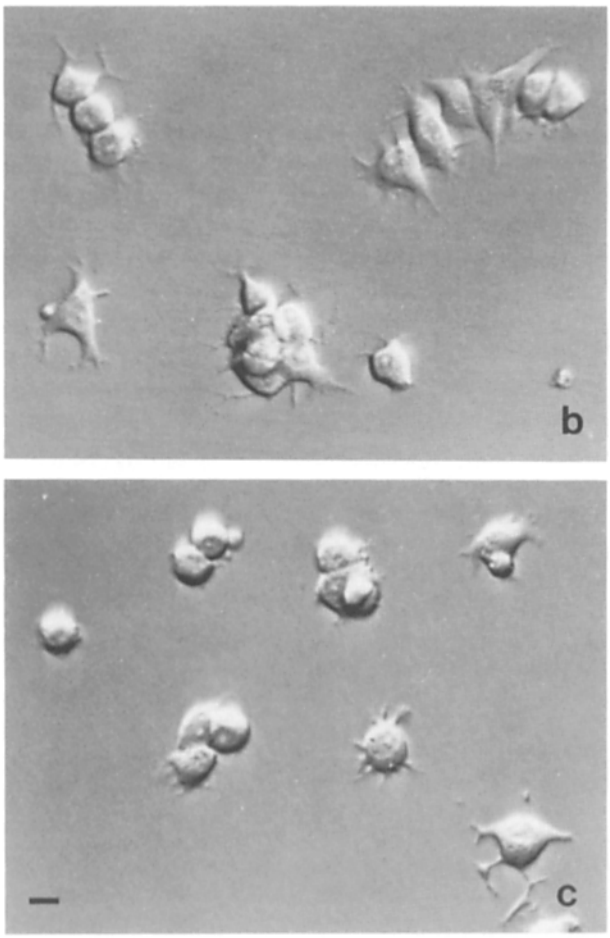


Figure 2. (*a*) Anti-GalTase IgG inhibits neurite initiation at early times (■; $n = 4$) and neurite formation at later times (□; $n = 3$). Values represent mean percent outgrowth in anti-GalTase IgG vs. normal rabbit IgG control. Error bars denote SEM. Concentrations of antibody used ranged from 250 to 500 μg/ml. Neurite formation was assayed from 3 to 6 h with similar results for the various times. (*b*) Normal rabbit IgG (400 μg/ml) 45 min after plating. (*c*) Anti-GalTase IgG (400 μg/ml) after 45 min. Note the inhibition of cell flattening and sprouting in the anti-GalTase IgG-treated cells. Bar, 10 μm.

to the effects of anti-GalTase IgG. The morphological appearances of control IgG and anti-GalTase IgG treated cells are illustrated in Fig. 2, *b* and *c*, respectively.

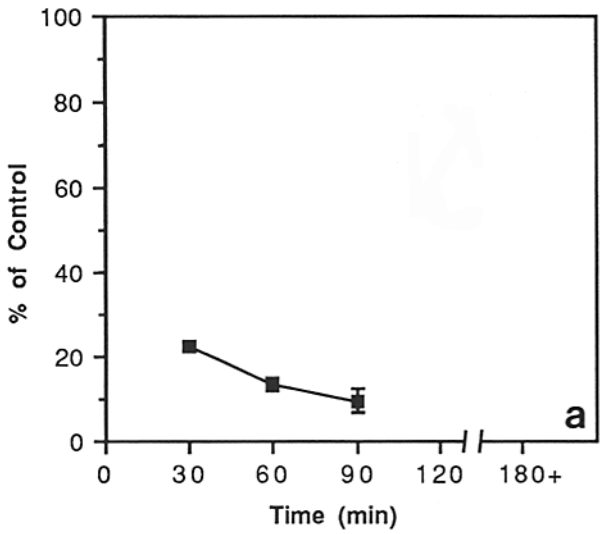
α-LA. α-LA is a GalTase modifier protein that shifts the substrate specificity of GalTase away from GlcNAc and toward glucose. α-LA proved to be the most effective inhibitor of neurite outgrowth. Control reagents for α-LA perturbation were lysozyme, a structurally homologous protein and carboxymethyl α-LA, a modified α-LA that is <1% as active as α-LA at modifying GalTase substrate specificity. As illustrated in Fig. 3 *a*, neurite initiation was reduced to 22% (±1%) of control carboxymethyl α-LA at 30 min, and this value decreased to <10% (±3%) of control after 90 min. With longer incubations up to 4 h, a negligible number of cells possessed definable neurites in the presence of α-LA. The inhibitory effects of α-LA at 90 min were dose dependent between 0.1 and 1.0% α-LA, relative to controls (data not shown). Furthermore, while initial cell attachment to laminin was not affected by α-LA, cells appeared more rounded with increasing α-LA concentration and time in culture. The morphological appearances of control and α-LA perturbed cells are depicted in Fig. 3, *b* and *c*, respectively.

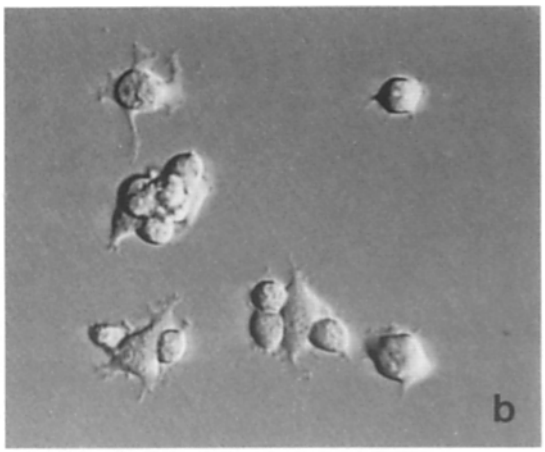
UDPGal. UDPGal is the sugar nucleotide donor required for the catalytic dissociation of the GalTase-GlcNAc complex. The addition of UDPGal to cultures may, therefore, increase the catalytic turnover of the GalTase-laminin complex and consequently enhance the ability of cells to extend neurites. As shown in Fig. 4 *a*, the addition of 200 μM UDPGal enhanced neurite initiation during early culture periods, ranging from 173% (±9%) of control UDP-glucose at 30 min, and decreasing to 121% (±5) of control UDP-glucose after 90 min. In longer term cultures (3–4 h), UDPGal enhanced neurite formation to 129% (±5%) of UDP-glucose controls, similar to the differences observed at 90 min for neurite initiation. A UDPGal concentration of 20 μM produced only a slight increase in neurite outgrowth, while 2 mM UDPGal produced results similar to those obtained with 200 μM UDPGal. The morphological appearances of UDP-glucose and UDPGal treated cells is depicted in Fig. 4, *b* and *c*, respectively.

An independent assessment of GalTase function during neurite outgrowth was obtained by incubating cells with the competitive substrate, ovalbumin. Ovalbumin (7.5 mg/ml) reduced neurite initiation to 22% (±3%) of control BSA (7.5 mg/ml) at 30 min and recovered to 85% (±1%) of control at 90 min. In one experiment, pretreatment of ovalbumin with β-*N*-acetylglucosaminidase, which was subsequently heat-inactivated, partly destroyed its ability to inhibit neurite outgrowth (i.e., 68% of BSA control at 30 min, recovering to 94% of control at 90 min).

Modification of Laminin Matrices

α-LA, anti-GalTase IgG, UDPGal, and ovalbumin are all reagents that affect cell surface GalTase activity and consequently, GalTase could function during neurite outgrowth by acting on substrates endogenous to the cell (i.e., *cis* effects) or by functioning as a laminin receptor by binding to substrates within laminin (i.e., *trans* effects). To distinguish between these possibilities, three methods were used to either consume or remove GalTase substrates in laminin, after which the extent of neurite initiation and neurite formation was determined.





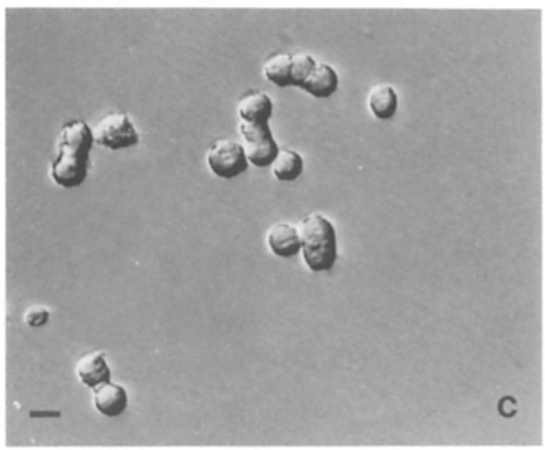
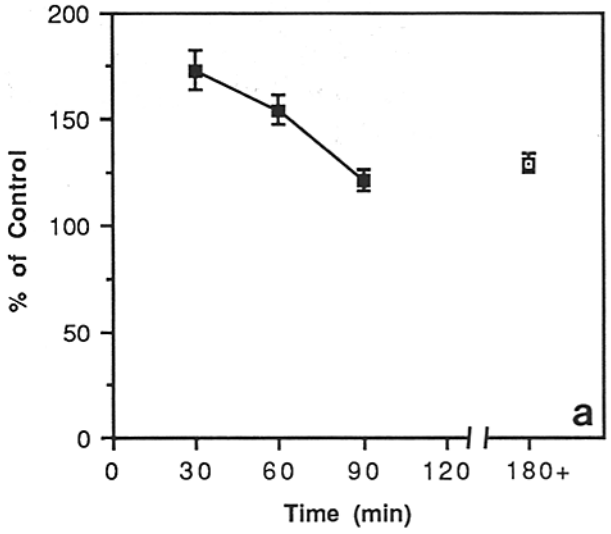
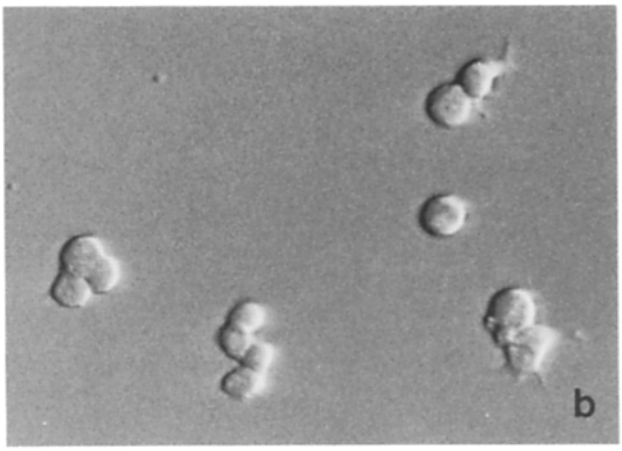


Figure 3. (*a*) α-LA inhibition of neurite initiation at early times (■; $n = 2$). Values represent the mean percent outgrowth in 0.5% α-LA vs. 0.5% carboxymethyl α-LA control. (*b*) 0.5% carboxymethyl α-LA after 70 min. (*c*) 0.5% α-LA after 70 min. There is increased cell flattening and process extensions in control (*b*) whereas the α-LA treated cells show a lack of flattening and sprouting. Bar, 10 μm.





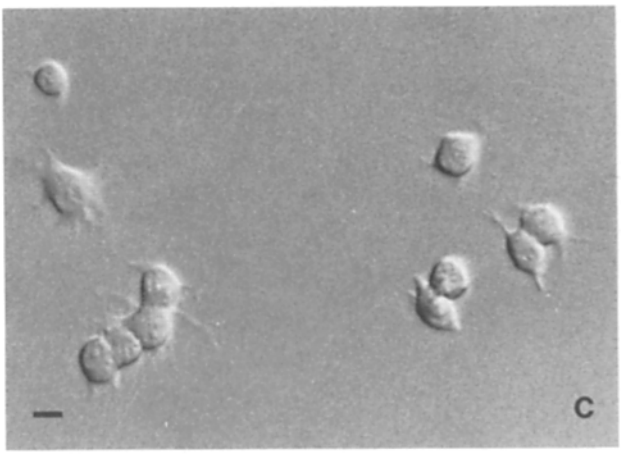


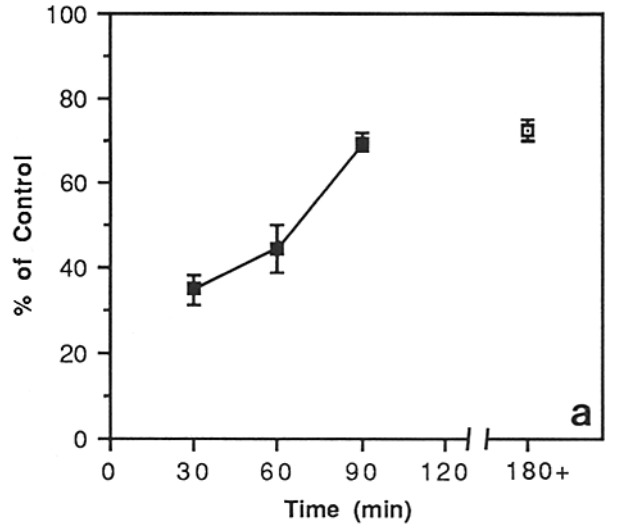
Figure 4. (*a*) UDPGal enhances neurite initiation at early times (■; $n = 8$) and neurite formation at later times (□; $n = 3$). Values represent the mean percent outgrowth in 200 μM UDPGal vs. 200 μM UDP-glucose control. Neurite formation was assayed after 3–4 h with similar results for the various times. Error bars denote SEM. (*b*) 200 μM UDP-glucose after 50 min incubation. (*c*) 200 μM UDPGal after 50 min. Note the enhanced flattening and greater process extensions in the UDPGal-treated group. Bar, 10 μm.

Table I. Modifying GalTase Substrates in Laminin Inhibits Neurite Initiation

Laminin pretreatment	Efficiency of blocking or removing GalTase substrate	Inhibition of neurite initiation*
	%	%
Earlier galactosylation	99 ± 0.6 (6)‡	65.3 ± 3.5
β-*N*-acetylglucosaminidase	74 ± 2.3 (5)	38.3 ± 5.4
N-glycanase	53 (1)	42.5 ± 4.5

* Assayed at 30 min after plating.
‡ Number of parenthesis represents the number of assays used to determine the efficiency of laminin modification.
Data shown are mean ± SEM.

Blocking GalTase Binding Sites. In the first series of experiments, available GlcNAc substrates in laminin were consumed by prior galactosylation of the matrix as described in Materials and Methods. The effectiveness of this earlier galactosylation was assessed by subsequent GalTase assays using UDP[^{3}H]Gal and confirmed that >99% of the available GlcNAc sites were blocked (Table I). Earlier galactosy-



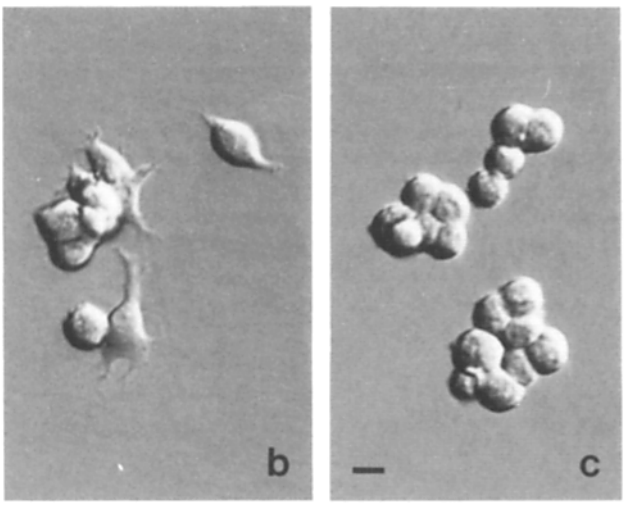
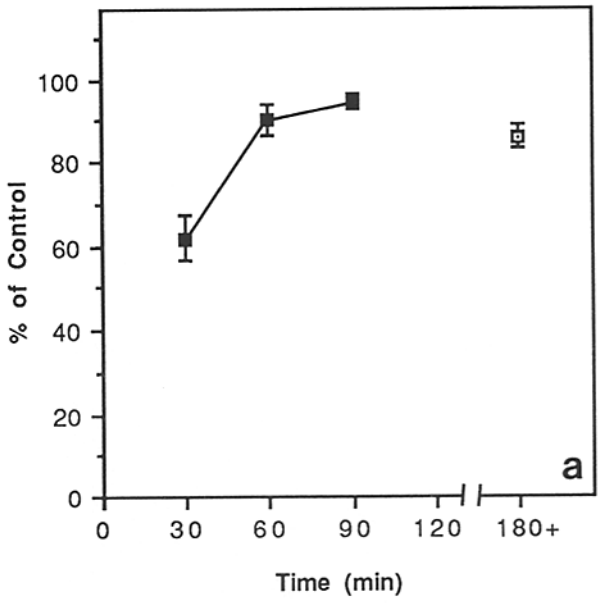


Figure 5. (*a*) Masking GalTase binding sites in laminin by earlier galactosylation inhibits neurite initiation at early times (■; $n = 6$) and neurite formation at later times (□; $n = 3$). Values represent the mean percent outgrowth on galactosylated laminin versus unmodified laminin. Error bars denote SEM. Neurite initiation on (*b*) unmodified laminin (control) or (*c*) galactosylated laminin after 45 min. Bar, 10 μm.



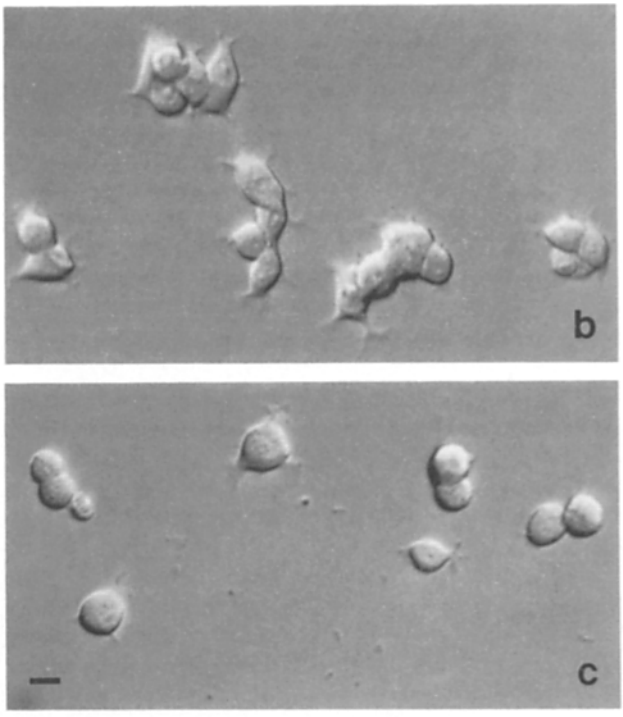


Figure 6. (*a*) Removal of GalTase substrates on laminin by β-*N*-acetylglucosamidase partially inhibits neurite initiation at early times (■; $n = 4$) and neurite formation at later times (□; $n = 4$). Values represent the mean percent outgrowth on treated laminin versus control laminin. Error bars denote SEM. Neurite initiation on (*b*) unmodified laminin (control) or (*c*) β-*N*-acetylglucosaminidase-treated laminin after 45 min. Bar, 10 μm.

lation reduced neurite initiation to 35% (±4%) of control at 30 min, recovering to 70% (±2%) of control after 90 min (Fig. 5 *a*). After 3–4 h of incubation, neurite formation was 73% (±3%) of control. Initial cell attachment was affected qualitatively to a minor extent with this treatment, but with time, cell attachment was established and neurite formation was initiated. Cells plated on galactosylated laminin (Fig. 5 *c*) remain round while cells on control laminin initiate neurite formation (Fig. 5 *b*).

Removal of GalTase Binding Sites. In the second modification of the laminin matrix, the matrix was first incubated with β-*N*-acetylglucosaminidase to remove terminal GlcNAc residues. Despite repeated attempts, the β-*N*-acetylglucosaminidase treatment was only partially effective at removing GalTase binding sites (Table I). Nevertheless, on this modified matrix, neurite initiation was reduced to 62% (±5%) of buffer control at 30 min becoming 95% (±2%) of control at 90 min (Fig. 6). In longer term cultures (3–5 h), neurite formation was reduced to 84% (±3%) of control. A reduction in neurite sprouting from cells is apparent on β-*N*-acetylglucosaminidase-treated laminin (Fig. 6 *c*) relative to unmodified laminin (Fig. 6 *b*).

The third modification of the laminin matrix was the enzymatic removal of *N*-linked oligosaccharide chains, which contain GalTase binding sites, by treatment with *N*-glycanase. This treatment reduced neurite initiation to 58% (±5%) of buffer control at 30 min and recovered to 89% (±3%) of control at 90 min (data not shown). The *N*-glycanase treatment, as with the β-*N*-acetylglucosaminidase treatment, was only partially effective at removing GalTase binding sites on laminin (Table I). Presumably this was due to limited accessibility of the *N*-glycanase to the oligosaccharide chains in native laminin.

Collectively, all three procedures inhibited neurite initiation, and to a lesser extent, neurite formation, on laminin. The magnitude of this inhibition roughly paralleled the relative efficiency of destroying GalTase binding sites within laminin.

Rate of Neurite Elongation

To distinguish between the effects of GalTase perturbation on neurite initiation from the effects on neurite formation, the rate of neurite elongation was determined (Table II). Perturbation of cell surface GalTase with anti-GalTase IgG produced a consistent reduction in the rate of neurite elongation to 65% (±2%) of control normal rabbit IgG. Similarly, modification of the laminin matrix by earlier galactosylation of GalTase substrates reduced the rate of neurite elongation to 66% (±5%) of control unmodified laminin. Therefore, the process of neurite elongation, subsequent to neurite initiation, is inhibited by GalTase perturbation though to a lesser extent.

Surface GalTase Activity and Immunolocalization

The presence of GalTase on the cell surface was established by two criteria: enzyme assay using exogenous GlcNAc as substrate as well as by indirect immunofluorescence. Under conditions that eliminated potential intracellular GalTase activity, surface GalTase activity was 100 pmol [^{3}H]Gal transferred per hour/10^6 cells. All cells used in these studies were cultured in the absence of NGF. Incubation of cells in NGF had no effect on the level of surface GalTase activity during the periods examined (i.e., 48 h incubation).

More importantly, the distribution of GalTase during neurite outgrowth was examined by indirect immunofluorescence using anti-GalTase IgG. GalTase was present on the surface of both the cell soma and the extending neurite processes. There were, however, focal concentrations of GalTase localized along the cell periphery in contact with the un-

Table II. Perturbing Surface GalTase Activity Inhibits the Rate of Neurite Elongation

Treatment	Experiment	Sample	*n**	Mean rate	Control
				μm/h; ±SEM	%
Anti-GalTase IgG	1	Anti-GalTase IgG‡	60	5.93 ± 0.4	63.0§
		Control IgG	41	9.42 ± 0.7	
	2	Anti-GalTase IgG	34	5.96 ± 0.6	62.5§
		Control IgG	59	9.54 ± 0.5	
	3	Anti-GalTase IgG	57	6.43 ± 0.4	68.5§
		Control IgG	64	9.39 ± 0.6	
	Average	Anti-GalTase IgG	3¶	6.11 ± 0.2¶	64.7¶
		Control IgG	3¶	9.45 ± 0.1¶	
Earlier Galactosylation	1	Galactosylated laminin	44	5.85 ± 0.3	65.3§
		Laminin	42	8.96 ± 0.5	
	2	Galactosylated laminin	60	6.49 ± 0.5	56.9§
		Laminin	58	11.41 ± 0.7	
	3	Galactosylated laminin	46	7.16 ± 0.5	72.7§
		Laminin	42	9.85 ± 0.5	
	Average	Galactosylated laminin	3¶	6.69 ± 0.4¶	66.4¶
		Laminin	3¶	10.07 ± 0.7¶	

* Number of neurites assayed.
‡ Experiments performed with anti-GalTase IgG or control normal rabbit IgG at concentrations of 500 μg/ml.
§ Significant at the 0.01% level when analyzed using the Student's *t*-test.
¶ Denotes average of three experiments.

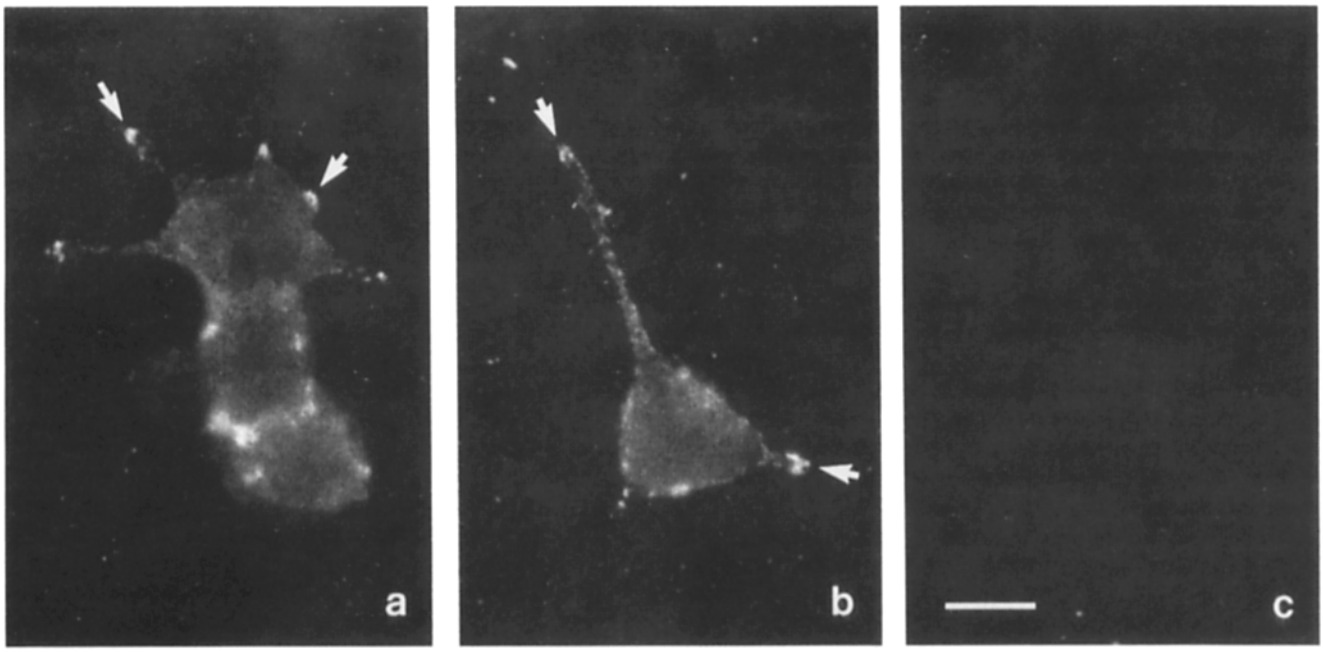


Figure 7. Indirect immunofluorescence localization of GalTase on primed PC12 cells plated on laminin. Note the focal GalTase concentrations at the growth cones (*arrows*) of early forming neurites (*a* and *b*). Control cells stained with normal rabbit IgG (*c*). Bar, 10 μm.

derlying matrix and at the growth cone of forming neurites (Fig. 7, *a* and *b*) consistent with its proposed function during neurite outgrowth. Focal GalTase localizations were also observed at contact sites between adjacent cells. Control normal rabbit IgG produced background levels of immunofluorescence (Fig. 7 *c*).

Discussion

This study demonstrates that surface GalTase functions during neurite outgrowth from PC12 cells on laminin. We have used two approaches, perturbation of cell surface GalTase and modification of GalTase substrates in laminin, to document the involvement of GalTase in neurite outgrowth. Both α-LA and anti-GalTase IgG, which bind GalTase and thus interfere with its binding to GlcNAc substrates, inhibited neurite outgrowth on laminin. These results are consistent with previous studies in which α-LA and/or anti-GalTase IgG were shown to inhibit neural crest cell migration (Runyan et al., 1986), B16-F10 melanoma cell spreading (Runyan et al., 1988), and 3T3 cell migration on laminin, but not on fibronectin (Eckstein and Shur, 1989). On the other hand, UDPGal, a reagent that increases the dissociation of the GalTase–GlcNAc complex, increased neural crest cell migration (Runyan et al., 1986) and B16-F10 melanoma cell spreading (Runyan et al., 1988). Likewise, in the present study, UDP-Gal increased neurite initiation relative to UDP-glucose controls. Thus, these results demonstrate that perturbing cell surface GalTase either negatively or positively results in a corresponding decrease or increase, respectively, in neurite outgrowth.

The perturbation of cell surface GalTase could produce intracellular effects that may have secondary consequences on neurite outgrowth. However, modification of the laminin substrate in a GalTase-specific manner, such as by earlier galactosylation and by enzymatic removal of GlcNAc substrates, established that the effects observed on neurite outgrowth do reflect a cell surface receptor association with laminin. These matrix modifications also specifically demonstrate that neurite outgrowth is dependent, to some extent, on terminal GlcNAc residues. Similarly, cell spreading on laminin has been shown to be dependent upon oligosaccharide residues on the basis of lectin inhibition studies (Dean et al., 1988) and laminin modifications (Runyan et al., 1988) similar to those in the present study.

The greatest inhibition of neurite outgrowth was observed after earlier galactosylation, which is the most GalTase-specific modification of laminin. The inhibition of neurite outgrowth after enzymatic removal of GlcNAc substrates, while not as great as with earlier galactosylation, nevertheless did parallel the results obtained with galactosylated laminin. In general, the degree of inhibition appears to be a consequence of the effectiveness of the particular laminin modification (Table I). Nevertheless, these experiments establish that modification of the laminin matrix in a GalTase-specific manner, as well as by reagents that directly perturb cell surface GalTase, result in an inhibition of neurite outgrowth.

An important finding of this study was that while neurite outgrowth was influenced by perturbing GalTase, cell attachment to laminin was not significantly affected. Only in experiments where laminin was treated by earlier galactosylation did cells appear to attach more slowly. This effect was not quantitated further. Laminin receptors (Douville et al., 1988) and integrins (Tomaselli et al., 1988) are present on PC12 cells and antibodies to integrins are capable of inhibiting adhesion as well as neurite outgrowth from PC12 cells (Tomaselli et al., 1987). While we cannot completely preclude GalTase involvement in cell attachment, it is likely that laminin receptors other than GalTase, including integrins,

mediate the initial binding to laminin, whereas other mechanisms are important in neurite sprouting and neurite extension. In this regard, B16-F10 melanoma cell attachment is also unaffected by perturbing GalTase interactions with laminin (Runyan et al., 1988), similar to our observations for PC12 cells. Thus, the GalTase effects on neurite outgrowth observed in the present study are independent of and subsequent to initial cell attachment phenomena.

The ability of cells to extend neurites is maximally inhibited by perturbing GalTase during the early phases of neurite initiation. By 90 min of incubation, the differences in neurite outgrowth between experimental and control groups are reduced but remain stable during prolonged incubation as evidenced by assaying the number of cells possessing neurites after 3–6 h. To determine the function of GalTase during neurite elongation as apposed to neurite initiation, the effect of perturbing GalTase on the rate of elongation of preexisting neurites was determined. Modification of both cell surface GalTase with anti-GalTase IgG or blocking GalTase substrates in laminin by earlier galactosylation reduced the rate of neurite elongation (Table II). This effect was of similar magnitude (i.e., 35% inhibition) to that observed after 90 min of incubation in the short-term assay (deviation from round) as well as that observed in the long-term assay (percent cells with neurites). Thus, while GalTase plays a more significant role during neurite initiation, it also has a secondary function during neurite elongation in conjunction with other laminin receptors, such as integrins, that have a more dominant role during neurite elongation.

Neurite outgrowth on muscle cell surfaces has been shown to be mediated by N-CAM, N-Cal-CAM, and the JG22 antigen (Bixby et al., 1987). Several molecules including N-cadherin, N-CAM and integrins collectively function during retinal neurite outgrowth on astrocytes (Neugebauer et al., 1988). Similarly, PC12 cell neurite outgrowth on laminin involves multiple receptors including the 67-kD laminin receptor (Kleinman et al., 1988; Douville et al., 1988), integrins (Tomaselli et al., 1987), and GalTase (this study). These studies support and emphasize the notion that multiple cell surface receptors are involved in neurite outgrowth (Reichardt et al., 1989).

In summary, our studies demonstrate that GalTase functions during neurite outgrowth, most significantly during neurite initiation but also during neurite elongation. While other receptors such as the integrins (Tomaselli et al., 1987) are known to play important roles during neurite outgrowth, additional cell surface molecules contribute to and have the ability to modulate neurite outgrowth as well. A further insight from this study is that different cell types such as fibroblasts and neuronal cells appear to be capable of using analogous receptor–matrix interactions during process extension involving terminal oligosaccharide residues in laminin. In this regard, the L2/HNK-1 carbohydrate is able to competitively inhibit neurite outgrowth on laminin (Kunemund et al., 1988) and anti-L2/HNK-1 antibodies inhibit cell migrations in vivo and in vitro (Bronner-Fraser, 1987). Further studies will be necessary to define any potential interaction with surface GalTase. It is apparent, however, that GalTase functions as a general cell surface receptor capable of mediating cell interactions with laminin in a variety of cell types including neural crest cells, mesenchymal cells, melanoma cells, and PC12 neuronal-like cells. We are presently defining the GalTase binding domain in laminin more precisely to compare it with laminin domains reported by others to promote neurite outgrowth.

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