Journal of Cell Science 108, 839-847 (1995)
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Overexpressing cell surface β1,4-galactosyltransferase in PC12 cells increases neurite outgrowth on laminin

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SUMMARY

Neurite outgrowth on cellular and extracellular matrices is mediated by a variety of cell surface receptors. Some of these receptors recognize peptide determinants, whereas others bind oligosaccharide ligands. Previous studies have suggested that cell surface β1,4-galactosyltransferase functions as one of these receptors during neurite outgrowth on basal lamina by binding to N-linked oligosaccharides in the E8 domain of laminin. However, these previous investigations have been limited to the use of galactosyltransferase inhibitory reagents to block neurite formation. Therefore, in this study, we investigated whether the level of surface galactosyltransferase directly affects the efficiency of neurite outgrowth, or rather, is incidental to neurite formation. Northern blot analysis and cell surface galactosyltransferase assays were used to select two stable PC12 transfectants that overexpress surface galactosyltransferase by approximately four-fold. Radiolabeled antibody binding to intact cells and indirect immunofluorescence confirmed the higher expression of surface galactosyltransferase on transfected cells, compared to controls. Both galactosyltransferase transfected cell lines exhibited markedly enhanced neurite initiation, neurite formation, and rates of neurite elongation by two- to three-fold. These studies demonstrate that the expression of laminin receptors can be rate-limiting during neurite outgrowth, and that the level of surface galactosyltransferase can modulate the frequency and rate of neurite formation from PC12 cells on laminin.

Key words: PC12 cell, neurite outgrowth, cell surface galactosyltransferase

INTRODUCTION

The outgrowth of an axon to its cellular target is critical for the development of the embryonic nervous system and the regeneration of nerve fibers during wound healing. During neurite outgrowth and pathway selection, neurons interact with adjacent neuronal and nonneuronal cells and with the extracellular matrix (Reichardt and Tomaselli, 1991; Doherty and Walsh, 1989). The distribution of particular extracellular matrix components in different regions of the developing vertebrate nervous system correlates spatially and temporally with the presence of axonal growth (Engvall et al., 1990; Rogers et al., 1986). One such extracellular matrix component known to facilitate neurite outgrowth is laminin, and several cell-binding domains of the laminin molecule have been mapped. The E1-4 fragment is believed to contain several cell adhesion domains (Mecham, 1991; Tomaselli et al., 1990), whereas P1 and E8 fragments promote the outgrowth of neurites (Liesi et al., 1990; Sephel et al., 1989; Engvall et al., 1986).

The various functional domains of laminin have been shown to interact with several classes of cell surface receptors (Mecham, 1991; Kleinman et al., 1990; Buck and Horwitz, 1987). One class is the integrins, a family of heterodimer cell surface receptors consisting of noncovalently associated α and β subunits (Hynes, 1987). Another cell surface receptor for laminin is β1,4-galactosyltransferase (GalTase), which participates in neurite outgrowth from PC12 cells, chick sympathetic ganglia, and sensory neurons (Begovac et al., 1991; Riopelle and Dow, 1991; Thomas et al., 1990; Begovac and Shur, 1990). GalTase is traditionally found in the *trans*-cisternae of the Golgi complex, where it engages in the biosynthesis of complex oligosaccharide moieties. GalTase has also been found on the plasma membrane of many cell types, where it serves as a cell surface receptor mediating various cell-cell and cell-matrix interactions (Shur, 1993; Roth, 1973; Roseman, 1970), including sperm-egg binding (Miller et al., 1992), cell spreading (Runyan et al., 1988), migration (Hathaway and Shur, 1992; Eckstein and Shur, 1992), and neurite outgrowth (Begovac et al., 1991; Riopelle and Dow, 1991; Thomas et al., 1990; Begovac and Shur, 1990).

The GalTase genes of all species examined thus far transcribe two classes of mRNA, each of which contains a unique in-frame AUG initiation codon (Lopez et al., 1991; Shaper et al., 1988). In mouse, translation from the first AUG encodes a

protein of 399 amino acids and is designated as the long form of GalTase, whereas translation from the second AUG encodes a protein of 386 amino acids and is designated as the short form of GalTase. The two GalTase proteins differ by a 13 amino acid extension at the amino-terminal cytoplasmic domain, which is present only on the long form of GalTase (Russo et al., 1990). Transfection of cells with appropriate GalTase cDNAs and S_1 nuclease protection analyses suggest that the short form of GalTase is confined to the Golgi complex, whereas a portion of the long form is transported out of the Golgi and to the cell surface where it functions as a cell adhesion molecule (Lopez et al., 1991). By immunofluorescence confocal microscopy, antiserum specific to the 13 amino acid cytoplasmic domain has been used to localize the long form to the cell surface in cell type-specific distributions (Youakim et al., 1994). Finally, overexpressing the cytoplasmic and transmembrane domains of the long form inhibits surface GalTase-dependent cellular interactions (Evans et al., 1993) and targets a reporter protein to the cell surface (S. C. Evans and B. D. Shur, unpublished), whereas the analogous portion of the short form does not.

That surface GalTase functions as a laminin receptor during neurite outgrowth has been suggested by a number of laboratories. Indirect immunofluorescence staining shows that surface GalTase is localized focally at the tips of the growing neurite during neurite outgrowth (Begovac and Shur, 1990). Blocking surface GalTase activity with anti-GalTase IgG or consuming GalTase substrates in laminin by pregalactosylation both inhibit neurite initiation and neurite elongation from PC12 cells. Similarly, perturbation of surface GalTase with antibodies, modifier proteins or competitive substrates, partially inhibits neurite outgrowth on laminin from sensory neurons (Riopelle and Dow, 1991) and from chick dorsal root ganglia (Thomas et al., 1990). The GalTase binding site in laminin has been mapped to the E8 domain, and E8's neurite promoting activity is at least partially dependent on surface GalTase binding, since blocking or removing GalTase binding sites in E8 destroys its neurite promoting activity (Begovac et al., 1991).

Although these studies provide evidence that surface GalTase functions as a laminin receptor during neurite outgrowth on laminin, they are indirect, in that they all rely upon the ability of GalTase-perturbants to inhibit neurite outgrowth. We felt it was important to assess GalTase function more directly and to do so by creating a positive, rather than a negative, result. Therefore, in this study, we examined the effects of overexpressing surface GalTase expression on neurite outgrowth in appropriately transfected PC12 cells.

MATERIALS AND METHODS

Cell culture

PC12 cells were maintained as previously described (Begovac and Shur, 1990). Briefly, PC12 cells were refed every 2-3 days and harvested with Ca^{2+}, Mg^{2+}-free phosphate buffered saline (CMF-PBS, pH 7.4) and replated weekly onto polystyrene dishes in fresh growth medium. For neurite outgrowth experiments, PC12 stock or transfected cells were primed by plating onto 1 μg/ml laminin-coated dishes and treating with 50 ng/ml nerve growth factor for 7-10 days.

Transfections

The long form of GalTase cDNA, designated pKJ-PDLGT, was obtained by subcloning a portion of the GalTase cDNA (−9/1265 bp) containing nine nucleotides of the 5′-untranslated region, 1200 nucleotides of the GalTase-coding sequence, and 65 nucleotides of the 3′-untranslated region into the PKJ-1 vector (Lopez et al., 1991).

PC12 cells were transfected using lipofectin (Muller et al., 1990). Briefly, 5×10^5 PC12 stock cells were plated onto polylysine-coated (50 μg/ml) 60 mm dishes 7 hours prior to DNA addition. After refeeding cells with 2 ml of Dulbecco's modified Eagle's medium (DMEM), 100 μl of a mixture containing 20 μg construct DNA, 2 μg pKJ-neo DNA, and 30 μg lipofectin (Gibco-BRL, Gaithersburg, MD) was added to the dish and the medium gently mixed. A 2 ml sample of PC12 growth medium was added to each dish 5 hours after DNA addition. The medium containing DNA and lipofectin was removed 18-24 hours after DNA addition and replaced by PC12 growth medium. Cells were allowed to grow in PC12 growth medium for 48 hours after DNA addition and then selected by growing in PC12 cell growth medium containing 400 μg/ml (final concentration) G418 antibiotic (Gibco-BRL). After 18-24 days, individual G418-resistant colonies were selected and expanded for further analysis.

Northern blot analysis

Total RNA was isolated from stable transfectants and mouse lactating mammary gland tissue by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987) as previously described (Lopez et al., 1991). The RNA samples were electrophoresed in a 1% agarose gel after glyoxylation (Sambrook et al., 1989). Briefly, 15-30 μg of total RNA per sample was resuspended in DEPC-treated water and denaturation solution, and heated at 50°C for 1 hour. After denaturation, RNA samples were chilled on ice, mixed with electrophoresis loading buffer, and loaded onto a 1% agarose gel in 10 mM NaH_2PO_4, pH 7.0. Samples were electrophoresed for about 3 hours at 55 V (constant voltage) in a circulating buffer (10 mM NaH_2PO_4, pH 7.0). Separated RNA was transferred onto nitrocellulose filters using capillary transfer in 20×SSC (Sambrook et al., 1989). The filters were dried under a heat lamp and baked at 80°C for 2 hours in a vacuum oven.

Hybridizations were performed by first prehybridizing in 25 ml of prehybridization solution (5× SSC, 20 mM Tris-HCl, pH 7.6, 10% dextran sulfate, 50% deionized formamide, 1× Denhardt's solution, 200 μg/ml denatured salmon sperm DNA, 0.1% SDS) at 43°C for 4-24 hours. Filters were hybridized at 43°C for 16-20 hours in prehybridization solution supplemented with the denatured radiolabeled probe prepared from gel-purified *Eco*RI-digested pMGT and radiolabeled by nick translation (Lopez et al., 1991). Following hybridization, the solution was removed and the filter was washed five times with 2×SSC containing 0.1% SDS at room temperature. Filters were then washed in 0.1×SSC, 0.1% SDS at 45°C for 30 minutes. Finally, the filters were visualized by autoradiography, using Kodak Diagnostic X-Ray film (Eastman Kodak Company, Rochester, New York). Densitometry was performed by digital scanning of the autoradiographs by using a video camera (Javelin Electronics, Torrance, CA) and Imagepro digitizing. Band intensities were determined by manually identifying an area of the image to be scanned and digitizing that area into pixal densities over a 0-256 density range for comparison to a control area.

Assay of cell surface GalTase expression

The expression of GalTase on the cell surface was monitored using three assays: enzyme activity on intact cells, radiolabeled antibody binding to intact cells, and indirect immunofluorescence. For enzyme assay, PC12 transfectants and stock cells were harvested with CMF-PBS, washed once with 1% BSA in DMEM, and twice with Medium B (127 mM NaCl, 5.3 mM KCl, 18.2 mM Hepes buffer, pH 7.2) containing a protease inhibitor cocktail (PIC; Begovac et al., 1991). The

cell pellet was resuspended in Medium B/PIC and 1.2×10^6 cells were added on ice to an assay tube containing 100 μM UDP[^{3}H]Gal (574 dpm/pmol; Dupont Co., Wilmington, DE), 10 mM $MnCl_2$, and 30 mM GlcNAc in a total volume of 300 μl Medium B/PIC. The assay tube was incubated at 37°C, and 50 μl aliquots were removed every 15 minutes for 1 hour. A 10 μl sample of ice-cold 0.2 M Tris-EDTA (pH 7.2) was added to each 50 μl aliquot to terminate the reaction. Product separation was carried out by high-voltage borate electrophoresis and quantitated by LSC as described previously (Lopez et al., 1991).

In some instances, radiolabeled antibody binding was used to quantify GalTase expression on the cell surface as previously described (Nguyen et al., 1994). Cells were prepared as above, and 2×10^5 cells were incubated in blocking buffer (Medium B, 4% BSA, and PIC) for 45 minutes at room temperature, washed once with Medium B, and incubated for 45 minutes at room temperature in either preimmune or anti-GalTase antiserum diluted to 1:25 in blocking buffer. The anti-GalTase antiserum was raised against bacterially expressed recombinant murine GalTase, and reacts specifically with the polypeptide backbone of GalTase (Nguyen et al., 1994). Cells were pelleted and washed three times with Medium B and incubated in 8.0 μCi/ml ^{125}I-labelled goat anti-rabbit IgG in Medium B with 2% BSA for 45 minutes at room temperature. The samples were washed three times with Medium B. Cells were lysed in 1 M NaOH and counted with a LKB 1275 mini gamma counter. Cell washes were monitored for removal of soluble ^{125}I-labelled goat anti-rabbit IgG.

Surface localization of GalTase was performed as previously detailed using anti-GalTase IgG, which recognizes GalTase on PC12 cells and which inhibits PC12 cell neurite outgrowth on laminin (Begovac and Shur, 1990). Briefly, primed PC12 cells were plated onto laminin-coated coverslips and allowed to extend neurites for 90-120 minutes. After rinsing once with PBS, the cells were incubated with 500 μg/ml of rabbit anti-GalTase IgG or preimmune IgG (as control) in DMEM containing 5% chicken serum for 45 minutes at room temperature. After rinsing with PBS, cells were incubated with fluorescein-conjugated goat anti-rabbit IgG (1:50 dilution; Boehringer Mannheim, Indianapolis, IN) in PBS containing 5% chicken serum for 40 minutes. Cells were then washed with PBS, fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 30 minutes, and washed again before coverslipping. The distribution of surface GalTase was evaluated with a fluorescence microscope (Nikon Optiphot, Melville, NY) and photographed using 400 ASA Tri-X film (Kodak).

Neurite outgrowth and elongation rate assays

Neurite outgrowth and neurite elongation rates were assayed as detailed previously (Begovac and Shur, 1990). Primed cells were harvested with CMF-PBS, washed three times with DMEM, and then plated onto the laminin-coated (10 μg/ml) 4-well dishes in DMEM. The neurite outgrowth process was videotape-recorded at 30 minutes and at 4 hours. Neurite initiation was defined as the percentage of the cells that had spikes extending from the cell body, whereas cells without neurite initiation had round morphology and no spikes. Neurite formation was defined as the percentage of the cells that possessed a neurite greater than one cell body in length. Quantitation was performed by counting cells with initiating neurites and formed neurites at 30 minutes and 4 hours, respectively. At least 200 cells were counted per timepoint.

The rate of neurite extension was measured from time-lapse video recordings of neurite outgrowth assays from PC12 stock and transfectants over 4 hours. The video field was printed onto transparencies at a 30-60 minute timepoint by which time process extensions were initiated, and at the 2-3 hour timepoint at which time pre-existing neurites had elongated. The transparencies were then projected onto an X-Y digitizing board and the differences in length of specifically identified neurites were calculated by computer program (SIGMA-SCAN, Jandel Corp., Corte Madera, CA). Neurite lengths were measured only on those cells for which pre-existing process extensions were observed at the 30-60 minute timepoint. The difference in process lengths between the two timepoints was used to generate the mean rate (μm/h) of neurite elongation. Data analysis was carried out using SIGMA-SCAN (Jandel Corp) using Student's *t*-test for statistical comparison.

RESULTS

Experimental strategy

In this study, we examined the effects of increasing cell surface GalTase expression on PC12 neurite outgrowth on laminin. PC12 cells were transfected with appropriate GalTase cDNAs and a series of screening assays, including Southern and northern blot analyses and GalTase enzyme activity, were used to select colonies overexpressing surface GalTase. Elevated surface GalTase expression was confirmed by radiolabeled antibody binding to intact cells and by indirect immunofluorescence. The biological consequences of increased surface GalTase expression were tested using neurite initiation, neurite formation, and neurite elongation assays.

Isolation of stable transfectants

Stably transfected PC12 cells containing the full-length PDLGT insert were initially selected by resistance to the neomycin analog G418. After 18-24 days exposure, G418-resistant colonies were isolated and expanded. Colonies were isolated after transfection with either pKJ-neo alone (control, 12 colonies) or with pKJ-neo and pKJ-PDLGT constructs (17 colonies). The pKJ-PDLGT transfectants were more flattened and had more spikes than did the neo control colonies, which were primarily round and had a smooth surface (data not shown). Two independent neo-transfected control clones (pKJ-neo 4, pKJ-neo 2-5) and two independent PDLGT-transfected clones (pKJ-PDLGT 3′, pKJ-PDLGT 2-5) were selected according to their morphological appearance for further analysis.

Northern blot analysis shows transfectants express high levels of GalTase transgene RNA

Northern blot analysis was used to determine the level of GalTase RNA in control cells and GalTase transfectants. Total RNA isolated from mouse lactating mammary gland (a positive control), PC12, pKJ-neo 4, pKJ-PDLGT 3′ and pKJ-PDLGT 2-5 cells was probed with radiolabeled GalTase cDNA fragment (30/2620 bp). The results revealed a 4.4 kb band, corresponding to endogenous GalTase mRNA (Lopez et al., 1991), present in all of the RNA samples (Fig. 1). RNA from pKJ-neo 2-5 also revealed a 4.4 kb band (data not shown). In addition to the endogenous GalTase mRNA, a transcript ~1.9 kb in size was observed in both pKJ-PDLGT 3′ and pKJ-PDLGT 2-5 colonies corresponding to the PDLGT transcript (Fig. 1). This transcript was absent in all other colonies tested as well as in lactating mammary gland. These results indicate that the introduced GalTase PDLGT gene was transcribed in the pKJ-PDLGT 3′ and pKJ-PDLGT 2-5 colonies. Quantitation of GalTase expression levels by scanning densitometry of northern blot films showed that the transgene mRNA was 4.1-fold (n=3 films) higher than endogenous GalTase mRNA in pKJ-PDLGT 3′ cells and 4.4-

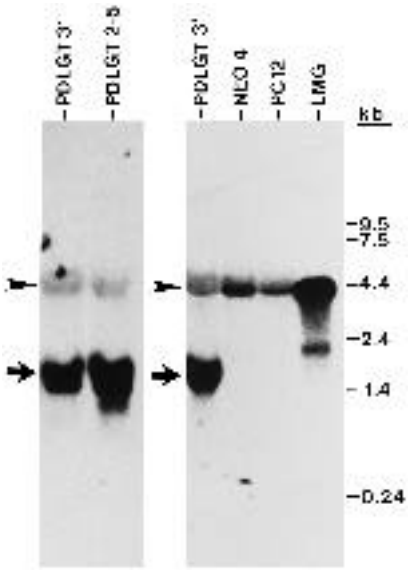

Fig. 1. Northern blot analysis of GalTase mRNA expression in stable transfectants. Northern blot analysis of total RNA from mouse lactating mammary gland (LMG; 1 μg), PC12 cells (PC12; 30 μg), pKJ-neo 4 control (NEO 4; 30 μg), pKJ-PDLGT 3′ cells (PDLGT 3′; 30 μg), and pKJ-PDLGT 2-5 cells (PDLGT 2-5; 30 μg). Blots were probed with ^{32}P-labeled GalTase cDNA (30/2620 bp). Arrowhead, the endogenous GalTase mRNA; arrow, the GalTase transgene mRNA; kb, RNA molecular mass markers (Gibco-BRL) in kilobases. Analysis of transfectants showed that compared to endogenous GalTase RNA, the GalTase transgene RNA was elevated 4.4-fold for pKJ-PDLGT 2-5 and 4.1-fold for pKJ-PDLGT 3′.

fold (n=3 films) higher in pKJ-PDLGT 2-5 cells. Southern blot analyses confirmed the integration of murine GalTase into PC12 genomic DNA in both pKJ-PDLGT 3′ and pKJ-PDLGT 2-5 cells (data not shown).

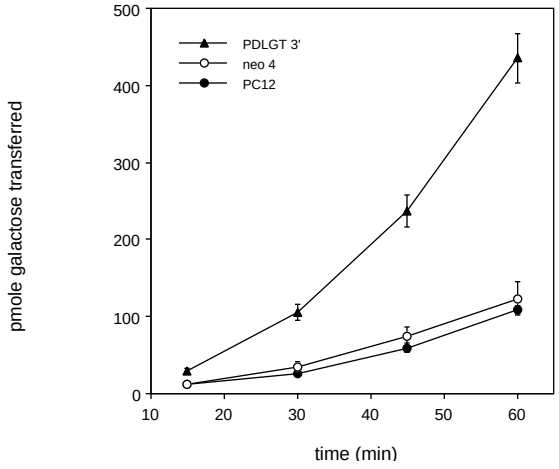

Fig. 2. Cell surface GalTase assays of PC12 transfectants. Intact cells were used as an enzyme source and the cell surface GalTase activities of PC12 (filled circles, n=3), pKJ-neo 4 (open circles, n=4) and pKJ-PDLGT 3′ (triangles, n=3) were determined as described in Materials and Methods. Each value represents the mean of at least triplicate assays and enzyme rates are expressed as pmoles galactose transferred per 10^6 cells at each timepoint. Error bars, s.e.m.

Cell surface GalTase levels are increased on PC12 transfectants

Since the GalTase transgene mRNA was apparently transcribed in pKJ-PDLGT transfectants, the expression of GalTase on the cell surface was monitored using three assays: enzyme activity associated with intact cells, radiolabeled antibody binding to intact cells, and indirect immunofluorescence. Viable, intact PC12 cells, pKJ-neo 4 cells and pKJ-PDLGT 3′ cells were assayed for cell surface GalTase activity under conditions optimized for surface-associated activity (Evans et al., 1993). Surface GalTase activity of PC12 cells was 109.37 ±5.25 pmol Gal transferred/hour per 10^6 cells, pKJ-neo 4 cell activity was 123.48 ±21.05 pmol Gal transferred/hour per 10^6 cells, and pKJ-PDLGT 3′ cell activity was 435.53 ±32.34 pmol Gal transferred/hour per 10^6 cells (Fig. 2). These results demonstrate that surface GalTase activity on pKJ-PDLGT 3′ cells was approximately four-fold higher than on control neo and parental PC12 cells.

As an independent means to assess GalTase expression on the cell surface, intact cells were incubated with anti-GalTase antiserum raised against bacterially expressed murine GalTase (Nguyen et al., 1994). Binding of anti-GalTase antiserum, or of preimmune serum in control assays, was quantified by ^{125}I-labeled goat anti-rabbit IgG. In agreement with assays of enzyme activity on intact cells (Fig. 2), pKJ-PDLGT 3′ and pKJ-PDLGT 2-5 cells bound 2.9 times and 2.7 times, respectively, more anti-GalTase IgG than did control cells (PC12, pKJ-neo 4) (Fig. 3). It is not readily apparent why assays using radiolabeled antibody binding gave slightly lowers levels of GalTase expression (3-fold increase) than did assays of GalTase enzymatic activity (4-fold increase). Possibly, not all of the GalTase expressed on the cell surface was as accessible to IgG as it was to low molecular mass enzyme substrates.

The increased expression of surface GalTase was localized by indirect immunofluorescence. As shown in Fig. 4, the level

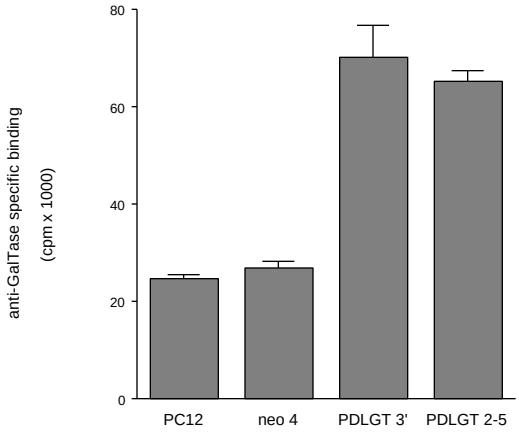

Fig. 3. Quantification of GalTase expression on the cell surface using radiolabeled antibody binding. Intact cells (2×10^5 of each type) were incubated with rabbit antiserum raised against bacterially expressed murine GalTase (Nguyen et al., 1994). The bound IgG was quantified by ^{125}I-labeled goat anti-rabbit IgG. Values represent the GalTase-specific immunoradioactivity (above preimmune controls) and are the average of triplicate determinations and representative of two independent assays. Control radioactivity (i.e. preimmune serum) ranged from 15,449 to 17,819 cpm. Error bars, s.e.m.

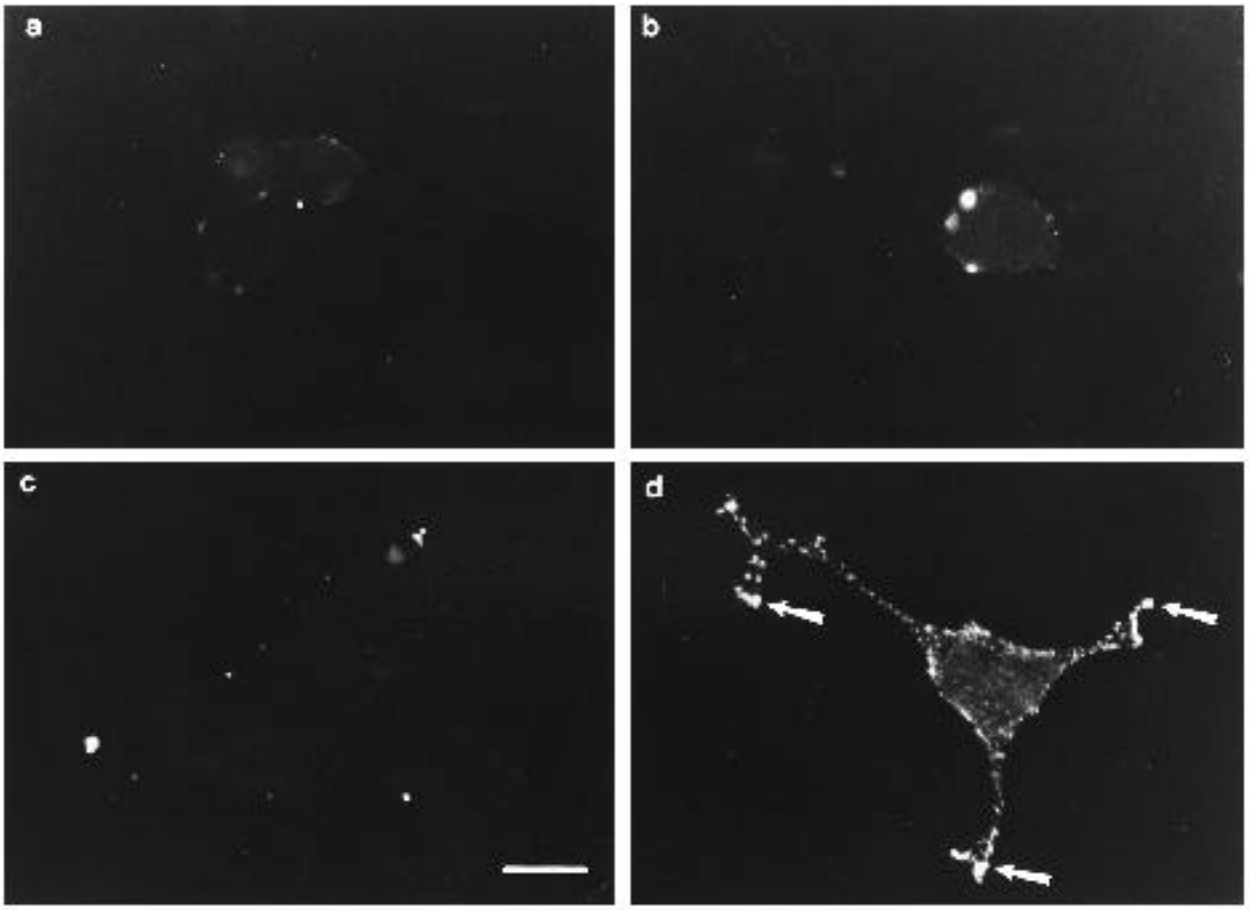

Fig. 4. Anti-GalTase immunofluorescence of PC12 transfectants. Indirect immunofluorescence with anti-GalTase antibodies was used to localize surface GalTase protein in pKJ-neo 4 (a and b) and pKJ-PDLGT 3′ (c and d) cells. In a and c, cells were incubated with pre-immune IgG (500 μg/ml), whereas the cells in b and d were incubated with anti-GalTase IgG (500 μg/ml). Arrows denote foci of GalTase on the tips of neurites. Bar, 10 μm.

of cell surface GalTase on pKJ-PDLGT 3′ cells was clearly higher than that on pKJ-neo 4 controls. Furthermore, GalTase was concentrated at the tips of neurites. Since anti-GalTase immunoreactivity was much more intense on PDLGT-transfected cells, the photographic conditions used minimize the appearance of the endogenous surface GalTase immunoreactivity on control cells as previously reported (Begovac and Shur, 1990). Collectively, all three assays agree with results from northern blot analyses and confirm that surface GalTase levels were increased three- to four-fold in PDLGT transfectants.

Neurite outgrowth is enhanced in cells expressing increased cell surface GalTase

We determined whether the increased cell surface GalTase levels on transfected PC12 cells affected neurite outgrowth on laminin. Neurites were scored at early (i.e., 30 minutes) and late (i.e., 4 hours) times of incubation to assess neurite initiation and neurite formation, respectively (Begovac and Shur, 1990). After 30 minutes, the percentages of neurite initiation in pKJ-PDLGT 3′ and pKJ-PDLGT 2-5 cells were 73.2% (±5.03%) and 76.3% (±6.8%), respectively, whereas PC12 and pKJ-neo 4 controls were only 28.8% (±1.57%) and 37.0% (±4.7%), respectively (Fig. 5a). Fig. 5b illustrates the morphology of PC12, pKJ-neo 4, pKJ-PDLGT 3′ and pKJ-PDLGT 2-5 cells at 30 minutes, showing the enhanced neurite initiation in transfectants compared to control cells.

After 4 hours of incubation, the percentages of neurite formation in pKJ-PDLGT 3′ and pKJ-PDLGT 2-5 cells were 79.5% (±5.1%) and 76.3% (±4.9%), respectively, whereas PC12 cells and pKJ-neo 4 controls were only 33.8% (±3.9%) and 31.8% (±7.6%), respectively (Fig. 6a). Fig. 6b illustrates the obvious difference in neurite formation between PC12 stock, pKJ-neo 4, pKJ-PDLGT 3′ and pKJ-PDLGT 2-5 cells at 4 hours. As seen in Fig. 7, in which results from multiple experiments are presented, the approximately three- to four-fold increase in cell surface GalTase levels (Figs 2 and 3) correlates well with the approximately three-fold enhancement in neurite initiation and formation on laminin.

Increased cell surface GalTase levels increase the rate of neurite elongation

We also determined if increased surface GalTase expression accelerated the process of neurite elongation from pre-existing neurites. The rate of neurite elongation among control cells was very similar, with PC12 cells elongating at 7.32 ±0.49

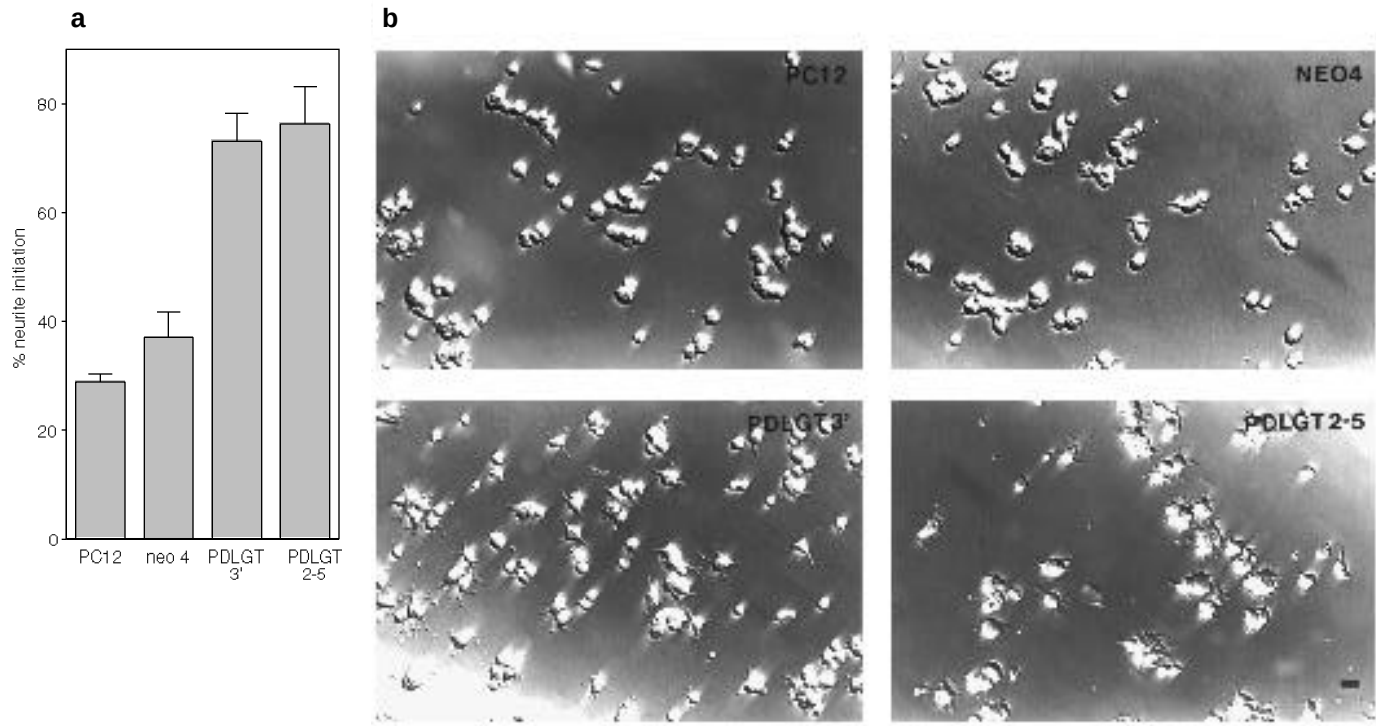

Fig. 5. Neurite initiation of PC12 transfectants. (a) Overexpression of surface GalTase on two independent clones, pKJ-PDLGT 3′ (*n*=6) and pKJ-PDLGT 2-5 (*n*=3), enhanced neurite initiation as compared to PC12 (*n*=6) and pKJ-neo 4 (*n*=5) controls. Values represent the mean % neurite initiation. Error bars, s.e.m. (b) Photomigraphs of PC12 (PC12), pKJ-neo 4 (NEO 4), pKJ-PDLGT 3′ (PDLGT 3′) and pKJ-PDLGT 2-5 (PDLGT 2-5) cells at 30 minutes after plating. Note the high neurite initiation frequency in transfected cells, relative to PC12 and pKJ-neo 4 controls. Bar, 10 μm.

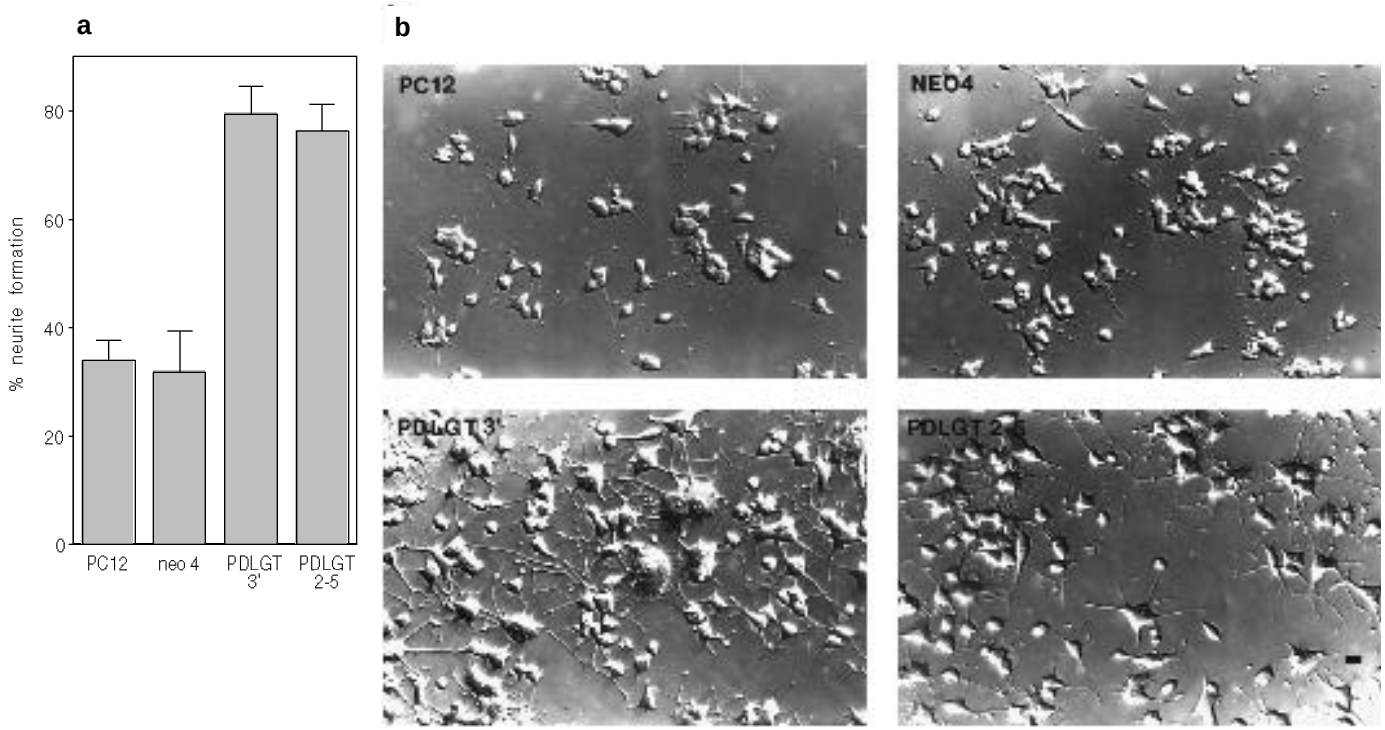

Fig. 6. Neurite formation of PC12 transfectants. (a) Overexpression of GalTase in two independent transfected clones, pKJ-PDLGT 3′ (*n*=6) and pKJ-PDLGT 2-5 (*n*=3), enhanced neurite formation as compared to PC12 (*n*=6) and pKJ-neo 4 (*n*=3) controls. Values represent the mean % neurite formation. Error bars, s.e.m. (b) Photomicrographs of PC12 (PC12), pKJ-neo 4 (NEO 4), pKJ-PDLGT 3′ (PDLGT 3′) and pKJ-PDLGT 2-5 (PDLGT 2-5) cells 4 hours after plating. Note that the neurites of pKJ-PDLGT 3′ and pKJ-PDLGT 2-5 are much longer and more abundant than those on control cells. Bar, 10 μm.

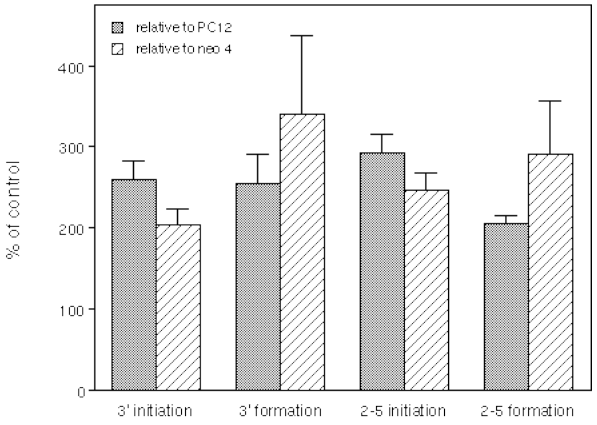

Fig. 7. Neurite initiation and formation frequencies relative to control. The percentages of neurite initiation at 30 minutes and neurite formation at 4 hours for pKJ-PDLGT 3′ cells (3′) are illustrated relative to PC12 (*n*=6) and pKJ-neo 4 (*n*=5) controls and for pKJ-PDLGT 2-5 cells (2-5) relative to PC12 (*n*=3) and pKJ-neo 4 (*n*=3) controls. Bars, s.e.m.

μm/h and pKJ-neo 4 cells elongating at 7.58 ±0.64 μm/h (Table 1). On the other hand, pKJ-PDLGT 3′ cells averaged 15.31 ±0.57 μm/h and pKJ-PDLGT 2-5 cells averaged 16.05 ±0.43 μm/h. The rate of neurite elongation of pKJ-PDLGT 3′ cells was 209% that of PC12 cells and 202% that of pKJ-neo 4 controls; neurite elongation of pKJ-PDLGT 2-5 cells was 219% of PC12 cells and 212% of pKJ-neo 4 controls (Table 1).

DISCUSSION

Previous studies have shown that blocking surface GalTase with GalTase perturbants decreases neurite initiation and neurite formation on laminin from PC12 cells and dorsal root ganglion cells (Thomas et al., 1990; Begovac and Shur, 1990). Although these studies support a role for surface GalTase in neurite outgrowth, they are all inhibitory by their very nature. In this study, we wished to increase cell surface GalTase levels through cellular transfection and examine the effects on neurite outgrowth. Previous studies have shown that expression of the pKJ-PDLGT construct, encoding the long form of GalTase, in transfected cells results in increased GalTase activity in both the intracellular and cell surface compartments. In contrast, when the short form of GalTase is overexpressed in transfected cells, the elevated activity remains intracellular (Lopez et al., 1991). These and other studies (Evans et al., 1993; Youakim et al., 1994; Nguyen et al., 1994) all show that the long form of GalTase serves in a multifunctional capacity, expressed in the Golgi complex and acting as the biologically relevant form on the cell surface; the short form performs a purely intracellular, biosynthetic role.

Two stably transfected PC12 cell lines were isolated expressing the full-length PDLGT long GalTase protein. Although we have not examined the effects on intracellular GalTase activity in this study, the critical issue here was whether transfected cells displayed increased levels of GalTase on their surface, and therefore, were suitable for analyses of neurite outgrowth. Three independent assays were used to confirm that GalTase levels were, in fact, elevated on the surfaces of PDLGT-transfectants, relative to controls: surface-associated enzyme activity, radiolabeled antibody binding to intact cells, and indirect immunofluorescence. The biological consequence of the three- to four-fold increase in surface GalTase expression was a three-fold increase in the rate of neurite initiation and neurite formation, coupled with an accelerated rate of neurite elongation. Thus, overexpressing surface GalTase increases neurite initiation, neurite formation, and the rate of neurite elongation. These results provide direct evidence that the level of cell surface GalTase is able to

Table 1. Overexpression of surface GalTase increases the rate of neurite elongation

Cell type	Expt	*n**	Mean rate (μm/h) (±s.e.m.)	% of control vs PC12	% of control vs neo 4
PC12	1	18	7.42±0.68		
	2	17	6.24±0.94		
	3	19	8.29±1.13		
	Average:		7.32±0.49	100	96
pKJ-neo 4	1	21	8.10±1.17		
	2	17	8.60±0.97		
	3	11	6.05±0.69		
	Average:		7.58±0.64	104	100
pKJ-PDLGT-3′	1	23	13.95±1.16		
	2	16	15.75±1.87		
	3	18	16.23±1.31		
	Average:		15.31±0.57	209†	202†
pKJ-PDLGT-2-5	1	20	16.93±2.21		
	2	20	15.10±1.33		
	3	20	16.13±1.81		
	Average:		16.05±0.43	219†	212†

*Number of neurites assayed.
†Significant at the 0.05% level when analyzed using Student's *t*-test.

modulate the rate and frequency of neurite outgrowth on laminin.

Surface GalTase is thought to function during neurite outgrowth by binding oligosaccharides within the E8 domain of laminin (Begovac et al., 1991). Unglycosylated laminin inhibits neurite outgrowth (Dean et al., 1990), illustrating the importance of available oligosaccharide substrates to support neurite outgrowth. The present study supports this conclusion by showing that increased surface GalTase levels enhance PC12 cell neurite outgrowth on laminin, indicating that surface GalTase levels are likely to be rate-limiting, rather than their binding sites in laminin. The integrins also participate in neurite outgrowth on laminin and bind to peptide components within the E8 domain (Tomaselli et al., 1990; Tashiro et al., 1989), emphasizing the importance of both carbohydrate and peptide elements within E8 for neurite outgrowth. The functional and molecular relationship of these different laminin receptors is unknown, but interestingly, another cell surface glycosyltransferase, *N*-acetylgalactosaminylphosphotransferase, interacts with and modulates the function of N-cadherin on neural retina cells (Balsamo and Lilien, 1990) and during neurite outgrowth (Gayá-González et al., 1991).

The enhanced neurite outgrowth observed in PDLGT transfectants raises questions as to how increased GalTase levels can affect the cell shape as is necessary for neurite extension. Although surface GalTase functions as a surface receptor for laminin by binding to N-linked oligosaccharides, little is known about any potential signal transduction pathway elicited by surface GalTase. One recent study showed that laminin induces neurite outgrowth by activating protein kinase C (Bixby and Jhabvala, 1990) and, in this regard, integrins have been suggested to interact with protein kinase C (Bixby, 1989; Banga et al., 1986). It is not yet known if surface GalTase also interacts with protein kinases during neurite outgrowth, although there is good evidence that the cytoplasmic domain of GalTase is phosphorylated (Strous et al., 1987; D. Dubois and B. D. Shur, unpublished). The fact that surface GalTase activity is thought to be regulated during PC12 cell differentiation by cyclic AMP and protein kinase A-dependent processes is particularly interesting (Roth et al., 1991). Surface GalTase may also elicit intracellular signals through its association with the cytoskeleton, as suggested for the integrins (Juliano and Haskill, 1993; Burn et al., 1988; Horwitz et al., 1986). The molecular components that link GalTase to the cytoskeleton are presently undefined. Finally, there is reason to believe that surface GalTase can affect G-protein-dependent signal cascades, since aggregation of surface GalTase on sperm elicits a pertussis toxin-sensitive exocytosis of the sperm acrosome (Macek et al., 1991; D. Miller and B. D. Shur, unpublished). In any event, the present study demonstrates that when surface GalTase levels are increased in transfected PC12 cells, the rate and frequency of neurite outgrowth are coincidentally increased, suggesting that the expression of surface GalTase can directly affect the degree of neurite outgrowth.

The work presented here was supported by grant 5-FY92-1105 from the Basil O'Connor Research Grant Program of the March of Dimes Birth Defects Foundation (P.C.B.) and by grant DE 07120 from the National Institutes of Health (B.D.S.).

REFERENCES

Balsamo, J. and J. Lilien. (1990). N-cadherin is stably associated with a cell surface N-acetylgalactosaminyl-phosphotransferase and is an acceptor for the enzyme. *J. Biol. Chem.* **265**, 2923-2928.

Banga, H., E. R. Simons, L. F. Brass and S. E. Rittenhouse. (1986). Activation of phospholipoases A and C in human platelets exposed to epinephrine: role of glycoproteins IIb/IIIa and dual role of epinephrine. *Proc. Nat. Acad. Sci. USA* **83**, 9197-9201.

Begovac, P. C. and B. D. Shur. (1990). Cell surface galactosyltransferase mediates the initiation of neurite outgrowth from PC12 cells on laminin. *J. Cell Biol.* **110**,461-470.

Begovac, P. C., D. E. Hall and B. D. Shur. (1991). Laminin fragment E8 mediates PC12 cell neurite outgrowth by binding to cell surface β1,4-galactosyltransferase. *J. Cell Biol.* **113**,637-644.

Bixby, J. L. (1989). Protein kinase C is involved in laminin stimulation of neurite outgrowth. *Neuron* **3**,287-297.

Bixby, J. L. and P. Jhabvala. (1990). Extracellular matrix molecules and cell adhesion molecules induce neurites through different mechanisms. *J. Cell Biol.* **111**,2725-2732.

Buck, C. and A. F. Horwitz. (1987). Cell surface receptors for extracellular matrix molecules. *Annu. Rev. Cell Biol.* **3**,179-205.

Burn, P., A. Kupfer and S. J. Singer. (1988). Dynamic membrane-cytoskeletal interactions: specific association of integrin and talin *in vivo* after phorbol ester treatment of peripheral blood lymphocytes. *Proc. Nat. Acad. Sci. USA.* **85**,497-501.

Chomczynski, P. and N. Sacchi. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**,156-159.

Dean, J. W. III, S. Chandrasekaran and M. L. Tanzer. (1990). A biological role of the carbohydrate moieties of laminin. *J. Biol. Chem.* **265**,12553-12562.

Doherty, P. and F. S. Walsh. (1989). Neurite guidance molecules. *Curr. Opin. Cell Biol.* **1**,1102-1106.

Eckstein, D. J. and B. D. Shur. (1992). Cell surface β1,4-galactosyltransferase is associated with the detergent-insoluble cytoskeleton on migrating mesenchymal cells. *Exp. Cell Res.* **201**,83-90.

Engvall, E., G. E. Davis, K. Dickerson, E. Ruoslahti, S. Varon and M. Manthorpe. (1986). Mapping of domains in human laminin using monoclonal antibodies: Localization of the neurite-promoting site. *J. Cell Biol.* **103**,2457-2465.

Engvall, E., D. Earwicker, T. Haaparanta, E. Ruoslahti and JR. Sanes. (1990). Distribution and isolation of four laminin variants; Tissue restricted distribution of heterodimers assembled from five different subunits. *Cell Regul.* **1**,731-740.

Evans, S. C., L. C. Lopez and B. D. Shur. (1993). Dominant negative mutation in cell surface β1,4-galactosyltransferase inhibits cell-cell and cell-matrix interactions. *J. Cell Biol.* **120**,1045-1057.

Gayá-González, L., J. Balsamo, N. Swaminathan and J. Lilien. (1991). Antibodies to the retina N-acetylgalactosaminylphosphotransferase inhibit neurite outgrowth. *J. Neurosci. Res.* **29**,474-480.

Hathaway, H. J. and B. D. Shur. (1992). Cell surface β1,4-galactosyltransferase functions during neural crest cell migration and neurulation *in vivo*. *J. Cell Biol.* **117**,369-382.

Horwitz, A., K. Duggan, C. Buck, M. Beckerle and K. Burridge. (1986). Interaction of plasma membrane fibronectin receptor with talin-a transmembrane linkage. *Nature* **320**,531-533.

Hynes, R. O. (1987). Integrins: a family of cell surface receptors. *Cell* **48**,549-554.

Juliano, R. L. and S. Haskill. (1993). Signal transduction from the extracellular matrix. *J. Cell Biol.* **120**,577-585.

Kleinman, H. K., G. C. Sephel, K. Tashiro, B. S. weeks, B. A. Burrous, S. H. Adler, Y. Yamada and G. R. Martin. (1990). Laminin in neuronal development. *Ann. NY Acad. Sci.* 302-310.

Liesi, P., A. Närvänen, J. Soos, H. Sariola and G. Snounou. (1990). Identification of a neurite outgrowth-promoting domain of laminin using synthetic peptides. *FEBS Lett.* **244**,141-148.

Lopez, L. C., A. Youakim, S. M. Carroll and B. D. Shur. (1991). Evidence for a molecular distinction between golgi and cell surface forms of β1,4 galactosyltransferase. *J. Biol. Chem.* **266**,15984-15991.

Macek, M. B., L. C. Lopez and B. D. Shur (1991). Aggregation of 1,4galactolytransferase on mouse sperm induces the acrosome reaction. *Dev. Biol. 1***47,** 440-44.

Mecham, R. P. (1991). Receptors for laminin on mammalian cells. *FASEB J.* **5**,2538-2546.

Miller, D., M. B. Macek and B. D. Shur. (1992). Complementarity between sperm surface β1,4-galactosyltransferase and egg coat ZP3 mediates sperm-edd binding. *Nature* **357**,589-593.

Muller, S. R., P. D. Sullivan, D. O. Clegg and S. C. Feinstein. (1990). Efficient transfection and expression of heterologous genes in PC12 cells. *DNA Cell Biol.* **9**,221-229.

Nguyen, T., D. Hinton, D. and B. D. Shur. (1994). Expressing murine β1,4 galactosyltransferase in HeLa cells produces a cell surface galactosyltransferase-dependent phenotype. *J. Biol. Chem.* **249**, 28000-28009.

Reichardt, L. F. and K. J. Tomaselli. (1991). Extracellular matrix molecules and their receptors: Functions in neural development. *Annu. Rev. Neurosci.* **14**,531-570.

Riopelle, R. J. and K. E. Dow. (1991). Neurite formation on laminin: effects of a galactosyltransferase on primary sensory neurons. *Brain Res.* **541**,265-272.

Rogers, S. L., K. J. Edson, P. C. Letourneau and S. C. Mcloon. (1986). Distribution of laminin in the developing peripheral nervous system of the chick. *Dev. Biol.* **113**,429-435.

Roseman, S. (1970). The synthesis of complex carbohydrates by multi-glycosyltransferase systems and their potential function in intra-cellular adhesion. *Chem. Phys. Lipids* **5**,270-297.

Roth, S. (1973). A molecular model for cell interactions. *Q. Rev. Biol.* **48**,541-563.

Roth, J. A., K. Marcucci, W. Lin, J. L. Napoli, J. A. Wagner and R. Rabin. (1991). Increase in β1,4-galactosyltransferase activity during PC12 cell differentiation induced by forskolin and 2-chloroadenosine. *J. Neurochem.* **57**,708-713.

Runyan, R. B., J. Versalovic and B. D. Shur. (1988). Functionally distinct laminin receptors mediate cell adhesion and spreading: The requirement for surface galactosyltransferase in cell spreading. *J. Cell Biol.* **107**,1863-1871.

Russo, R. N., N. L. Shaper and J. H. Shaper. (1990). Bovine β1-4-galactosyltransferase: Two sets of mRNA transcripts encode two forms of the protein with different amino-terminal domains. *J. Biol. Chem.* **265**,3324-3331.

Sambrook, J., E. F. Fritsch and T. Maniatis. (1989). *Molecular Cloning.* Second edition. Cold Spring Harbor Laboratory Press, New York.

Sephel, G. C., K.-I. Tashiro, M. Sasaki, S. Kandel, Y. Yamada and H. K. Kleinman. (1989). A laminin pepsin fragment with cell attachment and neurite outgrowth activity at distinct sites. *Dev. Biol.* **135**,172-181.

Shaper, N. L., G. F. Hollis, J. G. Douglas, I. R. Kirsch and J. H. Shaper. (1988). Characterization of the full length cDNA for murine β1,4-galactosyltransferase. *J. Biol. Chem.* **263**,10420-10428.

Shur, B. D. (1993). Glycosyltransferases as cell adhesion molecules. *Curr. Opin. Cell Biol.* **5**,854-863.

Strous, G. J., P. van Kerkhof, R. J. Fallon and A. L. Schwartz. (1987). Golgi galactosyltransferase contains serine-linked phosphate. *Eur. J. Biochem.* **169**,307-311.

Tashiro, K.-I., G. C. Sephel, B. Weeks, M. Sasaki, G. R. Martin, H. K. Kleinman and Y. Yamada. (1989). A synthetic peptide containing the IKVAV sequence from the A chain of laminin mediates cell attachment, migration and neurite outgrowth. *J. Biol. Chem.* **264**,16174-16182.

Thomas, W. A., A. W. Schaefer and R. M. Treadway, Jr (1990). Galactosyltransferase-dependence of neurite outgrowth on substratum-bound laminin. *Development* **110**,1101-1114.

Tomaselli, K. J., D. E. Hall, L. A. Flier, K. R. Gehlsen, D. C. Turner, S. Carbonetto and L. F. Reichardt. (1990). A neuronal cell line (PC12) expresses two β1-class integrins- α1β1 and α3β1-that recognize different neurite outgrowth-promoting domains in laminin. *Neuron* **5**,651-662.

Youakim, A., D. Dubois and B. D. Shur. (1994). Localization of long form β1,4 galactosyltransferase to the plasma membrane and Golgi complex in 3T3 and F9 cells by immunofluorescence confocal microscopy. *Proc. Nat. Acad. Sci. USA* **91**, 10913-10917.

(Received 1 June 1994 - Accepted 28 September 1994)