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Mouse fibroblasts null for the long isoform of β1,4-galactosyltransferase-I show defective cell-matrix interactions

Brooke H. Elder[a] and **Barry D. Shur**[a,1]

[a]Department of Cell Biology, Emory University School of Medicine, Atlanta, GA 30322

Abstract

β1,4 Galactosyltransferase-I (GalT-I) is expressed as two nearly identical polypeptides that differ only in the length of their cytoplasmic domains. The longer isoform has been implicated as a cell surface receptor for extracellular glycoside ligands, such as laminin. To more stringently test the function of the long GalT-I isoform during cell interactions with laminin, we created multiple independent fibroblastic cell lines that fail to express the long isoform, but which express the short GalT-I isoform normally and appear to have normal intracellular galactosylation. Cells devoid of the long GalT-I isoform are unable to adhere and spread on laminin substrates as well as control cells, but retain near normal interactions with fibronectin, which do not rely upon surface GalT-I function. The loss of the long GalT-I isoform also leads to a loss of actin stress fibers, focal adhesions and rac GTPase activation.

Keywords

cell-matrix interactions; laminin receptors; β1,4-galactosyltransferase-I; Cell surface GalT-I

1. Introduction

Cell interactions with the extracellular matrix (ECM) mediate a wide range of biological events and have been the subject of extensive study. The best characterized cell surface receptors for ECM ligands are the integrins, which bind extracellular glycoproteins, such as fibronectin and laminin, and orchestrate interactions with the ECM via association with a large cadre of signaling and structural proteins, collectively referred to as the ‘adhesome’ [1].

A less well-studied receptor for the ECM is β1,4-galactosyltransferase-I (GalT-I), which facilitates cell spreading and migration on laminin by binding to *N*-linked oligosaccharides in the E8 domain [2]. GalT-I is one of seven known β1,4-galactosyltransferase polypeptides that transfer galactose in a β1,4 linkage from uridine diphosphate galactose (UDP-Gal) to

[1]Corresponding author and present address: Barry D. Shur, barryshur@gmail.com, 556 Alta Vista Avenue, South Pasadena, CA 91030.

specific glycoprotein and glycolipid substrates [3]. The bulk of GalT-I is localized in the Golgi complex where it participates in the biosynthesis of complex glycoconjugates. However, a subset of GalT-I is directed to the cell surface where it facilitates binding to extracellular glycoconjugate substrates, or ligands [4]. The differential function of GalT-I appears to be due to the presence of two GalT-I polypeptides that differ only in their *N*-terminal cytoplasmic domains. The longer isoform has an additional 13 amino acid extension on the cytoplasmic tail that is required for GalT-I to function as a surface receptor for extracellular ligands. The shorter isoform, lacking these 13 amino acid residues, functions solely as a biosynthetic enzyme [5,6]. Expression of the long isoform on the cell surface is negatively regulated by serine/threonine phosphorylation [7].

Similar to other matrix receptors, GalT-I requires association with the cytoskeleton in order to function as a receptor for extracellular ligands [8,9]. Furthermore, ligand-induced GalT I aggregation activates cell-specific intracellular signaling cascades, such as transient activation of focal adhesion kinase leading to cytoskeletal reorganization during fibroblast migration [10], and heterotrimeric G-protein activation leading to vesicle exocytosis in sperm and *Xenopus* oocytes [11,12].

Thus far, studies that assess GalT-I function as a laminin receptor have relied upon traditional biochemical approaches as well as gain-of-function and loss-of-function studies. For example, expression of GalT-I on the surface of cells that do not normally bind laminin leads to GalT-I-dependent cell spreading on laminin [13]. On the other hand, the expression of a truncated GalT-I comprising the transmembrane and long cytoplasmic domain leads to a dominant negative phenotype by displacing GalT-I from the cytoskeleton, thus preventing it from serving as a receptor for laminin substrates [9]. Similarly, the rate of cell migration on laminin can be manipulated, both positively and negatively, by altering the level of surface GalT-I associated with the cytoskeleton [14].

Despite these studies, a more direct test of GalT-I's role during cell interactions with laminin requires cells that are devoid of the long GalT-I isoform, but which still express the short GalT-I protein and which have normal intracellular galactosylation. In this regard, mice have been created that are deficient for the long GalT-I isoform, but which still express the short isoform and appear to undergo normal intracellular galactosylation. Sperm from mice devoid of the long isoform are unable to bind their egg coat glycoprotein ligand, consistent with the long GalT-I isoform serving as a surface receptor for specific extracellular glycoside substrates [15]. In this study, we have taken advantage of these long GalT-I null mice to establish multiple independent fibroblastic cell lines that are devoid of the long isoform, but which still express the short GalT-I isoform normally. Cells lacking the long GalT-I isoform fail to adhere and spread on laminin, as do normal cells, thus confirming that GalT-I can serve as a surface receptor for specific extracellular ligands.

2. Materials and Methods

2.1. Establishing embryonic fibroblast cell lines

Mice made homozygous null for the long isoform of GalT-I have been previously described [15]. Twelve-day old embryos were harvested from matings between mice heterozygous for

the long GalT-I isoform and used for the isolation and cultivation of fibroblast cell lines [16]. The genotype of the isolated embryos and fibroblastic cell lines was determined using the previously described PCR-based strategy [15,17]. Three independent cell lines were established from both wild-type (+/+) and long GalT-I null (−/−) embryos. Cells were maintained in DMEM supplemented with 10% Fetal Bovine Serum (FBS). Cultures were passaged when confluency reached ~50–60%.

Cells null for the long GalT-I isoform were “rescued” by Lipofectamine (Invitrogen)-mediated transfection with the pKJ-PDLGT construct as described [6]. The pKJ-PDLGT construct has had the promoter sequences removed that are required for translation of the short GalT-I isoform, and thus pKJ-PDLGT specifically encodes the long GalT-I isoform. The expression of the long GalT-I isoform in transfected cultures was determined by indirect immunofluorescence using antiserum specific for the long GalT-I cytoplasmic domain [18]. Cultures in which >90% of the cells express the long GalT-I isoform were established by dilution cloning from the originally transfected cells.

2.2. GalT-I enzymatic activity

GalT-I catalytic activity was assayed under optimal enzymatic conditions as described [13]. The ^{3}H-product (^{3}H-Gal-GlcNAc) was isolated by high voltage borate electrophoresis and the radioactivity determined by liquid scintillation counting.

2.3. GalT-I immunolocalization

Cells were plated onto uncoated 4-well chamber slides (Nunc Lab-Tek), incubated overnight in a tissue culture incubator, washed twice, and fixed in 3.7% formaldehyde. Cells were washed three times, permeabilized with 0.1% saponin, washed, blocked with 5% normal goat serum (NGS) in PBS + 0.1% saponin (PBS/saponin), and incubated with 1:100 or 1:50 dilution of primary antibody in PBS/saponin + 5% NGS. After 1 h, cells were washed, incubated in 1:500 biotin-goat anti-rabbit IgG (Vector) for 45 min, washed again, incubated in 1:100 FITC-avidin (Vector) in PBS/saponin for 30 min, washed, and mounted with Vectashield before viewing by fluorescence microscopy. Primary antibodies included rabbit antiserum raised against recombinant GalT-I catalytic domain [13], rabbit antiserum raised against the long GalT-I cytoplasmic tail [18], or pre-immune serum from the corresponding rabbits.

To assess cell surface staining, cells were plated as above, rinsed, fixed and permeabilized in ice-cold methanol, rinsed, blocked with 5% BSA/PBS, and incubated in rabbit anti-GalT-I cytoplasmic tail antiserum (1:50) in 1% BSA/PBS for 1 h. Wells were washed three times, and incubated with biotin-goat anti-rabbit IgG (1:250) in 1% BSA/PBS for 1 h. Wells were washed again, and incubated with FITC-avidin (1:100) in 1% BSA/PBS for 30 min, followed by three rinses in PBS, rinsed in water, mounted with Vectashield, and viewed on a Zeiss 510 confocal microscope.

2.4. Lectin blot analysis

150 mm tissue culture plates of mouse fibroblasts were washed three times and lysed with 2 ml of 50 mM Na acetate, 2 mM $CaCl_2$, and 1% Triton. Particulate material was pelleted, the

supernatant transferred to a clean tube for protein determination by Bradford reagent, and heat- inactivated for 10 min at 56 °C. Some samples were treated with 200 mU of neuraminidase (#N5631, Sigma) for 1 hr at 37 °C. Ten μg protein was resolved by 10% SDS-PAGE, transferred to nitrocellulose, and blocked overnight in TBST + 1% gelatin at 4 °C. Blots were incubated with biotinylated-RCA I [Vector] (2 μg/ml blocking buffer) for 1 h, followed by HRP-Streptavidin (1:50,000 blocking buffer) for 45 min. Blots were washed three times and developed with ECL Reagent (Amersham). As indicated, some blots were pre-incubated with 300 mM galactose for 10 min prior to incubation with RCA I.

2.5. Cell adhesion assays

96-well tissue culture plates were coated with 100 μl of laminin (0.25 mg/ml) or fibronectin (0.25 mg/ml), the non-bound fluid aspirated and non-specific adhesion blocked with 200 μl of heat-denatured BSA (10 mg/ml PBS), and the wells washed. Cells were removed from tissue culture plates with EDTA, washed, resuspended in DMEM + 10% FBS, and the serum removed by washing in DMEM. Fifty μl of DMEM plus 50 μl of cells (@ 5×10^5 cells/ml) were added to each well. In parallel, 20%, 50% and 100% of the working cell suspension were added to wells and immediately fixed in 100 μl of 5% glutaraldehyde to create a "standard curve" of cell adhesion. The experimental assays were incubated for 1 h at 37 °C, the non-adherent cells removed by washing, and the cells fixed with glutaraldehyde. All wells (experimental and standards) were washed three times, and the adherent cells stained with 100 μl of 0.1% crystal violet for 60 min. Wells were washed three times, photographs taken of each well, and the dye solubilized with 100 μl of 10% acetic acid. Absorbance was measured at 560 nm. Each experimental assay was conducted in quadruplicate. Results are shown as a percentage of total cells adhered, as determined from the "standard curve" of cell adhesion.

2.6. Cell spreading assays

Each well in a 4-well chamber slide was coated with either laminin or fibronectin at 2 $\mu g/cm^2$ in PBS, and the wells rinsed. Cells were removed from tissue culture plates as above, washed, resuspended in DMEM without serum, and added to wells at one of four different concentrations (5×10^3, 10×10^3, 15×10^3, 20×10^3) to insure sufficient spreading cells to assay. After either 2 h or 24 h incubation, the wells were rinsed twice, and fixed with 3.7% formaldehyde. Wells were washed three times, stained with phalloidin to visualize the cells [8], rinsed, mounted with Vectashield, and analyzed by IPLab software (Scanalytics).

2.7 Integrin α6 expression

Cells were harvested with EDTA, pelleted, and resuspended three times in "wash" solution (5 mM Na azide, 3% sheep serum in PBS). The cell number and trypan blue exclusion were determined, and 0.5×10^6 cells were incubated with 25 μg rat anti-integrin α6 (Chemicon MAB1378) in 250 μl "wash" solution. After 1 h incubation on ice, cells were "washed" three times, and incubated in 250 μl of FITC anti-rat IgG (Santa Cruz) for 45 min on ice. The cells were "washed" three times again, trypan blue exclusion determined, fixed in 1 ml ice-cold 1% paraformaldehyde in PBS, and analyzed by flow cytometry. Control assays were incubated in parallel with rat IgG2α (Chemicon CBL605) instead of rat anti-integrin α6.

2.8. Cytoskeleton localization

Filamentous actin was visualized using rhodamine-conjugated phalloidin (Molecular Probes) as described [8]. Vinculin was imaged using a 1:50 dilution of mouse anti-vinculin MAB antibody (Sigma, V4504) followed by a 1:200 dilution of FITC conjugated goat anti-mouse IgG (Sigma, F-0257).

2.9. Rac activation

Rac activation was assayed using the GTPase Activation Kit (Cytoskeleton #BK034). Briefly, cells at ~40% confluency were rinsed twice, scraped in 1.5 ml ice-cold lysis buffer, pelleted, the supernatant collected, and protein concentration determined. To assess the basal level of activated rac, 0.65 mg of cell lysate was incubated in 1 ml lysis buffer with protease inhibitor and 20 μl of GST tagged PAK PBD protein beads. After 1 h incubation at 4 °C, the beads were pelleted, the supernatant discarded, the beads washed in 0.5 ml of wash buffer, and resuspended in 15 μl 2X Laemmle buffer. To assess the total amount of activatable rac in cell lysates, i.e., positive controls, 0.325 mg of cell lysate, in 1.1 ml of lysis buffer, was incubated with protease inhibitors, 0.18 mM GTPγS, and 14 mM EDTA. After 15 min incubation at 23 °C, the reaction was stopped on ice by adding 110 μl 600 mM $MgCl_2$ (STOP buffer). Twenty μl of GST tagged beads were added as above, incubated for 1 h at 4 °C and processed in parallel to the experimental samples. The samples (experimentals: 1.8 mg/lane; positive controls: 135 μg/lane) were resolved on a 15% polyacrylamide gel, transferred to nitrocellulose, and blocked with 5% non-fat milk in TBST. One μg GST-rac 1 fusion protein was run in a parallel lane. Blots were incubated overnight at 4 °C in 1 μg/ml mouse MAB anti-rac IgG (Upstate #05-389) in TBST, washed three times, incubated in 1:10,000 dilution of HRP-conjugated goat anti-mouse IgG (Santa Cruz) for 45 min, and washed three times again. The blots were developed using ECL reagent.

3. Results and Discussion

3.1 Fibroblastic cell lines established from wild-type and long GalT-I null embryos

Although extensive in vitro data indicate that GalT-I participates during cell-matrix interactions, the selective elimination of the long GalT-I isoform did not result in major morphological abnormalities in vivo [17]. Presumably, its requirement in vivo is either minimal, or can be compensated for by one of the six other GalT enzymes whose presences were unknown at the time [3]. Consequently, we felt it important to determine whether, in fact, the deletion of the long GalT-I isoform leads to defective cell-matrix interactions using defined in vitro analyses. We created multiple, independent fibroblastic lines from embryos resulting from GalT-I-null heterozygote matings: 3 wild-type cell lines (+/+) and 3 lines null for the long GalT-I isoform (long −/−).

As expected, cells lines established from wild-type embryos express both GalT-I isoforms in formaldehyde-fixed, saponin-permeabilized cells, as visualized using antibodies against either the catalytic domain [13], which cross reacts with both isoforms, or against sequences unique to the cytoplasmic domain of the long GalT-I isoform [18] (Fig. 1A). Furthermore, long −/− cells no longer react with antibodies against the long cytoplasmic tail, but do retain reactivity with the anti-catalytic antibody illustrating expression of the short isoform.

Although GalT-I immunoreactivity in the Golgi apparatus is clearly evident in formaldehyde-fixed, saponin-permeabilized cells, GalT-I immunoreactivity on the cell surface is not as evident under these fixation conditions. However, using methanol fixation, which is more suitable for membrane surface antigens [19], GalT-I is readily detectable on leading lamellipodia of well-spread cells when viewed by Z-section confocal microscopy (Fig. 1B). (Perinuclear florescence is an artifact of methanol fixation). As expected, the loss of the long GalT-I isoform leads to a loss of GalT-I immunoreactivity on lamellipodia (outlined in white), thus confirming that the long isoform is responsible for surface expression.

Cells devoid of the long GalT-I isoform still show near normal levels of enzymatic activity reflecting the expression of the short isoform (Fig. 1C). Consistent with this, lectin blot analysis confirms that intracellular galactosylation is grossly unaffected in long −/− cells, within the resolution of these assays, as was the case in long −/− tissues (Fig. 1D) [15]. Total cellular protein is also similar between wild-type cells and long −/− cells (dns).

3.2 Long GalT-I −/− fibroblasts show reduced adhesion and spreading on laminin

The ability to create stable cell lines devoid of the long isoform gave us the opportunity to clarify its function in cell-matrix interactions, devoid of secondary defects due to improper galactosylation. Previous studies indicated that GalT-I on the cell surface facilitates cell interactions with laminin [20,21]. Consistent with this, long −/− cells show greatly reduced, i.e., 27%, adhesion to laminin matrices after 1 h incubation, but behave near normal on plastic (dns) and fibronectin (Fig. 2A,B). Panel A illustrates the raw data from a representative assay containing quadruplicate replicates for each assay condition, whereas Panel B presents the aggregate data from two individual wild-type and three individual long −/− cell lines. Previous studies that indicated a specific role of GalT-I in cell spreading on laminin and not initial adhesion [21] used different cells types and relied upon a more stringent assay of ‘initial’ cell adhesion than the 1 h assay used here, which likely reflects both cell adhesion and the initial stages of cell spreading.

One of the predominant cell surface receptors for laminin is integrin α6 [22]. It was therefore important to determine if the levels of integrin α6 were diminished in long −/− cell lines and which could account for the reduced adhesion to laminin. FACS analysis demonstrates similar levels of integrin α6 expression on the surface of wild-type and long −/− cell lines, indicating that the reduced adhesion to laminin appears to be a reflection of the loss of the long GalT-I isoform and not due to a loss of integrin α6. To test this more directly, we determined whether re-expression of the long GalT-I isoform could rescue the cell adhesion to laminin. Long −/− cell lines were transfected with the pKG-PDLGT vector, which encodes the long GalT-I isoform [6]. These ‘rescued’ cell lines showed near normal, i.e., 82%, adhesion to laminin matrices (Fig. 2A,B), indicating that the loss of the long GalT-I isoform is responsible for the reduced adhesion to laminin.

We also determined if cell spreading on laminin is compromised in long −/− cells. Cells spreading after 2 h and 24 h of plating were fixed and stained with phalloidin to visualize the cell dimensions. The spread surface area, in μm^2, was calculated using IPLAB software. Representative images of cells spreading on laminin at 2 h and 24 h are shown (Fig. 3A), as

are the raw data from one representative assay using one wild-type and one long −/− cell line (Fig. 3B). The cumulative data from four individual assays, each composed of at least one wild-type and one long −/− cell line, is shown in Fig. 3C. As expected, both wild-type and long −/− cell lines showed rapid spreading on fibronectin within 2 h of plating; however, long −/− cells spread ~50% as well as wild-type cells on laminin. Although all cells continued to spread on laminin over the next 22 h of culture, the surface area of wild-type cells increased by 240%, whereas long −/− cells increased their surface area by only 50%.

3.3 Long GalT-I −/− fibroblasts show reduced stress fibers, focal adhesions and rac activation

Previous studies have shown that GalT-I-mediated cell spreading and migration on laminin relies upon similar molecular machinery as required for integrin-based cell-matrix interactions. In this regard, GalT-I requires association with the actin cytoskeleton to mediate cell spreading and migration on laminin [8,9], as does integrin-mediated cell spreading [23]. Consistent with this, expression of a dominant negative construct composed of the transmembrane and cytoplasmic domains of GalT-I displaces the endogenous GalT-I from its cytoskeletal association leading to a loss of GalT-I-dependent cell-matrix interactions [9]. GalT-I may associate with the cytoskeleton via an actin-associated scaffold protein, identified by yeast two-hybrid analysis, which directly binds the GalT-I cytoplasmic domain [24]. Finally, clustering of GalT-I by extracellular ligands induces a transient phosphorylation of Focal Adhesion Kinase and a loss of actin stress fibers [10].

In light of the similarity between integrin-mediated cell-matrix interactions and GalT-I-dependent interactions, we examined whether the defective spreading seen in long GalT-I −/− cells is associated with alterations in the actin cytoskeleton, focal adhesions, and/or GTPase activation. Similar to that seen when GalT-I is displaced from its cytoskeleton association by expression of a dominant negative construct [9], the loss of the long GalT-I isoform leads to a dramatic loss of actin stress fibers, as assayed by phalloidin staining, and focal adhesions, as determined by vinculin immunoreactivity (Fig. 4A). Long −/− cells rescued by expression of the pKG-PDLGT vector, which encodes the long GalT-I isoform [6], showed normal actin stress fibers and vinculin immunoreactivity (dns).

Furthermore, the constitutive (endogenous) levels of activated rac are markedly reduced in long −/− cells, relative to wild-type (Fig. 4B), consistent with the altered cytoskeleton and focal adhesions seen in these cells. However, both cell types have abundant rac protein that is capable of being activated by the non-hydrolyzable substrate GTPγS (lighter exposure shown relative to endogenous levels of rac activation). In aggregate, these results clearly demonstrate that long GalT-I facilitates cell spreading on laminin substrates relying upon similar molecular machinery as that employed during integrin-dependent cell spreading.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Elder and Shur, Highlights

- Cells devoid of the long isoform of GalT-I have been created.
- These cells retain grossly normal galactosylation via the short GalT-I isoform.
- Cells fail to express GalT-I on the surface and are unable to spread on laminin.
- This is associated with loss of stress fibers, focal adhesions and rac activation.
- Cells show normal interactions with fibronectin, which are GalT-I independent.

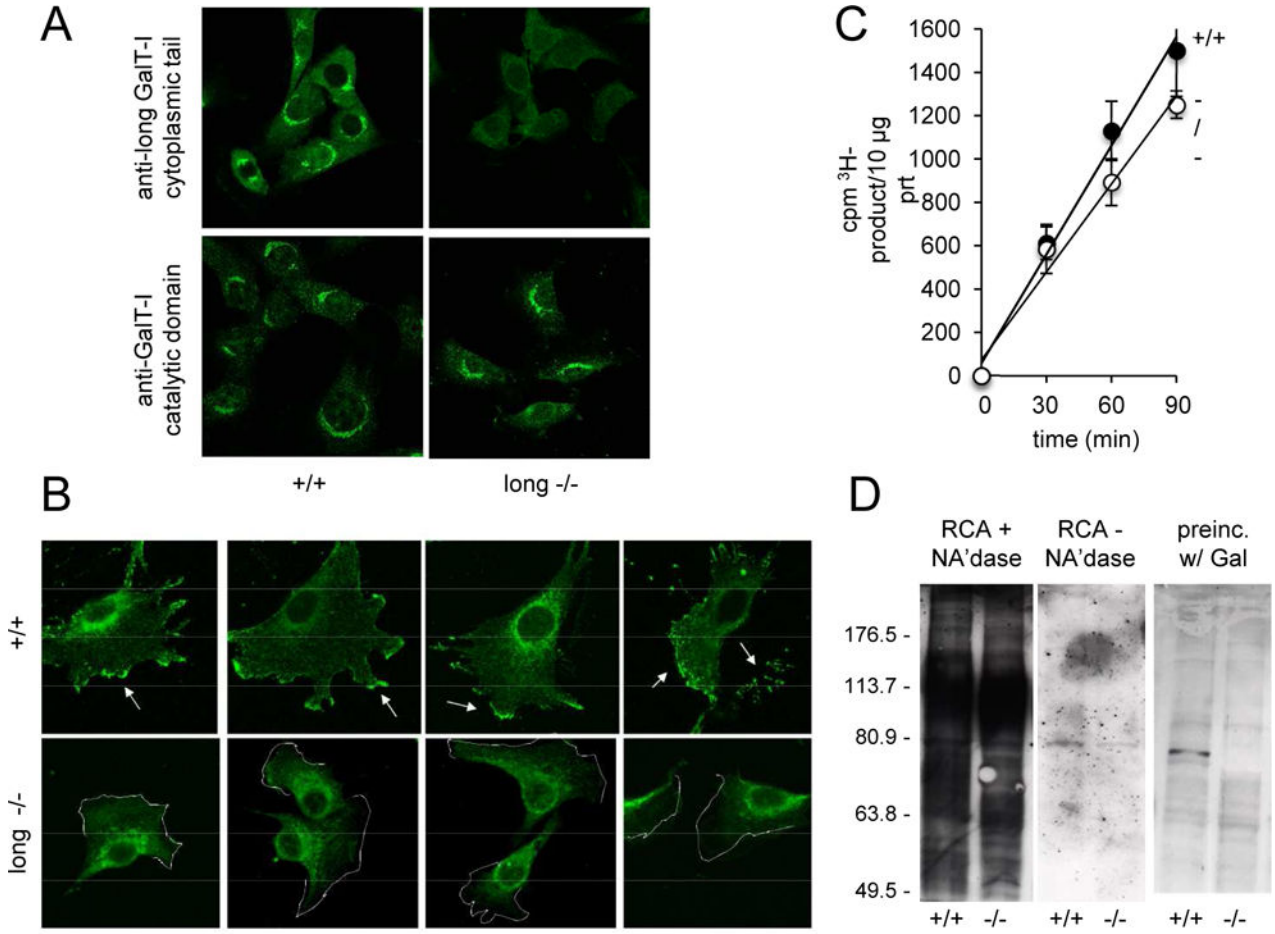

Figure 1.

Cells null for the long GalT-I isoform show normal levels of GalT-I enzyme activity, but fail to express GalT-I on the cell surface. A) Formalin-fixed wild-type (+/+) cells show Golgi-localized GalT-I immunoreactivity using antibodies against the catalytic domain as well as against the cytoplasmic domain unique to the long GalT-I isoform. Long GalT-I −/− (long −/−) cells fail to show immunoreactivity with anti-long cytoplasmic domain antibodies, but are still reactive to antibodies against the catalytic domain. Bar = 20 µm. B) Methanol-fixed wild-type (+/+) cells show GalT-I immunoreactivity on the leading lamellipodia of spreading cells (arrows) as viewed by confocal microscopy, whereas long −/− cells do not show surface immunoreactivity. Perinuclear fluorescence is an artifact of methanol fixation. Bar = 10 µm. C) GalT-I enzymatic activity is similar in wild-type (+/+) and long −/− cells, reflecting the activity of the short GalT-I isoform. Data represent the average of four assays. Bars = sem. D) Cellular galactosylation appears to be similar in wild-type (+/+) and long −/− cells as judged by RCA lectin blot analysis. RCA reactivity is greatly enhanced following neuraminidase (NA'dase) treatment of the cell lysate, and RCA immunoreactivity is decreased following pretreatment with galactose (Gal). Pretreatment with β-galactosidase removes the RCA reactivity (dns). The blots shown are representative of four similar assays.

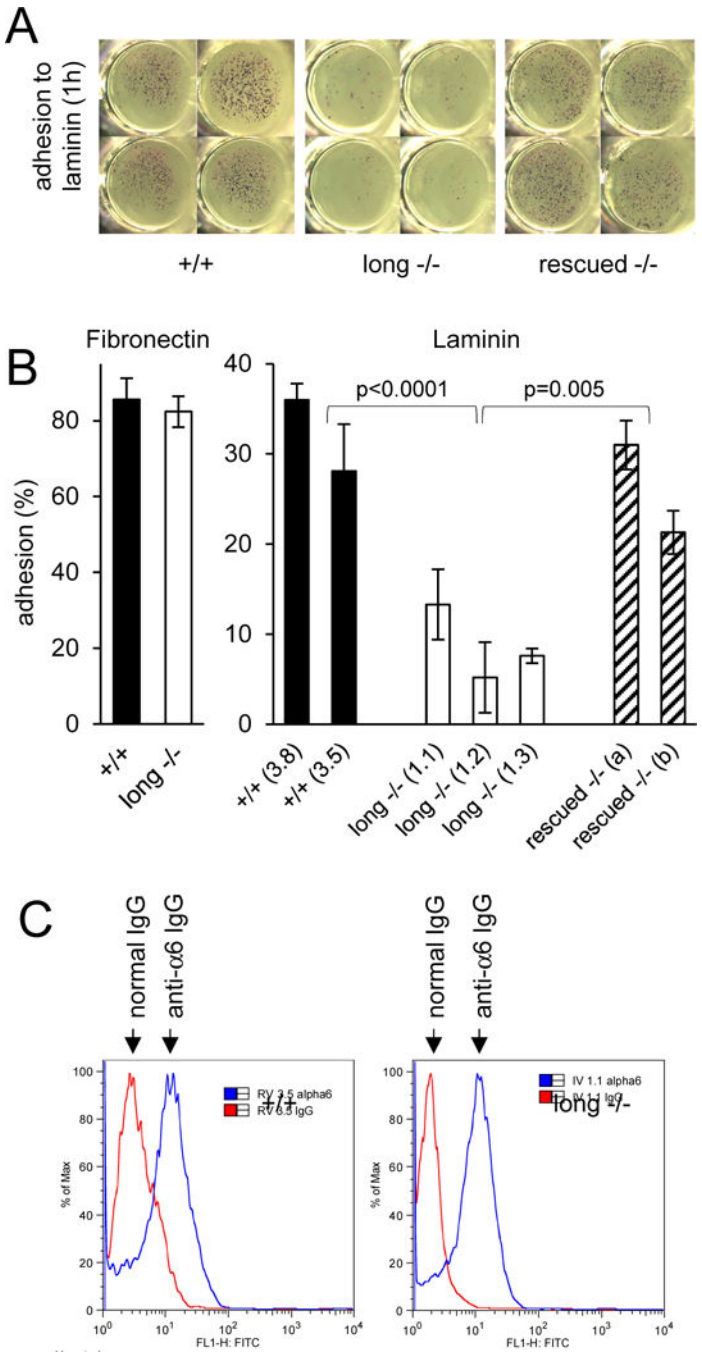

Figure 2.

Cells null for the long GalT-I isoform show normal adhesion to fibronectin, but reduced adhesion to laminin. A) Representative laminin-coated wells after 1 h assay using wild-type (+/+) cells, long −/− cells, and long −/− cells rescued by transfection with the long GalT-I isoform (rescued −/−). B) Quantification of cell adhesion to fibronectin and laminin using two wild-type (+/+) cell lines, three long −/− cell lines and two rescued long −/− cell lines. Bars = sem. C) The expression of the laminin receptor, integrin α6, is similar between wild-type (+/+) and long −/− cells.

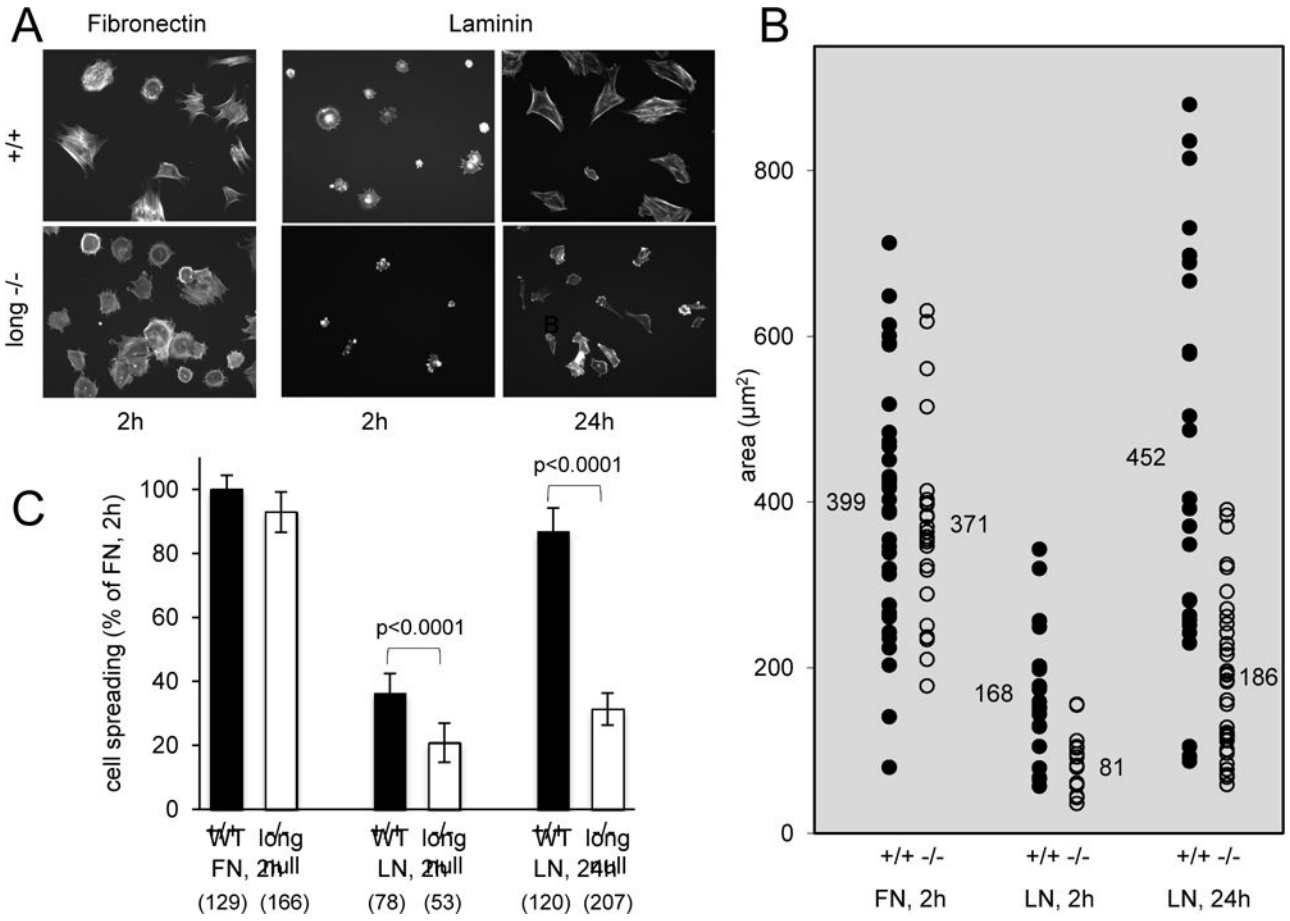

Figure 3.

Cells null for the long GalT-I isoform spread normally on fibronectin, but show reduced spreading on laminin. A) Representative images of phalloidin-stained cells after a 2 or 24 h incubation on fibronectin and laminin. Bar = 50 μm. B) Representative assay of spread cell surface areas after a 2 or 24 h incubation on fibronectin (FN) and laminin (LN). The mean surface area is indicated by the white bar. C) The cumulative data from 4 assays similar to that shown in (B). The number of cells assayed for each data point is shown in parentheses. Bars = sem.

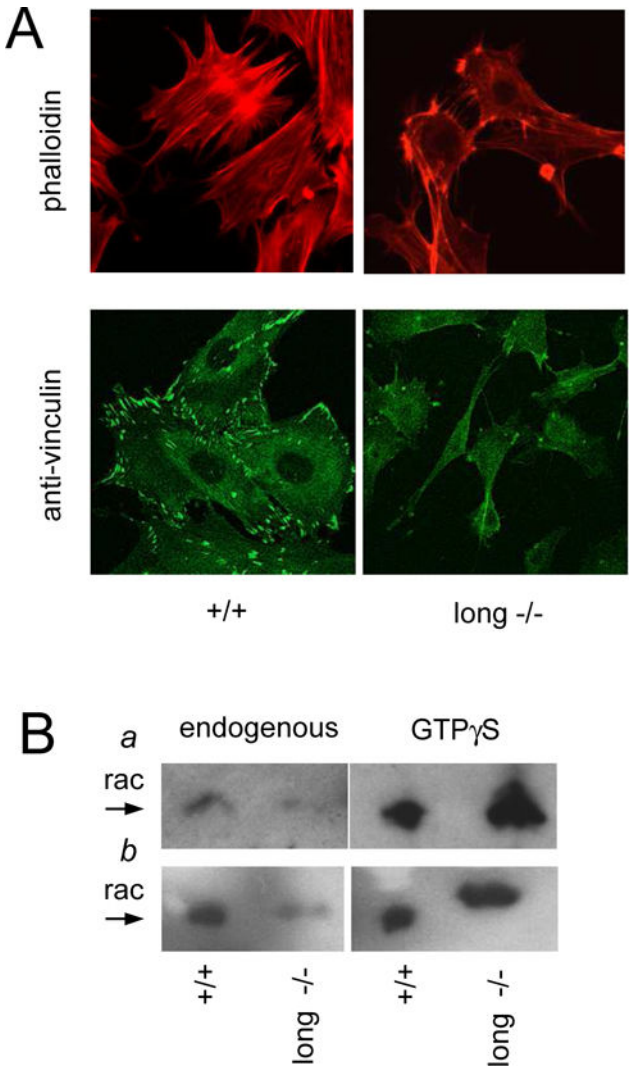

Figure 4.

The reduced adhesion and spreading of long GalT-I −/− cells on laminin is associated with reduced stress fibers, reduced focal adhesions, and reduced rac activation. A) Representative images of wild-type (+/+) and long −/− cells stained with phalloidin, to identify actin stress fibers, and vinculin, to identify focal adhesions (arrows.) Bar = 10 μm. B) Two (*a,b*) representative blots showing reduced levels of endogenous activated rac in long −/− cells relative to wild-type (+/+), which is associated with increased levels of rac protein in long −/− cells capable of being activated by GTPγS.