

Dominant Negative Mutation in Cell Surface β 1,4-Galactosyltransferase Inhibits Cell-Cell and Cell-Matrix Interactions

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Abstract. In addition to its traditional location within the Golgi complex, β 1,4-galactosyltransferase (GalTase) is also present on the cell surface, where it is thought to function as a cell adhesion molecule by binding to extracellular oligosaccharide ligands. Recent studies suggest that cells contain two forms of GalTase with distinct cytoplasmic domains. The longer form of GalTase contains a 13-amino acid cytoplasmic extension and is preferentially targeted to the plasma membrane, relative to the shorter GalTase protein that is confined primarily to the Golgi compartment. In this study, we created a dominant negative mutation that interferes with the function of cell surface GalTase by transfecting into cells cDNAs encoding truncated versions of the long form of GalTase containing the complete cytoplasmic and transmembrane domains, but devoid of the catalytic domain. In both F9 embryonal carcinoma cells and Swiss 3T3 fibroblasts, overexpressing the truncated long GalTase (TLGT) protein displaced the endogenous cell surface GalTase

from its association with the cytoskeleton, resulting in a loss of intercellular adhesion and cell spreading specifically on matrices that use GalTase as a cell surface receptor. In contrast, overexpressing the analogous truncated short GalTase (TSGT) protein did not affect cell morphology or GalTase activity. In control assays, inducing the TLGT protein had no effect on cell interactions with fibronectin (which is independent of GalTase), or on the cytoskeleton attachment of another matrix receptor (β 1 integrin), or on overall glycoprotein synthesis, thus eliminating nonspecific effects of the TLGT protein on cellular adhesion and metabolism. These results represent the first molecular manipulation of cell surface GalTase expression and confirm its function as a cell adhesion molecule. These studies further suggest that the cytoskeleton contains a defined, saturable number of binding sites for GalTase, which enables it to function as an adhesion molecule.

β 1,4-GALACTOSYLTRANSFERASE (GalTase)¹ is unusual among the glycosyltransferases in that it is found in two distinct subcellular pools, one within the *trans*-Golgi compartment and one on the plasma membrane (Shur, 1991). Each serves distinct biological functions. In the Golgi compartment, GalTase participates in the biosynthesis of membrane-bound and secretory glycoconjugates much like all other glycosyltransferases. Specifically, GalTase catalyzes the transfer of galactose from UDP-galactose (UDPGal) to terminal *N*-acetylglucosamine residues on elongating oligosaccharide chains (Schachter and Roseman, 1980; Paulson and Colley, 1989). On the cell surface, GalTase is thought to function as a recognition molecule during a variety of cell-cell and cell-matrix interactions by binding to specific oligosaccharide ligands on adjacent cell surfaces and in the extracellular matrix (Roseman, 1970; Roth, 1973; Shur, 1991). Cell interactions that use GalTase as a cell surface receptor

include murine fertilization (Miller et al., 1992), embryonal carcinoma (EC) cell adhesion (Shur, 1983), morula compaction (Bayna et al., 1988), mesenchymal cell migration (Hathaway and Shur, 1992), and neurite outgrowth (Begovac et al., 1991).

The cloning of GalTase has revealed that the gene encodes two in-frame ATG initiation codons (Shaper et al., 1988; Lopez et al., 1991). Two size classes of GalTase transcripts are specified by differential transcription initiation; long transcripts that initiate 5' to the first ATG encode a protein of 399 amino acids and short transcripts that initiate between the two ATGs encode a shorter protein of 386 amino acids. Both classes of mRNA are translated *in vitro* (Russo et al., 1990), which generate two GalTase proteins that are identical except that the longer protein possesses a 13-amino acid extension at its NH₂-terminal cytoplasmic domain.

We have presented evidence that the two forms of GalTase can be differentially regulated and are targeted to different subcellular compartments (Lopez et al., 1989, 1991). S1 nuclease protection analysis has shown that the levels of the short GalTase mRNA parallel the amount of Golgi GalTase activity, whereas the levels of the long GalTase paral-

1. *Abbreviations used in this paper:* EC, embryonal carcinoma; EDTA-BSS, 2 mM EDTA in Ca²⁺/Mg²⁺-free Eagle's balanced salt solution; GalTase, β 1,4-galactosyltransferase; MT-1, metallothionein-1; TLGT, truncated long GalTase; TSGT, truncated short GalTase.

lel GalTase-specific activity on the cell surface, suggesting that the long form of GalTase is preferentially targeted to the cell surface relative to the short GalTase. More directly, GalTase activity is significantly elevated on purified plasma membranes isolated by subcellular fractionation from stably transfected F9 EC cells that overexpress the long GalTase mRNAs, relative to cells that overexpress the short GalTase mRNAs (Lopez et al., 1991).

Recent evidence shows that the cell surface form of GalTase is anchored to the cytoskeleton, possibly through the 13-amino acid cytoplasmic extension unique to the long form (Eckstein and Shur, 1992). A portion of surface GalTase colocalizes with actin-containing microfilaments as assayed by double-label indirect immunofluorescence. Furthermore, GalTase partitions with the insoluble cytoskeleton fraction after detergent extraction of noncytoskeletally associated material. The degree of surface GalTase associated with the detergent-insoluble cytoskeleton is dependent upon the integrity of the cytoskeleton, since destabilizing cytoskeleton-protein interactions with high KCl, elevated pH, or cytochalasin B all reduce the amount of surface GalTase partitioning with the cytoskeleton. On the other hand, cross-linking surface GalTase with multivalent ligands or antibodies induces additional GalTase to associate with the cytoskeleton (Eckstein and Shur, 1992).

The role of surface GalTase during cellular interactions has been studied using various biochemical and immunological reagents to perturb GalTase activity and examining the consequences on specific cellular interactions (Shur, 1991). Although the results from these analyses suggest that surface GalTase participates in a variety of cellular interactions, definitive evidence that GalTase functions as a cell adhesion molecule awaits the manipulation of its expression on the cell surface and a consequent loss of cellular adhesion. In this study, we created a dominant negative mutation (for review see Herskowitz, 1987) that interferes with the function of cell surface GalTase by transfecting into cells cDNAs encoding truncated versions of GalTase devoid of the substrate-binding domain. Overexpressing the complete cytoplasmic and transmembrane domains of the long form of the GalTase protein, referred to as the truncated long GalTase (TLGT) protein, displaced the endogenous cell surface GalTase from its cytoskeletal attachment, leading to a loss of cellular adhesion. In contrast, overexpressing a truncated short GalTase (TSGT) protein containing the cytoplasmic and transmembrane domains of the short GalTase had no effect on cell adhesion. In both cases, GalTase activity remained unchanged. The nonadhesive phenotype of the TLGT transfectants was specific to GalTase, since the cytoskeletal attachment of $\beta 1$ integrin was unaffected. The results are consistent with previous studies showing that the long GalTase protein is preferentially targeted to the plasma membrane. In addition, these results suggest that the cell adhesive function of surface GalTase is dependent upon its association with the cytoskeleton, which contains a defined and saturable number of binding sites specific for GalTase.

Materials and Methods

Construction of pKJ-1 and EV-142 Expression Plasmids Containing the Truncated Forms of GalTase

The EV-142 expression vector contains the mouse metallothionein-1 (MT-1)

promoter fused by a unique BglII site to a fragment of the human growth hormone gene containing the polyadenylation signal (Low et al., 1985). The EV-142 vector was digested with BglII and treated with Klenow to fill the ends. XbaI linkers were added to the ends and then digested with XbaI for acceptance of the GalTase sequence. The GalTase cDNA fragments subcloned into EV-142 were as follows: TLGT (containing GalTase DNA sequence extending from -9 to 142 bp) was generated from the pUC 19 plasmid containing the SacII (-9)-SacII (239) fragment of GalTase DNA. The plasmid was digested at the BamHI and EcoRI sites at the multiple cloning region of pUC 19, which are 7 bp upstream of SacII (-9) and 14 bp downstream of SacII (239), respectively. The BamHI-EcoRI fragment was cut with DdeI (139) and treated with Klenow to generate blunt ends. The BamHI-DdeI fragment (158 bp) was isolated from an 8% polyacrylamide gel. XbaI linkers were added to the ends and then cut with XbaI to generate sticky ends; TSGT (+33/142) was constructed from the EcoRI (33)-DdeI (139) DNA fragment of pKJ-SGT (Lopez et al., 1991). The fragment was treated with Klenow to generate blunt ends, XbaI linkers were added, and then the fragment was digested with XbaI. The GalTase constructs were ligated into the EV-142 vector. A stop codon was generated in the XbaI linker region 23 bp downstream of the putative transmembrane domain. Orientation of insertion of the GalTase DNA fragments into the EV-142 vector was determined by restriction endonuclease digestion analysis.

Cotransfection of Truncated GalTase Constructs with pKJ-neo into Murine F9 EC Cells and Swiss 3T3 Fibroblasts

F9 and 3T3 cells were transfected by standard calcium phosphate precipitation methods (Chen and Okyama, 1987). Briefly, 50×10^3 F9 or 3T3 cells were plated on gelatinized or plastic 100-mm dishes, respectively, 8 h before DNA addition. Cells were cotransfected for 16–20 h with 10 μ g (for F9 cells) or 20 μ g (for 3T3 cells) of construct DNA and 2 μ g of pKJ-neo DNA per dish. DNA was isolated by two consecutive centrifugations on CsCl gradients (Sambrook et al., 1989). Cells were washed three times with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Eagle's balanced salt solution and allowed to recover in complete DME containing 15% heat-inactivated FBS (for F9 cells) or 10% heat-inactivated bovine calf serum (for 3T3 cells) and penicillin/streptomycin, before start of selection. After 24 h, cells were split (1:15) into 100-mm plates and selected by growth in complete DME containing 400 μ g/ml (final concentration) G418. After 10–14 d, individual G418-resistant colonies were selected and expanded. Swiss 3T3 clones expressing the TLGT construct under the control of the EV-142 MT-1 promoter were induced with 80 μ M ZnSO_4 for up to 20 h in DME supplemented with 5% bovine calf serum.

S1 Nuclease Protection Analysis

S1 nuclease protection analyses were performed as previously described (Battey et al., 1983). Uniformly ^{32}P -labeled DNA probes complementary to transcribed sequences were prepared by primer extension of M13 clone H5D in which the 531-bp HincII (multiple cloning region)-SmaI(287) DNA fragment from pMGT -239/2615 was cloned into HincII-SmaI-cut M13mp19 (Lopez et al., 1991). Primer extension of the M13 DNA and digestion with HincII resulted in a single-stranded DNA probe of 567 nucleotides, of which 38 are vector sequences (3 nt derived from linker). The single-stranded DNA probes were purified by electrophoresis on an 8-M urea 6% polyacrylamide gel and electroelution. The 20- μ l hybridization mixture contained 50,000 cpm of probe, 5–10 μ g of total cellular RNA, 70% formamide, 400 mM NaCl, 20 mM Tris, pH 7.5, and 1 mM EDTA. Control hybridizations contained equivalent amounts of partially hydrolyzed yeast RNA. Incubations were at 59°C for 16 h and were terminated by the addition of 800 U of S1 nuclease (BRL, Gaithersburg, MD) in 350 μ l of buffer containing 0.3 M NaCl, 30 mM sodium acetate, pH 4.5, 3 mM ZnSO_4 . After 1 h at 37°C, the digestion was terminated by the addition of 100 μ l of a stop solution containing 4 M ammonium acetate, 20 mM EDTA, and 1.2 mg/ml carrier yeast RNA followed by ethanol precipitation. The protected fragments were analyzed on an 8-M urea 6% polyacrylamide gel, with 300 cpm of undigested probe and 1,500 cpm of ^{32}P -labeled DNA ladder.

Isolation of Polysomal RNA

Polysomal RNA was isolated as previously described (Gold and Hecht, 1981). Briefly, 10×10^6 Swiss 3T3 cells transfected with the neo selectable marker, or a TLGT clone exposed to 80 μ M ZnSO_4 for 6 h, were har-

vested with 2 mM EDTA in Ca²⁺/Mg²⁺-free Eagle's balanced salt solution (EDTA-BSS) containing 2% chicken serum and washed three times at 4°C in wash buffer (50 mM Tris-HCl, pH 7.6, 100 mM KCl, and 1.5 mM MgCl₂). Cells were resuspended in wash buffer containing 0.1% Triton X-100 (vol/vol) and 0.1% diethylpyrocarbonate (vol/vol), and then lysed in a Teflon glass homogenizer. Unbroken cells, cell debris, and nuclei were pelleted and discarded after a 10-min centrifugation at 1,500 g. Mitochondria were pelleted and discarded after a 10-min centrifugation of the supernatant at 12,000 g. Postmitochondrial supernatants were loaded onto 10–50% sucrose (wt/vol) gradients containing 50 mM Tris-HCl, pH 7.6, 50 mM KCl, and 1.5 mM MgCl₂ and centrifuged in a type 50 Ti rotor (Beckman Instrs., Fullerton, CA) at 50,000 rpm for 2.5 h at 4°C to recover polyosomes. As a control for polysome isolation, EDTA was added in place of MgCl₂. Fractions were collected from the bottom and analyzed at 254 nm to determine polysomal and nonpolysomal fractions. SDS was added to a final concentration of 0.5% to the fractions; then each sample was extracted once with 2 vol cold phenol/chloroform (1:1) and twice with chloroform. The RNA samples were precipitated by the addition of sodium acetate and ethanol and stored overnight at -20°C. The samples were again extracted with phenol/chloroform and precipitated as above. The purified RNA was analyzed by S1 nuclease protection assays.

Immunofluorescence of TLGT Peptide

Approximately 10,000 cells were grown overnight in 8-well chamber slides (Miles Inc., Diags. Div., Kanakee, IL) coated with 1 µg/ml laminin or fibronectin. The cells were induced with 80 µM ZnSO₄ for various amounts of time (0–5 h) in DME supplemented with 5% bovine calf serum. Cells were washed twice in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄, pH 7.3), and then fixed for 5 min at -20°C in 5% glacial acetic acid/95% ethanol. The cells were washed twice with PBS, and then once with PBS/5% goat serum. Cells were incubated in 200 µl of rabbit serum made against the 13-amino acid NH₂ terminus of the long form of GalTase (anti-NH₂ terminal) or preimmune rabbit serum diluted 1:100 in PBS/5% goat serum for 1 h at 24°C in a humidified chamber. The cells were washed twice with PBS, and then once with PBS/5% goat serum. FITC-conjugated goat anti-rabbit antibody (Boehringer Mannheim, Indianapolis, IN) diluted 1:50 in PBS/5% goat serum was added to cells for 1 h at 24°C. For the competition assay, the cells were prepared and immunostained as described above; however, the primary antibody (anti-NH₂ terminal serum) was preincubated for 30 min on ice with 200 µg/ml of GalTase 13-amino acid NH₂-terminal peptide or an arbitrary peptide (15-amino acid myogenin peptide provided by Eric Olson, M. D. Anderson Cancer Center). Slides were washed three times with PBS, mounted with medium (90% [vol/vol] glycerol, 10% [vol/vol] PBS, and 4% [wt/vol] *n*-propylgalate), and viewed with a microscope equipped for epifluorescence (Dialux EB 22; Leitz, Rockleigh, NJ).

Determination of Cell Surface GalTase Activities in TLGT Clones

TLGT cells were cultured in complete DME or induced with 80 µM ZnSO₄ for 6–20 h. Cells were harvested with EDTA-BSS and washed three times in medium B (140 mM NaCl, 4 mM KCl, 20 mM Hepes, pH 7.2). Whole cells (at >95% viability) were assayed for surface GalTase activity as previously described (Shur, 1982). Briefly, 3–5 × 10⁵ cells were incubated at 37°C in a total volume of 300 µl of medium B containing 0.4% BSA and a mixture of protease inhibitors (PIC; 2 µg/ml antipain, 0.1% aprotinin, 10 µg/ml benzamide, 1 µg/ml chymostatin, 1 µg/ml leupeptin, 1 µg/ml pepstatin), 100 µM UDP[³H]Gal (17.6 cpm/pmol) (Dupont-New England Nuclear, Wilmington, DE), 1 mM 5'-AMP, 10 mM MnCl₂, and 30 mM GICNAc. 50-µl samples were removed at 0-, 20-, 40-, 60-, and 90-min time points and added to 10 µl of ice cold 0.2 M EDTA-Tris HCl, pH 7.2. A 50-µl sample of each reaction mixture was subjected to high-voltage borate electrophoresis to separate the ³H-labeled galactosylated product from unused UDP[³H]Gal and its breakdown products. The radiolabeled product remaining at the origin was quantitated by liquid scintillation spectroscopy.

Determination of GalTase Associated with the Cytoskeleton

Cells were assayed for surface GalTase association with the cytoskeleton as previously described (Eckstein and Shur, 1992). Briefly, uninduced or induced Swiss 3T3 transfectants were harvested with EDTA and washed twice with medium B, and aliquots of 10⁵ cells were placed in microfuge

tubes and incubated with 0.5 ml of 5% BSA in medium B for 45 min at 23°C. Cells were washed twice again with medium B and incubated for 45 min at 23°C in 0.5 ml of 0.5% BSA in medium B containing either preimmune rabbit serum, anti-GalTase serum (Bayna et al., 1988; Lopez et al., 1985), or anti-β1 integrin serum (Pat Brown, Department of Pharmacology, University of Texas Medical School at Houston) diluted 1:100. The anti-GalTase antiserum specifically recognizes GalTase on mouse cells as judged by immunoprecipitation and immunoblotting criteria (Eckstein and Shur, 1992; Macek et al., 1991; Bayna et al., 1988; Lopez et al., 1985). Cells were washed twice with 2% BSA in medium B and incubated for 45 min at 23°C in 0.5 ml of 0.5% BSA in medium B containing 4.1 µCi ¹²⁵I-goat anti-rabbit IgG (Dupont-New England Nuclear). Cells were again washed twice in 2% BSA in medium B. Cells were extracted in 0.5 ml of ice cold detergent buffer (150 mM NaCl, 1% Triton X-100, 50 mM Na acetate, 10 mM Tris, pH 5.0, and PIC). Cytoskeletal material was pelleted at 10,000 g for 10 min. The supernatant was removed and saved; the extraction was repeated two more times with each supernatant saved separately. Soluble radioactivity was reduced to background levels by the third supernatant. After the final extraction, 0.5 ml of 1 N NaOH was added to solubilize the pellet. ¹²⁵I activity was determined in a gamma counter (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The concentrations of both the primary (i.e., rabbit serum) and secondary (i.e., ¹²⁵I-goat anti-rabbit IgG) antibodies were shown to be in excess. Anti-GalTase or anti-β1 specific immunoreactivity was determined by subtracting the cpm associated with preimmune rabbit controls. In some experiments, the amount of TLGT protein associated with the cytoskeleton was determined using the anti-NH₂ terminal serum described above. Since this antiserum recognizes a cytoplasmic determinant, unlike the anti-GalTase and anti-β1 antisera, the protocol was used in reverse order; the cytoskeleton-associated material was first prepared by Triton extraction, and then blocked, incubated with primary (1:100 final dilution) and ¹²⁵I-secondary antibodies, washed three times, solubilized, and counted as above. Preimmune rabbit serum was used as control, and the anti-NH₂ terminal specific immunoreactivity was analyzed as above. To determine the amount of GalTase activity in the detergent-insoluble fraction of the uninduced and induced cells, the Triton extractions were performed as described above, after which GalTase activity in the third detergent extract and the detergent-insoluble pellet was assayed as described above. There was no detectable GalTase activity in the third detergent extract, showing that all detergent-soluble GalTase was extracted before assaying the detergent-insoluble pellet.

Metabolic Labeling with [³H]Leucine and [³H]Galactose

Swiss 3T3 cells growing on 100-mm plates in complete DME were labeled at 37°C with 30 µCi/ml [³H]leucine (142 Ci/mmol) in complete DME. After 1 h, 80 µM ZnSO₄ was added to the plates containing 5% bovine calf serum. All cells were incubated an additional 6 h, harvested with EDTA-BSS, and washed three times in medium B. Triton-soluble and -insoluble proteins were isolated from both uninduced and induced cells and then analyzed by electrophoresis on an 8–20% linear polyacrylamide gradient gel under reducing conditions (Fling and Gregerson, 1986). Alternatively, Swiss 3T3 cells were labeled with 5 µCi/ml [³H]galactose (60 Ci/mmol) for 20 h in the presence or absence of 80 µM ZnSO₄. Cells were washed three times in medium B, resuspended in sample buffer, and analyzed by electrophoresis on an 8% polyacrylamide gel under reducing conditions.

Cell Surface Labeling with Na¹²⁵I

Uninduced or induced Swiss 3T3 cells were surface labeled using Iodogen and Na¹²⁵I as described by the manufacturer (Pierce Chemical Co., Rockford, IL). Briefly, ~7 × 10⁶ cells were harvested with EDTA-BSS, washed three times with PBS, and then resuspended in 250 µl PBS. The washed cells and 500 µCi of Na¹²⁵I were added to Iodogen-coated tubes (250 µg) and incubated for 15 min on ice. Cells were washed three times in PBS, and then resuspended in 1% Triton X-100 detergent buffer. ¹²⁵I-labeled cell surface proteins were analyzed by electrophoresis on an 8% polyacrylamide gel under reducing conditions.

Analysis of Surface Galactosylated Proteins

Uninduced and induced Swiss 3T3 cells were dissociated with EDTA-BSS and washed three times in medium B. Approximately 10⁶ cells were incubated for 60 min at 37°C in a total volume of 100 µl of medium B-PIC-0.4% BSA, 100 µM UDP-[³H]Gal (1,990 cpm/pmol), 1 mM 5'-AMP, and 10 mM MnCl₂. After 60 min, cells were centrifuged out of solution,

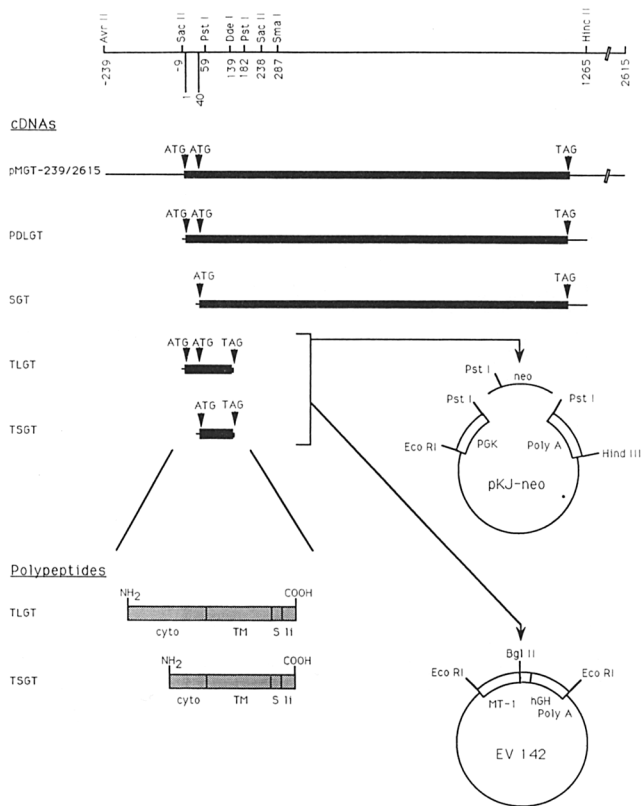


Figure 1. Schematic diagram of the GalTase cDNA clones and the truncated GalTase proteins. The full-length GalTase (–239/2615) cDNA, the promoter-deleted long GalTase (*PDLGT*; –9/1265) cDNA, and the short GalTase (*SGT*; +33/1265) cDNA clones were obtained as previously described (Lopez et al., 1991). The *TLGT* (–9/142) and the *TSGT* (+33/142) cDNA clones were obtained as described in Materials and Methods. The *TLGT* and *TSGT* constructs were cloned into either the *pKJ-neo* expression vector under the control of the constitutive phosphoglycerokinase (*PGK*) promoter or the *Bgl*III site of the *EV-142* expression vector under the control of the *MT-1*-inducible promoter. These plasmids were cotransfected with a *neo* selectable marker into either F9 EC cells or Swiss 3T3 fibroblasts. Both the *TLGT* and *PDLGT* cDNAs and both the *TSGT* and *SGT* cDNAs encode for proteins that have identical NH₂-terminal cytoplasmic (*cyto*) and transmembrane (*TM*) domains. However, the catalytic domain has been truncated three amino acids into the stem (*S*) region. The COOH-terminus of the truncated forms contains 14 bp derived from *XBa* linkers (*li*).

washed, lysed, and analyzed by electrophoresis on an 8–20% polyacrylamide gradient gel under reducing conditions.

Results

Constitutive Expression of a Truncated Form of the Long GalTase Protein Leads to a Loss of Cellular Adhesion in F9 EC Cells

Previously, we have shown that undifferentiated F9 EC cells have unusually high levels of surface GalTase activity that correlate with the expression of the long forms of the GalTase mRNA. In contrast, retinoic acid-differentiated F9 cells are characterized by greatly increased levels of Golgi GalTase activity and contain a concomitant increase in the levels of the short GalTase mRNA transcripts. Similarly, the

short GalTase mRNAs are the predominant mRNA species found in tissues that are enriched for Golgi GalTase activity, including parietal yolk sac cells and lactating mammary gland (Lopez et al., 1989, 1991). Collectively, these results suggest that the short GalTase protein, lacking the 13-amino acid cytoplasmic extension, is enriched in the Golgi compartment, whereas the long GalTase protein is enriched on the cell surface. Consistent with this, stable F9 EC cell transfectants that overexpress the long GalTase mRNAs have elevated levels of surface GalTase activity, relative to cells that overexpress the short GalTase mRNAs (Lopez et al., 1991).

To examine the biological effects of altering surface GalTase expression, truncated versions of the GalTase gene lacking sequences that encode the substrate-binding, catalytic domain were transfected into F9 EC cells under the control of the phosphoglycerokinase (*PGK*) constitutive promoter (Fig. 1). 20 independent clones expressing the cDNA encoding the *TLGT* protein lost their adhesive capabilities, in contrast to 20 independent clones expressing *TSGT* or control cells (*neo*), which remained adhesive (Fig. 2 A). S1 nuclease protection analysis showed that the appropriate size mRNA fragment, representing either the *TLGT* or *TSGT* transgene, was expressed in the corresponding F9 EC transfected clones (Fig. 2 B). However, because of the strong *PGK* promoter, the *TLGT* clones were constitutively nonadhesive and difficult to analyze further. To overcome this obstacle, an inducible promoter was used to allow the controlled expression of the transfected gene and its associated phenotype. cDNAs encoding the truncated long and short forms of GalTase were cloned into the *EV-142* vector containing the inducible *MT-1* promoter. Unfortunately, the *MT-1* promoter was uninducible in F9 transformants; thus, Swiss 3T3 fibroblasts were chosen for further analysis.

Inducible Expression of the *TLGT* Protein Leads to a Loss of Cellular Adhesion in Swiss 3T3 Fibroblasts

Similar to the F9 EC *TLGT* clones, Swiss 3T3 *TLGT* clones lost their adhesive capabilities coincident with expression of the *TLGT* construct (Fig. 3 A). Even in the absence of exogenous Zn²⁺, the *MT-1* promoter had a basal level of expression (Fig. 3 C) that resulted in reduced cellular adhesion, i.e., more rounded cells with shorter cytoplasmic extensions (Fig. 3 A, arrows at 0 h induction). After the *MT-1* promoter was induced for 6 h with Zn²⁺, most of the cells lost their adhesion to the substratum, although a few cells retracted their filopodia and remained bound (Fig. 3 A, arrows at 6 h induction). Dose-dependency studies in which the concentrations of Zn²⁺ and serum were varied showed that 80 μM Zn²⁺ in 5% serum gave a reproducible loss of cell adhesion in *TLGT* transfectants, without significantly affecting cell viability or the adhesion of control cells (*neo*) (data not shown). Prolonged incubation (~20 h) of control cells in 80 μM Zn²⁺ resulted in a slightly retracted morphology that was distinctly different from the nonadhesive phenotype seen in *TLGT* clones (Fig. 3 A). When Zn²⁺ was removed from *TLGT* cells, as well as from control cells, they regained their original adhesive phenotype within 24 h (Fig. 3 A).

12 independently selected *TLGT* clones showed this characteristic loss of cell adhesion after induction of the *MT-1* promoter; 2 independent clones (*TLGT61* and *TLGT64*) were chosen for further analysis. *TLGT61* had a reproduc-

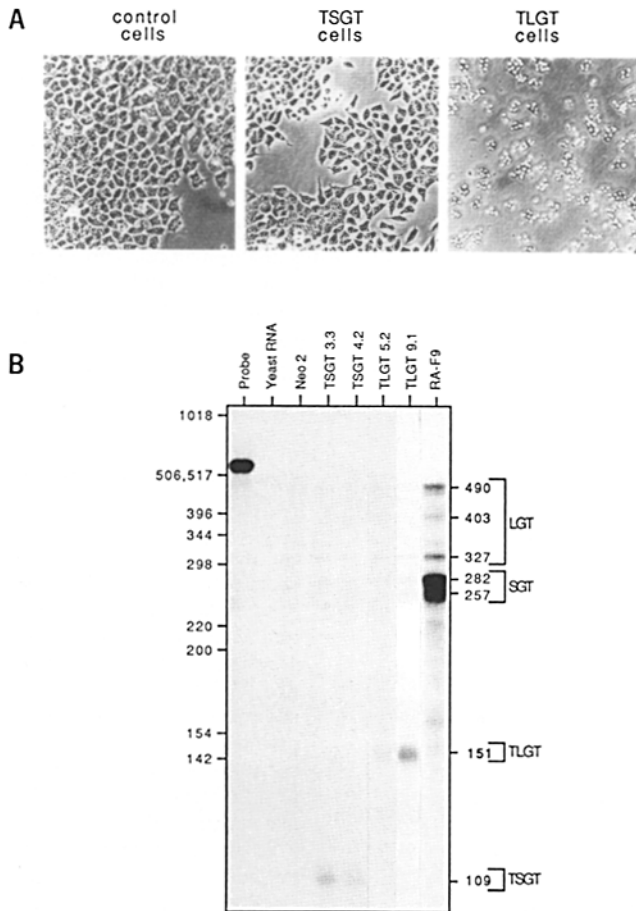


Figure 2. F9 EC cells transfected with the TLGT cDNA, in contrast to those transfected with the TSGT cDNA, have an altered adhesive morphology. (A) Shown is a representative clone transfected with the *neo* selectable marker, a representative TSGT clone, and a representative TLGT clone all under the control of the PGK promoter. The F9 EC cells transfected with the TLGT cDNA became nonadhesive after stable transfection, whereas the control cells (*neo* and TSGT) remained adhesive. (B) S1 nuclease protection analysis of 10 μ g of partially hydrolyzed yeast RNA, a representative control clone transfected with the *neo* selectable marker (*Neo2*), two independent TSGT cell transfectants (*TSGT3.3*, *TSGT4.2*), two independent TLGT cell transfectants (*TLGT5.2*, *TLGT9.1*), and retinoic acid-induced F9 EC cells (*RA-F9*). Undigested radiolabeled DNA probe was also analyzed on the gel. The 567-nucleotide (nt) probe contains 239 nt of GalTase 5'-untranslated sequence and 290 nt translated sequence. Bars to the right represent the major protected species, and the regions corresponding to endogenous long GalTase (*LGT*), endogenous short GalTase (*SGT*), truncated long GalTase (*TLGT*), and truncated short GalTase (*TSGT*) transcripts. Molecular size markers are 1 kb DNA ladder (BRL) in nt and are indicated on the left.

ibly stronger phenotype than did TLGT64, characterized by a less adherent morphology before induction and a more rapid and extensive loss of adhesion after induction. In contrast to all of the TLGT clones, all of the clones expressing the TSGT transgene appeared indistinguishable from control cells (*neo*) (Fig. 3 A) before and after induction with Zn^{2+} , as was the case for the F9 EC TSGT clones (Fig. 2). Therefore, subsequent experiments focused specifically on the TLGT clones.

Since surface GalTase mediates fibroblast spreading and migration on laminin but does not participate during cell interactions with fibronectin (Runyan et al., 1988; Eckstein and Shur, 1989), TLGT clones were plated on either laminin or fibronectin and subsequently induced with Zn^{2+} . In contrast to cells plated on fibronectin which remained adhesive, cells grown on laminin retracted their filopodia and became nonadhesive (Fig. 3 B). The TLGT protein was shown to be translated in both culture conditions by indirect immunofluorescence (see below). These results indicate that induction of the TLGT construct only affects those cell-matrix interactions known to be dependent upon surface GalTase and clearly eliminate any consideration of nonspecific effects of the TLGT protein on cell-matrix adhesion.

S1 nuclease protection analysis showed that the TLGT mRNA was transcribed in these cells (Fig. 3 C). The MT-1 promoter proved to be partially active even in the absence of Zn^{2+} , since TLGT transcripts (151 bp) were present in the uninduced TLGT clones (Fig. 3 C). As will be shown later (see Fig. 6), this expression reflects the heterogeneous nature of the uninduced cell population, which includes cells with normal morphology having low levels of TLGT expression, as well as rounded, partly retracted cells expressing higher levels of TLGT. More complete induction of the MT-1 promoter with Zn^{2+} resulted in consequent increases in the level of TLGT transcripts. Interestingly, after 12 h of Zn^{2+} induction, and even more significantly at 20 h, a specific up-regulation of the endogenous full-length long form of GalTase mRNA was observed (Fig. 3 C). There was no apparent increase in the levels of the endogenous short GalTase transcript. Since it has been suggested that the long GalTase protein is preferentially targeted to the cell surface, relative to the short GalTase protein (Lopez et al., 1991), one would predict that surface GalTase levels would be selectively increased after 20 h of induction in the TLGT clones due to the increased expression of the long GalTase RNA. This was shown to be the case; surface GalTase levels were increased over threefold, whereas total cellular (i.e., Golgi and surface) GalTase levels increased only $\sim 10\%$, which could be accounted for by the increased activity associated with the cell surface (Fig. 4).

The molecular mass of the protein encoded by the TLGT transgene is predicted to be 5.7 kD. SDS-PAGE proved to be an unreliable assay for proteins of this small size. Therefore, S1 nuclease protection assays were used to determine if the TLGT mRNA was associated with polyribosomes in TLGT cells, which is diagnostic of mRNAs that are being actively translated into protein (Alberts et al., 1989). Protected fragments corresponding to the endogenous full-length GalTase mRNAs in control cells (Fig. 5 A) and endogenous GalTase and TLGT mRNAs in the induced TLGT clones (Fig. 5 B) were present only in the polysomal RNA fractions. In control assays, EDTA was added to the gradient in place of $MgCl_2$ to dissociate the polysomes, which resulted in a displacement of both the endogenous GalTase and TLGT mRNAs into the nonpolysomal fractions (Fig. 5 C). Thus, the presence of the TLGT transcript, along with the endogenous full-length GalTase transcript, on polyribosomes suggests that the TLGT protein was being translated.

To verify expression of the TLGT protein, immunofluorescence analysis was performed on the TLGT clones using an antibody raised against the 13-amino acid NH_2 terminus

of the long form of GalTase (Fig. 6). As expected, the expression of the TLGT protein was heterogeneous in the uninduced (i.e., $-Zn^{2+}$) cell population, and correlated well with the heterogeneous morphology of these cells (Fig. 3A). In cells with normal morphology, low levels of the TLGT protein were detected and confined predominantly to the perinuclear region (Fig. 6, 0 h induction, *arrow*). More rounded, retracted cells with reduced substrate adhesion showed increased expression of TLGT immunoreactivity (*arrowhead*). Upon the addition of Zn^{2+} , the fluorescence intensity dramatically increased over time, correlating with the increased expression of the TLGT transcript (Fig. 3C) and became concentrated at the cell surface, coincident with

the loss in cell adhesion (Fig. 6). (A darker exposure of the fully induced, nonadherent cells is shown to better illustrate the surface-associated TLGT immunoreactivity [Fig. 6, 5 h induction, *arrowheads*].)

The NH_2 -terminal antibody was shown to react specifically with TLGT epitopes, since control *neo* transfectants failed to show any Zn^{2+} -induced immunofluorescence, and an excess of NH_2 -terminal peptide competed for the NH_2 -terminal antibody immunofluorescence, whereas an equimolar concentration of an irrelevant peptide did not affect immunofluorescence intensity (Fig. 6). The lower level of immunoreactivity in *neo* transfectant control cells was confined to the perinuclear region and what appear to be focal

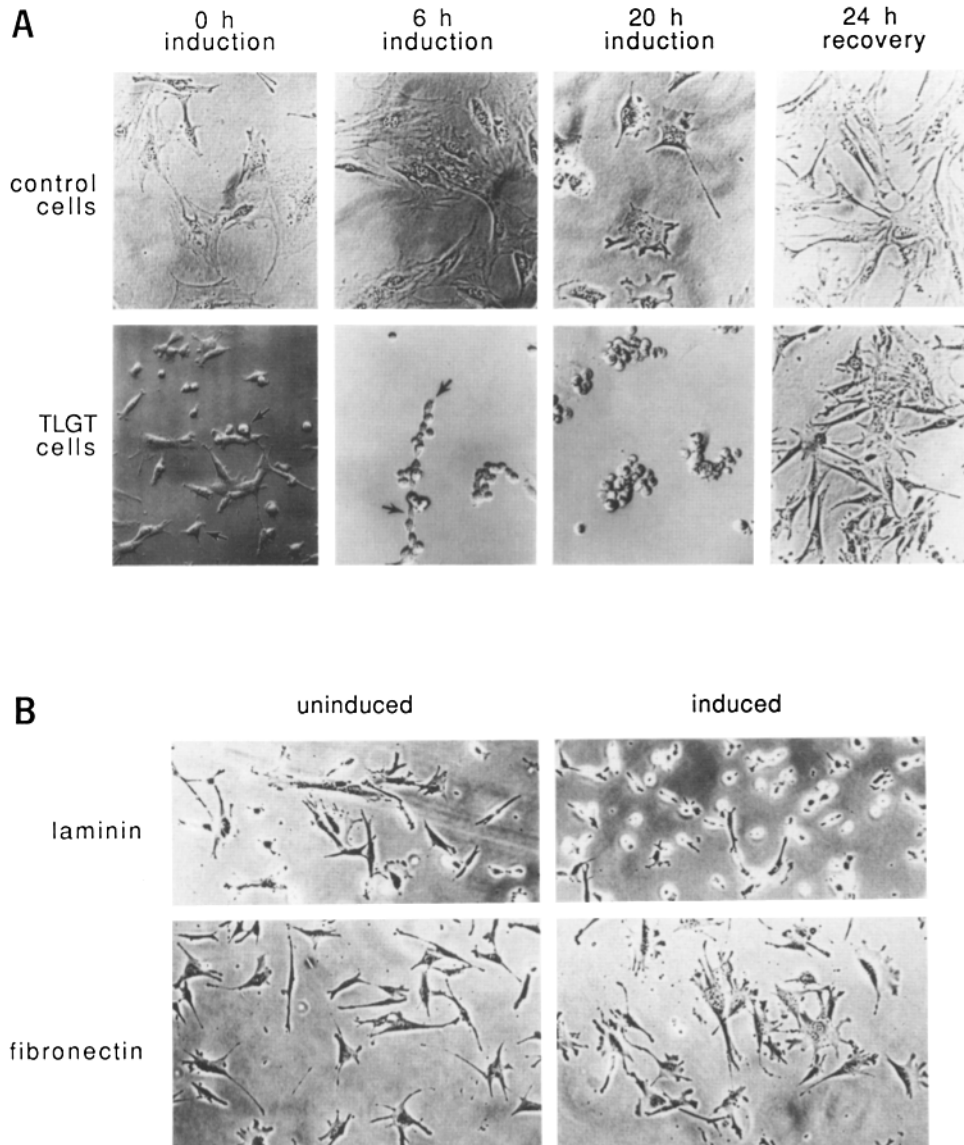


Figure 3. Induction of the TLGT mRNA in 3T3 TLGT clones inhibits cell adhesion and results in upregulation of endogenous LGT mRNA. (A) A representative control clone transfected with the selectable *neo* marker, and a representative TLGT clone (TLGT61) under the control of the MT-1 promoter in 3T3 cells were exposed to Zn^{2+} for 0, 6, or 20 h. Unlike control cells that remained adhesive, TLGT cells became nonadhesive after Zn^{2+} induction. The TLGT clone regained its original adhesive phenotype within 24 h after removal of Zn^{2+} . Notice that even in the absence of Zn^{2+} (0 h) TLGT clones are heterogeneous, with some cells showing reduced adhesion to the substratum with retracting filopodia (*arrows*). After 6 h of induction, many TLGT cells have detached from the substratum, some of which adhere to the few cells that remain bound to the dish (*arrows*). (B) The morphology of a representative TLGT clone (TLGT61) grown overnight on laminin or fibronectin is shown before induction with Zn^{2+} (*uninduced*) and 5 h after induction (*induced*). Note that GalTase-dependent adhesion on laminin is reduced compared with adhesion on fibronectin, which is not dependent upon GalTase. (C) S1 nuclease protection analysis of 10 μ g of partially hydro-

lyzed yeast RNA, a representative control clone transfected with the *neo* selectable marker (*N7*), two independent TLGT cell transfectants (*TLGT61*, *TLGT64*) analyzed at various time points (0, 6, or 12 h) after Zn^{2+} induction, and lactating mammary gland (*LMG*), which produces exclusively short transcripts. Bands corresponding to endogenous long GalTase (*LGT*), endogenous short GalTase (*SGT*), and TLGT-protected fragments are indicated on the right. (*) A shorter autoradiographic exposure of the TLGT61 (12-h Zn^{2+}) sample. A similar S1 nuclease protection analysis of TLGT61 and TLGT64 20 h after Zn^{2+} induction is shown in the right panel at a much lower autoradiographic exposure (1/36 that of the left panel). Note that the levels of the endogenous full-length LGT transcripts are significantly increased at 12 h of induction and are further upregulated at 20 h of induction. Probe and size standards are as described in Fig. 2.

adhesion plaques, and presumably reflects the endogenous full-length long form of GalTase—a possibility which is presently being pursued at the ultrastructural level.

Expression of the TLGT Protein Selectively Displaces Surface GalTase from Its Cytoskeletal Association

Results reported above indicate that overexpressing the TLGT protein resulted in a nonadhesive phenotype specifically on matrices that utilize surface GalTase as a receptor. This effect was presumed to result from a loss or downregulation of surface GalTase levels. Two independent assays were used to quantify surface GalTase levels, direct enzyme assay on intact cells and radiolabeled anti-GalTase antibody binding. Surprisingly, we found that the loss of cell adhesion in induced TLGT clones was not due to changes in the levels of surface GalTase: they were similar in induced and uninduced TLGT cells using either assay (Fig. 7 A).

In light of previous studies suggesting that surface GalTase is associated with the detergent-insoluble cytoskeleton (Eckstein and Shur, 1992), we determined whether the loss of cell adhesion in TLGT clones reflected changes in surface GalTase association with the cytoskeleton. As before, radiolabeled anti-GalTase antibody binding and direct enzyme assay of the detergent-insoluble pellet were used to quantify GalTase associated with the cytoskeleton. Both assays showed that the amount of surface GalTase associated with the cytoskeleton was reduced in TLGT cells before Zn²⁺ addition (48% reduction by antibody binding; 43% by enzyme activity), presumably due to the basal expression of the MT-1 promoter (see Figs. 3 C and 6); subsequent induction with Zn²⁺ caused a further 25% decrease in GalTase association with the cytoskeleton (Fig. 7 B).

The amount of surface GalTase that remained associated with the cytoskeleton in independent TLGT clones paralleled the intensity of their nonadhesive phenotype before and after induction. In the more responsive, i.e., less adhesive, TLGT61 clone, approximately half of GalTase remained

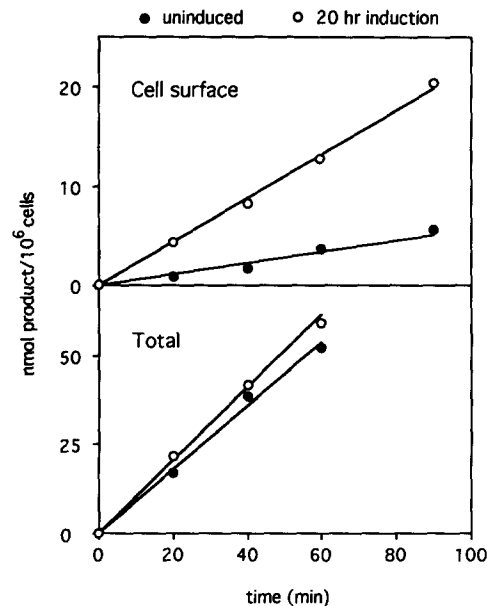
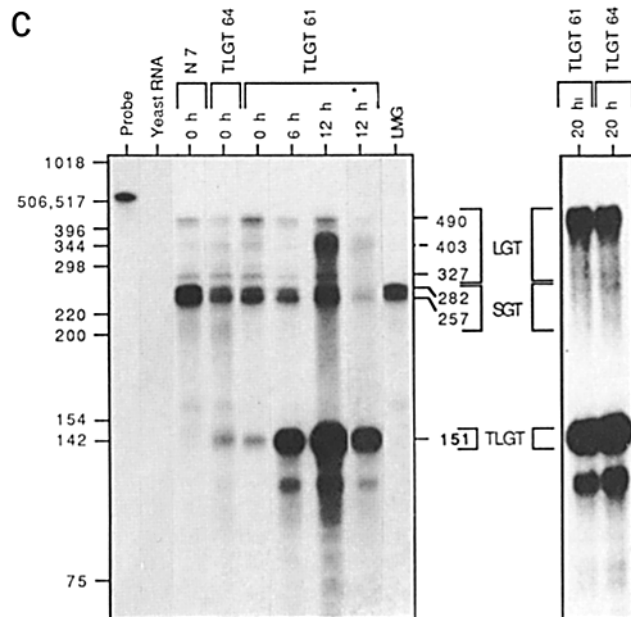
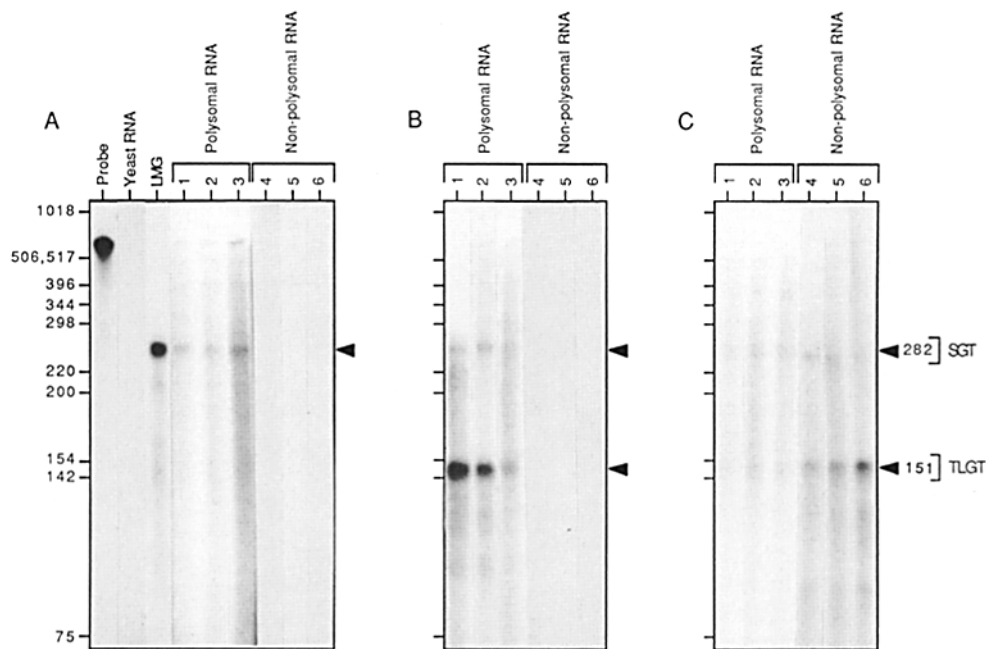


Figure 4. Upregulation of the endogenous long GalTase transcript results in a specific increase in surface GalTase activity. Cell surface and total GalTase activities were assayed as described in Materials and Methods. Cell surface GalTase activity is elevated 3.1-fold on TLGT64 cells after 20 h of induction with Zn²⁺, whereas total cellular (i.e., Golgi and surface) GalTase activity increases only ~10%, which is likely due to the increased activity associated with the cell surface. Identical results were obtained with TLGT61 cells.

bound to the cytoskeleton before induction (Fig. 7 B), while slightly higher levels of GalTase remained bound to the cytoskeleton in the more adhesive TLGT64 clone (66% by antibody binding; 63% by enzyme activity).

These results suggest that the number of cytoskeleton-binding sites for GalTase must be limited, since they could be saturated by an excess of competing TLGT protein, thus displacing the endogenous full-length GalTase from its cytoskeleton anchor. Consistent with this, the basal expression of the TLGT transcript in the uninduced cell population was approximately equal to the level of the endogenous full-length long GalTase transcript (Fig. 3 C), which was sufficient to reduce surface GalTase association with the cytoskeleton by 34–48% and produce a consequent partial reduction in cell–matrix adhesion (Fig. 3 A). As the level of TLGT protein increased, there was a coincident decrease in the amount of surface GalTase associated with the cytoskeleton. However, it is not clear at this time why ~25% of the surface GalTase remained associated with the detergent-insoluble cytoskeleton (Fig. 7 B) despite the vast overproduction of the TLGT protein (Figs. 3 C and 6). The assays used may not be sensitive enough to detect a complete loss of GalTase association with the cytoskeleton, and it is possible that longer induction times (>6 h) would result in a more complete loss of GalTase association with the cytoskeleton. Alternatively, the residual surface GalTase associated with the cytoskeleton may occur independent of its endogenous TLGT sequence, or may be stabilized by other cytoskeleton-accessory proteins; finally, this residual GalTase association may be of such high affinity that it cannot be displaced by competing TLGT protein.



protected fragments are indicated by the arrowheads on the right. Bands corresponding to endogenous long GalTase (*LGT*) cannot be seen at this exposure. Bands representing *SGT* (in *A*) and both *SGT* and *TLGT* (in *B*) are present only in the polysomal fractions. However, when *EDTA* was added in place of $MgCl_2$ to disrupt polysomes, the bands corresponding to *SGT* and *TLGT* mRNAs moved into the non-polysomal fractions (*C*), indicating that they were actively involved in translation. Probe and size standards are as described in Fig. 2.

Nevertheless, these results predict that the amount of GalTase NH_2 termini that are able to associate with the cytoskeleton is relatively constant, irrelevant of whether they are part of the full-length GalTase protein or the *TLGT* protein. Thus, the level of NH_2 termini associated with the detergent-insoluble cytoskeleton, assayed with anti- NH_2 terminal antibody, would be expected to be similar between induced and uninduced, control and *TLGT* cells. This was shown to be the case; despite the loss of full-length surface GalTase associated with the cytoskeleton (assayed with antibodies against the extracellular domain), the levels of NH_2 termini associated with the cytoskeleton remained relatively constant (Fig. 7 *B*). A slight increase ($\sim 20\%$) was observed in *TLGT* clones, relative to *neo* transfected controls, suggesting that in 3T3 cells, the cytoskeleton contains a slight excess ($\sim 20\%$) of binding sites for GalTase NH_2 termini. This is supported by recent studies of 3T3 cells overexpressing the full-length long form of GalTase; although the amount of surface GalTase increased up to 10-fold, the amount associated with the detergent-insoluble cytoskeleton increased by only $\sim 30\%$ (Appeddu, P., S. C. Evans, and B. D. Shur, unpublished observations).

Finally, the loss of GalTase association with the cytoskeleton was found to be at least partly specific, since the cytoskeletal attachment of another matrix receptor, $\beta 1$ integrin (Hynes, 1992), was similar to control levels before and after induction (Fig. 7 *B*), consistent with the inability of the *TLGT* protein to affect cell interactions with fibronectin (Fig. 3 *B*). Collectively, these data suggest that overexpression of the *TLGT* protein competes for a defined and saturable number of cytoskeleton binding sites specific for GalTase and that surface GalTase must remain bound to the cytoskeleton in order to function as an adhesion molecule.

Figure 5. S1 nuclease protection analysis of polysomal RNA from a *TLGT* clone shows that the *TLGT* mRNA is being actively translated into protein. Polysomal RNA was isolated by sucrose density centrifugation from cells transfected with either the *neo* selectable marker (*A*) or *TLGT61* cells exposed to Zn^{2+} for 6 h (*B* and *C*). As a control for polysome isolation, *EDTA* was added to the gradient in place of $MgCl_2$ (*C*). $10 \mu g$ of partially hydrolyzed yeast RNA, total RNA obtained from both polysomal (lanes 1–3) and nonpolysomal (lanes 4–6) fractions, and lactating mammary gland (*LMG*) RNA were analyzed by S1 nuclease protection analysis. Bands corresponding to endogenous short GalTase (*SGT*) and *TLGT*

Although the adhesive capabilities of the *TLGT* clones were altered after induction with Zn^{2+} , their overall metabolic capabilities remained unaffected. As shown above, *TLGT* clones recovered a normal adhesive phenotype after Zn^{2+} removal, and more significantly, Zn^{2+} induction of the *TLGT* protein had no effect on cell adhesion when plated on fibronectin. Metabolic labeling with [3H]leucine and [3H]galactose, to assess protein synthesis and intracellular galactosylation, respectively, were not different from control cells (Fig. 8, *A* and *B*). Furthermore, essentially the same proteins were expressed on the plasma membrane of control cells and *TLGT* clones either before or after induction, as determined by surface [^{125}I] labeling of intact cells (Fig. 8 *C*). However, displacement of surface GalTase from the cytoskeleton was associated with a change in the profile of plasma membrane proteins that were available for surface galactosylation with exogenous $UDP[^3H]Gal$ (Fig. 9). The displacement of surface GalTase from the cytoskeleton resulted in reduced galactosylation of a 177-kD glycoprotein, whereas galactosylation of glycoproteins of 88 kD and 47 kD was increased. The significance of these changes in surface GalTase interaction with endogenous cell surface glycoproteins is currently being addressed.

Discussion

This study reports the production and biological characterization of a dominant negative mutation that specifically affects the expression and function of the cell surface form of GalTase (Herskowitz, 1987). The results are significant for several reasons and are schematized in Fig. 10. First, this study provides the first demonstration that GalTase expression on the cell surface can be manipulated at the molecular

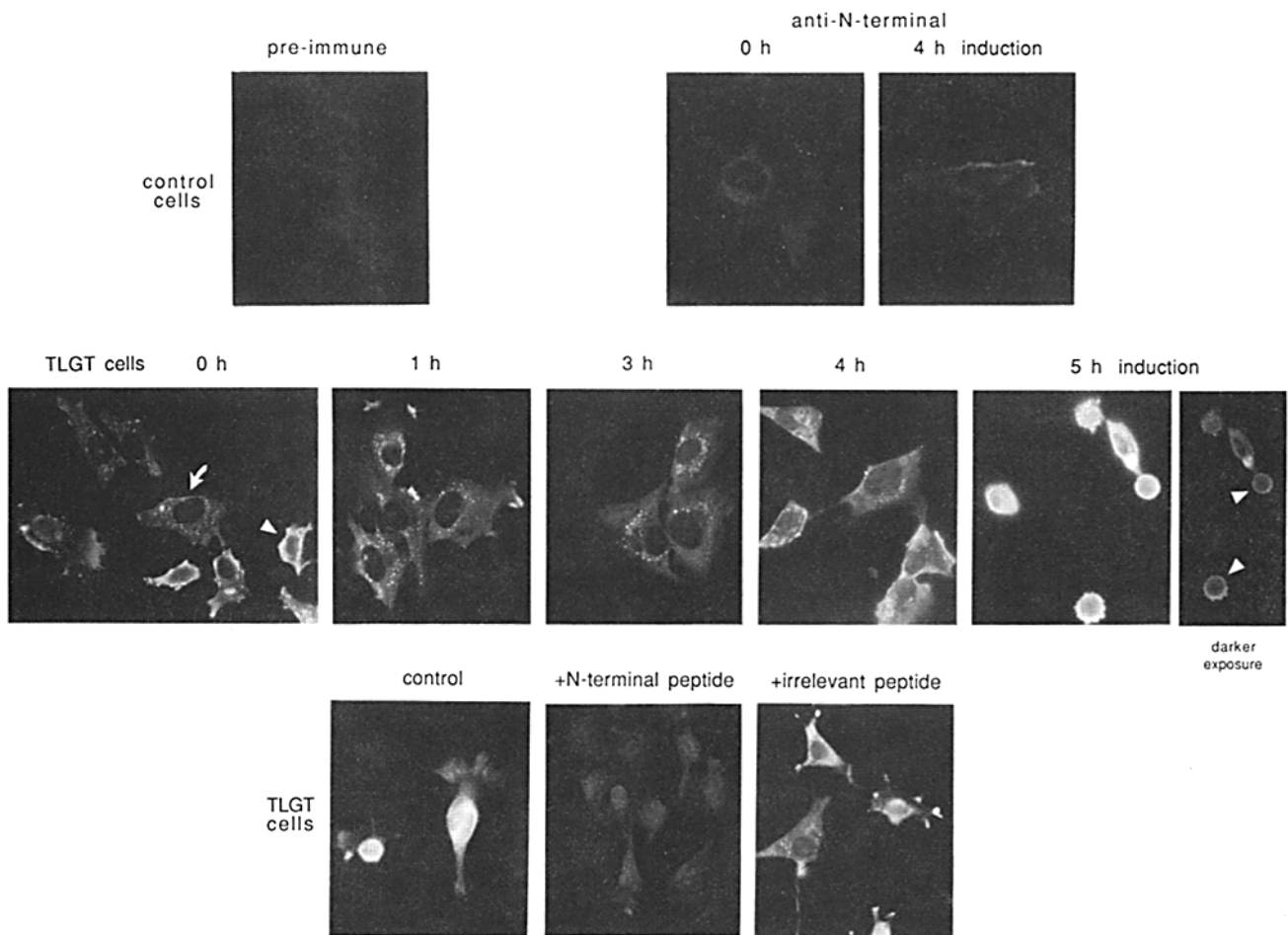


Figure 6. Immunofluorescence analysis localizes the TLGT protein during induction. Immunofluorescence analysis of TLGT61 cells using anti-NH₂-terminal antiserum (made against the 13-amino acid NH₂-terminus of the long form of GalTase) is illustrated at 0, 1, 3, 4, and 5 h after Zn²⁺ induction. Control cells (transfected with the *neo* selectable marker) labeled with anti-NH₂-terminal antiserum are shown before and after 4 h of exposure to Zn²⁺. To further assess specificity, TLGT cells were incubated in anti-NH₂-terminal antiserum alone, or with antiserum plus either GalTase NH₂-terminal peptide or an irrelevant peptide; immunofluorescence signal is inhibited specifically by the GalTase 13-amino acid peptide. Note the coincidence between the heterogeneous morphology and TLGT immunoreactivity in uninduced TLGT cells (0 h induction; *arrow*: cell with normal morphology having low levels of TLGT expression; *arrowhead*: retracted cell with reduced substrate adhesion having higher levels of TLGT expression). TLGT immunoreactive fluorescence intensity increases over time with Zn²⁺ induction (1–5 h induction). By 5 h of induction, the fluorescence intensity obscures subcellular location; a darker exposure at 5 h of induction is shown to better illustrate the surface-associated TLGT immunoreactivity. All other photographs were exposed and developed under similar conditions.

level to produce predictable consequences in cell–cell and cell–matrix interactions, thus confirming its role as a cell adhesion molecule. Second, the results show that the long form of GalTase is the biologically relevant form of the enzyme at the cell surface and is consistent with previous findings that it is preferentially targeted to the plasma membrane, since overexpressing the TLGT protein, but not the TSGT protein, produced a nonadhesive phenotype. Third, overexpressing the TLGT protein displaced GalTase from the cytoskeleton, thus suggesting a mechanism for localizing and/or stabilizing GalTase on the cell surface by virtue of an association between the 13-amino acid NH₂-terminal extension unique to the long form of GalTase and the cytoskeleton. Fourth, it appears that the cytoskeleton contains a defined and saturable number of binding sites specific for GalTase, since overexpressing the TLGT protein displaced the endogenous long form of GalTase from the cytoskeleton but did

not affect the cytoskeletal association of another matrix receptor, i.e., β 1 integrin. Similarly, when the long form of GalTase is greatly overexpressed in either F9 EC cells (our unpublished data) or in Swiss 3T3 fibroblasts (Appeddu, P., S. C. Evans, and B. D. Shur, unpublished data), the amount of GalTase associated with the cytoskeleton is only slightly increased above control cells, again suggesting that the cytoskeleton contains a limited number of binding sites for GalTase. Finally, the results suggest that GalTase must be associated with the cytoskeleton in order to function as a cell adhesion molecule, since displacement of GalTase from the cytoskeleton into the detergent-soluble pool of the plasma membrane resulted in a loss of cell adhesion, even though the noncytoskeletally associated pool of GalTase was still able to bind its glycoside substrate when assayed catalytically (Figs. 7 and 9). Furthermore, the displacement of GalTase from the cytoskeleton not only inhibits cellular adhesions,

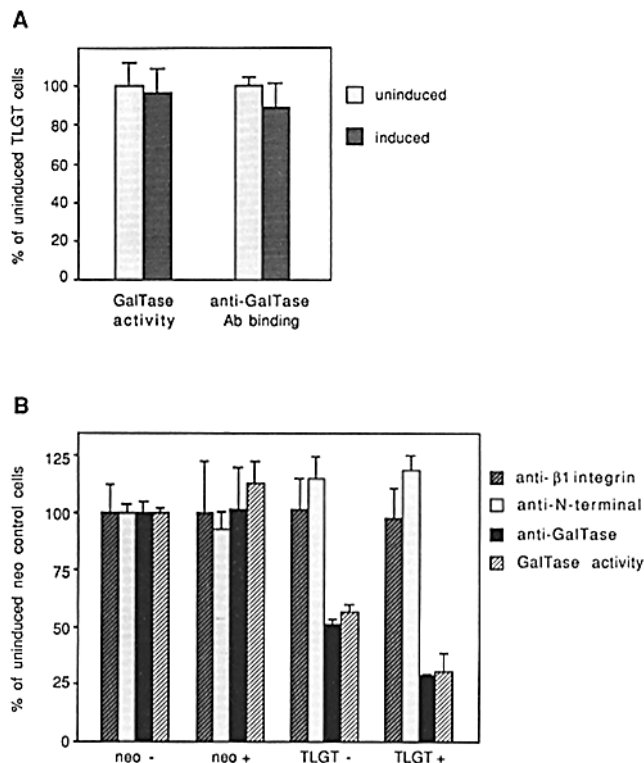


Figure 7. Swiss 3T3 cells expressing the TLGT protein have less GalTase associated with the cytoskeleton than do control cells, but the level of cell surface GalTase activity is unaffected. (A) Levels of cell surface GalTase on TLGT clones are similar before and after 6 h induction with Zn^{2+} , as assayed by either enzyme activity or by antibody binding. Results are the average of three experiments including two independent TLGT clones (TLGT61, TLGT64). GalTase levels on uninduced cells were 465 ± 10 cpm [3H]Gal-product/ 10^6 cells per h (GalTase activity) and 982 ± 82 cpm ^{125}I -goat anti-rabbit IgG bound/ 10^3 cells (anti-GalTase antibody binding). Errors bars, SEM. (B) Cell surface GalTase associated with the cytoskeleton is partially reduced in a representative TLGT clone (TLGT61) before induction with Zn^{2+} (-), due to the basal expression of the MT-1 promoter, and is further reduced after induction with Zn^{2+} for 6 h (+). Despite the loss of GalTase associated with the cytoskeleton, the levels of NH_2 termini associated with the cytoskeleton remain relatively constant. GalTase association with the cytoskeleton was unaffected in control (neo) cells. The effect of expressing the TLGT protein is specific for GalTase, since the level of $\beta 1$ integrin is unaffected. Quantitative antibody binding assays showed that the levels of $\beta 1$ integrin were approximately twice that of surface GalTase (anti- $\beta 1$ integrin antiserum: $2,026 \pm 15$ cpm ^{125}I -goat anti-rabbit IgG bound/ 10^3 vs. anti-GalTase antiserum: 982 ± 82 cpm bound/ 10^3 cells). The loss of GalTase associated with the cytoskeleton paralleled the loss of cellular adhesion both before and after induction with Zn^{2+} (see Fig. 3). Each determination was performed in duplicate and results are representative of two (antibody binding) or three (enzyme activity) experiments. The level of GalTase associated with the cytoskeleton, assayed either by anti-GalTase antibody binding or by GalTase activity, both before and after induction, is statistically different between TLGT and control (neo) clones ($P < 0.01$). Error bars, SEM.

but also changes its specificity toward endogenous cell surface glycoprotein substrates. Although it appears that the effects of the TLGT protein on cell adhesion are due primarily to displacing surface GalTase from its cytoskeleton attachment, we cannot eliminate the possibility that the loss of cell

adhesion is a consequence of the altered interaction between surface GalTase and its glycoprotein substrates.

Clearly, GalTase is one of many cell surface components that participate in cell adhesion, some of which recognize protein ligands and others, such as GalTase, which bind oligosaccharide ligands (Hynes, 1992; Mecham, 1991). Previous studies suggest that surface GalTase functions as a cell-cell and/or cell-matrix adhesion molecule in a cell type-specific manner; GalTase mediates intercellular adhesions in F9 EC cells (Shur, 1983), whereas it functions selectively as a laminin receptor during mesenchymal cell spreading and migration (Runyan et al., 1988; Eckstein and Shur, 1989). Initial cell adhesion to laminin is GalTase-independent and most likely requires integrin function (Hynes, 1992; Mecham, 1991). Interestingly, the effects of overexpressing the TLGT protein is compatible with GalTase's reported cell type-specific adhesive function. Overexpressing the TLGT protein in F9 cells resulted in totally nonadherent single cells, whereas overexpressing TLGT in 3T3 cells primarily inhibited cell spreading on laminin, as evidenced by filopodia retracting from the underlying matrix, although cells still remained weakly bound to the laminin and to other cells. Only when the TLGT protein was grossly overinduced did cells detach completely from the laminin substrate. Despite the loss of cell adhesion to laminin in TLGT clones, the level of another laminin receptor, $\beta 1$ integrin, remained unchanged, suggesting that the cells failed to compensate for their loss of adhesion by increasing integrin expression.

The actin cytoskeleton participates in adhesion and migration by associating directly or indirectly with a variety of cell surface receptors (Soranno and Bell, 1982; Hirano et al., 1987; Marcantonio et al., 1990), and it is therefore interesting that the cell adhesion function of GalTase appears to be dependent upon its association with the cytoskeleton. This is strikingly similar to that reported for E-cadherin in which mutagenesis of the cytoplasmic cytoskeletal (i.e., catenin)-binding domain eliminates its adhesive function despite its ability to bind extracellular ligands (Nagafuchi and Takeichi, 1988). In fact, overexpression of truncated cadherin proteins, analogous to that reported here for GalTase, results in a similar dominant negative nonadhesive phenotype, apparently by competing with endogenous cadherin for cytoskeleton (i.e., catenin) attachment (Kintner, 1992). It is possible that GalTase association with the cytoskeleton is due to a region of 9 amino acid residues (EQFLGGSAA) within the 13-amino acid extension that is homologous to a wide range of actin-binding proteins (Eckstein and Shur, 1992). The leucine residue at position 8 is particularly highly conserved and is likely to be critical for actin binding, since mutagenesis of this leucine residue in the actin-binding protein gelsolin results in a loss of actin-binding activity (Way et al., 1992). Specificity in actin binding may be conferred by amino acids flanking this critical leucine residue. Site-directed mutagenesis studies are in progress to define the amino acid sequence responsible for GalTase association with the cytoskeleton, as is affinity chromatography on immobilized NH_2 -terminal peptides to directly identify the cytoskeletal components that bind GalTase.

In normal Swiss 3T3 fibroblasts, $\sim 80\%$ of surface GalTase is associated with the cytoskeleton (Eckstein and Shur, 1992), suggesting that two separate pools of GalTase reside at the plasma membrane, one that is detergent-soluble and

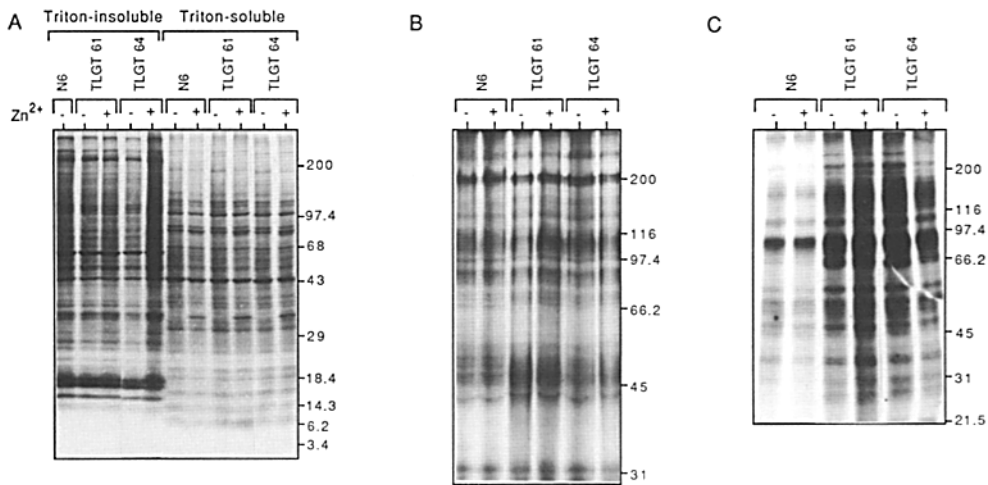


Figure 8. Metabolic and cell surface labeling of TLGT clones shows that normal metabolic functions are retained whether or not the TLGT protein is overexpressed. (A) Triton-insoluble and soluble [³H]-Leucine-labeled proteins were isolated from both uninduced (-) and induced (+) cell cultures and analyzed by electrophoresis on an 8–20% linear polyacrylamide gradient. (B) [³H]Galactose-labeled proteins from uninduced (-) or induced (+) cells were analyzed by electrophoresis on an 8% polyacrylamide gel. (C) [¹²⁵I]-labeled cell surface proteins

from uninduced (-) or induced (+) cells were analyzed by electrophoresis on an 8% polyacrylamide gel. Molecular weight standards (BRL) are indicated on the right of each gel. In all labeling procedures, both the uninduced and induced TLGT clones appear identical to the *neo* controls.

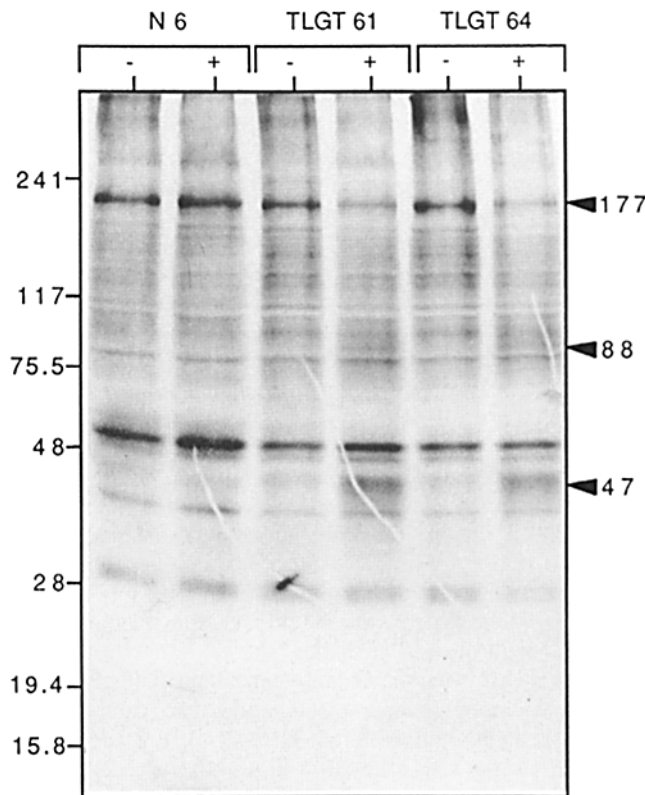


Figure 9. Different plasma membrane proteins are galactosylated by cytoskeletally bound and unbound cell surface GalTase. Cell surface glycoproteins were galactosylated by endogenous cell surface GalTase in uninduced (-) and induced (+) cell cultures, isolated, and analyzed by electrophoresis on an 8–20% linear polyacrylamide gradient. Molecular weight standards (BRL) are indicated to the left of the gel. The glycoproteins that are newly galactosylated (apparent molecular mass of 47 kD and 88 kD) or undergalactosylated (apparent molecular mass of 177 kD) as a result of Zn²⁺ induction in the TLGT clones are indicated by arrowheads to the right.

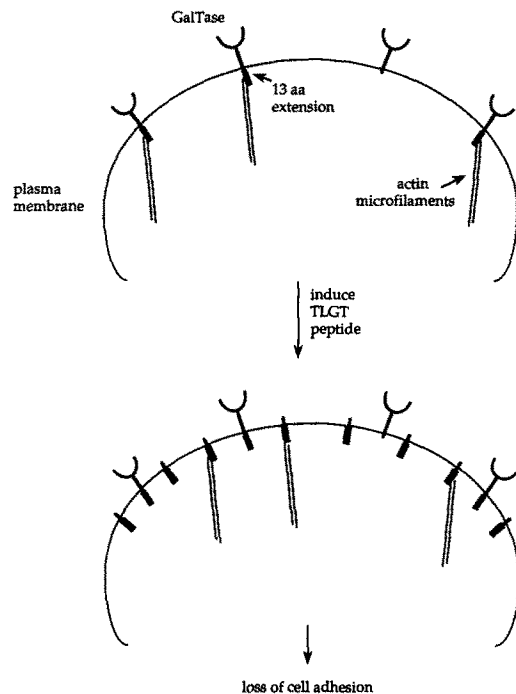


Figure 10. Schematic model accounting for the loss of cell adhesion by overexpressing the TLGT protein. Cells synthesize two forms of GalTase that differ only by a 13-amino acid (aa) extension in their cytoplasmic domains (shown by the shaded rectangle) (Shaper et al., 1988; Lopez et al., 1991). The longer form of GalTase, containing the 13-aa cytoplasmic extension, is suggested to be associated with actin-containing microfilaments and is enriched on the cell surface, relative to the shorter GalTase protein (Lopez et al., 1991; Eckstein and Shur, 1992). Overexpression of the TLGT protein, but not the analogous TSGT protein, corresponding to the cytoplasmic and transmembrane domains of the long form of GalTase, competes with the endogenous full-length long form of GalTase for a limited number of cytoskeleton binding sites. Thus, the full-length long form of GalTase, containing an intact oligosaccharide-binding site, is displaced off of the cytoskeleton where it is no longer able to function as a cell adhesion molecule.

not associated with the cytoskeleton and another that is bound to the cytoskeleton. Although the short form of GalTase, lacking the 13-amino acid extension at its NH₂ terminus, resides primarily in the Golgi compartment under normal circumstances, it can be transported to the plasma membrane when overexpressed in F9 EC cells (Lopez et al., 1991). Regardless, results presented here suggest that the biologically relevant form that functions in cell adhesion is the long form of GalTase that contains the additional 13-amino acid cytoplasmic extension, and which is suggested to be associated with the cytoskeleton. It is conceivable that both the detergent-soluble and -insoluble pools of surface GalTase participate in cell adhesion as has been postulated for the two cell surface pools of cadherin. The detergent-soluble cadherin pool has been suggested to mediate initial adhesion (Wheelock et al., 1987), whereas the cytoskeleton-associated form may stabilize cell adhesion and subsequent cell spreading (Nagafuchi and Takeichi, 1988). That the two pools of surface GalTase may function similarly is suggested in this study by the inability of cells to spread properly when an intermediate level of GalTase is displaced off the cytoskeleton in uninduced TLGT fibroblasts, whereas additional loss of GalTase from the cytoskeleton after induction results in nonadherent cells.

Past studies using a variety of cell types have shown that while the levels of short GalTase mRNAs vary extensively and in proportion to the levels of Golgi GalTase, the endogenous long GalTase mRNAs are expressed at fairly constant levels. Cell surface GalTase may be under such rigorous control because of its requirement for cell adhesion (Shur, 1991) and possibly for growth control (Humphreys-Beher et al., 1987; Purushotham et al., 1992). In this study, we show for the first time that the endogenous long GalTase transcript can be upregulated independent of the short GalTase transcript, producing a consequent increase in surface GalTase activity. The cells appear to be compensating for the release of GalTase from the cytoskeleton and/or a loss of cellular adhesion by specifically increasing the long GalTase transcript after prolonged induction of the TLGT protein; levels of the normally more abundant short transcript remain unaffected. A presently undefined feedback mechanism originating at the cell surface may be responsible for transducing the loss of cell adhesion and/or the detachment of GalTase from the cytoskeleton to a marked increase in the level of the long form of GalTase mRNA. In this regard, a number of cell adhesion molecules, including the integrins and cadherins, are thought to communicate with the cell interior through tyrosine phosphorylation and G protein-coupled intracellular signaling cascades (Ferrell and Martin, 1989; Golden et al., 1990; Guan et al., 1991; Kornberg et al., 1991; Doherty et al., 1991).

Two recent studies have examined the expression of cell surface GalTase in transfected cells, one of which appears to contradict results presented here. Russo et al. (1992) suggest that both the long and short forms of GalTase are confined to the Golgi compartment, as assayed by immunohistochemical analysis of bovine GalTase expressed in transfected CHO cells. However, surface GalTase usually represents a small percentage (~10–25%) of the total cellular GalTase pool in both control and transfected somatic cells (Lopez et al., 1989, 1991; Youakim and Shur, 1993). It is important to emphasize that although transfected cells expressing long-form

GalTase cDNAs consistently show elevated GalTase-specific activity on the cell surface, relative to cells expressing the short form of GalTase, the amount of long GalTase expressed on the surface of transfected cells is only a small percentage of the total increase in cellular GalTase activity, most of which is retained in a Golgi-like compartment (Lopez et al., 1991; Youakim and Shur, 1993). Thus, it appears that the cell surface cannot accommodate all of the excess long-form GalTase protein synthesized by long GalTase cDNA transfectants. In comparison, untransfected control cells appear to express a larger proportion, relative to transfected cells, of the long form of GalTase on their cell surface as judged by S1 nuclease protection analysis (Lopez et al., 1991). Consequently, given the relative amount of long-form GalTase expressed on the surface of transfected cells, Russo et al. (1992) would not have detected surface GalTase activity in their CHO transfectants, since the level of transgene expression they observed in the Golgi compartment precluded detectable levels of GalTase on the cell surface (See Fig. 4, Russo et al., 1992). Finally, it is not known whether in fact CHO cells normally express surface GalTase; there are cell lines (e.g., HeLa) apparently devoid of surface GalTase activity (Hagopian et al., 1969).

Using more quantitative and sensitive techniques, Teasdale et al. (1992) have shown that bovine GalTase does behave similarly to that reported here for murine GalTase. Murine L cells transfected with the full-length bovine GalTase cDNA express a portion of bovine GalTase on their surface, as assayed by fluorescence-activated cell sorting using antibodies made monospecific against the recombinant bovine GalTase. Most of the bovine GalTase remains in a Golgi-like compartment, similar to that reported by Russo et al. (1992) and Youakim and Shur (1993). Furthermore, GalTase expression on the cell surface is reduced when 18 of the 24 cytoplasmic amino acids are removed. Although not interpreted by the authors in this way, these results suggest, as do those presented here, that cell surface expression is dependent upon specific cytoplasmic amino acid residues (see Fig. 5, Teasdale et al., 1992). Although there is evidence for a Golgi retention signal within the transmembrane domain of GalTase (Teasdale et al., 1992; Russo et al., 1992), results presented here and elsewhere (Lopez et al., 1991; Teasdale et al., 1992) suggest that the presence of the additional 13-amino acid cytoplasmic extension overrides any Golgi retention signal and allows at least a portion of the long GalTase protein to associate with the cytoskeleton and be stably associated with the cell surface.

It is noteworthy that the GalTase gene is the only glycosyltransferase cloned to date that encodes two catalytically identical proteins with different NH₂-terminal cytoplasmic domains (Paulson and Colley, 1989). All other glycosyltransferases cloned thus far contain a short cytoplasmic domain similar to that found in the short form of GalTase, which resides within the Golgi region along with the other glycosyltransferases. Interestingly, the 13-amino acid cytoplasmic extension unique to the long form of GalTase, which appears to be required for its cell adhesion function, is found in all three cloned mammalian GalTases, i.e., murine, bovine, and human (Russo et al., 1990). Collectively, these observations imply that GalTase is unique, at least among glycosyltransferases cloned thus far, in its association with the cytoskeleton and consequent cell adhesion function. It is not surprising,

therefore, that of a variety of glycosyltransferases examined, GalTase was the only glycosyltransferase detectable on the surface of embryonic mouse cells (Shur, 1982). The full extent to which GalTase functions as a cell adhesion molecule can now be deciphered in transfected cells and in transgenic animals where GalTase expression on the cell surface can be selectively increased or decreased, using full-length or truncated cDNAs, respectively.

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