INTRODUCTION

The extracellular matrix (ECM) plays a critical role in modulating the morphology, growth, migration and differentiation of cells (Streuli et al., 1991; Juliano and Haskill, 1993). An extensive body of work has addressed the signal transduction pathways elicited by the binding of specific ECM components to their cognate cell surface receptors. The most thoroughly studied receptors are the integrins, which when bound by ligands, can induce an array of cytoplasmic signals including calcium influx, potassium channel activation and tyrosine phosphorylation (Miyamoto et al., 1995; Rosales et al., 1995). During cell migration in vitro, plasma membrane receptors for the ECM cluster into specialized structures called focal adhesions. This receptor clustering is associated with a rapid tyrosine phosphorylation of a spectrum of cytosolic proteins, the most predominant one being focal adhesion kinase (FAK) (Hanks and Polte, 1996). Paxillin is a

SUMMARY

It is well appreciated that clustering of receptors for the extracellular matrix, most notably the integrins, elicits intracellular signal cascades. One of the first indications that integrin-dependent signaling has occurred is by the activation of focal adhesion kinase (FAK). Another, although less well understood, receptor for the extracellular matrix is β1,4-galactosyltransferase I (GalT). GalT participates during lamellipodia formation and cell migration by recognizing terminal N-acetylglucosamine residues on basal lamina glycosides. In this study, we investigated whether GalT is also capable of eliciting intracellular signal cascades, specifically FAK activation, in response to ligand binding and/or aggregation.

3T3 fibroblasts were treated with two different reagents capable of aggregating GalT, either antibodies raised against recombinant GalT or multivalent polymers of N-acetylglucosamine, and the effects on tyrosine phosphorylation were analyzed. Both reagents induced an initial tyrosine phosphorylation (1-2 minutes) and subsequent dephosphorylation (5-10 minutes) of proteins with molecular mass 67 and 125 kDa. These proteins were identified as paxillin and FAK, respectively, by immunoprecipitation with anti-paxillin and anti-FAK antibodies. Preimmune IgG, anti-GalT Fab fragments, irrelevant polymers and monomeric N-acetylglucosamine had no effect. The ability of GalT aggregation to induce transient tyrosine phosphorylation was dependent upon cell density. In addition, FAK dephosphorylation was found to be sensitive to the phosphatase inhibitor, sodium pervanadate.

Similar to the integrins, GalT requires association with the cytoskeleton in order to function as a matrix receptor. To determine if the transient tyrosine phosphorylation of FAK was dependent upon GalT binding to the cytoskeleton, stably transfected fibroblasts expressing different amounts of GalT were treated with polymeric N-acetylglucosamine. Cells expressing increased levels of GalT associated with the cytoskeleton showed increased levels of FAK tyrosine phosphorylation and prolonged dephosphorylation, relative to control cells. In contrast, cells in which a dominant negative form of GalT prevents association with the cytoskeleton showed no or weak response to polymeric N-acetylglucosamine. Concomitant with the GalT-stimulated dephosphorylation of FAK, cells treated with anti-GalT antibodies or polymeric N-acetylglucosamine showed a loss of actin stress fibers and focal adhesions. Pervanadate treatment inhibited the GalT-dependent loss of actin stress fibers.

To confirm the requirement of GalT in transient FAK phosphorylation and stress fiber reorganization in this system, we created cells homozygous null for the GalT isoform that functions as a matrix receptor. These cells were incapable of phosphorylating FAK in response to GalT agonists and, interestingly, showed a lack of lamellar stress fibers when cultured on basal lamina matrices. These data suggest that GalT function as a basal lamina receptor involves transient activation of FAK and an associated reorganization of stress fibers.

Key words: β1,4-galactosyltransferase, Focal adhesion kinase, Cytoskeleton

Cluster of cell surface β1,4-galactosyltransferase I induces transient tyrosine phosphorylation of focal adhesion kinase and loss of stress fibers

Michael J. Wassler and Barry D. Shur*

Department of Cell Biology, Emory University School of Medicine, 1648 Pierce Drive, Room 100, Atlanta, GA 30322, USA

*Author for correspondence (e-mail: barry@cellbio.emory.edu)

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downstream component of FAK signaling and has been shown to colocalize with FAK and become tyrosine phosphorylated in coordination with FAK. Some data, though not all, suggest that FAK regulates the cycle of assembly and disassembly of focal contacts during cell migration by influencing the stability of the actin cytoskeleton (Ilic et al., 1995, 1997; Burrage et al., 1997). Other data support a role for FAK as a regulator of anchorage-independent cell growth and survival (Frisch et al., 1996).

Another, although less well studied, receptor for the ECM is β1,4-galactosyltransferase I (GalT). GalT is unusual in that it resides in two distinct subcellular compartments, the trans-Golgi network and the plasma membrane (Shur et al., 1998). In the Golgi, GalT catalyzes the transfer of galactose from UDPGal to terminal N-acetylglucosamine (GlcNAc) residues on glycoconjugates. On the plasma membrane, GalT functions as a matrix receptor since UDPGal is not normally available to complete the catalytic reaction, enabling low affinity, stable binding between GalT and its extracellular glycoside ligand (Begovac et al., 1991). The receptor function of GalT appears to be due, at least in part, to a 13-amino-acid sequence within its cytoplasmic domain (Lu and Shur, 1997; Evans et al., 1994; Appeddu and Shur, 1994), although this is a matter of active debate.

GalT has been implicated in a spectrum of biological functions, including sperm-egg interactions, cell migration on basal lamina and neurite extension. During migration, GalT is localized to the leading and trailing edges of cells where it binds to specific N-linked oligosaccharides in the E8 domain of laminin (Begovac et al., 1991). Increasing the expression of GalT leads to increased cell spreading and decreased rates of migration, whereas decreasing GalT expression through the use of a dominant negative GalT construct leads to a loss of lamellipodia formation and accelerated migration rates (Appeddu and Shur, 1994). The dominant negative construct displaces the full-length GalT from the cytoskeleton, illustrating that GalT requires association with the cytoskeleton in order to function as a matrix receptor (Evans et al., 1994; Appeddu and Shur, 1994).

Although it is known that GalT requires association with the cytoskeleton, our knowledge of any downstream signal transduction pathways elicited by GalT is still lacking. Recent observations suggest that ligand binding to GalT elicits intracellular signal cascades. On sperm, where GalT functions as a receptor for egg coat glycosides, aggregation of GalT leads to activation of a heterotrimeric G protein, which culminates in the acrosome reaction. In this context, a peptide mimicking the cytoplasmic domain of GalT is able to bind the α and βγ subunits of a sperm heterotrimeric G protein in vitro (Gong et al., 1995). Furthermore, increasing the expression of GalT on sperm leads to accelerated G protein activation (Youakim et al., 1994). In other systems, the growth and differentiation of 3T3 fibroblasts and F9 embryonic carcinoma cells are inhibited by altering GalT expression or by using GalT-specific perturbants (Hinton et al., 1995; Maillet and Shur, 1994).

In this study, we investigated the ability of GalT aggregation to elicit a specific signal transduction pathway in Swiss 3T3 fibroblasts. By using either polymers of GlcNAc or antibodies to GalT we show that clustering of cytoskeletonally bound GalT triggers a transient tyrosine phosphorylation of FAK and an associated reduction in actin stress fibers and focal adhesion contacts.

**MATERIALS AND METHODS**

All reagents were purchased from Sigma Chemical Co. unless noted otherwise.

**Cell culture**

Swiss 3T3 fibroblasts were maintained at 37°C in 5% CO₂ on plastic tissue culture dishes (Corning) in Dulbecco’s Modified Essential Medium (DMEM) supplemented with 10% bovine calf serum (BCS) (Gibco BRL). Three types of 3T3 fibroblasts were used: wild type, stably transfected cells that overexpress GalT (D16) (Appeddu and Shur, 1994) and stably transfected cells that overexpress a GalT dominant negative construct (TL61) (Evans et al., 1994). Fibroblasts from wild-type and long GalT homozygous null mice were isolated and cultivated cells were made quiescent with 0.5% BCS. Cells were then treated at the indicated times with various GalT perturbants: 100 μM of rabbit IgG raised against bacterially expressed mouse GalT (Nguyen et al., 1994), preimmune IgG, Fab fragments (isolated according to Pierce), polyacrylamide polymers with either 200 μM terminal GlcNAc or galactose residues (350 μM/ml polyacrylamide containing a 0.4 molar ratio of monosaccharide; kindly supplied by Dr George Whitesides, Harvard University), or monomeric GlcNAc. When indicated, cells were pretreated for 20 minutes at 37°C with 20 μM sodium pervanadate (Helgadottir et al., 1997) or 10 μg/ml cytochalasin D (Eckstein and Shur, 1992). TL61 cells were precultured with 80 μM ZnCl₂ for 3 hours prior to the experiment to induce overexpression of the GalT dominant negative construct (Evans et al., 1994).

**Cell lysis and immunoblot analysis**

Following incubation at 37°C, cell cultures were rinsed with phosphate-buffered saline (PBS) (Gibco BRL) and lysed in 0.5 ml of ice-cold lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% NP40, 1% Triton X-100, 10 mM NaF, 10 mM Na₃P₂O₇, 4 mM Na₃VO₄, 20 μM calpain inhibitor I (Calbiochem) and protease inhibitor cocktail (Boehringer Mannheim)). After 30 minutes, cells were scraped into microcentrifuge tubes and pelleted (15,600 g, 15 minutes, 4°C) and the supernatant was transferred to clean tubes. Proteins were resolved by 12% SDS-PAGE and transferred to nitrocellulose membranes (BA 85, Schleicher & Schuell). The membranes were blocked with 5% BSA in Tris-buffered saline/Tween 20 (TBST) (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20) for phosphotyrosine blots or 5% dry milk/TBST. The membranes were then incubated for 2 hours at room temperature with anti-phosphotyrosine antibody conjugated to peroxidase (RC20, Transduction laboratories) in 5% BSA/TBS. After washing the filters with TBST, they were developed by the ECL method (Amersham International). Films were scanned using a UMAX Astra 1200 and analyzed with NIH Image 1.6 software.

**Immunoprecipitation**

Cell lysates were prepared as described above. Each sample (100 μg of protein) was incubated with antibodies against FAK (Santa Cruz) for 2 hours at 4°C. A 30 μl sample of protein A-coupled agarose beads (Santa Cruz) was added to each lysate and gently mixed for 1 hour. The beads were pelleted (500 g, 1 minute, room temperature)
and washed three times with 500 µl lysis buffer. The proteins were released from the beads by boiling in sample buffer, subjected to SDS-PAGE and assayed for tyrosine phosphorylation as described above. To ascertain that equal amounts of protein were immunoprecipitated, nitrocellulose membranes were washed using stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) for 30 minutes at 50°C, reprobed with anti-FAK antibodies, and scanned as described above to assess the amount of protein immunoprecipitated.

Indirect immunofluorescence microscopy

Cells were spread on uncoated or laminin-coated glass chamber slides (Nalge Nunc Intern), starved and treated with either antibodies or polymers as described above. After treatment, cells were washed twice with PBS and immediately fixed with 4% paraformaldehyde/PBS for 30 minutes at room temperature. Cells were permeabilized with 0.1% Triton X-100/ PBS for 3 minutes at room temperature and blocked with PBS/5% BSA for 20 minutes. To visualize actin stress fibers, cells were stained with Texas Red-labeled phalloidin (Molecular Probes) for 45 minutes at 37°C. After washing three times with PBS/0.1% Triton X-100 and PBS, coverslips were placed on top and slides examined using a Nikon Eclipse E800 microscope. To stain for focal adhesion contacts, cells were first blocked for 1 hour with 5% normal goat serum/0.1% Triton X-100/PBS, and then incubated with monoclonal antibodies against vinculin (diluted 1:5) for an additional 1 hour. After treatment with FITC-conjugated goat anti-mouse IgG, cells were washed and viewed as above.

Measurement of filamentous actin in polymeric GlcNAc-treated cells

To quantify the content of filamentous actin in cells, a modified version of the method of Machesky and Hall (1997) was used. Cells were allowed to spread on poly-lysine-treated (10 µg/ml) tissue culture dishes and subsequently starved in DMEM/0.5% BCS for 4 hours at 37°C. Cells were incubated for 10 minutes in the presence of 200 µM polymer only or polymer GlcNAc, and then immediately fixed in a 400 µl fixative containing a saturated amount of Texas Red-phalloidin (20 mM KPO4, 10 mM Pipes, 5 mM EGTA, 2 mM MgCl2, 0.1% Triton X-100, 3.7% paraformaldehyde, 50 U/ml Texas Red-phalloidin, pH 7.6). Cells were scraped off the plates, transferred to 1.5 ml tubes and incubated on a rotator for 1 hour at room temperature. After centrifugation, the supernatant was discarded and the pellets washed three times in wash buffer (20 mM KPO4, 10 mM Pipes, 5 mM EGTA, 2 mM MgCl2, 0.1% saponin). To extract the phalloidin, pellets were resuspended in methanol and allowed to incubate for 1 hour at room temperature. Texas Red-phalloidin was measured at a fluorescence emission of 563 nm and excitation of 542 nm.

RESULTS

Laminin induces tyrosine phosphorylation of 67 kDa and 125 kDa proteins

GaIT mediates several types of cellular interactions with the basal lamina by binding to N-linked oligosaccharides in the E8 domain of laminin (Shur et al., 1998). To analyze a plausible signal transduction pathway elicited by surface GaIT, Swiss 3T3 fibroblasts were made quiescent with 0.5% BCS, and plated on laminin-coated or uncoated plastic substrates. The degree of protein tyrosine phosphorylation was assessed using anti-tyrosine phosphate antibody (PY20). Cells plated on laminin for 2 hours have two predominant phosphotyrosine containing proteins, of molecular mass 67 kDa and 125 kDa (Fig. 1); their degree of tyrosine phosphorylation is significantly enhanced relative to cells cultured on plastic.

Polymeric GlcNAc stimulates transient tyrosine phosphorylation of paxillin and FAK

The tyrosine phosphorylation of 67 and 125 kDa proteins in response to laminin is similar to that reported for integrin-dependent signaling (Flinn and Ridley, 1996). We specifically wanted to determine whether the laminin-dependent tyrosine phosphorylation of 65 and 125 kDa proteins was also elicited by a GaIT-dependent mechanism, since some cellular interactions with laminin are GaIT-dependent (Appeddu and Shur, 1994; Evans et al., 1994). We asked whether treating cells with the GaIT ligand, polymeric GlcNAc, would mimic the phosphorylation of the 67 and 125 kDa proteins. After incubation with 200 µM polymeric GlcNAc for various time periods, tyrosine phosphorylation was assayed as described in Materials and Methods. In the presence of polymeric GlcNAc, more than twofold phosphorylation of a 125 kDa protein was induced after 1 minute (Fig. 2). After 5-10 minutes, phosphorylation declined to below initial levels. The same effect was seen for the 67 kDa band (result not shown). No significant effects were seen when cells were treated with undervariatized polyacrylamide polymer (Fig. 2), nor were any effects seen when polymeric GlcNAc was added to control inhibited cells taken from confluent cultures (results not shown).

Clustering of GaIT induces tyrosine phosphorylation/dephosphorylation of FAK

The most common cytoplasmic proteins to be tyrosine phosphorylated during receptor activation are paxillin (67 kDa) and FAK (125 kDa) (Hanks et al., 1992; Kornberg et al., 1992). To determine whether the 67 and 125 kDa bands seen in response to the addition of polymeric GlcNAc were paxillin and FAK, respectively, lysates from treated cells were immunoprecipitated using antibodies to paxillin and FAK. Immune complexes were subjected to immunoblotting with anti-phosphotyrosine antibodies (PY20).

After 1-2 minutes in the presence of 200 µM polymeric GlcNAc, tyrosine phosphorylation and subsequent dephosphorylation of immunoprecipitated FAK was seen (Fig. 3A). The same effect was seen with paxillin (not shown). To determine whether the effect was specific for GlcNAc, cells were treated with an irrelevant polymer containing terminal galactose residues. In contrast to polymeric GlcNAc,
polymeric Gal had no effect on FAK (Fig. 3B) or paxillin phosphorylation.

Although polymeric GlcNAc is a ligand for GalT, we thought it necessary to verify the involvement of GalT in this response by using antibodies raised against bacterially expressed recombinant GalT. When cells were incubated with 100 μg/ml of IgG raised against the extracellular domain of GalT, a similar response was seen (Fig. 3C). Treatment with preimmune IgG had no effect on FAK phosphorylation. The combined results of polymeric GlcNAc and anti-GalT IgG show that GalT is responsible for eliciting a transient phosphorylation of FAK. That GalT aggregation is required, as opposed to GalT occupancy, is shown by the inability of monovalent GlcNAc (Fig. 3B) or monovalent Fab fragments (Fig. 3C) to affect FAK phosphorylation.

**Tyrosine dephosphorylation of FAK is phosphatase-dependent**

Many signal transduction pathways are regulated by an interplay between protein tyrosine kinases and protein tyrosine phosphatases. To see whether the GalT-dependent decrease in FAK tyrosine phosphorylation was dependent on a phosphatase activity, cells were treated with 20 μM sodium pervanadate, a general inhibitor of tyrosine phosphatases, 20 minutes prior to the addition of ligand. As seen in Fig. 4, the presence of the inhibitor resulted in a loss of the characteristic decrease in tyrosine phosphorylation, as seen in the control.

An alternative explanation for the decrease in FAK phosphorylation is that FAK becomes insoluble, i.e. associates with the cytoskeleton, following phosphorylation, and is therefore removed from the pool for immunoprecipitation. This does not appear to be the case, since a similar amount of FAK protein could be immunoprecipitated at all time points examined (Fig. 4). Furthermore, no increase of tyrosine phosphorylated FAK was seen when pellet fractions were assayed after lysis (results not shown). Together, these results show that tyrosine dephosphorylation was dependent on a phosphatase activity, but whether this acts directly or indirectly on FAK has not been addressed.

**Fig. 2.** Tyrosine phosphorylation (p-Tyr) of the 125 kDa protein is induced by the GalT ligand, GlcNAc. Swiss 3T3 fibroblasts were treated with 200 μM polymeric GlcNAc (PAA-GlcNAc) or polymer only (PAA) for different times and assayed for tyrosine phosphorylation by immunoblot analysis (A). Values were normalized to the amount of protein in each lane and plotted relative to the amount of phosphorylated FAK at 0 minutes (B). Error bars represent s.e.m. from three independent experiments. The increase in FAK phosphorylation seen at 1 minute of incubation with PAA-GlcNAc is significant (P=0.024) relative to the control (PAA).

**Fig. 3.** Transient tyrosine phosphorylation of FAK is specific for reagents that aggregate surface GalT. (A) Lysates from cells treated with 200 μM polymeric GlcNAc (PAA-GlcNAc) or polymeric galactose (PAA-Gal) for different times were subjected to immunoprecipitation with antibodies against FAK. The immunoprecipitated FAK was immunoblotted with antibodies against either phosphotyrosine (p-Tyr) to assess levels of phosphotyrosine or FAK to assess levels of immunoprecipitated protein. The tyrosine phosphorylation of FAK, normalized to the amount of immunoprecipitated FAK protein, is plotted as % of control (0 minutes) in B and C. Error bars for the peak values represent the s.e.m. from three replicate experiments. (B) Polymeric GlcNAc induces transient phosphorylation of FAK, but an inappropriate polymeric saccharide (PAA-Gal) does not, nor does monomeric GlcNAc. (C) Similarly, antibodies against recombinant GalT (anti-GalT), but not preimmune (PI) IgG or anti-GalT Fab fragments (Fab), induce a rise in FAK tyrosine phosphorylation within 1 minute.
FAK phosphorylation/dephosphorylation is dependent on GalT association with the cytoskeleton

It has been shown previously that GalT requires association with the cytoskeleton in order to participate in lamellipodia formation and cell migration. In fact, the rate of lamellipodia formation and cell migration are dependent upon the amount of GalT that is available to bind the cytoskeleton (Appeddu and Shur, 1994). We therefore determined whether the GalT-dependent FAK phosphorylation requires GalT association with the cytoskeleton.

Cells that constitutively overexpress GalT on the surface (D16), and which produce larger lamellipodia on laminin with slower rates of migration, were incubated in the presence of 200 μM polymeric GlcNAc, solubilized and analyzed for FAK tyrosine phosphorylation as described. As shown in Fig. 5, FAK phosphorylation in D16 cells occurred more rapidly, reached higher levels and was sustained for longer times, relative to control 3T3 cells.

In contrast, cells that overexpress a dominant negative form of GalT (TL61), and that have <50% of their surface GalT associated with the cytoskeleton, produce smaller lamellipodia and migrate more rapidly than controls (Evans et al., 1994; Appeddu and Shur, 1994). FAK phosphorylation in these cells was more or less resistant to the addition of polymeric GlcNAc (Fig. 5). Collectively, these results suggest that the transient tyrosine phosphorylation of FAK is proportional to and dependent upon GalT association with the cytoskeleton. This is consistent with reports showing that phosphorylation of FAK requires an intact actin cytoskeleton. As shown by others (Abedi and Zachary, 1997), pretreatment with cytochalasin D prevented all FAK phosphorylation whether or not polymeric GlcNAc was included (data not shown).

GalT perturbants result in decreased actin stress fibers and focal adhesions

Recent observations have shown a correlation between FAK phosphorylation/dephosphorylation and stress fiber integrity. Dephosphorylation of FAK is associated with disassembly of stress fibers and focal adhesions (Tahir et al., 1995; Knight et al., 1995; Hall et al., 1994). To determine whether the GalT-dependent decrease in FAK tyrosine phosphorylation seen here also has an effect on the actin cytoskeleton, cells were treated for different times with polymeric GlcNAc or anti-GalT IgG, fixed and stained with phalloidin. In the presence of 200 μM polymeric GlcNAc, but not underivatized polymer, a pronounced decrease in the staining of filamentous actin could be seen after 10 minutes (Fig. 6A). The decrease appeared confined to the actin stress fibers, since the actin cortical fibers were unaffected by the polymeric GlcNAc treatment. In some experiments, a loss of actin stress fibers was noticeable within 2 minutes, but always recovered after 30 minutes of treatment. Treatment with antibodies against GalT had a similar effect (results not shown). The cellular content of filamentous actin was decreased by 45% as quantified by by fluorescence spectroscopy, consistent with a dramatic loss of stress fibers but an apparent retention of cortical fibers.

As expected from the loss of actin stress fibers, treatment with polymeric GlcNAc, but not underivatized polymer, lead to a decrease in focal adhesion contacts as visualized by vinculin staining (Fig. 6B). The decrease in stress fibers and focal adhesions appears to be downstream of FAK dephosphorylation, since the time course of stress fiber disassembly occurred following dephosphorylation of FAK. Consistent with this, inhibition of FAK dephosphorylation with pervanadate (Fig. 4) prevented the loss of actin stress fibers normally seen by the addition of polymeric GlcNAc (Fig. 6C).

Cells deficient in the long form of GalT are refractory to GalT agonists and have reduced lamellar stress fibers

The observations reported here suggest the hypothesis that...
clustering of surface GalT by multivalent ligands induces a transient phosphorylation of FAK and a concomitant destabilization of stress fibers and focal adhesions. We tested this possibility more directly by creating fibroblasts from either wild-type mice or mice homozygous null for the long GalT isoform (Lu and Shur, 1997). These cells retain the short, biosynthetic form of GalT, but are lacking the surface isoform that functions as a matrix receptor. As predicted, the degree of FAK phosphorylation in GalT long isoform null cells was unaffected by the addition of polymeric GlcNAc, further illustrating the requirement of GalT in mediating this transient phosphorylation (Fig. 7). Interestingly, fibroblasts derived from GalT long isoform null embryos showed a striking absence of filamentous actin in the leading lamella of cells spreading on laminin. Multiple cell lines created from independent GalT null mice showed the same phenotype. Furthermore, GalT null cells were compared to control cells created from wild-type litter mates.

DISCUSSION

The results presented in this paper show that clustering of cell surface GalT results in a transient tyrosine phosphorylation of FAK and a coincident decrease in the amount of actin stress fibers and focal adhesions. These events may ultimately facilitate GalT-dependent migration through reorganization of the actin cytoskeleton.

The hypothesis that GalT can elicit intracellular signal cascades upon ligand-induced aggregation is consistent with results from other systems. For example, aggregation of GalT on sperm leads to activation of a pertussis toxin-sensitive heterotrimeric G protein complex (Gong et al., 1995). In this regard, the cytoplasmic domain of sperm GalT is able to bind the α and βγ subunits of a sperm heterotrimeric G-protein; additionally, increasing the amount of GalT on sperm leads to accelerated G-protein activation (Gong et al., 1995). Furthermore, fibroblasts that overexpress a dominant negative construct of GalT are more sensitive to EGF stimulation and are associated with a more rapid growth rate than cells that overexpress the full-length counterpart (Hinton et al., 1995). Finally, treatment of F9 embryonic carcinoma cells with GalT perturbants induces precocious laminin synthesis (Maillet and Shur, 1994). These reports and others (Humphreys-Beher et al., 1987; Purushotham et al., 1992) suggest that GalT is able to function as a signaling receptor in response to ligand binding. The studies presented here illustrate this fact during fibroblast interactions with laminin.

During the past few years, it has become clear that GalT is one member of a family of β1,4-galactosyltransferases, of which there are currently six known members (GalT I-VI; Almeida et al., 1997; Lo et al., 1998; Sato et al., 1998). The transient tyrosine phosphorylations reported here are known to be a consequence of GalT I aggregation specifically, since transfection of cell lines with either full-length GalT I or dominant negative constructs of GalT I showed direct effects on the degree and kinetics of FAK phosphorylation. More directly, elimination of GalT I by homologous recombination eliminated the GalT-dependent transient tyrosine phosphorylation of FAK and paxillin. Nevertheless, the other
members of the GalT family possess provocative amino acid sequences within their cytoplasmic domains that are suggestive of signaling functions, but this has yet to be investigated (Almeida et al., 1997; Lo et al., 1998; Sato et al., 1998). The response of cells to matrix adhesive proteins has been most extensively studied using the integrin family of receptors (Rosales et al., 1995; Juliano and Haskill, 1993). Integrins elicit tyrosine phosphorylation of a very limited number of proteins during the first 10 minutes of fibroblast adhesion to the matrix. The most significant increase in phosphorytrosine content following cell adhesion occurs on FAK and paxillin (Hanks et al., 1992; Kornberg et al., 1992). In cultured cells, both FAK and paxillin localize to focal adhesion contacts, and the β subunit of integrin has been shown to associate directly with FAK (Schaller and Parson, 1994). The functions of FAK and paxillin are still unresolved, but data support the idea that FAK may be involved in the turnover of focal adhesions and actin stress fibers during cell migration (Katoh et al., 1995; Hanks and Polte, 1996; Gilmore and Romer, 1996; Burridge et al., 1997). FAK may also participate in a signal transduction cascade that informs the nucleus that the cell is anchored to the extracellular matrix, thus suppressing apoptosis (Hungerford et al., 1996; Wan et al., 1997).

Unlike the actions of the integrins and growth factors (Knight et al., 1995), GalT agonists induce a short term, transient phosphorylation of FAK. Interestingly, similar kinetics of FAK phosphorylation/dephosphorylation have been reported for the hyaluronan receptor following treatment of C3 fibroblasts with hyaluronic acid (Hall et al., 1994). Insulin has been shown to stimulate the prolonged dephosphorylation of FAK in rat fibroblasts (Tahir et al., 1995) and in CHO cells (Knight et al., 1995). All of these results suggest that different classes of extracellular effectors can produce distinctly different readouts from common intracellular signaling networks.

The mechanism by which GalTase aggregation induces FAK phosphorylation is currently unknown, since GalT does not possess any intrinsic tyrosine kinase activity. Although surface GalTase has been shown to localize to focal adhesion contacts (Appeddu and Shur, 1994), coimmunoprecipitation experiments do not reveal any direct association between GalT and FAK (M. J. W. and B. D. S., unpublished observations). Interestingly, a serine/threonine kinase (p58) important in cell cycle progression has been reported to copurify with GalT (Bunnell et al., 1990; Purushotham et al., 1991). It remains to be established whether this kinase, or a G-protein dependent mechanism as in sperm (Gong et al., 1995), are responsible for transducing GalT aggregation into FAK phosphorylation.

Our results indicate that the decrease in GalT-stimulated tyrosine phosphorylation of FAK is due to a phosphatase activity. At least one type of phosphatase, LAR, is associated with focal adhesions and may have an important regulatory role for FAK activation/deactivation (Serra-Pages et al., 1995). Similarly, the phosphatase PTP-PEST associates with paxillin (Shen et al., 1998). However, it is unclear whether any of these previously identified phosphatases are responsible for the effects observed here.

Although the mechanisms underlying GalT-induced FAK phosphorylation/ dephosphorylation remain unclear, it is apparent that GalT must associate with the cytoskeleton to impact the status of FAK phosphorylation. This is consistent with the observation that FAK phosphorylation is dependent upon the integrity of the cytoskeleton (Abedi and Zachary, 1997; Baron et al., 1998) and with the fact that GalT requires association with the cytoskeleton in order to affect cell migration (Appeddu and Shur, 1994). Altering the amount of GalT associated with the cytoskeleton had a direct effect on the kinetics and degree of FAK tyrosine phosphorylation and dephosphorylation. Cells that overexpress surface GalT had a higher level of phosphorylation compared to nontransfected cells. Moreover, the onset of the signal occurred faster and was sustained longer than in nontransfected cells. This effect may be due to a higher stability of the GalT-cytoskeleton complex. In contrast, the addition of polymeric GlcNAc to cells with reduced levels of GalT associated with the cytoskeleton had little effect on FAK tyrosine phosphorylation.

Concomitant with dephosphorylation of FAK, we observed a decrease in actin stress fibers and focal adhesions. The cortical stress fibers, however, were not influenced. After prolonged incubation with ligand, new stress fibers were formed. A similar correlation between FAK tyrosine dephosphorylation and actin cytoskeleton integrity has been reported by others. For example, insulin treatment of cells induces FAK dephosphorylation and a subsequent decrease in actin stress fibers (Knight et al., 1995; Konstantopoulos and Clark, 1996). Treatment of cells with ethacrynic acid, a
pharmaceutical agent that induces cellular retraction, leads to a similar transient FAK dephosphorylation and loss of actin stress fibers (O’Brien et al., 1997). The kinetics of FAK dephosphorylation in these systems, as well as that reported here, suggest that it is responsible, at least superficially, for the turnover of stress fibers and focal adhesions. The ability of pervanadate to simultaneously inhibit FAK dephosphorylation and GaIT-induced stress fiber disassembly is consistent with this possibility. However, it is difficult to draw cause and effect relationships between the status of FAK phosphorylation and stress fiber integrity, since the phosphorylation status of FAK itself is dependent upon the integrity of the cytoskeleton (Abedi and Zachary, 1997; Burridge et al., 1997).

Members of the Rho family of small guanosine triphosphatases have recently emerged as key regulators of the actin cytoskeleton through tyrosine kinases (Fincham et al., 1996; Hall, 1998; Ridley and Hall, 1994). Protein kinase C has also been shown to regulate actin stress fibers integrity (Habib et al., 1994; Mogi et al., 1995; Zhang et al., 1996; Defilippi et al., 1997). In this regard it is interesting to note that, using the two hybrid system, we have recently isolated a known PKC substrate as a potential binding partner for GaIT and regulator for GaIT-dependent signal transduction (M. J. W. and B. D. S., manuscript in preparation).

In summary, we show here that cell surface GaIT is capable of eliciting an intracellular signal transduction cascade following ligand-induced aggregation. This is indicated by a transient tyrosine phosphorylation of FAK and paxillin. The effect is specific for GaIT agonists and requires multivalent ligands as opposed to GaIT occupancy by monovalent ligand. Altering the association of GaIT with the cytoskeleton, either positively or negatively, leads to a consequent change in the kinetics and duration of FAK phosphorylation. Associated with this transient FAK phosphorylation is the disassembly of actin stress fibers and focal adhesions. Finally, the inability of surface GaIT-null cells to show GaIT-dependent changes in FAK phosphorylation confirms the ability of GaIT to impact FAK activation. Interestingly, surface GaIT-null cells fail to show lamellar stress fibers, consistent with a role for surface GaIT in generating adhesive forces during migration. Collectively, these results demonstrate that GaIT’s previously reported role in lamellipodia formation and cell migration on laminin is associated with a transient phosphorylation of FAK and a consequent reorganization of the actin cytoskeleton and focal adhesions.

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