Deletion of Mouse Embryo Fibroblast N-Acetylglucosaminyltransferase V Stimulates α5β1 Integrin Expression Mediated by the Protein Kinase C Signaling Pathway*

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An N-linked glycan often increased during oncogenic transformation contains β(1,6)-linked GlcNAc, synthesized by the N-acetylglucosaminyltransferase V (GnT-V). The progression of polyoma middle T-antigen oncoprotein-induced mammary carcinomas in GnT-V null mice was significantly retarded compared with that observed in wild-type mice. The matrix adhesion of mouse embryonic fibroblasts (MEF) from GnT-V null and wild-type mice was investigated to understand the mechanism by which deletion of GnT-V could retard tumor progression. GnT-V null MEF displayed enhanced adhesion and spreading on fibronectin-coated plates with concomitant inhibition of cell migration. GnT-V null MEF also showed increased focal adhesion kinase tyrosine phosphorylation, consistent with decreased cell motility on fibronectin-coated plates. Expression of GnT-V cDNA in the null MEF reversed these abnormal characteristics, indicating the direct involvement of N-glycosylation events in these phenotypic changes. The α5β1 fibronectin receptors exhibited increased clustering on the null MEF cell surfaces, consistent with previous studies that observed less integrin clustering in cells overexpressing GnT-V. Most surprisingly, GnT-V null MEF displayed increased expression levels of both α5 and β1 subunits in lysates and on the cell surface. Increased α5β1 expression in the null MEF was because of increased α5β1 transcript levels that declined after re-expression of GnT-V cDNA, confirming that increased α5β1 expression in null MEF was because of changes in GnT-V expression. The increased null MEF transcripts were shown to be caused at least in part by increased integrin promoter activity. Moreover, increased α5β1 integrin transcripts in GnT-V null MEF were not due to a differential response to fibronectin; rather, they appeared to be mediated by activation of a protein kinase C signaling pathway. These results demonstrate that deletion of MEF GnT-V resulted in enhanced integrin clustering and activation of α5β1 transcription by protein kinase C signaling, which in turn up-regulated levels of cell surface α5β1 fibronectin receptors that resulted in increased matrix adhesion and inhibition of migration.

Tumor progression is associated with changes in glycan structures on the cell surface. A common glycan up-regulated during malignant transformation is β(1,6)-GlcNAc on N-glycans1 (1), synthesized by N-acetylglucosaminyltransferase V (GnT-V or Mgat5, EC 2.4.1.155), a key enzyme in the processing of multiantennary N-glycans during glycoprotein biosynthesis (2, 3). The increase in GnT-V activity and its cell surface products during oncosenesis results from increased transcription driven by activation of the Ras-Ets and protein kinase B signaling pathways (4–6). Studies have demonstrated the association of increased GnT-V activity and its glycan products with enhanced cell invasiveness and in some cases metastatic potential (7–9). Consequently, mice that lack GnT-V expression because of a targeted deletion (GnT-V or Mgat5(–/–)) have been used to study the effects of eliminating GnT-V activity on tumor progression (10). The progression of mammary tumors in GnT-V(–/–) mice that express the polyoma middle T oncoprotein antigen (PyMT) under control of the mouse mammary tumor virus promoter was significantly reduced when compared with mammary tumor progression in murine mammary tumor virus-PyMT/GnT-V(+/–) mice. Expression of GnT-V in the null background resulted in tumors that progressed at levels similar to those observed in the wild-type background (11).

Despite extensive studies implicating GnT-V activity and its products with tumorigenesis and increased metastatic potential, the exact mechanisms underlying these effects on cellular behaviors caused by GnT-V expression are only now becoming clearer. Studies have shown that GnT-V null mouse embryo fibroblasts (MEF) displayed altered focal adhesion formation compared with GnT-V wild-type MEF (10), as well as increased cortical stress fibers, suggesting a more non-motile phenotype in GnT-V null MEF. Overexpression of GnT-V in several cell types (7–9) resulted in reduced cell-matrix adhesion and increased migration. GnT-V overexpression clearly resulted in reduced α5β1-mediated adhesion to fibronectin and increased motility, likely due to inhibition of integrin clustering on the cell surface (9). Overexpression of GnT-III is known to decrease N-linked β(1,6) branching due to competition with GnT-V for glycan substrates; consequently, increased GnT-III expression levels in several cell types have been shown to promote cell

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1 The abbreviations used are: N-glycans, asparagine-linked glycans; MEF, mouse embryo fibroblasts; GnT-V, N-acetylglucosaminyltransferase V (Mgat5); PyMT, polyoma middle T-antigen; ECM, extracellular matrix; L-PHA, leucoagglutinating phytohemagglutinin; FAK, focal adhesion kinase; CHO, Chinese hamster ovary; RT, reverse transcription; PKC, protein kinase C; FBS, fetal bovine serum; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Earle’s medium; PMSF, phenylmethylsulfonyl fluoride; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; P38, phosphatidylidyinositol 3-kinase; MESS, 4-morpholinoneethanesulfonic acid; HRP, horseradish peroxidase; FITC, fluorescein isothiocyanate; PVDF, polyvinylidene difluoride; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; NHS, N-hydroxysuccinimide; WT, wild type.
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adhesion and inhibit migration, as well as to negatively influence tumor progression (12, 13).

GnT-V expression levels also mediate cell-cell interactions mediated by cadherins. Overexpression of GnT-V demonstrably inhibited cadherin-mediated cell association and the ERK and catenin intracellular signaling pathways regulated by cadherin (14). Again, evidence suggests that these effects resulted at least in part by cadherin clustering at the cell surface. As would be predicted, MEF from GnT-V null mice showed greatly enhanced cadherin-mediated adhesion compared with MEF from GnT-V(+/-) mice, and expression of GnT-V cDNA in the null MEF reversed cadherin-mediated adhesion to normal levels (14).

In the present study, we focused on analyzing in detail the α5β1 integrin-mediated adhesion and migration of GnT-V null(-/-) and wild-type(+/-) MEF to elucidate further the cellular basis of decreased tumorigenesis and metastasis potential observed in the GnT-V null mice. We found that deletion of GnT-V resulted in increased fibronectin-induced MEF adhesion, spreading, tyrosine phosphorylation of focal adhesion kinase (FAK), and reduced cell motility on fibronectin. These phenotypic defects were reversed by re-expression of GnT-V in the null MEF, confirming that they were due to altered N-linked glycosylation. Moreover, significantly increased mRNA, protein, and cell surface expression levels of both α5 and β1 integrin subunits were observed in GnT-V null MEF, which were reversed after re-expression of GnT-V cDNA. Increased transcription of α5β1 integrin in GnT-V null MEF was fibronectin-independent and mediated by activation of the PKC signaling pathway. These results demonstrate that deletion of GnT-V and subsequent aberrant N-glycosylation in MEF resulted in up-regulated levels of cell surface α5β1 fibronectin receptors, as well as their increased clustering, which consequently increased fibronectin-mediated cell-matrix adhesion and inhibited migration.

EXPERIMENTAL PROCEDURES

Antibodies and Chemicals—Polyclonal antibody against FAK, α5 integrin, epidermal growth factor receptor, ERK1/2, monoclonal anti-phosphoobsynine (PY20), HRP-labeled anti-rabbit IgG, and anti-mouse IgG for were from Santa Cruz Biotechnology. Monoclonal antibody against β1 integrin and function blocking anti-rat CD29 (integrin β1 chain) were products of BD Biosciences. Anti-keratin against mouse α5β1 integrin were kindly provided by Dr. S. K. Akiyama. Rhodamine-conjugated anti-CD29, anti-integrin, or normal immune IgG (control IgG) was added to separate cell suspensions and incubated for 30 min at 4 °C and then 5 min at 37 °C, prior to addition of the cell suspension to the fibronectin-coated wells. The data were expressed as the mean of triplicate wells.

For assessment of cell spreading on fibronectin, similar cell preparation protocols were used. After the adherent cells were fixed and stained with crystal violet, the number of spread cells was quantified by counting the percentage of spread cells versus the total number of cells counted. Cells were scored as spread if they became flattened and lost nuclear refractivity. Each experimental point was the result of counting cells in 3–5 fields/well in at least two wells viewed at ×100 magnification.

The values from 6 to 10 fields were used to calculate the mean and standard error. Typically, 150–250 cells were present per field.

In Vitro Cell Migration Assay—Migration assays were performed using transwell units with 8-μm pores, suspended in 0.1% BSA in RPMI 1640 culture medium supplemented with 10% FBS and 1-glutamine.

Cells were trypsinized, pelleted, and lysed on a 4–20% polyacrylamide mini gel, and then transferred onto a PVDF membrane. After blocking with 3% BSA in PBS, the membrane was stained with a mixture of rabbit polyclonal anti-CD29, anti-integrin, or normal immune IgG (control IgG) was added to separate cell suspensions and incubated for 30 min at 4 °C, and then 5 min at 37 °C, prior to addition of the cell suspension to the fibronectin-coated wells. The data were expressed as the mean value of cells in five fields based on two independent experiments.

For the scratch-wound healing assay, cells (1 × 10⁶) were seeded on fibronectin-coated 4-well chamber slides in serum-free DMEM overnight, and then scratch-wounded with a 200-μm yellow plastic tip. After washing with serum-free DMEM, the chamber slide was incubated for 6 h at 37 °C in serum-containing media. Migration of cells into wounded areas was photographed, and then the cells were stained with rhodamine-phalloidin (1:40), as described below, followed by photography using an inverted fluorescence microscope.

Fluorescence Staining of α5β1 Integrin—MEF were cultured on coverslips coated with fibronectin (10 μg/ml) for 1 h and fixed with 4% paraformaldehyde in PBS. After blocking with 5% BSA, cells were stained with a mixture of rabbit polyclonal anti-α5 and mouse monoclonal anti-β1 integrin, followed by incubation with rhodamine-conjugated anti-rabbit or FITC-conjugated anti-mouse IgG (1:250). After washing with PBS, the coverslips were mounted, and the cells subjected to deconvolution fluorescence microscopy.

Immunoblotting and Lectin Blotting—Cells were harvested and lysed in sodium dodecyl sulfate sample buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.5% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 1 mM PMSF, 1 μg/ml peptatin, 10 μg/ml aprotinin and leupeptin). Twenty μg of total cell lysates protein, determined using BCA protein assay procedure (Pierce), were boiled in 2× SDS sample buffer for 5 min, electrophoresed on a 4–20% polyacrylamide mini gel, and then transferred onto a PVDF membrane. After blocking with 3% BSA in PBS, the membrane was incubated for 2 h at room temperature with primary antibodies.
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(1:1000) or biotinylated lectins (1:2500) in TBS buffer containing 0.05% Tween 20 (TBST). The membrane was then washed with TBS and probed with HRP-conjugated anti-rabbit or anti-mouse IgG (1:3000) or streptavidin HRP (1:1000) at room temperature. The membrane was washed with TBS, protein bands were developed with ECL reagents and exposed on x-ray film. Images of immunoblots and lectin blots were quantified using a Fluor-S imager (Bio-Rad).

Cell Surface Labeling and Immunoprecipitation—Subconfluent cells were washed and detached using 2 mM EDTA. Cells were then washed twice with ice-cold PBS and incubated with 1 mg/ml NHS-LC-biotin in PBS for 20 min at 4 °C on a rocking platform. After washing with PBS, cells were lysed by incubation with lysis buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 10 μg/ml aprotinin and leupeptin) for 30 min at 4 °C. For immunoprecipitation of α5β1 integrin, cell lysates were clarified by centrifugation and incubated with protein G- or G-agarose at 4 °C under agitation for 1–2 h to remove nonspecific adsorption to the agarose beads. After the determination of total protein using the BCA assay, cell lysates (350 μg of protein) were incubated either with 2 μg of antibody against β1 integrin for 3 h at 4 °C under agitation, followed by incubation with protein G-plus agarose at 4 °C for 2 h under agitation, or with 50 μl of streptavidin-agarose. The pellets were washed twice in lysis buffer and subjected to SDS-PAGE under reducing conditions. The gels were then transferred onto a PVDF membrane (BD Biosciences). For lectin precipitation of α5 integrin (16), cell extracts were treated by boiling in 100 μl of denaturant buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% SDS, and 5 mM EDTA) for 5–10 min, followed by addition of 900 μl of lysis buffer containing protease inhibitors as mentioned above. Lysates were then incubated with each biotinylated lectin (5 μg) overnight at 4 °C under agitation, followed by incubation with streptavidin-agarose at 4 °C for 2 h under agitation. Particular N-glycans on α5β1 integrin were then detected using antibody against β1 after SDS-PAGE and membrane transfer, as described above.

For immunoprecipitation of focal adhesion kinase (pFAK), serum-starved cells were grown on fibronectin (10 μg/ml)-coated plates for 10 or 30 min in Medium 25 done with SB204 (17). Cells were lysed in ice-cold lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 20 mM pyrophosphate, 1% Triton X-100, 100 mM NaF, 2 mM NaVO₃, 0.2 mM ammonium molybdate, 2 mM PMSF, 0.7 μg/ml pepstatin, 20 μg/ml aprotinin and leupeptin) for 30 min and centrifuged at 12,000 × g for 20 min at 4 °C. Supernatants (350 μg of protein) were used for immunoprecipitation of FAK as described above. Immunocomplexes were then separated by reducing SDS-PAGE (7.5%), transferred onto a PVDF membrane, and probed with mouse anti-PY20 and antibody against FAK.

Metabolic Labeling and Autoradiography—Radiolabeling was performed as described (16) with minor modifications. Fibroblasts were grown to confluency on 60-mm culture dishes, rinsed twice with PBS, and preincubated for 30 min in Met/Cys-free DMEM (Invitrogen) containing 2% dialyzed FBS. Cells were then labeled with 100 μCi/ml [35S]methionine/cysteine in 2 ml of the same growth media for 30 min and then chased for the indicated times. Labeled cells were harvested and lysed in ice-cold extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 2% Triton X-100, 20 μg/ml leupeptin, 20 μg/ml pepstatin, 1 μg/ml aprotinin, 2 μg/ml PMSF). Two hundred μg of protein was used for immunoprecipitation of α5β1 integrin using antisera against α5β1 as described above. The precipitates were then subjected to 7.5% SDS-polyacrylamide gels under nonreducing conditions. After fixation and drying, β1 integrin was detected by autoradiography, and the results were analyzed by the Fluor-S imager (Bio-Rad). The very low intensity of α5 radiolabeled bands detected in these experiments precluded definitive analysis of the biosynthesis.

Flow Cytometry Analysis—Cells were grown to subconfluence and detached with 2 mM EDTA in PBS. Cells (10⁵) were washed, resuspended in 100 μl of FACs buffer (PBS containing 1% BSA and 0.01% sodium azide), and then incubated with antibody against α5β1 integrin (1:100) and biotinylated L-PHA (3 μg) at 4 °C for 30 min, followed by washing three times with FACs buffer. Cells were then labeled with FITC-linked secondary antibodies or FITC-streptavidin (10 μl) at 4 °C for 30 min. Analyses were then performed on FACScan (BD Biosciences).

Quantitative Real Time RT-PCR Analysis—The RNaseasy kit (Qiagen) was used to isolate total RNA. Reverse transcription reactions were performed using Superscript III (Stratagene) and random primers. Primers used in the quantitative real time RT-PCR analysis were as below: mouse α5 integrin forward, 5′-ATGCTGATGACATGACACTCC-3′, and reverse, 5′-GGTCACTGACCCCTCTCTCCTCA-3′; β1 integrin forward, 5′-GGTGTGCTGTGTTTAGGTGTC-3′, and reverse, 5′-TGAGCCTAGATGACGACGAC-3′; and mouse glycerolaldehyde-3-phosphate dehydrogenase forward, 5′-TGCGGATTCACGGAACCCGCAAC-3′, and reverse, 5′-ATGGATGCGTAGATGAGTCCAC-3′. Real time reactions were performed using the iQTM SYBR Green Supermix (Bio-Rad) under the following conditions: 30 s at 95 °C for 1 cycle, 10 s at 95 °C, 30 s at 65 °C for 40 cycles; 95 °C for 1 min, 55 °C for 1 min, and 80 cycles of 55 °C for 10 s for melting curve analysis.

Assay of Integrin mRNA Stability—MEF were spread onto 6-well plates and grown to subconfluence in DMEM containing 10% FBS. The medium was then replaced with DMEM containing 5% FBS and actinomycin D (10 μg/ml), an inhibitor of RNA transcription, and cells were collected at the indicated time points for total RNA isolation. Real time RT-PCR was performed using total RNA to detect mRNA levels. Glycerolaldehyde-3-phosphate dehydrogenase was used as an internal control, and the results were plotted as the percent of RNA remaining versus the time after addition of actinomycin D.

Plasmid Construction, Transient Transfection, and Luciferase Assay—The human integrin α5 promoter containing upstream regulatory sequences from positions −954 to +23 has been described (17) and was obtained by PCR from a genomic template that was prepared from human MCF-7 cells. The primers designed for the amplification of the promoter sequence were as follows: forward, 5′-TGAGCCTAGATGACGACGAC-3′; and reverse, 5′-ACCACGACGTCTC-CTG-3′. The 977-bp PCR product was confirmed by sequencing and inserted into the KpnI and Xhol sites in the pGL3-basic luciferase reporter vector via a shuttle TOPO clonal vector, generating an integrin α5 promoter-controlled luciferase reporter construct pGL3/α5-954. MEF grown on 24-well plates were transiently co-transfected using Lipofectamine as suggested by the manufacturer. One μg of pGL3/α5-954, 0.5 μg of pRSV-lacZ as control, and 1 μg of pCMV/His-LucZ, were added to 10⁵ cells in each well, and the mixture was incubated at 37 °C for 4 h, followed by replacement with complete DMEM growth media. Twenty four h after transfection, cells were washed with cold PBS and harvested. The levels of luciferase activity for transfected cells were then determined by using a quantitative assay kit and luminometer. Results were expressed as relative luciferase units normalized for transfection efficiency using β-galactosidase activity.

RESULTS

Characterization of GnT-V-deficient MEF—GnT-V-deficient MEF were isolated from E13.5 embryos that resulted from breeding of heterozygous GnT-V (GnT-V(+/−)) mice and were identified by PCR of genomic DNA, based on the expression of lacZ and GnT-V genes (14). The activity of GnT-V toward a synthetic trisaccharide acceptor was about 400 ± 35 pmol/mg/h in wild-type MEF, whereas GnT-V activity was undetectable in GnT-V-deficient MEF. Wild-type MEF stained very strongly with fluorescent L-PHA, which specifically binds to N-linked β(1,6)-branched structures (18). The staining of GnT-V null MEF with L-PHA was negligible, confirming the lack of N-linked β(1,6) branches after deletion of GnT-V (14). RT-PCR revealed essentially no expression in the wild-type or null MEF of a related glycosyltransferase, GnT-V-B (also referred to GnT-I) (19, 20), that can synthesize sequences from positions 5′-CTGGCCAGAGC-3′ to 5′-CTG-3′ has been described (17) and was used in a quantitative assay kit and luminometer. Results were expressed as relative luciferase units normalized for transfection efficiency using β-galactosidase activity.
MEF; attachment of both null and wild-type MEF to laminin and collagen IV was insignificant (data not shown), consistent with a previous report (21) that described MEF adhesion to these matrices. Cell adhesion to fibronectin was markedly inhibited by the addition of function-blocking monoclonal antibody against the β1 integrin subunit (anti-CD29), whereas a function-blocking antibody against N-cadherin (anti-A-CAM), the most significantly expressed cadherin in MEF, did not inhibit the cell-fibronectin adhesion (Fig. 2B). Consistent with increased matrix adhesion, cell spreading on fibronectin was also increased. As shown in Fig. 2C, almost 70% of GnT-V null MEF was well spread on fibronectin-coated plates after 30 min; by contrast, less than 40% of wild-type cells showed spreading. We then studied the effect of the deletion GnT-V on cell motility using both scratch-wound and transwell migration experiments. In the scratch-wound assay, null MEF showed decreased migratory activity compared with wild-type MEF 6 h after wounding (Fig. 2, D1 and D2). F-actin staining revealed multiple protrusions and extension of the leading edge of wild-type MEF but a relatively flat edge on null MEF (Fig. 2, D3 and D4). A quantitative transwell assay for migration was then used for assessment of cell motility, where the ability of cells to migrate through a fibronectin-coated membrane was measured. As shown in Fig. 2E, a significant decrease in motility

![Image](https://example.com/image1.png)

**Fig. 1.** GnT-V null MEF display altered morphology in culture. Wild-type and GnT-V null MEF were plated onto culture dishes in DMEM containing 10% FBS, and images were taken by phase contrast microscopy at subconfluency (8 h, A and B) and confluency (72 h, C and D). KO, knock-out. Bars, 100 μm.

![Image](https://example.com/image2.png)

**Fig. 2.** Increased cell adhesion and spreading and decreased cell motility in GnT-V null MEF. A, cells (3 × 10⁴) were applied to fibronectin-coated 96-well plates and incubated at 37 °C for 30 min. Adherent cells were stained with crystal violet, and the absorbance of each well was determined at 595 nm. Each bar represents the mean (±S.D.) of triplicate determinations. Similar results were obtained from three separate experiments. KO, knock-out. B, cells (3 × 10⁴) were applied to 96-well plates coated with fibronectin (10 μg/ml) and incubated with different function-blocking antibodies as indicated at 37 °C for 30 min. Cell adhesion assays were then performed as described in A. C, cells (3 × 10⁴) were applied to 96-well plates coated with fibronectin (10 μg/ml) and incubated at 37 °C for 30 min. Cells were rinsed, fixed, and stained with crystal violet. The percentage of spread cells was calculated by dividing the number of spread cells by the total number of cells. Each bar represents the mean (±S.D.) of triplicate determinations. D, cells (3 × 10⁴) were seeded onto a fibronectin-coated (10 μg/ml) 6-well plate in serum-free medium for 24 h, and the monolayer was then scratched with a plastic pipette tip. The plate was then incubated for 6 h at 37 °C in serum-containing media, and areas of migration were photographed (top panel). Arrow, starting line of migration. Six h after wounding, cells at the migrating edge were fixed and stained for F-actin with rhodamine-phalloidin (bottom panel). Bars, 50 μm. E, cell migration was determined by using a transwell apparatus with the underside of the membrane insert coated with fibronectin (10 μg/ml). Cells were incubated with or without the indicated antibodies in the upper chamber and allowed to migrate for 3 or 6 h. Migrating cells on the underside of the membrane were then fixed, stained, and counted using a light microscope. Each bar represents the mean (±S.D.) number of migrating cells from five randomly selected fields. Similar results were obtained in three separate experiments.
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Fig. 3. Aberrant phenotypes were reversed by re-expression of GnT-V in null MEF. A, both null MEF and null MEF transfected with GnT-V were incubated with biotinylated L-PHA (1:100) followed by incubation with FITC-conjugated streptavidin (10 μl). Analysis was performed using FACSscan. B, cells (3 × 10⁴) were applied to fibronectin-coated 96-well plates and incubated at 37°C for 30 min. Cell adhesion assays were performed as described in Fig. 2. C, cell migration was determined using a transwell apparatus with the underside of the membrane insert coated with fibronectin (10 μg/ml) as described in Fig. 2. KO, knock-out; KO/GnT-V, null MEF with transient expression of GnT-V.

was observed in GnT-V null MEF, and the motility was inhibited by anti-β1 integrin antibody. These results indicate that loss of GnT-V results in a cell-matrix motility defect in embryonic fibroblasts mediated by β1 integrin.

Re-expression of GnT-V Reverses Altered Cell-Fibronectin Interaction of Null MEF—If deletion of GnT-V did indeed affect α5β1 integrin-mediated cell-matrix adhesion and cell motility, then phenotypic changes caused by GnT-V deletion should be rescued by re-expression of GnT-V in the GnT-V null MEF. To this end, GnT-V cDNA was first transiently expressed in GnT-V-/- MEF. Re-expression of GnT-V caused increased β1(1,6) branching on the cell surface, demonstrated by fluorescent L-PHA binding using flow cytometry (Fig. 3A). Consistent with changes in glycosylation after re-expression of GnT-V, reduced α5β1 integrin-mediated cell-fibronectin adhesion (Fig. 3B) and increased cell motility (Fig. 3C) were found in MEF re-expressing GnT-V.

Deletion of GnT-V Enhanced Tyrosine Phosphorylation of Focal Adhesion Kinase—Cell adhesion to the ECM leads to recruitment of structural proteins and tyrosine kinases to specialized sites beneath the plasma membrane to form focal adhesions that link the ECM and cytoskeleton through cell surface integrins (22, 23). ECM-integrin signaling events are prominently involved in regulating cell motility, and tyrosine phosphorylation of some focal adhesion proteins, such as FAK, is believed to be a crucial event in this process (24–27). To investigate the mechanism by which deletion of GnT-V affects cell-matrix adhesive properties, including cell adhesion and motility, we examined differences in FAK tyrosine phosphorylation in MEF grown on fibronectin-coated plates by using immunoprecipitation with anti-FAK antibody. Phosphorylation of FAK, detected by binding of an anti-phosphotyrosine antibody, was significantly enhanced (Fig. 4) in the null MEF, consistent with increased cell adhesion, spreading, and decreased cell motility (28). Increased tyrosine phosphorylation of FAK in GnT-V null MEF grown on fibronectin-coated plates is consistent with increased focal adhesion formation reported for GnT-V null MEF (10).

Increased Clustering of α5β1 Integrin on the Cell Surface of GnT-V Null MEF—FAK phosphorylation relies on the clustering of integrins on the cell surface as they interact with the cell matrix (23). To explore if deletion of GnT-V expression altered cell surface integrin clustering, MEF spread for 1 h on fibronectin-coated slides were incubated with antibodies against α5 and β1, followed by fluorescently labeled secondary antibodies. The intensity of cell surface fluorescence from both α5 and β1 was stronger in GnT-V null MEF compared with wild-type MEF. In addition, as shown in Fig. 5, cell staining showed clear differences in the clustered appearance of both integrin subunits on the GnT-V null MEF. This result is consistent with enhanced fibronectin adhesion, FAK phosphorylation (29, 30), and a less-motile phenotype in cells lacking GnT-V expression (31).

Deletion of GnT-V Results in Altered Glycosylation and Increased Expression of α5β1 Integrin—To determine further whether the altered cell-matrix adhesive properties of the null MEF were at all the result of altered α5β1 integrin expression levels, we first explored the α5β1 expression levels on Western blots of total cell lysates using anti-α5 and anti-β1 antibodies. The results of these experiments showed significantly increased expression of these subunits in null MEF (Fig. 6A), whereas, as a control, ERK1/2 expression levels were similar to wild-type MEF. Consistent with these results, increased cell surface expression of both α5 and β1 subunits in the GnT-V null MEF (Fig. 6B) was also observed by flow cytometry. This result was further confirmed by cell surface labeling experiments in which the surfaces of both types of MEF were biotin-labeled, followed by cell lysis and immunoprecipitation using streptavidin (Fig. 6C) or β1 integrin antibody (Fig. 6D). These results all indicated that increased levels of α5 and β1 subunits were present on the cell surface and show co-incident localization, consistent with increased cell-fibronectin adhesion in the null MEF.

Deletion of GnT-V also altered integrin N-glycosylation. As shown in Fig. 6E, the expression of both N-linked β1(1,6) branching and poly-N-acetyllactosamine was eliminated on the mature form (125 kDa) of α1 integrin in GnT-V null MEF, as evidenced by lectin precipitation using L-PHA and D. stramonium agglutinin, respectively, followed by SDS-PAGE, blotting, and staining with anti-β1 antibody. By contrast, little change in concanavalin A reactivity of the precursor form of β1 (105 kDa) was observed, indicating a lack of effect of deletion of GnT-V on the expression of either high mannose or biantennary N-linked oligosaccharides. When GnT-V was re-expressed in null MEF, shown in Fig. 6E, lower panel, the aberrant N-glycosylation of α5β1 integrins was at least partly corrected, as indicated by enhanced L-PHA reactivity, showing that changes in GnT-V expression levels were reflected in changes of the levels of integrin N-linked β1(1,6) branches.

Because N-glycans are involved in the sorting, folding, and stability of glycoproteins (32, 33), aberrant N-glycosylation might therefore lead to altered turnover of α5β1, which can also cause increased cell surface protein levels. To test this possibility, the metabolism of α5β1 was detected by a pulse-chase experiment. As shown in Fig. 6F, the β1 precursor form (105 kDa) was detected in both wild-type and null MEF after a...
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GnT-V activity and cell surface N-linked β(1,6) branches (34). As indicated in Fig. 6G, the expression of β(1,6) branching was completely inhibited on both α5 and β1 integrin in Lec4 cells, as detected by lectin precipitation using L-PHA (left panel). As observed in GnT-V null MEF, significantly increased protein levels of both α5 and β1 subunits were also observed in Lec4 cells (Fig. 6G, right panel). These results confirmed the up-regulation of α5β1 integrin expression levels in cells that lack GnT-V activity.

Increased Levels of α5β1 Integrin in GnT-V Null MEF Resulted from Enhanced Transcript Levels—To investigate further if increased protein levels of α5β1 resulted from changes of mRNA levels, we explored the expression of α5β1 transcripts by RT-PCR. The transcripts of both α5 and β1 integrin, determined by either RT-PCR (data not shown) or quantitative real time RT-PCR (Fig. 7A, left panel), were dramatically increased in GnT-V null MEF, consistent with the enhanced protein expression levels of α5β1. Also, increased integrin transcripts (α5) were observed in Lec4 cells (Fig. 7A, right panel). When GnT-V was re-expressed in null MEF, increased mRNA levels of both α5 and β1 integrin were decreased (Fig. 7A), indicating the direct involvement of GnT-V activity in regulating α5β1 transcripts. We next studied integrin mRNA stability and promoter activation. Both wild-type and null MEF were treated with actinomycin D (10 μg/ml) for different times, and total RNA was isolated for analysis of mRNA levels. As shown in Fig. 7B, actinomycin D caused a similar time-dependent loss of α5 integrin in both cell lines, with an almost 70% loss at 12 h, indicating that the stability of integrin mRNA was not significantly affected; therefore, the increased transcript levels of α5β1 integrin were not a result of increased stability of mRNA in GnT-V null MEF. In order to explore whether the increased transcripts were determined by discrete cis-acting elements in the upstream regulatory sequences, we isolated the α5 promoter sequence by PCR and constructed an α5 promoter sequence-controlled luciferase reporter plasmid to transiently transfect MEF and assay promoter activity. As indicated in Fig. 7C, the reporter activity driven by pGL3/α5-954 was significantly increased in GnT-V null MEF compared with wild-type MEF, demonstrating an enhanced activation of the α5 promoter in null MEF. These results suggest that increased mRNA levels in GnT-V null cells likely resulted at least in part from the stimulation of promoter activity, causing an enhancement in transcription of α5β1 integrin mRNA.

Increased α5β1 Transcription in GnT-V Null MEF Was Fibronectin-independent and Mediated via PKC Activation—Studies have shown a coordinated expression between fibronecin-
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Fig. 6. Increased protein levels of α5β1 integrin in GnT-V null MEF. A, subconfluent MEF were harvested and lysed for immunoblotting using antibodies against α5, β1, and ERK1/2, respectively. Images were quantitated from at least three independent experiments and expressed as band intensity. WB, Western blot; KO, knock-out. B, MEF were incubated with anti-α5 or β1 integrin antibody (1:100) followed by incubation with FITC-conjugated anti-rabbit or anti-mouse IgG (10 μl). Analysis was performed using FACSscan. C, subconfluent MEF were labeled with NHS-LC-biotin (1 mg/ml) and subjected to immunoprecipitation (IP) using streptavidin-agarose. Immunoprecipitated proteins were separated by SDS-PAGE, blotted, and probed with antibodies against α5, β1 integrin, and epidermal growth factor receptor (EGFR). Images were quantitated from at least three independent experiments and expressed as band intensity. D, subconfluent MEF were labeled with NHS-LC-biotin (1 mg/ml) and subjected to immunoprecipitation using anti-β1 integrin antibody. Immunoprecipitated proteins were separated by SDS-PAGE under nonreducing conditions, blotted, and probed with HRP-conjugated streptavidin. Images were quantitated from at least three independent experiments and expressed as band intensity. E, various lectins were used to precipitate glycoproteins from cell lysates, followed by SDS-PAGE, blotting, and detection using antibody against β1 integrin. Lane 1, null MEF transiently expressing GnT-V; lane 2, null MEF stably expressing GnT-V. F, MEF labeled with 100 μCi/ml 35S-labeled methionine/cysteine and chased for the indicated times. Similar results were obtained from three separate experiments. KO/GnT-V, null MEF stably expressing GnT-V; G, subconfluent CHO and Lec4 cells were harvested and lysed either for immunoblotting using antibodies against α5, β1, and ERK1/2 (right panel) or lectin precipitation (LP) using L-PHA followed by SDS-PAGE, blotting, and detection using antibody against α5 or β1 integrin (left panel). Images were quantitated from at least three independent experiments and expressed as band intensity.
The PI3K inhibitor, wortmannin, slightly inhibited α5 transcript levels but increased β1 transcripts in both types of MEF, suggesting an opposite effect of the PI3K pathway in regulating α5 and β1 integrin expression. By contrast, calphostin C, an inhibitor of PKC, completely reversed the enhanced expression levels of both α5 and β1 integrin transcripts in null MEF but had no effect on these transcript levels in the wild-type MEF (Fig. 8C, indicated by arrows). These results indicate that the increased gene transcripts of α5β1 integrin were mediated by PKC. Deletion of GnT-V likely activated the PKC signaling pathway and, consequently, up-regulated transcription of α5β1 integrin in GnT-V null MEF.

To confirm PKC regulation of α5β1 integrin protein levels in GnT-V null cells, we treated both types of MEF with calphostin C for 24 h and then performed Western blotting to detect changes in α5β1 integrin protein levels. As indicated in Fig. 8D, increased expression of both α5 and β1 integrin caused by deletion of GnT-V was extensively reversed by treatment of cells with calphostin C that blocked the PKC signaling pathway. This result is consistent with the lowering of α5β1 transcript levels caused by calphostin C treatment (Fig. 8C) and strongly supports a function for PKC in up-regulating integrin levels in the GnT-V-deficient MEF.

DISCUSSION

Because the interaction of cells with extracellular matrix regulates cell adhesion and migration, it was logical to compare in detail the ECM-adhesive interactions of GnT-V null and wild-type MEF. In the present study, we found that GnT-V null MEF displayed increased cell adhesion to and spreading on fibronectin, and this increased cell-matrix adhesion was markedly inhibited by anti-integrin β1 chain antibody (anti-CD29), indicating that the adhesion was integrin-related. Changes in the interactions of cells with the extracellular matrix can affect the migration of cells, as well as tumor cell invasiveness (37). We used two independent and complementary approaches, the scratch-wound and haptotaxis assays, to determine the impact of GnT-V deletion on cell migration. We observed that the rates of cell migration of MEF, either in wound filling areas on fibronectin or penetrating through haptotaxis membrane inserts using fibronectin as chemoattractant, were markedly decreased after deletion of GnT-V. Also, F-actin staining revealed a relatively flat edge without multiple protrusions and extension of the leading edge in GnT-V null MEF, consistent with a less motile phenotype (38, 39). A critical factor that regulates the rate of cell migration is the degree of adhesion to ECM substrata. Biphasic curves reflecting rates of cell migration as a function of cell-substratum adhesion have been reported by Palecek et al. (37) and showed that intermediate levels of adhesion were required for maximum cell migration. Our results showed increased cell-fibronectin adhesion in GnT-V null MEF; therefore, the lower rates of migration of these cells through fibronectin-coated membranes were likely due to the increased cell-matrix adhesion.

To test further the hypothesis that deletion of GnT-V and the
N-glycan changes caused by this deletion were implicated in the abnormal adhesive phenotypes of GnT-V null MEF, we re-expressed GnT-V cDNA in GnT-V null MEF. The results showed that cell-matrix adhesion and cell motility in the GnT-V-transfected cells were then similar to those observed for wild-type MEF. These results confirmed the direct involvement of GnT-V and N-linked \( \beta(1,6) \) branching in modulating fibronectin-mediated interactions.

Integrin-ECM interactions are prominently involved in the regulation of cell migration, and FAK is a crucial mediator of this process (25, 27). However, phosphorylation of FAK may also function to inhibit cell motility in particular cell types under certain conditions (28, 40–44), in addition to its established function for promoting motility (21, 38, 45, 46). In our study, GnT-V null MEF exhibited increased phosphorylation of FAK, consistent with increased focal adhesion formation (10) and the reduced cell motility of GnT-V null MEF (Fig. 2). These phenotypes of null GnT-V MEF were very similar to those reported for protein-tyrosine phosphatase mutant cells (40, 42) in which FAK dephosphorylation was significantly reduced due to deletion of the protein-tyrosine phosphatase gene, and cell motility was inhibited, supporting the role for FAK in negatively regulating the cell motility of MEF.

The interaction of cells with ECM proteins is largely mediated by the integrin family (22, 47–50). To explore further the mechanism whereby deletion of GnT-V caused a more adhesive and less motile phenotype in the GnT-V null MEF, we focused on characterizing the effects of GnT-V deletion on \( \alpha5\beta1 \) integrin function, the primary receptor of fibronectin, including \( \alpha5\beta1 \) cell surface expression levels and its glycosylation, par-
According to the reports, overexpression of Gnt-V in HT1080 cells was shown to reduce α5β1 integrin clustering, inhibit α5β1 integrin-mediated adhesion, and promote cell motility (9). Consistent with the overexpression experiments, elimination of Gnt-V in MEF stimulated clustering of α5β1 integrin on the cell surface and increased adhesion to fibronectin and FAK phosphorylation, resulting in decreased fibronectin-mediated cell motility. Overexpression of Gnt-V in HT1080 cells had no effect on the cell surface levels of α5β1 integrin nor did it affect cell surface levels of N-cadherin (9, 14). By contrast, however, MEF without Gnt-V activity showed not only increased levels of both α5 and β1 expression on the cell surface but also increased levels of both subunits in total cellular lysates. Deletion of Gnt-V also led to aberrant N-glycosylation of the β1 integrin, because L-PHA and D. strumatum agglutinin binding to the β1 subunit from Gnt-V null MEF were eliminated. L-PHA binding was restored after expression of Gnt-V cDNA in Gnt-V null cells, confirming the role of Gnt-V in regulating these N-glycan changes. Because it is well documented that N-glycans are involved in the folding and sorting of glycoproteins, as well as their cell surface rates of turnover (32, 33), increased levels of cell surface α5β1 integrin could be influenced by altered turnover of α5β1. To test this possibility, the metabolism of α5β1 was measured by a radiolabeling pulse-chase experiment. We confirmed an overall increased level of β1 integrin in Gnt-V null MEF, supporting the evidence for up-regulated levels of β1 protein in the Gnt-V null MEF. Re-introduction of Gnt-V into null MEF reversed the increased β1 synthesis, directly implicating Gnt-V in regulating this process. An additional alteration in the turnover of β1 in the null MEF, however, cannot be ruled out by these data. Nonetheless, several types of experiments all point to increased expression of α5β1 in the null MEF, particularly on their surfaces.

By using quantitative real time RT-PCR to investigate the mechanism by which α5β1 expression was up-regulated in the null MEF, we surprisingly found that transcript levels of both α5 and β1 integrin subunits were upregulated. Notably, the aberrantly high transcript levels of α5 and β1 integrin were significantly lowered after stable re-expression of Gnt-V in the null MEF, demonstrating the direct involvement of Gnt-V in modulating expression of α5β1 transcript levels. Results also showed that α5 transcript stability was not significantly affected in the null MEF. Promoter-reporter experiments, however, demonstrated that α5 integrin promoter activity was clearly up-regulated after deletion of Gnt-V, suggesting that the up-regulation of transcript levels in the null MEF is due at least in part to enhanced rates of transcript synthesis.

The observation that deletion of Gnt-V up-regulated α5β1 integrin expression at both protein and mRNA levels was further confirmed by using the CHO glycosylation mutant Le4 cells that have no detectable Gnt-V activity as do Gnt-V null MEF (34). These results likely represent a common mechanism to modulate integrin expression in the absence of Gnt-V activity.

There are reports that fibronectin interactions can positively regulate α5β1 integrin at both the protein and mRNA levels (51, 52). Therefore, increased binding of fibronectin by Gnt-V null MEF could possibly stimulate expression of integrin transcripts. Fibronectin stimulation of integrin expression was only found at early times (~3 h), however; while longer interactions with fibronectin (~12 h) always resulted in significant decreases of α5β1 transcripts in a fibronectin concentration-dependent manner. Obviously, up-regulation of integrin expression induced by fibronectin stimulation was therefore not a major cause of the sustained enhancement in α5β1 integrin transcription in the null MEF.

To elucidate further the signaling pathways involved in the up-regulation of gene expression of α5β1 integrins in the null MEF, different intracellular signaling pathway inhibitors were tested for their effects on transcript levels. We first found that treatment of MEF with the MAPK inhibitor, PD98059, led to a significant increase in transcript levels of α5β1 integrins in both wild-type and null MEF and abolishment of the suppression of gene expression induced by fibronectin, indicating that expression of α5β1 integrins was negatively regulated via the MAPK pathway in both fibronectin-stimulated and nonstimulated cells. These results were consistent with a recent report (53) that integrins (including α5 and β1) and integrin-mediated cell adhesion were markedly down-regulated in osteoblasts transfected with activated H-ras or Raf-1/MEK. In both wild-type and Gnt-V null MEF, the opposite effect of the PI3K inhibitor, wortmannin, was observed on the expression of α5β1 integrins. In both cell types, wortmannin slightly inhibited α5 gene expression but increased β1 gene expression, demonstrating differential regulation of the expression of α5 and β1 integrin by PI3K. Therefore, increased α5β1 expression in Gnt-V null MEF could not be explained by activation of either the PI3K- or MEK-mediated pathways.

Most intriguingly, however, when MEF were treated with calphostin C, an inhibitor of PKC, the increased transcript levels of both α5 and β1 in the Gnt-V null MEF were lowered to levels observed for the wild-type MEF. An examination of α5 and β1 protein expression levels by Western blotting confirmed that calphostin C decreased α5β1 levels in the null cells. These results indicate the direct involvement of the PKC signaling pathway in the regulation of integrin gene expression in null MEF but not in the wild-type MEF. Several groups have reported that the PKC signaling pathway can affect integrin expression. For example, lipopolysaccharide induced the expression of α5β1 integrin in human monocytes in a PKC-dependent fashion (54), whereas activation of PKC by phorbol esters (55) or exogenous expression of PKCs both stimulated expression of β1 integrin in human breast carcinoma cells (56).

Aberrant glycosylation can affect the functions of several types of cell surface receptors by altering the signaling pathways mediated by these receptors (9, 13, 14, 33, 57, 58). The modulation of receptor transcript and protein levels by abnormal N-glycosylation resulting from deletion of Gnt-V appears to be a novel observation, however. The mechanism by which deletion of Gnt-V activates the PKC signaling pathway and, consequently, up-regulates α5β1 transcript expression levels is unclear and must be further explored. The fact that α5β1 transcript levels are affected by calphostin C only in the Gnt-V null MEF suggests an interesting mechanism of integrin regulation by altered glycosylation. Because other types of receptors, including cadherins and growth factor receptors, are also glycosylated by Gnt-V, their expression levels may also be affected by deletion of Gnt-V. Indeed, altered fibroblast growth factor signaling has been shown to modulate α5β1 expression levels (59, 60). The observation that overexpression of Gnt-V in HT1080 cells reduces α5β1 clustering and inhibits fibronectin-mediated adhesion, yet does not alter cell surface levels of α5β1, suggests that changes in Gnt-V levels and its glycan products can affect cell adhesion by more than a single mechanism.

Tumor cells often display a reduction in or loss of integrin expression, which may reflect a suppressive role of integrins in tumor progression (61). For example, overexpression of α5β1 integrin in HT-29 human colon carcinoma cells caused an inhibitory action on cell proliferation and reduced formation of lung colonies and cutaneous metastases after intravenous injection into nude mice (62). Tani et al. (63) used CHO cells with different expression levels of α5 integrin to study the relation-
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ship of integrin levels and the invasive phenotype. They found that the growth of primary tumors inversely correlated with α5 expression levels on CHO cells, whereas increased α5 expression led to enhanced adhesion and spreading on fibronectin and reduced cell migration (63).

Of particular note, the progression of mouse mammary carcinomas induced by the PyMT oncoprotein in a mouse model of human breast cancer developed by Muller and co-workers (64) was clearly accompanied by the loss of β1 integrin expression, indicating that a loss of β1 integrin is strongly associated with mammary tumor progression. This report and our observations that GnT-V null MEF show increased expression of α5β1 integrin suggest a possible additional mechanism underlying the suppression of PyMT-induced mammary carcinoma invasion observed in GnT-V null mice (10, 11).

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Deletion of Mouse Embryo Fibroblast N-Acetylglucosaminyltransferase V Stimulates α5β1 Integrin Expression Mediated by the Protein Kinase C Signaling Pathway

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