Changes in the expression of glycosyltransferases that branch N-linked glycans can alter the function of several types of cell surface receptors and a glucose transporter. To study in detail the mechanisms by which aberrant N-glycosylation caused by altered N-acetylgalactosaminyltransferase V(GnT-V, GnT-Va, and Mgat5a) expression can regulate the invasiveness-related phenotypes found in some carcinomas, we utilized specific small interfering RNA (siRNA) to selectively knock down GnT-V expression in the highly metastatic and invasive human breast carcinoma cell line, MDA-MB231. Knockdown of GnT-V by siRNA expression had no effect on epidermal growth factor receptor expression levels but lowered expression of N-linked β(1,6)-branching on epidermal growth factor receptor, as expected. Compared with control cells, knockdown of GnT-V caused significant inhibition of the morphological changes and cell detachment from matrix that is normally seen after stimulation with epidermal growth factor (EGF). Decreased expression of GnT-V caused a marked inhibition of EGF-induced dephosphorylation of focal adhesion kinase (FAK), consistent with the lack of cell morphology changes in the cells expressing GnT-V siRNA. The attenuation of EGF-mediated phosphorylation and activation of the tyrosine phosphatase SHP-2 was dramatically observed in GnT-V knockdown cells, and these effects could be rescued by reintroduction of GnT-V into these cells, indicating that reduced EGF-mediated activation of SHP-2 was GnT-V related. Concomitantly, knockdown of GnT-V caused reduced EGF-mediated ERK signaling and tumor cell invasiveness-related phenotypes, including effects on actin rearrangement and cell motility. No changes in EGF binding were observed, however, after knockdown of GnT-V. Our results demonstrate that decreased GnT-V activity due to siRNA expression in human breast carcinoma cells resulted in an inhibition of EGF-stimulated SHP-2 activation and, consequently, caused attenuation of the dephosphorylation of FAK induced by EGF. These effects suppressed EGF-mediated downstream signaling and invasiveness-related phenotypes and suggest GnT-V as a potential therapeutic target.

The expression of specific glycan structures on several cell surface adhesion molecules, growth factor receptors, and the GLUT2 transporter is associated with an alteration of their functions (1–5). For example, studies have shown that aberrant N-glycosylation on several cell surface receptors, including integrins and cadherins, is associated with changes in carcinoma progression and metastasis (6–11). A glycan whose expression is often up-regulated during malignant transformation contains the β(1,6)-linked N-acetylgalactosamine found on some N-glycans (12), synthesized by N-acetylgalactosaminyltransferase V (GnT-V2) or Mgat5, also called GnT-Va; EC 2.4.1.155), a key enzyme in the processing of multiantennary N-glycans during glycoprotein biosynthesis whose expression is regulated by oncogene expression (13–18). Studies have demonstrated the association of increased GnT-V activity and its glycan products with enhanced cell or tumor invasiveness and, in some cases, metastatic potential (6, 7, 19, 20). Of note, mouse mammary carcinomas resulting from expression of the polyoma middle T oncprotein show diminished progression and metastasis in GnT-V null mice (21). Reexpression of GnT-V in the tumor cells from the null background resulted in tumors that progressed at levels similar to those observed for tumors growing in wild-type mice (10). Moreover, patients with colorectal or breast carcinomas that show GnT-V glycan product expression have lowered 5-year survival rates (22, 23), although this association has not been observed for other types of cancer (24, 25). Based on studies with breast and colorectal carcinomas and glioma and many additional results, however, GnT-V is a potentially promising target for development of a therapeutic.

Several cell surface receptors that express the β(1,6)-branched glycan product have been identified, including integrins (6, 20), cadherins (7), and growth factor receptors (10, 26). In each of these cases, GnT-V was overexpressed, and an altered cellular phenotype linked to tumor invasiveness was
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correlated with altered receptor glycosylation and function. The epidermal growth factor receptor (EGFR), a 170-kDa glycoprotein with a single transmembrane domain, is a member of the Erb-b family of receptor tyrosine kinases, that mediate cellular responses to EGF and transforming growth factor-α and play a crucial role in promoting tumor cell motility and invasion (27). The human EGFR contains 12 putative N-glycosylation sites located in extracellular domains I–IV (28), and N-linked glycosylation of EGFR appears to be essential for its functions, particularly the glycans on domain III, the major binding site for EGF and transforming growth factor-α (29–32).

Studies have shown that EGFR function can be modulated by changes in GnT-V-related N-glycan expression. Aberrant glycosylation of EGFR caused by overexpression of GnT-V was reported in human hepatocarcinoma cells, and modified N-glycans on the EGFR in these cells were associated with increased MAPK signaling mediated by EGF (26). In PyMT-induced mouse mammary tumor cells in the GnT-V null background, EGFR was reported to show a higher level of co-localization with EEA-1, an early endosomal marker, suggesting that altered N-glycosylation of EGFR may affect nonligand induced receptor endocytosis (10). The mechanism of how the downstream signaling of the EGFR in these cells with aberrant GnT-V activity is affected is not clear, however, nor whether these effects on signaling result in changes in the EGF-induced invasive phenotype of the tumor cells.

To test directly if decreased levels of GnT-V-modified N-glycan expression affect EGF-mediated invasiveness, we chose a human breast carcinoma cell line, MDA-MB231, used by many investigators for invasiveness studies, to produce a population of cells whose GnT-V expression was attenuated by the expression of small interfering RNA (siRNA). EGF treatment of these cells results in a relatively rapid loss of strong adhesion to substrate and a stimulation of motility and invasiveness. We found that knockdown of GnT-V by siRNA expression caused reduced N-linked (β1,6)-branching on glycoproteins, including EGFR, which resulted in significant inhibition of EGF-stimulated cell detachment from matrix and actin arrangement. Moreover, knockdown of GnT-V also decreased EGF-mediated activation of the tyrosine phosphatase SHP-2, which consequently inhibited the EGF-mediated dephosphorylation of focal adhesion kinase (FAK), consistent with the attenuation of invasiveness-related phenotypes, including decreased actin rearrangement and cell motility. Our results demonstrate for the first time that regulation of the activity of a specific tyrosine phosphatase, SHP-2, and dephosphorylation of FAK result from inhibition of GnT-V expression and mediate subsequent reduced carcinoma invasiveness-related properties in vitro.

EXPERIMENTAL PROCEDURES

Antibodies and Chemicals—The pSUPER.retro.neo expression plasmid was purchased from OligoEngine (Seattle, WA). Polyclonal antibodies against FAK, EGFR, ERK1/2, SH-PTP-2, monoclonal anti-phosphotyrosine (PY20), anti-phosphopaxillin, phospho-ERK, and horseradish peroxidase-labeled anti-rabbit IgG and anti-mouse IgG were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibodies against FAK and paxillin were products of BD Biosciences. Anti-phospho-FAK was from Biosources. AlexaFluor® 594 goat anti-rabbit IgG, AlexaFluor® 488 goat anti-mouse IgG, rhodamine-streptavidin, and rhodamine-phalloidin were products of Molecular Probes, Inc. (Eugene, OR). Monoclonal antibody against vinculin, swainsonine, and bovine serum albumin (BSA) were products of Sigma. NHS-LS-biotin and the ECL assay kit were products of Pierce. Streptavidin-horseradish peroxidase was obtained from Rockland. Biotinylated L-PHA, and streptavidin-agarose were products of Vector Laboratories. Protein A-agarose and protein G-plus-agarose were from Santa Cruz Biotechnology. Boyden (Transwell) chambers (24-well) were products of BD Biosciences. The tyrosine phosphatase assay system was a product of Promega. [125I]-Labeled EGF was purchased from Amersham Biosciences.

Cells and Cell Culture—Human breast carcinoma cell line MDA-MB231 was from the American Type Culture Collection (Manassas, VA). MDA-MB231 cells were cultured in complete Dulbecco’s modified Eagle’s medium (DMEM) with high glucose (Hyclone) containing 10% FBS and 2 mm l-glutamine.

Small Interfering RNA—The human GnT-V (NM_002410)-specific oligonucleotides that contained a 19-nucleotide sequence corresponding to nucleotides 791–809 downstream of the transcription start site (GAGAAAGCGGAAGAAGTC), which is separated by a 9-nucleotide noncomplementary spacer (TTCAAGAGA) from the reverse complement of the same 19-nucleotide sequence, were chosen for targeted suppression of GnT-V. The sequences of forward and reverse strands of oligonucleotides were 5′-GATCATCCCGAGAACGGAAAGAAGTCTTCAAGAGAGCTTTCCGCTTCTCTTTTTA-3′ and 5′-AGCTTTAAAAGAGAAAGCCGAAGCTCTCTGTGAGACTTTCCGCTTTCTCTGGG-3′, respectively. Control RNA strands were used as previously described (33). Forward control and oligonucleosomes were annealed to form a duplex and inserted into the BglII/HindIII cleavage site of pSUPER retrovector (pSUPER.retro.neo/gfp). The recombinant plasmids containing either control RNAs or GnT-V siRNAs were transformed into competent Escherichia coli cells, selected, and amplified for later use.

Transfection—Cell transfections were performed with Lipfectamine™ 2000 according to the manufacturer’s instructions using 5 μg of recombinant retroviral pSUPER/60-mm dish. 24 h after transfection, cells were selected for 3 weeks under G418 (800 μg/ml), and cells with the highest GFP expression were isolated using fluorescence-activated cell sorting. Nonclonal populations of transfected cells were used for all experiments. The human pcDNA3/GnT-V plasmid (34) was used for transient expression of GnT-V. Cells were incubated for 48 h after transfection and used for the indicated experiments.

Quantitative Real Time Reverse Transcription-PCR Analysis—The RNEasy 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 reverse transcription-PCR analysis were as follows: human GnT-V forward (5′-GAGCACATCTGGACCTCAG-3′) and reverse (5′-GCTGTCACTGACTCCAGCCTGA-3′); human glyceraldehyde-3-phosphate dehydrogenase forward (5′-CAGCTTCAAGATCATTAGCAG-3′) and reverse
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(5'-GTCTTTCTGTTGGCAGTTG-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.

**GnT-V Activity Assay**—Cells were trypsinized, pelleted, and lysed with 50 mM MES, pH 6.5, 150 mM NaCl, and 1% Triton X-100. Insoluble debris was pelleted by microcentrifugation (10 min at 4 °C), and the supernatant was used for the GnT-V activity assay using UDP-[3H]GlcNAc as donor substrate and a synthetic trisaccharide acceptor (35). Sep-Pak columns (Waters) were used to separate the substrates and products. Assays were performed in duplicate, the data were averaged, and the results were expressed as specific activity (cpm/h/mg). Protein concentration in the cell lysates was determined using the BCA assay (Pierce).

**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 FCM buffer (PBS containing 1% BSA and 0.01% sodium azide), and then incubated with phycoerythrin (PE)-conjugated anti-EGFR or biotinylated L-PHA, concanavalin A, and Datura stramonium agglutinin at 4 °C for 30 min, followed by incubation with PE-conjugated streptavidin (10 μl) at 4 °C for 30 min. After a wash with ice-cold FCN buffer, analysis was performed using the FACS Calibur (BD Biosciences) instrument.

**Cell Spreading Assay**—Subconfluent cells were detached and resuspended in DMEM culture medium containing 2% FBS. After incubation for 15 min at 37 °C, cells were transferred to 6-well plates and grown in DMEM containing 2% FBS with or without EGF (50 ng/ml) for 60 min. In some experiments, cells were pretreated with swainsonine for 24 h before being used for the spreading assay. The percentage of spread cells was calculated by the ratio of the number of spread cells versus the total number of cells. Cells were considered as spread if they became flattened and lost nuclear refractivity.

**Wound Healing Assay**—Cells (4 × 105) were seeded on 6-well plates and grown to confluence. A clear area was then scraped in the monolayer with a 200-μl yellow plastic tip. After being washed with serum-free DMEM, the plate was incubated at 37 °C in low serum (2%) DMEM containing EGF (100 ng/ml) for the indicated times. Migration of cells into wounded areas was evaluated with an inverted microscope and photographed.

**In Vitro Cell Motility Assay**—Motility assays were performed using 24-well Transwell units with 8-μm pore size polycarbonate inserts (6). Briefly, cells (5 × 105), suspended in 500 μl of DMEM containing 2% FBS and 100 ng/ml EGF, were added into the upper compartment of the Transwell unit, and 700 μl of DMEM containing 2% FBS was added into the lower compartment. Cells were allowed to migrate for 12 h at 37 °C in a humidified atmosphere containing 5% CO2. Cells on the upper side of the membrane were then removed, whereas the cells that migrated through the membrane to the underside were fixed and stained with crystal violet. Cell numbers were then counted in five separate fields using light microscopy at ×100 magnification. The data were expressed as the mean value of cells in five fields based on two independent experiments.

**Fluorescent Staining**—Cells were cultured on chamber slides, fixed with 4% paraformaldehyde in PBS for 10 min, and permeabilized with 0.05% Triton X-100. For actin and vinculin double staining, cells were starved overnight, treated with EGF (100 ng/ml) for the indicated times, and fixed in HistoChoice for 15 min at room temperature, followed by permeabilization with cold methanol at −20 °C for 3 min (36). After blocking with 10% goat serum, cells were stained with biotinylated L-PHA, rhodamine-phalloidin, and vinculin, respectively, followed by incubation with rhodamine-conjugated streptavidin or fluorescein-conjugated anti-mouse IgG (1:250). After washing with PBS, the chamber slides were mounted, and the cells were subjected to deconvolution fluorescence microscopy.

**Immunoblotting**—Cells were harvested and lysed in lysis buffer (10 mM Tris·HCl, pH 7.2, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 10 μg/ml aprotinin and leupeptin). Twenty μg of total cell lysate protein, determined using the BCA protein assay procedure (Pierce), were boiled in 20 μl of lysis buffer with 5 μg/ml Denhardt’s solution, and electrophoresed on a 7% polyacrylamide minigel, and then transferred onto a polyvinylidene difluoride membrane. After blocking with 3% BSA in PBS, the membrane was incubated for 2 h at room temperature with primary antibodies (1:1000) in TBS buffer containing 0.05% Tween 20 (TBST). The membrane was then washed with TBS and probed with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:3000) for 1 h at room temperature. After 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 phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 10 μg/ml aprotinin and leupeptin) for 30 min at 4 °C. For the detection of surface EGFR levels, body against EGFR after SDS-PAGE and membrane transfer, published by E3L reagents and exposed on x-ray film. Images of immunoblots and lectin blots were quantified using a Fluor-S imager (Bio-Rad).
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can alter the phosphorylation of FAK. Specifically, when treated with GnT-V knockdown, MDA-MB231 cells showed decreased cell spreading and decreased cell motility. This effect was reversed when cells were preincubated with swainsonine (Fig. 2B). Concomitantly, when EGF was employed as a stimulus in a Boyden chamber cell migration assay, MDA-MB231 showed increased motility. This stimulatory effect of EGF was largely negated when cells were preincubated with swainsonine (Fig. 1C). These results demonstrate that inhibition of the N-linked β-(1,6)-GlCNac glycan biosynthetic pathway upstream from GnT-V action can significantly reduce the EGFR-induced invasiveness of MDA-MB231 cells.

Targeted Knockdown of GnT-V by siRNA Expression Caused Aberrant N-Glycosylation of EGFR in MDA-MB231 Cells—To determine if specific inhibition of N-linked β-(1,6)-glycan branching would also reduce carcinoma invasiveness, we used the pSUPER system to stably suppress the expression of GnT-V in MDA-MB231 cells. After transfection with pSUPER constructs, cells were selected for neomycin resistance and sorted by fluorescence-activated cell sorting for the expression of green fluorescent protein, which indicated plasmid expression. As shown in Fig. 2, in the cells expressing GnT-V siRNA, GnT-V mRNA levels were significantly decreased (about 50%) compared with control cells expressing control RNAs (Fig. 2A, top). GnT-V activity toward a synthetic trisaccharide acceptor was shown to be decreased greater than 50% in GnT-V knockdown cells (Fig. 2A, bottom), demonstrating a correspondence of GnT-V mRNA levels and enzymatic activity. Consistent with decreased activity, siRNA knockdown cells showed a significant inhibition of L-PHA binding detected by either fluorescent staining (Fig. 2B) or flow cytometry (Fig. 2C). This lectin specifically binds to N-linked β-(1,6)-branched, galactosylated structures (39). Consistent with decreased L-PHA binding, D. stramonium agglutinin binding to knockdown cells was also decreased (Fig. 2C), suggesting reduced expression of poly-N-acetyllactosamine on the cell surface. This result suggests that in these cells poly-N-acetyllactosamine is synthesized preferentially on N-glycans expressing the β-(1,6)-branch.

RESULTS

Attenuation of a Specific N-Linked Glycosylation Pathway Can Regulate EGF-induced Human Breast Carcinoma Cell Spreading and Motility—EGFR activation is one of the most potent stimulators of tumor cell motility and invasiveness (27). To determine if changes in GnT-V-related N-glycosylation affected EGFR-induced alterations of adhesion and invasiveness in vitro, we chose the human breast carcinoma cell line, MDA-MB231, which shows high expression of EGFR and highly invasive phenotypes in vivo and in vitro. When treated with swainsonine, which inhibits Golgi α-mannosidase II and ultimately causes the inhibition of N-linked β-(1,6) oligosaccharide expression upstream of the action of GnT-V, SDS-PAGE reveals that the EGFR in MDA-MB231 showed a significant shift to a lower molecular weight (Fig. 1A), indicating alterations in the N-linked EGFR glycans, as expected. To study the effect of EGF stimulation on invasiveness-related phenotypes, cell spreading and cell motility were explored. When treated with EGF, MDA-MB231 cells showed decreased cell spreading, and this effect was reversed when cells were preincubated with swainsonine (Fig. 1B). Concomitantly, when EGF was employed as a stimulus in a Boyden chamber cell migration assay, MDA-MB231 showed increased motility. This stimulatory effect of EGF was largely negated when cells were preincubated with swainsonine (Fig. 1C). These results demonstrate that inhibition of the N-linked β-(1,6)-GlCNac glycan biosynthetic pathway upstream from GnT-V action can significantly reduce the EGFR-induced invasiveness of MDA-MB231 cells.

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FIGURE 1. Changes in N-glycosylation regulate EGF-induced cell spreading and motility of human breast carcinoma MDA-MB231 cells. A, immunoblots of lysates from MDA-MB231 cells with or without swainsonine (SW) treatment for 24 h probed with antibody against EGFR. ERK levels were detected as loading control. IB, immunoblot. The arrows indicate the shift of molecular weight of EGFR. B, MDA-MB231 cells (5 × 10^5) suspended in DMEM containing 2% FBS were added to 6-well plates, either alone or in the presence of EGF (50 ng/ml), and cultured at 37 °C for 1 h. Cells were photographed (top), and the number of spreading cells was calculated as described under “Experimental Procedures” (bottom). In some experiments, cells were pretreated with swainsonine (1 μg/ml) for 24 h before they were transferred into 6-well plates. Each column represents the mean ± S.D. of the percentage of spreading cells in 6–10 randomly selected fields. Similar results were obtained in three separate experiments. C, MDA-MB231 cells (5 × 10^5) were suspended in DMEM containing 2% FBS and added into the upper chamber of the Transwell apparatus, either alone or in the presence of EGF (50 ng/ml), and DMEM containing 2% FBS was added into the lower chamber. Migrating cells found on the underside of the membrane were fixed, stained, and counted by microscopy. Each bar represents the mean ± S.D. number of migrating cells from five randomly selected fields.

cells was compared with that of control cells using L-PHA precipitation, blotting, and anti-EGFR staining, a significant decrease was observed (Fig. 2F), demonstrating that knockdown of GnT-V expression caused decreased levels of β(1,6)-branched glycans on the EGFR. A significant decrease in L-PHA binding to other glycoproteins was also observed, as expected (data not shown). Consistent with decreased β(1,6)-branched glycans, the formation of poly-N-acetyllactosamine, determined by D. stramonium agglutinin binding, was also inhibited (Fig. 2F). Similarly, the expression of either high manose or biantennary N-linked oligosaccharides on EGFR was not significantly affected by knockdown of GnT-V, detected by concanavalin A precipitation. Interestingly, increased Sambucus nigra agglutinin binding was observed in GnT-V knockdown cells, indicating increased relative expression of α(2,6)-sialic acid on EGFR after GnT-V knockdown. In addition, the expression levels of α(2,3)-sialylation and fucosylation on EGFR were very low, as evidenced by M. ameurinnsi agglutinin and Lotus agglutinin binding (Fig. 2F), respectively. These results indicate that knockdown of GnT-V caused significant changes in GnT-V-related N-glycan expressions on EGFR.

EGF-induced Cell Morphology Changes and Detachment Were Inhibited in MDA-MB231 Cells after GnT-V Knockdown—After EGF treatment, many tumor cell lines with high levels of EGFR expression, including human breast carcinoma cells, display refractile morphological changes and detachment from the extracellular matrix (40, 41). To investigate if reduced β(1,6)-branching on EGFR caused by knockdown of GnT-V in MDA-MB231 cells affected EGF-induced morphological changes, cells were allowed to spread onto culture wells and then treated with EGF (100 ng/ml) for different times. In control cells, EGF-induced refractile morphological changes and cell detachment were observed at 10 min after EGF treatment and became more prominent at 20 min (Fig. 3A). After a 24-h treatment, the cells became quite elongated and spindle-shaped, a typical morphology of motile cells and often an indicator of a more motile and invasive phenotype (41). In GnT-V knockdown cells, by contrast, morphological changes induced by treatment of EGF were significantly inhibited, even after a 20-min treatment. The majority of cells with GnT-V knockdown were still spread evenly after 24 h of treatment, indicating a significant decrease in the motile phenotype. These results suggest that EGF-mediated morphological changes in the carcinoma cells were inhibited by suppression of GnT-V expression.

Consistent with the cell morphology changes, control cells stimulated with EGF for 20 min showed prominent, central vinculin staining and cortical actin ring formation around the cell periphery, as shown in Fig. 3B. After overnight (18-h) stimulation of EGF, spotlike vinculin staining appeared at the cell periphery, and actin stress fibers were formed across the cell, indicating increased actin rearrangement and significant motility (42, 43). In the GnT-V knockdown cells, however, a more spread morphology with disrupted actin fibers was observed, and vinculin spots were scattered across the cells after EGF treatment at both 20 min and 18 h, indicating reduced actin rearrangement and reduced reorganization of focal adhesions, consistent with a less motile phenotype.

EGF-induced Dephosphorylation of FAK Was Inhibited in GnT-V Knockdown Cells—To investigate further the mechanisms underlying the suppressed EGF-induced morphological changes in the GnT-V knockdown cells, we examined the levels of tyrosine phosphorylation of proteins involved in focal adhesions, in particular FAK, because the dephosphorylation of this kinase is reported to be causally involved in the morphological changes and loss of matrix adhesion due to EGFR activation...
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First, knockdown of GnT-V had no effect on the expression levels of several major focal adhesion proteins, including FAK, paxillin, and vinculin (Fig. 4A). Phosphotyrosine levels of FAK were examined by immunoprecipitating FAK from both cell lines, as shown in Fig. 4B. Unlike control cells, where the cells treated with EGF for 10 min at two different concentrations showed significantly reduced phosphotyrosine levels, little change in phosphotyrosine levels was observed in GnT-V knockdown cells after identical treatment (Fig. 4B). Furthermore, decreased levels of phosphorylation at Tyr-397, the major FAK autophosphorylation site, were observed in a time-dependent manner after EGF treatment in control cells but not in GnT-V knockdown cells (Fig. 4C). This result indicates that inactivation of FAK induced by EGF was attenuated as a result of the down-regulation of GnT-V activity. Paxillin is another important focal adhesion protein...
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molecule that is associated with and phosphorylated by FAK (45). To explore whether paxillin phosphorylation is also regulated by EGF treatment, phosphopaxillin levels were measured after stimulating the cells with EGF. As shown in Fig. 4D, similar to the effects seen on FAK phosphorylation, EGF-mediated down-regulation of paxillin phosphorylation was significantly inhibited in GnT-V knockdown cells. When total phosphorysorine-containing proteins were pulled down with anti-phosphotyrosine antibody and probed with anti-FAK or anti-paxillin antibodies, a similar pattern of decreased phosphorylation was observed on both FAK and paxillin after EGF treatment (Fig. 4E), consistent with the conclusion that EGF stimulation caused FAK dephosphorylation and dephosphorylation of an associated downstream signaling protein. This EGF-induced decrease of FAK phosphorylation was inhibited by the pretreatment of control cells with swainsonine (Fig. 4F). When GnT-V cDNA was reintroduced into knockdown cells, GnT-V activity showed a significant increase (Fig. 4G, left), and EGF-induced down-regulation of FAK phosphorylation was promoted (Fig. 4G, right), confirming the involvement of N-linked β(1,6)-branching in regulating EGF-induced FAK activation.

Attenuated Expression of GnT-V Decreased EGF-induced Activation of Protein-tyrosine Phosphatase SHP-2—To test if dephosphorylation of FAK induced by EGF treatment was protein-tyrosine phosphatase (PTP)-dependent, pervanadate, a general tyrosine PTP inhibitor, was applied before EGF treatment. EGF-induced morphological changes (Fig. 5A) and FAK SHP-2 phosphorylation was also observed in control cells upon EGF stimulation; by contrast, this EGF-mediated phosphorylation of SHP-2 was significantly attenuated in GnT-V knockdown cells (Fig. 5E). Consistent with the phosphorylation status of SHP-2, the activation of SHP-2 revealed by an in vitro activity assay was dramatically inhibited in GnT-V knockdown cells, compared with control cells (Fig. 5F). When GnT-V cDNA was reintroduced into knockdown cells, activation of SHP-2 was markedly promoted (Fig. 5G), indicating the direct involvement of GnT-V activity in the regulation of SHP-2 activation. These results demonstrate that SHP-2 is involved in EGF-induced dephosphorylation of FAK and paxillin in these cells and that the SHP-2 activation that results from EGF binding is inhibited in GnT-V knockdown cells, leading to a sustained activation of FAK and downstream signaling.

Knockdown of GnT-V Expression Attenuated EGF-mediated ERK Signaling and Cell Motility—Activation of SHP-2 is required for mediating ERK phosphorylation induced by growth factor binding (51–53), which consequently causes increased invasiveness-related cell motility. Therefore, we next explored whether reduced activation of SHP-2 caused by reduction of GnT-V glycosylation resulted in altered activation of ERK signaling and invasiveness-related cell motility. As shown in Fig. 6A, EGF treatment of cells for different times led to activation of ERK1 and ERK2, as expected. This effect was attenuated, however, in the GnT-V knockdown cells, indicating that the EGF-MAPK signaling pathway triggered by EGF treat-
ment was affected as a result of the down-regulation of GnT-V activity. Moreover, using a scratch-wound assay to measure cell migration activity, 6 h after wounding, almost 50% of the wound area was filled, determined by measuring the relative wound width, in control cells. Less than 30% of the wound area was filled in GnT-V knockdown cells stimulated by EGF, by contrast, showing decreased migratory activity compared with control cells (Fig. 6B). Consistent with these results, using the Boyden chamber motility assay in which EGF was used as a stimulus, GnT-V knockdown cells showed a significant decrease in rate of migration compared with control cells (Fig. 6C), demonstrating decreased cell motility in the knockdown cells. In conclusion, reduction of GnT-V expression and activity in an invasive breast carcinoma cell line resulted in an inhibition of EGF-induced SHP-2 activation and downstream signaling events, including ERK activation, ultimately causing a decrease in cell motility.

**Knockdown of GnT-V Expression Had No Effect on EGF Binding**—To further explore the mechanism whereby knockdown of GnT-V caused inhibition of EGF-induced activation of SHP-2 and dephosphorylation of FAK and concomitantly downstream ERK signaling, competitive EGF binding assays were performed. As shown in Fig. 7, no significant changes in the binding of 125I-EGF to EGFR were observed after knockdown of GnT-V expression compared with control cells, suggesting that decreased N-linked β(1,6)-branching level on EGFR caused by knockdown of GnT-V had no effect on receptor binding and that attenuated invasiveness-related signaling
in GnT-V knockdown cells probably did not result from decreased EGF binding.

**DISCUSSION**

Studies have suggested the involvement of GnT-V levels in regulating cell-matrix and cell-cell adhesion as well as cell migration and invasion by affecting the N-glycosylation of cell surface receptors, including the integrins (6), cadherins (7), and growth factor receptors (10, 26). EGFR is expressed by most mesenchymal and epithelial cells, and enhanced expression of the EGFR was associated with aggressive progression of many kinds of human tumors, including breast carcinomas, by promoting tumor cell proliferation and motility (27). Changes in the N-glycans on this receptor are associated with effects on its function and EGF-mediated signaling pathways (3, 10, 26, 38, 54). In the present study, in order to study the mechanisms by which N-linked β(1,6)-branching levels may regulate EGF-induced carcinoma invasiveness, we suppressed GnT-V expression in MDA-MB231 cells, an invasive human breast cancer cell line, by knockdown of GnT-V using the siRNA technique.
Expression levels of several cell surface receptors can be modulated by changes in specific N-linked oligosaccharide expression through different mechanisms (6, 10, 55, 56). For example, mouse embryonic fibroblasts from GnT-V null embryos exhibited an enhanced integrin clustering and activation of αβ1 integrin transcription by protein kinase C signaling, which, in turn, up-regulated levels of cell surface α5β1 fibronectin receptors, resulting in increased matrix adhesion and inhibition of migration (55). Another recent report showed that N-glycan changes on EGFR and other cytokine receptors in GnT-V null, PyMT-induced mouse mammary carcinoma cells was associated with an increase in the nonligand-induced endocytosis of these receptors, causing lower expression levels of receptors on the cell surface (10). In the present study, however, knockdown of GnT-V in MDA-MB231 cells did not significantly affect the steady-state level of EGFR expressed on the cell surface, as determined by experiments employing surface biotinylation and anti-EGFR antibody detection. The β(1,6)-branched glycans on EGFR, detected by L-PHA binding, were found significantly reduced in the knockdown cells, indicating a significant change in the N-linked glycans on the EGFR after knockdown of GnT-V.

Morphological change and detachment from extracellular matrix play essential roles in regulating tumor cell motility and invasion (41, 42, 44). Our results show that EGF-mediated refractile changes and cell detachment from matrix were significantly inhibited after the knockdown of GnT-V expression in MDA-MB231 cells, indicating a less motile phenotype compared with control cells. Consistent with the effects on cell morphology changes induced by EGF in the GnT-V siRNA cells, the knock-out cells exhibited an inhibition of actin rearrangement and stress fiber formation, and vinculin-positive spots were scattered across the surface of the cells. In the control cells induced with EGF, by contrast, formation of typical focal adhesion contacts was observed at the cell periphery, consistent with a less motile phenotype caused by GnT-V knockdown. Decreased EGF-mediated cell motility in GnT-V knockdown cells was further confirmed by two independent and complementary approaches using the scratch-wound and Transwell migration assays.

FAK, a nonreceptor protein-tyrosine kinase localized to substrate focal contact structures, is one of the most prominent tyrosine-phosphorylated proteins in many types of cells (57–59). FAK is associated with transmembrane integrin receptors within focal adhesions, and the phosphorylation of FAK leads to its activation and spreading. Recent studies suggest that FAK is more likely to regulate cell motility by modulation of the turnover of focal adhesions rather than by simply eliciting their formation (43, 60–63). In addition, FAK has been implicated in growth factor-induced cell motility. FAK was dephosphorylated and inactivated upon EGF stimulation in a variety of human tumor cells as well as in NIH 3T3 cells overexpressing EGFR (41). In MCF-7 cells (64), IGF-1 receptor-induced migration and invasion was also associated with the dephosphorylation of FAK, p130Cas, and paxillin, suggesting that dephosphorylation of adhesion-related proteins may be a common phenotype associated with tumor invasion. Moreover, EGF-mediated morphological changes and increased cell motility in several types of tumor cells were regulated by the dephosphorylation of focal adhesion molecules, including FAK, paxillin, and p130Cas (41, 42). In our study, EGF-induced dephosphorylation of both FAK
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FIGURE 7. Effect of knockdown of GnT-V expression on EGF binding. Competitive EGF binding assays were carried out in both control and GnT-V knockdown cells. Cell-bound ¹²⁵I-EGF was detected using a γ-counter after incubation of cells at 4 °C for 2 h with ¹²⁵I-EGF as described under "Experimental Procedures." Each point represents the mean ± S.D. cpm/well. The figure shows one representative experiment of three.

and paxillin, the major components of focal adhesions, was dramatically inhibited in the GnT-V knockdown cells. This inhibition was consistent with the retention of substrate adhesion and a more flattened morphology in the knockdown cells compared with control cells.

Swainsonine inhibits Golgi α-mannosidase II and thus prevents the trimming of mannose residues and formation of complex type glycans from the high mannose type. Swainsonine-treated A431 cells have been shown to express an EGFR with mainly high mannose-type and hybrid-type glycans (65). In our study, EGF-induced dephosphorylation of FAK was greatly inhibited by pretreatment of control cells with swainsonine, supporting the hypothesis that changes in the expression of complex N-glycans are directly involved in regulating EGFR signaling. Furthermore, EGF-induced dephosphorylation of FAK was promoted by reexpression of GnT-V in the knockdown cells, consistent with the conclusion that GnT-V and N-linked β(1,6)-glycan branching can regulate this event. Based on these results, we propose a mechanism for EGF-induced cell motility and invasiveness, regulated by the levels of N-linked branching, that directly involves changes in the dephosphorylation of FAK and other focal adhesion molecules.

Consistent with the results from previous studies (41, 49), we found that EGF-induced morphology changes and dephosphorylation of FAK in control cells were abolished by pretreatment of cells with a PTP inhibitor, indicating that these events were PTP-dependent. Many PTPs, including SHP-2, PTP1B, PTP-PEST, and PTEN, have been implicated in modulating focal adhesion turnover by dephosphorylating FAK, paxillin, and p130cas (46, 48, 49, 66, 67). SHP-2 contains two Src homology 2 domains located at its N terminus and a phosphatase domain at its C terminus (68, 69). SHP-2 was found to bind to a variety of receptor tyrosine kinases in response to stimulation by growth factors and cytokines and to play an important role in regulating cell spreading, migration, and focal adhesion (70–74). Therefore, it was logical to determine if the attenuated dephosphorylation of FAK and paxillin in the GnT-V knockdown cells resulted from an inhibition of phosphatase SHP-2 activity. A decrease in the formation of a complex between FAK and SHP-2 upon EGF stimulation was observed in GnT-V knockdown cells by co-immunoprecipitation, indicating a decrease in association of FAK with SHP-2 (Fig. 5D) and, consequently, a likely reduction of FAK phosphorylation by SHP-2, as has been suggested (42, 49). To assess the enzymatic activity of SHP-2, two different methods were utilized, including detection of SHP-2 phosphorylation and an in vitro phosphatase activity assay. Studies have shown that in both A431 and h1HER cells in which the EGFR was overexpressed, SHP-2 was phosphorylated on tyrosine after EGF treatment (70, 75). This EGF-stimulated phosphorylation of SHP-2 was, however, not significant in mouse fibroblasts (76). We found that knockdown of GnT-V in a human breast cancer line caused a marked decrease in EGF-induced phosphorylation of SHP-2, consistent with decreased SHP-2 activity. In addition, consistent with decreased SHP-2 phosphorylation levels, a significant inhibition of SHP-2 activation by EGF stimulation was observed in the knockdown cells, which could be reversed by reexpression of GnT-V. These results strongly suggest that the inhibition of FAK dephosphorylation induced by EGF resulted most likely from attenuated SHP-2 activity and that GnT-V expression levels are directly implicated in regulating these events.

It has also been reported that SHP-2 is involved in regulating the turnover of focal adhesions as well as the phosphorylation state of FAK and other focal adhesion–related proteins (47, 77). In SHP-2 knock-out mouse embryonic fibroblasts, an increased level of phosphorylated FAK was observed, compared with wild type cells, supporting the conclusion that SHP-2 may possibly regulate turnover of FAK and consequently affect cell spreading and motility (42, 47). The phenotypes observed for the GnT-V knockdown cells, including decreased rearrangement of actin and focal adhesion formation, FAK dephosphorylation, and cell motility, were very similar to those reported for SHP-2 mutant cells (42, 47). We postulated, therefore, that the silencing of GnT-V in our study inhibited EGF-stimulated SHP-2 activation, which protected FAK from dephosphorylation and, consequently, led to a cellular phenotype of low motility and invasiveness.

In response to EGF stimulation, EGFR becomes autophosphorylated on C-terminal tyrosine residues. These phosphorytrosines act as docking sites for recruitment of Src homology 2-containing proteins, including SHP-2, to activate downstream signaling cascades, such as mitogen-activated protein kinase and protein kinase B pathways (51–53). The phosphatase activity of SHP-2 has been shown to be required for EGF-induced activation of ERK in various cell lines (69, 76, 78). We found that knockdown of GnT-V caused decreased activation of ERK, which was consistent with the decreased activation of SHP-2, indicating that inhibition of ERK pathway in knockdown cells might result, at least in part, from decreased activity of SHP-2.

A previous study showed decreased EGF binding after knockdown of GnT-V using an antisense technique in human
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hapatocarcinoma cells, concomitantly causing decreased EGFR phosphorylation (26). Therefore, one possibility to explain how changes in N-glycosylation caused abnormal EGFR function, which resulted in reduced activation of phosphatase SHP-2 and dephosphorylation of focal adhesion molecules, is that the altered N-glycan expression affected the affinity of the receptor for binding EGF. To test this hypothesis, competitive EGF binding assays were performed. No significant changes in EGF binding to receptor were observed, however, after knockdown of GnT-V expression, indicating that reduced EGF-induced signaling caused by knockdown of GnT-V was not due to decreased receptor binding affinity. This result was further confirmed by the observation that the relative levels of tyrosine phosphorylation of the EGFR appeared to be unaltered in the knockdown cells (data not shown). These results are also consistent with an earlier study that showed that the ligand binding and tyrosine kinase activities of EGFR were relatively unaffected in swainsonine-treated cells (79) and concluded that the ligand binding affinity of EGF was not affected in GnT-V null PyMT-induced mammary tumor cells. This study did conclude, however, that deletion of β(1,6)-N-glycan branching on the EGFR increased receptor internalization into the early endosomes in the absence of EGF induction, which resulted in the attenuation of EGFR signaling. Another study showed that overexpression of GnT-III, forming a bisecting GlcNAC on the β1-mannoside of the trimannose core, enhanced EGF-induced phosphorylation of ERK in HeLaS3 cells by up-regulating the internalization rate of EGFR without affecting receptor phosphorylation (38). Therefore, altered ligand-induced endocytosis of EGFR is a likely possibility implicated by the reduced signaling pathway caused by decreased expression of GnT-V in MDA-MB231 cells. On the other hand, aberrant N-glycosylation of the EGFR itself may alter receptor conformation such that the recruitment of SHP-2 to EGFR is hindered, causing reduced complex formation of FAK and SHP-2, reduced dephosphorylation of FAK and paxillin, and attenuated downstream signaling pathways linked to cell motility and invasion. Experiments are in progress to test these possibilities.

In conclusion, our results show that suppression of GnT-V expression and activity resulted in decreased β(1,6) N-linked glycan branching of EGFR as well as other glycoproteins, inhibition of EGF-induced phosphatase SHP-2 activation, and dephosphorylation of focal adhesion molecules. Consequently, cell morphology changes and cell detachment from matrix were inhibited, leading to a cellular phenotype with decreased cell motility and invasiveness. These findings suggest that GnT-V is a significant target for the development of an inhibitor that could display therapeutic utility.

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