N-Acetylglucosaminyltransferase III Antagonizes the Effect of N-Acetylglucosaminyltransferase V on α3β1 Integrin-mediated Cell Migration

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N-Acetylglucosaminyltransferase V (GnT-V) catalyzes the addition of β1,6-GlcNAc branching of N-glycans, which contributes to metastasis. N-Acetylglucosaminyltransferase III (GnT-III) catalyzes the formation of a bisecting GlcNAc structure in N-glycans, resulting in the suppression of metastasis. It has long been hypothesized that the suppression of GnT-V product formation by the action of GnT-III would also exist in vivo, which will consequently lead to the inhibition of biological functions of GnT-V. To test this, we draw a comparison among MKN45 cells, which were transfected with GnT-III, GnT-V, or both, respectively. We found that α3β1 integrin-mediated cell migration on laminin 5 was greatly enhanced in the case of double transfectants of GnT-III and GnT-V. Conversely, GnT-III knockdown resulted in increased migration on laminin 5, concomitant with an increase in β1,6-GlcNAc-branching N-glycans on integrin α3 subunit was observed in the double transfectants of GnT-III and GnT-V. Conversely, GnT-III knockdown resulted in increased migration on laminin 5, concomitant with an increase in β1,6-GlcNAc-branching N-glycans on the α3 subunit in CHP134 cells, a human neuroblastoma cell line. Therefore, in this study, the priority of GnT-III for the modification of the α3 subunit may be an explanation for why GnT-III inhibits GnT-V-induced cell migration. Taken together, our results demonstrate for the first time that GnT-III and GnT-V can competitively modify the same target glycoprotein and furthermore positively or negatively regulate its biological functions.

Malignant transformation is accompanied by increased β1,6-GlcNAc branching of N-glycans attached to Asn-X-Ser/Thr sequences in mature glycoproteins (1–3). N-Acetylglucosaminyltransferase V (GnT-V) catalyzes the addition of β1,6-linked GlcNAc (see Fig. 8) and defines this subset of N-glycans (4, 5). A relation between GnT-V and cancer metastasis has been reported by Dennis et al. (6) and Yamashita et al. (1). Studies on transplantable tumors in mice indicate that the product of GnT-V directly contributes to the growth of cancer and subsequent metastasis (7, 8). On the other hand, somatic tumor cell mutants that are deficient in GnT-V activity produce fewer spontaneous metastases and grow more slowly than wild-type cells (6, 9). The suppression of tumor growth and metastasis has been reported in GnT-V-deficient mice (3). Moreover, Partridge et al. (10) reported that GnT-V-modified N-glycans with poly-N-acetyllactosamine, the preferred ligand for galectin-3, on surface receptors oppose their constitutive endocytosis and result in promoting intracellular signaling and consequently cell migration and tumor metastasis. These results indicate that inhibition of GnT-V might be useful in the treatment of malignancies by targeting their roles in metastasis.

N-Acetylglucosaminyltransferase III (GnT-III) participates in the branching of N-glycans (see Fig. 8), catalyzing the formation of a unique sugar chain structure-bisecting GlcNAc (11). GnT-III is generally regarded to be a key glycosyltransferase in the N-glycan biosynthetic pathway, since in vitro the introduction of the bisecting GlcNAc results in the suppression of further processing and the elongation of N-glycans as the result of catalysis by other glycosyltransferases, which are unable to use the bisected oligosaccharide as a substrate (12, 13). It is interesting to note that the metastatic capabilities of B16 mouse melanoma cells are down-regulated by introduction of the GnT-III gene (14). E-cadherin, a homophilic type of adhesion molecule (15), is highly associated with the prevention of metastasis (16), and E-cadherin on GnT-III-transfected cell

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3 The abbreviations used are: GnT-V, N-acetylglucosaminyltransferase V; GnT-III, N-acetylglucosaminyltransferase III; ECM, extracellular matrix; LN5, laminin 5; FN, fibronectin; COL, collagen I; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; LC, liquid chromatography; MS, mass spectrometry; FT-ICR, Fourier transform ion cyclotron resonance.
surfaces was found to be resistant to proteolysis, resulting in an
extended half-life of turnover (17). Thus, GnT-III, contrary to
GnT-V, has long been thought to inhibit cancer metastasis.

Cell-extracellular matrix (ECM) interactions play essential
roles during the acquisition of migration and invasive behavior
of cells. Cell surface transmembrane glycoprotein-integrin is a
major receptor for ECM and connects many biological func-
tions, such as development, control of cell proliferation, protec-
tion against apoptosis, and malignant transformation (18).
Integrin α3β1, the major laminin 5 (LN5) receptor, is widely
distributed in almost all tissues, and it has been proposed to be
involved in tumor invasion (19–21). In some malignant
tumors, α3β1 integrin was found to be the most predominant
integrin expressed (22) and made an important contribution to
pulmonary metastasis (23). On the other hand, the glycosyla-
tion of integrins contributes to the tumor metastasis. Guo et al.
reported that an increase in β1,6-GlcNAc sugar chains of the
integrin β1 subunit resulted in the stimulation of cell migration
(24). Interestingly, it has also been reported that the α3β1 inte-
grin expressed by the metastasis human melanoma cell lines,
contained a higher level of β1,6-branched structures than that
expressed in a nonmetastasis parent cell line (25).

Although it had been assumed that the reaction of GnT-V
can be inhibited by the action of GnT-III, as evidenced by sub-
strate specificity studies in vitro, the hypothesis of competition
between GnT-III and GnT-V in cell migration and tumor
metastasis has not been directly verified so far. In the present
study, we examined the functions of α3β1 integrin, which is
believed to be highly associated with tumor metastasis, and
found that α3β1 integrin can be modified by either GnT-III or
GnT-V. Our finding clearly shows that GnT-III inhibits the
effects of GnT-V on α3β1 integrin-mediated cell migration by
competing with GnT-V for the modification of α3 subunit.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Antibodies against integrin α3
subunit (P1B5, I-19), monoclonal antibody against β-actin,
mouse control IgG, and peroxidase-conjugated rabbit antibody
against goat IgG were obtained from Santa Cruz Biotechnology,
Inc. (Santa Cruz, CA). Functional blocking antibody against the
integrin β1 subunit was purchased from Chemicon Interna-
tional, Inc. (Temecula, CA). A peroxidase-conjugated goat anti-
body against mouse IgG was from Promega (Madison, WI).
Biotinylated leukoagglutinating phytohemagglutinin (L4-
PHA), biotinylated erythroagglutinating phytohemagglutinin
(E4-PHA), and monoclonal antibodies against GnT-III and
GnT-V were from Seikagaku Corp.

**Cell Culture**—Transfected MKN45 Cells were established as
previously reported (26). Human gastric cancer cell line
MKN45 cells were cultured in RPMI 1640 medium (Sigma)
containing 10% fetal bovine serum (Invitrogen), penicillin (100
units/ml), and streptomycin (100 µg/ml) under a humidified
atmosphere containing 5% CO2.

**Plasmin and Transient Virus Transfection**—cDNAs encod-
ing full-length human GnT-III or GnT-V inactive mutant
(D317A) were ligated into adenoviral vector, constructed using
an adenoviral expression vector kit (Takara Bio). The 3 × 10^5
MKN45 GnT-V transfectants were then infected with 150 µl of
virus solution (2 × 10^9 plaque-forming units/ml). After a 24-h
incubation, the cultured medium was replaced with a fresh
medium. 48 h later after infection, cells were subjected to vari-
ous experiments.

**Construction of Small Interfering RNA Vector and Retrovi-
ral Infection**—Small interfering oligonucleotides specific for
GnT-III were designed on the Takara Bio site on the World
Wide Web, and the oligonucleotide sequences used in the
construction of the small interfering RNA vector were as fol-
ows: 5’-GATCCGTCAACCCAGTTTCAGCTTCA-
AGAGAGGTCGAACTCGTGGTTGACTTTTTAT-3’ and
5’-CAAACAAAGATCCACCGAGTTGACTCTCTCTCT-
TGAAGGTCGAACTCGTGGTTGACG-3’. The oligonucleo-
tides were annealed and then ligated into BamHI/ClaI sites of
the pSINsi-hU6 vector (Takara Bio). A retroviral supernatant
was obtained by transfection of human embryonic kidney 293
cells using the retrovirus packaging kit Ampho (Takara Bio)
according to the manufacturer’s protocol. CHP134 cells were
infected with the viral supernatant, and the cells were then
selected with 500 µg/ml G418 for 2–3 weeks. Stable GnT-III
knockdown clones were selected and confirmed by GnT-III
activity and gene expression. Quantitative real time PCR anal-
yses of GnT-III mRNA expression in these clones were per-
formed with a Smart Cycler II System and the SYBR premix Taq
(Takara Bio). Reverse transcription was carried out at 42 °C
for 10 min, followed by 95 °C for 2 min using random primers,
followed by PCR for 45 cycles at 95 °C for 5 s and 60 °C for 20
s with the following primers: 5’-GGCTCATCAACCGCCAT-
CAA-3’ and 5’-TGACTCGACCAACACCAAG-3’. Normaliza-
tion of the data were performed using the glyceraldehyde-3-
phosphate dehydrogenase mRNA levels.

**GnT-III and GnT-V Activity Assay**—The activities of GnT-
III and GnT-V were assayed as described previously (29, 30).
Briefly, cell lysates were homogenized in phosphate-buffered
saline (PBS) containing protease inhibitors. The supernatant,
after removal of the nucleus fraction by centrifugation for 15
min at 900 × g, was used in the assays, which involved high
performance liquid chromatography methods using a pyri-
dylaminated biantennary sugar chain as an acceptor substrate.
Protein concentrations were determined using a bicinchoninic
acid kit (BCA kit) (Pierce) with bovine serum albumin as a
standard.

**Western Blot and Lectin Blot Analysis**—Cells cultures were
harvested in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM
NaCl, 1% Triton, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM
phenylmethylsulfonyl fluoride). Cell lysates were centrifuged
at 15,000 × g for 10 min at 4 °C, the supernatants were collected,
and the protein concentrations were determined using a BCA
protein assay kit. Proteins were then immunoprecipitated from
GnT-III Counteracts the Effect of GnT-V

the lysates using a combination of 2 μg of anti-integrin α3 subunit antibody and 15 μl of protein G-Sepharose 4 Fast Flow (Amersham Biosciences) for 1 h at 4 °C. Immunoprecipitates were suspended in reducing sample buffer, heated to 100 °C for 3 min, resolved on 7.5% SDS-PAGE, and electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell). The blots were then probed with anti-α3 antibody or biotinylated E4, or L4-PHA. Immunoreactive bands were visualized using the Vectastain ABC kit (Vector Laboratories, CA) and an ECL kit (Amersham Biosciences). For GnT-III, GnT-V, cell lysate, and actin blotting, an equal amount of cell lysates was subjected to SDS-PAGE and then transferred to nitrocellulose membranes.

The membranes were incubated with the corresponding primary antibodies and secondary antibodies for 1 h each, and detection was performed by an ECL kit.

Cell Surface Biotinylation—Cell surface biotinylation was performed as described previously with minor modifications (31). Briefly, various semiconfluent transfected MKN45 cells were washed twice with ice-cold PBS and then incubated with ice-cold PBS containing 0.2 mg/ml sulfo-SNARCD (Pierce), for 3 h at 4 °C. After incubation, the cells were washed three times with ice-cold PBS, scraped, and lysed with radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 150 mM NaCl, 1 mM sodium orthovanadate, 2 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The resulting cell lysates were immunoprecipitated with anti-α3 antibody, as described above. The immunocomplex was subjected to 7.5% SDS-PAGE and then transferred to a nitrocellulose membrane. After blocking the membranes with 3% (w/v) skim milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST, pH 7.5), the biotinylated proteins were visualized using a Vectastain ABC kit (Vector Laboratories, CA) and an ECL kit.

Migration Assay—Transwells (BD Biosciences) were coated with 5 μg/ml human plasma FN, and collagen I (COL) (Sigma) in PBS by an incubation overnight at 4 °C. Serum-starved cells (2 × 10^5 cells/well in 500 μl) were transferred to the upper chamber of the plates. After incubation overnight at 37 °C, cells in the upper chamber of the filter were removed with a wet cotton swab. Cells on the lower side of the filter were fixed and stained with 0.5% crystal violet. Each experiment was performed in triplicate, and counting was done in three randomly selected microscopic fields within each well.

Functional Blocking Assay—To identify which integrin is involved in cell migration on LN5, functional blocking antibodies against different types of integrins were individually preincubated with cells for 10 min at 37 °C. The preincubated cells were transferred into transwells coated with LN5 and then incubated overnight at 37 °C. The migrated cells were then quantified as described above.

Statistical Analysis—Statistical evaluations were performed using Student’s t test; differences among experimental groups were considered significant for p < 0.05. Data were expressed as mean values ± S.D.

Purification of α3β1 Integrin—The purification of α3β1 integrin was performed as described previously (33). Briefly, cells in confluent were detached with TBS(+) (20 mM Tris-HCl, pH 7.5, 130 mM NaCl, 1 mM CaCl_2, and 1 mM MgCl_2) and washed with TBS(+). The cell pellets were extracted with 50 mM Tris/HCl containing 15 mM NaCl, 1 mM MgCl_2, 1 mM MnCl_2, pH 7.4, and protease inhibitor mixture (Roche Applied Science), 100 mM octyl-β-D-glucopyranoside at 4 °C. The cell extract was applied to an affinity column prepared by coupling 5 mg of the GD6 peptide of laminin α1 chain (33) (QNKLSSRAFRGCVVRNLRLSR residues numbered 3011–3032) (Peptide Institute, Inc., Osaka, Japan) to 1 ml of activated CH-Sepharose (Sigma). The bound α3β1 integrin was eluted with 20 mM EDTA in 50 mM Tris/HCl, pH 7.4, containing 100 mM octyl-β-D-glucopyranoside. The elutes containing α3β1 integrin were further purified on a 1-ml wheat germ agglutinin-agarose column (Seikagaku Corp., Tokyo, Japan) and eluted with 0.2 μM N-acetyl-d-glucosamine containing 100 mM octyl-β-D-glucopyranoside.

Analysis of N-Glycan Structures by Mass Spectrometry (LC/MS^n)—Purified α3β1 integrin was applied to SDS-PAGE, and the α3 subunit was excised from the gel and then cut into pieces. The gel pieces were destained and dehydrated with 50% acetonitrile. The protein in the gel was reduced and carboxymethylated by the incubation with dithiothreitol and sodium monooiodoacetate (34). N-Glycans were extracted from the gel pieces as reported by Kustar et al. (35) and reduced with NaBH_4. Half of the extracted oligosaccharides were incubated with α-neuraminidase from Arthrobacter ureafaciens in 50 mM phosphate buffer, pH 5.0, at 37 °C for 18 h and desalted with Envi-carb (Sulpco, Bellefonte, PA). LC/MS and LC/multistage MS (MS^n) was carried out on a quadrupole ion trap-Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR MS; Finnigan LTQ FTTM, Thermo Electron Corp., San Jose, CA) connected to a nano-LC system (Paradigm, Michrom BioResource, Inc., Auburn, CA). The eluents were 5 mM ammonium acetate, pH 9.6, 2% CH_3CN (pump A) and 5 mM ammonium acetate, pH 9.6, 80% CH_3CN (pump B). The borohydride-reduced N-linked oligosaccharides were separated on a Hypercarb (0.1 × 150 mm, Thermo Electron Corp.) with a linear gradient of 5–20% B in 45 min and 20–50% B in 45 min. A full MS^1 scan (m/z 450–2000) by FT-ICR MS followed by data-dependent MS^n for the most abundant ions was performed in both negative and positive ion modes as previously reported (36).

RESULTS

Overexpression of GnT-V Stimulated α3β1 Integrin-mediated Cell Motility—It has been reported that overexpression of GnT-V in epithelial cells results in a loss of contact inhibition, increased cell motility in athymic nude mice (7), and an enhanced metastasis (8). In this study, experiments were first designed to determine whether GnT-V overexpression could affect cell migration on different ECMs. The extent of haptotaxis toward LN5, FN, and COL, specific ligands for α3β1, α5β1, and α1β1 and α2β1 integrin, respectively, was observed in MKN45 cells transfected with mock, GnT-III, or GnT-V. In the case of the GnT-V transfecants on LN5, the number of transwell cells migrating to the lower surface of the membrane was considerably increased (p = 0.001), the overexpression of
GnT-III resulted in a decrease in cell migration on LN5 compared with mock (p = 0.0013) (Fig. 1A). However, the migration of these three types of cells on FN was barely detectable. Although GnT-III transfection resulted in a decreased cell migration on COL compared with mock (p = 0.007), GnT-V transfection failed to induce a significant in increase in cell migration on COL (Fig. 1B), suggesting that MKN45 cells may favor LN5 as an ECM for cell migration induced by GnT-V. These results further supported the view that α3β1 integrin, one of the most abundant integrins in epithelial cells, is distinct from other integrins, such as α5β1 integrin, and preferentially promotes cell migration (37). Moreover, the cell migration of GnT-V transfectant on LN5 was strongly inhibited by the presence of function-blocking antibodies against integrin α3 or/and β1 subunit, suggesting that the GnT-V-induced cell migration on LN5 was mainly mediated by α3β1 integrin (Fig. 2). These results indicated that overexpression of GnT-V resulted in an increase in α3β1 integrin-mediated motility.

**Overexpression of GnT-III Inhibited α3β1 Integrin-mediated Cell Migration Induced by GnT-V**—The Overexpression of GnT-III has been reported to inhibit cell migration by enhancement of E-cadherin-mediated homotypic adhesion (17) and by inhibiting α5β1 integrin-mediated cell migration (38). In addition, in vitro GnT-V cannot use the product of GnT-III, a bisected oligosaccharide, as a substrate (12), so experiments were then designed to determine whether the introduction of GnT-III prevents α3β1 integrin-mediated cell migration enhanced by GnT-V. The efficiency of transfection was confirmed by immunostaining with anti-GnT-III antibody and determined to be more than 80% (data not shown). As shown in Fig. 3, the transfection of GnT-III into the GnT-V transfectant resulted in a significant decrease in cell migration compared with the GnT-V transfectant (p = 0.002). However, the inhibition was not observed after transfection of the GnT-III-inactive mutant, suggesting that the activity of GnT-III was essential for the negative regulation of GnT-V-induced cell migration. Therefore, we proposed that GnT-III directly counteracted the effect of GnT-V on α3β1 integrin-mediated cell migration.

**Transfection of GnT-III Had No Effect on the Expression of GnT-V and Integrin α3 Subunit**—To explore the possible mechanisms involved in the inhibition of GnT-III to GnT-V-induced cell migration, we first attempted to determine whether the overexpression of GnT-III affected the expression of GnT-V and α3 subunit expressed on the cell surface by means of blotting a total cell lysate with the GnT-III antibody and determined to be more than 80% (data not shown). As shown in Fig. 3, the transfection of GnT-III into the GnT-V transfectant resulted in a significant decrease in cell migration compared with the GnT-V transfectant (p = 0.002). However, the inhibition was not observed after transfection of the GnT-III-inactive mutant, suggesting that the activity of GnT-III was essential for the negative regulation of GnT-V-induced cell migration. Therefore, we proposed that GnT-III directly counteracted...
the expression of integrin α3 subunit on the cell surface also remained unchanged among the transfectants of GnT-III plus GnT-V, GnT-III mutant plus GnT-V, and GnT-V (Fig. 4B). These results suggested that the inhibition of GnT-III to GnT-V-induced cell migration could not be ascribed to a change in the expression levels of GnT-V and/or α3 subunit on the cell surface.

Transfection of GnT-III Had No Effect on the Activity of GnT-V—Since the introduction of GnT-III had no effect on the expressions of GnT-V and α3 subunit, we further determined if the overexpression of GnT-III suppressed the activity of GnT-V. Since this was a transient transfection, the activity of GnT-III was checked at six time points from 24 to 144 h after the transfection. We found that GnT-III activity reached the highest level 48 h after transfection (Fig. 5A), and there was no corresponding activity in GnT-III mutant (data not shown). The expression level of GnT-III mutant was similar to that of wild-type GnT-III confirmed by blotting with GnT-III antibody, and equivalent amounts of loaded proteins were verified by blotting with anti-actin antibody (Fig. 5B). As shown in Fig. 5C, GnT-V activity was found to be stable, even in the period (48 h after transfection) where the activity of GnT-III reached the highest level in these double-transfected cells. This result indicated that GnT-III inhibited GnT-V-induced cell migration not due to the suppression of GnT-V activity.

Increased GnT-III Product but Decreased GnT-V Product on Integrin α3 Subunit—The modification of N-glycosylation contributes to the functions of integrins (39). Here, we checked whether changes of α3β1 integrin modification had occurred in these transfectants. The integrins were immunoprecipitated from these transfectants and then probed with Eα4-PHA lectin, which preferentially binds to bisecting GlcNAc residues in N-glycans, or Lα4-PHA lectin, which binds to β1,6-branched GlcNAc. Fig. 6A (top) shows that the transfection of GnT-III to the GnT-V transfectant resulted in an increase in the GnT-III product on the integrin α3 subunit. More interestingly, the level of GnT-V product on α3 was decreased in the double transfectants (Fig. 6A, middle). Consistent with this observation, transfection of the GnT-III mutant failed to induce such changes. Equivalent amounts of the α3 subunit were verified by blotting α3-immunoprecipitated lysates (Fig. 6A, bottom). Moreover, cell lysates were subjected to SDS-PAGE, followed by a lectin blot. A comparison of bands especially around 117–200 and 60–89 kDa among these transfectants consistently indicated that increased GnT-III products but decreased GnT-V products presented on the glycoproteins after the introduction of GnT-III to the GnT-V transfectant (Fig. 6B). Furthermore, to further confirm such competition on the α3 sub-
unit, we purified this integrin from GnT-III, GnT-V, and GnT-III plus GnT-V transfectants using a GD6 peptide affinity column combined with a wheat germ agglutinin affinity column. The purity was evaluated by SDS-PAGE followed by silver staining (data not shown). The purified α3 subunit was cut from gels and then subjected to LC/MS as described under “Experimental Procedures.” As shown in Fig. 6, C and D, mass spectra of desialylated N-glycans were obtained from the α3 expressed in GnT-III, GnT-V, and GnT-III plus GnT-V transfectants, respectively, by a full MS1 scan (m/z 450–2000). Carbohydrate structures of the major peaks were deduced from the m/z values of protonated ions in the full MS1 spectra obtained by FT-ICR MS and product ions in MS2,3 spectra (Fig. 6D). Based on the presence of [HexNAc-Hex-HexNAc-HexNAc-OH + H]+ (m/z 792) and [HexNAc-Hex-HexNAc-(dHex)HexNAc-OH + H]+ (m/z 938) in MS2,3 spectra, peaks 4, 5, 7, 8, 10, and 11 were determined as bisected glycans. Peak 4 was deduced to be a biantennary oligosaccharide, the major peak in the GnT-III transfectant. After the transfection of GnT-III into the GnT-V transfectant, peak 4 was increased compared with that of the GnT-V transfectant, whereas peak 6, which is the major peak in the GnT-V transfectant, was decreased. For the present technique, the branched form is determined by analyzing the sialylated oligosaccharides by LC/MS in the negative ion mode. Referring to the result of the L3-PHA lectin blot and the fact that peak 6 is the major one in the GnT-V transfectant, peak 6 could be deduced the β1,6-branched GlcNAc form, although only bisialylated forms were detected by MS. The MS data also revealed that peaks 7 or 8 and 9, 10, or 11 were triantennary and tetraantennary oligosaccharides, respectively, from the presence of their corresponding trisialylated and tetrasialylated forms. Peaks 1 and 2 were high mannos oligosaccharides. To further quantify the competition, we used the MS results to show that for GnT-III products (represented by the sum of the peaks 4, 5, 7, 8, 10, and 11), the proportion was, respectively, 79.5, 29.5, and 48.5% among the transfectants of GnT-III, GnT-V, and GnT-III plus GnT-V; for GnT-V products (represented by the sum of peaks 6 and 9), the proportion was 1.2, 34.9, and 18.1%, respectively, among the transfectants of GnT-III, GnT-V, and GnT-III plus GnT-V. Consistent with the results shown in Fig. 6A, these data strongly suggested that GnT-III transfection resulted in increased bisecting GlcNAc but decreased β1,6-branched GlcNAc on the α3 subunit. However, the N-glycan proportions partially, but not totally, are correlated with the extent of the modification in cell migration observed (Fig. 3), since only N-glycans located on some motifs of integrins have been proposed to influence their conformations and therefore to regulate their functions (40). Taken together, these results suggested the following; α3 was a common target of GnT-III and GnT-V, and the priority taken by GnT-III in the competition resulted in the inhibition of GnT-V modification.

Increased β1,6-Branched GlcNAc as Well as Cell Migration in GnT-III Knockdown Cells—To further identify the competition of GnT-III and GnT-V definitely, we developed an RNA interference strategy to efficiently silence GnT-III expression in CHPI34 cells, which express endogenous GnT-III and GnT-V. After retroviral infection, CHPI34 cells were selected based on their resistance to G418 as described under “Experimental Procedures.” GnT-III activity was effectively down-regulated by 70%, compared with those in parent and mock cells (Fig. 7A), whereas GnT-V activity, as a control, showed no significant changes (data not shown). A quantitative real time PCR analysis also indicated the down-regulation of RNA interference-directed GnT-III mRNA expression in these cells (Fig. 7B). We then tested cell migration on LN5 and found that GnT-III knockdown resulted in an increased cell migration compared with mock cells (Fig. 7C and D). We further investigated the N-glycans on the α3 subunit. As shown in Fig. 7E, increased β1,6-branched GlcNAc but decreased bisecting GlcNAc on α3 was found in the GnT-III knockdown cells, compared with those in the mock cells. Together with the data in Fig. 6, these data provided the evidence to show that GnT-III inhibited

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the functions of GnT-V by competing for the modification of the same protein in living cells, resulting in the positive or negative regulation of its biological functions.

DISCUSSION

It has long been thought that the product of GnT-V, β1,6-GlcNac branching of N-glycans, contributes directly to cancer progression and metastasis (6). Animal studies have shown that GnT-V-deficient transgenic mice experience attenuated tumor growth and metastasis (3). In human, the activity and/or expression of GnT-V is elevated in multiple types of tumors (41, 42), and high levels of these enzymes or their cognate sugars are correlated with metastasis and a poor patient prognosis (41, 43). In addition, GnT-V-modified cell surface receptors prolonged the turnover by inhibiting endocytosis (10) or resistance to degradation by protease (26). These results suggest that GnT-V may contribute to cancer metastasis through stabilizing target proteins. On the other hand, the introduction of GnT-III leads to a reduced metastatic potential. Moreover, those transfectants displayed decreased cell motility and attachment to laminin and collagen (14). Thus, it appears that GnT-V and GnT-III regulate cell migration and invasion as well as metastasis in opposite manners. In fact, GnT-III could be considered to be an antagonist of GnT-V, because bisecting GlcNAc renders the biantennary substrate inaccessible to GnT-V (13).

The α3β1 integrin is one of the most important proteins that mediate cell motility and invasion and appears to be one of the plausible target proteins of GnT-V in promoting cancer metastasis. In fact, Pochec et al. (25) reported that β1,6-branched structures were highly expressed in high metastatic melanoma, compared with low metastatic melanoma. In the present study, we found for the first time that GnT-III and GnT-V competitively modify the same target, integrin α3 subunit, thereby regulating its functions. We demonstrated that GnT-III transfection to the GnT-V transfectant resulted in the inhibition of α3β1 integrin-mediated cell migration, due to an increase of

![Image](Image.png)

**FIGURE 6. Increased product of GnT-III but decreased product of GnT-V on integrin α3 subunit after transfection of GnT-III into GnT-V transfectant.** A, whole cell lysates were immunoprecipitated (IP) with anti-α3 antibody, and the resulting immunocomplexes were subjected to 7.5% SDS-PAGE under reducing conditions. The blots were probed by E4-PHA (top), L4-PHA (middle) and anti-α3 antibody (bottom), respectively. B, lectin blotting was performed with E4-PHA and L4-PHA using cell lysates from different transfectants. C, base peak chromatograms and observed m/z values (charge number) obtained by a full MS1 scan (m/z 450–2000) of N-linked oligosaccharides extracted from the gel-separated α3 subunit expressed in transfectants: GnT-III transfectant (top), GnT-V transfectant (middle), and GnT-III plus GnT-V transfectant (bottom). D, deduced structures of peaks 1–11. △, fucose; ●, galactose; ○, mannose; ■, N-acetylgalactosamine. WB, Western blot.
Increased cell migration and increased GnT-V product on α3 subunit in GnT-III knockdown cells. A, activities of GnT-III in GnT-III knockdown CHP134 cells. B, mRNA expression of GnT-III in knockdown cells. Quantitative analysis was performed by real time PCR. C, cell migration on LN5 (5 nM). Representative fields were photographed using a phase-contrast microscope. Arrowheads, migrated cells. D, quantification of migration of mock and GnT-III knockdown cells. The number of migrated cells were quantified and expressed as the means ± S.D. from three independent experiments. E, whole cell lysates were immunoprecipitated (IP) with anti-α3 antibody, and the resulting immunocomplexes were subjected to 7.5% SDS-PAGE under reducing condition. The blots were probed by E4-PHA (top), L3-PHA (middle), and anti-α3 antibody (bottom), respectively. KD, GnT-III knockdown cells.

Hypothetical model for the competition of GnT-III and GnT-V for integrin α3 subunit modification. The product of GnT-V contributes to the promotion of cell migration. The reaction represented by a dashed line may not be predominant in vivo, whereas it could occur in vitro. On the other hand, the product of GnT-III suppresses cell migration. More importantly, this product cannot be utilized as a substrate by GnT-V, therefore causing the decreased cell migration. Details of the effect of glycans on this integrin are a subject of further investigation in our future study.

It is noteworthy that GnT-III and GnT-V do not always oppositely regulate all glycoproteins. In this study, we found that GnT-III transfection causes a similar decrease, but to a lesser extent, in cell migration on COL compared with the result on LN5. However, on COL, GnT-V transfection did not result in an increase in cell migration, compared with mock. This suggested that β1,6-GlcNAc modification has little effect or only mild effects on α1β1 and α2β1 integrin, which are receptors for COL. In fact, we reported that the introduction of the bisection GlcNAc to the α5 subunit resulted in a reduced affinity in

bisecting GlcNAc, but a decreased β1,6-GlcNAc on the α3 subunit. However, the transfection of the GnT-III inactive mutant failed to induce such changes. Conversely, the competition was further confirmed by an RNA interference strategy to silence GnT-III in CHP134 cells, which express endogenous GnT-V and GnT-III. We found that GnT-III knockdown resulted in increased GnT-V product on the α3 subunit. Taken together, to the best of our knowledge, we presented a previously uncharacterized demonstration of the existence of competition for the same substrate between GnT-III and GnT-V in living cells (Fig. 8).

Two mechanisms have been proposed for the inhibition of cell motility by the overexpression of GnT-III: an enhancement in cell-cell adhesion and the down-regulation of cell-ECM adhesion (39). Our present study suggested one more; GnT-III competed with GnT-V for the modification of α3 subunit, causing a decrease in the product of GnT-V on α3 subunit. Luo et al. (40) had suggested that the changes in the glycan structure of integrin can affect its conformation and activity. They reported that in CHO-K1 cells, the addition of a glycan at the β1 I-like domain caused an increase in the distance between the β1 head and stalk domains, therefore inducing the integrin dimer to be a more extended (activated) integrin conformation (40). We suggested that the competition of GnT-III and GnT-V for the modification of α3 may cause changes in the glycan within key regions of this integrin, therefore causing the decreased cell migration. Details of the effect of glycans on this integrin are a subject of further investigation in our future study.

Concerning metastasis, other important glycosyltransferases cannot be overlooked (e.g. sialyltransferases). The modification of the β1 subunit by sialyltransferase makes this integrin capped with the negatively charged sugar, sialic acid. The abundance of sialic acids, especially elevated α2,6-sialylation (44), contributes...
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to cell motility and invasion (25, 45–47). Thus, it is possible that GnT-V mediates at least some of its effects on cell behavior via increased sialylation (41). The effect of GnT-III on sialylation is a topic that also merits further exploration.

In conclusion, this study reports for the first time that GnT-III competes with GnT-V for the modification of integrin α3 subunit in living cells (Fig. 8). This competition results in the inhibition of α3β1 integrin-mediated cell migration induced by GnT-V. The finding suggests that the competition between both enzymes occurs not only in vitro but also in vivo and might provide a new insight into unraveling the molecular mechanism of tumor metastasis.

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