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Author(s)	Isaji, Tomoya; Gu, Jianguo; Nishiuchi, Ryoko et al.
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Introduction of Bisecting GlcNAc into Integrin $\alpha_5\beta_1$ Reduces Ligand Binding and Down-regulates Cell Adhesion and Cell Migration*

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Tomoya Isaji[‡], Jianguo Gu^{‡§}, Ryoko Nishiuchi[¶], Yanyang Zhao[‡], Motoko Takahashi[‡],
Eiji Miyoshi[‡], Koichi Honke[¶], Kiyotoshi Sekiguchi[¶], and Naoyuki Taniguchi[‡]

From the [‡]Department of Biochemistry, Osaka University Graduate School of Medicine, B1, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan, the [¶]Division of Protein Chemistry, Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan, and the [§]Department of Molecular Genetics, Kochi University Medical School, Kohasu, Oko-cho, Nankoku, Kochi 783-8505, Japan

The enzyme β 1,4-*N*-acetylglucosaminyltransferase III (GnT-III) catalyzes the addition of a bisecting GlcNAc residue to glycoproteins, resulting in a modulation in biological function. Our previous studies showed that the transfection of the GnT-III gene into B16 melanoma cells results in a suppression of invasive ability and lung colonization. The suppression has been postulated to be due to an increased level of E-cadherin expression on the cell surface, which in turn leads to the up-regulation of cell-cell adhesion. In this study, we report on the effects of overexpression of GnT-III on cell-matrix adhesion. The overexpression of GnT-III, but not that of an enzymatic inactive GnT-III (D323A), inhibits cell spreading and migration on fibronectin, a specific ligand for integrin $\alpha_5\beta_1$, and the focal adhesion kinase phosphorylation. E₄-PHA lectin blot analyses showed that the levels of bisecting GlcNAc structures on the integrin α_5 subunit as well as α_2 and α_3 subunits immunoprecipitated from GnT-III transfectants were substantially increased. In addition, the affinity of the binding of integrin $\alpha_5\beta_1$ to fibronectin was significantly reduced by the introduction of the bisecting GlcNAc, to the α_5 subunit. These findings suggest that the modification of *N*-glycan of integrin by GnT-III inhibits its ligand binding ability, subsequently leading to the down-regulation of integrin-mediated signaling.

Nearly all secreted and cell surface proteins are glycosylated. The attached sugar chains have many biological functions, for example, cell-cell communication, signal transduction, protein folding, and stability (1). Oligosaccharides are synthesized through the action of glycosyltransferase, which catalyzes the formation of glycosidic bonds. β 1,4-*N*-Acetylglucosaminyltransferase (GnT-III)¹ catalyzes the transfer of GlcNAc residues in a β 1,4-linkage to the β 1,4-mannose residue in the core

region of *N*-glycans, producing a bisecting GlcNAc (Fig. 1). We previously purified GnT-III from rat kidney and cloned the cDNA from both the rat and human (2, 3). In a quest for the biological significance of GnT-III, we found that GnT-III suppresses the dimerization of TrkA, the receptor for nerve growth factor, and EGF receptor phosphorylation (4, 5). It is interesting to note that the metastatic capabilities of B16 mouse melanoma cell are down-regulated by the introduction of the GnT-III gene (6). This anti-metastatic effect has been partly ascribed to the effect of GnT-III on an increase in E-cadherin-mediated homotypic adhesion and a suppression of the phosphorylation of the E-cadherin- β -catenin complex on the cell-cell adhesion (7, 8).

Cell-extracellular matrix (ECM) interactions play essential roles during the acquisition of migration and invasive behavior of the cells. The integrin family consists of α and β heterodimeric transmembrane receptors for ECM and connects many biological functions, such as development, control of cell proliferation, protection against apoptosis, and malignant transformation (9). The fibronectin (FN) receptor integrin $\alpha_5\beta_1$ is one of representative ECM receptors and has particularly important roles in tumor growth invasion and malignant behavior (10). Although $\alpha_5\beta_1$ integrin-mediated adhesion is based on the binding of α_5 and β_1 subunits to a defined peptide sequence of FN, the strength of this binding is modulated by various factors including the glycosylation status of integrin. Integrin $\alpha_5\beta_1$ contains 14 and 12 potential asparagine-linked glycosylation sites on the α_5 and β_1 subunits, respectively. When human fibroblasts were cultured in the presence of 1-deoxymannojirimycin, an inhibitor of α -mannosidase II that prevents *N*-linked oligosaccharide processing, immature $\alpha_5\beta_1$ integrin receptors appeared at the cell surface, and FN-dependent adhesion was greatly reduced (11). The treatment of purified integrin $\alpha_5\beta_1$ with *N*-glycosidase F resulted in blocking of $\alpha_5\beta_1$ binding to FN and the inherent association of both subunits (12). Those studies suggest that the interaction of cell-surface integrin $\alpha_5\beta_1$ with FN is dependent on the glycosylation and processing status of integrin.

To elucidate the effects of the bisecting GlcNAc structure on the biological functions of integrin, $\alpha_5\beta_1$ integrin-mediated cell spreading, cell migration, and focal adhesion kinase (FAK) phosphorylation were investigated in GnT-III-overexpressed cells. The molecular mechanisms underlying the changes in these biological events were addressed by characterizing the integrin $\alpha_5\beta_1$ purified from GnT-III transfectants.

EXPERIMENTAL PROCEDURES

Antibodies—Monoclonal antibodies (mAbs) against the human integrin α_5 (8F1) and integrin β_1 subunits (4G2) were established and have been characterized previously (13, 14). A polyclonal antibody against

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§ To whom correspondence should be addressed. Tel.: 81-6-6879-3421; Fax: 81-6-6879-3429; E-mail: jgu@biochem.med.osaka-u.ac.jp.

¹ The abbreviations used are: GnT-III, *N*-acetylglucosaminyltransferase III; EGF, epidermal growth factor; ECM, extracellular matrix; FN, fibronectin; FAK, focal adhesion kinase; mAb, monoclonal antibody; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; FBS, fetal bovine serum; E₄-PHA, erythroagglutinating lectin; GnT-V, *N*-acetylglucosaminyltransferase-V; COL, collagen I; LN, laminin; BSA, bovine serum albumin.

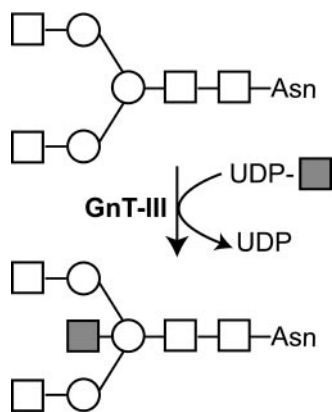


FIG. 1. Reaction pathway for biosynthesis of the bisecting GlcNAc by GnT-III. Square, N-acetylglucosamine; circle, mannose.

FAK and mAb against the integrin α_2 , α_3 , or α_5 subunit for a Western blot were obtained from Transduction Laboratories (Lexington, KY). A mAb against the human integrin β_1 subunit (TS2/16) was purchased from Pierce. A mAb against the human integrin α_3 subunit (P1B5) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A polyclonal antibody against the integrin α_2 subunit for immunoprecipitation was obtained from Chemicon International, Inc. (Temecula, CA). Monoclonal anti-phosphotyrosine antibody (4G10) and peroxidase-conjugate goat antibody against rabbit IgG were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). A peroxidase-conjugate goat antibody against mouse IgG was obtained from Promega (Madison, WI). A mouse control IgG and a fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin were obtained from DAKO (Glostrup, Denmark). A mouse mAb against the FN N-terminal domain (FN9-1) was obtained from Takara Bio, Inc. (Kyoto, Japan). Nonfunctional blocking mAb against the integrin α_5 subunit (5G10) was kindly provided by Dr. Toshinaga Maeda (Department of Medical Biochemistry, Shiga University of Medical Science, Shiga, Japan).

Cell Culture—A HeLa S3 cell line that stably expresses human GnT-III and mock transfectants was previously established by limited dilution using the LipofectAMINE reagent (Invitrogen) in our laboratory (15). This cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS).

Cell Adhesion Assay—Cell spreading assays were performed as described previously with minor modifications (13). Briefly, 96-well microtiter plates (Nunc, Wiesbaden, Germany) were coated with or without human plasma FN, collagen I (COL) (Sigma) or α_5 chain-containing laminin (LN) (Chemicon International, Inc., Temecula, CA) at different concentrations in phosphate-buffered saline (PBS) overnight at 4 °C and blocked with 1% BSA in PBS for 1 h at 37 °C. The cells were detached with PBS containing 1 mM EDTA with or without trypsin, washed with serum-containing DMEM, and then resuspended in serum-free DMEM with 0.1% BSA at a concentration of 4×10^4 cells/ml. The cells were preincubated with functional blocking mAbs against α_5 (8F1), β_1 (4G2), or mouse control IgG at a final concentration at 10 μ g/ml at room temperature for 10 min before plating. After an additional 20 min of incubation, the nonadherent cells were removed by washing with PBS, the attached cells were fixed with 3% formaldehyde, and three random fields were then scored using phase contrast microscopy. Only cells that contained a fully formed lamellipodia were counted as spread cells. The percentage of spreading cells was determined from the ratio of the number of spreading cells and the number of total cells in each field.

Migration Assay—Transwells (BD Biosciences) were coated with 15 nM of FN, COL, or LN in PBS by an overnight treatment at 4 °C followed by an incubation with 1% BSA for 1 h at 37 °C. The cells were detached with trypsin/EDTA, washed once with DMEM containing 10% FBS, and resuspended in DMEM containing 1% FBS at 1×10^6 cells/ml. 100- μ l aliquots of the cell suspension were preincubated with the functional blocking mAb against integrin α_5 (8F1) or mouse control IgG at a final concentration at 10 μ g/ml for 10 min and then added to the upper side of each chamber. After 3 h of incubation at 37 °C, the remaining cells on the upper side of the chamber were carefully scraped off with a cotton swab. The cells that had migrated to the lower surface of the membrane were fixed with 3% formaldehyde and stained with 0.3% Crystal Violet for 30 min. The cells on the lower surface of the mem-

brane were observed under a phase contrast microscope.

Flow Cytometry Analysis—Flow cytometry analysis was performed as described previously with minor modifications (16, 17). Briefly, the cells in semi-confluent conditions were detached from 10-cm culture dishes using trypsin containing 1 mM EDTA and resuspended in 50 μ l of PBS. The suspended cells ($5\text{--}10 \times 10^6$ cells) were incubated with and without a primary antibody (8F1 for the α_5 subunit or TS2/16 for the β_1 subunit) at a final concentration of 4 μ g/ml for 1 h on ice. The cells were washed three times with PBS, then resuspended in 50 μ l of PBS containing fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin, and further incubated for 1 h on ice. After washing three times with PBS, flow cytometry analyses were performed using a FAC-Scan instrument (BD Biosciences) operated with CELLquest software.

Immunoprecipitation and Western Blot—The cells were washed with ice-cold PBS, lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 130 mM NaCl, 1% (w/v) Nonidet P-40, 2 μ g/ml aprotinin, 5 μ g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 10 mM NaF) on ice and centrifuged at $12,000 \times g$ for 15 min at 4 °C. The supernatants were collected, and the protein concentrations were determined using a protein assay CBB kit (Nacalai tesque, Kyoto, Japan) with a BSA standard. The samples were incubated with 2 μ g of each antibody for 1 h and then with 15 μ l of protein G-Sepharose 4 Fast Flow (Amersham Biosciences) for 1 h at 4 °C. The immunoprecipitate was washed three times with lysis buffer. For a Western blot, equal amounts of proteins were subjected to SDS-PAGE and then transferred to nitrocellulose membranes (Schleicher & Schuell). The membrane was incubated with primary and secondary antibodies for 1 h each, and detection was performed by ECL (Amersham Biosciences) according to the manufacturer's instructions.

Cell Surface Biotinylation—Cell surface biotinylation was performed as described previously with minor modifications (15). Briefly, semi-confluent HeLa S3 cells were washed twice with ice-cold PBS and then incubated with ice-cold PBS containing 0.2 mg/ml sulfo-succinimidobiotin (Pierce), for 1 h at 4 °C. After incubation, the cells were washed three times with ice-cold PBS, scraped, and lysed with RIPA 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 μ g/ml aprotinin, 5 μ g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The resulting cell lysates were immunoprecipitated with the anti-integrin α_2 , α_3 (P1B5), or α_5 (8F1) antibodies as described above. The immunocomplex was subjected to 7.5% SDS-PAGE and 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, Inc., Burlingame, CA) and an ECL kit.

Purification of Integrin $\alpha_5\beta_1$ —The purification of integrin $\alpha_5\beta_1$ was performed as described previously, with minor modifications (13, 18, 19). Briefly, HeLa S3 cells were cultured with DMEM containing 10% FBS and 20 mM HEPES buffer (pH 7.4) in a roller bottle (Corning Costar, Osaka, Japan). Confluent HeLa S3 cells were detached with TBS (20 mM Tris-HCl, pH 7.5, 130 mM NaCl) containing 2 mM EDTA and washed with TBS(+) (20 mM Tris-HCl, pH 7.5, 130 mM NaCl, 1 mM CaCl_2 , and 1 mM MgCl_2). The cell pellets (~ 40 ml) were extracted with 160 ml of TBS(+) containing 100 mM 1-*O*- α -octyl- β -D-glucoside and 1 mM phenylmethylsulfonyl fluoride at 4 °C. The cell extract was applied to an affinity column prepared by coupling 20 mg of the 155/145-kDa thermolysin fragments of human plasma FN to 2 ml of cyanogen bromide-activated Sepharose (Amersham Biosciences). The bound integrin $\alpha_5\beta_1$ was eluted with TBS(+) containing 0.5 mg/ml synthetic GRGDS peptide (Peptide Institute, Inc., Osaka, Japan). The elutes containing integrin $\alpha_5\beta_1$ were further purified on 1 ml of wheat germ agglutinin-agarose column (Seikagaku Corp., Tokyo, Japan) and eluted with TBS(+) containing 0.2 M *N*-acetyl-D-glucosamine. The purity of the integrin was verified by SDS-PAGE by means of a silver staining kit (Daichi Pure Chemicals Co., Ltd., Tokyo, Japan).

Lectin Blot Analysis—The purified or immunoprecipitated integrins were subjected to 7.5% SDS-PAGE and transferred to nitrocellulose membranes as described above. The membrane was blocked with 3% BSA (w/v) in TBST and then incubated with 2 μ g/ml solution of biotinylated E₄-PHA (Seikagaku Corp., Tokyo, Japan) in TBST for 30 min at room temperature. After washing with TBST three times, lectin-reactive proteins were detected using a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA) and an ECL kit.

Integrin Liposome Binding Assay—The purified integrins (5 μ g/ml) were reconstituted into liposomes as described previously (13). The radioactive labeled integrin liposomes were added to a 96-well microtiter plate coated with 50 μ l of 0–100 nM FN or 20 nM mAb against the integrin α_5 subunit (8F1) or the integrin β_1 subunit (TS 2/16) and then

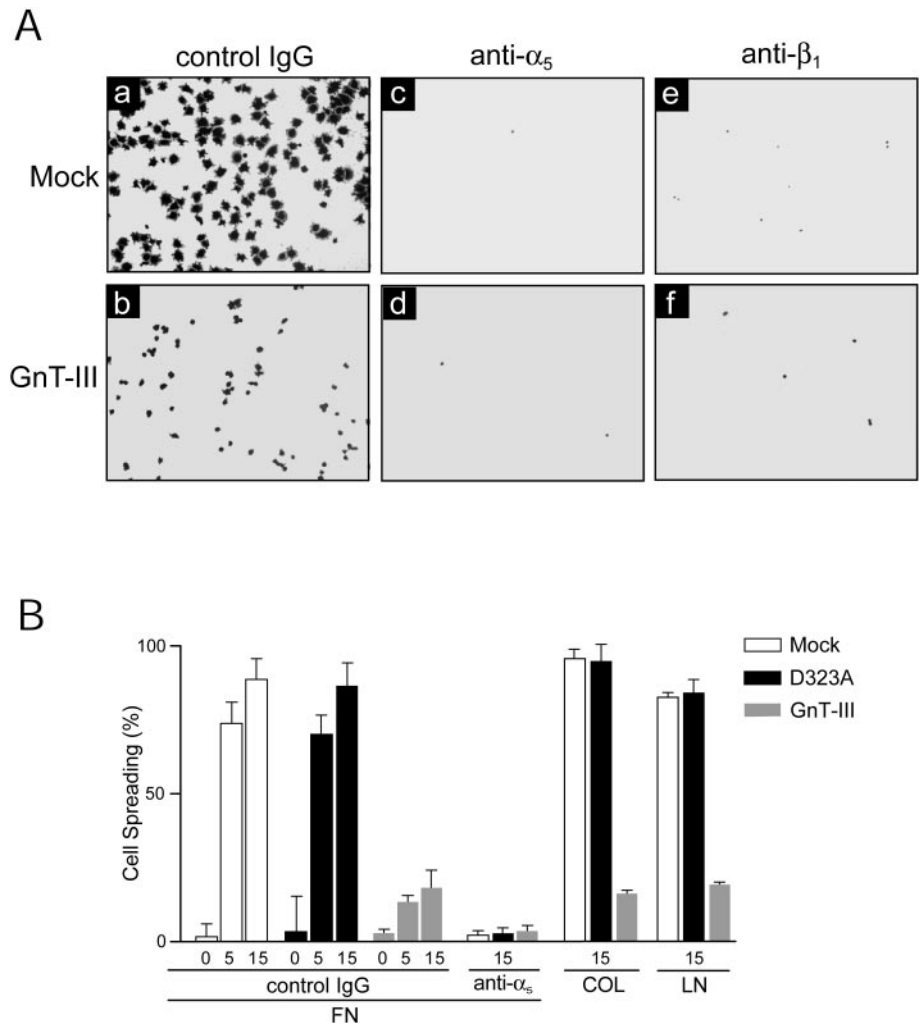


FIG. 2. Effects of the overexpression of GnT-III on cell spreading. HeLa S3 cells were detached, preincubated with mouse control IgG (A, panels a and b) or function-blocking mAbs against α_5 (A, panels c and d) or β_1 (A, panels e and f) integrin for 10 min, and then replated on 96-well plates coated with FN (5 or 15 nM), COL (15 nM), or LN (15 nM). After incubation (20 min for FN and COL or 60 min for LN), the attached cells were then fixed and stained with 0.3% Crystal Violet. A, representative fields were photographed using a phase contrast microscope. The rounded cells shown in panel b were not considered to be spread cells. B, the percentages of spread cells were quantified and expressed as the means \pm S.D. from three independent experiments. D323A, an enzymatic inactive mutant of GnT-III.

incubated at room temperature for 6 h. The plates were washed twice with TBS(+), and the bound liposomes were recovered in 1% SDS (100 μ l/well). The radioactivities of the bound liposomes were quantified using a Packard TRI-CARB 1500 liquid scintillation counter (Research Parkway Meriden, CT).

Binding Assay for Integrin $\alpha_5\beta_1$ and Soluble FN—The purified integrins were immobilized on 96-well microtiter plates via nonfunctional blocking anti-integrin α_5 antibody (5G10) precoating the plates. Amounts of the purified integrins captured on plates were verified by an enzyme-linked immunosorbent assay with biotinylated anti β_1 (TS2/16) as described (19). Those wells were incubated with TBS(+) containing various concentrations of FN (0–100 nM) for 2 h at room temperature, washed twice with 25 mM HEPES (pH 7.6) containing 1 mM CaCl_2 and MgCl_2 , and then fixed by incubation with 2.5% glutaraldehyde for 10 min. The bound FN was quantified by enzyme-linked immunosorbent assay with biotinylated anti-FN antibody (FN9-1).

RESULTS

Overexpression of GnT-III Inhibited Integrin-mediated Cell Adhesion—To date, several lines of evidence have shown that the overexpression of GnT-III inhibits growth factor receptor functions. For example, in U373 MG cells overexpressing GnT-III, the binding of EGF to its receptor and receptor autophosphorylation are inhibited (5). On the other hand, in PC12 cells, the N-glycan of TrkA, when modified by a bisecting GlcNAc causes functional changes by disrupting the dimerization of TrkA (4). In present study, the effects of GnT-III overexpression on cell adhesion were examined. After 20 min of incubation on FN, cell spreading was observed in HeLa S3 cells that had been transfected with mock or a GnT-III mutant, D323A, which serves as a negative molecule involved in competition with the

corresponding endogenous one as described previously (20). Interestingly, the $\alpha_5\beta_1$ integrin-mediated cell spreading was completely blocked in cells overexpressing wild-type GnT-III (Fig. 2). Because HeLa cell predominantly expresses integrin $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_5\beta_1$ (21), we also performed a spreading assay on COL and LN, which are ligands of integrin $\alpha_2\beta_1$ and $\alpha_3\beta_1$, respectively, and found that integrin $\alpha_2\beta_1$ - or integrin $\alpha_3\beta_1$ -mediated cell spreading was also inhibited in GnT-III transfectants (Fig. 2). The inhibition of cell spreading that was modulated by overexpression of GnT-III is not an artifact of proteolysis of those integrins, because a similar result was also observed in those cells detached by EDTA without trypsin (data not shown). In addition, the inhibition of cell spreading on FN by the overexpression of GnT-III was confirmed in other two HeLa S3 cell clones as well as in human mammary carcinoma MCF7 cells (data not shown).

Expression Levels of Integrin $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_5\beta_1$ on the Cell Surface Are Not Influenced by Overexpression of GnT-III—One possible explanation for the effects of GnT-III on integrin-mediated cell adhesion could be changes in the expression levels of integrin $\alpha_2\beta_1$, $\alpha_3\beta_1$, or $\alpha_5\beta_1$ on the cell surface. To test this possibility, expression levels of integrins $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_5\beta_1$ on cell surface were biochemically investigated by the means of the biotinylation of cell surface proteins and subsequent immunoprecipitation using anti-integrin antibodies. The levels of expression of integrin $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_5\beta_1$ on the cell surface were not influenced by the overexpression of GnT-III (Fig. 3A). Furthermore, expression levels of integrin α_5 and β_1

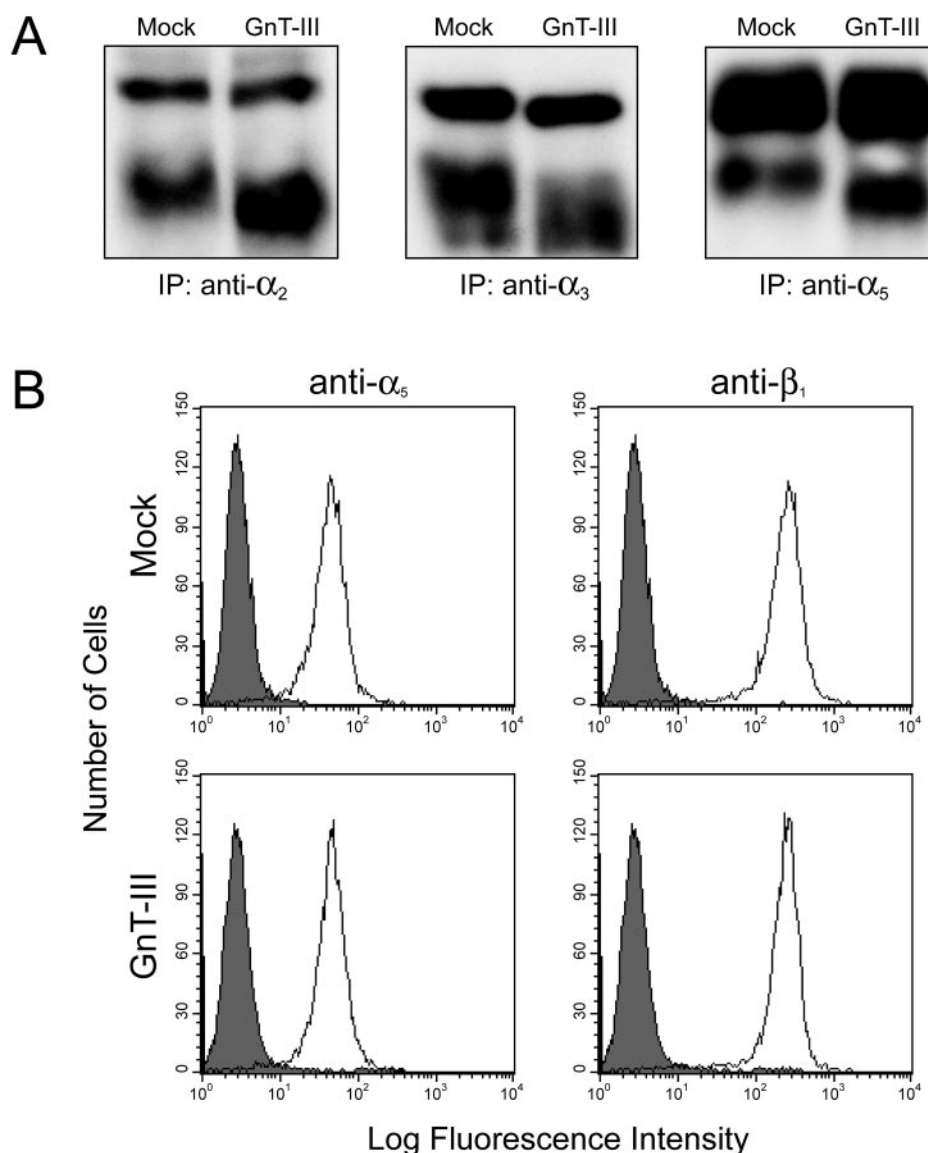


FIG. 3. Expression levels of integrin α_2 , α_3 , α_5 , and β_1 on cell surface were not affected by overexpression of GnT-III. *A*, cells were biotinylated, and whole lysates were immunoprecipitated (IP) with anti-integrin α_2 , α_3 , or α_5 antibodies, respectively. The samples were subjected to 7.5% SDS-PAGE and transferred to a nitrocellulose membrane, and the biotinylated proteins were detected as described under "Experimental Procedures." *B*, those mock (upper panels) and GnT-III (lower panels) transfectants were incubated with mAbs specific to α_5 (left panels) and β_1 (right panels) subunits and then stained with fluorescein isothiocyanate-conjugated secondary antibody for flow cytometry analysis. Negative control staining (shaded histogram) was done without the first antibody.

on cell surface were also quantified by flow cytometric analysis. Consistent with the assay by biotinylation, the level of expression of integrin $\alpha_5\beta_1$ on the cell surface was unchanged in the GnT-III transfectants (Fig. 3*B*).

Comparison of Glycosylation of $\alpha_2\beta_1$, $\alpha_3\beta_1$, or $\alpha_5\beta_1$ Integrin Immunoprecipitated from Mock and GnT-III Transfectants—To analyze alterations in the carbohydrate structure of integrin $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_5\beta_1$, those integrins were immunoprecipitated from GnT-III and mock transfectants. The blot was probed with E_4 -PHA lectin, which preferentially binds to bisecting GlcNAc residues in *N*-glycans. Each α subunit from GnT-III transfectants showed a substantially increased reactivity to E_4 -PHA, compared with that from the mock transfectants (Fig. 4, upper panels). Equivalent amounts of loaded proteins were verified by reprobings the corresponding membranes with anti integrin α_2 , α_3 , and α_5 antibodies (Fig. 4, lower panels).

Overexpression of GnT-III Inhibited Integrin $\alpha_5\beta_1$ -mediated Cell Migration—Experiments were then designed to determine whether the overexpression of GnT-III could affect cell migration, which was assayed by the extent of haptotaxis toward FN, COL, or LN. In the case of GnT-III transfectants on FN, Transwell cells migrating to the lower surface of the membrane were considerably decreased, compared with the mock transfectants

(Fig. 5*A*) and the D323A GnT-III mutant (data not shown). However, cell migrations of both mock and GnT-III transfectants on COL or LN were barely detected (Fig. 5*B*), suggesting that HeLa S3 may favor FN as an ECM for cell migration. Cell spreading and migration of mock transfectants on FN were completely blocked by the presence of function-blocking antibodies against α_5 or β_1 but not by mouse control IgG (Figs. 2 and 5), suggesting that the cell spreading and migration on FN were mainly mediated by $\alpha_5\beta_1$ integrin.

The Level of FAK Phosphorylation Was Decreased in GnT-III Transfectants—Cell adhesion to ECM leads to intracellular phosphorylation. The activation of integrin $\alpha_5\beta_1$ on cell adhesion to FN preferentially enhances tyrosine kinase FAK (16, 22). To address the effects of GnT-III overexpression on integrin-mediated signaling, FAK phosphorylation was compared between mock transfectants and GnT-III transfectants. The results show that the level of FAK phosphorylation was decreased in GnT-III transfectants (Fig. 6), suggesting that GnT-III was able to negatively regulate the integrin $\alpha_5\beta_1$ -mediated signaling pathway. A similar attenuation in FN-transduced FAK phosphorylation was also observed in the two other GnT-III-overexpressed HeLa S3 clones (data not shown).

Ligand Binding of Integrin $\alpha_5\beta_1$ Purified from GnT-III Transfectants—To elucidate the mechanism by which GnT-III

FIG. 4. Glycosylation analysis of integrin α_2 , α_3 , and α_5 from GnT-III transfectants. The whole cell lysates were immunoprecipitated (IP) with anti-integrin α_2 , α_3 , or α_5 antibody, and those immunocomplexes were subjected to 7.5% SDS-PAGE under reducing condition. After electroblotting, the blots were probed by E₄-PHA (upper panel), and corresponding blots were reprobed with anti-integrin α_2 , α_3 or α_5 antibody (lower panel), respectively. The arrow indicates the migrated position of each corresponding α subunit integrin. WB, Western blot.

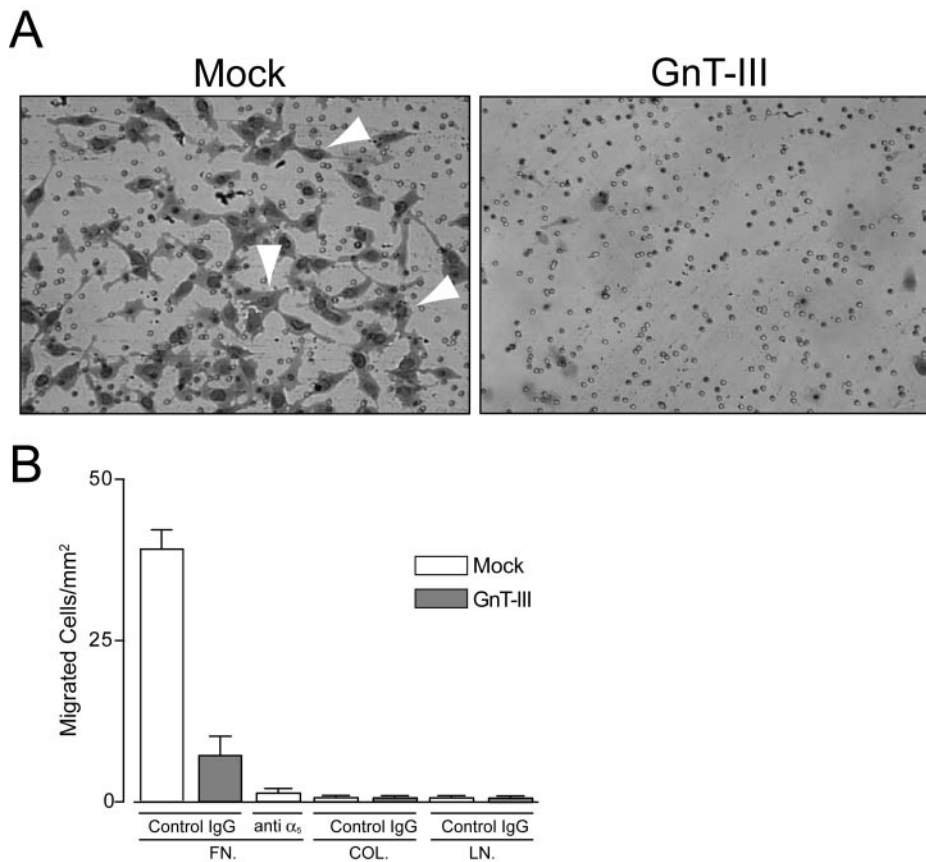
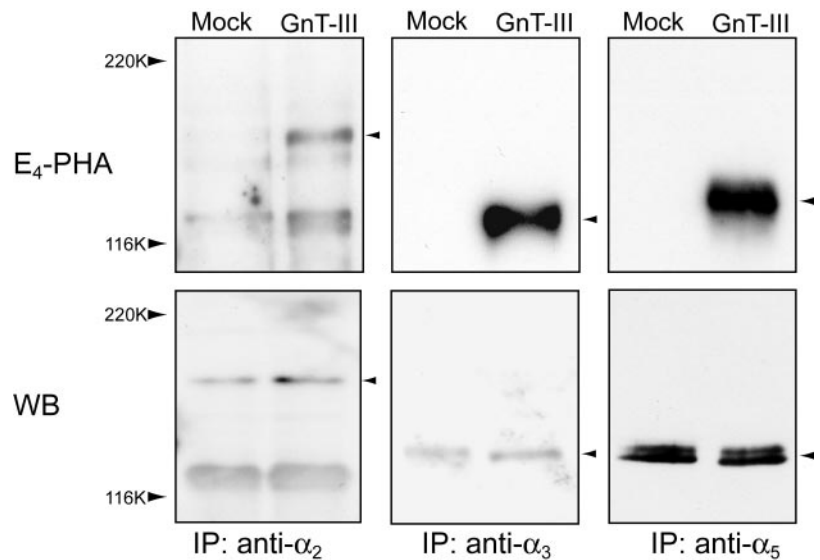


FIG. 5. Overexpression of GnT-III inhibited cell migration on FN. HeLa S3 cells were replated on upper chamber in the presence of 1% FBS and 10 μ g/ml mouse control IgG or anti- α_5 antibody. Cell migration was determined using the Transwell assay described under "Experimental Procedures." After incubation for 3 h, the cells that had migrated to the lower surface of the membrane were fixed and stained with 0.3% Crystal Violet. A, representative fields were photographed using a phase contrast microscope. The arrowheads indicate migrated cells. B, the numbers of migrated cells were quantified and expressed as the means \pm S.D. from three independent experiments.

transfection suppresses $\alpha_5\beta_1$ integrin-mediated cell spreading, migration, and FAK phosphorylation, we purified integrin $\alpha_5\beta_1$ from GnT-III and mock transfectants by a FN affinity column combined with a wheat germ agglutinin affinity column, and an integrin-liposome binding assay was carried out. The purity was evaluated by SDS-PAGE followed by silver staining. Two major bands, migrating at \sim 150 and 110 kDa on SDS-PAGE under nonreducing conditions (Fig. 7A, inset, left panel), corresponding to the immunoreactivity with the anti- α_5 and anti- β_1 antibodies, respectively, were detected (data not shown). In addition, we also analyzed glycosylation of the purified integrin $\alpha_5\beta_1$ by a FN affinity column. Surprisingly, the reactivity of E₄-PHA lectin was barely detected in the integrin β_1 subunit, suggesting that GnT-III selectively modifies some N-glycans

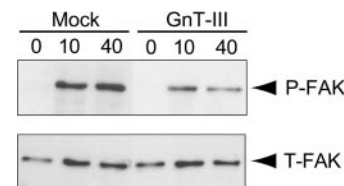


FIG. 6. Comparison of phosphorylation levels of FAK between GnT-III and mock transfectants. Serum-starved HeLa S3 cells were detached and held in suspension for 60 min and then replated on dishes coated with FN (15 nM) for the indicated times. The cell lysates were immunoprecipitated with anti-FAK antibody. The immunoprecipitates were blotted with either anti-phosphotyrosine antibody to detect phosphorylated FAK (P-FAK) or anti-FAK antibody to verify total amount of FAK (T-FAK).

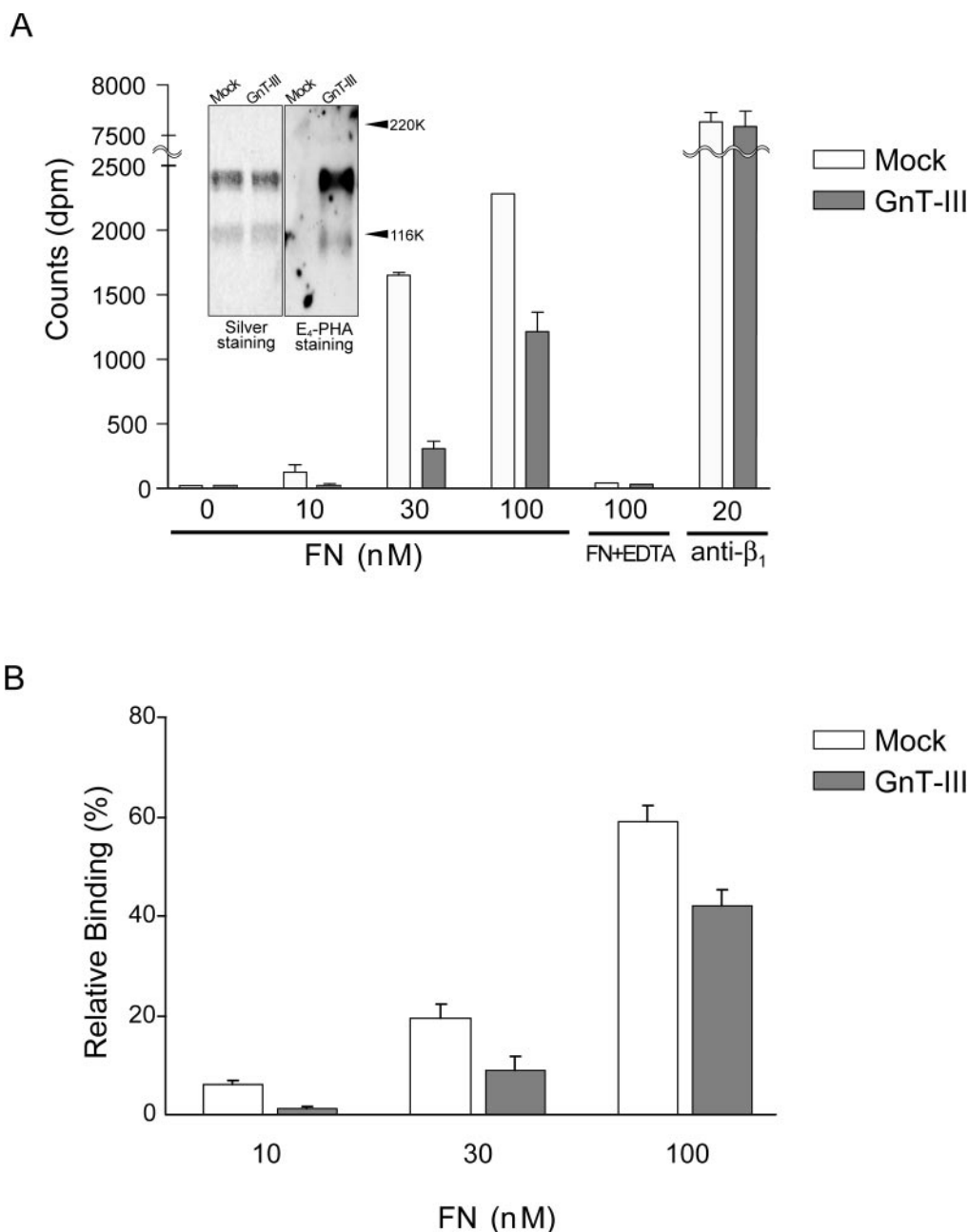


FIG. 7. **Inhibition of ligand binding for integrin $\alpha_5\beta_1$ purified from GnT-III transfectants.** A, the same amounts of purified integrin $\alpha_5\beta_1$ from mock and GnT-III transfectants were reconstituted in [3 H]phosphatidylcholine liposomes. The radioactively labeled liposomes were added to microtiter plates coated with FN or 20 nM mAb against integrin β_1 subunit. Net liposome binding was calculated by subtracting nonspecific binding to plates coated with BSA. The purity of integrin $\alpha_5\beta_1$ purified from mock and GnT-III transfectants was verified by silver staining under nonreducing condition as shown in the left panel of the inset, and the corresponding blots were probed by E₄-PHA as shown in the right panel of the inset. B, the purified integrins were immobilized on 96-well plates precoated with mAb 5G10, and FN at indicated concentrations were added to plates. The quantities of bounded FN were detected by enzyme-linked immunosorbent assay and expressed as percentages of TS2/16 binding to β_1 integrin, as described under "Experimental Procedures."

but not entire sugar chains (Fig 7A, inset, right panel). The same amounts of receptors were reconstituted in 3 H-labeled liposomes. The liposomes were incubated with various concentrations of FN precoated on 96-well plates. The binding of liposomes containing $\alpha_5\beta_1$ purified from GnT-III transfectants was significantly inhibited, compared with that of mock transfectants (Fig. 7A). Those amounts of integrins on the liposome surface were similar as verified by adhesion to the anti-integrin β_1 subunit antibody, TS 2/16 (*anti- β_1*). The specificities of the binding of $\alpha_5\beta_1$ integrin-FN were confirmed by treatment with 10 mM EDTA, which completely blocked integrin-FN interactions (*FN+EDTA*). On the other hand, we also performed a binding assay using solution phase FN and inte-

grin $\alpha_5\beta_1$ captured by antibody (5G10) precoating plates, and found the binding of soluble FN to the integrin $\alpha_5\beta_1$ -purified form GnT-III transfectants was also inhibited, compared with that purified from mock transfectants (Fig. 7B). Together, these results clearly indicate that the introduction of bisecting GlcNAc structures into integrin $\alpha_5\beta_1$ results in the inhibition of ligand binding.

DISCUSSION

Integrins are associated with many biological functions, such as development, control of cell proliferation, protection against apoptosis, and malignant transformation via cell-ECM adhesion processes (9, 23, 24). It is well known that synthetic

peptides and specific antibodies are capable of inhibiting integrin-ECM interactions, thereby modulating cell biological functions (25, 26). In the present study, we used an approach involving the modulation of the *N*-glycan structure by the overexpression of GnT-III and found that GnT-III substantially blocked $\alpha_5\beta_1$ integrin-mediated cell spreading, cell migration, and FAK phosphorylation. The reduction in affinity for FN, as evidenced by an integrin-liposome binding assay and FN-soluble binding assay, provides an explanation of the molecular mechanism underlying the down-regulation of these biological functions. In addition, integrin $\alpha_2\beta_1$ and $\alpha_3\beta_1$ -mediated cell spreading was also down-regulated, suggesting that GnT-III may have a wide spectrum of effects on cell-ECM interactions. Several lines of evidence suggest that *N*-glycans are required for integrin activation. The blocking of *N*-linked oligosaccharide processing of $\alpha_5\beta_1$ integrin results in an increase in immature integrins on the cell surface and a reduction in FN-dependent adhesion (11). Recently, Luo *et al.* (27) reported that the introduction of an *N*-glycosylation site into β_3 stabilizes the active conformation of $\alpha_{IIb}\beta_3$ integrin. In the present study, we show that the binding activity of $\alpha_5\beta_1$ integrin could also be modulated by introducing a single GlcNAc residue (bisecting GlcNAc). Collectively, these results strongly suggest that oligosaccharides attached to integrins play a crucial role in the conformational changes of integrins.

Integrin-mediated cell adhesion cooperates with growth factor receptors in the control of cell biological functions. Interestingly, the overexpression of GnT-III modulates not only integrin functions as described above but growth factor receptors as well. In fact, our previous studies showed that the overexpression of GnT-III suppressed the biological functions of growth factor receptors, including the EGF receptor (5, 28) and the nerve growth factor receptor, TrkA (4). U373 MG cells overexpressing GnT-III exhibit an inhibition of EGF binding to the cell surface and EGF receptor autophosphorylation. In contrast to the EGF receptor, although there is no significant difference in the capacity of nerve growth factor to bind to TrkA in PC12 cells overexpressing GnT-III, the *N*-glycans of TrkA that are modified by a bisecting GlcNAc causes functional changes by disrupting the dimerization process. Although the precise reason for why the bisecting GlcNAc negatively regulates these molecular functions remains to be elucidated, GnT-III may play an important role in biological functions such as growth factor- and integrin-mediated signalings.

N-Acetylglucosaminyltransferase V (GnT-V), which catalyzes the synthesis of β 1,6-GlcNAc branched *N*-linked oligosaccharides, has been proposed as one of the most important glycosyltransferase associated with tumor metastasis (29–32). The transfection of GnT-V enhances invasion and metastasis in mammary carcinoma cells (17). Conversely, tumor cell mutants that are selected for leucoagglutinating isoelectin resistance and found to be deficient in GnT-V activity are also deficient for metastasis (33, 34). On the other hand, GnT-III could be considered to be an antagonist to GnT-V, because bisecting GlcNAc rendered the biantennary substrate inaccessible to GnT-V, raising the possibility that β 1,6-GlcNAc branch formation could be suppressed by the induction of the GnT-III gene (35, 36). In an earlier study, we demonstrated that GnT-III suppresses lung metastasis in an animal model using highly metastatic B16 melanoma cells (6). In that case, GnT-III transfectants displayed both an increase in E-cadherin-mediated cell adhesion and a suppression of phosphorylation of β -catenin. Here, we clearly demonstrate that the overexpression of GnT-III significantly blocks cell adhesion on FN, thereby down-regulating cell migration. Accordingly, the overexpression of GnT-III inhibits tumor metastasis by at least two mechanisms:

enhancement of cell-cell adhesion and the down-regulation of cell-ECM adhesion. Interestingly, Pierce and co-workers (37) recently reported that the overexpression of GnT-V led to stimulation of $\alpha_5\beta_1$ integrin-mediated cell migration on FN. The current study is not inconsistent with their observation, considering the fact that GnT-III is an antagonist of GnT-V. The issue of whether cell adhesion on FN was decreased not only by the introduction of GnT-V into HT1080 cells but also by the overexpression of GnT-III in HeLa S3 cells (in this study) is unclear. Cell migration can be viewed as a process that is regulated by counterbalanced signals that control the rates of motility by multiple mechanisms. The strength of cell adhesion represents one such mechanism. In fact, extensive formation of focal adhesions has been linked to the slowing of cell migration (38, 39). However, cytoskeletal systems are also likely to play important roles in modulating the rates of cell migration (40, 41). It is noteworthy that the cell adhesion of HeLa S3 cells transfected with mock was obviously not sufficiently high to retard cell migration on FN, whereas it might be the case for HT1080 cells.

In conclusion, our results strongly suggest that the modulation of *N*-glycans of $\alpha_5\beta_1$ integrin by a bisecting GlcNAc significantly blocks FN binding, thereby inhibiting integrin-mediated cell spreading, migration, and signaling transduction. This study also provides a new insight into the molecular basis for the inhibition of tumor metastasis, because it is regulated by GnT-III. Further studies on identifying that site(s) of *N*-glycans bearing bisecting GlcNAc could shed light on the function of GnT-III and the structure of integrins.

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Introduction of Bisecting GlcNAc into Integrin $\alpha_5\beta_1$ Reduces Ligand Binding and Down-regulates Cell Adhesion and Cell Migration

Tomoya Isaji, Jianguo Gu, Ryoko Nishiuchi, Yanyang Zhao, Motoko Takahashi, Eiji Miyoshi, Koichi Honke, Kiyotoshi Sekiguchi and Naoyuki Taniguchi

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