ated cell migration and signaling. This study provides new insights into the biological functions of core fucosylation and the significance of the modification of *N*-glycans for α3β1 integrins.

Acknowledgments—We thank Takatoshi Nakagawa (Dept. of Glycotherapeutics, Osaka University Graduate School of Medicine) and Yoko Mizuno (Dept. of Biochemistry, Osaka University Graduate School of Medicine) for generous assistance.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 43, pp. 32122–32130, October 27, 2006

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

Received for publication, August 1, 2006 Published, JBC Papers in Press, August 28, 2006, DOI 10.1074/jbc.M607274200

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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 GnT-V transfectant. This enhanced cell migration was significantly blocked after the introduction of GnT-III. Consistently, an increase in bisected GlcNAc but a decrease in β1,6-GlcNAc-branched *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-branched *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)[3] 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

* This work was supported in part by Core Research for Evolution Science and Technology, Japan Science and Technology Agency, and the 21st Century Center of Excellence program from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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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 functions, such as development, control of cell proliferation, protection against apoptosis, and malignant transformation (18). Integrin $\alpha3\beta1$, 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, $\alpha3\beta1$ 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 glycosylation of integrins contributes to the tumor metastasis. Guo *et al.* reported that an increase in $\beta1$,6-GlcNAc sugar chains of the integrin $\beta1$ subunit resulted in the stimulation of cell migration (24). Interestingly, it has also been reported that the $\alpha3\beta1$ integrin expressed by the metastasis human melanoma cell lines, contained a higher level of $\beta1$,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 substrate 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 $\alpha3\beta1$ integrin, which is believed to be highly associated with tumor metastasis, and found that $\alpha3\beta1$ 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 $\alpha3\beta1$ integrin-mediated cell migration by competing with GnT-V for the modification of $\alpha3$ subunit.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Antibodies against integrin $\alpha3$ subunit (P1B5, 1-19), 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 $\beta1$ subunit was purchased from Chemicon International, Inc. (Temecula, CA). A peroxidase-conjugated goat antibody against mouse IgG was from Promega (Madison, WI). Biotinylated leukoagglutinating phytohemagglutinin (L_4-PHA), biotinylated erythroagglutinating phytohemagglutinin (E_4-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% CO_2. Human GnT-V cDNA (27) or GnT-III cDNA was inserted into a mammalian expression vector pCXNII (28). Vectors were then transfected into MKN45 cells by means of Lipofectamine (Invitrogen). Selection was performed by the addition of 500 μg/ml G418 (Sigma). CHP134 cells, a human neuroblastoma cell line expressing endogenous GnT-III and GnT-V, were cultured in RPMI 1640 medium (Sigma) containing 10% fetal bovine serum and penicillin (100 units/ml) and streptomycin (100 μg/ml) under a humidified atmosphere containing 5% CO_2.

Plasmids and Transient Virus Transfection—cDNAs encoding full-length human GnT-III or GnT-III 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 various experiments.

Construction of Small Interfering RNA Vector and Retroviral 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 follows: 5′-GATCCGTCAACCACGAGTTCGACCTTCA-AGAGAGGTCGAACTCGTGGTTGACTTTTTTAT-3′ and 5′-CGATAAAAAAGTCAACCACGAGTTCGACCTCTCT-TGAAGGTCGAACTCGTGGTTGACG-3′. The oligonucleotides 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 analyses of GnT-III mRNA expression in these clones were performed 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′-GCGTCATCAACGCCAT-CAA-3′ 5′-TGGACTCGCACACCACAAAG-3′. Normalization 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 pyridylaminated 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—Cell 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 phenymethylsulfonyl 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

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 E_4- or L_4-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 sulfosuccinimidobiotin (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, Inc., Burlingame, CA) and an ECL kit.

Migration Assay—Transwells (BD Biosciences) were coated with 5 nM recombinant LN5 as described previously (32), 10 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 of 5% fetal calf serum medium) were seeded in 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) (KQNCLSSRASFRGCVRNLRLSR 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 M *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 monoiodoacetate (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 (Supelco, Bellefonte, PA). LC/MS and LC/multistage MS (MS^n) was carried out on a quadrupole liner 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 $MS^{2,3}$ 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 transfectants on LN5, the number of transwell cells migrating to the lower surface of the membrane was considerably increased ($p = 0.001$), the overexpression of

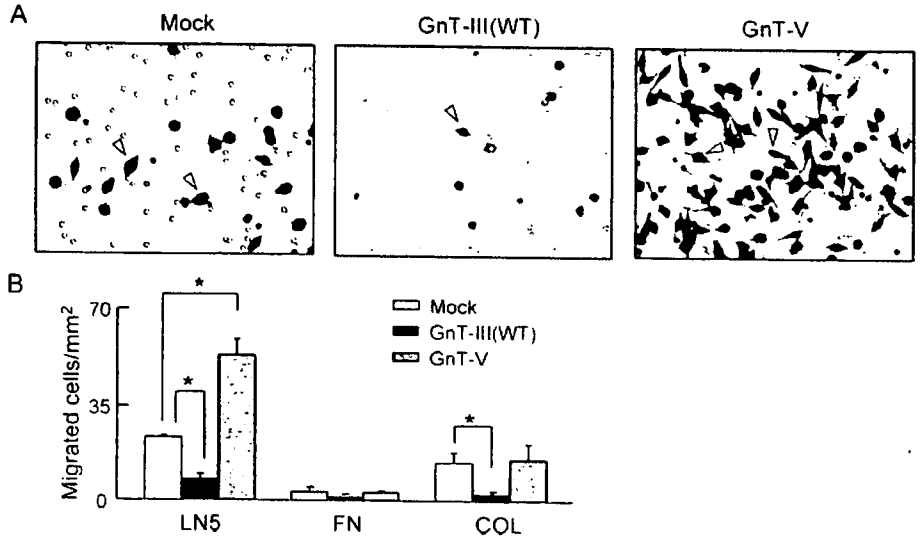

FIGURE 1. **Increased cell migration induced by GnT-V on LN5.** MKN45 cells were replated on the upper chamber in the presence of 5% fetal bovine serum. Cell migration was determined using the Transwell assay as described under "Experimental Procedures." After incubation overnight, the cells that had migrated to the lower surface of the membrane were fixed and stained with 0.3% Crystal Violet. *A*, cell migration on LN5 (5 nM). Representative fields were photographed using a phase-contrast microscope. The *arrowheads* indicate migrated cells. *B*, quantification of migration on LN5 (5 nM), COL (15 nM), and FN (15 nM). The numbers of migrated cells were quantified and expressed as the means ± S.D. from three independent experiments. *WT*, wild type.

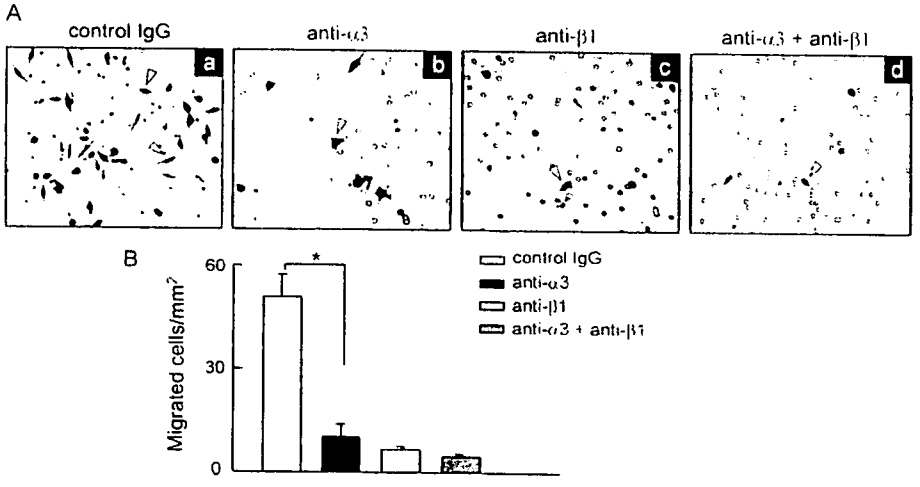

FIGURE 2. **GnT-V induced cell migration was mediated by $\alpha3\beta1$ integrin.** *A*, GnT-V-transfected MKN45 cells were detached, preincubated with mouse control IgG (*a*) or function-blocking monoclonal antibodies against $\alpha3$ (*b*) or $\beta1$ (*c*) or both (*d*) for 10 min, and then replated on the upper chamber coated with LN5 (5 nM) and checked by Transwell assay. Representative fields were photographed using a phase-contrast microscope. The *arrowheads* indicate migrated cells. *B*, quantification of migration on LN5 (5 nM). The numbers of migrated cells were quantified and expressed as the means ± S.D. from three independent experiments.

GnT-III resulted in a decrease in cell migration on LN5 compared with mock ($p = 0.0013$) (Fig. 1*A*). 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 increase in cell migration on COL (Fig. 1*B*), suggesting that MKN45 cells may favor LN5 as an ECM for cell migration induced by GnT-V. These results further supported the view that $\alpha3\beta1$ integrin, one of the most abundant integrins in epithelial cells, is distinct from other integrins, such as $\alpha5\beta1$ 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 $\alpha3$ or/and $\beta1$ subunit, suggesting that the GnT-V-induced cell migration on LN5 was mainly mediated by $\alpha3\beta1$ integrin (Fig. 2). These results indicated that overexpression of GnT-V resulted in an increase in $\alpha3\beta1$ integrin-mediated cell motility.

Overexpression of GnT-III Inhibited $\alpha3\beta1$ 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 $\alpha5\beta1$ 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 $\alpha3\beta1$ 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 $\alpha3\beta1$ integrin-mediated cell migration.

Transfection of GnT-III Had No Effect on the Expression of GnT-V and Integrin $\alpha3$ 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 $\alpha3$ subunit expressed on the cell surface by means of blotting a total cell lysate with the GnT-III antibody and the biotinylation of cell surface proteins followed by immunoprecipitation of $\alpha3$ using the corresponding antibody, since *N*-glycosylation plays an important role in the quality control of

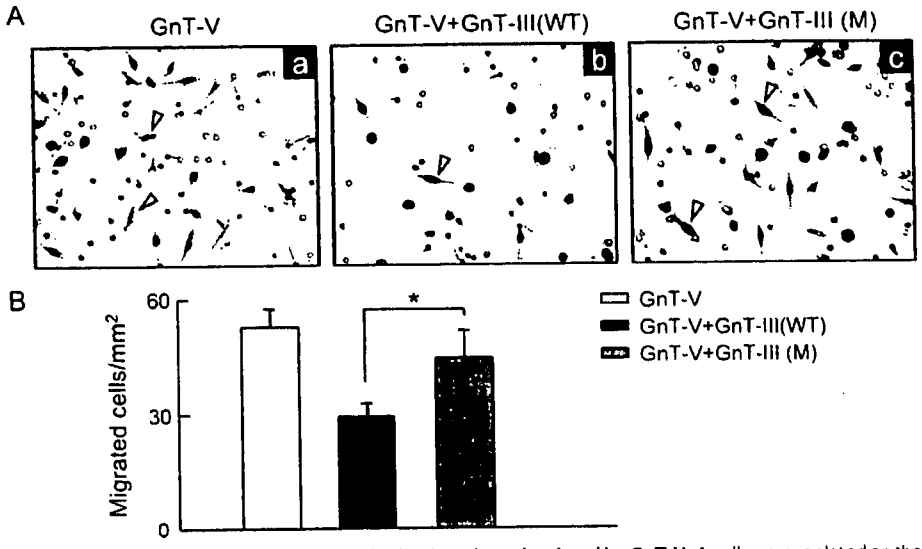

FIGURE 3. **GnT-III transfection suppressed cell migration stimulated by GnT-V.** *A*, cells were replated on the upper chamber coated with LN5 (5 nM). Cell migration was investigated by the GnT-V transfectant (*a*), GnT-III transfection to GnT-V transfectant (*b*), and GnT-III mutant transfection to GnT-V transfectant (*c*). 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 ± S.D. from three independent experiments. *WT*, wild type.

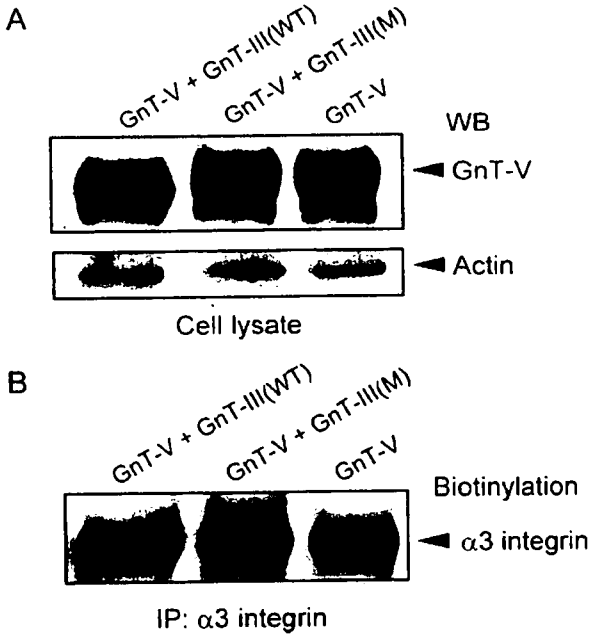

FIGURE 4. **No effects of GnT-III transfection on expression levels of both GnT-V and integrin α3 subunit expressed on cell surface.** *A*, double-transfected cells were lysed, and whole lysates were subjected to 7.5% SDS-PAGE and then transferred to a nitrocellulose membrane and blotted with GnT-V antibody (*top*) or actin antibody (*bottom*). *B*, transfected cells were biotinylated, whole lysates were immunoprecipitated (*IP*) with anti-α3 antibody, and the samples were subjected to 7.5% SDS-PAGE and transferred to a nitrocellulose membrane. The biotinylated proteins were then detected as described under "Experimental Procedures." *WT*, wild type; *WB*, Western blot.

the expression of glycoproteins. As shown in Fig. 4*A*, the levels of expression of GnT-V were not influenced by the introduction of GnT-III, and equivalent amounts of loaded proteins were verified by blotting an actin antibody. On the other hand, 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. 4*B*). 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. 5*A*), 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. 5*B*). As shown in Fig. 5*C*, 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 E_4-PHA lectin, which preferentially binds to bisecting GlcNAc residues in *N*-glycans, or L_4-PHA lectin, which binds to β1,6-branched GlcNAc. Fig. 6*A* (*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. 6*A*, *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. 6*A*, *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. 6*B*). Furthermore, to further confirm such competition on the α3 sub-

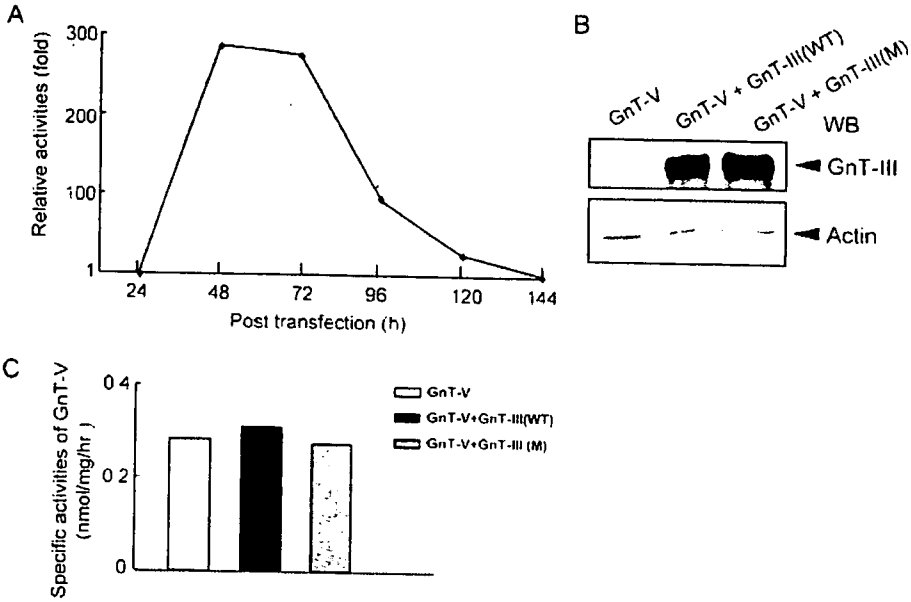

FIGURE 5. **No effects of GnT-III transfection on activity of GnT-V.** *A*, GnT-III activity of transient transfection of adenoviral expression vector to the GnT-V transfectant was examined by high performance liquid chromatography using a fluorescence-labeled agalactobiantennary sugar chain as a substrate. GnT-III activity was determined at various time points (6 days). Data are expressed as a ratio to the first time point (24 h after transfection). *B*, double-transfected cells were lysed, and whole lysates were subjected to 7.5% SDS-PAGE and then transferred to a nitrocellulose membrane and blotted with GnT-III antibody (*top*) or actin antibody (*bottom*). *C*, GnT-V activity of the transient transfection of the adenoviral expression vector to GnT V transfectant was investigated using the method mentioned in *A* at the time point of 48 h after transfection. Data are expressed as specific activity (nmol of product/mg of lysate/h). *WT*, wild type; *WB*, Western blot.

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 MS^1 spectra obtained by FT-ICR MS and product ions in $MS^{2,3}$ spectra (Fig. 6*D*). Based on the presence of $[HexNAc\text{-}Hex\text{-}HexNAc\text{-}HexNAc\text{-}OH + H]^+$ (*m/z* 792) and $[HexNAc\text{-}Hex\text{-}HexNAc\text{-}(dHex)HexNAc\text{-}OH + H]^+$ (*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 L_4-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 mannose 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. 6*A*, 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).[4] 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 CHP134 cells, which express endogenous GnT-III and GnT-V. After retroviral infection, CHP134 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. 7*A*), 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. 7*B*). We then tested cell migration on LN5 and found that GnT-III knockdown resulted in an increased cell migration compared with mock cells (Fig. 7, *C* and *D*). We further investigated the *N*-glycans on the α3 subunit. As shown in Fig. 7*E*, 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

[4] Isaji, T., Sato, Y., Zhao, Y., Miyoshi, E., Wada, Y., Taniguchi, N., and Gu, J. (2006) *J. Biol. Chem.*, in press.

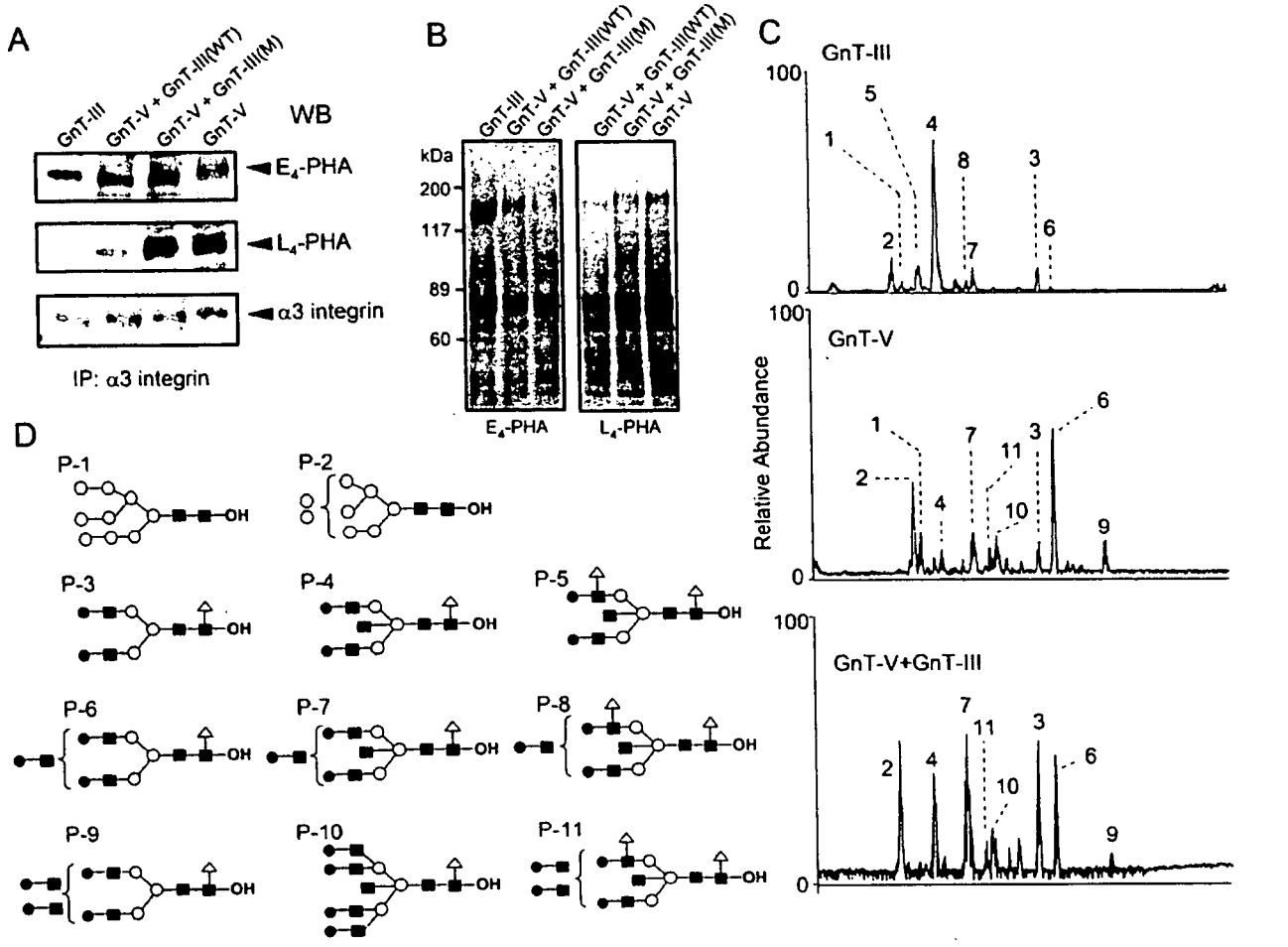

FIGURE 6. **Increased product of GnT-III but decreased product of GnT-V on integrin α3 subunit after transfection of GnT-III into GnT-V transfectant.** *A*, whole cell lysates were immunoprecipitated (*IP*) with anti-α3 antibody, and the resulting immunocomplexes were subjected to 7.5% SDS-PAGE under reducing conditions. The blots were probed by E_4-PHA (*top*), L_4-PHA (*middle*) and anti-α3 antibody (*bottom*), respectively. *B*, lectin blotting was performed with E_4-PHA and L_4-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*-acetylglucosamine. *WB*, Western blot.

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 *in vitro* (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

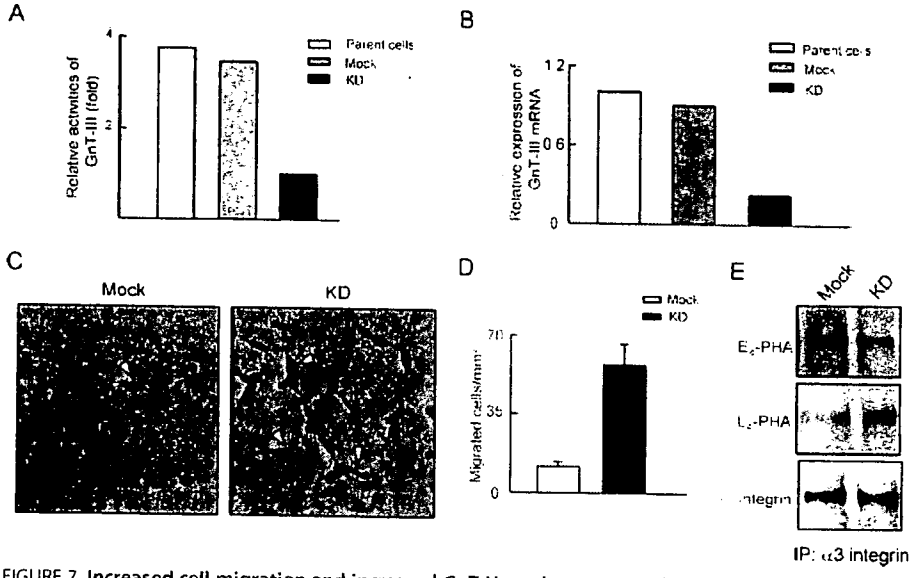

FIGURE 7. **Increased cell migration and increased GnT-V product on α3 subunit in GnT-III knockdown cells.** *A*, activities of GnT-III in GnT-III knockdown CHP134 cells. *B*, mRNA expression of GnT-III in knockdown cells. Quantitative analysis was performed by real time PCR. *C*, cell migration on LN5 (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 E_4-PHA (*top*), L_4-PHA (*middle*), and anti-α3 antibody (*bottom*), respectively. *KD*, GnT-III knockdown cells.

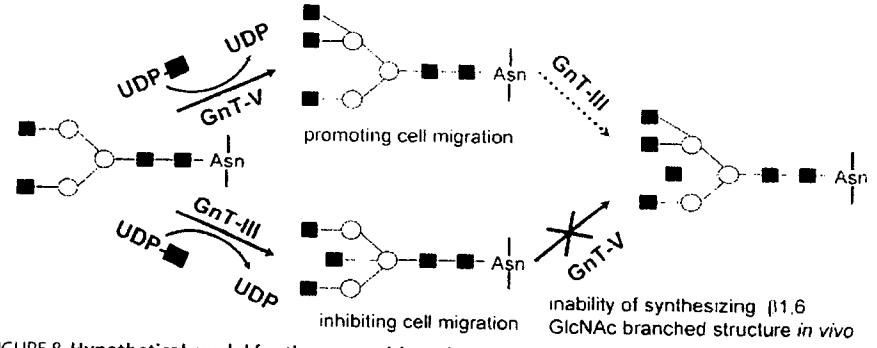

FIGURE 8. **Hypothetical model for the competition of GnT-III and GnT-V for integrin α3 subunit modification.** The product of GnT-V contributes to the promotion of cell migration. The reaction represented by a *dashed line* may not be predominant *in vivo*, whereas it could occur *in vitro*. On the other hand, the product of GnT-III suppresses cell migration. More importantly, this product cannot be utilized as a substrate by GnT-V, which is represented with a *cross*. Therefore, α3β1-mediated cell migration induced by GnT-V can be blocked due to competition with GnT-III. ○, mannose; ■, *N*-acetylglucosamine.

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.

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 bisecting GlcNAc to the α5 subunit resulted in a reduced affinity in the binding of α5β1 integrin to FN, resulting in a decreased cell migration (38). We thus assumed that the GnT-III affects the α1, α2, and α3 subunits similarly, which caused the decreased cell migration on LN5 and COL. However, the modification of β1,6-GlcNAc to α1 and α2 subunits may not affect their binding to COL. Considering that α3β1 integrin is a strong adhesive receptor that promotes cell migration (37), the selective competition between GnT-III and GnT-V for α3 might play an important role in cancer metastasis.

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

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 $\alpha 3$ subunit in living cells (Fig. 8). This competition results in the inhibition of $\alpha 3\beta 1$ 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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——資　　料——

医薬品研究
Pharm. Regul. Sci.
37(7)438～447(2006)

平成15年度「日本薬局方の試験法に関する研究」研究報告

糖タンパク質性医薬品の試験法に関する研究**
—LC/MS/MSを用いたペプチドマッピング—

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1. はじめに

ペプチドマッピングは，タンパク質性医薬品の一次構造や翻訳後修飾等の有無を確認することを目的とした品質試験法の一つである．従来はUV検出法によって得られたクロマトグラム中のいくつかのピークの保持時間や面積を標準物質のそれらと比較し，大きな違いがないことが判定基準として設定されてきた．しかし最近では，液体クロマトグラフィー質量分析法（LC/MS）と組み合わせ，MSで測定されたペプチドのm/z値が，理論値と一致することを判定基準に加えた試験も設定されるようになってきた．更には，LC/MS/MSによって得られたプロダクトイオンのいくつかをペプチドの配列を確認する目的で規格に取り入れることも検討されるようになってきている．このMS/MSを利用したペプチド配列解析は，プロテオミクスの技術として知られる方法で，MS/MSによってペプチドから生じたbイオン及びyイオン[1]を，データベースに登録されているタンパク質から生じるペプチドに由来するb及びyイオンの理論値と照合することによって，ペプチドの配列を推定するものである[2]．この方法を品質試験に取り入れることによって，より確かな定性的な確認が可能になると思われる[3,4]．

多くのバイオ医薬品の本質は糖タンパク質である．従来の糖タンパク質のペプチドマッピングでは，糖ペプチドは糖鎖の不均一性のためピーク形状がブロードになる傾向があり，判定の対象からはずされることが多かった．また，LC/MS/MSを用いた糖タンパク質のペプチドマッピングにおいては，MS/MSによって糖ペプチドの糖鎖部分が優先的に開裂するためデータベースとの照合が難しく，データベース検索による糖ペプチドの同定は避けられてきた．しかし，最近のプロテオミクス/グライコミクスの進展により，糖ペプチドのMS及びMS/MSスペクトルからペプチド部分の配列や部位特異的な糖鎖構造情報を得ることが可能になってきた[5~9]．

本研究では，糖タンパク質性医薬品の糖鎖結合部位を含むペプチドの一次構造確認，及び糖鎖構造確認におけるLC/MS/MSを用いたペプチドマッピングの応用可能性を検証した．糖タンパク質性医薬品のモデルとして，ヒト血清セルロプラスミンを選んだ．セルロプラスミンは1046アミノ酸残基からなる糖タンパク質で（Fig. 1），7箇所の推定糖鎖結合部位のうち，Asn119，339，378及び743に主にシアル酸が結合した2本鎖及び3本鎖複合型糖鎖が結合していることが報告されているが[10]，結合部位毎の糖鎖構造に関する報告はない．

2. 実験方法

【試薬・材料】

セルロプラスミンはCalbiochem社より購入した．トリプシンは，Promega社製の修飾トリプシンを使用した．

【トリプシン消化】

セルロプラスミン（200 μg）を8 Mグアニジン塩酸，5 mM EDTAを含む0.5 M Tris-HCl，pH 8.6（270 μL）に溶解し，2-メルカプトエタノール2 μLを加え，室温で2時間放置した．モノヨード酢酸ナトリウム5.7 mgを試料溶解溶液45 μLに溶かして試料溶液に加え，遮光下，室温にて2時間放置した．

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** 本研究は日本公定書協会の「日本薬局方の試験法に関する研究」により行ったものである．

KEKHYYIGII ETTWDYASDH GEKKLISVDT EHSNIYLQNG PDRIGRLYKK ALYLQYTDET
FRTTIEKPVW LGFLGPIIKA ETGDKVYVHL KNLASRPYTF HSHGITYYKE HEGAIYPDNT
TDFQRADDKV YPGEQYTYML LATEEQSPGE GDGNCVTRIY HSHIDAPKDI ASGLIGPLII
CKKDSLDKEK EKHIDREFVV MFSVVDENFS WYLEDNIKTY CSEPEKVDKD NEDFQESNRM
YSVNGYTFGS LPGLSMCAED RVKWYLFGMG NEVDVHAAFF HGQALTNKNY RIDTINLFPA
TLFDAYMVAQ NPGEWMLSCQ NLNHLKAGLQ AFFQVQECNK SSSKDNIRGK HVRHYYIAAE
EIIWNYAPSG IDIFTKENLT APGSDSAVFF EQGTTRIGGS YKKLVYREYT DASFTNRKER
GPEEEHLGIL GPVIWAEVGD TIRVTFHNKG AYPLSIEPIG VRFNKNNEGT YYSPNYNPQS
RSVPPSASHV APTETFTYEW TVPKEVGPTN ADPVCLAKMY YSAVDPTKDI FTGLIGPMKI
CKKGSLHANG RQKDVDKEFY LFPTVFDENE SLLLEDNIRM FTTAPDQVDK EDEDFQESNK
MHSMNGFMYG NQPGLTMCKG DSVVWYLFSA GNEADVHGIY FSGNTYLWRG ERRDTANLFP
QTSLTLHMWP DTEGTFNVEC LTTDHYTGGM KQKYTVNQCR RQSEDSTFYL GERTYYIAAV
EVEWDYSPQR EWEKELHHLQ EQNVSNAFLD KGEFYIGSKY KKVVYRQYTD STFRVPVERK
AEEEHLGILG PQLHADVGDK VKIIFKNMAT RPYSIHAHGV QTESSTVTPT LPGETLTYVW
KIPERSGAGT EDSACIPWAY YSTVDQVKDL YSGLIGPLIV CRRPYLKVFN PRRKLEFALL
FLVFDENESW YLDDNIKTYS DHPEKVNKDD EEFIESNKMH AINGRMFGNL QGLTMHVGDE
VNWYLMGMGN EIDLHTVHFH GHSFQYKHRG VYSSDVFDIF PGTYQTLEMF PRTPGIWLLH
CHVTDHIHAG METTYTVLQN EDTKSG

Fig. 1　ヒトセルロプラスミンのアミノ酸配列
LC/MS/MS及びデータベース検索により同定されたアミノ酸配列部分を下線で示す．□，糖鎖結合部位

PD-10カラム（Amersham Bioscience）を用いて脱試薬し，得られた試料溶液を凍結乾燥した．還元カルボキシメチル化したセルロプラスミンを50 mM重炭酸アンモニウム（200 μL）に溶かした．試料溶液100 μLをとり，1 μg/μLの修飾トリプシン溶液を1 μL加え，37℃で16時間消化した．消化後，測定まで−20℃に保存し酵素反応を停止させた．

【ペプチドマッピング】

セルロプラスミンのトリプシン消化産物を0.1%ギ酸水溶液で適度に稀釈し，以下の条件でLC/MS/MSを行った．サンプル量は，m/z 400〜2000で測定した場合はタンパク量に換算して0.2 μg，また，m/z 1000〜2000で測定した場合は，タンパク量に換算して0.4 μgを使用した．

HPLC：

装置：Paradigm MS4（Michrom BioResource社）

カラム：MAGIC C18（Michrom BioResource社，0.2×50 mm，3 μ）

溶離液A：0.1%ギ酸を含む2%アセトニトリル水溶液

溶離液B：0.1%ギ酸を含む90%アセトニトリル水溶液

グラジエントプログラム：

B液：5%（0〜10分）

5〜65%（10〜70分）

流速：ポンプ2 μL/min，スプリッター使用

MS

装置：ハイブリッド型LC/MS/MSシステム Qstar Puler i（Applied Biosystem社）

イオン化：ESI

測定モード：ポジティブイオンモード

スプレー電圧：2,500 V

検出：Q/TOF-MS

MSスキャン範囲（m/z）：
ペプチド検出：400～2,000
糖ペプチド検出：1000～2,000
MS/MSスキャン範囲：100～2,000（データ依存的にMS/MSを行った．コリジョンエネルギーはイオンの価数及び大きさに応じて自動的に変化させた）．

【データベース検索】

LC/MS/MSで得られたデータを，Mascot検索エンジンを用いてSwissPlotのヒトデータベースに対して検索を行い，ペプチドイオンを帰属した．検索の条件として，システイン残基のカルボキシメチル化，消化ミスの許容数を1，プリカーサーイオン及びプロダクトイオンの質量数の理論値との差の許容範囲をそれぞれ2u及び0.8uと設定した．また，別途，検索エンジンProIDを用いて同様に検索を行い，片方の検索エンジンのみでヒットしたヒトセルロプラスミン由来ペプチドについては，目視によりスペクトルを確認した．

3. 実験結果

3.1 ペプチド同定

セルロプラスミンを還元カルボキシメチル化し，そのトリプシン消化物をC18カラムを接続したLC/MS/MS装置を用いて分析した．Fig. 2はフルMSスキャン（m/z 400～2000）によって得られたベースピーククロマトグラムである．各スキャンで検出されたイオンのうち強度の高い2価の分子イオンは，自動的なデータ依存的MS/MSに供されている．そこで，データベース検索エンジンMASCOT及びProIDを用いて，ヒトデータベース（Swiss-Plot）検索を行ったところ，セルロプラスミンのアミノ酸残基の約70%がデータベースと一致した（Fig. 1下線）．検索結果を基に，ペプチドマップの各ピークの帰属を行い（Table 1），主なピークのアミノ酸配列を確認することができた．三個の推定N結合型糖鎖結合部位を含むペプチドも検出されていた．データベース検索によって同定されなかったペプチドは，分子量の小さなペプチド，糖ペプチド，又は親水性が高く，C18カラムに吸着されなかったペプチドと考えられた．

3.2 糖ペプチド同定

トリプシン消化によって生じたペプチドの多くは分子量2000 Da以下であるが，N結合型糖鎖は900 Da以上であるので，糖ペプチドの分子量はペプチドに比べて大きくなる．したがって，スキャン範囲をm/z 1000以上に設定すると，多くのペプチドの2価イオンは検出されず，主に糖ペプチドが検出されることになる．また，糖ペプチドのMS/MSでは，GlcNAcのBイオンに由来するm/z 204のプロダクトイオンが強く検出される．そこで，フルMSスキャンの範囲をm/z 1000～2000に設定し，更にデ

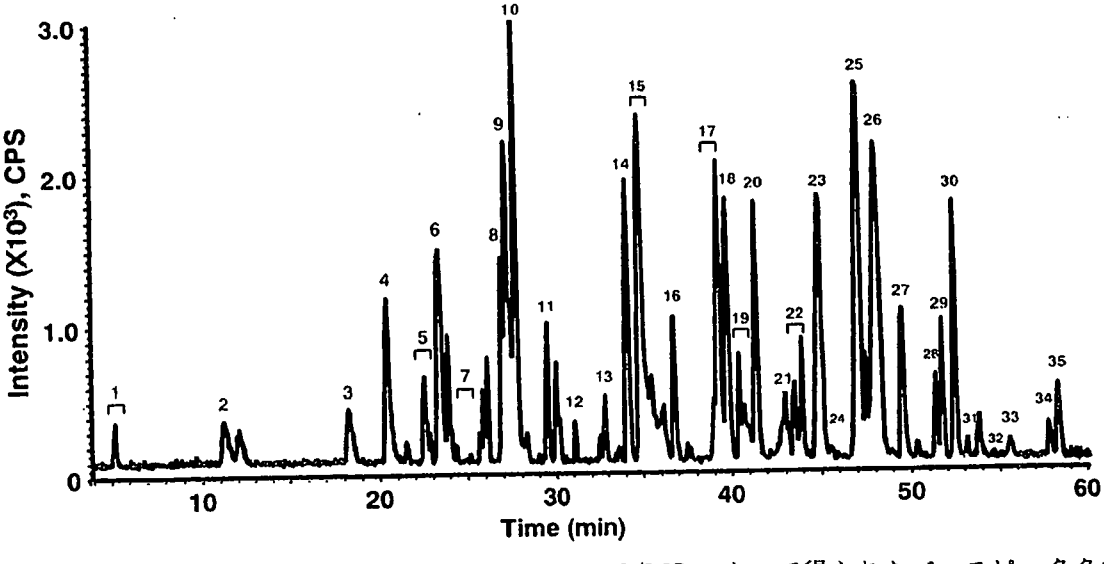

Fig. 2 ヒトセルロプラスミントリプシン消化物のLC/MSによって得られたベースピーククロマトグラム（m/z 400～2000）

ータ依存的なMS/MSで生じたm/z 204のイオンを抽出することによって，糖ペプチドの溶出位置を推定することができた．Fig. 3Aにm/z 1000～2000で測定したフルMSスキャンのベースピーククロマトグラム，Fig. 3Bにデータ依存的なMS/MSのトータルイオンクロマトグラム（TIC），また，Fig. 3CにMS/MSで生じたm/z 204のマスクロマトグラムを示す．この方法で浮かび上がった4つのピーク近傍からm/z 204のイオンが検出されているMS/MSスペクトル（ピークA，B，C，及びD）を選び出し，解析を行った．

Fig. 4は4つのピーク周辺から選び出した代表的なMS/MSスペクトルである．Fig. 4Aに示す糖ペプチド（ピークA）のペプチド部分は，ペプチドイオン（m/z 1892.8）から，Asp110-Arg125と同定された．更に，MS/MSスペクトルを詳細に調べた

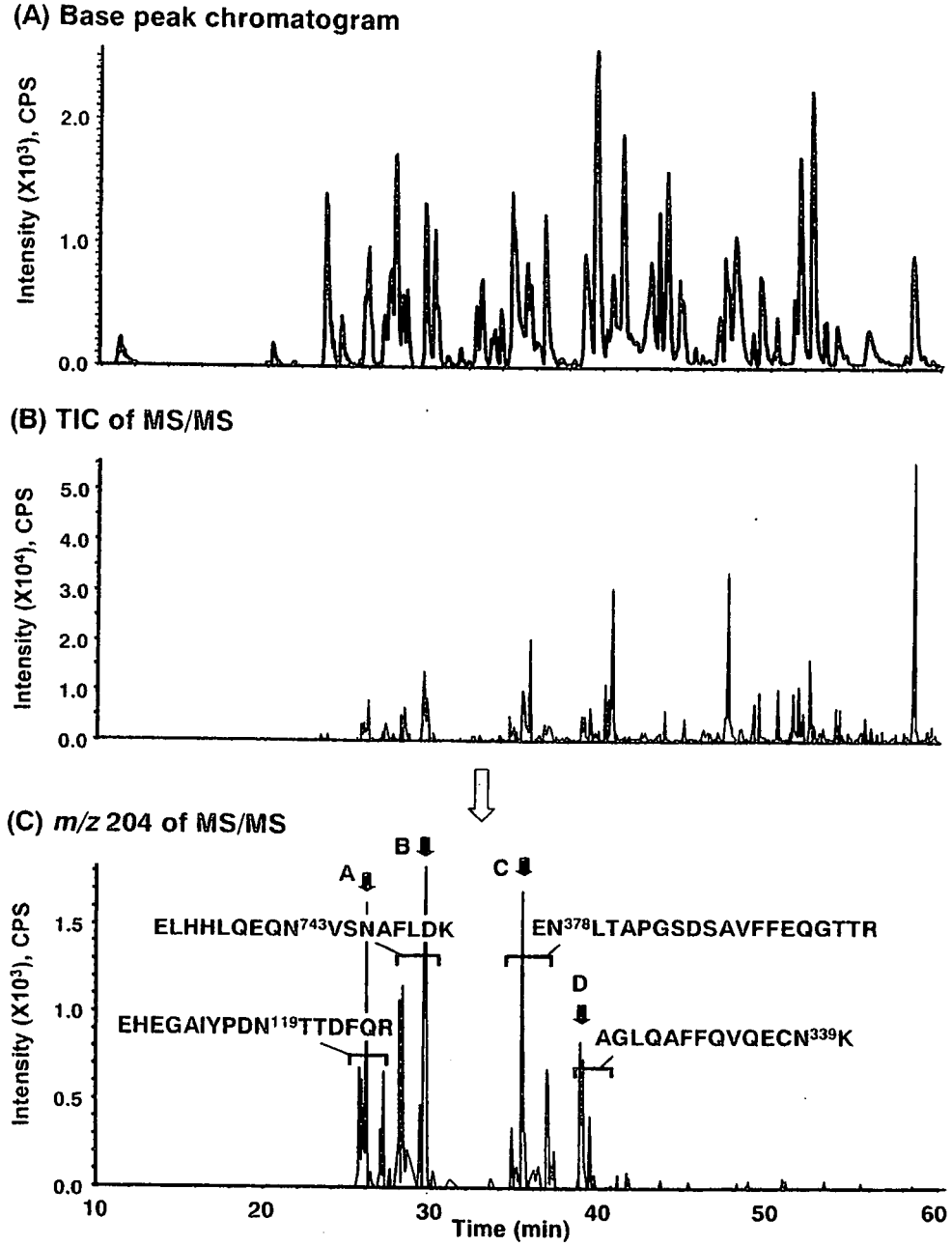

Fig. 3　ヒトセルロプラスミントリプシン消化物のLC/MSによって得られたベースピーククロマトグラム（m/z 1000～2000）（A），LC/MS/MSによって得られたTIC（B），及びLC/MS/MSによって生じたm/z 204のマスクロマトグラム（C）

Table 1 ヒトセルロプラスミンのトリプシン

Retention time (min)	Peak No.	*m/z*	Charge	Calculated MW	Peptide Sequence	amino acid residue	Theoretical MW	No. of missed cleavage
5	1	590.8	+2	1179.7	IYHSHIDAPK	159-168	1179.6	0
5	1	798.4	+2	1594.8	VDKDNEDFQESNR	227-239	1594.7	1
5	1	532.6	+3	1594.8				
11	2	509.2	+2	1016.5	QYTDSTFR	767-774	1016.5	0
18	3	783.9	+2	1565.8	VNKDDEEFIESNK	926-938	1565.7	1
18		522.9	+3	1565.8				
20	4	602.3	+2	1202.6	EYTDASFTNR	408-417	1202.5	0
22	5	450.7	+2	899.5	GEFYIGSK	752-759	899.4	0
22	5	626.8	+2	1251.6	MFTTAPDQVDK	580-590	1251.6	0
23	6	952.4	+2	1902.9	NNEGTYYSPNYNPQSR	466-481	1902.8	0
24	7	529.9	+3	1586.8	RQSEDSTFYLGER	701-713	1586.7	1
24	7	719.1	+3	2154.2	NLASRPYTFHSHGITYYK	92-109	2154.1	0
24	7	765.0	+3	2292.1	FNKNNEGTYYSPNYNPQSR	463-481	2292.0	1
27	8	716.3	+2	1430.7	QSEDSTFYLGER	702-713	1430.6	0
27	8	868.0	+2	1733.9	LVYREYTDASFTNR	404-417	1733.8	1
27	9	736.4	+2	1470.8	EVGPTNADPVCLAK	505-518	1470.7	0
27	9	752.8	+3	2255.3	KAEEEHLGILGPQLHADVGDK	780-800	2255.2	1
27		564.8	+4	2255.3				
27	10	1237.6	+2	2473.1	MFTTAPDQVDKEDEDFQESNK	580-600	2473.1	1
27		825.4	+3	2473.1				
28	10	1150.1	+2	2298.2	KLISVDTEHSNIYLQNGPDR	24-43	2298.2	1
28		575.6	+4	2298.2				
29	11	1064.6	+2	2127.1	AEEEHLGILGPQLHADVGDK	781-800	2127.1	0
29		710.0	+3	2127.1				
29		532.8	+4	2127.1				
29	11	1086.1	+2	2170.1	LISVDTEHSNIYLQNGPDR	25-43	2170.1	0
29		724.4	+3	2170.1				
31	12	550.0	+3	1646.9	KALYLQYTDETFR	50-62	1646.8	1
32	13	1103.0	+2	2204.0	MHSMNGFMYGNQPGLTMCK	601-619	2203.9	0
32		735.7	+3	2204.0				
34	14	760.4	+2	1518.8	ALYLQTDETFR	51-62	1518.7	0
35	15	686.4	+2	1370.8	GAYPLSIEPIGVR	450-462	1370.8	0
35	15	1266.2	+2	2530.3	SVPPSASHVAPTETFTYEWTVPK	482-504	2530.2	0
35	15	1291.7	+3	3872.0	NMATRPYSIHAHGVQTESSTVTPTLPGETLTYVWK	807-841	3871.9	0
35		969.0	+4	3872.1				
37	16	842.8	+3	2525.3	HYYIGIIETTWDYASDHGEKK	4-24	2525.2	1
39	17	1199.6	+2	2397.2	HYYIGIIETTWDYASDHGEK	4-23	2397.1	0
39		800.1	+3	2397.2				
39		600.3	+4	2397.2				
39	18	836.1	+3	2505.2	SGAGTEDSACIPWAYYSTVDQVK	846-868	2505.1	0

All masses are monoisotopic. The peptides including the potential *N*-glycosylation site are in bold type. Cystein

ところ，ペプチドに由来するｂイオン及びｙイオンが生じていることが明らかになった．このことから，糖ペプチドであっても，ペプチドの配列を確認できることが明らかになった．

同様にして，Fig. 4B の糖ペプチド（ピーク B）は，ペプチドの 2 価イオン（m/z 1011.7）から，Asp735-Lys751 と推定され，その配列由来のｂ及びｙイオンが検出されていることを確認した．Fig. 4C に示す糖ペプチド（ピーク C）の MS/MS スペクトルには，ペプチドイオン及びペプチドに糖鎖が結合したイオンは検出されなかったが，ｙイオンシリーズから，Asp379-Arg396 と同定された．また，Fig. 4D の糖ペプチド（ピーク D）は，ペプチドイオン（m/z 1640.8）及び GlcNAc が結合したペプチドイオン（m/z 1843.9）から，Ala327-Lys340 と同定された．推定糖鎖結合部位である 227，588，及び 929 番目の Asn を含む糖ペプチドは検出されなかったことから，糖鎖が結合していないことが確認された．

3.3 糖鎖解析

糖鎖の構造は，各糖ペプチドの MS/MS スペクトルと糖鎖部分の分子量から推定した．Fig. 4A に

消化ペプチドのLC/MS/MSによる同定

Retention time (min)	Peak No.	m/z	Charge	Calculated MW	Peptide Sequence	amino acid residue	Theoretical MW	No. of missed cleavage
40	19	1240.3	+3	3717.9	TPGIWLLHCHVTDHIHAGMETTYTVLQNEDTK	1013-1044	3717.8	0
40		930.7	+4	3718.9				
40	19	1232.3	+3	3693.8	ADDKVYPGEQYTYMLLATEEQSPGEGDGNCVTR	126-158	3693.6	1
41	20	596.4	+2	1190.7	DIFTGLIGPMK	529-539	1190.6	0
41	20	1045.5	+2	2089.0	TYYIAAVEVEWDYSPQR	714-730	2089.0	0
43	21	1089.2	+3	3264.5	VYPGEQYTYMLLATEEQSPGEGDGNCVTR	130-158	3264.4	0
43	22	1228.6	+2	2455.1	MYSVNGYTFGSLPGLSMCAEDR	240-261	2455.0	0
43		819.4	+3	2455.2				
44	22	1174.1	+2	2346.2	MYYSAVDPTKDIFTGLIGPMK	519-539	2346.2	1
44		783.1	+3	2346.3				
44	22	1426.7	+2	2851.4	WYLFGMGNEVDVHAAFFHGQALTNK	264-288	2851.4	0
44		713.9	+4	2851.5				
44	23	735.9	+2	1469.9	DIASGLIGPLIICK	169-182	1469.8	0
45	23	1122.3	+4	4485.2	RDTANLFPQTSLTLHMWPDTEGTFNVECLTTDHYTGGMK	653-691	4485.0	1
45	24	1386.7	+2	2771.5	ERGPEEEHLGILGPVIWAEVGDTIR	419-443	2771.4	1
45		924.8	+3	2771.5				
45		693.9	+4	2771.5				
46	25	1168.3	+3	3501.8	QSEDSTFYLGERTYYIAAVEVEWDYSPQR	702-730	3501.6	1
47	25	788.9	+2	1575.9	DLYSGLIGPLIVCR	869-882	1575.8	0
47		526.3	+3	1575.9				
47	25	956.6	+2	1911.2	TTIEKPVWLGFLGPIIK	63-79	1911.1	0
47		638.1	+3	1911.2				
48	26	1083.3	+4	4329.1	DTANLFPQTSLTLHMWPDTEGTFNVECLTTDHYTGGMK	654-691	4328.9	0
48	26	1244.2	+2	2486.3	GPEEEHLGILGPVIWAEVGDTIR	421-443	2486.3	0
48		829.8	+3	2486.4				
48		622.6	+4	2486.4				
49	27	1357.7	+2	2713.4	HYYIAAEEIIWNYAPSGIDIFTK	354-376	2713.3	0
49		905.5	+3	2713.5				
51	28	1141.9	+3	3422.8	GDSVVWYLFSAGNEADVHGIYFSGNTYLWR	620-649	3422.6	0
51	29	1054.2	+3	3159.6	DVDKEFYLFPTVFDEN^{569}ESLLLEDNIR	554-579	3159.5	1
52	30	1335.2	+2	2668.4	GVYSSDVFDIFPGTYQTLEMFPR	990-1012	2668.3	0
52		890.4	+3	2668.3				
53	31	1352.2	+2	2702.4	EFYLFPTVFDEN^{569}ESLLLEDNIR	558-579	2702.3	0
54	32	1077.9	+3	3230.7	HIDREFVVMFSVVDEN^{208}FSWYLEDNIK	193-218	3230.5	1
55	33	1355.7	+2	2709.4	EFVVMFSVVDEN^{208}FSWYLEDNIK	197-218	2709.3	0
58	34	1481.3	+2	2960.5	KLEFALLFLVFDEN907ESWYLDDNIK	894-917	2960.5	1
58		987.9	+3	2960.7				
58	35	1356.0	+3	4065.1	IDTINLFPATLFDAYMVAQNPGEWMLSCQNLNHLK	292-326	4065.0	0
58		1017.3	+4	4065.2				
61		1417.2	+2	2832.5	LEFALLFLVFDEN^{907}ESWYLDDNIK	895-917	2832.4	0

residue was carboxymethylated.

は m/z 204 ($HexNAc^+$) 及び 366 ($Hex\text{-}HexNAc^+$) などの糖鎖に由来するフラグメントに加え，m/z 292 のイオンが認められることから，この糖ペプチドにはシアル酸含有糖鎖が結合していることが推定された．さらに，糖ペプチド全体の計算分子量 4096.7 Da からペプチドの理論分子量 1891.8 Da を引いて得られた糖鎖部分の分子量から単糖組成を推定し，糖鎖構造は，ジシアロ2本鎖糖鎖と推定された．同様にして，ピークA周辺から m/z 204 が検出されているMS/MSスペクトルを選び出して解析したところ，糖鎖分子量の異なる糖ペプチドが存在しており，それぞれフコシルジシアロ2本鎖糖鎖やトリシアロ3本鎖糖鎖などが結合してることが推定された（Table 2）．

同じ要領で，他の3つのピークについても糖鎖構造解析を行った．Fig. 4Bの糖ペプチドの糖鎖部分は，糖ペプチド分子量 4882.1 Da からペプチドの理論分子量 2021.0 Da を引いた値 2879.1 Da から，トリシアロ3本鎖糖鎖と推定された．また，Fig. 4C から Asn743 にはジシアロ2本鎖糖鎖が結合していることが推定された．更に，Fig. 4D から，Asn339 に結合している糖鎖についても，フコースが結合し

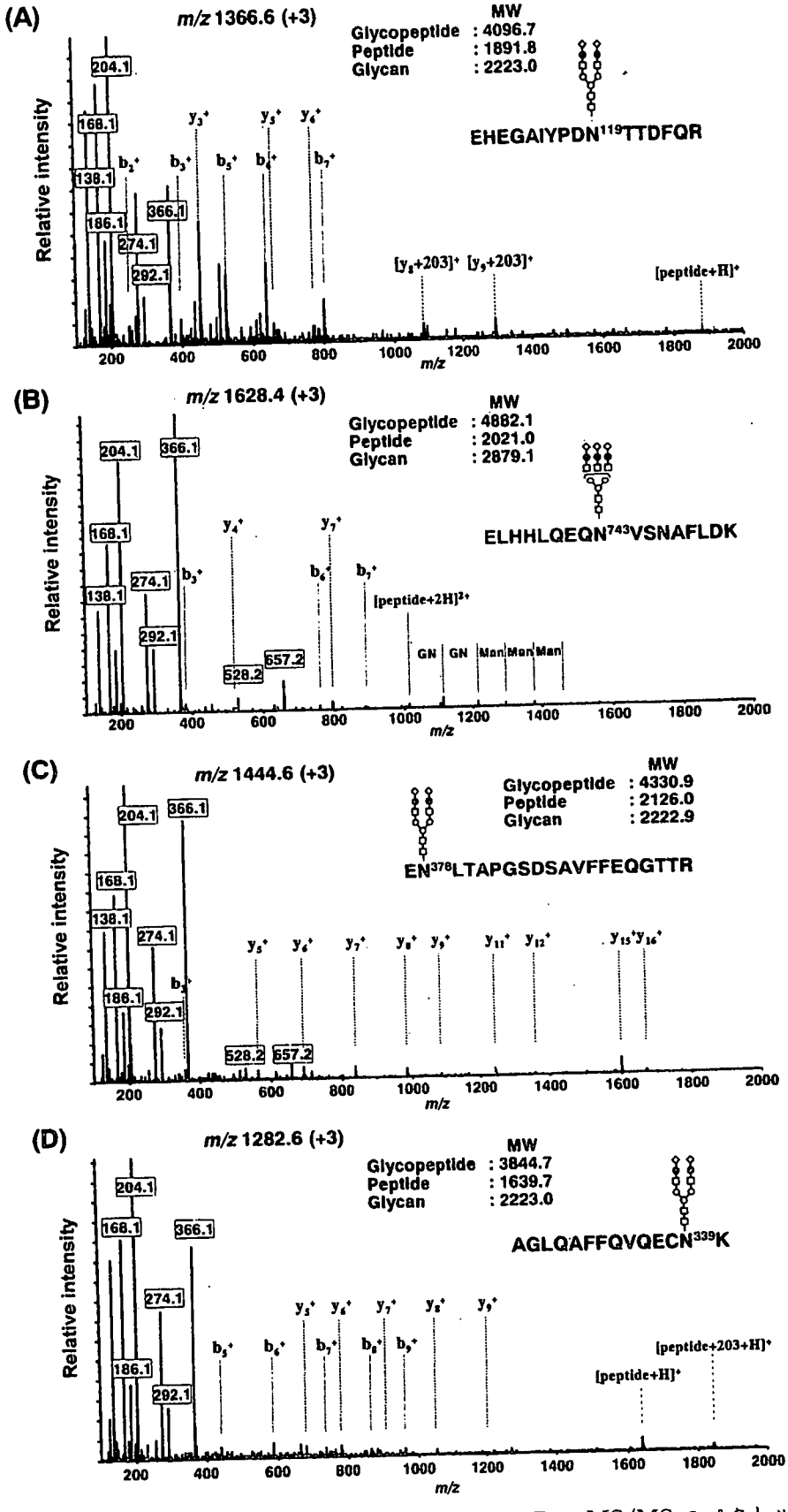

Fig. 4 Fig. 3C で示したピーク A, B, C, 及び D の MS/MS スペクトル

Table 2 ヒトセルロプラスミンの部位特異的糖鎖解析の結果

Retention time (min)	Glycopeptides *m/z*	Charge	Calculated MW	Relative peak intensity[a] %	Peptide Sequence	Theoretical MW	Oligosaccharide Calculated MW	Theoretical MW	Composition[b,c]
26	1415.3	+3	4242.8	52	EHEGAIYPDN^{119}TTDFQR	1891.8	2351.0	2350.8	[HexNAc]4[Hex]5[NeuAc]2[Fuc]1
26	1366.6	+3	4096.7	100	EHEGAIYPDN^{119}TTDFQR	1891.8	2204.9	2204.8	[HexNAc]4[Hex]5[NeuAc]2
27	1682.7	+3	5045.1	6	EHEGAIYPDN^{119}TTDFQR	1891.8	3153.3	3153.1	[HexNAc]5[Hex]6[NeuAc]3[Fuc]2
27	1634.0	+3	4899.0	21	EHEGAIYPDN^{119}TTDFQR	1891.8	3007.2	3007.1	[HexNAc]5[Hex]6[NeuAc]3[Fuc]1
27	1585.3	+3	4753.0	24	EHEGAIYPDN^{119}TTDFQR	1891.8	2861.2	2861.0	[HexNAc]5[Hex]6[NeuAc]3
28	1458.3	+3	4372.0	35	ELHHLQEQN^{743}VSNAFLDK	2021.0	2351.0	2350.8	[HexNAc]4[Hex]5[NeuAc]2[Fuc]1
28	1409.6	+3	4225.9	100	ELHHLQEQN^{743}VSNAFLDK	2021.0	2204.9	2204.8	[HexNAc]4[Hex]5[NeuAc]2
29	1725.8	+3	5174.5	5	ELHHLQEQN^{743}VSNAFLDK	2021.0	3153.5	3153.1	[HexNAc]5[Hex]6[NeuAc]3[Fuc]2
29	1677.1	+3	5028.2	29	ELHHLQEQN^{743}VSNAFLDK	2021.0	3007.2	3007.1	[HexNAc]5[Hex]6[NeuAc]3[Fuc]1
29	1628.4	+3	4882.1	43	ELHHLQEQN^{743}VSNAFLDK	2021.0	2861.1	2861.0	[HexNAc]5[Hex]6[NeuAc]3
31	1895.8	+3	5684.4	2	ELHHLQEQN^{743}VSNAFLDK	2021.0	3663.4	3663.3	[HexNAc]6[Hex]7[NeuAc]4[Fuc]1
31	1847.1	+3	5538.4	3	ELHHLQEQN^{743}VSNAFLDK	2021.0	3517.4	3517.2	[HexNAc]6[Hex]7[NeuAc]4
35	1493.3	+3	4477.0	6	EN^{378}LTAPGSDSAVFFEQGTTR	2126.0	2351.0	2350.8	[HexNAc]4[Hex]5[NeuAc]2[Fuc]1
35	1444.6	+3	4330.9	100	EN^{378}LTAPGSDSAVFFEQGTTR	2126.0	2204.9	2204.8	[HexNAc]4[Hex]5[NeuAc]2
37	1712.1	+3	5133.2	8	EN^{378}LTAPGSDSAVFFEQGTTR	2126.0	3007.2	3007.1	[HexNAc]5[Hex]6[NeuAc]3[Fuc]1
37	1663.4	+3	4987.1	23	EN^{378}LTAPGSDSAVFFEQGTTR	2126.0	2861.1	2861.0	[HexNAc]5[Hex]6[NeuAc]3
39	1331.3	+3	3990.8	14	AGLQAFFQVQECN^{339}K	1639.7	2351.1	2350.8	[HexNAc]4[Hex]5[NeuAc]2[Fuc]1
39	1282.6	+3	3844.7	100	AGLQAFFQVQECN^{339}K	1639.7	2205.0	2204.8	[HexNAc]4[Hex]5[NeuAc]2
41	1501.3	+3	4500.8	6	AGLQAFFQVQECN^{339}K	1639.7	2861.1	2861.0	[HexNAc]5[Hex]6[NeuAc]3

[a] Relative peak intensity was calculated by comparing triply-charge state glycopeptide ions. The intensity of the glycoform with maximum at each glycosylation site was taken as 100 %.

[b] The oligosaccharide composition was deduced from the molecular weight of the oligosaccharide.

[c] Fuc, fucose; Hex, Hexose; HexNAc, N-acetylhexosamine; NeuAc, N-acetylneuraminic acid.

All masses are monoisotopic.

Cystein residue was carboxymethylated.

ていないジシアロ2本鎖糖鎖と推定された．

4．考　察

本研究では，LC/MS/MSを用いたペプチドマッピングの糖タンパク質性医薬品の品質試験法としての応用可能性を検証した．まず，データベース検索を利用することによって，ペプチドマップ中の糖鎖非結合ペプチドを容易に帰属できることを確認した．溶出位置からペプチドを推定する方法と比較して，より確実に一次構造を確認できること，また，プロセッシングやその他の翻訳後修飾の有無を確認できることから，品質試験法として優れていると思われる．

データベースを用いた方法のみでは糖ペプチドを同定することはできなかった．そこで，糖ペプチドのMS/MSスペクトルを選び出し，ペプチド部分のアミノ酸配列と糖鎖部分の構造を推定することを検討した．糖ペプチドのMS/MSスペクトルを選び出すためには，まず，ペプチドマップにおける糖ペプチドの溶出位置を特定する必要がある．複雑なペプチドマップにおいて糖ペプチドの溶出位置を推定する方法として，糖鎖に特徴的なプロダクトイオン（m/z 204，366など）を診断イオンとしたインソースフラグメンテーションやプリカーサーイオンスキャンが知られている[10,11]．これらは，糖ペプチドの溶出位置の推定には適しているが，前駆イオンの分子量や糖ペプチドの開裂情報が得られないため，ペプチドや糖鎖の配列を推定するためには，別途データ依存的MS/MSが要求される．そこで我々は，データ依存的に取り込んだMS/MSスペクトルで生じた糖鎖由来イオン（m/z 204，HexNAc）を指標として糖ペプチドのMS/MSスペクトルを探し出す方法を見出した[8,9]．この方法を用いて選び出したMS/MSスペクトルを基にペプチド配列と糖組成を推定し，セルロプラスミンの4箇所の糖鎖結合位置に付加している糖鎖の特徴を明らかにすることに成功した．

これまで，糖ペプチドのMS/MSでは糖鎖部分が優先的に開裂するため一次構造情報が得られにくく，MS/MSを糖ペプチドの配列確認や糖鎖構造確認に利用するのは難しいといわれてきた．しかし，今回用いた分析システムとコリジョンエネルギーの条件ではペプチドの一次構造情報が得られていることを確認した．これは，糖ペプチドであってもアミノ酸配列を確認できることを意味している．また，ペプチドを特定することによって，糖鎖構造を確認することも可能であった．

以上のことから，糖タンパク質のペプチドマッピングにおいて，ペプチド部分はデータベースを利用することによって容易に配列を確認できること，また，糖ペプチドの場合は，MS/MSスペクトルを選び出して解析することによって，ペプチド部分の配列と部位特異的な糖鎖構造の概略を確認できることが明らかになった．この方法は，一次構造と糖鎖を含む翻訳後修飾を同時に確認できるものであり，糖タンパク質性医薬品の品質試験法として応用可能であると思われる．

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