An N-Glycosylation Site on the β-Propeller Domain of the Integrin α5 Subunit Plays Key Roles in Both Its Function and Site-specific Modification by β1,4-N-Acetylglucosaminyltransferase III*

Yuya Sato†, Tomoya Isaji‡, Michiko Tajiri‡, Shumi Yoshida-Yamamoto§, Tsuyoshi Yoshinaka*, Toshiaki Somehara*, Tomohiko Fukuda*, Yoshinao Wada*, and Jianguo Gu†

From the †Division of Regulatory Glycobiology, Institute of Molecular Biomembrane and Glycobiology, Tohoku Pharmaceutical University, 4-4-1 Komatsushima, Aoba-ku, Sendai, Miyagi 981-8558, the ‡Department of Molecular Medicine, Osaka Medical Center and Research Institute for Maternal and Child Health, 840 Murodo-cho Izumi, Osaka 594-1101, and §Laboratory Chemicals Division, Wako Pure Chemical Industries, Ltd., 1-2 Doshomachi 3-chome, Chuo-ku, Osaka 540-8605, Japan

Recently we reported that N-glycans on the β-propeller domain of the integrin α5 subunit (S-3,4,5) are essential for α5β1 heterodimerization, expression, and cell adhesion. Herein to further investigate which N-glycosylation site is the most important for the biological function and regulation, we characterized the S-3,4,5 mutants in detail. We found that site-4 is a key site that can be specifically modified by N-acetylglucosaminyltransferase V (GnT-V). The introduction of bisecting GlcNAc into the S-3,4,5 mutant catalyzed by GnT-III decreased cell adhesion and migration on fibronectin, whereas overexpression of N-acetylglucosaminyltransferase V (GnT-V) promoted cell migration. The phenomenon is similar to previous observations that the functions of the wild-type α5 subunit were positively and negatively regulated by GnT-V and GnT-III, respectively, suggesting that the α5 subunit could be duplicated by the S-3,4,5 mutant. Interestingly GnT-III specifically modified the S-4,5 mutant but not the S-3,5 mutant. This result was confirmed by erythroagglutinating phytohemagglutinin lectin blot analysis. The reduction in cell adhesion was consistently observed in the S-4,5 mutant but not in the S-3,5 mutant cells. Furthermore, mutation of site-4 alone resulted in a substantial decrease in erythroagglutinating phytohemagglutinin lectin staining and suppression of cell spread induced by GnT-III compared with that of either the site-3 single mutant or wild-type α5. These results, taken together, strongly suggest that N-glycosylation of site-4 on the α5 subunit is the most important site for its biological functions. To our knowledge, this is the first demonstration that site-specific modification of N-glycans by a glycosyltransferase results in functional regulation.

Glycosylation is a crucial post-translational modification of most secreted and cell surface proteins (1). Glycosylation is involved in a variety of physiological and pathological events, including cell growth, migration, differentiation, and tumor invasion. It is well known that glycans play important roles in cell-cell communication, intracellular signal transduction, protein folding, and stability (2, 3).

Integrins comprise a family of receptors that are important for cell adhesion. The major function of integrins is to connect cells to the extracellular matrix, activate intracellular signaling pathways, and regulate cytoskeletal formation (4). Integrin α5β1 is well known as a fibronectin (FN)3 receptor. The interaction between integrin α5 and FN is essential for cell migration, cell survival, and development (5–8). In addition, integrins are N-glycan carrier proteins. For example, α5β1 integrin contains 14 and 12 putative N-glycosylation sites on the α5 and β1 subunits, respectively. Several studies suggest that N-glycosylation is essential for functional integrin α5β1. When human fibroblasts were cultured in the presence of 1-deoxynajnorinomycin, which prevents N-linked oligosaccharide processing, immature α5β1 integrin appeared on the cell surface, and FN-dependent adhesion was greatly reduced (9). Treatment of purified integrin α5β1 with N-glycosidase F, which cleaves between the innermost N-acetylgalactosamine (GlcNAc) and asparagine N-glycan residues of N-linked glycoproteins, prevented the inherent association between subunits and blocked α5β1 binding to FN (10).

A growing body of evidence indicates that the presence of the appropriate oligosaccharide can modulate integrin activation. N-Acetylglucosaminyltransferase III (GnT-III) catalyzes the addition of GlcNAc to mannose that is β1,4-linked to an underlying N-acetylgalactosamine, producing what is known as a “bisecting” GlcNAc linkage as shown in Fig. 1B. GnT-III is generally regarded as a key glycosyltransferase in N-glycan biosynthetic pathways and contributes to inhibition of metastasis. The introduction of a bisecting GlcNAc catalyzed by GnT-III suppresses additional processing and elongation of N-glycans.

* This work was supported in part by Core Research for Evolutional Science and Technology; the Japan Science and Technology Agency; the “Academic Frontier” Project for Private Universities from the Ministry of Education, Culture, Sports, Science and Technology of Japan; the core to core program (Japan Society for the Promotion of Science); and Takeda Science Foundation, Japan.

† Present address: Dept. of Food Science and Nutrition, Mukogawa Women’s University, Nishinomiya, Hyogo 663-8558, Japan.

‡ To whom all correspondence should be addressed. Tel.: 81-2-727-0216; Fax: 81-2-727-0078; E-mail: jgu@tohoku-pharm.ac.jp.

§ The abbreviations used are: FN, fibronectin; BSA, bovine serum albumin; E4-PHA, erythroagglutinating phytohemagglutinin; GFP, green fluorescent protein; GlcNAc, N-acetylgalactosamine; GnT-I, N-acetylglucosaminyltransferase II; GnT-V, N-acetylglucosaminyltransferase V; L4-PHA, leukoagglutinating phytohemagglutinin; CHO, Chinese hamster ovary; DMEM, Dulbecco’s modified Eagle’s medium; RT-CES, real time cell electronic sensing; WT, wild type; POMT, protein O-mannosyltransferase.
These reactions, which are catalyzed in vitro by other glycosyltransferases, such as N-acetylglucosaminyltransferase V (GnT-V), which catalyzes the formation of β1,6 GlcNAc branching structures (Fig. 1B) and plays important roles in tumor metastasis, do not proceed because the enzymes cannot utilize the bisected N-glycans as a substrate. Introduction of the bisecting GlcNAc to integrin α5β1 by overexpression of GnT-III resulted in decreased in ligand binding and down-regulation of cell adhesion and migration (11–13). Contrary to the functions resulted in decreased ligand binding and down-regulation of integrin α5β1-mediated cell migration on FN (14). These observations clearly demonstrate that the alteration of N-glycan structure affected the biological functions of integrin α5β1. Similarly characterization of the carbohydrate moieties in integrin α5β1 from non-metastatic and metastatic human melanoma cell lines showed that expression of β1,6 GlcNAc branched structures was higher in metastatic cells compared with non-metastatic cells, confirming the notion that the β1,6 GlcNAc branched structure confers invasive and metastatic properties to cancer cells. In fact, Partridge et al. (15) reported that GnT-V-modified N-glycans containing poly-N-acetyllactosamine, the preferred ligand for galectin-3, on surface receptors oppose their constitutive endocytosis, promoting intracellular signaling and consequently cell migration and tumor metastasis.

In addition, sialylation on the non-reducing terminus of N-glycans of α5β1 integrin plays an important role in cell adhesion. Colon adenocarcinomas express elevated levels of α2,6 sialylation and increased activity of ST6Gall sialyltransferase. Elevated ST6Gall positively correlated with metastasis and poor survival. Therefore, ST6Gall-mediated hypersialylation likely plays a role in colorectal tumor invasion (16, 17). In fact, oncogenic ras up-regulated ST6Gall and, in turn, increased sialylation of β1 integrin adhesion receptors in colon epithelial cells (18). However, this is not always the case. The expression of hypersialylated integrin α5β1 was induced by phorbol ester-stimulated differentiation in myeloid cells in which the expression of the ST6Gall was down-regulated by the treatment, increasing FN binding (19). A similar phenomenon was also observed in hematopoietic or other epithelial cells. In these cells, the increased sialylation of the β1 integrin subunit was correlated with reduced adhesiveness and metastatic potential (20–22). In contrast, the enzymatic removal of α2,8-linked oligosialic acids from the α5 integrin subunit inhibited cell adhesion to FN (23). Collectively these findings suggest that the interaction of integrin α5β1 with FN is dependent on its N-glycosylation and the processing status of N-glycans.

Because integrin α5β1 contains multipotential N-glycosylation sites, it is important to determine the sites that are crucial for its biological function and regulation. Recently we found that N-glycans on the β-propeller domain (sites 3, 4, and 5) of the integrin α5 subunit are essential for α5β1 heterodimerization, cell surface expression, and biological function (24). In this study, to further investigate the underlying molecular mechanism of GnT-III-regulated biological functions, we characterized the N-glycans on the α5 subunit in detail using genetic and biochemical approaches and found that site-4 is a key site that can be specifically modified by GnT-III.

**Experimental Procedures**

**Reagents and Antibodies**—A monoclonal antibody against human integrin α5 subunit (clone 1) for Western blot analysis was obtained from BD Biosciences. For immunoprecipitation, the agarose-conjugated anti-green fluorescent protein (GFP) antibody (RQ2) was obtained from Medical & Biological Laboratories Co. Ltd. (Nagoya, Japan). Peroxidase-conjugated antimouse IgG was obtained from Cell Signaling Technology, Inc. (Danvers, MA). A VECTASTAIN ABC kit was purchased from Vector Laboratories, Inc. (Burlingame, CA). Antibodies against GnT-III (33A8) and GnT-V (24B11) were obtained from FUJIREBIO Inc. (Tokyo, Japan). Biotinylated erythroagglutinating phytohemagglutinin (E4-PHA), biotinylated leukoagglutinating phytohemagglutinin (L4-PHA), and biotinylated Datura stramonium lectin were purchased from Sekagaku Corp. (Tokyo, Japan). For fluorescence-activated cell sorting analysis, mouse anti-human α5β1 integrin monoclonal antibody (HA5, MAB1999) was purchased from Chemicon (Temecula, CA).

**Cells and Cell Culture**—The integrin α5 subunit-deficient CHO K1 cell line (CHO-B2) was a gift from Dr. Rudolf Juliano (School of Medicine, University of North Carolina, Chapel Hill, NC) (25). The CHO-B2 stable expression cells containing various integrin α5 with altered N-glycosylation sites were established in our laboratory (24). As shown in Fig. 1A, wild type (WT) indicates CHO-B2 expressing wild-type (full N-glycosylation sites) integrin α5; S-3,4,5, S-3,5, and S-4,5 show that all N-glycosylation sites were removed with site-directed mutagenesis except the indicated sites; and D-3 or D-4 represent single mutations at the indicated site. A HeLa cell line was purchased from RIKEN BioResource Center (Tsukuba, Japan). The stable expression of S-3,5 and S-4,5 mutants in HeLa cells was obtained by viral expression vector as mentioned below. These mutants and cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum, non-essential amino acids (Invitrogen), penicillin (100 units/ml), and streptomycin (100 μg/ml) (Nacalai Tesque, Inc., Kyoto, Japan) under a humidified atmosphere containing 5% CO2.

**GnT-III, GnT-V, and α5 Mutant (S-3,5 and S-4,5) Expression with Viral Vectors**—The cDNAs encoding human GnT-III and GnT-V were amplified for cloning into pENTR-D-Topo for the Gateway Conversion System (Invitrogen) according to the manufacturer’s protocol. The cloned genes were inserted into the virus expression vector, pBABE-puro (Addgene, Inc. Cambridge, MA), accommodated into the Gateway Conversion System using LR Clonase reaction. The GnT-III and GnT-V constructs were transfected into Phoenix-Ampho cells with Lipofectamine 2000 (Invitrogen) for production of viral supernatants. The various α5 integrin mutants were infected with the resulting viral supernatant containing 10 μg/ml Polybrene (Sigma-Aldrich) and selected with 13 μg/ml puromycin for 2 weeks. In the case of HeLa cells expressing S-3,5 and S-4,5 mutants, after virus infection the infected cells were selected with 2.5 μg/ml puromycin. For mock transfection, the same protocol was performed using the empty virus expression vector only.
Cell Adhesion Assay Using 96-well Plate—96-well plates (Corning Inc.) were coated with 3 μg/ml FN at 37 °C for 1 h and blocked with 1% bovine serum albumin (BSA) in DMEM at 37 °C for 1 h. The cells were detached with trypsin containing 1 mM EDTA, resuspended with 0.5 mg/ml trypsin inhibitor (Nacalai Tesque, Inc.) in DMEM. The suspended cells were centrifuged at 1,000 rpm for 3 min and diluted to 4 × 10^5 or 8 × 10^5 cells/ml with assay medium, 0.1% BSA in DMEM. One hundred-microliter aliquots of cell suspension were added to each well, and the plates were incubated at 37 °C for 20–25 min. After incubation, attached cells were fixed with 25% glutaraldehyde (Nacalai Tesque, Inc.) and stained with 0.5% crystal violet. The absorbance at 590 nm was measured using an automated microtiter plate spectrometer, Powerscan® HT (Dainippon Sumitomo Pharma Co., Ltd. Osaka, Japan) operated with Microplate Data Analysis Software, KC4™ (BioTek Instruments, Inc., Winooski, VT). Cell spreading assays were performed as described previously (12, 24). After a 20-min incubation, representative fields were observed using phase-contrast microscopy, and spread cells were counted. The rounded cells were not considered as spread cells.

Cell Adhesion Kinetics Assay Using the Real Time Cell Electronic Sensing (RT-CES™) System—The cell adhesion kinetics assay was performed using a RT-CES system (ACEA Biosciences, Inc.) (26). Briefly ACEA Biosciences, Inc. electrosensory (RT-CESTM) System and L4-PHA lectin blotting (Fig. 2 A). Comparison of N-Glycosylation Patterns on S-3,4,5 α5 Subunit Mutant in GnT-III and GnT-V Transfectants—N-Glycosylation is essential for integrin α5β1 heterodimer formation and therefore plays an important role in the biological function of integrin. GnT-III-modified integrin α5β1 decreased cell adhesion and cell migration on FN (12). In contrast to GnT-III, GnT-V specifically modified only the β1 subunit and up-regulated integrin α5β1-mediated cell migration (14). Recently we found that three N-glycosylation sites, sites 3, 4, and 5 from the N terminus of the α5 subunit, were essential for the biological functions of integrin, such as cell adhesion and migration on FN and heterodimerization.

The purpose of the present study was to determine whether the S-3,4,5 mutant, which contained only three potential N-glycosylation sites (i.e. sites 3, 4, and 5), had characteristics similar to those of the wild-type α5 subunit, such as modification by GnT-III and GnT-V as described above. Various α5 subunit mutants were used in this study as shown in Fig. 1A. First the expression levels of GnT-III and GnT-V in S-3,4,5 mutant cells that had been transfected with a retrovirus system were examined by Western blotting (Fig. 2A). Their products were detected by E4-PHA lectin, which specifically recognizes bisecting GlcNAc, and by L4-PHA lectin, which selectively recognizes β1,6-branched GlcNAc, blots (Fig. 2B) (27, 28). As expected, bands corresponding to GnT-III and GnT-V as well as lectin reactivities of E4-PHA and L4-PHA were increased in the GnT-III and GnT-V transfectants, respectively (Fig. 2, A and B). Equal amounts of protein (20 μg) were loaded in each lane, and α-tubulin was used as the loading control. Next we immunoprecipitated α5 and detected N-glycans using E4-PHA and L4-PHA lectin blotting (Fig. 2C). The E4-PHA reactivities
were much stronger in GnT-III transfectants than those in mock or GnT-V transfectants. This result indicates that both the α5 and β1 subunits are targets of GnT-III. In contrast, results of L4-PHA lectin staining indicated that only the β1 subunit could be modified by GnT-V, consistent with a previous study (14). The reactivities of α5 subunits could be modified by GnT-V, consistent with a previous study (14). The reactivities of α5 subunits could be modified by GnT-V, consistent with a previous study (14).

Effects of GnT-III and GnT-V on Integrin-mediated Cell Adhesion and Migration in S-3,4,5 Transfectants—It is well known that wild-type integrin α5β1-mediated cell migration can be positively and negatively regulated by GnT-V and GnT-III, respectively. Therefore, we determined whether modifications of S-3,4,5 mutants could mimic wild-type α5 to affect its biological functions, such as cell adhesion and cell migration. As shown in Fig. 3A, cell adhesion on FN was down-regulated in GnT-III transfectants compared with mock and GnT-V transfectants. The cell adhesion kinetics assay using RT-CES also indicated nonspecific staining for E4-PHA or L4-PHA. Asterisks indicate nonspecific staining for L4-PHA lectin, which was used as loading control (A and B, lower panels).

FIGURE 1. Potential N-glycosylation sites on the α5 subunit and its modification by GnT-III and GnT-V. A, schematic diagram of potential N-glycosylation sites on the α5 subunit. Putative N-glycosylation sites are indicated by triangles, and point mutations are indicated by crosses (N84Q, N182Q, N297Q, N307Q, N316Q, N524Q, N530Q, N593Q, N609Q, N675Q, N712Q, N724Q, N773Q, and N868Q). B, illustration of the reaction catalyzed by GnT-III and GnT-V. Square, GlcNAc; circle, mannos. TM, transmembrane domain.

FIGURE 2. Comparison of N-glycosylation patterns on α5 subunits modified by GnT-III and GnT-V. A, list of N-glycosylation sites on the α5 subunit. B, schematic diagram of potential N-glycosylation sites on the α5 subunit. Putative N-glycosylation sites are indicated by triangles, and point mutations are indicated by crosses (N84Q, N182Q, N297Q, N307Q, N316Q, N524Q, N530Q, N593Q, N609Q, N675Q, N712Q, N724Q, N773Q, and N868Q). B, illustration of the reaction catalyzed by GnT-III and GnT-V. Square, GlcNAc; circle, mannos. TM, transmembrane domain.

FIGURE 2. Comparison of N-glycosylation patterns on α5 subunits modified by GnT-III and GnT-V. A, list of N-glycosylation sites on the α5 subunit. B, schematic diagram of potential N-glycosylation sites on the α5 subunit. Putative N-glycosylation sites are indicated by triangles, and point mutations are indicated by crosses (N84Q, N182Q, N297Q, N307Q, N316Q, N524Q, N530Q, N593Q, N609Q, N675Q, N712Q, N724Q, N773Q, and N868Q). B, illustration of the reaction catalyzed by GnT-III and GnT-V. Square, GlcNAc; circle, mannos. TM, transmembrane domain.
formed fluorescence-activated cell sorting analysis using anti-α5β1 integrin antibody. As shown in Fig. 3D, there were no significant differences in the levels of cell surface expression among the three cell types, indicating that the functional alterations shown in Fig. 3 were due to N-glycosylation of the integrin modified by GnT-III or GnT-V.
**GnT-III Selectively Modifies N-Glycosylation Site-4 on the α5 Subunit**—As described above, the characteristics of the S-3,4,5 mutant are similar to those of wild-type α5. We therefore determined whether GnT-III could specifically modify the N-glycosylation site among site-3, site-4, and site-5. Because the N-glycosylation site-5 is essential for its expression on the cell surface, the mutant did not exhibit biological function such as cell adhesion (24). Thus, we chose the S-3,5 and S-4,5 mutants for use in further studies.

First GnT-III was overexpressed in both transfectants. The expression levels of GnT-III were almost the same in S-3,5 as in S-4,5 transfectants, which were examined by Western blot using anti-GnT-III antibody (Fig. 4A). It is of particular interest that the mutant S-4,5, but not the S-3,5 mutant, was clearly detected using E4-PHA lectin blot. The intensity of the lectin staining was comparable to that of S-3,4,5 (Fig. 4B). These results, taken together, suggest that site-3 may not be modified by GnT-III. Because introduction of bisecting GlcNAc into the α5 subunit down-regulates cell adhesion as described above, we checked whether this phenomenon occurred in these mutants. Overexpression of GnT-III in S-4,5 cells consistently inhibited cell adhesion on FN, whereas the inhibition of cell adhesion was not observed in S-3,5 cells overexpressing GnT-III (Fig. 4C and D). It should be noted that cell adhesion activities of the S-3,5 mutant was similar to that of S-4,5 mutant because CHO-B2 cells do not express enough endogenous GnT-III to modify integrin as shown in Fig. 2.

To confirm whether the site-specific modification also happens in endogenous conditions, we introduced the S-3,5 and S-4,5 mutants into HeLa cells that express a relatively higher level of endogenous GnT-III to examine the products of GnT-III as confirmed by E4-PHA lectin blot. Consistent with the results of overexpressing GnT-III, the E4-PHA lectin staining was clearly detected in S-4,5 but not in S-3,5 α5 subunit transfectants (Fig. 4E). Taken together, these results strongly suggest that GnT-III may specifically modify site-4 on the α5 subunit, which down-regulates its biological functions.

To further elucidate the importance of site-4 for GnT-III modification, we compared the E4-PHA staining patterns of WT with single mutants, such as site-3 (D-3) or site-4 (D-4), as shown in Fig. 1A. These three expression plasmids were co-transfected with GnT-III, and the α5 integrins were immuno-
**N-Glycosylation Site-4 on α5 Is Important for Its Function**

In the present study, we intensively investigated the effects of N-glycosylation on the β-propeller of the integrin α5 subunit on its biological functions such as cell adhesion and cell migration and found that site-4 is essential and effective for GnT-III modification among 14 potential N-glycosylation sites. To our knowledge, this is the first report to clearly demonstrate that a glycosyltransferase of N-glycosylation can specifically modify an N-glycosylation site among multiple potential sites and effectively regulate its biological functions.

Integrins can be activated by inside-out signaling mechanisms that trigger global conformational changes, which ultimately modulate integrin-ligand affinity. It is apparent that integrin activity can be regulated by other mechanisms, such as posttranscriptional modification, N-glycosylation. Altered integrin glycosylation has been associated with tumorigenesis, autoimmune disease, chronic inflammation, and cell adhesion events (11, 30). In particular, N-glycosylation of the integrin α5 and β1 subunits appears to be important for both structure and function. It has been reported that N-glycosylation of both the α5 and β1 subunits is necessary for α5β1 heterodimerization and its binding to FN. Moreover, changes in integrin glycan composition, resulting from forced expression of selected glycosyltransferases, i.e., “remodeling,” reportedly modulate integrin functions as described above. However, most of these earlier studies examined only total changes without individual information. Therefore, the exact molecular mechanisms by which N-glycosylation of site(s) or glycan(s) occurs remain unknown. Recently, we used site-directed mutagenesis to determine that N-glycosylation site-5 on the β-propeller plays an important role in the assembly of the integrin for its expression on the cell surface (24). These observations prompted us to determine whether there are specific N-glycosylation sites that regulate its biological functions. Here we clearly showed that site-4 is a key N-glycosylation site for the biological function of α5 subunit that is effectively modified by GnT-III. Taken together these results indicate that individual N-glycosylation sites may have unique functions.

Although the molecular mechanism by which bisecting GlcNAc is introduced into site-4, inhibiting its biological function, remain unknown, we speculate that the effect of altered glycosylation of site-4 may be related to conformational changes in the key functional regions of the β-propeller domain of the α5 subunit that are critical for integrin activation. In fact, the β-propeller domain has been postulated to be required for effective interaction between α5β1 integrin and its ligand (31). In contrast, the crystal structure of integrin αVβ3 has been successfully determined, and the main contact between the αV and β3 subunits is the β-propeller on the α and A domain on β3 with hydrophobic, ionic, and mixed contacts (32, 33). Because the α5 subunit has 47% homology to αV, Mould et al. (34) made a homologous modeling structure of α5β1. Based on the model, the α5 subunit seems to be surrounded by N-glycans, explaining the dissociation of the αβ heterodimer that occurs when α5β1 is deglycosylated by treatment with peptide-N-glycosidase F or removal of N-glycans on the β-propeller. Very recently, Liu et al. (35) used a molecular modeling approach to study the effects of altered glycosylation on the I-like domain of...
the β1 subunit, which is the partner of the β-propeller of the α subunit. These researchers found that α2,6 sialic acid affected the interactions between N-glycans and the I-like domain, which in turn altered the accessibility of the loop that determines specificity of ligand binding. In fact, the remodeling of N-glycans by GnT-III affects either the branching formations catalyzed by GnT-V and GnT-IV or the sialylation on the terminus of the N-glycans (11, 36). Therefore, a possible mechanism by which N-glycans are involved in the αβ interaction or conformational arrangement is that an unknown lectin domain may exist on the α or β subunit. The lectin domain of αMβ2 integrin is associated with GlcNAc on the non-reducing terminus of sugar chains on platelets, facilitating their phagocytosis (37, 38). These studies further support the observation that modification of bisecting GlcNAc on site-4 of the β-propeller may be critical for the regulation of its biological functions, which may shed light on the structural studies.

It is of interest to understand why GnT-III specifically and effectively modifies site-4 of the 14 putative N-glycosylation sites. There is currently no detailed information available regarding this observation, but several explanations have been proposed. First, N-glycosylation occurs on site-4 because it provides the easiest access for GnT-III. Because the integrin α5 crystal structure is currently unavailable this hypothesis cannot be proven. Second, GnT-III may associate with some other molecules, which define the specificities for protein or peptide substrates. Reportedly protein O-mannosyltransferase 1 (POMT1) and its homolog POMT2 are responsible for catalyzing the first step in O-mannosyl glycan, which is important for muscle and brain development (39). Interestingly Manya et al. (40) reported that formation of a POMT1-POMT2 complex is essential for POMT activity. Only two peptides derived from the mucin domain of α-dystroglycan are highly O-mannosylated by POMT, but no O-mannosylation occurs in mucin tandem repeat peptides (41). Similarly complex formation is also important for T-synthase (core 1 BL galactosyl transferase) activity. Ju et al. (42, 43) reported that Tn syndrome, a rare autoimmune disease, in which subpopulations of blood cells of all lineages carry an incompletely glycosylated membrane glycoprotein, known as the Tn antigen, is associated with a somatic mutation in Cosmic, a gene on the X chromosome that encodes a molecular “chaperone” that is required for the proper folding and hence full activity of T-synthase. Indeed it has been reported that caveolin-1 may co-localize with GnT-III to regulate its localization and activity (44). Those results, taken together, suggest that glycosyltransferase complex formation may play a crucial role in determination of both activity and substrate specificity. The detailed molecular mechanism requires further study.

This study specifically focused on N-glycosylation of the integrin α5 subunit. To fully understand the effects of the N-glycans on integrin structure and function, it will be necessary, in future studies, to investigate the interaction of glycans with glycans or peptides of integrin. The current study also has implications for engineering α5 that contains the glycans necessary for its activation that may facilitate the study of its crystal structure.

REFERENCES

1. Apweiler, R., Hermjakob, H., and Sharon, N. (1999) Biochim. Biophys. Acta 1473, 4–8
2. Dwek, R. A. (1995) Biochem. Soc. Trans. 23, 1–25
3. Saxon, E., and Bertozzi, C. R. (2001) Annu. Rev. Cell Dev. Biol. 17, 1–23
4. Hynes, R. O. (2002) Cell 110, 673–687
5. George, E. L., Georges-Labouesse, E. N., Patel-King, R. S., Rayburn, H., and Hynes, R. O. (1993) Development 119, 1079–1091
6. Goh, K. L., Yang, J. T., and Hynes, R. O. (1997) Development 124, 4309–4319
7. Watt, F. M., and Hodivala, K. J. (1994) Curr. Biol. 4, 270–272
8. Yang, J. T., Rayburn, H., and Hynes, R. O. (1993) Development 119, 1093–1105
9. Akiyama, S. K., Yamada, S. S., and Yamada, K. M. (1989) J. Biol. Chem. 264, 18011–18018
10. Zheng, M., Fang, H., and Hakomori, S. (1994) J. Biol. Chem. 269, 12325–12331
11. Gu, J., and Taniguchi, N. (2004) Glycoconj. J. 21, 9–15
12. Isaji, T., Gu, J., Nishiuchi, R., Zhao, Y., Takahashi, M., Miyoshi, E., Honke, K., Sekiguchi, K., and Taniguchi, N. (2004) J. Biol. Chem. 279, 19747–19754
13. Zhao, Y., Nakagawa, T., Itoh, S., Inamori, K., Isaji, T., Kariya, Y., Kondo, A., Miyoshi, E., Miyazaki, K., Kawasaki, N., Taniguchi, N., and Gu, J. (2006) J. Biol. Chem. 281, 32122–32130
14. Guo, H. B., Lee, I., Kamar, M., Akiyama, S. K., and Pierce, M. (2002) Cancer Res. 62, 6837–6845
15. Partridge, E. A., Le Roy, C., Di Guglielmo, G. M., Pawling, J., Cheung, P., Granovsky, M., Nabi, I. R., Wñana, J. L., and Dennis, J. W. (2004) Science 306, 120–124
16. Seales, E. C., Jurado, G. A., Brunson, B. A., Wakefield, J. K., Frost, A. R., and Bellis, S. L. (2005) Cancer Res. 65, 4645–4652
17. Shaikh, F. M., Seales, E. C., Clem, W. C., Hennessy, K. M., Zhuo, Y., and Bellis, S. L. (2008) Exp. Cell Res. 314, 2941–2950
18. Seales, E. C., Jurado, G. A., Singhal, A., and Bellis, S. L. (2003) Oncogene 22, 7137–7145
19. Semel, A. C., Seales, E. C., Singhal, A., Eklund, E. A., Colley, K. J., and Bellis, S. L. (2002) J. Biol. Chem. 277, 32830–32836
20. Pretzflaß, R. K., Xue, W. V., and Rowin, M. E. (2000) Cell Adhes. Commun. 7, 491–500
21. Kawanot, T., Takasaki, S., Tao, T. W., and Kobata, A. (1993) Int. J. Cancer 53, 91–96
22. Dennis, J., Waller, C., Timpl, R., and Schirmacher, V. (1982) Nature 300, 274–276
23. Nadanaka, S., Sato, C., Kitajima, K., Katagiri, K., Irie, S., and Yamagata, T. (2001) J. Biol. Chem. 276, 33657–33664
24. Isaji, T., Sato, Y., Zhao, Y., Miyoshi, E., Wada, Y., Taniguchi, N., and Gu, J. (2006) J. Biol. Chem. 281, 33258–33267
25. Schreiner, C. L., Bauer, J. S., Danilov, Y. N., Hussein, S., Sczekan, M. M., and Juliano, R. L. (1989) J. Cell Biol. 109, 3157–3167
26. Solli, K., Wang, X., Xu, X., Strulovicz, B., and Zheng, W. (2004) Assay Drug Dev. Technol. 2, 363–372
27. Cummings, R., and Kornfeld, S. (1982) J. Biol. Chem. 257, 11230–11234
28. Yamashita, K., Kitai, H., and Kobata, A. (1983) J. Biol. Chem. 258, 14753–14755
29. Yamashita, K., Totani, K., Ohkura, T., Takasaki, S., Goldstein, I. J., and Kobata, A. (1987) J. Biol. Chem. 262, 1602–1607
30. Bellis, S. L. (2004) Biochim. Biophys. Acta 1663, 52–60
31. Mould, A. P., Askari, J. A., and Humphries, M. J. (2000) J. Biol. Chem. 275, 20324–20336
32. Xiong, J. P., Stehle, T., Zhang, R., Joachimacki, A., Frech, M., Goodman, S. L., and Arnaout, M. A. (2002) Science 296, 151–155
33. Xiong, J. P., Stehle, T., Dieffenbach, B., Zhang, R., Dunker, R., Scott, D. L., Joachimacki, A., Goodman, S. L., and Arnaout, M. A. (2001) Science 294, 339–345
34. Mould, A. P., Symonds, E. J., Buckley, P. A., Grossmann, J. G., McEwan, P. A., Barton, S. J., Askari, J. A., Craig, S. E., Bella, J., and Humphries, M. J. (2003) J. Biol. Chem. 278, 39993–39999
35. Liu, Y., Pan, D., Bellis, S. L., and Song, Y. (2008) *Proteins* 73, 989–1000
36. Koyota, S., Ikeda, Y., Miyagawa, S., Ihara, H., Koma, M., Honke, K., Shirakura, R., and Taniguchi, N. (2001) *J. Biol. Chem.* 276, 32867–32874
37. Hoffmeister, K. M., Josefsson, E. C., Isaac, N. A., Clausen, H., Hartwig, J. H., and Stossel, T. P. (2003) *Science* 301, 1531–1534
38. Josefsson, E. C., Gebhard, H. H., Stossel, T. P., Hartwig, J. H., and Hoffmeister, K. M. (2005) *J. Biol. Chem.* 280, 18025–18032
39. Endo, T., and Manya, H. (2006) *Methods Enzymol.* 417, 137–152
40. Manya, H., Chiba, A., Yoshida, A., Wang, X., Chiba, Y., Jigami, Y., Margolis, R. U., and Endo, T. (2004) *Proc. Natl. Acad. Sci. U. S. A.* 101, 500–505
41. Manya, H., Suzuki, T., Akasaka-Manya, K., Ishida, H. K., Mizuno, M., Suzuki, Y., Inazu, T., Dohmae, N., and Endo, T. (2007) *J. Biol. Chem.* 282, 20200–20206
42. Ju, T., and Cummings, R. D. (2002) *Proc. Natl. Acad. Sci. U. S. A.* 99, 16613–16618
43. Ju, T., and Cummings, R. D. (2005) *Nature* 437, 1252
44. Sasai, K., Ikeda, Y., Ihara, H., Honke, K., and Taniguchi, N. (2003) *J. Biol. Chem.* 278, 25295–25301