N-Glycosylation of the β-Propeller Domain of the Integrin α5 Subunit Is Essential for α5β1 Heterodimerization, Expression on the Cell Surface, and Its Biological Function*

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The N-glycosylation of integrin α5β1 is thought to play crucial roles in cell spreading, cell migration, ligand binding, and dimer formation, but the underlying mechanism remains unclear. To investigate the importance of the N-glycans of this integrin in detail, sequential site-directed mutagenesis was carried out to remove single or combined putative N-glycosylation sites on the α5 integrin. Removal of the putative N-glycosylation sites on the β-propeller, Thigh, Calf-1, or Calf-2 domains of the α5 subunit resulted in a decrease in molecular weight compared with the wild type, suggesting that all of these domains contain attached N-glycans. Importantly, the absence of N-glycosylation sites (sites 1–5) on the β-propeller resulted in the persistent association of integrin subunit with calnexin in the endoplasmic reticulum, which subsequently blocked heterodimerization and its expression on the cell surface. Interestingly, the activities for cell spreading and migration for the α5 subunit carrying only three potential N-glycosylation sites (3–5 sites) on the β-propeller were comparable with those of the wild type. In contrast, mutation of these three sites resulted in a significant decrease in cell spreading as well as functional expression, although the total expression level of the Δ3–5 mutant on the cell surface was comparable with that of wild type. Furthermore, we found that site 5 is a most important site for its expression on the cell surface, whereas the S5 mutant did not show any biological functions. Taken together, this study reveals for the first time that the N-glycosylation on the β-propeller domain of the α5 subunit is essential for heterodimerization and biological functions of α5β1 integrin and might also be useful for studies of the molecular structure.

The fibronectin (FN)2 receptor, integrin α5β1, is heterodimeric glycoprotein that consists of an α5 subunit and a β1 subunit. The interaction between α5β1 and FN is essential for cell migration, development, as well as cell viability, because the genetic lack of integrin α5 or FN results in early embryonic lethality (1–3). The most general feature of integrin is that the interaction of integrin with its ligand can activate intracellular signaling pathways and cytoskeletal formation (outside-in signaling) (4). Another important feature of integrin is inside-out signaling, in which intracellular signals received by integrin or other receptors, in turn, activate its extracellular domain and contribute to the assembly of the extracellular matrix (4, 5). It is also well known that functions of integrins related to cell spreading and migration can be regulated by specific peptides such as Arg-Gly-Asp containing peptides or specific antibodies that can efficiently block or activate such integrin-ligand interactions (6–8).

Integrin is a major carrier of N-glycans. An increasing body of evidence exists to suggest that cell surface carbohydrates contribute to a variety of interactions between the cell and its extracellular environment, as well as a wide variety of biological functions such as cell-cell communication, signal transduction, protein folding, or stability (9–11). Among the integrin superfamily, α5β1 is one of the best characterized integrins. It has been reported that the presence of N-glycans on integrin α5β1 is required for αβ heterodimer formation and proper integrin-matrix interactions (12, 13). Indeed, the integrin cannot bind to its substrate or be normally transported to the cell surface in the presence of the glucosylation inhibitor tunicamycin (14). Moreover, treatment of the purified integrin with N-glycosidase F resulted in blocking the inherent association of both subunits and the interaction between integrin and FN, suggesting that N-glycosylation is essential for the integrin to be functional (15).

Integrin-mediated biological functions such as cell spreading and cell migration can be modulated as a consequence of an aberrant change in the N-glycosylation of integrins, which is often associated with a carcinogenic process (12, 13, 16–18). Several research groups, including our group, recently reported that alterations in the oligosaccharide portion of integrin α5β1, which are modulated by the expression of each glycosyltransferase gene such as N-acetylgalactosaminyltransferase-V (GnT-V), FBS, fetal bovine serum; PBS, phosphate-buffered saline; BSA, bovine serum albumin; WT, wild type; ER, endoplasmic reticulum; FACS, fluorescence-activated cell sorter.
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V), Gnt-T-III, and α2,6-sialytransferase, regulate cell malignant phenotypes such as α5β1-mediated cell migration and cell spreading (19–21). It has also been reported that alterations in the glycosylation state on the integrin affect its binding affinity to FN. In the case of the addition of a bisecting GlcNAc, a product of Gnt-T-III, to the α5 subunit, its binding to FN was reduced substantially (20). Conversely, the expression of hyposialylated integrin α5β1 was reported to be induced by phorbol ester-stimulated differentiation in myeloid cells, resulting in an increase FN binding (21). Alterations of N-glycans on integrins could also regulate their cis interactions with membrane-associated proteins, including the epidermal growth factor receptor, the urokinase-type plasminogen activation receptor, and the tetraspanin family of proteins (22–25).

Although the N-glycosylation of integrin α5β1 plays crucial roles in heterodimer formation and its biological functions, it contains 26 potential N-linked glycosylation sites, 14 in the α subunit and 12 in the β subunit. In this study, to determine which of the N-glycosylation sites on the α5 subunit are essential for these functions, we sequentially mutated one or combined asparagine residues in the putative N-glycosylation sites to glutamine residues, and then transfected these mutant genes into α5-deficient Chinese hamster ovary (CHO) cells (CHO-B2). We found that the N-glycosylation on the β-propeller domain of the α5 subunit, in particular sites number 3–5 sites, is essential for its heterodimer formation and its biological functions such as cell spreading and cell migration, as well as the proper folding of the α5 subunit.

MATERIALS AND METHODS

Antibodies—A hybridoma producing monoclonal antibody (mAb) against the human integrin α5 subunit (BIIG2) and the supernatant of the hybridoma of hamster integrin β1 subunit (7E2) were purchased from Developmental Studies Hybridoma Bank, University of Iowa (26, 27). The BIIG2 antibody was purified from the hybridoma supernatant with protein G-Sepharose™ 4 Fast Flow (Amersham Biosciences). For Western blot analysis, mAb against human integrin α5 subunit (clone1) was obtained from BD Biosciences. The rabbit antibody anti-integrin α5 carboxy-terminal domain (AB1949) and a non-functional blocking antibody (HA5, MAB1999) were purchased from Chemicon (Temecula, CA). The peroxidase-conjugate goat antibody against mouse IgG was obtained from Promega (Madison, WI). Alexa Fluor 546 goat anti-mouse IgG and phalloidin Alexa Fluor 546 were obtained from Invitrogen. The goat antibody against the green fluorescent protein (GFP) was obtained from Rockland Immunochemicals, Inc. (Gilbertsville, PA).

Integrin α5 Subunit Expression Vector—The cDNA of the human integrin α5 subunit was amplified by PCR from the reverse-transcribed product of human placenta total RNA (OriGene Technologies, Inc., Rockville, MD) to yield the fragment flanked by the Nhel and Xhol sites for 5’ and just before the stop codon using a mutagenic PCR primer. This complete cDNA of the integrin α5 subunit was cloned into a cloning vector (pGEMT-Easy; Promega, Madison, WI). The sequence of the full length of cDNA was confirmed using an ABI PRISM 3100 genetic analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan). Mutations were introduced into the cDNA for α5 using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA.) according to the manufacturer’s instructions. For expression in mammalian cells, these α5 cDNAs were digested by Nhel and Xhol and ligated to multiple cloning sites of Nhel and Sall (Xhol-compatible cohesive end) of pEGFP-N1 (BD Biosciences) with using the T4 ligase. The GFP-tagged α5 subunit contains a 13-aminos acid linker (STVPRARDPPVPAT) between the carboxy-terminal domain of α5 and the GFP tag. The coding regions of all constructs of cDNA of the α5 subunit were sequenced, to confirm the presence of the desired mutations as shown Fig. 1 without any additional mutation.

Cell Culture—Integrin α5 subunit-deficient CHO K1 mutant cells (CHO-B2) were a gift from Dr. Rudolf Juliano (School of Medicine, University of North Carolina, Chapel Hill) (28). CHO-B2 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS). The cDNA of integrin α5-GFP WT and mutants were transfected into CHO-B2 cells with Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were selected in the presence of 1.5 mg/ml G418 disulfate (Nacalai Tesque, Kyoto, Japan). The antibiotic-resistant and GFP-positive colonies were picked up and used in subsequent studies.

Cell Adhesion—Cell spreading assays were performed as described previously with minor modifications (20). Briefly, 96-well microtiters plates (Nunc, Wiesbaden, Germany) were coated with a solution of 10 μg/ml human serum FN (Sigma) in phosphate-buffered saline (PBS) overnight at 4 °C and blocked with 1% bovine serum albumin (BSA) in DMEM for 1 h at 37 °C. The cells were detached with trypsin containing 1 mM EDTA, washed with serum-containing DMEM, and then suspended in serum-free DMEM with 0.1% BSA at 4 × 10⁴ cells/ml. To confirm whether or not the cell spreading on FN was α5β1 integrin-dependent, cells were preincubated with the functional blocking mAb against α5 (BIIG2) or rat control IgG at final concentrations at 10 μg/ml at room temperature for 10 min before plating. After a 20-min incubation, nonadherent cells were removed by washing with PBS, and the attached cells were fixed with 3.7% paraformaldehyde in PBS, and representative fields were then observed by phase contrast microscopy.

Cell Migration—Transwell (BD BioCoat™ Control Inserts, 8.0-μm inserts; BD Biosciences) were coated by incubation in 10 μg/ml FN in PBS overnight at 4 °C followed by an incubation with 1% BSA for 1 h at 37 °C. Cells were detached with trypsin containing 1 mM EDTA, washed once with DMEM containing 10% FBS, and then suspended in DMEM containing 1% FBS at 1 × 10⁶ cells/ml. The cell suspension (100 μl) was preincubated with the anti-α5 integrin antibody (BIIG2) or rat control IgG at a final concentration of 10 μg/ml for 10 min, and then added to each upper side of the chamber. After 3 h of incubation at 37 °C, the remaining cells on the upper side of the chamber were carefully scraped off with a cotton swab. Cells that migrated to the lower surface of the membrane were fixed with 3.7% paraformaldehyde in PBS, stained with 0.3% crystal violet for 30 min, and then observed under a phase-contrast microscope and counted as migrated cells.

Immunofluorescence Microscopy—Glass coverslips (Iwaki, Tokyo, Japan) were coated with FN (20 μg/ml) in PBS overnight

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at 4 °C and then blocked with 1% BSA. The wild type and mutant of CHO-B2 cells were replated on the coverslips by incubation for 2 h in DMEM containing 1% BSA. The cells were then fixed with 3.7% paraformaldehyde in PBS for 20 min and permeabilized with 0.1% Triton X-100 at room temperature for 5 min. The integrin β1 subunit was visualized by incubating cells with the mAb against the hamster integrin β1 subunit (7E2), followed by incubation with Alexa Fluor 546 goat antimouse IgG. Actin filaments were stained with Alexa Fluor 546-conjugated phalloidin.

**Immunoprecipitation and Western Blot**—Cells were washed with ice-cold PBS and lysed in lysis buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% (w/v) Nonidet P-40, Complete™ EDTA-free protease inhibitor mixture (Roche Diagnostics). The cell lysates were centrifuged at 12,000 × g for 15 min at 4 °C. The supernatants were collected, and protein concentrations were determined by means of a protein assay Coomassie Brilliant Blue kit (Nacalai Tesque). Equal amounts of protein samples were incubated with 2 μg of each antibody for 1 h and then 15 μl of protein G-Sepharose was added for another 1-h incubation at 4 °C. To avoid antibody (IgG) contamination in the immunocomplexes, because IgG migrated at a rate similar to the integrins under nonreducing conditions on SDS-PAGE, we cross-linked goat antibody with protein G-Sepharose by means of disuccinimidyl substrate (Pierce) according to the manufacturer’s instructions. The immunoprecipitates were washed three times with lysis buffer. Equal amounts of proteins were subjected to 7.5% SDS-PAGE and then transferred to a nitrocellulose membrane (Schleicher & Schuell). The membrane was incubated with primary and secondary antibodies for 1 h each, and detection was performed using an ECL kit (Amersham Biosciences) according to the manufacturer’s instructions.

**Cell Surface Biotinylation**—Cell surface biotinylation was performed as described previously with minor modifications (20). Briefly, semi-confluent cells were washed twice with ice-cold PBS, and then incubated with ice-cold PBS containing 0.2 mg/ml sulfo-NHS-LC-biotin (Pierce) for 1 h at 4 °C. After washing three times with ice-cold PBS, the cells were harvested and lysed in lysis buffer as described above. The resulting cell lysate was immunoprecipitated with the anti-integrin α5 antibody. The immunocomplex was subjected to 7.5% SDS-PAGE and transferred to a nitrocellulose membrane. The biotinylated proteins were detected with a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA).

**Metabolic Labeling**—For pulse-chase experiments, cells grown at 90% confluence on 6-well dishes were washed three times with PBS-free medium and then starved for 30 min in DMEM by excluding methionine and cysteine (Sigma). After starvation, the cells were pulse-labeled in 500 μl of DMEM containing 200 μCi of [35S]methionine and cysteine (Amer- sham Biosciences) for 30 min, and then chased with complete DMEM containing 10% FBS at the indicated times. The cells were lysed, and the cell lysates were immunoprecipitated with the goat anti-GFP polyclonal antibody or anti-hamster β1 subunit antibody. The immunoprecipitates were separated on 7.5% SDS-PAGE. After drying the gels, radioactive bands were visualized with a Fuji BAS 2500 Bio-Image Analyzer.

**Flow Cytometry Analysis**—Flow cytometry analysis was performed as described previously with minor modifications (20). Briefly, the cells in semi-confluent conditions were detached from 10-cm culture dishes using trypsin containing 1 mM EDTA and stained with and without a primary antibody (BIIG2, HA5, or P1D6), followed by incubation with Alexa Fluor 647 goat anti-rat IgG (for BIIG2) or anti-mouse IgG (for HA5 and P1D6). After washing three times with PBS, flow cytometry analyses were performed using a FACSCalibur instrument (BD Biosciences) operated with CELLQuestPro software.

**RESULTS**

**Construction of Various Integrin α5 Mutants by the Mutagenesis of Potential N-Glycosylation Sites**—Human integrin α5 contains 14 N-glycosylation sites (Asn-Xaa-Ser/Thr) (Asn-84, Asn-182, Asn-297, Asn-307, Asn-316, Asn-524, Asn-530, Asn-593, Asn-609, Asn-675, Asn-712, Asn-724, Asn-773, and Asn-868), as shown in Fig. 1, which are located in the extracellular
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FIGURE 2. Comparison of cell spreading on FN for various unglycosylated mutants of α5 subunit. Cells were detached and then replated on dishes that had been precoated with 10 μg/ml of FN. After incubation for 30 min, cells were fixed with 3.7% paraformaldehyde, and representative fields were photographed using a 200-fold phase contrast microscope. The bar denotes 120 μm.

Effects of the Removal of N-Glycosylation Sites on Integrin α5 on FN-mediated Cell Spreading, Migration, and Cytoskeleton Formation—Although it is known that the N-glycosylation of integrin α5β1 is essential for its functions (14, 15, 29), the distinctive roles of N-glycosylation on each domain are not completely understood. To examine the effects of unglycosylation in the β-propeller domain or Thigh and Calf domain of α5, corresponding to Δ1–5 and Δ6–14, respectively, on cell spreading and migration, we transfected these mutated cDNAs into CHO-B2 cells. As expected, the expression of wild type (WT) α5 subunit, but not GFP control, extensively rescued cell adhesion on FN (Fig. 2), supporting that CHO-B2 cells are a useful cell model for studies of integrin α5 functions (28, 30–32). The cell spreading on FN was completely inhibited by an anti-α5 functional blocking antibody but not by normal rat IgG (data not shown), indicating that the initial cell spreading on FN is mediated through integrin α5. On the other hand, overexpression of the unglycosylation mutant of β-propeller Δ1–5 did not rescue cell spreading. In contrast, the transfection of the Δ6–14 mutant significantly induced cell adhesion and cell spreading on FN-coated dishes, as did the overexpression of the WT. Of particular interest, cells expressing the S3-5 mutant, in which the 3–5 N-glycosylation sites on α5 subunit are present only, showed comparable abilities for cell spreading as cells expressing the WT. In addition, cells expressing the Δ6–9 mutant on the Thigh domain or the Δ10–14 mutant on the Calf-1,2 domains were also able to rescue cell spreading on FN (data not shown). These results strongly suggest that the N-glycosylation on the β-propeller of the α5 subunit is essential for biological function. It is noteworthy that the overexpression of each 14 single unglycosylation mutant efficiently rescued cell spreading (data not shown).

Consistent with cell spreading, cell migration on FN was observed in cells expressing WT of the α5 subunit using a Border chamber assay (Fig. 3). Cell migration was completely blocked by incubation with a functional blocking antibody of integrin α5, but not normal rat IgG, suggesting that the integrin α5 plays a major role in cell migration on FN. Interestingly, cell migration was completely blocked in Δ1–5 transfectants as observed in mock transfectants. On the other hand, cell migration for Δ6–14 as well as the S3-5 transfectants had comparable activities to the WT transfectants. Integrin-mediated cell adhesion on the extracellular matrix usually activates small G proteins such as Rho, Rac, and Cdc42 to promote cytoskeletal formation. In fact, stress fiber formation was clearly observed in cells expressing the WT, Δ6–14, or S3-5 of the α5 subunits but
not the Δ1–5 mutant or the mock control (Fig. 4). These findings strongly suggest that N-glycosylation on the β-propeller of integrin α5 is essential for integrin α5-mediated cell spreading, migration, and cytoskeletal formation.

N-Glycosylation on β-Propeller of α5 Subunit Is Essential for Its Expression on the Cell Surface—To explore the molecular mechanisms associated with the reduced abilities for cell spreading and migration in transfectants expressing the Δ1–5 subunit, we examined the expression levels of each mutant in total cell lysates on cell surfaces. As shown in Fig. 5A, each mutant expressed comparable levels of α5 subunit, as confirmed by the use of an anti-integrin α5 antibody. It was also clear that each domain of the α5 subunit carries N-glycans, because the band for each mutant migrated faster than that for the WT. Moreover, after treatment with N-glycosidase F, all bands of the mutated or WT α5 subunits shifted to around 90 kDa under reducing conditions (data not shown). On the other hand, the expression levels of α5 subunits on the cell surface were examined by biotinylating the cell surface. Biotin-labeled cells were lysed and immunoprecipitated with an anti-α5 subunit antibody. Surprisingly, the expression level of the Δ1–5 mutant on the cell surface was significantly decreased, compared with those of the WT as well as the Δ5–14 mutant (Fig. 5B). It is important to note that the expression level of the S3–5 mutant was comparable with that of WT, suggesting that N-glycosylation on the β-propeller of the α5 subunit may be essential for its expression on a cell surface. These phenomena were also observed for 293T cells transfected with all of these mutants.

N-Glycosylation on β-Propeller Is Required for α/β Heterodimerization—It has been reported that functional integrin α5β1 is required for not only the heterodimerization of α5 and β1 subunits (4) but also for the maturation of N-glycans on the integrin (33). Here we investigated the heterodimeric formation of α5 and β1 subunits by immunoprecipitation and an immunostaining assay. The α subunits expressed in WT, Δ6–14, and the S3-5 transfectants were clearly observed in integrin β1 immunocomplexes. However, the α subunits expressed by Δ1–5 or vector control cells were under the detectable levels in integrin β1 immunocomplexes (Fig. 5C). The β1 subunits expressed in WT, Δ6–14, and S3-5, but not in the Δ1–5 transfectants, were consistently detected in α5 immunocomplexes (Fig. 5D). Surprisingly, the levels of β1 subunits expressed in total cell lysates of the Δ1–5 and GFP control transfectants were significantly decreased, compared with cells that expressed WT, Δ6–14, or S3-5 mutants (Fig. 5E). The phenomenon was also confirmed by co-transfection of α5 (WT, Δ6–14, S3-5, or Δ1–5) plus the β1 subunit in 293T cells. The findings showed that the expression levels of the β1 integrin in the Δ1–5 and GFP control transfectants were reduced, compared with those in the WT, Δ6–14, or S3-5 transfectants (data not shown). The association or dissociation of integrin α and β subunits was confirmed by immunostaining. Heterodimer formation of α5 and β1 was clearly observed in WT, Δ6–14, or S3-5, but not the Δ1–5 transfectants, as shown in Fig. 5F, arrowheads. Collectively, these results suggest that the N-glycosylation of the β-propeller domain of the α5 subunit is involved in the formation of αβ heterodimers.

Effects of N-Glycosylation on β-Propeller of α5 on Post-translational Processing and Stability for β1 Integrin—To elucidate the underlying mechanisms of impaired αβ heterodimer formation and the decreased expression of the β1 subunit in Δ1–5, the kinetics of the biosynthesis of the α5 and β1 subunits in WT and the mutant transfectants were examined by a pulse-chase method. When chased at 0 h, one band of the α5 subunit precursor was clearly observed in both the WT and Δ1–5 mutant transfectants. The contents of the mature forms of the α5 subunit were progressively increased during chases, reaching a maximum at over an 8-h chase in the α5 WT transfectants (Fig. 6A, upper panel). Concomitantly, the maturation of the β1 subunit with doublet bands as described in a previous study (34) was also observed in the WT transfectants. In contrast, the maturation of the α5 subunit was not detectable in the Δ1–5 transfectants, even in an 8-h chase (Fig. 6A, lower panel). Surprisingly, the immunocomplexes of the α5 subunit completely lacked the β1 subunit in the mutant transfectants. These results suggest that the N-glycosylation of the β-propeller may play an important role in heterodimer formation of α and β subunits in the ER. On the other hand, when endogenous β1 subunit was immunoprecipitated with the anti-integrin β1 antibody (7E2), the precursors of β1 subunits were clearly observed in the both transfectants at a 0-h chase (Fig. 6B). The α5 subunit was consistently detected in WT but not the Δ1–5 transfectants, further supporting the notion that N-glycosylation of the β-propeller of α5 subunit is required for heterodimer formation, as described above. The processing pattern of the β1 subunit precursor was similar to the α5 subunit, which was gradually converted to the mature form, showing bands shifted up in the WT transfectants. Such maturation could not be detected in Δ1–5 mutant cells. Interestingly, the rate of degradation of the β1 subunit was much faster in the Δ1–5 mutant cells than in the WT cells (Fig. 6B). In fact, it has been reported that the excess β1 integrin could be degraded via the proteasome-dependent pathway (35, 36). To examine whether this is the case, a proteasome inhibitor, MG-132, was added to the culture media. In fact, the degradation of the precursors of the β1 integrin was
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Three N-Glycosylation Sites on $\beta$-Propeller of Integrin $\alpha_5$ Are Required for Its Functional Expression—To define which one or two of these three sites are important for $\alpha_5\beta_1$ integrin expression on the cell surface and its biological functions, we constructed some additional mutants as shown Fig. 1, and we then examined the expression levels on the cell surface by FACS analysis. The expressions levels of the S3,5 and S4,5 mutants, but not the S3,4 mutant, on the cell surface were comparable with that of the S3-5 mutant. Furthermore, in comparing the S3 and S4 mutants, the S5 mutant was expressed at a relative higher level (Fig. 7B). These results suggest that the site 5 (Asn-316) plays an important role in the expression of the $\alpha_5$ integrin. Unexpectedly, although there were significant differences in cell spreading (Fig. 8) as well as functional epitope expressions on the cell surface, as analyzed by FACS using BiIG2, a functional blocking antibody (Fig. 7, A and B), the total expression levels on the cell surface were comparable between the $\Delta3–5$ mutant and WT. We speculate that the mutation of the 3–5 sites ($\Delta3–5$) might result in a large conformation change and/or compensation by N-glycosylation of the putative 1 and 2 sites, which could cause the mutant assembly with $\beta1$ subunit to allow its expression on the cell surface. Although S5 alone can be efficiently expressed on the cell surface, as shown in Fig. 7C, it is also noteworthy that the mutant may not have biological functions that are detected by a cell spreading assay (Fig. 8).

Taken together, these results indicate that the S3-5 mutant might be the minimum requirement for N-glycosylation of $\alpha_5$ subunit in terms of its functional expression and biological function, because a mutation in any of these three sites significantly affects its biological function.

Association between Calnexin and Unglycosylated Mutant of $\beta$-Propeller—To determine the possible involvement of chaperones such as calnexin and calreticulin in the folding of these mutants, we examined the association of these mutants with calnexin and calreticulin by co-immunoprecipitation. Calnexin and calreticulin are ER chaperone proteins that are associated with the monoglucosidated N-glycans on a misfolded glycopro-

rabbit anti-integrin $\alpha_5$ antibody. The samples were then subjected to 7.5% SDS-PAGE, and biotinylated proteins were detected as described under “Materials and Methods.” Asterisks and bracket indicate the position of migration of each corresponding integrin $\alpha_5$ and $\beta1$ subunits, respectively. Cells were lysed and immunoprecipitated (IP) with the anti-hamster $\beta1$ (C) or the anti-GFP (D) antibody, and immunoprecipitated samples were detected with the anti-human $\alpha_5$ at reducing conditions or the anti-hamster $\beta1$ at nonreducing conditions according to “Materials and Methods.” E, the expression level of the $\beta1$ sc, cells was detected in total lysates. $\bar{F}$, cells suspended in DMEM without fetal calf serum were replated on coverslips that had been pre-coated with 20 $\mu$g/ml FN. After incubation for 2 h, the cells were replaced with the normal culture media containing 10% fetal calf serum and then incubated overnight at 37 °C. The cells were stained with the mAb against hamster $\beta1$ subunits (7E2) and then visualized with the goat antibody for mouse IgG-conjugated Alexa 549. Integrin $\alpha_5$ subunit was visualized with GFP. Arrowheads represent co-localization of $\alpha$ and $\beta$ subunits. The bar denotes 10 $\mu$m. WB, Western blot.

FIGURE 5. Effects of unglycosylation of the $\alpha_5$ subunit on its expression and association with the $\beta1$ subunit. A, total cell lysates from different transfectants expressing GFP tag as a control, WT, and several unglycosylated mutants as indicated ($\Delta1-5$, $\Delta6-15$, and S3-5) were blotted with the anti-integrin $\alpha_5$ antibody (clone 1). $\bar{B}$, biotinylated cells were lysed and immunoprecipitated with the the presence of MG-132 as observed in the 8-h chase (Fig. 6B, lower panel). The degradation of the $\beta1$ subunit precursor, but not its mature form, was also inhibited by treatment of WT $\alpha5$ cells with MG-132 (Fig. 6B, upper panel). These results clearly demonstrate that N-glycosylation on the $\beta$-propeller domain of the $\alpha5$ is essential for maturation, heterodimer formation, and the stability of $\alpha5\beta1$ integrin.

In this study, we also examined the association of each corresponding integrin $\alpha5$ and $\beta1$ subunits, respectively. Cells were lysed and immunoprecipitated (IP) with the anti-hamster $\beta1$ (C) or the anti-GFP (D) antibody, and immunoprecipitated samples were detected with the anti-human $\alpha5$ at reducing conditions or the anti-hamster $\beta1$ at nonreducing conditions according to “Materials and Methods.” E, the expression level of the $\beta1$ sc, cells was detected in total lysates. $\bar{F}$, cells suspended in DMEM without fetal calf serum were replated on coverslips that had been pre-coated with 20 $\mu$g/ml FN. After incubation for 2 h, the cells were replaced with the normal culture media containing 10% fetal calf serum and then incubated overnight at 37 °C. The cells were stained with the mAb against hamster $\beta1$ subunits (7E2) and then visualized with the goat antibody for mouse IgG-conjugated Alexa 549. Integrin $\alpha5$ subunit was visualized with GFP. Arrowheads represent co-localization of $\alpha$ and $\beta$ subunits. The bar denotes 10 $\mu$m. WB, Western blot.
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FIGURE 6. Comparison of expression patterns of α5 and β1 subunits in WT and Δ1–5 transfectants by pulse-chase assay. After metabolic labeling with [35S]methionine for 30 min, cells were washed with fresh medium and then chased at the indicated times. The cells expressing WT (upper panel) or Δ1–5 (lower panel) of α5 subunits were lysed and immunoprecipitated (IP) with anti-GFP (A) and anti-β1 integrin antibody (B) at the indicated times. Open arrows, closed arrows, and arrows marked with star indicate the migrated position of precursor of α5, β1, and the corresponding mature forms, respectively. MG-132 is a proteasome inhibitor.

mutants, and we found that the localization of the Δ1–5 mutant was quite different from that of WT, although similar to that of calnexin, which is localized in the ER (Fig. 9C), further supporting the view that N-glycosylation of β-propeller is important for its export from the ER to the cell surface. Consistent with this, the expression level of the Δ3–5 mutant on the cell surface was comparable with that of WT as shown in Fig. 7, and the localization of Δ3–5 was also quite similar to that of WT (data not shown).

DISCUSSION

In this study, we report for the first time that N-glycosylation on the β-propeller but not the other domains of the integrin α5 subunit is essential for efficient heterodimer formation, maturation, and integrin-mediated biological function. In fact, the putative N-glycosylation sites on the β-propeller are completely conserved in human, mouse, rat, and Xenopus, but this is not true for the other domains. Although alteration of the oligosaccharide portion on integrin α5β1 could affect cis- and trans-interactions caused by GnT-III, α2,6-sialyltransferase, and GnT-V, respectively (19–21), the molecular mechanism remains unclear. Therefore, a detailed study of N-glycans such as presented here may be useful in revealing the underlying mechanisms of the remodeling of N-glycans on integrin α5β1. To date, several functional mutation sites have been mapped on α5 integrin (42), and the present study demonstrates, for the first time, that mutations within putative N-glycan sites could also regulate its function. Seales et al. (43) recently reported that the I-like domain on the β subunit, which could be the partner of the β-propeller of the α subunit, contains N-glycans, supporting the importance of N-glycans on the β-propeller in this study. Considering that the β-propeller domain has been postulated to be required for the accurate interaction between α5β1 integrin and its ligand (44), this study may shed light on such structural studies.

It has been reported or speculated that N-glycosylation facilitates conformational maturation by promoting the glycoprotein folding machinery and functions as tags for ER retention and targeting to the ER-associated degradation pathway (45, 46). Yoshida et al. (46) reported that the F-box protein Fbx2, a novel ubiquitin ligase, specifically interacted with the precursor of integrin β1 that contains a high mannose-type oligosaccharide. It is thought that the β1 subunit can associate with de novo synthesized α subunit, otherwise the excess of noncomplexed β1 would be either degraded immediately or remain in the ER (35, 36). We have shown that treatment with MG-132 resulted in the inhibition of β1 subunit degradation, suggesting that the reduction of the expression level of the β1 subunit in the Δ1–5 mutant transfectants was because of degradation probably through a proteasome pathway. It is clear that the impaired αβ assembly and processing of the α5 subunit was not because of the down-regulation of mRNA of the β1 integrin, because the protein expression levels of the β1 subunit in the Δ1–5 mutants as shown in pulse-chase experiments (Fig. 6) did not show any significant changes compared with that in the WT transfectants. The disassembly of αβ was also confirmed by the transient transfection of the Δ1–5 mutant, and the β1 subunit in
293T cells (data not shown) further supports the notion that N-glycosylation on the β1 propeller is essential for its assembly.

Calnexin is thought to function as a membrane-bound chaperone facilitating the assembly of glycoprotein complexes such as major histocompatibility complex class I, the T cell receptor, and integrin complexes as well (45). Lenter and Vestweber (47) reported that the immature β1 subunit, as well as the α6 subunit, transiently associates with a calnexin, prior to the maturation of the α6 and the β1 subunit, suggesting that a calnexin is involved in integrin assembly. The loss of αβ pairing caused by unglycosylation in this study supports this conclusion. The significantly enhanced calnexin binding to the Δ1–5 mutant could be due to this mutant being a misfolded protein that cannot escape from the calnexin cyclic machinery to be exported from the ER, which was also confirmed by a pattern of localization shown in Fig. 9C. Conversely, the ratios of calnexin binding to the α5 subunit were significantly decreased in S3-5 or S5, compared with that in the Δ1–5 mutant. These results strongly suggest that site 5 (Asn-316) plays an important role in the assembly of the integrin for its expression on the cell surface. Although S5 alone could be efficiently expressed on the cell surface, it did not show any biological function such as cell spreading. Taken together, this study clearly showed that these three N-glycosylation sites (S3–5) in the β1 propeller play important roles not only in its expression but also its biological functions. Although the effects of N-glycosylation on integrin α5 are complicated, it would be very important for studies of the molecular structure of the integrin.

Considering that the three sites (3–5 sites) are sufficiently close, either 10 or 9 residues to the neighboring N-glycosylation site, there might exist in these three sites a cross-competition for the transferase complexes as described by Karamyshev et al. (48). We examined the bands of these mutants migrated on SDS-PAGE by Western blotting, and we found that there were no band shifts among the S3, S4, S5, and S3,5 mutants, except for the S3-5 mutant (data not shown), suggesting that no apparent competition exists for the transferase complex in the three sites of α5 subunit. 

FIGURE 7. Comparison of expression levels on the cell surface among the unglycosylated mutants. The functional epitope expression on the cell surface was examined by its reactivity with the functional blocking anti-α5 antibody (BLIG2), whereas total expression was examined by the nonfunctional blocking anti-α5 antibody (HA5). The indicated cells were labeled with BLIG2 (A, left panel) or HA5 (A, right panel, and C), followed by a Alexa 647-labeled secondary antibody, prior to analysis by FACS, as described under “Materials and Methods.” Negative control staining (shaded histogram) was done without the first antibody. The relative ratios of functional expression of α5 integrin were determined by dividing the mean fluorescence intensity of BLIG2 by that of HA5, with WT as 100% (B).

FIGURE 8. Comparison of cell spreading on FN for unglycosylated mutants. Cells were detached and then replated on dishes that had been pre-coated with 10 μg/ml FN. After incubation for 30 min, cells were fixed with 3.7% paraformaldehyde. Relative cell spreading was determined by the percentage of cells spread, with WT as 100%. Data were obtained from three of independent experiments (mean values ± S.D.).
An analysis of crystal structure of integrins appears to be a challenge. However, the crystal structure of integrin αVβ3 has been successfully revealed, and the main contact between the αV and β3 subunit is the β-propeller on the α and A domain on β3 with hydrophobic, ionic, and mixed contacts (49, 50). Because the α5 subunit has a 47% homology to αV, Mold et al. (51) performed a homology modeling structure of α5β1. Based on the model, the α5 subunit seems to be surrounded by N-glycans. We therefore speculate that the structural environment of the αβ interfaces could be affected by the presence of N-glycans. In fact, it has been reported that the dissociation of the αβ heterodimer occurs when α5β1 is deglycosylated by treatment with N-glycosidase F and that N-glycan was required for its normal expression on the cell surface, as confirmed by using of tunicamycin (15). Another possible mechanism for the involvement of N-glycan in the αβ interaction is that an unknown lectin domain may exist on the β subunit, because the lectin domain of αMβ2 integrin is associated with GlcNAc on the nonreducing terminal of sugar chains on chilled platelets for its phagocytosis (52, 53).

Detailed structural studies of integrins have been consistently hampered because of the small amounts of purified protein available, the large size and the conformational flexibility of integrins, and the presence of transmembrane domains and N-linked glycosylation sites in both receptor subunits. To date, no atomic resolution structure is available for integrin α5β1, the non-l domain integrin. It has been reported recently that the structures of N-glycan on integrin α5β1 may be present in a site-specific and dependent manner (54). Therefore, we believe that mutants of the α5 subunit, such as S3-5 or S5, might be useful for a crystal structural study in the future.

In conclusion, this study clearly reports, for the first time, that N-glycosylation on the β-propeller domain of the α5 subunit is essential for the αβ dimer formation and its biological function, and might also shed light on structure-based molecular mechanism study.

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