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*N*-glycosylation of the I-like domain of  $\beta 1$  integrin is essential for  
 $\beta 1$  integrin expression and biological function:  
Identification of the minimal *N*-glycosylation requirement for  $\alpha 5\beta 1^*$

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Running title: Importance of *N*-glycosylation of  $\beta 1$  integrin

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*N*-glycosylation of integrin  $\alpha 5\beta 1$  plays a crucial role in cell spreading, cell migration, ligand binding, and dimer formation, but the detailed mechanisms by which *N*-glycosylation mediates these functions remain unclear. In a previous study, we showed that three potential *N*-glycosylation sites ( $\alpha 5S3-5$ ) on the  $\beta$ -propeller of the  $\alpha 5$  subunit are essential to functional expression of the subunit. In particular, site-5 ( $\alpha 5S5$ ) is the most important for its expression on the cell surface. In the present study, the function of the *N*-glycans on the integrin  $\beta 1$  subunit was investigated using sequential site-directed mutagenesis to remove the combined putative *N*-glycosylation sites. Removal of the *N*-glycosylation sites on the I-like domain of the  $\beta 1$  subunit (i.e., the  $\Delta 4-6$  mutant) decreased both the level of expression and heterodimeric formation, resulting in inhibition of cell spreading.

Interestingly, cell spreading was observed only when the  $\beta 1$  subunit possessed these three *N*-glycosylation sites (i.e., the S4-6 mutant). Furthermore, the S4-6 mutant could form heterodimers with either  $\alpha 5S3-5$  or  $\alpha 5S5$  mutant of the  $\alpha 5$  subunit. Taken together, the results of the present study reveal, for the first time, that *N*-glycosylation of the I-like domain of the  $\beta 1$  subunit is essential to both the heterodimer formation and biological function of the subunit. Moreover, because the  $\alpha 5S3-5/\beta 1S4-6$  mutant represents the minimal *N*-glycosylation required for functional expression of the  $\beta 1$  subunit, it might also be useful for the study of molecular structures.

Integrin is a heterodimeric glycoprotein that consists of both an  $\alpha$  and a  $\beta$  subunit (1). The interaction between integrin and the extracellular matrix (ECM) is essential to

both physiologic and pathologic events, such as cell migration, development, cell viability, immune homeostasis, and tumorigenesis (2,3). Among the integrin superfamily,  $\beta 1$  integrin can combine with 12 distinct  $\alpha$  subunits ( $\alpha 1-11$ ,  $\alpha v$ ) to form heterodimers, thereby acquiring wide variety of ligand specificity (1,4). Integrins are thought to be regulated by inside-out signaling mechanisms that provoke conformational changes, which modulate the affinity of integrin for the ligand (5). However, an increasing body of evidence suggests that cell-surface carbohydrates mediate a variety of interactions between integrin and its extracellular environment, thereby affecting integrin activity and possibly tumor metastasis, as well (6-8).

Guo *et al.* reported that an increase in  $\beta 1$ -6-GlcNAc sugar chains on the integrin  $\beta 1$  subunit stimulated cell migration (9). In addition, elevated sialylation of the  $\beta 1$  subunit, due to ras-induced STGal-I transferase activity also induced cell migration (10,11). Conversely, cell-migration and -spreading were reduced by the addition of a bisecting GlcNAc, which is a product of *N*-acetylglucosaminyltransferase III (GnT-III), to the  $\alpha 5\beta 1$  and  $\alpha 3\beta 1$  integrins (12,13). Alterations of *N*-glycans on integrins might also regulate their cis interactions with membrane-associated proteins, including the epidermal growth factor receptor, the galectin family, and the tetraspanin family of proteins (14-19).

In addition to the positive and negative

regulatory effects of *N*-glycan, several research groups have reported that *N*-glycans must be present on integrin  $\alpha 5\beta 1$  for the  $\alpha\beta$  heterodimer formation and proper integrin-matrix interactions. Consistent with this hypothesis, in the presence of the glycosylation inhibitor, tunicamycin, normal integrin-substrate-binding and transport to the cell surface are inhibited (20). Moreover, treatment of purified integrin with *N*-glycosidase F blocked both the inherent association of the subunits and the interaction between integrin and fibronectin (FN) (21). These results suggest that *N*-glycosylation is essential to the functional expression of  $\alpha 5\beta 1$ . However, because integrin  $\alpha 5\beta 1$  contains 26 potential *N*-linked glycosylation sites, 14 in the  $\alpha$  subunit and 12 in the  $\beta$  subunit, identification of the sites that are essential to its biological functions is key to understanding the molecular mechanisms by which *N*-glycans alter integrin function. Recently, our group determined that *N*-glycosylation of the  $\beta$ -propeller domain on the  $\alpha 5$  subunit is essential to both heterodimerization and biological functions of the subunit. Furthermore, we determined that sites 3-5 are the most important sites for  $\alpha 5$  subunit-mediated cell spreading and migration on FN (22). The purpose of the present study was to clarify the roles of *N*-glycosylation of the  $\beta 1$  subunit. Therefore, we performed combined substitutions in the putative *N*-glycosylation sites by replacement of asparagine residues with glutamine residues. We subsequently introduced these

mutated genes into  $\beta 1$ -deficient epithelial cells (GE11). The results of these mutation experiments revealed that the *N*-glycosylation sites on the I-like domain of the  $\beta 1$  subunit, sites number 4–6 (S4-6), are essential to both heterodimer formation and biological functions, such as cell spreading.

### EXPERIMENTAL PROCEDURES

**Reagent**—The Phoenix cell line was purchased from ATCC (Manassas, VA). A monoclonal antibody (mAb) against the human integrin  $\beta 1$  subunit (P5D2), which was obtained from Developmental Studies Hybridoma Bank, University of Iowa, was purified from the hybridoma supernatant using a protein A-Sepharose<sup>TM</sup> 4 Fast Flow column (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). For Western blot analysis, mAbs against amino acids 82-87 of the human integrin  $\beta 1$  subunit (MAB1965, JB1A) and  $\alpha$ -tubulin (DM1A) were obtained from Chemicon (Temecula, CA) and Sigma (St. Louis, MO), respectively. Polyclonal rabbit antibodies against the carboxyl-terminal of human  $\beta 1$  integrin (AB1952P),  $\alpha 5$  integrin (H-104),  $\alpha 5$  integrin (4705S) and calnexin (SPA-860) were purchased from Chemicon, Santa Cruz (Santa Cruz, CA), Cell Signaling Technology, Inc (Danvers, MA) and Stressgen (Ann Arbor, MI), respectively. A goat antibody against the green fluorescent protein (GFP) was obtained from Rockland Immunochemicals, Inc. (Gilbertsville, PA). Peroxidase-conjugated anti-rabbit and

anti-goat antibodies were obtained from Cell Signaling Technology, Inc. A peroxidase-conjugated goat anti-mouse IgG antibody was obtained from Chemicon (Temecula, CA). A rat antibody against mouse  $\alpha 5$  integrin subunit (Sc-23941) was purchased from Santa Cruz (Santa Cruz, CA).

**Cell Cultures**—Epithelial GE11 cells, derived from  $\beta 1$  integrin knock-out embryonic stem cells (23), were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin and 20 nM trichostatin A. Phoenix cells and integrin  $\alpha 5$ -deficient cells (CHO-B2) (22) were grown in DMEM supplemented with 10% FBS, penicillin/streptomycin. CHO Lec 3.2.8.1 cells, a GnT-I-deficient cell line, were a gift from Dr. Pamela Stanly (Albert Einstein College of Medicine, New York) (24).

**cDNA constructs**—The cDNA of integrin  $\beta 1$  subunit was amplified by PCR from the reverse-transcribed product of human placenta total RNA (OriGene Technologies, Inc., Rockville, MD), and then was inserted into a cloning vector (pENTR-D-Topo, Invitrogen), according to the manufacturer's protocol. Mutations were introduced into the  $\beta 1$  cDNA using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA.) according to the manufacturer's instructions.

The retrovirus vector, pBabe-Puro plasmid (25) was made to be Gateway-compatible using the Gateway Conversion System kit (Invitrogen), resulting in pBabe-puro-Rfa. The LR clonase enzyme (Invitrogen) was used to transfer the cDNA of either  $\beta 1$  integrin or GFP from the cloning vectors. The coding regions of all cDNA constructs were sequenced using an ABI PRISM 3130 sequencer (Applied Biosystems Japan Ltd., Tokyo, Japan) and compared with the human gene ITGB1 (Accession # BC020057) to confirm both the presence of the desired mutations (Fig.1) and the absence of any additional mutations. The cDNA constructs of soluble  $\alpha 5$  and  $\beta 1$  subunits were designed according to previous studies (26-29). The soluble expression system of  $\alpha 5\beta 1$  integrin was clearly described by Takagi, et al. (27,28). Briefly, the expression vectors of  $\alpha 5$  (pEF1- $\alpha 5$ - $\Delta 664$ -tev-AHCys) and  $\beta 1$  (pEF1-puro- $\beta 1$ - $\Delta 501$ -tev-BHCys-6XHis) subunits were kind gifts provided by Dr. Junichi Takagi (Institute for Protein Research, Osaka University, Japan). We further shorten the extracellular domain of  $\alpha 5$  to residue Asp<sup>644</sup> based on the extensive study carried by Coe, et al. (26). These cDNAs were then cloned into the pENTR-D-topo cloning vector, resulting in  $\alpha 5$ - $\Delta 644$ -tev-AHCys (residues from Met<sup>1</sup> to Asp<sup>644</sup>) and  $\beta 1$ - $\Delta 501$ -tev-BHCys-6XHis (residues from Met<sup>1</sup> to Glu<sup>501</sup>) in pENTR as shown in Fig.7A. These constructs of soluble integrin  $\alpha 5\beta 1$  contained 9 putative *N*-glycosylation sites on each subunit. Cloning vectors for the

*N*-glycosylation mutants of the soluble  $\alpha 5$  and  $\beta 1$  subunits were prepared using overlap extension PCR. The wild type (WT) and mutants constructs were recombined with a lentivirus vector plasmid, CSII-EF-Rfa using LR clonase (30).

#### **Retrovirus production and**

**transduction**—The pBabe-Puro-based vectors described above were transfected into the Phoenix ecotopic retrovirus-producing cells using Lipofectamine 2000 (Invitrogen) for the production of viral supernatants. The retroviruses for CHO-B2 cells were produced by co-transfection of both the pBabe-puro-based vector and pLP/VSVG (Invitrogen) into Phoenix cells. The lentivirus vectors were co-transfected with pCAG-HIVgp and pCMV-VSV-G-RSV-Rev into 293T cells. Forty-eight hours after transfection, retrovirus and lentivirus supernatants were filtered, and then incubated with target cells in the presence of 10  $\mu$ g/ml polybrene (Sigma) at 32°C overnight, respectively. Two days after infection, GE11 cells and CHO-B2 cells were selected using puromycin. Pooled stable cell lines were used in this study.

#### **Immunoprecipitation and Western**

**Blot**—Cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% (w/v) Triton-X100 and Protease Inhibitor Cocktail (Nacalai Tesque, Kyoto, Japan). The supernatants

were collected, and protein concentrations were determined using the BCA Protein Assay kit (Pierce, Rockford, IL). Samples containing equal amounts of protein were incubated with 2  $\mu$ g of each antibody and 15  $\mu$ l of protein G-Sepharose<sup>TM</sup> 4 Fast Flow (GE Healthcare Bio-Sciences Corp.) for 2 h at 4°C. The immunoprecipitates were washed three times with lysis buffer and subjected to 6% SDS-PAGE. After electrophoresis, the proteins were transferred to a nitrocellulose membrane (Whatman, Dassel, Germany). The membrane was incubated with primary and secondary antibodies, each for 1 h; detection was performed using an ECL kit (GE Healthcare Bio-Sciences Corp.), according to the manufacturer's instructions.

**Flow Cytometric Analysis**—Flow cytometric analysis was performed as described previously (12,22) with minor modifications. Briefly, semi-confluent cells were detached from the culture dishes using trypsin containing 1 mM EDTA and were subsequently stained with or without the primary mouse anti- $\beta$ 1 or rat anti- $\alpha$ 5 antibody, followed by incubation with Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 633 goat anti-rat IgG (Invitrogen), respectively. After washing three times with PBS, flow cytometric analysis was performed using an FACS Calibur flow cytometer and Cell Quest Pro software (BD Biosciences).

**Metabolic Labeling**—The pulse-chase experiments were performed described

previously with minor modifications (22).  $5 \times 10^5$  cells on 6-well dishes were washed three times with PBS and then starved for 30 min in DMEM by excluding methionine and cysteine (Sigma). After starvation, the cells were pulse-labeled in 500  $\mu$ l of DMEM containing 200  $\mu$ Ci of [<sup>35</sup>S] methionine and cysteine (Express protein labeling mix, PerkinElmer Japan, Yokohama, Japan) 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  $\beta$ 1 subunit antibody (JB1A). The immunoprecipitates were separated on 6% SDS-PAGE. After drying the gels, radioactive bands were visualized with a Fuji BAS 5000 Bio-Image Analyzer.

**Immunofluorescence Microscopy**—Cells on the Glass coverslips (Iwaki, Tokyo, Japan) were fixed with ice-cold methanol. The fixed cells were permeabilized by incubation in 0.05% Triton X-100 in PBS. After blocking with 3% BSA, cells were incubated with primary antibodies. Cells were visualized with Alexa488- or Alexa546-conjugated secondary antibody. Fluorescence images were observed by confocal microscopy using a FluoView FV1000 (Olympus, Tokyo Japan).

**Cell Adhesion and Spreading**—Cell spreading assays were performed as described previously with minor modifications (12,22). Briefly, 96-well

flat-bottom microtiter plates (Corning, Corning, NY) were coated with a solution of 5  $\mu\text{g/ml}$  purified human FN (Sigma) in 50  $\mu\text{l}$  of PBS overnight at 4°C and were subsequently blocked with 1% bovine serum albumin (BSA). The cells were detached with trypsin containing 1 mM EDTA, washed with FBS-containing DMEM, and then suspended in serum-free DMEM with 0.1% BSA at  $1 \times 10^4$  cells/ml. After a 20-min incubation, representative fields were observed using a phase-contrast microscopy. Cell adhesion was monitored dynamically using an RT-CES apparatus (ACEA, San Diego, CA) (31,32). The cells were re-suspended in serum-free DMEM with 0.1% BSA and then were seeded at  $5 \times 10^4$  cells/ml in the electrode sensors (16X E-plate, ACEA), which had been coated with 10  $\mu\text{g/ml}$  purified human FN and blocked with 1% BSA. Cell adhesion that occurred during the subsequent 3-h period was continually monitored by taking a measurement with the RT-CES apparatus operated with RT-CES SP Software every 5 min.

**Expression and Purification of WT and Unglycosylated Mutant of the Recombinant Integrins**—Recombinant integrins were produced using the CHO LEC 3.2.8.1 cells and were purified from cell culture supernatant as previously described with minor modifications (27,28). Briefly, CHO LEC 3.2.8.1 cells were co-infected with the lentiviruses for the expression of both the soluble  $\alpha 5$  and  $\beta 1$  subunits and were

subsequently grown in serum-free ASF 104 media (Ajinomoto Co., Inc., Tokyo, Japan). The conditioned media were collected and clarified by centrifugation and then precipitated with a saturated solution of ammonium sulfate (80%). Concentrated culture media was dialyzed against Tris-buffered saline (TBS), and then passed through a gelatin-Sepharose column (GE Healthcare Bio-Sciences Corp.). The eluted fractions were subjected to affinity chromatography using Ni-NTA agarose (QIAGEN Inc., Valencia, CA, USA). The columns were washed with Tris-buffered saline containing 1 mM  $\text{CaCl}_2$  and 1 mM  $\text{MgCl}_2$  (TBS(+)) and the bound proteins were eluted with TBS(+) containing 250 mM imidazole.

**Integrin Binding Assay**—The integrin binding assay was performed as described previously (12), with minor modifications. Briefly, 96-well flat bottom microtiter plates were coated with a solution of 5  $\mu\text{g/ml}$  purified human FN (Sigma) in 50 ml of PBS overnight at 4°C. Non-specific binding was then blocked with 1% BSA for 1 h at room temperature. Integrins were added to the plates and allowed to bind for 3 h in the presence of either 1 mM  $\text{MnCl}_2$  or 10 mM EDTA at 37 °C. Next, the plates were washed with TBS containing 1 mM  $\text{MnCl}_2$ , 0.1% BSA and 0.02% Tween-20 or TBS containing 0.1% BSA, 0.02% Tween-20 and 10 mM EDTA and the bound integrins were quantified using an enzyme-linked

immunosorbent assay. The plates were incubated with the mouse anti- $\alpha 5$  antibody (HA; diluted 1:1000) for 30 min at room temperature in TBS containing 1 mM  $MnCl_2$ , and then were washed three times followed by incubation with the biotinylated anti-mouse antibody (Vector Laboratories, Inc., Burlingame, CA). After three washes, the wells were incubated with avidin D conjugated alkaline phosphatase (Vector Laboratories, Inc.). The bound antibodies were quantified by measurement of the absorbance at 402 nm after incubation with *p*-Nitrophenyl phosphate (Calbiochem, Gibbstown, NJ).

## RESULTS

**Construction of Various Integrin  $\beta 1$  Mutants by the Mutagenesis of Potential *N*-Glycosylation Sites**—The human integrin  $\beta 1$  subunit contains 12 potential *N*-glycosylation sites, as shown in Fig. 1. Reportedly, 10 of these sites are normally *N*-glycosylated, while sites 2 and 11 do not normally carry *N*-linked glycans (33). However, which of the 12 *N*-glycosylation sites are important for biological function of the subunit remains unknown. Recently, we used a site-directed mutagenesis to determine that the *N*-glycans on the  $\beta$ -propeller domain of the  $\alpha 5$  subunit are essential to  $\alpha 5 \beta 1$  heterodimerization, cell surface expression, and biological function (22). In the present study, we mutated the asparagine residues within the NX(S/T) glycosylation consensus sequence to glutamine in various domains of

$\beta 1$  subunit (Fig. 1) to glutamine residues. These mutated cDNAs were then introduced into integrin  $\beta 1$  subunit-deficient GE11 cells. The expression of the constructs was detected using Western blotting. We hypothesized that the *N*-glycosylation sites on the  $\beta 1$  subunit could also be divided into indispensable and dispensable sites, as previously observed for the  $\alpha 5$  subunit. The underglycosylated mutant constructs of  $\beta 1$  integrin used in this study are listed in Fig. 1:  $\Delta 1-3$ ,  $\Delta 4-6$ ,  $\Delta 7,8$  and  $\Delta 9-12$ . These constructs correspond to the following unglycosylated sites on the PSI and in the upstream region of the hybrid domains: the I-like domain, the downstream region of the hybrid domain, EGF-repeat and  $\beta$ -tail domains of the  $\beta 1$  subunit, respectively.

**Effects of the Removal of *N*-Glycosylation Sites on the  $\beta 1$  Subunit on Cellular Expression, Heterodimer Formation and Expression on Cell Surface**—As described above, although *N*-glycosylation of integrin  $\alpha 5 \beta 1$  is essential to its function (20,21,34), the distinct roles of *N*-glycosylation of each domain of the  $\beta 1$  subunit are largely unknown. To examine the effects of the underglycosylated mutations shown in Fig. 1 on cellular expression, heterodimer formation and expression on the cell surface, we introduced these mutated cDNAs into GE11 cells. First, we compared the level of expression in total cell lysates among the mutants. As shown in Fig. 2A, the expression of  $\Delta 4-6$  mutant was less than that of other mutants, as evaluated using the anti- $\beta 1$



antibody (JB1A). Similar results were observed using another anti- $\beta 1$  antibody, which recognizes the C-terminal epitope of the  $\beta 1$  subunit (data not shown). These results suggest that *N*-glycans on the I-like domain of  $\beta 1$  integrin are required for efficient expression of the  $\beta 1$  subunit. We verified equivalent amounts of proteins were loaded by blotting with the  $\alpha$ -tubulin antibody (Fig.2A lower panel). It should be noted that the similar mobility on the SDS-PAGE between  $\Delta 1-3$  and  $\Delta 7,8$  mutants, or between  $\Delta 4-6$  and  $\Delta 9-12$  mutants, further supported the notion that site-2 and site-11 do not carry *N*-linked glycans as reported by Seals et al (33). The integrin  $\beta 1$  subunit associates with multiple  $\alpha$  subunits to form various heterodimers, which may stabilize its expression. To determine whether underglycosylated mutation in a specific domain of the  $\beta 1$  subunit affects integrin  $\alpha\beta$  heterodimerization, we performed Western blotting of immunoprecipitated samples prepared from GE11 cells expressing WT or an underglycosylated  $\beta 1$  subunit. Reportedly, the  $\alpha 5$  subunit is the main constituent in  $\beta 1$  integrin-containing dimers in  $\beta 1$  subunit-rescued GE11 cells (35). Therefore, we evaluated  $\alpha 5\beta 1$  heterodimerization. In the cells expressing WT  $\beta 1$  integrin, the  $\alpha 5$  subunit was clearly detected in the  $\beta 1$  immunocomplexes, which were immunoprecipitated with anti- $\beta 1$  antibody (Fig. 2B). However, expression of the  $\alpha 5$  subunit in the  $\beta 1$  immunocomplexes prepared from  $\Delta 4-6$  was considerably less than that in

cells expressing WT,  $\Delta 1-3$ ,  $\Delta 7,8$  or  $\Delta 9-12$  mutations (Fig. 2B and 2C). These results suggest that *N*-glycosylation on the I-like domain of  $\beta 1$  integrin is essential to efficient  $\alpha/\beta$  heterodimer formation.

Of particular interest, the  $\alpha 5$  subunit was only expressed in the  $\beta 1$  immunocomplexes of cells expressing the S4-6 mutation, which was devoid of all glycosylation sites, other than those in the I-like domain (Fig.3A). Although the level of S1-3 expression was similar to that in the S4-6 mutant (Fig.3A, middle panel), the  $\alpha 5/\beta 1$  heterocomplex was hardly detected in S1-3-expressing cells (Fig.3A, upper panel). Furthermore, the level of cell-surface expression on these mutants was investigated using flow cytometric analysis. FACS analysis showed that the level of cell-surface expression by the S4-6 mutant was the highest among the removal mutants (Fig.3B). Similarly, the level of cell-surface expression by the  $\Delta 4-6$  mutant was considerably less than that of WT (43% of WT); while expression by the  $\Delta 1-3$ ,  $\Delta 7,8$  and  $\Delta 9-12$  mutants were 91%, 104% and 79% of WT, respectively. On the other hand, FACS analysis showed that the expression of  $\alpha 5$  was clearly observed only in S4-6, but not in either S1-3, S7,8 or S9-12, as shown in Fig.3C. Taken together, these results further support our hypothesis that *N*-glycosylation on the I-like domain is indispensable for both  $\alpha/\beta$  dimerization and efficient expression on the cell surface.

#### *Effects of N-glycosylation on I-like Domain*

*of  $\beta 1$  on Post-translational Processing, Stability and Cellular Localization*—To elucidate the underlying mechanisms of impaired  $\alpha\beta$  heterodimeric formation and the decreased expression levels in the  $\beta 1$  subunits of underglycosylated mutants, such as  $\Delta 4-6$ , the biosynthetic kinetics of the  $\alpha 5\beta 1$  integrin in WT and in underglycosylated mutant transfectants were examined using a pulse-chase method. When chased at 0 h, the precursors for the  $\beta 1$  subunit were observed in all of these transfectants. However, the precursors for  $\alpha 5$  were clearly detected only in the WT and S4-6 (Fig. 4A). It seems that there were no significant differences in total expression levels of the nascent  $\beta 1$  subunits among these transfectants (chased at 0 or 4 h). Interestingly, the contents of the mature forms of the  $\beta 1$  subunit were clearly observed, even when chased at 4 h, in the WT and S4-6 transfectants, but obviously not in the other mutants. Coincidentally, the mature forms of the  $\alpha 5$  subunit were also clearly observed only in the WT and S4-6 transfectants. On the other hand, the protein degradation rates were much faster in  $\Delta 4-6$ , S7,8 and S9-12, compared with that of the WT or the S4-6, as shown the chase data at 4 and 16 h, although it remains unclear why S1-3 was stable even in its precursor form. The degradation of  $\beta 1$  proteins might be partially mediated through a proteasome pathway as previously described (36), since treatment with MG-132, a proteasome inhibitor, increased expression levels of  $\beta 1$  in all mutants, with the noted exception of the

S1-3 mutant (Fig. 4A).

Next, we examined the localization of WT, S4-6 and  $\Delta 4-6$  of  $\beta 1$  by immunostaining. Interestingly, both the WT and the S4-6 of the  $\beta 1$  subunit were expressed mainly on the cell surface as usual, while the  $\Delta 4-6$  accumulated mainly in the ER colocalized with calnexin (Fig. 4B). These results partly support the notion that  $\beta 1$  can be associated with a de novo synthesized  $\alpha$  subunit, otherwise the excess of the noncomplexed  $\beta 1$  would either be degraded immediately or would remain in the ER (36,37). On the other hand, based on results shown in figure 3C and 4B, without association with  $\alpha 5$ , the  $\beta 1$  subunit appeared to be marginally expressed on the cell surface in these underglycosylated mutants. In fact, Meng, X., et al. reported the existence of a monomer  $\beta 1$  subunit on the cell surface detected by a specific anti- $\beta 1$  monomer antibody (38). Therefore, the expression levels of integrin on the cell surface may not always match total expression levels, since these levels could be influenced by several factors, such as  $\alpha/\beta$  assembly, maturation and degradation, as described above.

*Effects of the Removal of N-Glycosylation Sites on the Integrin  $\beta 1$  on FN-mediated Cell Spreading*

—Integrin-mediated biological functions, such as cell spreading and cell migration, can be modulated by aberrant changes in the N-glycosylation of integrins. In a study, we reported that N-glycosylation of the  $\beta$ -propeller, but not

the other domains, of the integrin  $\alpha 5$  subunit is essential to integrin-mediated biological functions. In the present study, we compared cell spreading among the *N*-glycosylation mutants. Cell spreading was assayed within 20 min after replating the cells on FN-coated dishes. As expected, overexpression of the WT  $\beta 1$  subunit gene, but not the GFP control, largely rescued cell spreading on FN (Fig. 5A,B), providing GE11 cells are a useful cell model for studies of integrin  $\beta 1$  function. Cell spreading was completely inhibited by an anti- $\beta 1$ -function blocking antibody (P5D2), but not by normal IgG. This result indicates that the initial cell spreading on FN is mediated mainly by integrin  $\alpha 5\beta 1$ . On the other hand, overexpression of the underglycosylated mutant,  $\Delta 4-6$ , did not result in cell spreading at the same time point. In contrast, overexpression of the  $\Delta 1-3$ ,  $\Delta 7,8$  and  $\Delta 9-12$  mutants induced cell spreading on FN-coated dishes, as did the overexpression of the WT gene. Interestingly, cells expressing the S4-6, but not the S1-3, S7,8 or S9-12 mutant, exhibited significant cell spreading. Furthermore, a cell-adhesion kinetics assay was performed using the RT-CES system (Fig. 5C). Cell adhesion kinetics, evaluated using RT-CES, was greatly suppressed in the  $\Delta 4-6$  mutant compared with other mutants, such as  $\Delta 1-3$  and  $\Delta 7,8$  mutants (Fig. 5C, upper panel). On the other hand, overexpression of the S4-6 mutant significantly increased cell adhesion. Cell adhesion was not enhanced by overexpression of S1-3, S7,8 or S9-12

(Fig. 5C, lower panel). Collectively, these results strongly suggest that the *N*-glycosylation of the I-like domain of the  $\beta 1$  subunit is essential to biological function of the subunit.

**The S4-6 Mutant of  $\beta 1$  can Form Dimers with the Minimally *N*-Glycosylated Mutants of the  $\alpha 5$  Subunit**—*N*-Glycosylation is essential to integrin  $\alpha 5\beta 1$  heterodimer formation, and, as such, it plays an important role in the biological function of the integrin. Integrin  $\alpha 5\beta 1$  modified by GnT-III demonstrated reduced cell adhesion and cell migration on FN (12). Unlike GnT-III, GnT-V specifically modified the  $\beta 1$  subunit, not the  $\alpha 5$  subunit, and up-regulated integrin  $\alpha 5\beta 1$ -mediated cell migration (9). Recently, we found that three *N*-glycosylation sites on the  $\alpha 5$  subunit, i.e.,  $\alpha 5S3-5$ , were essential to its biological functions, such as cell adhesion and cell migration on FN, and heterodimerization. Furthermore, we also found that the  $\alpha 5$  subunit *N*-glycosylation site,  $\alpha 5S5$ , is the most important site for cell-surface expression, although this site has no effect on biological function (22). To examine whether the S4-6 mutant of the  $\beta 1$  subunit could form heterodimer with the  $\alpha 5$  mutants shown in Fig. 6A, we introduced either WT or the  $\beta 1$  mutants into CHO-B2 cells expressing *N*-glycosylation mutants of the  $\alpha 5$  subunit. As expected, the WT  $\beta 1$  subunit efficiently formed heterodimer with S3-5 of the  $\alpha 5$  subunit, as previously reported. Of particular interest, the S4-6, but not the

$\Delta 4-6$  mutant, associated with S3-5 of the  $\alpha 5$  subunit (Fig. 6B). Furthermore, both WT and S4-6 formed heterodimer with the S5 mutant, which has only one *N*-glycosylation site on the  $\alpha 5$  subunit. These mutants in cell-surface expression were confirmed by FACS analysis (data not shown). These results, taken together, suggest that *N*-glycosylation on both the  $\beta$ -propeller of the  $\alpha 5$  subunit and I-like domain of the  $\beta 1$  subunit is the minimal requirement for  $\alpha\beta$  dimer formation.

**Purification and Characterization of Recombinant Underglycosylated Mutant of the Integrin**—Because cells deficient in both the  $\alpha 5$  and the  $\beta 1$ -subunits are not currently available, we constructed secret expression vectors to avoid of interference with endogenous expression of  $\alpha 5$  or  $\beta 1$  subunit. In addition, to minimize the influence of complex *N*-glycan structures on integrin function, we introduced the expression vectors into CHO LEC 3.2.8 cells, which do not expression GnT-I, since GnT-I catalyzes the GlcNAc to the terminal  $\alpha$ -1,3-linked Man in  $\text{Man}_5\text{GlcNAc}_2\text{Asn}$  to initiate the synthesis of hybrid and complex *N*-glycans in multicellular organisms (39). The WT and  $\alpha 5\text{S}3-4/\beta 1\text{S}4-6$  mutant integrins were purified from conditioned media using Ni-NTA affinity chromatography. As expected, the purified integrins gave a single homogeneous band under non-reducing conditions, while two bands were observed in the presence of 2-mercaptoethanol (Figure 7A). These results suggest that the  $\alpha$  and  $\beta$

subunits were covalently linked through a disulfide bond, since  $\alpha$  and  $\beta$  subunits each contain a C-terminal cysteine as described (27). These purified integrins were also confirmed by immunoblot analysis with antibodies against the  $\alpha 5$  (Fig. 7B) and  $\beta 1$  subunit (Fig. 7C) under both non-reducing and reducing conditions, respectively. Furthermore, we performed a solid-phase binding assay between the recombinant integrins and FN. The equivalent amounts of the recombinant integrins were added to FN-coated microtiter plates. Interestingly, the binding capacity of the  $\alpha 5\text{S}3-4/\beta 1\text{S}4-6$  underglycosylated mutant was comparable to that of WT, suggesting that *N*-glycosylations of both the  $\beta$ -propeller of the  $\alpha 5$  subunit and the I-like domain of the  $\beta 1$  subunit is important for biological function, such as ligand binding. The binding specificity between  $\alpha 5\beta 1$  integrin and FN was confirmed by treatment with 10 mM EDTA, which completely blocked integrin-FN interactions. These results strongly suggest that *N*-glycosylation of a specific domain is important for both dimer formation and functional expression.

## DISCUSSION

The present study examined the effects of underglycosylation of the  $\beta 1$  subunit. We determined that only the *N*-glycan on the I-like domain of the  $\beta 1$  subunit is essential to its biological functions, such as  $\beta 1$  integrin-mediated initial cell spreading and dimer formation. Furthermore, we proposed

that the  $\alpha 5 S 3-5 / \beta 1 S 4-6$  mutant presents the minimal *N*-glycosylation requirement for functional  $\alpha 5 \beta 1$  integrin expression. In fact, the putative *N*-glycosylation sites on the I-like domain are completely conserved in human, mouse, rat, *Xenopus* and chicken. Recently, Liu *et al.*, using a molecular dynamic modeling approach, determined that alteration in the glycosylation of the I-like domain of  $\beta 1$  might affect interactions between oligosaccharides and the I-like domain, which in turn alters the accessibility of the specificity-determining loop (SDL) to ligands (40). In addition, we found that *N*-glycosylation site-4 on the  $\beta$ -propeller of the  $\alpha 5$  subunit was specifically modified by GnT-III, thereby regulating  $\alpha 5 \beta 1$ -mediated cell spreading and cell migration (Sato *et al.*, manuscript in submission). These observations suggest that site-specific modulation of *N*-glycans on integrin affects its biological functions. Therefore, a detailed mutagenesis study of *N*-glycans on integrins is indispensable for the elucidation of the complicated mechanisms that are involved in its functional regulation by glycosyltransferases, such as GnT-III,  $\alpha 2,6$ -sialyltransferase, and GnT-V (9,12,13,41).

It is well known that integrin-mediated cell adhesion functions cooperatively with growth factor receptors to control cell proliferation, differentiation, survival, and migration of epithelial cells and fibroblasts (42). The association of integrins with growth factor receptors has been demonstrated in

co-clustering and co-precipitation studies (43,44). In fact, integrins enable growth factor signaling in many cases, as normal growth factor signaling does not occur unless cells are adhered to the ECM or to other cells through integrins. Hakomori *et al.* reported that the formation of integrin-tetraspanin complexes was affected by the *N*-glycosylation of both integrin and tetraspanins, as well as by gangliosides in the microdomain (45). For example, CD82 with complete *N*-glycosylation demonstrated a reduction its association with  $\alpha 3$  or  $\alpha 5$  integrin, whereas CD82 with incomplete *N*-glycosylation exhibited enhanced association (46). Conversely, the association of CD9 with either the  $\alpha 3$  or  $\alpha 5$  integrin subunits was not influenced by *N*-glycosylation, as CD9 contains no *N*-glycosylation sites. Based on these observations, we postulate that *N*-glycosylation of integrin may participate in supramolecular complex formation on the cell surface, which controls intracellular signal transduction. Recently, we demonstrated, using biochemical visualization method and antibody array, that many receptor tyrosine kinases, such as epidermal growth factor receptor, formed clusters with  $\beta 1$  integrin in living cells (47). We believe that these underglycosylated mutants of the  $\beta 1$  and  $\alpha 5$  subunits will prove very useful in clarification of the molecular mechanisms of supramolecular complex formation proximal to the cell membrane, which, in turn, might both positively and

negatively regulate cellular biological functions by modulation of *N*-glycans.

Based on both previous studies (21,22) and the results of the present study, it is clear that *N*-glycosylation is essential to  $\alpha 5\beta 1$  heterodimer formation. However, this may not always be the case. Purified  $\alpha 5\beta 1$  integrin, but not  $\alpha L\beta 2$ , treated with *N*-glycosidase F blocked the inherent association of both subunits. In addition, SDL within the I-like domain of the  $\beta 2$  subunit, an essential conserved region required for  $\alpha\beta$  dimer formation, does not contain potential *N*-glycosylation sites (48,49), indicating *N*-glycosylation may not participate in  $\beta 2$ -containing integrin assembly. In contrast, the SDL segment is not required for formation of  $\alpha 5\beta 1$ , suggesting that use of subunit interface residues is variable among integrins (48). On the other hand, both the binding and killing of target cells by cytotoxins, such as CyaA, LtxA, and HlyA, depended on recognition of the *N*-glycans on  $\beta 2$  integrin (50).

In the crystal structure of integrin  $\alpha V\beta 3$ , the main contact between the  $\alpha V$  and  $\beta 3$  subunit is the  $\beta$ -propeller on  $\alpha V$  and the I-like domain of  $\beta 3$  with hydrophobic, ionic, and mixed-type interactions (29,51). Mold et al. determined the structure of  $\alpha 5\beta 1$  using

homology modeling (52). Based on the model, the interfaces of the  $\alpha 5\beta 1$  dimer are surrounded by *N*-glycans. Furthermore, based on homology modeling of the I-like domain of the  $\beta 1$  subunit, the interfaces seem to be influenced by the surrounding *N*-glycans (data not shown). In fact, artificial introduction of an *N*-glycan edge at the dimer interface prevented the association of the two subunits in the case of the GABA receptor, which is a G-protein-coupled receptor. This result suggests that *N*-glycans act at the dimer interface (53). These studies strongly support the notion that the structural environment of the  $\alpha\beta$  interface can be affected by the presence of *N*-glycans. Currently, the atomic resolution structure of integrin  $\alpha 5\beta 1$  has not been determined for the following reasons: only small amounts of purified protein are available; the large size of the molecule; the conformational flexibility; and, the presence of both transmembrane domains and heavily *N*-glycosylated regions in both subunits. Therefore, we believe that integrin mutants, such as  $\alpha 5S3-5$  or  $\alpha 5S5$  and  $\beta 1S4-6$ , might greatly facilitate future studies of the crystal structure.

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#### FOOTNOTES

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The abbreviations used are as follows: ECM, extracellular matrix; FN, fibronectin; GnT, N-acetylglucosaminyltransferase; CHO, Chinese hamster ovary; mAb, monoclonal antibody; GFP, green fluorescence protein; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal

bovine serum; PBS, phosphate-buffered saline; BSA, bovine serum albumin; WT, wild type; mAb, monoclonal antibody; FACS, fluorescence-activated cell sorter.

#### FIGURE LEGENDS

**FIGURE 1. Schematic illustration of the potential *N*-glycosylation sites on the integrin  $\beta 1$  subunit.** The sites corresponding to the putative *N*-glycosylation sites on the  $\beta 1$  subunit (N50, N94, N97, N212, N269, N363, N406, N417, N481, N520, N584 and N669) are shown by gray the arrows. The crosses represent the removal of glycosylation at each *N*-glycan site by site-directed mutagenesis, i.e., Asn to Gln.

**FIGURE 2. Effects of underglycosylation on  $\beta 1$  subunit expression and association of the  $\beta 1$  subunit with the  $\alpha$  subunit.** *A*, total cell lysates from GFP-expressing cells (control), WT, and several underglycosylated mutants as indicated ( $\Delta 1-3$ ,  $\Delta 4-6$ , and  $\Delta 9-12$ ), were blotted with the anti-integrin  $\beta 1$  antibody (JB1A) and anti- $\alpha$  tubulin antibody (DM1A) as a loading control. *B*, Equivalent amounts of cell lysate were immunoprecipitated (IP) with the anti- $\beta 1$  integrin (P5D2). The immunoprecipitates were then subjected to 6% SDS-PAGE and blotted (IB) with the anti- $\alpha 5$  (H-104) and anti- $\beta 1$  integrin (JB1A) antibodies. Loading of equivalent amounts of protein in total cell lysates was verified using the anti  $\alpha$ -tubulin antibody. *C*, The quantitative data were expressed as the relative ratios of  $\alpha 5/\beta 1$  subunit in the anti- $\beta 1$  immunoprecipitate. The optical densities of the  $\alpha 5$ - and  $\beta 1$ - subunit bands were measured using Image J software. The ratio of  $\alpha 5$  versus  $\beta 1$  of wild type was set at 100. Data were obtained from three independent experiments (mean  $\pm$  S.D). \*\*  $P < 0.01$  according to Student's two-tailed *t* test.

**FIGURE 3. Comparison of cell-surface expression and association with the  $\alpha$  subunit among the underglycosylated mutants.** *A*, Equivalent amounts of cells lysates were immunoprecipitated (IP) with the anti- $\beta 1$  integrin (P5D2), and then the immunoprecipitates were subjected to 6% SDS-PAGE, blotted (IB) with the anti- $\alpha 5$  (H-104) or anti- $\alpha 1$  (JB1A) antibody. Loading of equivalent amounts of protein in the total cell lysate was verified using the anti  $\alpha$ -tubulin antibody. *B*, Expression of  $\beta 1$  integrin on the cell surface was examined using FACS analysis. Prior to analysis, the cells were incubated with anti- $\beta 1$  antibody (P5D2), followed by incubation with Alexa Fluor 488 goat anti-mouse IgG as described under "EXPERIMENTAL PROCEDURES." Negative control staining (shaded histogram) was done without the first antibody. *C*, Relative expression levels of  $\alpha$  subunit on the cell surface. The cells were stained with rat anti- $\alpha 5$  antibody, and followed by incubation with Alexa Fluor 633 goat anti-rat IgG. The expression levels of  $\alpha 5$  subunit were expressed as relative fluorescence intensities examined using FACS analysis. The fluorescence intensity of wild type of  $\alpha 5$  subunit

was set at 100.

**FIGURE 4.** Effects of N-glycosylation on I-like domain of  $\beta 1$  on post-translational processing, stability and cellular localization. *A*, After metabolic labeling with [ $^{35}\text{S}$ ] methionine and cysteine for 30 min, cells were washed with fresh medium with or without MG-132, a proteasome inhibitor, in final concentration at 8  $\mu\text{M}$ , and then chased at the indicated times. The cells were lysed and the same amounts of cell lysate were immunoprecipitated with anti- $\beta 1$  (JB1A) antibody at the indicated times. The visualizations were performed as described under "EXPERIMENTAL PROCEDURES." M and P indicate the migrated position of mature and precursor form of  $\alpha 5$  and  $\beta 1$  subunit, respectively. *B*, The cells were stained with mouse antibody against the  $\beta 1$  subunit (JB1A) and rabbit antibody against calnexin (SPA-860), followed by visualization with goat antibodies against mouse IgG-conjugated Alexa 488 and rabbit IgG-conjugate Alexa 546, respectively. The bar denotes 20  $\mu\text{m}$ .

**FIGURE 5.** Comparison of cell spreading on FN among various underglycosylated mutants of the  $\beta 1$  subunit. *A*, Cells were detached and replated on culture dishes pre-coated with 5  $\mu\text{g}/\text{ml}$  of FN. After incubation for 20 min, the cells were fixed with 3.7% paraformaldehyde, and representative fields were photographed using a 200-fold phase contrast microscope. The bar denotes 200  $\mu\text{m}$ . *B*, Quantification of cell spreading on FN expressed as the means  $\pm$  S.D. of three independent experiments. *C*, Cell adhesion kinetics assay using RT-CES system as described under "EXPERIMENTAL PROCEDURES." Cell index means extent of cell adhesion. The bars show standard deviation.

**FIGURE 6.** Minimal N-glycosylation requirement for  $\alpha 5\beta 1$  dimer formation. *A*, Schematic illustration of two underglycosylation mutants of the  $\alpha 5$  subunit containing a GFP tag. The sites corresponding to putative N-glycosylation sites (N84, N182, N297, N307, N316, N524, N530, N593, N609, N675, N712, N724, N773, and N868) on the  $\alpha 5$  subunit are shown by the gray arrows. The crosses represent the removal of glycosylation at each N-glycan site by site-directed mutagenesis.  $\alpha 5\text{S3-5}$  and  $\alpha 5\text{S5}$  indicate that the removal of all sites other than the indicated sites, i.e., N297/N307/N316 and N316, respectively. *B*, Cells expressing the indicated N-glycosylation mutants of  $\alpha 5$  ( $\alpha 5\text{S3-5}$  and  $\alpha 5\text{S5}$ ), were infected with viruses containing either WT or N-glycosylation mutants ( $\Delta 4-6$  and S4-6) of the  $\beta 1$  subunit as described under "EXPERIMENTAL PROCEDURES." The cell lysates were immunoprecipitated with anti-GFP antibody, and the immunocomplexes were subjected to SDS-PAGE and blotted with anti- $\beta 1$  subunit (JB1A) (*upper panel*). The total cell lysates were blotted using an anti- $\beta 1$  subunit

antibody (JB1A) (*middle panel*) and anti-GFP antibody (*lower panel*).

**FIGURE 7. Characterization of WT and underglycosylated mutant of soluble  $\alpha 5\beta 1$  integrin.** *A*, Extracellular portions of  $\alpha 5$  (1-644) and  $\beta 1$  (1-501) subunits (bold) were fused to acid and base peptides, respectively, and the boxed seven-amino acid is recognition sequence for TEV protease as described (27). SDS-PAGE and immunoblot analyses of purified recombinant integrins. The purified WT and underglycosylated mutant of the integrin  $\alpha 5\beta 1$ , prepared as described under "EXPERIMENTAL PROCEDURES," were subjected to 6% SDS-PAGE for visualization using Coomassie Brilliant Blue R-250 (*B*), immunoblotting with anti- $\alpha 5$  (#4705S) (*C*) or  $\beta 1$  mAb (JB1A) (*D*) under reducing (*left side*) and non-reducing (*right side*) conditions. The brackets indicate the positions of WT and the  $\alpha 5S3-5/\beta 1S4-6$  underglycosylated mutant of the recombinant integrin. *E*, Comparison of ligand-binding abilities between WT and the  $\alpha 5S3-5/\beta 1S4-6$  underglycosylated mutant of the integrin. Equivalent amounts of the recombinant integrins were added to microtiter plates that had been coated with FN (5  $\mu\text{g}/\text{ml}$ ). The plates were then incubated for 3 h at 37°C in the presence of 1mM  $\text{MnCl}_2$  (gray bar) or 10 mM EDTA (open bar). The bound integrins were quantified using anti- $\beta 1$  antibody (JB1A) and alkaline phosphatase-conjugated streptavidin as described under "EXPERIMENTAL PROCEDURES."