β-glucosyltransferase (B, PDB No. 1IIR) are indicated in orange and red respectively. FUT8 is indicated in blue.

Fig.5. **Site-directed mutagenesis of the conserved amino acid residues of FUT8.**

(A) An alignment analysis of the catalytic regions of FUT8 from several species was carried out using CLUSTAL W. The amino-acid residues which are conserved in all species are highlighted in bold. The conserved cysteine residues are indicated in red, and mutated residues are indicated in blue. The motif I (residues 358-370), motif II (residues 403-416) and motif III (451-477), which are conserved in three fucosyltransferases, α1,2-, α1,6- and protein O-fucosyltransferases, are indicated in a box. GenBank accession numbers for FUT8 are: *H.sapiens*, BAA19764; *M.musculus*, NP_058589; *S.scrofa*, BAA13157; *G.gallus*, CAH25853; *X.laevis*, AAH79978; *D.rerio*, CAH03675; *D.melanogaster*, AAF48079; *C.elegans*, AAN84870; *C.intestinalis*, CAD561622. (B) I, The overall structure is shown as a ribbon model. Locations of the mutated residues near Arg-365 of FUT8 are indicated in a box. Side chains of the mutated residues are shown by stick. Conserved disulfide bonds are indicated in yellow. II, the region around Arg-365 in box of Fig. 5B-I are closed up. His-363, Arg-365 and Arg-366 were examined, as reported previously (Takahashi *et al.*, 2000). The σ_A weighted $2F_o$ - F_c map contoured at 1σ is also indicated. (C) The wild-type and mutant enzymes were transiently expressed in COS-1 cells. Cell lysates were separated on 8% SDS-gels and analyzed by immunoblot using anti-(FUT8) IgG. COS and mock indicate nontransfected and vector-transfected COS-1 cells, respectively. H363A and R365A are the controlled mutants (D) Enzyme activities of FUT8 and its mutants were assayed using Asn-linked oligosaccharide acceptor labeled with *N*-[2-(2-pyridylamino)ethyl]-succinamic acid 5-norbornene-2,3-dicarboxyimide ester, as described in experimental procedures.

Fig.6. **Superimposition of SH3 domain of FUT8 onto various proteins containing SH3 domain.**

(A) SH3 domain of FUT8 at C-terminal was superimposed onto various proteins in a stereo view.

Residues 502-562 comprise the SH3 domain of FUT8. The main chain of the SH3 domain of FUT8 is colored in cyan in this figure. Other SH3 domains were indicated in red, Abp1, the yeast actin binding protein (PDB No. 1JO8); blue, SH3 domain of neutrophil cytosol factor 4, the $p40^{phox}$, component of NADPH oxidase, (PDB No. 1W6X); green, the SH3 domain of c-Crk, a viral oncogene product (PDB No. 1CKA); yellow, p56lck(Lck), a T-lymphocyte-specific member of the Src family of non-receptor tyrosine kinases (PDB No. 1LCK). (B) A ribbon model of the SH3 domain of FUT8 is indicated.

FOOTNOTES

Enzyme: α1,6-fucosyltransferase (EC 2.4.1.68)

The atomic coordinates and structure factors (code 2DE0) have been deposited in the Protein Data Bank, Research Collaboratory for Structual Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/)

Table 1. Data collection and refinement statistics for the structure of FUT8.

Data set	Native2	Pt derivative
Data collection		
Space group	$P6_522$	$P6_522$
Wavelength (Å)	0.9	0.9
Unit cell		
a (Å)	90.0	90.6
b (Å)	90.0	90.6
c (Å)	380.7	380.6
Resolution	50-2.6 (2.69-2.60)	50-3.0 (3.11-3.00)
No. of unique reflections	28706	19663
completeness (%)	97.7 (80.2)	99.9 (99.8)
R_{merge} (%) [b]	5.7 (39.5)	9.2 (68.6)
$I/\sigma(I)$	16.1 (1.4)	11.39 (1.34)
Phase determination		
R_{Cullis} [c] (acentric/centric, isomorphous)		0.86/0.85
R_{Cullis} [c] (anomalous)		0.98
Phasing Power [d] (acentric/centric)		0.81/0.78
Number of heavy atom sites		1
Mean overall figure of merit (after DM [e])		0.83
Refinement		
Resolution (Å)	50-2.61	
R_{work} (%) [f]	22.0	
R_{free} (%) [g]	28.3	
r.m.s.d.*		

Bond length (Å)	0.017
Bond angle (°)	1.75
Ramachandranplot	
Most favored (%)	88.1
Additional allowed (%)	11.4
Generously allowed (%)	0.5

[a]The number in parentheses represents statistics in the highest resolution shell.

[b]R_{merge} =Σ|I - <I>|/Σ<I>, where I is the observed intensity, and <I> is the averaging intensity of multiple symmetry-related observations of that reflection.

[c]R_{Cullis} = Σε/Σ|F_{PH}-F_P|, where ε = phase-integrated lack of closure.

[d]Phasing Power = <|F_{Hcalc}|/ε,>, where ε = phase-integrated lack of closure.

[e]DM means density modification performed with SOLOMON and DM implemented in SHARP.

[f]R_{work} = Σ||F_{obs}| - |F_{calc}||/Σ|F_{obs}|, where F_{obs} and F_{calc} are the observed and calculated structure factors for data used for refinement, respectively.

[g]R_{free} =Σ||F_{obs}|- |F_{calc}||/Σ|F_{obs} | for 5% of the data not used at any stage of the structural refinement.

*r.m.s.d. means the root mean square deviation.

Table 2. Structural similarities of FUT8 to other proteins.

Applied query of FUT8 (residues)	protein	PDB	Z score	r.m.s.d.*
The coiled-coil region (residues 108-174)	human lysine-specific demethylase-1	2H94	8.3	2.0
the open sheet α/β structure (residues 201-300)	*E. coli* carbamoyl phosphate synthetase	1C3O	3.6	3.3
Rossmann fold (residues 348-500)	ADP-heptose lipopolysaccharide heptosyltransferase II	1PSW	6.1	3.0
the catalytic region (residues 201-500)	ATP-binding hypothetical protein	1MJH	6.0	3.0
	Gtfb, β-glucosyltransferase	1IIR	5.2	3.3
	trehalose-6-phosphate synthase	1GZ5	5.1	3.9
	sialyltransferase from *Pasteurella multocida*	2EX0	5.0	3.3
SH3 domain (residues 502-562)	yeast actin binding protein	1JO8	11.0	1.3
	neutrophil cytosol factor 4	1W6X	11.0	1.2
	c-Crk, oncogene protein	1CKA	10.3	1.4
	p56 Lck, the Src family kinase	1LCK	11.6	1.1

*r.m.s.d. means the root mean square deviation.

Fig.1 Ihara *et al.*

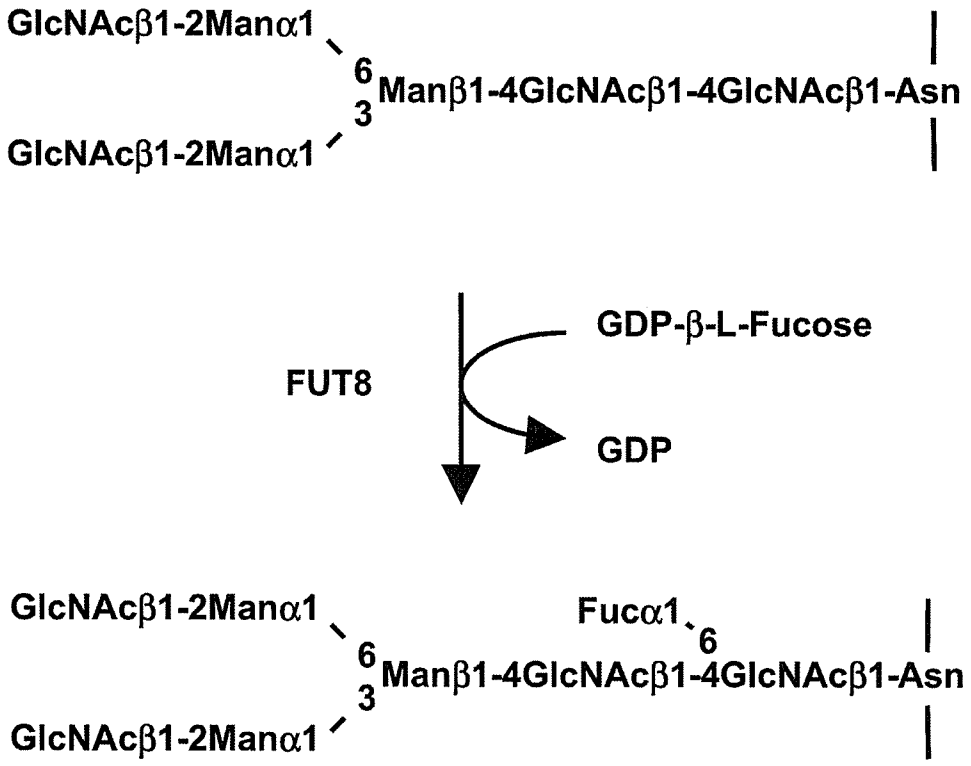


Fig.2 Ihara *et al.*

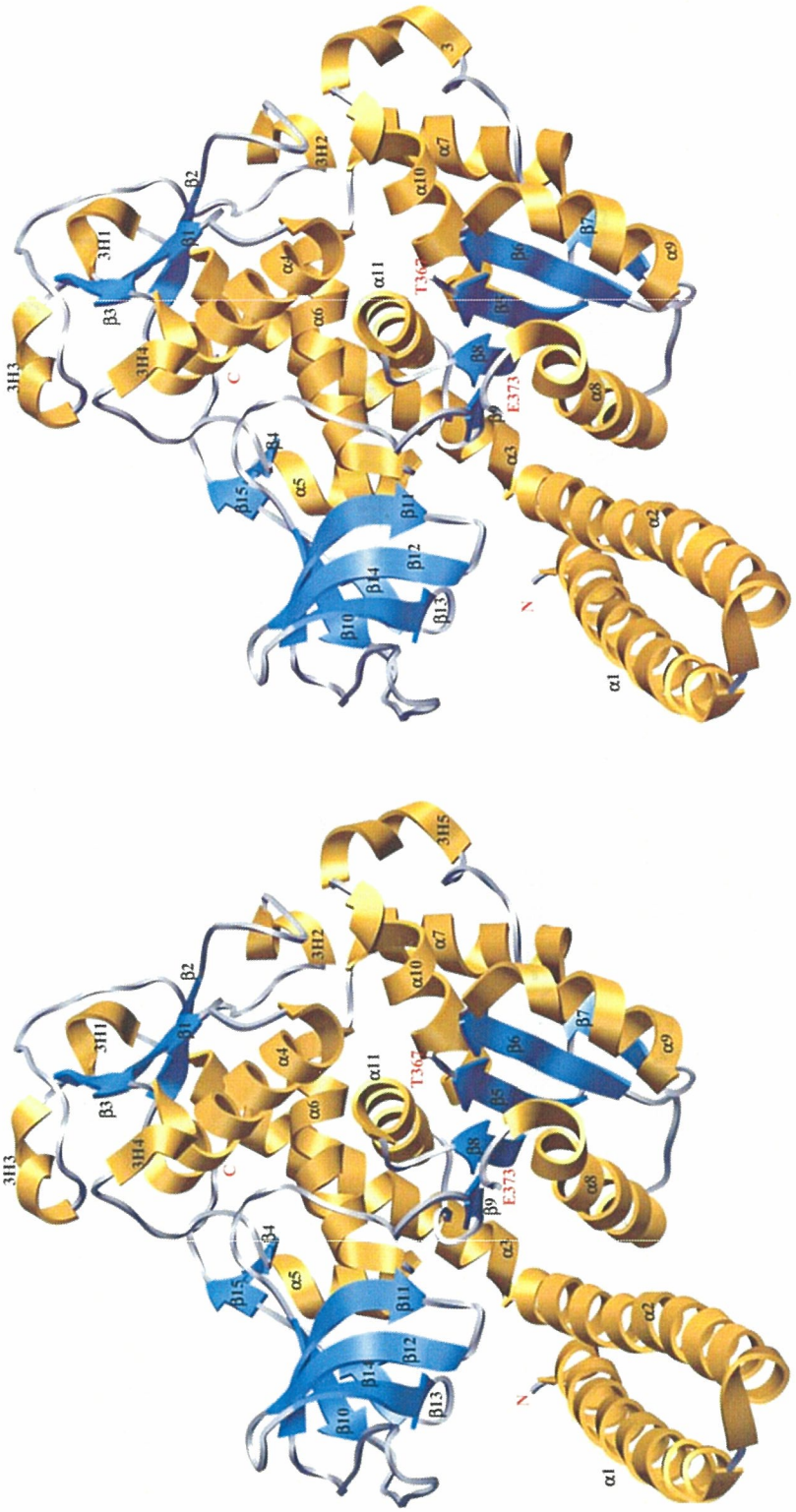


Fig.3 Ihara *et al.*

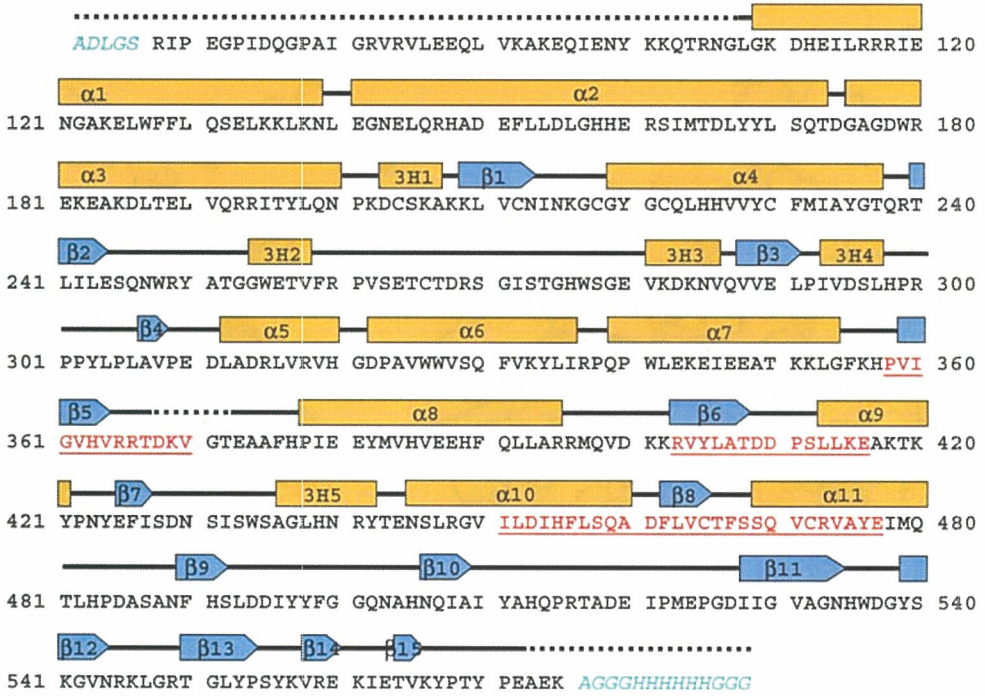


Fig.4A. Ihara *et al.*

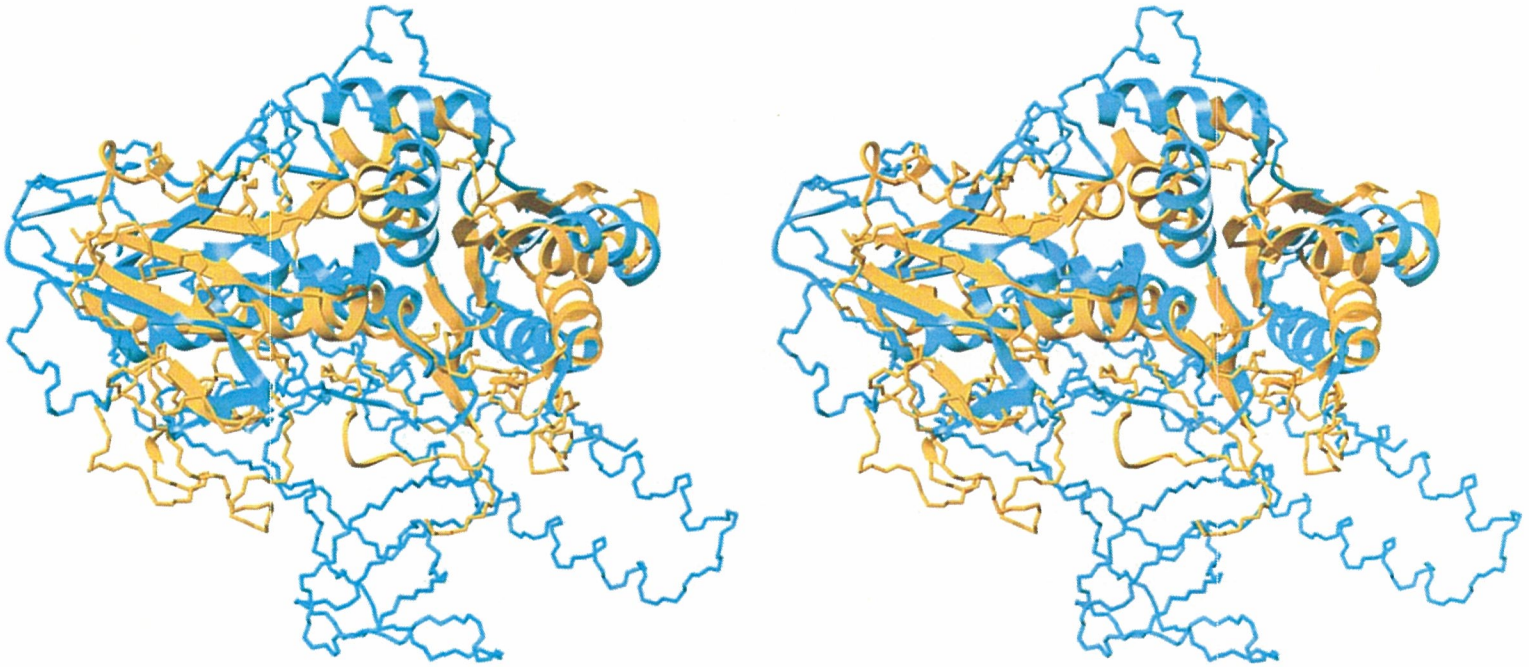


Fig.4B. Ihara *et al.*

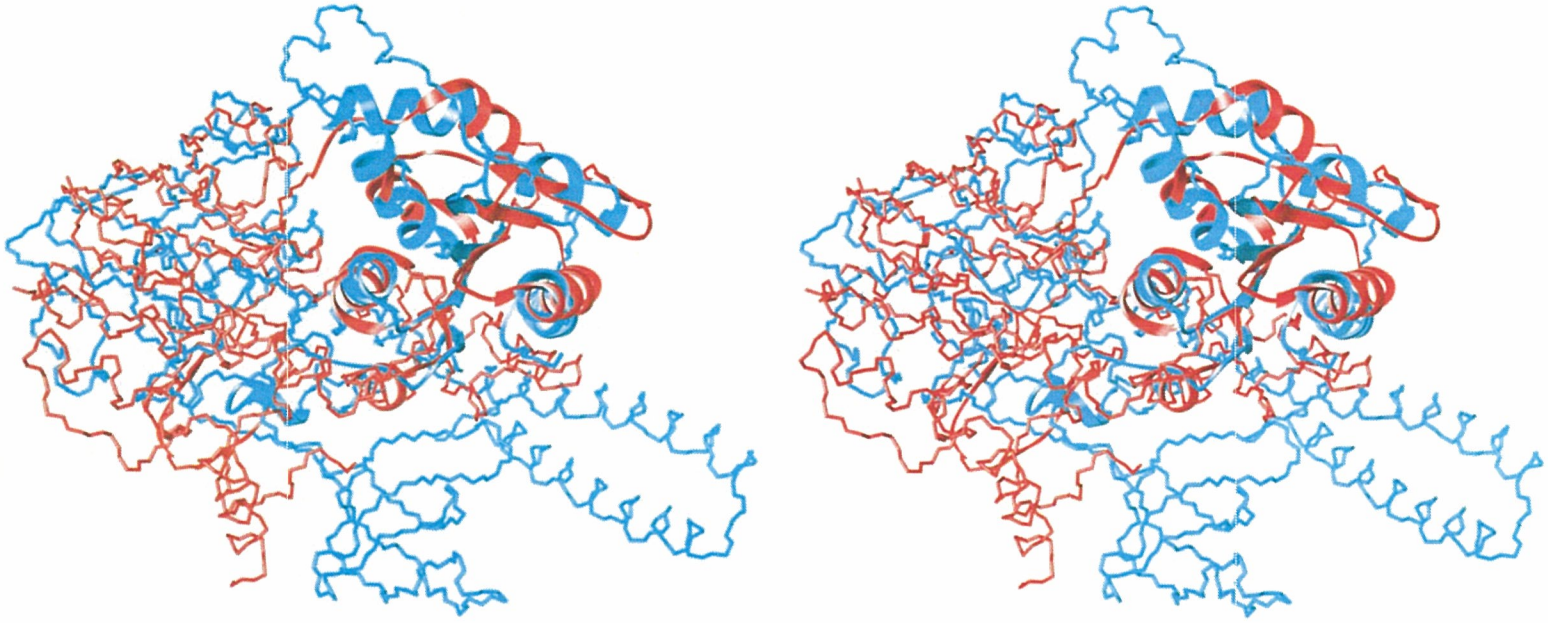
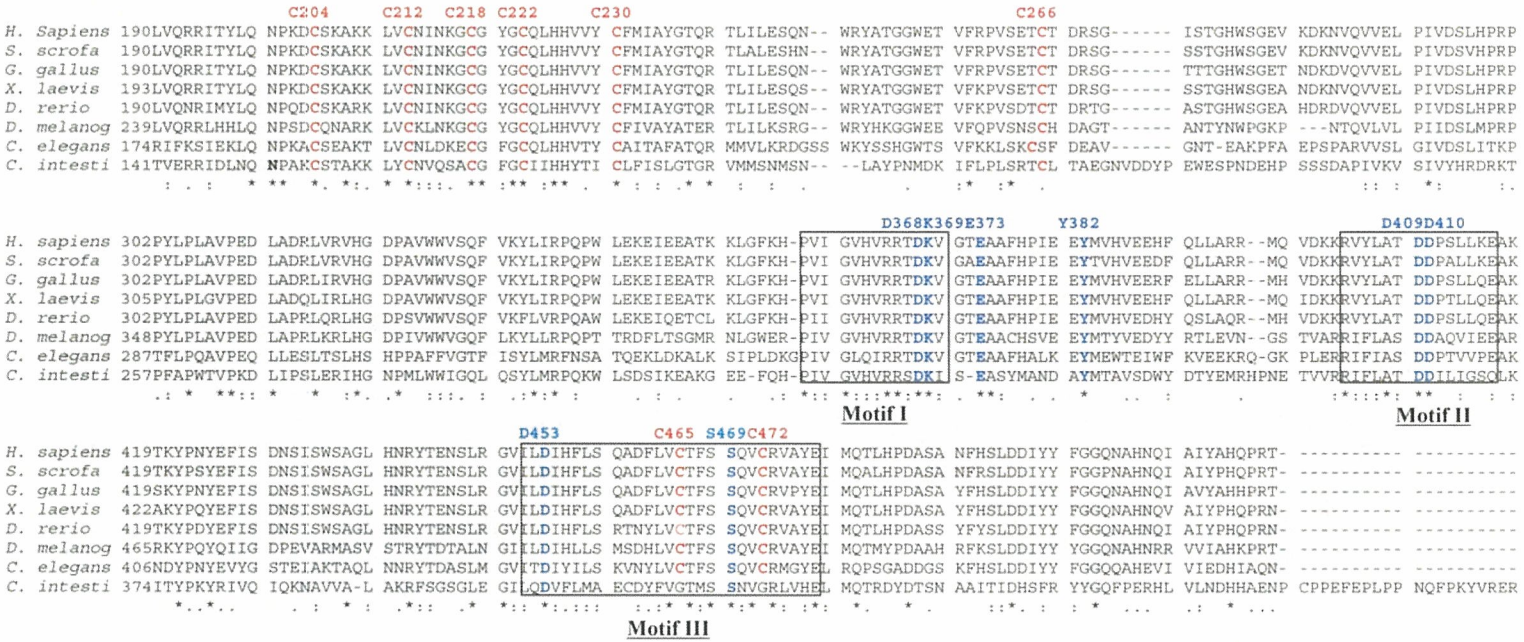


Fig.5A Ihara *et al.*



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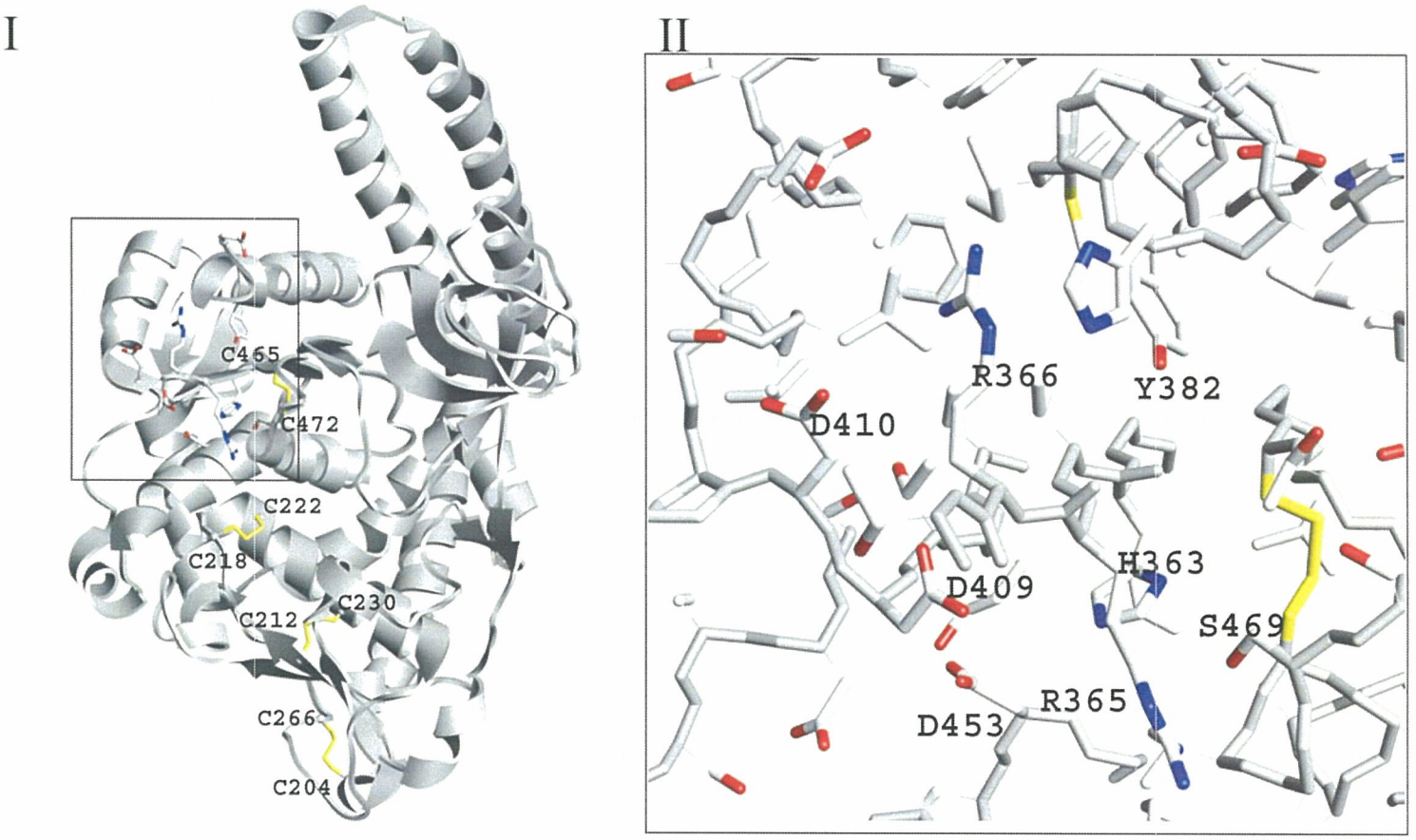

Fig.5C. Ihara *et al.*

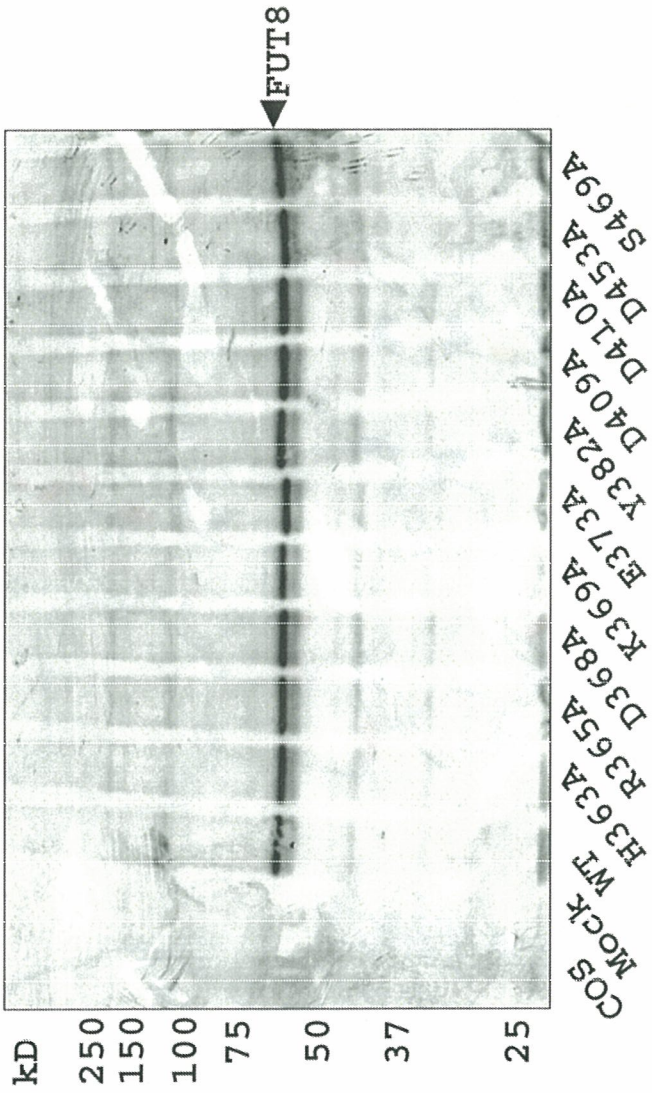

Fig.5D. Ihara *et al.*

Fig.6A. Ihara *et al.*

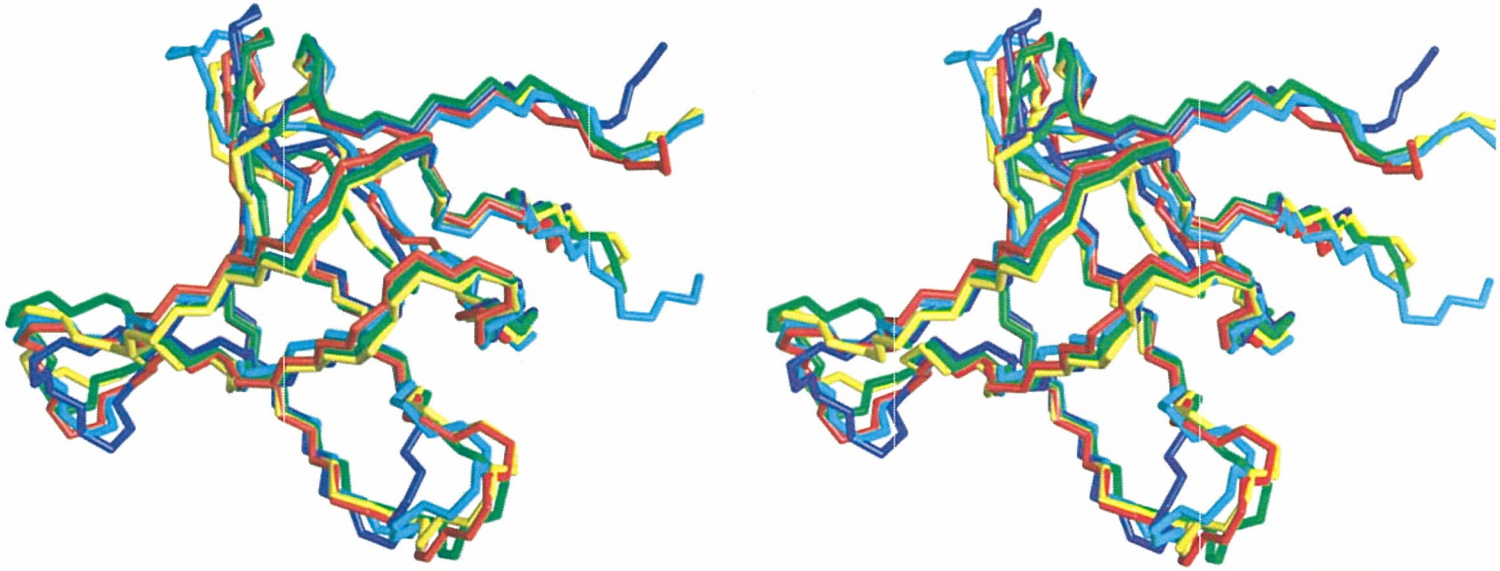

Fig.6B. Ihara *et al.*

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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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Tomoya Isaji[‡], Yuya Sato[‡], Yanyang Zhao[§], Eiji Miyoshi[§], Yoshinao Wada[¶], Naoyuki Taniguchi[‖], and Jianguo Gu[‡1]

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 Biochemistry, Osaka University Graduate School of Medicine, B1, 2-2 Yamadaoka, Suita, Osaka 565-0871, the [¶]Department of Molecular Medicine, Osaka Medical Center and Research Institute for Maternal and Child Health, 840 Murodo, Izumi, Osaka 594-1101, and the [‖]Department of Disease Glycomics, Research Institute for Microbial Diseases, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

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*-acetylglucosaminyltransferase-V (GnT-

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[1] To whom correspondence should be addressed. Tel.: 81-22-727-0078; Fax: 81-22-727-0216; E-mail: jgu@tohoku.pharm.ac.jp.

[2] The abbreviations used are: 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; ER, endoplasmic reticulum; FACS, fluorescence-activated cell sorter.

V), GnT-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-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 carboxyl-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 NheI and XhoI 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 NheI and XhoI and ligated to multiple cloning sites of NheI and SalI (XhoI-compatible cohesive end) of pEGFP-N1 (BD Biosciences) with using the T4 ligase. The GFP-tagged α5 subunit contains a 13-amino acid linker (STVPRARDPPVAT) between the carboxyl-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 microtiter 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 4×10^4 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 a trypsin containing 1 mM EDTA, washed once with DMEM containing 10% FBS, and then suspended in DMEM containing 1% FBS at 1×10^6 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

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 anti-mouse 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 sulfosuccinimidobiotin (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 FBS-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 [^{35}S]methionine and cysteine (Amersham 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.

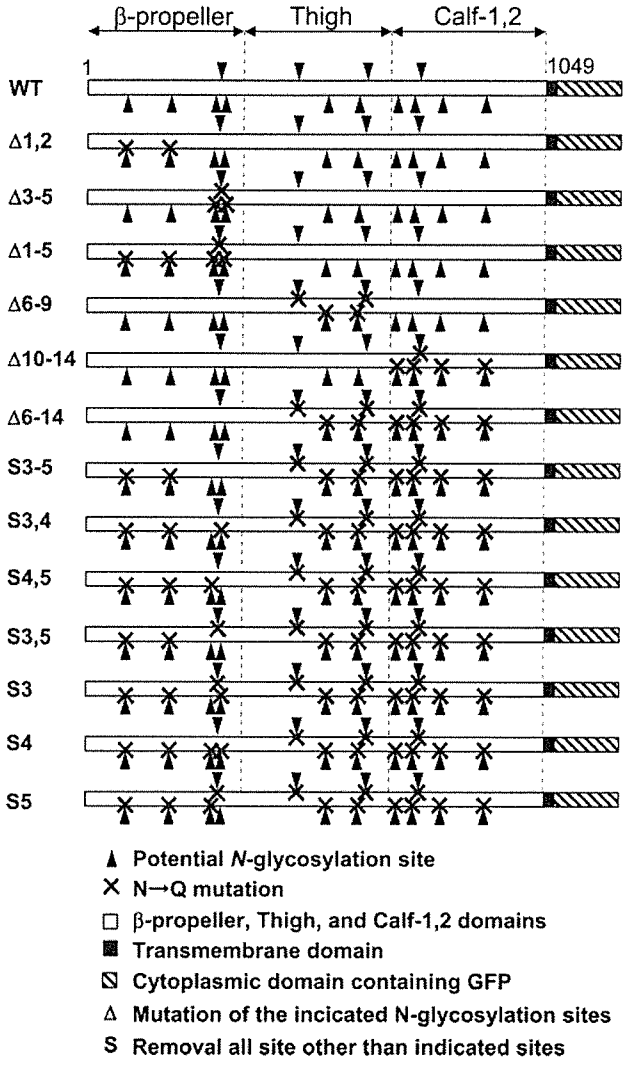

FIGURE 1. **Schematic illustration of potential *N*-glycosylation sites on the integrin α5 subunit.** The sites corresponding to putative *N*-glycosylation sites (N84Q, N182Q, N297Q, N307Q, N316Q, N524Q, N530Q, N593Q, N609, N675Q, N712Q, N724Q, N773Q, and N868Q) are shown by *arrows*. The *crosses* represent the unglycosylation of each *N*-glycan site by site-directed mutagenesis.

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

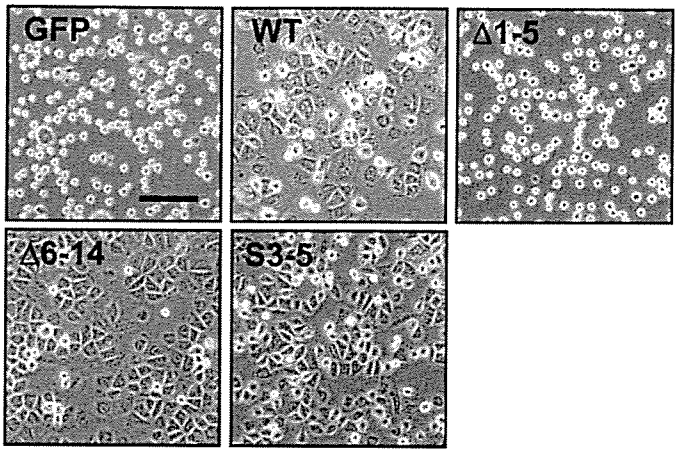

FIGURE 2. **Comparison of cell spreading on FN for various unglycosylated mutants of α5 subunit.** Cells were detached and then replated on dishes that had been pre-coated with 10 μg/ml of FN. After incubation for 30 min, cells were fixed with 3.7% paraformaldehyde, and representative fields were photographed using a 200-fold phase contrast microscope. The *bar* denotes 120 μm.

segment and are well conserved in the human, mouse, and rat. The potential *N*-glycosylation mutants were constructed as shown in Fig. 1 and as follows: 14 single mutants (N84Q, N182Q, N297Q, N307Q, N316Q, N524Q, N530Q, N593Q, N609Q, N675Q, N712Q, N724Q, N773Q, and N868Q), combined mutants (N87Q/N182Q/N297Q/N307Q/N316Q and N524Q/N530Q/N593Q/N609/N675Q/N712Q/N724Q/N773Q/N868Q), and the removal of all sites other than indicated sites (N297/N307/N316) as the S3-5 mutant in the presumed β-propeller, Thigh, Calf, and whole domains, respectively. The mutant cDNAs were transfected to CHO-B2 cells, an α5-deficient cell line, and stable cell lines for the expression of α5 integrin were selected with G418.

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 attachment 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

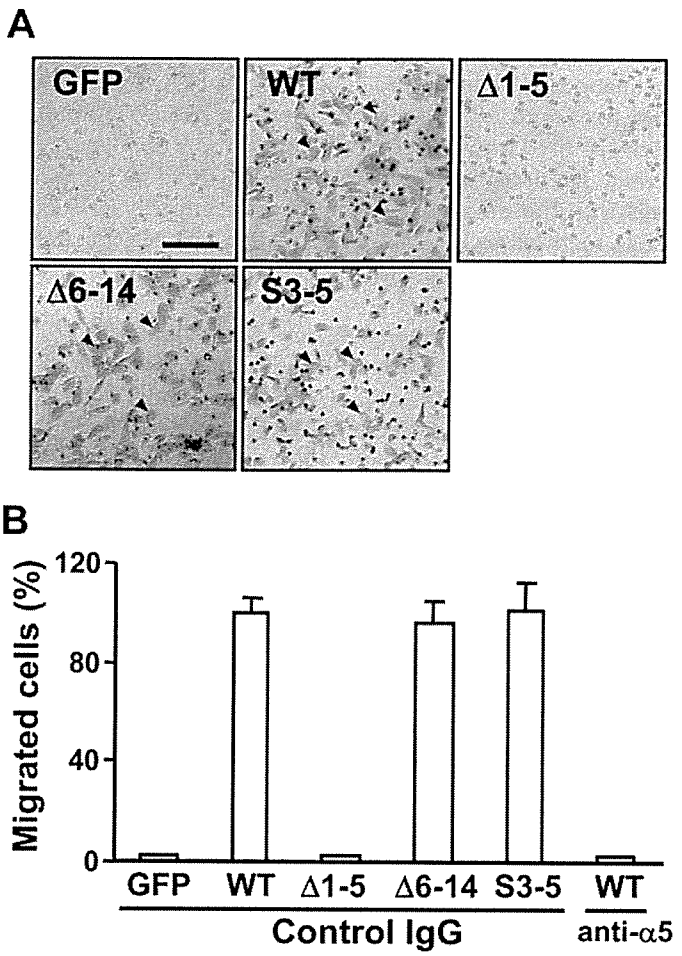

FIGURE 3. **Effects of unglycosylation of α5 subunit on cell migration.** Cell migration was determined using a transwell assay, as described under "Materials and Methods." After incubation for 3 h, migrated cells were fixed with 3.7% paraformaldehyde, stained with a 0.3% crystal violet, and photographed using a 200-fold phase contrast microscope. *Arrowheads* indicate migrated cells. The percentage of migrated cells was determined from the ratio of the number of migrated cells of each mutant to that of cells expressing the WT of α5 subunit. Data were obtained from three of independent experiments (mean values ± S.D.). The *bar* denotes 120 μm.

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 Borden 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