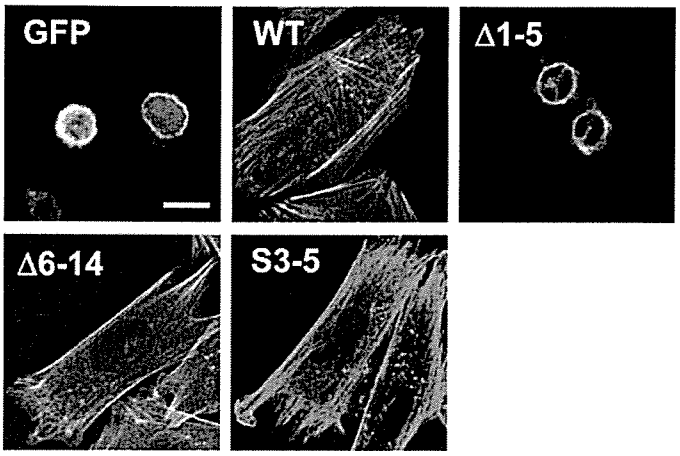



FIGURE 4. **Comparison of actin stress fiber formation among various transfectants spread on FN.** Cells were detached and then replated on coverslips that had been pre-coated with 20 μg/ml FN in PBS and blocked with a 1% BSA as described under "Materials and Methods." After incubation for 2 h, cells were fixed, permeabilized, and then visualized with phalloidin-Alexa 549 (actin) and GFP tag (α5 integrin), respectively. The *bar* is 10 μm.

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 or on cell surfaces. As shown in Fig. 5*A*, 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 Δ6–14 mutant (Fig. 5*B*). 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. 5*C*). 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. 5*D*). 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. 5*E*). 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. 5*F*, *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. 6*A*, *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. 6*A*, *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. 6*B*). 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 be not 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. 6*B*). 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

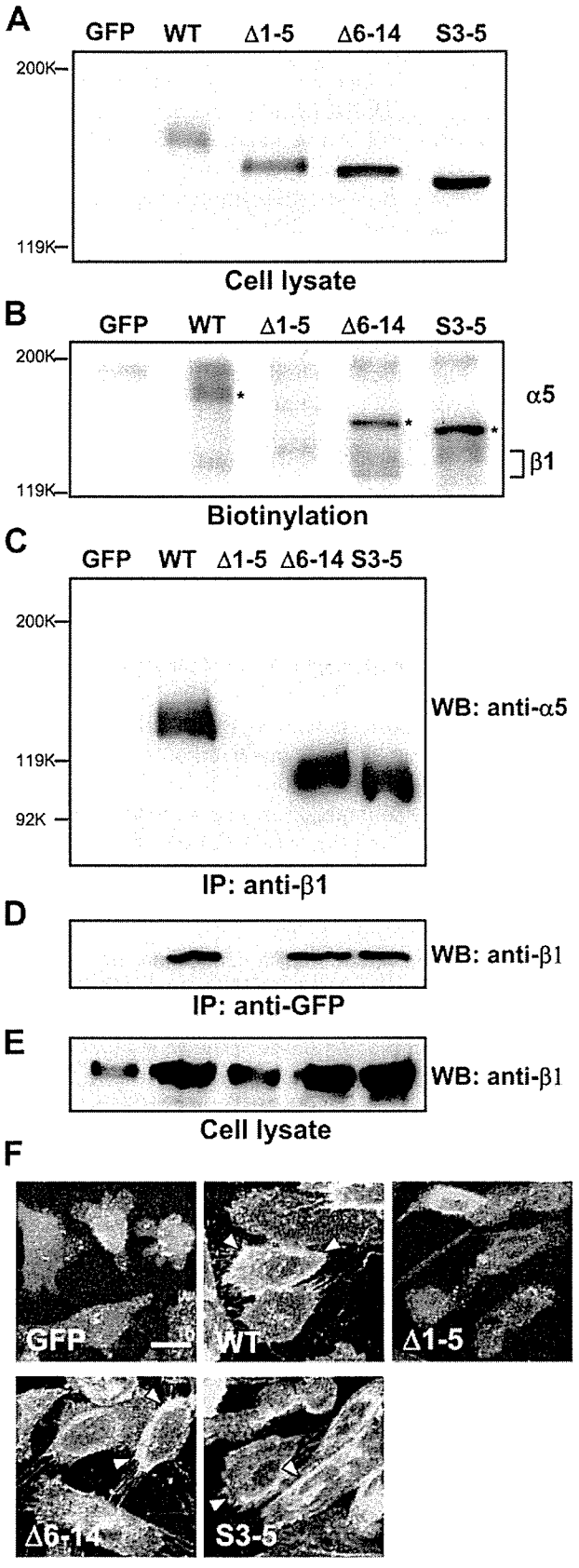



FIGURE 5. **Effects of unglycosylation of the α5 subunit on its expression and association with the β1 subunit.** *A*, total cell lysates from different transfectants expressing GFP tag as a control, WT, and several unglycosylated mutants as indicated (Δ1–5, Δ6–15, and S3-5) were blotted with the anti-integrin α5 antibody (clone 1). *B*, biotinylated cells were lysed and immunoprecipitated with the rabbit anti-integrin α5 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 α5 and β1 subunits, respectively. Cells were lysed and immunoprecipitated (*IP*) with the anti-hamster β1 (*C*) or the anti-GFP (*D*) antibody, and immunoprecipitated samples were detected with the anti-human α5 at reducing conditions or the anti-hamster β1 at nonreducing conditions according to "Materials and Methods." *E*, the expression level of the β1 subunit was detected in total lysates. *F*, cells suspended in DMEM without fetal calf serum were replated on coverslips that had been pre-coated with 20 μg/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 β1 subunits (7E2) and then visualized with the goat antibody for mouse IgG-conjugated Alexa 549. Integrin α5 subunit was visualized with GFP. *Arrowheads* represent co-localization of α and β subunits. The *bar* denotes 10 μm. *WB*, Western blot.

efficiently blocked in the presence of MG-132 as observed in the 8-h chase (Fig. 6*B*, *lower panel*). The degradation of the β1 subunit precursor, but not its mature form, was also inhibited by treatment of WT α5 cells with MG-132 (Fig. 6*B*, *upper panel*). These results clearly demonstrate that *N*-glycosylation on the β-propeller domain of the α5 is essential for maturation, heterodimer formation, and the stability of α5β1 integrin.

*Three N-Glycosylation Sites on β-Propeller of Integrin α5 Are Required for Its Functional Expression*—To define which one or two of these three sites are important for α5β1 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. 7*B*). These results suggest that the site 5 (Asn-316) plays an important role in the expression of the α5 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 Δ3–5 mutant and WT. We speculate that the mutation of the 3–5 sites (Δ3–5) 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 β1 subunit to allow its expression on the cell surface. Although S5 alone can be efficiently expressed on the cell surface, as shown in Fig. 7*C*, 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 α5 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 β-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 chaperon proteins that are associated with the monoglucosidated *N*-glycans on a misfolded glycopro-

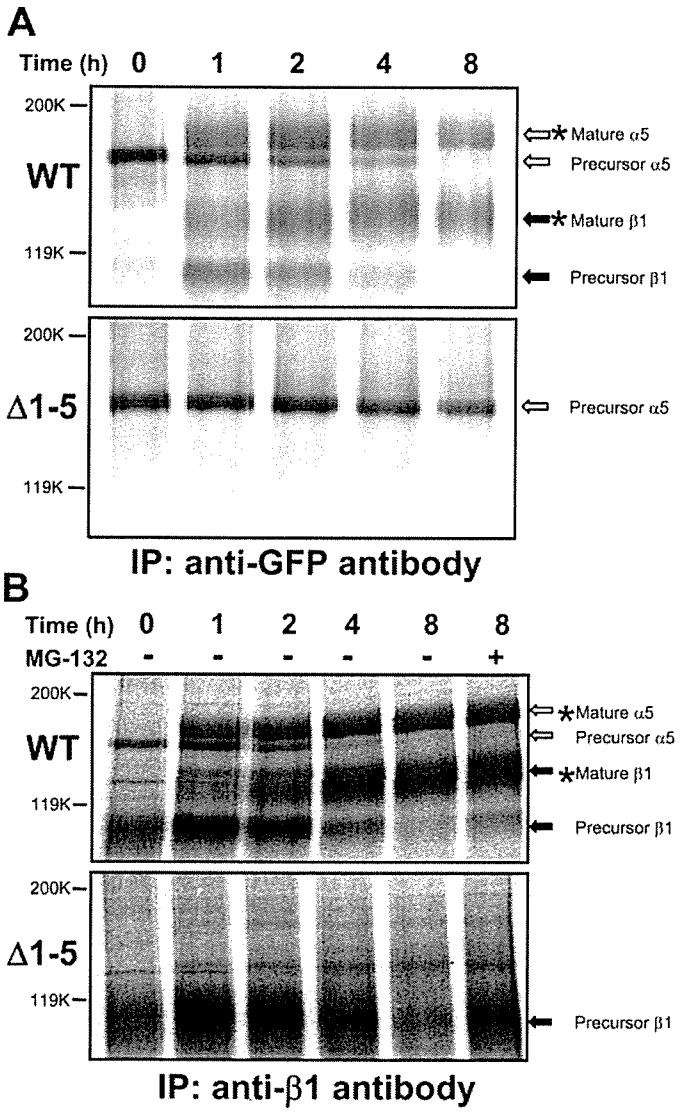



FIGURE 6. **Comparison of expression patterns of α5 and β1 subunits in WT and Δ1–5 transfectants by pulse-chase assay.** After metabolic labeling with [$^{35}$S]methionine for 30 min, cells were washed with fresh medium and then chased at the indicated times. The cells expressing WT (*upper panel*) or 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.

tein to offer the protein an opportunity to fold correctly and then to be exported out of the ER (37, 38). As shown in Fig. 9, increases in calnexin binding were clearly observed in these mutants, compared with that in WT of α5 subunit. In addition, the highest ratio of calnexin binding was found in Δ1–5 among these mutants, suggesting that this mutant was a misfolded protein and could not escape from the calnexin cyclic machinery to be exported from the ER. Moreover, it seemed that the ratios of relative binding of calnexin to these mutants were inversely correlated with their expression levels on the cell surface. On the other hand, no association of these mutants with calreticulin could be detected in this study (data not shown). In fact, several studies have suggested that various glycoproteins may specifically associate with calnexin or calreticulin, to achieve folding (37, 39–41). We next examined the localization of these 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. 9*C*), 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-sialytransferase, 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

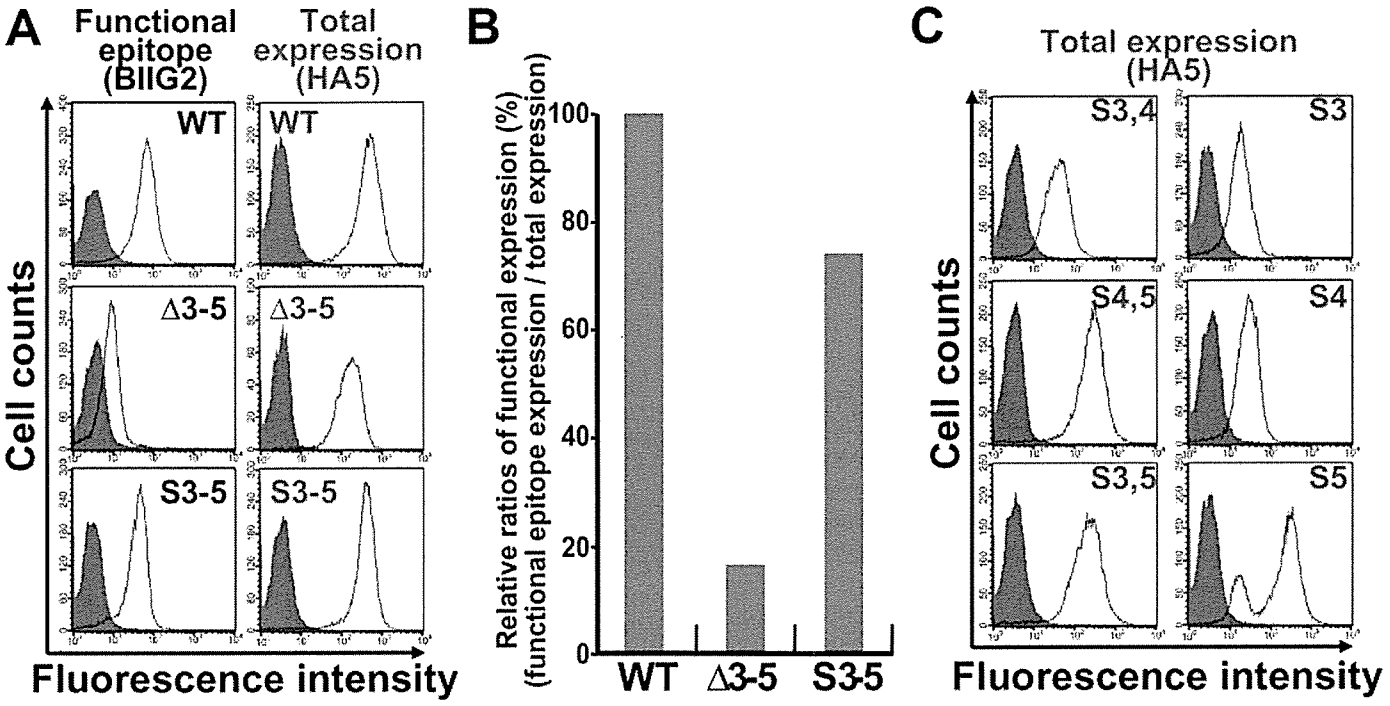



FIGURE 7. **Comparison of expression levels on the cell surface among the unglycosylated mutants.** The functional epitope expression on the cell surface was examined by its reactivity with the functional blocking anti-α5 antibody (BIIG2), whereas total expression was examined by the nonfunctional blocking anti-α5 antibody (HA5). The indicated cells were labeled with BIIG2 (*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 BIIG2 by that of HA5, with WT as 100% (*B*).

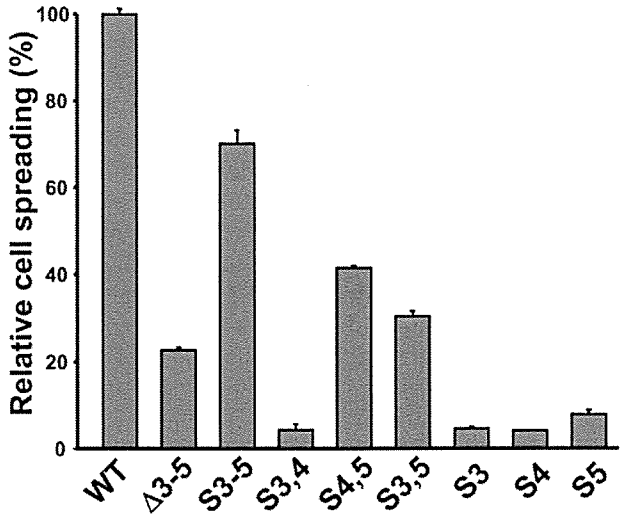



FIGURE 8. **Comparison of cell spreading on FN for unglycosylated mutants.** Cells were detached and then replated on dishes that had been pre-coated with 10 μg/ml FN. After incubation for 30 min, cells were fixed with 3.7% paraformaldehyde. Relative cell spreading was determined by the percentage of cells spread, with WT as 100%. Data were obtained from three of independent experiments (mean values ± S.D.).

293T cells (data not shown) further supports the notion that *N*-glycosylation on the β-propeller is essential for αβ 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. 9*C*. 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 β-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 S3,4, S4,5, 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.

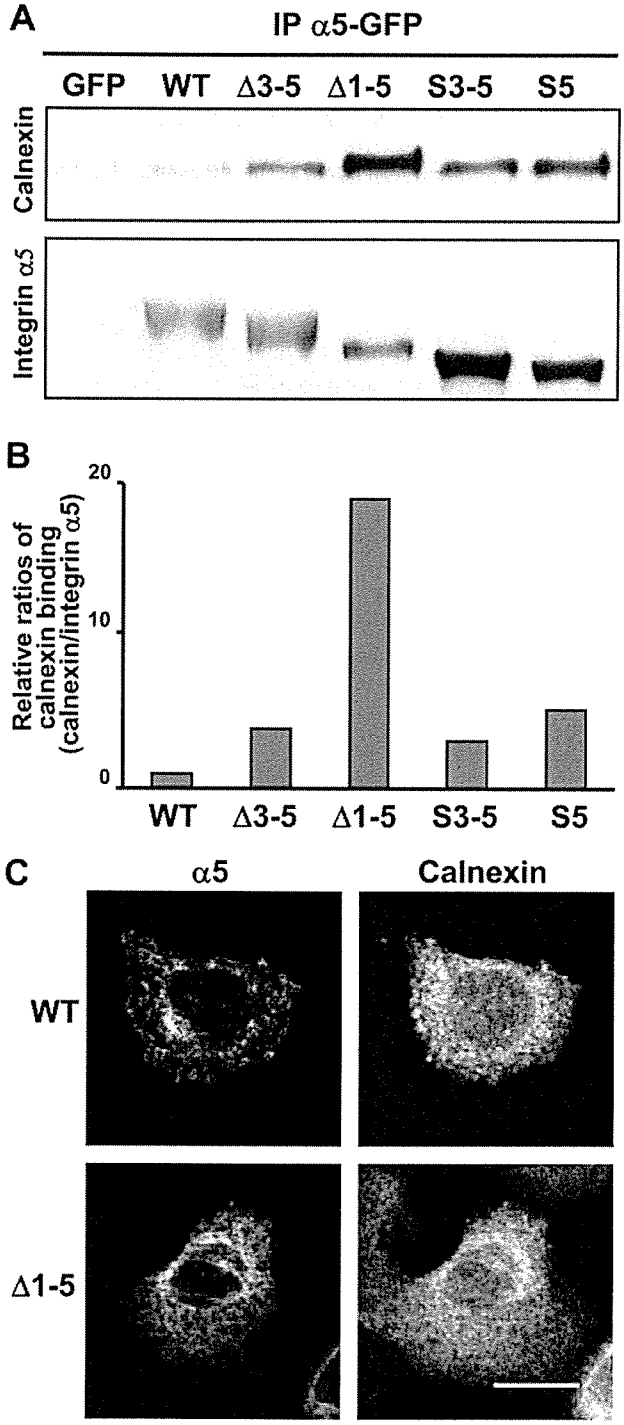



FIGURE 9. **Association of the unglycosylated mutants with calnexin.** *A*, the indicated cells were lysed and immunoprecipitated (*IP*) with a goat anti-GFP antibody. The samples were then subjected to 7.5% SDS-PAGE, and immune complexes were detected with rabbit anti-calnexin or mouse anti-integrin α5 as described under "Materials and Methods." *B*, relative ratios of calnexin binding were determined by dividing the mean intensity of calnexin calculated with Image J (NIH image) by that of integrin α5, with WT as 1.0. *C*, cells cultured on grass bottom dishes were stained with rabbit anti-calnexin and then visualized with the goat antibody for rabbit IgG-conjugated Alexa 549 (calnexin) and GFP (α5 integrin). The *bar* denotes 10 μm.

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-I 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.

## REFERENCES

1. Yang, J. T., Rayburn, H., and Hynes, R. O. (1993) *Development (Camb.)* **119,** 1093–1105
2. Goh, K. L., Yang, J. T., and Hynes, R. O. (1997) *Development (Camb.)* **124,** 4309–4319
3. George, E. L., Georges-Labouesse, E. N., Patel-King, R. S., Rayburn, H., and Hynes, R. O. (1993) *Development (Camb.)* **119,** 1079–1091
4. Hynes, R. O. (2002) *Cell* **110,** 673–687
5. Liddington, R. C., and Ginsberg, M. H. (2002) *J. Cell Biol.* **158,** 833–839
6. Adams, J. C., and Watt, F. M. (1993) *Development (Camb.)* **117,** 1183–1198
7. Akiyama, S. K., Olden, K., and Yamada, K. M. (1995) *Cancer Metastasis Rev.* **14,** 173–189
8. Ruoslahti, E. (1996) *Annu. Rev. Cell Dev. Biol.* **12,** 697–715
9. Varki, A. (1993) *Glycobiology* **3,** 97–130
10. Dwek, R. A. (1995) *Biochem. Soc. Trans.* **23,** 1–25
11. Saxon, E., and Bertozzi, C. R. (2001) *Annu. Rev. Cell Dev. Biol.* **17,** 1–23
12. Gu, J., and Taniguchi, N. (2004) *Glycoconj. J.* **21,** 9–15
13. Bellis, S. L. (2004) *Biochim. Biophys. Acta* **1663,** 52–60
14. Chammas, R., Veiga, S. S., Line, S., Potocnjak, P., and Brentani, R. R. (1991) *J. Biol. Chem.* **266,** 3349–3355
15. Zheng, M., Fang, H., and Hakomori, S. (1994) *J. Biol. Chem.* **269,** 12325–12331
16. Jasiulionis, M. G., Chammas, R., Ventura, A. M., Travassos, L. R., and Brentani, R. R. (1996) *Cancer Res.* **56,** 1682–1689
17. Miyoshi, E., Noda, K., Ko, J. H., Ekuni, A., Kitada, T., Uozumi, N., Ikeda, Y., Matsuura, N., Sasaki, Y., Hayashi, N., Hori, M., and Taniguchi, N. (1999) *Cancer Res.* **59,** 2237–2243
18. Dennis, J. W., Pawling, J., Cheung, P., Partridge, E., and Demetriou, M.

(2002) *Biochim. Biophys. Acta* **1573,** 414–422
19. Guo, H. B., Lee, I., Kamar, M., Akiyama, S. K., and Pierce, M. (2002) *Cancer Res.* **62,** 6837–6845
20. Isaji, T., Gu, J., Nishiuchi, R., Zhao, Y., Takahashi, M., Miyoshi, E., Honke, K., Sekiguchi, K., and Taniguchi, N. (2004) *J. Biol. Chem.* **279,** 19747–19754
21. Semel, A. C., Seales, E. C., Singhal, A., Eklund, E. A., Colley, K. J., and Bellis, S. L. (2002) *J. Biol. Chem.* **277,** 32830–32836
22. Gu, J., Zhao, Y., Isaji, T., Shibukawa, Y., Ihara, H., Takahashi, M., Ikeda, Y., Miyoshi, E., Honke, K., and Taniguchi, N. (2004) *Glycobiology* **14,** 177–186
23. Preissner, K. T., Kanse, S. M., and May, A. E. (2000) *Curr. Opin. Cell Biol.* **12,** 621–628
24. Berditchevski, F. (2001) *J. Cell Sci.* **114,** 4143–4151
25. Hemler, M. E. (2003) *Annu. Rev. Cell Dev. Biol.* **19,** 397–422
26. Damsky, C. H., Fitzgerald, M. L., and Fisher, S. J. (1992) *J. Clin. Investig.* **89,** 210–222
27. Brown, P. J., and Juliano, R. L. (1988) *Exp. Cell Res.* **177,** 303–318
28. Schreiner, C. L., Bauer, J. S., Danilov, Y. N., Hussein, S., Sczekan, M. M., and Juliano, R. L. (1989) *J. Cell Biol.* **109,** 3157–3167
29. Gipson, I. K., Kiorpes, T. C., and Brennan, S. J. (1984) *Dev. Biol.* **101,** 212–220
30. Zhang, Z., Vuori, K., Reed, J. C., and Ruoslahti, E. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 6161–6165
31. Cao, Z., Huang, K., and Horwitz, A. F. (1998) *J. Biol. Chem.* **273,** 31670–31679
32. Laukaitis, C. M., Webb, D. J., Donais, K., and Horwitz, A. F. (2001) *J. Cell Biol.* **153,** 1427–1440
33. Akiyama, S. K., Yamada, S. S., and Yamada, K. M. (1989) *J. Biol. Chem.* **264,** 18011–18018
34. Akiyama, S. K., and Yamada, K. M. (1987) *J. Biol. Chem.* **262,** 17536–17542
35. Heino, J., Ignotz, R. A., Hemler, M. E., Crouse, C., and Massague, J. (1989) *J. Biol. Chem.* **264,** 380–388
36. Yoshida, Y., Chiba, T., Tokunaga, F., Kawasaki, H., Iwai, K., Suzuki, T., Ito, Y., Matsuoka, K., Yoshida, M., Tanaka, K., and Tai, T. (2002) *Nature* **418,** 438–442
37. Williams, D. B. (2006) *J. Cell Sci.* **119,** 615–623
38. Helenius, A., and Aebi, M. (2004) *Annu. Rev. Biochem.* **73,** 1019–1049
39. Molinari, M., Eriksson, K. K., Calanca, V., Galli, C., Cresswell, P., Michalak, M., and Helenius, A. (2004) *Mol. Cell* **13,** 125–135
40. Gao, B., Adhikari, R., Howarth, M., Nakamura, K., Gold, M. C., Hill, A. B., Knee, R., Michalak, M., and Elliott, T. (2002) *Immunity* **16,** 99–109
41. Guo, L., Nakamura, K., Lynch, J., Opas, M., Olson, E. N., Agellon, L. B., and Michalak, M. (2002) *J. Biol. Chem.* **277,** 50776–50779
42. Humphries, M. J., Symonds, E. J., and Mould, A. P. (2003) *Curr. Opin. Struct. Biol.* **13,** 236–243
43. Seales, E. C., Shaikh, F. M., Woodard-Grice, A. V., Aggarwal, P., McBrayer, A. C., Hennessy, K. M., and Bellis, S. L. (2005) *J. Biol. Chem.* **280,** 37610–37615
44. Mould, A. P., Askari, J. A., and Humphries, M. J. (2000) *J. Biol. Chem.* **275,** 20324–20336
45. Ellgaard, L., and Helenius, A. (2003) *Nat. Rev. Mol. Cell Biol.* **4,** 181–191
46. Yoshida, Y., Adachi, E., Fukiya, K., Iwai, K., and Tanaka, K. (2005) *EMBO Rep.* **6,** 239–244
47. Lenter, M., and Vestweber, D. (1994) *J. Biol. Chem.* **269,** 12263–12268
48. Karamyshev, A. L., Kelleher, D. J., Gilmore, R., Johnson, A. E., von Heijne, G., and Nilsson, I. (2005) *J. Biol. Chem.* **280,** 40489–40493
49. Xiong, J. P., Stehle, T., Zhang, R., Joachimiak, A., Frech, M., Goodman, S. L., and Arnaout, M. A. (2002) *Science* **296,** 151–155
50. Xiong, J. P., Stehle, T., Diefenbach, B., Zhang, R., Dunker, R., Scott, D. L., Joachimiak, A., Goodman, S. L., and Arnaout, M. A. (2001) *Science* **294,** 339–345
51. Mould, A. P., Symonds, E. J., Buckley, P. A., Grossmann, J. G., McEwan, P. A., Barton, S. J., Askari, J. A., Craig, S. E., Bella, J., and Humphries, M. J. (2003) *J. Biol. Chem.* **278,** 39993–39999
52. Hoffmeister, K. M., Josefsson, E. C., Isaac, N. A., Clausen, H., Hartwig, J. H., and Stossel, T. P. (2003) *Science* **301,** 1531–1534
53. Josefsson, E. C., Gebhard, H. H., Stossel, T. P., Hartwig, J. H., and Hoffmeister, K. M. (2005) *J. Biol. Chem.* **280,** 18025–18032
54. Ethier, M., Krokhin, O., Ens, W., Standing, K. G., Wilkins, J. A., and Perreault, H. (2005) *Rapid Commun. Mass Spectrom.* **19,** 721–727

ELSEVIER

# Decoding sugar functions by identifying target glycoproteins

Naoyuki Taniguchi[1,2], Eiji Miyoshi[2], Gu Jianguo[2], Koichi Honke[2] and Akio Matsumoto[1]

Identification of the glycosyltransferase genes that are involved in the biosynthesis of glycoconjugates has opened up new avenues in glycobiology, the decoding of the function of sugar chains. Specific biosynthesis of branched *N*-glycan structures by glycosyltransferases functionally modifies target glycoproteins, as observed in the recognition of cancer cells. A mouse model with a specific defect in α1-6 fucosylation showed emphysema-like changes of the lung and severe degradation of lung alveoli that derived from the dysregulation of signaling through the transforming growth factor-β receptor. Functional glycomics and the identification of target proteins will provide a new way to elucidate the nature of disease in the post-genomic era.

**Addresses**
[1] Department of Disease Glycomics, Institute for Microbial Diseases, Osaka University, 2-1 Suita, Osaka 565-0871, Japan
[2] Department of Biochemistry, Graduate School of Medicine, Osaka University, 2-2 Suita, Osaka 565-0871, Japan

Corresponding author: Taniguchi, Naoyuki (tani52@wd5.so-net.ne.jp)

**Current Opinion in Structural Biology** 2006, **16**:561–566

This review comes from a themed issue on
Carbohydrates and glycoconjugates
Edited by Raymond A Dwek and Pauline M Rudd

Available online 12th September 2006

0959-440X/$ – see front matter


**DOI 10.1016/j.sbi.2006.08.011**

## Introduction

It is well known that half of all known proteins are glycosylated. Deciphering the function of the sugar chains of glycoproteins is an important focus in the post-genomic era. In addition, although targeting the genes of the individual glycosyltransferases that biosynthesize sugar chains has revealed various phenotypic changes [1•], few of the actual target proteins, namely the glycoproteins that are directly associated with the phenotypic changes, have been identified. The same is true for patients with congenital disorders of glycosylation (CDGs), even though mutation or lack of a certain glycosyltransferase gene has been identified as one of the causes [2••]. This review focuses on recent findings concerning the function of branched *N*-glycan structures, as revealed by the manipulation of glycosyltransferase genes, with some examples of the identification of target glycoproteins.

## Three glycosyltransferase genes involved in the biosynthesis of branched *N*-glycans

We have focused our work on the glycosyltransferases that are involved in the biosynthesis of branched *N*-glycan structures, and have successfully purified each enzyme to homogeneity and cloned each gene [3–6].

By identifying target proteins with branched sugar chains, the enzymatic products of such glycosyltransferases have been shown to have specific functions. This review focuses mainly on three enzymes, *N*-acetylglucosaminyltransferase III (GnT-III), *N*-acetylglucosaminyltransferase V (GnT-V) and α1-6 fucosyltransferase (Fut8). Each glycosyltransferase produces a β1-4 bisecting GlcNAc, a β1-6 branching GlcNAc and an α1-6 fucose, respectively (Figure 1).

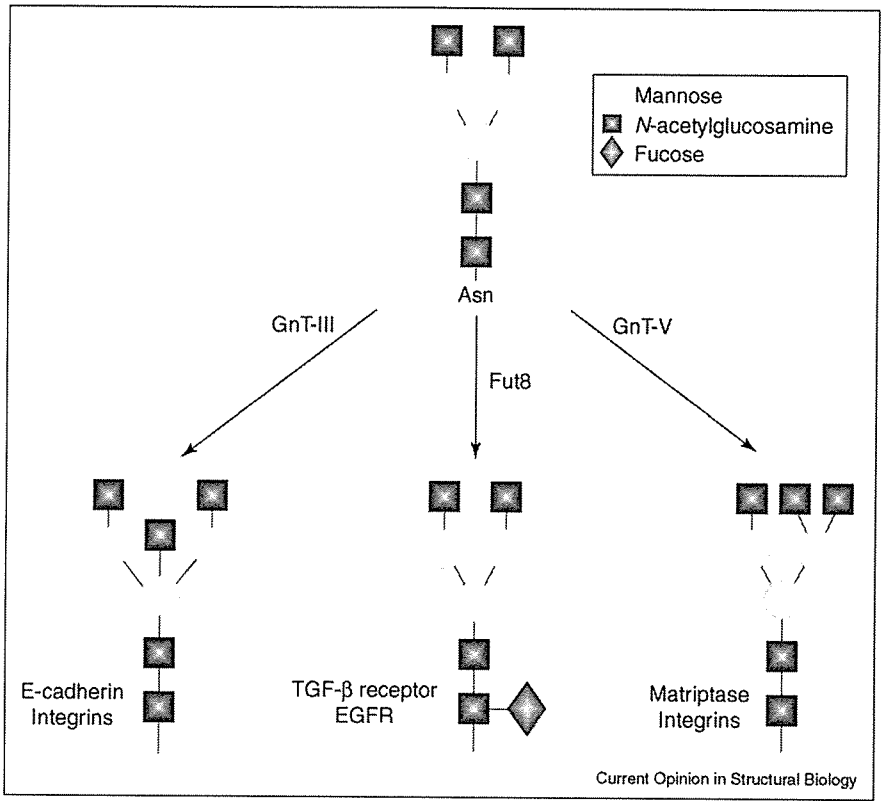
## Identification of target proteins for glycosyltransferases

### Target proteins with the β1-6 branching GlcNAc structure

A relationship between the formation of the β1-6 GlcNAc branching structure and cancer metastasis has been reported by several groups [7–9]. Transcriptional factors from the Ets family play a major role in the expression of the GnT-V gene, and oncogenes such as ras, src and ErbB2 are involved in the regulation of the gene [10–14].

A deficiency of GnT-V lowers T-cell activation thresholds by directly enhancing T-cell receptor (TCR) clustering [15]. When GnT-V-deficient mice are mated with tumorigenic mice, tumor growth and metastasis are severely suppressed, suggesting that GnT-V is essential for tumor metastasis [16]. GnT-V null mice embryonic fibroblasts (MEFs) displayed enhanced adhesion to and spreading on fibronectin-coated plates, with concomitant inhibition of cell migration. Moreover, galectins, proteins that are known to modulate T-cell proliferation and apoptosis, regulate receptor endocytosis. As galectin 3 binds to polylactosamine structures that are extended from β1-6 GlcNAc chains of growth factor receptors, GnT-V activity is involved in the regulation of receptor endocytosis. GnT-V knockout (KO) cells display deficiencies related to endocytosis [17]. MEFs from GnT-V null mice showed enhanced integrin clustering and activation of integrin α5β1 transcription by protein kinase C (PKC) signaling; this in turn up-regulated the levels of cell-surface fibronectin receptors for α5β1, resulting in increased matrix adhesion and inhibition of cell migration [17,18]. A decrease in β1-6 GlcNAc branching of α5β1

Figure 1




The activities of three glycosyltransferases. GnT-III, GnT-V and Fut8 produce a β1-4 bisecting GlcNAc, a β1-6 branching GlcNAc and an α1-6 fucose, respectively. These enzymes synthesize the branched structures of *N*-linked sugar chains. Possible target proteins are indicated.

integrins is directly linked to cisplatin resistance in neck and head tumor cells [19].

Different types of mechanisms exist for cancer metastasis [20]. We identified a matriptase, a metal-dependent serine protease, as a target glycoprotein for GnT-V in gastric cancer cells [21,22]. Matriptase has four asparagine residues that could be targets for glycosylation by GnT-V. Glycosylation of Asn772 by GnT-V mainly contributes to resistance to autolysis and trypsin digestion, resulting in a constitutively active form [23,24]. This may enhance cancer invasion and metastasis because matriptase converts proforms of hepatocyte growth factor (HGF) and urokinase-type plasminogen activator (uTA) to active forms that are involved in the regulation of metastatic migration of cancer cells. Thus, GnT-V participates in cancer metastasis.

GnT-V deletion mutants that lack catalytic activity retain the ability to induce the release of fibroblast growth factor 2 (FGF2), an angiogenic growth factor that contributes to the formation of blood vessels. These data suggest that GnT-V has bifunctional properties and that the angiogenic-factor-releasing function is separate from its glycosyltransferase activity [25]. Moreover, GnT-V is cleaved by protease(s), including γ-secretase, and then released in a soluble form that promotes angiogenesis [26]. GnT-V has a heparin-binding region with primary amino acid sequence KRKRKK; this sequence is commonly found in angiogenic factors such as vascular endothelial growth factor (VEGF) and heparin-binding EGF-like growth factor (HB-EGF). Thus, the cleaved form of GnT-V competes with other proteins through the heparin-binding region and may cause the release of angiogenic growth factors.

### Target proteins with the β1-4 bisecting GlcNAc structure

GnT-III and GnT-V act on the same substrate; once GnT-III synthesizes a bisecting GlcNAc structure, GnT-V cannot act on such bisected precursors [5,26–28]. Therefore, introducing the GnT-III gene results in not only gain of bisecting GlcNAc structure but also loss of β1-6 GlcNAc branching structure. Melanoma cells that overexpress GnT-III barely form a metastatic region in lungs following injection into immunologically compatible, syngeneic, mice [20]. Thus, GnT-III tends to compete with GnT-V, resulting in inhibition of metastasis. Interestingly, in the spleen, GnT-III overexpressed in erythroleukemia cells (K562) shows CD44-mediated

adhesion to hyaluronan, although wild-type K562 cells are rejected through recognition by natural killer (NK) cells in the spleen [29,30].

E-cadherin, a homophilic adhesion molecule, was identified as a target protein to which a bisecting GlcNAc was added. Overexpression of GnT-III in melanoma cells contributes to the stability of E-cadherin on the surface of the cell, as it prevents the degradation of E-cadherin. Thus, E-cadherin becomes resistant to proteolysis and remains on the cell–cell border after modification (sugar remodeling). Overexpression of GnT-III suppresses metastatic cell invasion in the lungs [20,31]. Different mechanisms exist in human cancer cells that explain this. Glycosylation of E-cadherin by GnT-III leads to a reduction in the phosphorylation of β-catenin following stimulation by EGF or Src. Therefore, β-catenin works as a physiological glue that continuously maintains a tight complex with E-cadherin; this prevents the initiation of cancer cell migration leading to metastasis [32]. It may also enhance the homophilic interactions of E-cadherin and contribute to the suppression of cancer metastasis.

Transfection of the GnT-III gene also resulted in the alteration of various functions of growth factor receptors. The *N*-glycans of EGF receptor (EGFR) are involved in receptor sorting, ligand binding and dimerization. The addition of a bisecting GlcNAc to EGFR *N*-glycans increases the rate of endocytosis of EGFR [33]. The *N*-glycans of Trk, a high-affinity nerve growth factor receptor, also affect its function [34]. EGFR, after modification by GnT-III, activates MAP kinase (also known as extracellular signal-regulated kinase; ERK) and PKC [33,35]. This may lead to the dramatic alteration of biological processes such as cell growth and apoptosis. Moreover, a deletion mutant of EGFR at Asn240, an *N*-glycosylation site, resulted in spontaneous oligomerization and signaling in the presence or absence of the EGF ligand [36].

The transfection of GnT-III was also found to modify the *N*-glycans and biological functions of the integrin family [37•]. Increased bisecting GlcNAc inhibits cell spreading and the migration of fibronectin, a specific ligand of integrin α5β1, and focal adhesion kinase phosphorylation. The modification of integrin *N*-glycans by bisecting GlcNAc inhibits its ligand-binding ability, subsequently leading to the down-regulation of integrin-mediated signaling [38].

*N*-glycans with a bisecting GlcNAc on target molecules such as β1 integrin play important roles in the regulation of neuritogenesis, an initial step in neurite formation [39]. The binding of annexin V to Hsp47 is mediated by a bisecting GlcNAc oligosaccharide structure; therefore Hsp47 is an intracellular glycoprotein ligand of annexin V [40].

The population of bisecting-GlcNAc-containing *N*-glycans is substantially increased in cells cultured under dense conditions compared with those cultured under sparse conditions. These effects are abolished in E-cadherin-deficient cells [41].

### Target proteins with core fucose

Glycoproteins modified by Fut8, a unique fucosyltransferase in mammals, to form α1-6 fucose are widely distributed in tissues and are altered under certain pathological conditions.

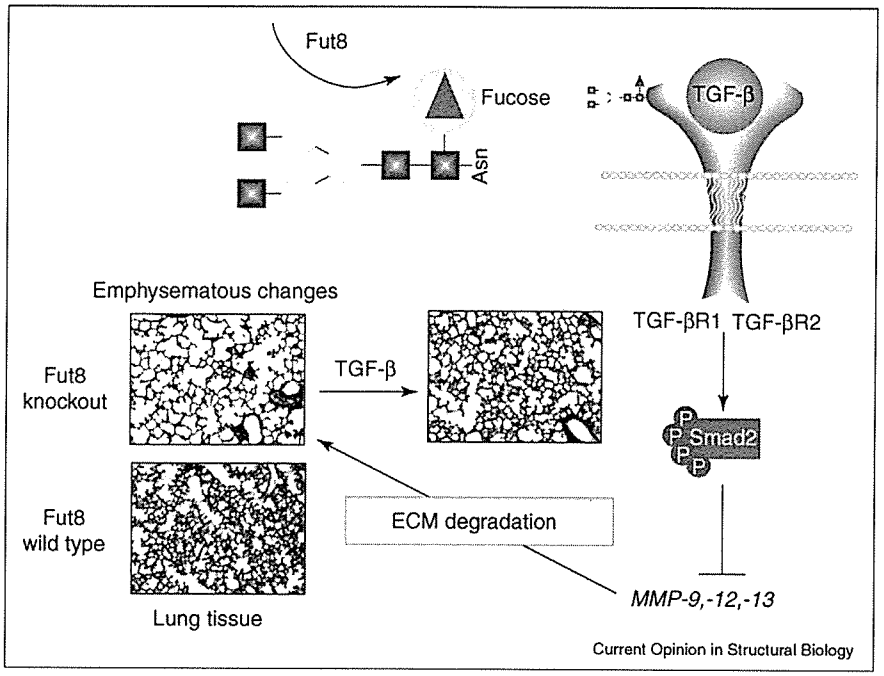
70% of Fut8 KO mice die during the neonatal period and eventually show emphysema-like degeneration of lung tissue. The abnormal production of metalloproteinases (MMPs) has been implicated in the induction of emphysema. The expression levels of MMP-1, -12 and -13 are greatly increased in Fut8 KO lung tissues, and there is a decreased level of elastin fibers. Signaling through transforming growth factor-β (TGF-β) receptors, which are α1-6 fucosylated, negatively regulates MMP expression, which is a key factor in the regulation of extracellular matrix (ECM) proteins. Signaling through the TGF-β1 receptor is down-regulated in Fut8 KO mice as ligand affinity for the receptor is less strong. Therefore, the degradation of the ECM overwhelms its synthesis, causing emphysema in Fut8 KO mice. Importantly, the administration of exogenous TGF-β1 resulted in significant rescue of the emphysema-like phenotype, stimulated the formation of elastin fibers and, concomitantly, reduced MMP-12 expression in Fut8 KO lung tissue [42••] (Figure 2).

Core fucosylation of *N*-glycans is required for the binding of EGF to its receptor. The EGF-induced phosphorylation of EGFR was substantially blocked in Fut8 KO cells. Consistent with this, EGFR-mediated JNK or ERK activation was significantly suppressed [43]. These results indicate that EGFR is a target protein for Fut8. Additionally, Li *et al.* [44] reported that Fut8 KO mice exhibit reduced expression of trypsinogen due to dysregulation of signaling via EGFR; this has possible implications for growth retardation of the KO mice.

Another important role of core fucose is related to tumor markers, substances produced by cancer cells. Alpha-fetoprotein (AFP) levels are increased in the serum of patients with a primary hepatoma, and are also increased in patients with acute and chronic hepatitis and liver cirrhosis. Fucosylated AFP, especially the L3 fraction of AFP that can be obtained by electrophoretic separation with LCA-lectin (lens culinaris agglutinin A lectin), is a specific marker for primary hepatoma [45]. Fucosylated haptoglobin is a good marker for pancreatic cancer, but its fucosylation is associated with α1-6 as well as α1-3/1-4 linkages [46].

Some NK cells have receptors for the Fc domain of IgG. They bind to the Fc portion of IgG antibodies on the

Figure 2




An example of a target protein with core fucose. Fut8 KO mice develop emphysema-like changes in lung tissue. One of the target proteins of Fut8 was identified as the TGF-β receptor. The lack of core fucosylation resulted in dysregulation of the TGF-β receptor. TGF-β receptor with core fucose acts as a negative regulator of MMP gene expression; in the Fut8 KO mice, dysregulated MMP expression led to degradation of ECM proteins in lung alveoli. Exogenous administration of TGF-β successfully rescued the histological changes of lungs in Fut8 KO mice.

surface of target cells, such as tumor cells, and release cytolytic components that kill the target cell. This mechanism of killing is referred to as antibody-dependent cell-mediated cytotoxicity (ADCC). It is considered to be the major mechanism of antibody therapeutics against tumors, such as trastuzumab (Herceptin®) and rituximab (Rituxan®), and requires both activation via FcγRIII and inhibition via FcγRIIB antibody receptors. The addition of bisecting GlcNAc to the Fc domain of the antibody leads to an increase in ADCC as a result of an up to 10–20-fold higher affinity for FCγRIII [47]. In a similar vein, two groups [48,49] reported the use of Lec13 cells, a variant CHO cell line, to produce human IgG1 that is deficient in the core fucose attached to the Asn297-linked carbohydrate. Deletion of the core fucose from IgG1 enhances ADCC activity by up to 50–100-fold. This indicates that the core fucose is the major sugar in terms of ADCC activity and strongly suggests that core fucosylation of *N*-glycans modifies the function of the glycoprotein. The strategy suggested by these reports should be applicable to optimizing the ADCC activity of other therapeutic IgGs and using conditional Fut8 KO mice would be the best way to develop these antibodies.

## Future perspectives

Following the isolation of glycosyltransferase genes, a promising strategy to elucidate specific functions of sugar chains is to identify target proteins for glycosyltransferases. Sugar chains, as reviewed in this article, modify biological functions of the target protein, resulting in pathophysiological changes of cells or tissues [42••,50,51]. Thus, integrated analyses of sugar chains to reveal the biological importance of glycosylation will give further insight into health and disease [52•].

## Acknowledgements

We apologize for having introduced only a fraction of the excellent work in this field because of the limited space. These studies were mainly supported by grants in aid from Matching Funds Aimed at the Promotion of Business-Academic-Public Sector Joint Research from MEXT, Japan and the JSPS 21st Century COE program.

## References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. • Lowe JB, Marth JD: **A genetic approach to mammalian glycan function.** *Annu Rev Biochem* 2003, **72**:643-691.
A review of the functions of glycosyltransferases in mice.

2. •• Freeze HH: **Genetic defects in the human glycome.** *Nat Rev Genet* 2006, **7**:537-551.
An overview of genetic defects related to the glycome.

3. Uozumi N, Yanagidani S, Miyoshi E, Ihara Y, Sakuma T, Gao CX, Teshima T, Fujii S, Shiba T, Taniguchi N: **Purification and cDNA cloning of porcine brain GDP-L-Fuc:N-acetyl-beta-D-glucosaminide alpha1 → 6fucosyltransferase.** *J Biol Chem* 1996, **271**:27810-27817.

4. Yanagidani S, Uozumi N, Ihara Y, Miyoshi E, Yamaguchi N, Taniguchi N: **Purification and cDNA cloning of GDP-L-Fuc:N-acetyl-beta-D-glucosaminide:alpha1-6 fucosyltransferase (alpha1-6 FucT) from human gastric cancer MKN45 cells.** *J Biochem (Tokyo)* 1997, **121**:626-632.

5. Gu J, Nishikawa A, Tsuruoka N, Ohno M, Yamaguchi N, Kangawa K, Taniguchi N: **Purification and characterization of UDP-N-acetylglucosamine: alpha-6-D-mannoside beta 1-6N-acetylglucosaminyltransferase (N-acetylglucosaminyltransferase V) from a human lung cancer cell line.** *J Biochem (Tokyo)* 1993, **113**:614-619.

6. Nishikawa A, Ihara Y, Hatakeyama M, Kangawa K, Taniguchi N: **Purification, cDNA cloning, and expression of UDP-N-acetylglucosamine: beta-D-mannoside beta-1,4N-acetylglucosaminyltransferase III from rat kidney.** *J Biol Chem* 1992, **267**:18199-18204.

7. Dennis JW, Laferte S, Waghorne C, Breitman ML, Kerbel RS: **Beta 1-6 branching of Asn-linked oligosaccharides is directly associated with metastasis.** *Science* 1987, **236**:582-585.

8. Miyoshi E, Ihara Y, Nishikawa A, Saito H, Uozumi N, Hayashi N, Fusamoto H, Kamada T, Taniguchi N: **Gene expression of N-acetylglucosaminyltransferases III and V: a possible implication for liver regeneration.** *Hepatology* 1995, **22**:1847-1855.

9. Yamashita K, Ohkura T, Tachibana Y, Takasaki S, Kobata A: **Comparative study of the oligosaccharides released from baby hamster kidney cells and their polyoma transformant by hydrazinolysis.** *J Biol Chem* 1984, **259**:10834-10840.

10. Pierce M, Buckhaults P, Chen L, Fregien N: **Regulation of N-acetylglucosaminyltransferase V and Asn-linked oligosaccharide beta(1,6) branching by a growth factor signaling pathway and effects on cell adhesion and metastatic potential.** *Glycoconj J* 1997, **14**:623-630.

11. Kang R, Saito H, Ihara Y, Miyoshi E, Koyama N, Sheng Y, Taniguchi N: **Transcriptional regulation of the N-acetylglucosaminyltransferase V gene in human bile duct carcinoma cells (HuCC-T1) is mediated by Ets-1.** *J Biol Chem* 1996, **271**:26706-26712.

12. Ko JH, Miyoshi E, Noda K, Ekuni A, Kang R, Ikeda Y, Taniguchi N: **Regulation of the GnT-V promoter by transcription factor Ets-1 in various cancer cell lines.** *J Biol Chem* 1999, **274**:22941-22948.

13. Miyoshi E, Nishikawa A, Ihara Y, Saito H, Uozumi N, Hayashi N, Fusamoto H, Kamada T, Taniguchi N: **Transforming growth factor beta up-regulates expression of the N-acetylglucosaminyltransferase V gene in mouse melanoma cells.** *J Biol Chem* 1995, **270**:6216-6220.

14. Saito H, Nishikawa A, Gu J, Ihara Y, Soejima H, Wada Y, Sekiya C, Niikawa N, Taniguchi N: **cDNA cloning and chromosomal mapping of human N-acetylglucosaminyltransferase V+.** *Biochem Biophys Res Commun* 1994, **198**:318-327.

15. Demetriou M, Granovsky M, Quaggin S, Dennis JW: **Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation.** *Nature* 2001, **409**:733-739.

16. Granovsky M, Fata J, Pawling J, Muller WJ, Khokha R, Dennis JW: **Suppression of tumor growth and metastasis in Mgat5-deficient mice.** *Nat Med* 2000, **6**:306-312.

17. Lagana A, Goetz JG, Cheung P, Raz A, Dennis JW, Nabi IR: **Galectin binding to Mgat5-modified N-glycans regulates fibronectin matrix remodeling in tumor cells.** *Mol Cell Biol* 2006, **26**:3181-3193.

18. Guo HB, Lee I, Bryan BT, Pierce M: **Deletion of mouse embryo fibroblast N-acetylglucosaminyltransferase V stimulates alpha5beta1 integrin expression mediated by the protein kinase C signaling pathway.** *J Biol Chem* 2005, **280**:8332-8342.

19. Nakahara S, Miyoshi E, Noda K, Ihara S, Gu J, Honke K, Inohara H, Kubo T, Taniguchi N: **Involvement of oligosaccharide changes in alpha5beta1 integrin in a cisplatin-resistant human squamous cell carcinoma cell line.** *Mol Cancer Ther* 2003, **2**:1207-1214.

20. Yoshimura M, Ihara Y, Matsuzawa Y, Taniguchi N: **Aberrant glycosylation of E-cadherin enhances cell-cell binding to suppress metastasis.** *J Biol Chem* 1996, **271**:13811-13815.

21. Ihara S, Miyoshi E, Ko JH, Murata K, Nakahara S, Honke K, Dickson RB, Lin CY, Taniguchi N: **Prometastatic effect of N-acetylglucosaminyltransferase V is due to modification and stabilization of active matriptase by adding beta 1-6 GlcNAc branching.** *J Biol Chem* 2002, **277**:16960-16967.

22. Ihara S, Miyoshi E, Nakahara S, Sakiyama H, Ihara H, Akinaga A, Honke K, Dickson RB, Lin CY, Taniguchi N: **Addition of beta1-6 GlcNAc branching to the oligosaccharide attached to Asn 772 in the serine protease domain of matriptase plays a pivotal role in its stability and resistance against trypsin.** *Glycobiology* 2004, **14**:139-146.

23. Takeuchi T, Shuman MA, Craik CS: **Reverse biochemistry: use of macromolecular protease inhibitors to dissect complex biological processes and identify a membrane-type serine protease in epithelial cancer and normal tissue.** *Proc Natl Acad Sci USA* 1999, **96**:11054-11061.

24. Lin CY, Anders J, Johnson M, Sang QA, Dickson RB: **Molecular cloning of cDNA for matriptase, a matrix-degrading serine protease with trypsin-like activity.** *J Biol Chem* 1999, **274**:18231-18236.

25. Saito T, Miyoshi E, Sasai K, Nakano N, Eguchi H, Honke K, Taniguchi N: **A secreted type of beta 1,6-N-acetylglucosaminyltransferase V (GnT-V) induces tumor angiogenesis without mediation of glycosylation: a novel function of GnT-V distinct from the original glycosyltransferase activity.** *J Biol Chem* 2002, **277**:17002-17008.

26. Schachter H: **Coordination between enzyme specificity and intracellular compartmentation in the control of protein-bound oligosaccharide biosynthesis.** *Biol Cell* 1984, **51**:133-145.

27. Taniguchi N, Ihara Y: **Recent progress in the molecular biology of the cloned N-acetylglucosaminyltransferases.** *Glycoconj J* 1995, **12**:733-738.

28. Nakahara S, Saito T, Kondo N, Moriwaki K, Noda K, Ihara S, Takahashi M, Ide Y, Gu J, Inohara H *et al.*: **A novel angiogenesis inducer, β1,6-N-acetylglucosaminyltransferase V (GnT-V), is processed as a secreted enzyme by γ-secretase.** *FASEB J* 2006, in press.

29. Sheng Y, Yoshimura M, Inoue S, Oritani K, Nishiura T, Yoshida H, Ogawa M, Okajima Y, Matsuzawa Y, Taniguchi N: **Remodeling of glycoconjugates on CD44 enhances cell adhesion to hyaluronate, tumor growth and metastasis in B16 melanoma cells expressing beta1,4-N-acetylglucosaminyltransferase III.** *Int J Cancer* 1997, **73**:850-858.

30. Yoshimura M, Ihara Y, Ohnishi A, Ijuhin N, Nishiura T, Kanakura Y, Matsuzawa Y, Taniguchi N: **Bisecting N-acetylglucosamine on K562 cells suppresses natural killer cytotoxicity and promotes spleen colonization.** *Cancer Res* 1996, **56**:412-418.

31. Yoshimura M, Nishikawa A, Ihara Y, Taniguchi S, Taniguchi N: **Suppression of lung metastasis of B16 mouse melanoma by N-acetylglucosaminyltransferase III gene transfection.** *Proc Natl Acad Sci USA* 1995, **92**:8754-8758.

32. Kitada T, Miyoshi E, Noda K, Higashiyama S, Ihara H, Matsuura N, Hayashi N, Kawata S, Matsuzawa Y, Taniguchi N: **The addition of bisecting N-acetylglucosamine residues to E-cadherin down-regulates the tyrosine phosphorylation of beta-catenin.** *J Biol Chem* 2001, **276**:475-480.

33. Sato Y, Takahashi M, Shibukawa Y, Jain SK, Hamaoka R, Miyagawa J, Yaginuma Y, Honke K, Ishikawa M, Taniguchi N: **Overexpression of N-acetylglucosaminyltransferase III enhances the epidermal growth factor-induced phosphorylation of ERK in HeLaS3 cells by up-regulation of the internalization rate of the receptors.** *J Biol Chem* 2001, **276**:11956-11962.

34. Ihara Y, Sakamoto Y, Mihara M, Shimizu K, Taniguchi N: **Overexpression of N-acetylglucosaminyltransferase III disrupts the tyrosine phosphorylation of Trk with resultant**

**signaling dysfunction in PC12 cells treated with nerve growth factor.** *J Biol Chem* 1997, **272**:9629-9634.

35. Shibukawa Y, Takahashi M, Laffont I, Honke K, Taniguchi N: **Down-regulation of hydrogen peroxide-induced PKC delta activation in N-acetylglucosaminyltransferase III-transfected HeLaS3 cells.** *J Biol Chem* 2003, **278**:3197-3203.

36. Tsuda T, Ikeda Y, Taniguchi N: **The Asn-420-linked sugar chain in human epidermal growth factor receptor suppresses ligand-independent spontaneous oligomerization. Possible role of a specific sugar chain in controllable receptor activation.** *J Biol Chem* 2000, **275**:21988-21994.

37. • Gu J, Taniguchi N: **Regulation of integrin functions by N-glycans.** *Glycoconj J* 2004, **21**:9-15.
Recent findings on the role of *N*-glycans in the function of integrins are presented.

38. Isaji T, Gu J, Nishiuchi R, Zhao Y, Takahashi M, Miyoshi E, Honke K, Sekiguchi K, Taniguchi N: **Introduction of bisecting GlcNAc into integrin alpha5beta1 reduces ligand binding and down-regulates cell adhesion and cell migration.** *J Biol Chem* 2004, **279**:19747-19754.

39. Shigeta M, Shibukawa Y, Ihara H, Miyoshi E, Taniguchi N, Gu J: **{beta}1,4-N-acetylglucosaminyltransferase III potentiates {beta}1 integrin-mediated neuritogenesis induced by serum deprivation in Neuro2a cells.** *Glycobiology* 2006, **16**:564-571.

40. Gao CX, Miyoshi E, Uozumi N, Takamiya R, Wang X, Noda K, Gu J, Honke K, Wada Y, Taniguchi N: **Bisecting GlcNAc mediates the binding of annexin V to Hsp47.** *Glycobiology* 2005, **15**:1067-1075.

41. Iijima J, Zhao Y, Isaji T, Kameyama A, Nakaya S, Wang X, Ihara H, Cheng X, Nakagawa T, Miyoshi E *et al.*: **Cell-cell interaction-dependent regulation of N-acetylglucosaminyltransferase III and the bisected N-glycans in GE11 epithelial cells. Involvement of E-cadherin-mediated cell adhesion.** *J Biol Chem* 2006, **281**:13038-13046.

42. •• Wang X, Inoue S, Gu J, Miyoshi E, Noda K, Li W, Mizuno-Horikawa Y, Nakano M, Asahi M, Takahashi M *et al.*: **Dysregulation of TGF-beta1 receptor activation leads to abnormal lung development and emphysema-like phenotype in core fucose-deficient mice.** *Proc Natl Acad Sci USA* 2005, **102**:15791-15796.
TGF-β receptor is identified as a target protein of Fut8.

43. Wang X, Gu J, Ihara H, Miyoshi E, Honke K, Taniguchi N: **Core fucosylation regulates epidermal growth factor receptor-mediated intracellular signaling.** *J Biol Chem* 2006, **281**:2572-2577.

44. Li W, Nakagawa T, Koyama N, Wang X, Jin J, Mizuno-Horikawa Y, Gu J, Miyoshi E, Kato I, Honke K *et al.*: **Down regulation of trypsinogen expression is associated with growth retardation in {alpha}1,6-fucosyltransferase-deficient mice: attenuation of proteinase-activated receptor 2 activity.** *Glycobiology* 2006, in press.

45. Sato Y, Nakata K, Kato Y, Shima M, Ishii N, Koji T, Taketa K, Endo Y, Nagataki S: **Early recognition of hepatocellular carcinoma based on altered profiles of alpha-fetoprotein.** *N Engl J Med* 1993, **328**:1802-1806.

46. Okuyama N, Ide Y, Nakano M, Nakagawa T, Yamanaka K, Moriwaki K, Murata K, Ohigashi H, Yokoyama S, Eguchi H *et al.*: **Fucosylated haptoglobin is a novel marker for pancreatic cancer: a detailed analysis of the oligosaccharide structure and a possible mechanism for fucosylation.** *Int J Cancer* 2006, **118**:2803-2808.

47. Davies J, Jiang L, Pan LZ, LaBarre MJ, Anderson D, Reff M: **Expression of GnTIII in a recombinant anti-CD20 CHO production cell line: expression of antibodies with altered glycoforms leads to an increase in ADCC through higher affinity for FC gamma RIII.** *Biotechnol Bioeng* 2001, **74**:288-294.

48. Shields RL, Lai J, Keck R, O'Connell LY, Hong K, Meng YG, Weikert SH, Presta LG: **Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human Fcgamma RIII and antibody-dependent cellular toxicity.** *J Biol Chem* 2002, **277**:26733-26740.

49. Shinkawa T, Nakamura K, Yamane N, Shoji-Hosaka E, Kanda Y, Sakurada M, Uchida K, Anazawa H, Satoh M, Yamasaki M *et al.*: **The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity.** *J Biol Chem* 2003, **278**:3466-3473.

50. Ohtsubo K, Takamatsu S, Minowa MT, Yoshida A, Takeuchi M, Marth JD: **Dietary and genetic control of glucose transporter 2 glycosylation promotes insulin secretion in suppressing diabetes.** *Cell* 2005, **123**:1307-1321.

51. Demetriou M, Granovsky M, Quaggin S, Dennis JW: **Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation.** *Nature* 2001, **409**:733-739.

52. • Taniguchi N: **From glycobiology to systems glycobiology: international network with Japanese scientists through consortia.** *IUBMB Life* 2006, **58**:269-272.
The author provides future perspectives on the field of glycobiology.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 50, pp. 38343–38350, December 15, 2006


# Deletion of Core Fucosylation on α3β1 Integrin Down-regulates Its Functions*

Received for publication, September 11, 2006, and in revised form, October 13, 2006 Published, JBC Papers in Press, October 16, 2006, DOI 10.1074/jbc.M608764200

**Yanyang Zhao[‡], Satsuki Itoh[§], Xiangchun Wang[‡], Tomoya Isaji[‡¶], Eiji Miyoshi[‡], Yoshinobu Kariya[‖], Kaoru Miyazaki[‖], Nana Kawasaki[§], Naoyuki Taniguchi[‡**1], and Jianguo Gu[‡¶2]**

*From the [‡]Department of Biochemistry, Osaka University Graduate School of Medicine, B1, 2-2 Yamadaoka, Suita, Osaka 565-0871, the [§]National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, the [‖]Division of Cell Biology, Kihara Institute of Biological Research, Yokohama City University, 641-12 Maioka-cho, Totsuka-ku, Yokohama 244-0813, the **Department of Disease Glycomics, Research Institute for Microbial Diseases, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, and the [¶]Division of Regulatory Glycobiology, Institute of Molecular Biomembrane and Glycobiology, Tohoku Pharmaceutical University, 4-4-1 Komatsusima, Aobaku, Sendai, Miyagi 981-8558, Japan*

**The core fucosylation (α1,6-fucosylation) of glycoprotein is widely distributed in mammalian tissues. Recently α1,6-fucosylation has been further reported to be very crucial by the study of α1,6-fucosyltransferase (*Fut8*)-knock-out mice, which shows the phenotype of emphysema-like changes in the lung and severe growth retardation. In this study, we extensively investigated the effect of core fucosylation on α3β1 integrin and found for the first time that *Fut8* makes an important contribution to the functions of this integrin. The role of core fucosylation in α3β1 integrin-mediated events has been studied by using $Fut8^{+/+}$ and $Fut8^{-/-}$ embryonic fibroblasts, respectively. We found that the core fucosylation of α3β1 integrin, the major receptor for laminin 5, was abundant in $Fut8^{+/+}$ cells but was totally abolished in $Fut8^{-/-}$ cells, which was associated with the deficient migration mediated by α3β1 integrin in $Fut8^{-/-}$ cells. Moreover integrin-mediated cell signaling was reduced in $Fut8^{-/-}$ cells. The reintroduction of *Fut8* potentially restored laminin 5-induced migration and intracellular signaling. Collectively, these results suggested that core fucosylation is essential for the functions of α3β1 integrin.**

α1,6-Fucosyltransferase (*Fut8*) catalyzes the transfer of a fucose residue from GDP-fucose to position 6 of the innermost GlcNAc residue of the hybrid and complex types of *N*-linked oligosaccharides on the glycoproteins (Fig. 1) (1). Core fucosylation (α1,6-fucosylation) of glycoprotein is widely distributed in mammalian tissues and altered under pathological conditions, such as hepatocellular carcinoma and liver cirrhosis (2, 3). A high expression of *Fut8* was observed in 33.3% of papillary carcinoma, and the incidence was directly linked to tumor size and lymph node metastasis, thus *Fut8* expression may be a key factor in the progression of thyroid papillary carcinomas (4). It has also been reported that the deletion of the core fucose from the IgG1 molecule enhances antibody-dependent cellular cytotoxicity activity by up to 50- to 100-fold. This indicates that the core fucose is an important sugar chain in terms of antibody-dependent cellular cytotoxicity activity (5). Recently, the physiological functions of the core fucose have been further investigated by our group using analysis of core fucose-deficient mice (6). The $Fut8^{-/-}$ mice showed severe growth retardation, and 70% died within 3 days after birth. The surviving mice suffered from emphysema-like changes in the lung that appear to be due to the lack of core fucosylation of transforming growth factor-β1 receptor, which consequently resulted in a marked dysregulation of transforming growth factor-β1 receptor activation and signaling. We also found that the loss of core fucosylation resulted in the down-regulation of EGF[3] receptor-mediated signaling pathway (7). These results together suggest that core fucose performs the important physiological functions through modification of some important functional proteins.

Cell-extracellular matrix (ECM) interactions play essential roles during the acquisition of migration and invasive behavior of the cells. The integrin family consists of α and β heterodimeric transmembrane receptors for ECM and connects many biological functions, such as development, the control of cell proliferation, protection against apoptosis, and malignant transformation (8). For example, α3β1 integrin, the major receptor for laminin 5 (LN5), is widely distributed in almost all tissues, and α3 knock-out mice have been reported to show the defects in kidney, lung, and skin (9). It has been reported that G-like repeats of LN5 constitute the favored ligand for α3β1 integrin, triggering haptotaxis (10). Especially, the G3 domain is essential for the unique activity of LN5, such as promotion of cell migration (11). Furthermore, α3β1 integrin has been proposed to be involved in tumor invasion (12, 13): the interaction

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

[1] To whom correspondence may be addressed. Tel.: 81-6-879-4137; Fax: 81-6-879-4137; E-mail: tani52@wd5.so-net.ne.jp.

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

[3] The abbreviations used are: EGF, epidermal growth factor; ECM, extracellular matrix; LN5, laminin 5; FN, fibronectin; COL, collagen; mAb, monoclonal antibody; PBS, phosphate-buffered saline; GnT-III, *N*-acetylglucosaminyltransferase III; GnT-V, *N*-acetylglucosaminyltransferase V; MEF, mouse embryonic fibroblast; ERK, extracellular signal-regulated kinase; AAL, *Aleuria aurantia* lectin; LC, liquid chromatography; MS, mass spectrometry; FT, Fourier transform; GM3, NeuAcα2,3Galβ1,4Glc-ceramide.

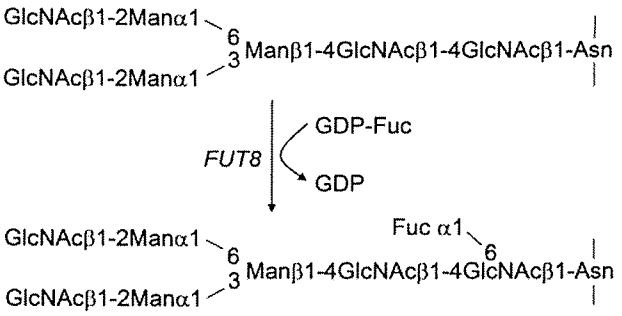



FIGURE 1. **Reaction pathway for the biosynthesis of core fucose by *Fut8*.** *Man*, mannose; *Fuc*, fucose; *GDP-Fuc*, guanosinediphospho-fucopyranoside; *Asn*, asparagine.

of α3β1 integrin with LN5 in exposed basement membrane provides both a molecular and a structural basis for cell arrest during pulmonary metastasis (14). In some malignant tumors, α3β1 integrin is found to be the most predominant integrin expressed (15), and cell invasion on ECM could be inhibited by antibodies against α3 integrin (13) and β1 integrin (14). Thus, α3β1 integrin, which mediates to laminins of basement membrane, preferentially promotes cell migration and metastasis (16–18). Given its various biological functions, α3β1 integrin, as one of most important extracellular adhesive molecules, deserves the more detailed investigation.

It has long been known that various factors can modulate integrin functions, including the status of glycosylation of integrin (19), the partnerships with tetraspanins, growth factor receptors (20–22), and the association with ganglioside GM3 (22), and others. Cell surface integrins are all major carriers of *N*-glycans, therefore *N*-glycosylation of integrins plays an important role in their biological functions (23). For example: the α3 and β1 subunits expressed by the metastasis human melanoma cell lines carry β1,6-branched structures, and these cancer-associated glycan chains may modulate tumor cell adhesion by affecting the ligand properties of α3β1 integrin (23). The linkage and expression levels of the terminal sialic acids of α3β1 integrin play an important role in cell-ECM interactions (24, 25). An increase in β1,6-GlcNAc sugar chains of β1 integrin resulted in the stimulation of cell migration and the organization of F-actin into extended microfilaments in cells plated on FN-coated plates (26). Moreover, a recent study has shown that introduction of bisecting GlcNAc into α5β1 integrin down-regulates cell adhesion and cell migration (27). These previous papers listed above have shown that the functions of integrins were positively or negatively regulated by *N*-glycans catalyzed by GnT-III, GnT-V, sialyltransferases, and others.

However, until now the effect of core fucosylation on integrin functions remains unclear. Here, we described studies comparing embryonic fibroblasts from wild-type and *Fut8*$^{-/-}$ mice to elucidate the role of core fucosylation in α3β1 integrin-stimulated events, and our finding for the first time showed that core fucosylation is required for the functions of α3β1 integrin.

## EXPERIMENTAL PROCEDURES

*Reagents and Antibodies*—A polyclonal antibody against mouse α3 integrin and functional blocking monoclonal antibody (mAb) against α2β1 integrin were obtained from Chemicon International, Inc. (Temecula, CA). mAbs against α3 integrin, FAK, FAK (pY397), and functional blocking mAbs against integrin α6 and β1 subunits were from BD Transduction Laboratories (Lexington, KY). A polyclonal antibody against rabbit ERK1/2 and peroxidase-conjugated goat antibody against rabbit IgG were obtained from Cell Signaling (Beverly, MA). A mouse control IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A peroxidase-conjugated goat antibody against mouse IgG was obtained from Promega (Madison, WI), and biotinylated *Aleuria aurantia* lectin (AAL) was from Seikagaku Corp., Japan.

*Cell Culture*—*Fut8*$^{+/+}$ and *Fut8*$^{-/-}$ mouse embryonic fibroblasts (MEFs) and restored cells were previously established in our laboratory (6). *Fut8*$^{+/+}$ and *Fut8*$^{-/-}$ embryonic fibroblasts and restored cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in the presence of 400 μg/ml Zeocin, and restored cells were maintained in Dulbecco's modified Eagle's medium in the presence of 400 μg/ml Zeocin and 400 μg/ml hygromycin.

*Western Blot and Lectin Blot Analysis*—Cell cultures were harvested in lysis buffer (20 mM Tris-HCl, pH 7.4, 10 mM EGTA, 10 mM $MgCl_2$, 1 mM benzamidine, 60 mM β-glycerophosphate, 1 mM $Na_3VO_4$, 20 mM NaF, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride). Cell lysates were centrifuged at 15,000 × *g* for 10 min at 4 °C, the supernatants were collected, and the protein concentrations were determined using a BCA protein assay kit (Pierce). Proteins were then immunoprecipitated from the lysates using a combination of 2 μg of anti-α3 integrin antibody and Protein G-Sepharose beads. Immunoprecipitates were suspended in nonreducing buffer, heated to 100 °C for 3 min, resolved on 7.5% SDS-PAGE, and electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell). The blots were then probed with anti-α3 integrin antibody and biotinylated AAL, respectively. Immunoreactive bands were visualized using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and an ECL kit (Amersham Biosciences).

*Cell Surface Biotinylation*—Cell surface biotinylation was performed as described previously (28). Briefly, cells were rinsed twice with ice-cold PBS and then incubated with ice-cold PBS containing 0.2 mg/ml sulfosuccinimidobiotin (Pierce) for 2 h at 4 °C. After incubation, 50 mM Tris-HCl (pH 8.0) was used for the initial wash to quench any unreacted biotinylation reagent, and the cells were washed three times with ice-cold PBS and then solubilized in lysis buffer. The resulting cell lysate was then immunoprecipitated with the anti-α3 integrin antibody as described above. The biotinylated proteins were visualized using a Vectastain ABC kit and an ECL kit.

*Migration Assay and Functional Blocking Assay*—Transwells (BD Bioscience) were coated with 5 nM of recombinant LN5, as described previously (29), or 15 nM of human plasma FN, 50 μg/ml collagen I (COL, Sigma) in PBS by an overnight treatment at 4 °C followed by an incubation with 1% bovine serum albumin for 1 h at 37 °C. Serum-starved cells ($2 \times 10^5$) per well in 500 μl of fetal calf serum-free medium were seeded in the upper compartment of the plates. After incubation for 3 h, the cells in the upper chamber of the filter were removed with a wet cotton swab. Cells on the lower side of the filter were fixed and

stained with 0.5% crystal violet. Each experiment was performed in triplicate, and counting was done in three randomly selected microscopic fields within each well. To identify which specific integrin mediates cell migration on LN5, monoclonal antibodies against different types of integrins at concentrations of 10 μg/ml were preincubated individually with fibroblasts for 10 min at 37 °C. Then cells were transferred into Transwells coated with LN5 and then incubated for 2 h 37 °C. The migrated cells were then quantified as described above.

*Construction of Small Interference RNA Vector and Retroviral Infection*—Small interfering oligonucleotides specific for integrin α3 subunit were designed on the Takara Bio website, and the oligonucleotide sequences used in the construction of the small interference RNA vector were as follows: 5′-GATCCGCTATGGAGAATCACACTGATTCAAGAGATCAGTGTGATTCTCCATAGCTTTTTTG-3′ and 5′-AATTCAAAAAAGCTATGGAGAATCACACTGATCTCTTGAATCAGTGTGATTCTCCATAGCG-3′. The oligonucleotides were annealed and then ligated into BamHI/EcoRI sites of the RNAi-Ready pSIREN-Retro Q vector (Takara Bio). A retroviral supernatant was obtained by transfection of human embryonic kidney 293 cells using a Retrovirus Packaging Kit Eco (Takara Bio) according to the manufacturer's protocol. Embryonic fibroblasts cells were infected with the viral supernatant, and the cells were then selected with 15 μg/ml puromycin for 2–3 weeks. Stable α3 integrin knockdown clones were therefore selected.

*Tyrosine Phosphorylation Assay of FAK*—Serum-starved cells were detached and held in suspension for 60 min to reduce the detachment-induced activation and then replated on dishes coated with LN5 (5 nM) for the indicated times, and the cell lysates were blotted with anti-phosphotyrosine FAK (pY397) antibody. Then the equal loading was confirmed by blotting with an antibody against total FAK.

*Purification of α3β1 Integrin*—The purification of α3β1 integrin was performed as described previously (30). Briefly, confluent cells were detached with TBS(+) (20 mM Tris-HCl, pH 7.5, 130 mM NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$) and washed with TBS(+). The cell pellets were extracted with 50 mM Tris/HCl containing 15 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, pH 7.4, and protease inhibitor mixture (Roche Applied Science), 100 mM octyl-β-D-glucopyranoside at 4 °C. The cell extract was applied to an affinity column prepared by coupling 5 mg of GD6 peptide of laminin α1 chain (30) (KQNCLSSRASFRGCVRNLRLSR residues numbered 3011–3032, Peptide Institute, Inc., Osaka, Japan) to 1 ml of activated CH-Sepharose (Sigma). The bound α3β1 integrin was eluted with 20 mM EDTA in 50 mM Tris/HCl, pH 7.4, containing 100 mM octyl-β-D-glucopyranoside. The elutes containing α3β1 integrin were further purified on 1 ml of a wheat germ agglutinin-agarose column (Seikagaku Corp.) and eluted with 0.2 M *N*-acetyl-D-glucosamine containing 100 mM octyl-β-D-glucopyranoside. The purity of the integrin was verified by SDS-PAGE by means of a silver staining kit (Daichi Pure Chemicals Co., Ltd., Tokyo, Japan).

*Analysis of N-Glycan Structure by Liquid Chromatography (LC/Tandem Mass Spectrometry (MS/MS))*—Purified α3β1 integrin was applied to SDS-PAGE and excised from the gel then cut into pieces. The gel pieces were destained and dehydrated with 50% acetonitrile. The protein in the gel was reduced and carboxymethylated with dithiothreitol and monoiodoacetic acid according to the reports described by Kikuchi *et al.* (31) with some modifications. *N*-Glycans were released and extracted from the gel pieces as reported by Kustar *et al.* (32). The extracted oligosaccharides were reduced with $NaBH_4$. LC/MS was performed using a quadrupole liner ion trap-Fourier transform (FT) ion cyclotron resonance mass spectrometer (Finnigan LTQ FT™, Thermo Electron Corp., San Jose, CA) connected to a nanoLC system (Paradigm, Michrom BioResource, Inc., Auburn, CA). The eluents were 5 mM ammonium acetate, pH 9.6/2% $CH_3CN$ (pump A), and 5 mM ammonium acetate, pH 9.6/80% $CH_3CN$ (pump B). The borohydride-reduced *N*-linked oligosaccharides were separated on a Hypercarb (0.1 × 150 mm, Thermo Electron Corp.) with a linear gradient of 5–20% of B in 45 min and 20–50% of B in 45 min. FT-full MS scan (*m/z* 450–2000) followed by data-dependent MS/MS for the most abundant ions was performed in both negative and positive ion modes as described in the previous report (33).

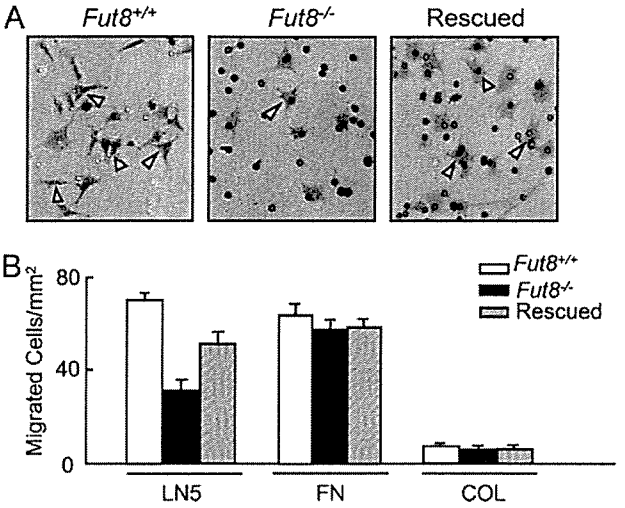



FIGURE 2. **Effects of deficient core fucosylation on cell migration on LN5 but not on FN.** *Fut8*$^{+/+}$, *Fut8*$^{-/-}$, and rescued cells were replated on the upper chamber coated with LN5 (5 nM), FN (15 nM), or 50 μg/ml COL. Cell migration was determined using the Transwell assay described under "Experimental Procedures." *A*, representative fields on LN5 were photographed using a phase-contrast microscope. The *arrowheads* indicate migrated cells. *B*, the numbers of migrated cells on LN5, FN, or COL were quantified and expressed as the means ± S.D. from three independent experiments.

## RESULTS

*Impaired α3β1 Integrin-mediated Cell Migration Was Found in Fut8*$^{-/-}$ *Cells*—One of the major functions of α3β1 integrin is promotion of cell migration. In some malignant tumors, α3β1 integrin was found to be the most predominant integrin expressed (15), and it has made an important contribution to metastasis (14); therefore, cell motility on different ECMs was firstly examined by utilizing a Transwell assay. Cells were applied into the chambers, the bottoms of which had been coated with LN5, FN, or COL. As shown in Fig. 2 (*A* and *B*), *Fut8*$^{-/-}$ cells showed impaired migration on LN5 by a decrease to 44% relative to *Fut8*$^{+/+}$ cells. Consistently, reintroduction of *Fut8* partly restored cell migration by an increase in the percentage of migrating cells from 44% to 74%, indicating that core fucosylation is required for LN5-stimulated cell migration. But in the case of cell migration on FN, a specific ligand for α5β1

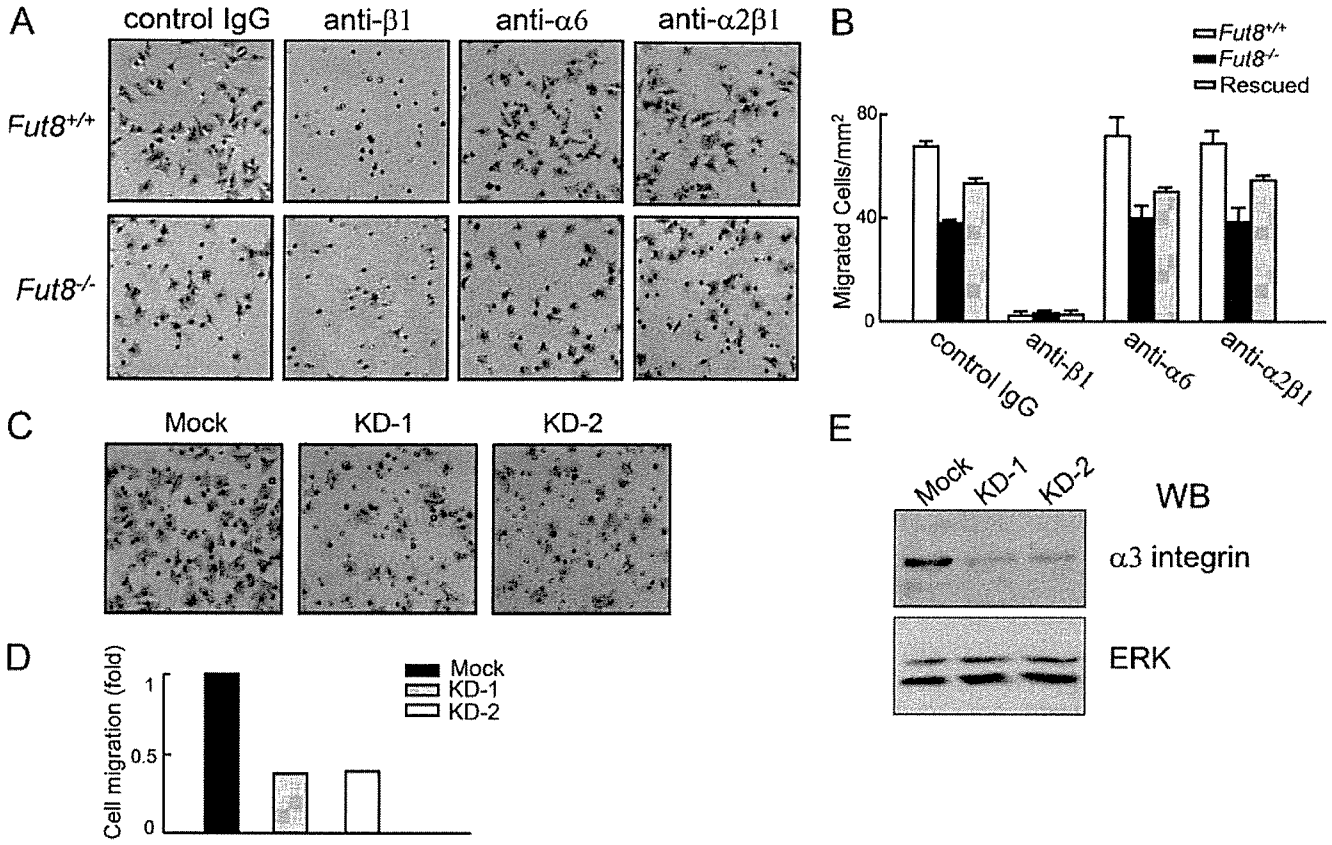



FIGURE 3. **Cell migration on LN5 was mediated by α3β1 integrin.** *A*, *Fut8*$^{+/+}$, *Fut8*$^{-/-}$, and rescued cells were detached, preincubated with mouse control IgG or function-blocking mAbs against β1, α6, or α2β1 integrin for 10 min, replated on the upper chamber coated with LN5 (5 nM), and checked by Transwell assay. Representative fields were photographed using a phase-contract microscope. *B*, the numbers of migrated cells were quantified and expressed as the means ± S.D. from three independent experiments. *C*, cell migration of α3-knockdown cells on LN5 (5 nM). Representative fields were photographed using a phase-contrast microscope. *Arrowheads* indicate migrated cells. *D*, quantification of migration of mock and α3-knockdown cells. The numbers of migrated cells were quantified and expressed as the means ± S.D. from three independent experiments. *E*, α3-knockdown was confirmed by blotting total cell lysates with anti-α3 antibody (*upper panel*), and equal loading was confirmed by probing with an antibody against total protein ERK1/2 (*lower panel*). *KD1* and *KD2*, α3-knockdown cells.

integrin, the obvious difference among *Fut8*$^{+/+}$, *Fut8*$^{-/-}$, and rescued cells was not found. In addition, the motility of these three types of cells on COL, a ligand for α1β1 and α2β1 integrins, was barely detectable (Fig. 2*B*). This suggested that α5β1, α1β1 and α2β1 integrins, unlike receptor of laminin 5, may be not strongly affected by *Fut8*. So MEF cells may favor LN5 as an ECM for cell migration. Furthermore, the cell migration on LN5 was completely blocked by the presence of function-blocking antibodies against β1 but not by α6 or α2β1 integrin antibodies (Fig. 3, *A* and *B*), further excluding the involvement of α6 and α2β1 integrin on LN5-stimulated cell migration. However, so far the function-blocking antibody against mouse α3 is unavailable. To definitely confirm the important function of integrin α3 subunit for the cell migration on LN5, we utilized an RNA interference strategy to silence α3 in MEF cells. After retroviral infection, the cells were selected based on their resistance to puromycin as described under "Experimental Procedures." Expression of α3 but not α5 (data not shown), or other proteins such as ERK, was effectively down-regulated, compared with those in mock cells (Fig. 3*E*). We then tested cell migration on LN5 and found that α3-knockdown resulted in a significant decreased cell migration compared with mock cells (Fig. 3, *C* and *D*). Together with the data in Fig. 3 (*A* and *B*), these results provided the evidence that in this study the cell migration on LN5 was mediated by α3β1 integrin. This result was consistent with the view of previous study that α3β1 integrin is distinct from other integrins and preferentially promotes cell migration (16). The result above was also supported by the previous observation that LN5 as well as LN10/11 promoted cell migration is mainly mediated by α3β1 integrin, but not α6β1 or α6β4 integrins (34). However, we cannot definitely exclude the involvement of syndecan-1 and -4, because it has been reported to have an interaction with LN5 (35, 36); therefore, they might regulate integrin functions in an indirect way. Collectively, these results suggested that α3β1 integrin is a key molecule for cell migration on LN5 in the embryonic fibroblasts and that core fucosylation regulates α3β1 integrin-mediated cell migration.

*Integrin-stimulated Phosphorylation of FAK Was Reduced in Fut8*$^{-/-}$ *Cells*—ECM-integrin signaling events are prominently involved in regulating cell migration (16). In particular, the protein-tyrosine kinase FAK plays a prominent role in integrin signaling (37–39). To address the effects of *Fut8* on α3β1 integrin-mediated signaling, we examined FAK phosphorylation in adherent cells on LN5. As shown in Fig. 4, the level of tyrosine phosphorylation was reduced in the *Fut8*$^{-/-}$ cells compared with *Fut8*$^{+/+}$ cells, moreover the down-regulation of phosphorylation in *Fut8*$^{-/-}$ cells was restored in the rescued cells, suggesting that deficient core fucosylation was able to neg-

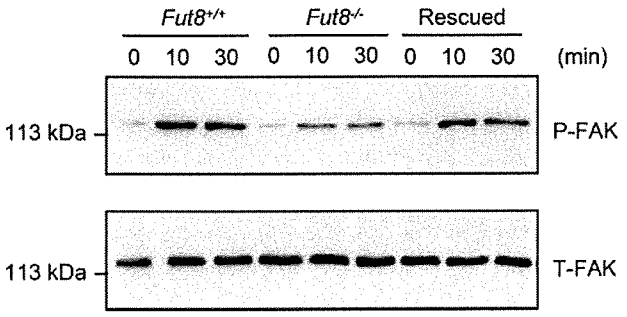



FIGURE 4. **Comparison of tyrosine phosphorylation levels of FAK among *Fut8*$^{+/+}$ and *Fut8*$^{-/-}$ and rescued cells on LN5.** Serum-starved cells were detached and held in suspension for 60 min to reduce the detachment-induced activation and then replated on dishes coated with LN5 (5 nM) for the indicated times, and the cell lysates were blotted with anti-phosphotyrosine FAK antibody to detect the amount of phosphorylation. Then the equal loading was confirmed with an antibody against total protein FAK.

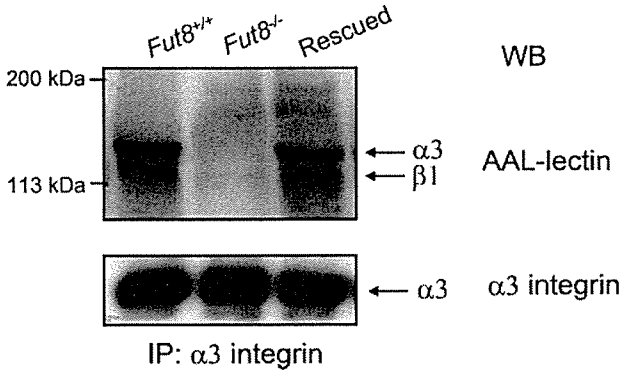



FIGURE 5. **Glycosylation analysis of α3β1 integrin from *Fut8*$^{+/+}$, *Fut8*$^{-/-}$, and rescued cells.** Whole cell lysates were immunoprecipitated (*IP*) with anti-α3 integrin antibody, and the resulting immunocomplexes were subjected to 7.5% SDS-PAGE under nonreducing condition. After electroblotting, the blots were probed, respectively, by AAL (*upper panel*) and an anti-α3 integrin antibody (*lower panel*).

atively regulate α3β1 integrin-mediated signaling pathway. Considerable evidence implicates FAK in the regulation of cell migration. Most notably, FAK-deficient cells exhibit poor migration ability in response to chemotactic and haptotactic migration (40, 41). Therefore, based on such evidence we suggested that the deficient signal transduction may account for the deficient cell migration on LN5 in *Fut8*$^{-/-}$ cells.

*Expression of α3β1 Integrin on the Cell Surface Was Not Influenced by Fut8*—Some important glycosyltransferases have been reported to modify and further regulate the functions of integrins by modulating the status of glycosylation on them such as GnT-III and GnT-V; however, there is no such data so far to show the relation of *Fut8* and integrins. Therefore, in Fig. 5, the fucosylation on α3β1 integrin among *Fut8*$^{+/+}$, *Fut8*$^{-/-}$, and rescued cells has been examined by using blotting of α3 integrin-immunoprecipitated lysates with AAL lectin (*upper panel*). Equal loadings were verified by blotting with α3 integrin antibodies (*lower panel*). As shown in Fig. 5, the levels of core fucosylation in both α3 and β1 subunits were abolished in *Fut8*$^{-/-}$ cells consistent with no *Fut8* activity in these cells (7), whereas they were rescued by reintroduction of *Fut8*, suggesting α3β1 integrin is the target of *Fut8*. Furthermore, the effect of deficiency of core fucosylation on the expression of α3β1 integrin on the cell surface was also determined, because *N*-glycosylation plays an important role in the quality control of the expression of glycoproteins. The biotinylation of cell surface proteins followed by immunoprecipitation of α3 integrin was examined by blotted. As shown in Fig. 6, the expression levels of α3β1 integrin on the cell surface remained unchanged among *Fut8*$^{+/+}$, *Fut8*$^{-/-}$, and rescued cells, indicating that the expression of α3β1 integrin on cell surface was not influenced by *Fut8*. Collectively, we suggested that the deficiency of core fucosylation resulted in the malfunctions of α3β1 integrin but not its expression level.

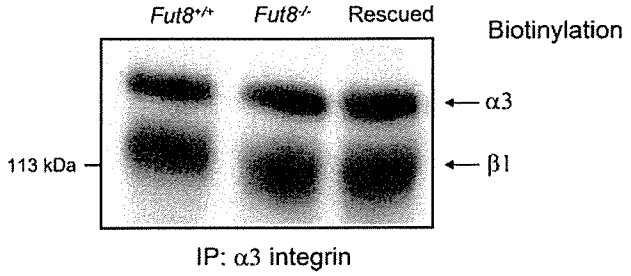



FIGURE 6. **Effects of core fucosylation on expression levels of α3β1 integrin on cell surface.** *Fut8*$^{+/+}$, *Fut8*$^{-/-}$, and rescued cells were biotinylated, whole lysates were immunoprecipitated (*IP*) with anti-α3 integrin antibody, the samples were subjected to 7.5% SDS-PAGE and transferred to a nitrocellulose membrane, and the biotinylated proteins were then detected as described under "Experimental Procedures."

*Purified α3β1 Integrin, Rich in Core Fucosylation, Was Shown by LC/MS/MS*—The analysis of glycan structural alteration in glycoproteins is becoming increasingly important in the discovery of therapies and diagnostic markers (42). To better understand the detailed modification of *Fut8* for α3β1 integrin, we purified α3β1 integrin from *Fut8*$^{+/+}$ and *Fut8*$^{-/-}$ cells by using a GD6 peptide affinity column combined with a wheat germ agglutinin affinity column. The purity was evaluated by SDS-PAGE followed by silver staining. Two major bands, migrating at 150 and 110 kDa on SDS-PAGE under nonreducing conditions (Fig. 7*A*, *inset, right panel*), corresponding to the immunoreactivity with the anti-α3 and anti-β1 antibodies, were detected, respectively (data not shown). Then we analyzed *N*-glycan profiles of purified α3β1 integrin by LC/MS and LC/MS/MS. The profiles of the *N*-linked oligosaccharides extracted from purified α3β1 integrin of *Fut8*$^{+/+}$ and *Fut8*$^{-/-}$, respectively, are shown in Fig. 7*A*. They were obtained by full MS scan (*m/z* 450–2000) in the negative ion mode. The FT MS spectra of the *peaks 1–7* (from *Fut8*$^{+/+}$) and *peaks 1′–7′* (from *Fut8*$^{-/-}$) are shown in Fig. 7*B*, respectively. The structures of carbohydrates in these peaks could be deduced from the *m/z* values of protonated ions obtained by FT MS and data-dependent MS/MS spectra. The oligosaccharides released from α3β1 integrin of *Fut8*$^{+/+}$ (*peaks 1–7*) were assigned to fucosylated complex and hybrid type oligosaccharides, whereas those released from α3β1 integrin of *Fut8*$^{-/-}$ (*peaks 1′–7′*) were nonfucosylated forms. The data correspond to that of AAL lectin blot, revealing that α3β1 integrin derived from *Fut8*$^{+/+}$ is highly modified by *Fut8* and suggesting loss of core fucosylation will result in the deficiency of α3β1 integrin function.

## DISCUSSION

The physiological importance of fucose modification on proteins has been highlighted by the description of human congen-

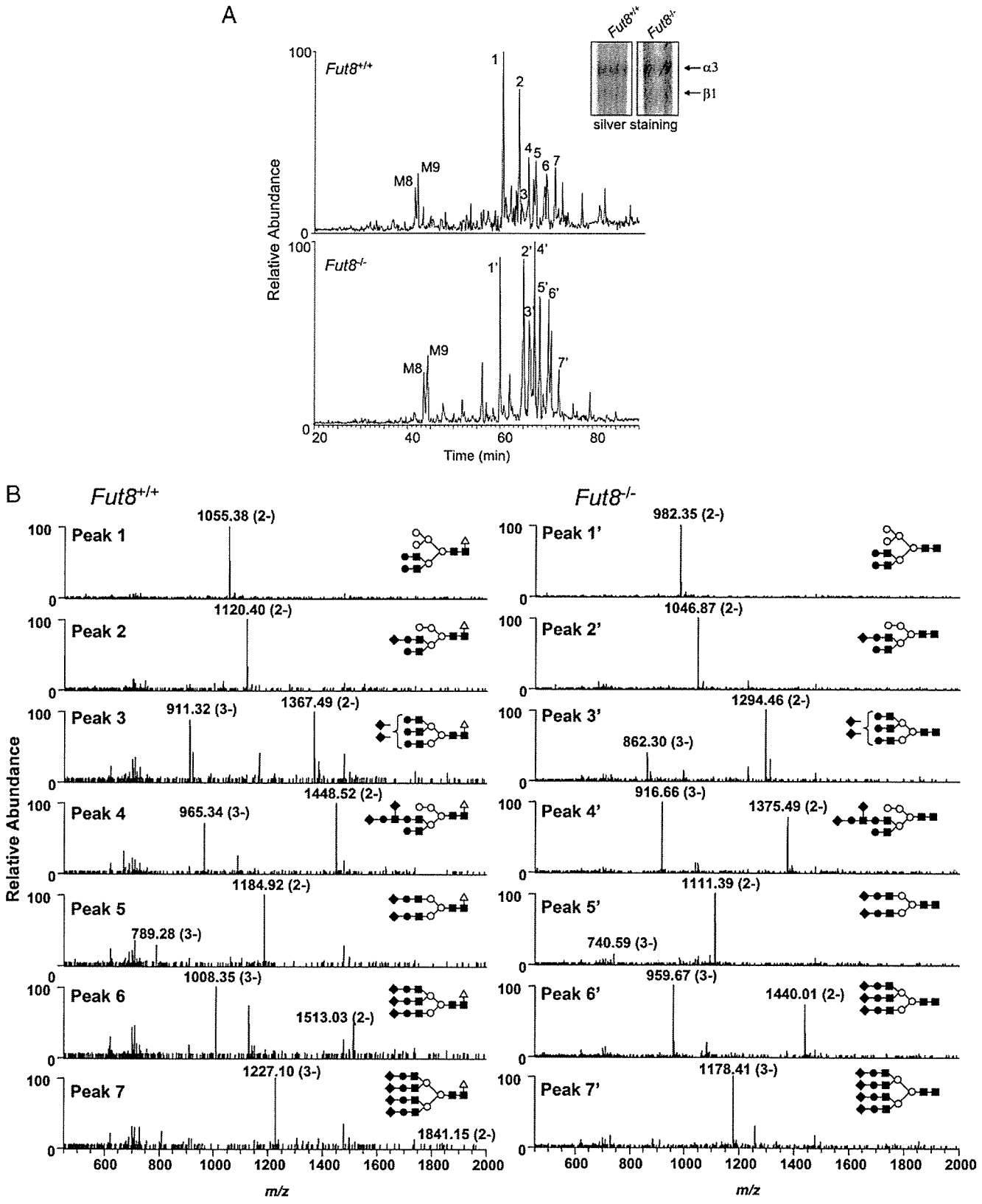



FIGURE 7. **Chromatograms of *N*-linked oligosaccharides extracted from purified α3β1 integrin from *Fut8*$^{+/+}$ and *Fut8*$^{-/-}$ cells.** In *A*: MS, full MS scan (*m/z* 450–2000) in the negative ion mode. LC, pump A, 5 mM ammonium acetate, pH 9.6/2% $CH_3CN$; Pump B, 5 mM ammonium acetate, pH 9.6/80% $CH_3CN$; column, hypercarb (0.1 × 150 mm); gradient, 5–20% of B (0–45 min) and 20–50% of B (45–90 min). The purity of α3β1 integrin was verified by silver staining under nonreducing condition as shown in the *right panel* of the *inset*. *B*, FT MS spectra of *N*-glycans from purified α3β1 integrin from *Fut8*$^{+/+}$ and *Fut8*$^{-/-}$ cells. *Peaks 1–7* in *Fut8*$^{+/+}$ cells, *peaks 1′–7′* in *Fut8*$^{-/-}$ cells, and carbohydrate compositions assigned by *m/z* values of protonated ions and MS/MS spectra. △, fucose; ●, galactose; ○, mannose; ■, *N*-acetylglucosamine; ♦, *N*-acetylneuraminic acid.

ital disorders of glycosylation (6). The congenital disorders of glycosylation-IIc disease is due to lack of the GDP-fucose transporter activity (43, 44), which mainly caused reduced terminal fucosylation of *N*-glycans (45, 46), and the core fucosylation is speculated to be responsible for the phenotype of congenital disorders of glycosylation-IIc (6). Recently, the loss of core fucosylation has been reported to down-regulate transforming growth factor-β1 receptor and EGF receptor functions, which is thought to be related to the phenotype of emphysema and growth retardation of *Fut8*$^{-/-}$ mice. In the present study, we found that the deficient core fucosylation results in the blockage of α3β1 integrin-mediated cell migration and cell signaling. These results showed for the first time that in addition to the important physiological functions mentioned above, core fucosylation is also essential for the functions of α3β1 integrin.

Several lines of evidence suggest that *N*-glycans are required for integrin activation. An increase in β1,6-branched sugar chains on α5β1 integrin by GnT-V promotes cell migration on FN (26). Although the overexpression of GnT-III has been reported to inhibit α5β1 integrin-mediated functions in HeLa S3 cells (27). It has also been reported that GnT-III and GnT-V can positively and negatively regulate α3β1 integrin-mediated cell migration on LN5 (47). The modification of β1 integrin by sialyltransferase makes this integrin capped with the negatively charged sugar, sialic acid, and contributes to cell motility and invasion (25). We found that cell migration on COL was barely detectable, suggesting that MEFs did not favor COL as an ECM for cell migration. In fact, we found that different cells may favor specific ECM for cell migration (27). We also found that core fucosylation had no significant difference in the cell migration on FN among wild-type, Fut8-KO, and rescued cells. This suggests that α1,6-fucose modification has little or only mild effects on α5β1 integrin, which is a receptor for FN. Actually, we previously reported that the introduction of the bisecting GlcNAc to the α5 subunit resulted in a reduced affinity in the binding of α5β1 integrin to FN, therefore resulting in decreased cell migration (27). Thus, we assumed that the core fucosylation affected α3 subunit in a similar manner, which caused the decreased cell migration on LN5. However, the modification of α1,6-fucose to α5 subunit may not affect their binding to FN. As described before, only *N*-glycans on some important domains of integrins, can contribute to the regulation of their functions (48). For example, the addition of a glycan at the β1 or β3 subunit I-like domains caused an increase in the distance between the head and stalk domains, therefore inducing the integrin dimer to adopt a more activated integrin conformation. Furthermore, it has recently been reported that the *N*-glycans only located on some specific sites of integrin α5 subunit play key roles in functional expression (49).

It has been reported that purified α5β1 integrin from human placenta and purified α3β1 integrin from the human ureter epithelium cell line HCV29 exhibited a highly heterogenous glycosylation pattern, and >50% of these were fucosylated (50, 51). In this study, the α3β1 integrin we purified from mouse embryonic fibroblast carried the bi-, tri-, and tetra-antennary complex types, and the majority of these were core-fucosylated. So it is easily postulated that core fucosylation may be important to integrin functions due to the abundance of it. However, to our knowledge, no reports showing that core fucosylation regulates integrin functions have appeared to date. The fact that integrin-mediated migration and cell signaling were decreased in *Fut8*$^{-/-}$ cells, and such inhibition was partly rescued by re-introduction of the *Fut8* gene to *Fut8*$^{-/-}$ cells, strongly suggested that core fucosylation is important to α3β1 integrin, and *Fut8*, like other important glycosyltransferases, plays an essential role in the regulation of integrin functions.

Although the precise reason for why the core fucosylation modifies these molecular functions remains to be elucidated, we proposed some possible mechanisms: *Fut8* may affect the cross-talk between growth factor receptors and integrin. It is well known that integrin mediated functions cooperatively with growth factor receptors in the control of cell proliferation, cell differentiation, cell survival, and cell migration in epithelial cells and fibroblasts (52), because integrins and growth factor receptors share many common elements in their signaling pathway (19). PC12 cells in a serum-free medium were plated on the plates without ECM coating and, when treated with EGF alone, failed to induce neurite formation (53), suggesting that the integration of the signaling pathway triggered by receptor and integrins is required for the regulation of PC12 cell differentiation. In our study, the association of integrin with EGF receptor was indicated by co-precipitation, and we found that the complex of α3β1 integrin and EGF receptor in *Fut8*$^{-/-}$ cells was decreased compared with *Fut8*$^{+/+}$ cells.[4] This may affect the signal integration of both partners and, thus, further affect the α3β1 integrin-stimulated signal and cell migration, or deficient core fucosylation may cause the conformation of integrin to change. Luo *et al.* (48) have suggested that the changes in the glycan structures of integrin can affect its conformation and activity. They reported that in Chinese hamster ovary-K1 cells, the addition of a glycan at β1 I-like domain caused an increase in the distance between the β1 head and stalk domains, therefore inducing the integrin dimmer to be a more extended (activated) integrin conformation (48). Consistently, the affinity of the binding of α5β1 integrin to fibronectin was significantly reduced by the introduction of the bisecting GlcNAc (27). So we supposed that core fucosylation contributes to stable conformation and normal activity of α3β1 integrin to its ligand. However, we cannot exclude additional reasons that still remain to be determined.

The α3 integrin gene is expressed during the development of many epithelial organs, including the kidney (54), lung (55), and others. As a major basement membrane receptor in both kidney and lung during embryogenesis, α3β1 integrin is likely to be involved in mediating signals between the mesenchyme and epithelial cells in the kidney and lung. The glomeruli of α3-KO mice showed the abnormality in kidney, including disorganized glomerular basement membrane and a dramatic absence of foot process formation by podocytes (9). Therefore, it could be worthy to extensively examine the effects of core fucosylation on α3β1 integrin *in vivo* in the future.

In conclusion, we demonstrate here some aspects of the biological significance of the core fucosylation of α3β1 integrin-medi-

[4] Y. Zhao, S. Itoh, X. Wang, T. Isaji, E. Miyoshi, Y. Kariya, K. Miyazaki, N. Kawasaki, N. Taniguchi, and J. Gu, unpublished data.

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

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

## REFERENCES


1. Wilson, J. R., Williams, D., and Schachter, H. (1976) *Biochem. Biophys. Res. Commun.* **72,** 909–916
2. Miyoshi, E., Noda, K., Yamaguchi, Y., Inoue, S., Ikeda, Y., Wang, W., Ko, J. H., Uozumi, N., Li, W., and Taniguchi, N. (1999) *Biochim. Biophys. Acta* **1473,** 9–20
3. Noda, K., Miyoshi, E., Gu, J., Gao, C. X., Nakahara, S., Kitada, T., Honke, K., Suzuki, K., Yoshihara, H., Yoshikawa, K., Kawano, K., Tonetti, M., Kasahara, A., Hori, M., Hayashi, N., and Taniguchi, N. (2003) *Cancer Res.* **63,** 6282–6289
4. Ito, Y., Miyauchi, A., Yoshida, H., Uruno, T., Nakano, K., Takamura, Y., Miya, A., Kobayashi, K., Yokozawa, T., Matsuzuka, F., Taniguchi, N., Matsuura, N., Kuma, K., and Miyoshi, E. (2003) *Cancer Lett.* **200,** 167–172
5. Shinkawa, T., Nakamura, K., Yamane, N., Shoji-Hosaka, E., Kanda, Y., Sakurada, M., Uchida, K., Anazawa, H., Satoh, M., Yamasaki, M., Hanai, N., and Shitara, K. (2003) *J. Biol. Chem.* **278,** 3466–3473
6. Wang, X., Inoue, S., Gu, J., Miyoshi, E., Noda, K., Li, W., Mizuno-Horikawa, Y., Nakano, M., Asahi, M., Takahashi, M., Uozumi, N., Ihara, S., Lee, S. H., Ikeda, Y., Yamaguchi, Y., Aze, Y., Tomiyama, Y., Fujii, J., Suzuki, K., Kondo, A., Shapiro, S. D., Lopez-Otin, C., Kuwaki, T., Okabe, M., Honke, K., and Taniguchi, N. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 15791–15796
7. Wang, X., Gu, J., Ihara, H., Miyoshi, E., Honke, K., and Taniguchi, N. (2006) *J. Biol. Chem.* **281,** 2572–2577
8. Hynes, R. O. (2002) *Cell* **110,** 673–687
9. Kreidberg, J. A., Donovan, M. J., Goldstein, S. L., Rennke, H., Shepherd, K., Jones, R. C., and Jaenisch, R. (1996) *Development* **122,** 3537–3547
10. Hintermann, E., and Quaranta, V. (2004) *Matrix Biol.* **23,** 75–85
11. Kariya, Y., Tsubota, Y., Hirosaki, T., Mizushima, H., Puzon-McLaughlin, W., Takada, Y., and Miyazaki, K. (2003) *J. Cell. Biochem.* **88,** 506–520
12. Melchiori, A., Mortarini, R., Carlone, S., Marchisio, P. C., Anichini, A., Noonan, D. M., and Albini, A. (1995) *Exp. Cell Res.* **219,** 233–242
13. Plopper, G. E., Domanico, S. Z., Cirulli, V., Kiosses, W. B., and Quaranta, V. (1998) *Breast Cancer Res. Treat* **51,** 57–69
14. Wang, H., Fu, W., Im, J. H., Zhou, Z., Santoro, S. A., Iyer, V., DiPersio, C. M., Yu, Q. C., Quaranta, V., Al-Mehdi, A., and Muschel, R. J. (2004) *J. Cell Biol.* **164,** 935–941
15. Tysnes, B. B., Larsen, L. F., Ness, G. O., Mahesparan, R., Edvardsen, K., Garcia-Cabrera, I., and Bjerkvig, R. (1996) *Int. J. Cancer* **67,** 777–784
16. Gu, J., Sumida, Y., Sanzen, N., and Sekiguchi, K. (2001) *J. Biol. Chem.* **276,** 27090–27097
17. Gu, J., Fujibayashi, A., Yamada, K. M., and Sekiguchi, K. (2002) *J. Biol. Chem.* **277,** 19922–19928
18. Kreidberg, J. A. (2000) *Curr. Opin. Cell Biol.* **12,** 548–553
19. Gu, J., and Taniguchi, N. (2004) *Glycoconj. J.* **21,** 9–15
20. Giancotti, F. G., and Ruoslahti, E. (1999) *Science* **285,** 1028–1032
21. Nishiuchi, R., Sanzen, N., Nada, S., Sumida, Y., Wada, Y., Okada, M., Takagi, J., Hasegawa, H., and Sekiguchi, K. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 1939–1944
22. Toledo, M. S., Suzuki, E., Handa, K., and Hakomori, S. (2005) *J. Biol. Chem.* **280,** 16227–16234
23. Pochec, E., Litynska, A., Amoresano, A., and Casbarra, A. (2003) *Biochim. Biophys. Acta* **1643,** 113–123
24. Yamamoto, H., Oviedo, A., Sweeley, C., Saito, T., and Moskal, J. R. (2001) *Cancer Res.* **61,** 6822–6829
25. Bellis, S. L. (2004) *Biochim. Biophys. Acta* **1663,** 52–60
26. Guo, H. B., Lee, I., Kamar, M., Akiyama, S. K., and Pierce, M. (2002) *Cancer Res.* **62,** 6837–6845
27. Isaji, T., Gu, J., Nishiuchi, R., Zhao, Y., Takahashi, M., Miyoshi, E., Honke, K., Sekiguchi, K., and Taniguchi, N. (2004) *J. Biol. Chem.* **279,** 19747–19754
28. Sato, Y., Takahashi, M., Shibukawa, Y., Jain, S. K., Hamaoka, R., Miyagawa, J., Yaginuma, Y., Honke, K., Ishikawa, M., and Taniguchi, N. (2001) *J. Biol. Chem.* **276,** 11956–11962
29. Kariya, Y., Ishida, K., Tsubota, Y., Nakashima, Y., Hirosaki, T., Ogawa, T., and Miyazaki, K. (2002) *J. Biochem. (Tokyo)* **132,** 607–612
30. Gehlsen, K. R., Sriramarao, P., Furcht, L. T., and Skubitz, A. P. (1992) *J. Cell Biol.* **117,** 449–459
31. Kikuchi, M., Hatano, N., Yokota, S., Shimozawa, N., Imanaka, T., and Taniguchi, H. (2004) *J. Biol. Chem.* **279,** 421–428
32. Kuster, B., Wheeler, S. F., Hunter, A. P., Dwek, R. A., and Harvey, D. J. (1997) *Anal. Biochem.* **250,** 82–101
33. Itoh, S., Kawasaki, N., Hashii, N., Harazono, A., Matsuishi, Y., Hayakawa, T., and Kawanishi, T. (2005) *J. Chromatogr. A* **1103,** 296–306
34. Kikkawa, Y., Sanzen, N., and Sekiguchi, K. (1998) *J. Biol. Chem.* **273,** 15854–15859
35. Okamoto, O., Bachy, S., Odenthal, U., Bernaud, J., Rigal, D., Lortat-Jacob, H., Smyth, N., and Rousselle, P. (2003) *J. Biol. Chem.* **278,** 44168–44177
36. Utani, A., Nomizu, M., Matsuura, H., Kato, K., Kobayashi, T., Takeda, U., Aota, S., Nielsen, P. K., and Shinkai, H. (2001) *J. Biol. Chem.* **276,** 28779–28788
37. Parsons, J. T. (2003) *J. Cell Sci.* **116,** 1409–1416
38. Schwartz, M. A., Schaller, M. D., and Ginsberg, M. H. (1995) *Annu. Rev. Cell Dev. Biol.* **11,** 549–599
39. Yamada, K. M., and Miyamoto, S. (1995) *Curr. Opin. Cell Biol.* **7,** 681–689
40. Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., and Yamamoto, T. (1995) *Nature* **377,** 539–544
41. Sieg, D. J., Hauck, C. R., and Schlaepfer, D. D. (1999) *J. Cell Sci.* **112,** 2677–2691
42. Hashii, N., Kawasaki, N., Itoh, S., Hyuga, M., Kawanishi, T., and Hayakawa, T. (2005) *Proteomics* **5,** 4665–4672
43. Lubke, T., Marquardt, T., Etzioni, A., Hartmann, E., von Figura, K., and Korner, C. (2001) *Nat. Genet.* **28,** 73–76
44. Luhn, K., Wild, M. K., Eckhardt, M., Gerardy-Schahn, R., and Vestweber, D. (2001) *Nat. Genet.* **28,** 69–72
45. Sturla, L., Rampal, R., Haltiwanger, R. S., Fruscione, F., Etzioni, A., and Tonetti, M. (2003) *J. Biol. Chem.* **278,** 26727–26733
46. Sturla, L., Fruscione, F., Noda, K., Miyoshi, E., Taniguchi, N., Contini, P., and Tonetti, M. (2005) *Glycobiology* **15,** 924–934
47. Zhao, Y., Nakagawa, T., Itoh, S., Inamori, K. I., Isaji, T., Kariya, Y., Kondo, A., Miyoshi, E., Miyazaki, K., Kawasaki, N., Taniguchi, N., and Gu, J. (2006) *J. Biol. Chem.* **281,** 32122–32130
48. Luo, B. H., Springer, T. A., and Takagi, J. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 2403–2408
49. Isaji, T., Sato, Y., Zhao, Y., Miyoshi, E., Wada, Y., Taniguchi, N., and Gu, J. (2006) *J. Biol. Chem.* **281,** 33258–33267
50. Litynska, A., Przybylo, M., Pochec, E., and Laidler, P. (2002) *Acta Biochim. Pol.* **49,** 643–650
51. Nakagawa, H., Zheng, M., Hakomori, S., Tsukamoto, Y., Kawamura, Y., and Takahashi, N. (1996) *Eur. J. Biochem.* **237,** 76–85
52. Schwartz, M. A., and Ginsberg, M. H. (2002) *Nat. Cell Biol.* **4,** E65–E68
53. Gu, J., Zhao, Y., Isaji, T., Shibukawa, Y., Ihara, H., Takahashi, M., Ikeda, Y., Miyoshi, E., Honke, K., and Taniguchi, N. (2004) *Glycobiology* **14,** 177–186
54. Korhonen, M., Ylanne, J., Laitinen, L., Cooper, H. M., Quaranta, V., and Virtanen, I. (1991) *Lab. Invest.* **65,** 347–356
55. Mette, S. A., Pilewski, J., Buck, C. A., and Albelda, S. M. (1993) *Am. J. Respir. Cell Mol. Biol.* **8,** 562–572