Occurrence of Oligosialic Acids on Integrin α5 Subunit and Their Involvement in Cell Adhesion to Fibronectin*

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Integrin α5β1, a major fibronectin receptor, functions in a wide variety of biological phenomena. We have found that α2–8-linked oligosialic acids with 5 ≤ degree of polymerization (DP) ≤ 7 occur on integrin α5 subunit of the human melanoma cell line G361. The integrin α5 subunit immunoprecipitated with anti-integrin α5 antibody reacted with the monoclonal antibody H.46 recognizing oligo/polysialic acid with DP ≥ 8. The occurrence of oligosialic acids was further demonstrated by fluorometric C5/C9 analysis on the immunopurified integrin α5 subunit. Oligosialic acids were also found in the α5 subunit of several other human cells such as foreskin fibroblast and chronic erythroleukemia K562 cells. These results suggest the ubiquitous modification with unique oligosialic acids occurs on the α5 subunit of integrin α5β1. The adhesion of human melanoma G361 cells to fibronectin was mainly mediated by integrin α5β1. Treatment of cells with sialidase from Arthrobacter ureafaciens cleaving α2–3, α2–6, and α2–8-linked sialic acids inhibited adhesion to fibronectin. On the other hand, N-acetylneuraminidase II, which cleaves α2–5 and α2–6 but not α2–8 linkages, showed no inhibitory activity. After the loss of oligosialic acids, integrin α5β1 failed to bind to fibronectin-conjugated Sepharose, indicating that the oligosialic acid on the α5 subunit of integrin α5β1 plays important roles in cell adhesion to fibronectin.

Integrins comprise a structurally and functionally complex family of integral membrane glycoproteins that mediate cell adhesion to extracellular matrices, intracellular adhesion, and binding of plasma proteins. Such interactions also seem crucial for many normal and disease processes including those of wound healing (1), embryonic development (2), the maintenance of tissue integrity (3), and metastasis of cancer cells (4, 5). The interaction of cells expressing integrin α5β1 with fibronectin results in augmented cell adhesion, migration, and differentiation and has been implicated in important events in early embryogenesis such as gastrulation (6). Although the molecular basis of the integrin-ligand interaction is not understood completely, a line of experimental evidence suggests that post-translational modifications of integrin α5β1 with carbohydrates are essential for cells to attach to fibronectin. The α and β subunits of integrin α5β1 contained 10 and 13 potential asparagine-linked glycosylation sites in their extracellular region, respectively (7). Previous studies indicated that each of the respective chains contained asparagine-linked carbohydrates accounting for a molecular mass of 20,000–30,000 daltons (8), and their carbohydrate modifications were involved in the binding to fibronectin (9–11). In the present study, we demonstrated the occurrence of α2–8 oligosialic acids on integrin α5β1 by applying highly sensitive immunochromatographic and chemical methods (12, 13).

Little attention has been paid to the occurrence and functions of such short sialyl oligomers on glycoproteins. Polysialic acids on glycoproteins were first found in a glycoprotein isolated from unfertilized eggs of rainbow trout (Oncorhynchus mykiss) (14). After this discovery, α2–8-linked oligopolysialic acid1 has been shown to occur in various animal specimens obtained from insect to human more frequently than hitherto recognized (12, 15–18). Although the functions of oligopolysialic acids are not fully understood, it has been shown that α2–8-linked polysialic chains are attached to the embryonic form of neural cell adhesion molecule (N-CAM)2 in mammals (16, 19, 20), and their chain length dramatically decreases to oligosialic acids in the adult brain (19). Polysialic acids are known to negatively modulate N-CAM-associated cell-cell interactions during neurite outgrowth and synaptogenesis because of weak adhesive properties as compared with N-CAM lacking polyamic polysialic acids (21, 22). Using highly sensitive chemical and immunochromatographic methods to detect oligosialic acids recently established by Sato et al. (12, 16), oligosialic acids have been found in embryonic and adult mammalian brains, and expression of oligosialic acids was shown to vary during development and cell differentiation in a stage-specific manner (17). Furthermore, oligosialic acids were expressed in a number of cultured mammalian cell lines such as HeLa, Hela cells, and murine NIH 3T3 cells.
as human myeloid cell line HL-60, tetracarcinoma cell line PA-1, mouse neuroblastoma cell line Neuro2A, myoblastoma cell line C2C12, and preadipose cell line 3T3-L1, and their expression was reported to change drastically by the induction of differentiation (23). Oligosialic acids are thus strongly suggested to play important roles in embryonic development and differentiation. Our present study demonstrates that α2–8-linked oligosialic acids on the α5 subunit of integrin α5β1 are integral for the integrin to bind to fibronectin.

**EXPERIMENTAL PROCEDURES**

**Materials—**Arthrobacter ureafaciens sialidase (EC 3.2.1.18), NANAse-II from Clostridium perfringens, and peptide-N-(4-acetyl-β-gluco-

asaminyl)asparagine amidase (Endo-N) was prepared from bacteriophage K1F as described previously (24). Rabbit anti-integrin α5 subunit polyclonal antibody and human anti-integrin α5 subunit monoclonal antibody (mAb) JBS5 were purchased from Chemicon International, Inc. (Temecula, CA). mAb KH/33 against the human integrin α5 subunit, mAb LY111 against chondroitin sulfate, and bovine plasma fibronectin were purchased from Seikagaku Kogyo Co. (Tokyo, Japan) and FluoroChem, Inc. (Aurora, OH) for the preparation of oligosialic acid, and the equine polyclonal antibody H4.6 raised against polylactic acid (developed by Dr. J. B. Robbins of the National Institutes of Health) were donated kindly (to K. K.) by Dr. T. Seki (Juntendo University School of Medicine, Tokyo, Japan) and Dr. Y. Inoue (Academia Sinica, Taipei, Taiwan), respectively. Horseradish peroxidase-conjugated goat anti-rabbit IgG fraction to rabbit IgG was used as a negative control. Peroxidase-conjugated goat anti-mouse IgG for 30 min at 4°C, and 0.01% Tween 20 for 10 min, blocked, and immunostained with 1.2-mm-diameter antibodies against integrin α5β1 (25). The blots were incubated for at least 2 h in 1% bovine serum albumin (BSA) in PBS (blocking buffer) following by the incubation with polyclonal rabbit antibodies against integrin α5 subunits (diluted 1:500 in the blocking buffer) at room temperature for 2 h, or mAb 12E3 and polyclonal antibody H4.6 against oligosialic acid and polylactic acid, respectively (diluted 1:500), at 4°C overnight. Secondary horseradish peroxidase-conjugated goat IgG fraction to rabbit IgG (diluted 1:5,000) and horseradish peroxidase-conjugated goat anti-

**Cell Culture—**Human melanoma G361 cells and human chronic myeloid leukemia Sinica, Taipei, Taiwan), respectively. Horseradish peroxidase-conjugated goat anti-mouse IgG was used as a negative control. Peroxidase-conjugated goat anti-mouse IgG for 30 min at 4°C, and 0.01% Tween 20 for 10 min, blocked, and immunostained with 1.2-mm-diameter antibodies against integrin α5β1 (25). The blots were incubated for at least 2 h in 1% bovine serum albumin (BSA) in PBS (blocking buffer) following by the incubation with polyclonal rabbit antibodies against integrin α5 subunits (diluted 1:500 in the blocking buffer) at room temperature for 2 h, or mAb 12E3 and polyclonal antibody H4.6 against oligosialic acid and polylactic acid, respectively (diluted 1:500), at 4°C overnight. Secondary horseradish peroxidase-conjugated goat IgG fraction to rabbit IgG (diluted 1:5,000) and horseradish peroxidase-conjugated goat anti-

**Chemical Analysis of α2–8-Linked Oligosialic Acids of Integrin α5β1 Blotted on PVDF Membranes—**Immunoblotting was performed as described previously with minor modifications (27). Briefly, 100 μl of bovine plasma fibronectin (10 μg/ml in PBS) were dispensed into each well of 96-well polystyrene microtiter plates (Costar Corp., Cambridge, MA) and adsorbed overnight at 4°C. The coated wells were washed three times with RPMI 1640 medium containing 10 mM HEPES, pH 7.4, and 0.03% BSA, blocked with 100 μl of RPMI 1640 medium containing 10 mM HEPES, pH 7.4, and 3% BSA at 37°C for 2 h, and washed with RPMI 1640 medium containing 10 mM HEPES, pH 7.4, and 0.03% BSA. Cells were harvested by washing four times with PBS (without Ca2+ and Mg2+ cations) and incubating in 2 ml of PBS (without Ca2+ and Mg2+ cations) containing 0.1% trypsin (Difco) and 0.01% EDTA for 1 min. The cells were dislodged by shaking, suspended in 10 ml of standard growth medium, allowed to recover from trypsin treatment for 15 min, and counted using a hemocytometer. The cells were then centrifuged and re-suspended in RPMI 1640 medium containing 10 mM HEPES, pH 7.4, and 0.3% BSA at a concentration of 1 × 106 cells/ml. Cells were labeled with 2,7'-bis(2-carboxyethyl)-5 (and -6)carboxyfluorescein (CFSE) for 3 h in 20 mM for 20 min. The cells were then added to precoated wells at 3 × 104 cells/well and then incubated at 37°C for 30 min. Nonadherent cells were removed with four 21-gauge needle aspirators. Adherent cells were fixed in the 96-well Cyto Fluor® multwell plate reader series 4000 (Perseptive Biosystems, Foster City, CA). Specific attachment was calculated by subtracting the number of cells attached to BSA from the number of cells attached to fibronectin. The percentage of adherence was calculated by the following formula: percentage adherence = (fluorescence intensity (FI) of experimental wells – FI of BSA-coated wells/ FI of unwashed wells – FI of BSA-coated wells) × 100.

**Inhibition of Cell Adhesion—**Labeled cells were preincubated with mAbs at 37°C for 20 min. The cells were then added to precoated wells and incubated for 30 min at 37°C. The cells were then washed and adherent cells were quantified as described above for the cell adhesion assay.

**Treatment of Cells with Sialidases—**The treatment of cells with sialidases was carried out before the cell adhesion assays. Cells (1 × 106 cells) were treated with 0.5 international units/ml exo-Nanase II from C. perfringens, or 0.1 international units/ml Endo-N from bacteriophage K1F (24) in 200 μl of buffered medium (RPMI 1640 medium/PBS) at a concentration of 10 μg/ml at 37°C for 2 h.

**Flow Cytometry Analysis—**Cells were treated with or without sialidase or mAb 12E3, suspended at 106 cells/ml and incubated with mAb JBS5 in PBS containing 0.3% BSA for 30 min at 4°C. The cells were washed with PBS containing 0.3% BSA, incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG for 30 min at 4°C, washed, and analyzed by EPICS XL (Coulter Corp., Miami, FL). Non-immune mouse IgG was used as a negative control.
Occurrence and Function of Oligosialic Acids of Integrin α5β1

RESULTS

Integrin α5 Subunit of Human Melanoma G361 Cells Susceptible to Exosialidase Capable of Cleaving α2–8-Linked Static Acids—To probe the occurrence of α2–8-linked oligosialic acids, the specimen was digested with several different sialidases. NANase-II can cleave α2–3 and α2–6 but not α2–8 linkages of Neu5Ac and N-glycolynerminic acid, whereas exosialidase from A. ureafaciens can split α2–3, α2–6, and α2–8 linkages of Neu5Ac and N-glycolynerminic acid. Treatment of the cell lysate of human melanoma G361 cells with NANase-II hardly affected the apparent Mr of integrin α5 subunit even if the incubation time was prolonged (Fig. 1). Digestion with exosialidase from A. ureafaciens yielded a sharp band with an Mr of 145,000 at the faster moving position than that of intact α5 subunit with an Mr of 150,000 (Fig. 1). Although optimal pH values for sialidases from A. ureafaciens and NANase-II were reported to be 5.0 and 6.0, respectively, enzyme digestion was performed at pH 7.5 in 20 mM Tris-HCl containing 0.15 mM NaCl, because oligosialic acids are sensitive to acidic conditions. The NANase-II-insensitive sialic acid linkages could have been cleaved by exosialidase from A. ureafaciens. Taking all these into consideration, the occurrence of α2–8-linked oligosialic acids of integrin α5 subunit of human melanoma G361 cells has been strongly suggested.

Immunochemical Evidence for the Occurrence of Oligosialic acids on Integrin α5 Subunit from Human Melanoma G361 Cell Line—The immunoprecipitate of human melanoma G361 cells with the anti-integrin α5 antibody mAb KH/33 was subjected to SDS-PAGE and subsequently transferred to a PVDF membrane. When the membrane was visualized with the anti-integrin α5 polyclonal antibody, a broad band with an apparent Mr value of ~150,000 was detected (Fig. 2). The band, corresponding to integrin α5 subunit, was also stained with the anti-oligo/ polysialic acid antibody mAb 12E3, which recognizes α2–8-linked Neu5Ac oligomers with a degree of polymerization (DP) ≥ 5. However, the integrin α5 subunit hardly reacted to equine polyclonal antibody H4.6, which recognizes α2–8-linked Neu5Ac polymers with a DP ≥ 8. These results suggest that the integrin α5 subunit possesses oligosialic acids with 5 ≤ DP ≤ 7. The electrobotted membranes were pretreated with exosialidase from A. ureafaciens, Endo-N, or peptide-N4-(N-acetyl-β-glucosaminy)asparagine amidase and then stained with mAb 12E3. Endo-N is known as an endosialidase that hydrolyzes α2–8-linked oligo/polysialic acids (24). The staining of the integrin α5 subunit with mAb 12E3 completely disappeared after the treatment with exosialidase from A. ureafaciens, Endo-N, and peptide-N4-(N-acetyl-β-glucosaminy)asparagine amidase. However, pretreatment of the membrane with NANase-II did not diminish the staining with mAb 12E3. Thus, the results indicate that the integrin α5 subunit contained N-linked glycan chains with unique oligosialic acids with an average degree of polymerization of 5–7. Integrin α5β1 can be immunoprecipitated as a heterodimer in the presence of a divalent cation. Although the integrin β1 subunit migrated to the position at an Mr of 120,000 on SDS-PAGE (data not shown), the immunoreactivity at the position of Mr 120,000 with 12E3 was not observed, indicating that oligosialic acid occurs in the integrin α5 but not β1 subunit. Although G361 cells express the 140-kDa N-CAM isoform with oligosialic acid (16, 19, 20), the immunoprecipitate with mAb KH/33 did not stain with the anti-N-CAM antibody (data not shown), showing that staining with mAb 12E3 was attributable solely to oligosialic acid of α5 subunit but not to N-CAM.

Fluorometric Detection of Internal Sialic Acid Residues—For chemical detection of α2–8-linked oligosialic acid, the periodate oxidation/fluorometric HPLC method (12) was applied to immunopurified integrin α5 subunit. The internal sialic acid residues in α2–8-linked oligosialyl chains were determined after sequential periodate oxidation, borohydride reduction, and mild acid hydrolysis according to a modified procedure of van Lenten and Ashwell (28). Briefly, when an α2–8-linked oligosialyl chain is subjected to periodate oxidation, the nonreducing terminal residue is oxidized to give rise to the C7 analogue of the Neu5Ac or N-glycolynerminic acid (5-acetoamido-3,5-dideoxy-2-heptulopyranose) and 5-hydroxycytosaminic-3,5-dideoxy-2-heptulosonic acid), whereas internal residues linked by α2–8 linkages remain intact as the C9 analogue of Neu5Ac or N-glycolynerminic acid (2-keto-5-hydroxycytosaminic-3,5-dideoxy-2-heptulosonic acid), whereas internal residues linked by α2–8 linkages remain intact as the C9 analogue of Neu5Ac or N-glycolynerminic acid (2-keto-5-acetoamido-3,5-dideoxy-2-hydroxyacetoamido-5-acetoamido-3,5-dideoxy-2-hydroxyacetoamido-5-acetoamido-3,5-dideoxy-2-hydroxyacetoamido). Thus, the detection of C9 analogues in the periodate oxidation products would further strongly support the presence of α2–8-linked oligosialic acids. Immunoprecipitated integrin α5 was electrophoresed and transferred to a PVDF membrane. When the membrane was stained with Coomasie Brilliant Blue, the band with an Mr value of 150,000 corresponding to integrin α5 was detected (Fig. 3). The membrane pieces containing integrin α5 were excised and subjected to fluorometric C7/C9 analysis. C7 and C9 derivatives

![Fig. 1. SDS-PAGE analysis of integrin α5 subunit digested with sialidases.](image1)

![Fig. 2. Western blotting analysis of immunopurified integrin α5 subunit of human melanoma G361 cells.](image2)
of Neu5Ac were detected in the molar ratio of 143:5 after complete hydrolysis of the oxidized and reduced integrin α5 subunit. Detection of the C9 derivative was a strong ground for the occurrence of oligomeric sialic acids in the integrin α5 subunit. Assuming that an oligo-sialic acid of the integrin α5 subunit consists of an average of five internal sialic acid residues (refer to the previous section), an oligo-sialic acid-containing chain occurs in 1 of 143 termini of N-linked oligosaccharides. According to the structural analysis of N-linked oligosaccharides of integrin α5β1 (30), one N-linked sugar chain is estimated to bear an average of 1.70 termini capped by α2-3 or α2-6 sialic acid residue. Because integrin α5 contains 14 potential N-linked glycosylation sites (7), integrin α5 contains 23.8 termini capped by α2-3 or α2-6 sialic acid residue. Thus, an oligo-sialic acid-containing chain appears at the frequency of one of six molecules of integrin α5β1. In the control experiment in which the immunoprecipitate was made with normal IgG in place of the mAb KH/33, the C9 derivative was not detected by fluorometric C7/C9 analysis.

Ubiquitous Modification of Integrin α5 Subunit with Oligo-sialic Acids—Whether integrin α5 subunits derived from other cells of different origins were likewise modified with oligosialic acid was determined using human foreskin fibroblast and chronic erythroleukemia K562 cells. The immunoprecipitate of each cell with the mAb KH/33 was analyzed using anti-integrin α5 polyclonal antibodies, mAb 12E3 and polyclonal antibody H.46. The immunoprecipitated integrin α5 subunit polyclonal antibody (mAb KH/33). The immunopurified integrin α5 subunit was analyzed by Western blotting using the anti-integrin α5 subunit polyclonal antibody mAb 12E3 and polyclonal antibody H.46. Thus, adhesion of human melanoma G361 cells to fibronectin after treatment with NANase-II was abrogated (Fig. 5A).

Adhesion of Human Melanoma G361 Cells to Fibronectin Mediated Mainly by Integrin α5β1—To establish the functional role of oligosialic acid of integrin α5 subunit in cell adhesion, the mediation of integrin α5β1 in cell adhesion to fibronectin was determined in human melanoma G361 cells. Integrin α5β1 is known as a major fibronectin receptor, recognizing the Arg-Gly-Asp (RGD) sequence in fibronectin (31). The adhesion of G361 cells to a 10 μg/ml fibronectin-immobilized plate was dose-dependently inhibited by the synthetic Gly-Arg-Gly-Asp-Ser-Pro peptide (Fig. 5A). Treatment of G361 cells with function-blocking anti-integrin α5 or β1 mAb resulted in the inhibition of adhesion of G361 cells to fibronectin. The anti-integrin β1 antibody mAb P4C10 (32) and anti-integrin α5 antibody mAb KH/33 inhibited cell adhesion by 70 and 90%, respectively (Fig. 5B).

Abolishment of the Fibronectin Binding Capacity of α5β1 after the Loss of Oligosialic Acids—We investigated the binding ability of integrin α5β1 to fibronectin after treatment with various sialidases to evaluate the roles of oligosialic acids of the integrin α5 subunit in fibronectin binding. G361 cell lysates were treated with sialidases and incubated with 5 mg/ml fibronectin-Sepharose at 4 °C for 2 h, and bound proteins were analyzed by Western blotting using an anti-integrin α5 polyclonal antibody. Binding of integrin α5β1 to fibronectin was not affected by NANase-II treatment, whereas integrin α5β1 treated with sialidase from A. ureafaciens failed to bind to fibronectin (Fig. 6, lanes 2 and 4, respectively). Because integrin α5β1 mixed with A. ureafaciens sialidase without incubation bound to fibronectin, it is suggested that A. ureafaciens sialidase effect on immobilized fibronectin was ruled out (Fig.
Occurrence and Function of Oligosialic Acids of Integrin \(\alpha_5\beta_1\)

**Involvement of Oligosialic Acids of Integrin \(\alpha_5\) Subunit in Cell Adhesion to Fibronectin**—Adhesion of G361 cells to a fibronectin-coated dish was not affected by pretreatment of cells with NANase-II (Fig. 7A). However, cell adhesion of cells pretreated with *A. ureafaciens* sialidase was decreased to 70% of the control. These results suggest that integrin \(\alpha_5\beta_1\)-mediated cell adhesion to fibronectin was suppressed by the loss of oligosialic acids. Contrary to the experiment shown in Fig. 2, Endo-N showed no inhibitory activity in the cell adhesion experiment. Endo-N is required to report a minimal DP of 5 to work on oligo/polysialic acids. Because the average DP of oligo- or polysialic acid of \(\alpha_5\) subunit has been shown to be 5–7, Endo-N could trim the oligosialic acid of \(\alpha_5\) subunit enough to lose its reactivity to mAb 12E3 but not enough to affect its binding capacity to fibronectin. Fig. 7A, inset, supports our notion by showing that Endo-N-treated \(\alpha_5\) subunit moved to the same position as that of the control and the \(\alpha_5\) subunit pretreated with NANase-II.

Whether the conformational change of integrin \(\alpha_5\) was induced by the treatment with *A. ureafaciens* sialidase was investigated. The reactivity of mAb JBS5 toward cells with or without *A. ureafaciens* sialidase treatment was investigated by flow cytometry. It has been proposed that the epitope of mAb JBS5 lies close to the RGD recognition site of \(\alpha_5\) integrin (33). The pretreatment of cells with *A. ureafaciens* sialidase decreased reactivity to mAb JBS5 (Fig. 7B), whereas NANase-II showed no effect (data not shown). These results suggest that the conformation of integrin \(\alpha_5\) was changed by the loss of oligosialic acids from integrin \(\alpha_5\) subunit, possibly resulting in the suppression of binding ability to fibronectin.

**Inhibition of Cell Adhesion to Fibronectin by the Treatment with Colominic Acids or mAb 12E3**—To further demonstrate that oligosialic acids of integrin \(\alpha_5\) subunit function in cell adhesion, the effects of colominic acids and mAb 12E3 on cell adhesion to fibronectin were investigated. Adhesion of G361 cells to fibronectin was not inhibited by colominic acid, a polymer of \(\alpha_2\)-8-linked sialic acid obtained from *E. coli* (Fig. 8A), suggesting that oligosialic acids of integrin \(\alpha_5\) do not reside in the interface between integrin and fibronectin binding. To obtain direct evidence that oligosialic acids are involved in binding between integrin \(\alpha_5\beta_1\) and fibronectin, cells were treated with the anti-oligosialic acid antibody mAb 12E3 by 40% (Fig. 8B), and the treatment with mAbs against integrin \(\alpha_5\) and \(\beta_1\) inhibited adhesion to fibronectin as discussed in the preceding section (Fig. 5B). Because mAb 12E3 is composed of IgM, nonspecific interaction caused by IgM should be excluded. Therefore, a control experiment was performed using mAb LY111 (subclass IgM) recognizing chondroitin sulfate proteoglycan expressed on the cell surface of G361 cells. Although heparan sulfate proteoglycan is reported to be involved in cell adhesion to fibronectin mediated by integrin \(\alpha_5\beta_1\) (34, 35), there has been no report yet on the involvement of chondroitin sulfate proteoglycan. The G361 cell adhesion to fibronectin was not affected by the treatment with mAb LY111, suggesting that activation of cell adhesion...
by mAb 12E3 should be ascribed to specific binding of mAb to oligosialic acid. Whether the stimulative effects of mAb 12E3 could be attributed to the exposure of the fibronectin recognition site of integrin α5β1 or the enhanced clustering of integrin α5β1 was investigated. The reactivity of mAb JBS5 toward cells with or without mAb 12E3 treatment was determined by flow cytometry. Pretreatment of cells with mAb 12E3 enhanced reactivity to mAb JBS5 (Fig. 8C). Therefore, it is highly likely that the treatment of cells with mAb 12E3 caused a conformational change in integrin that unmasked sites involved in ligand recognition. Furthermore, immunofluorescent microscopic observation indicated that clustering of integrin α5β2 was not induced by the treatment of cells with mAb 12E3 (data not shown). Taken together, it is suggested that the modification of integrin α5 with oligosialic acids enhances the binding capability of integrin α5β1 to fibronectin, possibly by holding the conformation in the high affinity form to the ligand.

**FIG. 8.** Inhibition of G361 cell adhesion to fibronectin by the treatment of colominic acids and mAb 12E3. A, G361 cells (3 × 10⁴ cells/well) were added to wells coated with 10 µg/ml fibronectin in the absence or presence of colominic acid. B, the cells (3 × 10⁴ cells/well) were preincubated at room temperature for 20 min in the presence of 50 µg/ml 12E3 or LY111, transferred to fibronectin-coated plates together with antibodies, and then incubated for 30 min at 37 °C. Cell adhesion was determined as described under “Experimental Procedures.” The data represent one of three independent experiments. C, flow cytometry analysis was carried out as described in the Fig. 7 legend. Control, cells incubated with normal mouse IgG instead of mAb JBS5. The fluorescence intensity of control cells was not affected with or without treatment of cells with mAb 12E3.

**DISCUSSION**

Glycoproteins with oligosialic acids have not yet been identified except for the adult form of N-CAM (16, 19, 20) and sodium channels (20). A previous structural study on N-linked oligosaccharides obtained from human placenta integrin α5β1 did not mention oligosialic acids (30). By applying immunohistochemical and chemical analyses (12, 16), we have succeeded in finding oligosialic acids of the integrin α5 subunit of human melanoma G361 cells (Fig. 2). In addition, modification of the integrin α5 subunit with oligosialic acids seems ubiquitous because occurrence of oligosialic acids was observed in the integrin α5 subunit of other cell types. Integrin α5β1 consists of α and β subunits, but oligosialic acids did not occur in the β1 subunit. Although the present study focused on oligosialic acids of the integrin α5 subunit, we do not rule out the possibility that oligosialic acids can also be present on proteins other than integrin α5 in human melanoma G361 cells. α5–8-Linked oligosialic acids in the α5 subunit were stained with mAb 12E3, which reacts with oligosialic acids with a DP ≥ 5, but not with polyclonal antibody H46, which recognizes polymers with a DP ≥ 8. Treatment of the α5 subunit with A. ureafaciens sialidase and Endo-N completely abolished the reactivity to mAb 12E3, but treatment with NANase-II did not decrease the reactivity. Endo-N requires a minimal DP of 5 to
work (24). From these studies, the degree of polymerization of α2–8-linked oligosaccharides in the α5 subunit was estimated to be 5–7. The treatment of G361 cells with A. ureafaciens sialidase brought about the loss of binding capability to fibronectin but failed to do so with Endo-N. Endo-N may attack the sialyl linkage near the nonreducing terminal of oligosaccharic acid chains, leading to a decrease in the degree of polymerization, which still allowed cells to interact with fibronectin, but did not support mAb 12E3 binding to the residual chains of oligosaccharic acids. The occurrence of internal sialic acids was confirmed further by the C2/C4 analysis. On the assumption that the average degree of polymerization is 6, it was calculated from the ratio that integrin α5 contains an oligosaccharic acid-bearing chain every six integrin α5 molecules in G361 cells and every two molecules in fibroblast and K562 cells.

In addition to the N-CAM and sodium channels being intensively studied, oligosaccharides were implied to play a role in embryonic development because their amount changed in a stage-specific manner (17). However, direct evidence on the functional roles of oligosaccharic acids is yet to be determined. We have investigated the function of oligosaccharic acids of integrin α5β1, known as a fibronectin receptor, and its interaction with fibronectin is considered to be involved in various biological phenomena. Previous studies showed that cell adhesion mediated by integrin was regulated by glycosylation. Akiyama et al. (9) reported that blockage of N-glycan processing by 1-deoxymannojirimycin, an inhibitor of Golgi mannosidases, resulted in inhibition of the fibronectin-binding capability of integrin α5β1 of mouse 3T3 cells. Zheng et al. (10) showed that N-glycosylation of both the α and β subunits of integrin α5β1 is essential for association of these subunits and for the receptor to function normally. Koyama and Hughes (36) described that cell adhesion to fibronectin was impaired in the N-acetylgalactosaminyl transferase I-deficient cells, which were unable to synthesize any hybrid or complex carbohydrates including those carrying oligosaccharic acids. In other studies, phorbol ester treatment of human multipotential hematopoietic cell line K562 and promonocytic cell lines U937 and THP-1 induced the decrease in sialylation of integrin α5β1, leading to the suppression of cell adherence to fibronectin (11, 37). In our study, we have demonstrated that unique α2–8-linked oligosaccharic acids located on N-linked outer chains of the integrin α5 subunit are involved in cell adhesion to fibronectin. Adhesion of human melanoma G361 cells to fibronectin mediated mainly by α5β1 was inhibited when cells were treated with sialidase from A. ureafaciens but not with NANase-II (Figs. 6 and 7). The decrease in binding to fibronectin was not caused by dissociation of integrin α5 and β1 subunits, because the β1 subunit was co-immunoprecipitated using integrin α5 mAb whether cell lysate had been treated with sialidases or not (data not shown).

Previous studies provided evidence that oligosaccharides of integrin α5 are indispensable for the binding to fibronectin. However, explanations have not been given on how the effect of the sugar moiety is achieved. In the present study, we showed that the treatment of cells with A. ureafaciens sialidase decreased reactivity of the α5 subunit to mAb JBS5, the epitope of which is closely located at the RGD recognition site of α5 (Fig. 7). Thus, it is suggested that the conformational change has been induced after the loss of oligosaccharic acids, leading to masking of the ligand-binding domain of α5. As shown in Fig. 8, the mAb 12E3 binding to α5 enhanced its reactivity to JBS5, suggesting that conformational change has also been induced. Treatment with mAb 12E3 may have exaggerated the conformational change introduced by the occurrence of oligosaccharic acid. Oligosaccharic acids of integrin α5 thus contribute to the binding capability of integrin α5β1 to fibronectin, possibly bringing the conformation into the high affinity form to the ligand.

α2–8-Linked oligosaccharic acids are known to be common structural units of gangliosides implicated in various biological processes such as cell adhesion, cell differentiation, signal transduction, and surface expression of stage-specific antigen. Cell adhesion regulated by integrins is modulated by co-existing gangliosides. It was reported that based on immunohistochemical and biochemical analyses (38), GD2 and GD3 gangliosides were closely associated with the vitronectin receptor (integrin α5β1) in human melanoma. Wang et al. (39) reported that GT1b regulated integrin α5β1-mediated adhesion of epithelial cells to fibronectin through carbohydrate-carbohydrate interactions between GT1b and the integrin α5 subunit. However, in human melanoma G361 cells, the occurrence of gangliosides containing α2–8 oligosaccharic acids such as GD2, GD3, or GT1b was not confirmed using thin layer chromatography analysis (data not shown). Thus, the loss of cell adhesion to fibronectin by sialidase treatment was suggested not to involve α2–8-oligosacchari- lated gangliosides. Koyama and Hughes (36) reported that adhesion of BHK cells to fibronectin was not affected by treatment of 1-deoxymannojirimycin. Their finding is in contrast to our and previous studies (9) that indicate that hybrid or complex carbohydrates are important for the normal function of integrin α5β1. Such a conflict may possibly be caused by gangliosides on the cell surface. The adhesion of G361 cells to fibronectin were remarkably inhibited by 1-deoxymannojirimycin treatment (data not shown), probably because G361 cells scarcely express gangliosides containing α2–8 sialic acids. In the study by Koyama and Hughes, it is conceivable that gangliosides expressed on BHK cells compensated the loss of function of integrin by the treatment with 1-deoxymannojirimycin.

Of the known α2–8 sialyltransferases (ST8Sia), ST8Sia III is most likely to be the enzyme responsible for the synthesis of oligosaccharic acids on glycoproteins. Recently, Angata et al. (40) reported that oligosaccharic acids could be synthesized by ST8Sia III. However, although integrin α5β1 is widely distributed, no expression of ST8Sia III was reported in tissues other than brain or testis. Thus, we may propose the presence of other types of ST8Sia for the oligosialylation of the integrin α5 subunit. It will be of interest to search for the biosynthetic enzyme of oligosaccharic acids by which the function of integrin is regulated.

The interaction of integrin α5β1 with fibronectin plays an essential role in development. The integrin α5 null and fibronectin null embryos have pronounced defects in mesodermal structures, suggesting the role of both interacting molecules in mesoderm formation (41). However, it remains to be determined whether oligosaccharic acids of integrin α5 function in the interaction between integrin α5β1 and fibronectin while mesoderm formation takes place. Based on the present study, the hypothesis that oligosaccharic acids are important for the interaction between integrin α5β1 and fibronectin in the developmental stage seems quite plausible, particularly taking into account the occurrence of and dynamic change in oligosaccharic acids and the regulation of N-CAM function by oligosialylation during embryonic development.

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