Bisecting GlcNAc Residues on Laminin-332 Down-regulate Galectin-3-dependent Keratinocyte Motility

Received for publication, June 29, 2009, and in revised form, October 27, 2009. Published, JBC Papers in Press, November 25, 2009, DOI 10.1074/jbc.M109.038836

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Laminin-332 (Lm332; formerly laminin-5) is a basement membrane protein in the skin, which promotes cell motility in wound healing and cancer invasion. In a previous study, we reported that the introduction of bisecting GlcNAc into Lm332 (GnT-III-Lm332), catalyzed by N-acetylgalcosaminyltransferase III (GnT-III), reduced cell migration (Kariya, Y., Kato, R., Itoh, S., Fukuda, T., Shibukawa, Y., Sanzen, N., Sekiguchi, K., Wada, Y., Kawasaki, N., and Gu, J. (2008) J. Biol. Chem. 283, 33036–33045). However, the underlying molecular mechanism by which GnT-III-Lm332 suppresses the normal biological functions of Lm332 remains to be elucidated. In this study, we show that galectin-3, which is a β-galactoside-binding protein, strongly bound to unmodified Lm332 but not to GnT-III-Lm332 and that binding of galectin-3 was completely blocked by lactose. Exogenous galectin-3 significantly enhanced keratinocyte cell motility on control Lm332 but not on GnT-III-Lm332. A functional blocking antibody against galectin-3 inhibited Lm332-induced α3β1 and α6β4 integrin clustering and focal contact formation. Co-immunoprecipitation revealed that galectin-3 associated with both β4 integrin and epidermal growth factor receptor, thereby cross-linking the two molecules. The associations were inhibited by either the presence of lactose or expression of GnT-III. Moreover, galectin-3 consistently enhanced ERK activation. Taken together, the results of this study are the first to clearly identify the molecular mechanism responsible for the inhibitory effects of GnT-III on extracellular matrix-integrin-mediated cell adhesion, migration, and signal transduction. The findings presented herein shed light on the importance of N-glycosylation-mediated supramolecular complex formation on the cell surface.

Tissue maintenance requires that cells be in communication with the surrounding microenvironment. During both physical and pathological conditions, cells receive extracellular matrix (ECM)2-mediated signals via cell surface receptors, including α3β1 integrin, migration, differentiation, and proliferation (1, 2). Significant research effort, using biochemical and genetic techniques, has advanced our understanding of how ECM signals are transduced and then translated into the cell. However, most studies have focused on the protein–protein interactions, namely on ECM-cell surface receptor interactions, rather than on carbohydrate–protein interactions.

Laminin-332 (Lm332; previously known as laminin-5) is a component of basement membranes in the skin and other stratified squamous epithelial tissues (2–4). Lm332, which consists of α3, β3, and γ2 subunits, associates with hemidesmosomes through integrin α6β4 (5). A null mutation for Lm332 causes a severe and lethal skin blistering disease (6, 7). On the other hand, Lm332 is overexpressed at the leading edge of wounds during healing (8–10) and in various types of squamous and other epithelial tumors (11, 12). In addition, Lm332 plays an essential role in a mouse model of human squamous cell carcinoma tumorigenesis (13). Consistent with the result of in vivo studies, Lm332 promotes various cellular activities in vitro, such as cell adhesion, spreading, migration, and proliferation, via association of the carboxyl-terminal globular domain of the α3 chain with cell surface receptors, such as α3β1 integrin, α6β4 integrin, and syndecans (14–16).

Although between 13 and 30% of the total molecular weight of laminins is N-linked glycosylated (17), most studies of laminin have not paid attention to the contribution of N-glycosylation to the functional activities of laminin. N-Acetylgalcosaminyltransferase V (GnT-V) catalyzes addition of the β1,6-linked GlcNAc branch and defines this subset of N-glycans (18, 19). In some cancers, an increase in β1,6-GlcNAc is correlated with cancer metastasis, which is supported by studies of patient-derived tissue (20) and GnT-V-deficient mice (21). In contrast, bisecting GlcNAc catalyzed by N-acetylgalcosaminyltransferase III (GnT-III) suppresses further processing by branching enzymes, such as GnT-V, and elongation of N-glycans (22–24), resulting in down-regulation of cell migration and cancer metastasis (25). In fact, GnT-III modification of α3β1 integrin inhibits GnT-V-promoted cell migration on Lm332 (26). Modification by GnT-III also down-regulates other cell surface receptors, e.g. α5β1 integrin (27, 28) and epidermal growth factor receptor (EGFR) (29). Interestingly, GnT-III can specifically modify a key N-glycosylation site of 14
potential N-glycosylation sites on the α5 subunit (30). In addition, we recently reported that expression of GnT-III also plays a role in ECM-mediated cell adhesion. An increased amount of bisecting GlcNAc on Lm332 suppressed Lm332-induced cell spreading and migration (31). These studies suggest that GnT-III usually inhibits cell adhesion, cell migration, and intracellular signaling by down-regulating the function of target proteins.

Recent studies suggest that carbohydrate-protein interactions are important for cell signal transduction. These interactions involve galectins, which can bind to β-galactosides on proteins (32). Galectin-3 is one of the best characterized galectins, and it is the only chimeric galectin reported to date (33). Galectin-3 is ubiquitously expressed in adult tissues. It is localized in the nucleus and cytoplasm as well as on the cell surface and in the extracellular space. Cytosolic galectin-3 is involved in the regulation of cell proliferation, differentiation, survival, and death (33). Galectin-3 is highly expressed in some cancers, including the human skin cancer squamous cell carcinoma (34, 35), and at the leading edge of wound sites (36). Galectin-3 forms a lattice or cell surface microdomain, which links cyto- 

kine receptors on the cell surface (37). The galectin-3 lattice up-regulates cytokine receptor signaling by down-regulation of receptor endocytosis (37). Galectin-3 binds to ECM proteins, including laminin-111, fibronectin, and type IV collagen, integrins α1β1 and α3β1, NG2 (a transmembrane chondroitin sulfate proteoglycan), and L6y family member C4.4A (33, 38, 39). Taken together, the results of these studies strongly suggest that extracellular galectin-3 plays an important role in modulation of cell adhesion and migration.

The purpose of this study was to identify the molecular mechanism responsible for the inhibitory effects of GnT-III. Thus, we thoroughly investigated the effects of galectin-3 on Lm332-mediated cell adhesion and migration. We found that the inhibitory effects of GnT-III could be ascribed to down-regulating the formation of molecular complexes composed of ECM, integrin, and other growth factor receptors, which are linked by galectin-3.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—The following antibodies were used in this study: rat monoclonal antibodies (mAbs) specific for galectin-3 (M3/38) and α6 (GoH3); rabbit polyclonal Abs to EGFR (1005), glutathione S-transferase (GST) (z-5), and β4 integrin (H101) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); mouse mAbs to the human laminin β3 chain (Kalinin B1), ERK1 (MK12), and paxillin (349) (BD Transduction Laboratories, Lexington, KY); and a mouse mAb to phospho-ERK1/2 (E10) from Cell Signaling Technology. A mouse mAb against α-tubulin was purchased from Sigma. The control rat and rabbit IgGs were obtained from Santa Cruz Biotechnology Inc.; Alexa Fluor 488 and 546 secondary antibodies were purchased from Invitrogen. A mouse mAb against the human laminin γ2 chain (D4B5) was a generous gift from Dr. Kaoru Miyazaki (Yokohama City University, Yokohama, Japan). Purified human Lm332 was prepared as described previously (31). A homo-bifunctional cross-linker 3,3’-dithiobis(sulfosuccinimidyl propionate) (DTSSP) was purchased from Thermo Scientific Inc. (Rockford, IL).

**Cell Culture**—Normal immortalized keratinocytes (NIK) and keratinocytes isolated from patients with junctional epidermolysis bullosa, which lack the gene for the Lm332 β3 chain, were a generous gift from Dr. M. Peter Marinkovich (Stanford University) (13). These keratinocytes were grown in a 50:50 mixture of defined keratinocyte medium (Invitrogen) and medium 154 (Cascade Biologics, Portland, OR) containing penicillin and streptomycin sulfate. The human gastric cancer cell line MKN45 was cultured in RPMI 1640 medium (Nacalai Tesque, Japan) containing 10% fetal calf serum, penicillin, and streptomycin sulfate. MKN45 transfectants were prepared as follows. Human GnT-III and GnT-V cDNA were inserted into a mammalian expression vector pCWX, and those cDNA expression vectors, as well as vector alone, were transfected into MKN45 cells by Lipofectamine reagent (Invitrogen) following the manufacturer’s instructions, respectively. Selection was performed in the medium containing 500 μg/ml neomycin (Sigma). After a 2-week selection, neomycin resistance clones were isolated by serially limited dilution. Positive cells were selected by immunoblotting.

**Protein Expression and Purification of Human Galectin-3**—Human galectin-3 cDNA corresponding to the coding region, i.e. nucleotides 19–753 (GenBank™ accession number M57710), was generated by reverse transcription-PCR using galectin-3-forward (5’-CACCGATCCTCCATGATGGCTTTATCGGG-3’) and galectin-3-reverse (5’-GGCGAATTC-TTATACTGTTATAGA-3’) primers and KOD Plus polymerase (Toyobo, Japan). PCR products were cloned into the pENTR™/D-TOPO vector (Invitrogen) according to the manufacturer’s instruction manual and then recombined in the pDEST15 vector (Invitrogen) for GST fusion protein expression via the Gateway LR reaction (Invitrogen). The cDNA sequence was verified by DNA sequencing. The GST-galectin-3 fusion protein was expressed in *Escherichia coli* BL21 (DE3) (BioDynamics Laboratory Inc., Japan). The BL21 cells transformed with the expression vector were grown at 37 °C until the *A* 600 reached 0.5. The expression of recombinant galectin-3 was induced by addition of isopropyl-1-thio-β-D-galactopyranoside at a final concentration of 1 mM. The culture was then incubated for an additional 4 h at 37 °C. Cells were harvested by centrifugation (6,000 rpm, 30 min, 4 °C), resuspended in sonication buffer (50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol), and lysed by sonication for 10–15 s pulses separated by 1-min intervals in an ice bath. After sonication, 10% Triton X-100 was added to the lysed solution at a final concentration of 1%. The lysate was clarified by centrifugation at 18,000 rpm for 30 min at 4 °C, and the supernatant was applied to a glutathione-Sepharose 4B column. After washing with PBS containing 0.5% Triton X-100 (v/v), followed by sonication buffer, bound proteins were eluted using sonication buffer containing 10 mM reduced glutathione. The eluted protein was dialyzed against PBS.

**Preparation of Cell Lysates and Immunoprecipitation**—Cell lysates were prepared as follows. Cells were washed twice with cold PBS and then lysed with lysis buffer (1% Triton X-100, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA) containing a protease inhibitor mixture (Nacalai Tesque) and a phosphatase inhibitor mixture (Nacalai Tesque). After incubation for 10
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min on ice, cell lysates were clarified by centrifugation at 15,000 rpm for 10 min at 4 °C. The resulting supernatant was used in the following experiments. The protein concentration was determined using a protein assay kit (Nacalai Tesque). For immunoprecipitation, protein G-Sepharose Fast Flow beads (Amersham Biosciences) were added to the cell lysate, followed by rotation for 1 h at 4 °C to remove nonspecific binding protein to the beads. After centrifugation, the primary Ab was added to the supernatant and rotated overnight at 4 °C. The protein G-Sepharose was then added, followed by a 2-h incubation at 4 °C. Immunoprecipitates were washed five times with STE buffer, and heated at 95 °C for 5 min. Pulldown Assay—After glutathione-Sepharose 4B beads were washed with sonication buffer (50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA) without dithiothreitol, beads were blocked with 2% BSA in Tris-buffered saline for 30 min at room temperature. After washing again with sonication buffer without dithiothreitol, GST or GST-galectin-3 was added to the beads, followed by rotation for 1 h at 4 °C. After washing the beads twice with STE washing buffer, purified Lm332 was added to the beads, followed by rotation for 1.5 h at 4 °C. Beads were thoroughly washed five times with STE washing buffer and then incubated with 0.2 M lactose dissolved in supplement-free keratinocyte growth medium for 20 min at room temperature. After centrifugation, the supernatant was collected, and the bound Lm332 was checked by immunoblotting with the anti-laminin-β3 chain mAb.

Immunoblotting—For the immunoblotting analyses, proteins resolved by SDS-PAGE were transferred to nitrocellulose membranes. The blots were probed with each Ab. Immunoreactive bands were detected using an ECL detection kit (GE Healthcare). The band intensity was calculated using NIH ImageJ software.

Cell Adhesion Assay—The cell adhesion assay was performed as described previously (14). Briefly, each well of a 96-well plate was precoated with 2 μg/ml Lm332 and then blocked with 1% BSA. Cells (2 × 10^4 cells) were added to each well in supplement-free keratinocyte growth medium. After nonadherent cells were removed by vigorous shaking, adherent cells were fixed with 25% (w/v) glutaraldehyde and stained with 0.5% crystal violet (w/v) in 20% (v/v) methanol for 10 min. The dye was extracted with 0.1 M sodium citrate in 50% methanol (v/v) for 30 min. The absorbance at 590 nm was then measured using a microplate reader. For the inhibition assay, cells were suspended in each conditioned buffer before plating.

Cell Migration Assay—A glass-bottom dish (Asahi Techno Glass, Japan) was precoated with 2 μg/ml Lm332 and then blocked with 1% BSA for 1 h at 37 °C. A 200-μl aliquot of the Lm332 null keratinocyte suspension (4 × 10^4 cells/ml) in growth medium was added to each Lm332 precoated glass-bottom dish. For the inhibition assays, cells were treated with 15 μg/ml rat control IgG or galectin-3 mAb for 20 min at room temperature. After incubation for 1 h at 37 °C, cell migration was monitored for 4 h using time-lapse video equipment (Carl Zeiss, Germany).

Would Healing Assay—NIK cells were cultured to confluence, and the cell monolayer was scratched with a plastic pipette tip. After washing twice with PBS to remove cell debris, cells were incubated in keratinocyte growth medium for 6 h and then subjected to immunostaining with the appropriate Abs.

ELISA—ELISA was performed as follows. The wells of a 96-well plate were coated with test proteins and then blocked with 1.2% BSA at room temperature for 1 h. The wells were then washed three times with PBS containing 0.05% Tween 20 (washing buffer) and then incubated with primary Ab for 1 h at room temperature. The wells were then washed three times and incubated with the biotin-conjugated secondary Ab (Vector Laboratories, Inc., Burlingame, CA) for 45 min at room temperature. Similarly, the wells were washed three times and incubated with alkaline phosphatase conjugated with avidin D (Vector Laboratories, Inc.) for 45 min at room temperature. After washing five times, the bound antibody was quantified by measurement of absorbance at 405 nm after incubation with p-nitrophenyl phosphate disodium salt in 100 mM diethanolamine (pH 9.8) containing 0.24 mM MgCl2.

Binding Assay—The wells of microtiter plates were coated with 2 μg/ml Lm332 overnight at 4 °C. The wells were then blocked with 1.2% BSA for 1 h at 37 °C. Recombinant galectin-3, at a concentration of 25 μg/ml in supplement-free keratinocyte growth medium, was added to the plates and allowed to bind to Lm332s for 1 h at 37 °C. The plates were washed three times with PBS containing 0.05% Tween 20 (washing buffer), and then bound galectin-3 was fixed with 2.5% glutaraldehyde in PBS for 10 min at room temperature. The wells were washed with washing buffer, and the bound proteins were quantified by ELISA.

Immunofluorescence Microscopy—A 200-μl aliquot of cell suspension (2 × 10^5 cells/ml of growth medium) was added to each glass-bottom dish (Asahi Techno Glass). After incubation for 24 h, the cells were washed with PBS and then fixed with 4% (w/v) paraformaldehyde in PBS for 10 min. For permeabilization, the cells were treated with 0.2% (v/v) Triton X-100 in PBS. The fixed cells were blocked with 2% BSA in PBS for 1 h before staining with appropriate primary and secondary antibodies. Fluorescence images were obtained using a fluorescence microscope (Olympus Corp., Tokyo) equipped with 100× /1.35 UPlan-Apochromat oil immersion objectives (Olympus Corp.).

Cell Surface Cross-linking—For cross-linking, cells were incubated in PBS containing 0.1 mg/ml DTSSP for 15 min at room temperature. After washing three times with ice-cold PBS, the cell lysate was prepared as described above and immunoprecipitated with the appropriate primary Ab. The immunoprecipitants were subjected to SDS-PAGE and transferred to a nitrocellulose membrane.

Flow Cytometry Analysis—Cells were detached from a 10-cm dish using trypsin containing 1 mM EDTA. After quenching trypsinization with medium containing 10% fetal bovine serum, cells were washed twice with PBS containing 1 mM EDTA and incubated with primary Ab or control IgG for 30 min on ice, followed by incubation with the appropriate secondary Ab. After washing three times with PBS containing 1 mM EDTA,
flow cytometric analysis was performed using CellQuest software on a FACSCalibur (BD Biosciences).

**Statistical Analysis**—Data collected were analyzed using GraphPad Prism and Microsoft Excel software. Data are means ± S.D. A Student's t test was used to compare the two groups. For the cell migration assay, a two-way analysis of variance was used when the mean cell migration distance between each condition of groups was compared followed by a Bonferroni post test to compare cell migration distance between each of the groups.

**RESULTS**

*Introduction of Bisecting GlcNAc into Lm332 Inhibits Its Association with Galectin-3*—Because galectin-3 has been co-purified with laminin-111 (previously known as EHS-laminin or laminin-1), we tried to determine whether galectin-3 is also co-purified with Lm332. To examine purity, the purified Lm332 from the human gastric cancer cell line MKN45 was separated by SDS-PAGE and then stained with Coomassie Brilliant Blue (Fig. 1A). The major bands migrated around 100–250 kDa, which were components of Lm332. Although we could not clearly find the galectin-3 band (around 25 kDa) in the purified fraction of Lm332 in the Coomassie Brilliant Blue staining, immunoblotting with an anti-galectin-3 mAb revealed that a very small amount of galectin-3 was bound to the purified Lm332 (Fig. 1B, vecLm332). To confirm this interaction, recombinant GST-galectin-3 was prepared as described under “Experimental Procedures.” Then, using the purified Lm332, the direct association was tested in a pulldown assay. The results of the pulldown assay indicated that GST-galectin-3, but not control GST, bound to Lm332 (Fig. 1C). Galectin-3 has a high affinity for β-galactoside sugar chains. To examine whether the binding of galectin-3 to Lm332 occurs via β-galactoside, binding ability was assessed using an ELISA in the presence of lactose, which is a competitive inhibitor of galectin binding. Binding of galectin-3 to Lm332 was completely blocked in the presence of lactose, but not in the presence of sucrose or EDTA, suggesting that the association of galectin-3 with Lm332 was mediated by β-galactoside residue (Fig. 1D).

GnT-V catalyzes the conversion of β1,6-GlcNAc to complex N-glycans, leading to elongation by the poly-N-acetyllactosamine sequence, which is a preferred ligand for galectins. By contrast, GnT-III catalyzes the introduction of bisecting GlcNAc, which suppresses further processing by branching enzymes such as GnT-V, and elongation of N-glycans. Our previous report showed that Lm332 could be modified by both enzymes (31). To check whether galectin-3 shows distinct binding capacities for different types of N-glycans on Lm332, we compared the abilities of galectin-3 to bind three types of Lm332, vector-Lm332, GnT-III-Lm332, and GnT-V-Lm332, which were purified from control vector-transfected MKN45 cells, GnT-III-overexpressing MKN45 cells, and GnT-V-overexpressing MKN45 cells, respectively. As a result, the binding ability of galectin-3 was significantly decreased for GnT-III-Lm332, as compared with vector-Lm332 and GnT-V-Lm332 (Fig. 1E). To exclude the possibility that the inability of GnT-III-Lm332 to bind to galectin-3 was due to the fact that the GnT-III-Lm332 preparation was saturated with endogenous
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Galectin-3, we did the immunoblotting for purified vector-, GnT-III-, and GnT-V-Lm332s using an anti-galectin-3 antibody (Fig. 1B). As a result, a similar amount of galectin-3 was bound to the purified vector Lm332 and GnT-V-Lm332, but it was difficult to detect galectin-3 in the purified GnT-III-Lm332. Therefore, GnT-III-Lm332 preparation was not saturated with endogenous galectin-3. Interestingly, the binding of GnT-V-Lm332 to galectin-3 was similar to that of vector-Lm332. In addition, cell migration on GnT-V-Lm332 was less than that on vector-Lm332 or GnT-V-Lm332, but there was no significant difference between vector-Lm332 and GnT-V-Lm332, as described previously (31). These results strongly suggest that the intrinsic GnT-V-mediated glycosylation in keratinocytes might be adequate for occupation of some important N-glycosylation sites on Lm332. In fact, such GlcNAc-branched N-glycans structures have been confirmed by mass spectrometry on both vector-Lm332 and GnT-V-Lm332 but not on GnT-III-Lm332 (31).

Galectin-3 Enhances Lm332-mediated Cell Migration—To assess whether binding of galectin-3 to Lm332 affects the functions of Lm332, we examined cell migration of Lm332-null keratinocytes on Lm332 in the presence or absence of exogenous galectin-3. Generally, it is quite difficult to measure the biological functions of other ECMs in Lm332-expressing cells, because endogenous Lm332 has stronger bioactivities for cell adhesion and migration. To avoid interference by endogenous Lm332, we utilized Lm332-null keratinocytes established from patients with genetic mutations of the laminin β3 chain (13). In fact, the immunoblotting using an anti-laminin β3 antibody showed that Lm332-null keratinocytes, unlike NIK cells, did not express Lm332 (Fig. 2A). When galectin-3 was added to the cell culture medium at 6.3 μg/ml, the cell motility of the keratinocytes on vector-Lm332 was greatly enhanced compared with addition of GST-tagged protein only (Fig. 2B, vecLm332). By contrast, addition of exogenous galectin-3 had no effect on cell migration on GnT-III-Lm332 (Fig. 2B, III-Lm332). Similar results were also obtained when galectin-3 was preincubated with Lm332 (data not shown). Functional blocking of galectin-3 using a mAb suppressed NIK cell migration on vector-Lm332 (Fig. 2C, vecLm332, gal3 mAb) to a level similar to that of GnT-III-Lm332 (Fig. 2C, III-Lm332, IgG). These results indicate that endogenous galectin-3 derived from keratinocytes can also promote cell motility on Lm332. By contrast, there were no obvious inhibitory effects of the functional blocking of mAb on cell motility induced by

![FIGURE 2](image-url)

**Galectin-3 promoted Lm332-dependent cell migration.** A, detection of Lm322 expression in NIK and laminin β3 chain null keratinocytes. Cell lysates (20 μg) from the indicated keratinocytes were run on a 7.5% SDS-polyacrylamide gel, blotted onto a nitrocellulose membrane, and then probed with an anti-laminin β3 Ab and then reprobed with an anti-α-tubulin Ab. IB, immunoblot. B, effect of galectin-3 on Lm332-dependent cell migration. Lm332 null keratinocytes in culture medium containing 6.3 μg/ml GST or purified galectin-3 (gal3) protein were plated onto dishes that had been precoated with 2 μg/ml vector-Lm332 (vecLm332) or GnT-III-Lm332 (III-Lm332), followed by incubation for 1 h. Cell migration on each substrate was monitored by time-lapse microscopy as described under “Experimental Procedures.” Each bar represents the mean ± S.D. of the migration distance of eight cells in each assay in three independent experiments. C, effect of anti-galectin-3 function-blocking mAb (gal3 mAb) on cell migration on the indicated Lm332 substrates were monitored by time-lapse microscopy. Rat IgG was used as a control (IgG). D, NIK cells and Lm332 null keratinocytes were grown to confluency on collagen-coated dishes. After the cell monolayer was scratched, cells were incubated in keratinocyte growth medium for 8.5 h, and photographs were then taken. Dashed lines indicate the borders between cell and wound cell-free area at 0 h.

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In the wound-healing assay, which used a pipette tip to make a straight scratch, the lack of Lm332 in keratinocytes resulted in suppression of wound healing, as compared with NIK, suggesting that Lm332 is indispensable for galectin-3-mediated cell motility during wound healing (Fig. 2D). These results further suggest that galectin-3 as well as Lm332 might play an important role in keratinocytes during wound healing.

**Exogenous Galectin-3 Weakens Initial Cell Spreading on Lm332**—Because galectin-3 enhanced cell migration on Lm332, it was likely that galectin-3 might also modulate both the cell attachment and spreading activities of Lm332. To verify the effects of galectin-3 on cell adhesion, we used ELISA to test cell adhesion toward Lm332 in the keratinocytes treated with either a functional blocking mAb against galectin-3 or lactose. Surprisingly, neither the functional blocking mAb nor lactose inhibited cell attachment on Lm332 compared with treatment with control IgG or sucrose, suggesting that galectin-3 was not involved in initial stages of cell attachment to Lm332 (Fig. 3A). By contrast, galectin-3 markedly suppressed cell spreading when cell morphology was observed 15 min after plating cells on Lm332 precoated dishes (Fig. 3, B and C). These data indicate that galectin-3 may down-regulate extensive cell spreading on Lm332, which contributes to up-regulation of cell migration, as shown in Fig. 2. In fact, the degree of cell adhesion to the
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Galectin-3 Affects Integrin Clustering and Focal Contact Formation—We previously reported that α3β1 integrin clustering and focal contact formation were impaired on GnT-III-Lm332, which showed weaker binding to galectin-3 compared with vector-Lm332 or GnT-V-Lm332, as shown in Fig. 1. Galectin-3 binds not only ECMs but also many membrane proteins such as integrin α3β1 (38). In fact, α3β1 integrin can be modified by either GnT-III or GnT-V (26). In addition, as shown in Fig. 3, galectin-3 affected cell spreading. Thus, we hypothesized that galectin-3 might partially link Lm332 with α3β1 integrin through a galectins/carbohydrate-protein interaction and thereby affect Lm332-induced α3β1 integrin clustering and focal contact formation. To confirm this hypothesis, we investigated the effect of galectin-3 on α3β1 integrin clustering and focal contact formation using immunofluorescence microscopy. As a result, the anti-galectin-3 functional blocking mAb, but not control IgG, greatly inhibited integrin α3 clustering (Fig. 4A, gal3 mAb, upper panel) and focal contact formation visualized by paxillin (Fig. 4B, gal3 mAb, upper panel) on vector-Lm332. Consistent with previous results, GnT-III-Lm332 did not show α3 integrin clustering (Fig. 4A, lower panel) or focal contact formation (Fig. 4B, lower panel) in Lm332-null-keratinocytes. These results further suggest that galectin-3 plays an important role in inhibition of cell spreading and migration by GnT-III as observed in both previous studies and this study.

Galectin-3-mediated Complex Formation and Cellular Signaling—It is well known that α6β4 integrin is a cell surface receptor for Lm332 besides α3β1 integrin. Actually, β4 integrin is a key factor for wound healing and tumor progression (42, 43). To examine whether galectin-3 affects the activity of α6β4 integrin, we compared the localization of α6β4 integrin in Lm332-null-keratinocytes spread on Lm332 in the presence or absence of an anti-galectin-3 functional blocking mAb. When Lm332-null-keratinocytes were plated on vector-Lm332, α6β4 integrin showed dot-like localization (Fig. 5A, vecLm332). By contrast, α6β4 integrin showed diffuse localization on GnT-III-Lm332 (Fig. 5A, III-Lm332), suggesting that modification of Lm332 by bisecting GlcNAc reduced the efficiency of α6β4 integrin clustering, as observed on α3β1 integrin (Fig. 4). α6β4 integrin clustering on vector-Lm332 was also impaired by treatment with anti-galectin-3 functional blocking mAb (Fig. 5B). These results suggest that Lm332-induced α6β4 integrin clustering is mediated through galectin-3.

β4 integrin plays a central role in intracellular signaling, because it both associates and cooperates with several growth factor receptors that can also interact with galectin-3 through β-galactoside (37), such as EGFR (42). To determine whether galectin-3 also participates in formation of these complexes, we performed co-immunoprecipitation using primary antibodies specific for galectin-3, EGFR, or β4 integrin. Galectin-3 associated with both EGFR and β4 integrin but not control IgG (Fig.

ECM is a critical determinant of the rate of cell migration, i.e. migration is maximal under conditions of intermediate levels of cell adhesion (40). For example, GnT-V expression is strongly associated with cell migration and cancer metastasis, as greater modification of branched sugar chains by GnT-V inhibited both extensive cell spreading and organization of actin cytoskeletal formation. Conversely, GnT-V-null cells displayed enhanced cell spreading with concomitant inhibition of cell migration (41). The cell spreading and cell migration were inhibited in the presence of both lactose and sucrose, even at 0.05 M, which is a concentration that should not inhibit galectin-3 binding to β-galactoside residue (37), which suggests that those sugars may nonspecifically affect an initial cell spreading of keratinocytes.

FIGURE 3. Galectin-3 suppressed cell spreading on Lm332 during the initial stage of cell adhesion. A, effects of anti-galectin-3 Ab and lactose on cell attachment. Keratinocytes in suspension were incubated with a control rat IgG (IgG), anti-galectin-3 functional blocking mAb (gal3 mAb), sucrose, or lactose for 20 min at room temperature and then added to the vector-Lm332 precoated plates for 10 min at 37 °C. Each bar represents the mean ± S.D. of triplicate assays. B, effects of galectin-3 on cell spreading. The cell morphology was photographed after cell spreading on Lm332 for 15 min in the presence of GST or galectin-3. C, relative value of cell spreading area in each condition. Each bar represents the mean ± S.D. of 25 cells.
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Effects of galectin-3 on Lm332-mediated α3β1 integrin clustering and focal contact formation. Lm332-null keratinocytes were treated with control IgG (IgG) or anti-galectin-3 functional blocking mAb (gal3 mAb) and then plated on the indicated Lm332s. After incubation for 1.5 h, cells were fixed and stained with α3 integrin (A) and paxillin (B) mAb and followed by secondary antibodies. Arrowheads indicate α3β1 integrin clustering (A) and focal contacts (B). Scale bar, 20 μm.

DISCUSSION

To date, several research groups, including our laboratory, have reported that modification by GnT-III inhibits the biological activity of some integrins, growth factor receptors, and the ECM. However, previous studies focused on a single target molecule. Therefore, which GnT-III-modified molecule(s) is/(are) the most important and the precise molecular mechanism have been the subject of debate in recent years. This study thoroughly investigated the inhibitory effects of GnT-III on cell adhesion and migration on Lm332. We found that GnT-III action is most likely dependent on galectin-3 binding and on galectin-3-mediated complex formation, as summarized in Fig. 8.

Galectin-3 is known to play important roles in many biological processes, including cell adhesion, migration, spreading, endocytosis, and signal transduction. Reportedly, galectin-3 modulates cell adhesion to ECM proteins such as laminin-111, type IV collagen, and fibronectin (45). Galectin-3-overexpressing Evasa-T human breast cancer cells showed increased adhe-
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In keratinocytes, suggesting that α3β1 integrin transduces a signal that trans-dominantly inhibits the cytoskeletal reorganization by other integrins such as fibronectin receptor, integrin α5β1 (50). Therefore, it may be reasonable to assume that the addition of galectin-3 inhibits cell spreading on Lm332 because galectin-3 induces integrin α3β1 clustering and activates cellular signaling.

Galectin-3 functional blocking of mAb inhibited both α3β1 and α6β4 integrin clustering on Lm332 (Figs. 4 and 5). Furthermore, as shown in supplemental Fig. 1, galectin-3 promoted β4 integrin clustering and complex formation between β4 integrin and EGFR compared with control GST. Accordingly, the effect of galectin-3 addition on integrin clustering probably has to do with the efficiency of integrin clustering. Surprisingly, the addition of a high concentration of galectin-3 (50 μg/ml) resulted in complete inhibition of cell adhesion because keratinocytes were aggregated by the effect of galectin-3 at more than 25 μg/ml (supplemental Fig. 2). Accordingly, we could not examine the effect of galectin-3 on integrin clustering at higher concentrations. Although the underlying mechanism remains unclear, it seems that galectin-3 may have dual roles that are concentration-dependent.

As described above, overexpression of GnT-V increases β1,6-linkage GlcNAc-branched N-glycan, which is the preferred intermediate for extension by poly-N-acetyllactosamine. Modification with poly-N-acetyllactosamine enhances galectin-binding affinity. By contrast, GnT-III adds GlcNAc to the inner β-linked mannose to form bisecting GlcNAc, which blocks further branching (22). Thus, it is reasonable to speculate that sugar chains may switch galectin binding on and off during oncogenesis and cancer metastasis, as alterations in glycosylation are usually observed during cell invasion (51). As shown in this study, galectin-3 bound to Lm332, which greatly enhanced Lm332-dependent keratinocyte motility. However, increased cell migration on GnT-III-Lm332 was not induced by exogenous galectin-3, because modification of Lm332 by GnT-III reduced its ability to bind to galectin-3. These results strongly suggest that galectin-3 may be a cofactor for Lm332-induced cell motility during wound healing and squamous cell carcinoma tumor progression conditions that are associated with GnT-V overexpression (35, 36). Therefore, the opposing galectin functions may be attributed to...
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the study of different cell types or cell states, which express different oligosaccharides and therefore have distinct responses to galectins. In fact, a recent report showed that sia-

lylation of β1 integrins blocks cell adhesion to galectin-3 (52). In addition, glycosylation of ECM proteins might influence the results. For example, galectin-3 binds to human amniotic fluid fibronectin but not plasma fibronectin, because poly-N-acetyllactosamine glycans as well as tri- and tetra-antennary complex-type glycans are present in the former but absent in the latter (47).

Galectin lattice formation has been proposed to delay glycoprotein turnover by constitutive endocytosis on the cell surface (37). In this study, exogenous galectin-3 did not affect the expression of integrins α3β1, α6β4, or EGFR on the keratinocyte surface (data not shown). However, galectin-3 modulated α3β1 and α6β4 integrin clustering, focal contact formation, as well as Lm332-integrins-EGFR complex formation. These effects of galectin-3 were inhibited by an anti-galectin-3 functional blocking mAb. In addition, the presence of lactose inhibited complex formation. On the other hand, galectin-3 has been detected in caveolae or lipid rafts (53, 54). EGFR (55) and α3β1 and α6β4 integrins (56) are also localized in lipid rafts. Thus, galectin-3 might play an important role in the ternary complex formation in lipid rafts. Compartmentalization of lipid rafts is necessary to couple α6β4 integrin and EGFR to active Src family kinase, which both prevents incorporation of α6β4 integrin into hemidesmosomes and promotes cell migration (42, 44, 57). Some studies have reported that integrins are localized in the so-called glycosphingolipid-enriched microdomain rather than existing in lipid rafts on the cell surface. However, integrins associate with other receptors through glycans (58, 59), further suggesting that galectin-glycan interactions are important for cell adhesion and signaling.

It is also worth noting that N-glycans regulate both cell-ECM association and cell-cell adhesion. Overexpression of GnT-III slowed E-cadherin turnover, resulting in increased E-cadherin expression on the surface of B16 melanoma cells (60). In contrast to GnT-III, overexpression of GnT-V decreased both N-cadherin clustering on the cell surface and down-regulation of cadherin-associated cell-cell adhesion (61). E-cadherin engagement at the point of cell-cell contact is known to suppress cell migration, an effect that has been best described in the context of tumorigenesis (62). Conversely, disruption of E-cadherin-mediated cell adhesion appears to be a central event in the transition from noninvasive to invasive carcinomas. We recently found that E-cadherin-mediated cell-cell adhesion up-regulates GnT-III expression, suggesting that regulation of GnT-III and E-cadherin expression may constitute a positive feedback loop (63, 64). Enhanced GnT-III expression as a result of cell-cell adhesion may modify glycoproteins expressed on the cell surface or

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**FIGURE 7.** Expression of GnT-III suppressed galectin-3-mediated complex formation in MKN45 transfectants. A, vector- and GnT-III-MKN45 transfectants were subjected to DTSSP cross-linking, and then the cell lysates were immunoprecipitated (IP) using anti-β4 integrin antibody to detect β4 integrin complex. Immunoprecipitates were run on either a 6% or 4 integrin antibody to detect the ratio of phospho-ERK1/2 to total ERK1 bands, and the ratio in 24 h. The collected lysate in each condition was probed with phospho-ERK Ab. Results of the densitometric analysis are shown as the integrated density of the ratio of phospho-ERK1/2 to total ERK1 bands, and the ratio in vector-MKN45 transfectants treated with GST was equal to 1.

**FIGURE 8.** Model for the inhibitory effects of GnT-III on cell migration and signal transduction. Under normal conditions, galectin-3 binds to integrins, ECM, and growth factor receptors to form a complex on the cell surface for cellular signaling and cell migration in cancer cells or normal cells, such as keratinocytes, which usually express high or moderate levels of GnT-V and, therefore, contain some poly-N-acetyllactosamine N-glycans. However, overexpression of GnT-III results in modification of glycoproteins by bisection GlcNAc, which inhibits GlcNAc branch formation catalyzed by GnT-IV and GnT-V, thereby suppressing addition of poly-N-acetyllactosamine N-glycans on these proteins. Therefore, in this case, galectin-3 cannot form the signaling platform consisting of molecules such as EGFR, Lm332, and α3β1 and α6β4 integrins, inhibiting both cellular signaling and cell migration.
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ECM, which would perturb galectin-mediated complex formation and inhibit both cell migration and signal transduction, as described here. Therefore, the results of this study and prior investigations support the novel hypothesis that cooperative cross-talk between cell-cell adhesion and cell-ECM adhesion, which is mediated by induction of GnT-III expression, modulates cell adhesion, migration, and signal transduction.

In conclusion, it is important to determine whether the phenomena observed in vitro are also observed in vivo. Use of animal models, such as Gnt-T-III-knock-out mice, will elucidate the relationships between GnT-III-catalyzed bisected N-glycosylation and cell adhesion and migration, which might ultimately lead to novel cancer therapies.

Acknowledgment—We thank the Scientific Editorial Services, Harrison, AR, for editing this manuscript.

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