Inverse Correlation between the Extent of N-Glycan Branching and Intercellular Adhesion in Epithelia

CONTRIBUTION OF THE Na,K-ATPase β1, SUBUNIT

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The majority of cell adhesion molecules are N-glycosylated, but the role of N-glycans in intercellular adhesion in epithelia remains ill-defined. Reducing N-glycan branching of cellular glycoproteins by swainsonine, the inhibitor of N-glycan processing, tightens and stabilizes cell-cell junctions as detected by a 3-fold decrease in the paracellular permeability and a 2–3-fold increase in the resistance of the adherens junction proteins to extraction by non-ionic detergent. In addition, exposure of cells to swainsonine inhibits motility of MDCK cells. Mutagenic removal of N-glycosylation sites from the Na,K-ATPase β1 subunit impairs cell-cell adhesion and decreases the effect of swainsonine on the paracellular permeability of the cell monolayer and also on detergent resistance of adherens junction proteins, indicating that the extent of N-glycan branching of this subunit is important for intercellular adhesion. The N-glycans of the Na,K-ATPase β1 subunit and E-cadherin are less complex in tight renal epithelia than in the leakier intestinal epithelium. The complexity of the N-glycans linked to these proteins gradually decreases upon the formation of a tight monolayer from dispersed MDCK cells. This correlates with a cell-cell adhesion-induced increase in expression of GnT-III (stops N-glycan branching) and a decrease in expression of GnTs IVC and V (promote N-glycan branching) as detected by real-time quantitative PCR. Consistent with these results, partial adhesion-induced increase in expression of GnT-III (stops N-glycan branching) and a decrease in expression of GnTs IVC and V (promote N-glycan branching) as detected by real-time quantitative PCR. Consistent with these results, partial adhesion-induced increase in expression of N-glycans linked to these proteins suggest epithelial cells can regulate tightness of cell junctions via remodeling of N-glycans, including those linked to the Na,K-ATPase β1-subunit.

The generation of a polarized epithelium depends on the establishment of cell-cell contacts that induce the formation of the adherens junctions followed by assembly of the tight junc-
N-glycan Branching of Na,K-ATPase β1, and Cell-Cell Adhesion

a significantly lower rate of formation of cell-cell contacts than non-transfected MDCK cells (11).

Recent studies indicate that as the mature monolayer develops, the degree of N-glycosylation of adhesion molecules is altered. The complexity of N-glycans linked to E-cadherin is decreased in densely populated MDCK cell cultures when compared with sparsely populated MDCK cell cultures (20). Consistent with these observations, the relative molecular weight of N-glycans linked to the N-cadherin decreases as nonconfluent RPE cells become confluent (21). Also, during development of cell-cell adhesion of epithelial GE11 cells, GnT2-III, the enzyme that stops branching of N-glycans is up-regulated (22).

These data suggest that changes in the structure of the N-glycans linked to adhesion molecules might be expected to affect intercellular adhesion. Because N-glycans linked to the Na,K-ATPase β1 subunit are required for the initial steps of formation of intercellular contacts (11), it is possible that complexity of N-glycans of this subunit also changes with development of cell-cell junctions and a specific N-glycan structure is necessary for normal cell to cell adhesion.

To examine these issues, we altered the extent of N-glycan branching either by exposure of MDCK cells in culture to swainsonine, an inhibitor of N-glycan branching, or by post-transcriptional silencing the gene encoding GnT-III, a critical N-glycan stop-branching enzyme, using RNA interference and assessed the impact of these perturbations on cell-cell adhesion. The results of these studies demonstrate that prevention of N-glycan branching by swainsonine tightens and stabilizes cell-cell junctions. Conversely, increased N-glycan branching due to partial silencing of the gene encoding GnT-III, loosens cell-cell contacts. Consistent with these observations, the normal development of cell-cell adhesion is associated with reduced complexity of N-glycans of the Na,K-ATPase β1 subunit and other adhesion proteins. These changes correlate with increased expression of GnT-III, the enzyme that reduces branching, and decreased expression of GnT-IVC and GnT-V, enzymes that promote branching. These results suggest tightness of cell junctions can be modulated through modification of N-glycans.

The tightening and stabilizing effects of swainsonine are attenuated in a cell line expressing the unglycosylated mutant of the Na,K-ATPase β1 subunit; findings indicating that the structure of the N-glycans linked to this subunit is important for normal cell-cell adhesion.

EXPERIMENTAL PROCEDURES

Construction of MDCK Stable Cell Lines—Stable cell lines expressing a fusion protein with YFP linked to the N terminus of the Na,K-ATPase β1 subunit (YFP-β1) and mutated YFP-β1 fusion protein lacking three N-glycosylation sites (N123) were obtained as described previously (11, 23). Non-transfected MDCK cells and cells expressing YFP-β1 and N123 were grown in Corning Costar polyester transwell inserts (Corning Inc.) in 6-well plates in Dulbecco’s Modified Eagle’s medium (DMEM) (Cellgro).

Primary Antibodies—The following monoclonal antibodies were used for Western blot analysis: against E-cadherin (Alexis Biochemicals), against the Na,K-ATPase β1 subunit, clone M17-P5-F11 (Affinity Bioreagents), against GFP, clones 7.1 and 13.1, that also recognized YFP (Roche Applied Sciences), against β1-integrin (BD Transduction Laboratories), and against the Na+/Ca2+ exchanger (Novus Biologicals, Inc). Also, a polyclonal antibody against polycystin-2 (Chemicon International) was used.

Cell Culture—Cells were grown in DMEM (Cellgro Media-tech) containing 4.5 g/liter glucose, 2 mM L-glutamine, 8 mg/liter phenol red, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 10% FBS unless specified otherwise.

Isolation of Basolateral or Total Plasma Membrane Proteins of MDCK Cells using Surface-specific Biotinylation—Cells were maintained for 6 days after becoming confluent in transwell inserts. Biotinylation of surface proteins was performed according to previously described procedures (24, 25). Cell monolayers were biotinylated with EZ-Link™ Sulfo-NHS-SS-biotin (Pierce) that was added either into the well only (basolateral surface of the tight cell monolayers) or into both insert and well (total surface of the dispersed cells). After quenching the biotinylation reaction, cells were washed and then lysed by incubation with 200 μl of 0.15 M NaCl in 15 mM Tris pH 8.0 with 1% Triton X-100 and 4 mM EGTA. Cell extracts were clarified by centrifugation (15,000 × g, 10 min) at 4 °C. To isolate surface-biotinylated proteins, the cell extract was incubated with 100 μl of streptavidin-agarose beads (Sigma) in a total volume of 1 ml of 0.15 M NaCl in 15 mM Tris, pH 8.0 with 0.5% Triton X-100 and 4 mM EGTA at 4 °C with continuous rotation for 60 min. The bead-adherent complexes were washed three times on the beads, and then proteins were eluted from the beads by incubation in 40 μl of SDS-PAGE sample buffer (4% SDS, 0.05% bromphenol blue, 20% glycerol, 1% β-mercaptoethanol in 0.1 M Tris, pH 6.8) for 5 min at 80 °C.

Preparation of Crude Microsomal Membranes from Kidney and Intestine—Rabbit kidney and small intestine were homogenized with a tight Dounce homogenizer (Wheaton, Millville, NY). The homogenate was collected, layered onto a 42% sucrose solution, and spun in a Beckman SW28 swinging bucket rotor at 25,000 rpm for 1 h at 4 °C. The fraction at the interface of buffer/sucrose was collected and diluted to a total volume of 15 ml in buffer A. This membrane-enriched fraction was collected by centrifugation in a Beckman 75Ti rotor (35,000 rpm, 4 °C, 1 h). The pellet was resuspended in 10 mM PIPES/TRIS buffer containing 2 mM EGTA and 2 mM EDTA, pH 7.0 by homogenization with a 2 ml of Teflon homogenizer (Wheaton). Protein concentration was determined by the modified Lowry protein assay reagent (Pierce). The typical protein concentration was 5–10 μg/μl. The membranes were aliquoted, flash-frozen, and stored at −80 °C.

Confocal Microscopy—Confocal microscopic images were acquired using the Zeiss LSM 510 laser scanning confocal microscope and LSM 510 software, version 3.2.
Western Blot Analysis of the Total and Plasma Membrane Resident Proteins of MDCK Cells and Microsomal Membranes from Kidney and Small Intestine—To equalize the amounts of proteins in cell extracts, the confluent cells from one well insert and the dispersed cells from three well inserts were collected in 200 µl of the lysis buffer. Samples containing 20 µl of the MDCK cell extract mixed with 15 µl of SDS-PAGE sample buffer, or 1 µg of microsomal membrane proteins in 20 µl of SDS-PAGE sample buffer, or biotinylated proteins eluted from the agarose-streptavidin beads were loaded onto 4–12% gradient SDS-PAGE gels (Invitrogen). Proteins were separated by SDS-PAGE using MES/SDS running buffer (0.05 M MES, 0.05 M Tris base, 0.1% SDS, and 1 mM EDTA free acid, pH 7.3), transferred onto a nitrocellulose membrane and detected by Western blot analysis using the appropriate primary antibody and the anti-mouse, anti-rabbit, or anti-rat IgG conjugated to alkaline phosphatase (Promega) as a secondary antibody. Alkaline phosphatase was detected using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate in alkaline phosphatase buffer (150 mM NaCl, 1 mM MgCl₂ in 10 mM Tris-HCl, pH 9.0). Immunoblots were quantified by densitometry using Kodak 1D 3.6 software.

Analysis of N-glycans of the E-cadherin and the Na,K-ATPase β₁ Subunit using Endoglycosidases—MDCK cells, either dispersed or organized into tight monolayers, were biotinylated as described above. The biotinylated proteins adherent to agarose-streptavidin beads obtained from a single well insert (tight monolayers) or three well inserts (dispersed cells) were treated with the following glycosidases: 30 milliunits/ml EndoH (Prozyme) or 30,000 units/ml PNGase F (New England Biolabs). The reaction mixtures containing bead-adherent biotinylated proteins of MDCK cells, the appropriate buffers and detergents in the total volume of 50 µl were incubated for 3 h at 37 °C according to the manufacturer’s instructions. Then the reaction mixtures were diluted with 30 µl of the SDS-PAGE sample buffer and incubated for 5 min at 80 °C. The aliquots were loaded onto SDS-PAGE gels followed by Western blot analysis using the appropriate antibodies.

Treatment of MDCK Cells by Tunicamycin, Swainsonine, and Sialidase—The MDCK cell monolayers, grown on the transwell inserts for 4 days after the cells became confluent, were incubated with 1 µg/ml tunicamycin C₁ homolog (Sigma) for 48 h, or 2 µg/µl swainsonine (Sigma) for 48 h, or 5 units/µl sialidase (Prozyme) for 18 h.

Paracellular Permeability of Cell Monolayers—MDCK cell monolayers grown on transwell porous inserts were incubated in DMEM without phenol red and without FBS (Cellgro Mediatech) that was added into the well (lower chamber) and insert (upper chamber). The fluorescent membrane-impermeable dye, BCECF free acid (10 µM), was added into the lower chamber. Accumulation of the dye in the upper chamber was determined by taking 50-µl aliquots, diluting them in 3 ml of PBS, pH 7.2 and measuring the fluorescence intensity every 30 min during cell incubation at room temperature for 2 h. Accumulation of BCECF in the upper chamber reflects paracellular flux of the dye through the monolayer since this dye is membrane-impermeable and, therefore, can penetrate the monolayer only between the cells. The slope of the linear regression of the fluorescence intensity plotted versus time was used as a measure of the paracellular permeability. Typically, the paracellular permeability of the tight monolayer is about 100-fold less than the paracellular permeability of subconfluent cells or cells incubated in Ca²⁺-free buffer where cell junctions are fully disrupted (supplemental Fig. S1).

Reverse Transcription (RT) Real-time Quantitative Polymerase Chain Reaction—Total RNA from MDCK cells, either sparsely plated, or organized into the tight monolayers, was isolated using RNAqueous (Ambion). Typical RNA concentrations were 100–200 ng/µl. The RNA was measured for purity and stability with a Bioanalyzer 2100 (Agilent Technologies). 2000 ng of total RNA in total 40 µl of volume were converted to cDNA by use of Omniscript RT Kit (Qiagen) and an oligo(dT) 12–18 primer (Invitrogen), according to the to the manufacturer’s protocol. 1 µl of RT product was amplified by real-time PCR using the SYBR Premix ExTaq Perfect Real-Time PCR kit (TaKaRa), according to the manufacturer’s protocol. The primers used are shown in supplemental Table S1. Real-time PCR was performed in eight-well strips using a DNA Engine Opticon 2 (MJ Research). The cycle of threshold (Ct) was determined for each primer set. The efficiency of amplification was determined by generating a standard curve for each primer pair using a corresponding PCR product as a template. The PCR products were purified by using the MinElute Gel Extraction Kit (Qiagen). The range of PCR product concentrations was chosen for each primer set so that the standard curve included the values of Cₑ observed for the RNA samples isolated from both tight monolayers and dispersed cells. The resulting values of Cₑ were plotted against the logarithm of the PCR product copy number (supplemental Fig. S2). Quantification of the mRNA levels in the tight monolayers relative to that in the dispersed cells was performed as described previously (26) using the Na,K-ATPase β₁ subunit mRNA as a reference. The cycle of threshold for the Na,K-ATPase β₁ subunit (Cₑ) was 13.3 ± 0.38 and 13.2 ± 0.25 in the dispersed cells and tight monolayers, respectively. All reactions were carried out in duplicates, and three separate mRNA isolations were performed.

GnT-III and GnT-IVC Gene Silencing using siRNA—MDCK cells were grown on porous well inserts for 2 days after becoming confluent in the regular DMEM with 10% FBS. Two then cells were rinsed with the medium without FBS and antibiotics and transfected with the dicer-substrate siRNA (IDT). The sequences of siRNAs are shown in supplemental Table S2. 100 pmol of siRNA were added to 250 µl of serum-free DMEM. In a separate tube, 6 µl of Lipofectamine 2000 (Invitrogen) were added to 250 µl of serum-free DMEM. Diluted siRNA and Lipofectamine were incubated at room temperature for 5 min, then mixed with each other and incubated for additional 20 min. The mixture was diluted with 1.5 ml of serum-containing DMEM and added to the cells (1.5 ml to the lower chamber and 0.5 ml to the upper chamber). The efficiency of transfection was close to 100% as detected by using a fluorescently labeled transfection control (IDT) (supplemental Fig. S3). The next day, the medium was changed to regular serum-containing DMEM. The transfection procedure was repeated 2 days later. The measurement of the paracellular permeability was performed on the day 6
RESULTS

Branching of N-glycans Is Reduced with Development of Cell-Cell Junctions—MDCK cells sparsely plated on the porous well inserts (Fig. 1A, dispersed cells) spread over the surface, replicate, and migrate. Cell migration is abruptly inhibited after formation of the confluent monolayer (Fig. 1A, Day 0). However, cells of the confluent monolayer continue to divide over about a week until they reach a critical density and stop replicating. They increase in number 6-fold, decrease in horizontal dimensions 2.4-fold (Fig. 1A, Day 6) and increase in height about 6-fold (not shown). In parallel, cells of the monolayer developed competent tight junctions as detected by a gradual decrease in paracellular permeability to the membrane-impermeable dye, BCECF, free acid, gradually decreases during maturation of the confluent cells for 3 days and then is maintained at the same level until day 6. As detected by a Western blot analysis, the gel mobility of E-cadherin and the Na,K-ATPase β1 subunit gradually increases during the development of the tight MDCK cell monolayer from the dispersed cells. (Fig. 2, A lane 1, B lane 1, and C lane 1) as does resistance to detergent extraction (lanes 2, 4, 6, and 8). Where indicated, the cells were treated with 0.25% Triton X-100 and washed with PBS before cell lysis. Error bars ± S.D. (n = 4), * significant difference with Day 6, p < 0.01, Student’s t test.

Also, gel mobility of E-cadherin and the Na,K-ATPase β1 subunit was greater in rabbit kidney that contains relatively tight epithelia as compared with the leaky epithelium of small intestine (Fig. 2, lanes 1 and 2). These results suggest that there is a correlation between the tightness of epithelia in both cultured cells and animal tissues and gel mobility of E-cadherin and the Na,K-ATPase β1 subunit. A similar correlation was detected for several other cellular glycoproteins, β1-integrin (Fig. 2, lanes 3 and 4), Na+/Ca2+ exchanger and polycystin-2 (not shown), suggesting that the N-glycosylation status of cellular glycoproteins is different in tight and leaky epithelia. Consistent with this interpretation, deglycosylation of the Na,K-ATPase β1 subunit from the dispersed cells and tight monolayers using PNGase F resulted in the products having the same gel mobility (Fig. 3, lanes 1 and 6), β1-integrin subunit had the same gel mobility (not shown).

Statistical analysis was performed using Student’s t test (GraphPad Prism 4 software and Microsoft Excel). Statistical significance is specified in the figure legends.

FIGURE 2. Electrophoretic gel mobility of E-cadherin, β1-integrin and the Na,K-ATPase β1 subunit isolated from kidney is greater than that of the proteins isolated from small intestine. Microsomal membrane proteins isolated from kidney and small intestine were analyzed by SDS-PAGE followed by Western blot analysis. The intestinal Na,K-ATPase β1 subunit, E-cadherin, and β1-integrin migrate more slowly (lanes 1 and 3, dotted arrows) than the corresponding proteins of the kidney (lanes 2 and 4, solid arrows).
It is known that virtually all types of N-linked glycans are removed from glycoproteins by PNGase F, while glycoproteins containing complex N-glycans are resistant to EndoH cleavage. The analytical cleavage of surface proteins of MDCK cells by these glycosidases showed that the Na,K-ATPase \( \beta_1 \) subunit contains only complex N-glycans (Fig. 3) in agreement with previously published results (27), while E-cadherin contains at least one hybrid or high-mannose N-glycan and at least one complex-type N-glycan (Fig. 3) consistent with recently published data on N-glycan composition of the human E-cadherin expressed in CHO cells (20). Importantly, susceptibility to glycosidases of E-cadherin and the Na,K-ATPase \( \beta_1 \) subunit is the same as in dispersed cells (lanes 1–3), showing that the type of N-glycosylation of both E-cadherin and the Na,K-ATPase \( \beta_1 \) subunit is not change during formation of the tight monolayer from the dispersed cells. The EndoH products of both E-cadherin and Na,K-ATPase \( \beta_1 \) subunit of dispersed cells migrate more slowly (lane 3, dotted arrow) than the corresponding proteins of the tight monolayers (lane 5, solid arrows) indicating that the composition of complex-type N-glycans becomes less complex during formation of the tight monolayer from the dispersed cells.

Reduced Branching of N-glycans Promotes Cell-Cell Adhesion

The analytical cleavage of surface proteins of MDCK cells by these glycosidases showed that the Na,K-ATPase \( \beta_1 \) subunit contains only complex N-glycans (Fig. 3) in agreement with previously published results (27), while E-cadherin contains at least one hybrid or high-mannose N-glycan and at least one complex-type N-glycan (Fig. 3) consistent with recently published data on N-glycan composition of the human E-cadherin expressed in CHO cells (20). Importantly, susceptibility to glycosidases of E-cadherin and the Na,K-ATPase \( \beta_1 \) subunit is the same as in dispersed cells (lanes 1–3), showing that the type of N-glycosylation of both E-cadherin and the Na,K-ATPase \( \beta_1 \) subunit is not change during formation of the tight monolayer from the dispersed cells. The EndoH products of both E-cadherin and Na,K-ATPase \( \beta_1 \) subunit of dispersed cells migrate more slowly (lane 3, dotted arrow) than the corresponding proteins of the tight monolayers (lane 5, solid arrows) indicating that the composition of complex-type N-glycans becomes less complex during formation of the tight monolayer from the dispersed cells.

Various glycosyltransferases generate highly diverse N-glycans. Four of them, Golgi GnT III to VI are known to be responsible for variations in the number of branches in N-glycans (Fig. 4A). GnTs IV, V, and VI promote branching of N-glycans, while GnT-III stops branching. The latter enzyme can add the bisection GlcNAc to hybrid or complex N-glycans and prevent the downstream action of the branching enzymes. For example, the action of this enzyme on the product of the glycosyltransferase II results in the formation of bi-antennary complex N-glycans (Fig. 4A, bottom).

All chains where the original mannos residues are substituted by GlcNAc residues, except for those formed by GnT-III, can be elongated due to the action of other various glycosyltransferases (Fig. 4A, dashed arrows) that compete with each other for the N-glycan substrate. As a result, individual branches vary in the length and carbohydrate composition. The most common extension of individual branches occurs due to the multiple linkages of sialic acid residues by sialyltransferases. Individual branches can be also significantly elongated as a result of polylactosamine extensions. The key enzyme that contributes to polylactosamine synthesis is \( \beta \)-1,4-galactosyltransferase.

To test which of these glycosyltransferases contribute to the changes in N-glycosylation observed upon intercellular adhesion, we compared the levels of mRNA encoding the enzymes in the tight monolayers to those in dispersed cells using real-time PCR. The level of the Na,K-ATPase \( \beta_1 \) subunit mRNA was the same in tight monolayers and dispersed cells, providing an internal control. Similarly, the levels of \( \beta \)-1,4-galactosyltransferase and sialyltransferase, the enzymes responsible for elongation of N-glycan branches, did not change upon cell-cell adhesion (Fig. 4B).

In contrast, the levels of mRNA encoding the enzymes that promote branching, GnT-IVC and GnT-V, were reduced by (83 ± 6)% and (50 ± 10)%, respectively, while the level of the mRNA encoding an enzyme that stops branching, GnT-III, was increased (4.4 ± 1.7)-fold at the day 6 of the tight monolayer development as compared with the dispersed cells (Fig. 4B). The increase in the mRNA level of GnT-III and decrease in GnT-IVC developed gradually, starting from the day when cells became confluent, while a significant decrease in GnT-V was observed only at the day 6 (supplemental Fig. S4). These changes in mRNA and corresponding changes in the amount of the three enzymes, GnT-III, GnT-IVC, and GnT-V, would result in an increase in gel mobility of glycoproteins upon formation of the tight cell monolayer from dispersed cells due to a reduction in the number of branches in N-glycans. Therefore, the data on the differential expression levels of glycosyltransferases in the dispersed cells and the tight cell monolayers are consistent with the observed differences in N-glycosylation of the E-cadherin and the Na,K-ATPase \( \beta_1 \) subunit in the dispersed cells and the tight cell monolayers (Fig. 3, lanes 2 and 4).

Reduced Branching of N-glycans Promotes Cell-Cell Adhesion in Epithelia—The following approaches to modify N-glycosylation of cellular proteins were employed. To prevent N-glycosylation of newly synthesized proteins, cells were treated with tunicamycin, an inhibitor of N-glycosylation which prevents the synthesis of the glycosylated lipid precursor of N-glycans. As expected, the glycosylated Na,K-ATPase \( \beta_1 \) subunit was replaced by non-glycosylated subunit in the inhibitor-treated
**N-glycan Branching of Na,K-ATPase β₁ and Cell-Cell Adhesion**

The decreased complexity of N-glycans in tight MDCK cell monolayers correlates with up-regulation of the genes encoding the enzymes that stop branching of N-glycans and down-regulation of the genes encoding enzymes that facilitate branching of N-glycans. A simplified scheme showing the role of GnT in N-glycan branching. Six different GnTs (I–VI) can add N-acetylglucosamine (GlcNAc) residues to the three-mannosyl core of N-glycans (green rectangle) and start the formation of diverse structures. Addition of GlcNAc residues, except for those added by GnT-III, allows elongation of the chains, referred as antennae, with additional monosaccharide linkages due to the action of other glycosyltransferases (dashed arrows). GnT-I is a key regulatory enzyme that initiates formation of hybrid and complex type N-glycans, while GnT-II initiates formation of the complex-type N-glycans. GnTs IV, V, and VI promote branching of N-glycans by adding GlcNAc into the positions shown by different shades of blue. The individual N-glycans can be modified by one, two, or all three of these enzymes resulting in two to five-antennary N-glycans. In contrast, GnT-III (red) stops branching by adding a bisecting GlcNAc to any hybrid or complex N-glycan structures shown on the scheme that prevent downstream action of GnTs II, IV, V, and VI. A similar stop-branching effect can be achieved by incubation of cells with the Golgi mannosidase II inhibitor, swainsonine (Sw), that prevents mannose trimming and consequent action of GnT-II, resulting in preservation of the hybrid type structure of N-glycans.

**FIGURE 4.** The decreased complexity of N-glycans in tight MDCK cell monolayers correlates with up-regulation of the genes encoding the Golgi enzymes stop branching of N-glycans and down-regulation of the genes encoding enzymes that facilitate branching of N-glycans. A, simplified scheme showing the role of GnT in N-glycan branching. Six different GnTs (I–VI) can add N-acetylglucosamine (GlcNAc) residues to the three-mannosyl core of N-glycans (green rectangle) and start the formation of diverse structures. Addition of GlcNAc residues, except for those added by GnT-III, allows elongation of the chains, referred as antennae, with additional monosaccharide linkages due to the action of other glycosyltransferases (dashed arrows). GnT-I is a key regulatory enzyme that initiates formation of hybrid and complex type N-glycans, while GnT-II initiates formation of the complex-type N-glycans. GnTs IV, V, and VI promote branching of N-glycans by adding GlcNAc into the positions shown by different shades of blue. The individual N-glycans can be modified by one, two, or all three of these enzymes resulting in two to five-antennary N-glycans. In contrast, GnT-III (red) stops branching by adding a bisecting GlcNAc to any hybrid or complex N-glycan structures shown on the scheme that prevent downstream action of GnTs II, IV, V, and VI. A similar stop-branching effect can be achieved by incubation of cells with the Golgi mannosidase II inhibitor, swainsonine (Sw), that prevents mannose trimming and consequent action of GnT-II, resulting in preservation of the hybrid type structure of N-glycans. B, formation of the tight monolayer from dispersed cells is accompanied by an increase in the levels of expression of the genes encoding GnT-III and a decrease in the levels of expression of GnT-IVC and GnT-V as determined by RT real-time PCR of RNA samples isolated from dispersed cells and from tight cell monolayers grown for 6 days after confluence. Quantification of the results was performed as described under "Experimental Procedures" and demonstrated in supplemental Fig. S2. Abbreviations: GT, UDP-GalbetaGlcNAc β-1,4-galactosyltransferase 1, membrane-bound form; ST, β-galactoside-α-2,3-sialyltransferase. * expression of GnT-VI has not been tested because the sequence of this enzyme in dogs is unknown. Error bars ± S.D. (n = 3). **, p < 0.001, Student's t test.

Both E-cadherin and the Na,K-ATPase β₁ subunit were resistant to treatment of the tight MDCK cell monolayers by 0.25% Triton X-100 (Fig. 6, lane 2) but were partially extracted by 0.5% detergent (Fig. 6, lane 3) and largely removed by 0.75% Triton X-100 (Fig. 6, lane 4). In the swainsonine-treated cells, the Na,K-ATPase β₁ subunit was more resistant to extraction.
N-glycan Branching of Na,K-ATPase β₁, and Cell-Cell Adhesion

A

B

C

FIGURE 5. Prevention of branching of N-glycans by swainsonine increases tightness of the MDCK cell monolayer, while silencing of the gene encoding a stop-branching enzyme, GnT-III, loosens cell-cell contacts. Tight monolayers of MDCK cells were grown on porous transwell inserts without inhibitors for 4 days after the cells became confluent and then for 2 days in the absence or presence of 2 μg/ml swainsonine or 1 μg/ml tunicamycin. As indicated, other cells were transfected with either scrambled siRNA (negative control) or with siRNA against GnT-III. Transfection was performed as described under “Experimental Procedures.” A, electrophoretic mobility of the Na,K-ATPase β₁ subunit was increased in the cells treated with tunicamycin and swainsonine and decreased in the cells transfected with siRNAs against GnT-III as detected by Western blot analysis. B, decrease in the mRNA level of GnT-III by siRNAs as detected by Western blot analysis. C, treatment of cells with tunicamycin significantly increased, while swainsonine decreased the paracellular permeability of the cell monolayers. Transfection of cells with three different siRNAs against GnT-III significantly increased the paracellular permeability. Abbreviations: Com., complex; Hyb., hybrid; H-M, high-mannose; and D-G, deglycosylated. Error bars ± S.D. (n = 3). *, p < 0.01, Student’s t test.

by increasing detergent concentrations, while E-cadherin was completely resistant to detergent extraction at concentrations up to 0.75% (Fig. 6, lanes 5–8). The relative resistance of cellular proteins to detergent extraction is probably due to their association with the cytoskeleton. Therefore, the increased detergent resistance of the E-cadherin and the Na,K-ATPase β₁ subunit in the swainsonine-treated cells suggests that reduced branching of N-glycans increases stability of association of the adherens junctional complex with the cytoskeleton.

To test whether the effect of swainsonine on cell-cell adhesion of MDCK cells is due to the reduced content of the sialic acid residues in the hybrid type N-glycans as compared with the complex-type N-glycans, MDCK cells grown and polarized on porous well inserts were incubated in the presence or absence of sialidase overnight. Sialidase treatment increased gel mobility of the Na,K-ATPase β₁ subunit and E-cadherin indicating that some sialic acid residues were cleaved from the surface N-glycans, but this did not affect the detergent resistance of the Na,K-ATPase β₁ subunit and E-cadherin and the paracellular permeability of the MDCK cell monolayer (data not shown). Therefore, the effect of swainsonine on cell-cell adhesion of MDCK cells does not appear to be a result of the reduced content of sialic acid residues in glycoproteins of the inhibitor-treated cells.

To test whether swainsonine affects cell motility, tight monolayers of the MDCK cells were damaged to provoke cell migration into the wound area (Fig. 7). Cell migration was inhibited by 46% in swainsonine-treated cells as quantified by measuring the area of the wound covered by migrated cells during “wound healing” process.

The Degree of Branching of N-glycans of the Na,K-ATPase β₁ Subunit Modulates the Efficacy of Cell-Cell Adhesion—Remodeling of N-glycan branching affects stability and integrity of the cell-cell junctions, suggesting that the structure of N-glycans linked to particular cellular glycoproteins is important for intercellular adhesion. It is possible that one of these glycoproteins is the Na,K-ATPase β₁ subunit, because N-glycans of this subunit were found important for cell-cell contact formation (11). To test if the structure of N-glycans of this subunit is important for cell-cell adhesion, we used the cell line, in which 55% of the endogenous Na,K-ATPase β₁ subunit is replaced by the unglycosylated mutant of this subunit, N123, while the abundance of the of the Na,K-ATPase α-subunits on the lateral membrane is not changed (11).

The unglycosylated mutant of the Na,K-ATPase β₁ subunit was less resistant to extraction by Triton X-100 from the basolateral membrane than the normally glycosylated β₁ subunit (Fig. 8A, lanes 1–4 and Fig. 6A, lanes 1–4) consistent with previous published results (11). Remarkably, E-cadherin was also more easily removed from the basolateral plasma membrane by Triton X-100 in the cell line expressing the N123 mutant com-
the detergent resistance of E-cadherin was decreased in tunicamycin-treated cells (not shown). These results suggest that the N-glycans of the Na,K-ATPase β₁ subunit are important for stabilization of adherens junctions by cytoskeletal elements. Consistent with this interpretation, both overexpression of unglycosylated mutant of the Na,K-ATPase β₁ subunit and exposure of cells to tunicamycin significantly distorted intercellular junctions in the middle section of the cells and slightly distorted them in the supapical region (Fig. 9).

As expected, detergent resistance of the unglycosylated form of the Na,K-ATPase β₁ subunit was not affected by swainsonine (Fig. 8). Susceptibility of E-cadherin to detergent also was not significantly changed in the cells exposed to swainsonine, suggesting that the presence and degree of branching of N-glycans of the Na,K-ATPase β₁ subunit are important for stability of E-cadherin to detergent extraction.

The paracellular permeability of the monolayer formed by the cells expressing the unglycosylated mutant of the Na,K-ATPase β₁ subunit was significantly increased compared with that in non-transfected cells (Fig. 10), suggesting that N-glycans of this subunit are also important for the integrity of the tight junctions. Swainsonine decreased the paracellular permeability in both non-transfected and the mutant-expressing cell lines (Fig. 10A). However, the swainsonine-induced decrease in the permeability was significantly less in the transfected cell line than in non-transfected cells (Fig. 10A, inset). Therefore, the tightening effect of swainsonine in non-transfected cells, at least in part, is related to reduction of N-glycans linked to the Na,K-ATPase β₁ subunit.

The lack of N-glycans on the β₁ subunit does not affect the activity of the Na,K-ATPase (28). Detergent resistance, the paracellular permeability and sensitivity to swainsonine of the MDCK cell line expressing the wild type YFP-β₁ was the same as in the non-transfected cells (not shown). Therefore, the decreased sensitivity to swainsonine in the mutant cells is related to the lack of N-glycans in the β₁ subunit and is not due to the nonspecific effects of transfection or impaired Na,K-ATPase activity.

**DISCUSSION**

The results of the present study show that the structure of N-glycans linked to adhesion proteins affect the process of intercellular adhesion and elaboration of a tight epithelium. Exposure of MDCK cell monolayers to the inhibitor of N-glycosylation, tunicamycin, significantly increases the paracellular

**N-glycan Branching of Na,K-ATPase β₁, and Cell-Cell Adhesion**

*JOURNAL OF BIOLOGICAL CHEMISTRY*
N-glycan Branching of Na,K-ATPase β1, and Cell-Cell Adhesion

FIGURE 8. Prevention of glycosylation of the Na,K-ATPase β1 subunit decreases detergent resistance of E-cadherin and abolishes a swainsonine-induced increase in detergent resistance of E-cadherin. A, tight monolayers of MDCK cells expressing unglycosylated mutant of the Na,K-ATPase β1 subunit were grown in the absence (lanes 1–4) or presence of swainsonine (lanes 5–8) and treated with Triton X-100 as described in the legend to Fig. 6. As expected, swainsonine did not change gel mobility of N123, but slightly increased gel mobility of E-cadherin. Swainsonine did not affect detergent stability of either E-cadherin or the mutated Na,K-ATPase β1 subunit. B and C, quantification of the results shows that both unglycosylated mutant of the Na,K-ATPase β1 subunit, N123 (B), and E-cadherin (C) resident in the basolateral plasma membrane are less resistant to extraction by Triton X-100 in the cell line expressing N123 mutant as compared with non-transfected MDCK cells (Fig. 6). Swainsonine did not change detergent resistance of the unglycosylated Na,K-ATPase β1 subunit (B) and E-cadherin (C) in the mutant-expressing cell line, in contrast to the protective effect of the inhibitor in non-transfected cells (Fig. 6). Error bars ± S.D. (n = 3); Significant difference with non-transfected MDCK cells: *p < 0.05; Student’s t test. **p < 0.001, Student’s t test.

Therefore, the lack or modification of N-glycans probably affects the tight junctions through changes in the adherens junctions.

This interpretation is consistent with numerous reports on cross-talk between the adherens and tight junctions. The assembly of the tight junctions is always preceded by the formation of the adherens junctions and can be inhibited by an E-cadherin-blocking antibody (30–32). Conversely, disruption or weakening of the adherens junctions by depletion of E-cadherin and catenins or by chelating Ca2+ in the culture medium causes partial or complete disassembly of the tight junctions (33, 34). Therefore, the integrity of the tight junctions depends on the intactness and stability of the adherens junctions. The integrity of the tight junctions is maintained and regulated by a variety of signaling pathways that are initiated by the cytoplasmic components of the adherens junctions such as β-catenin and afadin (7, 29).

Although swainsonine should modify all cellular glycoproteins, the increase in both tightness of the monolayer and stability of E-cadherin to detergent extraction observed after exposure to swainsonine is attenuated in the cell line expressing the unglycosylated Na,K-ATPase β1 subunit, observations confirming the tightening and stabilizing effects of the inhibitor in non-transfected cells are related, at least in part, to the action of swainsonine on the N-glycan branching of the Na,K-ATPase β1 subunit.
During the progression of MDCK cells from individually attached dispersed cells to a mature cell monolayer, the complexity of N-glycans linked to E-cadherin and Na-K-ATPase is reduced. This reduction appears to reflect the decrease in N-glycan branching due to changes in expression of the enzymes that determine the degree of N-glycan branching, GnT-III, GnT-IVC, and GnT-V. These changes occur in parallel with a gradual decrease in the paracellular permeability of the epithelium. Furthermore, inhibiting the expression of a stop-branching enzyme, GnT-III, by RNA interference causes an increase in paracellular permeability of the mature cell monolayer, indicating that reduced branching of N-glycans is an important element of the complex process of formation and maintenance of the tight junctions.

These findings suggest that modification of N-glycans could serve as a mechanism whereby inherent paracellular permeability of epithelia of different tissues is modified. In support of this possibility, the N-glycans of the Na,K-ATPase β1 subunit, E-cadherin, and other glycoproteins isolated from the renal collecting duct, epithelia known to have a high electrical resistance, are less complex than those of the glycoproteins obtained from the relatively leaky intestinal epithelium.

Reducing N-glycan branching by treatment with swainsonine suppresses motility of MDCK cells as detected by the “wound healing” assay. This observation is consistent with the suppression of fibroblastic cell motility and invasiveness of malignant cells induced by measures that reduce N-glycan branching (35–38).

Formation of a tight cell monolayer from dispersed MDCK cells is accompanied by changes in cell motility. Dispersed MDCK cells that are more motile compared with the cells comprising the tight monolayer manifest glycoproteins with a relatively high degree of N-glycan branching. This is consistent with the data on correlation between high motility of fibroblas-

tic and malignant cells and increased branching of N-glycans (39–43). Formation of cell-cell contacts between dispersed cells induces changes in the expression levels of GnTs III, IVc, and V and results in a decrease in N-glycan branching. Reduced branching would inhibit cell migration. In addition, reducing N-glycan branching facilitates intercellular adhesion by tightening and stabilizing cell-cell junctions, which, in turn, causes additional inhibition of cell motility.

Therefore, reducing N-glycan branching by modulating activities of glycosyltransferases may contribute to the contact inhibition of locomotion, an important property of normal epithelial cells. Cancer cells may lack the ability to regulate expression of glycosyltransferases upon cell-cell contact and fail to reduce branching of N-glycans when they make contact. As a consequence, tumor cells would stay motile even when they reach confluence and would not form stable intercellular junctions.

The findings that exposure of MDCK cells in culture to swainsonine tightens cell monolayers could have important implications for the treatment of various disorders characterized by disruption of cell adhesion. The healing of the disrupted skin epithelia after injury or tubular integrity with acute kidney injury might be facilitated by treatment with swainsonine. This possibility remains to be examined.

Thus, the correlation between N-glycan branching, cell-cell adhesion and cell motility in epithelia may explain the biological requirement for heterogeneity of complex N-glycans in vertebrates. Highly branched N-glycans are needed to promote cell motility, while less branched N-glycans facilitate intercellular adhesion. The proposed mechanism of modulation of intercellular adhesion via remodeling of N-glycans, in particular those of the Na,K-ATPase β1 subunit, suggests a regulatory role of specific glycosyltransferases. The precise signaling pathways involved in relationship between cell motility, cell-cell adhesion and expression of glycosyltransferases remain to be elucidated.

Acknowledgment—We thank Dr. Jeff Kraut for careful reading of the manuscript and helpful suggestions.

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