Epithelial Junctions Depend on Intercellular Trans-interactions between the Na,K-ATPase β_1 Subunits*

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N-Glycans of the Na,K-ATPase β_1 subunit are important for intercellular adhesion in epithelia, suggesting that epithelial junctions depend on N-glycan-mediated interactions between the β_1 subunits of neighboring cells. The level of co-immunoprecipitation of the endogenous β_1 subunit with various YFP-linked β_1 subunits expressed in Madin-Darby canine kidney cells was used to assess β_1-β_1 interactions. The amount of co-precipitated endogenous dog β_1 was greater with dog YFP-β_1 than with rat YFP-β_1, showing that amino acid-mediated interactions are important for β_1-β_1 binding. Co-precipitation of β_1 was also less with the unglycosylated YFP-β_1 than with glycosylated YFP-β_1, indicating a role for N-glycans. Mixing cells expressing dog YFP-β_1 with non-transfected cells increased the amount of co-precipitated β_1, confirming the presence of intercellular (YFP-β_1)-β_1 complexes. Accordingly, disruption of intercellular junctions decreased the amount of co-precipitated β_1 subunits. The decrease in β_1 co-precipitation both with rat YFP-β_1 and unglycosylated YFP-β_1 was associated with decreased detergent stability of junctional proteins and increased paracellular permeability. Reducing N-glycan branching by specific inhibitors increased (YFP-β_1)-β_1 co-precipitation and strengthened intercellular junctions. Therefore, interactions between the β_1 subunits of neighboring cells maintain integrity of intercellular junctions, and alterations in the β_1 subunit N-glycan structure can regulate stability and tightness of intercellular junctions.

Intercellular tight and adherens junctions link individual cells in an epithelial cell monolayer to retard transepithelial diffusion, thus allowing regulated transport of solutes through intercellular spaces in response to appropriate stimuli. The tight junctions also separate the plasma membrane into the apical and basolateral domains and maintain polar distribution of plasma membrane transporters and channels, permitting vectorial transepithelial transport. Epithelial cells employ numerous proteins to form, maintain, and regulate these intercellular junctions. Some of these proteins are involved in intracellular signaling and regulation of cell-cell adhesion, whereas others are intrinsic structural components of junctional complexes.

The Na,K-ATPase plays essential roles in formation, stabilization, and regulation of intercellular junctions. Numerous studies demonstrated co-localization of the Na,K-ATPase with junctional proteins in epithelial cell monolayers. Inhibition of Na,K-ATPase activity by ouabain in various epithelia prevented tight junction formation, triggered disassembly of existing junctions, or increased their permeability (reviewed in Ref. 1). The ouabain-dependent effects on cell adhesion were similar to the effects detected upon incubation of cells at low K^+ concentration or in the presence of the Na^+-ionophore gramicidin that increased the intracellular concentration of Na^+ (2, 3), demonstrating that the maintenance of the ion balance by the Na,K-ATPase is crucial for intercellular junctions. It is also possible that Na,K-ATPase plays a role in regulating intercellular junctions via signaling in response to binding of endogenous ouabain and ouabain-like compounds to the extracellular domain of the Na,K-ATPase α_1 subunit. Several Na,K-ATPase-mediated signaling pathways are implicated in the regulation of intercellular junctions (reviewed in Refs. 1 and 4).

In addition, substantial experimental evidence supports a direct structural role of the Na,K-ATPase in formation and stabilization of intercellular junctions. Similar to cell adhesion molecules of adherens and tight junctions, the Na,K-ATPase is resistant to extraction by non-ionic detergents from epithelial cell monolayers (5–7). This resistance is due to the linkage of the enzyme to the F-actin-spectrin cytoskeleton via ankyrin (5, 6). As with all cell adhesion molecules, the Na,K-ATPase becomes resistant to the detergent only after formation of intercellular junctions (7), suggesting that Na,K-ATPase molecules of neighboring cells interact with each other in a cell monolayer. In agreement with this interpretation, immunoprecipitation of the YFP-linked rat β_1 subunit overexpressed in canine MDCK[2] cells resulted in co-immunoprecipitation of the endogenous β_1 subunit of normal rat kidney epithelial cells in mixed MDCK/normal rat kidney epithelial cell monolayers (8). Furthermore, cell junction formation between surface-attached MDCK cells was inhibited by an antibody against the extracellular domain of the β_1 subunit (9). Also, overexpression

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[2] The abbreviations used are: MDCK, Madin-Darby canine kidney cells; YFP-β_1 and YFP-β_2, fusion proteins between the yellow fluorescent protein and Na,K-ATPase β_1 and β_2 subunits, respectively; DMJ, deoxymannojirimycin; nLC, nano-liquid chromatography; PNGase F, peptide:N-glycosidase F.

of the Na,K-ATPase β_1 subunit facilitated formation of tight junctions in MDCK cells transformed by the Moloney sarcoma virus (10) and increased adhesiveness of non-polarized cells (11).

Overexpression of the unglycosylated Na,K-ATPase β_1 subunit, which was normally associated with the endogenous α_1 subunit and delivered to the lateral membranes, delayed formation of cell-cell contacts between dispersed MDCK cells (9). In addition, the overexpressed unglycosylated mutant of the β_1 subunit was significantly less resistant to detergent extraction from mature cell monolayers as compared with the overexpressed wild type β_1 subunit (9). Moreover, the endogenous α_1 subunit and E-cadherin were less resistant to the detergent in mutant-expressing cells as compared with non-transfected cells or to cells overexpressing the wild type β_1 subunit (7). Therefore, the *N*-glycans of the Na,K-ATPase β_1 subunit are important for stability of the junctional complex, suggesting that they mediate the intercellular trans-interactions between the β_1 subunits of neighboring cells.

To determine whether β_1-β_1 interaction is dependent on the presence of *N*-glycans or particular amino acid residues and whether this interaction is required for normal cell-cell adhesion, we investigated the effects of removing or modifying *N*-glycans of rat or dog YFP-linked β_1 subunits overexpressed in MDCK cells on 1) co-immunoprecipitation of these subunits with the endogenous β_1 subunits, 2) detergent resistance of adhesion proteins, and 3) permeability of intercellular junctions. The results indicate that *N*-glycans of the β_1 subunit and their structure are critical for integrity of intercellular junctions in MDCK cell monolayers due to their stabilizing effect on direct amino acid-mediated interactions between the extracellular domains of the β_1 subunits of neighboring cells.

EXPERIMENTAL PROCEDURES

Construction of MDCK Stable Cell Lines—The Na,K-ATPase rat β_1, dog β_1, the unglycosylated mutated rat β_1, the unglycosylated mutated dog β_1 and human β_2 subunits linked with their N termini to YFP were constructed as described previously (9, 12). Stable MDCK cell lines expressing wild type and mutated YFP-β_1 and YFP-β_2 were obtained as described previously (13).

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

Confocal Microscopy—Confocal microscopy images were acquired using the Zeiss LSM 510 laser scanning confocal microscope and LSM 510 software, version 3.2.

Primary Antibodies—The following monoclonal antibodies were used for immunoprecipitation and/or Western blot analysis: against the Na,K-ATPase α_1 subunit, clone C464.6 (Millipore); against GFP, clones 7.1 and 13.1, which also recognizes YFP (Roche Diagnostics); against the Na,K-ATPase β_1 subunit, clone M17-P5-F11 (Affinity Bioreagents); against β-catenin (BD Transduction Laboratories); against E-cadherin, clone DECMA (Sigma); and against occludin (Zymed Laboratories Inc.. Also, a polyclonal antibody against GFP, which recognizes YFP (Clontech), was used.

Isolation of Separated MDCK Cells—Confluent monolayers of MDCK cells expressing the YFP-linked dog β_1 subunit grown in 35-mm^2 wells of a 6-well plate were rinsed twice with PBS containing 1 mM EDTA and incubated with PBS for 60 min at 37 °C in CO_2 incubator. This incubation resulted in disruption of intercellular contacts and weakening of cell adhesion to the well. These separated cells were resuspended in PBS, isolated by centrifugation (1,500 $\times$ *g* for 5 min), and further used for Western blot analysis or immunoprecipitation. Alternatively, PBS was gently removed from the well with weakly attached separated cells and replaced by a complete cell culture medium to allow cell re-adhesion to the bottom of the well and neighboring cells. Re-formation of intercellular contacts was monitored by live time-lapse confocal microscopy during cell incubation in a cell culture medium at room temperature.

Immunoprecipitation Followed by Western Blot Analysis—Confluent MDCK cell monolayers were rinsed twice with ice-cold PBS and lysed by incubation with 200 μl/well of 150 mM NaCl in 50 mM Tris, pH 7.5, containing 1% Nonidet P-40, 0.5% sodium deoxycholate, and Complete Protease Inhibitor Mixture, 1 tablet/50 ml (Roche Diagnostics), at 4 °C for 30 min followed by scraping cells. Where indicated, cell monolayers were incubated with 2 μg/ml of swainsonine (Sigma) or 100 μg/ml of deoxymannojirimycin (DMJ) (Sigma) for 72 h prior to cell lysis. Separated MDCK cells were lysed by incubation of a dispersed cell pellet with the same lysis buffer at 4 °C for 30 min in a tube. Cell extracts were clarified by centrifugation (15,000 $\times$ *g*, 10 min) at 4 °C. Then, the cell extracts (400 μg of protein) were incubated with 30 μl of the protein A-agarose suspension (Roche Diagnostics) in a total volume 1 ml of the lysis buffer at 4 °C with continuous rotation for at least 3 h (or overnight) to remove the components that non-specifically bind to protein A. The pre-cleared cell extract was mixed with 2 μl of polyclonal antibodies against GFP/YFP (Clontech) and incubated with continuous rotation at 4 °C for 60 min. After addition of 30 μl of the protein A-agarose suspension, the mixture was incubated at 4 °C with continuous rotation overnight. Where indicated, this mixture was incubated at room temperature for 3 h with or without 200 milliunits/ml of neuraminidase (Prozyme), 150 milliunits/ml of β-galactosidase (Prozyme), 1 mM Ca^{2+}, or 1 mM EGTA. The bead-adherent complexes were washed on the beads first with the lysis buffer, then with 500 mM NaCl in 50 mM Tris, pH 7.5, containing 0.1% Nonidet P-40 and 0.05% sodium deoxycholate and finally with 10 mM Tris, pH 7.5, containing 0.1% Nonidet P-40 and 0.05% sodium deoxycholate. The adherent proteins were eluted from the beads by incubation in 45 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.

Where indicated, the bead-adherent proteins were deglycosylated by incubation with 30 μl of 50 mM sodium phosphate, pH 7.5, containing 0.16% SDS, 13 mM DTT, 1% Nonidet P-40, and 1 μl of PNGase F from *Flavobacterium meningosepticum* (New England BioLabs) for 1 h at 37 °C. After addition of 30 μl of SDS-PAGE sample buffer, the mixture was incubated for 5 min at 80 °C. Proteins eluted from the beads were separated by SDS-PAGE and analyzed by Western blot to detect immunoprecipitated and co-immunoprecipitated proteins by using monoclonal antibodies against GFP/YFP, Na,K-ATPase α_1 subunit, and the Na,K-ATPase β_1 subunit.

Western Blot Analysis of Total and Immunoprecipitated Proteins of MDCK Cells—MDCK cell extracts containing 1–10 μg of protein mixed with the equal volume of SDS-PAGE sample buffer or 5–20 μl of proteins eluted from the protein A-conjugated agarose beads were loaded onto 4–12% gradient SDS-PAGE gels (Invitrogen). Where indicated, cell lysates were treated by PNGase F from *F. meningosepticum* (New England BioLabs) according to the manufacturer's instructions prior to loading on SDS-PAGE. 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, pH 7.3), transferred onto a nitrocellulose membrane (Bio-Rad) and detected by Western blot analysis using the appropriate primary antibody and the anti-mouse or anti-rabbit secondary antibody conjugated to alkaline phosphatase (Promega) or horseradish peroxidase (American Qualex). Alkaline phosphatase was detected using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate in alkaline phosphatase buffer (150 mM NaCl, 1 mM $MgCl_2$ in 10 mM Tris-HCl, pH 9.0). Horseradish peroxidase was detected using the Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific). Immunoblots were quantified by densitometry using Zeiss LSM 510 software, version 3.2.

Immunoprecipitation Followed by Nano-Liquid Chromatography with Tandem Mass Spectrometry (nLC-MS/MS)—80 μl of the rabbit polyclonal antibodies against GFP/YFP (Clontech) were cross-linked to protein A-agarose beads (200 μl of bead suspension) using the Seize X Protein A Immunoprecipitation Kit (Thermo Scientific) according to the manufacturer's instructions. One-half of the antibody cross-linked beads was used for immunoprecipitation from the pre-cleared cell extracts of YFP-β_1-expressing cells, and the other half of the antibody cross-linked beads was used for immunoprecipitation from the pre-cleared cell extracts of non-transfected cells as described above. The eluted proteins were loaded on 4–12% reducing SDS-PAGE and run until the front had moved 1 cm. Then the lane was excised to perform an in-gel trypsin digest. The products of this digest were analyzed by nLC-MS/MS (14). The proteins identified in YFP-β_1-expressing cells, but not in non-transfected cells, were considered putative interacting partners of the β_1 subunit.

Detergent Resistance Assay of the Na,K-ATPase and Adherens Junction Proteins in MDCK Cell Monolayers—Cells grown on transwell inserts (Corning Inc.) were washed with PBS containing 1 mM Ca^{2+} and 1 mM Mg^{2+} twice and incubated with the PBS containing 1% digitonin for 30 min at room temperature. The digitonin solution was discarded, and cells were lysed as described in the immunoprecipitation procedure. In a parallel control sample, cells were lysed without digitonin pre-treatment. Cell lysates were analyzed by SDS-PAGE followed by Western blot using monoclonal antibodies against GFP/YFP, the Na,K-ATPase α_1 subunit, β-catenin, and E-cadherin.

Paracellular Permeability of Cell Monolayers—This was determined using a previously described procedure (7). Briefly, MDCK cell monolayers grown for 6 days after becoming confluent 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, 2,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-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 2,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein in the upper chamber reflects paracellular flux of the dye through the monolayer because this dye is membrane-impermeable and, therefore, can penetrate the monolayer only between the cells. The fluorescence intensity in the upper chamber was plotted *versus* incubation time. The paracellular permeability for each insert was calculated as a slope of the linear regression of this plot. Typically, the paracellular permeability of the tight monolayer is about 100-fold less than the paracellular permeability of subconfluent cells or cells incubated in Ca^{2+}-free buffer where cell junctions are fully disrupted (7).

Statistical Analysis—Analysis was performed using Student's *t* test (GraphPad Prism 4 software and Microsoft Excel). Statistical significance and number of experiments are specified in the figure legends.

RESULTS

Endogenous Na,K-ATPase β_1 Subunits Associate with YFP-linked Dog β_1 Subunits in MDCK Cell Monolayers—In total lysates of dog YFP-β_1-expressing cells, both endogenous and exogenous β_1 subunits were detected on immunoblots as two bands (Fig. 1, *A* and *B*). The lower bands of both subunits represent their high mannose-glycosylated forms that are predominantly located in the ER, whereas the higher bands represent complex-type glycosylated forms that are predominantly located in the plasma membrane (15).

To determine interacting partners of dog YFP-β_1, this fusion protein was immunoprecipitated by polyclonal anti-YFP antibodies from cell lysates of MDCK cell monolayers. The immunoprecipitation of YFP-β_1 itself and co-immunoprecipitation of the endogenous Na,K-ATPase α_1 subunit was found by Western blot analysis using anti-YFP and anti-α_1 antibodies, respectively, in YFP-β_1-expressing cells, but not in non-transfected cells (Fig. 1*A*). The immunoblots probed with the anti-α_1 antibody were further developed using the antibody against β_1 subunit. This antibody detected both YFP-β_1 and endogenous β_1 subunit in cell lysates (Fig. 1*B*, *left lane*). In the immunoprecipitated fraction, in addition to the two bands of YFP-β_1, the anti-β_1 antibody also detected a smeared band that had the same electrophoretic mobility as the plasma membrane fraction of the endogenous β_1 subunit in cell lysates (Fig. 1*B*). After deglycosylation of immunoprecipitated proteins using PNGase F, YFP-β_1 and the endogenous β_1 subunit were detected at 60 and 35 kDa, respectively, as expected from their core protein molecular masses (Fig. 1*C*). The endogenous β_1 subunit was not found after immunoprecipitation from cell lysates of non-transfected MDCK cells (Fig. 1*B*, *right lane*), indicating specificity of its binding to YFP-β_1. Therefore, the endogenous β_1 subunits interact with YFP-linked β_1 subunits in MDCK cell monolayers.

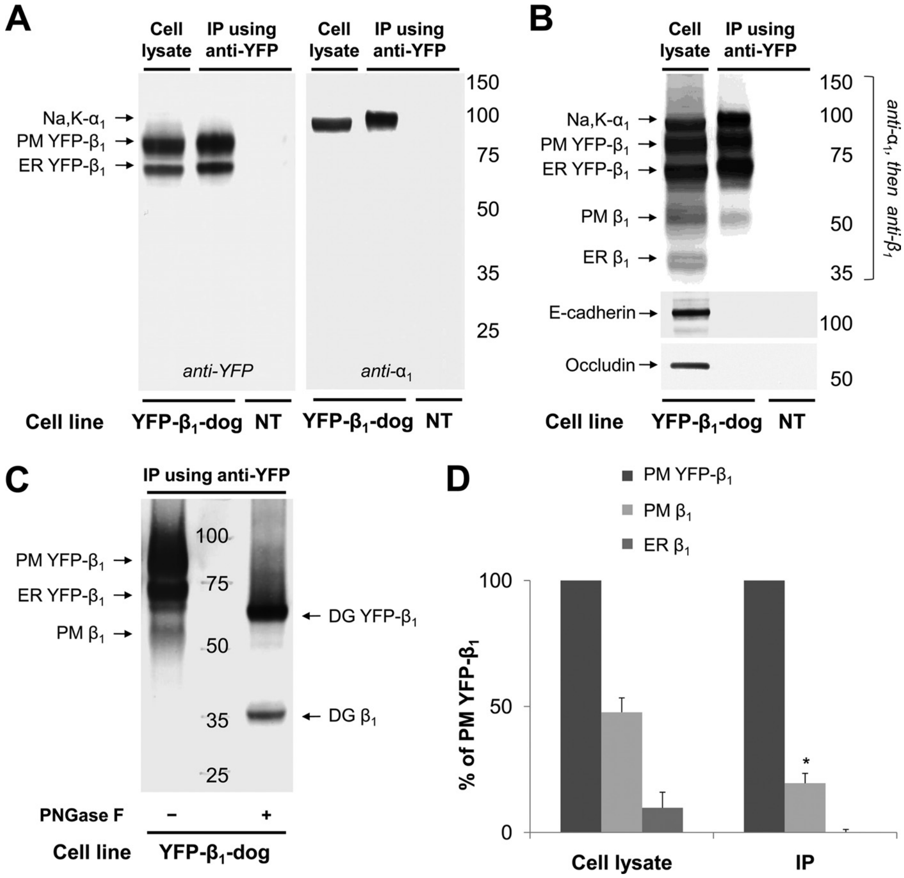

FIGURE 1. **The endogenous plasma membrane-resident Na,K-ATPase β_1 subunit is bound to YFP-linked Na,K-ATPase dog β_1 subunit in MDCK cell monolayers.** *A,* Western blot analysis using anti-YFP and anti-Na,K-ATPase α_1 antibodies shows that immunoprecipitation (*IP*) of dog YFP-β_1 resulted in co-precipitation of the endogenous Na,K-ATPase α_1 subunit from cell lysates of dog YFP-β_1-expressing cells, but not from non-transfected MDCK cells. In the *left lanes* of *A* and *B*, 10% of the total amount of cell lysate used for immunoprecipitation was loaded. *B,* immunoprecipitation of dog YFP-β_1 resulted also in co-precipitation of the Na,K-ATPase endogenous β_1 subunits, as detected by immunoblotting using the anti-β_1 antibody, which reacts with both dog YFP-β_1 and endogenous β_1 subunits. Both plasma membrane and ER forms of the endogenous β_1 subunit were detected in total cell lysates of transfected cells, whereas only the plasma membrane form was detected in the immunoprecipitated fraction. No β_1 subunits were found in the immunoprecipitated fraction from non-transfected cells. Both E-cadherin and occludin were detected in cell lysates, but not in the immunoprecipitated fractions from lysates of either transfected or non-transfected cells. *C,* PNGase F digestion of immunoprecipitated proteins prior to SDS-PAGE resulted in detection of deglycosylated YFP-β_1 at 60 kDa, and the deglycosylated endogenous β_1 subunit at 35 kDa. *D,* densitometry quantification of the results presented in *B*. In both cell lysate and immunoprecipitated fractions, density of the bands was calculated as a percentage of the density of the plasma membrane fraction of dog YFP-β_1. *Error bars,* ± S.D., $n = 3$; *, significant difference from *PM* β_i in cell lysate, $p < 0.001$, Student's *t* test.

Only the plasma membrane form of the endogenous β_1 subunit was co-immunoprecipitated with YFP-β_1 (Fig. 1*B*). The quantity of the plasma membrane fraction of the endogenous β_1 subunit in cell lysates accounted for about 50% of the plasma membrane fraction of YFP-β_1 (Fig. 1*D*). In the immunoprecipitated fraction, the amount of endogenous β_1 subunit was 20% of the amount of the plasma membrane YFP-β_1 (Fig. 1*D*). Therefore, about 40% of the endogenous β_1 subunits present in the plasma membrane were bound to YFP-β_1.

Because only the plasma membrane forms of the endogenous β_1 subunits interact with YFP-β_1, binding must occur at the sites of cell contact, where the major fraction of the plasma membrane Na,K-ATPase is present in MDCK cell monolayers (9). To determine whether junctional proteins that are co-localized with the Na,K-ATPase at these sites are involved in (YFP-β_1)-β_1 binding, total cell lysates and YFP-immunoprecipitated proteins were analyzed by immunoblotting using antibodies against the cell adhesion molecules of adherens and tight junctions, E-cadherin and occludin. Although E-cadherin and occludin were present in cell lysates, they were not detected in immunoprecipitated fractions in YFP-β_1-expressing or non-transfected cells (Fig. 1*B*). Therefore, the endogenous β_1 subunits interact with expressed YFP-β_1 at the lateral membrane, and this interaction is not mediated by the cell adhesion molecules of adherens junctions or tight junctions.

The β_1 Subunits of Neighboring Cells Interact with Each Other—To determine whether interactions between YFP-β_1 and endogenous β_1 subunits occur in the same membrane or between two neighboring cells in a cell monolayer, the amount of endogenous β_1 subunits co-immunoprecipitated with dog YFP-β_1 was compared in cell monolayers and single cells. To obtain single cells, the intercellular junctions in MDCK cell monolayers were disrupted by cell incubation in a Ca^{2+}-free buffer (Fig. 2*A*). After 1 h, the majority of cells separated from each other, but remained attached to the surface of the dish. At this stage, the process of cell separation was reversible. Replacement of PBS by a complete cell culture medium resulted in re-formation of a significant fraction of cell contacts after 1 h of cell incubation in the medium followed by a complete recovery of the cell monolayer (Fig. 2*A*).

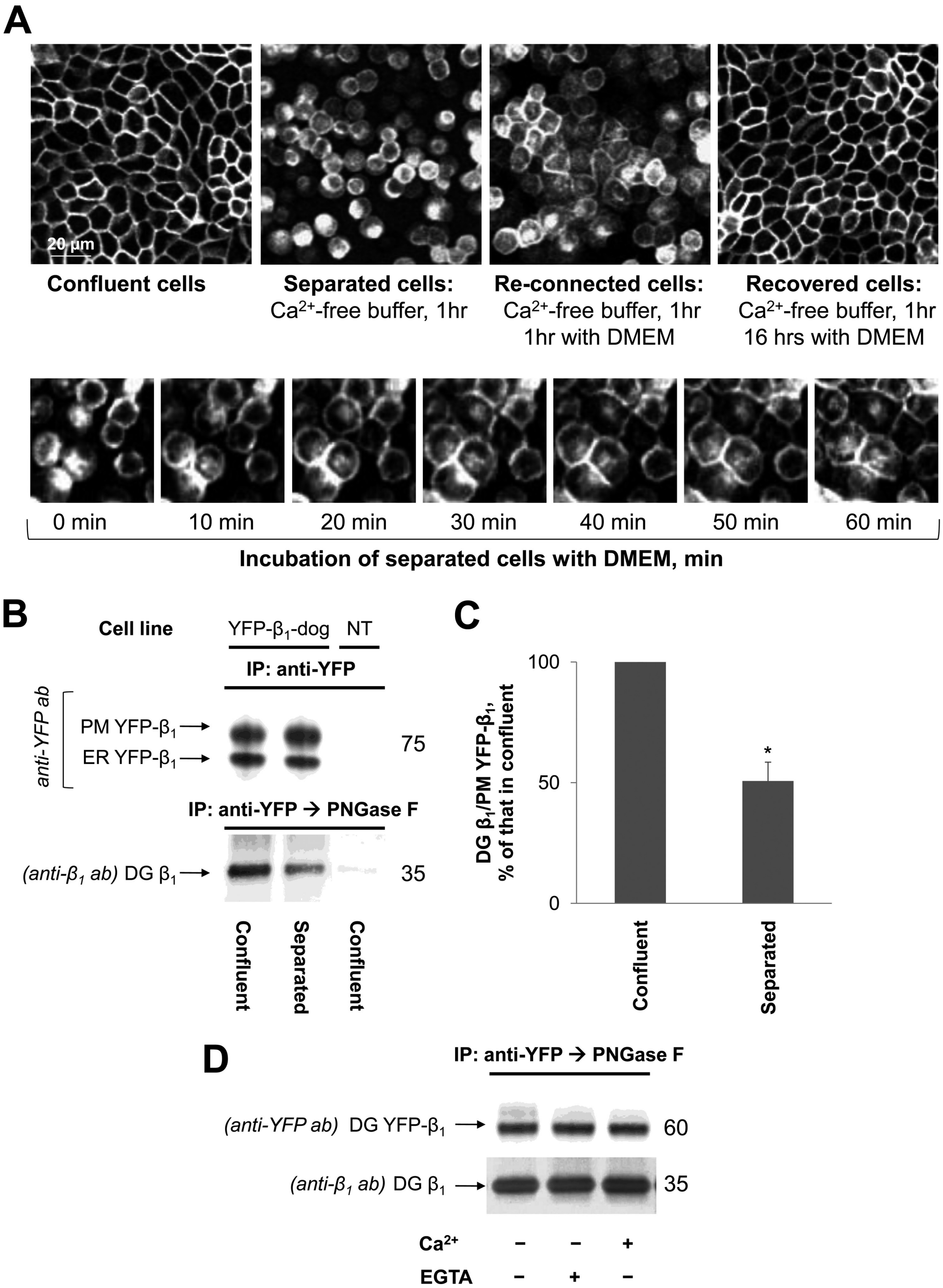

FIGURE 2. **Disruption of intercellular junctions decreases the amount of the endogenous Na,K-ATPase β_1 subunit bound to YFP-β_1.** *A,* confocal microscopy images of MDCK cells expressing dog YFP-β_1 show that a short incubation of confluent cell monolayers with 1 mM EDTA followed by a 1-h incubation with Ca^{2+}-free PBS resulted in almost complete disruption of intercellular junctions. When PBS was replaced with a complete cell culture medium, the intercellular contacts were reformed. Time lapse confocal microscopy images that document re-formation of cell-cell contacts are shown at the *bottom*. *B,* Western blot analysis of the proteins immunoprecipitated (*IP*) from lysates obtained either from confluent YFP-β_1-expressing cells (panel *Confluent cells* on *A*) or from YFP-β_1-expressing cells separated from each other by cell incubation with PBS for 60 min (panel *Separated cells* on *A*). Immunoprecipitated YFP-β_1 was detected by anti-YFP antibody. Co-precipitated endogenous Na,K-ATPase β_1 subunits were deglycosylated with PNGase F prior to SDS-PAGE and Western blot analysis using the anti-β_1 antibody to enable accurate quantification. *C,* densitometric quantification of the results presented in *B* was performed by dividing a signal from anti-β_1 antibody by the corresponding signal from anti-YFP antibody for PM YFP-β_1. The comparative bar graph shows these ratios as a percentage of the ratio obtained for confluent cells. *D,* immunoprecipitation of dog YFP-β_1 was performed in the absence or presence of 1 mM Ca^{2+} or 1 mM EGTA. To enable quantitative comparison of immunoprecipitated and co-immunoprecipitated subunits, immunoprecipitated protein complexes were deglycosylated prior to SDS-PAGE. *NT,* non-transfected cells; *DG*, deglycosylated by PNGase F; *error bars*, ± S.D. ($n = 3$); *, significant difference from *Confluent*, $p < 0.001$, Student's *t* test.

Immunoprecipitation of YFP-β_1 from lysates of both confluent and separated cells resulted in co-immunoprecipitation of the endogenous β_1 subunits (Fig. 2*B*). However, the amount of co-precipitated β_1 subunits was significantly less in separated cells. To enable accurate quantification of co-precipitated β_1 subunits, each immunoprecipitation reaction was performed in two parallel tubes. In the first tube, proteins were eluted from the beads and analyzed by immunoblotting using the anti-YFP antibody (Fig. 2*B*, *top panel*). In the second tube, the immunoprecipitated proteins were deglycosylated by PNGase F and

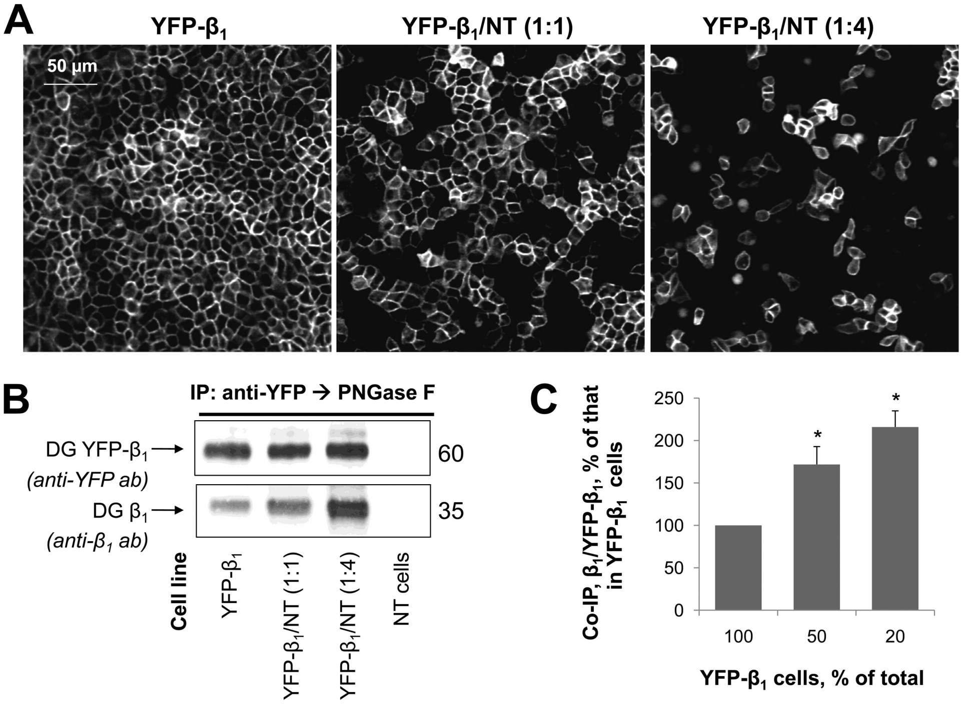

FIGURE 3. **Increasing the number of intercellular contacts between non-transfected and YFP-β_1-expressing MDCK cells increases the amount of YFP-β_1-co-immunoprecipitated endogenous β_1 subunits.** *A,* confocal microscopy images of the confluent monolayers formed by MDCK cells expressing dog YFP-β_1 and co-cultured dog YFP-β_1-expressing cells and non-transfected cells mixed at 1:1 and 1:4 ratios. Only YFP-β_1-expressing cells are visible in co-cultures with non-transfected cells. *B,* Western blot analysis of the proteins immunoprecipitated by anti-YFP antibody from lysates obtained from YFP-β_1-expressing cells, from co-cultures of YFP-β_1-expressing cells with non-transfected cells at a 1:1 ratio, or from co-cultures of YFP-β_1-expressing cells with non-transfected cells at a 1:4 ratio. To maintain a similar amount of the immunoprecipitated YFP-β_1, 0.2, 0.4, and 1 mg of total protein, respectively, was used for immunoprecipitation from YFP-β_1-expressing cells, 1:1 and 1:4 cell mixtures, whereas all other components of the immunoprecipitation reaction were the same. As a negative control, 1 mg of non-transfected cells was used in the immunoprecipitation assay. Immunoprecipitated proteins were deglycosylated with PNGase F prior to SDS-PAGE and Western blot analysis to enable accurate quantification. *C,* densitometric quantification of the results presented in *B* was performed by dividing a signal from anti-β_1 antibody by the corresponding signal from anti-YFP antibody. The comparative bar graph shows these ratios as a percentage of the ratio obtained in the dog YFP-β_1 cell line. The amount of co-immunoprecipitated endogenous Na,K-ATPase β_1 subunits was increased by adding non-transfected cells to β_1-expressing cells. *IP*, immunoprecipitation; *NT*, non-transfected cells; *DG*, deglycosylated by PNGase F; *error bars*, ±S.D. ($n = 3$); *, significant difference from YFP-β_1-expressing cells, $p < 0.01$, Student's *t* test.

then analyzed by immunoblotting of the endogenous β_1 subunits (Fig. 2*B*, *bottom panel*). Densitometric quantification of the amount of the β_1 subunits relative to the amount of the plasma membrane fractions of YFP-β_1 showed that the amount of YFP-β_1-bound β_1 subunits was 2-fold less in separated compared with confluent cells (Fig. 2*C*). There was no change in the amount of co-immunoprecipitated β_1 subunits when the immunoprecipitation reaction was performed with or without addition of 1 mM Ca^{2+} or 1 mM EGTA (Fig. 2*D*). Therefore, the smaller amount of co-precipitated β_1 subunits in separated cells was indeed due to disruption of cell-cell contacts, but not due to lack of Ca^{2+}. These results suggest that separation of the cells disrupted the intercellular interactions between YFP-β_1 and endogenous β_1 subunits. It seems unlikely that the loss of intercellular junctions would result in dissociation of the β_1 subunits from the oligomeric complexes of the Na,K-ATPase in the same membrane, resulting in a decreased amount of YFP-β_1-precipitated β_1 subunits. However, because such a possibility cannot be excluded, we used an alternative approach to confirm the presence of the intercellular β_1-β_1 interaction.

We compared the amount of YFP-β_1-co-immunoprecipited endogenous β_1 subunits in mixed cell monolayers of YFP-β_1-expressing cells and non-transfected cells with that in the monolayers of YFP-β_1-expressing cells only. If β_1-β_1 interactions are intercellular, more efficient co-precipitation of endogenous β_1 subunits is expected in mixed co-cultures. On the other hand, if YFP-β_1 interacts with the endogenous β_1 subunits only in the same cell, adding the non-transfected cells should not have an effect on co-precipitation of endogenous β_1 subunits with YFP-β_1. The results show that the amount of co-precipitated β was increased 70% by adding an equal amount of non-transfected cells to YFP-β_1-expressing cells and 116% by adding a 4-fold excess of non-transfected cells to YFP-β_1-expressing cells (Fig. 3), confirming the presence of the intercellular (YFP-β_1)-β_1 interactions.

The Lack of N-Glycans and Differences in the Amino Acid Sequence Weaken the Interaction between Endogenous and Exogenous β Subunits—To determine the importance of *N*-glycans and the polypeptide sequence in β_1-β_1 binding, we performed immunoprecipitation with anti-YFP antibody from lysates of cells expressing different variants of YFP-β and determined the amount of co-precipitated endogenous β_1 subunits. Five stable cell lines expressing YFP-β_2, dog YFP-β_1, rat YFP-β_1, the unglycosylated dog YFP-β_1, and the unglycosylated rat YFP-β_1 were used in these experiments. In all these cell lines, YFP-β was detected predominantly in the lateral membrane in MDCK cell monolayers by confocal microscopy (Fig. 4*A*). In all of these transfected cell lines, the level of expression of the endogenous β_1 subunits was similar, but less than in non-transfected MDCK cells (Fig. 4*B*). A decrease in the endogenous β_1

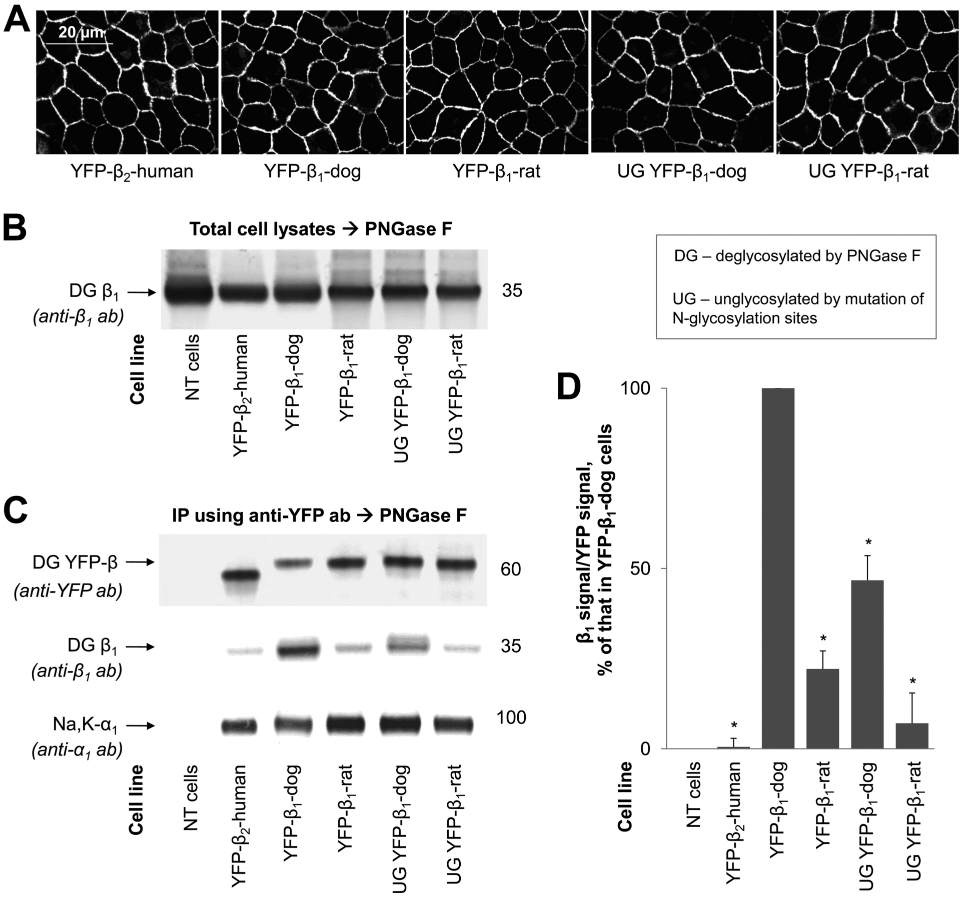

FIGURE 4. **Interactions between the endogenous and exogenous Na,K-ATPase β subunits in MDCK cell monolayers depend on their amino acid sequence and presence of *N*-glycans.** *A,* confocal microscopy images of MDCK cell monolayers (horizontal sections) show predominant localization of the YFP-linked wild type β_2 subunit and wild type or unglycosylated β_1 subunits (dog and rat) on the lateral membranes. *B,* Western blot analysis of total cell lysates pre-treated with PNGase F shows similar levels of expression of endogenous Na,K-ATPase β_1 subunits in all studied YFP-β-expressing cell lines. *C,* Western blot analysis of the proteins immunoprecipitated by anti-YFP antibody shows co-immunoprecipitation of the endogenous Na,K-ATPase β_1 and α_1 subunits with YFP-linked β_2 subunits and four variants of the β_1 subunits from total lysates of the corresponding transfected, but not from lysates of non-transfected MDCK cells. To enable comparative densitometric quantification of immunoprecipitated YFP-linked β_1 subunits and co-immunoprecipitated endogenous β_1 subunits, immunoprecipitated proteins were treated with PNGase F prior to SDS-PAGE and Western blot analysis. *D,* densitometric quantification for each cell line was performed by dividing a signal from anti-β_1 antibody by the corresponding signal from the anti-YFP antibody. The comparative bar graph shows these ratios as a percentage of the ratio obtained in the dog YFP-β_1 cell line. The amount of co-immunoprecipitated endogenous Na,K-ATPase β_1 subunits is less with rat than with dog exogenous β_1 subunits. For both dog and rat exogenous subunits, the amount of co-immunoprecipitated endogenous Na,K-ATPase β_1 subunits is less with the unglycosylated subunits than with the fully glycosylated subunits. *Error bars*, ±S.D. ($n = 3$); *, significant difference from dog YFP-β_1 dog, $p < 0.001$, Student's *t* test.

subunits in transfected cells was probably due to competition of the exogenous with endogenous β subunits for binding to the endogenous α_1 subunits. This binding is required for export of the β subunits from the ER and delivery to the plasma membrane (15). As a result, a fraction of the endogenous β_1 subunits in the α-β complexes in the plasma membrane is replaced by exogenous β subunits. Consistent with this interpretation, all five YFP-β variants bound endogenous α_1 subunits in the co-immunoprecipitation assay (Fig. 4*C*).

The amount of endogenous β_1 subunits that co-immunoprecipitated varied between different species of YFP-β. The highest amount of the endogenous β_1 subunits (normalized to the amount of immunoprecipitated YFP-β) was detected for dog YFP-β_1 (Fig. 4, *C* and *D*). The amount of β_1 subunits was less with rat YFP-β_1. For both dog and rat YFP-β_1, the amount of co-precipitated β_1 subunits was higher for fully glycosylated than for unglycosylated YFP-β_1 (Fig. 4, *C* and *D*). No co-precipitation of β_1 subunits was detected with expressed human YFP-β_2. These results show that both amino acid and carbohydrate residues of the β_1 subunits contribute to β_1-β_1 binding. These results also rule out the presence of complexes containing two α_1, one β_1, and one β_2 subunit.

Reducing N-Glycan Complexity Improves the Interaction between Endogenous and Exogenous β_1 Subunits—To determine whether the structure of *N*-glycans is important for β_1-β_1 interaction, we used inhibitors of *N*-glycan processing to change the type of *N*-glycans. Normally, the high-mannose *N*-glycans formed by trimming the co-translationally added oligosaccharide core by the ER glucosidases are transformed first to hybrid- and then to complex-type *N*-glycans due to the action of the Golgi mannosidases and glycosyltransferases (Fig. 5*A*). The inhibitor of mannosidase I, DMJ, prevents transformation of the high mannose-type *N*-glycans to hybrid-type *N*-glycans. As a result, in the cells exposed to this inhibitor, all newly synthesized glycoproteins contain only high mannose-type *N*-glycans (Fig. 5*A*). Also, the inhibitor of the Golgi man-

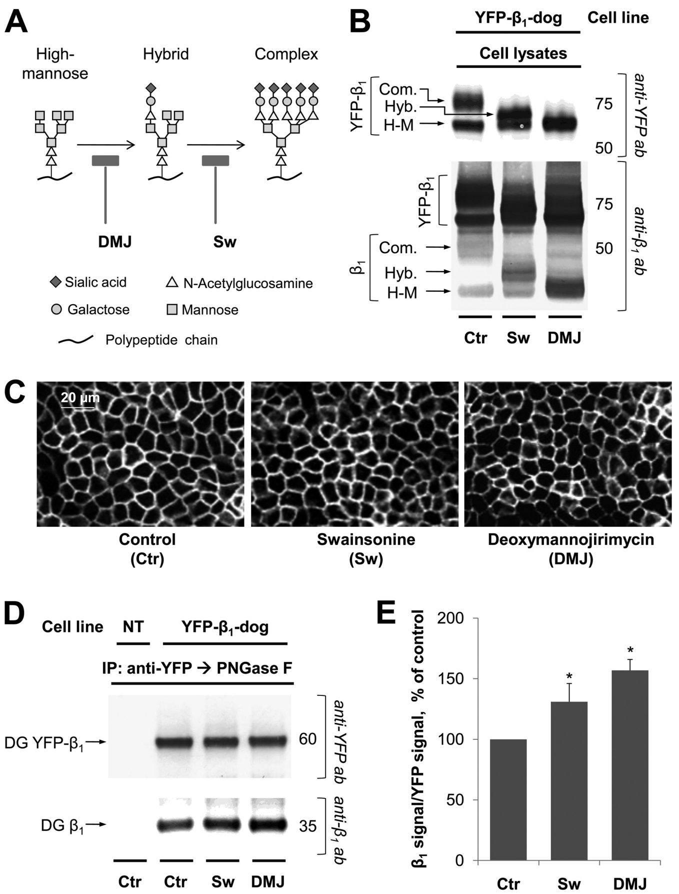

FIGURE 5. **Prevention of *N*-glycan processing that preserves high mannose- or hybrid-type structures of *N*-glycans increases the amount of the YFP-β_1-bound endogenous β_1 subunits.** *A,* a scheme showing that inhibition of *N*-glycan processing by DMJ and swainsonine (*Sw*) preserves high mannose- and hybrid-type structures of *N*-glycans, respectively. *B,* Western blot analysis of lysates of control and inhibitor-treated cells shows that cell incubation with DMJ or swainsonine for 72 h was sufficient to substitute the complex-type (*Com.*) glycosylated YFP-β_1 and endogenous β_1 subunits with the newly synthesized high mannose- (*H-M*) or hybrid-type (*Hyb.*) forms of the subunits. *C,* confocal microscopy images of dog YFP-β_1-expressing cells showing that cell exposure to swainsonine or DMJ did not prevent plasma membrane delivery of YFP-β_1. *D,* Western blot analysis of immunoprecipitated YFP-β_1 (anti-YFP antibody) and co-immunoprecipitated endogenous Na,K-ATPase β_1 subunits (anti-β_1 antibody). To enable accurate quantification, immunoprecipitated protein complexes were deglycosylated prior to SDS-PAGE and immunoblotting. *E,* densitometric quantification of the results presented in *D*, which was performed by dividing a signal from the anti-β_1 antibody by the corresponding signal from the anti-YFP antibody, shows an increase in the amount of YFP-β_1-bound endogenous β_1 subunits in immunoprecipitated fractions from cells pre-treated with swainsonine and DMJ. *IP*, immunoprecipitation; *DG*, deglycosylated by PNGase F; *error bars*, ±S.D. ($n = 3$); *, significant difference from control, $p < 0.01$, Student's *t* test.

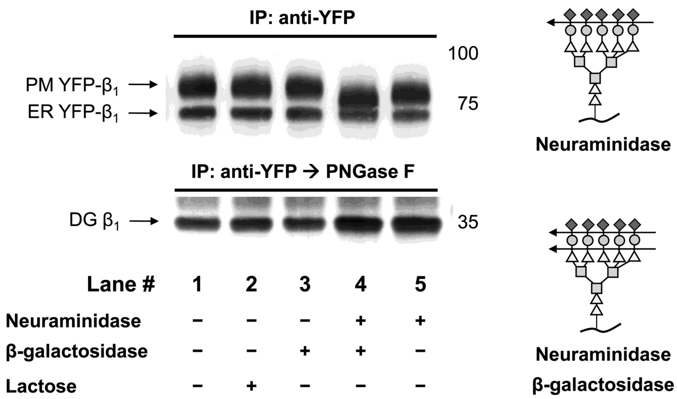

FIGURE 6. **Assessment of the possible involvement of sialic acid or galactose residues of *N*-glycans in (YFP-β_1)-β_1 interaction.** Immunoprecipitation (*IP*) of dog YFP-β_1 was performed for 3 h at room temperature in the absence or presence of neuraminidase or β-galactosidase, or after a 24-h incubation of cells with 50 mM lactose. Western blot analysis of immunoprecipitated YFP-β_1 using anti-YFP antibody shows a noticeable increase in the electrophoretic mobility of the complex type-glycosylated form of the neuraminidase-treated YFP-β_1 as compared with the untreated control, indicating that terminal sialic residues were cleaved from its *N*-glycans. In contrast, β-galactosidase did not change the electrophoretic mobility of YFP-β_1, whereas a combination of neuraminidase and β-galactosidase increased the electrophoretic mobility of YFP-β_1. With two enzymes this increase was greater than with neuraminidase alone, indicating that β-galactosidase can cleave galactose residues only after removal of sialic acid residues. Western blot analysis of co-immunoprecipitated endogenous Na,K-ATPase β_1 subunits (anti-β_1 antibody) shows an increase in the amount of co-precipitated β_1 subunits in the presence of neuraminidase or a mixture of neuraminidase and β-galactosidase and no change in co-precipitation in the presence of β-galactosidase, and also after cell incubation with lactose. To enable accurate quantification of co-immunoprecipitated β_1 subunits, immunoprecipitated protein complexes were deglycosylated prior to SDS-PAGE. *IP*, immunoprecipitation; *DG*, deglycosylated by PNGase F; symbols showing monosaccharide residues are the same as in Fig. 5.

nosidase II, swainsonine, prevents transformation of the hybrid-type *N*-glycans to complex-type *N*-glycans. Thus, in the presence of this inhibitor, cells can synthesize only high mannose- or hybrid-type glycoproteins (Fig. 5*A*).

Cell incubation with the inhibitors for 72 h was sufficient to substitute complex-type glycosylated YFP-β_1 and endogenous β_1 subunits with newly synthesized high mannose- or/and hybrid-type subunits (Fig. 5*B*). In cells exposed to DMJ, both endogenous and exogenous β_1 subunits were predominantly of the high mannose-type, whereas in cells exposed to swainsonine, both high mannose- and hybrid-type forms were present (Fig. 5*B*). Immunoprecipitation of YFP-β_1 resulted in co-immunoprecipitation of the endogenous β_1 subunits in cells pretreated with either inhibitor. The amount of co-precipitated β_1 subunits was greater in swainsonine-exposed cells and even more in DMJ-exposed cells as compared with the control cells (Fig. 5, *D* and *E*), indicating that a less complex structure of *N*-glycan favors β_1-β_1 interaction. Exposure to either DMJ or swainsonine did not affect plasma membrane delivery of YFP-β_1 (Fig. 5*C*).

Sialic acid residues are the major terminal residues in *N*-glycans linked to the β_1 subunit of the renal Na,K-ATPase (16). To determine whether these negatively charged residues are important for the β_1-β_1 interaction, we studied the effect of neuraminidase on co-immunoprecipitation of the endogenous β_1 subunits with YFP-β_1. Immunoprecipitation of dog YFP-β_1 in the presence of neuraminidase resulted in a noticeable increase in the electrophoretic mobility of its complex-type glycosylated form (Fig. 6, *top panel, lanes 1* and *5*), confirming the presence of sialic acid residues on the termini of its *N*-glycans. However, the amount of YFP-β_1-bound endogenous β_1 subunits was not decreased by neuraminidase. On the contrary, it was even greater than in the control sample (Fig. 6, *bottom panel, lanes 1* and *5*). Therefore, sialic acid residues are not involved in maintaining contacts between YFP-β_1 and β_1 subunits. Moreover, they seem to prevent efficient β_1-β_1 interaction. These results are in agreement with improvement of the β_1-β_1 interaction by swainsonine and DMJ, which partially and completely, respectively, prevent addition of sialic acid residues to *N*-glycans (Fig. 5*A*).

Assessment of the Possible Involvement of Other Molecules in the Interaction between Endogenous and Exogenous β_1 Subunits—To see whether the intercellular β_1-β_1 interaction is mediated by other molecules, we performed a search for β_1-interacting proteins using nLC-MS/MS of YFP-β_1-co-immunoprecipitated proteins. This analysis identified a number of intercellular proteins, including the ER chaperones, BiP and calnexin, which has been shown previously to be involved in maturation of the Na,K-ATPase β_1 subunit (12), several Rab and Rho GTPases, and cytoskeleton proteins. The search has identified a single secreted protein, galectin-3, but has not revealed any integral lateral membrane proteins that can be involved in the β_1-β_1 interaction. Galectin-3 is a galactose-binding lectin (17), which is expressed predominantly in the cytoplasm of MDCK cells with a minor fraction secreted via the basolateral membrane to the extracellular space by a non-conventional mechanism (18). Previously, galectin-3 has been found to be involved in apical sorting of several proteins in MDCK cells (19). To determine whether galectin-3 is involved in the β_1-β_1 interaction, we incubated cells with 50 mM lactose, which is a competitive inhibitor of galectin-3 binding to galactose-containing glycans (20). Immunoprecipitation of YFP-β_1, which was performed after a 24-h incubation of cells with lactose, resulted in no change of the amount of co-immunoprecipitated β_1 subunits (Fig. 6, *lanes 1* and *2*). As an alternative approach to determine the possible involvement of galectin-3, we performed immunoprecipitation of YFP-β_1 in the presence of β-galactosidase, which should remove terminal galactose residues, which are galectin-3 ligands, from *N*-glycan termini of both YFP-β_1 and β_1 subunits. However, immunoprecipitation performed in the presence of β-galactosidase did not change the amount of YFP-β_1-co-precipitated β_1 subunits (Fig. 6, *lanes 1* and *3*). Comparative Western blot analysis of YFP-β_1 immunoprecipitated without or with the enzyme did not show any visible change in the electrophoretic mobility of YFP-β_1 (Fig. 6, *top panel, lanes 1* and *3*), indicating either the lack of β-galactose residues on *N*-glycan termini of YFP-β_1, or the lack of the β-galactosidase activity. In contrast, the electrophoretic mobility of YFP-β_1 treated with two enzymes (neuraminidase and β-galactosidase) was greater than that of YFP-β_1 treated with neuraminidase alone (Fig. 6, *top panel, lanes 4* and *5*). These results show that β-galactosidase cleaved the galactose residues located below sialic acid residues in *N*-glycans. Therefore, no change in electrophoretic mobility with β-galactosidase alone indicates that there are no or very few galactose residues on *N*-glycan termini, explaining why galactosidase did not have an effect on (YFP-β_1)-β_1 co-precipitation. Removing the galactose residues located below the sialic acid residues did not have an effect on (YFP-β_1)-β_1 co-precipitation either: the effect of neuraminidase/β-galactosidase was similar to the effect of neuraminidase alone (Fig. 6, *lanes 4* and *5*). These results indicate that galactose residues in *N*-glycans are not important for the β_1-β_1 interaction and hence galectin-3 apparently does not link the β_1 subunits of neighboring cells.

Reduction in the Number of (YFP-β_1)-β_1 Complexes Decreases Both Stability and Tightness of Intercellular Junctions—To determine whether intercellular β_1-β_1 interactions are important for integrity of intercellular junctions in a cell monolayer, we performed an assay on detergent resistance of adherens junctions and also measured the paracellular permeability in mature monolayers of cells expressing different variants of YFP-β (Fig. 7).

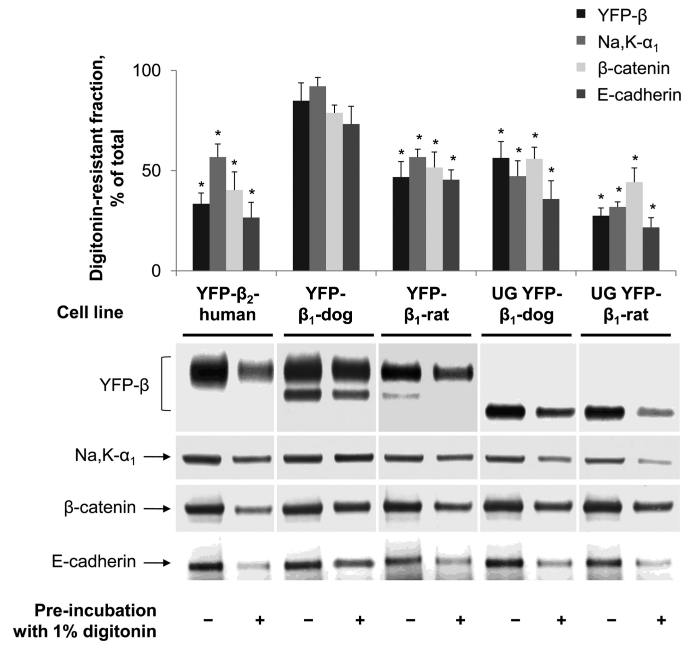

FIGURE 7. **The fully glycosylated dog YFP-β_1 is more resistant to removal from a cell monolayer by a non-ionic detergent than the rat YFP-β_1, or the unglycosylated mutants of YFP-β_1, or the wild type YFP-β_2.** Mature MDCK cell monolayers expressing various YFP-linked β subunits of the Na,K-ATPase were lysed as described under "Experimental Procedures" either before or after a 30-min preincubation with 1% digitonin, which was then replaced by cell lysis buffer. The amount of YFP-linked β subunits, Na,K-ATPase α_1 subunit, β-catenin, and E-cadherin before and after preincubation with 1% digitonin was determined by Western blot analysis of total cell lysates. Densitometric quantification for each cell line shows the amount of each protein in cells after digitonin treatment as a percentage of its amount before digitonin treatment. *Error bars*, ±S.D. ($n = 3$); *, significant difference from dog YFP-β_1-expressing cells, $p < 0.01$, Student's *t* test.

It is known that the cytoplasmic domain of Na,K-ATPase is linked to the cytoplasmic domain of E-cadherin via the ankyrin/spectrin cytoskeleton (5, 29, 30). E-cadherin is the main cell adhesion molecule of the adherens junctions. Its extracellular domain binds to the extracellular domain of the E-cadherin molecule of a neighboring cell, whereas its cytoplasmic domain interacts with the cytoskeleton via anchoring proteins, including β-catenin. Due to this linkage to the cytoskeleton, E-cadherin as all other cell adhesion molecules is resistant to the extraction by non-ionic detergents from epithelial cell monolayers (21–23) in contrast to the majority of other cellular proteins, which are removed from cells by this treatment. E-cadherin acquires resistance to non-ionic detergents only after formation of cell junctions, showing that the intercellular linkage between extracellular domains of two E-cadherin molecules is required for stable association of their cytoplasmic domains with the cytoskeleton (23). Similar to E-cadherin, Na,K-ATPase is resistant to detergent extraction in cell monolayers, but not in dispersed MDCK cells (7). If the intercellular β_1-β_1 interaction is important for association of Na,K-ATPase with the cytoskeleton, the weakening of β_1-β_1 binding by removing *N*-glycans or altering an amino acid sequence is expected to increase detergent extractability of the

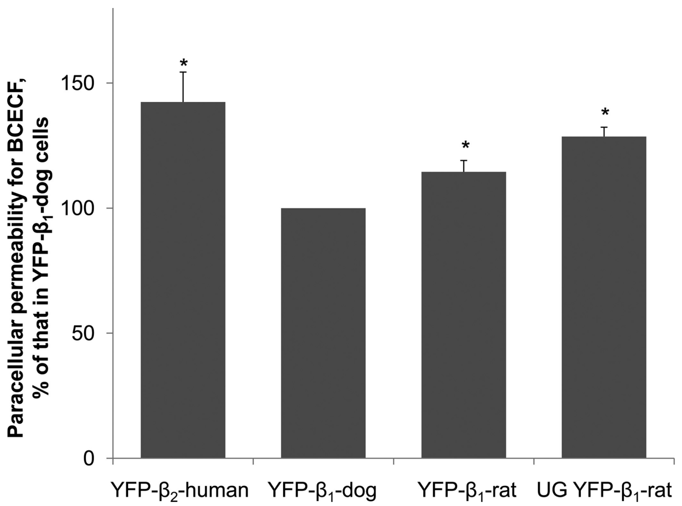

FIGURE 8. **MDCK cells expressing fully glycosylated dog YFP-β_1 form tighter monolayers than cells expressing rat YFP-β_1, its unglycosylated mutant, or the wild type YFP-β_2.** Cells expressing various YFP-β constructs were maintained on porous transwell inserts for 6 days after becoming confluent. Paracellular permeability for the membrane-impermeable fluorescent dye, which was added to the bottom of the well, was determined as a rate of dye accumulation in the upper chamber of the insert. *Error bars*, ±S.D. ($n = 3$); *, significant difference from dog YFP-β_1-expressing cells, $p < 0.01$, Student's *t* test.

Na,K-ATPase, as well as extractability of E-cadherin and β-catenin, because they are linked to the same cytoskeleton network.

Dog YFP-β_1 was more resistant to digitonin extraction from cell monolayers than the rat YFP-β_1 (Fig. 7). The unglycosylated YFP-β_1 subunits, both dog and rat, were less resistant than their fully glycosylated native forms (Fig. 7). YFP-β_2 was the least resistant among other YFP-β variants. Therefore, the stability of various YFP-linked β subunits to digitonin extraction from cell monolayers (Fig. 7) correlated with their ability to complex with endogenous β_1 subunits (Fig. 4, *C* and *D*). Stability of the endogenous α_1 subunit to digitonin in different cell lines was allied to the digitonin resistance of the expressed variants of YFP-β (Fig. 7). Similarly, detergent resistance of adherens junction proteins, E-cadherin and β-catenin, was greater in cells expressing fully glycosylated dog YFP-β_1 than in cells expressing rat YFP-β_1, YFP-β_2, or unglycosylated YFP-β_1 (Fig. 7).

The paracellular permeability for the membrane impermeable dye in the mature monolayers formed by cells expressing different variants of YFP-β showed the following order: dog YFP-β_1 < the wild type rat YFP-β_1 < the unglycosylated rat YFP-β_1 < the wild type YFP-β_2 (Fig. 8). Therefore, the ability of the expressed YFP-linked subunits to interact with the endogenous β_1 subunits directly correlates with detergent resistance of junctional proteins and inversely correlates with the paracellular permeability, indicating that the β_1-β_1 interaction is important for stability and integrity of intercellular junctions.

DISCUSSION

Intercellular Interaction between β_1 Subunits of Neighboring Cells Versus Oligomerization of Pumps in the Same Membrane—Immunoprecipitation of YFP-β_1 from cell lysates of YFP-β_1-expressing cell monolayers resulted in co-precipitation of the plasma membrane, but not the ER, fraction of the endogenous β_1 subunit (Fig. 1*B*), showing that binding between YFP-β_1 and β_1 subunits occurs in the lateral membranes. This binding may occur in the same membrane, between two membranes of neighboring cells, or both. The presence of intercellular interactions between the Na,K-ATPase β_1 subunits was previously documented in mixed monolayers of two cell types, in which endogenous β_1 subunits of rat cells were co-immunoprecipitated with rat YFP-β_1 expressed in MDCK cells (8). Here we present additional evidence for intercellular interactions between the β_1 subunits of neighboring MDCK cells in a monolayer. We found that the amount of YFP-β_1-co-precipitated β_1 subunits was increased in mixed monolayers of YFP-β_1-expressing cells with non-transfected cells as compared with the monolayers of YFP-$\beta_1$expressing cells only (Fig. 3). The results indicate that, at the borders between two YFP-β_1-expressing cells, YFP-β_1 interacts with both YFP-β_1 and the endogenous β_1 subunits present in the neighboring cells. However, at the mixed borders, YFP-β_1 interacts only with the endogenous β_1 subunits. As a result, increasing the proportion of mixed contacts increases the amount of YFP-β_1-bound β_1 subunits. Consistent with the presence of intercellular β_1-β_1 interactions in a cell monolayer, disruption of intercellular junctions resulted in a significant decrease in the amount of YFP-β_1-precipitated β_1 subunits (Fig. 2). A similar decrease in co-immunoprecipitated β_1 subunits in separated cells was observed with rat YFP-β_1 and the unglycosylated YFP-β_1 (not shown).

Therefore, our results confirm the presence of intercellular β_1-β_1 interactions. However, they do not exclude the possibility of β_1-β_1 interactions in the same cell in agreement with the data suggesting an oligomeric state of the Na,K-ATPase and homologous H,K-ATPase (24–26).

Amino Acid-mediated Interactions Are Important for β_1-β_1 Binding—In mixed cultures of cells of different species, the β_1 subunit was detected on homotypic, but not on heterotypic cell borders, suggesting that the β_1 subunits interact with each other in a species-specific mode (8, 11). Here, we compared the interactions between two dog subunits and between dog and rat subunits and confirmed that the β_1-β_1 interaction is indeed species-specific. The amount of co-precipitated β_1 subunits was less with rat YFP-β_1 than with dog YFP-β_1 (Fig. 4). The protein sequences of the extracellular domains of dog and rat β_1 subunits have some differences, whereas the number and positions of *N*-glycans are the same. No co-precipitation of dog β_1 subunits was detected with human YFP-β_2 (Fig. 4) that has even more differences in the amino acid sequence. Therefore, these results emphasize the importance of protein-protein interactions for β_1-β_1 binding. These results also indicate that the amino acid residues important for β_1-β_1 binding must be different in the dog and rat β_1 subunits. The most variable region between rat and dog subunits containing amino acid residues 199–207 includes the known epitope for the monoclonal antibody against the Na,K-ATPase β_1 subunit, clone M17-P5-F11 (27). This antibody recognizes the dog, but not the rat β_1 subunit, and inhibits formation of intercellular junctions between MDCK cells (9). Therefore, it is possible that particular amino acid residues of the 199–207 region are involved in β_1-β_1 interactions.

N-Glycans Stabilize and Strengthen Direct Protein-Protein Interactions between the Extracellular Domains of the β_1 Subunits—For both dog and rat YFP-β_1, the amount of co-precipitated β_1 subunits was significantly greater with normally glycosylated YFP fusion proteins than with their unglycosylated mutants (Fig. 4). Because *N*-glycans are not required for proper folding of the β_1 subunits (28), these results indicate that *N*-glycans are important for the β_1-β_1 interaction. Less complex *N*-glycans appear to be more suitable for this interaction, because (YFP-β_1)-β_1 co-immunoprecipitation is improved both by swainsonine and DMJ (Fig. 5, *D* and *E*). In addition, the presence of sialic acid residues appears to repel the interacting β_1 subunits from each other, because removal of terminal sialic acid residues improved (YFP-β_1)-β_1 co-immunoprecipitation (Fig. 6, *lane 5*). This result also indicates that sialic acid-binding lectins are not implicated in the β_1-β_1 interaction. The interaction does not involve galactose-binding lectins either, because β_1-β_1 binding is not impaired by removing terminal galactose residues or by the inhibitor of galactose-galectin binding, lactose (Fig. 6, *lanes 2* and *3*). Furthermore, the involvement of other lectins that bind to mannose or *N*-acetylglucosamine residues is doubtful, because these residues are not exposed on the *N*-glycan termini of the β_1 subunits (16). Therefore the β_1-β_1 interaction is unlikely mediated by lectins and is not mediated by E-cadherin or occludin (Fig. 1). Also, no other integral lateral membrane proteins or secreted non-lectin proteins have been identified by nLC-MS/MS of the β_1-intercting proteins. These data are consistent with the results of fluorescence resonance energy transfer showing that the β_1 subunits of neighboring cells in a cell monolayer have sufficient proximity to permit direct interaction (8).

Therefore, *N*-glycans are important for β_1-β_1 binding, most likely due to stabilizing and strengthening amino acid-mediated interactions. However, the contribution of direct glycan-glycan interactions cannot be excluded.

The β_1-β_1 Bridges between Neighboring Cells Are Essential for Regulating Stability and Tightness of Intercellular Junctions in Epithelia—The Na,K-ATPase β_1 subunits do not directly interact with cell adhesion molecules of adherens and tight junctions, E-cadherin and occludin (Fig. 1*B*). However, the cytoplasmic domains of Na,K-ATPase and E-cadherin are indirectly linked by the ankyrin/spectrin/F-actin cytoskeleton (5, 29, 30). The results presented here indicate that the Na,K-ATPase itself acts as a cell adhesion molecule, because it is connected to the Na,K-ATPase of neighboring cells via its β_1 subunit and to the cytoskeleton via its α_1 subunit. Weakening the β_1-β_1 interactions by removing *N*-glycans or changing the amino acid sequence of one of the two interacting β_1 subunits decreases resistance to detergent extraction not only for the Na,K-ATPase, but also for E-cadherin and β-catenin (Fig. 7). Therefore, the β_1-β_1 bridges are important for stability of the junctional complex. It is well known that stable adherens junctions are required to ensure functioning of the tight junctions. Accordingly, the paracellular permeability of cell monolayers formed by MDCK cells expressing various YFP-β subunits was inversely correlated with the ability of YFP-β variants to bind β_1 subunits (Fig. 8). Therefore, the intercellular homotypic interactions between the Na,K-ATPase β_1 subunits are important for stability of adherens junctions and integrity of the tight junctions.

Improved binding between β_1 subunits due to reduced complexity of the *N*-glycan structure in the presence of swainsonine and DMJ (Fig. 5) is consistent with the results showing that exposure of MDCK cell monolayers to swainsonine decreased paracellular permeability and increased resistance of the adherens junction proteins to extraction by a non-ionic detergent (7). The effects of swainsonine were attenuated in a cell line expressing the unglycosylated β_1 subunits, indicating that it is the decreased branching of β_1 subunit *N*-glycans that tightens and stabilizes cell-cell junctions. Because the expression of various glycosyltransferases and structure of *N*-glycans changes during formation and maturation of junctions (7, 31), intercellular adhesion may be regulated by glycosyltransferase-mediated remodeling of *N*-glycans of the β_1 subunit.

In conclusion, the bridges between the Na,K-ATPase β_1 subunits of neighboring cells are crucial for integrity of intercellular junctions in epithelia. Both amino acid residues and *N*-glycans are involved in β_1-β_1 binding. The results suggest an important role of regulated *N*-glycosylation of the Na,K-ATPase β_1 subunit in modulation of intercellular adhesion in epithelia.

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