EPITHELIAL JUNCTIONS DEPEND ON INTERCELLULAR TRANS-INTERACTIONS BETWEEN THE NA,K-ATPASE β1 SUBUNITS*  

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Running Head: Intercellular interactions between Na,K-ATPase β1 subunits  
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N-glycans of the Na,K-ATPase β1 subunit are important for intercellular adhesion in epithelia, suggesting that epithelial junctions depend on N-glycan-mediated interactions between the β1 subunits of neighboring cells. The level of co-immunoprecipitation of the endogenous β1 subunit with various YFP-linked β1 subunits expressed in MDCK cells was used to assess β1:β1 interactions. The amount of co-precipitated endogenous dog β1 was greater with dog YFP-β1 than with rat YFP-β1, showing that amino-acid-mediated interactions are important for β1:β1 binding. Co-precipitation of β1 was also less with the unglycosylated YFP-β1 than with glycosylated YFP-β1, indicating a role for N-glycans. Mixing cells expressing dog YFP-β1 with non-transfected cells increased the amount of co-precipitated β1, confirming the presence of intercellular YFP-β1:β1 complexes. Accordingly, disruption of intercellular junctions decreased the amount of co-precipitated β1 subunits. The decrease in β1 co-precipitation both with rat YFP-β1 and unglycosylated YFP-β1 was associated with decreased detergent stability of junctional proteins and increased paracellular permeability. Reducing N-glycan branching by specific inhibitors increased YFP-β1:β1 co-precipitation and strengthened intercellular junctions. Therefore, interactions between the β1 subunits of neighboring cells maintain integrity of intercellular junctions, and alterations in the β1 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, while 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 (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 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 the 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 α₁ subunit. Several Na,K-ATPase-mediated signaling pathways are implicated in the regulation of intercellular junctions (reviewed in (1,4)).

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 the 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 the 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 β₁ subunit over-expressed in canine MDCK cells resulted in co-immunoprecipitation of the endogenous β₁ subunit of normal rat kidney epithelial cells NRK cells in mixed MDCK/NRK cell monolayers (8). Further, cell junction formation between surface-attached MDCK cells was inhibited by an antibody against the extracellular domain of the β₁ subunit (9). Also, over-expression of the Na,K-ATPase β₁ subunit facilitated formation of tight junctions in MDCK cells transformed by the Moloney sarcoma virus (10) and increased adhesiveness of non-polarized cells (11).

Over-expression of the unglycosylated Na,K-ATPase β₁ subunit, which was normally associated with the endogenous α₁ subunit and delivered to the lateral membranes, delayed formation of cell-cell contacts between dispersed MDCK cells (9). In addition, the over-expressed unglycosylated mutant of the β₁ subunit was significantly less resistant to detergent extraction from mature cell monolayers as compared to the over-expressed wild type β₁ subunit (9). Moreover, the endogenous α₁ subunit and E-cadherin were less resistant to the detergent in the mutant-expressing cells as compared to non-transfected cells or to cells over-expressing the wild type β₁ subunit (7). Therefore, the N-glycans of the Na,K-ATPase β₁ subunit are important for stability of the junctional complex, suggesting that they mediate the intercellular trans-interactions between the β₁ subunits of neighboring cells.

To determine whether β₁:β₁ 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 β₁ subunits over-expressed in MDCK cells on (1) co-immunoprecipitation of these subunits with the endogenous β₁ subunits, (2) detergent resistance of adhesion proteins and (3) permeability of intercellular junctions. The results indicate that N-glycans of the β₁ 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 β₁ subunits of neighboring cells.

**Experimental Procedures**

**Construction of MDCK stable cell lines**- The Na,K-ATPase rat β₁, dog β₁, the unglycosylated mutated rat β₁, the unglycosylated mutated dog β₁ and human β₂ 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-β₁ and YFP-β₂ were obtained as described previously (13).

**Cell culture**- Cells were grown in DMEM medium (Cellgro Mediatech) containing 4.5 g/L glucose, 2 mM L-glutamine, 8 mg/L phenol red, 100 units/ml penicillin, 0.1 mg/ml 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 α₁ subunit, clone C464.6 (Millipore), against GFP, clones 7.1 and 13.1, which also recognizes YFP (Roche Diagnostics), against the Na,K-ATPase β₁ 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 YFP-linked dog β₁ subunit grown in 35 mm² 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 CO₂ 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 (1,500g, 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 to 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 a 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 P40, 0.5% sodium deoxycholate and Complete Protease Inhibitor Cocktail, 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 swainsonine (Sw) (Sigma) or 100 µg/ml deoxymannojirimycin (DMJ) (Sigma) for 72 hours 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 g, 10 min) at 4°C. Then, the cell extracts (400 µg 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 hours (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 hours with or without 200 µl/mi neuraminidase (Prozyme), 150 µM/ml β-galactosidase (Prozyme), 1 mM Ca²⁺ 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 P40 and 0.05% sodium deoxycholate and finally with 10 mM Tris pH 7.5 containing 0.1% Nonidet P40 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% bromophenol 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 P40 and 1 µl PNGase F from Flavobacterium meningosepticum (New England BioLabs) for 1 hour 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, the Na,K-ATPase α₁ subunit, and the Na,K-ATPase β₁ subunit.

Western blot analysis of the total and immunoprecipitated proteins of MDCK cells-MDCK cell extracts containing 1-10 µg 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 Flavobacterium meningosepticum (New England BioLabs) according to the manufacturer 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.
(BioRad) 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, 1mM MgCl$_2$ in 10mM Tris-HCl, pH 9.0). Horseradish peroxidase was detected by using 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** ($n$LC-MS/MS) – 80 µl of the rabbit polyclonal antibodies against GFP/YFP (Clontech) were cross-linked to the protein A-agarose beads (200 µl of bead suspension) by using 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-$\beta_1$-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 ran until the front had moved 1 cm. Then the lane was excised to perform in-gel trypsin digest. The products of this digest were analyzed by nLC-MS/MS (14). The proteins identified in YFP-$\beta_1$-expressing cells, but not in non-transfected cells were considered as putative interacting partners of the $\beta_1$ subunit.

**Detergent resistance assay of the Na,K-ATPase and adherens junction proteins in MDCK cell monolayers** - Cells grown on transwell inserts (Corning Incorporated) were washed with PBS containing 1mM 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 the digitonin pre-treatment. Cell lysates were analyzed by SDS-PAGE followed by Western blot using monoclonal antibodies against GFP/YFP, the Na,K-ATPase $\alpha_1$ subunit, $\beta$-catenin and E-cadherin.

**Paracellular Permeability of Cell Monolayers** 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, 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 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-** 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 $\beta_1$ subunits associate with YFP-linked dog $\beta_1$ subunits in MDCK cell monolayers.** In total lysates of dog YFP-$\beta_1$-expressing cells, both endogenous and exogenous $\beta_1$ subunits were detected on immunoblots as two bands (Fig. 1A and 1B). The lower bands of both subunits represent their high-mannose glycosylated forms that are predominantly located in the ER, while the higher bands represent complex-type
glycosylated forms that are predominantly located in the plasma membrane (15).

To determine interacting partners of dog YFP-β1, this fusion protein was immunoprecipitated by polyclonal anti-YFP antibodies from cell lysates of MDCK cell monolayers. The immunoprecipitation of YFP-β1 itself and co-immunoprecipitation of the endogenous Na,K-ATPase α1 subunit was found by Western blot analysis using anti-YFP and anti-α1 antibodies, respectively, in YFP-β1-expressing cells, but not in non-transfected cells (Fig. 1A). The immunoblots probed with the anti-α1 antibody were further developed by using the antibody against β1 subunit. This antibody detected both YFP-β1 and endogenous β1 subunit in cell lysates (Fig. 1B, left lane). In the immunoprecipitated fraction, in addition to the two bands of YFP-β1, the anti-β1 antibody also detected a smeared band that had the same electrophoretic mobility as the plasma membrane fraction of the endogenous β1 subunit in cell lysates (Fig. 1B). After deglycosylation of immunoprecipitated proteins using PNGase F, YFP-β1 and the endogenous β1 subunit were detected at 60 kDa and 35 kDa, respectively, as expected from their core protein molecular weights (Fig. 1C). The endogenous β1 subunit was not found after immunoprecipitation from cell lysates of non-transfected MDCK cells (Fig. 1B, right lane), indicating specificity of its binding to YFP-β1. Therefore, the endogenous β1 subunits interact with YFP-linked β1 subunits in MDCK cell monolayers.

Only the plasma membrane form of the endogenous β1 subunit was co-immunoprecipitated with YFP-β1 (Fig. 1B). The quantity of the plasma membrane fraction of the endogenous β1 subunit in cell lysates accounted for about 50% of the plasma membrane fraction of YFP-β1 (Fig. 1D). In the immunoprecipitated fraction, the amount of the endogenous β1 subunit was 20% of the amount of the plasma membrane YFP-β1 (Fig. 1D). Therefore, about 40% of the endogenous β1 subunits present in the plasma membrane were bound to YFP-β1.

Since only the plasma membrane forms of the endogenous β1 subunits interact with YFP-β1, binding must occur at the sites of cell contact, where the major fraction of the plasma membrane Na,K-ATPase is present in MDCK monolayers (9). To determine whether junctional proteins that are co-localized with the Na,K-ATPase at these sites are involved in YFP-β1:β1 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-β1-expressing or non-transfected cells (Fig. 1B). Therefore, the endogenous β1 subunits interact with expressed YFP-β1 at the lateral membrane, and this interaction is not mediated by the cell adhesion molecules of adherens junctions or tight junctions.

The β1 subunits of neighboring cells interact with each other. To determine whether interactions between YFP-β1 and endogenous β1 subunits occur in the same membrane or between two neighboring cells in a cell monolayer, the amount of endogenous β1 subunits co-immunoprecipitated with the dog YFP-β1 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 Ca2+-free medium resulted in re-formation of a significant fraction of cell contacts after one hour of cell incubation in the medium followed by a complete recovery of the cell monolayer (Fig. 2A).

Immunoprecipitation of YFP-β1 from lysates of both confluent and separated cells resulted in co-immunoprecipitation of the endogenous β1 subunits (Fig. 2B). However, the amount of co-precipitated β1 subunits was significantly less in separated cells. To enable accurate quantification of co-precipitated β1 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. 2B, top panel). In the second
tube, the immunoprecipitated proteins were deglycosylated by PNGase F and then analyzed by immunoblotting of the endogenous β₁ subunits (Fig. 2B, bottom panel). Densitometric quantification of the amount of the β₁ subunits relative to the amount of the plasma membrane fractions of YFP-β₁ showed that the amount of YFP-β₁-bound β₁ subunits was 2-fold less in separated compared to confluent cells (Fig. 2C). There was no change in the amount of co-immunoprecipitated β₁ subunits when the immunoprecipitation reaction was performed with or without addition of 1mM Ca²⁺ or 1mM EGTA (Fig. 2D). Therefore, the smaller amount of co-precipitated β₁ subunits in separated cells was indeed due to disruption of cell-cell contacts, but not due to lack of Ca²⁺. These results suggest that the separation of cells disrupted the intercellular interactions between YFP-β₁ and endogenous β₁ subunits. It seems unlikely that the loss of intercellular junctions would result in dissociation of the β₁ subunits from the oligomeric complexes of the Na,K-ATPase in the same membrane, resulting in a decreased amount of YFP-β₁-precipitated β₁ subunits. However, since such a possibility cannot be excluded, we used an alternative approach to confirm the presence of the intercellular β₁:β₁ interaction.

We compared the amount of YFP-β₁-co-immunoprecipitated endogenous β₁ subunits in mixed cell monolayers of YFP-β₁-expressing cells and non-transfected cells with that in the monolayers of YFP-β₁-expressing cells only. If β₁:β₁ interactions are intercellular, more efficient co-precipitation of endogenous β₁ subunits is expected in mixed co-cultures. On the other hand, if YFP-β₁ interacts with the endogenous β₁ subunits only in the same cell, adding the non-transfected cells should not have an effect on co-precipitation of endogenous β₁ subunits with YFP-β₁. The results show that the amount of co-precipitated beta was increased by 70% by adding the equal amount of non-transfected cells to YFP-β₁-expressing cells and by 116% by adding a 4-fold excess of non-transfected cells to YFP-β₁-expressing cells (Fig.3), confirming the presence of the intercellular YFP-β₁:β₁ interactions.

The lack of N-glycans and differences in the amino-acid sequence weaken the interaction between the endogenous and exogenous β subunits. To determine the importance of N-glycans and the polypeptide sequence in β₁:β₁ 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 β₁ subunits. Five stable cell lines expressing YFP-β₂, dog YFP-β₁, rat YFP-β₁, the unglycosylated dog YFP-β₁, and the unglycosylated rat YFP-β₁ 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. 4A). In all these transfected cell lines, the level of expression of the endogenous β₁ subunits was similar, but less than in non-transfected MDCK cells (Fig. 4B). A decrease in the endogenous β₁ subunits in transfected cells was probably due to competition of the exogenous with endogenous β subunits for binding to the endogenous α₁ subunits. This binding is required for the export of the β subunits from the ER and delivery to the plasma membrane (15). As a result, a fraction of the endogenous β₁ 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 α₁ subunits in the co-immunoprecipitation assay (Fig. 4C).

The amount of endogenous β₁ subunits that co-immunoprecipitated with different species of YFP-β varied. The highest amount of the endogenous β₁ subunits (normalized to the amount of immunoprecipitated YFP-β) was detected for dog YFP-β₁ (Fig. 4C and 4D). The amount of β₁ subunits was less with rat YFP-β₁. For both dog and rat YFP-β₁, the amount of co-precipitated β₁ subunits was higher for fully glycosylated than for unglycosylated YFP-β₁ (Fig. 4C and 4D). No co-precipitation of the β₁ subunits was detected with expressed human YFP-β₂. These results show that both amino-acid and carbohydrate residues of the β₁ subunits contribute to β₁:β₁ binding. These results also rule out the presence of complexes containing two α₁, one β₁, and one β₂ subunits.
Reducing N-glycan complexity improves the interaction between the endogenous and exogenous β1 subunits. To determine whether the structure of N-glycans is important for β1:β1 interaction, we used inhibitors of N-glycan processing to change the type of N-glycans. Normally, the high-mannose N-glycans formed by trimming of 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 mannosidase and glycosyltransferases (Fig. 5A). 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 the newly synthesized glycoproteins contain only high-mannose-type N-glycans (Fig. 5A). Also, the inhibitor of the Golgi mannosidase II, Sw, 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. 5A).

Cell incubation with the inhibitors for 72 hours was sufficient to substitute complex-type glycosylated YFP-β1 and endogenous β1 subunits with newly synthesized high-mannose or/and hybrid-type subunits (Fig. 5B). In cells exposed to DMJ, both endogenous and exogenous β1 subunits were predominantly of the high-mannose type, while in cells exposed to Sw, both high-mannose and hybrid type forms were present (Fig. 5B). Immunoprecipitation of YFP-β1 resulted in co-immunoprecipitation of the endogenous β1 subunits in cells pretreated with either inhibitor. The amount of the co-precipitated β1 subunits was greater in Sw-exposed cells and even more in DMJ-exposed cells as compared to the control cells (Fig. 5D and 5E), indicating that less complex structure of N-glycan favors β1:β1 interaction. Exposure to either DMJ or Sw did not affect plasma membrane delivery of YFP-β1 (Fig. 5C).

Sialic acid residues are the major terminal residues in N-glycans linked to the β1 subunit of the renal Na,K-ATPase (16). To determine whether these negatively charged residues are important for β1:β1 interaction, we studied the effect of neuraminidase on co-immunoprecipitation of the endogenous β1 subunits with YFP-β1. Immunoprecipitation of dog YFP-β1 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-β1-bound endogenous β1 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-β1 and β1 subunits. Moreover, they seem to prevent efficient β1:β1 interaction. These results are in agreement with improvement of β1:β1 interaction by Sw and DMJ, which partially and completely, respectively, prevent addition of sialic acid residues to N-glycans (Fig. 5A).

Assessment of the possible involvement of other molecules in the interaction between endogenous and exogenous β1 subunits. To see whether the intercellular β1:β1 interaction is mediated by other molecules, we performed a search for β1-interacting proteins using nLC-MS/MS of YFP-β1-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 β1 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 β1:β1 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 β1:β1 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-β1, which was performed after 24-hour
incubation of cells with lactose, resulted in no change of the amount of co-immunoprecipitated β₁ subunits (Fig. 6, lanes 1 and 2). As an alternative approach to determine the possible involvement of galectin-3, we performed immunoprecipitation of YFP-β₁ in the presence of β-galactosidase, which should remove terminal galactose residues, which are galectin-3 ligands, from N-glycan termini of both YFP-β₁ and β₁ subunits. However, immunoprecipitation performed in the presence of β-galactosidase did not change the amount of YFP-β₁, co-precipitated β₁ subunits (Fig. 6, lanes 1 and 3). Comparative Western blot analysis of YFP-β₁ immunoprecipitated without or with the enzyme did not show any visible change in the electrophoretic mobility of YFP-β₁ (Fig. 6, top panel, lanes 1 and 3), indicating either the lack of β-galactose residues on N-glycan termini of YFP-β₁, or the lack of the β-galactosidase activity. In contrast, the electrophoretic mobility of YFP-β₁ treated with two enzymes (neuraminidase and β-galactosidase) was greater than that of YFP-β₁ treated with neuraminidase alone (Fig. 6, top panel, lanes 4 and 5). These results show that β-galactosidase cleaved the galactose residues located underneath 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-β₁:β₁ co-precipitation. Removing of the galactose residues located underneath sialic acid residues did not have an effect on YFP-β₁:β₁ 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 β₁;β₁ interaction and hence galectin-3 apparently does not link the β₁: subunits of neighboring cells.

**Reduction in the number of YFP-β₁:β₁ complexes decreases both stability and tightness of intercellular junctions.** To determine whether intercellular β₁:β₁ 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-β₁ (Fig. 7-7).

It is known that the cytoplasmic domain of the Na,K-ATPase is linked to the cytoplasmic domain of E-cadherin via ankyrin/spectrin cytoskeleton (5,29,30). E-cadherin is a main cell adhesion molecule of the adherens junctions. Its extracellular domain binds to the extracellular domain of E-cadherin molecule of a neighboring cell, while 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 (22,23) 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, the Na,K-ATPase is resistant to detergent extraction in cell monolayers, but not in dispersed MDCK cells (7). If the intercellular β₁:β₁ interaction is important for association of the Na,K-ATPase with the cytoskeleton, the weakening of β₁:β₁ binding by removing N-glycans or by altering an amino-acid sequence is expected to increase detergent extractability of the Na,K-ATPase, as well as extractability of E-cadherin and β-catenin, since they are linked to the same cytoskeleton network.

Dog YFP-β₁ was more resistant to digitonin extraction from cell monolayers than the rat YFP-β₁ (Fig. 7). The unglycosylated YFP-β₁ subunits, both dog and rat, were less resistant than their fully glycosylated native forms (Fig. 7). YFP-β₂ 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 β₁ subunits (Fig. 4C and 4D). Stability of the endogenous α₁ 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 the adherens junction proteins, E-cadherin and β-catenin, was greater in cells expressing fully glycosylated dog YFP-β1 than in cells expressing rat YFP-β1, YFP-β2, or unglycosylated YFP-β1 (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-β1 < the wild type rat YFP-β1 < the unglycosylated rat YFP-β1 < the wild type YFP-β2 (Fig. 8). Therefore, the ability of the expressed YFP-linked subunits to interact with the endogenous β1 subunits directly correlates with the detergent resistance of junctional proteins and inversely correlates with the paracellular permeability, indicating that β1;β1 interaction is important for stability and integrity of intercellular junctions.

**DISCUSSION**

*Intercellular interaction between β1 subunits of neighboring cells versus oligomerization of pumps in the same membrane.* Immunoprecipitation of YFP-β1 from cell lysates of YFP-β1-expressing cell monolayers resulted in co-immunoprecipitation of the plasma membrane, but not of the ER fraction of the endogenous β1 subunit (Fig. 1B), showing that binding between YFP-β1 and β1 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 β1 subunits was previously documented in mixed monolayers of two cell types, in which endogenous β1 subunits of rat cells were co-immunoprecipitated with rat YFP-β1 expressed in MDCK cells (8). Here we present additional evidence for intercellular interactions between the β1 subunits of neighboring MDCK cells in a monolayer. We found that the amount of YFP-β1-co-precipitated β1 subunits was increased in mixed monolayers of YFP-β1-expressing cells with non-transfected cells as compared to the monolayers of YFP-β1-expressing cells only (Fig. 3). The results indicate that, at the borders between two YFP-β1-expressing cells, YFP-β1 interacts with both YFP-β1 and the endogenous β1 subunits present in the neighboring cells. However, at the mixed borders, YFP-β1 interacts only with the endogenous β1 subunits. As a result, increasing the proportion of mixed contacts increases the amount of YFP-β1-bound β1 subunits. Consistent with the presence of intercellular β1;β1 interactions in a cell monolayer, disruption of intercellular junctions resulted in a significant decrease in the amount of YFP-β1-precipitated β1 subunits (Fig. 2). A similar decrease in co-immunoprecipitated β1 subunits in separated cells was observed with rat YFP-β1 and with the unglycosylated YFP-β1 (not shown).

Therefore, our results confirm the presence of intercellular β1;β1 interactions. However, they do not exclude the possibility of β1;β1 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 β1;β1 binding.* In mixed cultures of cells of different species, the β1 subunit was detected on homotypic, but not on heterotypic cell contacts, suggesting that the β1 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 β1;β1 interaction is indeed species-specific. The amount of co-precipitated β1 subunits was less with rat YFP-β1 than with dog YFP-β1 (Fig. 4). The protein sequences of the extracellular domains of dog and rat β1 subunits have some differences, while the number and positions of N-glycans are the same. No co-precipitation of dog β1 subunits was detected with human YFP-β2 (Fig. 4) that has even more differences in the amino acid sequence. Therefore, these results emphasize the importance of protein-protein interactions for β1;β1 binding. These results also indicate that the amino acid residues important for β1;β1 binding must be different in the dog and rat β1 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 β1 subunit, clone M17-P5-F11 (27). This antibody recognizes the dog, but not...
the rat β1 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 β1:β1 interactions.

N-glycans stabilize and strengthen direct protein-protein interactions between the extracellular domains of the β1 subunits. For both dog and rat YFP-β1, the amount of co-precipitated β1 subunits was significantly greater with normally glycosylated YFP-fusion proteins than with their unglycosylated mutants (Fig. 4). Since N-glycans are not required for proper folding of the β1 subunits (28), these results indicate that N-glycans are also responsible for β1:β1 interaction. Less complex N-glycans appear to be more suitable for this interaction, since YFP-β1:β1 co-immunoprecipitation is improved both by Sw and DMJ (Fig. 5D and 5E). In addition, the presence of sialic acid residues appears to repel the interacting β1 subunits from each other, since removal of terminal sialic acid residues improved YFP-β1:β1 co-immunoprecipitation (Fig. 6, lane 5). This result also indicates that sialic-acid-binding lectins are not implicated in β1:β1 interaction. The interaction does not involve galactose-binding lectins either, since β1:β1 binding is not impaired by removing terminal galactose residues or by the inhibitor of galactose-galectin binding, lactose (Fig. 6, lanes 2 and 3). Further, the involvement of other lectins that bind to mannose or N-acetylglucosamine residues is doubtful, since these residues are not exposed on the N-glycan termini of the β1 subunits (16). Therefore the β1:β1 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 β1-interacting proteins. These data are consistent with the results of fluorescence resonance energy transfer showing that the β1 subunits of neighboring cells in a cell monolayer have sufficient proximity to permit direct interaction (8).

Therefore, N-glycans are important for β1:β1 binding, most likely due to stabilizing and strengthening amino-acid-mediated interactions. However, the contribution of direct glycan-glycan interactions cannot be excluded.

The β1:β1 bridges between neighboring cells are essential for regulating stability and tightness of intercellular junctions in epithelia. The Na,K-ATPase β1 subunits do not directly interact with cell adhesion molecules of adherens and tight junctions, E-cadherin and occludin (Fig. 1B). 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, since it is connected to the Na,K-ATPase of the neighboring cells via its β1 subunit and to the cytoskeleton via its α1 subunit. Weakening the β1:β1 interactions by removing N-glycans or by changing the amino-acid sequence of one of the two interacting β1 subunits decreases resistance to detergent extraction not only of the Na,K-ATPase, but also of E-cadherin and β-catenin (Fig. 7). Therefore, the β1:β1 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 inversely correlated with the ability of YFP-β variants to bind β1 subunits (Fig. 8). Therefore, the intercellular homotypic interactions between the Na,K-ATPase β1 subunits are important for stability of adherens junctions and integrity of the tight junctions.

Improved binding between β1 subunits due to reduced complexity of N-glycan structure in the presence of Sw and DMJ (Fig. 5) is consistent with the results showing that exposure of MDCK cell monolayers to Sw decreased the paracellular permeability and increased the resistance of the adherens junction proteins to extraction by a non-ionic detergent (7). The effects of Sw were attenuated in a cell line expressing the unglycosylated β1 subunits, indicating that it is the decreased branching of β1 subunit N-glycans that tightens and stabilizes cell-cell junctions. Since 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 β₁ subunit.

In conclusion, the bridges between the Na,K-ATPase β₁ subunits of neighboring cells are crucial for integrity of intercellular junctions in epithelia. Both amino-acid residues and N-glycans are involved in β₁:β₁ binding. The results suggest an important role of regulated N-glycosylation of the Na,K-ATPase β₁ subunit in modulation of intercellular adhesion in epithelia.

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FOOTNOTES

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The abbreviations used are: YFP-β1 and YFP-β2, the fusion proteins between the yellow fluorescent protein and the Na,K-ATPase β1 subunit and β2 subunit, respectively; Sw, swainsonine; DMJ, deoxymannojirimycin.

FIGURE LEGENDS

**Fig. 1.** The endogenous plasma membrane-resident Na,K-ATPase β1 subunit is bound to YFP-linked Na,K-ATPase dog β1 subunit in MDCK cell monolayers. A. Western blot analysis using anti-YFP and anti-Na,K-ATPase α1 antibodies shows that immunoprecipitation of YFP-β1-dog resulted in co-precipitation of the endogenous Na,K-ATPase α1 subunit from cell lysates of YFP-β1-dog-expressing cells, but not from non-transfected MDCK cells. In the left lane on A and B, 10% of the total amount of cell lysate used for immunoprecipitation was loaded. B. Immunoprecipitation of YFP-β1-dog resulted also in co-precipitation of the Na,K-ATPase endogenous β1 subunits, as detected by immunoblotting using the anti-β1 antibody, which reacts with both YFP-β1-dog and endogenous β1 subunits. Both the plasma membrane and ER forms of the of endogenous β1 subunit were detected in total cell lysates of transfected cells, while only the plasma membrane form was detected in the immunoprecipitated fraction. No β1 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-β1 at 60 kDa, and the deglycosylated endogenous β1 subunit at 35 kDa. D. Densitometry quantification of the results presented in B. In both cell lysate and immunoprecipitated fraction, density of the bands was calculated as a percentage of the density of the plasma membrane fraction of YFP-β1-dog. Error bars, ±s.d. (n=3); *, significant difference from “PM β1,” in cell lysate, P<0.001, Student’s t-test.

**Fig. 2.** Disruption of intercellular junctions decreases the amount of the endogenous Na,K-ATPase β1 subunit bound to YFP-β1. A. Confocal microscopy images of MDCK cells expressing dog YFP-β1 show that a short incubation of confluent cell monolayers with 1mM EDTA followed by 1-hr incubation with Ca2+-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 from lysates obtained either from confluent YFP-β₁-expressing cells (panel “Confluent cells” on A) or from YFP-β₁-expressing cells separated from each other by cell incubation with PBS for 60 min (panel “Separated cells” on A). Immunoprecipitated YFP-β₁ was detected by anti-YFP antibody. Co-precipitated endogenous Na,K-ATPase β₁ subunits were deglycosylated with PNGase F prior to SDS-PAGE and Western blot analysis using the anti-β₁ antibody to enable accurate quantification. C. Densitometric quantification of the results presented in B was performed by dividing a signal from anti-β₁ antibody by the corresponding signal from anti-YFP antibody for PM YFP-β₁. The comparative bar graph shows these ratios as a percentage of the ratio obtained for confluent cells. D. Immunoprecipitation of dog YFP-β₁ was performed in the absence or in the presence of 1 mM Ca²⁺ or 1 mM EGTA. To enable quantitative comparison of immunoprecipitated and co-immunoprecipitated subunits, immunoprecipitated protein complexes were deglycosylated prior to SDS-PAGE. DME, Dulbecco’s Modified Eagle Medium; IP, immunoprecipitation; 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.

**Fig. 3.** Increasing the number of intercellular contacts between non-transfected and YFP-β₁-expressing MDCK cells increases the amount of YFP-β₁-co-immunoprecipitated endogenous β₁ subunits. A. Confocal microscopy images of the confluent monolayers formed by MDCK cells expressing dog YFP-β₁ and by co-cultured dog YFP-β₁-expressing cells and non-transfected cells mixed at 1:1 and 1:4 ratios. Only YFP-β₁-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-β₁-expressing cells, from co-cultures of YFP-β₁-expressing cells with non-transfected cells at 1:1 ratio, or from co-cultures of YFP-β₁-expressing cells with non-transfected cells at 1:4 ratio. To maintain the similar amount of the immunoprecipitated YFP-β₁, 0.2, 0.4 and 1 mg of total protein, respectively, was used for immunoprecipitation from YFP-β₁-expressing cells, 1:1 cell mixture and 1:4 cell mixture, while 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-β₁ 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 YFP-β₁-dog cell line. The amount of co-immunoprecipitated endogenous Na,K-ATPase β₁ subunits was increased by adding non-transfected cells to β₁-expressing cells. IP, immunoprecipitation; NT, non-transfected cells; DG, deglycosylated by PNGase F. Error bars, ±s.d. (n=3); *, significant difference from YFP-β₁-expressing cells, P<0.01, Student’s t-test.

**Fig. 4.** Interactions between the endogenous and exogenous Na,K-ATPase β subunits in MDCK cell monolayers depend on their amino acid sequence and the presence of N-glycans. A. Confocal microscopy images of MDCK cell monolayers (horizontal sections) show predominant localization of the YFP-linked wild type β₂ subunit and wild type or unglycosylated β₁ subunits (dog and rat) on the lateral membranes. B. Western blot analysis of total cell lysate pre-treated with PNGase F shows similar levels of expression of endogenous Na,K-ATPase β₁ subunits in all studied YFP-β₁-expressing cell lines. C. Western blot analysis of the proteins immunoprecipitated by anti-YFP antibody shows co-immunoprecipitation of the endogenous Na,K-ATPase β₁ and α₁ subunits with YFP-linked β₁ subunits and with four variants of the β₁ subunits from total lysates of corresponding transfected, but not from lysates of non-transfected MDCK cells. To enable comparative densitometric quantification of immunoprecipitated YFP-linked β₁ subunits and co-immunoprecipitated endogenous β₁ subunits, immunoprecipitated proteins were treated with of PNGase F prior to SDS-PAGE and Western blot analysis. D. Densitometric quantification for each cell line is performed by dividing a signal from anti-β₁ 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 YFP-β₁-dog cell line. The amount of co-
immunoprecipitated endogenous Na,K-ATPase β₁ subunits is less with rat than with dog exogenous β₁ subunits. For both dog and rat exogenous subunits, the amount of co-immunoprecipitated endogenous Na,K-ATPase β₁ subunits is less with the unglycosylated subunits than with fully glycosylated subunits. Error bars, ±s.d. (n=3); *, significant difference from “YFP-β₁-dog”, P<0.001, Student’s t-test.

**Fig. 5.** Prevention of N-glycan processing that preserves high-mannose- or hybrid-type structure of N-glycans increases the amount of the YFP-β₁-bound endogenous β₁ subunits. A. A scheme showing that inhibition of N-glycan processing by deoxymannojirimycin (DMJ) and swainsonine (Sw) preserves high-mannose- and hybrid-type structure of N-glycans, respectively. B. Western blot analysis of lysates of control and inhibitor-treated cells shows that cell incubation with DMJ or Sw for 72 hrs was sufficient to substitute the complex-type (Com.) glycosylated YFP-β₁ and endogenous β₁ subunits with the newly synthesized high-mannose- (H-M) or hybrid-type (Hyb.) forms of the subunits. C. Confocal microscopy images of YFP-β₁-dog-expressing cells showing that cell exposure to Sw or DMJ did not prevent plasma membrane delivery of YFP-β₁. D. Western blot analysis of immunoprecipitated YFP-β₁ (anti-YFP antibody) and co-immunoprecipitated endogenous Na,K-ATPase β₁ subunits (anti-β₁ 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 anti-β₁ antibody by the corresponding signal from anti-YFP antibody, shows an increase in the amount of YFP-β₁-bound endogenous β₁ subunits in immunoprecipitated fractions from cells pre-treated with Sw 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.

**Fig. 6.** Assessment of the possible involvement of sialic-acid or galactose residues of N-glycans in YFP β₁-β₁ interaction. Immunoprecipitation of dog YFP-β₁ was performed for 3 hours at room temperature in the absence or in the presence of neuraminidase or β-galactosidase, or after 24 hr incubation of cells with 50mM of lactose. Western blot analysis of immunoprecipitated YFP-β₁ using anti-YFP antibody shows a noticeable increase in the electrophoretic mobility of the complex-type glycosylated form of the neuraminidase-treated YFP-β₁ as compared to 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-β₁, while a combination of neuraminidase and β-galactosidase increased the electrophoretic mobility of YFP-β₁. 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 β₁ subunits (anti-β₁ antibody) shows an increase in the amount of co-precipitated β₁ 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 β₁ 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.

**Fig. 7.** The fully glycosylated dog YFP-β₁ is more resistant to removal from a cell monolayer by a non-ionic detergent than the rat YFP-β₁, or the unglycosylated mutants of YFP-β₁, or the wild type YFP-β₂. Mature MDCK cell monolayers expressing various YFP-linked β subunits of the Na,K-ATPase were lysed as described in Methods either before or after a 30-min pre-incubation with 1% digitonin, which was then replaced by a cell lysis buffer. The amount of YFP-linked β subunits, Na,K-ATPase α₁ subunit, β-catenin and E-cadherin before and after pre-incubation with 1% digitonin was determined by a 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 YFP-β₁-dog-expressing cells, P<0.01, Student’s t-test.
Fig. 8. MDCK cells expressing fully glycosylated dog YFP-β₁ form tighter monolayers than cells expressing rat YFP-β₁, or its unglycosylated mutant, or the wild type YFP-β₁. 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 YFP-β₁-dog-expressing cells, P<0.01, Student’s t-test.
Fig. 1.
Fig. 2.

A

Confluent cells | Separated cells: Ca^{2+}-free buffer, 1hr | Re-connected cells: Ca^{2+}-free buffer, 1hr with DMEM | Recovered cells: Ca^{2+}-free buffer, 1hr 16 hrs with DMEM

Incubation of separated cells with DMEM, min

0 min 10 min 20 min 30 min 40 min 50 min 60 min

B

Cell line | YFP-β1-dog | NT
---|---|---
| IP: anti-YFP |

anti-YFP

PM YFP-β1 → ER YFP-β1 → 75

IP: anti-YFP → PNGase F

(anti-β, ab) DGβ1 → 35

Confluent | Separated | Confluent

C

DGβ1/PM YFP-β1, % of that in confluent

Confluent | Frames

D

IP: anti-YFP → PNGase F

(anti-YFPab) DG YFP-β1 → 60

(anti-β, ab) DGβ1 → 35

Ca^{2+} | - | - | +

EGTA | - | + | -

Fig. 2.
Fig. 3

A

B

C

Table: Co-IP of YFP-β1, β1, and β1/NT

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>YFP-β1</th>
<th>YFP-β1/NT (1:1)</th>
<th>YFP-β1/NT (1:4)</th>
<th>NT cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP: anti-YFP</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>PNGase F</td>
<td></td>
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</tr>
</tbody>
</table>

- DG YFP-β1 (anti-YFP) → PNGase F → 60
- DG β1 (anti-β, αβ) → 35

Graph: Co-IP of YFP-β1, β1, and β1/NT in YFP-β1 cells

- YFP-β1 cells, % of total
- 100, 50, 20

* Significant difference
Fig. 4.
Fig. 5.
Fig. 6.
Fig. 7.
Fig. 8. Paracellular permeability for BCECF, % of that in YFP-β₁-dog cells

- YFP-β₁-human
- YFP-β₁-dog
- YFP-β₁-rat
- UGFP-β₁-rat
Epithelial junctions depend on intercellular trans-interactions between the Na,K-ATPase β1 subunits
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