The Role of the $\beta_1$ Subunit of the Na,K-ATPase and Its Glycosylation in Cell-Cell Adhesion

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Based on recent data showing that overexpression of the Na,K-ATPase $\beta_1$ subunit increased cell-cell adhesion of nonpolarized cells, we hypothesized that the $\beta_1$ subunit can also be involved in the formation of cell-cell contacts in highly polarized epithelial cells. In support of this hypothesis, in Madin-Darby canine kidney (MDCK) cells, the Na,K-ATPase $\alpha_1$ and $\beta_1$ subunits were detected as precisely co-localized with adherens junctions in all stages of the monolayer formation starting from the initiation of cell-cell contact. The Na,K-ATPase and adherens junction protein, $\beta_1$-catenin, stayed partially co-localized even after their internalization upon disruption of intercellular contacts by Ca$^{2+}$ depletion of the medium. The Na,K-ATPase subunits remained co-localized with the adherens junctions after detergent treatment of the cells. In contrast, the heterodimer formed by expressed unglycosylated Na,K-ATPase $\beta_1$ subunit and the endogenous $\alpha_1$ subunit was easily dissociated from the adherens junctions and cytoskeleton by the detergent extraction. The MDCK cell line in which half of the endogenous $\beta_1$ subunits in the lateral membrane were substituted by unglycosylated $\beta_1$ subunits displayed a decreased ability to form cell-to-cell contacts. Incubation of surface-attached MDCK cells with an antibody against the extracellular domain of the Na,K-ATPase $\beta_1$ subunit specifically inhibited cell-cell contact formation. We conclude that the Na,K-ATPase $\beta_1$ subunit is involved in the process of intercellular adhesion and is necessary for association of the heterodimeric Na,K-ATPase with the adherens junctions. Further, normal glycosylation of the Na,K-ATPase $\beta_1$ subunit is essential for the stable association of the pump with the adherens junctions and plays an important role in cell-cell contact formation.

Specialized tight epithelia of multicellular organisms function as barriers that maintain the distinct molecular composition of apical and basolateral plasma membrane domains. Individual cells within the monolayer are linked with each other to maintain its structural integrity and to retard or prevent the diffusion of solutes through the intercellular space. The junctional complex in epithelial cells consists of tight junctions, adherens junctions, and desmosomes (1). Adherens junctions and desmosomes mechanically link adjacent cells, whereas tight junctions are responsible for intercellular sealing (2, 3). Gap junctions are important for communication between cells of the monolayer by allowing passage of small water-soluble molecules and ions from cell to cell.

Development of cell junctions is a key step in the evolution from individual cells to a functional epithelium. Moreover, disruption of these junctions and structural contacts between cells is the key event in the epithelial-mesenchymal transition, the process in which cells change from a highly polarized epithelial phenotype to a motile, fibroblast-like phenotype (4, 5). This transition occurs with inflammation, fibrosis, and malignant transformation of tissues (6, 7). Cell-to-cell contacts are also disrupted in ischemia-induced acute tubular necrosis (8, 9) and polycystic kidney disease (10).

Many of the factors essential for the development or maintenance of cell junctions remain undefined. Recent studies have shown that inhibition of Na,K-ATPase activity by exposure to ouabain or by K$^+$ depletion resulted in cell detachment in mature monolayers (11–13) or prevented tight junction formation between surface-attached Madin-Darby canine kidney (MDCK)$^2$ cells in culture (14, 15). Inhibition of the Na,K-ATPase also increased permeability of polarized monolayers of human retinal pigment epithelial cells and pancreatic polarized cell line to both ions and nonionic molecules (16, 17). These results were interpreted as showing that activity of the Na,K-ATPase is important for formation and maintenance of cell-cell contacts. How the Na,K-ATPase activity contributes to intercellular adhesion is not clear. It was suggested that the pump stimulates activity of the RhoA GTPase that is involved in F-actin stress fiber formation (14, 15). Since the Na,K-ATPase is important for establishment of transepithelial transport, maintenance of the normal membrane potential, and normal intracellular concentrations of K$^+$, Na$^+$, Ca$^{2+}$, and other ions and neutral molecules, cell adhesion could depend on any of these factors. The observations that 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 concent-

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† The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1 and Videos 1–5.

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$^2$ The abbreviations used are: MDCK, Madin-Darby canine kidney; CHO, Chinese hamster ovary; YFP, yellow fluorescent protein; GFP, green fluorescent protein; TRITC, tetramethylrhodamine isothiocyanate; PBS, phosphate-buffered saline; YFP-3 Subunit, the fusion protein between the yellow fluorescent protein and the Na,K-ATPase $\beta_1$ subunit.
**Na,K-ATPase β1, Subunit and Cell-Cell Adhesion**

Mutation of Na⁺ (12, 14) suggest that the maintenance of the ion balance by the Na,K-ATPase is crucial for cell-cell adhesion.

Overexpression of the Na,K-ATPase β1 subunit in nonpolarized CHO cells and MDCK cells transformed by Moloney sarcoma virus (MSV-MDCK) increased cell-cell adhesion in these cell lines (18, 19). These data could suggest that the Na,K-ATPase β1 subunit acts as an adhesive protein. However, since in both cell lines overexpression of the β1 subunit increased expression of the endogenous α1 subunit (18, 20), these data might be interpreted differently. It is possible that gain of adhesiveness by these two cell lines is a result of contribution of the increased Na,K-ATPase activity to cell adhesion or a signaling role of the α1 subunit. Recent results suggested that overexpression of the Na,K-ATPase β1 subunit in MSV-MDCK cells suppressed cell motility due to the signaling mechanism involving both subunits of the Na,K-ATPase, annexin-II, and phosphatidylinositol 3-kinase (21).

To examine the possibility that the Na,K-ATPase β1 subunit is directly involved in cell-cell adhesion by providing a structural link between neighboring cells, we studied association of the pump with constituents of the junctional complex during maturation of the monolayer and after disruption of cell to cell contacts. In addition, we analyzed the impact of removal of N-glycosylation sites from the β1 subunit on its attachment to adherens junctions. Finally, we determined the effect of exposure of MDCK cells to a β1 subunit-specific antibody and to ouabain on cell contact formation.

The results of these studies showed that the Na,K-ATPase associates with the adherens junctions upon the inception of cell junction formation. Upon disruption of cell contacts, the Na,K-ATPase is retrieved from the sites of cell contact together with adherens junction proteins. Furthermore, removal of N-glycosylation sites from the Na,K-ATPase β1 subunit loosens association of the enzyme with the adherens junctions and impairs the initial step of cell-cell adhesion. Finally, attachment of a specific antibody to the extracellular domain of the Na,K-ATPase β1 subunit inhibits cell-cell contact formation, whereas exposure to ouabain has no effect.

These novel data indicate that the Na,K-ATPase β1 subunit plays an important role in the establishment of contacts between MDCK cells, an important step in the maturation of a tight epithelium.

**EXPERIMENTAL PROCEDURES**

**Construction of DNAs Encoding YFP-linked Constructs of the Na,K-ATPase β1, Subunit and Its Mutants Lacking N-Glycosylation Sites**—The vector pEYFP-β1 encoding the fusion protein of YFP linked to the amino terminus of the rat Na,K-ATPase β1 subunit (YFP-β1) was constructed as described previously (22). Mutated YFP-β1 fusion proteins lacking one, two, or three N-glycosylation sites were generated by using the QuikChange mutagenesis kit (Stratagene). The vector encoding a fusion protein of the bile acid transporter, NTCP, and YFP (23) was kindly provided by Olga Mareninova.

**Stable Transfection—**MDCK cells were purchased from ATCC. In order to obtain cell lines stably expressing NTCP-YFP, wild type YFP-β1, or mutated YFP-β1 fusion proteins, MDCK cells were grown on 10-cm plates until 20% confluence and transfected with NTCP-YFP, the wild type or mutated pEYFP-β1 using the FuGENE 6 Transfection Reagent (Roche Applied Science). Stable cell lines were selected by adding, 24 h after transfection, the eukaryotic selection marker G-418 at a final concentration of 1.0 mg/ml. This concentration of G-418 was maintained until single colonies appeared. 15-20 colonies were isolated, expanded, and grown in the presence of 0.25 mg of G-418 per ml of medium in 24-well plates. Two clones with the highest expression of YFP-β1 or NTCP-YFP were selected and expanded for further studies.

**Confocal Microscopy Identification of the Site of Expression of YFP-linked Proteins—**Cells stably expressing NTCP-YFP or wild type or mutated YFP-β1 were grown for at least 5 days after becoming confluent on collagen-coated glass bottom microwell dishes (MatTek Corp.) or Corning Costar polyester transwell inserts (Corning Glass). Confocal microscopic images were acquired using the Zeiss LSM 510 laser-scanning confocal microscope using LSM 510 software, version 3.2.

**Primary Antibodies—**The following antibodies were used for immunostaining, Western blot analysis, and in cell-cell adhesion assay: TRITC-conjugated monoclonal antibody against β-catenin (BD Transduction Laboratories), a monoclonal antibody C464.6 against the Na,K-ATPase α1 subunit (Upstate Biotechnology, Inc., Lake Placid, NY), a monoclonal antibody against occludin (Zymed Laboratories Inc.), a monoclonal antibody against spectrin (Chemicon), a monoclonal antibody against E-cadherin (Sigma), a polyclonal antibody against EE1, the marker of early endosomes (Abcam), a monoclonal antibody against the Na,K-ATPase β1 subunit, clone M17-P5-F11 (Affinity Bioreagents), and the monoclonal antibody against GFP, clones 7.1 and 13.1, that also recognized YFP (Roche Applied Science) and a monoclonal antibody against transferrin receptor, clone OX-26 (Invitrogen).

**Immunofluorescent Staining of Cell Monolayers—**Cells were fixed by incubation with 3.75% formaldehyde in PBS for 15 min at 37 °C (before or after treatment with 0.25% Triton X-100 as indicated) and permeabilized by incubation with 0.1% Triton X-100 for 15 min. Then cells were incubated with Dako protein block serum-free solution (Dako Corp.) for 30 min. F-actin was stained by using Alexa633-conjugated phalloidin (Invitrogen). The β-catenin was visualized using TRITC-conjugated monoclonal antibody against β-catenin (BD Transduction Laboratories). Immunostaining of other proteins was performed by a 1-h incubation with the appropriate primary antibodies followed by a 1-h incubation with one of the following secondary antibodies: Alexa633-conjugated mouse or anti-rabbit antibodies or Alexa488-conjugated anti-mouse antibodies (Invitrogen) or TRITC-conjugated anti-rat antibodies (MP Biomedicals).

**Triton X-100 Treatment of MDCK Cells—**Triton X-100 treatment of MDCK cells was performed by a previously described procedure (24). Briefly, cells grown on glass bottom microwell dishes or Corning Costar polyester transwell inserts (Corning Glass) were washed with PBS containing 1 mM Ca⁺² and 1 mM Mg⁺² twice and incubated with the PBS containing 0.25% Triton X-100 for 5 min at room temperature. Then cells were washed twice with PBS, fixed by incubation with 3.75% formaldehyde for 15 min at room temperature, rinsed twice by PBS,
and subjected to the confocal microscopy studies or to further immunostaining procedures.

Analysis of Cellular Distribution and Detergent Resistance of the Na,K-ATPase Subunits Using Surface-specific Biotinylation and Western Blot Analysis—Nontransfected MDCK cells or MDCK cells stably expressing wild type or mutated YFP-linked β₁ subunits were maintained for at least 5 days after becoming confluent in Corning Costar polyester transwell inserts (Corning Glass) in 6-well plates. Biotinylation of the apical or basolateral membrane proteins was performed by previously described procedures (25, 26). Cell monolayers were biotinylated with EZ-Link™ Sulfo-NHS-SS-Biotin (sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate) (Pierce) that was added from either the apical or basolateral side. After quenching the biotinylation reaction, cells were washed and then lysed by incubation with 200 μl of 0.15 m NaCl in 15 mM Tris, pH 8.0, with 1% Triton X-100 and 4 mM EGTA. Cell extracts were clarified by centrifugation (15,000 × g, 10 min) at 4 °C. Samples containing 20 μl of supernatant mixed with 15 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) were loaded onto SDS-polyacrylamide gels to analyze proteins extracted from the whole cells by 1% Triton X-100-containing buffer. To isolate apical or basolateral biotinylated proteins, the rest of the cell extract (180 μl) was incubated with 100 μl of streptavidin-agarose beads (Sigma) in a total volume of 1 ml of 0.15 m NaCl in 15 mM Tris, pH 8.0, with 0.5% Triton X-100 and 4 mM EGTA at 4 °C with continuous rotation for 60 min. The bead-adherent complexes were washed three times on the beads, and then proteins were eluted from the beads by incubation in 40 μl of SDS-PAGE sample buffer for 5 min at 80 °C and loaded onto SDS-PAGE side by side with the corresponding extracts from the whole cells. Where indicated, cells on the transwell inserts were treated with 0.25% Triton X-100 in PBS for 15 min at room temperature after quenching biotinylation reaction and before cell lysis. Proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane and analyzed by the Western blot analysis using the appropriate primary antibody and the anti-mouse IgG conjugated to alkaline phosphatase (Promega) as a secondary antibody according to the manufacturer’s instructions. Immunoblots were quantified by densitometry using Eastman Kodak Co. 1D 3.6 software.

Cell-Cell Adhesion Assay—Nontransfected MDCK cells or cells expressing a YFP-linked protein (NTCP-YFP, wild type YFP-β₁, or mutant YFP-β₂) were trypsinized and sparsely plated on collagen-coated glass bottom microwell dishes. After a 1-h incubation in the regular culture medium, nonadherent cells were removed by rinsing. The areas having similar densities of the cells of ~300 cells/mm² on the plates were selected. Confocal microscopy (for cells expressing YFP-linked proteins) or light microscopy (for nontransfected cells) images of these fields were acquired. At this stage, antibodies, a blocking peptide, or ouabain were added into the culture medium where indicated. Cells were placed back into the tissue culture incubator. Intercellular adhesion was controlled by acquiring images of the same fields on microwell dishes at certain time points of cell incubation with or without the antibodies, the peptide, ouabain. Cell-cell adhesion was quantified by calculating the percentage of cells that did not form contacts with the neighboring cells at the indicated time intervals of incubation.

The monoclonal antibodies against the Na,K-ATPase β₁ subunit, E-cadherin, transferrin receptor, or GFP were purified from sodium azide and other low molecular mass contaminants using Amicon Ultra-4 30K centrifugal filter devices (Millipore) and transferred to PBS, pH 7.2, at 4 °C prior to their use in a cell-cell adhesion assay. Protein concentration before and after purification of the antibodies was determined using NanoDrop ND-1000 Spectrophotometer. The antibody against the Na,K-ATPase β₁ subunit was added at final concentrations of 5, 15, 25, or 35 μg/ml in the regular cell culture medium. The peptide KNESLETYPVM (Biopeptide Co., Inc.) containing amino acids 195–199 (shown in boldface type) of the sheep Na,K-ATPase β₁ subunit was added to the culture medium at a final concentration 2.5 μg/ml alone or together with 25 μg/ml of the antibody against the Na,K-ATPase β₁ subunit after a 1-h preincubation of the peptide and antibody in Dulbecco’s modified Eagle’s medium. The antibodies against E-cadherin, transferrin receptor, and GFP were added at the final concentration of 25 μg/ml. To compensate for a possible internalization of the antibody against transferrin receptor, fresh additions of the antibody (25 μg/ml) were made after 2 and 4 h of cell incubation. Ouabain was added at concentrations of 1, 5, or 10 μM.

RESULTS

Na,K-ATPase Is Resistant to Triton X-100 Extraction—The endogenous Na,K-ATPase α₁ and β₁ subunits were detected by surface-selective biotinylation almost exclusively in the basolateral plasma membrane domain of MDCK cells (Fig. 1A, lanes Ap and BL). To assure that biotinylated samples were not contaminated by intracellular proteins, the blot was stained by an antibody against a cytoskeletal protein, spectrin. The band corresponding to spectrin was not detected in the lanes containing surface biotinylated proteins (Fig. 1A, lanes Ap, BL, and BL*), but it was detected in the lanes containing intracellular proteins (Fig. 1A, lanes Ext and Ext*). Therefore, biotinylated proteins were not contaminated by intracellular proteins.

It has been shown previously that treatment of adherent cells with 0.25–0.5% Triton X-100 leaves the cytoskeleton and cytoskeleton-associated proteins attached to the surface but removes all soluble and most membrane proteins that are not linked to the cytoskeleton (24, 27). In agreement with these data, treatment of MDCK cells with 0.25% Triton X-100 similar to the procedures described previously (24) significantly reduced the total protein content in the whole cell lysate (Fig. 1B), indicating that many proteins were extracted from cells by the detergent. In contrast, Triton X-100 did not remove α₁ and β₁ subunits from the basolateral membranes (Fig. 1A, lanes BL*). Therefore, the Na,K-ATPase subunits residing in the basolateral plasma membrane domain are resistant to detergent extraction, suggesting that the enzyme is directly or indirectly associated with the cytoskeleton at the basolateral membrane. In contrast, the intracellular fraction of the Na,K-ATPase subunits was sensitive to the detergent treatment. The whole cell extract contained both Na,K-ATPase subunits (Fig. 1A, lane Ext). The β₁ subunit was represented by two bands in the extract. Only one of these two bands, the upper one, was
**Na,K-ATPase β1 Subunit and Cell-Cell Adhesion**

![Diagram](Image)

**FIGURE 1.** A Western blot analysis of surface and total proteins before and after extraction of cells with 0.25% Triton X-100. MDCK cells grown on porous transwell inserts were biotinylated from either apical or basolateral side and lysed using the buffer containing 1% Triton X-100. Then apical (Ap) or basolateral (BL) biotinylated proteins were precipitated by agarose-streptavidin beads and analyzed by SDS-PAGE and Western blot analysis. A, Western blot analysis of apical, basolateral, and total proteins of nontransfected MDCK cells. The dashed lines show how the blot was cut prior immunostaining. The upper, middle, and lower parts of the blot were stained using the antibodies against spectrin, the Na,K-ATPase α1 subunit, and the Na,K-ATPase β1 subunit, respectively. The proteins that were removed due to the treatment of cells with 0.25% Triton X-100 are marked by an asterisk (BL* and Ext*). B, Ponceau staining of total proteins extracted from untreated cells (Ext) and cells treated with 0.25% Triton X-100 (Ext*). C and D, apical and basolateral proteins of MDCK cells stably expressing the wild type YFP-β1. The blot was first immunostained using the antibody against YFP. Then the blot was cut along the dashed lines, and the upper, middle, and lower parts of the blot were stained using the antibodies against spectrin, the Na,K-ATPase α1 subunit, and the Na,K-ATPase β1 subunit, respectively. D, Na,K-α1, the Na,K-ATPase α1 subunit; Na,K-β1, the Na,K-ATPase β1 subunit.

Present in the basolateral membrane (Fig. 1A, lanes BL and BL*). Therefore, the upper band corresponds to the fully glycosylated form of the β1 subunit, whereas the lower band (marked by round brackets on Fig. 1A, lane Ext) presumably represents a high mannose fraction of the protein that resides in the ER. The extract obtained from the cells treated with 0.25% Triton X-100 also contained both subunits. However, the amounts of both the α1 and β1 subunits were slightly decreased, and the β1 subunit was detected as a single upper band (Fig. 1A, lane Ext*).

This suggests that the intracellular fraction of the pump consisting mostly of the α1 subunit assembled with the high mannosic fraction of the β1 subunit (Fig. 1A, lane Ext, round brackets) was removed by 0.25% Triton X-100. Similarly, the lower bands detected by anti-spectrin antibody in the whole cell extract (Fig. 1A, lane Ext, square brackets) disappeared in the lane corresponding to the cell extract after 0.25% Triton X-100 treatment of cells (Fig. 1A, lane Ext*). This observation suggests that some soluble proteins cross-reacting with the anti-spectrin antibody were removed by 0.25% Triton X-100.

Similar to the endogenous Na,K-ATPase subunits, the expressed YFP-β1 was also detected predominantly on the basolateral membrane (Fig. 1C). The basolateral fraction of YFP-β1 was also resistant to 0.25% Triton X-100. Expression of YFP-β1 did not change the detergent resistance of the endogenous Na,K-ATPase α1 or β1 subunits, as detected by immunostaining the lower part of the blot using the antibody against the α1 subunit (Fig. 1D). As expected, spectrin was not detected in the apical and basolateral fractions.

Resistance of YFP-β1 to the extraction by 0.25% Triton X-100 was also confirmed by confocal microscopy. Treatment of MDCK cells expressing YFP-β1 with Triton X-100 did not remove YFP-β1 from the lateral membranes (Fig. 2, D and E).

This resistance of the Na,K-ATPase to detergent extraction suggests that the enzyme is directly or indirectly associated with the cytoskeleton at the basolateral membrane. It has been shown that the Na,K-ATPase α1 subunit interacts with the...
membrane cytoskeletal protein, spectrin, via a cytoplasmic anchoring protein, ankyrin, in various cell types (28). Moreover, it has been suggested that this interaction is responsible for the polar distribution of the Na,K-ATPase in MDCK cells due to stabilization of the pump at the basolateral plasma membrane domain (29, 30). In agreement with these data, immunostaining of the tight monolayer of MDCK cells expressing YFP-β1, using antibodies against spectrin showed co-localization of YFP-β1 with spectrin in the lateral membrane (Fig. 2A–C). However, spectrin was also detected as a meshlike network inside the cells in contrast to YFP-β1 that was absent from the cytoplasm (Fig. 2A–C). This suggests that mechanisms other than spectrin association are involved in the specific localization of the Na,K-ATPase on the lateral membranes in MDCK cells.

The Na,K-ATPase Is Located at the Sites of Cell Contact in the Tight Monolayers of MDCK Cells—As seen from the vertical sections of MDCK cells, YFP-β1 is located predominantly in the lateral membranes and is virtually absent from the basal membrane (Fig. 3). This might suggest that the contacts with the neighboring cells are important for stable expression of the protein in the lateral membrane. As seen from the high resolution confocal microscopy images of horizontal sections of the cell monolayer, distribution of YFP-β1 in the lateral membranes is not uniform but has the appearance of a string of beads (Fig. 2D). The beaded appearance is also evident in a three-dimensional image of the cells (supplemental Video 1). This beaded appearance was retained after treatment of cells with 0.25% Triton X-100 (Fig. 2E). To test whether this appearance is a result of membrane junctions of the two neighboring cells, we incubated cells in Ca2+-free buffer. It is known that Ca2+ is required for formation and maintenance of cell-cell contacts. As expected, removal of Ca2+ from the medium resulted in separation of the lateral membranes of neighboring cells (Fig. 2F, white circle, and Supplemental Video 2). As a consequence, YFP-β1 now appeared evenly distributed in a single lateral membrane as a thin line and not as beads (Fig. 2F, white arrow). This suggests that the beaded appearance of the YFP-β1 in the lateral membranes is related to the presence of cell-to-cell contacts.

The Na,K-ATPase Is co-localized with Adherens Junctions—Epithelial cells connect to each other in the tight monolayer by formation of the tight junctions and adherens junctions. Immunostaining of MDCK cells using an antibody against the tight junction protein, occludin, showed that YFP-β1 did not co-localize with the tight junctions but resided in the lateral membrane below them (Fig. 3A).

In contrast, YFP-β1 displayed discrete co-localization with a marker of adherens junctions, β-catenin, as detected by immunostaining (Fig. 3B). Adherens junctions are formed as a result of interaction between the extracellular domains of the E-cadherin molecules that reside in the lateral membranes of two adjacent cells (31). A cytoplasmic protein β-catenin is associated with adherens junctions at the lateral membrane and binds with a high affinity to the cytoplasmic domain of E-cadherin (32). The complex of E-cadherin and β-catenin associates with the cytoskeleton due to interaction of β-catenin with α-catenin that binds in turn to actin filaments directly or via other actin-binding proteins. Due to this cytoskeleton association, the complex E-cadherin:β-catenin:α-catenin is resistant to extraction by Triton X-100 (33). Co-localization of YFP-β1 with β-catenin was retained after cell treatment with Triton X-100 in all sections of the cells below the tight junctions. The middle and the bottom horizontal sections of the cells are shown in Fig. 4A. In the middle plane of the cells, YFP-β1 also was co-localized with F-actin (Fig. 4B, top panels). However, in the basal section, YFP-β1 resided along actin filaments at the sites of contact between cells but not along the thick circumferential F-actin cables (Fig. 4B, bottom panels).

As expected, immunostaining of MDCK cells expressing YFP-β1 before and after extraction by Triton X-100 showed that the endogenous Na,K-ATPase α, subunit was also resistant to detergent extraction and co-localized with YFP-β1 (Fig. 4C). Therefore, the Na,K-ATPase spatially coincides with adherens junctions in the mature tight monolayer of MDCK cells. Association of the Na,K-ATPase with adherens junctions is retained after the detergent extraction.

To test whether or not association with adherens junctions is a common property of every basolateral membrane protein, we expressed the basolateral bile acid transporter, NTCP, as an YFP-linked fusion protein in MDCK cells. Distribution of NTCP-YFP in the mature tight monolayer of MDCK cells was very similar to that of YFP-β1 (Fig. 4D, left panel). However, in contrast to YFP-β1, NTCP-YFP was not resistant to the treatment of cells with Triton X-100. The major fraction of NTCP-YFP was removed by the extraction procedure (Fig. 4D). The minor fraction of the protein that was left in the cells after Triton X-100 extraction was not co-localized with the β-catenin as clearly seen in the zoomed image of Fig. 4D. Therefore, NTCP is not associated with the adherens junctions and cytoskeleton, indicating specificity of the interaction of the Na,K-ATPase with cell junctions.

Fig. 5 demonstrates the change in distribution of YFP-β1 that occurs during formation of cell contacts and transition from a single MDCK cell to a colony. YFP-β1 was detected mostly in the basal membrane of single cells and was virtually absent from
the rest of the plasma membrane (5A, left panels). In small colonies that contained two, three, or four cells, YFP-β₁ accumulated in the newly formed lateral membranes between neighboring cells (Fig. 5, white arrows). To visualize trafficking of YFP-β₁ during cell contact formation, we performed time lapse live imaging using confocal microscopy. MDCK cells expressing YFP-β₁ were plated sparsely on the glass bottom dishes and incubated in the regular culture medium in the CO₂ incubator overnight. Formation of cell contacts was visualized the next day by taking confocal microscopy images of the same microscopic fields at certain time points of incubation of cells in a regular medium at 37 °C. Three separate fields are shown on Fig. 5B. Accumulation of YFP-β₁ in the areas of cell contact occurred as soon as these contacts were formed. The field 1 shows accumulation of YFP-β₁ in the region of cell contact between two dividing cells (Fig. 5B, arrows). Fields 2 and 3 show accumulation of YFP-β₁ in a newly formed cell contact between two neighboring cells that adhere to each other (Fig. 5B, arrows). The YFP-β₁ present in newly formed lateral membranes coincided with adherens junction proteins, E-cadherin (Fig. 5C, arrows) and β-catenin (Fig. 5D, arrows). It also co-localized with actin filaments in the sites of cell contacts but not with the F-actin fibers in the basal plane of the cells (supplemental Videos 3 and 4). At this early stage of formation of a colony, the tight junctions were not present, as seen from the mostly intracellular location of the tight junctional protein, occludin (supplemental Video 5). Therefore, the association of YFP-β₁ with the adherens junctions and cytoskeleton starts at the very early stages of the development of the cell monolayer.

The next experiment was aimed at studying the distribution of the Na,K-ATPase upon cell detachment. It is known that adhesion molecules, such as E-cadherin, β-catenin, and occludin, are retrieved from the plasma membrane of polarized cells when they detach from each other and from the surface, either upon removal of Ca²⁺ from the bathing medium or upon the addition of specific inhibitor of Na,K-ATPase, ouabain (34). In agreement with these data, internalization of β-catenin in...
FIGURE 5. Accumulation of YFP-β₁ in the sites of adherens junctions during MDCK colony formation occurs simultaneously with the cell contact formation. A, confocal micrographs of the bottom, middle, and subapical horizontal sections of MDCK cells show that in a single MDCK cell most of the YFP-β₁ is present on the basal membrane and absent from the rest of the plasma membrane. In small colonies, YFP-β₁ is accumulated in the lateral membranes (white arrows). B, MDCK cells were plated sparsely on the glass bottom dishes and incubated in the CO₂ incubator overnight. Formation of new lateral membranes was followed the next day by taking confocal microscopy images of live cells upon their incubation in the regular medium. Accumulation of YFP-β₁ (white arrows) in the newly formed lateral membrane is seen either after cell division (field 1) or as a result of formation of new cell contacts (fields 2 and 3). C and D, immunostaining of MDCK cells expressing YFP-β₁ shows co-localization of YFP-β₁ with E-cadherin (C) and β-catenin (D) in the sites of newly formed cell contacts. The monoclonal antibody against E-cadherin was used as a primary antibody, and the TRITC-conjugated anti-rat IgG was used as a secondary antibody (C). TRITC-conjugated monoclonal antibody was used to stain β-catenin (D).

A single cell  Two cells  Three cells  Four cells

Field 1:
Cell division

Field 2:
Formation of a new contact

Field 3:
Formation of a new contact

C

YFP-β₁  E-cadherin  Merge

D

YFP-β₁  β-catenin  Merge
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in the presence of the anti-β₁ antibody in the culture medium at 37 °C for 2 h (Fig. 7B, b). Preincubation of the antibody with the peptide containing amino acids 195–199 of the Na,K-ATPase β₁ prior to immunostaining prevented the antibody binding to the surface of the cells as detected by the lack of immunofluorescence (Fig. 7B, c). The antibody against the extracellular domain of E-cadherin was used as a reference in cell-cell adhesion assay, because this antibody has been shown previously to inhibit specifically intercellular adhesion of MDCK cells (36). This antibody, similar to the anti-β₁ antibody, stained basal and lateral surfaces of sparsely plated MDCK cells (not shown). As a negative control, the monoclonal antibody against GFP was used, because this antibody does not react with any epitope on the surface of the cells and, therefore, should not specifically affect the adhesion between neighboring cells.

Use of cells expressing a live fluorescent lateral membrane protein for cell-cell adhesion assay allows visualization of formation of cell contacts in real time by confocal microscopy. However, we could not use the cells expressing YFP-β₁ to study the effect of the anti-β₁ antibody on cell-cell adhesion. This antibody binds to the extracellular domain of the endogenous canine β₁ subunit of MDCK cells but does not recognize the rat β₁ subunit and hence does not react with the extracellular domain of YFP-β₁ that contains the rat β₁ subunit. Therefore, instead of YFP-β₁, we used a nonrelated basolateral protein, NTCP-YFP, as a fluorescent marker of the lateral membranes. Accordingly, in the experiments on the effect of the anti-β₁ antibody and other control antibodies on cell-cell adhesion, we routinely used MDCK cells stably expressing NTCP-YFP and watched cell-cell adhesion by confocal microscopy. Control experiments using nontransfected MDCK cells and light microscopy to evaluate cell-cell adhesion showed that expression of NTCP-YFP did not affect the adhesion.

At the beginning of the adhesion assay, cells were attached to the surface but did not have any contacts with each other (Fig. 7A, a–c). Most cells were round shaped. The cells incubated in the presence of the antibodies against E-cadherin and the Na,K-ATPase β₁ subunit for 6 h displayed a significantly lower degree of intercellular adhesion as compared with the control (Fig. 7A, d–f). The percentage of cells that stayed single was calculated after 3 and 6 h of cell incubation in the presence of antibodies (Table 1). The percentage of single cells after incubation with anti-β₁ antibody was significantly higher than under control conditions (Table 1). The inhibitory effect of the antibody against the β₁ subunit on all cell adhesion was dose-dependent and reached its maximum at a concentration of 25 μg/ml. Preincubation of the antibody with the peptide containing amino acids 195–199 of the sheep Na,K-ATPase β₁ subunit almost completely prevented the inhibition of cell adhesion (Table 1). The inhibitory effect of the anti-β₁ subunit antibody on cell adhesion at concentrations of >25 μg/ml was not statistically different from that observed with antibody against E-cadherin (Table 1). Therefore, binding of the monoclonal antibody to the extracellular domain of the Na,K-ATPase β₁ subunit inhibited cell adhesion of MDCK cells to each other similarly to the well-known effect of the anti-E-cadherin antibody (36).

To exclude the possibility that the antibody against the β₁ subunit prevents contacts between cells not due to its binding...
to the β₁ subunit but due to nonspecific steric hindrance in the intercellular space, the effect on cell adhesion of the antibody against a transferrin receptor, a basolateral protein that is not implicated in cell-cell adhesion, was studied. The monoclonal antibody that binds to the extracellular domain of the endogenous transferrin receptor in MDCK cells (37) was utilized. This antibody labeled mostly lateral membranes in the confluent monolayer of MDCK cells (Fig. 7B, f), consistent with previously published data (37). In sparsely plated MDCK cells, the antibody stained basal and lateral surfaces of MDCK cells in a very similar pattern as found with the antibody against the Na⁺,K⁺-ATPase β₁ subunit (Fig. 7B, d and a). It is known that the transferrin receptor undergoes recycling between the plasma membrane and endosomes in MDCK cells (38). To test if endocytosis decreased the amount of the antibody bound to the surface, we incubated cells in the presence of the antibody in the medium at 37 °C for 2 h, fixed, and exposed them to the fluorescently labeled secondary antibody. The intensity of immunofluorescence was not significantly decreased (Fig. 7B, e). This suggests that the antibody was present in the medium in excess, and probably its binding to the new receptor molecules recycled to the surface could compensate for possible antibody internalization. To maintain the excess of the antibody in the medium during prolonged cell incubation, we supplemented the cell culture medium by fresh additions of the antibody (25 μg/ml) every 2 h during the adhesion assay. The percentage of single cells after 3 and 6 h of incubation with anti-transferrin receptor antibody was similar to that under control conditions (Table 1).

The next experiment ruled out the possibility that the anti-β₁ antibody inhibits cell-cell adhesion indirectly by blocking the extracellular domain of E-cadherin that might be located in close proximity to the extracellular domain of the Na⁺,K⁺-ATPase β₁ subunit. If this were the case, then the anti-β₁ antibody would inhibit binding of the anti-E-cadherin antibodies to the extracellular domain of E-cadherin in confluent MDCK cells. Immunostaining of MDCK cells using rat anti-E-cadherin antibody in a combination with anti-rat TRITC-conjugated secondary antibody detected bright fluorescence on the lateral membranes (Fig. 7C, a). The addition of a 10-fold excess of the anti-β₁ antibody together with the anti-E-cadherin antibody did not decrease the intensity of a fluorescent signal (Fig. 7C, b). As expected, a mouse monoclonal antibody against the β₁ subunit did not react with the anti-rat secondary antibody even when the anti-β₁ antibody was used at concentrations that were 10-fold higher than those of the anti-E-cadherin antibody (Fig. 7C, c). These results indicate that the anti-β₁ antibody does not interfere with the anti-E-cadherin antibody binding to the extracellular domain of E-cadherin. Alternatively, the antibody against the transferrin receptor antibody was similar to that under control conditions (Table 1).

FIGURE 7. Adhesion between adjacent MDCK cells is inhibited by the monoclonal antibody against the extracellular domain of the Na⁺,K⁺-ATPase β₁ subunit. A, cell-cell adhesion assay in the presence of the antibodies against E-cadherin, the Na⁺,K⁺-ATPase β₁ subunit, and the control monoclonal anti-GFP antibody. MDCK cells stably expressing NTCP-YFP were used to visualize cells by confocal microscopy during the adhesion assay. Adhesion of nontransfected MDCK cells was similar (not shown). Cells were trypsinized and plated on three glass bottom dishes. After a 1-h incubation of cells in the regular culture medium, the nonadherent cells were removed by rinsing. α–c, the selected fields on the three dishes before the addition of the antibodies as detected by confocal microscopy. Shown are the same fields after incubation of the cells in the presence of the monoclonal antibody against the extracellular domain of the E-cadherin (d), the monoclonal antibody against the extracellular domain of the Na⁺,K⁺-ATPase β₁ subunit (e), and the control monoclonal anti-GFP antibody (f) for 6 h. The number of cells that did not form cell-cell contacts after incubation in the presence of anti-E-cadherin and anti-Na⁺,K⁺-ATPase β₁ subunit antibodies is greater as compared with the control. B, control experiments showing that the antibodies used in the cell-cell adhesion assay bind to the surface of MDCK cells. Nonpermeabilized MDCK cells were stained using either the antibody against the β₁ subunit (α–c) or the antibody against the transferrin receptor (d–f) as a primary antibody. Cells were fixed and stained using the appropriate primary antibody and Alexa488-conjugated secondary antibody (a, c, d, and f). Alternatively, cells were incubated in the presence of the appropriate primary antibody in Dulbecco’s modified Eagle’s medium in the tissue culture incubator for 2 h, fixed, and exposed to the Alexa488-conjugated secondary antibody (b and e). To confirm that the antibody against the β₁ subunit specifically binds to its epitope in the extracellular domain of the β₁ subunit, immunostaining of fixed cells was performed after preincubation of the anti-β₁ antibody with the epitope-containing peptide for 2 h (c). Concentration of the primary antibodies was 25 μg/ml. Concentration of the peptide was 2.5 μg/ml. C, control experiments showing that the antibody against the extracellular domain of the Na⁺,K⁺-ATPase β₁ subunit does not inhibit binding of the anti-E-cadherin antibody to the extracellular domain of E-cadherin. Cells were stained using a 3 μg/ml concentration of the rat antibody against E-cadherin (a), 3 μg/ml rat antibody against E-cadherin, and 30 μg/ml mouse antibody against the β₁ subunit (b) or 30 μg/ml mouse antibody against the β₁ subunit (c). TRITC-conjugated anti-rat IgG was used as a secondary antibody in all three cases. Anti-β₁, the antibody against the β₁ subunit; anti-TIR, the antibody against the transferrin receptor.
Na,K-ATPase β₁ Subunit and Cell-Cell Adhesion

TABLE 1
Inhibition of the intercellular adhesion of MDCK cells by the monoclonal antibodies against E-cadherin and the Na,K-ATPase β₁ subunit

| Antibodies or inhibitors added to sparsely plated surface-attached MDCK cells | The percentage of cells that did not adhere to the adjoining cells after incubation * |
|---------------------------------------------------------------------------|----------------------------------------------------------------------------------|
| Regular medium (no additions)                                             | 9.0 ± 0.5                                                                         |
| Control anti-GFP antibody (25 μg/ml)                                       | 8.8 ± 0.3                                                                         |
| Antibody against E-cadherin (25 μg/ml)                                    | 34.9 ± 0.8                                                          |
| Antibody against the Na,K-ATPase β₁ subunit (5 μg/ml)                      | 12.6 ± 0.6                                                                        |
| Antibody against the Na,K-ATPase β₁ subunit (12 μg/ml)                     | 21.3 ± 1.5                                                                         |
| Antibody against the Na,K-ATPase β₁ subunit (25 μg/ml)                     | 31.5 ± 0.8                                                         |
| Antibody against the Na,K-ATPase β₁ subunit (35 μg/ml)                     | 33.5 ± 2.6                                                                         |
| Antibody against the Na,K-ATPase β₁ subunit (25 μg/ml) + blocking peptide (2.5 μg/ml) | 13.0 ± 1.7                                                                         |
| Blocking peptide (2.5 μg/ml)                                               | 8.6 ± 0.5                                                                         |
| Antibody against transferrin receptor (25 μg/ml)                           | 7.9 ± 0.7                                                                         |
| Ouabain (1 μM)                                                             | 6.8 ± 0.9                                                                         |
| Ouabain (5 μM)                                                             | 8.5 ± 0.4                                                                         |
| Ouabain (10 μM)                                                            | 8.9 ± 0.9                                                                         |

* The results of three independent adhesion assays.
  * Statistically significant difference with the control (anti-GFP antibody), p < 0.05 as calculated by CHITEST.

extracellular domain of the E-cadherin. Therefore, the effect of the anti-β₁ antibody on cell adhesion is related solely to its binding to the extracellular domain of the Na,K-ATPase β₁ subunit.

To test if cell-cell adhesion requires Na,K-ATPase activity at the sites of cell contact formation, we used the specific inhibitor of the Na,K-ATPase, ouabain. We performed a cell-cell adhesion assay in the presence of three different concentrations of ouabain (1, 5, and 10 μM) to cover the range of the inhibitor concentrations that were used by other investigators in functional assays related to cell-cell adhesion/detachment (12–14). We found that ouabain did not affect cell-cell adhesion for the first 6 h (Table 1) except for the highest concentration of ouabain, 10 μM, which caused slightly higher percentage of single cells after 6 h (Table 1). The differences between the control cells and the cells incubated with ouabain became detectable only after 6–10 h, depending on the concentration of the inhibitor. At that time, but not earlier, the ouabain-treated cells started to detach from each other and the surface (supplemental Fig. 1). After 20 h of incubation in the presence of the inhibitor, virtually all cells were detached because of the cytotoxicity of ouabain resulting from inhibition of the sodium pump, which agrees with previously published results (12, 13). These data show that fully functional Na,K-ATPase is not essential for the initial steps of cell-cell adhesion. Thus, the inhibition of contacts between cells observed in our cells is due to blocking of the extracellular domain of the β₁ subunit by the antibody and not to possible attenuation of activation of the pump.

N-Glycans Linked to the Na,K-ATPase β₁ Subunit Are Essential for Stable Association of the Pump with the Adherens Junctions—Each of the three N-glycosylation sites in the YFP-linked β₁ subunit was mutated by replacement of Asn with Gln residues. Double, single, and triple mutants were constructed (Fig. 8A). Mutated YFP-β₁ fusion proteins were stably expressed in MDCK cells. A Western blot analysis of cell lysates showed the expected gradual decrease in molecular mass of the mutants due to N-glycosylation site removal (Fig. 8B).

The effect of mutations of glycosylation sites on the location of YFP-β₁ in MDCK cells was studied using confocal microscopy. The wild type YFP-β₁ was detected exclusively in the lateral membranes in MDCK cells (Fig. 8C, left panels). Removal of one or two N-glycosylation sites resulted in only minor intracellular accumulation of YFP-β₁ (Fig. 8C, N1, N23, and N12). However, the presence of at least one glycosylation site in the β₁ subunit appears to be critical to ensure lateral localization of the subunit. The removal of all three sites resulted in a significant intracellular accumulation and distribution of the mutant between the lateral membrane and membrane-proximal vesicles (Fig. 8C, N123). As shown previously, the β₁ subunit lacking all three glycosylation sites retained the ability to assemble with the α₁ subunit and produce an active heterodimer when expressed in insect cells (39).

To identify the intracellular compartments in which the glycosylation mutant is accumulated, we fixed the cells and stained them by using antibodies against a specific marker of endosomes, EE1.3. EE1.3 (early endosomal antigen 1) is a membrane-bound protein specific to the early endosomes and is essential for fusion between early endocytic vesicles formed due to endocytosis from the plasma membrane (40, 41). The mutant N123 was partially co-localized with early endosomes (Fig. 8D). This observation suggests that the mutant is unstable in the membrane upon delivery and hence accumulates in early endosomes due to endocytosis.

In MDCK cells expressing the wild type YFP-β₁, the endogenous Na,K-ATPase α₁ subunit was localized exclusively in the lateral membranes similar to YFP-β₁ (Fig. 4C, left). Similarly, the mutants lacking one or two N-glycosylation sites were largely co-localized with the endogenous Na,K-ATPase α₁ subunit in the lateral membrane (not shown). The mutant N123 was co-localized with the α₁ subunit in the lateral membrane (Fig. 8E, arrows) and in intracellular vesicles in close proximity to the membrane (Fig. 8E, arrowheads). Thus, expression of the N123 mutant causes internalization of the endogenous Na,K-ATPase α₁ subunit.

The N1 mutant, similar to the wild type YFP-β₁, was resistant to extraction by Triton X-100 (Fig. 9A, two left panels). The detergent-resistant fraction of N1 was precisely co-localized with β-catenin similar to the wild type YFP-β₁ (Figs. 4A and 9A). Similar resistance was detected for the mutants N12 and N23 (not shown). In contrast, the N123 mutant was mostly removed from the cells by detergent extraction (Fig. 9B, two left panels). The Triton X-100-resistant fraction of the N123...
of the N123 mutant in this lane reflects the actual presence of the mutant on the apical membrane and is not an artifact resulting from the access of the biotinylation reagent to the basolateral surface of the cell monolayer. Further, it indicates that the mutant is present on the apical membrane alone, without the endogenous α1 subunit. A significant fraction of the mutant N123 resident in the basolateral membrane domain was removed by Triton X-100 extraction (Fig. 9C, lanes BL and BL*, and Fig. 9D). Moreover, expression of the unglycosylated mutant also decreased the resistance of the endogenous α1 subunit to the detergent extraction. A significant loss of the α1 subunit occurred after the extraction (Fig. 9, C and D). This is not due to different amounts of protein loaded on to the SDS-polyacrylamide gel, because the amount of the endogenous β1 subunit did not change after the Triton X-100 extraction (Fig. 9, C and D).

Therefore, intact N-glycosylation sites in the Na,K-ATPase β1 subunit are essential for stable association of the Na,K-ATPase with the adherens junctions in MDCK cells. Dissociation of the N123 mutant from the adherens junctions can explain the increased susceptibility of the mutant and, associated with it, the endogenous α1 subunit to endocytosis (Fig. 8, D and E). Further, removal of the glycosylation sites results in abnormal sorting and trafficking of this subunit.

**N-Glycans Linked to the Na,K-ATPase β1 Subunit Are Required for Cell-Cell Adhesion**—To see if glycosylation of the β1 subunit is important for cell-cell contact formation, we determined whether the lack of N-glycans in the N123 mutant affects cell-to-cell adhesion. In order to use a stable MDCK cell line expressing the N123 mutant for this purpose, it was necessary to determine the ratio between amounts of the expressed unglycosylated and endogenous normally glycosylated β1 subunits in this cell line. We analyzed cell lysates obtained from nontransfected cells and cells transfected with either the wild type or unglycosylated β1 subunit side by side using SDS-PAGE followed by a Western blot analysis. The amounts of total protein were the same for all three cell lysates loaded onto the gel as detected by Ponceau staining (Fig. 10A). After washing off the stain, the upper part of the blot was probed using the antibody against the α1 subunit, and the bottom part was probed using the antibody against the β1 subunit (Fig. 10B). Stable expression of the wild type or unglycosylated YFP-β1 proteins significantly decreased the amount of the endogenous β1 subunits, by 41 and 50% respectively, whereas the level of the endogenous α1 subunit was unchanged in the cells expressing the wild type YFP-β1 and even increased in the cells expressing the N123 mutant (Fig. 10, B and C). It is known that the α subunit of the Na,K-ATPase cannot be expressed alone; it requires the β subunit for normal folding, exit from the ER and maturation (42). This suggests that the expressed wild type or mutant YFP-β1 molecules partially substitute for the endogenous β1 subunits in the Na,K-ATPase α1,β-complexes. Then we compared the apical and basolateral membrane fractions isolated from nontransfected and transfected cells. As expected, no α1 subunit and only trace amounts of β1 subunit were detected in the apical fractions (Fig. 10D, lanes Ap) in all cells. In the basolateral membrane domain, the levels of expression of the endogenous Na,K-ATPase α1 subunit did not change, but the amount of the

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**FIGURE 8. Removal of N-glycosylation sites of YFP-β1 decreases the content of the protein in the lateral membrane and increases its accumulation in endosomes.** A, amino acid substitutions resulting in impairment of one, two, or all three N-glycosylation sites in the YFP-linked β1 subunit. B, Western blot analysis of the wild type and mutated YFP-linked β1 subunits of the Na,K-ATPase expressed in MDCK cells, showing a gradual decrease in molecular mass of the mutants as a result of N-glycosylation site removal. C, confocal micrographs of the horizontal sections of MDCK cells stably expressing the wild type or mutated YFP-β1 subunit are essential for stable association of the Na,K-ATPase α1 subunit with the lateral membrane; and the mutant N123 is distributed between the lateral membrane and intracellular vesicles proximal to the membrane. D, immunostaining of MDCK cells expressing the N123 mutant YFP-β1 revealed partial colocalization of the mutant with the endosomes. High resolution confocal microscopy allows distinction between the pools of the N123 mutant in the lateral membrane (arrows) and endosomes in a close proximity to the membrane (arrowheads). E, the N123 mutant is co-localized with the endogenous Na,K-ATPase α1 subunit in the lateral membrane (arrows) and in the membrane-proximal vesicles (arrowheads). The polyclonal antibody against the marker of early endosomes EE1 was used as a primary antibody, and the Alexa633-conjugated anti-rabbit IgG was used as a secondary antibody. The monoclonal antibody against the Na,K-ATPase α1 subunit was used as a primary antibody, and the Alexa633-conjugated anti-mouse IgG was used as a secondary antibody.

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mutant was only partially co-localized with β-catenin (Fig. 9B), indicating that N-glycans linked to the Na,K-ATPase β1 subunit are essential for stable association of the subunit with adherens junctions.

In contrast to the wild type YFP-β1, the N123 mutant was detected not only on the basolateral but also on the apical membrane (Fig. 9C, lanes Ap and BL). The absence of the endogenous Na,K-ATPase β1 and α1 subunits in the lane containing apical proteins (Fig. 9C, lane Ap) indicates that the appearance
Na,K-ATPase \( \beta_1 \) Subunit and Cell-Cell Adhesion

Control  After treatment of cells with 0.25% Triton X-100 for 15 min

A

N1  N1  \( \beta \)-catenin  Merge  5x-Zoom

N123  N123  \( \beta \)-catenin  Merge  5x-Zoom

B

C

Ap  BL  BL*  

109  79  60  

Na,K-\( \alpha_1 \)  N123  Na,K-\( \beta_1 \)

Ap  BL  BL*  

D

100%  BL*, % of BL

0  50%  100%

Na,K-\( \alpha_1 \)  N123  Na,K-\( \beta_1 \)

E

Tight junction

Na,K-\( \alpha_1 \)  Na,K-\( \beta_1 \)  E-cadherin  N-glycans  \( \alpha \)-catenin  F-actin  Spectrin  Ankyrin  Lamellin?  \( \beta \)-catenin
endogenous β₁ subunit decreased in cell lines expressing the wild type and mutant β₁ subunit by 39 and 55%, respectively (Fig. 10, D and E). We showed that the N123 mutant not only resided on the lateral membrane but also was present in endosomes (Fig. 8D). Moreover, expression of this mutant in MDCK cells resulted in accumulation of a fraction of the endogenous α₁ subunit in endosomes (Fig. 8E). These combined results suggest that in the N123-expressing cell line, the pump is distributed between the intracellular endosomes and the plasma membrane. The endosomal pool of the Na,K-ATPase is represented by the α₁ subunits assembled with the N123 mutant only. In the basolateral membrane, about half of the α₁ subunits are assembled with the endogenous β₁ subunits and the other half with the unglycosylated mutant. As discussed above, the unglycosylated mutant β₁ subunit forms a fully functional complex with the α₁ subunit (39). Therefore, the cell line expressing the N123 mutant is an appropriate model in which to study the role of glycosylation in intercellular adhesion. It has approximately the same number of the active pumps in the basolateral membrane as nontransfected cells, suggesting that this cell line contains approximately the same number of the active pumps in the basolateral membrane, indicating that half of the endogenous normally glycosylated β₁ subunits as compared with nontransfected cells or cells expressing the wild type YFP-β₁. If N-glycans linked to the Na,K-ATPase β₁ subunit are important for cell-cell adhesion, the mutant-expressing cells are expected to have impaired ability to form cell contacts. Accordingly, we performed a cell-cell adhesion assay for non-transfected MDCK cells, cells expressing the wild type YFP-β₁, and cells expressing N123. The results clearly demonstrate that the cell line expressing unglycosylated mutant displayed significantly slower progression of cell-to-cell adhesion than the non-transfected cells and cells transfected with wild type YFP-β₁ (Fig. 10F).

**DISCUSSION**

The mature electrically tight epithelium evolves as individual cells come into contact with each other, become less motile, and form tight junctions between individual cells. This property of increased cell adhesion is essential for normal maturation of the epithelium, and it is lost in various forms of carcinoma. This loss contributes to the tendency of carcinoma cells to metastasize or spread to other portions of the body.

**Na,K-ATPase β₁ Subunit and Cell-Cell Adhesion**

A role for the Na,K-ATPase β₁ subunit in cell adhesion was suggested by studies from our laboratory and those of others that showed that expression of Na,K-ATPase β₁ subunit is strikingly reduced in cells with impaired cell-cell adhesion, such as the gastric carcinoma cell line, HGT-1 (22), the cystic epithelia in polycystic kidney disease (43), bladder cancer cells, clear cell renal carcinoma cells, and poorly differentiated carcinoma cell lines derived from colon, breast, kidney, and pancreas (44–46). In gastric carcinoma cells and cystic epithelia, the Na,K-ATPase α₁ subunit associates with the β₁ subunit and resides in the apical membranes (22, 43). These data suggest that the lack of the β₁ subunit along with the high abundance of the β₁ subunit might be responsible for abnormal apical distribution of the Na,K-ATPase, the lack of the enzyme in the sites of cell contact, and loss of intercellular adhesive properties in these cell types.

In the present studies, we found that incubation of MDCK cells with an antibody against the extracellular portion of the Na,K-ATPase β₁ subunit specifically reduced cell-cell adhesion similar to the known effect of anti-E-cadherin antibody (36). Control experiments showed that the inhibitory effect of the anti-β₁ antibody is not due to nonspecific steric hindrance and not due to possible inhibition of the Na,K-ATPase activity. This suggests that the extracellular domain of the Na,K-ATPase β₁ subunit is directly involved in formation of cell contacts providing a structural link between adjoining cells.

Further, we found that N-glycans linked to the β₁ subunit are essential for cell-cell adhesion. We performed cell-cell adhesion assay using the stable MDCK cell line that expressed unglycosylated mutant N123 and demonstrated that cell contact formation occurred significantly more slowly in this cell line as compared with nontransfected cells or cells expressing the wild type YFP-β₁. Using surface-selective biotinylation followed by a Western analysis, we showed that the N123-expressing cell line has a normal amount of the α₁ subunit but a 2-fold decrease in the quantity of the endogenous β₁ subunit in the lateral membrane, indicating that half of the endogenous normally glycosylated β₁ subunits are substituted by unglycosylated subunits (Fig. 10). Therefore, normal glycosylation of the β₁ subunit is required for intercellular adhesion.

The requirement of normal N-glycosylation of the β₁ subunit for cell-cell adhesion is consistent with our data showing the importance of N-glycans linked to the extracellular domain of the subunit in stabilization of the pump at the sites of adherens junctions.

**FIGURE 9. Stable association between the Na,K-ATPase and adherens junctions depends on the presence of N-glycosylation sites in the Na,K-ATPase β₁ subunit.** The effect of treatment of MDCK cells expressing mutated YFP-β₁ by 0.25% Triton X-100 for 15 min on distribution of the endogenous and expressed Na,K-ATPase subunits was analyzed either by immunostaining using the TRITC-conjugated antibody against β-catenin (A and B) or by surface-selective biotinylation followed by a Western blot analysis (C and D). A, the N1 mutant, similar to the wild type YFP-β₁, is resistant to the extraction by Triton X-100. The detergent-resistant fraction of N1 is precisely co-localized with the β1-catenin. B, the N123 mutant is mostly removed from the cells by the detergent extraction. The Triton X-100-resistant fraction of N123 mutant is only partially co-localized with β-catenin, indicating that N-glycans linked to the Na,K-ATPase β₁ subunit are essential for a stable association of the subunit with adherens junctions. C and D, a Western blot analysis of apical (Ap) and basolateral (Bl) proteins that were isolated as described in the legend to Fig. 1. Samples originated from the cells treated with 0.25% Triton X-100 for 15 min are marked by the asterisk (Bl*). The blots were first stained using the antibody against YFP. Then the blots were cut along the dashed lines, and the upper and lower parts of the blots were stained by the antibodies against the Na,K-ATPase α₁ subunit and the Na,K-ATPase β₁ subunit, respectively. E, a model showing the role of glycosylation of the Na,K-ATPase β₁ subunit in stabilization of the pump at the sites of adherens junctions. Na,K-α₁, the Na,K-ATPase α₁ subunit; Na,K-β₁, the Na,K-ATPase β₁ subunit. Names of the mutants correspond to the description given in the legend to Fig. 8A.
Na,K-ATPase β1 Subunit and Cell-Cell Adhesion

juncts. We showed that the pump co-localized with adherens junctions at their inception as individual cells began to form a monolayer. This association was resistant to treatment with nonionic detergent, and upon disruption of cell contact, the Na,K-ATPase accompanied adherens junctions as both were endocytosed from the basolateral side. These results agree with previously published data on specific lateral localization and detergent resistance of the pump in MDCK cells (30, 47, 48). It was suggested that cell contact formation initiates recruitment of ankyrin/spectrin cytoskeleton elements at the sites of cell contact that in turn stabilize the Na,K-ATPase at the lateral membrane (47, 48). However, the results presented here demonstrate that the ankyrin/spectrin linkage, while important, is not sufficient to stabilize the Na,K-ATPase at the sites of cell contact. The other requirement that we have discovered is the presence of intact N-glycans on the extracellular domain of the β1 subunit. We found that the absence of a normal N-glycosylation of the β1 subunit results in loss of tight attachment of the pump to the lateral membrane and adherens junctions, since the pump can be easily removed from the lateral membrane by detergent extraction and is readily endocytosed. It is unlikely that the pump loses its ability to bind ankyrin/spectrin due to the lack of N-glycans on the extracellular domain of the β1 subunit. The glycosylation-deficient β1 subunit forms a functionally active complex with the α subunit (39) and traffics to the plasma membrane (Figs. 8 and 9), indicating that the absence of N-glycans does not affect the enzyme folding and hence the ankyrin-binding sites that reside in the cytoplasmic domain of the Na,K-ATPase α subunit (49).

It is known that linkage of E-cadherin to the cytoskeleton is not sufficient to ensure stability of the adherens junctional complex. The Ca²⁺-dependent interaction between the extracellular domains of the two E-cadherin molecules of two neighboring cells is another necessary linkage. Similar to E-cadherin, the Na,K-ATPase is stabilized in the lateral membrane by attachment at both cytoplasmic and extracellular sites as depicted in our theoretical model (Fig. 9E) and not just the cytoplasmic side as previously postulated. A cytoplasmic region of the α subunit is linked to spectrin via ankyrin, whereas the β1 subunit stabilizes the pump at the sites of cell contact due to glycosylation-dependent interaction with the β1 subunit of neighboring cells and/or with the extracellular domain of E-cadherin directly or via a putative multivalent lectin that links two proteins by binding to their N-glycans (Fig. 9E).

Our model is consistent with recent data showing that E-cadherin, the main component of adherens junctions, requires the presence of the Na,K-ATPase β1 subunit to induce epithelial polarization and suppress invasiveness and motility of carcinoma cells (19). Expression of the Na,K-ATPase β1 subunit in MSV-MDCK cells increased stabilization of E-cadherin in the plasma membrane as determined by Triton X-100 solubility assay (19), which is consistent with our hypothesis on glycan-dependent interaction between E-cadherin and the β1 subunit (Fig. 9E). A glycan-mediated interaction between the Na,K-ATPase β1 subunits of the neighboring cells (Fig. 9E) also can explain the increased stability of E-cadherin and the whole adherens junctional complex in the lateral membrane due to an additional support via the linkage of the Na,K-ATPase to the ankyrin/spectrin cytoskeleton that is in turn connected to F-actin cytoskeleton.

The latter model is also in agreement with the recent finding that the β1 subunit has an intrinsic glycan-binding capacity (50). The β subunit isoforms of the Na,K-ATPase possess properties of adhesive proteins. They are integral proteins with large glycosylated extracellular domains, 80% by mass for the β1 subunit, and 90% for the β2 subunit. Interestingly, the β1 subunit was originally discovered as an adhesion protein on glial cells (AMOG) in the rat brain (51). The model also is in agreement with data on distribution of the β1 subunit in mixed cell monolayers (18). MDCK cells co-cultured with nonpolarized CHO
cells expressed the β₁ subunit only on the borders between two MDCK cells and not on the borders between MDCK and CHO cells that do not contain a β₁ subunit (18). However, the β₁ subunit was detected on the borders between MDCK and CHO cells when CHO cells were transfected with the β₁ subunit. The linkage between the β₁ subunits of the neighboring cells can explain why highly polarized distribution of the Na,K-ATPase and accumulation in the sites of cell contact is observed with inception of cell-to-cell contact preceding formation of the tight junctions that function as barriers between the basolateral and apical plasma membrane domains and why the Na,K-ATPase complex localizes predominantly on the lateral but not on the basal membranes in MDCK cells.

The results of these studies suggest that the presence of the Na,K-ATPase β₁ subunit at the lateral membrane of adjoining cells is essential for normal intercellular adhesion. Moreover, normal glycosylation of this subunit is required for association with adherens junctions and maturation of polarized epithelia. The data suggest the intriguing possibility that abnormalities of glycosylation of the subunit could contribute to the malignant transformation of tissues or other disorders in which intactness of epithelia is disrupted. These possibilities remain to be explored.

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