Recombinant Addition of N-Glycosylation Sites to the Basolateral Na,K-ATPase β₁ Subunit Results in Its Clustering in Caveolae and Apical Sorting in HGT-1 Cells*

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In most polarized cells, the Na,K-ATPase is localized on the basolateral plasma membrane. However, an unusual location of the Na,K-ATPase was detected in polarized HGT-1 cells (a human gastric adenocarcinoma cell line). The Na,K-ATPase α₁ subunit was detected along with the β₂ subunit predominantly on the apical membrane, whereas the Na,K-ATPase β₁ subunit was not found in HGT-1 cells. However, when expressed in the same cell line, a yellow fluorescent protein-linked Na,K-ATPase β₁ subunit was localized exclusively to the basolateral surface and resulted in partial redistribution of the endogenous α₁ subunit to the basolateral membrane. The human β₂ subunit has eight N-glycosylation sites, whereas the β₁ isoform has only three. Accordingly, up to five additional N-glycosylation sites homologous to the ones present in the β₂ subunit were successively introduced in the β₁ subunit by site-directed mutagenesis. The mutated β₁ subunits were detected on both apical and basolateral membranes. The fraction of a mutant β₁ subunit present on the apical membrane increased in proportion to the number of glycosylation sites inserted and reached 80% of the total surface amount for the β₁ mutant with five additional sites. Clustered distribution and co-localization with caveolin-1 was detected by confocal microscopy for the endogenous β₂ subunit and the β₁ mutant with additional glycosylation sites but not for the wild type β₁ subunit. Hence, the N-glycans linked to the β₂ subunit of the Na,K-ATPase contain apical sorting information, and the high abundance of the β₂ subunit isoform, which is rich in N-glycans, along with the absence of the β₁ subunit, is responsible for the unusual apical location of the Na,K-ATPase in HGT-1 cells.

The asymmetric distribution of membrane proteins on distinct plasma membrane domains is a fundamental property of epithelial cells. This asymmetry determines vectorial transport, signal transduction mechanisms in development, cell-matrix interactions, and barrier function (1, 2). Sorting of proteins between the apical and basolateral membranes in epithelial polarized cells depends on the recognition of intrinsic sorting signals such as minimal amino acid motifs and carbohydrate or lipid moieties embedded within individual proteins. Specific sorting machinery present in TGN (3, 4) or endosomes (5, 6) recognizes these sorting signals and directs the proteins into specific apical or basolateral transport containers, which deliver proteins to their final destinations. Apical sorting signals are usually found in the ectodomain or the transmembrane domain of proteins and may include glycosylphosphatidylinositol anchors, N- and O-linked glycans, and transmembrane anchor signals (6, 7–9). Basolateral sorting signals are usually found within the juxtamembrane cytoplasmic region of membrane proteins and include tyrosine-based and dileucine-based motifs (4, 6–9). Several different sorting signals can be present in a protein, but they may vary in their importance in the final distribution of the protein. A particular motif might be preferentially recognized by the sorting machinery or be dominant over other signals (4). Sorting and trafficking of heterodimeric proteins may depend on signals embedded in one of the subunits, with the other subunit co-sorted after the formation of stable complexes in the ER (10). The presence of multiple sorting signals within a particular protein presumably enables it to traffic to different locations in the same cell type depending upon the physiologic conditions. Also it may allow the same protein to be distributed differently in various cell types that differ in cell-specific sorting machinery. Finally, the same protein may be sorted differently in cells at different stages of development because of variability in the signal recognition system.

An example of a protein with multiple sorting signals is the gastric H,K-ATPase, the enzyme responsible for acid secretion in the stomach. The H,K-ATPase is a heterodimer consisting of two subunits, an α subunit that contains the catalytic site and a glycosylated β subunit that is necessary for normal maturation of the enzyme (11). The H,K-ATPase is located in tubulovesicular cytoplasmic elements in the resting parietal cell and relocates to the secretary canalicular (apical) membrane upon stimulation of acid secretion (12). Stimuli-dependent recycling of the pump between intracellular tubulovesicles and the apical membrane is thought to be a crucial step in the regulation of acid secretion. Trafficking, sorting, and recycling of the H,K-ATPase must depend on the presence of the intrinsic sorting signals within its subunits. Expression studies in MDCK and LLC-PK1 cells revealed the presence of an apical sorting signal in the α subunit (13) as well as basolateral, endocytic, and apical sorting signals in the β subunit (14). Multiple biosynthetic, endocytic, recycling, and transcytotic pathways of H,K-ATPase β subunit trafficking in polarized cells were identified (14).

The homologous Na,K-ATPase shares a number of structural and catalytic similarities with the H,K-ATPase. However, it usually resides exclusively in the basolateral domain in most epithelial cells (15). Similar to the H,K-ATPase, the Na,K-ATPase is a transport enzyme that undergoes phosphorylation and dephosphorylation coupled to ion transport and is a heterodimer consisting of a catalytic α subunit and an N-glycosylated β subunit (16). Four isoforms of the Na,K-ATPase α subunit (α₁, α₂, α₃, and α₄) and three isoforms of the Na,K-ATPase β subunit (β₁, β₂, and β₃) are known. The presence of a basolateral sorting signal was demonstrated in the α₁ subunit (10). The role of the other α isoforms in...
sorting of the pump has not been studied. Nothing is known about sorting signals in any of the β isoforms of the Na,K-ATPase. However, it appears that the β subunit might affect sorting of the enzyme. The Na,K-ATPase that contains either β1 or β2 isoforms localizes exclusively in the basolateral membrane (15). The unusual apical localization of Na,K-ATPase β subunits in a number of cell types appears to correlate with the high abundance of the β2 isoform (17–20). Of the three isoforms of the Na,K-ATPase β subunits, the β2 is the most homologous to the β subunit of the gastric H,K-ATPase (16). Both the H,K-ATPase β subunit and the Na,K-ATPase β2 subunit have multiple N-glycosylation sites (up to seven and up to nine sites, respectively), whereas β1 or β3 isoforms have only two or three N-glycosylation sites. The high degree of glycosylation in the H,K-ATPase β and Na,K-ATPase β2 subunits might imply a role of N-glycosylation in the apical sorting of the corresponding α,β complexes.

Recently, we have shown that removal of specific N-glycosylation sites from the H,K-ATPase β subunit results in a significant decrease in apical delivery of the subunit in polarized LLC-PK1 cells (14). This implies that N-glycosylation sites likely encode apical sorting information in the H,K-ATPase β subunit. However, the removal of N-glycosylation sites significantly affects other trafficking steps, including protein folding and quality control in the ER, endocytosis, recycling, and degradation (14). This complicates analysis and interpretation of the results, because the effect of N-glycosylation site removal on an apical sorting event has to be differentiated from a possible alteration of other trafficking steps (14).

To overcome drawbacks related to removal of N-glycosylated sites, here we used an opposite approach to study the role of N-glycans in apical sorting of P2-type ATPases, namely the recombiant insertion of N-glycosylation sites homologous to the sites unique for the β2 subunit into the basolateral Na,K-ATPase β2 subunit. A gradual increase in the amount of mutant subunit detected on the apical membrane was observed in parallel to the stepwise increase in the number of additional glycosylation sites in the mutants.

**EXPERIMENTAL PROCEDURES**

**Construction of cDNAs Encoding YFP-linked Constructs of the Na,K-ATPase β1 and Na,K-ATPase β2 Subunits and Mutants with Engineered Glycosylation Sites**—The cDNA of the rat Na,K-ATPase β2 subunit was a kind gift from Dr. Steven Karlsh. The cDNA encoding the Na,K-ATPase β2 subunit was inserted into the multiple cloning site of the expression vector pEYFP-C1 (Clontech) using EcoRI and XbaI restriction sites to form pEYFP-β2, which encodes YFP-β2, a fusion protein of YFP linked to the amino terminus of the Na,K-ATPase β2 subunit. Mutants with engineered N-glycosylation sites were generated with the QuikChange mutagenesis kit (Stratagene), using pEYFP-β2 as a template. The cDNA of the rat Na,K-ATPase β2 subunit was a kind gift from Kathi Geering. The cDNA encoding the Na,K-ATPase β2 subunit was inserted into the multiple cloning site of the expression vector pEYFP-C1 (Clontech) using EcoRI and BamHI restriction sites to form pEYFP-β2. This encodes YFP-β2, a fusion protein of YFP linked to the amino terminus of the Na,K-ATPase β2 subunit.

**Stable Transfection**—MDCK and LLC-PK1 cells were purchased from the American Type Culture Collection. HGT-1 cells (human gastric carcinoma cell line) were a kind gift of Dr. C. L. Laboisse (21). To obtain cell lines stably expressing wild type YFP-β1 or YFP-β2 or mutant YFP-β1 fusion proteins, HGT-1 cells were grown on 10-cm plates until 20% confluence and transfected with the wild type or mutant pEYFP-β1 or the wild type pEYFP-β2 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 milliliter of medium in 24-well plates. Two clones with the highest expression of YFP-β were selected and expanded for further studies.

**Confocal Microscopy Identification of the Site of Expression of YFP-linked β Subunits**—Cells stably expressing wild type or mutant YFP-β1 or wild type YFP-β2 were grown for at least 5 days after becoming confluent on glass-bottomed micro-well dishes (MatTek Corporation). Confocal microscopic images were acquired using the Zeiss LSM 510 laser-scanning confocal microscope with LSM 510 software, version 3.2.

**Immunofluorescent Staining of Cell Monolayers**—Immunofluorescent staining was performed by using the Zenon labeling kit (Invitrogen) according to the manufacturer’s instructions. As primary antibodies we used the monoclonal antibody C464.6 against the Na,K-ATPase α1 subunit (Upstate), the monoclonal antibody against caveolin-1 (BD Transduction Laboratories), and a polyclonal antibody against the Na,K-ATPase β2 subunit (Upstate).

**Triton X-100 Treatment of HGT-1 Cells**—This treatment was performed by a previously described procedure (22). Briefly, cells grown on glass-bottomed micro-well dishes (MatTek Corporation) were washed with phosphate-buffered saline twice on ice and treated with the buffer containing 1% Triton X-100 for 20 min. Then cells were washed twice with 3.75% formaldehyde in phosphate-buffered saline, incubated with formaldehyde for 15 min at room temperature, rinsed twice with phosphate-buffered saline, and subjected to confocal microscopy studies.

**Estimation of Surface Content of ATPase Subunits by Surface-specific Biotinylation**—Cells stably expressing wild type or mutant YFP-linked β1 or β2 subunits were maintained for at least 5 days after becoming confluent in Corning Costar polyester Transwell inserts (Corning Incorporated) in 6-well plates. Biotinylation of the apical or basolateral membrane proteins was performed using previously described procedures (23, 24). Cell monolayers were biotinylated with sulfoaminomethyl 2-(biotinamido)-ethyl-1,3-dithiopropionate (EZ-Link™ sulfo-NHS-SS-biotin from Pierce), which was added from either the apical or the basolateral side. After washing the biotinylation reaction, cells were washed and then lysed, and the membranes were solubilized 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 lysates were clarified by centrifugation (15,000 × g for 10 min). Samples containing 20 μl of supernatant mixed with 15 μl of SDS-containing sample buffer were loaded onto SDS-PAGE gels to determine the total YFP-β content in the supernatant. To isolate biotinylated proteins, the rest of each supernatant was incubated with 100 μl of streptavidin agarose beads (Sigma-Aldrich) in a total volume of 800 μl of the lysing buffer for 1 h at 4 °C with continuous rotation. The bead-adherent complexes were washed three times on the beads, and then proteins were eluted from the beads by incubation in 40 μl of SDS-PAGE sample buffer (4% SDS, 0.05% bromphenol blue, 20% glycerol, and 1% mercaptoethanol in 0.1 M Tris, pH 6.8) for 5 min at 80 °C, separated on SDS-PAGE gels, and analyzed by Western blot analysis using the following primary antibodies: the monoclonal antibody 464.8 against the Na,K-ATPase β1 subunit (Novus Biologicals); the monoclonal antibody C464.6 against the Na,K-ATPase α1 subunit (Upstate); the monoclonal antibody against green fluorescent protein, clones 7.1 and 13.1 (Roche Molecular Biochemicals); and a polyclonal antibody against the Na,K-ATPase β2 subunit (Upstate). The corresponding anti-mouse or anti-rabbit IgG conjugated to alkaline phosphatase (Promega) was used as the secondary antibody according.
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FIGURE 1. Endogenous and expressed Na,K-ATPase subunits reside in the basolateral plasma membrane domains in polarized LLC-PK1 and MDCK cells. A, steady-state apical (Ap) and basolateral (BL) distribution of endogenous Na,K-ATPase α1 (NaKα1) and β1 (NaKβ1) subunits in LLC-PK1 cells (lanes 1–4) and MDCK cells (lanes 5–8) as detected by surface-selective biotinylation. B, apical (Ap) and basolateral (BL) distribution of expressed YFP-linked β1 (YFP-β1) and β2 (YFP-β2) Na,K-ATPase subunits in LLC-PK1 (lanes 1–4) and MDCK cells (lanes 5–8) as detected by surface-selective biotinylation followed by Western blot using anti-green fluorescent protein antibody that also recognizes YFP. C, confocal microscopy images of XY and Z sections of MDCK cells show that the expressed YFP-linked β1 subunit (YFP-β1) and the endogenous α1 subunit (NaKα1) co-localize on the lateral membranes. Green, YFP; red, anti-α1 antibody conjugated with the Alexa 546-Fab fragment of mouse IgG.

FIGURE 2. Steady-state surface distribution of endogenous and expressed Na,K-ATPase subunits in polarized HGT-1 cells as detected by surface-selective biotinylation. A, apical (Ap) and basolateral (BL) distribution of endogenous Na,K-ATPase α1 (NaKα1), β1 (NaKβ1), and β2 (NaKβ2) subunits. B, apical (Ap) and basolateral (BL) distribution of expressed YFP-linked β subunits (YFP-β1 and YFP-β2) of Na,K-ATPases in HGT-1 cells.

Results

Steady-State Surface Distribution of the Endogenous Na,K-ATPase Subunits in HGT-1 Cells—The Na,K-ATPase usually resides in the basolateral plasma membrane domains in native tissues and cultured polarized epithelial cells. Thus, MDCK and LLC-PK1 cells showed typical basolateral location of both the α1 subunit and β1 subunit of Na,K-ATPase (Fig. 1A) as detected by surface-selective biotinylation. When expressed as a YFP N-terminal fusion protein, the β1 subunit was also detected almost exclusively in the basolateral domains in both MDCK and LLC-PK1 cells (Fig. 1B, lanes 1–4) indicating that YFP linkage did not change trafficking and sorting behavior of the subunit. The YFP-linked β2 isoform of the Na,K-ATPase was also detected mainly in the basolateral domains in both cell types as detected by surface-selective biotinylation (Fig. 1B, lanes 5–8). The subunit was predominantly located in the lateral membrane in the regions of cell-to-cell contacts as seen from the confocal microscopy horizontal and vertical sections of MDCK cells expressing the YFP-linked β2 isform (Fig. 1C, left). Immunostaining using the monoclonal antibody against the endogenous Na,K-ATPase α1 subunit showed co-localization of the YFP-linked β2 and endogenous α1 subunits in MDCK cells (Fig. 1C, central and right).

In contrast to MDCK and LLC-PK1 cells, HGT-1 cells expressed the Na,K-ATPase α1 subunit predominantly on the apical surface (Fig. 2A, lanes 1–2). The Na,K-ATPase β1 subunit was not detected (Fig. 2A, lanes 3–4), even though the monoclonal antibody used has been shown to be reactive to human isoform according to the manufacturer’s instructions. The Na,K-ATPase β2 subunit was highly expressed in HGT-1 cells and distributed, similarly to the Na,K-ATPase α1 subunit, predominantly on the apical surface (Fig. 2A, lanes 3–6).

Expressed YFP-linked Na,K-ATPase β1 and β2 Subunits Reside in Opposite Plasma Membrane Domains in HGT-1 Cells—A fusion protein of YFP and the Na,K-ATPase β2 subunit was expressed predominantly on the apical surface of HGT-1 cells (Fig. 2B, lanes 1–2). Steady-state distribution of the expressed YFP-linked β2 subunit was similar to that of the endogenous β2 subunit in these cells (Fig. 2A, lanes 5–6). This indicates that YFP linkage to the N terminus of the β2 subunit did not affect trafficking and sorting of the subunit. However, the YFP-linked Na,K-ATPase β2 subunit was localized exclusively in the basolateral membrane domain as detected by surface-selective biotinylation (Fig. 2B, lanes 3–4) and confocal microscopy (Fig. 6, left).

Expression of the YFP-linked Na,K-ATPase β2 subunit caused partial redistribution of the endogenous Na,K-ATPase α1 subunit from the apical to the basolateral domain, as detected by surface-selective biotinylation.
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Addition of N-Glycosylation Sites to the YFP-linked Na,K-ATPase β1 Facilitates Apical Sorting of the Subunit—Alignment of the amino acid sequences of the rat β1 subunit and human β2 subunit of Na,K-ATPase shows only 36.5% identity. A schematic presentation of this alignment is drawn to scale in Fig. 4A. The human Na,K-ATPase β2 subunit has nine potential NXS or NXXT N-glycosylation sites (Fig. 4B, right column). In contrast, the β1 subunit has only three natural N-glycosylation sites (Fig. 4B, left column). These three sites, N1, N2, and N3, are homologous to sites 4, 5, and 8 in the β2 subunit (Fig. 4A and B). By using site-directed mutagenesis, additional N-glycosylation sites homologous to sites 1, 2, 3, 6, 7, and 9 in the β2 subunit were introduced into the YFP-linked β1 subunit as shown by arrows in Fig. 4A. Amino acid sequences of the N-glycosylation sites in the subunit and the corresponding amino acid substitutions that were made in the YFP-linked β1 subunit to create additional glycosylation sites are shown in Fig. 4B. The ninth potential N-glycosylation site in the β2 subunit, 209NKT301, resides in the very C terminus of the subunit. According to the alignment of the β1 and β2 subunits, the β1 subunit is shorter. Therefore, to create in the β1 subunit the N-glycosylation site homologous to 209NKT301, the terminal codon in YFP-β1 was mutated to serine (Fig. 4B, left column), and the next codon was mutated to the terminal codon. Six different mutants containing from one to six engineered N-glycosylation sites in addition to the three natural sites were constructed (Fig. 4C).

Mutants were stably expressed in HGT-1 cells. Western blot analysis of the cell lysates showed that cells expressing the wild type YFP-linked β1 and β2 subunits or the mutants of YFP-β1 contain two fractions corresponding to the complex glycosylated subunit and high mannose-type glycosylated subunit (Fig. 5). The high mannose-type fraction was sensitive to endoglycosidase H (EndoH), whereas the complex-glycosylated form was resistant to EndoH cleavage (not shown). As seen from Western blot analysis of surface-biotinylated YFP-β1 and YFP-β2, only the complex-glycosylated forms of the subunits reached the plasma membrane (Fig. 2B, lanes 1 and 2 and lanes 3 and 4, respectively). The high mannose form, therefore, represents the immature fraction of the protein that either undergoes transit from the ER to Golgi or is retained in the ER and destined for ER-associated degradation because it did not pass quality control. Hence, the percentage of the high mannose form in the total amount of glycosylated protein in the mutants compared with that in the wild type can be used as a criterion for the effect of mutations on the ER retention of the protein (14). The relative content of the ER (high mannose-type glycosylated) fraction was slightly increased in the YFP-β1 mutants with an additional 1–5 N-glycosylation sites and significantly increased in the mutant with six N-glycosylation sites (Fig. 5). This indicates that the addition of the last N-glycosylation site to the β1 subunit resulted in a significant retention of the protein in the ER probably due to the impaired folding. Successive addition of 1–5 N-glycosylation sites resulted in gradual increase in molecular mass of the mutants (Fig. 5, lanes β1, 1, 2, 3, 4, and 5). This indicates that each of added sites, E1, E2, E3, E6, and E7 (Fig. 4), becomes glycosylated in the mutants. The YFP-β1 mutant with six additional glycosylation sites had the same molecular mass as the mutant with five additional sites and the same molecular mass as YFP-β2 as well (Fig. 5, lanes 5, 6, and 7β, showing that the ninth site is not glycosylated in either the mutant with six additional sites or in the YFP-linked β2 subunit. Probably, the very C-terminal location of that site does not allow proper binding of the oligosaccharyltransferase. Hence, eight sites of the YFP-linked human β2 subunit are glycosylated in HGT-1 cells.

Surface-selective biotinylation of the cell lines expressing the wild type YFP-β1, the mutants of YFP-β1 with additional glycosylation sites, and YFP-β2 are shown in Fig. 6. The wild type YFP-β1 was detected exclusively on the basolateral membrane (Fig. 6, lane YFP-β1). Insertion of additional N-glycosylation sites resulted in distribution of the subunit between the two plasma membrane domains, apical and basolateral. The fraction of the total surface amount of the subunit detected on the apical membrane gradually increased with insertion of each additional glycosylation site and reached its maximum, 80%, in the YFP-β1 mutant with five engineered N-glycosylation sites (Fig. 6). YFP-β2 was predominantly localized at the apical membrane (Fig. 6, lane YFP-β2).

The effect of addition of N-glycosylation sites to the β1 subunit on its surface distribution in HGT-1 cells is evident from the comparison of the confocal microscopy images of the cell lines expressing the wild type YFP-β1 and the mutant YFP-β1 with five additional glycosylation sites (Fig. 7). YFP-β1 resides almost exclusively in the basolateral domain and mostly in the regions of cell-to-cell contacts as seen from the top and middle XY sections and vertical Z section of the cell monolayer (Fig. 7, left). In contrast, the mutant with five engineered glycosylation sites was predominantly detected on the apical surface (Fig. 7, right). A significant amount of the mutant protein was also detected inside the cells in the perinuclear region as seen from the middle XY section of the cells. Some of this intracellular accumulation can be explained by a slightly greater fraction of the mutant being retained in the ER as compared with the wild type YFP-β1 (Fig. 5). However, the major intracellular fraction has a clustered pattern that is different from the homogeneous distribution of the protein usual for ER retention.
It is known that some apical proteins, particularly glycosylphosphatidylinositol-anchored proteins, have a tendency to cluster in lipid rafts in the TGN, endosomes, and the plasma membrane. It is known that lipid rafts have higher resistance to the cold Triton X-100 extraction as compared with other membrane lipids. To check if the clustering of the YFP-β1/H92521 mutant with additional glycosylation sites is related to the lipid rafts, we treated cells with an ice-cold 1% Triton X-100 for 20 min, fixed the cells, and studied the cell components resistant to the extraction procedure by confocal microscopy. No fluorescence was detected after Triton X-100 treatment in the cells expressing the wild type YFP-β1/H92521 (Fig. 8, left). However, in the cells expressing the YFP-β1 mutant with five additional glycosylation sites, a small fraction of fluorescent patches was still visible after the extraction procedure (Fig. 8, right).

Immunostaining of the HGT-1 cells expressing either the wild type YFP-β1 or the YFP-β1 mutant with five engineered glycosylation sites, using the antibody against the caveolin-1, the marker of caveolae (25), showed that the mutant but not the wild type was accumulated in caveolae. No co-localization of the wild type YFP-β1 with caveolin-1 was detected in either the plasma membrane or the cytoplasm (Fig. 9, column A). The distribution patterns of the mutant and caveolin-1 are very similar, and the merged image shows clearly that there is co-localization of the mutated β subunit and caveolin-1 (Fig. 9, column B). Similar co-localization was found by using double immunostaining for the endogenous β2 subunit and caveolin-1 (Fig. 9, column C). The difference between the wild type YFP-β1 on one hand (no co-localization with the caveolin-1) and the YFP-β1 mutant with additional glycosylation sites and endogenous β2 subunit on the other hand (co-localization with the caveolin-1) is clearly evident in the zoomed merged images shown at the bottom of Fig. 9.

**DISCUSSION**

Primary Role of the β Subunit Isoform in Apical-Basolateral Distribution of the Na,K-ATPase in HGT-1 Cells—Sorting of the Na,K-ATPase, a heterodimeric membrane protein, may depend on sorting signals present in both α and β subunits. Expression studies in LLC-PK1 cells demonstrated the presence of basolateral sorting information in the Na,K-ATPase β1 subunit (10). The Na,K-ATPase α1 subunit was shown to interact with a membrane cytoskeleton element, spectrin, through association with ankyrin (26–28). It has been suggested that this interaction plays a role in both biosynthetic trafficking pathway and stabilization in basolateral membranes (29, 30). The presence of sorting information in the α1 subunit was thought to be sufficient to determine basolateral location of the Na,K-ATPase α1β1 enzyme in most polarized cells (2, 31), whereas a possible role of the β subunit isoforms in
sorting of the Na,K-ATPase was generally discounted. However, the
results presented here clearly demonstrate that the β isoform is crucial
for sorting of the pump in HGT-1 cells. The high abundance of the
endogenous β2 isoform and the lack of the endogenous β1 subunit are
very likely responsible for the apical location of the Na,K-ATPase 1,2,
complexes in HGT-1 cells. Our data are in agreement with previously
published data on unusual apical localization of the Na,K-ATPase in a
number of epithelial cell types with a relatively high abundance of the β2
isoform, such as retinal pigment epithelium (19), and kidney cystic epithelia in the
patients with polycystic kidney disease (18). The data presented here
also indicate that the apical sorting information encoded in the β2 sub-
unit overrides the basolateral sorting information present in the Na,K-
ATPase α1, subunit in HGT-1 cells. One of the reasons for this could be
the lack of specific basolateral distribution of spectrin and ankyrin in
these cells. Another reason could be that the HGT-1 cell line may
express the apical sorting machinery more effectively than the MDCK
and LLC-PK1 cell lines. In the parietal cells that gave rise to the HGT-1
cell line (21), the H,K-ATPase is sorted exclusively to the apical mem-
brane compartment, and much more of this ATPase is expressed as

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FIGURE 5. Five of the six engineered N-glycosylation sites in the mutants of YFP-β1 become
glycosylated in HGT-1 cells. A, Western blot analysis of the lysates of HGT-1 cells expressing YFP-β1,
(lane β1), YFP-β2, mutants with various number of engineered glycosylation sites (lanes 1–6), or YFP-
β2 (lane β2). A gradual increase in size in five mutants with successively added glycosylation
sites (lanes 1–5) indicates that five of the six engineered glycosylation sites are occupied by N-gly-
cans. B, densitometry quantification of the relative amount of high mannose glycosylated fraction in
YFP-β1 (lane β1), YFP-β2, mutants with engineered glycosylation sites (lanes 1–6), and YFP-β2 (lane β2).
The addition of up to five glycosylation site (lanes 1–5) only slightly increased the relative
amount of the high mannose form, indicating that mutations had a minor effect on folding, quality
control, and exit of YFP-β1 from the ER. The addition of the last N-glycosylation site (lane 6) caused
significant ER retention. Lanes β1 (in panels A and B), YFP-β1; lanes 1, YFP-β1 +1 site; lanes 2, YFP-β1
+ 2 sites; lanes 3, YFP-β1 + 3 sites; lanes 4, YFP-β1 + 4 sites; lanes 5, YFP-β1 + 5 sites; lanes 6, YFP-
β2. Names of the mutants of YFP-β1 correspond to the descriptions given in Fig. 4C.

FIGURE 6. Successive addition of glycosylation sites increases apical distribution of the
mutants of YFP-β1. A, steady-state apical (Ap) and basolateral (BL) distribution of YFP-β1 (lane β1),
YFP-β2, mutants with various number of engineered glycosylation sites (lanes 1–6), and YFP-β2 (lane β2)
in HGT-1 cells as detected by surface-selective biotinylation. B, densitometry quantification results of three independent biotinylation
experiments. YFP-β1 and YFP-β2 are YFP-linked constructs of the β1 and β2 subunits of Na,K-
ATPase, respectively. Names of the mutants of YFP-β1 correspond to the descriptions given in
Fig. 4C.
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FIGURE 7. Recombinant addition of five N-glycosylation sites to the basolateral YFP-linked \( \beta_1 \) subunit results in apical sorting of the mutant subunit. Confocal microscopy images of HGT-1 cells showing that the wild type YFP-\( \beta_1 \), predominantly expressed on the lateral membranes (left), whereas the YFP-\( \beta_1 \) mutant with five additional N-glycosylation sites is located mostly on the apical membrane (right).

FIGURE 8. Recombinant addition of five N-glycosylation sites to the basolateral YFP-linked \( \beta_1 \) subunit increases subunit resistance to the Triton X-100 treatment. HGT-1 cells expressing either wild type YFP-\( \beta_1 \) or the mutant YFP-\( \beta_1 \) with five additional glycosylation sites were treated with an ice-cold 1% Triton X-100 for 20 min and fixed. Confocal microscopy images of the live cell before the Triton X-100 treatment (top row) were compared with the confocal images of the fixed cell after the Triton X-100 extraction (bottom) that were acquired under identical conditions. No fluorescence was detected after the Triton X-100 treatment in the cells expressing the wild type YFP-\( \beta_1 \) (bottom left section). By contrast, in the cells expressing the YFP-\( \beta_1 \) mutant with five additional glycosylation sites a small fraction of fluorescent patches was still visible after the Triton X-100 treatment (bottom right section).

Compared with the basolateral Na,K-ATPase. When the YFP-linked Na,K-ATPase \( \beta_1 \) subunit was expressed in HGT-1 cells, the Na,K-ATPase \( \alpha_1 \) subunit was redistributed between the apical and the basolateral membranes according to the the apical location of the endogenous \( \beta_1 \) isofrom and basolateral location of the expressed \( \beta_1 \) isofrom (Fig. 3). This result suggests that sorting of the Na,K-ATPase in HGT-1 cells depends mainly on the \( \beta_1 \) isofrom present. The \( \beta_1 \) isofrom determines basolateral location of the enzyme, and the \( \beta_1 \) isofrom determines its apical location in these cells. This finding indicates that the \( \beta_1 \) and \( \beta_2 \) subunits contain basolateral and apical sorting signals, respectively. The basolateral sorting information present within the \( \alpha_1 \) subunit is ignored in these cells. This makes HGT-1 cells a good model system for elucidating the nature of the sorting information embedded in the \( \beta \) isoforms.

The relative importance of sorting signals in the \( \alpha_1 \) and \( \beta_2 \) subunits is different in other polarized cells, such as MDCK and LLC-PK1. In these cell types, the expressed YFP-linked \( \beta_2 \) subunit localizes exclusively to the basolateral domain, similarly to the endogenous or expressed \( \beta_1 \) isofrom (Fig. 1, B and C). Probably, in MDCK and LLC-PK1 cells the basolateral sorting signal of the Na,K-ATPase \( \alpha_1 \) subunit dominates over the apical sorting information present in the \( \beta_2 \) subunit. As shown previously, this basolateral sorting signal is dominant over the apical sorting information present in the H,K-ATPase \( \beta \) subunit in MDCK (32) and LLC-PK1 cells (10). As mentioned above, the interaction of the \( \alpha_1 \) subunit with ankyrin and the spectrin cytoskeleton determine specific basolateral sorting and stable expression of the Na,K-ATPase in these cells (29, 30). Therefore, neither MDCK nor LLC-PK1 cells can be used to study the apical sorting signals in the Na,K-ATPase \( \beta_2 \) subunit.

However, our data on basolateral location of the expressed human \( \beta_2 \) subunit in MDCK cells do not agree with previously reported results on its apical localization (18). It is possible that the expressed \( \beta_2 \) subunit in the latter study was not properly folded. An indication for that is the mostly intracellular retention of the expressed protein as detected by immunostaining (18). Misfolding could have happened either because of the fusion of the expressed \( \beta_2 \) subunit with the Myc-epitope or because of the several errors in the cDNA sequence of the \( \beta_2 \) subunit used for cloning and expression (GenBank TM accession number M81181). These errors were corrected in NCBI data base recently (GenBank TM accession number NM_001678, July 1, 2004).

The Role of N-Glycans in Apical Sorting—Removal of N-glycosylation sites in a number of secreted and membrane apical proteins resulted in a loss of apical sorting (reviewed in Refs. 1 and 2). The recombinant addition of N-glycosylation sites to rat growth hormone, which is usually secreted in a non-polarized fashion, promoted apical release of rat growth hormone from MDCK cells (33). The addition of N-glycans was also shown to promote apical delivery of three membrane proteins (34). However, these data are difficult to interpret, because these all three proteins were either truncated or chimeric proteins that were retained inside the cells in the absence of N-glycans, and proper folding and ability to exit the ER were not assessed (34).

The data presented here clearly demonstrate that the addition of N-glycosylation sites to the Na,K-ATPase \( \beta_2 \) subunit not only promotes apical sorting but redirects the sorting pathway from the basolateral to the apical surface in the HGT-1 cell line. The effect of N-glycans on apical sorting is cumulative, because the fraction of the mutant protein detected on the apical membrane gradually increased proportionally to the number of glycosylation sites inserted and reached 80% of the total surface amount for the \( \beta_2 \) mutant with five additional sites (Fig. 6). No significant folding impairment or ER retention was detected for any of the mutants with up to five sites added.

These data indicate that N-glycans provide apical sorting information. The current concept of protein sorting suggests that sorting signals present within the proteins are specifically recognized by the specific sorters or sorting receptors that, with the involvement of other components of sorting machinery, place the proteins into apical or basolateral transport containers in the TGN or endosomes (1, 2). These containers are then delivered to the apical or basolateral plasma membrane. Basolateral and endocytic sorting receptors bind specifically to the basolateral and endocytic sorting signals and promote clathrin-dependent basolateral delivery or endocytosis (1, 2).

However, the constituents of the apical sorting pathway are unknown. A candidate lectin, VIP36, was shown to enhance apical sorting of several N-glycosylated proteins (35–37). But VIP36 was found in both apical and basolateral containers (38). Another argument against the role of VIP36 as an apical sorting receptor comes from its specificity to high mannose N-glycans rather than complex-glycosylated chains.
However, mature proteins, sorted in the TGN or endosomes and delivered to the apical membrane in mammalian cells, are predominantly of complex type, whereas the high mannose fraction is mostly present in the ER and cis-Golgi (14, 32, 40, 41). This makes the involvement of the mannose-specific lectin VIP36 in apical sorting unlikely.

An alternative model suggests that apical sorting occurs as a result of accumulation of the apical proteins in lipid-rafts (42, 43), which would then bud from the TGN or endosomes to form apical transport containers (42, 44). Rafts are rigid membrane microdomains enriched in glycolipids, sphingolipids, and cholesterol embedded in the fluid membrane (45). The cholesterol-binding protein caveolin-1, which forms homooligomers on the cytoplasmic side of lipid rafts, appears to be involved in raft clustering, the budding process, caveolae formation, and organization of the apical transport containers (25). Apical transport containers move along the microtubules and fuse with the apical membrane (2). It is not clear what links the apical containers and the microtubules. Most of the proteins that have been found in lipid rafts are glycosylphosphatidylinositol-anchored proteins (46). They associate with rafts via their glycosylphosphatidylinositol anchors. Our data suggest that association with lipid rafts and caveolae may occur also via N-glycans (Figs. 8 and 9).

The data presented here clearly indicate that not all N-glycans attached to the Na,K-ATPase β2-subunit have the same roles in sorting and trafficking. The three N-glycans attached to the Na,K-ATPase β1-subunit do not cause clustering of the Na,K-ATPase molecules in caveolae and obviously do not interfere with a recognition of the basolateral sorting information present in the Na,K-ATPase β1-subunit and basolateral delivery. However, addition of the glycosylation sites that are unique for the homologous Na,K-ATPase β2-subunit override the basolateral sorting information present in the Na,K-ATPase β1-subunit and result in apical sorting of the mutant subunit in HGT-1 cells. These additional N-glycans appear to facilitate clustering of the mutant subunit in lipid rafts and caveolae. This could happen because of their interaction with unidentified raft-resident membrane multivalent lectins that would react with several β subunits at the same time and “cross-link” neighboring molecules of Na,K-ATPase. Alternatively,
these N-glycans could be oriented in a way that allows their interactions with the N-glycans of the mutant β2 subunits of the neighboring α/β2 complexes. Involvement of N-glycans in protein–protein interactions and protein oligomerization has been shown previously (47, 48). As a result of clustering in caveolae, the mutant escapes basolateral sorting signal recognition in the TGN or endosomes and basolateral delivery. Instead, caveolae containing mutant β1 subunits become inserted into apical transport containers and are delivered to the apical membrane. Similarly, the α/β2 complexes are sorted apically.

In summary, using the expression system in which basolateral sorting information present in the Na,K-ATPase α1 subunit is ignored allowed us to reveal independent sorting signals in the two Na,K-ATPase β isoforms, an apical sorting signal in the β1 subunit and a basolateral sorting signal in the β2 subunit. Apical sorting information in the β2 subunit might be encoded by its five unique N-glycosylation sites that are absent from the β1 subunit. A basolateral sorting signal in the β1 subunit is still to be identified.

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