A dynamic equilibrium between multiple sorting pathways maintains polarized distribution of plasma membrane proteins in epithelia. To identify sorting pathways for plasma membrane delivery of the gastric H,K-ATPase β subunit in polarized cells, the protein was expressed as a yellow fluorescent protein N-terminal construct in Madin-Darby canine kidney (MDCK) and LLC-PK1 cells. Confocal microscopy and surface-selective biotinylation showed that 80% of the surface amount of the β subunit was present on the apical membrane in LLC-PK1 cells, but only 40% was present in MDCK cells. Nondenaturing gel electrophoresis of the isolated membranes showed that a significant fraction of the H,K-ATPase β subunits associate with the endogenous Na,K-ATPase α subunits in MDCK but not in LLC-PK cells. Hence, co-sorting of the H,K-ATPase β subunit with the Na,K-ATPase α subunit to the basolateral membrane in MDCK cells may determine the differential distribution of the β subunit in these two cell types. The major fraction of unassociated monomeric H,K-ATPase β subunits is detected in the apical membrane. Quantitative analysis showed that half of the apical pool of the β subunit originates directly from the trans-Golgi network and the other half from transcytosis via the basolateral membrane in MDCK cells. A minor fraction of monomeric β subunits detected in the basolateral membrane represents a transient pool of the protein that undergoes transcytosis to the apical membrane. Hence, the steady state distribution of the H,K-ATPase β subunit in polarized cells depends on the balance between (a) direct sorting from the trans-Golgi network, (b) secondary associative sorting with a partner protein, and (c) transcytosis.

Integral membrane proteins located on the apical and basolateral membranes of epithelial cells reach their destination by one of two pathways. They can be sorted within the trans-Golgi network (TGN) into specific containers, which are then delivered directly either to the apical or basolateral membranes (1–5). Alternatively, they are initially delivered first to one membrane (e.g. the basolateral membrane) but then retrieved from that membrane by the process of endocytosis and then delivered to the opposite membrane (i.e. the apical membrane). This latter indirect process has been termed transcytosis (3–5).

Both of these direct and indirect pathways are regulated. Specific machinery essential for the sorting process is present within the cell and recognizes sorting signals such as minimal amino acid motifs, carbohydrate or lipid moieties, embedded within individual proteins (1–5). 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 (1–5). Basolateral sorting signals are usually found within the juxtanuclear cytoplasmic region of membrane proteins and include tyrosine-based and dileucine-based motifs (1–5). Several different sorting signals can be present in a protein, but they may vary in their importance in the targeting process. A particular motif might be preferentially recognized by the sorting machinery or be dominant over other signals (1).

Targeting of heterodimeric proteins may depend on signals embedded in one of the subunits, with the other subunit tagging along after synthesis of stable complexes in the ER (6).

The gastric H,K-ATPase and the Na,K-ATPase are two homologous transport enzymes that go through a cycle of phosphorylation and dephosphorylation coupled to ion transport, protons or hydronium in exchange for potassium (H,K-ATPase) and sodium in exchange for potassium (Na,K-ATPase). Both enzymes are heterodimers consisting of an α subunit, which contains the catalytic site, and a glycosylated β subunit, which is thought to be necessary for normal maturation and delivery of the enzyme out of the ER (7). The Na,K-ATPase α subunit was shown to form a stable complex also with the H,K-ATPase β subunit that, similar to its natural partner, enabled maturation and plasma membrane delivery of the Na,K-ATPase α subunit in various cell expression systems (8–13). Despite the similarities in structure and catalytic properties, the Na,K-ATPase and H,K-ATPase reside in the opposite membrane domains in epithelial cells. The Na,K-ATPase is targeted to basolateral surfaces in most polarized cells (14). In contrast, the gastric H,K-ATPase, the enzyme responsible for acid secretion by the stomach, is located in tubulovesicular elements in the resting parietal cell and relocates to the secretory canalicular (apical) membrane upon stimulation of acid secretion (15, 16). The nature of the targeting information contained within each subunit has been deduced from studies of MDCK and LLC-PK1 cells in which both subunits are coexpressed or the β subunit is expressed alone. Studies in which the α subunit is expressed alone cannot be done, because this subunit is degraded and fails to be delivered to the plasma membrane (7).

Coexpression studies in LLC-PK1 cells demonstrated the presence...
ence of apical sorting signals in both the α and β subunits (17) of the H,K-ATPase and a basolateral sorting signal in the α1 subunit of the Na,K-ATPase (6). Sorting signals in both Na,K-ATPase and H,K-ATPase catalytic subunits were shown to reside in their N-terminal halves (6, 17). An apical sorting signal of the H,K-ATPase β subunit was demonstrated to be encoded in its N-glycosylation sites (18). On the other hand, when the H,K-ATPase β subunit is expressed alone, it can be distributed to either the apical or the basolateral membrane, depending on the cell type examined. It is distributed to the apical membrane in LLC-PK1 cells and mostly to the basolateral membrane in MDCK cells (19). These results indicate the β subunit may contain both apical and basolateral sorting signals.

Therefore, the gastric H,K-ATPase β subunit has several properties that make it an excellent model to use in studies of the sorting pathways involved in distribution of membrane proteins and the factors modulating the sorting process. It can be expressed alone or in association with its natural partner or other subunits in cultured cells, it has been inserted in both apical and basolateral sorting signals, and it is delivered predominantly to the apical or basolateral membranes in different polarized cell systems.

In the present studies, the H,K-ATPase β subunit was expressed as a YFP N-terminal fusion protein in MDCK and LLC-PK1 cells. Using confocal microscopy, surface-selective biotinylation, and biotin cleavage, we were able to assess the steady state distribution of the protein, determine its fate after it was inserted into either membrane domain, and measure accumulation of the newly delivered protein in the apical membrane. The results indicate the presence of both direct and indirect routes for apical delivery of the protein. By selectively inhibiting a direct or indirect apical pathway, we were able to evaluate the relative quantity of the β subunit, which was directly delivered from the TGN to the apical membrane compared with that which arrived there by transcytosis from the basolateral membrane. Using nondenaturing gel electrophoresis, we determined whether association of the expressed protein with the endogenous partner affects its sorting and final distribution. We found that promiscuous association with the endogenous Na,K-ATPase α1 subunit in MDCK but not LLC-PK1 cells is probably responsible for differential distribution of the H,K-ATPase β subunit in the two cell types.

The results of these studies indicate that membrane proteins containing both apical and basolateral sorting signals, such as the gastric H,K-ATPase β subunit, which are resident in the apical membrane, can arrive there by both direct delivery from the TGN and transcytosis via the basolateral membrane. Moreover, association with another subunit, if this has a dominant sorting signal, can cause it to be delivered to a membrane different than in its native state. Furthermore, these data emphasize that sorting is a complex process involving an interaction between sorting machinery unique to specific cells, signals embedded in proteins, and, in the case of heterodimeric proteins, the relative dominance of the sorting information in the particular subunit.

**EXPERIMENTAL PROCEDURES**

**Construction of cDNAs Encoding YFP-β Fusion Proteins and Mutants Lacking Glycosylation Sites**—pcDNA3(+)/β (20) was used as a source for cDNA encoding the rabbit H,K-ATPase β-subunit (21) (GenBank™ accession number M35544). The cDNA encoding the β-subunit was inserted into the multiple cloning site of the expression vector pEYFP-C1 (BD Bioscience Clontech) using BgIII and BamHI restriction sites to form pEYFP-β that encodes YFP-β, a fusion protein of YFP linked to the amino terminus of the H,K-ATPase β-subunit. Mutants were generated by using the QuikChange mutagenesis kit (Stratagene), using pEYFP-β as a template.

**Stable Transfection**—In order to obtain cell lines stably expressing wild type YFP-β or mutant YFP-β fusion proteins, LLC-PK1 cells were grown on 10-cm plates until 20% confluent and transfected with wild type or mutant pEYFP-β using 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/ml of medium in a 24-well plate. Two clones with the highest expression of YFP-β were selected and expanded for further studies.

The cell lines expressing YFP-β were subjected to a second transfection with pcDNA3/H9252 encoding the rabbit H,K-ATPase α subunit (GenBank™ accession number X64694). By addition of the second selection marker zeocin at a concentration of 0.4 mg/ml in addition to maintenance of a concentration of G-418 of 0.25 mg/ml, 15–20 cell lines expressing both α- and β-subunits were selected. Two clones with the best ratio of expressed H,K-ATPase to total protein were expanded for biotinylation experiments. The maintenance concentration for zeocin was 0.1 mg/ml.

**Confocal Microscopy Identification of Site of Expression of YFP-β**—Cells stably expressing wild type or mutant YFP-β were grown for at least 5 days after becoming confluent on glass bottom microwell dishes (MatTek Corp.). Confocal microscopic images were acquired using the Zeiss LSM 510 laser-scanning confocal microscope using LSM 510 software version 3.2.

**Estimation of Surface YFP-β Content by Surface-specific Biotinylation**—LLC-PK1 cells stably expressing wild type or mutant YFP-β were maintained for at least 5 days after becoming confluent in Costar Costar brand polyester transwell inserts (Corning Glass) in 6-well plates. Biotinylation of the apical or basolateral membrane proteins was performed by previously described procedures (22, 23). Briefly, cell monolayers were biotinylated with EZ-Link™ sulfo-NHS-SS-biotin (22) (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, and membranes were solubilized by incubation with 200 μl of 0.15 mM 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, 10 min). Samples containing 20 μl of supernatant were incubated with 15 μl of streptavidin-agarose beads (Amersham Pharmacia Biotech) diluted onto SDS-polyacrylamide gel 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-aragose beads (Sigma) in a total volume of 800 μl of the lysis 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, 1% mercaptoethanol in 0.1 mM Tris, pH 6.8) for 5 min at 80 °C, separated on SDS-polyacrylamide gel, and analyzed by Western blot using 2B6 monoclonal antibody against the H,K-ATPase β subunit (MBL, Inc.) or the monoclonal antibody against the Na,K-ATPase β1 subunit (Novus Biologicals), the monoclonal antibody against the Na,K-ATPase α1 subunit (Upstate Biotechnology, Inc., Lake Placid, NY), or the monoclonal antibody against the H,K-ATPase subunit (monoclonal antibody 12.18; generous gift from Dr. A. Smolka) as a primary antibody and anti-mouse IgG conjugated to alkaline phosphatase (Promega) as the secondary antibody according to the manufacturer’s instructions. Immunoblots were quantified by densitometry using Kodak ID 3.0 software.

In all experiments, the specific basolateral location of the Na,K-ATPase β1 subunit was considered as a control for intact tight junctions, and the absence of high mannose type YFP-β in biotinylated samples was used as an indication of plasma membrane integrity during biotinylation. The presence of biotinylated high mannose type YFP-β protein would show that the biotinylation reagent had access to the intracellular pool of high mannose type YFP-β because of plasma membrane leak to the reagent during the experiment (18).

**Endocytosis and Recycling Assay by Surface-selective Biotinylation and Surface-selective Biotin Cleavage**—Polarized cells stably expressing wild type YFP-β were biotinylated from either apical or basolateral side as described above with different incubation times. Cells were then incubated at 18 °C to impede further apical membrane delivery of the protein for 20 min. After this low temperature incubation, apical biotin was stripped off (Fig. 4A) by incubating with 50 mM reduced glutathione (Sigma) in 100 mM NaCl with 10% fetal bovine serum, pH 8.4, twice for 20 min. This procedure selected for internalized biotinylated protein. After cell lysis, the previously internalized biotinylated proteins were precipitated, washed, eluted from streptavidin-aragose beads, and analyzed by SDS-polyacrylamide gel and Western blot analysis as described above. In the negative control, biotin was

Downloaded from http://www.jbc.org/ by guest on August 31, 2017
stripped off immediately after biotinylation. To determine the total biotinylated YFP-β, in the positive control, cells were lysed immediately after biotinylation. To account for any instability of biotinylated protein, in a separate experiment, cells were incubated at 18 °C for 120 min and then lysed. To measure recycling, cells were incubated at 18 °C for 120 min, and the surface biotin was removed as above. Then these cells that now only contained internalized YFP-β were incubated at 37 °C for 30 min (Fig. 4A) to estimate delivery of this fraction to the plasma membrane. After this procedure, in the control, cells were lysed immediately, whereas in the experimental cells, surface biotin was stripped off again to account for the fraction of internalized YFP-β that was returned to the plasma membrane. After cell lysis, biotinylated proteins were precipitated, washed, eluted, and analyzed as described above.

**Transcytosis Assay by Surface-selective Biotinylation and Surface-selective Biotin Cleavage**—A diagram of the experimental procedure to measure transcytosis from the apical to the basolateral membrane is shown in Fig. 5A. Filter-grown MDCK cells expressing the wild type YFP-β were biotinylated from either apical or basolateral side. After a 4-h incubation at 37 °C in the control experiment, cells were lysed immediately. In the three separate experiments, biotin was stripped off by incubating with 50 mM reduced glutathione from the apical surface only, from the basolateral surface only, or from both the apical and basolateral surfaces before cell lysis. Biotinylated proteins were precipitated as described above. The amount of protein that was transcytosed to the opposite membrane domain was calculated as the difference between the amount detected in the experiment where biotin was stripped off from the side of biotinylation and the amount detected in the experiment where biotin was stripped off from both sides. Transcytosis from the basolateral to the apical membrane was measured according to a similar scheme, with the only difference being that the cells were biotinylated from the basolateral surface rather than the apical surface.

**Blue Native Gel of Microsomal Membranes Isolated from MDCK and LLC-PK1 Cells to Detect Associated Na,K-ATPase**—The microsomal membranes from MDCK and LLC-PK1 cells stably expressing YFP-β were isolated as described before (24). Briefly, cells were collected in buffer A (10 mM Pipes/Tris, pH 7.0, with 2 mM EDTA and 2 mM EDA) containing 10 mM dithiothreitol (DTT), homogenized, layered onto the 42% sucrose solution, and spun in the Beckman SW28 swinging bucket rotor at 100,000 rpm for 1 h at 4 °C. The fraction at the buffer/sucrose interface was collected, diluted with buffer A, and centrifuged in a Beckman 75Ti rotor (35,000 rpm, 4 °C, 1 h). The pellet was resuspended in 2 ml of buffer A and homogenized with a Teflon homogenizer (Wheaton, Millville, NJ). The total protein concentration was determined using a modified protein assay reagent (Pierce). The typical protein concentration was 5–10 mg/ml.

Blue native gel electrophoresis was performed as previously described (25). All buffers and solutions were at pH 7.0 at 4 °C. 1 mg of protein of microsomal membranes isolated from MDCK or LLC-PK1 cells was resuspended in 100 μl of 50 mM BisTris buffer containing 0.5 M aminoacproic acid (Fluka). After adding 12.5 μl of 10% n-dodecyl-β-D-maltoside (Boehringer-Mannheim) and a 20-min incubation on ice with vortexing every 5 min, samples were centrifuged at 14,000 × g for 10 min. Then 6.3 μl of a 5% suspension of Coomassie Brilliant Blue G-250 (Serva, Germany) in 0.5 M aminoacproic acid was added to 100 μl of supernatant. Samples were then stored on ice for no more than 30 min prior to gel loading. A 4–12% gradient gel with 4% stacker was used (Invitrogen). The anode buffer contained 50 mM BisTris. The cathode buffer contained 50 mM BisTris, 15 mM BisTris, and 0.01% Coomassie Brilliant Blue G-250. The gel, buffers, and electrophoretic apparatus were chilled to 4 °C before samples were loaded (35 μl/well with 40–80 μg of protein). Electrophoresis was carried out at 34 V and 0.05 mA overnight. Then the cathode buffer was exchanged for one-tenth the amount of 0.001% Coomassie Blue, and electrophoresis was resumed at 60 V, 0.05 mA. Staining was carried out as described above for the various ATPase subunits.

**RESULTS**

**Steady State Surface Distribution of the Wild Type and Mutant YFP-β in Polarized MDCK and LLC-PK1 Cells**—Expression of the gastric H,K-ATPase β subunit as a YFP N-terminal construct in eukaryotic cells results in a synthesis of a fusion protein that retains correct folding, post-translational modifications, and ability to assemble with its α subunit and supports the active conformation of the αβ complex, as was shown before in nonpolarized HEK-293 cells (24) and polarized LLC-PK1 cells (18). The expressed fusion protein of YFP and the H,K-ATPase β subunit (YFP-β) was detected by Western blot in cell lysates of MDCK cells as two bands, a major band at 80–100 kDa and a minor band at ~75 kDa (Fig. 1A, MDCK, wt, lane T) similar to the pattern found previously in HEK-293 and LLC-PK1 cells (18, 24). A similar pattern was observed in the cell lines expressing the wild type protein and Y20A and Y20F mutants (Fig. 1A, lanes T). The lower H YFP-β band is endoglycosidase H-sensitive and represents the immature high mannosyl glycospolyl ER-residing fraction of YFP-β, as was shown before (18, 24). The YFP-β band represents the mature complex-glycosylated YFP-β that transits from the ER to the Golgi and undergoes stepwise mannos trimming and terminal glycopeolation in the Golgi (18, 24). Only the complex-glycosylated fraction of YFP-β is able to reach the plasma membrane as detected by surface-selective biotinylation (Fig. 1A, lanes A and B) in MDCK cells, as found previously in HEK-293 and LLC-PK1 cells (18, 24). The total level of expression of the wild type and mutant YFP-β was quantified by normalization to the level of expression of the enogenous Na,K-ATPase α1 subunit in the same cell line. As shown in the bar graph (Fig. 1D), the levels of expression of Y20F and Y20A mutants were 115 and 87% of the wild type YFP-β expression level, respectively.

Two alternative methods, confocal microscopy and surface-selective biotinylation, were used to study localization of YFP-β in MDCK and LLC-PK1 cells. Confocal microscopy visualizes the spatial distribution of YFP-β on the surface between apical, basal, and lateral membranes as well as inside the cells, whereas surface-selective biotinylation allows quantitative comparison of the amounts of YFP-β present in apical and basolateral membrane domains. As seen from the confocal micrograph showing a vertical section of the MDCK cell monolayer, the wild type YFP-β was localized mostly on the plasma membrane (Fig. 1B). The major fraction of expressed YFP-β was on the lateral membranes, and a minor fraction was on the apical surface. The weak fluorescence signal inside the cells probably represents the immature ER fraction of YFP-β that corresponds to the high mannosyl glycospolylated fraction of YFP-β detected as the low molecular weight band by Western blot of cell lysates (Fig. 1A, band H YFP-β). Surface biotinylation of cell monolayers from either the apical or basolateral side (Fig. 1A) and quantification of the Western blot analysis data (Fig. 1C) indicated that about 40% of the total surface YFP-β was present in the apical membrane domain and about 60% in the basolateral domain of the MDCK cells. This distribution of YFP-β is different from that found in LLC-PK1 cells (18), where the major fraction (~80%) was detected on the apical membrane (Fig. 1, A–C). The preferential apical distribution of the H,K-ATPase β in LLC-PK1 cells in contrast to the mostly basolateral distribution in MDCK cells has also been found by others (19, 26). These authors suggested that the reason for the differential sorting of the β subunit in these two cell types is the tyrosine 20-based motif in the cytoplasmic tail of the subunit that is interpreted as a basolateral signal in MDCK but not in LLC-PK1 (19).

In our experiments, mutation of tyrosine 20 to alanine also resulted in an increase of the relative apical content of the protein from 41 to 58% of total surface content in MDCK cells compared with the wild type. However, a significant amount of YFP-β, 42%, still remained in the basolateral domain (Fig. 1, A–C), indicating that the presence of the tyrosine 20-based motif is not the only reason for basolateral distribution of YFP-β in MDCK cells. When the tyrosine was substituted by phenylalanine, the apical content of YFP-β was even less than in the wild type (Fig. 1, A–C).
Detection of a Complex between the Expressed YFP-β/H9252 and the Endogenous Na,K-ATPase/β1 Subunit in MDCK Cells—The H,K-ATPase/β2 subunit has been shown to form stable complexes with the Na,K-ATPase/β1 subunit in various cell expression systems as reviewed in Ref. 7. It is known that the Na,K-ATPase/β1 subunit is restricted to basolateral domains in most polarized cells, including MDCK and LLC-PK1 cells (14). Furthermore, the Na,K-ATPase/β1 subunit was shown to contain in its N-terminal half a basolateral sorting signal that is dominant over the apical sorting information within the H,K-ATPase/β2 subunit (6). Based on these findings, we thought that the H,K-ATPase/β2 subunit might assemble with the endogenous Na,K-ATPase/β1 subunit in the ER and then be co-sorted to the basolateral membrane due to the recognition of the basolateral sorting information in the β1 subunit by the sorting machinery of MDCK cells. To test this hypothesis, we isolated the microsomal membranes from MDCK and LLC-PK1 cells expressing the wild type YFP-β/H9252 and analyzed them by nondenaturing gel electrophoresis, followed by Western blot analysis. In LLC-PK1 cells, the antibody against the H,K-ATPase/β subunit detected YFP-β as a monomer with a molecular mass of about 90 kDa (Fig. 2, lane 1). A minor fraction of YFP-β was detected as a band at ~380 kDa. The same two bands were detected in MDCK cells using the antibody against the H,K-ATPase/β subunit. However, in contrast to LLC-PK1 cells, the two bands were of almost equal intensity (Fig. 2, lane 2).

Detection of a Complex between the Expressed YFP-β and the Endogenous Na,K-ATPase α1 Subunit in MDCK Cells—The H,K-ATPase/β subunit has been shown to form stable complexes with the Na,K-ATPase/α1 subunit in various cell expression systems as reviewed in Ref. 7. It is known that the Na,K-ATPase/α1 subunit is restricted to basolateral domains in most polarized cells, including MDCK and LLC-PK1 cells (14). Furthermore, the Na,K-ATPase/α1 subunit was shown to contain in its N-terminal half a basolateral sorting signal that is dominant over the apical sorting information within the H,K-ATPase/β subunit (6). Based on these findings, we thought that the H,K-ATPase/β subunit may assemble with the endogenous Na,K-ATPase/α1 subunit in the ER and then be co-sorted to the basolateral membrane due to the recognition of the basolateral sorting information in the α1 subunit by the sorting machinery of MDCK cells. To test this hypothesis, we isolated the microsomal membranes from MDCK and LLC-PK1 cells expressing the wild type YFP-β and analyzed them by nondenaturing gel electrophoresis, followed by Western blot analysis. In LLC-PK1 cells, the antibody against the H,K-ATPase/β subunit detected YFP-β as a monomer with a molecular mass of about 90 kDa (Fig. 2, lane 1). A minor fraction of YFP-β was detected as a band at ~380 kDa. The same two bands were detected in MDCK cells using the antibody against the H,K-ATPase/β subunit. However, in contrast to LLC-PK1 cells, the two bands were of almost equal intensity (Fig. 2, lane 2).
band at 380 kDa was also detected when an identical lane was probed with the antibody against the Na,K-ATPase α1 subunit (Fig. 2, lane 3). This indicates that this band corresponds to a protein complex composed of the YFP-β and the Na,K-ATPase α1 subunits. The major fraction detected with the antibody against the Na,K-ATPase α1 subunit in MDCK cells was the band at 300 kDa. The same band was detected using the antibody against the Na,K-ATPase β1 subunit (Fig. 2, lane 4), showing that it corresponds to the endogenous Na,K-ATPase α1β1 complex. Taken together, these data indicate that the band at 300 kDa represents a tetramer consisting of two Na,K-ATPase α1 and two Na,K-ATPase β1 subunits, and the band at 380 kDa represents the tetramer consisting of two Na,K-ATPase α1 subunits and two YFP-β subunits. Therefore, whereas YFP-β in LLC-PK1 cells is predominantly found as a monomer, a significant fraction of it in MDCK cells is a heterodimer associated with the endogenous Na,K-ATPase α1 subunit. These data support the hypothesis that the endogenous Na,K-ATPase α1 subunit contributes significantly to basolateral co-sorting of the H,K-ATPase β subunit in MDCK cells but minimally in LLC-PK1 cells.

The absence of the band corresponding to the monomeric Na,K-ATPase β1 on the native gel (Fig. 2, lane 4) suggests that the Na,K-ATPase α1 subunit is synthesized in excess relative to the β1 in MDCK cells. Only the α1 subunits that assemble with the β1 subunits exit the ER and reach the plasma membrane. It has been shown in various expression systems that the α1 subunit alone is not able to exit the ER and is degraded (27). If the expressed H,K-ATPase β subunit assembles with the endogenous Na,K-ATPase α1 subunit in MDCK cells, the amount of the Na,K-ATPase α1 subunit present in the plasma membrane must be greater in the cells expressing the H,K-ATPase β subunit compared with nontransfected cells. In contrast, in LLC-PK1 cells where the H,K-ATPase β-Na,K-ATPase α1 complex is hardly detectable (Fig. 2, lane 1), the level of the Na,K-ATPase α1 subunit should not change as a result of expression of YFP-β. To test this hypothesis, nontransfected MDCK and LLC-PK1 cells as well as cells expressing the wild type YFP-β were biotinylated from either the apical or basolateral side. Biotinylated proteins from these two cell lines were analyzed side by side on the same SDS-PAGE and transferred...
to nitrocellulose. The upper part of the blot was probed, one after another, by the antibodies against the H,K-ATPase β subunit and against the Na,K-ATPase α1 subunit. The lower part of the blot was immunostained using the antibody against the Na,K-ATPase β1 subunit. The expression level of the Na,K-ATPase α1 subunit was higher in transfected MDCK cells compared with that in nontransfected cells, as seen from the Western blot (Fig. 3A). Quantification of the three parallel biotinylation experiments presented as a total intensity of the Na,K-ATPase α1 bands normalized to the total intensity of the Na,K-ATPase β1 bands detected a 1.4-fold higher relative amount of the endogenous Na,K-ATPase α1 subunit in MDCK cells expressing YFP-β compared with nontransfected cells (Fig. 3B). However, the difference is within the variability of the three parallel biotinylation experiments. Expression levels of the Na,K-ATPase α1 subunit in LLC-PK1 cells and levels of the Na,K-ATPase β1 subunit in both cell types did not change upon transfection (Fig. 3, A and B). Intensities of the bands corresponding to the endogenous Na,K-ATPase β1 subunit are about 3-fold higher in MDCK cells compared with that in LLC-PK1 cells in both nontransfected cells and in the cells transfected with YFP-β (Fig. 3C). The intensity of the Na,K-ATPase α1 band is 3-fold higher in nontransfected MDCK cells and 4-fold higher in MDCK cells transfected with YFP-β as compared with that in LLC-PK1 cells (Fig. 3C). These data show that constitutive expression level of both Na,K-ATPase subunits is higher in MDCK cells compared with LLC-PK1 cells. However, the quantitative comparison of two cell types might be affected by the differential recognition of the Na,K-ATPase from different species, hog (LLC-PK1) and dog (MDCK), by the antibodies in Western blot analysis.

Therefore, one possible reason for the complex formation with the Na,K-ATPase α1 subunit in MDCK cells but not in LLC-PK1 cells could be the much higher constitutive expression level of the Na,K-ATPase in MDCK cells as compared with that in LLC-PK1 cells. It is also possible that the excess of the newly synthesized Na,K-ATPase α1 subunit over the endogenous Na,K-ATPase β1 subunit in MDCK but not LLC-PK1 cells allows assembly with the expressed YFP-β and basolateral delivery of the Na,K-ATPase α1 subunit that would otherwise be degraded.

Indirect evidence supporting H,K-ATPase β-Na,K-ATPase α1 complex formation in MDCK cells was also obtained by co-transferring the cell line expressing YFP-β with the H,K-ATPase α subunit. When the H,K-ATPase α subunit was co-expressed in the MDCK cell line stably expressing YFP-β, the apical content of YFP-β was increased from 41 to 75% of total surface amount (Fig. 3D). This result shows that the expressed H,K-ATPase α subunit effectively competes with the endogenous Na,K-ATPase α1 subunit for assembly with YFP-β in the ER. As a result, the fraction of YFP-β promiscuously co-sorted with the Na,K-ATPase α1 subunit to the basolateral membrane is decreased. The alternative model suggesting that the apical sorting signal present in the H,K-ATPase α subunit dominates over the basolateral tyrosine 20-containing sorting signal does not explain the low (25%) basolateral YFP-β content in the YFP-β/H,K-ATPase α cell line, since even after the mutation of tyrosine 20 to alanine, 42% of total surface YFP-β was detected on the basolateral membrane (Fig. 1C).

Endocytosis, Recycling, Transcytosis, and Direct Sorting of YFP-β—Next, we assessed other trafficking and sorting pathways in MDCK cells, such as endocytosis, recycling, transcytosis, and direct sorting. To detect endocytosis, either apical or basolateral surfaces of the cells expressing YFP-β were treated with a membrane-impermeable, cleavable biotinylation reagent. Then cells were incubated up to 2 h at 18 °C to impede plasma membrane delivery of internalized proteins. After a given time of incubation, surface biotin was cleaved off. The remaining biotinylated proteins represent the proteins that had been endocytosed (Fig. 4A). Internalized YFP-β was gradually accumulated over the 120-min period of incubation from both apical and basolateral membranes (Fig. 4, B and C). Basolateral endocytosis was more evident. After 2 h, 57% of the basolateral YFP-β was internalized compared with 45% of the apical YFP-β.

To determine the fate of internalized YFP-β in MDCK cells, we measured both recycling (delivery of the internalized protein back to the membrane of origin) and transcytosis (delivery of the internalized protein to the opposite plasma membrane domain). To define recycling, the cells after biotinylation from either the apical or basolateral side were incubated at 18 °C for 2 h, and then the surface biotin was stripped off from the side of biotinylation, and cells were placed back at 37 °C. After a 30-min incubation, the surface biotin was stripped off again from the side of biotinylation to determine the fraction of the internalized YFP-β that was returned back to the same membrane (Fig. 4A). A comparison of the fraction that was retained inside after this treatment (R) with the control (I) allowed determination of the YFP-β amount that was recycled back. Quantification indicated that 12 and 14% of total apical and basolateral YFP-β, respectively, were recycled back to the membrane of origin in these conditions (Fig. 4, B and C).

To assess transcytosis from the apical to the basolateral membrane, cells were biotinylated from the apical side and incubated at 37 °C for 4 h (Fig. 5A). Then surface biotin was stripped off either from the apical side only or from the basolateral side only or from both apical and basolateral surfaces (Fig. 5A). This surface-selective biotin cleavage allows determination of the fate of the apically biotinylated YFP-β after a 4-h cell incubation. Western blot analysis and densitometry quantification results are shown in Fig. 5B. Comparison of the total amount of biotinylated YFP-β before incubation (T0) and after incubation (T) shows that there is a 13% decrease in its total amount, probably due to the intracellular degradation of the protein and intracellular biotin cleavage. Therefore, the distribution of YFP-β between the apical membrane, the basolateral membrane, and an intracellular pool was determined as a percentage of T. After a 4-h incubation, 37% of total biotinylated YFP-β became intracellular (I) as measured directly by biotin cleavage from both surfaces, quantified by densitometry (Fig. 5B), and presented as a percentage of T in Fig. 5C (table). The amounts of YFP-β present in the apical and basolateral membranes can be calculated as shown in Fig. 5C. The difference between the total biotinylated YFP-β (T) and biotinylated YFP-β present on the apical membrane and inside the cells (T − A) reflects the amount of YFP-β that was transcytosed from the apical membrane to the basolateral membrane in 4 h (Fig. 5C). Alternatively, the amount of transcytosed YFP-β could be calculated from the difference between biotinylated YFP-β present inside the cells and in the basolateral membrane (T − A) and internal biotinylated YFP-β (I) (Fig. 5C). The amount of YFP-β that stays in the apical membrane (this amount also includes YFP-β that recycles back to the apical membrane after internalization) also can be calculated by two alternative ways (Fig. 5C). As seen from the table, the two methods produce very similar values. The average value from these two numbers is shown in the last column in each case (Fig. 5C). Thus, 58% of apical protein was retained in the apical membrane, 36% was internalized, and 5% was transcytosed to the basolateral membrane in these conditions (Fig. 5C).

Transcytosis from the basolateral to the apical membrane was determined using a similar approach, but cells were ini-
FIG. 4. Detection of apical and basolateral endocytosis and recycling in MDCK cells using surface-selective biotinylation and surface-selective biotin cleavage. A, a scheme showing the experimental procedure. The filter-grown MDCK cells expressing the wild type YFP-β were biotinylated from the apical side. $T_0$, total biotinylated YFP-β (positive control); $N$, biotin was stripped off immediately after biotinylation from apical side (negative control); $T$, cells were incubated at 18 °C for 120 min (control for stability of biotinylated YFP-β); $I_0$, biotin was stripped off from the apical surface after incubation of cells at 18 °C for 120 min at 18°C; control for stability of biotinylated YFP-β; $R$, biotin was stripped off after incubation of cells at 18 °C for 120 min, and then cells were incubated at 37 °C for 30 min, and biotin was stripped off again (remainder of internalized biotinylated YFP-β after its partial return to the apical membrane); $I$, biotin was stripped off after incubation of cells at 18 °C for 120 min, and then cells were incubated at 37 °C for 30 min (control for stability of internalized biotinylated YFP-β at 37 °C). After completion of the above procedures, cells were lysed. Biotinylated proteins were precipitated on streptavidin-agarose beads and analyzed by Western blot. Basolateral endocytosis and recycling were assayed according to a similar scheme, but cell biotinylation and biotin cleavage were performed from the basolateral side. B and C, representative immunoblots for apical (B) and basolateral (C) endocytosis and recycling of the wild type YFP-β. Data from three independent experiments were quantified as a percentage of $T$. 

---

Direct Apical Sorting and Transcytosis of the H,K-ATPase

14747

by guest on August 31, 2017
http://www.jbc.org/ Downloaded from

---
Fig. 5. Detection of transcytosis from the apical to the basolateral membrane in MDCK cells. MDCK cells expressing the wild type YFP-β were grown on transwell inserts. A, scheme showing the experimental procedure. Cells were biotinylated from the apical side. T₀, total biotinylated YFP-β (positive control); N, biotin was stripped off immediately after biotinylation (negative control); T, cells were incubated at 37 °C for 4 h (control for stability of biotinylated YFP-β); in the next three inserts, after cell incubation at 37 °C for 4 h, biotin was stripped off from the apical side only (T − A), from both apical and basolateral sides (I), or from the basolateral side only (T − B). After completion of the above procedures and cell lysis, biotinylated proteins were precipitated on streptavidin-agarose beads and analyzed by Western blot. B, a representative immunoblot and quantification of the data from three independent experiments. C, calculation of the amounts of YFP-β that were transcytosed to the basolateral membrane, internalized, or retained in the apical membrane after a 4-h incubation.

Partially biotinylated from the basolateral side (Fig. 6A). The results and densitometry quantification are shown in Fig. 6B. The distribution of the basolateral YFP-β between two plasma membrane domains and the intracellular pool after a 4-h cell incubation was determined and calculated using the approach described above for the apical biotinylation. After a 4-h incubation, one-half of the basolateral protein (53%) remained on the basolateral membrane (Fig. 6C). This amount also includes the fraction of the protein that was internalized and recycled back to the basolateral membrane. The other half of the basolateral YFP-β was split into two fractions, 26% became internal, and 21% were transcytosed to the apical membrane (Fig. 6C).

Transcytosis from the basolateral membrane to the apical membrane is much more intense compared with the transcytosis in the opposite direction. Based on these data and the data on detection of both apical and basolateral YFP-β recycling to the membrane of origin (Fig. 4, B and C), one can conclude that the YFP-β that is internalized from the apical membrane recycles mostly back and forth between the apical membrane and apical endosomes (Fig. 7). In contrast, YFP-β that is endocyto
tosed from the basolateral membrane is split into two fractions, the one that recycles between the basolateral membrane and basolateral endosomes and the other that is transcytosed to the apical membrane (Fig. 7). This implies that YFP-β must be delivered directly from TGN to the basolateral membrane in order to maintain the steady state apical/basolateral surface distribution 41–59%, whereas a direct delivery of YFP-β from TGN to the apical membrane is uncertain (Fig. 7).

Deglycosylation and Cycloheximide Treatment to Show Apical Delivery from the TGN—The approaches employed to study endocytosis, recycling, and transcytosis (Figs. 4–6) followed the fate of surface YFP-β. To study direct delivery from TGN to the plasma membrane, it was necessary to distinguish between newly synthesized protein and the protein that was already present on the surface. We found that deglycosylation of surface YFP-β using PNGF treatment could be used as a tool to selectively remove the protein from either plasma membrane domain in order to study accumulation of the newly delivered protein on the surface. As shown recently in LLC-PK1 cells, treatment of the apical surface of the cells with PNGF overnight resulted in disappearance of YFP-β from the apical membrane (18). A similar result was obtained in MDCK cells expressing YFP-β (Fig. 8A). Removal of N-glycans from YFP-β appears to destabilize the protein in the membrane and make it susceptible to intracellular degradation after its internalization, as shown schematically in Fig. 8D (panels 1–3). As a result, no deglycosylated product of YFP-β (band D YFP-β) was detected on the apical surface (Fig. 8A, lane 2) or in the cell lysate (Fig. 8A, lane 4). The high mannose glycosylated fraction of YFP-β was unchanged after PNGF treatment (Fig. 8A, lanes 3 and 4, bands H YFP-β), since this fraction represents the intracellular ER portion of the YFP-β pool that was not exposed to PNGF. The amount of complex-glycosylated fraction of YFP-β in the cell lysate was almost halved after PNGF treatment (Fig. 8A, lanes 3 and 4, bands C YFP-β). The complex-glycosylated fraction that is retained after deglycosylation corresponds to the basolateral pool of YFP-β that was not exposed to the enzyme.

Hence, PNGF treatment results in clearance of YFP-β from the apical membrane and allows detection of YFP-β delivery from either TGN or basolateral membrane. (Fig. 8D, panel 3). The addition of cycloheximide blocks protein synthesis (28) and, hence, delivery from the TGN and allows detection of only transcytosis from the basolateral membrane (Fig. 8D, panel 4). This approach is another means of studying transcytosis from the basolateral to the apical membrane (Fig. 8, B and C) in addition to the transcytosis assay shown in Figs. 5 and 6. The advantage of this approach is the direct measurement of the YFP-β amount in the apical membrane upon its accumulation and the possibility to compare quantitatively its two possible sources, direct delivery from the TGN and transcytosis (Fig. 8D, panels 3 and 4).

MDCK cells were incubated in the presence of cycloheximide for up to 2 h after apical YFP-β clearance with PNGF. A gradual accumulation of YFP-β occurred in the apical membrane (Fig. 8B). The accumulated amount, about one-half of the
steady state apical content, was higher compared with the quantity detected by the alternative transcytosis assay, one-third of the steady state apical content (Fig. 6C). This can be explained by the fact that the method in Fig. 6 takes into account only the surface protein, whereas the method in Fig. 8 detects accumulation of the protein from both the basolateral membrane and the basolateral endosomes (Fig. 8D, panel 1). In parallel with accumulation of YFP-β in the apical membrane, the amount of basolateral YFP-β was decreased, indicating that YFP-β was transcytosed from the basolateral to the apical membrane under those conditions. The same immunoblot was probed with the antibodies against the Na,K-ATPase α1 and β1 subunits. In contrast to the basolateral YFP-β, the amount of both Na,K-ATPase α1 and β1 subunits on the basolateral membrane was not changed after the 2-h incubation, indicating that Na,K-ATPase was not transcytosed. The total amounts of biotinylated proteins loaded onto the gel before and after the 2-h incubation were the same (Fig. 8B, bands NaKα and YFP-β). No Na,K-ATPase α1 and almost no Na,K-ATPase β1 subunits were detected in the apical membrane after PNGF treatment, similar to the control before PNGF treatment, indicating that intensive deglycosylation of apical surface proteins did not impair tight junctions and polarity of the cell monolayer. This also suggests that only the monomeric forms of YFP-β undergo transcytosis from the basolateral membrane, whereas the fraction of YFP-β that is present as heterodimers with the endogenous Na,K-ATPase α1 is retained in the basolateral mem-

FIG. 6. Detection of transcytosis from the basolateral to the apical membrane in MDCK cells. A, a scheme showing the experimental procedure. The experimental procedure was similar to that described in the legend to Fig. 5A, with the only difference being that cells were biotinylated from the basolateral side. B, a representative immunoblot and quantification of the data from three independent experiments. C, calculation of the amounts of YFP-β that were transcytosed to the apical membrane, internalized, or retained in the basolateral membrane after a 4-h incubation.

FIG. 7. A model summarizing the data shown in Figs. 4–6. YFP-β that is internalized from the apical membrane recycles mostly back and forth between the apical membrane and apical endosomes (AE). In contrast, YFP-β that is endocytosed from the basolateral membrane is split into two fractions, the one that recycles between basolateral membrane and basolateral endosomes (BE) and the other that is transcytosed to the apical membrane. Much more intense transcytosis from the basolateral membrane indicates the presence of a direct basolateral delivery of YFP-β, whereas a direct apical delivery is uncertain.
brane. Similar results were obtained for YFP-β expressed in LLC-PK1 cells (Fig. 8C), indicating that even in LLC-PK1 cells where the major amount of YFP-β is detected at steady state in the apical membrane, the transcytosis from the basolateral membrane to the apical membrane occurs in the same way as that in MDCK cells.

Transcytosis from the basolateral membrane may be the only source of the apical YFP-β, or a fraction of YFP-β may also be sorted directly from TGN to the apical membrane. To address this question, we compared YFP-β accumulation in the apical membrane upon cell incubation in the presence and in the absence of cycloheximide (Fig. 9A, panels 1 and 4). The amount of YFP-β that was accumulated at this location in the absence of cycloheximide was greater compared with that in the presence of the inhibitor (Fig. 9B, wt, CHX and wt, no CHX). This suggests that both direct and transcytotic pathways are present in MDCK cells. However, to obtain firm evidence for the presence of a direct pathway, it was necessary to block transcytosis to determine the apical delivery of YFP-β directly from the TGN per se. A tannic acid treatment of the basolateral surface that, according to the procedure described recently, specifically blocks basolateral transcytosis (29) was not successful in our protocol, since this treatment makes membranes leaky to the biotinylation reagent. Instead of chemical inhibitors of transcytosis or endocytosis, we took advantage of the mutants with impaired basolateral endocytosis.

When Y20A and Y20F mutants were assayed using PNGF and cycloheximide to determine transcytosis, as described above for the wild type YFP-β, no accumulation of YFP-β in the apical membrane and no change in the amount of YFP-β observed for 2 h, the densitometry quantification shows that for 2 h, the amount of Y20A reached 60% of the TGN per se. A tannic acid treatment of the basolateral surface that, according to the procedure described recently, specifically blocks basolateral transcytosis (29) was not successful in our protocol, since this treatment makes membranes leaky to the biotinylation reagent. Instead of chemical inhibitors of transcytosis or endocytosis, we took advantage of the mutants with impaired basolateral endocytosis.

When Y20A and Y20F mutants were assayed using PNGF and cycloheximide to determine transcytosis, as described above for the wild type YFP-β, no accumulation of YFP-β in the apical membrane and no change in the amount of YFP-β observed for 2 h, the densitometry quantification shows that for 2 h, the amount of Y20A reached 60% of
the steady state level in this cell line (Fig. 9B, Y20A, no CHX). No decrease in the basolateral content of YFP-β was detected in the mutants (Fig. 9A, panels 5 and 6). These data indicate that the mutation of tyrosine 20 to either alanine or phenylalanine completely prevents basolateral endocytosis and transcytosis to the apical membrane as shown in the scheme (Fig. 9C, left). Consequently, accumulation of the mutant Y20F or Y20A YFP-β in the apical membrane upon incubation in the absence of cycloheximide must reflect direct apical sorting of the newly synthesized protein from TGN without the contribution of transcytosis (Fig. 9C, right). This shows the presence of both a direct and transcytotic pathway of YFP-β delivery to the apical membrane in MDCK cells.

**DISCUSSION**

The results of the present studies demonstrate that the gastric H,K-ATPase β subunit when expressed in polarized epithelial cells is delivered to the apical membrane by two alter-
In MDCK cells, the basolateral abundance of the gastric H,K-ATPase subunit is due to passive co-sorting of the H,K-ATPase subunit with its natural partner, resulting in a lower abundance of the YFP-β-NaKα complex.

Thus, taken as a whole, the data support the contention that the formation of a substantial quantity of complexes of the H,K-ATPase β with the Na,K-ATPase α subunit in MDCK cells but not in LLC-PK1 cells explains why the former subunit is primarily delivered to the basolateral membrane in MDCK cells but not LLC-PK1 cells.

Of note, these results could explain the findings of studies by Duffield et al. (26). These investigators postulated that the reason for the greater basolateral abundance of the H,K-ATPase β subunit in MDCK than in LLC-PK1 cells was the lack of recognition of the tyrosine 20-based basolateral sorting signal in the β subunit in LLC-PK1 cells, since these cells were deficient in μ1B, the subunit of AP1B clathrin adaptor necessary to identify the presumed basolateral sorting signal (19, 26). However, they found that, even after the μ1B subunit was expressed in LLC-PK1 cells, the β subunit was still sorted predominantly to the apical membrane (26). Since we have shown that differences in the Na,K-ATPase α subunit available for association with the H,K-ATPase β subunit in MDCK and LLC-PK may explain lower basolateral distribution of this subunit in the latter cell type, it would be expected that supplying the μ1B subunit would not augment the quantity of the H,K-ATPase β subunit delivered to the basolateral membrane.

Presumably, promiscuous association with the Na,K-ATPase α and H,K-ATPase β subunits occurs at the level of the ER in MDCK cells. These results, as well as data from other laboratories detecting stable complexes between the H,K-ATPase β subunit and Na,K-ATPase α subunit in various cell expression systems (8–13), raises the question as to why such a complex is not formed in the gastric parietal cell. Most importantly, several studies have indicated preferential association of the gastric H,K-ATPase β subunit with its natural partner, the H,K-ATPase α subunit (7). Given that the expression of the latter subunit is in excess of the Na,K-ATPase α subunit, it is likely that there would be little opportunity for substantial cross-association of the H,K-ATPase β with the Na,K-ATPase α subunit in the ER. It is also possible that the parietal cells have an as yet undefined stringent regulatory control mechanism that prevents promiscuous complex formation.

Sorting Mechanisms for the H,K-ATPase β Subunit in MDCK Cells: Evidence for Active and Passive Pathways—Recent stud-
ies have shown that plasma membrane delivery of proteins in polarized cells is a tightly regulated process that depends on the recognition of the sorting signals within the proteins by sorting machinery in the TGN and endosomes (3–5, 30). Even in nonpolarized cells, some proteins are sorted into distinct containers upon the exit from the TGN rather than delivered by a default pathway (31). Therefore, detection of several distinct routes for plasma membrane delivery of YFP-β in MDCK cells, namely basolateral delivery of YFP-β with the Na,K-ATPase α1 subunit, direct apical and basolateral delivery, and transcytosis (Fig. 10, left), indicates the presence of distinct sorting pathways of the protein. Basolateral delivery of YFP-β with the Na,K-ATPase α1 subunit in MDCK cells can be considered as “passive” sorting of YFP-β, since it appears to be guided by the basolateral sorting information encoded within the Na,K-ATPase α1 subunit. “Active” sorting based on the recognition of the sorting signals within the H,K-ATPase β subunit itself must be responsible for direct apical and basolateral delivery from the TGN as well as for transcytosis of the unassociated monomeric form of YFP-β. Recently N-glycans linked to the extracellular loop of the β subunit have been shown to be essential for apical sorting of the subunit in LLC-PK1 cells (18). Hence, it is possible that N-glycans act as apical sorting signals in MDCK cells, although the contribution of other unidentified apical sorting signals cannot be excluded. The data presented here suggest that the tyrosine 20-based motif in the cytoplasmic region of the α1 subunit is recognized as a basolateral signal and as an endocytic signal in MDCK cells. Further support for an essential role for the tyrosine 20-based motif as an endocytic signal was indicated by studies that found that the H,K-ATPase in parietal cells of transgenic mice expressing the Y20A mutant H,K-ATPase β subunit was located exclusively in the canalicular (apical) membrane and that these parietal cells lacked intracellular tubulovesicular compartments (32). The latter result could be explained in part by an impairment of proper endocytic signals caused by the tyrosine 20 mutation.

Delivery of the H,K-ATPase β Subunit to the Apical Membrane in MDCK Cells: Evidence for Direct and Indirect Pathways—The use of tyrosine 20 mutants allowed for evaluation of the relative contribution of a direct apical delivery versus transcytosis. Mutation of tyrosine 20 to phenylalanine or alanine completely prevented transcytosis from the basolateral to the apical membrane (Fig. 9A, panels 2 and 3). Therefore, the YFP-β present in the apical membrane in cells expressing these mutants must originate entirely from the TGN without participation of transcytosis. The steady state amounts of YFP-β detected in Y20F and Y20A mutants are different, 20 and 58% of total surface YFP-β, respectively. It is unlikely that the point mutation in the intracellular N terminus of the H,K-ATPase β subunit would change its association with the endogenous Na,K-ATPase α1 subunit. It is known that the major association between the α and β subunits occurs in the extracellular domain (5), and some interaction is found in the transmembrane region (33) but not in the intracellular region. Therefore, the fraction of YFP-β that is co-sorted with the endogenous Na,K-ATPase α1 subunit must be similar in these two mutants, since the total expression levels of YFP-β relative to the expression level of the endogenous Na,K-ATPase α1 subunit in the mutants are similar to that in the wild type (Fig. 1D). The easiest explanation for a different apical content of YFP-β in Tyr20 mutants is shown in the model (Fig. 10, middle and right). Probably the Tyr20-based motif is recognized as both an endocytic and basolateral sorting motif. It has been shown that tyrosine-based basolateral sorting signals identified in a number of proteins are similar but not the same as endocytic tyrosine-based motifs (34, 35). These motifs are recognized by the homologous but distinct adaptor proteins (36, 37). Our data might suggest that the mutation Y20F affected only endocytosis and retained basolateral sorting (Fig. 10, middle). In other words, conservative substitution of the tyrosine to phenylalanine impaired the recognition of the endocytic signal at the plasma membrane but did not affect its recognition as a basolateral signal in TGN. Therefore, the quantity of Y20F mutant YFP-β present in the apical membrane, 20% of total surface YFP-β, reflects the amount of the protein that is delivered directly from TGN to the apical membrane. The rest of Y20F mutant YFP-β is delivered to the basolateral membrane. The basolateral YFP-β pool consists of two fractions, heterodimers with the endogenous Na,K-ATPase α1 subunits and the monomers of YFP-β that are delivered to the basolateral membrane due to a recognition of a basolateral sorting signal that contains Phe20 instead of Tyr20 (Fig. 10, middle). The mutation Y20A impaired not only basolateral endocytosis but also basolateral sorting (Fig. 10, right). As a result, the basolateral pool of YFP-β consists only of the fraction of YFP-β that was co-sorted with the Na,K-ATPase α1 subunit. All of the monomers of YFP-β in the Y20A mutant are directed to the apical membrane (Fig. 10, right).

In the wild type, the pool of YFP-β monomers in the TGN is split into two fractions (Fig. 10, left). One is delivered directly from the TGN to the apical membrane, probably due to the recognition of N-glycans or other yet unidentified apical signals. The other is delivered first to the basolateral membrane due to the recognition of the Tyr20-based basolateral sorting signal and then is internalized due to the recognition of the Tyr20-based endocytic signal and sorted again in endosomes based on the recognition of the above apical and basolateral sorting signals. One fraction of the endosomal pool of YFP-β is returned back to the basolateral membrane, and the other is delivered to the apical membrane (Fig. 10, left). The apical content of YFP-β in the wild type is greater compared with that in Y20F, since it contains not only the fraction that was delivered directly from the TGN but also the fraction that was indirectly delivered by transcytosis via the basolateral membrane (Fig. 10, left and middle). On the other hand, the apical content of YFP-β in the wild type is lower compared with that in Y20A, since a certain fraction of transcytosed YFP-β is transiently present in the basolateral membrane (Fig. 10, left and right).

Therefore, our model for the H,K-ATPase β subunit transcytosis suggests recognition of apical and basolateral sorting signals in the TGN and similar sorting signal recognition in endosomes (Fig. 10, left). However, a model for transcytosis of glycosylphosphatidylinositol-anchored proteins in MDCK cells suggested that the whole pool of newly synthesized glycosylphosphatidylinositol-anchored proteins is delivered to the basolateral membrane first and then is selectively endocytosed into raft-enriched transport carriers and delivered to the apical membrane (29). This raises the question as to why the apical signal recognition and segregation of glycosylphosphatidylinositol-anchored proteins into lipid rafts occurs only at the basolateral membrane and not in the TGN that is also enriched in lipid rafts (38).

Quantification of the Contributions of the Direct, Transcytotic, and Co-sorting Pathways in Delivery of the H,K-ATPase β to the Apical and Basolateral Membranes of the MDCK Cell—The results of our studies have enabled us to obtain a relative quantification of the contributions of the individual sorting pathways responsible for surface distribution of YFP-β in MDCK cells (Fig. 10, left). Co-sorting with the endogenous Na,K-ATPase α1 subunit appears to be a prevailing sorting pathway in these cells. The predominant fraction of the baso-
lateral pool of YFP-β is represented by heterodimers with the Na,K-ATPase α1 subunits. This is consistent with the results of non-denaturing gel electrophoresis that showed that about half of the YFP-β molecules form complexes with the Na,K-ATPase α1 subunits (Fig. 2).

A minor portion of the basolateral pool is represented by YFP-β monomers that are transiently present on the basolateral surface, since they undergo transcytosis to the apical membrane via the basolateral membrane (Fig. 10, left). This is consistent with the results presented in Fig. 8. When de novo synthesis is blocked by CHX and direct sorting does not occur, the β subunit accumulates in the apical membrane and decreases in the basolateral membrane, whereas the contents of the Na,K-ATPase α1 do not change (Fig. 8, B and C). These data suggest that only the YFP-β monomers undergo transcytosis, whereas the heterodimers YFP-βNaKα stay in the basolateral membrane.

About one-half of the apical steady state content of the β subunit originates from the TGN and the other half from transcytosis (Fig. 10, left). This is consistent with the results shown in Fig. 9A (panels 4 and 1), where accumulation of the wild type YFP-β on the apical surface in the absence of CHX (direct apical sorting plus transcytosis) is twice as much as the accumulation in the presence of CHX (transcytosis only). It also explains why the steady state apical content in Y20F (direct sorting only) is 2-fold lower compared with the wild type (direct sorting plus transcytosis) (Fig. 1C).

In conclusion, the distribution of the H,K-ATPase β subunit in polarized cells involves multiple sorting pathways including direct apical sorting of the newly synthesized protein from TGN, transcytosis via the basolateral membrane and “passive” co-sorting with the endogenous Na,K-ATPase α1 subunit. In MDCK cells, the large amount of co-sorting with the endogenous Na,K-ATPase α1 subunit is responsible for the greater basolateral content of the H,K-ATPase β subunit compared with LLC-PK1 cells. The tyrosine 20 in the cytoplasmic tail of the H,K-ATPase β subunit is important for two distinct steps of the transcytotic pathway, basolateral sorting of the newly synthesized subunit from TGN and endocytosis from the basolateral membrane. The sorting information encoded by tyrosine 20-based motif must be recognized by distinct components of sorting machinery, since alanine substitution of tyrosine 20 impairs both basolateral sorting and basolateral endocytosis, whereas the phenylalanine mutation impairs basolateral endocytosis but retains basolateral sorting.

Moreover, these studies emphasize the value of using the expressed gastric H,K-ATPase β subunit in cultured polarized epithelial cells as a model for defining the factors essential for cell sorting.

Acknowledgment—We acknowledge the careful reading of the manuscript and helpful suggestions of Jeffrey A. Kraut, M.D.

REFERENCES

Use of the H,K-ATPase β Subunit to Identify Multiple Sorting Pathways for Plasma Membrane Delivery in Polarized Cells
Olga Vagin, Shahlo Turdikulova, Iskandar Yakubov and George Sachs

doi: 10.1074/jbc.M412657200 originally published online February 4, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M412657200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 38 references, 12 of which can be accessed free at
http://www.jbc.org/content/280/15/14741.full.html#ref-list-1