The role of N-glycosylation in trafficking of an apical membrane protein, the gastric H,K-ATPase β subunit linked to yellow fluorescent protein, was analyzed in polarized LLC-PK1 cells by confocal microscopy and surface-specific biotinylation. Deletion of the N-glycosylation sites at N1, N3, N5, and N7 but not at N2, N4, and N6 significantly slowed endoplasmic reticulum-to-Golgi trafficking, impaired apical sorting, and enhanced endocytosis from the apical membrane, resulting in decreased apical expression. Golgi mannosidase inhibition to prevent carbohydrate chain branching and elongation resulted in faster internalization and degradation of the β subunit, indicating that terminal glycosylation is important for stabilization of the protein in the apical membrane and protection of internalized protein from targeting to the degradation pathway. The decrease in the apical content of the β subunit was less with mannosidase inhibition compared with that found in the N1, N3, N5, and N7 site mutants, suggesting that the core region sugars are more important than the terminal sugars for apical sorting.

Sorting of proteins between apical and basolateral membranes in polarized cells depends on the recognition of intrinsic sorting signals within the proteins by specific sorting machinery in the trans-Golgi network (TGN) or endosomes (1–3, 4). Basolateral sorting signals often contain tyrosine-based, dileucine, or other hydrophobic motifs that are recognized by clathrin coat proteins that package them into basolateral transport vesicles (5, 6). Apical sorting signals are less well defined. N-Linked glycans are considered as possible candidates based on the findings that some proteins gain the ability to reach the apical membrane after recombinant addition of N-glycosylation sites (7) and that other proteins entirely or partially lose apical expression as a result of removal of N-glycans by mutation or treatment with glycosylation inhibitors (5, 8–10). N-Glycosylation might significantly affect other trafficking steps, including protein folding and quality control in the endoplasmic reticulum (ER), endocytosis, recycling, and degradation. Because the effect of N-glycan removal on an apical sorting event has not been differentiated from a possible impairment of other trafficking steps, the role of N-glycans as apical sorting signals remains unclear.

An interesting paradigm for the study of the role of glycosylation in protein maturation, trafficking, sorting, and degradation is an apically targeted multiply glycosylated protein, the H,K-ATPase β subunit. The gastric H,K-ATPase, the enzyme responsible for acid secretion in the stomach, consists of two subunits, a catalytic α subunit and an accessory β subunit, which has seven N-glycosylation sites. Glycosylation of the β subunit has been shown to be critical for the quality control of the H,K-ATPase isoforms in the ER (11, 12). Specific N-glycosylation sites have been found to be essential for plasma membrane delivery of the H,K-ATPase β subunit in non-polarized HEK293 (13) and for delivery of both α and β subunits in COS-7 cells (12), suggesting that they may also play a role in apical sorting. The homologous Na,K-ATPase also consists of α and β subunits. Four of known isoforms of the Na,K-ATPase β subunits, β2 is the most homologous to the H,K-ATPase β subunit, has up to nine glycosylation sites (14), and appears to result in apical sorting of the Na,K-ATPase α/β complex in a number of tissues (15–17). The Na,K-ATPase that contains either the β1 or β3 isoform with only two or three N-glycosylation sites localizes exclusively in the basolateral membrane (18). 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 αβ complexes.

N-Glycosylation occurs in various stages. N-Linked oligosaccharides appear when the 14-saccharide core is transferred from the dolichol phosphate precursor to the nascent membrane protein that remains associated with the translocon in the ER. Immediately after coupling the core to the asparagine of the specific amino acid NXS or NXT motif, the N-glycosylation site, the terminal glucose residues are trimmed by ER glucosidases. Various chaperones such as calnexin bind to sugar chains as quality control elements at this and perhaps later stages. Subsequently, the mannose residues are trimmed in the ER and then in the Golgi by mannosidases I and II, respectively. This is followed by elongation of the carbohydrate chains due to addition of terminal sugars by the action of various glycosyltransferases in the trans-Golgi (19). There is considerable variation in the number and composition of terminal chains in the mature complex oligosaccharides, giving rise to heterogeneity of glycosylated proteins. The core region that contains five residues originating from the initial 14-saccharide core is the same in all molecular species of mature glycoproteins. Deletion of any N-glycosylation site in a protein abrogates the whole oligosaccharide tree at that particular locus, leaving the others intact. Inhibition of glucose or mannose trimming prevents addition of the terminal sugar chains but leaves core regions intact at all N-glycosylation sites. This enables distinctions to be made concerning the roles of core...
Glycosylation and Apical Sorting of the H,K-ATPase

**EXPERIMENTAL PROCEDURES**

Construction of cDNAs Encoding Yellow Fluorescent Protein (YFP)-H,K-ATPase β Subunit Fusion Proteins and Mutants Lacking Glycosylation Sites—pCDNA3+ β (20) was used as a source for CDNADNA encoding the rabbit H,K-ATPase β subunit (GenBank™/EBI accession number M35544) [21]. The cDNA encoding the β subunit was inserted into the multiple cloning site of the expression vector pEYFP-C1 (Clontech) using BglII and BamHI restriction sites to form pEYFP-β, which encodes YFP-β, a fusion protein of YFP linked to the N terminus of the H,K-ATPase β subunit. Mutants were generated using the QuikChange mutagenesis kit (Stratagene). This technique was used to generate the mutants F193Q (N6), and N222Q (N7), which are the non-glycosylated forms of YFP-β and are denoted as pEYFP-N6 and pEYFP-N7, respectively.

Stable Transfection—To obtain cell lines stably expressing wild-type or mutant YFP-β fusion proteins, LLC-PK1 cells were grown on 10-cm plates until 20% confluent and transfected with wild-type YFP-β or mutant YFP-β using FuGENE 6 transfection reagent (Roche Applied Science). 24 h after transfection, stable cell lines were selected by addition of the eukaryotic selection marker G418 at a concentration of 1.0 mg/ml. This concentration of G418 was maintained until single colonies appeared. 15–20 colonies were isolated, expanded, and grown in the presence of 0.25 mg of G418/ml of medium in 24-well plates. Cell lines with the highest expression of YFP-β were selected and expanded for further studies.

Confocal Microscopy Studies—Cells stably expressing wild-type or mutant YFP-β were grown for at least 5 days before becoming confluent on glass bottom micro-well dishes (Mattek Corp.). Confocal microscopy images were acquired using a Zeiss LSM 510 laser scanning confocal microscope with LSM 5.10 Version 3.2 software.

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 Corning Costar polyester Transwell inserts (Corning Inc.) in 6-well plates. Biotinylation of the apical or basolateral membrane proteins was performed by previously described procedures [22, 23]. Briefly, cells were incubated with EZ-Link™ sulfosuccinimidyl-2-(biotinamido)ethyl 1,3-dithiopropionate (Pierce), which was added from either the apical or basolateral side. After quenching the biotinylation reaction, cells were washed and then lysed by incubation with 200 μl of 0.15 M NaCl in 15 mM Tris (pH 8.0) containing 1% Triton X-100 and 4 mM EGTA. Cell lysates were clarified by centrifugation at 15,000 × g for 10 min. Samples containing 20 μg of supernatant mixed with 15 μl of SDS-containing sample buffer were loaded onto SDS-polyacrylamide gel to determine the total YFP-β content in the supernatant. To precipitate biotinylated proteins, the rest of each supernatant was incubated with 100 μl of streptavidin-agarose beads (Sigma) in a total volume of 800 μl of the biotinylation buffer for 1 h at 4 °C with continuous rotation. Precipitated 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% bromophenol blue, 20% glycerol, and 1% β-mercaptoethanol in 0.1 M Tris (pH 6.8)) for 5 min at 80 °C, separated on SDS-polyacrylamide gel, and analyzed by Western blotting using a monoclonal antibody 2B8 against the H,K-ATPase β subunit (MBL, Inc.) or the monoclonal antibody against the Na,K-ATPase β subunit (Novus Biologicals) as the primary antibody and anti-mouse IgG conjugated to alkaline phosphatase (Promega) as the secondary antibody according to the manufacturer's instructions.

Endocytosis Assay by Apical Surface Biotinylation—Polarized cells stably expressing wild-type or mutant YFP-β were biotinylated from the apical side as described above. Cells were incubated at 18 °C to prevent apical membrane delivery for 20, 60, or 120 min. After that, apical biotin was stripped off by incubation with 50 mM reduced glutathione (Sigma) in 100 mM NaCl with 10% fetal bovine serum (pH 8.4) twice for 20 min. After cell lysis, the internalized biotinylated proteins were precipitated, washed, and eluted from streptavidin-agarose beads, and analyzed by SDS-PAGE and Western blot analysis as described above. In the negative control, biotin was stripped immediately before biotinylation. The initial apical content was determined by lysing cells immediately after biotynilation. In the positive control, cells were incubated at 18 °C for 60 or 120 min to account for any instability of biotinylated protein and then lysed. After cell lysis, biotinylated proteins were precipitated, washed, eluted, and analyzed as described above. For the mutants with very low apical content of YFP-β (N1, N3, and N5), each experimental condition was repeated in three wells, and cell lysates from three identical wells were combined before precipitation on streptavidin-agarose beads. Endocytosis efficiency was calculated using YFP-β that was not in the apical membrane.

Treatment of Cells with Glycosidases Inhibitors—Cells were incubated with castanospermine (Sigma), the Golgi mannosidase I inhibitor deoxymannojirimycin (dMAN) (Sigma), or the Golgi mannosidase II inhibitor swainsonine (Sigma) at a concentration of 1, 2, or 200 μg/ml, respectively, for 48 h prior to apical biotinylation.

Glycosidase Cleavage—Where indicated, the total cell lysates or proteins precipitated by streptavidin-agarose beads were treated with peptide N-glycosidase F (PNGase F) from Flavobacterium meningo-septicum (New England Biolabs Inc.) or endoglycosidase H (Endo H) from Streptomyces plicatus (Glyco-Prozyme Inc.) according to the manufacturer's instructions.

In experiments with live cells, sialidase from Salmonella typhi murium recombinant in Escherichia coli (Glyco-Prozyme Inc.), β1,4galactosidase from Streptococcus pneumonia (Glyco-Prozyme Inc.), or PNGase F from F. meningo-septicum was added to the cell medium from the apical side at a concentration of 1 unit/ml, 68 milliunits/ml, or 7500 New England Biolabs units/ml, respectively, and incubated for 1 h. Prior to completion of the cleavage, cycloheximide (Sigma) at a concentration of 10 μg/ml was added to the medium to inhibit de novo synthesis of YFP-β.

**RESULTS**

Analysis of N-Glycosylation Alteration—The H,K-ATPase β subunit has seven N-glycosylation sites. The mature oligosaccharide linked to each of the seven sites consists of the three-mannosyl core region and terminal chains (Fig. 1). Three approaches were used to analyze the effect of N-glycosylation deficiency in the β subunit. By mutating each of these seven N-glycosylation sites and expressing these mutants in polarized cells, we obtained β subunits lacking all core and terminal sugars at only a single N-glycosylation site, with six sites still normally glycosylated. Alternatively, cells expressing the wild-type β subunit were treated with a glucosidase inhibitor (castanospermine), a Golgi mannosidase I inhibitor (dMAN), or a Golgi mannosidase II inhibitor (swainsonine). The inhibitors prevent glucose or mannose trimming and further digestion of all the carbohydrate chains and thus result in expression of the
14-saccharide core forms of YFP-β can be clearly distinguished from each other upon SDS-PAGE due to the difference in molecular masses, as shown in Fig. 1B.

**Characterization of Glycosylated Forms of YFP-β Expressed in LLC-PK1 Cells—**YFP-β was detected in cell lysates of LLC-PK1 cells as two bands, one at 80–100 kDa and the other at ~75 kDa (Fig. 2, lane 1). After PNGase F treatment of the cell lysates, the bands at 80–100 and 75 kDa both disappeared, and a single band was seen at ~55 kDa, corresponding to deglycosylated YFP-β (lane 3). A similar product was detected after treatment of the cell lysates with Endo H, but this treatment resulted in the disappearance of only the lower band on the Western blot, whereas the higher band was retained (lane 2). It is known that Endo H cleaves only high mannose- or hybrid-type glycoproteins, whereas complex-type chains are Endo H-resistant. Therefore, the 80–100-kDa band represents the complex-type glycosylated fraction of YFP-β. The 75-kDa Endo H-sensitive band represents high mannose-type YFP-β (Fig. 1B, fourth lane), as can be concluded from comparison with the lysates prepared from cells preincubated with the glycosylation inhibitors dMAN, which led to formation of both hybrid- and high mannose-type glycoproteins (third lane).

In contrast to the total cell lysate, the apically biotinylated protein fraction contained only the complex-type glycosylated fraction of YFP-β (Fig. 2, lane 4), which was Endo H-resistant (lane 5). Thus, the mature YFP-β component in LLC-PK1 cells...
Glycosylation and Apical Sorting of the H,K-ATPase

**Effect of N-Glycosylation Site Mutations on the Internalization Efficiency of YFP-β in LLC-PK1 Cells—**To compare the effect of mutations on the internalization efficiency of YFP-β, the apical surface of cells expressing wild-type or mutant YFP-β was biotinylated, and cells were incubated at 18 °C to prevent apical membrane delivery of internalized proteins. Any apical biotin was then cleaved off, and internalized biotinylated proteins were detected as described under “Experimental Procedures.” Internalization efficiency (viz. the fraction of apical YFP-β internalized after 1 h) was increased from 2- to 3-fold in the N1, N3, and N5 mutants, but was only slightly increased in the N1, N4, and N6 mutants (Fig. 6). The internalization efficiency in N7 could not be measured due to the very low apical content of YFP-β in this mutant.

**Effect of N-Glycosylation Site Mutations on Apical Sorting—**The surface distribution of mutants lacking the N3 or N5 site was different from that of the wild-type protein. The major plasma membrane-located fraction of the mutant proteins was detected on the basolateral (but not apical) membrane (Fig. 3). This might imply that removal of the particular N-glycosylation site impaired apical sorting. However, a steady-state distribution between two distinct surface domains is not a result only of apical sorting in the TGN and/or endosomes, but also reflects a balance between apical and basolateral sorting as well as apical and basolateral endocytosis.

To quantify the effect of mutations solely on apical sorting, we compared their effect on the relative apical content and the efficiency of endocytosis and calculated the effect of mutations on the apical sorting efficiency. The apical content of YFP-β normalized by comparison with the mature complex-type fraction of YFP-β (Cw/Cu) (Fig. 5) reflects a steady-state distribution between the apical and internal mature complex-type pools of YFP-β. We found that the rate of degradation of the mature complex-type fraction was not changed by N-glycosylation site mutations. Therefore, the distribution between apical and internal YFP-β could be shifted toward the internal pool in the mutants either because of enhanced endocytosis or because of the impaired apical membrane delivery (see Fig. 10). The relative decrease in the apical content was calculated for each mutant by dividing the apical content in the wild-type protein, (Cw/Cu)wt, by the apical content in the mutant, (Cw/Cu)mut (Table I, second column). Similarly, the relative increase in the efficiency of endocytosis in each mutant was calculated by dividing the endocytosis efficiency in the mutant by the endocytosis efficiency in the wild-type protein as assessed by apical biotinylation as described above (Table I, third column). If the apical content was decreased by the same factor as the endocytosis efficiency was increased, then the enhanced endocytosis would be the only reason for the lowered apical content in this mutant. However, in all mutants, the apical content was dramatically decreased in N1, N3, and N5 by 14.5-, 7.1-, and 5.5-fold, respectively, and was not detectable in N7. In contrast, mutation of N2, N4, and N6 only moderately decreased the relative apical content (from 1.4- to 1.7-fold).

We found that mature YFP-β contains complex-type oligosaccharides only and that the high mannose-type form of the protein corresponds to the ER fraction of the cellular YFP-β pool (Fig. 2). Therefore, the ratio between the complex-type form and total YFP-β can be used as a measure of ER-to-Golgi trafficking. In all the mutants except N6, the relative content of the complex-type glycosylated fraction of YFP-β was decreased, indicating that these mutations slowed down ER-to-Golgi trafficking and caused more ER retention. The most significant effect on ER-to-Golgi trafficking was observed in the N1 and N7 mutants (2.3- and 6.4-fold decreases, respectively).

![Fig. 3. Surface distribution of wild-type or mutant YFP-β in LLC-PK1 cells as assessed by surface-specific biotinylation. Cells were grown on Transwell filters. Upon polarization, cell layers were biotinylated from the apical (A) or basolateral (B) side and lysed. Biotinylated proteins were precipitated by streptavidin-agarose beads.](image)

The apical region in LLC-PK1 cells (Fig. 4, *inset*) was predominantly localized to the perinuclear region, presumably in the ER and Golgi.

To quantify the apical content in the mutants compared with the wild-type protein, cells expressing wild-type or mutant YFP-β were biotinylated from the apical side in the same experiment to prevent variations in biotinylation, streptavidin-agarose precipitation, and immunoblotting efficiencies. The apical content was normalized to the total YFP-β content in the corresponding cell lysate for each mutant and compared with that in the wild-type protein, as shown in Fig. 5. The relative apical content was dramatically decreased in N1, N3, and N5 by 14.5-, 7.1-, and 5.5-fold, respectively, and was not detectable in N7. In contrast, mutation of N2, N4, and N6 only moderately decreased the relative apical content (from 1.4- to 1.7-fold).

![Table I](table)

| Mutation | Apical Content (normalized to total YFP-β) | Relative Apical Content | Endocytosis Efficiency |
|----------|--------------------------------------------|------------------------|------------------------|
| wt       | 1.00                                       | 1.00                    | 1.00                   |
| N1       | 0.08                                       | 0.35                    | 0.29                   |
| N3       | 0.10                                       | 0.30                    | 0.28                   |
| N5       | 0.10                                       | 0.30                    | 0.28                   |
| N2       | 0.70                                       | 0.61                    | 0.61                   |
| N4       | 0.50                                       | 0.49                    | 0.49                   |
| N6       | 0.50                                       | 0.49                    | 0.49                   |
| N7       | 0.02                                       | 0.02                    | 0.02                   |

![Diagram](diagram)
decreased to a greater extent than the endocytosis efficiency. For example, in the N5 mutant, the apical content was decreased by 4.5-fold, but the internalization efficiency was increased by only 2-fold. This indicates that the apical membrane delivery was also affected by the mutation because the relative apical content reflects a balance between apical membrane delivery and endocytosis. If the apical content in the mutant is decreased by $X$-fold compared with the wild-type protein and the endocytosis efficiency is increased by $Y$-fold, then the apical membrane delivery rate must be decreased by $Z = X/Y$-fold. Thus, as shown in Table I, the effect of each mutation on the apical sorting efficiency was calcu-
lanes 4

The apical sorting efficiency was decreased in all the mutants compared with wild-type YFP-β (Table I). The very low content of mannose-type glycosylated YFP-β in cell lysates (Fig. 1). In the presence of dMAN, the immature ER portion of the protein and the mature form of YFP-β were indistinguishable since dMAN led to formation of high mannose-type form, as shown in Fig. 1B. Apical biotinylation of cells treated with castanospermine did not show any detectable amount of YFP-β on the surface (data not shown), indicating that the untrimmed protein is unable to exit the ER and to be processed to the Golgi and plasma membrane.

Preincubation of cells expressing wild-type YFP-β with the two Golgi mannosidase inhibitors resulted in a decrease in the molecular mass of mature YFP-β in cell lysates (Fig. 7). In the presence of swainsonine, two fractions of YFP-β were detected. The high mannose-type fraction represents the ER portion of the protein, and hybrid-type glycosylated YFP-β was the only form of the mature protein formed in the Golgi in the presence of this inhibitor (Fig. 1). In the presence of dMAN, the immature ER portion of the protein and the mature form of YFP-β were indistinguishable since dMAN led to formation of high mannose-type glycosylated YFP-β only (Fig. 1). The apical content was decreased as a result of preincubation with swainsonine or dMAN (Fig. 7). However, the effect of the inhibitors on the relative apical content was less than that of the N1, N3, N5, and N7 glycosylation site mutations (compare Figs. 5 and 7).

In contrast, internalization in the presence of swainsonine was higher compared with that in the wild-type protein and all the mutants (Fig. 6). Both inhibitors lowered the total cellular content of YFP-β, suggesting that they promote its degradation.

To compare the degradation rates of YFP-β preincubated with and without swainsonine, we measured the mature YFP-β

| TABLE I Effect of N-glycosylation site mutations on the relative apical content, endocytosis, and apical sorting of mutant YFP-β compared with wild-type YFP-β |
|----------------|----------------|----------------|----------------|----------------|
| YFP-β          | Decrease in apical content (C₈/C₇) | Increase in endocytosis efficiency | Decrease in apical sorting efficiency (calculated) |
|                | fold | fold | fold | fold |
| N1             | 6.2  | 3.1  | 2.0  |       |
| N2             | 1.2  | 1.2  | 1.0  |       |
| N3             | 5.2  | 2.6  | 2.0  |       |
| N4             | 1.4  | 1.1  | 1.3  |       |
| N5             | 4.5  | 2.0  | 2.3  |       |
| N6             | 1.6  | 1.1  | 1.4  |       |
| N7             | ND   | ND   | ND   |       |
| Wild-type      | 1.0  | 1.0  | 1.0  |       |

* C₈ YFP-β on the apical membrane; C₇ complex-type glycosylated YFP-β in the cell lysate; ND, not detectable.

† Decrease in apical sorting efficiency = (decrease in C₈/C₇) (increase in endocytosis efficiency).

labeled as a ratio between the factor in the second column, (C₈/C₇)wt, and the factor in the third column, (endocytosis efficiency)mut/(endocytosis efficiency)wt.

The apical sorting efficiency was decreased in all the mutants except N2 up to 2.3-fold (Table I). The very low content of complex-type YFP-β in N7 did not allow determination of the relative apical content in the cell line expressing this mutant.
content upon incubation of cells with cycloheximide to inhibit de novo protein biosynthesis. Swainsonine significantly increased the degradation rate (Fig. 8). By contrast, mutation of any single glycosylation site did not change the degradation rate of the complex-type glycosylated fraction of YFP-β. As an example, data for the N5 mutant are shown in Fig. 8. Effect of Treatment of the Apical Surface of LLC-PK1 Cells

with Glycosidases on YFP-β Internalization and Degradation—Apical treatment of cells expressing wild-type YFP-β with sialidase resulted in a decrease in the molecular mass of the protein (Fig. 9), indicating that terminal sialic acid residues were cleaved from the terminal chains of YFP-β. The amount of YFP-β found on the apical membrane and in the total cell lysate after desialylation was the same as in the control (Fig. 9), indicating that sialic acid is not important for the stability of the protein in the apical membrane and for recycling.

Treatment of the cells with sialidase in combination with galactosidase resulted in a decrease in the molecular mass of YFP-β as a result of a sequential cleavage of sialic acid and β-galactose residues. The amount of YFP-β after cleavage did not change in either the apical membrane or the total cell lysate (Fig. 9), showing that galactose residues also are not important for endocytosis and recycling.

Apical treatment of cell layers with PNGase F resulted in an almost complete disappearance of YFP-β from the apical membrane (Fig. 9). This result indicates that the deglycosylated protein is not stable in the membrane and internalizes and is not recycled to the apical membrane. A significant decrease in the complex-type glycosylated form of YFP-β was detected in the cell lysate (Fig. 9), whereas the high mannose-type form reflecting the newly synthesized ER fraction of the protein remained unchanged. Surprisingly, the deglycosylated form was not detected, suggesting that it was rapidly degraded during incubation of cells with the enzyme.

**DISCUSSION**

*N-Glycosylation and Degradation as a Quality Control System in Mammalian Cells—Glycosylation is indispensable for functional expression of the H,K-ATPase in mammalian cells. In HEK-293 cells, prevention of β subunit glycosylation due to tunicamycin treatment or mutation of all seven glycosylation sites results in almost complete loss of protein expression (12). Removal of all the sugars from apical YFP-β by PNGase F in LLC-PK1 cells resulted in internalization of the protein from the apical membrane and rapid degradation (Fig. 9), showing that carbohydrates are crucial for stabilization of both newly synthesized and recycling proteins. When expressed in insect cells, the H,K-ATPase αβ complex is not subject to degradation in the presence of tunicamycin even though the absence of glycosylation of the β subunit results in a complete loss of the H,K-ATPase activity, indicating probable impairment of the proper conformation (24).

Mammalian cells apparently have more stringent quality control that responds to more subtle changes in protein conformation, such as changes due to lack of N-glycosylation, and that allows for selective elimination of nonconforming proteins. It appears also that degradation pathways were evolved in parallel with complex-type glycosylation as additional quality control steps for additional regulation of trafficking and sorting. For example, the hybrid- and high mannose-type glycosylated β subunits formed in the presence of swainsonine and dMAN in LLC-PK1 cells were degraded much faster compared with the normal complex-type glycosylated protein (Figs. 7 and 8). In insect cells, however, the expressed β subunit is not degraded but instead forms a functionally active complex with the α subunit (24) even though it contains only high mannose-type carbohydrate chains due to the lack of terminal glycosylation.

**N-Glycosylation Site Mutations Slow Down ER-to-Golgi Trafficking and Apical Membrane Delivery and Enhance Internalization of the β Subunit—Mutation of any one of the seven N-glycosylation sites reduced the apical content of YFP-β (Fig. 5). The degradation rate was approximately the same in the wild-type protein and mutants (Fig. 8, compare wt and N5). This leaves three possible steps that could be affected by mutations: ER-to-Golgi trafficking, apical membrane delivery from the TGN (or endosomes), and internalization from the**

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**Fig. 8. Effects of a glycosylation inhibitor (swainsonine) and mutation of a single N-glycosylation site on the degradation rate of YFP-β in LLC-PK1 cells.** Cells expressing wild-type or N5 mutant YFP-β were incubated with or without cycloheximide (CHX; 20 μg/ml), a protein synthesis inhibitor. In half of the wells containing wild-type YFP-β-expressing cells, swainsonine (2 μg/ml) was applied 48 h prior to addition of cycloheximide. After 3 or 6 h of incubation with cycloheximide, the apical and total cellular contents of complex-type YFP-β (wild-type protein (wt) and N5) or hybrid-type YFP-β (wild-type protein + swainsonine) were determined (see “Experimental Procedures”) as a fraction of the YFP-β content in a corresponding control well with no cycloheximide. A decreased amount of YFP-β upon incubation of cells with cycloheximide reflected the degradation rate of the protein. The bar graphs demonstrate that, in the presence of the glycosylation inhibitor swainsonine, the intracellular degradation of wild-type YFP-β was significantly enhanced, whereas the degradation rate of the mutant lacking the N5 glycosylation site remained unchanged compared with that of wild-type YFP-β in the absence of swainsonine.

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**Fig. 9. Effect of cell treatment with glycosidases on internalization and stability of YFP-β.** Cells expressing wild-type YFP-β were grown and polarized on Transwell inserts and incubated with sialidase (1 units/ml), β-galactosidase (66 milliunits/ml), or PNGase F (7500 units/ml), which was added to the medium from the apical surface for 16 h. Cycloheximide (20 μg/ml) was added to both sides 60 min prior to biotinylation to inhibit de novo synthesis of YFP-β. Apical and total cellular fractions of YFP-β were determined as described under “Experimental Procedures.” C, complex-type YFP-β; H, high mannose-type YFP-β. The sites of cleavage by glycosidases in the glycoprotein are shown by the arrows.

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**Glycosylation and Apical Sorting of the H,K-ATPase**

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Distinct Roles of Core Region and Terminal Sugars in Apical Sorting, Internalization, and Degradation of the β Subunit—

Mature complex-type glycosylated YFP-β, which undergoes sorting and trafficking from the TGN to the plasma membrane, internalization, recycling, and degradation, contains the three-mannosyl core and terminal chains (Fig. 1). The effect of the Golgi mannosidase inhibitors compared with that of the core region sugars contributes more to apical sorting compared with the terminal sugars. Swainsonine significantly increased the rate of internalization of YFP-β. The internalization rate of wild-type YFP-β in the presence of swainsonine was higher compared with that of any of the mutants. This suggests that terminal sugars are important for stabilization of the β subunit in the apical membrane. The enhanced rate of endocytosis in the N1, N3, and N5 mutants is probably a result of a lack of terminal sugars in these particular sites. To find out which of the terminal sugars are essential, we treated cells with specific terminal glycosidases. It appears that terminal sialic acid and β-galactose residues are not essential for stabilization of the β subunit in the apical membrane since sialidase and galactosidase treatment of the cells did not change the amount of apical YFP-β (Fig. 9). Thus, some other terminal carbohydrate residues such as α-galactose, fucose, and terminal N-acetylglucosamine might be important for β subunit stabilization.

Both swainsonine and dMAN significantly decreased the total amount of YFP-β (Fig. 7), suggesting that they promote degradation of the protein. Indeed, the rate of YFP-β degradation that was seen as a decrease in protein content upon incubation with cycloheximide was much higher after swainsonine treatment (Fig. 8). Therefore, terminal glycosylation protects the protein from being targeted to degradation pathways. The degradation rate was not increased in the mutants lacking both core region and terminal sugars at one of seven N-glycosylation sites (Fig. 8). This might imply that specific mannose residues exposed at the chain termini in the high mannose- and hybrid-type glycoproteins (after dMAN and swainsonine treatment) act as degradation targeting signals. Without inhibitors, in complex-type wild-type or mutant YFP-β, these mannose residues are not present due to the trimming by Golgi mannosidases, and the mannosides of the three-mannosyl core are concealed by the terminal sugars (see Fig. 1). The mannose residues are also concealed in the products of deglycosylation by sialidase and galactosidase, perhaps explaining why sialidase and galactosidase treatment of the cells did not decrease the total content of YFP-β (Fig. 9).

Role of N-Glycans as Apical Sorting Signals—Certain N-glycosylation sites in the β subunit appear to be more important for trafficking and apical sorting than the others. For example, the N7 site seems to be the most critical in the β subunit since its removal resulted in complete ER retention of the protein. The other mutations that significantly increased the ER retention of the protein (N1, N3, and N5) also affected the other two trafficking steps, apical sorting and internalization. On the other hand, the N2, N4, and N6 mutations only slightly affected all three steps. This might suggest that the carbohydrate chains located at N1, N3, N5, and N7 are critical for the conformation of the protein necessary for the molecular recognition of trafficking signals for ER exit and then of apical sorting signals in the TGN and endosomes. A specific conformation might also be required for stability of the protein in the apical membrane, which would explain why the N1, N3, and N5 mutants were internalized faster. This is compatible with the suggestion that N-glycans provide a structural support for apical sorting (25).

However, the data presented here show that particular carbohydrate residues may also play a specific role in trafficking and apical sorting. We found that terminal sugars are essential for stabilization of the protein in the apical membrane, whereas the core region sugars are more important for apical sorting in the TGN and endosomes. This might imply that specific carbohydrate residues act as sorting signals. It is possible that terminal sugars anchor the protein in the apical membrane by interacting with unknown apical proteins or lipids in lipid rafts, whereas the particular core region carbohydrate residues...
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(core N-acetylglucosamine or mannose) act as primary apical sorting signals that are recognized by lectin cargo receptors in the TGN and endosomes, in agreement with the proposed earlier model (8).

Role of N-Glycosylation of the β Subunit in Stability and Apical Sorting of the Gastric H,K-ATPase αβ Holoenzyme—The assembly of the α and β subunits of the gastric H,K-ATPase occurs in the ER (26). Expression studies in various cell types have shown that the presence of the β subunit is required for the correct folding and membrane insertion of the α subunit in the ER and its stabilization and subsequent trafficking (12, 26, 27). If expressed alone, the α subunit is retained in the ER and is degraded. In contrast, even in the absence of the α subunit, the β subunit is able to fold properly, to undergo full maturation, and to traffic to the plasma membrane in both non-polarized and polarized cells (13, 28, 29). Carbohydrate residues linked to the β subunit play a significant role in quality control of the β subunit alone in non-polarized cells (13) and in polarized LLC-PK1 cells or in assembled αβ complexes (12, 24) in non-polarized cells. Mutation of particular N-glycosylation sites impairs membrane targeting of the β subunit in non-polarized HEK-293 cells (13) and apical sorting in LLC-PK1 cells. Recent findings indicate that mechanisms of membrane targeting in non-polarized cells are similar to sorting in polarized cells (5, 7, 30–32) and that apical and basolateral proteins even in non-polarized cells are sorted into different transport containers. Data on H,K-ATPase expression in non-polarized cells favor the primary role of the β subunit and not the α subunit in the apical sorting of the H,K-ATPase since the presence of the α subunit does not restore the plasma membrane targeting of the H,K-ATPase αβ complexes containing N-glycosylation site-mutated β subunits (12). This might suggest that N-glycosylation in the β subunit is essential for apical sorting of the H,K-ATPase αβ holoenzyme in parietal cells. In conclusion, N-glycosylation, in particular at the N1, N3, N5, and N7 glycosylation sites, is crucial for apical localization of the H,K-ATPase β subunit in polarized LLC-PK1 cells. Core glycosylation and trimming in the ER are critical for proper β subunit folding and quality control. Core region sugars are more important than terminal sugars for apical sorting of the protein in the TGN and/or endosomes. Terminal glycosylation is important for stabilization of the protein on the apical membrane and protection of the internalized protein from degradation. Presumably, these conclusions extend to other apically targeted glycosylated proteins.

REFERENCES

1. Traub, L. M., and Kornfeld, S. (1997) Curr. Opin. Cell Biol. 9, 527–533
2. Ikonen, E., and Simons, K. (1998) Semin. Cell Dev. Biol. 9, 503–509
3. Yeaman, C., Grindstaff, K. K., and Nelson, W. J. (1999) Physiol. Rev. 79, 73–98
4. Nelson, W. J., and Rodriguez-Boulan, E. (2004) Nat. Cell Biol. 6, 282–284
5. Matter, K., and Mellman, I. (1994) Curr. Opin. Cell Biol. 6, 545–554
6. Keller, P., and Simons, K. (1997) J. Cell Sci. 110, 3001–3009
7. Gut, A., Kapperl, F., Hyka, N., Balda, M. S., Hauri, H. P., and Matter, K. (1998) EMBO J. 17, 1919–1929
8. Scheiffele, P., Peranen, J., and Simons, K. (1995) Nature 378, 96–98
9. Peter, B. A., Burke, G., Bruns, J. R., Wezel, K. M., and Weisz, O. A. (2004) Mol. Biol. Cell 15, 1407–1416
10. Hendriks, G., Koudijs, M., van Balkom, B. W., Oorschot, V., Klumperman, J., Deen, P. M., and van der Stuijs, P. (2004) J. Biol. Chem. 279, 2975–2983
11. Geering, K. (2001) J. Bioenerg. Biomembr. 33, 425–438
12. Asano, S., Kawada, K., Kimura, T., Grishin, A. V., Caplan, M. J., and Takeguchi, N. (2000) J. Biol. Chem. 275, 8324–8330
13. Vagin, O., Denewich, S., and Sachs, G. (2003) Am. J. Physiol. 285, C968–C976
14. Blanco, G., and Mercer, R. W. (1998) Am. J. Physiol. 275, F633–F650
15. Wilson, P. D. (2004) N. Engl. J. Med. 350, 151–164
16. Wilson, P. D., Devroy, O., Li, X., Gatti, L., Falkenstein, D., Robinson, S., Fambrough, D., and Burrow, C. R. (2000) Am. J. Pathol. 156, 253–368
17. Mohasheri, A., Oukrif, D., Dawodi, S. P., Sinha, M., Greenwell, P., Stewart, D., Djonga, M. B., Foster, C. S., Martin-Vasallo, P., and Mohasheri, R. (2001) Histol. Histopathol. 16, 141–154
18. Caplan, M. J. (1997) Am. J. Physiol. 272, G1304–G1313
19. Hellenius, A., and Aebi, M. (2001) Science 291, 2364–2369
20. Lambrecht, N., Munson, K., Vagin, O., and Sachs, G. (2000) J. Biol. Chem. 275, 4041–4048
21. Reuben, M. A., Laseter, L. S., and Sachs, G. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6767–6771
22. Gottardi, C. J., Dunbar, L. A., and Caplan, M. J. (1995) Am. J. Physiol. 268, F285–F290
23. Kreep, J. P., and Gardinier, M. V. (2001) J. Neurochem. 77, 1301–1309
24. Klaassen, C. H., Fransen, J. A., Swarts, H. G., and De Pont, J. J. (1997) Biochem. J. 321, 419–424
25. Rodriguez-Boulan, E., and Gonzalez, A. (1999) Trends Cell Biol. 9, 291–294
26. Beguin, P., Hasler, U., Staub, O., and Geering, K. (2000) Mol. Biol. Cell 11, 1657–1672
27. Kimura, T., Tabuchi, T., Takeguchi, N., and Asano, S. (2002) J. Biol. Chem. 277, 20671–20677
28. Gottardi, C. J., and Caplan, M. J. (1993) J. Cell Biol. 121, 283–293
29. Roux, D. L., Gottardi, C. J., Naim, H. Y., Roth, M. G., and Caplan, M. J. (1998) J. Biol. Chem. 273, 28662–28669
30. Keller, P., Toomre, D., Diaz, E., White, J., and Simons, K. (2001) Nat. Cell Biol. 3, 140–149
31. Musch, A., Xu, H., Shields, D., and Rodriguez-Boulan, E. (1996) J. Cell Biol. 133, 543–558
32. Yoshimori, T., Keller, P., Roth, M. G., and Simons, K. (1996) J. Cell Biol. 133, 247–256
