Plasma membrane delivery of the gastric H,K-ATPase: the role of β-subunit glycosylation

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Vagin, O., S. Denevich, and G. Sachs. Plasma membrane delivery of the gastric H,K-ATPase: the role of β-subunit glycosylation. Am J Physiol Cell Physiol 285: C968–C976, 2003. First published May 28, 2003; 10.1152/ajpcell.00068.2003 — The factors determining trafficking of the gastric H,K-ATPase to the apical membrane remain elusive. To identify such determinants in the gastric H,K-ATPase, fusion proteins of yellow fluorescent protein (YFP) and the gastric H,K-ATPase β-subunit (YFP-β) and cyan fluorescent protein (CFP) and the gastric H,K-ATPase α-subunit (CFP-α) were expressed in HEK-293 cells. Then plasma membrane delivery of wild-type CFP-α, wild-type YFP-β, and YFP-β mutants lacking one or two of the seven β-subunit glycosylation sites was determined using confocal microscopy and surface biotinylation. Expression of the wild-type YFP-β resulted in the plasma membrane localization of the protein, whereas the expressed CFP-α was retained intracellularly. When coexpressed, both CFP-α and YFP-β were delivered to the plasma membrane. Removing each of the seven glycosylation sites, except the second one, from the extracellular loop of YFP-β prevented plasma membrane delivery of the protein. Only the mutant lacking the second glycosylation site (Asn103Gln) was localized both intracellularly and on the plasma membrane. A double mutant lacking the first (Asn99Gln) and the second (Asn103Gln) glycosylation sites disperse intracellular accumulation of the protein. Therefore, six of the seven glycosylation sites in the β-subunit are essential for the plasma membrane delivery of the β-subunit of the gastric H,K-ATPase, whereas the second glycosylation site (Asn103), which is not conserved among the β-subunits from different species, is not critical for plasma delivery of the protein. 

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 apical membrane upon stimulation (25, 27). Parietal cells, as well as other polarized cells, are able to sort newly synthesized proteins transiting the trans-Golgi network (TGN) into two delivery routes either to apical or basolateral domains of the plasma membrane. Thus exit from the Golgi and subsequent surface delivery of proteins depend on the presence of intrinsic sorting signals within the proteins (10, 26, 30). The H,K-ATPase consists of two subunits, namely α and β, each of them containing sorting signals. The information on the sorting signals within the H,K-ATPase subunits has been inferred from expression studies. When expressed in LLC-PK1 cells, which do not contain tubulovesicular elements, the gastric H,K-ATPase is located exclusively on the apical membrane (6). Studies in which the H,K-ATPase β-subunit or chimeric complexes of the H,K-ATPase α- or β-subunit with the appropriate subunit of the Na,K-ATPase were expressed in LLC-PK1 cells have shown that both α- and β-subunits of the H,K-ATPase contain apical trafficking signals (6, 22). The apical sorting signal in the α-subunit appears to reside in the fourth transmembrane domain and to act through long-range interactions with its flanking cytoplasmic loop domains (5). However, the nature of the apical sorting signal(s) within the β-subunit remains unknown. N-glycosylation sites might be considered potential candidates for the apical signals because the gastric H,K-ATPase β-subunit is heavily glycosylated and N-glycosylation sites have been found to act as apical signals in a number of secreted and membrane proteins (8, 18, 24). However, the involvement of N-glycosylation sites in apical sorting of the gastric H,K-ATPase has not been directly tested as yet. Besides unidentified apical sorting signal(s), the H,K-ATPase β-subunit contains a tyrosine-based “endocytosis” motif in the cytoplasmic tail that appears to govern retrieval of the H,K-ATPase from the apical membrane (3). It was suggested that the same motif might be recognized as a basolateral sorting signal when the H,K-ATPase β-subunit was expressed in Madin-Darby canine kidney (MDCK) cells (22). However, it has been shown that the tyrosine-based motif in the β-subunit does not serve as a conventional basolateral signal recognized by a clathrin adaptor complex in MDCK cells (4).

In contrast to polarized cells, nonpolarized cells were thought to have a default pathway for surface delivery of plasma membrane proteins (29). However, there is increasing evidence that apical and basolateral sorting
and trafficking pathways exist not only in polarized but also in nonpolarized cells (11). When apical and basolateral proteins are expressed in nonpolarized cells, in TGN they are sorted into different containers that travel separately until they fuse with the plasma membrane (12, 19, 23, 32). Therefore, at least for some proteins, surface delivery in nonpolarized cells does not occur by a default mechanism but rather as a result of a sorting event in TGN (12, 19, 32). However, it is unclear whether a signal-based sorting event in TGN is an absolute requirement for subsequent surface delivery of the proteins in nonpolarized cells.

If expressed in nonpolarized HEK-293 cells, the H,K-ATPase is localized on the plasma membrane (13, 16). The α-subunit is not able to leave the endoplasmic reticulum (ER) without the β-subunit, and, when expressed alone in HEK-293 cells, it does not reach the plasma membrane (13, 16). In contrast, plasma membrane delivery of the β-subunit does not require the presence of the α-subunit. Hence, it is the β-subunit that must be responsible for plasma membrane delivery of the gastric H,K-ATPase in HEK-293 cells. It is unknown whether surface delivery of the gastric H,K-ATPase β-subunit occurs by a default mechanism or whether it depends on the presence of intrinsic sorting or trafficking signals.

To address these issues, we examined intracellular trafficking of the gastric H,K-ATPase β-subunit, normally an apical protein, in nonpolarized HEK-293 cells using a fusion protein of the β-subunit and yellow fluorescent protein (YFP). By generating mutants with successive exclusion of glycosylation sites, we found that six of the seven glycosylation sites in the β-subunit are essential for the plasma membrane targeting in HEK-293 cells. The data strongly indicate that plasma membrane delivery of the gastric H,K-ATPase β-subunit in nonpolarized HEK-293 cells is sorting or trafficking signal dependent.

METHODS

Construction of cDNAs encoding cyan fluorescent protein-α and YFP-β fusion proteins and glycosylation site-lacking mutants. pcDNA3.1/zeo+α (16) was used as a source for cDNA encoding the rabbit H,K-ATPase α-subunit (2) (GenBank accession no. X64694), and pcDNA3(+)/β (16) was used as a source for cDNA encoding the rabbit H,K-ATPase β-subunit (21) (GenBank accession no. M35544). cDNA encoding the α-subunit was inserted into the multiple cloning site of the expression vector pECFP-C1 (BD Bioscience Clontech) using BgIII and EcoRI restriction sites to form pECFP-α. 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.

Mutants were generated by using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). The rabbit gastric H,K-ATPase β-subunit has seven N-glycosylation sites in the extracellular COOH-terminus that we numbered from one to seven from Asn99 (the first) to Asn222 (the seventh). Seven single mutants of YFP-β fusion protein lacking a single N-glycosylation site, one double mutant lacking the first and the second glycosylation sites, and one triple mutant lacking the first, the second, and the fourth glycosylation sites were constructed using pEYFP-β as a template (Table 1). Two single mutants of the gastric H,K-ATPase β-subunit lacking the fourth or the seventh sites and one double mutant lacking the first and the second sites were constructed using pcDNA3 (+)/β as a template (Table 1). Some of the single mutants were used as the templates for creation of double mutants. The double mutant was used as the template for construction of a triple mutant. Amino acid residues are numbered according to the sequence of the rabbit gastric H,K-ATPase β-subunit.

**Table 1. Mutations of N-glycosylation sites in YFP-β fusion protein and in the nonlabeled H,K-ATPase β-subunit**

<table>
<thead>
<tr>
<th>Plasmid Used as a Template for Mutations</th>
<th>Mutant</th>
<th>Amino Acid Substitution</th>
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<tbody>
<tr>
<td>pEYFP-β</td>
<td>N1</td>
<td>Asn99Gln</td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td>Asn103Gln</td>
</tr>
<tr>
<td></td>
<td>N3</td>
<td>Asn130Gln</td>
</tr>
<tr>
<td></td>
<td>N4</td>
<td>Asn146Gln</td>
</tr>
<tr>
<td></td>
<td>N5</td>
<td>Asn161Gln</td>
</tr>
<tr>
<td></td>
<td>N6</td>
<td>Asn193Gln</td>
</tr>
<tr>
<td></td>
<td>N7</td>
<td>Asn222Gln</td>
</tr>
<tr>
<td></td>
<td>N1/N2</td>
<td>Asn99Gln/Asn103Gln</td>
</tr>
<tr>
<td></td>
<td>N1/N2/N4</td>
<td>Asn99Gln/Asn103Gln/Asn146Gln</td>
</tr>
<tr>
<td>pcDNA3(+)-β</td>
<td>βN4</td>
<td>Asn146Gln</td>
</tr>
<tr>
<td></td>
<td>βN7</td>
<td>Asn222Gln</td>
</tr>
<tr>
<td></td>
<td>βN1/N2</td>
<td>Asn99Gln/Asn103Gln</td>
</tr>
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EYFP, enhanced yellow fluorescent protein.

**Transient transfection.** HEK-293 cells were grown on 10-cm plates till 80% confluent or on coverslips in six-well plates till 60% confluent and transiently transfected with pECFP-β alone, pEYFP-β alone, or with both pECFP-α and pEYFP-β using FuGENE 6 transfection reagent (Roche). Stable cell lines were selected by adding, 24 h after transfection, the eukaryotic selection marker G-418 at a concentration of G-418 was maintained until single colonies appeared. Fifteen to twenty colonies were isolated, expanded, and grown in the presence of 0.25 mg G-418/ml medium. The stable expression of the YFP-β fusion protein or H,K-ATPase β-subunit was confirmed by Western blot analysis using monoclonal antibody against green fluorescent protein (GFP) (BD Bioscience Clontech) and a 2B6 monoclonal antibody against the H,K-ATPase β-subunit (MBL) as described in SDS-PAGE and Western blot analysis. Two clones with the best ratio of expressed protein (YFP-β fusion protein or mutant H,K-ATPase β-subunit) to total protein were grown on coverslips in six-well plates for confocal studies and in the flasks for isolation of membranes.

The cell lines expressing mutant H,K-ATPase β-subunits were subjected to a second transfection with pcDNA3.1-(zeo+α)α. By addition of the second selection marker zeocin at
a concentration 0.4 mg/ml in addition to maintenance 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 isolation of membranes. Maintenance concentration for zeocin was 0.1 mg/ml.

Confocal microscopy studies. Cells stably expressing wild-type or mutant YFP-β were grown on coverslips in six-well plates till 80% confluent and fixed with methanol at –20°C. Cells subjected to transient transfection on coverslips (see Transient transfection) were fixed with methanol 24 h after transfection. Confocal microscopic images were acquired using the Zeiss LSM 510 laser scanning confocal microscope.

Preparation of crude membranes. The cells stably expressing the wild-type or mutant YFP-β were grown till confluent in flasks and then collected. The cells transiently expressing CFP-α and YFP-β fusion proteins were collected from 10-cm plates. Cells were collected by suspending them in the sodium-free buffer A (10 mM PIPES/Tris, pH 7.0, with 2 mM EGTA and 2 mM EDTA) containing 250 mM sucrose. The cell suspension was homogenized with a tight Dounce homogenizer (Wheaton, Millwille, NY). The homogenate was collected by centrifugation in a Beckman 75Ti rotor (35,000 rpm, 4°C). The fraction at the buffer/sucrose interface was collected and diluted to the total volume of 15 ml in buffer A. The membrane fraction was collected by centrifugation 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). The total protein concentration was determined by using modified Lowry protein assay reagent (Pierce). The typical protein concentration was 5–10 μg/μl. The membranes were aliquoted, flash frozen, and stored at –80°C. When indicated, the membrane fraction was treated with PNGase F (New England BioLabs) or EndoH (Prozyme) according to the manufacturers’ instructions.

Biotinylation and estimation of surface YFP-β expression. HEK-293 cells stably expressing wild-type or mutant YFP-β were grown for 2 days after becoming confluent in six-well plates. Biotinylation of the plasma membrane proteins was performed by previously described procedures (7, 15). Briefly, cell monolayers were rinsed with cold phosphate-buffered saline containing 1 mM Ca2+ and 1 mM Mg2+ (PBS2+). Cold PBS2+ (2 ml) containing 1 mg/ml sulfosuccinimidyl-6-(biotinamido)hexanoate (EZ-Link Sulfo-NHS-LC-biotin; Pierce, Rockford, IL) was added to each well. After 30 min of incubation at 4°C, the PBS2+/biotin solution was discarded, and the biotinylation reaction was quenched by adding cold PBS2+ with 50 mM NH4Cl (30 min, 4°C). Cells were washed twice with PBS2+ and then incubated with 60 μl of lysing buffer (1% SDS, 4 mM EGTA, and 10 mM Tris, pH 8.0) for 30 min with agitation at room temperature. Cell lysates were transferred into Eppendorf tubes and clarified by centrifugation (15,000 g for 5 min). Samples containing 15 μl of supernatant mixed with an equal volume of SDS-containing sample buffer were loaded onto SDS-PAGE gel to determine the total YFP-β content in the supernatant. To precipitate biotinylated proteins, 40 μl of each supernatant were incubated with 100 μl of streptavidin-agarose beads (Sigma) in a total volume of 800 μl of the lysing buffer for 1 h at 4°C with continuous rotation. Precipitated complexes were washed twice in 15 mM Tris, pH 8.0, containing 0.5% (vol/vol) Triton X-100, 4 mM EGTA, and 0.5 M NaCl and then in the same buffer without NaCl. Proteins were eluted from the beads by incubation in 40 μl of SDS-PAGE sample buffer (4% SDS, 0.05% bromophenol blue, 20% glycerol, 1% β-mercaptoethanol in 0.1 M Tris, pH 6.8) for 5 min at 80°C. After centrifugation, 30 μl of supernatant were loaded onto an SDS-PAGE gel next to the lane with the sample containing 15 μl of cell lysate obtained from the same cell line.

After SDS-PAGE and Western blotting (see below), nitrocellulose membranes were scanned and the bands were quantified using the AMBIS optical imaging system (AMBIS, San Diego, CA). A ratio between the density of the biotinylated YFP-β band and the density of the YFP-β fully glycosylated band from the total cell lysate for each cell line was calculated as a percentage of that in the wild type.

SDS-PAGE and Western blot analysis. The microsomal membrane fraction (5–15 μg), cell lysates, or biotinylated proteins were loaded on the 10% Tris-glycine or 4–12% NuPAGE Bis-Tris gels (Invitrogen). As a standard for native α- and β-subunits, 40–170 ng of purified gastric vesicles (G1 fraction) (20) were used and molecular weight standards (Bio-Rad Laboratories, Hercules, CA) were run on each gel.

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ATP. The reaction was terminated after 1 h at 37°C by adding 100 μl of stop solution containing 0.046% (wt/vol) malachite green, 1.43% (wt/vol) ammonium molybdate, 1.36 N HCl, and 1.26% (vol/vol) NP-10. After 1 min, 25 μl of 34% (wt/vol) sodium citrate were added. After 30 min, the absorbance at 680 nm was measured in a plate reader. Ion-stimulated ATPase activity was calculated as a difference between P_i release in the presence and in the absence of NH4Cl. The activity was expressed in μmol P_i·h⁻¹·mg total protein⁻¹ and then converted to specific H,K-ATPase activity (μmol P_i·h⁻¹·mg H,K-ATPase⁻¹) relative to a purified gastric vesicle preparation (G₁) using the content of expressed H,K-ATPase calculated from the quantitative Western blot analysis (see above).

**Determination of Vₚₛ, Kₘ,app for NH₄⁺, and Kᵢ for the SCH28080 inhibitor.** Data for the specific H,K-ATPase activities at various NH₄Cl concentrations (21–36 points) in the absence of inhibitor or at the fixed concentration of inhibitor were fitted by nonlinear regression to the Michaelis-Menten equation (Enzfitter, BIOSOFT, 1999), and the maximal velocity (Vₚₛ) ± SE and the apparent Kₘ (Kₘ,app) ± SE for NH₄Cl were obtained. Each set of data points was plotted as an inverse plot to determine the mechanism of SCH28080 inhibition (competitive, noncompetitive, or mixed). The inhibitor constant (Kᵢ) ± SE was obtained from linear regression of the Kₘ,app/Vₚₛ values plotted vs. inhibitor concentration. The intersection of the linear regression with x-axis gives the negative Kᵢ.

**RESULTS**

**Expression and characterization of CFP-α and YFP-β fusion proteins.** Expression of the wild-type CFP-α and YFP-β fusion proteins in HEK-293 cells was analyzed by Western blot. HEK-293 cells were transiently transfected with pECFP-α. Microsomal membranes were isolated and analyzed by Western blot using monoclonal antibodies 12.18 against the H,K-ATPase α-subunit or against the GFP (Fig. 1, lanes 2 and 3). As control, microsomal membranes isolated from HEK-293 cells stably expressing the gastric H,K-ATPase (28) were loaded (Fig. 1, lane 1). The H,K-ATPase α-subunit was detected as a ~100-kDa band with the 12.18 antibody. The CFP-α fusion protein was detected as a ~125-kDa band with both 12.18 and anti-GFP antibodies (lanes 2 and 3). The difference in molecular weights corresponds to the CFP portion of the CFP-α fusion protein.

The microsomal membranes isolated from the cells stably expressing YFP-β were analyzed using monoclonal antibodies against GFP and against the β-subunit (Fig. 1, lanes 4-7). Both antibodies detected two bands, one at 80–100 kDa and another at ~75 kDa (Fig. 1, lanes 4 and 5), presumably representing complex- and core-glycosylated YFP-β fusion proteins, respectively. After PNGase F treatment of the membrane fraction, bands at 80–100 and 75 kDa disappeared, and a single band of ~55 kDa was detected using the monoclonal antibody against the β-subunit (Fig. 1, lane 6). A band of the same molecular weight was detected using anti-GFP antibodies (data not shown). This band represents the nonglycosylated YFP-β fusion protein. Treatment of the membranes with EndoH resulted in the disappearance of only the lower band on the Western blot, whereas the band corresponding to complex-glycosylated YFP-β fusion protein was retained (Fig. 1, lane 7). A small shift in the molecular weight of the EndoH cleavage product compared with the PNGase F product reflects the difference in the cleavage sites for these two enzymes in the glycoprotein. In Fig. 1, lanes 8-10, the membranes isolated from the HEK-293 cells stably expressing the gastric H,K-ATPase with and without treatment with PNGase F and EndoH are shown. A pattern similar to the cleavage by glycosidases of YFP-β fusion protein (Fig. 1, lanes 5-7) was observed. The difference in molecular weights of nonglycosylated YFP-β fusion protein (Fig. 1, lane 6) and nonglycosylated β-subunit (Fig. 1, lane 9) corresponds to YFP portion of the fusion protein.

The microsomal membranes isolated from HEK-293 cells transfected with pECFP-α alone did not possess ion-stimulated H,K-ATPase activity. However, the microsomes isolated from HEK-293 cells cotransfected with pECFP-α and pEYFP-β displayed ion-stimulated SCH28080-inhibited ATPase activity (Fig. 2), indicating that CFP and YFP tags of α- and β-subunits did not disrupt the proper folding and association of the two subunits and only moderately changed the Vₚₛ (85 vs. 132 μmol·h⁻¹·mg⁻¹) and Kₘ,app [NH₄⁺]₁ (1.0 vs. 2.4 mM) compared with the wild-type H,K-ATPase (28).

**Characterization of glycosylation site mutants of the YFP-β fusion protein and the H,K-ATPase β-subunit.** The Western blot of the membrane fractions isolated from the cells expressing wild-type and mutant YFP-β fusion proteins is shown in Fig. 3. All the mutants, similar to the wild type, were represented by both complex- and core-glycosylated fractions of YFP-β fusion proteins, indicating that all the mutants were able to leave the ER and reach the Golgi. A stepwise decrease in molecular weights of mutants lacking one, two, or three glycosylation sites was observed compared with the wild-type YFP-β fusion protein. PN-

**Fig. 1.** Expression of cyan fluorescent protein (CFP)-α and yellow fluorescent protein (YFP)-β fusion proteins in HEK-293 cells analyzed by Western blot analysis. Microsomal membranes isolated from HEK-293 cells coexpressing α- and β-subunits of the gastric H,K-ATPase were loaded onto the gel (lanes 1 and 8-10), CFP-α (lanes 2 and 3), or YFP-β (lanes 4-7). Detection with anti-α 12.18 antibody: lane 1, α; lane 2, CFP-α. Detection with anti-green fluorescent protein (GFP) antibody: lane 3, CFP-α; lane 4, YFP-β. Detection with anti-β 2B6 antibody: lane 5, YFP-β; lane 6, YFP-β + PNGase F; lane 7, YFP-β + EndoH; lane 8, β; lane 9, β + PNGase; lane 10, β + EndoH.
Gase F cleavage of all the mutants resulted in a single product correspondent to the size of nonglycosylated YFP-β fusion protein (only N1 mutant treated with PNGase F is shown; Fig. 2, lane 11). The ratio between complex-glycosylated and core-glycosylated portions of the protein was decreased in most of the mutants, especially in N3, N4, N7, and triple mutant N1/N2/N4.

To determine whether removal of one or two glycosylation sites from the β-subunit affects the activity of the H,K-ATPase, three cell lines, stably expressing the β-subunits lacking the fourth, the seventh, or the first and the second glycosylation sites were obtained (Table 1). These cell lines were transfected with cDNA of the wild-type β-subunit, and stable lines expressing β-subunit in a complex with each mutant β-subunit (α + βN4, α + βN7, and α + βN1/N2) were selected as described in METHODS. Microsomal membranes from the cells were isolated and assayed for the H,K-ATPase activity and sensitivity to SCH28080. All three mutants possessed ion-stimulated, SCH28080-inhibited H,K-ATPase activity. The results of SCH28080 inhibition kinetics for α + βN1/N2 are presented in Fig. 4. Similar to that in the wild type, SCH28080 inhibition of the mutant was purely competitive in respect to the stimulating ion. Mutants α + βN4, α + βN7 also displayed competitive kinetics of SCH28080 inhibition (data not shown). Calculated kinetic parameters for all three mutants were not significantly different from those in the wild type (Fig. 4). These data indicate that removing the fourth, the seventh, or first and the second glycosylation sites from the β-subunit did not disrupt folding and proper association of the two H,K-ATPase subunits.

Plasma membrane expression of CFP-α and YFP-β fusion proteins in HEK-293 cells. Transient expression of CFP-α fusion protein in HEK-293 cells resulted in the intracellular retention of the protein (Fig. 5A), whereas expression of the wild-type YFP-β fusion protein resulted in plasma membrane localization of the protein (Fig. 6A). When HEK-293 cells stably expressing the H,K-ATPase β-subunit were transfected with pECFP-α, the fusion protein was partly localized intracellularly and partly on the plasma membrane (Fig. 5B). In HEK-293 cells cotransfected with pECFP-α and pEYFP-β, both CFP-α and YFP-β fusion proteins were targeted to the plasma membrane with partial intracellular retention (Fig. 5, C and D).

The effect of glycosylation site mutations on surface expression of YFP-β was studied using confocal microscopy (Fig. 6). The confocal XY images of the middle plane of the cells stably expressing the wild-type and mutant YFP-β fusion proteins are shown on Fig. 6. The wild-type YFP-β predominantly was found on the plasma membrane (Fig. 6A). A small fraction of the protein pool was detected intracellularly in the perinuclear region. The mutant lacking the second glycosylation site, N2, showed both intracellular accumulation of the protein and plasma membrane localization (Fig. 6C). Removing each of the other six glycosylation sites from the exoplasmic loop of YFP-β resulted in a loss of plasma membrane localization and intracellular accumulation of the protein (Fig. 6). Mostly, accumulation of YFP-β was observed as bright areas near the nucleus, which appeared to represent Golgi localization. A double mutant lacking the first and the second glycosylation sites displayed intracellular accumulation of the protein.

In addition to confocal microscopy studies, the effect of mutating glycosylation sites on trafficking and plasma membrane expression of YFP-β was studied by using Western blot analysis of biotinylated surface proteins vs. total cell proteins. Biotinylated plasma membrane proteins and total cell lysate obtained from the same cell line were run side by side on SDS-PAGE and analyzed by Western blot with the anti-GFP antibody (Fig. 7). Biotinylated plasma membrane proteins of the wild-type and mutant YFP-β contained only the fully glycosylated portion of YFP-β. By contrast, total cell lysates contained both fully- and core-glycosylated fractions of the protein. In agreement with the confocal microscopy studies, the amount of YFP-β detected in the plasma membrane was dramatically reduced in most of the mutants, except N2, compared with the wild type. Normalization of the YFP-β plasma membrane expression in mutants compared with the wild type.

Fig. 2. NH₄Cl-stimulated H,K-ATPase activity of the membranes isolated from HEK-293 cells cotransfected with YFP-β and CFP-α. Vₘₐₓ = 85.0 ± 3.7 μmol·h⁻¹·mg⁻¹; Kₘₕₜₜ [NH₄⁺] = 1.0 ± 0.2 mM.

Fig. 3. Western blot analysis of wild-type and mutant YFP-β fusion proteins. Lanes 1, wild type; 2, N1; 3, N2; 4, N3; 5, N4; 6, N5; 7, N6; 8, N7; 9, N1/N2; 10, N1/N2/N4; 11, N1 treated with PNGase F.
type yielded a quantitative interpretation of the effect of glycosylation site mutations on plasma membrane expression of YFP-β (Fig. 7). Mutation of the second glycosylation site resulted in a 2.5-fold reduction of the relative surface expression compared with the wild type. All other mutants showed a 13- to 55-fold reduction of relative plasma membrane YFP-β content.

**DISCUSSION**

*Fusion proteins of the gastric H,K-ATPase α- and β-subunits with CFP and YFP as the tools for intracellular sorting and trafficking studies.* It is important to assess the structural integrity of the expressed fusion proteins. The results of this study clearly demonstrate
that after fusion of α- and β-subunits of the gastric H,K-ATPase with fluorescent proteins, the complex CFP-α + YFP-β is catalytically active (Fig. 2), which implies that proper folding and association of the subunits are not impaired. Another important finding is that, when expressed in HEK-293 cells, YFP-β, similar to the nonlabeled β-subunit, represents a mixture of core-glycosylated and complex-glycosylated proteins with a higher content of the complex-glycosylated portion (Fig. 1), indicating that the fusion protein undergoes normal processing from ER to Golgi. As seen from the confocal images of the YFP-β-expressing HEK-293 cells (Fig. 6A), the fusion protein is delivered to the plasma membrane. Therefore, the CFP-α and YFP-β fusion proteins, as well as CFP-α and YFP-β complex, are in their native states and proceed through the effective intracellular processing steps necessary for sorting and membrane delivery, which makes them physiologically relevant and reliable tools with which to study cellular sorting mechanisms.

Six of the seven N-glycosylation sites in the H,K-ATPase β-subunit are essential for its plasma membrane expression in HEK-293 cells. Removing each of the glycosylation sites, except the second one, from the YFP-β results in a loss of plasma membrane localization of the protein (Figs. 6 and 7). A double mutant

![Fig. 6. Confocal images of HEK-293 cells stably expressing the wild-type YFP-β (A) and mutants lacking 1 or 2 N-glycosylation sites: N1 (B), N2 (C), N3 (D), N4 (E), N5 (F), N6 (G), N7 (H), and N1/N2 (I) (see Table 1). Wild-type YFP-β is located predominantly on the plasma membrane (A), N2 is detected both intracellularly and on the plasma membrane (C), and the rest of the mutants are located exclusively inside the cells with accumulation in bright areas near the nucleus, which appear to represent Golgi localization (marked by arrows).](image)

![Fig. 7. Effect of glycosylation site mutations on relative surface YFP-β expression in HEK-293 cells. Biotinylated surface proteins (S) and the correspondent amount of total cell lysate (T) obtained from the same cell line were run side by side on SDS-PAGE and analyzed by Western blot analysis using anti-GFP antibody. The results of 3 independent experiments were quantified using AMBIS optical imaging system (AMBIS, San Diego, CA). The ratio between the density of the band corresponding to biotinylated YFP-β (S) and the density of the band corresponding to the fully glycosylated fraction of YFP-β in the total cell lysate (T) for each mutant was plotted as a percentage of that in the wild type (wt).](image)
lacking the first and second glycosylation sites also shows intracellular retention. This is consistent with earlier data showing the loss of surface delivery of α- and β-subunits in COS-7 cells as a result of removing three glycosylation sites—the first, the third, and the seventh (1).

One of the possible reasons for the loss of surface delivery of the mutants could be impaired protein folding with subsequent retention of the protein in the ER and the ER-dependent degradation cascade. However, the experimental data show that N-glycosylation site defective mutants are properly folded. The α/β complexes containing the β-subunits lacking the fourth, the seventh, or the first and the second glycosylation sites are catalytically active and display kinetic properties, which are similar to the wild-type enzyme (Fig. 4), indicating that they retain the proper folding and assembly between α- and β-subunits. These data are consistent with preservation of H,K-ATPase activity after removal of any one of the seven N-glycosylation sites from the β-subunit (1). Serial removal of N-glycans from the β-subunit resulted in a progressive loss of activity. Removing all the seven N-glycosylation sites or inhibition of glycosylation resulted in prevention of α/β assembly and complete loss of H,K-ATPase enzymatic activity in HEK-293 cells (1) or insect cells (14).

N-glycosylation site defective mutants not only retain the native structure of the enzyme heterodimer but also undergo normal processing and reach the Golgi. The complex-glycosylated, EndoH glycosidase-insensitive form of the protein is present in all the mutants, indicating that the mutations do not prevent trafficking of the β-subunit from the ER to the Golgi. The ratio between complex-glycosylated and core-glycosylated portions of the protein is, however, less in some of the mutants compared with the wild-type β-subunit, indicating that mutations increase the fraction of the protein pool that does not pass quality control and is thus retained in the ER (31). Nevertheless, all the mutant fusion proteins undergo complex glycosylation, which is clear evidence that they are able to reach the Golgi. As seen from Fig. 6, accumulation of YFP-β in Golgi occurs with all glycosylation site mutants.

The experimental observations described above allow the conclusion that the trafficking step that is affected by the removal of N-glycosylation sites is the route from the Golgi to the plasma membrane and not from ER to Golgi. This implies that TGN-plasma membrane delivery depends on the presence or absence of trafficking information encoded by particular glycosylation sites within the extracellular domain of the β-subunit. It is possible that, similar to polarized cells, HEK-293 cells have machinery that recognizes this trafficking signal and places the β-subunit into specific cargo vesicles in TGN that deliver the protein to the plasma membrane. The alternative explanation is that N-glycosylation sites are essential not for the exit from TGN and surface delivery but for the stabilization of the protein on the plasma membrane. However, recent data showing that nonpolarized cells are capable of sorting basolateral and apical proteins to the appropriate vesicles in TGN based on the sorting signals present within the proteins (8, 12, 18, 19, 32) and that N-glycosylation can mediate apical sorting for a number of secreted and membrane proteins (8, 18, 24) favor the former explanation. Recent data on the role of N-glycans in trafficking of the homologous Na,K-ATPase β-subunit, a basolateral protein, also can be interpreted according to this hypothesis. In contrast to H,K-ATPase β-subunit, removing N-glycans from the Na,K-ATPase β1-subunit does not prevent its ability to reach the plasma membrane (17). Our interpretation of this result suggests that the presence of a basolateral signal within the Na,K-ATPase β1-subunit allows trafficking to the plasma membrane in nonpolarized cells. Similar interpretation may explain why a basolateral bile acid transporter retains plasma membrane targeting in HEK-293 cells after complete removal of its two glycosylation sites (9).

In summary, these results indicate that the fusion proteins of the gastric H,K-ATPase α- and β-subunits with CFP and YFP, respectively, are appropriate tools for intracellular sorting and trafficking studies. Plasma membrane delivery of the gastric H,K-ATPase β-subunit in HEK-293 cells does not occur by a default pathway but is highly sensitive to structural peculiarities of the normal or mutated proteins. Six of the seven glycosylation sites in the gastric H,K-ATPase β-subunit are essential for TGN to plasma membrane delivery, suggesting their role either in the exit of the protein from TGN or in the stabilization on the plasma membrane. They are, however, not required for movement into the TGN. The second glycosylation site (Asn103), which is not conserved among the β-subunits from different species, is not essential for sorting and trafficking of the gastric H,K-ATPase.

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DISCLOSURES

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