The Roles of Carbohydrate Chains of the β-Subunit on the Functional Expression of Gastric H⁺,K⁺-ATPase*

(Received for publication, September 17, 1999, and in revised form, December 11, 1999)

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Gastric H⁺,K⁺-ATPase consists of α and β-subunits. The α-subunit is the catalytic subunit, and the β-subunit is a glycoprotein stabilizing the α/β complex in the membrane as a functional enzyme. There are seven putative N-glycosylation sites on the β-subunit. In this study, we examined the roles of the carbohydrate chains of the β-subunit by expressing the α-subunit together with the β-subunit in which one, several, or all of the asparagine residues in the N-glycosylation sites were replaced by glutamine. Removing any one of seven carbohydrate chains from the β-subunit retained the H⁺,K⁺-ATPase activity. The effects of a series of progressive removals of carbohydrate chains on the H⁺,K⁺-ATPase activity were cumulative, and removal of all carbohydrate chains resulted in the complete loss of H⁺,K⁺-ATPase activity. Removal of any single carbohydrate chain did not affect the α/β assembly; however, little α/β assembly was observed after removal of all the carbohydrate chains from the β-subunit. In contrast, removal of three carbohydrate chains inhibited the surface delivery of the β-subunit and the α-subunit assembled with the β-subunit, indicating that the surface delivery mechanism is more dependent on the carbohydrate chains than the expression of the H⁺,K⁺-ATPase activity and α/β assembly.

Gastric proton pump, H⁺,K⁺-ATPase, consists of two kinds of subunits. One is a catalytic α-subunit with a molecular mass of 114 kDa (1), which contains an ATP-binding site, an acylphosphorylation site (2), binding sites for proton pump inhibitors (3, 4), and sites responsible for ion recognition (5, 6). The other is a glycoprotein, β-subunit, with a molecular mass of 60–80 kDa (7, 8), which is heavily glycosylated on a protein core with a molecular mass of 33 kDa (9). Both subunits are essential for the functional expression of H⁺,K⁺-ATPase, which has been observed in SF9 cells (10), Xenopus oocytes (11), and HEK-293 cells (5). The β-subunit is involved in the structural and functional maturation of the holozyme and intracellular transport of the ATPase (12).

Although the sugar content of the H⁺,K⁺-ATPase β-subunit is very high and almost all of the N-glycosylation sites of the β-subunit are conserved among different animals, the role of carbohydrate chains in the function of H⁺,K⁺-ATPase has not been clearly understood. Rabbit H⁺,K⁺-ATPase β-subunit contains seven putative N-glycosylation sites (Asn-Xaa-Ser and Asn-Xaa-Thr); Asn-99, Asn-103, Asn-130, Asn-146, Asn-161, Asn-193, and Asn-222 (9), all of which are conserved in the β-subunits among rats (13, 14), humans (15), and hogs (16), except that hog β-subunit does not have Asn-103. Recently, it was reported that all of these putative glycosylation sites in rabbit H⁺,K⁺-ATPase β-subunit prepared from gastric mucosa are fully modified with carbohydrate chains (8, 17). The structures of the oligosaccharide chains of the gastric H⁺,K⁺-ATPase β-subunit are unique in that they contain no sialic acid (17). It has been suggested that the unique structure of oligosaccharide chains of the β-subunit reflects the adaptation of the H⁺,K⁺-ATPase to the acidic environment of the stomach (18).

Recently, Klaassen et al. (19) reported that the N-linked carbohydrate chains are essential for the activity of the H⁺,K⁺-ATPase expressed in SF9 cells using a glycosylation inhibitor, tunicamycin. However, there has been no study that shows the functional role of the carbohydrate chains of the H⁺,K⁺-ATPase in an expression system derived from mammalian cells. The significance of each individual carbohydrate chain on the β-subunit also has not been studied yet. In this study, we removed the carbohydrate chains of rabbit H⁺,K⁺-ATPase β-subunit in a stepwise fashion by mutating its N-glycosylation sites and expressed it together with the wild-type α-subunit in human HEK-293 and COS-1 cells. We studied the functional role of each of the carbohydrate chains on the H⁺,K⁺-ATPase activity, the assembly between α- and β-subunits, and the intracellular localization of each subunit.

EXPERIMENTAL PROCEDURES

Materials—HEK-293 cells (human embryonic kidney cell line) were a kind gift from Dr. Jonathan Lytton (University of Calgary, Calgary, Canada). pcDNA3 vector was obtained from Invitrogen Co. (San Diego, CA). Restriction enzymes and other DNA and RNA modifying enzymes were from Toyobo (Osaka, Japan), New England Biolabs, Life Technologies, Inc., or Amersham Pharmacia Biotech. Inc. Anti-gastric H⁺,K⁺-ATPase β-subunit antibody (2B6) was purchased from Molecular Biological Laboratories (Nagoya, Japan). Another anti-gastric H⁺,K⁺-ATPase β-subunit antibody (2G11) was kindly provided by Drs. Chow and Forte (University of California, Berkeley, CA). SCH 28080 was obtained from Schering-Plow Co. (Kenilworth, NJ). Endoglycosidase H (Endo H)1 and N-glycosidase F (PNGase F) were obtained from Roche Molecular Biologicals. Tunicamycin was obtained from Sigma. All other reagents were of molecular biology grade or the highest grade of purity available.

cDNAs of α- and β-Subunits of H⁺,K⁺-ATPase—cDNAs of the α- and β-subunits were amplified using a TA Cloning Kit (Invitrogen Co.) and cloned into pBluescript II SK- (Stratagene). The α-subunit cDNA was inserted in-frame 5′ to the E. coliotoxin signal sequence, and the β-subunit cDNA was inserted 5′ to the signal sequence of the α-subunit.

* This work was supported in part by a Grant-in-Aid for Scientific Research (to S. A. and N. T.) from the Ministry of Education, Science, Sports and Culture in Japan and a fellowship from Takeda Scientific Research (to S. A. and N. T.) from the Ministry of Education, Science, Sports and Culture in Japan and a fellowship from Takeda Scientific Research (to S. A. and N. T.) from the Ministry of Education, Science, Sports and Culture in Japan.

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β-subunits of H⁺,K⁺-ATPase were prepared from rabbit gastric mucosa as described elsewhere (5). The α- and β-subunit cDNAs were digested with EcoRI and XhoI. The obtained fragments were each ligated into pcDNA3 vector treated with EcoRI and XhoI.

Site-directed Mutagenesis—Introduction of site-directed mutations was carried out by sequential polymerase chain reaction steps as described elsewhere (20), in which appropriately mutated β-subunit cDNAs (segments between nucleotide 302 ( Aph III site) and 1036 (Eco 47 III site) were prepared. The 5’-flanking sense and 3’-flanking antisense primers were 5’-GCTGAACTGCAGCTAGATAC-3’ (nucleotides 281–301) and 5’-CCACGGGA AGCCAGGCGGC-3’ (nucleotides 1049–1068), respectively. Sense and antisense synthetic oligonucleotides, each 21 bases long containing one mutation base near the center, were designed. The cDNA of H⁺,K⁺-ATPase β-subunit in pBluescript SK(-) was used as was a polymerase chain reaction template. Polymerase chain reaction was routinely carried out in the presence of 200 μM each dNTP, 500 mM primers, 10 mM KCl, 50 mM (NH₄)₂SO₄, 2 mM MgSO₄, 20 mM Tris-HCl, pH 8.8, 0.1% Triton X-100, 100 μg/ml bovine serum albumin, and 2.5 units of Pfu DNA polymerase for 30 cycles. DNA sequencing was done by the dideoxy chain termination method using an Autoread and Autocycle DNA sequencing kits and an ALFexpress DNA sequencer (Amersham Pharmacia Biotech). After sequencing, the fragment amplified in the final polymerase chain reaction was digested with Aph III plus Hind III or Hind III plus Eco 47 III and ligated back into the relevant position of the wild-type H⁺,K⁺-ATPase β-subunit construct.

Cell culture, Transfection, and Preparation of Membrane Fractions—Cell culture of HEK-293 was carried out as described previously (5). α- and β-subunit cDNA transfection was performed by the calcium phosphate method with 10 μg of cesium chloride-purified DNA/10-cm dish. Cells were harvested 2 days after the DNA transfection. Membrane fractions of HEK cells were prepared as described previously (5). SDS-Polyacrylamide Gel Electrophoresis and Western Blot—SDS-polyacrylamide gel electrophoresis was carried out as described elsewhere (21). Membrane preparations (30 μg of protein) were incubated in a sample buffer containing 2% SDS, 2% β-mercaptoethanol, 10% glycerol, and 10 mM Tris-HCl, pH 6.8, at room temperature for 2 min and applied to the SDS-polyacrylamide gel. Western blot was carried out as described previously (5). Densitometric analysis of the blots was carried out using the ATTO Densitographic software (ATTO, Tokyo, Japan).

Antibody Ab1024 was previously raised against the carboxyl-terminal peptide (residues 1024–1034) of the H⁺,K⁺-ATPase β-subunit (PGSWWDQELYY) (22). Monoclonal antibody 2B6 was derived from the splenocytes of mice with autoimmune gastritis (16). The epitope was located on the carboxyl-terminal portion of the H⁺,K⁺-ATPase β-subunit with a monoclonal antibody, 2G11 (8). Both antibodies were used at dilutions of 1:100. Goat anti-mouse IgG conjugated to fluorescein isothiocyanate and goat anti-rabbit IgG conjugated to rhodamine were used as the secondary antibodies at dilution of 1:100. Immunostaining was visualized with a Zeiss LSM 410 laser scanning confocal microscope. Contrast and brightness settings were chosen to ensure that all pixels were in the linear range. All images were the product of 8-fold line averaging.

Glycosidase Treatment—Thirty micrograms of membrane fraction was treated with Endo H or PNGase F following the manufacturer’s instructions. For Endo H digestion, 30 μg of membrane fraction was treated with 0.01 units of Endo H in a solution containing 0.1% SDS, 1 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, and 50 mM sodium phosphate, pH 6.0 at 37 °C overnight. For PNGase F digestion, 30 μg of membrane fraction was treated with 1 unit of PNGase F in a solution containing 0.1% SDS, 1% n-octylglucoside, 1 mM 2-mercaptoethanol, 30 mM EDTA, and 50 mM sodium phosphate, pH 6.0, at 37 °C overnight.

Assay of H⁺,K⁺-ATPase Activity—ATPase activity was assayed in 1 ml of a solution containing 50 μg of membrane protein, 3 mM MgCl₂, 1 mM ATP, 5 mM NaN₃, 15 mM KCl, 2 mM ouabain, and 40 mM Tris-HCl, pH 6.8, in the presence and absence of 50 μM SCH 28098, which is an inhibitor of H⁺,K⁺-ATPase activity.
hydrate chains play an important role in the function of gastric H\(^+\)\(\cdot\)ATPase. After incubation at 37 °C for 30 min, the reaction was terminated by the addition of ice-cold stop solution containing 12% perchloric acid and 3.6% ammonium molybdate. Inorganic phosphate released was measured from the absorbance at the wavelength of 320 nm as described elsewhere (25). The K\(^+\)-ATPase activity was calculated as the difference between activities in the presence and absence of SCH 28080. Inorganic phosphate released in the enzyme reaction with the wild-type H\(^+\)\(\cdot\)K\(^+\)-ATPase was 4–5-fold higher than the background level of inorganic phosphate released in the absence of enzyme. Protein was measured using the BCA protein assay kit from Pierce with bovine serum albumin as a standard.

RESULTS

Construction of the β-Subunits with Mutated N-Glycosylation Sites—Rabbit gastric H\(^+\)\(\cdot\)K\(^+\)-ATPase β-subunit contains seven putative N-glycosylation sites (Asn-Xaa-Ser and Asn-Xaa-Thr); Asn-99, Asn-103, Asn-130, Asn-146, Asn-161, Asn-193, and Asn-222 as shown in Fig. 1, which are located in the extracellular segment and are well conserved in rat and human gastric H\(^+\)\(\cdot\)K\(^+\)-ATPases. Therefore, it is likely that the carbohydrate chains play an important role in the function of gastric H\(^+\)\(\cdot\)K\(^+\)-ATPase. Klaassen et al. (19) studied the role of N-linked carbohydrate chains by expressing gastric H\(^+\)\(\cdot\)K\(^+\)-ATPase in insect Sf9 cells in the presence of an N-glycosylation inhibitor, tunicamycin. They reported that N-glycosylation was essential to produce a catalytically active recombinant H\(^+\)\(\cdot\)K\(^+\)-ATPase, whereas the expression of the H\(^+\)\(\cdot\)K\(^+\)-ATPase α- and β-subunit proteins was observed even in the presence of tunicamycin. In a preliminary study, we found that the effect of tunicamycin on the expression of the wild-type H\(^+\)\(\cdot\)K\(^+\)-ATPase α- and β-subunits in mammalian cells is quite different from that in insect Sf9 cells. In addition, there has been no report that tests the influence of each carbohydrate chain by mutating the putative glycosylation sites. In this study, we mutated one, several, or all of the asparagine residues in the putative N-glycosylation sites to glutamine residues, co-expressed the mutant β-subunit with the α-subunit, and studied the role of N-linked carbohydrate chains on the subunit interaction, enzyme activity, and intracellular localization of H\(^+\)\(\cdot\)K\(^+\)-ATPase. Of the 127 possible β-subunit mutants to be analyzed, we prepared mutants as shown in Table I; seven single mutants (N99Q, N103Q, N130Q, N146Q, N161Q, N193Q, and N222Q); two double mutants (N99Q/N103Q and N130Q/N222Q); mutant III (N99Q/N103Q/N130Q/N146Q/N161Q/N193Q/N222Q); mutant IV (N99Q/N103Q/N130Q/N146Q/N161Q/N193Q/N222Q); mutant VI (N99Q/N103Q/N130Q/N146Q/N193Q/N222Q); and mutant VII (N99Q/N103Q/N130Q/N146Q/N161Q/N193Q/N222Q). Each of these mutant cDNAs was co-transfected with the α-subunit cDNA to HEK-293 cells.

Expression of α- and β-Subunits—Fig. 2 (A and B) shows Western blot patterns of the membrane fractions of the transfected, detected by using the anti-β-subunit antibody, 2B6. When the cells were co-transfected with both the wild-type α-subunit and β-subunit cDNAs, a dense band with a lower molecular mass (48 kDa) (β\(_m\)) and a smear band with a higher molecular mass (60–70 kDa) (β\(_m\)) was resistant to Endo H, whereas the β-subunit with a lower molecular mass (40–48 kDa) (β\(_m\)) was digested with Endo H (data not shown). These results indicate that the 60–70-kDa band represents the β-subunit with complex-type (resistant to Endo H) carbohydrate chains and that the bands with a lower molecular mass (40–48 kDa) represent the β-subunits with high mannose-type (sensitive to Endo H) carbohydrate chains (23). When the cells were co-transfected with the wild-type α-subunit and each of the seven single mutant β-subunit cDNAs, two bands, β\(_m\) and β\(_m\) were observed (Fig. 2A). The molecular mass of these two bands were slightly lower than...
The expression of the $\alpha$-subunit increased 7.9, 6.1, 5.4, and 5.5 times for the N130Q/N222Q, mutants III, IV, and V, respectively, compared with the expression of the $\alpha$-subunit in the absence of the $\beta$-subunit (Fig. 3B). However, there was only a 2.4-fold increase in expression of the $\alpha$-subunit for the mutants VI, and little or no (1.1-fold) increase for the mutant VII.

$H^+,K^+$-ATPase Activity of the Glycosylation Site Mutants—$H^+,K^+$-ATPase activity was assayed in 1 ml of a solution containing 50 $\mu$g of membrane protein, 3 mM MgCl$_2$, 1 mM ATP, 5 mM NaN$_3$, 15 mM KCl, 2 mM ouabain, and 40 mM Tris-HCl, pH 6.8, in the presence and absence of 50 $\mu$M SCH 28080. After incubation at 37°C for 30 min, inorganic phosphate released was measured from the absorbance at a wavelength of 320 nm. $H^+,K^+$-ATPase activity was calculated as the difference between the ATPase activity in the presence and absence of 50 $\mu$M SCH 28080. $H^+,K^+$-ATPase activities of the single-site mutants (A) and multi-site mutants (B) were shown. The values are shown as the percentage of the $H^+,K^+$-ATPase activity of the wild-type $\alpha/\beta$ complex, 1.00 $\pm$ 0.07 $\mu$mol/mg h (means $\pm$ S.E.) ($n = 19$).
membrane fractions were solubilized in 1% Nonidet P-40, 150 mM NaCl, or N222Q (lane 4), mutant IV (lane 5), N130Q/N222Q (lane 6), or N222Q (lane 7), or N130Q (lane 8) cDNAs. B, HEK-293 cells were co-transfected with the wild-type H\(^+\),K\(^-\)-ATPase \(\alpha\)-subunit cDNA plus N222Q (lane 1), N130Q/N222Q (lane 2), mutant III (lane 3), mutant IV (lane 4), mutant V (lane 5), mutant VI (lane 6), or mutant VII (lane 7) cDNAs. The membrane fractions were solubilized in 1% Nonidet P-40, 150 mM NaCl, 0.5 mM EDTA, and 50 mM Tris-HCl, pH 7.4, and centrifuged. The supernatant was incubated with an anti-\(\alpha\)-subunit antibody, Ab1024, and protein A-coated beads. The precipitated preparations were separated on SDS-polyacrylamide gel and blotted with anti-\(\beta\)-subunit antibody, 2B6.

**Fig. 5.** Western blots with anti-\(\beta\)-subunit antibody 2B6 of membrane fractions of HEK cells immunoprecipitated with the anti-\(\alpha\)-subunit antibody Ab1024. A, HEK-293 cells were co-transfected with the wild-type H\(^+\),K\(^-\)-ATPase \(\alpha\)-subunit cDNA plus the wild-type H\(^+\),K\(^-\)-ATPase \(\beta\)-subunit (lane 1), N99Q (lane 2), N103Q (lane 3), N130Q (lane 4), N146Q (lane 5), N161Q (lane 6), N193Q (lane 7), or N222Q (lane 8) cDNAs. B, HEK-293 cells were co-transfected with the wild-type H\(^+\),K\(^-\)-ATPase \(\alpha\)-subunit cDNA plus N222Q (lane 1), N130Q/N222Q (lane 2), mutant III (lane 3), mutant IV (lane 4), mutant V (lane 5), mutant VI (lane 6), or mutant VII (lane 7) cDNAs. The membrane fractions were solubilized in 1% Nonidet P-40, 150 mM NaCl, 0.5 mM EDTA, and 50 mM Tris-HCl, pH 7.4, and centrifuged. The supernatant was incubated with an anti-\(\alpha\)-subunit antibody, Ab1024, and protein A-coated beads. The precipitated preparations were separated on SDS-polyacrylamide gel and blotted with anti-\(\beta\)-subunit antibody, 2B6.

**Fig. 6A.** Western blot patterns of the samples immunoprecipitated with anti-\(\alpha\)-subunit antibody, Ab1024, and detected with anti-\(\beta\)-subunit antibody 2B6 (Fig. 5A). The molecular mass of these two bands was slightly lower than those observed in the wild-type \(\alpha/\beta\) complex. When the cells were co-transfected with the \(\alpha\)-subunit plus N130Q/N222Q mutant \(\beta\)-subunit cDNAs, a similar \(\beta\)-subunit pattern was observed (Fig. 5B, lane 2). Mutants III, IV, V, and VI were also co-precipitated with the \(\alpha\)-subunit (Fig. 5B, lanes 3–6). However, in the case of mutants V and VI, the amounts of precipitated \(\beta\)-subunits were significantly lower than that of the wild-type \(\beta\)-subunit. Mutant VII was hardly co-precipitated with the \(\alpha\)-subunit. These results suggest that each carbohydrate chain of the \(\beta\)-subunit is not directly involved in \(\alpha/\beta\) assembly of the H\(^+\),K\(^-\)-ATPase; however, only a small amount of \(\alpha/\beta\) complex was formed after removal of all seven carbohydrate chains.

**Localization of \(\alpha\)- and \(\beta\)-Subunits in COS Cells—**The wild-type \(\beta\)-subunit, seven single mutants, N130Q/N222Q, and mutants III and IV can leave the ER compartment for the Golgi apparatus as evidenced by the observation that they have not only high mannose-type but also complex-type carbohydrate chains, as shown in Fig. 2B. We tried to examine the subcellular localization of the \(\alpha\)- and \(\beta\)-subunits in HEK cells directly using immunocytochemistry. It was difficult to perform subcellular localization on proteins in HEK cells because HEK cells grew in fairly dense islands, and the individual cells in these islands were small and closely packed against their neighbors. In this study, therefore, we used COS-1 cells because COS cells were larger and more spread out, which made it much easier to distinguish intracellular from cell-surface distributions. We have previously shown that pump subunit proteins behave similarly with respect to surface delivery in these cell lines (26).

When the COS cells were transfected with H\(^+\),K\(^-\)-ATPase \(\alpha\)-subunit cDNA alone, a perinuclear pattern was observed, consistent with retention of the \(\alpha\)-subunit in the ER (Fig. 6B). When the H\(^+\),K\(^-\)-ATPase \(\alpha\)-subunit was expressed with its wild-type \(\beta\)-subunit, the \(\alpha\)-subunit was observed primarily at the cell surface (Fig. 6D). Therefore, the H\(^+\),K\(^-\)-ATPase \(\alpha\)-subunit requires its \(\beta\)-subunit for efficient cell surface expression as reported previously (24). The single mutant N99Q (Fig. 6F) and the double mutants N99Q/N103Q (not shown) and N130Q/N222Q (Fig. 6H) also supported the \(\alpha\)-subunit expression on the cell surface, although the extent of the surface delivery was lower in the case of the mutant \(\beta\)-subunits compared with the wild-type \(\beta\)-subunit. However, no cell surface expression of the \(\alpha\)-subunit was detected when it was co-expressed with mutant III (Fig. 6J).

When the cells were transfected with H\(^+\),K\(^-\)-ATPase \(\beta\)-subunit cDNA alone, the \(\beta\)-subunit was observed at the cell surface as well as in intracellular vesicles (Fig. 6A). Therefore, the H\(^+\),K\(^-\)-ATPase \(\beta\)-subunit reaches the cell surface with (Fig. 6C) and without (Fig. 6A) its \(\alpha\)-subunit as reported previously (24). The single mutant N99Q (Fig. 6E) and the double mutants N99Q/N103Q (not shown) and N130Q/N222Q (Fig. 6G) also reach the cell surface. However, no cell surface distribution of the \(\beta\)-subunit was detected for mutant III (Fig. 6F). Thus, carbohydrate chains are also important for surface delivery of the \(\alpha\)- and \(\beta\)-subunits.

**DISCUSSION**

H\(^+\),K\(^-\)-ATPase \(\beta\)-subunit shows a number of structural similarities with Na\(^+\),K\(^-\)-ATPase \(\beta\)-subunit. Both \(\beta\)-subunits consist of a short amino-terminal cytoplasmic domain, one transmembrane domain, and a large extracellular domain. This extracellular domain contains six conserved cysteine residues involved in the formation of three disulfide bonds and several carbohydrate chains. The cytoplasmic and transmembrane domains are replaceable between these two ATPases for the functional expression of H\(^+\),K\(^-\)-ATPase, but the majority of the extracellular domain is not replaceable (23). The number and
glycosylation of the b-drug chains. Tamkun and Fambrough (27) have shown that the deglycosylated form and transport activities of the Na\(^{+},K\(^{+}\))-ATPase in mammalian cells, because there are striking differences in the glycosylation state of the α-subunit and the localization of α/β complex between wild-type H\(^{+},K\(^{+}\))-ATPase expressed in insect and mammalian cells. When the H\(^{+},K\(^{+}\))-ATPase was expressed in SF9 cells, the molecular mass of carbohydrate chains was smaller than that reported in the native H\(^{+},K\(^{+}\))-ATPase (10); a large percentage of the β-subunit contained high mannose-type carbohydrate chains, and there was little β-subunit-associated complex-type carbohydrate chains (19). Nonglycosylated β-subunit (protein core) was also observed on the Western blot. Catalytically active H\(^{+},K\(^{+}\))-ATPase α/β complexes were localized to intracellular membranes, and the α-subunit was not observed on the plasma membrane of SF9 cells. In contrast to these findings in SF9 cells, we found that H\(^{+},K\(^{+}\))-ATPase β-subunits expressed in HEK-293 cells contained carbohydrate chains, a large fraction of which were high mannose-type carbohydrate chains. Nonglycosylated β-subunit was not observed on the Western blot. The α-subunits associated with the β-subunit were observed to be delivered to the plasma membrane.

In the expression system using mammalian cells, no H\(^{+},K\(^{+}\))-ATPase activity was observed in the membrane fraction of HEK cells expressing the α/β mutant VII, in which all seven carbohydrate chains were removed. This result is consistent with the previous finding that no H\(^{+},K\(^{+}\))-ATPase activity was observed in SF9 cells incubated with tunicamycin (19). However, each carbohydrate chain was not specifically essential for the H\(^{+},K\(^{+}\))-ATPase function, because any single mutation in the glycosylation sites of the β-subunit did not abolish the K\(^{-}\)-ATPase activity. The effect of a series of progressive removals of carbohydrate chains from the β-subunit on the enzyme activity was cumulative, although the effect of isocharge substitution (from Asn to Gln) on the enzyme activity could not be completely excluded. The present results suggest that the carbohydrate chains of the β-subunit are collectively important for the catalytic activity of H\(^{+},K\(^{+}\))-ATPase.

Each carbohydrate chain on the β-subunit was not directly involved in the α/β assembly. However, very small amounts of α/β complex were observed when the cells were co-transfected with the α-subunit and mutant VI cDNAs. The α/β assembly was not observed after removal of all seven carbohydrate chains. Therefore, the carbohydrate chains of the β-subunit are collectively important for assembly between the α- and β-subunits. The β-subunits of single mutants, double mutants, mutant III, and mutant IV contained both complex-type and high mannose-type carbohydrate chains, indicating that these subunits are able to leave the ER compartment.

The carbohydrate chains on the β-subunit are important for the surface delivery of the β-subunit and the α-subunit that had assembled with the β-subunit. In COS cells, no surface delivery occurred in the mutant III complex. This result con-
tracts with the previous finding that N-glycosylation of Na\(^+\),K\(^-\)-ATPase β-subunit is not necessary for its transport to the plasma membrane (27, 28). Further study of this difference between H\(^+\),K\(^-\)-ATPase and Na\(^+\),K\(^-\)-ATPase would be interesting because they have different destinations in polarized cells (apical and basolateral membranes, respectively).

Rabbit gastric H\(^+\),K\(^-\)-ATPase β-subunit contains seven glycosylation sites. Therefore, theoretically, it is necessary to prepare all the combinations of mutants (127 mutants) and analyze the functions of all mutants to precisely study the roles of each carbohydrate chain and the possible interactions between the carbohydrate chains. Here, we prepared one series of representative mutants and analyzed their functions because all seven single mutants showed almost equivalent effects. It cannot be completely excluded that some specific interactions between certain combination of carbohydrate chains are more important than others for some functions of the ATPase.

In conclusion, from the present series of experiments, it has been found that different levels of glycosylation of the β-subunit are necessary for the ATPase activity, assembly, and the surface delivery of the β-subunit. Surface delivery of the α- and β-subunits is more dependent on the carbohydrate chains than the expression of the H\(^+\),K\(^-\)-ATPase activity and α/β assembly.

Acknowledgments—We thank Drs. Dar C. Chow and John G. Forte for generously providing a monoclonal antibody 2G11 against H\(^+\),K\(^-\)-ATPase β-subunit.

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*J. Biol. Chem. 2000, 275:8324-8330.*
doi: 10.1074/jbc.275.12.8324

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