Significance of Lysine/Glycine Cluster Structure in Gastric H\(^+\),K\(^+\)-ATPase

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Abstract: Gastric H\(^+\),K\(^+\)-ATPase consists of α- and β-subunits. The catalytic α-subunit consists of a very unique structure consisting of lysine and glycine clusters, KKK(or KKKK)AG(G/R)GGGK-(K/R)K, in the amino-terminal cytoplasmic region. This structure is well conserved in all gastric H\(^+\),K\(^+\)-ATPases from different animal species, and was postulated to be the site controlling the access of cations (or proton) to its binding site. In this report, we studied the role of this unique structure by expressing several H\(^+\),K\(^+\)-ATPase mutants of the α-subunit together with the wild-type β-subunit in HEK-293 cells. Even after replacing all the positively-charged amino acid residues (six lysines and one arginine) in the cluster with alanine or removing all the glycine residues in the cluster, the mutants preserved the H\(^+\),K\(^+\)-ATPase activity, and showed similar affinity for ATP and K\(^+\) as well as similar pH profiles as those of wild-type H\(^+\),K\(^+\)-ATPase, indicating that the cluster is not indispensable for H\(^+\),K\(^+\)-ATPase activity and not directly involved in determination of the affinity for cation (proton).

Key words: proton pump, H\(^+\),K\(^+\)-ATPase, site-directed mutagenesis, chimera, ion recognition.

The gastric proton pump, H\(^+\),K\(^+\)-ATPase, consists of two kinds of subunits. One is the catalytic α-subunit, which spans the membrane 10-times and contains sites responsible for ion recognition [4–7]. The other is the glycoprotein, β-subunit, which spans the membrane once, and is also essential for the functional expression of H\(^+\),K\(^+\)-ATPase [5, 8, 9] and involved in the structural and functional maturation, intracellular transport and stabilization of the functional holoenzyme [10, 11]. The α-subunit has a very unique structure, a “lysine/glycine cluster,” KKK(or KKKK)AG(G/R)GGGK(K/R)K, in the amino-terminal region located in the cytoplasm (amino acids 28–40 in rabbit H\(^+\),K\(^+\)-ATPase) as shown in Fig. 1. This structure is conserved in the gastric H\(^+\),K\(^+\)-ATPase α-subunits from different animals (rats [1], rabbits [12], pigs [13], humans [14] and dogs [15]). However, the cluster is absent in colonic isoforms of H\(^+\),K\(^+\)-ATPase [16, 17]. Differences in mechanical and physiological regulations between gastric and colonic H\(^+\),K\(^+\)-ATPases are interesting, but they have not yet been clarified. Na\(^+\),K\(^+\)-ATPase also consists of α- and β-subunits. Amino acid identities of the α- and β-subunits between gastric H\(^+\),K\(^+\)-ATPase and Na\(^+\),K\(^+\)-ATPase are 62 and 30%, respectively. In the Na\(^+\),K\(^+\)-ATPase α-subunits, the lysine block of the lysine/glycine cluster was also conserved, whereas they do not have the glycine block. Because the basic amino acids (Lys and Arg) in the lysine/glycine cluster contain positive charges in the physiological pH ranges, and because the lysine/glycine cluster was partly conserved between gastric H\(^+\),K\(^+\)-ATPase and Na\(^+\),K\(^+\)-ATPase, this structure was postulated to act as the cation discrimination site or the site controlling the
METHODS

Materials. HEK-293 cells (human embryonic kidney cell line) were a kind gift from Prof. Jonathan Lytton (University of Calgary, Calgary, Canada). The pcDNA3 vector was obtained from Invitrogen (San Diego, CA). The pcIS2 vector was obtained from Genentech (San Francisco, CA). The MutanK kit was from Takara Shuzo (Kyoto, Japan). Restriction enzymes and other DNA modifying enzymes were from Toyobo (Osaka, Japan), New England Biolabs. (Beverly, MA), Life Technologies, Inc. (Rockville, MD), or Amersham Pharmacia-Biotech. (Tokyo, Japan). Anti-gastric H⁺,K⁺-ATPase antibody (2B6) was purchased from Molecular Biology Laboratories (Nagoya, Japan). 2-Methyl-8-(phenylmethoxy)imidazo[1,2-a]pyridine-3-acetonitrile (SCH 28080) was obtained from Schering-Plough (Kenilworth, NJ). All other reagents were of molecular biology grade or the highest grade of purity available.

cDNAs of α- and β-subunits of gastric H⁺,K⁺-ATPase. cDNAs of the α- and β-subunits of gastric 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 with the pcDNA3 vector treated with EcoRI and XhoI.

cDNA of colonic H⁺,K⁺-ATPase α-subunit. Guinea pig colonic H⁺,K⁺-ATPase α-subunit cDNA, in which a portion of the 5′-noncoding region (−144 to −37 nucleotides) was removed, was prepared as described elsewhere [16]. The α-subunit cDNA was subcloned in the pCIS2 vector.

Site-directed mutagenesis, chimera construction, and DNA sequencing. Rabbit gastric H⁺,K⁺-ATPase α-subunit contains the lysine/glycine cluster, KKKKAGGGGGKRK, between amino acids 28 and 40. To study the role of the lysine/glycine cluster on the functions of the H⁺,K⁺-ATPase, in this study, we prepared three kinds of mutants: A(28–31) mutant, 28KKK31K; A(38–40) mutant, 38AA40A; and A(28–31)/A(38–40) mutant, 28AAA31A plus 38KR40K. We also prepared one deletion mutant, Δ(31–40), which lacks amino acids from 31K to 40K including the glycine block, and a chimeric H⁺,K⁺-ATPase α-subunit, CGHK, in which the amino-terminal 86 amino acid block of gastric H⁺,K⁺-ATPase includes...
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ing the lysine/glycine cluster was replaced with the counterpart of colonic H\(^+\),K\(^-\)-ATPase (84 amino acids). Introduction of site-directed mutations in the lysine/glycine cluster of the H\(^+\),K\(^-\)-ATPase \(\alpha\)-subunit was carried out by sequential polymerase chain reaction (PCR) steps as described elsewhere [18], in which appropriately mutated \(\alpha\)-subunit cDNAs [segments between EcoRI site (nucleotide −28) and BstEII site (nucleotide 456)] were prepared. The 5′-flanking sense and 3′-flanking antisense primers were 5′-CCGAATTCAGGGAGGCGACGGCAGCGAG-3′ (nucleotides −28 to −9, EcoRI site is underlined) and 5′-GCCTCGAGCTCGGATCACGGACGC-3′ (nucleotides 534–553, XhoI site is underlined), respectively. Sense and antisense synthetic oligonucleotides, each 21 to 27 bases long containing one to six mutated bases near the center, were designed. The cDNA of H\(^+\),K\(^-\)-ATPase \(\alpha\)-subunit in pBluescript SK(−) was used as a PCR template. PCR was routinely carried out in the presence of 200 μM each dNTP, 500 nM primers, 10 mM KCl, 10 mM (NH\(_4\))\(_2\)SO\(_4\), 2 mM MgSO\(_4\), 20 mM Tris/HCl, pH 8.8, 0.1% Triton X-100, 100 μg/ml bovine serum albumin and 2.5 U Pfu DNA polymerase for 30 cycles. DNA sequencing was done by the dideoxy chain termination method using an Autocycle DNA sequencing kit and an ALFexpress DNA sequencer (Amersham Pharmacia Biotech). After sequencing, the fragment amplified in the final PCR was digested with EcoRI and BstEII, and ligated back into the relevant position of the wild-type H\(^+\),K\(^-\)-ATPase \(\alpha\)-subunit construct.

A chimeric \(\alpha\)-subunit cDNA between rabbit gastric and guinea pig colonic H\(^+\),K\(^-\)-ATPases was constructed by replacing the cDNA segment of the gastric H\(^+\),K\(^-\)-ATPase \(\alpha\)-subunit between EcoRI (in pBlue- script SK(−) vector) and ApaI sites (nucleotide 259) by the corresponding segment of colonic H\(^+\),K\(^-\)-ATPase \(\alpha\)-subunit.

Cell culture, transfection and preparation of membrane fractions. Cell culturing of HEK-293 cells was carried out as described previously [5]. \(\alpha\)- and \(\beta\)-subunit cDNA transfection was performed by the calcium phosphate method with 10 μg of cesium chloride-purified DNA per 10 cm dish. Cells were harvested 2 d after cDNA transfection. Membrane fractions of HEK cells were prepared as described previously [5]. Briefly, cells in a 10-cm Petri dish were washed with phosphate-buffered saline and incubated with 2 ml of low-ionic salt buffer [0.5 mM MgCl\(_2\), 10 mM Tris/HCl (pH 7.4)] at 0°C for 10 min. After the addition of phenylmethylsulfonfluoride (1 mM) and aprotinin (0.09 U/ml), the cells were homogenized in a Dounce homogenizer, and the homogenate was diluted with an equal volume of a solution comprising 500 mM sucrose and 10 mM Tris/HCl (pH 7.4). The homogenized suspension was centrifuged at 800×g for 10 min. The supernatant was centrifuged at 100,000×g for 90 min, and the pellet was suspended in a solution comprising 250 mM sucrose and 5 mM Tris/HCl (pH 7.4).

SDS-polyacrylamide gel electrophoresis and Western blot. SDS-polyacrylamide gel electrophoresis was carried out as described elsewhere [19]. Membrane preparations (30 μg of protein) were incubated in a sample buffer comprising 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 blotting was carried out as described previously [5].

Antibody. Ab1024 was previously raised against the carboxy terminal peptide (residues 1,024–1,034) of the H\(^+\),K\(^-\)-ATPase \(\alpha\)-subunit (PGSWSDEELYY) [20]. Monoclonal antibody 2B6 was derived from the splenocytes of mice with autoimmune gastritis. The epitope was located on the carboxy-terminal portion of the \(\beta\)-subunit [11].

Assay of protein and H\(^+\),K\(^-\)-ATPase activity. Gastric H\(^+\),K\(^-\)-ATPase activity was assayed by the following two different methods depending on the purpose and conditions of the experiments.

1) Measurement of the amount of NADH coupled with the regeneration of ATP from ADP ("coupled-enzyme method"). H\(^+\),K\(^-\)-ATPase activity was assayed in 1.2 ml of a reaction mixture comprising membrane protein (50 μg), 3 mM MgCl\(_2\), 160 μM NADH, 0.8 mM phosphoenolpyruvate, 3 U/ml pyruvate kinase, 2.75 U/ml lactate dehydrogenase, 5 mM NaN\(_3\), 1 mM ouabain, 15 mM KCl, 40 mM Tris/HCl (pH 7.4) and various concentrations of ATP. The decrease in the amount of NADH was measured at 37°C as the absorbance at 340 nm with a Beckman spectrophotometer as described elsewhere [21]. H\(^+\),K\(^-\)-ATPase activity was calculated as the difference between the K\(^+\)-stimulated ATPase activity in the presence and absence of 50 μM SCH 28080.

2) Measurement of inorganic phosphate released from ATP. H\(^+\),K\(^-\)-ATPase activity was assayed in 1 ml of a solution comprising membrane protein (50 μg), 3 mM MgCl\(_2\), 1 mM ATP, 5 mM NaN\(_3\), 1 mM ouabain, 15 mM KCl, and 40 mM Tris/HCl (pH 7.4) in the presence and absence of 50 μM SCH 28080. After reaction at 37°C for 30 min, the inorganic phosphate released was measured as described elsewhere [22]. H\(^+\),K\(^-\)-ATPase activity was calculated as the difference between activity in the presence and absence of

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SCH 28080.

We routinely measured the ATPase activity using both methods. We usually showed results measured by the latter method unless indicated. When the effect of ATP concentration on ATPase activity was studied, we determined the activity by the former method to maintain a constant ATP concentration during the incubation period.

Colonic H⁺,K⁺-ATPase activity was assayed in 1 ml of a solution comprising membrane protein (50 μg), 3 mM MgCl₂, 1 mM ATP (Tris salt), 5 μM oligomycin, and 40 mM Tris/HCl (pH 7.4) in the presence and absence of 15 mM KCl. H⁺,K⁺-ATPase activity was calculated as the difference between activity in the presence and absence of KCl, which was inhibited by 1 mM ouabain, however insensitive to 50 μM SCH 28080.

Protein was measured using a BCA protein assay kit from Pierce (Rockford, IL) with bovine serum albumin as the standard.

RESULTS

Expression of the mutant α-subunit and β-subunit in the membrane

The expression levels of the mutant α-subunits (including alanine mutants, deletion mutant and chimera) were studied using Western blotting with an anti-α-subunit antibody (Fig. 2A). The molecular mass of the A(28–31), A(38–40), A(28–31)/A(38–40), Δ(31–40) mutants and CGHK chimera was about 95 kDa, apparently identical to that of the wild-type α-subunit. The expression levels of all the mutant α-subunits were not significantly different from that of the wild-type H⁺,K⁺-ATPase α-subunit (Fig. 2A). Therefore, the positive charges in the lysine/glycine cluster were not involved in controlling the expression level of the α-subunit. Furthermore, the structure of the lysine/glycine cluster itself was not involved in controlling the expression level of the α-subunit because the mutant α-subunit was expressed in the membrane even after removing 10 amino acid residues around the lysine/glycine cluster in the Δ(31–40) mutant or replacing the 86 amino acid residues in the amino-terminal portion of gastric H⁺,K⁺-ATPase by the counterpart of colonic H⁺,K⁺-ATPase.

Figure 2B shows the expression levels of the H⁺,K⁺-ATPase β-subunits co-expressed with the wild-type or mutant α-subunits by Western blotting with an 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) (β₁) and a smear band with a higher molecular mass (60–70 kDa) (β₂) were observed (lane 1). The band with a lower molecular mass (48 kDa) represents the β-subunits with high mannose-type carbohydrate chains, which are located in the endoplasmic reticulum, and the band with a higher molecular mass represents the β-subunits with complex-type carbohydrate chains, which leave the endoplasmic reticulum for transGolgi and plasma membrane [11]. Similar patterns were observed when the cells were co-transfected with the mutant α-subunit and wild-type β-subunit cDNAs. Therefore, the positive charges and glycine block in the lysine/glycine cluster were not involved in the expression level of the β-subunit, modification of the β-subunit with carbohydrate chains or intracellular localization of the β-subunit.
**H⁺,K⁺-ATPase activity of the mutant α/β complexes**

The H⁺,K⁺-ATPase activities of all the mutant α/β complexes are shown in Table 1. The A(28–31), A(38–40), A(28–31)/A(38–40) and Δ(31–40) mutant complexes retained 84, 108, 97 and 82% of the activity of the wild-type H⁺,K⁺-ATPase, respectively, indicating that the lysine/glycine cluster of the H⁺,K⁺-ATPase α-subunit is not involved in H⁺,K⁺-ATPase activity. The CGHK chimera, which lacks the lysine/glycine cluster, also retained 77% of the activity of the wild-type H⁺,K⁺-ATPase.

**pH profile of the mutant H⁺,K⁺-ATPases**

If the lysine/glycine cluster is really involved in the activity. The CGHK chimera, which lacks the lysine/glycine cluster, also retained 77% of the activity of the H⁺,K⁺-ATPase activity. The CGHK chimera, which lacks the lysine/glycine cluster, also retained 77% of the activity of the wild-type H⁺,K⁺-ATPase.

**Table 1. H⁺,K⁺-ATPase activity of the mutant α/β complexes.**

<table>
<thead>
<tr>
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<th>H⁺,K⁺-ATPase activity (μmol/mg/h)</th>
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<tbody>
<tr>
<td>Wild-type</td>
<td>1.46±0.21 (n=7)</td>
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<tr>
<td>A(28–31)</td>
<td>1.22±0.19 (n=5)</td>
</tr>
<tr>
<td>A(38–40)</td>
<td>1.58±0.23 (n=7)</td>
</tr>
<tr>
<td>A(28–31)/A(38–40)</td>
<td>1.41±0.13 (n=5)</td>
</tr>
<tr>
<td>Δ(31–40)</td>
<td>1.19±0.10 (n=3)</td>
</tr>
<tr>
<td>CGHK chimera</td>
<td>1.12±0.14 (n=3)</td>
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[22]. H⁺,K⁺-ATPase activity was calculated as the difference between the ATPase activity in the presence and absence of 15 mM KCl, and expressed as the percentage of the control value measured at pH 6.5. The control value is 1.70±0.10 μmol/mg/h for wild-type gastric H⁺,K⁺-ATPase, 1.66±0.13 μmol/mg/h for mutant A(28–31)/A(38–40), and 1.53±0.26 μmol/mg/h for mutant Δ(31–40), respectively. The values are mean±SE for three observations. (B) The H⁺,K⁺-ATPase activity of wild-type gastric H⁺,K⁺-ATPase (●), wild-type colonic H⁺,K⁺-ATPase (■) and CGHK chimera (▲) was assayed. H⁺,K⁺-ATPase activity was expressed as a percentage of the control value measured at pH 6.5 for wild-type gastric H⁺,K⁺-ATPase and CGHK chimera, and at 7.5 for wild-type colonic H⁺,K⁺-ATPase. The control value is 1.70±0.10 μmol/mg/h for wild-type gastric H⁺,K⁺-ATPase, 0.44±0.02 μmol/mg/h for wild-type colonic H⁺,K⁺-ATPase, and 1.15±0.09 μmol/mg/h for CGHK chimera, respectively. The values are mean±SE for three observations.

![Fig. 3. pH dependence of the expressed H⁺,K⁺-ATPase activity.](image)

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ATPase, whereas CGHK chimera showed a pH profile similar to that of gastric H\(^+\),K\(^+\)-ATPase, having maximal activity around pH 6.5 to 6.8. This result further indicates that the amino-terminal region of gastric H\(^+\),K\(^+\)-ATPase including the lysine/glycine cluster is not involved in determining the affinity for protons.

**Cation sensitivity of the mutant H\(^+\),K\(^+\)-ATPases**

Figure 4 shows the effect of K\(^+\) concentrations on the H\(^+\),K\(^+\)-ATPase activity of wild-type and mutant [A(28–31)/A(38–40) and Δ(31–40)] gastric H\(^+\),K\(^+\)-ATPases. The wild-type and two mutant gastric H\(^+\),K\(^+\)-ATPases showed similar affinity for K\(^+\). The \(K_m\) values are 1.5, 1.6 and 1.4 mM, respectively. Therefore, the cluster structure is not directly involved in determining the affinity for K\(^+\). CGHK chimera also showed a \(K_m\) value of 1.4 mM, which is closer to that of gastric H\(^+\),K\(^+\)-ATPase than that of colonic H\(^+\),K\(^+\)-ATPase (\(K_m\) value of 0.7 mM) [16]. This result further indicates that the amino-terminal region of gastric H\(^+\),K\(^+\)-ATPase including the lysine/glycine cluster is not involved in determining the affinity for K\(^+\).

It has been reported that Na\(^+\) acts as a proton or K\(^+\) analogue in the reaction of gastric H\(^+\),K\(^+\)-ATPase [24, 25]. However, the H\(^+\),K\(^+\)-ATPase activity of gastric vesicles was not affected by Na\(^+\) in the presence of K\(^+\). In our experiments, the K\(^+\)-stimulated ATPase activity of the wild-type gastric H\(^+\),K\(^+\)-ATPase was not affected by Na\(^+\) up to 30 mM (2% inhibition by 30 mM Na\(^+\)). The K\(^+\)-stimulated ATPase activity of mutant enzymes [A(28–31)/A(38–40) and Δ(31–40)] was not affected by 30 mM Na\(^+\) (9 and 12% inhibition for A(28–31)/A(38–40) and Δ(31–40) mutants, respectively). Therefore, modification of the lysine/glycine cluster of gastric H\(^+\),K\(^+\)-ATPase did not confer the Na\(^+\)-stimulated ATPase activity, indicating that the lysine/glycine cluster structure is not involved in discriminating Na\(^+\) and protons.

**ATP dependence of the mutant H\(^+\),K\(^+\)-ATPases**

Figure 5 shows the effects of ATP concentrations on the expressed H\(^+\),K\(^+\)-ATPase activity of wild-type and mutant [A(28–31)/A(38–40) and Δ(31–40)] enzymes. Both the wild-type and mutant H\(^+\),K\(^+\)-ATPases showed almost similar affinities for ATP. The apparent \(K_{1/2}\) values are 42, 52 and 34 \(\mu\)M for the wild-type, A(28–31)/A(38–40) and Δ(31–40) mutant enzymes, respectively. Therefore, the cluster structure is not involved in determining the affinity for ATP.
Gastric H⁺,K⁺-ATPase is a member of the P-type ATPase family which actively transports ions coupled with the hydrolysis of ATP by forming an acid-stable acylphosphate compound as a high-energy intermediate. P-type ATPases generally have common structures in the catalytic center including the acyl-phosphorylation site and the ATP-binding site. The location of domains containing their cation recognition sites and transport pathways is also common to some extent. However, the roles of individual amino acid residues in the cation recognition sites are supposed to be different depending on the species of the transporting cations. In fact, several acidic or polar amino acid residues in the fourth, fifth and sixth transmembrane segments were identified as the binding sites or the sites involved in determining the affinity for the cations in the Ca²⁺-ATPase [26, 27], Na⁺,K⁺-ATPase [27–29] and H⁺,K⁺-ATPase [4–7].

One of the most unique structures in the catalytic α-subunits which are divergent between different ATPases is located in the cytoplasmic amino-terminal region. In rabbit gastric H⁺,K⁺-ATPase, the cluster of lysine residues interspersed by the glycine block was observed from amino acid no. 28 to 40. This characteristic structure was conserved in all of the gastric H⁺,K⁺-ATPases from different animal species. Therefore, it was postulated that this cluster structure plays an important role(s) on the function of gastric H⁺,K⁺-ATPase. One of the hypotheses is that the amino-terminal structure including the lysine/glycine cluster regulates the ATPase function by binding with or near the ion pathway as in the case of the “ball and chain model” in the voltage-gated potassium channels [30] as schematically illustrated in Fig. 6. However, there have been no reports on the actual role of this lysine/glycine cluster in the functions of H⁺,K⁺-ATPase.

A similar structure, a “lysine cluster,” was only found in the amino-terminal regions of Na⁺,K⁺-ATPase α-subunits. The lysine cluster is not interspersed with the glycine block. This structure is also conserved among different isoforms of the α-subunits (α1, α2, α3) and different animal species [31] except frog (Rana catesbeiana) enzyme [32]. It has been proposed that the lysine cluster in Na⁺,K⁺-ATPases controls the passage of Na⁺ and K⁺ ions to and from cation-binding sites on the cytoplasmic side of the membrane [32]. The roles of the whole N-terminal region of the α-subunit (amino acid no. 1 to 30) including the lysine cluster on the functions of Na⁺,K⁺-ATPase were studied by limited tryptic digestion of native enzyme [33, 34] or preparation of deletion mutant α-subunits by manipulation of the cDNA [35–39]. A former study showed that removing the amino-terminal 30 residues from hog kidney Na⁺,K⁺-ATPase α-subunit preserved Na⁺,K⁺-ATPase activity, increased the apparent affinity for Na⁺ at low pH, and that the amino-terminal region played a role on the transition between the E₁–E₂ conformations. The lat-

**Fig. 6. Schematic illustration of putative ball and chain model in gastric H⁺,K⁺-ATPase.** Left and right illustrations show active and inactive H⁺,K⁺-ATPases, respectively. Eleven cylinders show the transmembrane sequences of gastric H⁺,K⁺-ATPase; ten for the α-subunit, and one for the β-subunit. Black spots in the transmembrane sequences represent the cation binding sites. A shaded ball with plus charge signs represents the amino-terminal domain of the α-subunit including the lysine/glycine cluster.
The resulting mutant involving the membrane fraction as judged by Western blotting. C is involved in endocytosis of the lysine and arginine residues with alanines, the glycine cluster in the gastric \( H_{\alpha} \), \( K_{\alpha} \)-ATPase \( \alpha \)-subunit, and demonstrated that the lysine/glycine cluster as well as its positive charges are not essential for the function of gastric \( H^{+}, K^{+} \)-ATPase. Even after removing the lysine/glycine block of the \( \alpha \)-subunit or replacing the lysine and arginine residues with alanines, the \( \beta \)-subunit assembled with the mutant \( \alpha \)-subunits. As a consequence, the mutant \( \alpha \)-subunits were stabilized in the membrane fraction as judged by Western blotting. The resulting mutant \( \alpha/\beta \) complexes retained \( H^{+}, K^{+} \)-ATPase activity comparable with that of wild-type \( H^{+}, K^{+} \)-ATPase. The mutants showed apparently similar affinity for ATP, \( K^{+} \) and proton as that of the wild type, respectively. In fact, the A(28–31)/A(38–40) mutant complex showed a pH profile similar to that of wild-type \( H^{+}, K^{+} \)-ATPase although the mutant lost seven positive charges compared to the wild type. Therefore, the lysine/glycine cluster is not directly involved in the interaction with the cations and protons in the reaction of \( H^{+}, K^{+} \)-ATPase. This conclusion was supported by the finding that CGHK chimera showed a similar pH profile and affinity for \( K^{+} \) as that of wild-type gastric \( H^{+}, K^{+} \)-ATPase. The pH sensing site may be localized on another part of the \( H^{+}, K^{+} \)-ATPase than the amino-terminal region.

Although it is clear that the lysine/glycine cluster is not involved in determination of the affinity for ions in the overall ATP hydrolysis reaction, unfortunately, we cannot study the contribution of the cluster on the current generated by the ATPase as in several studies of \( Na^{+},K^{+} \)-ATPase because gastric \( H^{+}, K^{+} \)-ATPase is non-electrogenic. In addition, it cannot be completely excluded that the lysine/glycine cluster structure plays a role in the secondary regulation of \( H^{+}, K^{+} \)-ATPase activity. It is interesting that there are several phosphorylation sites on the amino-terminal region of the \( H^{+}, K^{+} \)-ATPase \( \alpha \)-subunit; Tyr-7 and Tyr-10 for tyrosine kinase, and Ser-27 for protein kinases A and C, although the significance of phosphorylation is not yet understood [40, 41]. Phosphorylation of Ser-18 of the \( Na^{+},K^{+} \)-ATPase \( \alpha \)-subunit (rat \( \alpha \)1) by protein kinase C is involved in endocytosis of the \( \alpha \)-subunit into endosomes [42]. Therefore, phosphorylation in the amino-terminal region of \( H^{+}, K^{+} \)-ATPase \( \alpha \)-subunit might also be involved in the intracellular transport of \( H^{+}, K^{+} \)-ATPase. It is worth studying whether some interaction occurs between the negative charge(s) of the phosphate group attached to Tyr-7, Tyr-10 or Ser-27 and the positive charges of the lysine/glycine cluster. In this paper, we reported that all of the mutant \( H^{+}, K^{+} \)-ATPases expressed in HEK cells retained their \( H^{+}, K^{+} \)-ATPase activity in the membrane fraction. However, it is not clear whether these mutants can secrete protons when expressed in parietal cells or not.

In conclusion, the lysine/glycine cluster does not play an important role in determination of the affinity for ATP, \( K^{+} \) and protons in the ATP hydrolysis reaction.

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