Chimeric Domain Analysis of the Compatibility between H\(^+\), K\(^+\)-ATPase and Na\(^+\), K\(^+\)-ATPase \(\beta\)-Subunits for the Functional Expression of Gastric H\(^+\), K\(^+\)-ATPase*

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Gastric H\(^+\), K\(^+\)-ATPase consists of \(\alpha\)-subunit with 10 transmembrane domains and \(\beta\)-subunit with a single transmembrane domain. We constructed cDNAs encoding chimeric \(\beta\)-subunits between the gastric H\(^+\), K\(^+\)-ATPase and Na\(^+\), K\(^+\)-ATPase \(\beta\)-subunits and co-transfected them with the H\(^+\), K\(^+\)-ATPase \(\alpha\)-subunit cDNA in HEK-293 cells. A chimeric \(\beta\)-subunit that consists of the cytoplasmic plus transmembrane domains of Na\(^+\), K\(^+\)-ATPase \(\beta\)-subunit and the ectodomain of H\(^+\), K\(^+\)-ATPase \(\beta\)-subunit assembled with the H\(^+\), K\(^+\)-ATPase \(\alpha\)-subunit and expressed the K\(^+\)-ATPase activity. Therefore, the whole cytoplasmic and transmembrane domains of H\(^+\), K\(^+\)-ATPase \(\beta\)-subunit were replaced by those of Na\(^+\), K\(^+\)-ATPase \(\beta\)-subunit without losing the enzyme activity. However, most parts of the ectodomain of H\(^+\), K\(^+\)-ATPase \(\beta\)-subunit were not replaced by the corresponding domains of Na\(^+\), K\(^+\)-ATPase \(\beta\)-subunit. Interestingly, the extracellular segment between Cys\(^{152}\) and Cys\(^{176}\), which contains the second disulfide bond, was exchangeable between H\(^+\), K\(^+\)-ATPase and Na\(^+\), K\(^+\)-ATPase, preserving the K\(^+\)-ATPase activity intact. Furthermore, the K\(^+\)-ATPase activity was preserved when the N-terminal first 4 amino acids \(\text{Gly}^6\text{Phe}^7\text{Trp}^8\text{Thr}^9\) in the ectodomain of H\(^+\), K\(^+\)-ATPase \(\beta\)-subunit were replaced by the corresponding amino acids \(\text{Gly}^6\text{Ser}^7\text{Asp}^8\text{Phe}^9\) of Na\(^+\), K\(^+\)-ATPase \(\beta\)-subunit. The ATPase activity was abolished, however, when 4 amino acids \(\text{Gly}^6\text{Lys}^7\text{Leu}^8\text{Lys}^9\) in the ectodomain of H\(^+\), K\(^+\)-ATPase \(\beta\)-subunit were replaced by the counterpart \(\text{Glu}^6\text{Val}^7\text{Asp}^8\text{Val}^9\) of Na\(^+\), K\(^+\)-ATPase \(\beta\)-subunit, indicating that this region is the most N-terminal one that discriminates the H\(^+\), K\(^+\)-ATPase \(\beta\)-subunit from that of Na\(^+\), K\(^+\)-ATPase.

Gastric proton pump, H\(^+\), K\(^+\)-ATPase, consists of two kinds of subunits. One is the catalytic \(\alpha\)-subunit, which has 10 transmembrane domains and contains sites for ATP-binding (1, 2) and its phosphorylation (3), binding sites of proton pump inhibitors (4–6), and sites responsible for ion recognition (6–9). The other is the glycoprotein, \(\beta\)-subunit, which has a single transmembrane domain and is also essential for the functional expression of H\(^+\), K\(^+\)-ATPase (7, 10, 11) and involved in the structural and functional maturation, intracellular transport and stabilization of the functional holoenzyme (12). Gastric H\(^+\), K\(^+\)-ATPase is closely related to Na\(^+\), K\(^+\)-ATPase from the structural and functional viewpoints. Amino acid identity of the \(\alpha\)-subunits of these ATPases is as high as 62% (2), whereas that of the \(\beta\)-subunit is 29–37% (13). H\(^+\), K\(^+\)-ATPase \(\beta\)-subunit was stably assembled with Na\(^+\), K\(^+\)-ATPase \(\alpha\)-subunit in the microsomal membrane of Xenopus oocytes (14) and also led to an increased ouabain binding at the plasma membrane, accompanied by increased Rb\(^+\) uptake and Na\(^+\), K\(^+\) pump current (15). Similar results were obtained from co-expression in yeast cells (16). On the contrary, H\(^+\), K\(^+\)-ATPase was not functionally expressed in HEK-293 cells under co-transfection with the H\(^+\), K\(^+\)-ATPase \(\alpha\)-subunit and the Na\(^+\), K\(^+\)-ATPase \(\beta\)-subunit cDNAs (17). Therefore, there should be some difference in stringency for the functional \(\alpha\)-\(\beta\) assembly between H\(^+\), K\(^+\)-ATPase and Na\(^+\), K\(^+\)-ATPase.

Recently, there have been several reports for the co-expression of the Na\(^+\), K\(^+\)-ATPase \(\alpha\)-subunit with the chimeric \(\beta\)-subunits between H\(^+\), K\(^+\)-ATPase and Na\(^+\), K\(^+\)-ATPase. Chimeric cRNAs between the Na\(^+\), K\(^+\)- and H\(^+\), K\(^+\)-ATPase \(\beta\)-subunits and the Na\(^+\), K\(^+\)-ATPase \(\alpha\)-subunit cRNA were co-injected in Xenopus oocytes, and it was found that the transmembrane domain of Na\(^+\), K\(^+\)-ATPase \(\beta\)-subunit was important for efficient assembly with the Na\(^+\), K\(^+\)-ATPase \(\alpha\)-subunit, and both the transmembrane domain and the ectodomain of the \(\beta\)-subunit modulated the transport activity of Na\(^+\), K\(^+\) pump (18). Similar chimeric \(\beta\)-subunits together with the Na\(^+\), K\(^+\)-ATPase \(\alpha\)-subunit were expressed in yeast cells, and it was found that the ectodomain of H\(^+\), K\(^+\)-ATPase \(\beta\)-subunit was primarily responsible for the effect on K\(^+\) affinity (19), and the structure of \(\beta\)-subunit was involved in the interaction of Na\(^+\), K\(^+\)-ATPase with Na\(^+\) (20). From the replacement of the first S-S loop segment \(\text{Cys}^{127} \text{Cys}^{150}\) of the Na\(^+\), K\(^+\)-ATPase \(\beta\)-subunit with the corresponding segment of the H\(^+\), K\(^+\)-ATPase, Arg\(^{148}\) in the \(\beta\)-subunit was found to be essential for the functional expression of Na\(^+\), K\(^+\)-ATPase (21). More recently, several chimeric \(\beta\)-subunits between H\(^+\), K\(^+\)-ATPase and Na\(^+\), K\(^+\)-ATPase were prepared and expressed with the Na\(^+\), K\(^+\)-ATPase \(\alpha\)-subunit to study the compatibility of these \(\beta\)-subunits for the \(\alpha\)-\(\beta\) assembly and Na\(^+\), K\(^+\)-ATPase activity (22).

However, there have been no reports for the functional expression study on H\(^+\), K\(^+\)-ATPase using chimeric \(\beta\)-subunits between H\(^+\), K\(^+\)-ATPase and Na\(^+\), K\(^+\)-ATPase. In this study, we expressed the chimeric \(\beta\)-subunits, some of which had been constructed previously (22), with the H\(^+\), K\(^+\)-ATPase \(\alpha\)-subunit in HEK-293 cells and studied the compatibility between the H\(^+\), K\(^+\)-ATPase and Na\(^+\), K\(^+\)-ATPase \(\beta\)-subunits in \(\alpha\)-\(\beta\) assembly and functional expression of H\(^+\), K\(^+\)-ATPase.
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). Pfu DNA polymerase was from Stratagene. Restriction enzymes and other DNA and RNA modifying enzymes were from Toyobo (Osaka, Japan) and New England Biolabs (Beverly, MA). Endoglycosidase H (Endo H) and N-glycosidase F (PNGase F) were obtained from Roche Molecular Biochemicals (Tokyo, Japan). Anti-gastric H+—K+-ATPase β-subunit monoclonal antibody, 2B6, was obtained from Molecular Biologial Laboratories (Nagoya, Japan). SCH 28080 was obtained from Schering Co. (Kenworth, NJ). All other reagents were of molecular biology grade or the highest grade of purity available.

Cloning of Chimeric β-Subunits of H+—K+-ATPase—H+—K+-ATPase α-subunit cDNA was prepared from rabbit gastric mucosa and cloned in pCDNA3 vector as described elsewhere (7).

Construction of Chimeric β-Subunits cDNAs—Chimeric β-subunit cDNAs between hog gastric H+-K+-ATPase (23) and Torpedo californica Na+—K+-ATPase (24) were constructed as described elsewhere (22). In the processes to create restriction sites such as EcoRI, SphI, and EcoT22I in the hog H+-K+-ATPase β-subunit cDNA, mutations such as Asp95→Glu, Ser151→ Ala, and Phe179→Ile were incorporated, respectively (22).

Site-directed Mutagenesis—Introduction of site-directed mutations between SnaBI and EcoRV sites of the H+-K+-ATPase β-subunit was carried out by sequential polymerase chain reaction (PCR) steps as described elsewhere (9). Two kinds of flanking sequence primers were prepared in this step: one between the 5′ flanking sense primer, 5′-GCAATACGCTTACATAAGG-3′ (sequence in pBluescript II vector), and the other is the 3′-flanking antisense primer, 5′-CGTGTCGTCGACACGTGTG-3′ (close to the EcoRI site of the H+-K+-ATPase β-subunit cDNA). Additionally, sense and antisense oligonucleotides, each 21 bases long containing mutated bases near the center, were designed (referred as the sense mutating primer and antisense mutating primer). In the first PCR amplification step, the NH2 chimeric β-subunit cDNA or H+-K+-ATPase β-subunit cDNA was used as a template DNA. Two fragments were prepared in this step: one between the 5′-flanking sense primer and the antisense mutating primer and the other between the sense mutating primer and the 3′-flanking antisense primer. Each amplified fragment was purified by gel electrophoresis, combined, and incubated with the 5′-flanking sense primer and the 3′-flanking antisense primer in the second PCR amplification. The amplified fragment was purified by gel electrophoresis, subcloned in pCR-Script Amp SK(+) vector (Stratagene), and sequenced. PCR was routinely carried out in the presence of 200 μM each dNTP, 500 nM primers, 10 mM KCl, 10 mM (NH4)2SO4, 2 mM MgSO4, 20 mM Tris-HCl, pH 8.9, 0.1% Triton X-100, 100 μg/ml bovine serum albumin, and 2.5 U of Pfu DNA polymerase for 30 cycles. After sequencing, the amplified fragment in the second PCR was digested with AatII and EcoRI and ligated back into the pcDNA3 vector for further amplification. The chimeric β-subunit construct was then sequenced by dideoxy chain termination method using an Autoread DNA sequencing kit and an ALFexpress DNA sequencer (Amersham Pharmacia Biotech).

Cell Culture, Transfection, and Preparation of Membrane Fractions—Cell culture of HEK-293 was carried out as described previously (7). 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 (25).

SDS-Polyacrylamide Gel Electrophoresis and Western Blot—SDS-polyacrylamide gel electrophoresis was carried out as described elsewhere (25). Membrane preparations (50 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 7.5% SDS-polyacrylamide gel. Western blot was carried out as described previously (7).

Antibody—Anti-gastric H+-K+-ATPase α-subunit antibody, Ab1024, was previously raised against the C-terminal peptide (residues 1024–1034) of the H+-K+-ATPase α-subunit (PGSWQWDQELLY) (26).

Glycosidase Treatment—30 μg 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 10

milliliters of Endo H in a solution containing 0.1% SDS, 1 μg 2-mercaptoethanol, 0.5 mM phenylmethylsulfonl 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 μg 2-mercaptoethanol, 50 mM sodium phosphate, and 50 mM sodium chloride, pH 7.4, at 4 °C for 30 min. After centrifugation at 16,000 × g for 20 min, the supernatant was incubated with an anti-α-subunit antibody, Ab1024, at a 1:100 dilution, and 10 μl of ImmunoPure immobilized protein A (Pierce) at 4 °C for 12 h. After centrifugation, the pellet was washed four times with the lysis buffer followed by two washes in 0.1% Nonidet P-40, 150 mM NaCl, 0.5 mM EDTA, and 50 mM Tris-HCl, pH 7.4. The pellet was solubilized in the sample buffer for SDS-polyacrylamide gel electrophoresis and incubated at room temperature for 10 min. The proteins were separated on SDS-polyacrylamide gel and blotted. The β-subunit in the blot was detected by an anti-β-subunit antibody, 2B6, in combination with a peroxidase-conjugated anti-mouse antibody, which was preabsorbed with rabbit serum. When indicated, the precipitated proteins were deglycosylated, that is, immunoprecipitated samples were treated with PNGase F as described above, solubilized in the sample buffer for SDS-polyacrylamide gel electrophoresis, and blotted.

Assay of H+-K+-ATPase Activity—H+-K+-ATPase activity was measured from the decrease in the amount of NADH coupled with regeneration of ATP from ADP ("coupled-enzyme assay") in 1.2 ml of a reaction mixture containing 50 μg of membrane protein, 3 mM MgCl2, 800 μM ATP, 160 μM NADH, 0.8 mM phosphoenolpyruvate, 3 units/ml pyruvate kinase, 2.75 units/ml lactate dehydrogenase, 5 mM NaF, 1 mM ouabain, 15 mM KCl, and 40 mM Tris-HCl, pH 7.4. The decrease in the amount of NADH was measured at 37 °C from the absorbance at 340 nm in a Beckman spectrophotometer as described elsewhere (27). H+-K+-ATPase activity, defined as the SCH 28080-sensitive K+-ATPase, was calculated as the difference between the K+-ATPase activities in the presence and absence of 50 μM SCH 28080.

ATPase activity was measured as a function of K+ concentrations. The ATPase activity was measured from the measurement of inorganic phosphate released from ATP. K+-ATPase activity was measured in 1 ml of solution containing 50 μg of membrane protein, 3 mM MgSO4, 1 μM ATP, 5 mM NaF, 2 mM ouabain, and 40 mM Tris-HCl, pH 6.8, in the presence and absence of various concentrations of KCl. 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 at the absorbance of the wavelength of 320 nm as described elsewhere (28). The K+-ATPase activity was calculated as the difference between the activities in the presence and absence of KCl. The K+-ATPase activity was sensitive to 50 μM SCH 28080. Inorganic phosphate released in the enzyme reaction with the wild-type H+-K+-ATPase was 5–10 times higher than the background level of inorganic phosphate released in the absence of enzyme. Values of K+-ATPase activity measured from colorimetric assay of released inorganic phosphate were comparable with those measured in coupled enzyme assay. Protein was measured using the BCA protein assay kit from Pierce with bovine serum albumin as a standard.

RESULTS

Construction of Chimeric β-Subunits between H+—K+-ATPase and Na+—K+-ATPase—First, we used two series of chimeric β-subunits that were constructed using β-subunits of hog gastric H+—K+-ATPase and T. californica Na+—K+-ATPase as shown in Fig. 1. One set of chimeras (NaXh series) was prepared by successively exchanging 5′-portion of the H+—K+-ATPase β-subunit cDNA with the corresponding portion of the Na+—K+-ATPase β-subunit cDNA (Fig. 1A). The other set of chimeras (HxNyH series) was prepared by replacing a middle portion of the H+—K+-ATPase β-subunit (between adjacent unique restriction sites) with the corresponding portion of the Na+—K+-ATPase β-subunit (Fig. 1B).

Expression of α- and β-Subunits—Fig. 2 shows Western blot patterns of the membrane fractions of the transfecteds, detected by using an anti-gastric H+—K+-ATPase α-subunit antibody. When the cells were transfected with the wild-type α-sub-
Chimeric Analysis of Gastric H\(^{+}\),K\(^{-}\)-ATPase β-Subunit

**Fig. 1.** Construction of chimeric β-subunits between H\(^{+}\),K\(^{-}\)-ATPase and Na\(^{+}\),K\(^{-}\)-ATPase. Schematic representations of NxH series (A) and HxNyH series (B) of β-subunit chimeras are shown. NNH and HeN chimeras are also shown in A and B, respectively. The capital letters H and N used for chimera symbols represent fragments of unit cDNA in the absence of the β-subunit cDNA, a single faint band was detected around 95 kDa, which represents the expression of the H\(^{+}\),K\(^{-}\)-ATPase α-subunit (lane 8 in both panels A and B in Fig. 2). The expression of the α-subunit increased when the cells were co-transfected with the wild-type H\(^{+}\),K\(^{-}\)-ATPase β-subunit cDNA (lane 1, chimeric β-subunit NsH (lane 2), NvH (lane 3), NmH (lane 4), NpH (lane 5), NeH (lane 6), or wild-type Na\(^{+}\),K\(^{-}\)-ATPase β-subunit (lane 7) cDNAs; transfected only with the wild-type H\(^{+}\),K\(^{-}\)-ATPase α-subunit cDNA (lane 8) or mock-transfected (lane 9). These cell membrane fractions (30 μg) were applied on the gel and blotted with Ab1024, which is an anti-H\(^{+}\),K\(^{-}\)-ATPase α-subunit antibody. Bands representing H\(^{+}\),K\(^{-}\)-ATPase α-subunit are shown by a bold arrow. B, HEK-293 cells were co-transfected with the wild-type H\(^{+}\),K\(^{-}\)-ATPase α-subunit cDNA plus wild-type H\(^{+}\),K\(^{-}\)-ATPase β-subunit (lane 6 in both panels A and B in Fig. 2). A similar increase in expression of the α-subunit was also observed when the cells were co-transfected with the Na\(^{+}\),K\(^{-}\)-ATPase β-subunit cDNA (lane 2 in both panels A and B in Fig. 2) or HpNeH chimeric β-subunit cDNA (lane 6 in Fig. 2B). However, there was no increased expression of the α-subunit when co-expressed with other chimeric β-subunits including NsH, NvH, NmH, NeH (Fig. 2A), HsNvH, HvNmH, HmNpH, and HeN chimeras (Fig. 2B) and the wild-type Na\(^{+}\),K\(^{-}\)-ATPase β-subunit (lane 7 in Fig. 2A).

Fig. 2 shows Western blot patterns of the membrane fractions of the transfectedants, detected by using an anti-gastric H\(^{+}\),K\(^{-}\)-ATPase antibody, 2B6 (23). The epitope of

H\(^{+}\),K\(^{-}\)-ATPase and Na\(^{+}\),K\(^{-}\)-ATPase, respectively. Lowercase letters show the restriction sites that are used as the joining points for chimeric construction; s, v, m, p, and e represent SnaBI, EcoRV, MunI, SphI, and EcoT22I sites, respectively. H\(^{+}\),K\(^{-}\)-ATPase (closed bar) and Na\(^{+}\),K\(^{-}\)-ATPase (open bar) and their transmembrane domains (hatched and cross-hatched bars) are shown schematically. The locations of disulfide bonds in the ectodomain are shown with brackets. The numeric numbers show the connecting points between the two β-subunits and refer to the hog H\(^{+}\),K\(^{-}\)-ATPase β-subunit. The total numbers of amino acids in these chimeras are shown on the right. Glycosylation sites are shown with sugar chain symbols.

**Fig. 2.** Western blots with Ab1024 of the membrane fraction of HEK cells cotransfected with the wild-type H\(^{+}\),K\(^{-}\)-ATPase α-subunit and chimeric β-subunit cDNAs. A, HEK-293 cells were co-transfected with the wild-type H\(^{+}\),K\(^{-}\)-ATPase α-subunit cDNA plus wild-type H\(^{+}\),K\(^{-}\)-ATPase β-subunit (lane 1), chimeric β-subunit NsH (lane 2), NvH (lane 3), NmH (lane 4), NpH (lane 5), NeH (lane 6), or wild-type Na\(^{+}\),K\(^{-}\)-ATPase β-subunit (lane 7) cDNAs; transfected only with the wild-type H\(^{+}\),K\(^{-}\)-ATPase α-subunit cDNA (lane 8) or mock-transfected (lane 9).
A band represents the (data not shown). These results indicate that the 60–70-kDa ATPase NsH (in Fig. 3). The cell membrane fractions (30 μg) were digested with both Endo H and PNGase F, or wild-type Na⁺,K⁺-ATPase β-subunit cDNA; transfected only with the wild-type H⁺,K⁺-ATPase α-subunit cDNA (lane 2); or mock-transfected (lane 9). The cell membrane fractions (30 μg) were applied on the gel and blotted with 2B6. βm and βc represent the β-subunit with complex-type (mature) carbohydrate chains and that with high mannose type (core) carbohydrate chains, respectively. B, HEK-293 cells were co-transfected with the wild-type H⁺,K⁺-ATPase α-subunit cDNA plus wild-type H⁺,K⁺-ATPase β-subunit (lane 1); chimeric β-subunit NsH (lane 2), NvH (lane 3), NmH (lane 4), NpH (lane 5), NeH (lane 6), or wild-type Na⁺,K⁺-ATPase β-subunit (lane 7) cDNA; transfected only with the wild-type H⁺,K⁺-ATPase α-subunit cDNA (lane 8); or mock-transfected (lane 9).

Fig. 3. Western blots with an antibody against gastric H⁺,K⁺-ATPase β-subunit (280 kDa) of the membrane fraction of HEK cells co-transfected with the wild-type α-subunit plus chimeric β-subunit cDNAs. A, HEK-293 cells were co-transfected with the wild-type H⁺,K⁺-ATPase α-subunit cDNA plus wild-type H⁺,K⁺-ATPase β-subunit (lane 1), chimeric β-subunit NsH (lane 2), NvH (lane 3), NmH (lane 4), NpH (lane 5), NeH (lane 6), or wild-type Na⁺,K⁺-ATPase β-subunit (lane 7) cDNA; transfected only with the wild-type H⁺,K⁺-ATPase α-subunit cDNA (lane 8); or mock-transfected (lane 9). The cell membrane fractions (30 μg) were applied on the gel and blotted with 2B6. βm and βc represent the β-subunit with complex-type (mature) carbohydrate chains and that with high mannose type (core) carbohydrate chains, respectively. B, HEK-293 cells were co-transfected with the wild-type H⁺,K⁺-ATPase α-subunit cDNA plus wild-type H⁺,K⁺-ATPase β-subunit (lane 1). This pattern was similar to that observed in the Western blot of the β-subunits in the membrane fractions (Fig. 3). On the other hand, no β-subunit-related band was observed when the cells were transfected only with the H⁺,K⁺-ATPase β-subunit cDNA (data not shown). When the cells were co-transfected with the H⁺,K⁺-ATPase α-subunit cDNA plus chimeric β-subunits NsH, NvH, NmH, NpH, NeH (Fig. 4A), or HpNPH cDNA (lane 5 in Fig. 4B). This pattern was similar to that observed in the Western blot of the β-subunits in the membrane fractions (Fig. 3).

The anti-a-antibody co-precipitated proteins with molecular masses of 70 kDa (βm panels A and B in Figs. 4) and 48 kDa (βc panels A and B in Fig. 4) when the cells were co-transfected with the wild-type H⁺,K⁺-ATPase α-subunit cDNA plus wild-type H⁺,K⁺-ATPase β-subunit (lane 1 in both panels A and B in Fig. 4), chimeric β-subunit NsH (lane 2 in Fig. 4A), or HpNPH cDNA (lane 5 in Fig. 4B). This pattern was similar to that observed in the Western blot of the β-subunits in the membrane fractions (Fig. 3). On the other hand, no β-subunit-related band was observed when the cells were transfected only with the H⁺,K⁺-ATPase β-subunit cDNA (data not shown). When the cells were co-transfected with the H⁺,K⁺-ATPase α-subunit cDNA plus chimeric β-subunits NsH, NvH, NmH, NpH, NeH (Fig. 4A), or HpNPH cDNA (Fig. 4B) cDNAs, a single band or doublet bands with molecular mass of 40–48 kDa (βm) were observed. The amount of these bands was much smaller than those observed in the samples with the wild-type H⁺,K⁺-ATPase β-subunit (lane 1 in both panels A and B in Fig. 4). Na⁺,K⁺-ATPase chimera (lane 2 in Fig. 4A), or HpNPH chimera (lane 5 in Fig. 4B). When the precipitated samples were treated with PNGase F, the molecular mass of the bands shifted to 30–35 kDa on the blot (CP in panels C and D in Fig. 4), indicating that the precipitated proteins detected with 2B6 were the wild-type H⁺,K⁺-ATPase β-subunit or its chimeric β-subunits. The bands with molecular masses of 70 kDa represent the β-subunits with complex-type carbohydrate chains (βm) and those around 48 kDa represent the β-subunits with high mannose-type carbohydrate chains (βc). These results indicate that all the chimeric β-subunits as well as the wild-type H⁺,K⁺-ATPase β-subunit assembled with H⁺,K⁺-ATPase α-subunit.

Detailed Chimeric and Mutational Analysis of the N-terminal Domain of the β-Subunit—As shown in Fig. 2, the Na⁺,K⁺-ATPase chimera β-subunit increased the expression of the α-subunit in the membrane, whereas the Na⁺,K⁺-ATPase β-subunit did not. Therefore, it is likely that some structure that is important for
The stabilization of the α-subunit is located in the portion between Tyr<sup>45</sup> (SnaBI site) and Ile<sup>96</sup> (EcoRV site) of the β-subunit. Because Tyr<sup>45</sup> is in the transmembrane domain (from Trp<sup>37</sup> to Ile<sup>96</sup>) located about one quarter of the domain length from the cytoplasmic/membrane boundary, it is not clear whether the whole transmembrane domain of the H<sup>+</sup>,K<sup>+</sup>-ATPase β-subunit is replaceable with the corresponding domain of the Na<sup>+</sup>,K<sup>+</sup>-ATPase β-subunit, preserving its ability to stabilize the α-subunit in the membrane. Here, we constructed a new chimera termed NNH (Fig. 1A) by replacing the whole cytoplasmic plus transmembrane domains of H<sup>+</sup>,K<sup>+</sup>-ATPase β-subunit with the corresponding domains of Na<sup>+</sup>,K<sup>+</sup>-ATPase β-subunits. As shown in Fig. 5, the expression level of the α-subunit was similar between the cells expressing the wild-type H<sup>+</sup>,K<sup>+</sup>-ATPase α-β complex (lane 1) and α-NNH complex (lane 2). Therefore, the chimeric H<sup>+</sup>,K<sup>+</sup>-ATPase β-subunit, in which the whole cytoplasmic plus transmembrane domains were replaced with the corresponding domains of Na<sup>+</sup>,K<sup>+</sup>-ATPase β-subunit, stabilized the α-subunit in the membrane. The anti-α-antibody also co-precipitated β-subunit proteins with molecular masses of 70 kDa (β<sub>m</sub> in Fig. 6A) and 48 kDa (β<sub>c</sub> in Fig. 6A) when the cells were co-transfected with the H<sup>+</sup>,K<sup>+</sup>-ATPase α-subunit cDNA plus NNH chimera (lane 1 in Fig. 6).

From the above findings, it is likely that the segment of H<sup>+</sup>,K<sup>+</sup>-ATPase β-subunit from Asp<sup>67</sup> to Ile<sup>96</sup> is important for the stabilization of the α-subunit. To study this point further, we prepared two additional β-subunit mutants. One is the mutant in which only the first four amino acids (67DPYT70) in the beginning of the ectodomain of the H<sup>+</sup>,K<sup>+</sup>-ATPase β-subunit were replaced by the corresponding amino acids of the H<sup>+</sup>,K<sup>+</sup>-ATPase β-subunit (lanes 1), chimeric β-subunit NsH (lanes 2), NvH (lanes 3), NmH (lanes 4), NpH (lanes 5), or NeH (lanes 6). B and D, HEK-293 cells were co-transfected with the wild-type H<sup>+</sup>,K<sup>+</sup>-ATPase α-subunit cDNA plus wild-type H<sup>+</sup>,K<sup>+</sup>-ATPase β-subunit cDNA (lanes 1), chimeric β-subunit HsNH (lanes 2), HvNH (lanes 3), HmNH (lanes 4), or HpNH (lanes 5). The solubilized membrane fractions were incubated with an anti-α-subunit antibody, Ab1024, and protein A-coated beads. The precipitated preparations were treated with (C and D) or without PNGase F (A and B), separated on SDS-polyacrylamide gel and blotted with anti-β-subunit antibody, 2B6. β<sub>m</sub>, β<sub>c</sub>, and CP represent the β-subunit with complex-type (mature) carbohydrate chains and that with high mannose type (core) carbohydrate chains and core protein of the β-subunit, respectively.
Na⁺,K⁺-ATPase β-subunit, SDFE⁶⁶ (termed SDFE mutant). The other is the one in which only four amino acids ⁷ＱLKS⁷⁹ in the ectodomain of H⁺,K⁺-ATPase β-subunit were replaced by the counterpart of Na⁺,K⁺-ATPase β-subunit, RVAP⁷⁹ (termed RVAP mutant). As shown in Fig. 7, these eight amino acids of H⁺,K⁺-ATPase differ from those of Na⁺,K⁺-ATPase. As shown in Fig. 5, the expression level of the α-subunit was similar between the cells expressing the wild-type H⁺,K⁺-ATPase α-β complex (lane 1) and α-SDFE complex (lane 3). However, the expression level of the α-subunit for α-RVAP complex (lane 4) was significantly lower than that for the wild-type α-β complex (lane 1) and similar to that expressed in the absence of β-subunit (lane 5).

The anti-α-antibody co-precipitated β-subunit proteins with molecular masses of 70 kDa (βₘ in Fig. 6A) and 48 kDa (βₐ in Fig. 6A) when the cells were co-transfected with the H⁺,K⁺-ATPase α-subunit cDNA plus SDFE mutant (lane 2). When the precipitated samples were treated with PNGase F, the molecular mass of these bands shifted to 30–35 kDa on the blot (CP in Fig. 6B). Therefore, the SDFE β-subunit assembled with the α-subunit to form a stable α-β complex. On the other hand, RVAP mutant β-subunit did not form a stable α-β complex (Fig. 5) although weakly assembled with the α-subunit (Fig. 6, A and B).

**H⁺,K⁺-ATPase Activity of α-β Complex**—Fig. 8 shows the H⁺,K⁺-ATPase activity in the membrane fractions expressing chimeric α-β complex and the wild-type H⁺,K⁺-ATPase. As the wild-type complex, we used a heterocomplex of rabbit gastric H⁺,K⁺-ATPase α-subunit and hog gastric H⁺,K⁺-ATPase β-subunit. The H⁺,K⁺-ATPase activity found in the membrane fraction was sensitive to a gastric proton pump inhibitor, SCH 28080 (data not shown). There is no significant difference between the activities of this rabbit-hog α-β heterocomplex and rabbit H⁺,K⁺-ATPase α-β homocomplex (data not shown), suggesting that rabbit gastric H⁺,K⁺-ATPase α-subunit assembled with hog gastric H⁺,K⁺-ATPase β-subunit and formed a functional H⁺,K⁺-ATPase complex despite the species difference. In the NsH series (x indicates s, v, m, p, or e), only the α-NsH complex showed the H⁺,K⁺-ATPase activity (Fig. 8A). This α-NsH complex exhibited a 40% higher H⁺,K⁺-ATPase activity than that of the wild-type enzyme. From these results, it became clear that the segment from the N terminus in the cytoplasm to Tyr⁴⁵ (SnaBI site) in the transmembrane domain was replaceable with the corresponding segment of the Na⁺,K⁺-ATPase β-subunit for the functional expression of H⁺,K⁺-ATPase. However, the segment from Tyr⁴⁵ (SnaBI site) to Ile⁶⁸ (EcRV site), which is located in the transmembrane domain and ectodomain of the H⁺,K⁺-ATPase β-subunit, was not replaceable. This result is comparable with the finding that the corresponding segment of the Na⁺,K⁺-ATPase β-subunit is not replaceable with that of the H⁺,K⁺-ATPase β-subunit for stable complex formation with the Na⁺,K⁺-ATPase α-subunit and for the functional expression of Na⁺,K⁺-ATPase (22).

The α-HeN complex showed no H⁺,K⁺-ATPase activity, indicating that the C-terminal 110 amino acids of the H⁺,K⁺-ATPase β-subunit were not replaceable with the counterpart of the Na⁺,K⁺-ATPase β-subunit (Fig. 8B). In the HsNyH series of chimeras, only the α-HpNeH complex retained the H⁺,K⁺-ATPase activity, 75% of the wild-type enzyme activity (Fig. 8B).

Furthermore, the α-NNH complex also retained the H⁺,K⁺-
ATPase activity, 27% higher than that of the wild type (Fig. 8C). This result indicates that the whole transmembrane domain and the cytoplasmic domain of the H$^+\cdot$K$^+$-ATPase $\beta$-subunit are replaceable with the corresponding domains of the Na$^+\cdot$K$^+$-ATPase $\beta$-subunit. The $\alpha$-SDFE complex retained the H$^+\cdot$K$^+$-ATPase activity, 75% of the wild-type enzyme activity, whereas the $\alpha$-RVAP complex almost lost it (Fig. 8C). Therefore, 76QLKS79 block in the ectodomain of H$^+\cdot$K$^+$-ATPase $\beta$-subunit is important for the expression of H$^+\cdot$K$^+$-ATPase activity by stabilizing the $\alpha$-subunit.

Fig. 9 shows that double-reciprocal plots between the K$^+$ concentration and the H$^+\cdot$K$^+$-ATPase activity of the membrane fractions of yeast cells resulted in the appearance of high affinity ouabain binding sites in the membrane (16). Na$^+\cdot$K$^+$-ATPase $\beta$-subunit, on the contrary, did not support the functional expression of gastric H$^+\cdot$K$^+$-ATPase in HEK cells (17).

In the present study, we showed that a chimeric H$^+\cdot$K$^+$-ATPase $\beta$-subunit (Na$^+\cdot$H chimera), which contains the cytoplasmic and transmembrane domains of the $\alpha$-Na$^+$, and $\alpha$-HpNeH complexes. The $K_m$ values for K$^+$ of the wild type, $\alpha$-NNH, $\alpha$-Na$^+$, and $\alpha$-HpNeH complexes were 0.32, 0.27, 0.30, and 0.40 mM, respectively, indicating that the replacements of the cytoplasmic and transmembrane domains and the short extracellular segment from Cys$^{172}$ to Cys$^{178}$ with the corresponding ones of Na$^+\cdot$K$^+$-ATPase did not change the K$^+$ affinity of the enzyme. These results may suggest that these domains and the segment are not involved in determining affinity for K$^+$.

**DISCUSSION**

H$^+\cdot$K$^+$-ATPase $\beta$-subunit shows a number of structural similarities with Na$^+\cdot$K$^+$-ATPase $\beta$-subunit. Both $\beta$-subunits consist of a short N-terminal cytoplasmic domain (about 40 amino acids) and one transmembrane domain followed by a large ectodomain (13). They contain six conserved cysteine residues in the ectodomain, which form three disulfide bonds (32). These disulfide bonds are important for the protein folding for the maintenance of the ATPase function, because the ATPase activities were abolished by reduction with dithiothreitol or 2-mercaptoethanol (33–35). Na$^+\cdot$K$^+$-ATPase activity was also abolished when one of the three disulfide bonds was broken by mutation of the conserved cysteine residue(s) (36). From these similarities between H$^+\cdot$K$^+$-ATPase and Na$^+\cdot$K$^+$-ATPase $\beta$-subunits, we may deduce a possibility that H$^+\cdot$K$^+$-ATPase $\beta$-subunit acts as a surrogate for the Na$^+\cdot$K$^+$-ATPase $\beta$-subunit for the functional expression of Na$^+\cdot$K$^+$-ATPase and vice versa for the functional expression of H$^+\cdot$K$^+$-ATPase. In fact, H$^+\cdot$K$^+$-ATPase $\beta$-subunit was assembled with Na$^+\cdot$K$^+$-ATPase $\alpha$-subunit in Xenopus oocyte to form an $\alpha$-$\beta$ complex exhibiting functional Na$^+\cdot$K$^+$ pump, although the affinity of this pump for K$^+$ was lower compared with that of the wild-type Na$^+\cdot$K$^+$ pump (15, 37). Heterologous expression of H$^+\cdot$K$^+$-ATPase $\beta$-subunit together with Na$^+\cdot$K$^+$-ATPase $\alpha$-subunit in yeast cells resulted in the appearance of high affinity ouabain binding sites in the membrane (16). Na$^+\cdot$K$^+$-ATPase $\beta$-subunit, on the contrary, did not support the functional expression of gastric H$^+\cdot$K$^+$-ATPase in HEK cells (17).
Chimeric Analysis of Gastric $H^+\cdot K^+$-ATPase $\beta$-Subunit

The cytoplasmic domain is not important for the functional assembly of the $\alpha$-complex. The other is that the role of the cytoplasmic domain of $H^+\cdot K^+$-ATPase $\beta$-subunit was similar to that of $Na^+\cdot K^+$-ATPase $\beta$-subunit; therefore, this domain is compatible between $H^+\cdot K^+$-ATPase and $Na^+\cdot K^+$-ATPase despite the low amino acid homology. It should be pointed out that the $\alpha$-NaH complex showed a significantly (40%) higher $K^+$-ATPase activity than the wild-type $H^+\cdot K^+$-ATPase $\alpha$-$\beta$ complex. Because there was no difference in the affinity for $K^+$ and the apparent expression level of $H^+\cdot K^+$-ATPase $\alpha$-subunit on the blot between $\alpha$-NaH complex and the wild-type $H^+\cdot K^+$-ATPase $\alpha$-$\beta$ complex, the NaH chimeric $\beta$-subunit may stabilize the $H^+\cdot K^+$-ATPase $\alpha$-subunit in the membrane more efficiently than the wild-type $\beta$-subunit, suggesting that the cytoplasmic domain has an assisting role.

A chimeric $\beta$-subunit (NNH), which consists of the cytoplasmic plus transmembrane domains of $Na^+\cdot K^+$-ATPase and the ectodomain of $H^+\cdot K^+$-ATPase, also formed a stable complex with the $H^+\cdot K^+$-ATPase $\alpha$-subunit to exhibit the $H^+\cdot K^+$-ATPase activity (Figs. 5, 6, and 8). Therefore, the whole transmembrane domain of the $\beta$-subunit is also compatible between $Na^+\cdot K^+$-ATPase and $H^+\cdot K^+$-ATPase to form a functional $H^+\cdot K^+$-ATPase. However, the stabilization of the $\alpha$-subunit by the $\beta$-subunit, and $H^+\cdot K^+$-ATPase activity were abolished when the sequence 76QLKS79 in the ectodomain of $H^+\cdot K^+$-ATPase $\beta$-subunit was replaced by the counterpart, 79ENVP75, of $Na^+\cdot K^+$-ATPase $\beta$-subunit. Therefore, this portion is the first N-terminal amino acid block that is not conserved between the $H^+\cdot K^+$-ATPase and $Na^+\cdot K^+$-ATPase. It is also noteworthy that this portion was modified when restriction sites were introduced in the preparation of chimeras between the $H^+\cdot K^+$-ATPase and $Na^+\cdot K^+$-ATPase $\beta$-subunits in the previous study done by Jaunin et al. (18).

When the parts of the whole ectodomain of $H^+\cdot K^+$-ATPase $\beta$-subunit were replaced by the counterparts of $Na^+\cdot K^+$-ATPase $\beta$-subunit in this study, chimeric $\beta$-subunits such as NaVH, HcN, and HnN didn't stabilize the $\alpha$-subunit in the membrane, resulting in loss of the $H^+\cdot K^+$-ATPase activity. However, one small extracellular segment located between Cys152 (Sph1 site) and Cys178 (EcoT22I site) was replaceable. This segment contains the second S-S loop (Cys162 and Cys178). The amino acid identity of this segment is around 53% between $H^+\cdot K^+$-ATPase and $Na^+\cdot K^+$-ATPase, higher than the overall amino acid identity. It is likely that this segment is compatible between the $H^+\cdot K^+$-ATPase and $Na^+\cdot K^+$-ATPase because the structure of this segment is relatively conserved between two ATPases, although it is not completely excluded that this segment is not directly involved in the stabilization of the $\alpha$-subunit or the function of the enzyme.

Hamrick et al. (40) prepared chimeric proteins between the $Na^+\cdot K^+$-ATPase $\beta$-subunit and dipetidyl peptidase IV and found that the ectodomain of the $Na^+\cdot K^+$-ATPase $\beta$-subunit was sufficient for assembly with the $Na^+\cdot K^+$-ATPase $\alpha$-subunit. They also prepared deletion mutants that lack extracellular C-terminal portions of the $Na^+\cdot K^+$-ATPase $\beta$-subunit and reported that deletions of up to 146 extracellular amino acids from the C terminus of the $\beta$-subunit allow reduced assembly with the $Na^+\cdot K^+$-ATPase $\alpha$-subunit. This deletion mutant had the first S-S loop but lacked the second and third S-S loops, and the C terminus was Asn159, which was close to Sph1 site used in the present work. Recently, Colonna et al. (41) reported that the segment from Glu63 (Phe68 in the present work) to Asp125 (Asn130, MunI site in the present work) of the $Na^+\cdot K^+$-ATPase $\beta$-subunit was critical in $\alpha\beta$ assembly of the $Na^+\cdot K^+$-ATPase using a two-hybrid assay system in yeast. It is noteworthy that the tryptic cleavage site between Arg134 and Gly135 (located on the first disulfide loop) of the ectodomain of $Na^+\cdot K^+$-ATPase $\beta$-subunit is hidden in the presence of Rb+ and exposed in the presence of Mg2+/Pi, suggesting that this region is close to the site of interaction between $\alpha\beta$-subunits and the K+ binding pocket (42). The sequence (Arg-Gly) was not conserved in $H^+\cdot K^+$-ATPase $\beta$-subunit. Melle-Milovanovic et al. (43) identified two different segments in the ectodomain of $H^+\cdot K^+$-ATPase $\beta$-subunit, from Glu64 to Asn130 and from Ala156 to Arg188 as possibly associated with the $\alpha$-subunit from the yeast two-hybrid analysis. The former segment includes 72RVAP75 sequence reported in the present work, and the latter partly overlaps the segment found in this study to be replaceable by the $H^+\cdot K^+$-ATPase and $Na^+\cdot K^+$-ATPase $\beta$-subunits. Fig. 10 shows the segments in $H^+\cdot K^+$-ATPase $\beta$-subunit replaceable with those of $Na^+\cdot K^+$-ATPase $\beta$-subunit.

Recently, rat colonic $H^+\cdot K^+$-ATPase cRNA and guinea pig colonic $H^+\cdot K^+$-ATPase cDNA were functionally expressed in Xenopus oocyte (44) and HEK-293 cells (17), respectively. In these studies, 86Rb uptake and $K^+$-ATPase activity were observed under co-expression of the colonic $H^+\cdot K^+$-ATPase catalytic subunit ($\alpha$-subunit) with either $Na^+\cdot K^+$-ATPase $\beta$-subunit or gastric $H^+\cdot K^+$-ATPase $\beta$-subunit. It is of interest that both rat and guinea pig colonic $H^+\cdot K^+$-ATPase $\alpha$-subunits assembled with either $\beta$-subunit to functionally express the ATPase.

In conclusion, we have shown that the whole cytoplasmic and transmembrane domains of $H^+\cdot K^+$-ATPase $\beta$-subunit can be replaced by those of $Na^+\cdot K^+$-ATPase $\beta$-subunit, retaining the $\alpha\beta$ assembly capacity, $H^+\cdot K^+$-ATPase activity and affinity for $K^+$.
The ATPase activity was almost abolished when 4 amino acids (T9QLKS79) in the early N-terminal part of the ectodomain of H⁺,K⁺-ATPase β-subunit were replaced by the counterpart of Na⁺,K⁺-ATPase β-subunit. The segment between Cys152 and Cys178 of H⁺,K⁺-ATPase β-subunit was also replaceable with the corresponding segment of Na⁺,K⁺-ATPase β-subunit, preserving αβ assembly and H⁺,K⁺-ATPase activity almost intact.

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