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Stable Expression of Gastric Proton Pump Activity at the Cell Surface[1]

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Stable cell lines expressing the gastric proton pump α- and/or β-subunits were constructed. The cell line co-expressing the α- and β-subunits showed inward Rb^+ transport, which was activated by Rb^+ in a concentration-dependent manner. In the α+β-expressing cell line, rapid recovery of intracellular pH was also observed after acid load, indicating that this cell line transported protons outward. These ion transport activities were inhibited by a proton pump inhibitor, 2-methyl-8-(phenylmethoxy)imidazo[1,2-*a*]pyridine-3-acetonitrile (SCH 28080). In a membrane fraction of the α+β-expressing cell line, K^+-stimulated ATPase (K^+-ATPase) activity and the acylphosphorylation of the α-subunit were observed, both of which were also inhibited by SCH 28080. The specific activity and properties of the K^+-ATPase were comparable to those found in the native gastric proton pump. In the stable cell lines, the α-subunit was retained in the intracellular compartment and was unstable in the absence of the β-subunit, but it was stabilized and reached the cell surface in the presence of the β-subunit. On the other hand, the β-subunit was stable and able to travel to the cell surface in the absence of the α-subunit. These cell lines are ideal for the structure-function study of ion transport by the gastric proton pump as well as for characterization of the cellular regulation of surface expression of the functional proton pump.

Key words: active transport, functional expression, gastric acid, H^+,K^+-ATPase, proton pump.

The gastric proton pump, H^+,K^+-ATPase, actively transports protons and K^+ ions, coupled with the hydrolysis of ATP. This pump consists of a catalytic α- and a non-catalytic β-subunit. The α-subunit with a molecular mass of 114 kDa contains sites for ATP-binding (*1, 2*) and its acylphosphorylation (*3*), binding sites of proton pump inhibitors (*4–7*) and sites responsible for ion recognition (*6, 8–11*). The β-subunit with a molecular mass of 33 kDa (as a protein core) contains six or seven carbohydrate chains and three disulfide bonds in the ectodomain (*12–14*). This subunit is also essential for the functional expression of H^+,K^+-ATPase through the interaction with the catalytic α-subunit (*8, 15, 16*). The interaction between the α- and β-subunits seems to be important for the conformational stability of the functional holoenzyme (*17*). The β-subunit contains a Tyr-based sorting signal for internalization (*18*), and it is involved in intracellular trafficking of the α- and β-subunits.

To perform mutational studies on the roles of specific domains or amino acid residues in the reaction mechanism or in the cellular regulation of enzymes, construction of good functional expression systems is necessary. There have been several reports of transient expression of functional gastric H^+,K^+-ATPase in insect Sf9 cells, *Xenopus* oocytes and human HEK-293 cells (*8, 15, 16*). Recently, stable cell lines expressing the ammonium-stimulated ATPase activity have also been constructed; ammonium ions easily permeate the cell membrane and stimulate the H^+,K^+-ATPase activity in a manner similar to K^+ (*19*). However, there have been no reports of the construction of stable cell lines containing ion transport activity, and this lack has hampered the structure-function studies of the sites directly involved in ion transport or energy coupling between ATP hydrolysis and ion transport. In this report, we constructed stable cell lines co-expressing the H^+,K^+-ATPase α- plus β-subunits that showed inward Rb^+ transport as well as outward proton transport. We also studied the expression levels of the α- and β-subunits and partial reaction steps of the expressed H^+,K^+-ATPase, and showed that the expressed H^+,K^+-ATPase retained similar properties and potency to native gastric H^+,K^+-ATPase.

In this study, we also constructed stable cell lines expressing the α-subunit alone and the β-subunit alone, and precisely compared the intracellular localization and the stability of each subunit by immunofluorescence analysis and pulse-chase labeling experiments, respectively.

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Abbreviations: SCH 28080, 2-methyl-8-(phenylmethoxy)imidazo-[1,2-*a*]pyridine-3-acetonitrile; PBS, phosphate-buffered saline; BCECF, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein; FITC, fluorescein isothiocyanate; ER, endoplasmic reticulum.

EXPERIMENTAL PROCEDURES

Materials—HEK-293 cells (human embryonic kidney cell line) were a kind gift from Prof. Jonathan Lytton (University of Calgary, Calgary, Canada). The pcDNA3.1/ZEO(+) and pcDNA3 vectors were obtained from Invitrogen (San Diego, CA). Effectene Transfection Reagent and EndoFree Plasmid Maxi and Mega kits were obtained form Qiagen (Tokyo). Restriction enzymes and other DNA and RNA modifying enzymes were from Toyobo (Osaka), New England Biolabs (Beverly, MA) or Amersham Pharmacia Biotech. (Tokyo). 2-Methyl-8-(phenylmethoxy)imidazo[1,2-*a*]-pyridine-3-acetonitrile (SCH 28080) was a kind gift from Dr. Peter Chiu (Schering-Plough, Kenilworth, NJ). Anti-gastric H^+,K^+-ATPase α-subunit monoclonal antibody 1H9 and anti–β-subunit monoclonal antibody 2B6 were obtained from Molecular Biological Laboratories (Nagoya). Anti–H^+,K^+-ATPase β-subunit monoclonal antibody 2/2E6 was a kind gift from Dr. Curtis Okamoto (University of Southern California, Los Angeles, CA). Anti–H^+,K^+-ATPase α-subunit antibody HK9 was a kind gift from Prof. Michael Caplan (Yale University School of Medicine, New Haven, CT). 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 of H^+,K^+-ATPase were prepared from rabbit gastric mucosae as described elsewhere (*8*). The α- and β-subunit cDNAs were digested with *Eco*RI and *Xho*I. The obtained fragments were each ligated into the pcDNA3 vector (for the β-subunit cDNA) or pcDNA3.1/ZEO(+) (for the α-subunit cDNA) treated with *Eco*RI and *Xho*I.

DNA Sequencing—DNA sequencing was done by the dideoxy chain termination method using an ABI Prism 377 DNA sequencer (Applied Biosystems, Tokyo).

Transfection and Selection of Stable Cell Lines—Cell culture of the HEK-293 cell line was carried out as described previously (*6, 8*). Stable cell lines expressing gastric H^+,K^+-ATPase subunit(s) were constructed as follows. For construction of the β-expressing cell line, HEK-293 cells were transfected with the pcDNA3-HKβ cDNA construct by lipofection using an Effectene Transfection Reagent, and stable cell lines were selected in the presence of 1 mg/ml Geneticin® (G-418 sulafate). Single colonies were isolated, expanded and maintained in the presence of 0.5 mg/ml Geneticin®. For construction of the α-expressing cell line, HEK-293 cells were transfected with the pcDNA3.1/ZEO(+)-HKα cDNA construct, and stable cell lines were selected in the presence of 0.1 mg/ml Zeocin. Single colonies were isolated, expanded and maintained in the presence of 0.1 mg/ml Zeocin. Cloning was performed by limited dilution in 96-well microplates. The expression of the α- or β-subunit was confirmed by immunofluorescence and Western blotting. For construction of the α+β-expressing cell line, the β-expressing cell lines were transfected with the pcDNA3.1/ZEO(+)-HKα cDNA construct, and stable cell lines were selected in the presence of 0.2 mg/ml Zeocin plus 0.5 mg/ml Geneticin®. Single colonies were isolated, ex- panded and maintained in the presence of 0.5 mg/ml Geneticin® and 0.2 mg/ml Zeocin. The expression of the α- and β-subunits was confirmed by immunofluorescence and Western blotting.

Preparation of Membrane Fractions—Membrane fractions from stable cell lines were prepared as described previously (*8*). Briefly, cells in a 10-cm Petri dish were washed with phosphate-buffered saline (PBS) and incubated in 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 addition of phenylmethylsulfonyl fluoride (1 mM) and aprotinin (0.09 unit/ml), the cells were homogenized in a Dounce homogenizer, and the homogenate was diluted with an equal volume of a solution composed of 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 composed of 250 mM sucrose and 5 mM Tris/HCl (pH 7.4).

SDS–Polyacrylamide Gel Electrophoresis and Western Blotting—SDS–polyacrylamide gel electrophoresis was carried out as described elsewhere (*20*). Membrane preparations (30 μg protein) were incubated in a sample buffer composed of 2% SDS, 2% β-mercaptoethanol, 10% glycerol, and 10 mM Tris/HCl (pH 6.8) at room temperature for 2 min, and separated on an SDS–polyacrylamide gel. Western blotting was carried out as described previously (*8*).

Antibodies—Anti–α-subunit antibody Ab1024 was previously raised against the carboxy-terminal peptide (residues 1024–1034) of the H^+,K^+-ATPase α-subunit (*21*). Anti–β-subunit monoclonal antibody 2B6 and anti–α-subunit monoclonal antibody 1H9 were derived from the splenocytes of mice with autoimmune gastritis (*22*). The epitope of 2B6 was located on the carboxy-terminal portion of the β-subunit. None of the antibodies used cross-reacted with endogenous Na^+,K^+-ATPase α- and β-subunits.

Immunohistochemistry—Transfected HEK cells were fixed for 7 min in cold methanol (–20°C) and washed three times with PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ [PBS(+)]. Cells were permeabilized in a permeabilization buffer of 0.3% Triton X-100 and 0.1% BSA in PBS(+) for 15 min at room temperature. Nonspecific antibody binding was blocked by preincubating in a goat serum dilution buffer solution [16% goat serum, 0.3% Triton X-100, 0.9% NaCl, and 20 mM Na phosphate (pH 7.4)] for 30 min. All antibody incubations were carried out using the goat serum dilution buffer solution. Cells were incubated for 1 h at room temperature with anti–α-subunit (HK9) or anti–β-subunit (2B6) antibody, followed by three washes with the permeabilization buffer. Rhodamine-conjugated anti–rabbit IgG and fluorescein isothiocyanate (FITC)–conjugated anti–mouse IgG secondary antibodies were used for 1 h at room temperature at a 1:100 dilution. After washing with PBS(+) three times, immunofluorescence images were visualized using a Zeiss LSM 510 laser scanning confocal microscope. When the cells were incubated with the 2/2E6 antibody, cells were fixed with 3.5% formaldehyde at room temperature for 30 min instead of methanol, and the antibody incubation was carried out without permeabilization.

Pulse-Chase Labeling and Immunoprecipitation—Stable cell lines were cultured on collagen-coated 6-well plates. Cells were washed and incubated at 37°C for 30 min in methionine-free medium. Cells were labeled for 60 min with [^{35}S]Met, Cys labeling mixture (Express) (NEN), and chased in complete Dulbecco's modified Eagle medium for indicated periods. Cells were washed with washing buffer composed of 150 mM NaCl, 0.5 mM EDTA, and 50 mM Tris/HCl (pH 7.4), and incubated in 500 μl of lysis buffer composed of 1% Nonidet P-40, 150 mM NaCl, 0.5 mM EDTA, and 50 mM Tris/HCl (pH 7.4) at 4°C for 30 min.

After centrifugation at 16,000 ×*g* for 20 min, the supernatant was incubated with anti–α-subunit antibody 1H9 or anti–β-subunit antibody 2B6 at a 1:100 dilution, and 10 μl of ImmunoPure Immobilized Protein A (Pierce, Rockford, IL) 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 in-cubated at room temperature for 10 min. The proteins separated on the SDS–polyacrylamide gel were visualized by digital autoradiography of dried gels using a Bio Imaging Analyzer BAS 2000 (Fuji Photo Film, Tokyo).

Quantification of Expressed H^+,K^+-ATPase in the Membrane Fraction—A hog gastric vesicle preparation containing gastric H^+,K^+-ATPase was prepared as described elsewhere (*23*). The H^+,K^+-ATPase α-subunit comprises about 60% of the protein content of the gastric vesicle preparation judged by the densitometric analysis of the SDS-polyacrylamide gel using the ATTO Densitographic software (ATTO, Tokyo). The content of expressed H^+,K^+-ATPase α- and β-subunits was quantified by comparing with the subunits in the gastric vesicle preparation. The content of β-subunit was quantified after treatment with *N*-glycosidase F. Membrane fractions from the stable cell lines were separated on the same SDS–polyacrylamide gel as a series of diluted gastric vesicle preparations and subjected to Western blotting. The blots were scanned using an optical scanning image system. The content of H^+,K^+-ATPase in the membrane fractions was estimated from the standard curve of the gastric vesicle preparation.

Glycosidase Treatment—Thirty micrograms of membrane fraction was treated with 5 units of *N*-glycosidase F in a solution composed of 0.1% SDS, 1% *n*-octylglucoside, 1 M 2-mercaptoehanol, 30 mM EDTA, and 50 mM sodium phosphate (pH 6.0) at 37°C overnight as described previously (*24*).

Rubidium Transport Assay—Cells were grown to confluence in 6-well collagen-coated plates. The medium was then removed, and the wells were washed with 4 ml of ice-cold wash solution composed of 144 mM NaCl and 5 mM HEPES/NaOH (pH 7.4). Each well was incubated in 1 ml of solution composed of 144 mM NaCl, 5 mM HEPES/NaOH (pH 7.4), 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM RbCl (3×10^6 cpm ^{86}Rb), 500 μM ouabain, and 10 μM furosemide at 37°C for 10 min. Rubidum transport was assayed in the presence and absence of various concentrations of SCH 28080. After a 10-min incubation, the supernatant was removed, and each well was washed with 4 ml of ice-cold wash solution. Cells were solubilized with 2 ml of lysis buffer composed of 1% Nonidet P-40, 150 mM NaCl, 0.5 mM EDTA, and 50 mM Tris/HCl (pH 7.4). One milliliter of solution was mixed with 5 ml of aqueous counting scintillant, ACS-II (Amersham Pharmacia Biotech), and the radioactivity was counted. When the effect of Rb^+ concentrations on ^{86}Rb transport was studied, RbCl concentration was varied from 0.1 to 10 mM.

Measurement of pH_i—Intracellular pH (pH_i) of the mock-transfected cells and the stable cell lines was measured by monitoring the fluorescence of 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) as described previously (*25*). The pH_i recovery from acid loading by treatment with an NH_3/NH_4 pulse was compared in α+β-expressing cells and mock-transfected cells. Stable cell lines were plated on a coverslip coated with poly-L-lysine and grown to confluency for one day. The cells were incubated with 5 μM acetoxymethyl ester of BCECF (Molecular Probes, Junction City, OR) in the loading buffer composed of 145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, 2 mM NaH_2PO_4, 10.5 mM glucose, and 32 mM HEPES (pH 7.4) for 30 min at 37°C. The coverslip was placed in the chamber (1 ml volume) of a spectrofluorometer (RF-5000 Shimadzu) equipped with a continuous buffer flow system. Intracellular BCECF was alternately excited with 505 and 448 nm light, and the emitted light was measured at 535 nm.

The cells were acid-loaded by successively incubating in the NH_3/NH_4-containing buffer [125 mM NaCl, 20 mM NH_4Cl, 5 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, 2 mM NaH_2PO_4, 10.5 mM glucose, and 32 mM HEPES (pH 7.4)], and the Na^+-free buffer [145 mM *N*-methyl-D-glucamine, 5 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, 2 mM NaH_2PO_4, 10.5 mM glucose, and 32 mM HEPES (pH 7.4)]. The time course of pH_i recovery after acid loading was monitored. For the intracellular pH calibration, the pH_i value was monitored in buffers with various pHs containing nigericin and monensin.

Assay of H^+,K^+-ATPase Activity—H^+,K^+-ATPase activity was measured in 1 ml of solution composed of 50 μg of membrane protein, 3 mM $MgCl_2$, 1 mM ATP, 5 mM NaN_3, 2 mM ouabain, and 40 mM Tris/HCl (pH 6.8) in the presence and absence of 15 mM KCl. After incubation at 37°C for 30 min, inorganic phosphate released was measured as described elsewhere (*26*). The K^+-ATPase activity was calculated as the difference between activities in the presence and absence of KCl.

Protein was measured using the BCA Protein Assay Kit from Pierce with bovine serum albumin as a standard.

Phosphorylation and Dephosphorylation—A 50-μg portion of membrane proteins was phosphorylated in 110 μl of solution composed of 1 μM ATP ([γ-^{32}P]ATP 4×10^6 cpm), 2 mM $MgCl_2$, 1 mM EGTA, 3 mM ouabain, and 40 mM Tris/HCl (pH 6.8) at 0°C for 15 s. The reaction was quenched by the addition of 590 μl of ice-cold stop solution composed of 10% trichloroacetic acid and 10 mM inorganic phosphate. The protein was collected by centrifugation at 13,000 ×*g* at 4°C for 3 min, and the pellet was washed successively with 500 μl of ice-cold stop solution and 30% sucrose, solubilized in a sample buffer composed of 2% SDS, 2.5% dithiothreitol, 10% glycerol, and 50 mM Tris/HCl (pH 6.8), and subjected to the SDS–polyacrylamide gel electrophoresis under acidic conditions at pH 6.5 (*27*). The radioactivity associated with the separated H^+,K^+-ATPase α-subunit was visualized and quantified by digital autoradiography of the dried gels using a Bio-Imaging Analyzer BAS2000.

In the dephosphorylation experiments, membrane proteins were phosphorylated as mentioned above, then incubated with various concentrations of KCl and non-radioactive ATP at 0°C for 15 s. The reaction was stopped as described above, and the proteins were separated on the SDS–polyacrylamide gel. The radioactivity associated with the H^+,K^+-ATPase α-subunit was visualized as described above.

RESULTS

Expression of the α- and β-Subunits in Stable Cell Lines—Stable cell lines expressing the gastric H^+,K^+-ATPase α-subunit alone, the β-subunit alone, and the α- plus β-subunits were constructed. The expression and localization of the α- and β-subunits in these cells were studied by the immunofluorescence technique with a laser scanning confocal microscope.

Figure 1 shows immunofluorescent patterns of the α- and β-subunits in representative stable cell lines. In the α-expressing cells, the α-subunit was observed only in the intracellular compartment, and no cell-surface expression was observed (A). The expression level of the α-subunit was low. In spite of the twice limited dilution for cloning, the expression pattern of the α-subunit was heterogeneous. In the β-expressing cells, the expression pattern of the β-subunit was homogeneous. The β-subunit was observed at the cell surface as well as the intracellular compartment (B). In the α+β-expressing cells, both subunits were expressed at the cell surface and intracellularly (C, D). The distribution of the α-subunit overlapped well with that of the β-subunit (E). These findings indicate that the β-subunit by itself can

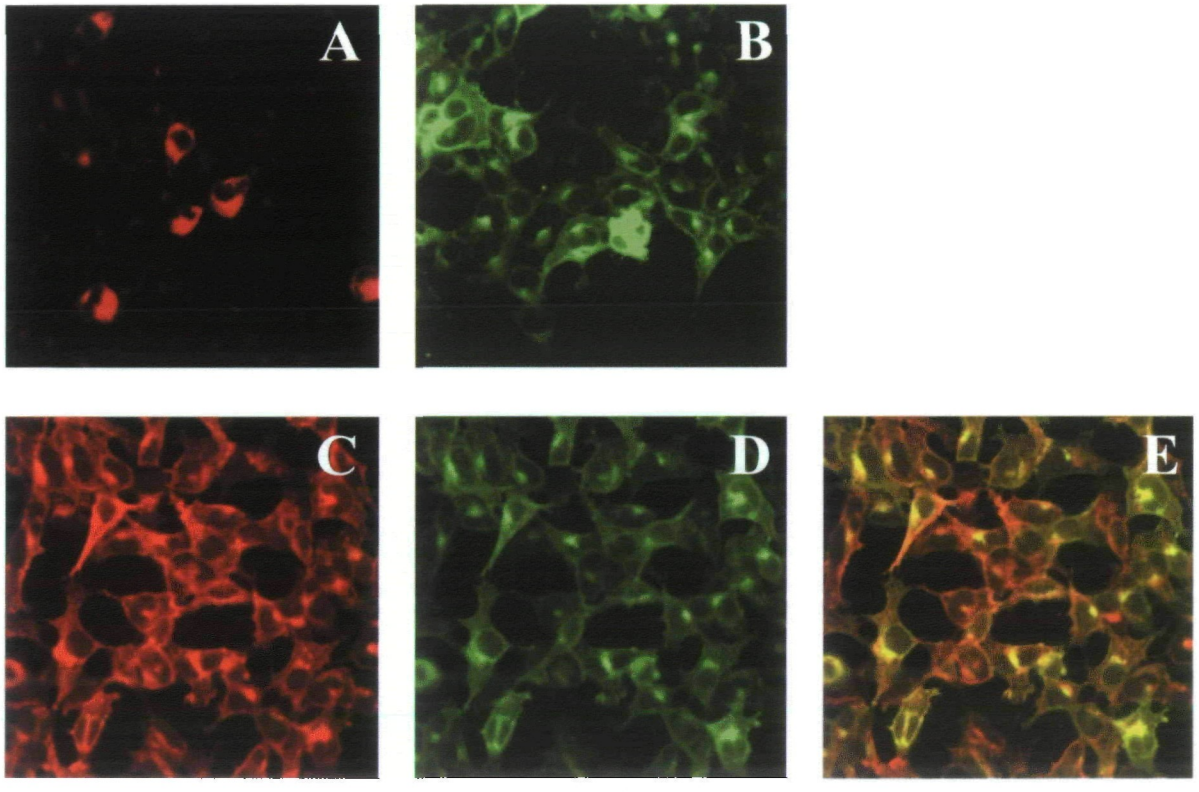

Fig. 1. **Immunolocalization of the H^+,K^+-ATPase α- and β-subunits expressed in stable cell lines.** HEK-293 cells were stably transfected with the cDNAs encoding the H^+,K^+-ATPase α-subunit alone (A), the β-subunit alone (B), or the α-plus β-subunits (C–E). Immunofluorescence analysis was performed using an anti-α antibody (HK9) and an anti-β antibody (2B6) under permeabilizing conditions. The α- (A, C) and β-subunits (B, D) were detected with secondary antibodies conjugated to rhodamine and FITC, respectively. The merged pattern of figures (C) and (D) is presented (E).

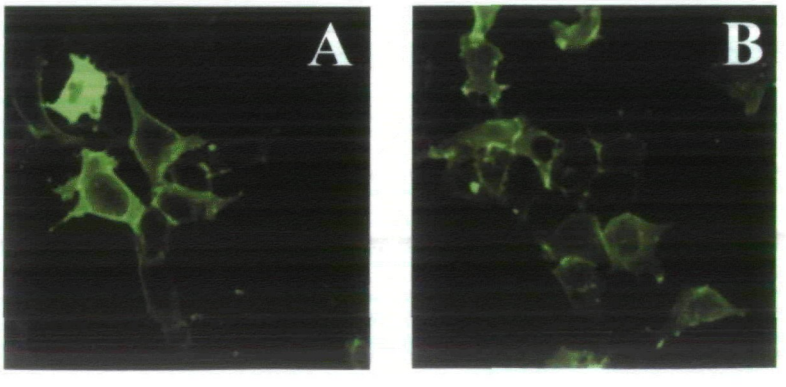

Fig. 2. **Immunofluorescence analysis of the H^+,K^+-ATPase β-subunit expressed in stable cell lines with an anti-β-subunit antibody, 2/2E6.** The β-expressing (A) or α+β-expressing cells (B) were stained with antibody 2/2E6 under non-permeabilizing conditions. The β-subunits were detected with a secondary antibody conjugated to FITC.

leave the endoplasmic reticulum (ER) to reach the cell surface, whereas the α-subunit cannot reach the cell surface without the β-subunit, as previously reported in the transient expression systems in HEK-293 cells and COS-7 cells (*13, 25, 28*). The expression level of the α-subunit was significantly higher in the α+β-expressing cells than in the α-expressing cells, whereas the expression level of the β-subunit in the α+β-expressing cells was apparently unchanged from that in the β-expressing cells.

Monoclonal antibody 2/2E6 against the H^+,K^+-ATPase β-subunit recognizes the epitope $S^{226}LHY^{229}$ located in the ectodomain of the β-subunit. This epitope seems to overlap the site involved in assembly between the α- and β-subunits (*29*), because antibody 2/2E6 binds only to the unassembled or free β-subunit. We tried to stain the β-expressing and α+β-expressing cell lines with antibody 2/2E6 to study whether the β-subunit assembled with the endogenous Na^+,K^+-ATPase or the exogenous H^+,K^+-ATPase α-subunit at the cell surface in these cell lines. As shown in Fig. 2, antibody 2/2E6 stained the β-subunit at the surface of the β-expressing cells (A) and the α+β-expressing cells (B) without any treatment for denaturation. Therefore, in the β-expressing cells, the β-subunit seems to reach the cell surface unassembled with the endogenous Na^+,K^+-ATPase α-subunit, although the H^+,K^+-ATPase β-subunit is able to assemble with the Na^+,K^+-ATPase α-subunit to act as a surrogate for the Na^+,K^+-ATPase β-subunit in *Xenopus* oocytes (*30*) and in transfected MDCK cells (*29*). In the α+β-expressing cells, some portion of the β-subunit seems to be located at the cell surface unassembled with the exogenous H^+,K^+-ATPase α-subunit or the endogenous Na^+,K^+-ATPase α-subunit.

To study the steady-state expression levels of the α- and β-subunits in membrane fractions prepared from each cell line, Western blotting was performed with antibodies against the α- and β-subunits, respectively. The expression level of the α-subunit in the membrane fractions was quantitatively compared with those in a series of diluted gastric vesicle preparation used as an internal standard (Fig. 3). The expression of the α-subunit in the α-expressing cells was significantly lower than that in the α+β-expressing cells. The α-subunit content in 30 μg of membrane fractions from the α-expressing and α+β-expressing cell lines was equivalent to the α-subunit content present in 0.06 and 0.48 μg of a gastric vesicle preparation, respectively. Since the H^+,K^+-ATPase α-subunit comprises about 60% of the protein content of the gastric vesicle preparation, we estimate that 1 mg of membrane fraction of the α+β-expressing cells contains 9.6 μg of the α-subunit.

Figure 4A shows the Western blot with an anti–β-subunit antibody of membrane fractions prepared from the α+β-expressing and β-expressing cell lines, and from the cells transiently co-transfected with the α- and β-subunit cDNAs. A major proportion of the β-subunits found in membrane fractions of the α+β-expressing and β-expressing cell lines (lanes 1 and 2) was modified with complex-type carbohydrate chains with a molecular mass of 60–70 kDa, which were resistant to endoglycosidase H treatment (data not shown). These patterns of the β-subunits are considerably different from that found in membrane fractions of the cells transiently expressing the α- and β-subunits, in

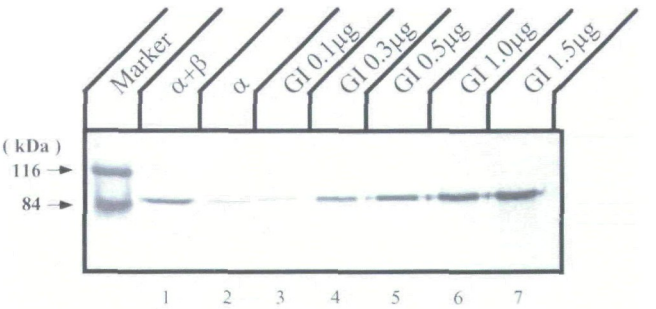

Fig. 3. **Western blots with an anti-α-subunit antibody of the membrane fractions prepared from the α+β-expressing and α-expressing cells.** Portions of 30 μg of membrane fractions prepared from the α+β-expressing cell lines (lane 1), the α-expressing cell lines (lane 2), and a series of diluted gastric vesicle preparations (0.1–1.5 μg) (lanes 3–7) were separated on a gel and subjected to Western blotting with anti α-subunit antibody Ab1024.

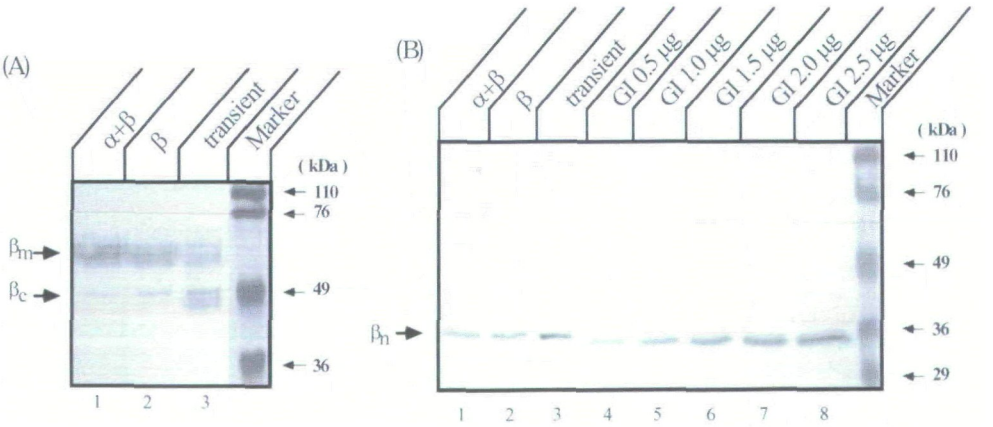

Fig. 4. **(A) Western blots with an anti β-subunit antibody of membrane fractions prepared from the α+β-expressing and β-expressing cell lines, and cells transiently co-transfected with the α- and β-subunit cDNAs.** Portions of 30 μg of membrane fractions prepared from the α+β-expressing cell line (lane 1), the β-expressing cell line (lane 2), and from the cells transiently co-transfected with the α- and β-subunit cDNAs (lane 3) were separated on a gel and subjected to Western blotting with anti–β-subunit antibody 2B6. β_m and β_C represent the β-subunits with complex-type (mature) carbohydrate chains, and that with high-mannose type (core) carbohydrate chains, respectively. **(B) Western blots with an anti–β-subunit antibody of the membrane fractions and gastric vesicle preparations after *N*-glycosidase F treatment.** Portions of 30 mg of membrane fractions prepared from the a+b-expressing cell line (lane 1), the b-expressing cell line (lane 2), from the cells transiently co-transfected with the α- and β-subunit cDNAs (lane 3), and a series of diluted gastric vesicle preparations (0.5–2.5 mg) (lanes 4–8) were treated with N-glycosidase F as described in the "EXPERIMENTAL PROCEDURES." The proteins were separated on a gel and subjected to Western blotting with anti–β-subunit antibody 2B6. β_n represents the protein core of the β-subunit.

which the β-subunits modified with high mannose-type carbohydrate chains with a molecular mass of 50 kDa were more abundant. These results indicate that a major fraction of the β-subunits in these stable cell lines leaves the ER and is expressed at the transGolgi or the cell surface, whereas those in the transient expression system accumulate in the ER. These results are in good agreement with the results of the immunofluorescence study shown in Figs. 1 and 2.

The band representing the H^+,K^+-ATPase β-subunit is broad due to the heterogeneity of sugar chains, especially of the β-subunit in native gastric vesicles (data not shown). Therefore, the expression level of the β-subunit in the membrane fractions was quantitatively compared with those in a series of diluted gastric vesicle preparations after treatment with *N*-glycosidase F as shown in Fig. 4B. The molecular mass of the protein core of the β-subunit was slightly different between the membrane fractions (lanes 1–3) and the gastric vesicle preparations (lanes 4–8) due to the difference in animal species (rabbit and pig). The β-subunit content in 30 μg of membrane fractions from the α+β-expressing (lane 1) and the β-expressing cells (lane 2) was equivalent to the β-subunit content present in 0.73 and 0.67 μg of a gastric vesicle preparation, respectively. Therefore, the expression level of the β-subunit in the α+β-expressing cells was similar to that in the parental β-expressing cells.

Pulse-Chase Labeling of the α- and β-Subunits—To precisely study the stability of the α- and β-subunits, we performed pulse-chase labeling experiments of the α-expressing, β-expressing, and α+β-expressing cells. Cells were labeled with [^{35}S]Met/Cys for 1 h, followed by cold chase for various periods, and their membrane fractions were immunoprecipitated with either the anti–α-subunit or anti–β-subunit antibody. The immunoprecipitated proteins were separated on SDS–polyacrylamide gels and visualized by digital autoradiography (Fig. 5). Expression of the α-subunit found in the α-expressing cells in a 1-h pulse period (chase time, 0) was higher than that found in the α+β-expressing cells. In the α-expressing cells, degradation of the α-subunit was apparent within 3 h and almost complete within 6 h. However, in the α+β-expressing cells, the α-subunit was much more stable. More than 70% of the α-subunit was observed after a 6-h chase period.

In both the β-expressing and α+β-expressing cells, the β-subunits were modified with complex-type carbohydrate chains (β_m) within 3 h of chase. The β_m was stable at chase times of 3 to 6 h. The modification process and stability of the β-subunit were similar in the β-expressing cells and the α+β-expressing cells (Fig. 5).

Rubidium Transport of the Stable Cell Lines—Transport of ^{86}Rb into stable cell lines was studied in the presence of 500 μM ouabain and 10 μM furosemide to inhibit Na^+,K^+-ATPase and NaK2Cl transporter, respectively. ^{86}Rb transport activities found in the α-expressing and β-expressing cells were similar to that found in the mock-transfected cells in the presence and absence of 100 μM SCH 28080 (Fig. 6). The background ^{86}Rb transport activities found in these cells were not inhibited by SCH 28080. The ^{86}Rb transport activity of the α+β-expressing cells in the absence

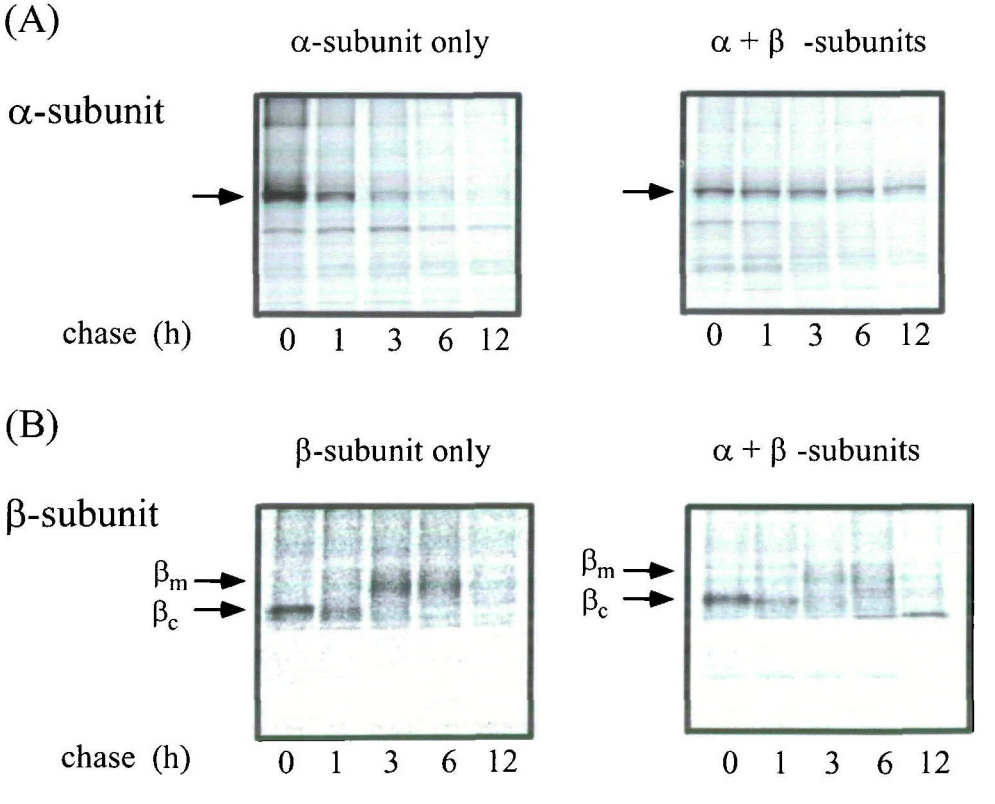

Fig. 5. **Pulse chase labeling of the α-expressing, β-expressing and α+β-expressing cells, followed by immunoprecipitation with an anti–α-subunit (A) or an anti–β-subunit antibody (B).** Stable cell lines were labeled for 60 min with [^{35}S]Met, Cys labeling mixture (Express) (NEN) in methionine-free, cysteine-free Dulbecco's modified Eagle medium, followed by a chase in complete medium for indicated periods. Cells were washed with washing buffer composed of 150 mM NaCl, 0.5 mM EDTA, and 50 mM Tris/HCl (pH 7.4), and incubated in 500 μl of lysis buffer buffer composed of 1% Nonidet P-40, 150 mM NaCl, 0.5 mM EDTA, and 50 mM Tris/HCl (pH 7.4) at 4°C for 30 min. After centrifugation at 16,000 ×*g* for 20 min, the supernatant was incubated with anti–α-subunit antibody 1H9 (A) or anti–β-subunit antibody 2B6 (B), and 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, separated on a gel, and visualized by digital autoradiography.

of SCH 28080 was 0.6 nmol/10^6 cells/min, 2.5-fold higher than that found in the mock-transfected cells. The ^{86}Rb transport activity of the α+β-expressing cells was inhibited by SCH 28080 with an IC_{50} value of 0.2 μM (Fig. 6), and was stimulated by Rb^+ in the extracellular medium in a concentration-dependent manner with a K_m value of 0.8 mM (Fig. 7).

Intracellular pH of the Stable Cell Lines—Intracellular pH (pH_i) values of the α+β-expressing and mock-transfected cells were measured by using a pH-sensitive fluorescence probe, BCECF. The average pH value of the α+β-expressing cells (7.47 ± 0.07) was higher than that of the mock-transfected cells (7.32 ± 0.12); however, this difference was not significant.

Measurement of pH_i Recovery of the Stable Cell Lines after Acid-Loading—To study the capacity of acid extrusion from the stable cell lines, the recovery of pH_i in cells acid-loaded through an ammonium pulse was observed (*25*). The pH_i recovery was monitored in the absence of Na^+ in order to exclude the contribution of Na^+/H^+ exchanger. The pH_i was monitored by the change in fluorescence of BCECF loaded in the cells (Fig. 8). The pH_i recovery process was very slow in the α-expressing (curve A), β-expressing (curve B) and mock-transfected (curve D) cells; and no clear difference was observed in the slope of pH_i recovery process between these three types. On the other hand, the α+β-expressing cells showed a rapid pH recovery after the medium was changed to the Na^+-free buffer solution (curve C). The rapid pH_i recovery was not observed when the α+β-expressing cells were incubated with 50 μM SCH 28080 (curve E). These findings suggest that the proton pump expressed at the cell surface of the α+β-expressing cells secretes protons when the pH_i is decreased. However, the proton pump activity was not detectable without the ammonium pulse. The change in the pH_i value due to the expressed proton pump under usual conditions may be compensated by the action of other ion transport systems expressed in the cell.

K^+-ATPase Activity of the Expressed H^+,K^+-ATPase—The K^+-ATPase activity was found in the membrane fraction prepared from the α+β-expressing cells. The specific activ-

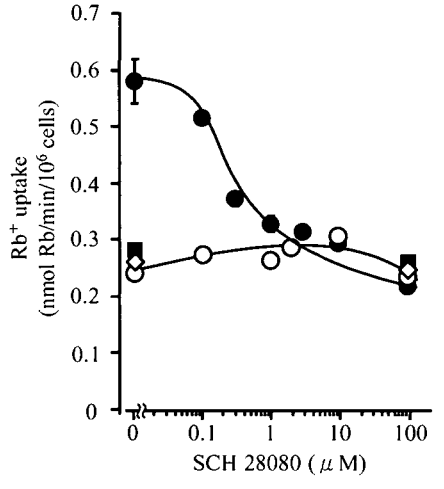

Fig. 6. **^{86}Rb transport activity of stable cell lines in the presence or absence of various concentrations of SCH 28080.** The α-expressing (■), β-expressing (◇), α+β-expressing cells (●), or mock-transfected cells (○) were grown to confluence in 6-well collagen-coated plates. The cells were incubated in 1 ml of solution composed of 144 mM NaCl, 5 mM HEPES/NaOH (pH 7.4), 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM RbCl (3 × 10^6 cpm ^{86}Rb), 500 μM ouabain, and 10 μM furosemide in the presence and absence of various concentrations of SCH 28080 at 37°C for 10 min. Cells were washed with 4 ml of ice-cold wash solution composed of 144 mM NaCl and 5 mM HEPES/NaOH (pH 7.4), and solubilized with 2 ml of lysis buffer composed of 1% Nonidet P-40, 150 mM NaCl, 0.5 mM EDTA, and 50 mM Tris/HCl (pH 7.4). One milliliter of lysate was mixed with 5 ml of aqueous counting scintillant, and its radioactivity was counted. The ^{86}Rb transport activity was expressed as Rb transported/10^6 cells in 1 min. The values are mean ± SE for three experiments.

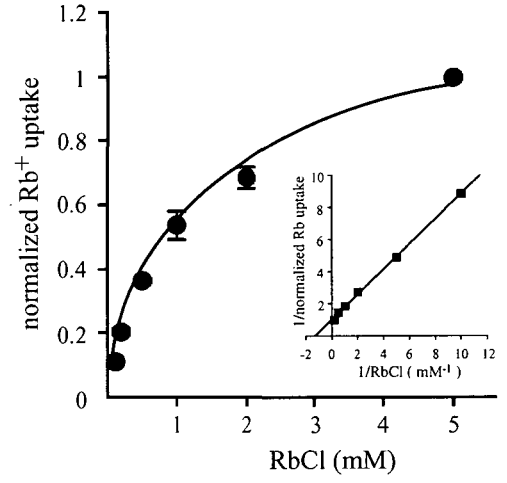

Fig. 7. **Effects of Rb^+ concentrations on ^{86}Rb transport activity of the α+β-expressing cells.** The α+β-expressing cells or the mock-transfected cells were grown to confluence in 6-well collagen-coated plates. The cells were incubated in 1 ml of solution composed of 144 mM NaCl, 5 mM HEPES/NaOH (pH 7.4), 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 500 μM ouabain, 10 μM furosemide, and various concentrations of RbCl (3 × 10^6 cpm ^{86}Rb) at 37°C for 10 min. The ^{86}Rb transport activity was measured as shown in Fig. 6. Results are corrected for specific activity of ^{86}Rb transport by subtracting the ^{86}Rb transport of the mock-transfected cells from that of the α+β-expressing cells, and normalized to activity at 5 mM Rb^+. The values are mean ± SE for 3 experiments. (Inset) The results are shown as the double-reciprocal plot of ^{86}Rb transport activity against Rb^+ concentration.

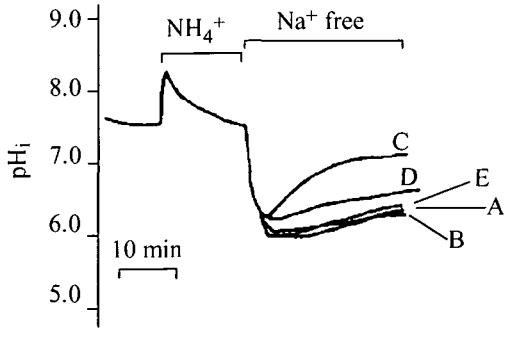

Fig. 8. **pH_i recovery of the stable cell lines and the mock-transfected cells from an acid load.** Intracellular pH recovery was monitored in the absence of extracellular Na^+ after an ammonium pulse of the α-expressing (A), β-expressing (B) and α+β-expressing cell (C, E) and the mock-transfected cell (D). The cells were incubated in the presence (E) or absence of 50 μM SCH 28080 (A–D). Intracellular pH of the cells was measured by monitoring the fluorescence of BCECF as described in "EXPERIMENTAL PROCEDURES."

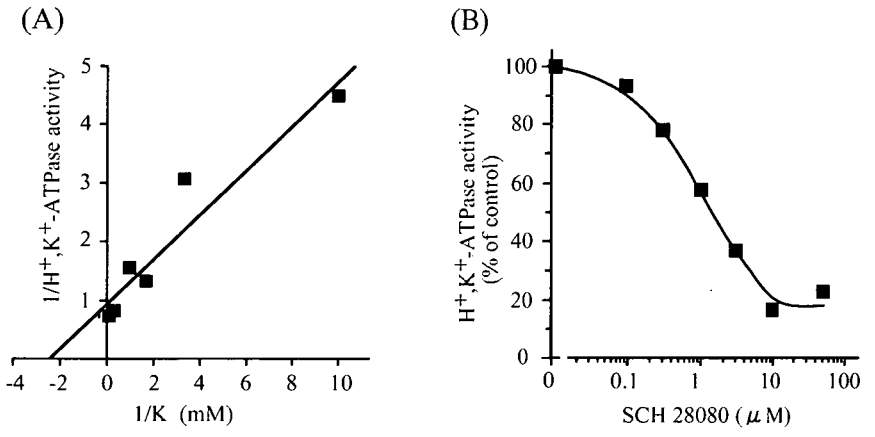

Fig. 9. **Effects of K^+ and SCH 28080 concentrations on the expressed K^+-ATPase activity.** (A) The K^+-ATPase activity of a membrane fraction prepared from the α+β-expressing cells was measured as a function of K^+ concentration. The K^+-ATPase activity was calculated as the difference between the ATPase activities in the presence and absence of various concentrations of KCl. The results are shown as the double-reciprocal plot of K^+-ATPase activity against K^+ concentration. A typical result of one of three experiments is shown. (B) The K^+-ATPase activity of a membrane fraction prepared from the α+β-expressing cells was measured as a function of SCH 28080 concentration. The values are mean ± SE for three experiments.

ity is 1.32 μmol/mg protein/h. Since 1 mg of the membrane fraction contains 9.6 μg of H^+,K^+-ATPase α-subunit, the specific activity of K^+-ATPase of the membrane fractions is equal to 137 μmol/mg α-subunit/h. This value is comparable or slightly higher than that found in the gastric vesicle preparation (*31*). The ATPase activity was stimulated by K^+ in a concentration-dependent manner with a K_m value of 0.41 mM (Fig. 9A), and inhibited by SCH 28080 in a concentration-dependent manner with an IC_{50} value of 1.5 μM (Fig. 9B). No significant K^+-ATPase activity was found in the membrane fraction prepared from the α-expressing or β-expressing cells (data not shown).

Phosphorylation and Dephosphorylation Capacity of the Expressed H^+,K^+-ATPase—Membrane fractions prepared from the α+β-expressing, α-expressing, and β-expressing cells were phosphorylated with [γ-^{32}P]ATP in the presence of 1 mM ouabain at 0°C for 15 s, and separated on an SDS–polyacrylamide gel under weakly acidic conditions. Doublet bands with a molecular mass of 100 kDa were observed in each membrane fraction. The lower band representing the H^+,K^+-ATPase α-subunit was clearly observed only in the membrane fraction prepared from the α+β-expressing cells (Fig. 10A). The phosphorylation of the α-subunit was inhibited by 50 μM SCH 28080.

The same membrane fraction phosphorylated with [γ-^{32}P]ATP was followed by the incubation with KCl in the presence of 1 mM non-radioactive ATP at 0°C (Fig. 10B). Even in the absence of KCl, the upper 100-kDa band in the membrane fractions disappeared during incubation with cold ATP. The phosphorylated α-subunit was dephosphorylated by K^+ in a concentration-dependent manner. The half-maximally effective concentration of K^+ for the dephosphorylation was about 0.2 mM (Fig. 10C). These properties are comparable with those of gastric H^+,K^+-ATPases transiently expressed in HEK-293 cells or found in the gastric vesicle preparation.

DISCUSSION

In order to study the structure-function relationship of enzymes by site-directed mutagenesis, construction of good functional expression systems is absolutely necessary. There have been several reports of transient expression of the functional gastric proton pump H^+,K^+-ATPase in insect Sf9 cells (*15*), *Xenopus* oocytes (*16*) and human kidney cell line, HEK-293 (*8*). There are several advantages and disadvantages in each expression system.

In the expression system using Baculovirus and Sf9 cells, expression level of gastric H^+,K^+-ATPase is very high. However, the content of active enzyme in the fraction is quite low. The phosphorylation level of the expressed H^+,K^+-ATPase was as low as 5 pmol/mg of protein, indicating that only 5% of the recombinant H^+,K^+-ATPase is active, as stated by the authors (*11*). The value of H^+,K^+-ATPase activity found in the membrane fraction was 0.3 μmol/mg protein/h (*32*). This value corresponds to 20 μmol/mg H^+,K^+-ATPase/h, which is 7 to 10 times lower than the specific activity of native gastric H^+,K^+-ATPase. Furthermore, the catalytic α-subunit of H^+,K^+-ATPase was primarily expressed in the intracellular membrane fractions, and there was little or no cell surface expression (*33*).

Xenopus oocytes expressing gastric H^+,K^+-ATPase take up ^{86}Rb and secrete protons, resulting in intracellular alkalization of the oocytes and acidification of the bathing media (*16*). However, unfortunately, no data were reported regarding the absolute expression level of the H^+,K^+-ATPase and the specific activity of the expressed enzyme.

We also transiently expressed the H^+,K^+-ATPase in HEK-293 cells. The K^+-ATPase activity found in a crude membrane fraction was comparable with that found in native gastric H^+,K^+-ATPase (*8*). The α- and β-subunits were expressed mainly in intracellular compartments rather than at the cell surface. The cells prepared in this transient expression system are actually a mixture of the α-expressing, β-expressing, α+β-expressing, and untransfected cells. Therefore, it was difficult to measure ion transport activity in this system.

Very recently, stable cell lines expressing gastric H^+,K^+-ATPase were newly constructed by successive transfections with the α- and β-subunit cDNA constructs (*19*). In this stable expression, the catalytic α-subunit was observed mainly at the cell surface, as judged by immunofluorescence, and the ammonium-stimulated ATPase activity was observed in a membrane fraction. However, there was no detailed characterization of gastric H^+,K^+-ATPase expressed in these stable cell lines, especially with respect to its ion transport activities.

In this report, we constructed stable cell lines expressing the gastric H^+,K^+-ATPase α- and β-subunits at the cell surface. The expressed H^+,K^+-ATPase was represented by K^+-dependent ATPase activity, which was inhibited by SCH 28080. Its specific activity was comparable to that found in

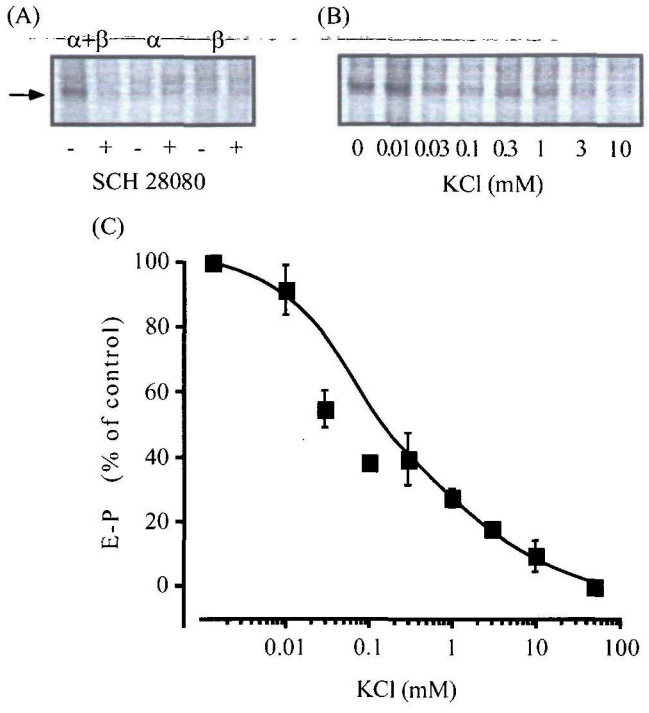

Fig. 10. **A: Phosphorylation of the membrane fractions prepared from the α+β-expressing, α-expressing, and β-expressing cells.** A 50-μg portion of membrane fractions prepared from each cell line was phosphorylated with 1 μM [γ-^{32}P]ATP at 0°C for 15 s. Prior to phosphorylation, the samples were preincubated with or without 50 μM SCH 28080 (shown as SCH 28080 + or –) at 0°C for 30 min. The phosphorylation reaction was quenched by the addition of ice-cold stop solution composed of 10% trichloroacetic acid and 10 mM inorganic phosphate. The protein was collected by centrifugation, washed successively with the ice-cold stop solution and 30% sucrose, and solubilized in a sample buffer for SDS–polyacrylamide gel electrophoresis. After gel electrophoresis at pH 6.5, the radioactivity was visualized by digital autoradiography of the dried gel. **B and C: Effects of K$^+$ concentration on the dephosphorylation reaction of the phosphorylated intermediates of the enzyme.** A 50-μg portion of membrane fractions prepared from the α+β-expressing cells was phosphorylated with 1 μM [γ-^{32}P]ATP at 0°C for 15 s. The phosphorylation was followed by the incubation with 1 mM non-radioactive ATP and indicated concentrations (0–50 mM) of KCl at 0°C for 15 s. The reaction was quenched by the addition of the ice-cold stop solution. Precipitated proteins were separated on an SDS–polyacrylamide gel, and the radioactivity associated with the H$^+$,K$^+$-ATPase α-subunit was visualized by digital autoradiography (B) and expressed as the percentage of the control values measured in the absence of KCl (C).

the native gastric H$^+$,K$^+$-ATPase samples prepared from hog gastric mucosae. The expressed H$^+$,K$^+$-ATPase underwent partial reactions, that is, formation of phosphorylated intermediate from ATP and K$^+$-dependent dephosphorylation, similar to the native H$^+$,K$^+$-ATPase preparation. Above all, the cells transported Rb$^+$ inward and protons outward. Therefore, our stable cell lines have many advantages in studying the effects of mutation on the transport activity and cellular regulation of the proton pump.

In addition to the α+β-expressing cells, we constructed two kinds of stable cell lines expressing individually the gastric H$^+$,K$^+$-ATPase subunit(s), the α-expressing and the β-expressing cells, and studied the interaction of the α- and β-subunits in the process of acquisition of stability and cell surface delivery. The expression level and location of the α-subunit was significantly changed in the presence of the β-subunit. The expression levels of the α-subunit in the α-expressing cells and in their membrane fraction were lower than those in the α+β-expressing cells, as judged by immunofluorescence and Western blotting. The α-subunit unassembled with the β-subunit was very unstable as indicated in the pulse chase labeling experiment. The α-subunit was observed only in the intracellular compartment, indicating that it cannot leave the ER and migrate to the cell surface in the absence of the β-subunit. However, the expression level of the α-subunit was significantly increased by co-expression of the β-subunit due to the acquisition of stability. The α-subunit can reach the cell surface in the presence of the β-subunit. On the other hand, the expression level of the β-subunit was almost unchanged in the presence of the α-subunit. The β-subunit can leave the ER and reach the cell surface by itself (unassembled with the Na$^+$,K$^+$-ATPase α-subunit). This behavior is similar to that found in stable cell lines constructed in LLC-PK1 (*29*). In fact, the majority of the β-subunit was expressed at the cell surface, as judged by the immunofluorescence and the glycosylation pattern of the β-subunit on the Western blot. It should be noted that the majority of β-subunit was expressed in intracellular compartments in the transient expression system. The reason for the difference in location of the β-subunit between these two expression systems remains to be studied.

In conclusion, stable cell lines expressing the α- and β-subunits showed Rb$^+$ and proton transport at the cell surface accompanying the K$^+$-dependent ATP hydrolysis. This is the first report of stable cell lines expressing the ion transport activities of the gastric proton pump.

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