C-terminal topology of gastric H⁺,K⁺-ATPase

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An antibody was prepared against a peptide corresponding to residues 1024–1034 (the putative C-terminus) of the α -subunit of hog gastric H⁺,K⁺-ATPase. The antibody bound to a 95 kDa band of H⁺,K⁺-ATPase that was solubilized in SDS, but not to that of Na⁺,K⁺-ATPase. It also bound to products of tryptic digestion that included C-terminal fragments of the H⁺,K⁺-ATPase α -subunit. The same amount of the antibody bound to both intact (tight) and lyophilized (leaky) inside-out gastric vesicles, indicating that its epitope is present on the cytosolic side of the vesicles. This finding was further confirmed by using fluorescence-immunolocalization techniques and streptolysin-O

to permeabilize newt oxyntic cells. Stimulation of isolated newt oxyntic cells with dibutyryl cyclic AMP induces fusion of tubulovesicles with the apical membrane, so that the luminal domains of the H⁺,K⁺-ATPase α -subunit directly face the cell-suspension medium. The antibody did not bind to the stimulated intact cell, but bound to cells permeabilized with streptolysin-O, indicating that it binds from the cytoplasmic side to the C-terminus of the H⁺,K⁺-ATPase α -subunit in apical and tubulovesicular membrane, and also that the H⁺,K⁺-ATPase α -subunit has an even number of transmembrane domains.

INTRODUCTION

In the last decade, amino acid sequences of catalytic subunits of P-type cation-transporting ATPases, such as Na⁺,K⁺-ATPase [1,2], Ca²⁺-ATPase [3,4] and H⁺,K⁺-ATPase [5,6], have been determined by cloning their cDNAs. The predicted structures of these ATPases are considerably different from each other, especially at their C-terminal halves, although their hydropathy profiles are similar to each other [7]. The C-terminus of the Na⁺,K⁺-ATPase α -subunit was proposed to be located in the extracellular space [8] or in the cytosolic space [9]. As to sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPases (SERCAs), the C-terminus of SERCA1 is present in the cytosolic space [10,11], but SERCA2 is expressed as two forms by alternative splicing, with their C-termini on opposite sides of the reticulum membrane; SERCA2a's is in the cytosolic space and SERCA2b's is in the reticulum lumen [12–14]. From the hydropathy plot [5], the C-terminus of the gastric H⁺,K⁺-ATPase α -subunit has been speculated to be in the cytosolic space, but a recent immunochemical study failed to provide confirmative evidence [15]. Therefore in this study we have tried to obtain definitive immunocytochemical evidence for the C-terminal topology of the H⁺,K⁺-ATPase α -subunit.

To define the topology with immunological tools, we used isolated newt oxyntic cells. Our recent study has shown that intracellular secretory canaliculi which are present in mammalian parietal cells are absent from newt oxyntic cells and therefore secrete acid directly into the medium [16]. Furthermore, the H⁺,K⁺-ATPase-specific inhibitor E3810 bound to part of the external cell surface when isolated newt oxyntic cells were stimulated by dibutyryl cyclic AMP. This fact indicates that the luminal domain of the H⁺,K⁺-ATPase α -subunit in the apical membrane directly faces the cell-suspension medium [16]. By raising a polyclonal antibody against the C-terminal peptide (residues 1024–1034) of the hog H⁺,K⁺-ATPase α -subunit, which bound to tryptic C-terminal fragments [17], we determined the topology of the C-terminus by immunofluorescent image analysis

of single intact and permeabilized newt oxyntic cells together with e.l.i.s.a. and immunoblots. In order to permeabilize the plasma membrane, we used bacterial toxin streptolysin-O (SLO), which was also employed in recent topological studies on Ca²⁺-ATPase [14] and Na⁺,K⁺-ATPase [14,18].

EXPERIMENTAL

Materials

Peroxidase-conjugated sheep anti-rabbit Ig(G + A + M), and alkaline phosphatase-conjugated sheep anti-rabbit Ig(G + A + M) were obtained from Binding Site (Birmingham, U.K.). Freund's complete adjuvant was from Difco Laboratories (Detroit, MI, U.S.A.), and Ribi adjuvant was from Ribi Immunochemical Research Inc. (Hamilton, MT, U.S.A.). Collagenase was obtained from Wako Pure Chemical Industries (Osaka, Japan), Actinase E from Kaken Pharmaceutical Co. (Tokyo, Japan) and ranitidine from Sankyo Co. (Tokyo, Japan). SLO and dog kidney Na⁺,K⁺-ATPase were obtained from Sigma (St. Louis, MO, U.S.A.). Fluorescein 5'-isothiocyanate (FITC)-conjugated swine anti-rabbit immunoglobulins was obtained from DAKO Japan Co. (Kyoto, Japan), and peroxidase-conjugated anti-rabbit IgG antibodies were from Cappel (West Chester, PA, U.S.A.). Japanese red belly newts were purchased from Mr. Kazuo Ouchi (Saitama, Japan).

Preparation of hog gastric vesicles

Tight gastric vesicles enriched in H⁺,K⁺-ATPase were prepared from mucosa in the fundic region of hog stomachs by differential and density-gradient centrifugation as described elsewhere [19]. These vesicles are essentially inside-out: the cytosolic domains of H⁺,K⁺-ATPase including the ATP-binding site are present on the external side of the vesicles, and the luminal domains on the internal side [20,21]. Where indicated, leaky vesicles were used, and these were prepared as follows. Tight vesicles were diluted with 10 vol. of distilled water followed by immediate freezing in

liquid nitrogen. They were then lyophilized and resuspended with the original volume of distilled water.

Enzyme assays

K⁺-ATPase activity was measured in 0.4 ml of solution containing lyophilized leaky gastric vesicles (4 µg of protein), 3 mM MgSO₄, 3 mM ATP and 40 mM Tris/HCl (pH 7.4) in the presence or absence of 15 mM KCl as described elsewhere [22]. Activity was calculated as the difference between the activities in the presence and absence of KCl.

Peptide synthesis and immunization

A peptide corresponding to residues 1024–1034 (Pro-Gly-Ser-Trp-Trp-Asp-Gln-Glu-Leu-Tyr-Tyr) of the hog H⁺,K⁺-ATPase α -subunit was synthesized by the solid-phase method [23] by using an Applied Biosystems model 430 peptide synthesizer (Foster City, CA, U.S.A.). The peptide was coupled to carrier protein keyhole limpet haemocyanin as described elsewhere [24]. The carrier-conjugated peptide was emulsified with Freund's complete adjuvant, and the emulsion injected subcutaneously into Japanese white rabbits five times at intervals of 7–10 days (for one booster injection, Freund's complete adjuvant was replaced by Ribi adjuvant). Blood was taken 10 days after the final injection, and the antisera titre was monitored by e.l.i.s.a. using plates coated with the peptide.

The IgG protein was purified by a Protein A MASS device (Nygene Corp., Yonkers, NY, U.S.A.), and the IgG fraction was obtained.

E.l.i.s.a.

This was performed in Corning polystyrene e.l.i.s.a. plates (Corning, NY, U.S.A.) at room temperature. Gastric vesicles (2.5 µg of protein/50 µl of PBS per well) or 1024–1034 peptide (2.5 µg/50 µl per well) were adsorbed overnight. The wells were washed three times with washing solution containing 10 mM Tris/HCl (pH 8.0), 0.05 % Tween 20 and 0.05 % NaN₃ and then incubated in PBS supplemented with 1 % BSA for 30 min. After being washed three times with the washing solution, the wells were incubated with antisera for 2 h. The wells were washed again, and 50 µl of PBS supplemented with alkaline phosphatase-conjugated anti-rabbit IgG and 1 % BSA was introduced into each well followed by incubation for 25 min. The wells were washed yet again, and 1 mg/ml *p*-nitrophenyl phosphate in 100 mM aminomethylpropanediol/HCl (pH 9.8) was introduced into the wells and incubated at 37 °C for 60 min. The reaction was stopped by the addition of 50 µl of 2 M NaOH, and the absorbance at 405 nm was measured in a microplate reader (model 450; Japan Bio-Rad Laboratory, Tokyo, Japan).

Tryptic digestion

Gastric vesicles (1.5 mg/ml protein) were prewarmed in 0.5 ml of solution containing 10 mM Pipes/Tris (pH 7.4), 250 mM sucrose, 1 mM EDTA, 1 M glycerol in the presence of either 10 mM ATP or 100 mM KCl at 37 °C for 10 min and then incubated together with trypsin (15 µg/ml) for 30 min. The reaction was stopped by the addition of trypsin inhibitor (150 µg/ml), and the solution was frozen in liquid nitrogen.

SDS/PAGE

This was carried out by the method of Laemmli [25]. Isolated gastric vesicles (1 mg/ml protein), their tryptic fragments

(0.6 mg/ml) or Sigma Na⁺,K⁺-ATPase (1 mg/ml) were incubated with a solution containing 2 % SDS, 5 % 2-mercaptoethanol and 10 % glycerol for 10 min at room temperature and applied to an SDS/polyacrylamide gel.

Immunoblotting

Proteins were blotted to nitrocellulose filters as described elsewhere [26] in a solution of 20 % methanol, 192 mM glycine and 25 mM Tris (pH 8.3). The filters were incubated for 1 h in 10 mM Tris-buffered saline (pH 7.4) containing 4 % Block Ace (Dainippon Pharmaceutical Co., Osaka, Japan). They were incubated for 1 h with the antiserum diluted in 10 mM Tris-buffered saline containing 1 % BSA, diluted 500-fold for the experiments with gastric vesicles and Na⁺,K⁺-ATPase, and 1000-fold for the experiment with trypsin-digested products. The blots were washed and then incubated with the secondary antibody (peroxidase-conjugated anti-rabbit IgG antibodies). The immunoblots were stained with a peroxidase immunostain kit (Wako) containing Nitro Tetrazolium Blue, NADH and H₂O₂.

Isolation of Japanese newt oxyntic cells

Before isolation of fundic mucosa, Japanese red-belly newts (*Cynops pyrrhogaster pyrrhogaster*) were pithed. The mucosa was stripped off the external muscle layer, stretched and fixed with its luminal surface upwards on the bottom of a glass dish. A thin layer of oxygenated standard amphibian Ringer's solution (87 mM NaCl, 18 mM NaHCO₃, 4 mM KCl, 1 mM KH₂PO₄, 1 mM MgCl₂, 2 mM CaCl₂, 5 mM Hepes, pH 7.4) supplemented with 1 mg/ml collagenase, 1 mg/ml Actinase E, 2 mg/ml BSA and 10 µM ranitidine was added to the dish. Ranitidine was added because amphibian oxyntic cells of newt and *Necturus* retain distinct polarity between the apical and basolateral membranes when the cells are isolated in the presence of a histamine H₂ antagonist [16,27]. The dish was covered with a lid, gassed with 95 % O₂/5 % CO₂ and incubated in a shaking bath at 27 °C for 90 min. After the removal of dispersed surface epithelial cells, the mucosa was further treated with fresh enzyme solution for 30 min, and the resulting isolated cells were used. The content of oxyntic cells in the cell suspension was 50–70 %. Oxyntic cells rich in mitochondria were easily recognized under a phase-contrast microscope.

Permeabilization of the plasma membrane of isolated oxyntic cells

Newt oxyntic cells were incubated in amphibian Ringer's solution supplemented with 1 mM dibutyl cyclic AMP at 25 °C for 30 min. They were separated by sedimentation at 170 g (0 °C for 5 min) and resuspended in the Ringer's solution. They were then placed on a coverslip coated with poly-L-lysine and fixed in cold methanol for 1 min. SLO was used to permeabilize the plasma membrane only [28]. SLO was activated by incubation with 2 mM dithiothreitol for 10 min at 37 °C. The cells were incubated in PBS containing 2.5 units/ml SLO for 5 min at 0 °C. After this toxin-binding step, the cells were washed to remove excess toxin. The reaction of SLO adsorbed on the plasma cell membrane was allowed to proceed for 15 min at 37 °C.

Immunofluorescence staining of oxyntic cells

Methanol-fixed SLO-permeabilized newt oxyntic cells were incubated in PBS containing 0.5 % BSA, 0.02 % NaN₃, and a 100-fold dilution of anti-peptide serum (or preimmune serum) at

room temperature for 2 h. After being washed three times with PBS, the cells were incubated with a FITC-conjugated anti-rabbit IgG and 0.5% BSA for 1 h. After a further three washes with PBS, the cells were examined in a Nikon (Tokyo, Japan) TMD microscope equipped with a Spex (Edison, NJ, U.S.A.) spectrophotometer. To excite FITC fluorescence, the excitation wavelength was set at 490 nm, and a filter cassette B of the TMD microscope (the eyepiece-side absorption filter was of 520–700 nm) was used. The images were stored in an SIT-camera as previously described [29]. Pseudocolouring images of the relative fluorescence scale of FITC were hard-copied.

For immunofluorescence of intact (unpermeabilized) oxyntic cells, isolated cells were incubated with the primary antibody in frog Ringer's solution containing 0.5% BSA in the presence of 1 mM dibutyryl cyclic AMP for 1 h at room temperature, followed by washing and another incubation with the secondary antibody. The cells were washed and sedimented on a coverslip which was not coated with poly-L-lysine. The distribution of FITC fluorescence intensity in a single cell was measured under the microscope.

RESULTS

E.I.s.a. study

The presence of anti-peptide antibodies in sera was checked by e.i.s.a. against synthetic peptide 1024–1034 of the α -subunit of hog gastric H^+,K^+ -ATPase. We obtained three sera which showed high titres. Figure 1 shows the results obtained with the serum (Ab1024) showing the highest titre.

In Figure 2, the assay plates were coated with native or lyophilized gastric vesicles. Ab1024 shows high titres against both the peptide and native gastric vesicles. Even when the serum was diluted 2000-fold with PBS, the binding of Ab1024 to the peptide and native vesicles was significantly higher than that of preimmune serum. Because the polarity of native gastric vesicles is almost completely inside-out (i.e. the external surface of the vesicle is cytoplasmic [20,21]), the present result indicates that the antibody binds to the cytoplasmic surface of the vesicles. Furthermore, Figure 2 shows that the same amount of antibody binds to lyophilized leaky vesicles as to intact tight vesicles.

Figure 1 Binding of antibody Ab1024 to the C-terminal peptide of the H^+,K^+ -ATPase α -subunit (residues 1024–1034)

The assay wells were precoated with synthetic peptide. Antiserum Ab1024 (●) and preimmune serum (□) were successively diluted with PBS and incubated in the peptide-coated wells. Antibody binding was detected by incubating alkaline phosphatase-conjugated anti-rabbit IgG (A + M) and *p*-nitrophenyl phosphate. The absorbance of released *p*-nitrophenol was read at 405 nm.

Immunoblot study

Figure 3 shows immunoblots of gastric vesicles and dog kidney Na^+,K^+ -ATPase with antibody Ab1024. The antibody bound to the 95 kDa band of the H^+,K^+ -ATPase α -subunit, but not to Na^+,K^+ -ATPase at all. Preimmune serum did not bind to proteins of the vesicles (results not shown). These results suggest that antibody Ab1024 can specifically react with the gastric H^+,K^+ -ATPase α -subunit.

In the next experiment, we studied whether antibody Ab1024 bound to C-terminal fragments of the H^+,K^+ -ATPase α -subunit which were obtained by limited digestion of the enzyme with trypsin. Trypsin digestion of gastric H^+,K^+ -ATPase depends on the conformation of the enzyme. On the basis of the N-terminal sequence of the digestion products of hog gastric H^+,K^+ -ATPase, Van Uem et al. [17] reported that tryptic digestion produces two large fragments which vary depending on the conformation: 67 kDa N-terminal and 35 kDa C-terminal fragments in the presence of ATP, and 42 kDa N-terminal and 56 kDa C-terminal fragments in the presence of K^+ .

Figure 4 shows that antibody Ab1024 binds to a 35 kDa fragment when the enzyme is digested in the presence of ATP and to a 56 kDa fragment when digested in the presence of K^+ , indicating that the antibody binds to C-terminal fragments.

Image analysis of isolated oxyntic cells

To confirm further the polarity of the epitope of antibody Ab1024, we examined whether the antibody bound to the luminal surface of stimulated intact oxyntic cells or the cytosolic membrane surface of permeabilized newt oxyntic cells. Note that the partial amino acid sequence of the H^+,K^+ -ATPase α -subunit in the region of the C-terminus is well conserved in all reported species such as hog [6], rat [5], human [30] and rabbit [31]. Because the outer surface of single parietal cells isolated from mammalian species such as rabbit and guinea pig is almost completely surrounded by the basolateral membrane, we cannot use mammalian parietal cells for the present experimental purpose. Our recent study using fluorescence image analysis has shown that stimulation with dibutyryl cyclic AMP of isolated

Figure 2 Binding of antibody Ab1024 to tight and leaky gastric vesicles

The assay wells were precoated with tight (intact; ○, □) or leaky (lyophilized; ×) vesicles. Binding of antibody Ab1024 (○, ×) or preimmune serum (□) to the wells was measured by e.i.s.a. Binding of preimmune serum to leaky vesicles was similar to that to tight vesicles (results omitted for simplicity). Typical results from one of three experiments are shown.

Figure 3 Immunoblots of gastric vesicles and Na^+, K^+ -ATPase stained with antibody Ab1024

Hog gastric vesicles (lane a, 1 μg) and dog kidney Na^+, K^+ -ATPase (lane b, 1 μg) were run on a 7.5% Laemmli gel and then transferred to a nitrocellulose filter. The blot was stained with antibody Ab1024. The molecular mass is shown in kDa.

newt oxyntic cells induces fusion of endoplasmic reticulum (so-called tubulovesicles or smooth-membrane tubules) with the apical membrane, so that the extracytoplasmic region of H^+, K^+ -ATPase in the apical membrane directly faces the cell-suspension medium [16].

Antibody Ab1024 did not bind to an isolated intact cell stimulated by dibutyryl cyclic AMP (Figure 5b), but did bind to a permeabilized cell stimulated by dibutyryl cyclic AMP (Figure 5a). Preimmune serum stained neither permeabilized (Figure 5c) nor intact (results not shown) cells. Furthermore, antibody Ab1024 stained a permeabilized unstimulated cell that had been isolated in the presence of a histamine H_2 antagonist (Figure 5d). SLO treatment presumably permeabilized only the external cell membrane under the experimental conditions. On the basis of these results, the epitope of antibody Ab1024 is suggested to be present on the cytoplasmic surface of both the apical membrane and tubulovesicles.

K^+ -dependent ATPase activity

Here we used antibody Ab1024 purified by a Protein A MASS device because unpurified antisera interfered with measurements of enzyme activity. The purified antibody Ab1024 also showed a high titre against gastric vesicles in an e.l.i.s.a. experiment (Figure 6). Lyophilized gastric vesicles (10 $\mu\text{g}/\text{ml}$) were incubated at

Figure 4 Immunoblots of tryptic fragments of gastric H^+, K^+ -ATPase stained with antibody Ab1024

Hog gastric vesicles (2 $\mu\text{g}/\text{lane}$) were digested with trypsin for 30 min in the presence of ATP (lane a) or K^+ (lane b). Samples were run on 10% Laemmli gels and transferred to nitrocellulose filters. The blots were stained with antibody Ab1024. The molecular mass is shown on the right in kDa.

room temperature for 1 h in a solution containing 3 mM MgSO_4 , 40 mM Tris/HCl (pH 7.4) and the IgG fraction of Ab1024 or that of preimmune serum (200 $\mu\text{g}/\text{ml}$). The enzyme reaction was then started by the addition of 3 mM ATP in the presence or absence of 15 mM K^+ . The K^+ -ATPase activity in the absence of antibodies was 36 μmol of P_i/h per mg of protein, that in the presence of antibody Ab1024 was also 36 $\mu\text{mol}/\text{h}$ per mg of protein, and that in the presence of the preimmune serum was 38 $\mu\text{mol}/\text{h}$ (average of two experiments), indicating that binding of the antibody to the C-terminus of the H^+, K^+ -ATPase α -subunit did not affect the enzyme activity. Besides antibody Ab1024, two other antisera raised against the C-terminus peptide were obtained and showed high titres against gastric vesicles. Their IgG fractions (200 $\mu\text{g}/\text{ml}$) also did not inhibit the enzyme activity.

DISCUSSION

The topological structure of the catalytic subunits of P-type ATPases including H^+, K^+ -ATPase, Na^+, K^+ -ATPase and sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPases are relatively conserved in their N-terminal halves [1–7] and are well established.

In the present study, we determined the location of the C-terminus of the gastric H^+, K^+ -ATPase α -subunit. Polyclonal antibodies were raised against the C-terminal teleopeptide (residues 1024–1034) of the α -subunit of hog gastric H^+, K^+ -ATPase. Antibody Ab1024 specifically bound to a 95 kDa band of SDS-solubilized hog H^+, K^+ -ATPase, but not to that of dog Na^+, K^+ -ATPase. Furthermore, the antibody bound to the C-terminal fragments of the H^+, K^+ -ATPase α -subunit that were produced by limited tryptic digestion in the presence of ATP or

Figure 5 Immunofluorescence image analysis of newt oxyntic cells

Isolated single oxyntic cells of red belly newt were stimulated by dibutyryl cyclic AMP (**a–c**), but that in (**d**) was not stimulated. The cells in (**a**), (**c**) and (**d**) were permeabilized with SLO, that in (**b**) was not permeabilized. Cells were stained with antibody Ab1024 (**a, b** and **d**) or preimmune serum (**c**). Data show typical examples; contradictory examples were not observed. The coloured strip indicates the scale of relative intensity of the FITC fluorescence. Bar = 20 μ m.

Figure 6 Binding of the IgG fraction of antibody Ab1024 to intact gastric vesicles

The assay wells were precoated with intact vesicles. Binding of the total IgG fraction of antibody Ab1024 (●) or preimmune serum (□) to the wells was measured by e.l.i.s.a.

K^+ . The present e.l.i.s.a. studies using inside-out gastric vesicles show that the antibody bound to the cytosolic surface of tubulovesicles.

Further evidence that the C-terminus of the H^+,K^+ -ATPase α -subunit is present not on the luminal side of the membrane but on the cytosolic side was obtained using an immunocytochemical technique and a procedure to permeabilize the plasma membrane selectively with SLO. SLO produces lesions of 15 nm in the plasma membrane with endoplasmic reticulum membranes left intact, permitting the entrance of macromolecules (up to 400 kDa) into the cytoplasm [32]. When oxyntic cells isolated from Japanese red-belly newt were stimulated by dibutyryl cyclic AMP, part of the tubulovesicles fused with the apical membrane, and the luminal domains of the H^+,K^+ -ATPase α -subunit directly faced the cell-suspension medium [16]. The present antibody which binds to the C-terminus of the H^+,K^+ -ATPase did not bind to the external surface of the stimulated intact cells. However it did bind to the cells permeabilized with SLO. Furthermore, the antibody also bound to the permeabilized cells that had been in the resting state.

The fact that the C-terminus is located in the cytoplasm suggests that the total number of transmembrane domains of the α -subunit is even because the N-terminus is also present in the cytoplasm [15]; this number may be 8 or 10. A recent study in which a chimera composed of the Na^+, K^+ -ATPase α -subunit and SERCA1 was co-expressed with the Na^+, K^+ -ATPase β -subunit has shown that the Na^+, K^+ -ATPase α -subunit and Ca^{2+} -ATPase have the same topology with ten membrane-spanning domains [18]. Therefore catalytic subunits of all these P-type ATPases, including Na^+, K^+ -ATPases, SERCAs (except SERCA2b) and gastric H^+, K^+ -ATPase, seem to have the same transmembrane topology. SERCA2b is reported to have an extended C-terminus which spans the membrane an additional (i.e. 11th) time [12–14].

The amino acid sequence of the gastric H^+, K^+ -ATPase α -subunit was determined by cloning its cDNA [5,6], but not directly sequencing the polypeptide. Furthermore, the molecular mass of the H^+, K^+ -ATPase α -subunit calculated from its cDNA (114 kDa) is substantially larger than that (95 kDa) determined by SDS/PAGE. Therefore it is not clear whether the full sequence predicted from its cDNA exists in parietal cells as a mature protein. In fact, there are many putative sites for tryptic processing in the sequence of the H^+, K^+ -ATPase α -subunit. In the present paper we have shown that the sequence corresponding to the putative C-terminus (residues 1024–1034) actually does exist in the H^+, K^+ -ATPase α -subunit.

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