Functional coupling of chloride–proton exchanger CIC-5 to gastric H⁺,K⁺-ATPase

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Summary

It has been reported that chloride–proton exchanger CIC-5 and vacuolar-type H⁺-ATPase are essential for endosomal acidification in the renal proximal cells. Here, we found that CIC-5 is expressed in the gastric parietal cells which secrete actively hydrochloric acid at the luminal region of the gland, and that it is partially localized in the intracellular tubulovesicles in which gastric H⁺,K⁺-ATPase is abundantly expressed. CIC-5 was co-immunoprecipitated with H⁺,K⁺-ATPase in the lysate of tubulovesicles. The ATP-dependent uptake of 36Cl⁻ into the vesicles was abolished by 2-methyl-8-(phenylmethoxy)imidazo[1,2-a]pyridine-3-acetonitrile (SCH28080), an inhibitor of H⁺,K⁺-ATPase, suggesting functional expression of CIC-5. In the tetracycline-regulated expression system of CIC-5 in the HEK293 cells stably expressing gastric H⁺,K⁺-ATPase, CIC-5 was co-immunoprecipitated with H⁺,K⁺-ATPase, but not with endogenous Na⁺,K⁺-ATPase. The SCH28080-sensitive 36Cl⁻ transporting activity was observed in the CIC-5-expressing cells, but not in the CIC-5-non-expressing cells. The mutant (E211A-CIC-5), which has no H⁺ transport activity, did not show the SCH28080-sensitive 36Cl⁻ transport. On the other hand, both CIC-5 and its mutant (E211A) significantly increased the activity of H⁺,K⁺-ATPase. Our results suggest that CIC-5 and H⁺,K⁺-ATPase are functionally associated and that they may contribute to gastric acid secretion.

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Key words: CIC-5, H⁺,K⁺-ATPase, Gastric acid, Tubulovesicle, Parietal cell

Introduction

CIC-5 was previously suggested to be an electrically shunting Cl⁻ channel in early endosomes, facilitating intraluminal acidification (Günther et al., 2003; Piwon et al., 2000). Thereafter, CIC-5 has been characterized as a voltage-dependent Cl⁻/H⁺ exchanger rather than a Cl⁻ channel (Picollo and Pusch, 2005; Scheel et al., 2005; Zifarelli and Pusch, 2009). CIC-5 may compensate the charge accumulation by the endosomal V-ATPase via coupling directly vesicular pH gradients to Cl⁻ gradients as a secondary active ion transporter (Picollo and Pusich, 2005; Scheel et al., 2005). On the other hand, it has been suggested that CIC-5 is directly involved in endosomal acidification by exchanging endosomal Cl⁻ to H⁺ (Smith and Lippiat, 2010); that is, H⁺ transport of CIC-5 is not coupled to that of V-ATPase.

Expression of CIC-5 was also reported in the rat intestinal tissues (duodenum, jejunum, ileum and colon), although its
expression level was lower than that in the kidney (Vandewalle et al., 2001). CIC-5 was found to be colocalized with V-ATPase in a vesicle-rich region beneath the apical brush border of enterocytes, suggesting the significant role of CIC-5 in the endocytic pathways of epithelial intestinal cells (Vandewalle et al., 2001).

So far, it has not been reported about function of CIC-5 in the stomach. Gastric parietal cells secrete hydrochloric acid (HCl) into the lumen of the stomach. On activation of parietal cells, large proportion of the tubulovesicles, which are rich in gastric proton pump (H⁺,K⁺-ATPase), fuse each other and connect with the apical canicular membrane. The tubulovesicular and apical membranes may not mix but remain separate and distinct in this stimulated phase (Fujii et al., 2009; Nishi et al., 2012). Cystic fibrosis transmembrane conductance regulator (CFTR) Cl⁻ channel (Sidani et al., 2007) and solute carrier 26A9 (SLC26A9), which functions as a Cl⁻ channel and Cl⁻/HCO₃⁻ exchanger (Xu et al., 2008) in tubulovesicles and K⁺-Cl⁻ cotransporter-4 (KCC4) in apical membranes (Fujii et al., 2009) are reported as a candidate of Cl⁻ transporting molecules for HCl secretion.

In the present study, we have found that CIC-5 is expressed in gastric tubulovesicles and that CIC-5 and H⁺,K⁺-ATPase are functionally associated. Our results suggest the novel function of CIC-5 in gastric acid secretion.

Results

Expression of CIC-5 in gastric tubulovesicles

First, we checked expression levels of CIC-5 mRNA in brain, kidney and stomach of rabbits (Fig. 1A). Northern blotting with the CIC-5 cDNA probe gave a single band of 9.5 kb. This size is the same as that of rat kidney CIC-5 (9.5 kb) (Steinmeyer et al., 1995). Expression level of CIC-5 mRNA in the stomach was lower than that in the kidney (Fig. 1A). For detection of CIC-5 protein, two anti-CIC-5 antibodies (SS53 and SS58) were used. The SS53 (Sakamoto et al., 1999) and SS58 (Fig. 4A) were demonstrated to have no cross reactivity to CIC-3 or CIC-4. In Fig. 1B, Western blotting with SS58 and SS53 gave a single band of 85 kDa in hog gastric samples, which contain H⁺,K⁺-ATPase as shown by using two anti-H⁺,K⁺-ATPase κ-subunit (HKα) antibodies (1H9 and Ab1024; Fig. 1B). The size of the gastric CIC-5 protein bands (85 kDa) is close to those of CIC-5 proteins observed in the kidneys of rats (83 kDa) (Steinmeyer et al., 2001) and mice (85 kDa) (Sakamoto et al., 1999). The specificity of anti-CIC-5 antibodies for the 85-kDa band was confirmed in the presence of the corresponding blocking peptide (Fig. 1B).

Significant expression of CIC-5 protein was observed in gastric samples of hogs, rabbits and humans (Fig. 1C). Gastric H⁺,K⁺-ATPase is abundantly expressed in parietal cells and localized in intracellular tubulovesicles and apical canalicular membrane of the cells (Fujii et al., 2009). In the present study, two types of hog gastric vesicles were prepared; one is intracellular tubulovesicles (TV) and the other is stimulation-associated vesicles (SAV) derived from the apical canalicular membrane. HKα was rich in both TV and SAV (Fig. 1D). β-actin was rich in SAV as previously reported (Fujii et al., 2009). Interestingly, expression level of CIC-5 in TV was much higher than that in SAV (Fig. 1D). This expression pattern of CIC-5 is apparently different from that of KCC4 which is functionally associated with H⁺,K⁺-ATPase in the apical canalicular membrane (Fujii et al., 2009) (Fig. 1D). In hog parietal cells, CIC-5 was found to be localized in both the endosome marker (Rab5)-positive and negative areas (Fig. 1E), suggesting that CIC-5 was expressed in both endosomes and tubulovesicles (TV). In fact, no significant expression of Rab5 was observed in TV (Fig. 1F).

Localization of CIC-5 in gastric parietal cells at the luminal region of the glands

In the immunohistochemistry of isolated hog gastric mucosa, the distribution pattern of CIC-5 mostly accorded with that of HKα (Fig. 2A–F), but not completely overlapped (Fig. 2F). Taking into account the results in Fig. 1, areas exhibited the yellow and
Association of CIC-5 with H⁺,K⁺-ATPase in TV

To study whether CIC-5 is associated with HKα in TV of hogs, immunoprecipitation was performed with an anti-CIC-5 antibody. The subsequent Western blotting of the immune pellets with anti-CIC-5 and anti-HKα antibodies gave a clear band for CIC-5 (85 kDa) and HKα (95 kDa), respectively (Fig. 3A). In contrast, the blotting with an anti-Rab11 antibody (as a negative control) gave no band for Rab11 (27 kDa) which is present in TV and related to the vesicular trafficking machinery in gastric parietal cells (Calhoun and Goldenring, 1997) (Fig. 3A). These results suggest that CIC-5 and H⁺,K⁺-ATPase are located close together in the TV.

Caveolae are known to be insoluble for treatment with detergents such as Triton X-100 and CHAPS at low temperature and form detergent resistance membrane (DRM) fractions with low density. In the present study, caveolae were isolated from hog TV by using CHAPS and sucrose gradient. Caveolin-1 was used as a marker for caveolae (Rothberg et al., 1992). As shown in Fig. 3B, CIC-5 and HKα were mainly distributed in the DRM fractions, in which caveolin-1 was expressed. In non-DRM fractions, in which clathrin was expressed, no significant distribution of CIC-5 and HKα was observed (Fig. 3B).

Most of TVs are inside-out vesicles (Asano et al., 1987): that is, ATP-binding site of H⁺,K⁺-ATPase faces outside and H⁺ is transported from outside to inside (Fig. 3C). Here we measured 36Cl⁻ uptake into TV in the presence of valinomycin, a potassium ionophore. In Fig. 3C, the presence of ATP significantly increased the 36Cl⁻ uptake into TV. SCH28080, a specific inhibitor of H⁺,K⁺-ATPase, significantly inhibited the ATP-dependent 36Cl⁻ uptake, while no SCH28080-induced inhibition was observed in the absence of ATP (Fig. 3C). These results suggest that H⁺,K⁺-ATPase-coupled secondary active Cl⁻ transporter(s) are present. Although it has been reported the expression of Cl⁻ channels such as CFTR (Fujii et al., 2009) and SLC26A9 (Xu et al., 2008) in TV, these proteins are not secondary active transporters coupled with the ATPase. Since CIC-5 can functionally couple to H⁺,K⁺-ATPase via H⁺ transport, the SCH28080-sensitive 36Cl⁻ uptake in TV may be, at least, partly mediated by CIC-5. On the other hand, SCH28080-insensitive 36Cl⁻ uptake was also observed (Fig. 3C). This passive Cl⁻ transport may be mediated by CFTR and SLC26A9.

Stable coexpression of CIC-5 and H⁺,K⁺-ATPase in HEK293 cells

It is noted that amino acid sequence of the antigen peptide used for preparation of the anti-hog, rabbit and human CIC-5 antibody (KHIAQMANQDPISLFN) is slightly different from that of the corresponding region of rat CIC-5 (KHIAQMANQDPESILFN);
that is, one amino acid residue is different (E741 in rat ClC-5). Here, rat ClC-5 cDNA was cloned. In Fig. 4A, the ClC-5 antibody (SS58) did not bind to WT-ClC-5 but to E741D-ClC-5 of rats. We also constructed the I732M/L744M double mutant (I732M/L744M-ClC-5) to check whether the SS58 antibody can also react with ClC-3 or ClC-4. The antibody did not bind to the I732M/L744M-ClC-5 (Fig. 4A). These results confirmed again high specificity of the antibody. No significant expression of endogenous human ClC-5 protein was observed in mock-transfected HEK293 cells (Fig. 4A, lower left) in which β-actin was normally expressed (Fig. 4A, lower right). On the other hand, the anti-Xpress antibody binds to WT-, E741D-, and I732M/L744M-ClC-5 because the Xpress sequence is tagged at the N-terminus of the cloned ClC-5 (Fig. 4A, lower middle).

Next, the tetracycline-regulated expression systems of WT-ClC-5 and E211A-ClC-5 which has no H⁺ transport activity (Picollo and Pusch, 2005) were constructed in the HEK293 cells that stably expressing α- and β-subunits of gastric H⁺,K⁺-ATPase. Exogenous expressions of WT- and E211A-ClC-5 were observed in the cells treated with tetracycline (Tet-on cells), but not in the cells treated without tetracycline (Tet-off cells) (Fig. 4B). Expression levels of HKz in the Tet-on cells were not significantly different from those in the Tet-off cells (Fig. 4C). WT- and E211A-ClC-5 and HKz were found to be partially present in the plasma membrane of the Tet-on cells (Fig. 4D). To check whether WT/E211A-ClC-5 and H⁺,K⁺-ATPase are located close together in the Tet-on cells as is the case in the TV (Fig. 3A), immunoprecipitation was performed with an anti-His tag antibody (for ClC-5). The subsequent Western blotting of the immune pellets with an anti-HKz antibody gave a band for HKz (Fig. 4E), suggesting the association between WT/E211A-ClC-5 and HKz. On the other hand, an anti-NKz1 antibody gave no band for endogenous Na⁺,K⁺-ATPase (Fig. 4E).

H⁺,K⁺-ATPase-dependent Cl⁻ transport activity by ClC-5 in HEK293 cells

To assess function of WT- and E211A-ClC-5 in the HEK293 cells, it is necessary to know expression levels of ClC-5 protein in the plasma membrane. Therefore, the cell surface biotinylation assay was performed with the cells. As control experiments, expression of myosin, an intracellular protein, was checked (Fig. 5A). In the Tet-on cells, biotinylated level of WT-ClC-5 was not significantly different from that of E211A-ClC-5 (Fig. 5B). Furthermore, biotinylated level of HKz in the Tet-on cells was not significantly different from that in the Tet-off cells (Fig. 5A). In the Tet-on cells, biotinylated level of WT-ClC-5 expression was not significantly different from those in the Tet-off cells (Fig. 5A, C). Expression level of ClC-5 in the plasma membrane was estimated to be about 15% of total ClC-5 expressed in the HEK293 cells.

Next, we estimated Cl⁻ transport activities of WT- and E211A-ClC-5 in the Tet-on and Tet-off cells. The SCH28080-sensitive 36Cl⁻ transport activity in the WT Tet-on cells was significantly greater than that in the WT Tet-off cells (Fig. 5D). In contrast, no activity of the SCH28080-sensitive 36Cl⁻ transport was observed in the E211A-ClC-5 Tet-on cells (Fig. 5D). It is noted that amounts of incorporated 36Cl⁻ before starting the transport experiments were not significantly different in these Tet-on and Tet-off cells (not shown). These results suggest that the ClC-5-derived 36Cl⁻ transport depends on the H⁺,K⁺-ATPase activity, and that ClC-5 can functionally couple to gastric H⁺,K⁺-ATPase via H⁺ transport in the HEK293 cells.

Upregulation of H⁺,K⁺-ATPase activity by coexpression of ClC-5

Interestingly, the SCH28080-sensitive K⁺-ATPase activity (H⁺,K⁺-ATPase activity) in the Tet-on cells was significantly greater than that in the Tet-off cells (Fig. 6A, middle), although the total expression level (Fig. 4C) and biotinylated level (Fig. 5C) of HKz in the Tet-on cells were not significantly different from those in the Tet-off cells. It is noted that expression of E211A-ClC-5 also increased the H⁺,K⁺-ATPase activity based on Sch28080-sensitive 36Cl⁻ transport activity (Fig. 5D).
activity (Fig. 6A, right). Treatment of tetracycline had no significant effects on the H^+,K^+-ATPase activity in the control HEK293 cells (Fig. 6A, left). The upregulatory effect in the Tet-on cells depended on the expression level of ClC-5 (Fig. 6B). Corresponding to these results, the SCH28080-sensitive 86Rb^+ transport activity in the Tet-on cells was significantly greater than that in the Tet-off cells (Fig. 6C).

Discussion
In the present study, we found the following. 1) ClC-5 protein is expressed partially in intracellular tubulovesicles of gastric parietal cells. 2) ClC-5 is associated with H^+,K^+-ATPase in tubulovesicles, and both of them are highly localized in caveolae. 3) In tubulovesicles, an H^+,K^+-ATPase inhibitor suppresses the secondary active Cl^- transport. 4) In the HEK293 cells stably expressing ClC-5 and H^+,K^+-ATPase, the ClC-5-derived 36Cl^- transport was suppressed by the H^+,K^+-ATPase inhibitor. 5) ClC-5 increases the H^+,K^+-ATPase activity in the membrane fraction of the cells stably expressing these proteins.

Previously, it has been reported that ClC-5 mRNA (Sakamoto et al., 1996) and protein (Günther et al., 1998) are not detected in the rat stomach. This may be due to low expression level of ClC-5 in the stomach, compared with predominant expression of it in the kidney (Günther et al., 1998; Sakamoto et al., 1996). In fact, expression level of ClC-5 mRNA in the gastric parietal cells was considerably lower than that in the kidney (Fig. 1A). In Xenopus laevis stomach, lower but detectable amounts of ClC-5 mRNA were found (Lindenthal et al., 1997). ClC-5 protein was expressed in the parietal cells more abundantly at the luminal region of the glands than at the basal
region. Gastric parietal cells migrate from the luminal to the basal region of the glands. It has been suggested that the luminal parietal cells more actively secrete acid than do the basal parietal cells (Bamberg et al., 1994; Karam et al., 1997; Sachs, 2001). Therefore, CIC-5 is suggested to be involved in the mechanism of gastric acid secretion. KCC4 was also found to be predominantly expressed in the luminal region (Fujii et al., 2009).

Function of tubulovesicles dramatically changes between resting and stimulated phases in gastric parietal cells. In resting parietal cells, tubulovesicles are present in intracellular compartments underlying the apical canalicular membrane. Upon stimulation, the tubulovesicles fuse each other and connect with the canalicular membrane, resulting in massive acid secretion (Fujii et al., 2009; Nishi et al., 2012). So far, several Cl− channels and transporters such as CFTR (Sidani et al., 2007), SLC26A9 (Xu et al., 2008), CLIC-6 (Nishizawa et al., 2000; Sachs et al., 2007) and KCC4 (Fujii et al., 2009) have been suggested as candidates that could be involved in the luminal Cl− efflux for gastric acid (HCl) secretion. CFTR is localized predominantly in the tubulovesicles (Fujii et al., 2009). SLC26A9 is also expressed in tubulovesicles and plays an essential role in HCl secretion by regulating Cl− secretion and/or by affecting the viability of tubulovesicles/secretory canaliculi in parietal cells (Xu et al., 2008). CLIC-6 is distributed throughout the cytosol (Nishizawa et al., 2000). KCC4 is expressed in the apical canalicular membrane and suggested to contribute to basal HCl secretion in resting parietal cells (Fujii et al., 2009). In the present study, we have found that CIC-5 is expressed in tubulovesicles, suggesting that CIC-5 is involved in massive acid secretion in stimulated parietal cells. In future, it will be necessary to clarify whether CFTR, SLC26A9 and CIC-5 are expressed in all tubulovesicles or in part of tubulovesicles if further separation of tubulovesicles into different subtypes becomes possible. It will be also important to examine the expression and function of other ClC members such as ClC-3 and ClC-4 in parietal cells.

So far, there have been no reports that focus on the morphology and function of stomach in the CIC-5-deficient mice and the Dent’s disease patients. This probably means that no severe gastric failures occur in these mice and patients. It is noted that no difference in the character of gastric secretion was found between WT- and CFTR-deficient mice (McDaniel et al., 2005). Sidani et al. suggested that complete ablation of the CFTR gene may potentially cause activation of compensatory mechanisms such as up-regulation of non-CFTR regulated K+ channels.
a, expression levels of ClC-5 and HKα estimated as in Fig. 6A, and the data were shown as means ± s.e.m. An equation: Normalized 86Rb+ transport activity was measured in the presence and absence of 50 μM SCH28080, and the SCH28080-sensitive (H+,K+-ATPase-dependent) transport activity was calculated. The score was calculated by using the following equation: Normalized 86Rb+ transport activity = (the activity in the Tet-on cells)/(the activity in the Tet-off cells). The 86Rb+ transport activity of the Tet-off cells was 0.21 ± 0.02 μmol Pi/mg of protein/h, respectively (n = 6). Calibrated H+,K+-ATPase activity of the Tet-off cells is normalized as 1. n = 6. **, P < 0.01. (B) The upregulatory effect depends on the expression level of ClC-5 protein. The WT cells were treated with (Tet-on) and without (Tet-off) 2 μg/ml tetracycline for various periods (6, 9, 12 and 15 h). A expression levels of ClC-5 and HKα proteins in the Tet-on (right) and Tet-off (left) cells are shown. In b, the H+,K+-ATPase activity of each sample was estimated as in Fig. 6A, and the data were shown as means ± s.e.m. Tet-off; ○, Tet-on; n = 6. **, significantly different (P < 0.01) compared with Tet-off. (C) 86Rb+ transport activity of the WT Tet-on and Tet-off cells. The 86Rb+ transport activity was measured in the presence and absence of 50 μM SCH28080, and the SCH28080-sensitive (H+,K+-ATPase-dependent) transport activity was calculated. The score was calculated by using the following equation: Normalized 86Rb+ transport activity = (the activity in the Tet-on cells)/(the activity in the Tet-off cells). The 86Rb+ transport activity of the Tet-off cells was 0.34 ± 0.07 nmol 86Rb+/min/10⁶ cells (n = 5). The score for Tet-off cells is normalized as 1. n = 5. **, P < 0.01.

In conclusion, ClC-5 is expressed in the tubulovesicles of gastric parietal cells, and its Cl– transport may be regulated by H+,K+-ATPase. It is interesting that ClC-5 is functionally associated with two types of proton pump; V-ATPase in the kidney and H+,K+-ATPase in the stomach.

Materials and Methods

Chemicals

HEK293 cells stably expressing α- and β-subunits of gastric H+,K+-ATPase were established as previously described (Kimura et al., 2002). Abs2024 (Asano et al., 2002).

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1994) and IHP (Medical & Biological Laboratories Co., Nagoya, Japan) were used as the anti-H+, K+-ATPase α-subunit (HKα) antibodies. Anti-ClC-5 monoclonal antibodies (SS53 and SS58) were prepared as described elsewhere (Sakamoto et al., 1999). The synthetic peptide corresponding to amino acids 730–746 (KIAQMANQDPDSLFF) in the C-terminus of CIC-5 was purchased from Takara Bio Inc. (Osaka, Shiga, Japan), and used for checking specificity of the anti-ClC-5 antibodies. The anti-ClC-5 antibodies were prepared as previously described (Fujii et al., 2000). Platinum Taq DNA Polymerase High Fidelity, Lipofectamine 2000, anti-Xpress antibody, Alexa Fluor 488-conjugated anti-mouse, anti-rabbit and anti-goat IgG antibodies and Alexa Fluor 546-conjugated anti-rabbit and anti-rat IgG antibodies were obtained from Invitrogen (Carlsbad, CA, USA). Anti-Na+, K+-ATPase α1-subunit (NKα1), anti-AQP4 (H-19) and anti-caveolin-1 antibodies and protein A/g-agarose beads were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Immobilized Protein A and sulfo-NHS-sil-biotin were from Pierce (Rockford, IL, USA). Anti-β-actin, anti-Rab5 and anti-mycos antibodies, avidin and SCH28080 were purchased from Sigma-Aldrich (St Louis, MO, USA). Anti-clathrin heavy chain (X22) antibody was from Affinity BioReagents (Golden, CO, USA). Anti-Rab11 antibody was from Cell Signaling Technology Japan (Tokyo, Japan). All other reagents were of the molecular biology grade or the highest grade of purity available.

Northern blotting

Poly A+ RNAs of tissue samples were prepared by using PolyATtract mRNA isolation system II (Promega, Madison, WI, USA). The amplified products were sequenced and used for preparation of the 32P-labeled cDNA probes. The rabbit CIC-5 probe was 305 bp long and corresponded to nucleotides 499–803 of the CIC-5 cDNA. The rabbit GAPDH probe was 493 bp long and corresponded to nucleotides 443–935 of the GAPDH cDNA. For Northern blot analysis, poly A+ RNA of each sample (2.5 µg) was separated on a 1% agarose/formaldehyde gel and transferred onto a nylon membrane (Zeta-probe GT, Bio-Rad). The membrane was hybridized with the 32P-labeled cDNA fragment of CIC-5 or GAPDH, and exposed to the Imaging Plate (Fuji Film) for 6 h (GAPDH) or 48 h (CIC-5).

Preparation of gastric samples

Animals were humanely killed in accordance with the guidelines presented by the Animal Care and Use Committee of University of Toyama. Human gastric specimens were obtained from surgical resection of a Japanese patient with gastric cancer (70 years, male) in accordance with the recommendations of the Declaration of Helsinki. Informed consents were obtained from the patient at University of Toyama. The normal gastric mucosae used for the experiments were 10–20 cm apart from the carcinoma. H. pylori and human gastric tubulovesicles (TV) (Fujii et al., 2009), hog stimulation-associated vesicles (SAV) (Fujii et al., 2009) and rabbit gastric P3 fraction (Hori et al., 2004) rich in H+, K+-ATPase were prepared as described previously.

Preparation of membrane fractions

For preparing membrane fractions of HEK293 cells, the cells were incubated in low ionic salt buffer (0.5 mM MgCl2 and 10 mM Tris-HCl, pH 7.4) supplemented with the protease inhibitor cocktail (10 µg/ml aprotinin, 1 µg/ml leupeptin and 1 µg/ml peptatin A) at 0˚C for 10 min. Subsequently, they were homogenized with 40 strokes in a Dounce homogenizer, and the homogenate was diluted with an equal volume of a solution containing 500 mM sucrose and 10 mM Tris-HCl (pH 7.4). The cell suspension was rich in caveolin-1, a marker of caveolae (Fig. 3B).

Isolation of detergent resistant membrane (DRM)

The membrane proteins were lysed with ice-cold MBS buffer (150 mM NaCl and 25 mM MES-NaOH, pH 6.5) containing 1% CHAPS and the protease inhibitor cocktail for 15 min. The solution was mixed with equal volume of 66% sucrose in MBS buffer, the mixture was placed at the bottom of an ultracentrifuge tube, and a discontinuous gradient was formed by overlaying with the 30% sucrose and 5% sucrose solutions. The sample was centrifuged at 261,000 × g in SW41Ti rotor (Beckman) for 18 h at 4˚C. Ten fractions of 1 ml each were collected from the top of the gradient, and proteins were precipitated by acetone before SDS-polyacrylamide gel electrophoresis and Western blotting. The DRM fractions were rich in caveolin-1, a marker of caveolae (Fig. 3B).

Western blotting

Western blotting was carried out as described previously (Sakai et al., 2004). The signals were visualized with ECL system (GE Healthcare) or West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). To quantify the chemiluminescence signals on the membranes, a FujiFilm LAS-1000 system and the Multi Gauge software (Fuji Film) were used. Anti-HKα antibodies were used at 1:5,000 (IHP) and 1:3,000 dilution (Ab1024). Anti-CIC-5 antibodies (SS53 and SS58) were used at 1:2,000 dilution. For the negative control, 1 µl of the anti-clathrin heavy chain antibodies was used at 1:2,000 dilution. Anti-Xpress and anti-mycos antibodies were used at 1:5,000. Anti-Rab5 antibody was used at 1:10,000 dilution. When indicated, anti-CIC-5 (SS58), anti-Xpress and anti-HKα antibodies were labeled with horseradish peroxidase (HRP) using Peroxidase Labeling Kit-NH2 (Dojindo Laboratories, Kumamoto, Japan).

Immunohistochemistry

The gastric mucosa isolated from hog stomach was fixed in PLP (Periodate-Lowicryl) paraformaldehyde containing 10 mM sodium periodate, 75 mM lysine and 2% paraformaldehyde for 12 h at 4˚C. The PLP-fixed mucosa was incubated with a series of PBS containing 5% sucrose (for 4 h), 10% sucrose (for 4 h), 15% sucrose (for 4 h) and 20% sucrose (for 12 h) at 4˚C. The mucosa embedded in the optimum cutting temperature compound (Sakura Finetechical Co., Tokyo, Japan) and cut at 6 µm. The sections were pre-treated with 3% BSA (in PBS) for 1 h at room temperature to prevent nonspecific binding of antibodies. Subsequently, the sections were incubated with anti-CIC-5 (SS53; 1:50 dilution), anti-HKα (Ab1024; 1:100 dilution), anti-Rab5 (1:100 dilution), anti-NKα1 (1:100 dilution), or anti-AQP4 antibody (1:100 dilution) for 15 h at 4˚C. Alexa Fluor 488-conjugatedAlexa Fluor 546-conjugated Anti-rabbit and Anti-rat IgG antibodies were used as secondary antibodies. Immunofluorescence images were visualized using a Zeiss LSM 510 laser scanning confocal microscope.

Measurement of 36Cl- transport in gastric tubulovesicles

Hog gastric tubulovesicles (100 µg of protein) were incubated with a solution containing 250 mM sucrose, 15 mM KCl, 3 mM MgSO4, 1 mM ATP, 20 µM ouabain, 10 µM R-(+)-[2R,3S]-6,7-dichloro-2-cyclopentenyl-2,3-dihydro-1-oxo-4Hinden-5-yl]oxoy acetic acid (DIOA), 10 µM furosemide and 100 mM Pipes-Tris (pH 7.4) for 2 min at 37°C. The solution was supplemented with and without 10 µM SCH280800a, a specific inhibitor of gastric H,K+-ATPase. Then, 5 µc/ml 11Cl and 10 µg/ml valinomycin were added to reaction mixtures, and they were incubated for 5 min at 37°C. The samples were rapidly filtered through a 0.45-µm HAWP filter (Millipore Co., Bedford, MA, USA). To calibrate nonspecific binding of H1Cl to the vesicles and the filter, the experiment was performed in the absence of ATP. The filter was washed with a solution containing 5 mM KCl, 250 mM sucrose and 100 mM Pipes-Tris (pH 7.4); transferred to a counting vial; and solubilized with 5 ml of ACS II scintillant. Then the radioactivity of 36Cl- was measured.

Plasmid construction

A full-length cDNA encoding rat CIC-5 was inserted into the pcDNA4/His vector containing the Xpress tag sequence at N-terminal side (Invitrogen) by using BamHI and NolI restriction sites (CIC-5-pcDNA4/His vector). The DNA corresponding Xpress-tagged CIC-5 from the pcDNA4/His was introduced into the pcDNA5/TO vector by using AfII and NolI restriction sites (CIC-5-pcDNA5/TO vector). Site-directed mutagenesis for preparing the E211A (E211A-ClC-5-pcDNA5/TO vector), E741D (E741D-ClC-5-pcDNA5/TO vector), and I732M/I732V (I732M/I732V-ClC-5-pcDNA5/TO vector) mutants were performed and the mutated cDNA sequences were verified by sequencing. The mutated cDNAs were verified using an ABI PRISM 310 sequencer (Applied Biosystems).

Expression of ClC-5 in HEK293 cells

For establishing the tetracycline-regulated expression system of CIC-5 in the HEK293 cells stably expressing gastric H,K+-ATPase α- and β-subunits, the cells were cotransfected with the CIC-5-pcDNA5/TO or E211A-ClC-5-pcDNA5/TO plus pcDNA6/TR vectors (Invitrogen) using Lipofectamine 2000, and cultured in D-MEM supplemented with 10% FBS for 24 h. Then, the transfected cells were selected in the presence of 0.4 mg/ml hygromycin B (Wako Pure Chemical Industries, Osaka, Japan), 7 µg/ml blasticidin S (Kaken Pharmaceutical Co., Tokyo, Japan), 0.25 mg/ml enzootic (Envyo Life Sciences) and 20 µg/ml puromycin (Invitrogen). To check the tetracycline-regulated CIC-5 expression, each cell line was treated with 2 µg/ml tetracycline (Invitrogen) for 12 h. The expression of CIC-5 was confirmed by immunocytochemistry and Western blotting.

Immunocytochemistry

The HEK293 cells were fixed in ice-cold methanol for 7 min at room temperature, and the cells were permeabilized with 0.3% Triton X-100 and 0.1% BSA (in PBS)
for 15 min. Then, the samples were pre-treated with the GSDB buffer (20 mM phosphate buffer (pH 7.4), 450 mM NaCl, 16.7% goat serum and 0.3% Triton X-100) for 30 min, and incubated with anti-Xpress (1:100 dilution) and anti-HKx (Ab1024; 1:100 dilution) antibodies for 60 min at 25˚C. Alexa Fluor 546-conjugated IgG and Alexa Fluor 488-conjugated IgG antibodies were used as secondary antibodies (1:100 dilution).

Immunoprecipitation

H+Og hog tubogolosomes (100 μg of protein) and membrane fractions from HEK293 cells (0.5–2 mg of protein) were lysed with 0.5–1 ml of the solution containing the 0.5% Triton X-100 (for vesicles) or 1% Nonidet P-40 (for membrane fractions), 150 mM NaCl, 0.5 mM EDTA and 50 mM Tris-HCl (pH 7.4) for 1 h on ice. The lysate was centrifuged at 100,000 g for 30 min at 4˚C. The supernatant was pre-cleared by protein A-agarose beads for 30 min at 4˚C. The eluted sample was used for Western blotting.

Measurement of 36Cl– transport assay

36Cl– transport activity was measured as difference between the activity in the presence and absence of SCH28080. Then, the cells were washed and lysed with 2 ml of the solution containing 150 mM NaCl, 0.5 mM EDTA, 1% Nonident P-40 and 50 mM Tris-HCl (pH 7.4). The samples were solubilized with 5 ml of ACS II scintillant and radioactivity of 36Cl– was measured.

H+K+ATPase activity

H+K+ATPase activity was determined in 1 ml of solution comprising 50 μg of membrane protein, 3 mM MgCl2, 1 mM ATP, 5 mM NaCl, 2 mM ouabain, 15 mM KCl and 40 mM Tris-HCl (pH 6.8), in the presence and absence of 50 μM SCH28080. After incubation for 30 min at 37˚C, the inorganic phosphate released was measured as described elsewhere (Yoda and Hokon, 1970).

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Cell surface biotinylation

Cell surface biotinylation was performed as described previously (Wang et al., 2005). HEK293 cells on 6-well collagen-coated plates were treated with 0.5 mg/ml sulfo-NHS-ss-biotin for 30 min at 4˚C. Then, the cells were washed with 500 μl of the solution containing the 1% Triton X-100, 150 mM NaCl, 0.5 mM EDTA and 50 mM Tris-HCl (pH 7.4) for 1 h on ice. The lysate was centrifuged at 15,000 × g for 20 min at 4˚C. The supernatant (1 mg of protein) was rocked with 40 μl of avidin-agarose beads for 4 h at 4˚C. The sample was then incubated with protein A/G-agarose beads (for vesicles) or protein A-agarose beads (for membrane fractions) for 5 h at 4˚C, and reacted with anti-CIC-5 antibody SS58 (for vesicles) or anti-His tag antibody (for membrane fractions) for 15 h at 4˚C. The sample was then incubated with protein A/G-agarose (for vesicles) or protein A-agarose (for membrane fractions) beads for 4-6 h at 4˚C. The beads were washed and eluted into 250 mM Tris-HCl (pH 6.8) supplemented with 8% SDS, 4% glycerol and 10% β-mercaptoethanol. After centrifugation, the supernatant was used for Western blotting.

Measurement of 36Cl– transport in CIC-5 Tet-on and Tet-off cells

The cells were seeded on a collagen type I-coated 6-well culture plate (1.5×105 cells per well) and cultured for 12 h in the D-MEM supplemented with 10% FBS followed by additional 12-h incubation in the presence of 2 μg/ml tetracycline (for Tet-off cells) and 2 μg/ml HisCl2. In the case for Tet-off cells, tetracycline was omitted. Subsequently, the 36Cl–-loaded cells were washed and incubated in the reaction medium containing 129 mM sodium gluconate, 15 mM NaCl, 1 mM KCl, 0.5 mM MgSO4, 0.5 mM CaCl2, 20 mM ouabain, 10 mM D-glucose, 10 mM furosemide and 5 mM HEPES-NaOH (pH 7.4) for 3 min at 25˚C. At the appropriate time, the sample was reacted with protein A/G-agarose beads for 4 h at 4˚C. The eluted sample was used for Western blotting.

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Author Contributions

Y.T., T.F., K.F., T.S., T.H., Y.T. and A.I. performed experiments and analyzed the data. His.S., I.N. and K.M. generated anti-CIC-5 antibodies, and contributed to the discussion of the manuscript. K.T. prepared clinical samples. S.U. and S.S. contributed to the experimental plan and discussion of the manuscript. Hid.S. contributed to supervision of the project, interpretation of the data and writing the paper.

Competing Interests

The authors have no competing interests to declare.
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