Is the ClC-2 chloride channel involved in the Cl^- secretory mechanism of gastric parietal cells?

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Abstract **It has been controversial whether the ClC-2 chloride channel is involved in hydrochloric acid secretion of gastric parietal cells. Here, we investigated whether ClC-2 is the apical Cl^- channel associated with gastric acid secretion. Two anti-ClC-2 antibodies used in this study reacted with cloned ClC-2 protein expressed in HEK293 cells. In isolated rabbit gastric glands, significant expression of ClC-2 mRNA was observed, but the presence of ClC-2 protein was not clear. Furthermore, no expression of ClC-2 protein was observed in isolated rat and human gastric mucosa. Immunohistochemistry on the rat gastric mucosa showed no significant expression of ClC-2 protein in the parietal cells which showed abundant expression of H^+,K^+-ATPase. These results indicate that ClC-2 may not be a Cl^--transporting protein for gastric acid secretion in parietal cells.**

Keywords: ClC-2; Chloride channel; Gastric acid

1. Introduction

ClC-2 is a broadly expressed Cl^- channel activated by hyperpolarization, cell swelling and acidic pH (for review see [1,2]). This channel has been suggested to contribute to epithelial Cl^- secretion in human and rat airways [3] and rat fetal lung [4–6] because the channel protein is expressed in the apical membrane of these epithelia. Although ClC-2 knockout mice showed no gross anatomical or histological changes during the lung development, it does not rule out the possibility that ClC-2 participates in pulmonary transepithelial Cl^- transport [7]. ClC-2 has also been suggested to be involved in maintaining the inhibitory GABA response (for review see [1]). The ClC-2 knockout mice revealed a degeneration of photoreceptors and male germ cells that led to the total loss of both cell types in adults, suggesting an important role of ClC-2 for cells depending on close cell–cell interactions [7].

In the stomach, protons are actively secreted by H^+,K^+-ATPase present in the apical membrane of gastric parietal cells, but it has not been established what molecule contributes to apical Cl^- transport for hydrochloric acid (HCl) secretion. Concerning the involvement of ClC-2 in gastric acid secretion, the results so far reported are divided and controversial.

One group has reported that ClC-2 is the apical Cl^- channel that may be involved in gastric acid secretion of parietal cells based on the following results: (1) ClC-2 was cloned from rabbit gastric mucosa [8] and its electrophysiological properties including anion selectivity ($I^- > Cl^-$) are similar to those of the native channel in H^+,K^+-ATPase-containing gastric vesicles [9]. (2) ClC-2 of rabbits [8] and humans [10] is activated by cAMP-dependent phosphorylation, and this mechanism is consistent with an essential role of intracellular cAMP in the HCl secretory mechanism. (3) Anti-ClC-2 antibody showed signals in rabbit gastric parietal cells [11].

In contrast, another group suggested that ClC-2 is not associated with gastric acid secretion [1] based on the following results: (1) Histamine-stimulated gastric acidification in ClC-2-deficient mice was not significantly different from that in wild-type mice [7]. (2) Anion selectivity of ClC-2 cloned from rats and rabbits is $Cl^- > I^-$ [12–14]. (3) ClC-2 of rats [1] and rabbits [14] cannot be activated by cAMP-dependent phosphorylation.

We investigated whether ClC-2 is expressed in the gastric mucosa of rabbits, rats and humans in the present study, where positive and negative control experiments were performed. We found no significant expression of ClC-2 protein in the gastric mucosa.

2. Materials and methods

2.1. Preparation of gastric samples

Rabbit gastric glands and parietal cells were prepared from male Japanese white rabbits weighing 2–2.5 kg as previously described [15], and rat gastric mucosa was obtained from female Sprague–Dawley rats weighing 200–300 g [16]. Animals were humanely killed in accordance with the guidelines presented by the Animal Care and Use Committee of Toyama Medical and Pharmaceutical University [15,16]. Hog gastric vesicles rich in H^+,K^+-ATPase were prepared as described elsewhere [17]. Human gastric specimens were obtained from surgical resection of two Japanese patients with gastric cancer (76 years, male and 60 years, female) in accordance with the recommendations of the Declaration of Helsinki. Informed consents were obtained from the patients at Toyama Medical and Pharmaceutical University Hospital. The normal gastric mucosae used for the experiments were 10–20 cm apart from the carcinoma. Rat and human preparations of mucosa were free from muscle layer.

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2.2. RNA isolation and Northern blot analysis

Poly (A^+) RNA was prepared from isolated rabbit gastric parietal cells as previously described [15]. A set of primers based on the rabbit ClC-2 sequence (nucleotide positions 490–512: 5′-GGGTC-CGGCATCCCCGAGATGAA-3′ and nucleotide positions 781–803: 5′-ACCTCAATGCTGAATAGGACGCC-3′) was used for PCR. The amplified products were sequenced and used for the preparation of the ^{32}P-labeled cDNA probes. Northern blot analysis was performed as described elsewhere [18].

2.3. Cell culture and transfection

HEK293 cells were maintained in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. The cells were transfected with rabbit ClC-2 cDNA subcloned into pcDNA3.1 (+) [19] by using Lipofectamine 2000 (Invitrogen). The cells were cultured for two days and used for the following fractionation.

2.4. Fractionation of gastric samples and cultured cells

Isolated rat and human gastric mucosa, isolated rabbit gastric glands or HEK cultured cells were homogenized in the buffer (250 mM sucrose, 1 mM EDTA and 20 mM Tris–HCl, pH 7.4) supplemented with 10 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A and 15 μg/ml aprotinin. The P1, P2, P3 and S fractions were prepared by the following three steps at 4 °C [20]. (1) The homogenate was centrifuged at $1400 \times g$ for 10 min. The supernatant was collected, and the pellet was homogenized and centrifuged again. This procedure was repeated three times. The resulting pellet was suspended with the buffer (P1 fraction). (2) The supernatant from the step 1 was centrifuged at $5900 \times g$ for 15 min. Then, the supernatant was collected, and the pellet was homogenized and centrifuged again. This procedure was repeated twice. The resulting pellet was suspended with the buffer (P2 fraction). (3) The supernatant from the step 2 was centrifuged at $105\,000 \times g$ for 60 min. Then, the pellet (P3 fraction) and the supernatant (S fraction) were separated. These four fractions were rich in cell debris and nuclei (P1), mitochondria (P2), microsome and plasma membrane (P3) and cytosol (S), respectively [20,21]. In the case of human preparation and HEK cultured cells, step 2 was skipped.

2.5. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and Western blotting

The fractions P2, P3, P2 + P3 and S (each 30 μg of protein) or hog gastric vesicles (1 μg of protein) were incubated in a sample buffer containing 2% SDS, 5% β-mercaptoethanol, 10% glycerol and 65 mM Tris–HCl (pH 6.8) at room temperature for 5 min and applied to the SDS–polyacrylamide gel. Western blotting was carried out as described previously [22]. We used two polyclonal anti-ClC-2 antibodies, CLC21-A (1:500 dilution; Alpha Diagnostic, TX, USA) and ACL-002 (1:400 dilution; Alomone Labs, Jerusalem, Israel), and a monoclonal anti-H^+,K^+-ATPase α subunit antibody, 1H9 (1:200 dilution; Medical & Biological Laboratories, Nagoya, Japan). For negative control, 10 μg of the anti-ClC-2 antibody was pre-incubated with 10 μg of the corresponding blocking peptide. The CLC21-A blocking peptide (Alpha Diagnostic) that corresponded to 22 amino acid residues near the C-terminus of rat ClC-2 is 100% conserved in rabbit and 90% in human, and the ACL-002 blocking peptide (Alomone Labs) that corresponded to amino acid residues 888–906 of rat ClC-2 is 95% conserved in rabbit and 90% in human. Horseradish peroxidase-conjugated anti-rabbit IgG (1:5000 dilution) was used as a secondary antibody.

2.6. Immunohistochemistry

Fresh-frozen rat gastric tissues embedded in the O.C.T. compound were cut at 5 μm. The ClC-2-transfected HEK cells and the corresponding mock cells were cultured on the collagen-coated cover glass. The tissue sections and the cultured cells were fixed in 4% paraformaldehyde for 30 min at room temperature, and the cells were subsequently permeabilized with the phosphate buffered saline (pH 7.4) containing 0.3% Triton X-100 and 0.1% bovine serum albumin. These samples were treated with 20 mM phosphate buffer (pH 7.4) containing 450 mM NaCl, 16.7% goat serum and 0.3% Triton X-100 for 30 min. Then, the samples were reacted with the anti-ClC-2 antibody (ACL-002 or CLC21-A) or the anti-H^+,K^+-ATPase α subunit antibody (1H9) for 15–20 h at 4 °C. These primary antibodies were used at 1:100 dilution. As a secondary antibody, Alexa Fluor 546-conjugated anti-rabbit IgG (1:100 dilution) or Alexa Fluor 488-conjugated anti-mouse IgG (1:100 dilution) was used. The stained samples were observed under the fluorescent microscope (BX50-34-FLA-2, Olympus, Tokyo, Japan).

3. Results and discussion

Gastric acid (HCl) secretion is one of the fundamental physiological functions in all mammals. The amino acid sequence of the H^+,K^+-ATPase, which actively secretes H^+ in the apical membrane of gastric parietal cells, is highly conserved among different species of animals [23,24], but the Cl^--transporting molecule for HCl secretion has not been established yet. A potential candidate is the ClC-2 Cl^- channel [8–11], but this is still controversial [1]. In the present study, we examined if ClC-2 protein is expressed in parallel with the H^+,K^+-ATPase in the gastric mucosa of rabbits, rats and humans.

First, we checked the expression of the H^+,K^+-ATPase α subunit protein in several gastric fractions from rabbits, rats and humans. As expected, the H^+,K^+-ATPase is abundantly expressed in the P3 fraction which is rich in microsomes and plasma membrane (Fig. 1).

Fig. 2A shows that ClC-2 mRNA (3.3 kb) is significantly expressed in rabbit gastric parietal cells. This result is in agreement with the previous report showing that ClC-2 mRNA is localized to the parietal cells by in situ hybridization and by direct in situ RT-PCR [11]. In the Western blotting using two anti-ClC-2 antibodies (CLC21-A and ACL-002), several bands similar to ClC-2 protein (~97 kDa), at least in molecular size, were observed in P2, P3 and S fractions of rabbit (Fig. 2B and C). Surprisingly, these bands did not disappear in the presence of the blocking peptide, while the specific band for cloned ClC-2 (97 kDa) completely disappeared (Fig. 2B and C). In the blot with ACL-002, the non-specific band of 130 kDa in the S fraction unexpectedly disappeared in the presence of the blocking peptide (Fig. 2C). These results indicate that the bands (~97 kDa) in P2, P3 and S fractions are not due to ClC-2 protein.

Sherry et al. [11] reported the presence of ClC-2 protein in rabbit gastric parietal cells as determined by Western blotting and by immunohistochemistry using ACL-002 antibody.

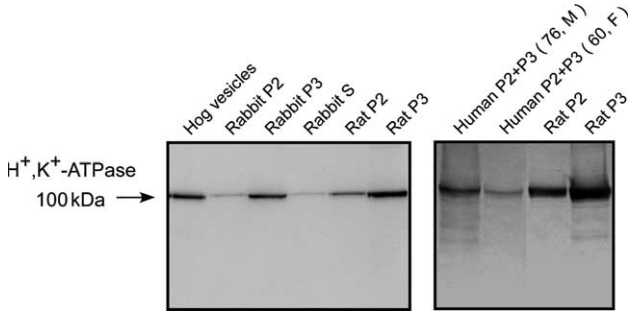

Fig. 1. High expression of the protein of H^+,K^+-ATPase α subunit in the P3 fraction from rabbit gastric glands, and from rat and human gastric mucosa. Western blotting was performed in hog gastric vesicles (a positive control), rabbit P2, P3 and S fractions (left panel), rat P2 and P3 fractions (left and right panels) and human P2 + P3 fraction (right panel). Monoclonal antibody 1H9 was used for the detection of H^+,K^+-ATPase α subunit (100 kDa). In the human sample, age (years) and sex (M or F) of the patient are shown in the upper parenthesis.

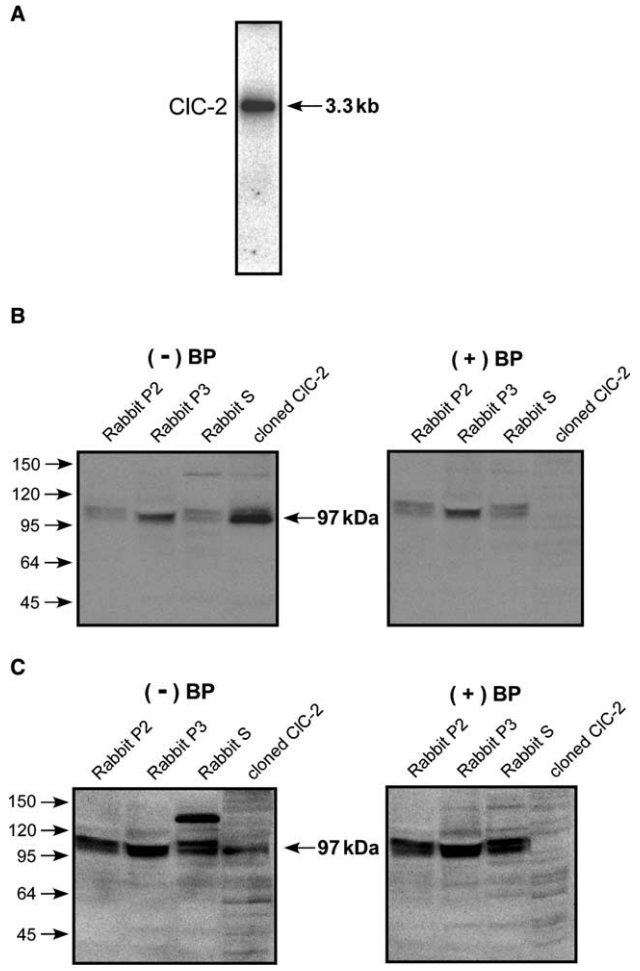

Fig. 2. Expression of ClC-2 in the rabbit gastric samples. (A) Detection of ClC-2 mRNA on poly (A^+) RNAs (2.5 μg) of isolated rabbit gastric parietal cells. A single band of 3.3 kb was detected. (B,C) No significant expression of ClC-2 protein in rabbit gastric glands. Western blotting was performed with rabbit P2, P3 and S fractions using CLC21-A (B) and ACL-002 (C) polyclonal antibodies. As a positive control (indicated as cloned ClC-2), the P2 + P3 fraction from the HEK293 cells that were transfected with cloned rabbit ClC-2 was used. The specific bands for the cloned ClC-2 (97 kDa) disappeared in the presence of the corresponding blocking peptide (+BP; right panels).

Although their findings seem to be opposite to our results, there are several possible reasons to generate the differences. (1) In the Western blotting, they observed a 93-kDa protein band in rabbit gastric vesicles, but they performed no control experiment using the blocking peptide [11]. Therefore, it is likely that the 93-kDa band may be non-specific as observed in the present experiment (~97-kDa band; Fig. 2B and C). (2) In the immunohistochemistry, they found that major staining was present in the parietal cells, and the staining was weakened by pre-incubating the antibody with the blocking peptide [11]. This weakening may be due to contribution of the 130-kDa protein (Fig. 2C) to the staining. In fact, the 130-kDa band is highly sensitive to the blocking peptide (Fig. 2C). It seems to be inappropriate to use ACL-002 antibody for obtaining the positive evidence for the presence of ClC-2 protein from the immunohistochemical study on the rabbit gastric tissue because of its non-specific reactivity (Fig. 2C). Taken together, it is suggested that ClC-2 mRNA is expressed, but the presence of ClC-2 protein is not clear in rabbit gastric parietal cells.

Next, we studied the presence of ClC-2 protein in rat and human gastric mucosa. In the Western blotting using CLC21-A and ACL-002 antibodies, no signal for ClC-2 protein was detected in P2 and P3 fractions from rat gastric mucosa (Fig. 3) and the P2 + P3 fraction from human gastric mucosa (Fig. 4).

In the immunohistochemistry, both CLC21-A and ACL-002 antibodies reacted with the cloned ClC-2 protein expressed in HEK293 cells, while these antibodies showed no reactivity with endogenous proteins in the mock cells (Fig. 5A). Fig. 5B shows that CLC21-A and ACL-002 antibodies gave no signals in the parietal cells of the rat gastric tissue section. Only the

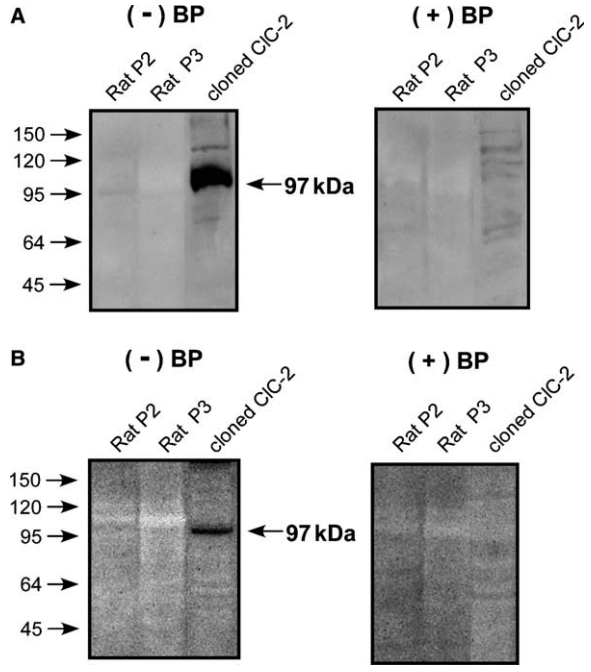

Fig. 3. No expression of ClC-2 protein in rat gastric mucosa. Western blotting was performed with rat P2 and P3 fractions using CLC21-A (A) and ACL-002 (B) polyclonal antibodies. As a positive control, the P2 + P3 fraction from the HEK293 cells that were transfected with cloned rabbit ClC-2 was used. The specific bands for the cloned ClC-2 (97 kDa) disappeared in the presence of the corresponding blocking peptide (+BP; right panels).

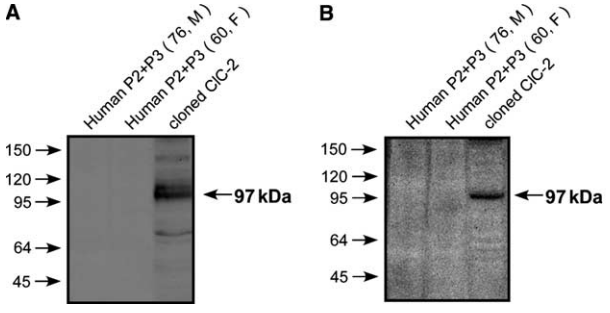

Fig. 4. No expression of ClC-2 protein in human gastric mucosa. Western blotting was performed with human P2 + P3 fraction using CLC21-A (A) and ACL-002 (B) polyclonal antibodies. Age (years) and sex (M or F) of the patient are shown in the upper parenthesis. As a positive control, the P2 + P3 fraction from the HEK293 cells that were transfected with cloned rabbit ClC-2 was used.

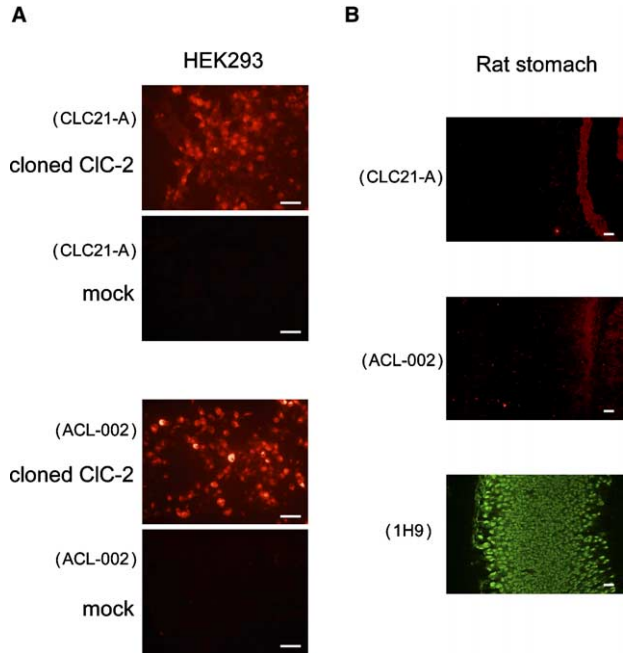

Fig. 5. Immunohistochemical detection of ClC-2 protein. (A) Immunostaining was performed with the HEK293 cells expressing the cloned ClC-2 and the corresponding mock cells using CLC21-A (upper two panels) and ACL-002 (lower two panels) primary antibodies and the Alexa Fluor 546-conjugated secondary antibody. Positive signals were observed only in the ClC-2-transfected cells. Bar = 50 μm. (B) Immunostaining was performed with the rat gastric tissue using the CLC21-A (top panel) and ACL-002 (middle panel) primary antibodies and the Alexa Fluor 546-conjugated secondary antibody. These two panels show signals of ClC-2 in the muscularis mucosae but not them in the gastric parietal cells. As a positive control, the tissue was stained with 1H9 primary antibody against H^+,K^+-ATPase and the Alexa Fluor 488-conjugated secondary antibody (bottom panel). Specific signals of H^+,K^+-ATPase are observed in the parietal cells. On these panels, the surface epithelial layers are present on the left side and the submucosal layers on the right side. Bar = 50 μm.

weak signals were observed in muscularis mucosae of the tissue (Fig. 5B). On the other hand, the anti-H^+,K^+-ATPase antibody (1H9) showed clear signals in the parietal cells of the tissue section (Fig. 5B).

Our present results suggest that no significant amount of ClC-2 protein is expressed in rat and human gastric mucosa. Therefore, ClC-2 may not be a Cl^- channel in the apical membrane of the gastric parietal cells, although the channel mRNA is expressed in the cells. At present, the Cl^--transporting molecule for gastric acid secretion is unknown and it would be an interesting subject to be clarified in a future study.

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