Cellular and subcellular immunolocalization of ClC-5 channel in mouse kidney: colocalization with H⁺-ATPase

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Sakamoto, Hisato, Yoshikazu Sado, Ichiro Naito, Tae-Hwan Kwon, Shinichi Inoue, Kenichi Endo, Masanobu Kawasaki, Shinichi Uchida, Søren Nielsen, Sei Sasaki, and Fumiaki Marumo. Cellular and subcellular immunolocalization of ClC-5 channel in mouse kidney: colocalization with H⁺-ATPase. Am. J. Physiol. 277 (Renal Physiol. 46): F957–F965, 1999.—To determine the immunolocalization of ClC-5 in the mouse kidney, we developed a ClC-5-specific rat monoclonal antibody. Immunoblotting demonstrated an 85-kDa band of ClC-5 in the kidney and ClC-5 transfected cells. Immunocytochemistry revealed significant labeling of ClC-5 in brush-border membrane and subapical intracellular vesicles of the proximal tubule. In addition, apical and cytoplasmic staining was observed in the type A intercalated cells in the cortical collecting duct. In contrast, the staining was minimal in the outer and inner medullary collecting ducts and the thick ascending limb. Western blotting of vesicles immunosolated by the ClC-5 antibody showed the presence of H⁺-ATPase, strongly indicating that these two proteins were present in the same membranes. Double labeling with antibodies against ClC-5 and H⁺-ATPase and analysis by confocal images showed that ClC-5 and H⁺-ATPase colocalized in these ClC-5-positive cells. These findings suggest that ClC-5 might be involved in the endocytosis and/or the H⁺ secretion in the proximal tubule cells and the cortical collecting duct type A intercalated cells in mouse kidney.

proximal tubule; endocytosis; proton pump; Dent’s disease; chloride channel

THE CLC-5 CHLORIDE CHANNEL (human gene symbol: CLCN5) is a molecule responsible for four hereditary kidney stone diseases: Dent’s disease (33), X-linked recessive hypophosphatemic rickets (XLRH) (1), X-linked recessive nephrolithiasis (XRN) (7), and idiopathic low-molecular-weight proteinuria (15). All four of these diseases are associated with mutations in the ClC-5 gene and have common phenotypic features characterized by excessive low-molecular-weight proteinuria, hypercalciuria, and either nephrocalcinosis or nephrolithiasis (19, 20, 23, 25, 28). Accordingly, these four related X-linked syndromes have been considered phenotypic variants of a single disease. However, other renal tubular dysfunctions such as phosphaturia, glycosuria, uricosuria, and impairment of urinary acidification may also occur, and the mutation in the ClC-5 gene does not always correlate with the severity of the phenotype. To date, we have no pathophysiological explanation of how and why a molecular dysfunction of the single channel ClC-5 causes such different renal tubular dysfunctions in these related syndromes. Localization of ClC-5 expression along the nephron is of particular importance in understanding the physiological roles of ClC-5 in the kidney.

In a RT-PCR study performed on microdissected tubules, Steinmeyer et al. (29) first demonstrated that the ClC-5 message was ubiquitously expressed along the nephron, and this expression was predominant in the cortical collecting tubules, the S3 segment of the proximal tubules, and the thick ascending limb. However, contamination of the samples in the dissection procedure might disturb the accurate localization. A subsequent in situ hybridization study of rat kidney showed expression of ClC-5 in the collecting duct type A intercalated cells from cortex through the upper portion of the inner medulla, but not in the proximal tubule cells (26). The absence of the message in proximal tubule cells is not consistent with the presence of low-molecular-weight proteinuria in the ClC-5-defective syndromes. Recently, Gunther et al. (13) described the cellular and subcellular localization of ClC-5 in rat kidney using ClC-5-specific monoclonal antibodies. They further showed partly overlapping labeling patterns of ClC-5 and the vacuolar-type H⁺-ATPase. In the present study, we examined the localization of ClC-5 in the mouse kidney using a monoclonal antibody. In addition, we examined whether ClC-5 colocalizes with H⁺-ATPase in the same membrane vesicles and membrane domains. For this purpose, two series of experiments were carried out. First double-labeling confocal microscopy of ClC-5 and H⁺-ATPase was undertaken. Second, ClC-5-bearing vesicles were immunoisolated and subjected to immunoblotting.

METHODS

Preparation of monoclonal antibodies. The anti-peptide antibodies were generated against a synthetic peptide (CC54: CKHIAQMANQDPDSILFN) corresponding to the COOH-terminal 17 amino acids of ClC-5, and cysteine was added for coupling to NH₂ terminus. Eight-week-old female WKY/NCrj rats were injected via hind footpads with 1 mg/ml of authentic peptide (CC54) conjugated with keyhole limpet hemocyanin and Freud’s complete adjuvant. After 2 wk, the rats were killed, and peritoneal lymph nodes were removed. Isolated lymphocytes (1 × 10⁶) were fused with mouse myeloma cell line SP2/0-Ag14 as previously described (26). The cells were
buffer, frozen in liquid N2, and stored at

without the pretreatment; lane 2

for nonimmune rat IgG in ClC-5-transfected cells pretreated with 5 µM DEX for 12 h; and

ClC-3-transfected cells pretreated with 5 µM DEX for 12 h.

was rehomogenized and centrifuged at 100,000

and any remaining large cellular fragments. The supernatant

tant pellet was resuspended in

primary antibody (SS53) for nonimmune rat IgG.

synthetic peptide of COOH-terminal of ClC-5.

CT4, synthetic peptide of COOH-terminal region of ClC-4; and CC54,

peptides. CT3, synthetic peptide of COOH-terminal region of ClC-3;

high value indicates a high reactivity of antibodies and synthetic

tes. The supernatant was removed magnetically, and the remaining sample buffer was used for immunoblotting to detect ClC-5 and H+-ATPase. The only difference in the treatment of the controls was the substitution of the anti-ClC-5 antibody with nonimmune rat IgG.

Electrophoresis and immunoblotting. The membrane samples were solubilized in the loading buffer (2% SDS, 30% glycerol, 10% 2-mercaptoethanol, and 250 mM Tris·HCl, pH 6.8). Samples were loaded at 10–20 µg/lane onto a 7.5% or 10–20% SDS-polyacrylamide gel and run on a minigel system, and the proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane. After blocking with 5% nonfat milk and 0.1% Tween 20 in PBS (PBS-T) for 1 h, the blots were incubated overnight at 4°C with antibodies (1:1,000). Blots were visualized by the enhanced chemiluminescence procedure (ECL Plus; Amersham, Arlington Heights, IL). Controls in which the primary antibody was substituted with nonimmune rat IgG or the primary or secondary antibody was omitted revealed no labeling. To confirm the specificity of the primary antibody, it was preincubated with the immunizing peptide (1 mg/ml) overnight at 4°C prior to exposure to the PVDF membranes.

Immunohistochemistry. The mouse kidneys were perfusion-fixed with PLP fixative containing 2% paraformaldehyde, 75 mM lysine, and 10 mM sodium periodate in phosphate buffer (pH 7.4). The blocks were postfixed in the same fixative for an additional 4 h at 4°C and cryoprotected in 20% sucrose in a phosphate buffer. After freezing the blocks in liquid nitrogen,

Table 1. Cross-reactivity between monoclonal antibodies and synthetic peptides

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>CC54</th>
<th>CT3</th>
<th>CT4</th>
<th>(–)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS53</td>
<td>1.885 ± 0.001</td>
<td>0.808 ± 0.006</td>
<td>0.909 ± 0.000</td>
<td>0.806</td>
</tr>
<tr>
<td>SS54</td>
<td>2.039 ± 0.005</td>
<td>0.800 ± 0.005</td>
<td>0.782 ± 0.000</td>
<td>0.134</td>
</tr>
<tr>
<td>2nd Ab alone</td>
<td>0.084 ± 0.021</td>
<td>0.074 ± 0.010</td>
<td>0.098 ± 0.032</td>
<td>0.070</td>
</tr>
</tbody>
</table>

Values are means ± SD and are absorbance values of ELISA; a high value indicates a high reactivity of antibodies and synthetic peptides. CT3, synthetic peptide of COOH-terminal region of ClC-3; CT4, synthetic peptide of COOH-terminal region of ClC-4; and CC54, synthetic peptide of COOH-terminal of ClC-5.

plated in four 96-well tissue culture plates (Becton-Dickinson Labware), and supernatants from individual wells were collected and screened by enzyme-linked immunosorbent assay (ELISA). The epitope of each isolated monoclonal antibody was mapped using twenty kinds of overlapping 10-residue peptides corresponding to the immunizing peptide (CC54) (11).

Preparation of membrane fractionations. Crude membrane fractions were prepared from the mouse kidneys. The kidneys were minced finely and homogenized in an isolation buffer (0.3 M sucrose, 5 mM Tris·HCl, and 2 mM EDTA, pH 7.2, containing the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml leupeptin, and 2 µg/ml pepstatin) with five strokes of a motor-driven Teflon-glass homogenizer at 1,000 rpm. The homogenate was centrifuged at 4,000 g for 15 min at 4°C to remove nuclei, mitochondria, and any remaining large cellular fragments. The supernatant was rehomogenized and centrifuged at 100,000 g (Beckman Optima L-70K: SW41Ti rotor) for 60 min at 4°C. The resulting pellet was resuspended in ~100 µl of resuspension buffer, frozen in liquid N2, and stored at −80°C until use. The cultured cells were harvested after three rinses with ice-cold PBS. The cell suspension was centrifuged at 1,000 rpm for 5 min at 4°C and then treated in the same manner described above.

A

116 kDa

cont

82 kDa

← ClC-5

B

cont

1

2

3

CIC-5

Fig. 1. Western blots of the crude membrane preparation from the mouse kidney, and of the membranes from the transfected CHO-K1 cells expressing ClC-5 (J2702) or ClC-3 (C21) using dexamethasone (DEX)-inducible mammalian expression vector (pMAM-neo). SS53 monoclonal antibody specifically recognizes a band of approximately 85 kDa in mouse kidney (A) and in ClC-5-transfected cells (B). A: control (cont) indicates substitution of primary antibody (SS53) for nonimmune rat IgG. B: control (cont) indicates substitution of primary antibody (SS53) for nonimmune rat IgG in ClC-5-transfected cells pretreated with 5 µM DEX for 12 h; lane 1, CIC-5-transfected cells without the pretreatment; lane 2, CIC-5-transfected cells pretreated with 5 µM DEX for 12 h; and lane 3, CIC-3-transfected cells pretreated with 5 µM DEX for 12 h.
they were cut into 5-µm sections and mounted on Silane-coated slides and the slides were stained by antibodies. In addition, the transfected cultured cells expressing CIC-5 (J2702) or CIC-3 (C21) using dexamethasone (DEX)-inducible mammalian expression vector (pMAM-neo) were used to determine the specificity of anti-CIC-5 antibodies (18, 27). After overnight treatment with 5 µM DEX, the cells were fixed for 10 min in 2% paraformaldehyde in PBS, and permeabilized for 2–5 min in 0.1% Triton X-100 at room temperature. The primary antibody was visualized with secondary antibodies [FITC-conjugated F(ab')2 fragment rabbit anti-rat IgG (H+L) diluted 1:200, Cy5-conjugated goat anti-rabbit IgG (H+L) diluted 1:500, or Cy3-conjugated goat anti-mouse IgG (H+L) diluted 1:500], or Cy3-conjugated goat anti-mouse IgG (H+L) diluted 1:500].

The fluorescence signal of labeled specimens was observed first with a Zeiss Axivert microscope and then analyzed by a laser confocal microscope (Zeiss LSM 510). Digitized images were produced with a Mirus Film Printer GALLERIA using Raster Plus 95 software (version 1.01). In some studies, we used the affinity-purified polyclonal antibody against the aquaporin-2 (AQP2) water channel, an antibody which has been previously described and characterized (8). We also examined the staining of the monoclonal antibody against the 31-kDa subunit of H⁺-ATPase (E11, kindly provided by S. L. Gluck, Gainesville,

Fig. 2. Immunocytochemistry of stably transfected CHO-K1 cells expressing CIC-5 (J2702) or CIC-3 (C21) using DEX-inducible mammalian expression vector (pMAM-neo). After overnight treatment with 5 µM DEX, the SS53 monoclonal antibody recognized J2702 cells (A) but not C21 cells (B).

Fig. 3. Localization of CIC-5 channel in proximal tubule of mouse kidney by confocal laser microscope (A) and transmission electron microscope (B and C). Cross section of proximal tubules was stained with the SS53 monoclonal antibody against CIC-5. A: labeling for CIC-5 is concentrated beneath the apical cytoplasm in proximal tubule cells. B and C: transmission electron micrographs showing presence of CIC-5 (detected by SS53 and peroxidase/diaminobenzidine) in a region rich in vesicles below the apical invaginations of the brush-border membrane. Two different proximal tubules are shown at different magnifications (B: ×4,900; C: ×8,800).
FL) (14). E11 was diluted 1:1 and visualized with Cy3-conjugated goat anti-mouse IgG (H+L) diluted 1:500 (CyDye; Amersham, Buckinghamshire, UK).

Immunoelectron microscopy. For electron microscopy, 5- to 7-µm thick sections of kidneys fixed by the PLP fixative containing 2% paraformaldehyde were initially incubated with the SS53 antibody for 12 h and then with peroxidase-conjugated F(ab\(^2\))\(_2\) fragment goat anti-rat IgG (Cappel, Aurora, OH) for 3 h. The sections were then fixed in 2.5% glutaraldehyde for 10 min at 4°C, and immersed in 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution for 30 min and DAB containing 0.005% H\(_2\)O\(_2\) for 10 min, respectively. Tissue slices were postfixed by 2% OsO\(_4\) and embedded in Quetol 812. Ultrathin sections were examined with a Hitachi transmission electron microscope.

RESULTS

ClC-5-specific monoclonal antibodies. Rats were immunized with the 18-amino-acid synthetic peptide CKHIAQMANQDPDSL FN. The last 17 amino acids of this sequence, KHIAQMANQDPDSL FN, are conserved among human (6), rat (27, 29), and mouse (unpublished data). The criteria for selection of monoclonal antibodies were specificity for ClC-5, epitope specificity and length, a good ability to stain the mouse kidney sections, and classification as an IgG subclass. Two monoclonal antibodies (SS53 and SS54) were selected and characterized (Table 1). Since ClC-5 is highly homologous (~80% amino acid sequence identity) to ClC-3 and ClC-4 chloride channels (2, 16, 17, 31), the antibodies were evaluated for the cross-reactivities to corresponding COOH-terminal peptides of ClC-3, -4, and -5 (Table 1). The result showed that SS53 was selective to ClC-5, whereas SS54 had a considerable cross-reactivity to ClC-3 and ClC-4. The determined epitopes were DSIF and HIAQMA in SS53 and SS54, respectively. In ClC chloride channel family, the alignment of DSIF is not conserved except for the amino acid sequences in ClC-5. Thus, we used SS53 for further studies.

In the Western blots (Fig. 1), SS53 recognized a band of about 85 kDa in the crude membranes prepared from both the mouse kidney and the stably ClC-5-transfected cultured cells with DEX treatment. This size of the band conformed well with that demonstrated by Gunther et al. (13). Indirect immunofluorescence staining of the stably transfected CHO-K1 cells of rat ClC-3 (C21) or ClC-5 (J2702) also confirmed the isoform specificity of our monoclonal antibodies. The SS53 antibody recognized J2702 cells (Fig. 2A) but not C21 cells (Fig. 2B), indicating the isoform specificity of SS53 for ClC-5.

Cellular and subcellular localization of ClC-5 along the nephron. Immunostaining of the mouse kidney by SS53 showed that ClC-5 was strictly localized in the proximal tubule cells and subpopulation of cortical collecting duct (CCD) cells. The specificity of antibody
(SS53) was evaluated using the preadsorbed antibody with synthesized peptide (CC54) or nonimmune rat IgG (data are not shown). No staining was seen, even though a high concentration of the antibody was applied in the glomerulus and the other renal tubule cells. ClC-5 appeared to be broadly expressed from the S1 to S3 segments in all proximal tubules examined. Subcellular localization determined by a high magnification showed a marked staining beneath the brush-border membrane, indicating the presence of ClC-5 in the apical cytoplasmic vesicles. Staining was also found in the brush-border membrane itself (Fig. 3A). Transmission electron microscopy showed the presence of immunoreactive material in the brush-border membrane and the subapical regions containing endocytotic vesicles (Fig. 3, B and C). These subcellular localizations were confirmed by immunogold labeling (data are not shown).

ClC-5 was localized broadly in the apical cytoplasmic regions of the subpopulation of collecting duct cells in the cortex. Double staining with FITC-conjugated SS53 and Cy5-conjugated anti-AQP2 antibody showed the staining of ClC-5 (green) in the apical site of the cells which lacked the AQP2 staining (red) (Fig. 4). No overlapping of the staining was observed (Fig. 4C). These findings indicate that CIC-5 is strictly localized in the intercalated cells and not in the principal cells in the CCD. Most of the CIC-5-positive intercalated cells were stained as apical predominant pattern, indicating that these cells were type A intercalated cells. This was confirmed using an antibody to the H\(^{+}\)-ATPase in parallel sections demonstrating strong apical labeling in type A intercalated cells, as described previously (3). Occasionally, a reverse pattern, i.e., staining of ClC-5 and H\(^{+}\)-ATPase at the basolateral region, was observed (see Fig. 7). In contrast, the antibody did not stain intercalated cells in the outer medullary region, and positive staining of the proximal S3 segments in the same section confirmed that this was not due to a technical error (Fig. 5A). In the other nephron segments, we could not detect any significant staining with anti-CIC-5 antibody compared with those with preadsorbed antibody with synthesized peptide (CC54) or with nonimmune rat IgG (Fig. 5, B and C).

Fig. 5. Overview of distribution of CIC-5 and AQP2 localization in outer medullary region (A). In outer stripe of outer medulla (right side), proximal S3 segments are stained exclusively for CIC-5 (green). However, staining of CIC-5 is weak or absent in intercalated cells (arrowheads) and thick ascending limb in outer (right side) and inner (left side) stripes of outer medullary region. Red, staining for AQP2 by the polyclonal antibody against AQP2. ClC-5 localization determined by a low magnification showed a negligible staining in the other nephron segments except for proximal tubule and collecting duct (B and C). The glomerulus partially showed a weak staining with SS53, but this was not different from that with secondary antibody alone.
Colocalization of CIC-5 with H\(^+\)-ATPase. Since the localization of CIC-5 was similar to that of H\(^+\)-ATPase, especially in the rat (3) but also to some extent in the mouse (30), we examined whether these two proteins colocalize. For this purpose double immunostaining was performed, and two-color confocal images of CIC-5 (green) and H\(^+\)-ATPase (red) demonstrated overlapping labeling patterns (resulting in a yellow color; Figs. 6 and 7) in proximal tubule and type A intercalated cells. In the proximal tubule cells, the region beneath the brush border was extensively stained (Fig. 6). In addition, green color at the brush-border membrane was evident after merging two color images, indicating presence of CIC-5 in this region in addition to the presence in subapical cytoplasmic vesicles.

In the collecting duct, the staining pattern for H\(^+\)-ATPase and CIC-5 was essentially the same. The intercalated cells characterized by their protuberance into the lumen predominantly expressed both H\(^+\)-ATPase and CIC-5 (Fig. 7).

To test the colocalization of CIC-5 and H\(^+\)-ATPases in the same membranes, membrane vesicles were immunoisolated with the SS53 antibody and then analyzed by immunoblotting. As shown in Fig. 8, vesicles immunoisolated by the anti-CIC-5 antibody showed abundant CIC-5 labeling in low-speed and high-speed sedimented fractions, whereas no CIC-5 or H\(^+\)-ATPase was recovered when nonimmune rat IgG was used for vesicle isolation (Fig. 8, A and B). Immunoblotting of CIC-5-immunoisolated vesicles revealed a clear presence of the 31-kDa H\(^+\)-ATPase in both fractions (Fig. 8B). Although the separation of the membrane fraction is not perfect, these results indicate that H\(^+\)-ATPase and CIC-5 colocalize in the same membranes in the plasma membrane and intracellular vesicle membrane.

**DISCUSSION**

Localization of CIC-5 along the nephron in the mouse kidney. In this study, we successfully made a CIC-5-specific rat monoclonal antibody and examined its cellular and subcellular localization in the mouse kidney by use of immunoblotting and light- and electron-microscopic immunohistochemistry.

Immunoblotting demonstrated selective and specific labeling of CIC-5 using membrane fraction of mouse kidney. In mouse CIC-5 localized throughout all proximal tubule segments (S1, S2, and S3 segments). In the proximal tubule cells, the intense staining of CIC-5 was evident in both the brush-border membrane and subapical region (Figs. 3 and 6). The intercalated cells in the CCD were also clearly stained. In contrast, the staining of thick ascending limb of Henle’s loop was minimal. According to a recent report by Teng-umnuay et al. (30), intercalated cells in the cortical and outer medullary collecting ducts of the mouse are composed of three

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**Fig. 6.** Localization of CIC-5 and H\(^+\)-ATPase in proximal tubule by confocal laser microscope. Cross section of several straight proximal tubules (S3) stained with E11 monoclonal antibody against the 31-kDa subunit of H\(^+\)-ATPase (A) and with SS53 antibody against CIC-5 (B). The two images A and B are merged in C. Red, staining for H\(^+\)-ATPase. Green, staining for CIC-5 channel. Costaining for H\(^+\)-ATPase and CIC-5 appears yellow.
morphologically and immunologically distinct cell types in a pattern similar to that of the rat. Most of the ClC-5-positive intercalated cells were type A intercalated cells characterized by an apical staining of H\textsubscript{1}-ATPase.

The immunohistological properties of ClC-5 localization along the nephron in the mouse kidney were basically similar to those of rat recently reported by Gunther et al. (13). However, the cellular and subcellular localization was somewhat different in two points: 1) in the proximal tubule cells, the labeling of the brush-border membrane was stronger in our study, suggesting that the physiological roles of ClC-5 are important in the brush-border membrane as well as in the cytoplasmic vesicles; 2) the ClC-5 expression was evident in the intercalated cells in the cortex, but the intercalated cells in outer medullary collecting duct (OMCD) almost completely lacked immunoreactive ClC-5 (Fig. 5). However, Gunther et al. showed the staining of ClC-5 in the type A intercalated cells in OMCD as well as in CCD. Although the reason for this discrepancy between rat and mouse is not clear, we should keep in mind the following possibilities: 1) the partitioning of membrane transport protein between brush-border membrane and subapical vesicles is quite dynamic, 2) the differences in relative distribution among these two membranes between different studies may be differences in the conditions under which the

![Image](https://example.com/image1.png)

**Fig. 7.** H\textsuperscript{+}-ATPase and ClC-5 in the collecting duct. Cross section of several CCDs stained with E11 monoclonal antibody against the 31-kDa subunit of H\textsuperscript{+}-ATPase (A) and with SS53 antibody of ClC-5 (B). The two images A and B are merged in C. Red, staining for H\textsuperscript{+}-ATPase; green, staining for ClC-5 channel. Costaining for H\textsuperscript{+}-ATPase and ClC-5 appears yellow.

![Image](https://example.com/image2.png)

**Fig. 8.** Immunoblots demonstrating labeling for ClC-5 and H\textsuperscript{+}-ATPases in vesicles immunoisolated with SS53 antibody against ClC-5. A: immunoblot labeled for ClC-5, demonstrating that the immunoisolation procedure efficiently isolated vesicles bearing ClC-5 from LS and HS fractions. In contrast, the signal from SP fraction was weak. B: immunoblot labeled for H\textsuperscript{+}-ATPases (anti-E11), showing labeling of ClC-5-immunoisolated vesicles from both LS, HS, and SP fractions. No ClC-5 or H\textsuperscript{+}-ATPase was recovered when nonimmune rat IgG was used for vesicle isolation as shown in control (cont). LS, low-speed fraction (17,000 g pellet), containing mainly plasma membranes; HS, high-speed fraction (200,000 g pellet), containing mainly intracellular vesicles; SP, supernatant of ClC-5-immunoisolated HS fraction.
animals are kept (diet, hydration, etc.). In addition, our data may also suggest that there is heterogeneity between the type A intercalated cells in CCD and OMCD in the mouse kidney.

The similarity between the localizations of CLC-5 and H^+-ATPase reported in the literature led us to examine whether they are colocalized (3, 30). According to the analysis by confocal microscope, H^+-ATPase was always present in the tubular cells in which CLC-5 was expressed, but CLC-5 was not always stained in the tubular cells in which H^+-ATPase was expressed. The intercalated cells in OMCD also showed this character. It is tempting to speculate that there are other ClC chloride channels together with H^+-ATPase in these cells. Other outwardly rectifying chloride channels are known to be expressed in the kidney, e.g., we have recently demonstrated that ClC-3 is expressed in the kidney and that it evokes outwardly rectified currents in the same manner as CLC-5 (17).

Physiological roles of CLC-5 in relation to Dent's disease. When we consider that mutations in the CLCN5 gene cause Dent's disease and that functional expression of these mutations causes the loss of function, we gain insights into the physiological roles of CLC-5. Low-molecular-weight proteinuria is a distinct characteristic of patients associated with mutations of the CLCN5 gene. This phenotypic characteristic implies that CLC-5 might play an important role in the reabsorption of low-molecular-weight protein in the proximal tubule. Previous studies have demonstrated that the low-molecular-weight proteins filtered by the glomerulus were reabsorbed by receptor-mediated endocytic uptake in the proximal tubule cells (4, 21). In this process, the endosomal acidification mediated by H^+-ATPase has been considered to play important roles in maintaining the endocytic activity (5, 9, 12, 32). Chloride channel(s) has been regarded as an indispensable element of this process in the dissipation of the electrical gradient generated by H^+ pump in the endosomes vesicles, but the molecular identity of such chloride channels remains elusive (10). The striking colocalization of CLC-5 and H^+-ATPase in the apical cytoplasmic vesicles in the proximal tubule strongly suggests that CLC-5 plays such a role.

However, another unsolved question remains about the channel characteristic of CLC-5. In the heterologous expression system using Xenopus oocytes (19, 29) or CHO cells (27), CLC-5 channel elicits strong outwardly rectifying Cl^- currents passing large currents only above +20 mV. Its inward currents were almost completely inactivated at negative potentials in the Xenopus oocytes, whereas small inward currents could be detected in the stably transfected mammalian cultured CHO cells. If CLC-5 was the channel to shunt the voltage created by the H^+ pump, then the expected orientation of Cl^- currents should be inward (Cl^- efflux). Thus, the strict outward rectification of the Cl^- currents evoked in the expression studies were oriented in the direction opposite to that needed for endosomes. The reason for this discrepancy is not clear at present, and we speculate that some undefined factor(s) that regulates the rectification of the CLC-5 channel is missing in the heterologous expression system.

In summary, CLC-5 was abundantly present not only in proximal tubule cells, but also in type A intercalated cells of CCD in parallel to H^+-ATPase. CLC-5 seems likely to work cooperatively with H^+ pump as an essential element for acidification of endosomes and proton secretion into the lumen. These findings suggest that the molecular abnormalities of the CLC-5 channel cause the dysfunction of proximal tubule and type A intercalated cells.¹

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¹ During the submission and revision of this article, two independent studies were published reporting the immunolocalization of CLC-5 in rat kidney (Luyckx, V. A., F. O. Goda, D. B. Mount, T. Nishio, A. Hall, S. C. Hebert, T. G. Hammond, and A. S. L. Yu. Am. J. Physiol. 275 (Renal Physiol. 44): F761–F769, 1998) and human kidney (Dedyuyst, O., P. T. Christie, P. J. Courtoy, R. Beauwens, and R. V. Thakker. Hum. Mol. Genet. 8: 247–257, 1999). In these studies, polyclonal antibodies were raised against the CLC-5 peptides, in which CLC-5 isomorphism antibody fractions were prepared by immunoadsorption against the corresponding fusion proteins or synthetic peptides of CLC-3 and CLC-4. Our finding is quite different in immunolocalization in the thick ascending limb of Henle's loop and/or intercalated cells of the collecting ducts. It is not clear whether the difference is due to the species and/or methodology, especially the modification of antibody properties following repeat immunoadsorption.

REMARKS


