Intrarenal and subcellular localization of rat CLC5

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Intrarenal and subcellular localization of rat CLC5. Am. J. Physiol. 275 (Renal Physiol. 44): F761–F769, 1998.—Dent’s disease, an inherited disorder characterized by hypercalciuria, nephrolithiasis, nephrocalcinosis, rickets, low-molecular-weight proteinuria, Fanconi’s syndrome, and renal failure, is caused by mutations in the renal chloride channel, CLC5. The normal role of CLC5 is unknown. We have investigated the intrarenal and subcellular localization of CLC5 in rat kidney by in situ hybridization and immunohistochemistry. By in situ hybridization, CLC5 mRNA was detected predominantly in cortical medullary ray and outer medullary tubule epithelial cells. Polyclonal antisera was generated against a CLC5 fusion protein, affinity purified, and immunoadsorbed against CLC3 and CLC4 to yield a CLC5 isoform-specific antisera. By immunohistochemistry, CLC5 protein was localized to the intracellular domain of tubular epithelial cells in the S3 segment of the proximal tubule and the medullary thick ascending limb. By subcellular membrane fractionation and flow cytometry, CLC5 expression was found in outer medullary endosomes. These findings are consistent with a model in which CLC5 encodes an endosomal chloride channel that facilitates acidification and trafficking of renal epithelial endosomes.

chloride channel; calcium; nephron; endosome; Dent’s disease

DENT’S DISEASE, a disorder characterized by hypercalciuria, nephrolithiasis, nephrocalcinosis, rickets, low-molecular-weight proteinuria, Fanconi’s syndrome, and renal failure (33), has recently been mapped to the renal chloride channel gene, CLC5 (15, 16, 19, 33). Chloride currents expressed by mutant CLC5 in Xenopus oocytes are significantly reduced or absent compared with those of wild-type CLC5 (15), suggesting a causal role for loss of CLC5 function in this disease. The mode of presentation of Dent’s disease and its clinical severity is quite variable, with affected members in some families developing end-stage renal disease or rickets and others having only persistent hypercalciuria or low-molecular-weight proteinuria.

Exactly how loss of CLC5 function leads to the broad clinical spectrum encompassing Fanconi’s syndrome, hypercalciuria, and low-molecular-weight proteinuria has not yet been explained. We (8) and others have postulated that CLC5 is expressed on the renal tubular endosomal membrane where it would provide the chloride conductance required for endosomal acidification, a prerequisite for efficient membrane protein recycling. Interference with apical membrane transport protein recycling and endocytosis would lead to defective reabsorption of glucose, amino acids, phosphate, uric acid, and low-molecular-weight proteins in the proximal tubule and of calcium in distal nephron segments. Dent’s disease is rare, but the problems of “idiopathic” hypercalciuria and nephrolithiasis are significant in the broader population, in a subset of whom CLC5 dysfunction may be a contributing factor. The nephron segment localization of CLC5 may provide important clues to its physiological function.

The intrarenal distribution of CLC5 is controversial. By RT-PCR of microdissected tubules (30), CLC5 was found to be expressed predominantly in the cortical collecting tubule, S3 segment of proximal tubule, and medullary thick ascending limb of the loop of Henle (MTAL). RT-PCR, however, is not entirely specific, because cross-contamination of tubule samples cannot unequivocally be excluded. Also, the technique is not quantitative and so cannot distinguish between physiological levels of expression and minute amounts of contamination. By contrast, a recent in situ hybridization study localized CLC5 mRNA only to type A intercalated cells of the collecting duct (17). No studies of CLC5 protein expression and subcellular localization have been reported. In the present study, we have determined the nephron segment and subcellular localization of CLC5 mRNA and protein by in situ hybridization and immunohistochemistry.

METHODS

Preparation of riboprobes and Northern blot analysis. A CLC5 cDNA containing the full coding region (corresponding to nucleotides 11–2550, GenBank accession no. D50497; Ref. 24) was cloned by RT-PCR from rat kidney cortex. A 770-bp CLC4 cDNA sequence was cloned in the same manner (corresponding to nucleotides 1034–1798, GenBank accession no. Z49916; Ref. 23). Digoxigenin (Dig)-labeled sense and antisense riboprobes were prepared by in vitro transcription with T3 or T7 RNA polymerase. For in situ hybridization, riboprobes were treated with alkaline hydrolysis to yield fragments of ~200 bp. Northern blots were performed at high stringency using the Dig Genius System (Boehringer-Mannheim, Indianapolis, IN) as described previously (35).

Preparation of tissue cryosections. Adult male Sprague-Dawley rats were anesthetized with pentobarbital and perfused via the infrarenal aorta with 4% paraformaldehyde followed by 750 mosmol/kg of sucrose-PBS. Perfused kidneys were then removed, equilibrated in 30% sucrose overnight at 4°C, embedded in Tissue-Tek (Sakura, Torrance, CA), and frozen in isopentane. For immunohistochemistry, 5-μm thick sections were cut on a cryostat, mounted on Superfrost-Plus glass slides (Fisher, Pittsburgh, PA), and air dried for 20 min. For in situ hybridization, unperfused kidneys were used, which were prepared similarly except that cryosections were 10-μm thick.
In situ hybridization. Our protocol was modified from previously published methods (25). Cryosections were fixed for 15 min in 4% paraformaldehyde at room temperature, washed three times in PBS, acetylated for 10 min, washed twice in PBS and once in 2× SSC. Sections were then prehybridized at room temperature for 6–8 h. Hybridization was performed in a humidified chamber overnight at 70°C with 400 ng/ml hydrolyzed riboprobes in the presence of 50% formaldehyde. High-stringency washes were performed at 70°C with 2× SSC for 1 and 5 min, followed by 0.2× SSC twice for 30 min. Immunodetection of Dig-labeled probes was performed using the Dig Genius System. Sections were rinsed in washing buffer and incubated for 1 h in blocking solution, followed by 1 h in blocking solution containing alkaline phosphatase-conjugated anti-digoxigenin antibody at 1:500 concentration. Following two 30-min washes in washing buffer, sections were transferred to detection buffer for 5 min. For the color reaction, sections were incubated upside-down in the dark with detection buffer containing nitro blue tetrazolium chloride, 30 mg/ml, and 5-bromo-4-chloro-3-indolylphosphate, 15 mg/ml, for up to 8 h, washed, air dried, mounted with Vectashield (Vector Laboratories, Burlingame, CA), and examined under light microscopy.

Generation and affinity-purification of the C1 antiserum. A cDNA encoding a 108-amino acid polypeptide from the region of the carboxy terminus of rat CLC5 residues 570–677 (GenBank accession no. Z56277; Ref. 30) that shares the least identity to the homologous region of either of the closely related isoforms CLC3 (62% identity, GenBank accession no. D17521; Ref. 14) and CLC4 (65% identity, GenBank accession no. Z36944; Ref. 11) was cloned into the bacterial expression vector, pMALT-c2 (New England Biolabs, Beverly, MA), downstream of the maltose-binding protein (MBP) coding sequence and a recognition site for the specific protease, factor Xa. Synthesis of the MBP-CLC5 fusion protein under the control of a lacZ promoter was induced by isopropyl-β-D-thiogalactopyranoside. The protein was purified by binding to an amyllose column and used to immunize two rabbits by footpad injection, followed by four booster injections, and tested for reactivity to the fusion protein by ELISA.

Serum from the rabbit with the highest titer of specific antibody was collected and affinity purified in a two-step, positive-negative selection procedure. Antibodies with specificity for MBP were adsorbed away by incubation of serum with an MBP-galactose fusion protein (MBP-gal) immobilized on polyvinylidene difluoride (PVDF) membrane. Antibodies with specificity for CLC5 were then selected by incubation of the negatively selected serum with MBP-CLC5 fusion protein bound to PVDF and elution with 0.2 M glycine-HCl, pH 2.6. The eluate was neutralized to pH 7.4 and dialyzed against PBS, to yield C1 antiserum.

Preparation of the isoform-specific C2 antibody fraction. To select a CLC5 isoform-specific antibody fraction, C1 serum was subjected to a further round of selection in which cross-reacting antibodies to the CLC3 and CLC4 isoforms were removed by immunoabsorption. MBP fusion proteins containing the homologous carboxy-terminal regions of CLC3 (residues 584–691) and CLC4 (residues 571–678) were immobilized on PVDF and incubated with C1 overnight at 4°C. The unbound serum fraction was designated C2.

Western blotting. Protein samples were denatured by boiling in 2% SDS, electrophoresed in discontinuous polyacrylamide gels, electrophoretically transferred to PVDF membrane, immunoblotted with the C1 antibody at 1:100 dilution or C2 at 1:25 dilution, and detected using the ECL enhanced chemiluminescence system (Amersham, Arlington Heights, IL). The following items were used as negative controls. 1) C1 serum was preincubated overnight at 4°C with equimolar MBP-CLC5 fusion protein (peptide-blocked C1). 2) Premunere serum was diluted 1:1,000 to the equivalent immunoglobulin concentration to C1 (preimmune serum). 3) Antibodies with specificity for MBP (anti-MBP) were isolated from the eluate of the first adsorption step in affinity purification described above, and used at 1:5,000.

The specificity of the C1 and C2 antibodies was tested by immunoblotting against MBP-CLC3, -CLC4, and -CLC5 digested with factor Xa, which proteolytically cleaves these fusion proteins into separate MBP and CLC polypeptides. Immunoblots were also performed on crude kidney membranes prepared from rat and mouse kidneys by a standard differential centrifugation technique (6). The 47,000 g pellet and supernatant were considered to be the crude membranes and cytosolic fraction, respectively.

Immunohistochemistry. C1 antibody staining was performed using fluorescein isothiocyanate (FITC) or cyanine-3 (CY3) with the tyramide signal amplification system (NEN Life Science Products, Boston, MA). Cryosections, 5 µm thick, were thawed and rehydrated in PBS. Sections were permeabilized for 5 min in 1% SDS, washed three times, then incubated for 1 h in 5% goat serum/1% BSA in PBS at 37°C to reduce nonspecific background staining. Sections were then blocked sequentially with a drop of avidin and biotin (Vector Laboratories), each for 5 min at 37°C. After one rinse in PBS, antibody-labeled sections were incubated overnight at 4°C with a 1:50 dilution of the primary antibody (C1). Sections were sequentially washed in high-salt PBS then regular PBS and then incubated at 37°C with 1:800 dilution of the secondary antibody, biotinylated goat anti-rabbit IgG, for 1 h. Sections were then washed and incubated with 0.3% H2O2-90% methanol for 30 min at room temperature to block endogenous peroxidase activity. After four washes in PBS, tyramide amplification was carried out according to manufacturer’s recommendations, except that FITC-conjugated tyramide was used at 1:100 and the CY3-conjugated tyramide was used at a 1:250 concentration. Sections were mounted in Vectashield (Vector Laboratories) and viewed by epifluorescence microscopy.

C2 antibody staining was performed without amplification at a 1:2 dilution and detected with FITC- or Texas Red-conjugated secondary antibody at 50 ng/ml concentration. To evaluate the specificity of staining of C1 and C2 antibodies on tissue sections, C1 and C2 were peptide-blocked by overnight preincubation at 4°C with up to fourfold molar excess of MBP-conjugated CLC5 peptide.

In double-labeling experiments, the polyclonal antibody to calbindin-D28K (CBB) (2) (generous gift from Dr. René Bindels) was used at a concentration of 1:250, polyclonal anti-buromante-sensitive Na-K-2Cl cotransporter (BSC1) (13) at 1:500, and polyclonal anti-thiazide-sensitive NaCl cotransporter (TSC) (18) at 1:500; and the E11 monoclonal antibody against the 31-kDa subunit of the vacuolar H+-ATPase (9) (generous gift from Dr. Stephen Gluck) was applied undiluted. Artifacts due either to cross-reactivity of the second secondary antibody to the first primary antibody (when the two primary antibodies were derived from the same species) or to spectral overlap between the two fluorophores were excluded by one or more of the following control experiments. 1) The pattern of staining of sections with each primary antibody when applied individually was demonstrated to be identical to the staining by the same antibody in double-labeled sections. 2) The staining pattern was unchanged when the order of application of the two primary antibody was reversed. 3) The first primary antibody was applied at a very low concentration (undetectable without amplification) and amplified with tyramide, whereas the second primary antibody was detected with unamplified fluorescence-conjugated secondary antibody.
Flow cytometry analysis of endosomes. True endosomes were isolated from rat kidneys and labeled with C1 or C2 antisera by the indirect sandwich technique, as previously published (7). In brief, the kidneys of adult male Sprague-Dawley rats were harvested without pretreatment or 10 min after injection of intravenous FITC-dextran (average molecular mass, 10 kDa) as an entrapment marker for endosomes or with nonfluorescent dextran as a negative control. The cortex and outer medulla were isolated by macroscopic dissection and homogenized, and membranes of low buoyant density were enriched by successive rounds of differential centrifugation followed by separation on an 18% Percoll gradient at 48,000 g. Light endosomes, which have previously been shown to represent a true endosomal population (7), were removed from the top of the gradient.

Blocking of nonspecific binding sites was performed on aliquots of endosomes by incubation with 50% clarified normal goat serum for 2 h at 4°C. Endosomes were then incubated overnight at 4°C in C1 or C2 antisera at dilutions ranging from 1:33 to 1:10,000, washed, incubated for 4 h at room temperature with the phycoerythrin-conjugated goat anti-rabbit secondary antibody and washed again. Flow cytometry was performed on a Becton-Dickinson FACStar flow cytometer using 100-mW excitation of blue 488-nm light from a 5-W argon laser, as described previously (12). Data were collected on 2,000 individual endosomes in a list mode file. The binding of antibodies to different endosomal populations was compared using Kolmogorov-Smirnoff summation statistics (34).

RESULTS

Intrarenal localization of CLC5 by in situ hybridization. The intrarenal distribution of CLC5 mRNA was determined by high-stringency in situ hybridization. A CLC5 riboprobe that encompasses the full coding region was prepared and shown to be isoform specific by high-stringency Northern blot. As shown in Fig. 1A, the CLC5 probe hybridizes to a single band of ~9.5 kb, the expected size for CLC5 (30), with no cross-hybridization to the predominant 4.4-kb transcript for CLC4 (11). Figure 2 shows rat kidney cryosections hybridized to antisense and sense CLC5 probes. Prominent staining with the antisense probe was seen within tubule epithelial cells in the medullary rays and outer stripe of outer medulla, consistent with the distribution of the S3 segments of proximal tubules. In the inner stripe of the outer medulla, strongly positive staining was seen in a distinct subset of tubules that appeared to be the thick ascending limbs of the loop of Henle. Staining in the inner medulla and papilla was sparse. Minimal background staining was observed in control sections hybridized with the sense probe.

Generation of a polyclonal antisera to CLC5. An affinity-purified polyclonal antibody (C1) was prepared against a CLC5 fusion protein, so that the cellular and subcellular localization of CLC5 could be further refined. As shown by Western blotting in Fig. 1B, the C1 antibody binds to a single peptide of ~80 kDa (the predicted molecular mass for rat CLC5 is 83 kDa) in both mouse and rat kidney. As expected for a membrane transport protein, immunoreactivity is confined to the crude membrane fraction and is absent from the cytosolic fraction. C1 also recognizes the 55-kDa intact MBP-CLC5 fusion protein against which the antisera was originally raised. Preimmune serum, as well as C1 serum blocked with peptide, showed no immunoreactivity in negative control blots. This indicates that C1 contains antibodies specific for CLC5. C1 binds neither to the negative control construct, MBP-gal, nor to a 42-kDa degradation fragment found in the MBP-CLC5 peptide sample. MBP antisera was used to demonstrate that the 42-kDa fragment is in fact the MBP peptide, spontaneously cleaved off from its CLC5 portion. This confirms that our affinity purification method effectively removed all traces of MBP-reactive antibody from the C1 serum.

Although we specifically selected the region of the CLC5 protein against which our antisera was raised on the basis of least similarity to homologous isoforms,
the C1 antiserum does in fact cross-react with CLC3 and CLC4 (Fig. 1C). We therefore further prepared a CLC5 isoform-specific antibody fraction by immunoadsorption of C1 against CLC3 and CLC4 fusion proteins immobilized on solid supports. The resultant C2 serum fraction was found to be highly specific for CLC5 by Western blot (Fig. 1C).

Immunohistochemical localization of CLC5. Immunofluorescence was performed on perfusion-fixed kidney sections with C1 and C2 antisera. To confirm the identity of specific nephron segments, double labeling was performed with either anti-CBP antibody (marker for distal convoluted tubule and connecting segment), anti-BSC1 (marker for apical membrane of thick ascending limb), anti-TSC (marker for apical membrane of distal convoluted tubule), or anti-vacuolar-type H\(^+\)-ATPase (marker for apical membrane of type A and basolateral membrane of type B intercalated cells of collecting duct). As shown in Fig. 3, C1 staining is present in tubule epithelial cells of the renal cortex and outer medulla but absent from glomeruli, blood vessels, or the interstitium. C1 staining is seen strongly on the apical membrane and subapical region of proximal convoluted and straight tubules with a faint intracellular signal. The specificity of C1 binding was confirmed by demonstrating that preincubation with CLC5 peptide blocks binding in both the cortex and outer medulla (Fig. 3, b and d). In double-labeled sections (Fig. 4, a-d), C1 staining is clearly absent from calbindin-D\(_{28k}\)-positive cells in the renal cortex but is present on the apical membrane of calbindin-D\(_{28k}\)-negative cells, which are presumed to be intercalated cells of the connecting segment (Fig. 4a). C1 stains intracellularly in distal convoluted tubule cells, identifiable by apical TSC staining (Fig. 4b). C1 staining is absent from cortical BSC1-positive tubules (data not shown), but in the outer medulla, intracellular C1 staining is observed in MTAL cells that are also positive for apical BSC1 (Fig. 4d). In the cortical collecting duct, C1 staining colocalizes with the vacuolar-type H\(^+\)-ATPase to the apical.
membrane of type A intercalated cells and the apical and basolateral membranes of type B intercalated cells (Fig. 4c). In summary, C1 staining is present on the apical membrane domain of the proximal tubule and type A intercalated cells, on the apical and basolateral membranes of type B intercalated cells, and in the intracellular compartments of the proximal tubule, MTAL, and distal convoluted tubule.

Because C1 cross-reacts with CLC3 and CLC4, we repeated our immunohistochemistry studies with the highly CLC5-specific C2 antiserum. C2 staining is seen intracellularly in tubules of the medullary ray and outer medulla and is absent from the inner medulla (Fig. 3). In double-labeled cortical sections, C2 staining is seen to localize to S3 segments of proximal tubule but, in contrast to C1, is absent from distal convoluted tubule, connecting segment, cortical thick ascending limb, and cortical collecting duct (Fig. 4, e–i). In outer medulla, C2 stains MTAL and colocalizes to tubules with apical BSC1 staining (Fig. 4j).

In summary, our results indicate that CLC5 is expressed in the intracellular compartments of the S3 segment of the proximal tubule and the MTAL (Fig. 5). CLC5 is expressed in outer medullary endosomes. To determine whether intracellular CLC5 is located in endosomes, rats were preloaded with intravenous FITC-dextran as a freely filtered luminal entrapment marker (or nonfluorescent dextran as a negative control) and apical membrane-derived endosomal vesicles isolated from the outer medulla, where the majority of CLC5 immunoreactivity was identified. CLC5 epitopes exposed on the cytoplasmic side of the endosomal membrane were labeled with either C1 or C2 primary antibody and indirectly conjugated with a phycoerythrin-labeled secondary antibody, and the vesicles were subjected to two-color fluorescence flow cytometry. The results are displayed as three dimensional histograms in Fig. 6A. Vesicles that were isolated from rats loaded with nonfluorescent dextran and not labeled with any primary antibody exhibit minimal background fluorescence, as shown by the low values on both horizontal axes in Fig. 6. Vesicles derived from rats loaded with FITC-dextran demonstrate significant fluorescence in the bandwidth for FITC emission (left horizontal axis in Fig. 6), demonstrating entrapment of the fluorophore by endocytosis, as shown previously (7). When FITC-dextran-loaded vesicles were labeled with C1 antiserum, substantial antibody binding was observed, as evidenced by the high phycoerythrin fluorescence values (right horizontal axis). Furthermore, there was a close correlation between phycoerythrin and FITC fluorescence, indicating colocalization of C1 immunoreactivity and entrapped FITC-dextran to the same vesicle population. The dilution curve of the amount of antibody bound to endosomal vesicles plotted against the dilution of C1 used was nonlinear (data not shown) and highly suggestive of specific binding.

To confirm that CLC5 is expressed in endosomes, these experiments were subsequently repeated using the CLC5 isoform-specific antibody fraction, C2 (Fig. 6B). Outer medullary light endosomal vesicles demonstrated a bimodal phycoerythrin fluorescence distribution (right horizontal axis), with approximately one-third of vesicle binding in a highly specific manner to C2. Furthermore, the size distribution (as estimated by side scatter, left horizontal axis) of the subpopulation of C2-positive endosomal vesicles was similar to that of the whole population, excluding vesicle aggregation as an artifactual cause of increased fluorescence. These data confirm that CLC5 protein is expressed on endosomes.

**DISCUSSION**

Using in situ hybridization and immunohistochemistry, we have localized CLC5 within the rat kidney. In
initial immunohistochemical studies using C1 antiseraum, staining was seen in several different nephron segments on both the plasma membrane and in intracellular compartments. The staining observed with C1 could not, however, be assumed to represent only CLC5 expression, because although attempts were made to maximize its specificity, C1 was found to cross-react with CLC5’s closest homologs, CLC3 and CLC4 (Fig. 1C). Accordingly, it was necessary to purify our antiseraum by immunoadsorption with CLC3 and CLC4 peptides to remove cross-reacting antibodies.

Using the purified antiserum, C2, CLC5 was found to be strongly expressed in the intracellular compartments of both S3 segments of proximal tubules and the MTAL. These findings are likely to represent true CLC5 staining, because 1) C2 was shown to be CLC5 isoform-specific by Western blot analysis (Fig. 1C); 2) the results are in full agreement with our in situ hybridization, which was performed at high stringency, under which conditions we have clearly demonstrated that our CLC5 riboprobe does not cross-react with CLC4 mRNA (Fig. 1A); and 3) our results are in general agreement with previous work in the rat kidney in which CLC5 mRNA was localized by RT-PCR (30). Because our immunoadsorption technique would be expected to remove antibodies reactive to epitopes common to CLC5 and either CLC3 or CLC4, we cannot formally exclude the possibility that CLC5 may exist in several antigenically distinct forms and that reactivity to one or more of these forms may have been lost with immunoadsorption. Some CLC5 staining may therefore have been lost with C2 antiserum.

Our results by both in situ hybridization and immunohistochemistry do not agree with those of a recent in situ hybridization study in which CLC5 was reported to be expressed only in type A intercalated cells (17). There are two main differences in in situ hybridization methodology between that study and ours, which might perhaps explain this discrepancy. First, Obermüller et al. (17) perfusion-fixed their kidneys with paraformaldehyde, which, although this does indeed improve tissue morphology, in our experience appears to hinder access of riboprobe to cellular RNA and reduces signal-to-noise ratio. Second, our hybridization conditions were more stringent than those used by Obermüller et al. (42°C in 50% formamide; Ref. 17).

By flow cytometry analysis of subcellular membrane fractions, we found that CLC5 was expressed in the endosomal compartment of cells in the outer medulla (as expected for the S3 segment of proximal tubule and MTAL). Very few electrophysiological studies of rat kidney endosomal chloride channels have been performed (21, 27), and these report widely differing properties. It is difficult to speculate whether any of the reported channels correspond to CLC5, since the electrophysiological properties of CLC5 have only been characterized briefly, only at the whole cell level, and only in the unphysiological setting of heterologous cell expression systems.

The proximal tubule is the site of sodium-coupled reabsorption of solutes such as glucose, amino acids, and phosphate as well as the site of endocytosis of low-molecular-weight proteins. It is therefore not surprising that CLC5 is expressed in this tubule segment, given that a partial Fanconi’s syndrome and low-molecular-weight proteinuria are prominent features of Dent’s disease. The localization of CLC5 to the endosomal compartment in this tubule segment is consistent with a role in providing counter-ion conductance for the electrogenic vacuolar H^+−ATPase pump, thereby permitting adequate endosomal acidification. Indeed, optimal endosomal acidification has been shown to require the presence of cytoplasmic chloride ions and to be inhibited by nonspecific chloride channel blockers (1). However, if CLC5 is a strong outward rectifier (as observed when expressed on the plasma membrane of Xenopus oocytes; Ref. 30), then it would not be predicted to provide a significant conductance for chloride entry into the endosome. This apparent paradox may be explained if the in vivo physiological properties of CLC5 differ from those observed in Xenopus oocytes, either simply because of artifacts arising from the use of an amphibian expression system or because CLC5 normally associates with other peptides to form a multi-subunit channel with different functional properties. Since endosomal acidification is necessary for the normal recycling of plasma membrane proteins (5, 20), the proximal reabsorption defects in Dent’s disease might therefore be due to defective endocytosis of...
Our results also raise some interesting hypotheses for the pathogenesis of hypercalciuria in Dent’s disease. Transepithelial calcium transport and its regulation by calcitropic hormones is conventionally believed to be primarily mediated by the distal convoluted tubule and the connecting segment (3, 10, 28, 29), yet C2 staining was absent from these cells. As discussed previously, it is possible, although unlikely, that C2 may have missed CLC5 expression that was detected, for instance in the distal convoluted tubule, with C1. C2 staining, and therefore CLC5 expression, is, however, clearly present in the MTAL. This staining is unlikely to represent cross-reactivity with CLC-K1 or -K2 (also expressed in the MTAL), as these channels share less than 20% homology with CLC5 in the carboxy-terminal region. The thick ascending limb is not only an important site for paracellular calcium reabsorption, but it may also mediate active, transepithelial calcium transport in certain species, when stimulated by various peptide hormones such as glucose, amino acids, and phosphate.

In conclusion, CLC5 is predominantly expressed intracellularly in tubule epithelial cells of the renal outer medulla and is found on endosomal membranes derived from this region. The finding that CLC5 is expressed in endosomes of the MTAL and S3 segment of the proximal tubule lends significant weight to the hypothesis that endosomal dysfunction may be a unifying cause of the clinical features of Dent’s disease.

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1 Shortly after this manuscript was accepted for publication, a study by W. Günther, A. Lüchow, F. Cluzeaud, A. Vandewalle, and T. J. J. Ents (Proc. Natl. Acad. Sci. USA 95: 8075–8080, 1998) reporting the immunolocalization of CLC5 in rat kidney was published. In this study, polyclonal antibodies were raised against two CLC5 peptides that share identity over at least 12 of 15 amino acid residues with either CLC3 or CLC4. The authors attempted to remove cross-reactivity by immunoadsorbing their serum against CLC4 peptide and testing the specificity of the resultant antibodies against Western blots of membranes isolated from Xenopus oocytes injected with CLC3, CLC4, and CLC5 cRNA. However, these studies were inconclusive, because no evidence was presented that CLC3 or CLC4 protein was expressed in the oocytes, and indeed this group have previously reported absence of functional expression of CLC3 and CLC4 in this preparation (J. Physiol. 482: 195–255, 1995). Of note, their immunohistochemistry findings with these antibodies are strikingly similar to our initial findings with our C1 antibody (which cross-reacts with CLC3 and CLC4), namely, staining of the subapical domain of proximal tubule with predominance in the S1 segment, and staining of the apical membrane of α-intercalated cells, as well as apical and basolateral membranes of β-intercalated cells of the collecting duct. In the light of our quite different findings with the CLC5-specific C2 antibody, it is possible that their antibodies may have stained primarily CLC3 or CLC4.