Receptor-mediated endocytosis and endosomal acidification is impaired in proximal tubule epithelial cells of Dent disease patients

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Receptor-mediated endocytosis, involving megalin and cubilin, mediates renal proximal-tubular reabsorption and is decreased in Dent disease because of mutations of the chloride/proton antiporter, chloride channel-5 (CLC-5), resulting in low-molecular-weight proteinuria, hypercalciuria, nephrolithiasis, and renal failure. To facilitate studies of receptor-mediated endocytosis and the role of CLC-5, we established conditionally immortalized proximal-tubular epithelial cell lines (ciPTECs) from three patients with CLC-5 mutations (30:insH, R637X, and del132-241) and a normal male. Confocal microscopy using the tight junction marker zona occludens-1 (ZO-1) and end-binding protein-1 (EB-1), which is specific for the plus end of microtubules, demonstrated that the ciPTECs polarized. Receptor-mediated endocytic uptake of fluorescent albumin and transferrin in 30:insH and R637X ciPTECs was significantly decreased, compared with normal ciPTECs, and could be further reduced by competition with 10-fold excess of unlabeled albumin and transferrin, whereas the del132-241 ciPTEC, receptor-mediated endocytic uptake was abolished. Investigation of endosomal acidification by live-cell imaging of pHluorin-VAMP2 (vesicle-associated membrane protein-2), a pH-sensitive-GFP construct, revealed that the endosomal pH in normal and 30:insH ciPTECs was similar, whereas in del132-241 ciPTECs, receptor-mediated endocytic uptake was abolished. Investigation of endosomal acidification by live-cell imaging of pHluorin-VAMP2 (vesicle-associated membrane protein-2), a pH-sensitive-GFP construct, revealed that the endosomal pH in normal and 30:insH ciPTECs was similar, whereas in del132-241 ciPTECs, receptor-mediated endocytic uptake was abolished. The role of CLC-5 in Cl− conductance and endosomal acidification has been investigated in vitro by expression of wild-type and mutant CLC-5s in Xenopus oocytes (9, 14) and human embryonic kidney (HEK) cells (15), and in vivo by generating mice that harbor a CLC-5 mutation (E211A) that converts the CLC-5 (chloride/proton exchanger) into a pure Cl− conductance that some, but not all, CLC-5 mutants may also impair endosomal acidification (17). Thus, the in vitro expression studies of Dent disease in man provide new reagents/analytic tools; C.M.G., M.J.W., S.E.P., J.D.L., L.P., and R.V.T. contributed new reagents/analytic tools; C.M.G., M.J.W., S.E.P., B.H., L.P., P.S.J., J.D.L., E.N.L., and R.V.T. contributed new reagents/analytic tools; C.M.G., M.J.W., S.E.P., B.H., L.P., P.S.J., J.D.L., E.N.L., and R.V.T. edited the manuscript; and C.M.G., S.E.P., B.H., J.D.L., and R.V.T. analyzed the data; C.M.G., S.E.P., and R.V.T. wrote the paper. The authors declare no conflict of interest.

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Results
Establishment of Dent Disease ciPTECs. Three males with known CLCN5 mutations, comprising 30:insH, deletion of codons 132–241 (del132-241) and R637X (Table 1) that affected different functional domains of CLCN5 (Fig. 1A) were identified (9, 14, 20, 21), first void morning urine samples were obtained, and primary cultures were established (19). After conditional immortalization by infecting with SV40-T and human telomerase reverse transcriptase (hTERT), the presence of the CLCN5 mutation was confirmed by DNA sequence analysis of each cell line and in matched leukocyte DNA samples from each patient. Cultures of immortalized urinary cells may contain different types of urinary tract cells, including proximal tubular cells (18). To determine the content of proximal tubular cells in these cultures, flow cytometry using fluorescein-isothiocyanate (FITC)-labeled antibody against cluster determinant (CD)13, which is aminopeptidase-N and an established marker for the proximal tubule brush border (22), and flow cytometry using fluorescein isothiocyanate (FITC)-labeled antibody against CD13 (22). Readings were initially made in unstained cells (left), and then in CD13-FITC-treated cells (center). Forward scatter was plotted (on y axis) against the CD13-FITC count (on x axis). The readings from the unstained (red peak) and CD13-FITC stained cells (blue peak) are representative for each ciPTEC (right). (C) RT-PCR analysis of genes expressed in the renal proximal tubule (ABCC4, ABCC8, loop of Henle (NKCC2 and KCNJ1), distal tubule (CALB1 and TRPV5), and bladder (UPKIIIa)). RNA extracted from the four ciPTECs (three Dent disease patients and one control, wild-type) was used. RNA from a normal kidney and a human bladder cell line, and a water blank were used as positive and negative controls, respectively. Calmodulin (Cam) expression was used as a loading control. (D) Western blot analysis of proteins expressed in the renal proximal tubule (AQP1), distal tubule (TRPV5), collecting duct (AQP2), and bladder (UPKIIIa). Cell lysates from each of the four ciPTECs were used. Cell lysates from a normal human kidney and a bladder cell line were used as positive controls, and α-tubulin was used as a loading control. (E) Confocal microscopy of the four ciPTECs stained with anti-ZO-1 (green) and anti-EB-1 (red). The presence of tight junctions (green) at the apical surface with EB-1–labeled microtubules below (red) demonstrates that the ciPTECs are polarized. (F Upper) XY image. (F Lower) Orthogonal view. (Scale bars: 10 μm.)
Expression of these receptor-mediated endocytic pathway facilitators such as kinesin family member 3B (KIF3B), and water channel, aquaporin-1 (AQP1), but not the collecting duct analysis revealed that the ciPTECs expressed the proximal tubular kidney and bladder, respectively (Fig. 1).

Characterization of the ciPTECs from the patients with Dent disease and a normal (wild-type) male by RT-PCR using extracted total RNA showed the following: the presence of the proximal tubular expressed genes for ATP-binding cassette family member multidrug resistance protein 4 (ABCB4) and P-glycoprotein (ABCB1), and an absence of genes that are expressed in: the thick ascending limb, Na\(^+\)-K\(^+\)-Cl\(^-\) cotransporter (NKCC2) and K\(^+\) inwardly-rectifying channel, subfamily J, member 1 (KCNJ1); distal tubule, Calbindin-1 (CALB1) and Transient receptor potential cation channel, subfamily V, member 5 (TRPV5); and bladder, Uroplakin IIIA (UPKIIIA). The appropriate renal or bladder expression of these genes was shown in total RNA from human kidney and bladder, respectively (Fig. 1C). Similarly, Western blot analysis revealed that the ciPTECs expressed the proximal tubular water channel, aquaporin-1 (AQP1), but not the collecting duct specific water channel, aquaporin-2 (AQP2), the distal tubular TRPV5, or the bladder UPKIIIA (Fig. 1D). Thus, the ciPTECs from the patients with Dent disease and the normal male were demonstrated only to express markers of proximal tubule cells. A critical function of PTECs is reabsorption of solutes from the glomerular filtrate, by the receptor-mediated endocytic pathway, whose components include the multiligand receptors megalin, also referred to as LRP2, and cubilin (CUBN) and intracellular facilitators such as kinesin family member 3B (KIF3B), and V-ATPase subunits A2 and D (ATP6V0A2 and ATP6V1D). RT-PCR using total RNA obtained from the ciPTECs revealed expression of these receptor-mediated endocytic pathway components (Fig. 1E) as well as CLCN5 (Table S1), thereby indicating that the ciPTECs may be able to reabsorb molecules such as albumin and transferrin by endocytosis. However, Western blot analysis revealed that CLCN5 was markedly degraded, absent, or truncated in the mutant 30:insH, del132-241, and R637X ciPTECs, respectively (Fig. S3).

Polarization of ciPTECs. Proximal tubular cells, which are polarized, facilitate endocytosis of proteins at the apical brush border membrane. The apical endocytosed proteins are then transferred, which is most probably mediated by anterograde microtubule transport, to lysosomes for degradation (3–5). The anterograde transport is facilitated by orientation of the microtubules such that their minus ends are toward the apical side and their plus ends, which have the microtubule associated protein end-binding protein-1 (EB-1), are extending through the cell to the basolateral side (4, 23). Confocal microscopy and analysis of orthogonal sections from Z-stack images obtained of the four ciPTECs stained with antibodies to zona occludens-1 (ZO-1) and EB-1 showed the presence of ZO-1 containing tight junctions (green) at the apical side, and the location of EB-1–labeled microtubules (red) below the tight junctions (Fig. 1F), thereby demonstrating that the growing ends of microtubules are predominantly toward the basolateral surface of the polarized ciPTECs. Thus, these results reveal that all four ciPTECs form polarized cells (Fig. 1F), consistent with their derivation from proximal tubular cells (Fig. 1B–E).

### Dent Disease ciPTECs Have Impaired Uptake of Albumin and Transferrin.
Proximal tubular cells reabsorb >80% of the filtered protein load, including albumin and transferrin, by receptor-mediated endocytosis that involves the megalin–cubilin receptor complex and is impaired in patients with Dent disease (24). Albumin is a ligand for both megalin and cubilin, whereas transferrin is a ligand for cubilin only. We therefore investigated the ability of the polarized ciPTECs (Fig. 1F) that express megalin and cubilin (Fig. 1E) to absorb albumin and transferrin. All of the Dent disease ciPTECs, compared with the wild-type ciPTECs, were found to have significant reductions in albumin uptake (Fig. 2A); thus, the 30:insH, del132-241, and R637X ciPTECs had mean ± SEM fluorescent albumin uptakes of 57.0 ± 4.6%, 23.3 ± 4.0%, and 64.9 ± 6.1%, respectively, compared with the wild-type (control) ciPTECs (100.0 ± 8%, n = 10, P < 0.02). However, the Dent disease ciPTECs showed different responses in the uptake of fluorescently labeled albumin when challenged with competition by the presence of excess unlabeled albumin or transferrin in the medium.

Thus, in control ciPTECs, fluorescent albumin uptake in the presence of excess unlabeled albumin or transferrin was significantly reduced to 38.4 ± 2.6%, or 43.7 ± 4.1%, compared with the uptake with no competition, respectively (n = 6, P < 0.02), and the 30:insH and R637X ciPTECs also showed significantly reduced albumin uptake in the presence of excess unlabeled albumin (30:insH: 13.1 ± 2.1%, P < 0.02, and R637X: 37.6 ± 5.8%, P < 0.05, n = 6) or excess unlabeled transferrin (30:insH: 17.2 ± 4.2%, P < 0.02 and R637X: 39.6 ± 4.4%, P < 0.02, n = 6). In contrast, the del132-241 ciPTECs did not demonstrate a further reduction in fluorescent albumin uptake (31.3 ± 3.2% and 27.4 ± 5.2%, in the presence of excess unlabeled albumin or transferrin, respectively). These different responses in albumin uptake correlated with differences in cell surface expression and

### Table 1. Comparison of clinical and cellular phenotypes and mouse models with CLC-5 mutations

<table>
<thead>
<tr>
<th>Origin of Dent Disease</th>
<th>CLC-5 mutation*</th>
<th>CLC-5 transport†</th>
<th>Dent disease phenotype‡</th>
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<tr>
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*CLC-5 mutation details and location provided with reference to helix or CBS domain (Fig. 1A).
†CLC-5 transport activity assessed by heterologous expression of CLC-Ss in HEK293 cells. Cl\(^-\) conductance and H\(^+\) efflux were measured. ++, normal, +, impaired; −, absent; (−) likely absent, but not measured; ?, not known.
‡ALP, alkaline phosphatase; Ca, hypercalciuria; Nc, nephrocalcinosis; Pr, low-molecular-weight proteinuria; Rf, renal failure; Ri, rickets; glycosuria and phosphaturia were not reported in any of the patients; UP, hyperphosphaturia; +, present; −, absent; ?, not known.
§EAc, endosomal acidification; FPE, fluid-phase endocytosis; MGI, megalin internalization; RME, receptor-mediated endocytosis. ++, normal, +, impaired; −, absent; ?, not known.

*Genetic abnormalities in patients and mouse models previously reported are as follows: patient 1 (14), patient 2 (9, 27), patient 3 (20), mouse model A (10), mouse model B (11, 12, 31), mouse model C (32), and mouse model D (16).
internalization of megalin by the ciPTECs (Fig. 2B). Thus, in control ciPTECs, megalin was predominantly expressed at the cell surface at 2 min after exposure to albumin, but at 5 min, internalized megalin was observed and, at 15 min, most of the megalin was in the cytoplasm. However, in the mutant 30:insH and R637X ciPTECs, which initially had similar megalin cell surface expression to control ciPTECs, megalin internalization at 15 min was reduced and in the del132-241 ciPTECs, megalin was located predominantly in the cytoplasm at all time points (Fig. 2B). Thus, these data indicate that the CLC-5 mutation in del132-241 results in a complete disruption of receptor-mediated endocytosis, whereas the 30:insH and R637X cause a partial loss of receptor-mediated endocytosis.

The studies of fluorescently labeled transferrin uptake revealed similar abnormalities (Fig. 2C) to those observed for fluorescent-labeled albumin uptake (Fig. 2A). Thus, all of the Dent disease ciPTECs, compared with the wild-type ciPTECs, were found to have significant reductions in transferrin uptake (Fig. 2C); the 30:insH, del132-241, and R637X ciPTECs had mean ± SEM fluorescent transferrin uptake of 60.1 ± 4.8%, 29.8 ± 6.5%, and 62.9 ± 5.9%, respectively, compared with the control ciPTECs (100.0 ± 6%) (n = 8, P < 0.02). However, the Dent disease ciPTECs showed differences in uptake in response to competition with excess unlabeled albumin or transferrin. Thus, in control ciPTECs in the presence of excess unlabeled albumin or transferrin, fluorescent transferrin uptake was significantly reduced to 29.8 ± 5.3% or 60.0 ± 4.8%, respectively, compared with uptake without competition (n = 6, P < 0.02); the 30:insH and R637X ciPTECs also showed significantly reduced transferrin uptake in the presence of excess unlabeled albumin (30:insH: 44.1 ± 5.5%, P < 0.02 and R637X: 44.7 ± 3.7%, P < 0.05, n = 6) or excess unlabeled transferrin (30:insH: 39.9 ± 6.2%, P < 0.02 and R637X: 36.4 ± 3.6%, P < 0.02, n = 6). In contrast, competition with excess unlabeled albumin or transferrin had no effect on fluorescent transferrin uptake in del132-241 cells (38.7 ± 2.1% and 39.1 ± 3.2%, respectively, n = 6) similar to the effects observed with fluorescent albumin.

The combined results demonstrate that the ciPTECs with the del132-241 mutation had a complete disruption of receptor-mediated endocytosis, whereas the ciPTECs with the 30:insH and R637X mutations had active but reduced receptor-mediated endocytosis, compared with that in normal ciPTECs. Investigation of fluid-phase endocytosis using FITC-labeled dextran revealed that the del132-241 ciPTECs, but not the 30:insH and R637X ciPTECs, had significantly reduced dextran uptake compared with control ciPTECs (24.4 ± 1%, n = 44, P < 0.02) (Fig. 2D). The addition of excess unlabeled albumin during the FITC-labeled dextran uptake assays did not affect dextran uptake by the control or Dent disease ciPTECs, thereby confirming that dextran uptake was not via the receptor-mediated endocytic pathway involving the megalin-cubilin complex.
Fig. 3. Endosomal acidification in ciPTECs. Endosomal pH was determined by using the ratiometric GFP variant pHluorin-VAMP2, by comparison with a pH standard curve constructed using surface-exposed GPI-tagged pHluorin in buffers with different pHs. (A) Live-cell imaging using confocal microscopy of the four ciPTECs [three from Dent disease patients and one normal control (wild-type) male]. Images with 488 nm and 760 nm excitation were collected, the fluorescence intensity of individual vesicles was measured, and the ratios of 760 nm/488 nm were calculated. (Scale bars: 10 μm.) (B) Endosomal pH values were determined for each of the four ciPTECs that were either untreated (black bars) or treated with bafilomycin-A1 (white bars). Data are expressed as mean ± SEM for untreated Dent disease ciPTECs versus control ciPTECs (P < 0.05 and #P < 0.02); and for ciPTECs treated with bafilomycin versus the respective untreated ciPTECs (##P < 0.02). Values were calculated by Student’s unpaired, two-tailed t test. The number of vesicles and cells from which recordings were collected are indicated below each bar. Horizontal dashed line at pH 7 represents the pH of the extracellular solution.

del132-241 and R637X ciPTECs was significantly increased to 6.36 ± 0.19 and 6.63 ± 0.27, respectively (P < 0.05) (Fig. 3B), thereby demonstrating defective endosomal acidification. The addition of bafilomycin-A1, an inhibitor of the V-ATPase (15), raised the pH significantly in all ciPTECs, demonstrating that the differences in acidification were likely due to differences between wild-type and mutant CLC-5, and not changes in V-ATPase. Thus, the combined results of these studies show that the CLC-5 mutant 30:insH is associated with reduced Cl− conductance but normal H+ efflux and normal endosomal acidification, compared with wild-type CLC-5; and the del132-241 and R637X CLC-5 mutants result in loss of Cl− conductance and H+ efflux with impaired endosomal CLC-5 acidification (Table 1, Figs. 2 and 3, and Figs. S1 and S4).

Discussion

Our studies, which establish human renal proximal tubular cells that harbor endogenous CLC-5 mutations, provide human cell models for Dent disease, a disorder manifesting with defects in receptor-mediated endocytosis and endosomal acidification. Thus, these ciPTECs provide valuable resources for the investigation of the receptor-mediated endocytic pathway, the mechanisms and roles of CLC-5 in endosomal acidification, and the assessment of compounds that may be of potential benefit in treating Dent disease and other human renal proximal tubular disorders due to defects in receptor-mediated endocytosis and endosomal acidification. Such applications are illustrated by use of ciPTECs derived from patients with nephropathic cystinosis (OMIM:218800) due to mutations in the lysosomal transporter cystinosin (25) for studies of cell metabolism in the disease (25).

Our investigations of the three human ciPTECs from Dent disease patients who had CLC-5 mutations involving different domains (Fig. 1) reveal that each CLC-5 mutation resulted in a different cellular phenotype (Table 1 and Figs. 2 and 3). Thus, the R637X ciPTECs had impaired receptor-mediated endocytosis, defective endosomal acidification, but normal fluid-phase endocytosis; the 30:insH ciPTECs had impaired receptor-mediated endocytosis, but normal endosomal acidification and normal fluid-phase endocytosis; whereas the del132-241 ciPTECs had a lack of receptor-mediated endocytosis, decreased fluid-phase endocytosis, and defective endosomal acidification. These findings indicate a correlation between the CLCN5 genotype and cellular phenotype, which is consistent with the findings in mouse models with different Clcn5 mutations (Table 1), in which detailed studies have revealed the presence of genotype-phenotype correlations (10, 11, 16, 26). For example, mice deleted for CLC-5 helices C-F were reported to have defective receptor-mediated and fluid-phase endocytosis and impaired endosomal acidification (10) (Table 1), and these defects resemble the defects observed in del132-241 ciPTECs, which would have deletion of helices C-G; whereas Clcn5y/211A mice had defects in receptor-mediated and fluid-phase endocytosis without impairment of endosomal acidification (16) (Table 1), resembling the phenotypic defects observed in the 30:insH ciPTECs. Our observations in the ciPTECs of a possible genotype-cellular phenotype correlation contrasts to the reported lack of a genotype-phenotype correlation in Dent disease patients (2, 27). However, the phenotypic features reported in studies of Dent disease patients, which are usually retrospective and from multiple centers, require cautious interpretation, because the data: could not be collected using uniformly standard conditions but instead was ascertained at different ages and stages of the disease; often lacked detailed assessment of receptor-mediated endocytosis; and could not have undertaken measurements of fluid-phase endocytosis or endosomal acidification. However, our results from these ciPTECs indicate that a detailed and prospective study of Dent disease patients examining for such genotype-phenotype correlation is required. In addition, these ciPTECs will be valuable for studying the mechanisms whereby CLC-5 mutations cause Dent disease as well as for assessing therapeutic compounds, which are not available for Dent disease.

Our investigations of the ciPTECs with CLC-5 mutations indicate that receptor-mediated endocytosis is not always coupled to endosomal acidification, which is consistent with the observations of in vitro studies that have expressed the CLC-5 mutants—G57V, S270R, G513E, R516W, I524K, and E527D—in HEK cells (17), and in vivo studies of Clcn5y/211A mice (16). An examination of the locations of these CLC-5 mutants and their effects on receptor-mediated endocytosis and endosomal acidification helps to provide insights into the structure–function relationships of the CLC-5 domains. Thus, the G57V mutant was reported to be associated with reduced Cl− currents without a defect in endosomal acidification, whereas the E527D mutant was associated with...
abstraction of Cl− currents and impairment of endosomal acidification, and the mutants S270R, G513E, R516W, and I524K were retained in the ER and degraded and, hence, with absent Cl− currents and a lack of endosomal acidification (17).

The 30:insH mutation, which is located in helix A, had similar abnormalities to the G57V mutant, also in helix A (Fig. 1A), in being associated with reduced but not absent Cl− currents (Fig. 1A), and impaired trafficking in a loss of receptor-mediated endocytosis (Fig. 2) that occurred without a defect in endosomal acidification (Fig. 3) or proton transport (Fig. S4 and Table 1) (17). These abnormalities also parallel the E211A knock-in mouse where a defect in endocytosis is present, although acidification is intact (16). The R637X mutation, which is located in CBS1, had similar abnormalities to the E527D mutant, located in helix P (Fig. L4) in being associated with abolition of Cl− currents (Fig. S1) and proton transport (Fig. S4) and resulting in defective endosomal acidification (Fig. 3), and impaired receptor-mediated endocytosis (Fig. 2) (Table 1). Finally, the del132-241 mutation (which involves helices C-E) (L4) had similarities to the S270R (in H-I loop), G513E (in helix O), R516W (in helix O), and I524K (in helix P) (17) and mice deleted for CLC-5 helices C-F (10), in resulting in an absence of CLC-5 protein (Fig. 1B).

In conclusion, our studies have established human cell models (ciPTECs) for the proximal tubular defects observed in Dent disease. These ciPTECs provide valuable resources for the investigation of receptor-mediated endocytosis and the mechanisms of endosomal acidification.

Materials and Methods

Detailed methods can be found in SI Materials and Methods.

Supplementary Information

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SI Materials and Methods

Primary Cell Culture and Immortalization. Primary cell cultures were established from morning first voided urine samples and maintained in supplemented DMEM-F12 HAMS medium (Gibco), at 37 °C, in 5% (vol/vol) CO2, as described (1, 2). Cells were immortalized by infection with SV40-T (U19tsA58) and hTERT, and grown at 33 °C, 5% (vol/vol) CO2 until confluent, by using methods described (3). Proximal tubule cells were isolated from cell populations by fluorescence-activated cell sorting (FACS) using mouse-anti-human CD13-FITC antibody (Dako) (1:100 dilution in PBS). CD13-positive cells were immediately seeded on NIH 3T3 (ATCC, LGC Standards) fibroblast feeder layers and grown for ~12 d. Colonies were picked by using cloning discs (Sigma-Aldrich) soaked in trypsin/EDTA, seeded into new flasks, and grown to confluency.

Flow Cytometry. Confluent subcloned cells were seeded in six-well plates, trypsinized, and single cell suspensions were treated with CD13-FITC (1:100 dilution in PBS) (Dako) before investigation by flow cytometry (1). Unlabeled cells were used as negative controls. Data were gathered by using Cytomation Summit (version 3.0) and analyzed by using FlowJo (version 9.0.2) software (4).

Mutational Analysis. DNA was extracted from peripheral leukocytes and conditionally immortalized proximal-tubular epithelial cell lines (ciPTECs) by using Gentra Puregene Blood Kit (Qiagen) and used for PCR amplification of the coding region exons and intron-exon boundaries of CLCN5 (primer sequences available on request) as described (5). The DNA sequences of the PCR products were determined by using DYEnamic ET Terminators (Amerham Biosciences) and the ABI377 semiautomated sequencer (Applied Biosystems) (6).

RT-PCR and Quantitative RT-PCR Analysis. First-strand cDNA was generated from 1 μg of total RNA from each cell line extracted by MirVana (Ambion), using Quantscript reverse transcriptase in the Quantitect Reverse Transcription kit (Qiagen) (7). PCR was performed on cDNA by using primers specific for ATP-binding cassette family member multidrug resistance protein 4 (ABCC4), ATP-binding cassette sub-family B member 1, P-glycoprotein (ABCB1), Na+K+Cl− cotransporter-2 (NKCC2), K+- inwardly-rectifying channel, subfamily J, member 1 (KCNJ1), Calbindin-1 (CALB1), Transient receptor potential cation channel, subfamily V, member 5 (TRPV5), Urolakin-IIIA (UPKIIIA), Calmodulin (CaM), Cubilin (CUBN), Megalin, also referred to as Low-density lipoprotein related protein 2 (LRP2), Kinesin family member 3B (KIF3B), V-ATPase subunits A2 (ATP6V0A2) and D (ATP6V1D) and Cyclin D1 (CCND1) (primer sequences available on request), as described (8). Human kidney RNA (Clontech) and bladder RNA (extracted from ATCC CRL-1473 cells) were used as controls. Quantitative RT-PCRs (qRT-PCRs) were carried out by using the Rotorgene Sybr Green Kit (Qiagen) in triplicate in four independent samples for each ciPTEC using a Rotorgene 5 (Qiagen), as described (9). All qRT-PCR test samples were normalized to levels of the geometric mean of three reference genes, GAPDH, CCND1, and CCND2. Threshold cycle (Ct) values were obtained from the start of the log phase on Rotorgene Q Series Software, and Ct values were analyzed in Microsoft Excel 97–2010 by using the Pfaffl method (10). Data for each ciPTEC was normalized to the control ciPTEC.

Western Blot. Protein was extracted from confluent cell lines by using RIPA buffer (50 mM Tris at pH 7.4, 150 mM NaCl, 1% (vol/vol) Igepal CA630, 0.5% Na+-deoxycholate, 0.1% SDS, 0.01% phenylmethane sulphonylfluoride, 3% (wt/vol) aprotinin and 1 mM Na-orthovanadate), eluted in Laemmli buffer, and analyzed by Western blotting using 12% (vol/vol) sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) (11). Following transfer to polyvinylidene difluoride membrane (Amersham), blots were probed with rabbit anti-AQP1 (1:2,000; Chemicon International, Millipore), goat–anti-AQP2 (1:250; Santa Cruz), rabbit–anti-TRPV5 (1:250; Santa Cruz), rabbit–anti-UPKIIA (1:1,500; Abcam), rabbit–anti-CLC-5 (1:500; Abcam), rabbit–anti-HRP (1:2,000, Abcam), and HRP-conjugated secondary antibodies goat–anti-rabbit (1:3,000; Bio-Rad) or donkey–anti-goat (1:3,000; Bio-Rad) (11). Blots were visualized by using Pierce ECL Western blotting substrate (Thermo Fisher Scientific) on a Bio-Rad Chemidoc XRS+ system (12).

Confocal Imaging. Confluent ciPTECs were plated in six-well plates containing poly-l-lysine–treated coverglasses and cultured at 37 °C for 7–10 d (13). For Z-stack imaging, cells were fixed in ice-cold methanol and coimmunostained with rabbit polyclonal anti-ZO-1 (Invitrogen) and mouse monoclonal anti-microtubule-associated protein, RB/EB family, member 1 (MAPRE1) (EB-1; Abcam), and secondary antibodies anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 594 (Molecular Probes), respectively (13). For megalin localization studies, cells were incubated in serum-free media, before exposure to 5 mg/mL unlabeled albumin, fixed in 4% paraformaldehyde/PBS, permeabilized in 1% Triton X-100/PBS, and immunostained with primary goat–anti-megalin (Santa Cruz) and secondary anti-goat Alexa Fluor 594 antibodies. The images and Z-stacks were recorded by using a confocal laser-scanning microscope (Zeiss, LSM 10 META) with a Plan-Achromat 63×/1.4 N.A. oil differential interference contrast (DIC) objective (13). An argon laser λ = 488 nm was used to excite Alexa Fluor 488 fluorescence and a HeNe laser λ = 543 nm for Alexa Fluor 594 (13). Emission of Alexa Fluor 488 and Alexa Fluor 594 was detected in the Multitrack mode in META channels of the confocal system within the following spectral detection ranges: from 509 to 550 nm for Alexa Fluor 488, and from 580 to 620 nm for Alexa Fluor 594 as described (13). Images were merged and orthogonal sections were analyzed by using Zeiss LSM Image browser software (13).

Confocal images were also collected from live HEK293 cells transiently transfected with wild-type (WT) or mutant p-enhanced yellow fluorescent protein (pEYFP)–CLC-5 constructs and seeded onto Fluorodishes, using a 100×/1.49 N.A. oil-immersion objective (Nikon) and selecting a 200-μm pinhole on the same imaging system (Thornlabs). EYFP was excited with a 488-nm laser, fluorescence collected with a 535-nm filter, and three consecutive images were averaged.

Endocytosis Assays. Albumin and transferrin uptake were measured to investigate receptor-mediated endocytosis, and dextran uptake was measured to investigate fluid-phase endocytosis by using modifications of standard methods, as described (14–16). Cells were seeded in 24-well plates and grown for 7 d until polarized. To measure albumin uptake, cells were exposed to 50 μg/mL albumin conjugated to Alexa Fluor 488 (Molecular Probes) for 120 min (13). To measure transferrin uptake, cells were exposed to 50 μg/mL transferrin conjugated to Alexa Fluor 594 (Molecular Probes) for 15 min (13). To measure dextran uptake, cells were exposed to
5 mg/mL dextran conjugated to FITC (Sigma-Aldrich) for 15 min (13). At the end of the uptake period, cells were washed in ice-cold PBS and solubilized in imidazole buffer (1 mM EDTA, 20 mM imidazole at pH 7.2, and 250 mM sucrose). The Alexa Fluor 488, Alexa Fluor 594, and FITC fluorescence was determined by using a CytoFluor microplate reader (PerSeptive Biosystems) at 485-nm excitation and 530-nm emission wavelengths for albumin-488 and FITC-dextran and 566-nm excitation and 615-nm emission wavelengths for transferrin-594, respectively (13). Total Alexa Fluor 488-albumin or Alexa Fluor 594-transferrin uptake was standardized to total cellular protein. For competition experiments, cells were simultaneously incubated with either 5 mg/mL unlabeled albumin or 5 mg/mL unlabeled transferrin during the endocytosis assay, and the uptake of fluorescent albumin or transferrin determined (13). Fluorescence uptake in Dent disease ciPTECs and competition assays expressed relative to uptake in control ciPTECs without competition. Significance values were calculated by unpaired, two-tailed Student’s t test.

**Acidification Studies.** Endosomal pH was determined by using a modification of methods described (17, 18). To generate a standard curve, control ciPTECs were first plated in six-well plates and transfected with pHluorin-vesicle-associated membrane protein (VAMP2), which is expressed on the cell surface, using FuGene6 (Roche), and bathed in buffers of differing pH. Fluorescence was measured in live cells by using a Zeiss LSM510-META confocal microscope 21–23 h after transfection. pHluorin was excited at 488 nm by using a diode laser and at 760 nm by using an argon two-photon laser. Emitted fluorescence was collected through a 505-nm long-pass filter. ImageJ software was used to measure the fluorescence intensity at each wavelength and the 760 nm to 488 nm ratio calculated in each buffer to construct a standard curve (18). To determine endosomal pH, ciPTECs were plated in six-well plates and transfected with pHluorin-vesicle-associated membrane protein (VAMP2), which is expressed at the endosomal membrane. pHluorin-VAMP2–expressing cells were imaged at 488 nm and 760 nm, and the 760 nm/488 nm ratios were compared with the standard curve to determine vesicular pH (18). For bafilomycin experiments, 25 μM Bafilomycin-A1 (Sigma-Aldrich) was applied to the cells and incubated at 37°C for 1 h before recordings were made. Significance values were calculated by unpaired, two-tailed Student’s t test.

**Electrophysiological Studies.** The WT pEYFP–CLC-5 construct (18) was used to generate the His insertion at codon 30 (30:insH) and R637X mutants by site-directed mutagenesis (QuikChange; Stratagene). HEK293 cells were transiently transfected with wild-type or mutant pEYFP–CLC-5 constructs by using FuGene6 6 reagent (Promega) and incubated for 2–3 d. Transfected cells were identified by EYFP epifluorescence, and whole-cell currents were recorded at room temperature (20–22°C) by using an EPC-10 patch clamp amplifier under control of Patchmaster software (HEKA). The bath solution contained 150 mM NaCl, 5 mM KOH, 10 mM Hepes, 2.6 mM MgCl2, 1.2 mM CaCl2, 5 mM glucose at pH 7.4 with NaOH, and patch pipettes (2–3 MΩ) were filled with solution containing 42 mM NaCl, 98 mM aspartic acid, 10 mM EGTA, 10 Hepes, and pH 7.4 with CsOH. Cells were held at ~30 mV and 10–ns voltage pulses (P) were applied from ~100 to +200 mV. A leak-subtraction protocol, which uses voltage pulses (P (−4)) to measure the Ohmic membrane properties at voltages below the threshold for CLC-5 activation, was used to subtract residual capacitance and leak currents.

Data were analyzed by plotting the relationship between the current density (steady-state current divided by whole-cell capacitance) and voltage. Conductance density was calculated by dividing the current density by the driving force, \( V_{rev} \), where \( V \) is the membrane voltage and \( E_{rev} \) the calculated reversal potential (of ~20.5 mV), and derived by using the \( [C^+]_p \) and pH of the solutions and assuming 2\( C^+ / H^+ \) exchange transport, as described (18). Recordings were measured in \( n = 11, 10, \) and 6 cells for WT, 30:insH, and R637X, respectively. Conductance density (G)–voltage (V) relationships from each cell were fitted with a Boltzmann function in the form \( G = G_{max}/(1 + e^{-\frac{(V-V_{1/2})}{k}}) \), where \( G_{max} \) is the maximum conductance density of the cell membrane, \( V_{1/2} \) the half-maximal activation voltage, and \( k \) a factor relating to the steepness of the voltage dependence. Because recordings from cells expressing R637X contained a greater proportion of endogenous HEK293 cell currents, which might distort the measured activation parameters, and because the analysis assumed that 2\( C^+ / H^+ \) transport is preserved in the mutant CLC-5, an alternative method of measuring relative transporter activity was used. CLC-5 activation was quantified by integrating the inward current recorded upon repolarization to ~30 mV as a measure of the relative gating charge movement \( (Q_{OFF}) \) (19). Charge \((Q)–voltage (V) \) relationships were analyzed by using a similar Boltzmann function, substituting \( Q \) for \( G \). Data are expressed as mean ± SEM of number of cells. Comparisons between data and parameters obtained from cells expressing the different CLC-5 variants were compared by using ANOVA and Bonferroni post hoc analysis of individual means. Statistical significance is stated when \( P < 0.05 \) between individual datasets.

**Proton-Transport Function Assessed by Intracellular pH Changes.** HEK293 cells were transfected with wild-type or mutant pEYFP–CLC-5 constructs as described above and seeded onto 35-mm Fluorodishes (World Precision Instruments). The same electrophysiological solutions and apparatus were used as above, but 50 μM of the pH-sensitive dye 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF; Biotium) was added to the pipette solution. After establishing the whole-cell configuration, cells were held at ~30 mV and left for at least 5 min for the BCECF to load into the cell from the patch pipette. Fluorescence was imaged on a Nikon TE-2000 microscope by using a 40× objective lens and a laser scanning imaging system (Thorlabs). Images were collected over 30 s at 1 frame per s, exciting with a 488-nm laser and collected with 535-nm filter. A pinhole of 2 mm was selected to collect images from an optical slice containing the whole cell. Between the 10- and 20-s timepoints, 100-ms voltage pulses to +150 mV were applied at 5 Hz to activate CLC-5. Fluorescence intensity from a region of interest enclosed by the cell was analyzed by using ImageJ software. Data were background subtracted and corrected for bleaching by extrapolating the change in intensity over the first 9 s, which upon inspection was very close to linear. For comparison between cells, fluorescence intensity was expressed as a percentage change relative to baseline.


**Fig. S1.** Electrophysiological studies of CLC-5 mutants. (A) Confocal microscopy of HEK293 cells transiently transfected with CLC-5-YFP WT, 30:insH, and R637X constructs to study cell surface expression. The del132-241 CLC-5 mutant was not assessed because no CLC-5 protein was present in the del132-241 cell line expressing functional in Drosophila. Activation curves generated by normalizing current density to conductance density functions to conductance density relationships. The mean (Gmax) for R637X was significantly smaller than that for WT CLC-5 (P < 0.05 compared with WT CLC-5). (E) The activation curve generated by normalizing current density-to-conductance density relationships to the fitted Gmax. The activation of voltage dependence for both CLC-5 mutants was significantly shifted to more positive potentials, with half-maximal activation voltages (V1/2) of 102 ± 1.7 mV, 125 ± 2.5 mV, and 115 ± 1.8 mV for WT, 30:insH, and R637X CLC-5, respectively (n = 6-11). (F) Relative Goff currents measured as the charge moved during the transient inward currents recorded at the end of the voltage pulse. To measure this relative transporter activity, integration of the transient inward “tail” current, representative of the conformational change from active to inactive state (19) was performed. The symbols in E and F representing data from cells expressing WT, 30:insH, and

Legend continued on following page
**Fig. S2.** Examination of Dent disease and control cell lines by flow cytometry. Cells were examined for the presence of the proximal tubular marker CD13 (aminopeptidase-N) by flow cytometry. Readings were initially made in unstained cells, then in CD13-FITC-treated cells. Forward scatter was plotted against CD13-FITC in histograms to compare unstained and stained cells. Cell lines with the mutation 30:insH had mixed cell populations with <45% of cells being CD13-FITC positive. In contrast, the del132-241 and R637X cells were comparable to the control cells with >90% of cells being CD13-FITC positive.

**Fig. S3.** CLC-5 expression. Western blot analysis of control (wild-type) ciPTEC lysates revealed the presence of the 80-kDa CLC-5 protein. However, CLC-5 expression in the mutant ciPTECs was abnormal compared with wild-type ciPTEC. Thus, CLC-5 protein was markedly degraded in 30:insH ciPTECs, absent in del132-241 ciPTECs, and truncated in R637X ciPTECs. Expression of α-tubulin was used as a control to confirm equal loading of protein.
Table S1. CLCN5 expression detected by qRT-PCR in WT and Dent Disease mutant ciPTECs

<table>
<thead>
<tr>
<th>ciPTEC</th>
<th>Fold change</th>
<th>t test compared with WT</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>Wild-type</td>
<td>1.00 ± 0.31</td>
</tr>
<tr>
<td>Mutant</td>
<td>30:insH</td>
<td>0.51 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>del132-241</td>
<td>0.47 ± 0.14</td>
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<tr>
<td></td>
<td>R637X</td>
<td>1.01 ± 0.12</td>
</tr>
</tbody>
</table>

CLCN5 expression was assessed by qRT-PCR using RNA obtained from control (WT) and Dent disease mutant (30:insH, del132-241, and R637X) ciPTECs. Fold changes are expressed relative to WT levels (n = 12, obtained from replicates for each of four biological replicates per ciPTEC). P values were calculated by Student’s unpaired, two-tailed t test.