Characterization of Dent’s disease mutations of CLC-5 reveals a correlation between functional and cell biological consequences and protein structure

Andrew J. Smith,1 Anita A. C. Reed,2 Nellie Y. Loh,2 Rajesh V. Thakker,2 and Jonathan D. Lippiat1

1Institute of Membrane and Systems Biology, Faculty of Biological Sciences, University of Leeds, Leeds; and 2Academic Endocrine Unit, Nuffield Department of Medicine, University of Oxford, Oxford Centre for Diabetes, Endocrinology and Metabolism, Churchill Hospital, Oxford, United Kingdom

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DENT’S DISEASE IS AN X-LINKED renal tubulopathy that is characterized by low molecular weight proteinuria, hypercalciuria, nephrocalcinosis/nephrolithiasis, and progressive renal failure (18, 31, 36). The disease is primarily associated with inactivating mutations in the CLCN5 gene (Dent disease 1; OMIM 300009; Ref. 18), although more recently it has also been reported to be due to mutations of the OCRL1 gene that encodes a phosphatidylinositol-4,5-bisphosphate-5-phosphatase (Dent disease 2; OMIM 300555; Ref. 13). It is generally thought that the principal cause of Dent’s disease (31) results from a loss of functional CLC-5 from the subapical endosomes of kidney proximal tubule cells leading to a defect in endosomal acidification and impaired protein reabsorption (14, 16).

CLCN5 encodes CLC-5, a voltage-dependent chloride/proton exchanger of the voltage-gated chloride channel (CLC) family (8, 27, 30). CLC-5 forms a dimer of two identical subunits, each of which contains a complete ion conduction pathway and is composed of 18 α-helices (A-R). It is found abundantly in the nephron, particularly in the cells of the proximal tubule (5). The majority of CLC-5 is located in numerous subapical endosomes, with only a small fraction present at the apical membrane (∼8%; Refs. 5, 10, 32, 38). Correct function of these endosomes is vital to facilitate the endocytosis of low molecular weight proteins from the primary urine. In subapical endosomes, CLC-5 colocalizes with v-ATPase and is thought to contribute a Cl− conductance to the endosomal lumen to counterbalance the accumulation of H+ due to the action of v-ATPase (10). Consistent with this, CLC-5 knockout (KO) mice exhibit a reduced rate of endosomal acidification than that observed in wild-type (WT) mice (11).

Dent’s disease results from missense mutations in CLC-5 as well as nonsense mutations and base insertions/deletions that lead to truncation of the protein (15, 19, 22, 40). Previous investigations (17, 18, 21) focused on the impact of missense mutations on the function and cell surface targeting of CLC-5 heterologously expressed in Xenopus oocytes. Consequently, there is little understanding of how disease-causing mutations disrupt trafficking or the function of CLC-5 in membranes and organelles where they may play important physiological roles, for example, in endosomal acidification. To further elucidate the underlying causes of Dent’s disease, we investigated the functional and cell biological consequences of known Dent’s disease mutations in a mammalian expression system and, through the use of molecular modeling, examined the relationship between the nature of the defects and the structure of the protein.

MATERIALS AND METHODS

DNA constructs and cell lines. Human CLC-5 was cloned into either pEYFP-N1 (Clontech) to be expressed with a fused C-terminal yellow fluorescent protein (CLC-5-YFP) or into pcDNA6 with an N-terminal HA tag (HA-CLC-5). Mutations were generated using the Quikchange site-directed mutagenesis protocol (Stratagene) and verified by DNA sequencing (University of Leeds Facility). Ratiometric pHluorin fused to either VAMP2 or GPI-anchor (24) was kindly donated by Dr. G. Miesenböck (Yale University). HEK-MSR cells were obtained from Invitrogen and were cultured in DMEM supplemented with 10% (vol/vol) FBS. HEK-MSR cells stably expressing WT or mutant HA-CLC-5 were produced by transfection using Fugene 6 (Roche) followed by selection of positive transfectants with

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5 μg/ml blasticidin (Invitrogen). After selection, stably expressing cells were maintained in medium supplemented with blasticidin and expression was confirmed by Western blotting.

**Electrophysiology.** HEK-MSR cells were transiently transfected with CLC-5-YFP using Fugene 6. Transfected cells were identified by YFP epifluorescence. Patch pipettes were pulled from thin-walled borosilicate glass (Harvard Apparatus) and polished. Electrode resistances ranged from 2 to 3 MΩ in experimental solutions. Currents were recorded using the whole cell patch clamp configuration with an EPC-10 amplifier (HEKA Electronics) with >80% series resistance compensation where appropriate. Currents were filtered at 10 kHz and digitized at 50 kHz using PatchMaster software (HEKA Electronics). Cells were held at −30 mV and 10-ms pulses from −100 to +200 mV at 10-mV increments that were applied at 1-s intervals. P/4 leak subtraction was used. The bath solution contained the following (in mM): 140 CsCl, 1 CaCl₂, 1 MgCl₂, and 10 HEPES-CsOH at pH 7.4. The pipette solution contained the following (in mM): 42 CsCl, 49 Cs₂SO₄, 10 EGTA, and 10 HEPES-CsOH at pH 7.4. Subtraction was used. The bath solution contained the following (in mM): 100 NaCl, 1 CaCl₂, 1 MgCl₂, and 10 HEPES-NaOH at pH 7.4.

**Confocal microscopy.** HEK-MSR cells were transfected with WT or mutant CLC-5-YFP using Fugene 6 and seeded onto poly-L-lysine-coated borosilicate glass cover slips. Cells were fixed with 3% paraformaldehyde, and the coverslips were mounted onto microscope slides using Vectorshield mounting medium (Vector Laboratories). Where necessary, cells were permeabilized with 0.05% Triton, blocked with 5% serum, and stained with organelle-labeling antibodies. The antibodies used were mouse anti-calnexin (Abcam), mouse anti-EEA1 (BD Biosciences), and mouse anti-Cation-independent mannoside-6-phosphate receptor (Abcam). Secondary antibodies used were anti-mouse Cy3 conjugates (Sigma). Labeled cells were examined using a Zeiss LSM510-META laser scanning confocal microscope under an oil-immersed ×63 objective lens (numerical aperture = 1.40). YFP was excited using an argon laser fitted with 488-nm filters, and Cy3 was excited using a He/Ne laser fitted with 543-nm filters.

**Cell surface expression assay.** The cell surface expression of mutant relative to WT CLC-5 was examined using HEK-MSR cells stably expressing WT or mutant HA-CLC-5 and a modification of previously published methods (23). Cell surface proteins were labeled in suspension with an antibody raised against an extracellular CLC-5 epitope (Sigma) at 4°C for 1 h with rotation. After being washed extensively with chilled PBS, cells were labeled with anti-rabbit horseradish peroxidase (HRP)-conjugated antibodies (Thermo Scientific) for 30 min at 4°C with rotation. The cells were again washed extensively with chilled PBS and then lysed by sonication. Cell lysates were examined for HRP content by chemiluminescence using a POLARstar OPTIMA microplate reader (BMG Labtech). Steady-state luminescence readings were normalized to the total protein content of the lysate using a Bradford assay (Bio-Rad), with mean values from untransfected cells subtracted from those stably expressing CLC-5.

**Western blotting.** HEK-MSR cells were transiently transfected with CLC-5-YFP as described in Electrophysiology. Where required, cells were treated with either 1 μM MG132 (Calbiochem) or 200 μM leupeptin (Sigma) for 24 h before lysis. Forty-eight hours posttransfection the cells were harvested and lysed using RIPA buffer (100 mM Tris·HCl, 150 mM NaCl, 0.2 mM EDTA, 1% NP-40, 1% Nonidet P-40, and 0.1% SDS at pH 7.4), and the samples were prepared in 2× SDS-PAGE sample buffer (2.5% SDS, 60 mM Tris·HCl, 20% glycerol, and 8 mM EDTA at pH 6.8, supplemented with 100 mM DTT). After incubation at room temperature for 20 min, proteins were resolved by SDS-PAGE before transfer onto nitrocellulose membrane. The membrane was blocked with 5% nonfat milk/PBS-Tween, and antibody incubations were performed in 0.5% nonfat milk/PBS-Tween and washed in PBS-Tween. The antibodies used were mouse anti-green fluorescent protein (GFAP; Abcam) and antimouse HRP-conjugated secondary antibody (Pierce). Labeled proteins were visualized by ECL reagent (Pierce) and exposure to photographic film.

**Vesicular acidification assay.** The intracellular pH of endosomal compartments was determined using a modification of previously published methods (24). The fluorescence of pHluorin was imaged in live cells using a Zeiss LSM510-META confocal microscope. pHluorin was excited at 405 nm using a diode laser and at 488 nm using an argon laser. Emitted fluorescence was collected through a 505-nm long-pass filter. The fluorescence intensity of ratiometric-pHluorin with each laser was measured using ImageJ software, and the 405- to 488-nm ratio was calculated. The pH corresponding to each ratio value was determined by comparing against a pH/ratio standard curve constructed using GIP-pHluorin (surface exposed) expressing cells bathed in buffers of various pH.

**Homology model construction.** A CLC-5 homology model was constructed by threading residues 21-565 of human CLC-5 (Genbank NP_000075) onto the coordinates of the CLC-ecl1 crystal structure (PDB 1KP) using SWISS-MODEL (33). The large extracellular loop between helices B and C (101–120) of CLC-5 was omitted due to a lack of sequence homology with CLC-ecl1. The resulting models were manipulated using DeepView Swiss-pdb viewer.

**RESULTS**

**Differential effects of Dent’s disease mutations on whole cell currents.** We examined seven previously identified Dent’s disease mutations. Five of these mutations were chosen because they were shown to display a range of functional defects in Xenopus oocytes from moderately reduced (G57V), to severely reduced (R280P and R516W), and to abolished (R280R and E527D) whole cell currents. The WT currents of all of the mutants, since differences between the two expression systems have been noted (6, 26) and also provide a reference to which the uncharacterized mutations can be directly compared.

WT or mutant CLC-5-YFP was expressed in HEK-MSR cells, and whole cell currents were examined by patch-clamp electrophysiology. Cesium-based solutions were used to eliminate endogenous potassium channel currents. Cells transfected with WT CLC-5 gave robust currents that activated at test potentials above approximately +30 mV and displayed strong outward rectification (Fig. 1, A and C), consistent with previous reports (9). Since CLC-5 exhibits C¹/H⁺ exchange activity (27, 30), the outward currents correspond to the electrogenic flux of C¹ into the cell and H⁺ out from the cell. The currents were rapidly activating (<1 ms), displayed no inactivation, and then rapidly deactivated, giving rise to a small, brief (<1 ms) inward tail current. In contrast, currents recorded from untransfected cells were 10-fold smaller with no tail current upon repolarization (Fig. 1, A and B). Compared with...
WT CLC-5 transfected cells a slight, but not significant ($P > 0.05$), reduction was seen in the amplitude of currents in cells expressing G57V, whereas introduction of R280P led to a ~50% reduction in amplitude (Fig. 1B; $P < 0.05$). Both G57V and R280P give rise to currents that are qualitatively similar to those observed with WT (Fig. 1A), and normalization of the current-voltage relationships to the maximum current revealed no change in the voltage dependence (Fig. 1C, inset). Whole cell currents in cells expressing S270R, G513E, R516W, I524K, and E527D were not significantly different from untransfected cells ($P > 0.05$) but were significantly reduced compared with WT ($P < 0.05$). It should be noted that the reduction in current amplitude observed in the present study is much greater than previously reported for R516W in *Xenopus* oocytes (21).

**Subcellular distribution of CLC-5.** The subcellular distribution and membrane targeting of WT and mutant CLC-5-YFP were investigated by expression in HEK-MSR cells (Fig. 2A). WT was located in perinuclear structures as well as the plasma membrane. In contrast, the mutants S270R, G513E, R516W, and I524K were diffusely spread throughout the cell in a pattern characteristic of endoplasmic reticulum retention (Fig.

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**Fig. 1.** A: representative traces from whole cell recordings of untransfected (Untrans) HEK-MSR cells or those expressing wild-type (WT) and mutant CLC-5 as indicated. B: means ($\pm$SE; $n$ values indicated above each bar) current densities at +200 mV are summarized ($^*P < 0.05$ vs. WT; $^#P < 0.05$ vs. untransfected; ANOVA). C: mean ($\pm$SE; $n$ values as indicated in B) current density-voltage relationships of whole cell currents from untransfected HEK-MSR cells (○) or those expressing WT (●), or G57V (○), S270R (●), R280P (○), or R516W (●) mutant CLC-5. For clarity, only those mutants with whole cell currents greater than untransfected cells are shown. Inset: mean current-voltage relationship of whole cell currents normalized to the amplitude evoked by steps to +200 mV (III-200). Symbols are the same as those in the main graph.

**Fig. 2.** A: confocal images of HEK-MSR cells expressing WT and mutant CLC-5- yellow fluorescent protein (YFP) as indicated. Scale bars = 10 μm. Inset: endoplasmic reticulum was stained by anti-calnexin antibodies (red). Colocalization with mutant CLC-5 (green) is denoted by yellow fluorescence. Arrowheads denote areas surface fluorescence. B: cell surface expression of CLC-5 mutants determined by chemiluminescence. Surface expression in each repeat was normalized to WT and expressed as means ($\pm$SE; $n = 4$; $**P < 0.05$ vs. WT; ANOVA).
2A). The endoplasmic reticulum (ER) localization of these mutants was confirmed by colabeling with the ER-resident protein calnexin (Fig. 2A, insets). These mutants were not expressed at the cell surface to any significant extent (Fig. 2B).

Perinuclear structures and plasma membrane staining were also evident in cells expressing the mutants G57V and R280P (Fig. 2A). E527D was also distributed in vesicular structures within the cell, although these were more dispersed and less distinct than those observed in WT-expressing cells (Fig. 2A). The surface expression of the mutants G57V, R280P, and E527D was significantly (P < 0.05) reduced by ~30, ~35, and ~70%, respectively, compared with WT (Fig. 2B). The endosomal distributions of WT, G57V, R280P, and E527D were investigated by colabeling with markers for early and late endosomes (Fig. 3). After confocal microscopy, offline analysis was performed to gauge the extent of colocalization with each marker. WT CLC-5 was found to colocalize equally well with the early endosome marker EEA1 and the late endosome marker cation independent mannose-6-phosphate receptor (Cl-M6PR). The endosomal localization of E527D was similarly distributed equally well in both early and late endosomes. In contrast, G57V colocalized very poorly with EEA1 but displayed a high degree of overlap with Cl-M6PR, suggesting redistribution from early to late endosomes. The opposite was true of R280P, with substantial colocalization with EEA1 but little with Cl-M6PR.

Dent’s disease mutations are associated with protein degradation, which is increased with ER retention. The relative expression and stability of WT and mutant CLC-5 were investigated by Western blotting. Examination of CLC-5-YFP protein using anti-GFP antibody revealed a strong band at the predicted size for full-length CLC-5-YFP (~110 kDa) in cells expressing WT, with the higher molecular weight glycosylated forms also observed (Fig. 4A). Each of the Dent’s disease mutants displayed several discrete bands ranging in size from ~40- to 90-kDa in addition to the band corresponding to the full-length protein, except for G513E in which only the lowest molecular weight bands were apparent. These bands were absent in WT-expressing and untransfected cells, suggesting that they each correspond to C-terminal degradation products of CLC-5 (Fig. 4A). In general, the intensity of the ~110-kDa band also appeared to be reduced in the ER-retained mutants (S270R, G513, R516W, and I524K), consistent with increased degradation after ER retention (summarized in Table 1). The bands corresponding to the full-length, glycosylated forms of CLC-5 appeared most strongly with the mutants that exhibited membrane currents (Fig. 1). The involvement of the proteasome and lysosome in mediating the degradation was investigated. R516W was used as a representative ER-retained mutant. The presence of the lower molecular weight bands was not affected by treatment of cells for 24 h with either leupeptin (an inhibitor of lysosomal degradation) or MG132 (an inhibitor of proteasomal degradation), although MG132 treatment led to an increase in the density of each band (Fig. 4B). This suggests that neither the proteasome nor lysosome is involved in the initial degradation process but that the proteasome is involved in the final breakdown of the truncated protein fragments.

![Fig. 3. Endosomal distribution of WT and mutant CLC-5-YFP examined by colabeling with anti-EEA1 antibodies (early endosomes) and anti-cation-independent mannose-6-phosphate receptor (Cl-M6PR) antibodies (late endosomes). Merged images (rows 1 and 3) show the relative distribution of CLC-5 (green) and organelle markers (red). Images obtained from offline analysis (rows 2 and 4) showing areas of colocalization (white) are also shown. Cell outlines are denoted by dotted lines. Scale bars = 10 μm.](http://ajprenal.physiology.org/)

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**Table 1**

<table>
<thead>
<tr>
<th>Dent’s Disease Mutations</th>
<th>Western Blot Band Intensity</th>
<th>ER Retention</th>
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<tbody>
<tr>
<td>S270R</td>
<td>~70%</td>
<td>~30%</td>
</tr>
<tr>
<td>G513</td>
<td>~40%</td>
<td>~35%</td>
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<tr>
<td>R516W</td>
<td>~10%</td>
<td>~70%</td>
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<tr>
<td>I524K</td>
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<td>~90%</td>
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**Fig. 4.** Western blotting of WT and mutant CLC-5-YFP expressed in HEK293 cells. (A) Immunoblot of CLC-5-YFP protein using anti-GFP antibody showed a strong band at the predicted size for full-length CLC-5-YFP (~110 kDa) in cells expressing WT (left lane). Higher molecular weight glycosylated forms were also observed. Each of the Dent’s disease mutants displayed several discrete bands ranging in size from ~40- to 90-kDa in addition to the band corresponding to the full-length protein (right lanes). (B) Treatment of cells for 24 h with leupeptin (an inhibitor of lysosomal degradation) or MG132 (an inhibitor of proteasomal degradation) led to an increase in the density of each band (right lanes).
CLC-5 mutations causing Dent’s disease do not necessarily result in impaired endosomal acidification. Dent’s disease is thought to result from a defect in endosomal acidification associated with a loss of CLC-5 function in the early endosome. Since two of the mutations examined, G57V and R280P, are both able to pass currents that are qualitatively similar to WT and both display similar endosomal distribution compared with WT, it is unclear if endosomal acidification would be impaired in these cases. Endosomal acidification was assayed using the pH-sensitive GFP variant pHluorin, which was targeted to the lumen of endosomes by fusion to the N terminus of VAMP2 (24). Immunolabeling shows that the endosomal structures to which VAMP2·pHluorin is targeted also express EEA1 and CI-M6PR, suggesting that these intracellular compartments represent a mixed population of early and late endosomes (Fig. 5A) similar to CLC-5. The mean intralumenal pH of these endosomes in untransfected cells was 6.94 (±0.05). Expression of WT or G57V significantly (P < 0.05) lowered the mean intralumenal pH to 6.40 (±0.18) and 6.25 (±0.19), respectively, while with expression of R280P this was further lowered to 5.46 (±0.15). In cells expressing E527D, the mean intralumenal pH was significantly (P < 0.05) increased to 7.56 (±0.09) compared with the external starting pH of 7.4 (Fig. 5B).

DISCUSSION

Based on our data, we classified these Dent’s disease mutants into three different functional classes (Table 1). Class 1 Dent’s disease mutations, S270R, G513E, R516W, and I524K, result in retention of CLC-5 in the endoplasmic reticulum; class 2 comprises the functionally defective E527D; and class 3 mutations, G57V and R280P, result in alterations in the subcellular distribution of CLC-5. The consequences of each class of mutation are discussed in more detail below.

The locations of the seven mutations studied in the present investigation were examined on a homology model of the human CLC-5, which was based on the crystal structure of the prokaryotic homologue CLC-ec1 (7; Fig. 6). Previous modeling studies (39) have highlighted the proximity of the majority of Dent’s disease missense mutations to the interface between the two subunits. Again, in our homology model all mutations were located in helices forming the subunit interface (Fig. 6). The four class 1 mutations (S270R, G513E, R516W, and I524K) are located in regions of the subunit interface that would be buried during dimer formation. The only class 2 mutation identified was E527D, and it is not clear if its location is representative of a “hot-spot” for functional defects. However, E527 is not located in close proximity to either the known chloride (7) or proposed proton (1) conduction pathways. The two class 3 mutations (G57V and R280P), despite being well separated in the primary protein sequence, are located adjacent to each other at the periphery of the subunit interface facing into the cytoplasm. This area of the protein may therefore prove to be important for interactions with the cytoplasmic C terminus or with various accessory and chaperone proteins.

ER retention of ion channels is a common mechanism of disease and has been well characterized for the ΔF506 cystic
fibrosis transmembrane conductance regulator mutation in cystic fibrosis (4) and several mutations of K\textsubscript{ATP} channels responsible for congenital hyperinsulinism (3, 35). This ER retention is often associated with degradation of the mutant subunits, and our data suggest that this is true of CLC-5. Rather than appearing as a smear of degradation products, as might be expected of an indiscriminate proteolytic process, the discrete banding pattern observed suggests a tightly regulated mechanism involving specific protease(s) cutting at specific sites. This partial digestion appears to be independent of both proteosomal and lysosomal degradation, although the proteasome may be involved in the final removal of the resulting CLC-5 fragments since its inhibition by MG132 causes these protein fragments to accumulate. It appears that all of the Dent’s disease mutants that were tested are susceptible to partial digestion to some extent but that the WT protein appears not to be affected. The most resistant mutants, i.e., those that have large molecular weight glycosylated protein, appear to be those that are able to traffic to the cell surface and pass current, suggesting a link between the stability of the protein and its ability to dimerize and escape the ER efficiently. The location of the mutations close to the dimer interface (Fig. 6), thus potentially destabilizing dimer formation, supports this hypothesis.

The only example from the present study of a class 2 mutation, where we can say for certain that function is abolished, is E527D. Macroscopic CLC-5 currents were wholly absent in cells expressing E527D, despite its presence at the cell surface (Figs. 1 and 2). Similarly, endosomal E527D failed to enhance endosomal acidification in HEK-MSR cells. In fact, instead of giving a null effect that was similar to untransfected HEK-MSR cells, expression of E527D prevented the endogenous endosomal acidification (Fig. 5B). This dominant effect may explain the unusual presence of Dent’s disease symptoms in females who carry this mutation (17) where the mosaic expression of this allele may inhibit CLC-5-independent acidification and endocytosis in a proportion of proximal tubule cells. Interestingly, E527 in CLC-5 is well conserved throughout the CLC family and mutation of the corresponding residue in CLC-0 leads to reversed voltage dependence: currents were activated by hyperpolarization rather than depolarization (20). If this were to be the case, chloride flux through CLC-5 would be absent or even reversed, resulting in the loss of the “chloride shunt” conductance. This would compromise the function of v-ATPase, thus severely disrupting endosomal acidification.

The reduction in whole cell currents observed with both class 3 mutations (G57V and R280P) correlates well with the corresponding reductions in cell surface density, as determined by the chemiluminescence assay, suggesting that these mutations do not result in any functional defect. Because ion conduction through CLC-5 at the cell surface is not thought to be physiologically relevant, Dent’s disease is not likely to result from these alterations in surface density. Rather, the

**Fig. 5.** A: subcellular distribution of VAMP2-pHluorin (green fluorescence) examined by colabeling with anti-EEA1 (left) and anti-CI-M6PR (right) antibodies (red fluorescence), demonstrating targeting to early and late endosomes. Scale bars = 10 \mu m. B: means (± SE; n > 20 cells) endosomal pH as indicated by pHluorin fluorescence ratio (see MATERIALS AND METHODS) in untransfected (untrans) HEK-MSR cells and HEK-MSR cells stably expressing WT and mutant HA-CLC-5 as indicated. Horizontal dashed line at pH 7.4 represents the pH of the extracellular solution (**P < 0.05 vs. untransfected; #P < 0.05 vs. WT; ANOVA).

![Fig. 5](http://ajprenal.physiology.org/)

**Fig. 6.** Three-dimensional homology model of human CLC-5 based on the structure of ClC-ec1 (7) showing the locations of each mutated residue. Mutations are color coded according to their class; class 1 (red), class 2 (blue), and class 3 (green) and are shown with side chains in space fill. Views of the CLC-5 dimer observed from the side (A) demonstrate the location of all mutations near the subunit interface. Views from the extracellular face (B) and of the subunit interface (C) demonstrate a clear distinction in the distributions of each class of mutations. Only a single subunit is shown in B and C for clarity.
alterations in surface density may point towards a more general defect in intracellular trafficking. This appears to be the case since, relative to WT, both mutants display alterations in endosomal distribution. It is likely, however, that the decreased protein stability and alterations in subcellular distribution are responsible for the deleterious effects associated with the onset of the disease in these cases. CLC-5 has been proposed to act as part of a macromolecular protein complex that influences the targeting and subsequent stability of key proteins in endosomal compartments (14). For example, CLC-5 KO mice show a downregulation of the megalin/cubilin receptor-mediated endocytosis complex at the cell surface (29) and the endosomal distribution of v-ATPase in several Dent’s disease patients lacking functional CLC-5 has been shown to be significantly altered (25). We can speculate that altered endocytic trafficking is responsible for the redistributions observed with both mutants. After endocytosis from the cell surface, proteins normally recycle back to the plasma membrane unless diverted, via signal sequences, into alternative pathways, e.g., to lysosomes (28); however, further investigations are required to determine if this is the case with WT CLC-5. Disruption of the normal recycling process may lead to an accumulation in early endosomes (R280P) where increased levels of CLC-5 would be ideally situated to support and enhance endosomal acidification. The elevated level of acidification (Fig. 5B) observed with the R280P mutant, which was preferentially targeted to early endosomes (Fig. 3), supports this idea. The G57V mutant accumulated predominantly in the late endosome. To reach this compartment, it must be endocytosed from the cell surface and then traverse the early endosome, supporting acidification in the process. Proteins arriving at the late endosome from the early endosome are usually contained within intraluminal vesicles, which are small vesicles contained within the lumen of late endosomes (28). Because they are contained entirely within the lumen and not contiguous with the late endosomal membrane, the membrane proteins contained within intraluminal vesicles are not able to influence the luminal contents. This may explain why CLC-5 has not been seen to affect late endosome pH (12) and also why late endosomal retention of G57V does not increase the acidification further.

An appreciation of the heterogeneity of effects resulting from Dent’s disease mutations may be important in understanding the variable nature of disease symptoms observed in Dent’s disease. Unfortunately, sufficient clinical data are not available to be able to investigate any correlation between the severity and type of the observed symptoms. While there does appear to be some variation in the extent of proteinuria, nephrolithiasis, and kidney failure and in the appearance of secondary symptoms such as rickets (2, 15, 17, 19, 34), it is not clear how much of this variation is related to the progression of the disease as opposed to being a patient/mutation-specific degree of severity. It is clear, however, that the severity of the disease is closely related to age (42) and long-term clinical studies would be required for any correlation to be made. The heterogeneity in the properties of the different class of mutation may prove to be more important when considering the potential for therapeutic intervention. Functional correction of class 2 (E527D) and class 3 mutations (G57V and R280P) is likely to be difficult to achieve due to the nature of the defects. In the case of class 2 mutations, where CLC-5 is functionally defective, correct function may only be restored by replacing the defective protein with one that has normal function. The correction of class 3 mutations, which display altered subcellular distributions, would require the manipulation of intracellular trafficking machineries but to do so specifically to CLC-5 while not altering the trafficking of other proteins may prove a challenge. Class 1 mutations, however, are likely to be more amenable to therapeutic intervention. The ER export of mutant channels may be improved by exposure to chemical or pharmacological chaperones that improve forward trafficking by allosteric means. This principle has been shown in vitro with cystic fibrosis transmembrane conductance regulator, HERG, and K<sub>ATP</sub> channels (26, 37, 41, 43). The identification of specific allosteric modulators of CLC-5 in the future may provide an effective method for the treatment of Dent’s disease caused by class 1 mutations.

In summary, we have described a range of experimental techniques that are suitable for analyzing Dent’s mutations in CLC-5. In applying these to a subset of previously described mutants, we identified three distinct classes of effect. These classes appear to correlate with the structure of the protein, although a similar analysis of a wider range of disease-causing mutations would further test or refine this correlation. These data begin to reveal the heterogeneity of effects that Dent’s disease causing mutations have on CLC-5, which are not apparent when studying membrane current alone. These also support the developing ideas that CLC-5 has a complex physiological role in the proximal tubule epithelium that extends beyond simply supporting endosomal acidification.

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REFERENCES


