Cl\(^-\) and H\(^+\) coupling properties and subcellular localizations of wildtype and disease-associated variants of the voltage-gated Cl\(^-\)/H\(^+\) exchanger CIC-5

Dent disease 1 (DD1) is caused by mutations in the CLCN5 gene encoding a voltage-gated electrogenic nCl\(^-\)/H\(^+\) exchanger CIC-5. Using ion-selective microelectrodes and Xenopus oocytes, here we studied Cl\(^-\)/H\(^+\) coupling properties of WT CIC-5 and four DD1-associated variants (S244L, R345W, Q629*, and T657S), along with trafficking and localization of CIC-5. WT CIC-5 had a 2Cl\(^-\)/H\(^+\) exchange ratio at a V\(_h\) of +40 mV with a Cl\(^-\) out of 104 mM, but the transport direction did not reverse with a Cl\(^-\) out of 5 mM, indicating that CIC-5-mediated exchange of two Cl\(^-\) out for one H\(^+\) in is not permissible. We hypothesized that CIC-5 and H\(^+\)-ATPase are functionally coupled during H\(^+\)-ATPase-mediated endosomal acidification, crucial for CIC-5 activation by depolarizing endosomes. CIC-5 transport that provides three net negative charges appeared self-inhibitory because of CIC-5's voltage-gated properties, but shunt conductance facilitated further H\(^+\)-ATPase-mediated endosomal acidification. Thus, an on-and-off “burst” of CIC-5 activity was crucial for preventing Cl\(^-\) exit from endosomes. The subcellular distribution of the CIC-5:S244L variant was comparable with that of WT CIC-5, but the variant had a much slower Cl\(^-\) and H\(^+\) transport and displayed an altered stoichiometry of 1:6:1. The CIC-5:R345W variant exhibited slightly higher Cl\(^-\)/H\(^+\) transport than CIC-5:S244L, but co-localized with early endosomes, suggesting decreased CIC-5:R345W membrane trafficking is perhaps in a fully functional form. The truncated CIC-5:Q629* variant displayed the lowest Cl\(^-\)/H\(^+\) exchange and was retained in the endoplasmic reticulum and cis-Golgi, but not in early endosomes, suggesting the nonsense mutation affects CIC-5 maturation and trafficking.

Dent disease 1 (DD1)\(^2\) is an X-linked kidney disorder caused by mutations in the CLCN5 gene, and is characterized by low molecular weight proteinuria (LMWP), hypercalciuria, nephrocalcinosis, nephrolithiasis, rickets, and, most importantly, progressive renal failure in the majority of affected males (1–3). As of 2019 a total of 226 pathogenic CLCN5 mutations have been reported consisting of nonsense, missense, splice site, insertion and deletion mutations (4). Yet, very little is known regarding how these mutations lead to specific disease manifestations.

CLCN5 encodes the CIC-5 protein expressed abundantly in kidney and intestinal epithelial cells (5, 6). In the kidney, immunohistochemistry has localized CIC-5 expression predominantly to epithelial cells lining the proximal tubule (PT), the thick ascending limb of Henle’s loop, and to α-intercalated cells of the collecting duct (5, 7, 8). In the PT, expression of CIC-5 is highest below the brush border, where urinary LMW protein is reabsorbed by endocytotic vesicles. CIC-5 colocalizes here with the vacuolar-type (V-type) proton pump (H\(^+\)-ATPase). The H\(^+\)-ATPase is ubiquitously expressed in intracellular organelles such as endosomes, lysosomes, secretory granules, and the trans-Golgi network of all eukaryotic cells (9). H\(^+\)-ATPase pumps H\(^+\) across membranes using energy generated by ATP hydrolysis to provide an acidic intracellular compartment that is critical for normal membrane trafficking, receptor-mediated endocytosis, and lysosomal degradation of macromolecules (10–12). Interestingly, Clcn5-deficient mice from two independent groups displayed altered endosomal acidification, recapitulating a typical DD1 phenotype including LMWP (13–16). Taken with the fact that CIC-5 colocalizes with H\(^+\)-ATPase in early endosomes, this evidence strongly suggests that H\(^+\)-ATPase and CIC-5 are functionally coupled during endosomal acidification and/or endocytosis, likely explaining LMWP in DD1.

Initially, CIC-5 was electrophysiologically characterized as a Cl\(^-\) channel, a common feature of the CIC gene family (17).
More recent studies demonstrated that CIC-5 functions not as a Cl\(^{-}\) channel but rather as a voltage-gated, electrogenic nCl\(^{-}/H^{+}\) exchanger (antiporter) similar to the prokaryotic homologue from *Escherichia coli* (CIC-ec1) and CIC-4 (18–22). The CIC-5 protein sequence is most closely related to the CIC-3 and CIC-4 in the CLC family, however, despite functional differences the overall protein architecture of the whole CLC family is conserved and is made up by two subunits each bearing an ion translocation pathway (23–25).

It is generally assumed that altered endosomal acidification due to dysfunctional CIC-5 impairs proximal tubular endocytosis and degradation of reabsorbed proteins resulting in the characteristic LMWP of DD1. This hypothesis is supported by the altered endosomal acidification observed in *Clcn5* KO mice (13–16). Nevertheless, our knowledge of CIC-5 molecular function and biophysical properties remains limited. Further investigation of the exchanger transport stoichiometry, and the effects of DD1 mutations on Cl\(^{-}/H^{+}\) exchanger activity are therefore needed. The bacterial homologue of CIC transporters, CIC-ec1, was the first demonstrated to have strict exchange stoichiometry of 2 Cl\(^{-}\) to 1 H\(^{+}\) ratio (26–29). The initial estimates for the Cl\(^{-}/H^{+}\) stoichiometry of CIC-5 were very rough, ranging from 1 to 5 because of extreme outward-rectifying currents (18, 20). Later the relative coupling efficiency of CIC-5 was determined as 2 Cl\(^{-}\)/1 H\(^{+}\) by the fluorescence-based measurements using pH-sensitive dye, BCECF, to measure proton flux outside of *Xenopus* oocytes expressing CIC-5 (30).

Given its initial characterization as a Cl\(^{-}\) channel (20, 31), CIC-5 was first hypothesized to transport Cl\(^{-}\) to counter and dissipate positive charge (H\(^{+}\)) accumulation generated by H\(^{+}\)-ATPase, thereby facilitating efficient endosomal acidification. In this model, Cl\(^{-}\) shunting by CIC-5 to facilitate H\(^{+}\)-ATPase was considered essential for normal endocytosis (32). However, recent studies demonstrate that CIC-5 is a voltage-gated electrogenic Cl\(^{-}/H^{+}\) exchanger (transporter) (18, 20). Valuable cellular energy (ATP) is consumed by H\(^{+}\)-ATPase pumping H\(^{+}\) from the cytoplasm into the endosome thereby acidifying it. Why would CIC-5 then move H\(^{+}\) out of the endosome? This would seem to be a very counterproductive, or at least inefficient, process. Therefore, a comprehensive reassessment of the purported CIC-5 physiological roles in endosomal acidification and/or endocytosis and its interaction with H\(^{+}\)-ATPase remain important yet unanswered questions (33).

Previously published data on CIC-5 and DD1 mutations have focused on its electrogenic properties (elicited currents) at positive voltages (+60 to +100 mV) (34–38). Consequently, a systematic study of the effects of CIC-5 mutations on Cl\(^{-}/H^{+}\) exchanger activity and coupling properties is lacking. To fill this gap, we used ion selective microelectrodes to monitor intracellular pH (pH\(_{i}\)) and Cl\(^{-}\) concentration ([Cl\(^{-}\)]) to investigate Cl\(^{-}/H^{+}\) coupling properties of wildtype (WT) and mutated CIC-5 in *Xenopus* oocytes under voltage-clamped conditions. We focused on common (S244L) and novel CLCN5 variants (R345W, Q629*) identified by the Rare Kidney Stone Consortium (RKSC). We also examined protein trafficking and cellular localization of WT and these patient-specific mutations in human renal epithelial cells by immunofluorescence microscopy. Together, these findings shed light on functional properties of CIC-5 and the molecular mechanisms leading to Dent disease pathogenicity.

**Results**

**Cl\(^{-}/H^{+}\) coupling properties of patient-specific mutations**

Oocytes were injected with WT or selected mutant CIC-5 cRNAs to examine effects of mutations on H\(^{+}\) and Cl\(^{-}\) transport properties while clamping to +40 mV. WT CIC-5 exhibited a robust influx of Cl\(^{-}\) (47.0 ± 7.9 μM s\(^{-}\)) and efflux of H\(^{+}\) (56.7 ± 9.9 × 10\(^{-3}\) pH units s\(^{-}\)) in response to 104 mM [Cl\(^{-}\)]\(_{\text{out}}\) (ND96) in the media (Figs. 1–2 and Table 1). The exchange of Cl\(^{-}\) for H\(^{+}\) was effectively inhibited by reduction of extracellular [Cl\(^{-}\)] to 5 mM. The final [Cl\(^{-}\)] for WT CIC-5 oocytes increased by 12.4 mM and pH\(_{i}\) increased by 0.13 pH units over 5 min. As previously reported (4), the T657S variant exhibited no significant differences from WT CIC-5 in Cl\(^{-}/H^{+}\) exchange activity and in response to extracellular [Cl\(^{-}\)] manipulations. In contrast, the S244L variant exhibited defective Cl\(^{-}\) for H\(^{+}\) exchange activity. Specifically, the S224L variant blunted H\(^{+}\) transport (16.8 ± 2.7 × 10\(^{-5}\) pH units s\(^{-}\)) by 70% (p < 0.05) and significantly impaired Cl\(^{-}\) influx (8.9 ± 5.0 μM s\(^{-}\)) by 81% (p < 0.001) compared with WT CIC-5 in 104 mM [Cl\(^{-}\)]\(_{\text{out}}\) buffer. S224L-expressing oocytes also exhibited no significant change in [Cl\(^{-}\)]\(_{\text{i}}\), nor pH\(_{i}\) in response to experimental solution manipulation from 104 to 5 mM [Cl\(^{-}\)]\(_{\text{out}}\). Similar trends were observed with the R345W variant demonstrating a decrease in Cl\(^{-}\) for H\(^{+}\) exchange activity by 49 (p = 0.07) and 54% (p < 0.05) compared with WT CIC-5, respectively. With these same conditions, the nonsense mutation Q629* dramatically altered both H\(^{+}\) (87% decrease relative to WT; p < 0.01) and Cl\(^{-}\) (89%) decrease relative to WT; p < 0.01) transport.

**Stoichiometry**

Because CIC-5 is a voltage-gated transporter that does not have obvious reversal potential to indicate an energetic steady-state, the Nernst equation expression cannot be used to calculate the transport stoichiometry at equilibrium. Instead, we use the more general Gibbs free energy, ΔG, equation to estimate the transport stoichiometry. This ΔG equation for each ion species transport through the CIC-5 is the sum of two energies: the solute concentration gradient and the solute electrical gradient,

\[
\Delta G = RT \ln \frac{C_{\text{i}}}{C_{\text{out}}} + zF\Delta \psi
\]

where \(C_{\text{i}}\) and \(C_{\text{out}}\) are the respective ion concentration inside and outside the specific compartment, \(z\) is the ion valence, \(T\) is the absolute temperature (Kelvin), \(R\) is the ideal gas constant (8.314 J/mol/K), and \(F\) is the Faraday’s constant (9.6485 × 10\(^{4}\) coulombs/mol), \(\Delta \psi\) is the holding membrane potential at +40 mV. Of note, when expressed in an oocyte, CIC-5 would not be actively transporting under normal circumstances because the resting membrane potential of an oocyte is around ~30 to ~60 mV. In other words, using the initial pH\(_{i}\) and [Cl\(^{-}\)], (the measurements taken at t = 0) to calculate stoichiometry does not actually represent the state of CIC-5 transport activity. Thus to portray CIC-5 transport activity during active state (V\(_{H}\) = +40 mV) and to account for the biological variations, the ΔpH and...
measurements from each oocyte were used in the equation as $C_{in}$ to offset the difference of initial pH and $[\text{Cl}^-]_i$ among oocytes.

The movement of any molecule or ion up or down a concentration gradient involves a change in free energy, $\Delta G$. When $\Delta G$ is positive, the reaction consumes energy, \textit{i.e.} is not spontaneous. However, if $\Delta G$ is negative the reaction releases energy, \textit{i.e.} is spontaneous. Because both $\Delta G_{\text{Cl}^-}$ and $\Delta G_{\text{H}^+}$ were negative the transporter is predicted to be exchanging spontaneously (active) at the given condition. Thus the ratio between $\Delta G_{\text{Cl}^-}$ and $\Delta G_{\text{H}^+}$ is not the same for all conditions.
and Δ$G_{H^+}$ describe how efficiently the transporter is spontaneously transporting the respective ion species (i.e., represents the coupling ratio).

Therefore the apparent stoichiometry was calculated as: ($ΔG_{Cl^-}$)/($ΔG_{H^+}$), or Equation 2.

$$\frac{RT \ln \frac{[Cl^-]_{in}}{[Cl^-]_{out}} + zFΔψ}{RT \ln \frac{[H^+]_{in}}{[H^+]_{out}} + zFΔψ}$$

(Eq. 2)

The calculated apparent Cl$^-$:H$^+$ coupling ratio (stoichiometry) for WT ClC-5 and the nonpathogenic T657S variant were 2:1 (Fig. 3) at $V_h = +40$ mV with $[Cl^-]_{out} = 104$ mM and $[H^+]_{out} = 3.16 \times 10^{-5}$ mM. Under the same conditions, the Cl$^-$:H$^+$ coupling ratio of the S244L and R345W variants were reduced to 1.6:1 and 1.7:1, respectively, consistent with decreased current. The Q629* variant has a further decreased apparent stoichiometry of 1.4:1. With 104 mM [Cl$^-$/] in the extracellular solution, at +40 mV CIC-5 functions as Cl$^-$/H$^+$ exchanger. However, after lowering extracellular [Cl$^-$/] to 5 mM, the discernable Δ$H^+$ and Δ[Cl$^-$/] were too small to make ΔG calculations meaningful. Therefore the Cl$^-$/H$^+$ coupling ratios were not calculated under this condition.

Of note, this “apparent stoichiometry” is a calculated parameter to describe how efficiently the transporter is spontaneously transporting Cl$^-$ relative to transporting H$^+$ within the same CIC-5 variants. The decimal number was the result from mathematical normalization, not the molecular count of the ion species. The Δ$H^+$ recorded with WT ClC-5 was 0.13 pH units versus the Δ$H^+$ for S244L, R345W, and Q629* were 0.03, 0.06, and 0.01 pH units, respectively (Table 1). In other words, the 1 in the apparent stoichiometry of WT CIC-5 is not equivalent to the 1 in the apparent stoichiometry of S244L.

**Transport function of EGFP/HA double-tagged CIC-5**

To determine whether defective CIC-5 transporter activity is due to failure of CIC-5 trafficking to the plasma membrane, we expressed WT and mutant CLCN5 constructs containing a N-terminal intracellular EGFP tag, as well as an extracellular HA tag for surface labeling, in human immortalized renal cortical tubular epithelial (RCTE) cells. We first sought to validate that transporter activity of these constructs was maintained. We expressed EGFP/HA double-tagged WT CIC-5 (pEGMHE expression vector) in oocytes and detected EGFP signal using an epifluorescence microscope (Fig. 4) indicating that the protein was successfully synthesized by the oocyte. When extracellular [Cl$^-$/] is 104 mM, a strong outward-rectifying current was observed that diminished in a 5 mM Cl$^-$/ solution. These results were not significantly different from the untagged version of CIC-5 counterpart reported previously (4), verifying that the tags (EGFP/HA) do not interfere with innate CIC-5 transport function.

**Subcellular localization of CIC-5**

In human RCTE cells, WT CIC-5 and the S244L variant are both localized to the plasma membrane as illustrated by a robust EGFP signal distributed in a punctate fashion along the cell membrane, as well as positive surface staining of the extracellular HA tag (Fig. 5). Neither cell membrane expression of CIC-5 (EGFP signal) nor surface HA tag labeling were detected in R345W and Q629* expressing cells. Rather, R345W and Q629* proteins appeared to co-localize primarily with the endoplasmic reticulum marker KDEL compared with low co-
localization of S244L and WT ClC-5 (Fig. 6). Finally, WT CIC-5, S244L, and R345W variants, but not the Q629* variant, also co-localize with EEA1 (Fig. S1), an early endosome marker, and the signal from the Q629* variant overlapped strongly with a cis-Golgi marker, GM130 (Fig. 7).

Discussion

The current study used ion-selective microelectrodes to directly measure Cl\(^-\)/H\(^+\) transport by ClC-5. The apparent stoichiometry calculated from the Gibbs free energy of Cl\(^-\)/H\(^+\) versus H\(^+\)/Cl\(^-\) transport reveal a 2 Cl\(^-\)/1 H\(^+\) ratio in agreement with previous findings (30). WT ClC-5 acts as 2Cl\(^-\)/1H\(^+\) exchanger at +40 mV with 104 mM Cl\(^-\) and pH 7.5 in the extracellular solution. Lowering extracellular [Cl\(^-\)] to 5 mM, creating a chemical gradient in favor of outward Cl\(^-\) movement, did not reverse the transport direction because there were no apprecia-

Figure 4. EGFP/HA double-tagged ClC-5 expressed in *Xenopus* oocyte. A, strong green fluorescent signal was observed compared with water-injected control oocyte using an epifluorescence microscope (488/509) indicating EGFP/HA-CIC-5 was successfully synthesized. B, transport function of EGFP/HA double-tagged ClC-5 under voltage-clamp (V\(_h\) = −60 mV) experimental condition. Current-voltage relationship of EGFP/HA double-tagged ClC-5 in response to extracellular Cl\(^-\) maneuver is no different from the untagged ClC-5.

Figure 5. EGFP/HA double-tagged ClC-5 expression in RCTE cells. Human immortalized RCTE cells were transfected with GFP/HA double-tagged ClC-5 WT or patient-specific mutations and incubated overnight at 37 °C. EGFP signal, representing total ClC-5, and Alexa 594-HA tag (red), indicating surface ClC-5 expression, were co-localized along the cell membrane distributed in a punctate fashion in cells expressing WT and S244L. No plasma membrane distribution of ClC-5 protein was detected in R345W and Q629* variant-expressing cells.

Figure 6. ClC-5 expression and co-localization of ER in RCTE cells. RCTE cells expressing GFP/HA double-tagged ClC-5 WT or patient-specific mutations immunostained with ER marker (KDEL 10C3). Strong ClC-5 GFP signal co-localized with ER (red arrow) were evident in R345W and Q629* mutants.

Cl\(^-\)/H\(^+\) coupling properties of CLCN5 mutations

Figure 7. EGFP/HA double-tagged ClC-5 expression in RCTE cells. Human immortalized RCTE cells were transfected with GFP/HA double-tagged ClC-5 WT or patient-specific mutations and incubated overnight at 37 °C. EGFP signal, representing total ClC-5, and Alexa 594-HA tag (red), indicating surface ClC-5 expression, were co-localized along the cell membrane distributed in a punctate fashion in cells expressing WT and S244L. No plasma membrane distribution of ClC-5 protein was detected in R345W and Q629* variant-expressing cells.

Cl\(^-\)/H\(^+\) coupling properties of CLCN5 mutations

Is the altered Cl\(^-\)/H\(^+\) transport stoichiometry of ClC-5 mutants due to (a) more H\(^+\) being now needed to exchange for the same amount of Cl\(^-\) or (b) due to less Cl\(^-\) being needed to exchange for the same amount of H\(^+\)? Our data demonstrated that both H\(^+\) transport (ΔpH\(_i\) and dpH\(_i\)/dt) and Cl\(^-\) transport (Δ[Cl\(^-\)]\(_i\) and d[Cl\(^-\)]\(_i\)/dt) by ClC-5 mutants were much smaller and slower than those recorded for WT ClC-5 or the T657S variant assuming the buffering capacity of the oocytes is consistent (Table 1). Even though mutations have an overall defective ionic exchange, the calculated Cl\(^-\)/H\(^+\) coupling ratio

**Table 1**

| Mutation | ΔpH\(_i\) | dpH\(_i\)/dt | Δ[Cl\(^-\)]\(_i\) | d[Cl\(^-\)]\(_i\)/dt |
|----------|----------|-------------|-----------------|------------------|
| WT       |          |             |                 |                  |
| S244L    |          |             |                 |                  |
| R345W    |          |             |                 |                  |
| Q629*    |          |             |                 |                  |

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remained >1, suggesting that the mutated ClC-5 transporters still exchange more Cl\(^-\) for H\(^+\) and thus remained electrogenic. We believe the changed amino acids in ClC-5 mutants have altered ClC-5 protein 3D structure. Accordingly, we hypothesize that these changes affect the Cl\(^-\)/H\(^+\) ion binding and releasing by the ClC-5 protein, which ultimately interfere with the ion transporting pathways. A working ClC-5 protein structure model and additional experiments are needed to further test this speculation.

### Subcellular localization of ClC-5

Even though the S244L variant displayed a decreased apparent Cl\(^-\)/H\(^+\) transport ratio, its cell surface expression was not significantly different from WT (previously quantified by chemiluminescence in oocytes (4) or currently by immunocytochemistry in mammalian cells (Fig. 5). Given there is ample expression of the S244L protein at the membrane, the defect of the S244L variant appears solely due to decreased Cl\(^-\)/H\(^+\) transport. The R345W and Q629* variants did not appear at cell surfaces but rather in the ER and/or cis-Golgi suggesting impaired protein trafficking (Figs. 5–7). Although we did not rigorously demonstrate the exact subcellular localization of each variant, our data are consistent with our previous findings using an HEK293 cell expression model (4). It is likely these specific mutated ClC5 proteins never fully matured allowing them to leave the ER and/or Golgi apparatus because Q629* localized to the ER and/or cis-Golgi but not to early endosomes.

Interestingly, despite having lower surface expression, the R345W variant functions slightly better than the S244L variant in terms of current magnitude and Cl\(^-\)/H\(^+\) exchange activity. The R345W variant also localized to early endosomes (Fig. 7B, Fig. S1), suggesting that this variant may be trafficked to the membrane, albeit to a lesser degree, and that R345W molecules that successfully traffic to the plasma membrane are fully functional. This speculation is supported by the slightly higher calculated apparent stoichiometric Cl\(^-\)/H\(^+\) coupling ratio (1.7:1) than that of S244L (1.6:1). These data demonstrate that studying ClC-5 mutational effects on both transport function and cellular localization is essential to better understand divergent pathogenesis of DD1.

### Biophysical mechanisms ClC-5 ion transport

The H\(^+\)-ATPase and ClC-5 are functionally coupled during endosomal-to-lysosomal acidification. However, this critical endosomal acidification does not rely on ClC-5 directly, rather active H\(^+\) entry occurs via H\(^+\)-ATPase with the hydrolysis of ATP. What then is the role of ClC-5 as a 2Cl\(^-\)/H\(^+\) exchanger? It is this fundamental question that requires elucidating the “control mechanisms” of WT and mutant ClC-5 transport. First, voltage-gating prevents ClC-5 transport at negative voltages (4, 18, 22) as neither inward nor outward rectifying currents were observed in any cellular experimental condition. Second, as illustrated in Figs. 1 and 2 and Table 1, merely creating a chemical gradient (by reducing extracellular [Cl\(^-\)] to 5 mM) does not result in ClC-5 reversing its transport direction explicitly for membrane potential held at +40 mV, at which the transporter should be active state. However, the physiological role of ClC-5 during endosomal acidification is still more complicated and elusive because impaired endosomal acidification was reported in proximal tubule cells of ClC-5 deficient mice (15).

Immediately after internalization, luminal chloride concentration, [Cl\(^-\)]\(_{\text{lumen}}\), drops from an extracellular value of 120–150 to ~20 mM inside early endosomes (39). This drop has been
attributed to Cl− expulsion by a Donnan potential produced by negative membrane protein charges \((40)\) that face the outside solution of the plasma membrane and the lumen of forming of vesicles (as depicted in Fig. 8) \((41)\). It was also hypothesized that the emerging endosomes were formed as flattened structures with a very high surface/volume ratio to mandate substantial Donnan potential \((42)\).

The difference in \([\text{Cl}^-]\) from cytoplasm \((30–70 \text{ mM})\) \((39, 43–45)\) to nascent endosomes \((20 \text{ mM})\) continues to provide a favorable chemical gradient for Cl− entry. However, because CIC-5 is a voltage-gated transporter, it only activates when net positive charges inside the endosomes are sustained, the energy (driving force) from the \([\text{Cl}^-]\) gradient alone is not sufficient to activate CIC-5 transport \((43, 46)\). This critical characteristic was previously reported \((4, 18, 20)\) as strong, outward currents that rectified only when membrane potential inside more positive \((i.e.\) oocytes were depolarized) was controlled by voltage clamping \((41)\).

Energy consuming active \(\text{H}^+\) entry driven by the \(\text{H}^+\)-ATPase (making the charge inside early endosomes positive) is crucial for secondary active transport by CIC-5. As portrayed in the model \((42)\), CIC-5 removes one \(\text{H}^+\) from the endosomal lumen to the cytoplasm in exchange for 2 \(\text{Cl}^-\) per transport-cycle providing 3 net negative charges \((2\text{Cl}^-/\text{H}^+)\) to dissipate \(\text{H}^+\) buildup generated by \(\text{H}^+\)-ATPase. Thus the total free energy for CIC-5 transport \(\Delta G_{\text{CIC-5}} = 2\times(\Delta G_{\text{Cl}^-}) - 1\times(\Delta G_{\text{H}^+})\) dissipate the net negative charge accumulation inside the endosome and thereby provides a shunt conductance that facilitates further endosomal acidification by the \(\text{H}^+\)-ATPase without making the endosomal membrane potential \(V_{\text{endo}}\) too positive. This balance-counterbalance means that CIC-5 transport will resume once \(V_{\text{endo}}\) becomes positive due to active \(\text{H}^+\) entry via \(\text{H}^+\)-ATPase \((42)\). CIC-5 activity switched on and off by a gating process is consistent with the “burst” hypothesis proposed by Jentsch and Pusch and colleagues \((22)\) as transport activity of CIC-5 occurs in bursts and transport is very fast within each burst \(\left(10^5 \text{ ions/s}\right)\).

Endosomal \([\text{Cl}^-]\) increases due to \(2\text{Cl}^-/\text{H}^+\) exchange by CIC-5 as the endosome becomes mature. The \(\text{Cl}^-\) concentration in early endosomes \([\text{Cl}^-]_{\text{EE}}\) was reported as 17 or 28 mM in J774 and CHO cells, respectively, quantified using a fluorescent \(\text{Cl}^-\) indicator \((39, 41)\). The \(\text{Cl}^-\) concentration in late endosomes \([\text{Cl}^-]_{\text{LE}}\) increased to 58 (J774 cells) and 73 mM (CHO cells). Consistent with these results, an increase in \([\text{Cl}^-]_{\text{lumen}}\) during endosomal maturation, with a mean \([\text{Cl}^-]_{\text{EE}}\) and \([\text{Cl}^-]_{\text{LE}}\) of 37.0 and 60.4 mM, respectively, was also observed in Drosophila S2R+ cells \((46)\). Accompanying the \([\text{Cl}^-]_{\text{lumen}}\) increase, a parallel pH decrease from 6.91–7.1 to 5.2 in J774 cells and from 6.7 to 5.4 in CHO cells occurs \((41, 47)\). Lower intracellular pH (increasing intracellular \([\text{H}^+]\)) further stimulates CIC-5 transport in an allosteric manner with an apparent pK of ~7.2 as reported by Zifarelli and Pusch \((30)\). This model is supported by studies that demonstrated impaired endosomal acidification and less \(\text{Cl}^-\) accumulation in proximal tubular cell cultures from CIC-5–deficient mice compared with WT mice \((15)\).

2\(\text{Cl}^-/\text{H}^+\) exchangers in the CLC family contain a critical glutamate residue that plays a key role in the coupling of \(\text{H}^+\) exchange to \(\text{Cl}^-\) transport \((48, 49)\). An artificial mutation of this
“gating glutamate” to alanine in CIC-5 (c.632A>C, p.Glu-211–Ala) and in other ClCs (Glu-148 in CIC-ecl and Glu-224 in CIC-4) abolishes H⁺ coupling and allows the conversion of ClCs to pure Cl⁻ conductance, i.e. Cl⁻ channel (18, 20, 26). However, the removal of gating glutamate not only abolished H⁺ coupling, but also changes the voltage-gating property, the key characteristic of the CIC-5 transporter. When the “gating” glutamate is removed, Cl⁻ “leaks” out of endosome under a hyperpolarized condition due to a favorable electrochemical gradient (i.e. ΔG_ClC-5 < 0) through E211A-CIC-5 (20). Intriguingly, mice carrying the E211A mutation displayed the same renal phenotype as Clcn5 knock-out mice (50) including LMWP, despite normal endosomal acidification, suggesting that endosomal chloride-proton exchange rather than chloride conductance is crucial for renal endocytosis and proper protein degradation. Another pathogenic mutation identified in DD1 patients that affects the gating glutamate of CIC-5 (c.632A>G, p.Glu-211–Gly, E211G) displays normal endosomal acidification when expressed in HEK293T cells, further indicating that impaired endosomal acidification is not the cause of defective endocytosis in the PT of DD1 patients (21). Nevertheless, it is still possible that the endosomal Cl⁻ concentration in E221A or E211G cells is lower than WT cells because of Cl⁻ leakage, resulting in the LMWP.

Based on the above model, the pathogenic CLCN5 mutations identified in DD1 patients displaying an altered Cl⁻/H⁺ transport stoichiometry, have overall lower transport function and apparently do not provide sufficient net negative charges within endosomes to dissipate H⁺ buildup generated by the H⁺-AT-Pase activity. Thus, in these decreased apparent stoichiometry cases, functional coupling of V-ATPase and ClC-5 will be disrupted. As a consequence, impaired endosomal acidification and Cl⁻ accumulation occur, as is seen in PT cell cultures from CIC-5–deficient mice. Finally, and most importantly, how altered endosomal Cl⁻ accumulation impairs renal endocytosis remains to be determined.

Experimental procedures

Molecular biology

Human WT CIC-5 (GenBank NM_000084.4) ORF was sub-cloned into the pGEMHE expression vector for Xenopus laevis oocyte expression, or into the pEGFP-C2 expression vector for renal epithelial cell expression. The HA epitope (YPYDVPDYA) was introduced into the extracellular loop of CIC-5 between transmembrane domains B and C (48). Four representative mutant CLCN5 constructs (S244L, R345W, Q629*, and T657S) were generated by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as previously described (4). Capped cRNAs were synthesized in vitro from WT and mutant CIC-5 expression vectors linearized with Mlu using the T7 mMessage mMachine Kit (Ambion, Austin, TX).

Expression in X. laevis oocytes

Frogs were housed and cared for in accordance and approval of the Institutional Animal Care and Use Committee of the Mayo Clinic College of Medicine. Defolliculated stage V/VI Xenopus oocytes were injected with 10 ng of the specific cRNAs. The oocytes were then kept at 16 °C in OR3 media.

Microelectrodes

The voltage electrodes fabricated from a P-97 Flaming Brown Micropipette Puller (Sutter Instrument, Novato, CA) with a borosilicate fiber capillary had a resistance of 0.5–1 MΩ and were back filled with 3 M KCl (51). For ion-selective experiments, the electrodes were silanized with bis-(dimethyl-amino)-dimethylsilane, and filled with the Fluka H⁺ ionophore I, mixture B (pH), or Fluka Cl⁻ ionophore I, mixture A (aCl⁻).

The finished electrodes were backfilled with buffer solution (pH backfill is phosphate buffer, pH 7.0; Cl⁻ backfill is 500 mM KCl).

Two-electrode voltage-clamp electrophysiology

Two-electrode voltage-clamp experiments were performed 2 or 3 days after cRNAs injection at room temperature using an OC-725C voltage clamp (Warner instruments, Hamden, CT) and HEKA software (Wiesenstrasse, Germany). A CIC-5 expressing oocyte, visualized with a dissecting microscope, was held on a nylon mesh in a chamber, through which saline flows continuously. Currents were recorded in either 104 mM Cl⁻ (ND96) solution (high Cl⁻: 96 mm NaCl, 2.0 mm KCl, 1.8 mm CaCl₂, 1.0 mm MgCl₂, 5.0 mm HEPES, pH 7.5) or 5 mM Cl⁻ (5 Cl⁻-ND96) solution (low Cl⁻: iso-osmotic Cl⁻ replacements with gluconate). Currents were recorded in response to a voltage protocol consisting of 20-mV steps from −120 to +80 mV during 75 ms/step from a holding potential of −60 mV; the resulting I-V traces were filtered at 2 kHz (8 pole Bessel filter) and sampled at 10 kHz. Data were acquired and analyzed using Pulse and PulseFit (HEKA Instruments, Germany).

Measurement of ion transport in oocytes

pH electrodes were calibrated to standard solutions (pH 6.0 and 8.0) and Cl⁻ electrodes were calibrated to 10 and 100 mM NaCl standard solutions. All ion-selective microelectrodes had slopes of −54 to −57 mV/decade ion concentration (or activity). Ion-selective electrodes were connected to a high-impedance electrometer (WPI FD-223). The Vm signal (from the voltage-clamp apparatus) was subtracted from the ion selective electrode voltage yielding a true voltage change due solely to pHi or aCl⁻. With all three electrodes impaled into the oocyte, Vm and pHi, or Cl⁻, were allowed to stabilize. The oocyte was then clamped to a holding potential (Vh) at +40 mV. At the steady state, clamping current and pHi, or Cl⁻, were filtered (20 Hz) and continuously monitored. Solutions were switched by computer, which records intracellular pH (pHi), intracellular Cl⁻ activity (aCl⁻), membrane potential (Vm), and membrane current (Im) at 0.5–1 Hz; and controls the voltage-clamp (52–54).

Cell culture and transfection

Human immortalized RCTE cell lines were cultured in Dulbecco’s modified Eagle’s medium (supplemented with GlutaMAX, 5% fetal bovine serum and 5% penicillin-streptomycin; GIBCO, Invitrogen) as previously described (55) and maintained in a humidified atmosphere containing 5% CO₂ at 37 °C.
Cl\(^-\)-H\(^+\) coupling properties of CLCN5 mutations

For transient expression, RCTC cells were grown to 80–90% confluence and transfected with 1.25 μg of DNA/million cells in 100 μl of transfection buffer (135 mM KCl, 2 mM MgCl\(_2\), 20 mM HEPES, 0.5% Ficoll 400, pH 7.6) using Gene Pulser Xcell™ Electroporation Systems (Bio-Rad).

**Immunocytochemistry staining**

Transiently transfected RCTE cells expressing EGFP/HA double-tagged ClC-5 were grown on glass coverslips for immunostaining. Primary and secondary antibody incubations were performed in a humidified chamber at room temperature unless specified otherwise. For surface HA tag staining, cells were cooled to 4 °C prior to staining to prevent endocytosis of antibodies. Monoclonal anti-HA antibody produced in mouse (HA-7, IgG1; Sigma) was diluted to 1:500 in serum-free media and applied to the cells and incubated at 4 °C for 1 h. After fixing with 4% paraformaldehyde in PBS (10 min in room temperature), cells were blocked (1% BSA, 10% normal growth serum in PBS) and stained with a secondary antibody. For organelle labeling, cells were first fixed in 4% paraformaldehyde and permeabilized using 0.5% Triton X-100, 1% BSA, 10% normal growth serum in PBS. All primary antibodies were diluted to 1:200 as a working concentration. Mouse monoclonal KDEL antibody (10C3, IgG2a; Novus) was used for endoplasmic reticulum (ER) labeling, rabbit monoclonal GM130 antibody (EP892Y, IgG; Abcam) was used as a Golgi marker. For early endosome labeling, rabbit polyclonal EEA1 antibody (IgG; Abcam) was used. Secondary antibodies (goat anti-mouse Alexa Fluor® 594 or goat anti-rabbit Alexa Fluor® 647) were used at a dilution of 1:1000. Controls for specificity and autofluorescence staining were performed using secondary antibodies alone. Labeled cells were imaged using an inverted epifluorescence microscope system (Zeiss, Germany) and analyzed using ImageJ software.

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