The Chloride Channel ClC-4 Contributes to Endosomal Acidification and Trafficking*

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Mutations in the gene coding for the chloride channel ClC-5 cause Dent's disease, a disease associated with proteinuria and renal stones. Studies in ClC-5 knockout mice suggest that this phenotype is related to defective endocytosis of low molecular weight proteins and membrane proteins by the renal proximal tubule. In this study, confocal micrographs of proximal tubules and cultured epithelial cells revealed that the related protein ClC-4 is expressed in endosomal membranes suggesting that this channel may also contribute to the function of this organelle. In support of this hypothesis, specific disruption of endogenous ClC-4 expression by transfection of ClC-4 antisense cDNA acidified endosomal pH and altered transferrin trafficking in cultured epithelial cells to the same extent as the specific disruption of ClC-5. Both channels can be co-immunoprecipitated, arguing that they may partially contribute to endosomal function as a channel complex. These studies prompt future investigation of the role of ClC-4 in renal function in health and in Dent's disease. Future studies will assess whether the severity of Dent's disease relates not only to the impact of particular mutations on ClC-5 but also on the consequences of those mutations on the functional expression of ClC-4.

There are nine members of the ClC family of chloride channels in mammals, and several members have been implicated in congenital diseases. For example, mutations in ClCN1 cause congenital myotonia (1–4); mutations in ClCN2 cause idiopathic generalized epilepsy (5); ClCN5 is mutated in Dent's disease, a renal disease characterized by low molecular weight proteinuria, hypercalciuria, and in some severe cases renal failure (6–11); and mutations in ClCN7 are associated with osteopetrosis (12). Each of these particular chloride channels belongs to distinct subgroups of the ClC family, defined on the basis of their degree of sequence similarity. ClC-1 is grouped with ClC-2, ClC-Ka, and ClC-Kb (7). ClC-7 is most closely related to ClC-6 (7). ClC-5, ClC-4, and ClC-3 form a distinct subgroup, sharing close to 80% sequence identity.

The subgroup of ClC channels, including ClC-3, ClC-4, and ClC-5, is thought to function in intracellular compartments.

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For example, ClC-3 is localized in synaptic vesicles in neurons where it has been shown to contribute to vesicular acidification probably by providing an electrical shunt permissive to V-type ATPase activity (13). In non-neuronal tissue, the shorter isoform (ClC-3A) has been localized to late endosomes and lysosomes where it is presumed to function in regulating the pH of these compartments (14, 15). The function of the longer Golgi-localized isoform (ClC-3B) has yet to be determined (14).

ClC-5 is localized primarily in early endosomes in native tissues of rodent renal proximal tubules and in various heterologous expression systems (16, 17). Disruption of Clcn5 in mice leads to defective fluid phase and receptor-mediated endocytosis by the renal proximal tubule, arguing that ClC-5 contributes to endocytosis in vitro (16, 18–20). On the basis of these studies, it has been hypothesized that ClCN5 mutations may lead to low molecular weight proteinuria and hypercalciuria in patients with Dent's disease because of defective internalization of protein and membrane receptors from the apical surface of the renal proximal tubule (19). Furthermore, it was proposed that ClC-5 normally contributes to endocytosis by facilitating endosomal acidification, a phenomenon essential for appropriate vesicular trafficking (21–23). As for ClC-3A, it has been suggested that ClC-5 may provide an electrical shunt for charge dissipation, thereby permitting endosomal acidification through the action of V-type ATPases (15, 24). In fact, endosomal vesicles purified from Clcn5 knockout mice exhibited slower rates of acidification than vesicles purified from their wild type siblings (18). However, a direct contribution of ClC-5 to this function in vivo has not yet been demonstrated.

ClC-4 shares 78% sequence identity with ClC-5, and these two channels exhibit almost identical channel properties when studied in heterologous expression systems (25). However, unlike ClC-5, very little is known about the native expression and function of ClC-4 in epithelial cells. Recently, we generated a specific antibody against ClC-4, and we showed that in rodent and human intestinal epithelia, ClC-4 channels co-localize with the cystic fibrosis transmembrane conductance regulator in the apical membrane and in subapical vesicles (26). Significantly, a proportion of the intracellular vesicles bearing ClC-4 appeared to co-localize with the endosomal marker EEA1 (26), raising the possibility that ClC-4 may participate with ClC-5 in regulating the function of this organelle.

The primary goal of the present project was to assess the role of ClC-4, relative to ClC-5, in endosomal trafficking in epithelial cells and to determine the mechanism underlying this putative function. We show for the first time that the specific depletion of ClC-4 reduces the rate of transferrin receptor recycling and that this reduction is associated with a defect in endosomal acidification. Hence, normal endosomal trafficking...
in epithelial cells may require the functional expression of ClC-4 as well as ClC-5.

MATERIALS AND METHODS

Constructs—The antisense murine ClC-4 was generated as described previously (26). Similarly, the antisense human cDNA ClC-5 (ClC-5 cDNA was a gift from Dr. T. J. Jentsch) was generated by cloning the ClC-5 open reading frame with BamHI (5′) and EcoRI (3′) linkers on the forward and reverse primers, respectively. As described previously, the resulting PCR fragment was cloned into pcDNA3.1 (+) to create the antisense plasmid. The HA1 tag was inserted onto the amino terminus of ClC-5. This antibody was originally characterized by guest on July 27, 2018http://www.jbc.org/Downloaded from
tibody disulfide bands and analyzed for the presence of ClC-4 and ClC-5 proteins.

Patch Clamp Electrophysiology—Caco-2 cell membrane currents were measured using conventional whole cell patch clamp technique as described (26). Data were collected and analyzed with an Axopatch-200A amplifier and pCLAMP software (Axon Instruments, Foster City, CA). The bath solution contained (in mM): 140 N-methyl-D-glutamine chloride, 2 MgCl2, 2 CaCl2, 5 HEPES, whereas the pipette solution contained (in mM) 140 N-methyl-D-glutamine-Cl, 2 MgCl2, 2 EGTA, and 5 HEPES. Both pipette and bath solutions were adjusted to pH 7.4. The tip resistance was 3–5 MΩ when filled with the pipette solution.

Endosomal pH measurements—Endosomal pH was determined using fluorescence ratio imaging as described (30, 31). Caco cells plated on coverslips were co-transfected with enhanced blue fluorescence protein and either vector control or ClC-4 or ClC-5 antisense and serum-starved overnight. Endosomes were loaded with 150 μM FITC-transferrin in sodium-rich saline (140 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, pH 7.4) for 30 min. Coverslips were washed and pulsed briefly with PBS (pH 5.0) to remove surface-bound FITC-transferrin before transferring to Leiden chambers for analysis. Transfected cells were identified using UV filters using a Zeiss IM-35 microscope. Internalized FITC-transferrin was then excited at alternating wavelengths of 490 (700 ms) and 440 nm (100 ms) using a Sutter filter wheel. The fluorescence light was directed to a 535-nm emission filter placed before a cooled CCD camera used for fluorescent detection using 8 × 8 binning. Image acquisition was controlled using the Metafluor software (Universal Imaging Corp.). Images were captured at 2-min intervals. The fluorescence ratio versus pH was calibrated by equilibrating the cells in K+-rich medium (140 mM KCl, 10 mM NaCl, 5 mM glucose, 1 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, pH 7.4) adjusted to varying pH values (ranging from 7.3 to 5.7) containing 10 μM of nigericin and monensin. Endosomes were defined as regions of interest, and pixel intensity values were determined for resting endosomes and defined calibration pH acquisitions following background subtraction for both 490 (pH-dependent) and 440 nm (pH-independent). Calibration curves of the fluorescence ratio (490:440) were then plotted against pH, and endosomal pH values were extrapolated from the curve.

**Fig. 2.** Antisense ClC-5 and antisense ClC-4 cDNA specifically reduce ClC-5 and ClC-4 protein, respectively. A, confocal images of ClC-5-specific immunofluorescence in Caco-2 cells co-transfected with vector (Vtr) and GFP (upper panel) or with antisense ClC-5 (aClC-5) and GFP (lower panel). Ab, antibody. Scale bar corresponds to 10 μm. Bar graph shows quantitated immunofluorescence (IP) (Scion Image); mean ± S.E. There was a marked and significant reduction of ClC-5 signal in antisense ClC-5-transfected cells (open bar *, n = 56) relative to vector-transfected cells (solid bar, p < 0.0001, n = 57) or antisense ClC-4-transfected cells (hatched bar, p < 0.0001, 26, n = 49, included for comparison). B, confocal image of ClC-4 immunofluorescence in Caco-2 cells co-transfected with Vtr and GFP (upper panel) or with antisense ClC-5 (aClC-5) and GFP (lower panel). Scale bar = 10 μm. Bar graphs show quantitated immunofluorescence; mean ± S.E. There was a marked effect on ClC-4 signal by antisense ClC-4 transfection (hatched bar *, 26, n = 48, included for comparison) relative to antisense ClC-5-transfected cells (open bar, n = 41, p < 0.0001) or vector-transfected cells (solid bar, n = 48, p < 0.0001). The data shown were obtained from three different transfections for each construct.

**Fig. 3.** Antisense ClC-4 and antisense ClC-5 cDNA are effective in reducing endogenous depolarization chloride currents in Caco-2 cells. A, whole cell chloride currents evoked by voltage steps ranging from −100 to +100 mV by 20-mV increments in an empty vector-transfected Caco-2 cell and an antisense ClC-5 (aClC-5)-transfected Caco-2 cell. B, current-voltage relationships determined for vector-transfected (solid square, n = 6) and antisense ClC-5 transfected cells (solid triangle, n = 9) relative to antisense C4 transfection (hatched bar, * p < 0.0125). Mean values ± S.E. shown. C, inhibition of depolarization-activated chloride current density (measured at +100 mV) by aClC-5 (open bar, *, p < 0.0125) and aClC-4 transfection (hatched bar, *, p < 0.0026) relative to control (solid bar). Mean values ± S.E. shown.

**Immunofluorescence**—Immunofluorescence labeling was performed on 5-μm cryosections of rat kidney tissues and on Caco-2, CHO, COS-7, and LLC-PK1 cells as described previously (28). The following primary antibodies were used: rabbit anti-CIC-5 (1:150), rabbit anti-CIC-4 antibody (1:200), and mouse HA antibody (1:1000, Babco, Richmond, CA).
vesicles in Caco-2 cells.

localize with transferrin-positive
ClC-4-specific staining (green) in Caco-2
state distribution. During a 60-min pulse to assess steady
Tfn-Rhd ferrin-Fe-Rhd (green) in Caco-2 cells relative to trans-
lar puncta in which there is co-localiza-
tion between Tfn-Rhd and ClC-5 or ClC-4.
The scale bar for all figures represents 10

Cy3- or Cy5-conjugated or FITC-conjugated anti-rabbit or anti-mouse secondary antibodies (1:1000, Molecular Probes, Leiden, The Netherlands) were used for immunodetection.

To study the localization of ClC-4 and ClC-5 proteins relative to transferrin-positive compartments, cells were serum-starved for 1 h at 37 °C in free serum medium (Invitrogen) and then loaded with 50 μg/ml transferrin conjugated to iron and tetramethylrhodamine (transferrin-Fe²⁺-Rhd, Molecular Probes, Leiden, The Netherlands) for 60 min. Following fixation, cells were labeled with ClC-4 or ClC-5 antibodies for subsequent immunolocalization. For kinetic analyses of transferrin internalization in control and ClC-4- and ClC-5-depleted (antisense-treated) cells, cells were serum-starved as above and then pulse-labeled for 2.5, 5, 10, 20, 40, and 60 min with 50 μg/ml transferrin-Fe²⁺-Rhd. To examine recycling, cells were pulse-labeled with 50 μg/ml transferrin-Fe²⁺-Rhd for 1 h and then washed and chase for 5, 20, 40, 60, and 80 min in medium containing 10% fetal calf serum. At the end of each labeling and chase interval, cells were washed and then fixed in cold 4% paraformaldehyde. Slides were viewed with a ×100 objective on a Carl Zeiss LSM 510 equipped with an Axiovert 100 confocal microscope.

Quantification of Immunofluorescence—GFP-transfected cells were analyzed with respect to CIC channel-specific immunofluorescence (labeled with Cy3-conjugated secondary antibodies) or rhodamine-transferrin. Although cross-talk of the fluorophores into the wrong detectors was negligible, GFP fluorescence was specifically subtracted prior to ClC-4 or rhodamine immunofluorescence measurements. All images were acquired using the palette function of LSM510 confocal acquisition software, which allows verification that the fluorescence intensity is in the linear range. The linear range is defined as 0 units = white and 255 units = black. The Cy3 or rhodamine-specific immunofluorescence was converted to a gray scale image, and the background (determined from the mean intensity detected in cell nuclei) was subtracted prior to quantitation. Mean pixel intensity of the gray scale image was measured (Scion Image software as described under “Materials and Methods.”

Table II

| Cell type | Caco2 | LLC-PCK-1 |
|-----------|-------|----------|
| aClC-4    | 66% (n = 100) | 63% (n = 84) |
| aClC-5    | 65% (n = 74) | 60% (n = 77) |

The effect of antisense ClC-4 cDNA (aClC-4) and antisense ClC-5 cDNA (aClC-5) transfection is relative to empty vector cDNA transfection on rhodamine-transferrin (Tfn) uptake after a 20-min pulse in Caco-2 cells and LLPCK cells. The number of single cells studied are shown in parentheses and were obtained from four separate experiments (transfections). Rhodamine-Tfn fluorescence in transfected cells was quantitated using Scion Image software as described under “Materials and Methods.”

Electron Microscopy—CHO cells co-transfected with both Rab5a-
GFP and HA-CIC-5 cDNA were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. They were then harvested with a rubber policeman, drawn off the culture dish, and lightly centrifuged into a 1 mm2 pellet in a microcentrifuge tube. They were fixed for an additional 4 h and washed in PBS containing 20 mM azide and stored at 4 °C until further processing. Prior to cryo-ultramicrotomy, the sections were infiltrated with a solution of 10% gelatin in PBS at 37 °C, allowed to gel at 4 °C, and infused with 2.3% sucrose overnight. Blocks of gelatin containing cells were attached to aluminum pins, frozen in liquid nitrogen, and cut at ~95 °C on a diamond knife ~100 nm thick using a Leica Ultracut R with a cryo-chamber (Leica Canada, Willowdale, Ontario, Canada). Sections were transferred to Formvar-coated grids in a drop of molten sucrose, and the aldehyde residues were blocked with a solution of PBS containing 0.15% glycine and 0.5% BSA. Samples were rinsed several times with PBS with just BSA prior to incubation in a polyclonal rabbit antibody against CIC-4 for 1 h. Grids were then washed in PBS/BSA thoroughly and incubated for another hour in goat anti-rabbit IgG 10-nm gold complexes (Amersham Biosciences). Again after rinses in PBS/BSA samples were incubated for 1 h in a monoclonal antibody against GFP. This was followed by more washes and another 1 h of incubation in goat anti-mouse IgG 5-nm gold complexes (Amersham Biosciences). Grids were thoroughly washed with PBS followed by distilled water. They were then stabilized in a thin film of methylcellulose containing 0.2% uranyl acetate. Controls included the omission of the primary antisera or the IgG gold complexes. Specimens were examined and images acquired with a JEOL JEM transmission electron microscope (JEOL, Peabody, MA) equipped with a digital camera (AMT Corp. Danvers, MA).

Statistics—Statistical analyses, analysis of variance followed by Bonferroni’s non-paired “t” test, were conducted using Prism software, and p values of 0.05 or less were considered significant.

RESULTS

CIC-4 Is Expressed in the Renal Proximal Tubule—Previously, we showed that a polyclonal, affinity-purified antibody directed against the amino terminus of CIC-4 labels a 90–97-kDa protein in immunoblots of rat brain tissue. This band was competed using the amino terminus of ClC-5, attesting to its specificity (26). In Fig. 1A, we show that only this band is specifically detected in sub-apical vesicles of the proximal renal tubule epithelium. These same vesicles were labeled using the CIC-4 antibody described above, and we observed that the pattern of CIC-4-specific immunofluorescence overlapped with that of FITC-dextran (Fig. 1C). These findings are similar to those reported for the related protein CIC-5 (16) and suggest that both channels are expressed in the proximal tubule, probably in the apical membrane and sub-apical membrane vesicles.

Expression of CIC-4 or CIC-5 Channels Can Be Specifically Depleted in Caco-2 Cells Using an Antisense Strategy—We used an antisense strategy to determine the relative functional expression of CIC-4 and CIC-5 endogenously expressed in epithelial cells. As both channel proteins are endogenously expressed in the Caco-2 cell line (26), we initiated our comparative studies using Caco-2 cells transfected with empty vector (Vtr), aCIC-4, or aCIC-5 after a 20-min pulse. Tfn-biotin was detected in cell lysates following SDS-PAGE using Extravidin. β-Actin immunoreactivity was assessed as sample loading control. A bar graph shows relative Tfn-biotin uptake by vector and antisense cDNA-transfected Caco-2 cells. Quantitation of β-actin was relative to β-actin signals for two separate transfections. The reductions in Tfn-biotin uptake caused by aCIC-4 (hatched bar) and aCIC-5 (open bar) transfection relative to vector transfection are statistically significant (*, p < 0.05). Mean values ± S.E. shown.

Fig. 6. Antisense CIC-4 and antisense CIC-5 cDNA reduce uptake of biotinylated transferrin (Tfn-biotin) by Caco-2 cells. A, Tfn-biotin internalized by Caco-2 cells transfected with empty vector (Vtr), aCIC-4, or aCIC-5 after a 20-min pulse. Tfn-biotin was detected in cell lysates following SDS-PAGE using Extravidin. β-Actin immunoreactivity was assessed as sample loading control. B, bar graph shows relative Tfn-biotin uptake by vector and antisense cDNA-transfected Caco-2 cells. Quantitation of β-actin was relative to β-actin signals for two separate transfections. The reductions in Tfn-biotin uptake caused by aCIC-4 (hatched bar) and aCIC-5 (open bar) transfection relative to vector transfection are statistically significant (*, p < 0.05). Mean values ± S.E. shown.
Having shown that antisense ClC-4 and antisense ClC-5 were capable of specifically depleting ClC-4 and ClC-5 proteins, respectively, we then assessed the efficacy of antisense transfection in depleting the functional expression of these channels. As expected for channels that traffic to and from the cell surface, the channel function of ClC-4 and ClC-5 can be measured at the plasma membrane by patch clamp electrophysiology (25, 26). Expression of either ClC-4 or ClC-5 is associated with the appearance of depolarization-activated chloride-selective conductance paths (25, 26). The biophysical properties of these channels are virtually identical with respect to their voltage dependence, kinetics of activation, and ionic selectivity. Furthermore, these channels cannot be distinguished pharmacologically, as both are insensitive to classic chloride channel inhibitors (25). As shown previously, Caco-2 cells endogenously express depolarization-activated chloride conductances with properties consistent with the functional expression of ClC-4 and/or ClC-5. Specific "knock-down" of ClC-4 using antisense cDNA was shown to reduce significantly depolarization-activated chloride-selective currents (26). In the present work we show that specific knock-down of ClC-5 also reduced these endogenous depolarization-activated currents (Fig. 3), providing quantitative evidence that both channels contribute to this function. Current density (at +100 mV) in antisense ClC-5 cDNA-transfected cells was 4.4 ± 0.8 (n = 9) versus 9.4 ± 1.5 pA/pF (n = 6) in vector-transfected cells (p < 0.0125). Together with our previous studies, these findings suggest that our an-
antisense ClC-4 and ClC-5 constructs are effective in depleting functional expression of these channels.

**CIC-4 and CIC-5 Participate in Trafficking of the Transferrin Receptor**—CIC-5 has been implicated in endosomal trafficking of membrane proteins (8, 10, 18–20), and our goal is to assess the relative role of CIC-4 in this function. However, the specific trafficking events that require CIC-5 function have not been determined. Therefore, we designed experiments to assess the role of CIC-4 and CIC-5 in transferrin trafficking via the transferrin receptor, as this pathway has been studied extensively (34–36). In Fig. 4A, we show confocal images of CIC-4 and CIC-5 localization relative to endosomes loaded with rhodamine-labeled transferrin for 60 min. At this time, transferrin is expected to label both early and recycling endosomes (34, 36, 37). CIC-5-specific immunofluorescence in Caco-2 cells appears as small punctate structures. Although the pattern associated with rhodamine-labeled transferrin is more extensive, most of CIC-5-positive structures overlap with those structures containing rhodamine-labeled transferrin internalized via the receptor-mediated endocytosis pathway (merged image). In Fig. 4B, we show that CIC-4-specific immunofluorescence also appears in punctate structures and most of these structures overlap with vesicular structures bearing rhodamine-transferrin (merged image). These images suggest that both CIC-5 and CIC-4 partially co-localize with transferrin-positive endosomes, including both early and recycling endosomes.

In order to determine the relative function of these channels in endosomal trafficking, we assessed whether depletion of these proteins affects transferrin receptor trafficking in Caco-2 cells (Fig. 5). Antisense cDNA was co-transfected with cDNA encoding GFP, and GFP fluorescence was used to identify transfected cells. Rhodamine-labeled transferrin uptake by antisense CIC-5 (Fig. 5A, lower panel) or antisense CIC-4-transfected cells (Fig. 5A, middle panel) was compared with that measured in vector-transfected cells (Fig. 5A, upper panel) as described under “Materials and Methods.” We observed that transfection with either antisense CIC-4 (36 units ± 2, n = 100; p < 0.0001) or antisense CIC-5 (37.3 units ± 2.5, n = 74; p < 0.0001) caused significant reductions in transferrin accumulation (measured 20 min after transferrin addition to cultures) relative to control, vector-transfected cells (106.8 ± 3.3 units, n = 67, Table II). These results suggest that both CIC-4 and CIC-5 participate in transferrin receptor trafficking in Caco-2 cells. Furthermore, the degree of inhibition by either antisense construct was greater than would be expected if CIC-4 and CIC-5 proteins were independent (i.e. 50% inhibition, Table II). Hence, these data support a model wherein a proportion of CIC-4 and CIC-5 molecules may form a functional heteromeric complex that participates in endosomal trafficking.

The fidelity of our methods for reporting the relative rhodamine-transferrin uptake by control and antisense-transfected cells could be substantiated using a biochemical assay (Fig. 6). In this assay, biotinylated transferrin (Tfn-biotin) was added to transfected Caco-2 cell cultures, and after a 20-min interval, endocytosis was stopped, and cells were washed with weak acid to remove surface-associated Tfn-biotin. Internalized Tfn-biotin was assessed following cell lysis and analysis by SDS-PAGE. As for our measurements of Rh-dTfn immunofluorescence, we found that antisense CIC-4 and antisense CIC-5-transfected cells accumulated significantly less Tfn-biotin than the control, vector-transfected cells, i.e. 58.5 ± 4 and 69 ± 0.5% of control (Fig. 6). The percent reduction caused by antisense expression in this biochemical assay is less than in the single cell immunofluorescence assay as the biochemical assay reflects uptake by both transfected and non-transfected cells. However, these biochemical data clearly support the utility and validity of our single-cell immunofluorescence assay in assessing the effect of CIC channel antisense expression on transferrin trafficking.

In order to probe the relative function of CIC-4 and CIC-5 in transferrin-mediated endocytosis and recycling in renal proximal tubule cells, we manipulated endogenous CIC-4 and CIC-5 expression in the LLPKC-1 cell line, a cell line that has been described previously (39) as a useful model of proximal renal tubule cells and shown to express both CIC-4 and CIC-5. As in the studies using Caco-2 cells, we found that there was a significant reduction in rhodamine-labeled transferrin fluorescence intensity in antisense-transfected cells relative to the vector-transfected cells after a 20-min pulse (Fig. 7A). At 20 min, transferrin accumulation was markedly decreased in CIC-4 (37.8 units ± 3, n = 84, p < 0.0001) and CIC-5 antisense-treated cells (41.7 units ± 3, n = 77, p < 0.0001) relative to control (vector-transfected) cells (103.4 units ± 7.2, n = 83) (Fig. 7B and Table II).

To determine whether CIC channel knock-down affected the rate of transferrin internalization or recycling, we conducted pulse-chase experiments (Fig. 7, C and D). The maximal accumulation of rhodamine-transferrin (Tfn) fluorescence intensity is significantly decreased in antisense CIC-4 (41.3 ± 2.4) and antisense CIC-5 (44.8 ± 2.3) cDNA-transfected cells relative to
membrane recycling is consistent with measurements (1/2) cDNA-transfected cells (Fig. 7). In vector-transfected, ClC-4 or ClC-5 antisense cDNA-transfected cells (Fig. 7D). In vector-transfected LLCPK-1 cells, greater than 50% of the transferrin recycled to the cell surface with rapid kinetics (t1/2 = 2.4 min). This rapid rate of membrane recycling is consistent with measurements (t1/2 < 5 min) obtained in the renal proximal tubule and certain cultured cells (34, 36, 40). On the other hand, there was a marked delay in rhodamine-transferrin recycling with no apparent recycling by 5 min of “chase” in ClC-4 or ClC-5-depleted cells. After this 5-min lag period, recycling in antisense-transfected cells commenced although it was still less efficient than in control cells at 40 min, with 39% transferrin recycled for both antisense ClC-4 and ClC-5 versus 72% in vector-transfected cells (p < 0.007). Overall, these studies suggest that in renal epithelial cells, ClC-4 and ClC-5 may contribute to multiple steps in transferrin receptor trafficking. However, these channels appear to play a primary role in a fast component of receptor recycling.

ClC-4 and ClC-5 Contribute to the Regulation of Endosomal pH—It has been hypothesized that ClC-5 participates in endosomal trafficking because it functions to permit acidification of this compartment (16, 23). Furthermore, in comparative in vitro studies of endosomes purified from wild type and ClC-5 knockout mice, it was shown that disruption of ClC-5 expression was associated with endosomal alkalization (18). Therefore, we evaluated the relative importance of ClC-4 expression in endosomal pH regulation. Endosomal pH was determined in situ by fluorescence ratio imaging of endosomes from single cells by preloading the endocytic compartment with the pH-sensitive fluorophore FTTC conjugated to transferrin. We compared the pH of endosomes from cells with depleted ClC-4 or ClC-5 expression, by antisense transfection, versus vector-transfected Caco-2 cells (Fig. 8). As depicted in Fig. 8, the endosomal pH values of both ClC-4 and ClC-5 antisense-transfected cells were significantly more alkaline than vector control endosomal pH (6.78 ± 0.07 and 6.90 ± 0.06 versus 6.1 ± 0.11, respectively; p < 0.05 for both antisense treatments). Therefore, both ClC-4 and ClC-5 contribute to the regulation of endosomal pH in living cells.

ClC-4 and ClC-5 Can Be Co-immunoprecipitated and Co-localize in Endosomes Expressing Rab5—The dominant effect of disrupting either ClC-4 or ClC-5 by antisense transfection on rhodamine-transferrin trafficking suggests that these two channels may be capable of forming a complex. To test this hypothesis we assessed whether the two channel-forming proteins could be co-immunoprecipitated. We found that our ClC-4 polyclonal antibody (but not preimmune sera) can co-immunoprecipitate ClC-5 protein from whole rat kidney (Fig. 9A, i), providing the first biochemical evidence that these two channels can assemble in nature. In order to further evaluate this concept, we reconstituted ClC-5, engineered to possess an aminoterminal HA tag, into ClC-5-deficient CHO cells (16) (Fig. 9B, i). ClC-4 protein is expressed endogenously in these cells and can be detected by immunoblotting as a 90–97-kDa protein (Fig. 9B, ii). We show in Fig. 9C that the polyclonal antibody directed against ClC-4 could co-precipitate HA-ClC-5 (Fig. 9C, i) and the antibody directed against the HA epitope co-precipitated both the HA-ClC-5 and ClC-4 (Fig. 9C, ii). Hence, these studies support the notion that ClC-4 can form a complex with ClC-5. As predicted from these biochemical findings, the confocal micrographs in Fig. 9D revealed that both ClC-4 and HA-ClC-5 are expressed in similar vesicular structures in CHO cells. It was shown previously (16) in COS-7 cells that ClC-5 protein co-localizes with Rab5a-GFP expressing early endosomes. Hence, in order to assess further whether ClC-4 and ClC-5 co-localize in early endosomal membranes, we compared HA-ClC-5 and ClC-4-specific immunofluorescence in COS-7 cells expressing Rab5a-GFP (Fig. 10A). Rab5a is a key regulator of endocytosis and mediates endosomal membrane fusion (41, 42) such that the overexpression of Rab5a leads to enlarged endosomal vesicles (43). As predicted on the basis of previous studies (16), we found that a subpopulation of swollen puncta expressing Rab5a-GFP also labeled with HA-ClC-5 (blue)-specific immunofluorescence (Fig. 10A). Furthermore, of those puncta expressing both Rab5a-GFP and HA-ClC-5, a significant proportion were labeled by ClC-4-specific immunofluorescence. In
addition, localization of ClC-4 in Rab5a-GFP-containing endosomal compartments was detected in electron micrographs of transfected CHO cells double-labeled with immunogold directed against ClC-4 (10 nm grains) and GFP (5 nm). Large vesicular structures, reminiscent of the swollen puncta expressing Rab5a-GFP in the previous confocal images, were also evident in electron micrographs. These swollen vesicular structures were decorated with 5 nm gold particles, supporting the notion that they are associated with the expression of Rab5a-GFP. As shown in Fig. 10B, 10 nm gold particles (indicative of ClC-4 expression) are detected on Rab5a-GFP-labeled swollen vesicles. ClC-4-specific label was associated with 32 of 41 (78%) Rab5a-positive endosomal structures. On the other hand, only 10 of 48 (20%) Rab5a-negative endosomal structures were labeled with ClC-4-specific immunogold (Table III). These studies suggest that both ClC-4 and ClC-5 are partially localized in early endosomes.

DISCUSSION
In this report, we show that ClC-4 participates with ClC-5 in endosomal trafficking of the transferrin receptor in renal and intestinal epithelial cells. Our kinetic analyses argue that these channels possibly contribute to multiple steps in transferrin receptor trafficking but predominantly to a rapid recy-
ClC-4 and ClC-5 currents are activated by membrane depolarization, by expression of either ClC-4 or ClC-5 into HEK293 cells. Both contribute to the chloride permeability of this branch of the ClC chloride channel family. Based on the similarity of their conductance and activation properties, it seems improbable that heteromerization between ClC-4 and ClC-5 would confer a distinct activity (25). If there is a functional advantage to the interaction of these two channels, it may relate to protein biosynthesis and/or stability, and these properties will be studied in detail in our future work.

The molecular basis for the interaction between ClC-4 and ClC-5 remains unclear. The biochemical studies described in this report do not allow us to distinguish between a direct or indirect basis for interaction. However, in our future work, we plan to test the prediction that ClC-4 and ClC-5 interact directly using information provided by the high resolution structure of procaroytic ClC channels recently solved by x-ray diffraction (48). This structure supported previous biophysical, biochemical, and electron diffraction studies (33) and showed that ClC-type channels are dimeric, with each polypeptide forming a single pore of a double-barreled channel (48). The x-ray structure shows that the dimerization interface in the membrane is extensive, and we would predict that mutation of residues residing in the putative interfacial helices in ClC-5 would be expected to disrupt homodimerization and possibly ClC-4/ClC-5 heterodimerization if assembly occurs at this interface.

Finally, mutations in CIC5 are thought to cause proteinuria in Dent's disease because CIC-5 channels normally contribute to endocytosis of low molecular weight proteins from the lumen of the proximal renal tubule (8, 10, 16, 38). The current studies show that ClC-4 also contributes to endosomal trafficking by epithelial cells of the renal proximal tubule and other absorptive epithelia and hence provide the rationale for future in vivo studies of the role of ClC-4 in renal function in health and disease. We speculate that the severity of Dent's disease may relate not only to the impact of particular mutations on the structure and function of ClC-5 but also on the consequences of those mutations on the functional expression of ClC-4.

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