ClC-5: role in endocytosis in the proximal tubule

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Wang, Yinhong, Hui Cai, Liudmila Cebotaru, Deanne H. Hryciw, Edward J. Weinman, Mark Donowitz, Sandra E. Guggino, and William B. Guggino. ClC-5: role in endocytosis in the proximal tubule. Am J Physiol Renal Physiol 289:F850–F862, 2005. First published June 7, 2005; doi:10.1152/ajprenal.00011.2005.—The proper functioning of the Cl− channel, ClC-5, is essential for the uptake of low molecular mass proteins through receptor-mediated endocytosis in the proximal tubule. Dent’s disease patients with mutant ClC-5 channels and ClC-5 knockout (KO) mice both have low molecular mass proteinuria. To further understand the function of ClC-5, endocytosis was studied in LLC-PK1 cells and primary cultures of proximal tubule cells from wild-type (WT) and ClC-5 KO kidneys. Endocytosis in the proximal tubule cells from KO mice was reduced compared with that in WT animals. Endocytosis in WT but not in KO cells was inhibited by bafilomycin A-1 and Cl− depletion, whereas endocytosis in both WT and KO cells was inhibited by the NHE3 blocker, S3226. Infection with adenovirus containing WT ClC-5 rescued receptor-mediated endocytosis in KO cells, whereas infection with any of the three disease-causing mutants, myc-W22G-ClC-5, myc-S520P-ClC-5, or myc-R704X-ClC-5, did not. WT and the three mutants all trafficked to the apical surface, as assessed by surface biotinylation. WT-CIC-5 and the W22G mutant were internalized similarly, whereas neither the S520P nor the R704X mutants was. These data indicate that ClC-5 is important for Cl− and proton pump-mediated endocytosis. However, not all receptor-mediated endocytosis in the proximal tubule is dependent on ClC-5. There is a significant fraction that can be inhibited by an NHE3 blocker. Our data from the mutants suggest that defective targeting and trafficking of mutant ClC-5 to the endosomes are a major determinant in the lack of normal endocytosis in Dent’s disease.

knockout mice; albumin; dextran; Na-H exchanger type 3; bafilomycin A-1; gluconate; adenovirus

EPITHELIAL CELLS OF THE RENAL proximal tubule have a high capacity for endocytosis of a broad range of extracellular proteins (29). The renal glomerular membrane retains cells and large proteins within the blood, whereas small proteins pass into the primary urine and are subsequently removed by receptor-mediated endocytosis at the brush-border membrane of the proximal tubules (27). As in every eukaryotic cell, endocytic vesicles in proximal tubular epithelial cells are progressively acidified (31). Dissipation of the intravesicular pH gradients inhibits the overall process of endocytosis (7). A Cl− channel, ClC-5, is expressed in the renal proximal tubule, the thick ascending limb, and the intercalated cells of the collecting duct (12). It is abundant in renal proximal tubular subapical endosomes where it colocalizes with the V type H+-ATPase (20, 34). This suggests that ClC-5 may be involved in proximal tubular endocytosis by providing an electrical shunt that is necessary for the efficient acidification of vesicles in the endocytic pathway by neutralizing the positive charges produced by the H+-ATPase.

Studies have shown that ClC-5 may have a role in endocytosis in addition to acting as a Cl− shunt. We found previously that the COOH-terminal tail of ClC-5 binds to the actin depolymerizing protein, coflin (23). Coflin is an ubiquitously expressed member of the coflin/ADF family of actin-associated proteins that binds to both filamentous (F-actin) and monomeric (G-actin) actin to stimulate depolymerization of the actin microfilaments (2). Phosphorylation of coflin in two proximal tubule cell models [LLC-PK1 and opossum kidney (OK)] is also accompanied by a pronounced inhibition of albumin uptake. We proposed that the interaction of coflin with ClC-5 at the plasma membrane plays a crucial role in mediating actin depolymerization, leading to a highly localized dissolution of the terminal actin web and facilitating budding of the endosome from the plasma membrane. In another study, the COOH-terminal tail of ClC-5 was shown to interact with the ubiquitin ligases Nedd4/Nedd4–2 (22). This study also showed that Nedd4–2 is a physiological regulator of constitutive albumin uptake. The results suggested that in response to the increased endocytosis, cells produce more ClC-5 and Nedd4–2 thereby increasing membrane turnover/endocytosis, proteosomal activity, and resultant degradation of albumin. These two studies suggested that in addition to its role as a shunt pathway for Cl−, ClC-5 may function as a structural component of the endocytic apparatus important both in budding of the endosome and in the processing of its cargo.

Dent’s disease is an X-chromosome-linked disorder characterized by low molecular mass proteinuria, aminoaciduria, glycosuria, and hypercalcuiuria, which leads to nephrocalcinosis, nephrolithiasis, and progressive renal failure (14). These patients have abnormal proximal tubule endocytosis of albumin and vitamin D-binding protein, which is caused by mutations in the gene encoding ClC-5, predominantly expressed in the kidney (25, 37). There are many single amino acid mutations in ClC-5, which can cause Dent’s disease with similar symptoms (26). The physiological properties of some of these mutants have been studied in Xenopus laevis oocytes and some basic properties have been characterized (25, 26, 37).

Defects in the ability of ClC-5 to act as a shunt pathway for Cl− and subsequent disruption of endocytosis have been suggested as the explanation of the low molecular mass proteinuria observed in Dent’s patients (12, 33). In addition, it has been shown that the brush-border components megalin and cubilin are altered in proximal tubules.
are dramatically reduced in knockout (KO) mice (6, 33). Defects in the trafficking of megalin and cubilin in the proximal tubule would also significantly impair the uptake of low molecular mass proteins into the body.

Studies with the CIC-5 KO mouse model show impaired endocytosis in the proximal tubules, which results in low molecular mass proteinuria, aminoaciduria, and glycosuria as occurs in Dent’s disease (33, 38). To understand the role of CIC-5 in the endocytic process, we studied both LLC-PK1 cells, in which CIC-5 is expressed abundantly thereby allowing detailed biochemical studies, as well as primary renal proximal tubule cells derived from normal and KO mice. In addition, to further elucidate the mechanism of three different CIC-5 mutations found in human patients, we studied their expression, trafficking, and endocytic function in transfected LLC-PK1 cells and infected primary renal tubule cells. Our data show that CIC-5 is important for Cl− and proton pump-mediated endocytosis. Importantly, not all receptor-mediated endocytosis in the proximal tubule is dependent on CIC-5, with a significant fraction dependent on NHE3. Our data from the proximal tubule is dependent on ClC-5, with a multiplicity of infection (MOI) of 250 in the culture medium 48 h before the studies.

Antibodies and immunofluorescent staining. Mouse monoclonal antibody (9E10) for c-myc was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal and mouse monoclonal antibodies for ZO-1 were from Zymed Laboratory (South San Francisco, CA). Rabbit anti-AQP-1 antibody was from Alpha Diagnostic International (San Antonio, TX). FITC-albumin and FITC-dextran (molecular mass 40,000) were obtained from Sigma (St. Louis, MO). Rabbit antibodies for aminopeptidase N (38), megalin (459) (10), and ClC-5 (38) were generously provided by Dr. A. Hubbard (Johns Hopkins University), Dr. R. Orlando (UCSD), and Dr. O. Devuyst (Université Catholique de Louvain), respectively. Fluorescent-labeled secondary antibodies were from Jackson ImmunoResearch Lab (West Grove, PA). For immunostaining, cells were fixed with 4% paraformaldehyde at room temperature for 15 min, permeabilized with 0.1% Triton X-100 for 1–2 min, and blocked with 3% nonfat milk in PBS for 30 min. The cells were incubated with primary antibody in the blocking solution for 30 min to 1 h, washed with PBS three times, and incubated with secondary antibody for 1 h. Then, the cells were washed thoroughly and mounted in Vectashield medium (Vector Lab). Finally, the slides were sealed with cytosel 60 (Richard-Allan Scientific, Kalamazoo, MI) and viewed with an Ultraview confocal microscope (PerkinElmer Life Sciences, Boston, MA) fitted with a ×63 oil immersion objective lens (Zeiss Plan-Apochromat). In all the immunofluorescent experiments, negative controls were performed by staining the cells with the secondary antibody only or without transfection. For the double-labeling experiments, specimens stained with a single label were examined under the other fluorescent channel to determine bleed-through. The intensity of both dyes in the double-stained specimens was balanced to reduce artifacts.

Endocytosis assay. Ringer solution, pH 7.4 (140 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 12.4 mM HEPES, and 5 mM glucose), was used for all assays. For low-CI− solutions, NaCl was replaced with equimolar sodium gluconate or NaBr. The NHE3 blocker S3226 (1 μM) was made in Ringer solution. LLC-PK1 cells were plated on Transwell inserts in regular medium for 3–5 days to ensure that the cells were polarized before the experiments. Before endocytosis assays, the cells were incubated in the solutions containing chloride, gluconate, or bromide at 37°C for 30 min. Next, the cells were exposed to prewarmed fluorescent-labeled endocytic markers, as described below, from their apical surfaces at 37°C for 0, 1, 2, 5, 10, 15, and 30 min. After the incubation, the cells were put on ice and washed with ice-cold PBS eight times to stop the endocytic process. The residual fluorescent markers on the cell surface were removed with acid-stripping buffer (50 mM glycine, 2 M urea, 30 g/l BSA, 100 mM NaCl, pH 2.5). Then, the cells were fixed and immunostained as described above with specific mouse anti-ZO-1 primary antibody and cy3-anti-mouse secondary antibody. All the negative controls were done at 4°C.

The primary cells (both the WT and CIC-5 KO) were treated with the same solutions as above at 37°C for 2 h, which was shown to have the best effect from series of tests with different time points. Then, the endocytosis assay was performed as above for LLC-PK1 cells except that only the 15-min time point was used. Images of fluorescent-labeled markers were acquired by a Zeiss Axiovert microscope.
microscope (Achrostigmat objective lens \times 40), and the average intensity per cell was calculated using IPlab software from multiple images after background subtraction. The endocytic curve consisting of average total intensity per cell vs. time was plotted using Origin.

Pulse chase endocytosis assay in primary proximal tubular cells. Primary tubular cells were incubated with fluorescent-labeled dextran (5 or 50 mg/ml) for 1 or 5 min at 37°C, then the dextran was washed away and the cells were incubated with prewarmed medium for 15 or 30 min. Then, cells were fixed and stained with anti-megalin or anti-AQP-1 antibody. FITC-conjugated secondary antibody was used for the detection of the primary antibody.

Western blot analysis. Cells were harvested in lysis buffer (60 mM HEPES, pH 7.5, 150 mM NaCl, 3 mM KCl, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, and complete protease inhibitor from Roche Diagnostics, Indianapolis, IN) and syringe homogenized. Lysates were centrifuged at 6,000 g for 10 min to remove the insoluble fraction, and the supernatant protein was quantified using a Bio-Rad Protein Assay Kit (Pierce, Rockford, IL). After incubation in Laemmli...
buffer (Bio-Rad, Hercules, CA) at 37°C for 30 min, the protein sample was separated by SDS-PAGE and transferred to PVDF membranes (Amersham Biosciences, Piscataway, NJ) for Western blot analysis. After antibody labeling, detection was performed with ECL (Amersham Biosciences, Piscataway) or Super Signal (Pierce) according to instructions from the suppliers.

**Surface biotinylation.** Cell-surface biotinylation experiments were performed on LLC-PK1 cells 1 day after transfection with myc-ClC-5 constructs. LLC-PK1 cells were incubated with 5 ml PBS with 0.8 mg/ml NHS-S-S-biotin (Pierce) at 4°C with rocking for 30 min. Then, the free biotin in PBS was quenched with 100 mM glycine. Cells were washed with ice-cold PBS three times before the collection of lysates. Six-hundred micrograms of lysate protein were rocked with Neutravidin-beads (Pierce) at 4°C overnight. Ten micrograms of lysate protein were used to determine the total expression. The beads were washed with lysis buffer and eluted into Laemmli sample buffer and separated by SDS-PAGE for Western blot analysis using an anti-myc antibody. The images were acquired with Fuji film Image Reader (LAS-1000 Lite), and quantification was done with ImageGauge 4.0. The percentage of the protein on the surface compared with the total cell lysate was calculated after the subtraction of background. Background was measured by exposing the beads to myc-ClC-5 lysate without biotin exposure.

Internalization of surface biotinylated myc-ClC-5 in LLC-PK1 cells. LLC-PK1 cells were transfected with myc-ClC-5 1 day before the assay. The cells were incubated with NHS-S-S-Biotin (Pierce) at the apical surface at 4°C for 30 min to allow biotin binding. The free biotin in the incubation solution was then quenched by adding 100 mM glycine solution. The cells were recovered to 37°C by incubation with prewarmed medium for 30 min. After allowing endocytosis to occur at 37°C, cells were put on ice and washed with ice-cold PBS to stop the endocytosis. The biotin that was not internalized was cleaved off by exposure to 150 mM glutathione (Sigma) for 30 min. The rest of the procedure was the same as described in surface biotinylation.

The percentage of myc-ClC-5 that was internalized from the surface was compared with the total ClC-5 in the lysate. The fraction of internalized ClC-5 in the surface pool was quantified by comparing the internalization and surface biotinylation assays. Background was assessed again by exposing the beads to myc-ClC-5 lysate without the surface biotinylation.

**RESULTS**

**Primary cultures of proximal tubule cells.** After 2 wk, primary cultures from mouse kidneys were confluent with dome formation in some areas. WT cells and ClC-5 KO cells had similar differentiated cobblestone morphology (Fig. 1A). Several lines of evidence confirmed that the primary cultures retain characteristics consistent with their proximal tubule origin. Immunostaining and biochemical studies confirmed that the cells express AQP-1 (Fig. 1B), ClC-5 (Fig. 1C), APN (Fig. 1D), and megalin (Fig. 1E).

CIC-5 antibodies also detected the expression of CIC-5 in cultured proximal tubule cells grown both on plastic dishes and Transwell inserts. LLC-PK1 and OK cells were used as positive controls because both have been shown to express CIC-5 (23, 35), and Cos-7 cell lysate, a cell line shown to be negative for CIC-5 expression (39), was used as a negative control (Fig. 1C).

Because APN is a proximal tubule cell marker (38), for these experiments, lysates from LLC-PK1 cells and proximal tubules isolated from mouse kidneys were used as positive controls.
Fig. 3. Endocytosis assay with albumin and dextran in primary cells confirms the impairment of endocytic function in the proximal tubule cells of ClC-5 KO mice. A: endocytosis of FITC-albumin was detected in proximal tubule cells (PTC) and medullary collecting duct cells (MCDC). The cells internalized FITC-albumin at 37°C for 15 min and then were washed with ice-cold PBS to stop the endocytosis. Compared with the uptake in WT PTC, ClC-5 KO cells have much less albumin endocytosis (Aa, bar 20 μm). Ab: endocytosis of MCDC under the same conditions. No noticeable difference in the endocytosis of albumin was found between WT and ClC-5 KO MCDC. B: negative controls of the assay were done at 4°C. Ba: PTC, Bb: MCDC. ZO-1 staining in all images was in red. C: quantification of the endocytosis of FITC-albumin in PTC and MCDC under different conditions. The cells were treated with normal Ringer solution, bafilomycin A-1 (500 nM), gluconate-containing solution, or Bafilomycin A-1 plus gluconate solution for 2 h at 37°C before the endocytosis assays were performed. Bafilomycin A-1 and gluconate treatments significantly reduced the endocytosis of albumin in WT cells. No additive inhibitory effect was detected by the double treatments with bafilomycin A-1 plus gluconate. In contrast, ClC-5 KO cells were not sensitive to the same treatments (#P < 0.001 is the difference between WT and KO cells under the control conditions, n = 15). Right: endocytosis of albumin in MCDC measured at 4 and 37°C. No difference in endocytosis was found between WT and KO MCDC. D: quantification of the endocytosis of FITC-dextran in primary PTC under different conditions. WT cells were sensitive to the bafilomycin A-1 and gluconate treatments, whereas KO cells were not (†P < 0.01, difference between WT and KO cells under the control condition, n = 15. ‡P < 0.01, difference between control condition and different treatments in WT cells, n = 15).
Lysates from MCDC from mouse kidneys were used as negative controls (Fig. 1D). The expression of APN in WT and KO primary cells is comparable, which indicates the ratio of cells expressing proximal tubule markers in both WT and KO primary cells is the same.

Cultured cells from both WT and KO animals express the proximal tubule marker megalin (Fig. 1E), verifying further their proximal tubule origin (3, 5). Megalin expression in KO cells is 24.2% of that of WT cells (shown in the bar graph in Fig. 1E), which is similar to the difference in megalin expression between WT and KO proximal tubules. Lysates from LLC-PK1 cells and proximal tubules were used as positive controls, whereas lysates from MCDC and Cos-7 cells were used as negative controls. The reduction of megalin expression in KO compared with WT primary cultures is also evident from the immunofluorescence data shown in Fig. 2.

Endocytosis of dextran in primary proximal tubular cells. To determine that primary cells do indeed undergo endocytosis, cells were treated either with dextran or albumin as markers of fluid phase and receptor-mediated endocytosis, respectively (15, 33, 38). An example of dextran-treated cells is shown in Fig. 2. WT cells rapidly took up labeled dextran when cells were treated with 5 mg/ml dextran for 1 min at 37°C, washed, and allowed to warm for 15 min, whereas no label could be detected in the KO cells during the same time period. To test whether the endocytosis was completely abolished in KO cells, the incubation time was increased to 5 min, the cells were washed as before and allowed to warm for 30 min, and the concentration of dextran was increased to 50 mg/ml. Under this condition, label could be detected sporadically in the KO cells but always in lower abundance than in WT cells. This verifies that the primary cultures of proximal tubule cells are capable of endocytosis in this experimental setting.

To more comprehensively understand endocytosis in proximal tubules, cultured cells were incubated with 10 mg/ml of fluorescently labeled albumin or dextran for 15 min at 37°C, then washed and stained with anti-ZO-1 antibody (red), and the amount of uptake was quantified by measurement of fluorescence intensity normalized per cell. Shown in Fig. 3Aa, both WT and KO primary proximal tubular cells take up significant quantities of albumin (green), although the amount in KO cells was visually much less than that in WT cells. Note that cells cultured from the MCDC also take up albumin but there is no apparent difference between WT and KO cells (Fig. 3Ab). Figure 3, Ba and Bb, shows the images acquired from cells exposed to albumin at 4°C as negative controls.

Quantification of albumin and dextran uptake in those cells is presented in Fig. 3, C and D. WT primary proximal tubular cells endocytose both albumin and dextran. KO primary proximal tubule cells also take up both markers but at reduced levels, 48 and 61% of WT, respectively, indicating that receptor-mediated and fluid phase endocytosis is not completely abolished in the KO cells. Note also that there is no difference in endocytosis between WT and KO measured in MCDC.

To further explore the role of ClC-5 in endocytosis, cells were treated with the vacuolar proton pump inhibitor Bafilomycin A-1 (4) or Cl−/H+1002 was replaced by gluconate (19), both for 2 h at 37°C and then endocytosis of albumin or dextran was measured (Fig. 3C). Bafilomycin A-1 is expected to inhibit the proton pump and thus endosomal acidification. Because gluconate is not permeable to most anion channels (including...

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

**Fig. 4.** Bafilomycin A-1, gluconate, or bromide treatment reduces the endocytosis of albumin and dextran in LLC-PK1 cells. The average intensity of fluorescent-labeled markers per cell was quantified under different treatment conditions as described in EXPERIMENTAL PROCEDURES. Control was done at 4°C. A: bafilomycin A-1 and gluconate treatments inhibited the endocytosis of albumin in LLC-PK1 cells. See the detailed protocol in EXPERIMENTAL PROCEDURES. B: inhibitory effects of bafilomycin A-1 and gluconate treatments on the endocytosis of dextran in LLC-PK1 cells. C: inhibitory effects of bromide and gluconate treatments on the endocytosis of albumin in LLC-PK1 cells. Bromide inhibited endocytosis of albumin compared with the normal control level, but the effect was not as great as that of gluconate.
CLC-5 (13), replacing the Cl-containing solution with a gluconate-containing solution is expected to inhibit albumin endocytosis presumably due to the lack of Cl⁻ available in the cytosol for CLC-5 to transport across the endosomal membrane. In WT primary proximal tubular cells, uptake of both albumin (Fig. 3C) and dextran (Fig. 3D) was significantly reduced by bafilomycin A-1 treatment and gluconate replacement. The effects of bafilomycin A-1 and gluconate were indistinguishable, which indicated that their effects were on the same pathway. In contrast, in KO primary proximal tubular cells, the same treatments did not significantly reduce endocytosis of either albumin or dextran.

Endocytosis in LLC-PK₁ cells. To further assess the role of CLC-5 in the time course of endocytosis, we studied LLC-PK₁ cells, which afforded us a large number of cells from an established and well-studied cell line known to express CLC-5 (8, 13). LLC-PK₁ cells also took up both albumin and dextran. When Cl⁻ was replaced by gluconate or treated with Bafilomycin A-1, endocytosis was reduced (Fig. 4, A and B). Bromide (Br⁻) is another anion that is not highly selective for CLC-5 (8, 37). Figure 4C showed that replacement of Cl⁻ with Br⁻ decreased endocytosis of albumin, although the effect of Br⁻ was not as robust as gluconate.

ClC-5-independent endocytosis of albumin involves NHE3 in LLC-PK₁ cells and primary proximal tubule cells. Because 48% of endocytosis of albumin is CLC-5 independent, we explored the role of NHE3 in the residual endocytosis. Both in vivo and in vitro studies showed that NHE3 blockade interferes with apical receptor-mediated endocytosis of albumin (16, 18) and that NHE3 is a source of endosomal acidification at an intracellular vesicular compartment in different cell models (1, 11, 15, 30).

In WT primary cells, shown in Fig. 5A, albumin endocytosis was reduced by ~46% by the NHE3 inhibitor, S3226. Bafilomycin A-1 treatment or Cl⁻ replacement by gluconate further reduced endocytosis to 25% of the control level. In CLC-5 KO primary cells, S3226 was still effective in decreasing endocytosis to 56% of that in the untreated KO cells. In contrast to WT cells, in the KO cells treatment with bafilomycin A-1 or Cl⁻ replacement by gluconate did not reduce endocytosis beyond that of S3226.

Similar results were obtained in LLC-PK₁ cells, which contain endogenous CLC-5, S3226 (1 µM) reduced the endocytosis of albumin to less than 50% of the control level (Fig. 5B). Likewise, when cells were treated with bafilomycin A-1 or Cl⁻ was replaced by gluconate in the presence of S3226, there...
was an additional 30% inhibitory effect. The total endocytosis remaining after the double treatments was ~20% of the control level. These data show that both the H\(^+\)-ATPase and NHE3 play roles in receptor-mediated endocytosis in WT primary proximal tubular cells and LLC-PK\(_1\) cells, but only NHE3 contributes to endocytosis in the CIC-5 KO mouse cells.

**Infection with adenovirus containing WT CIC-5 gene restores endocytosis in KO primary proximal tubule cells.** To test whether the addition of normal CIC-5 to KO primary proximal tubule cells could rescue endocytosis, we constructed an adenovirus (Ad) vector containing WT-CIC-5 gene. Figure 6A is a Western blot showing that myc-CIC-5 protein expression was present in WT and KO cells infected with the vector. The uptake of albumin was not enhanced after infection of WT cultures with Ad WT-CIC-5 vector. In contrast, there was a dramatic increase in the uptake in the KO cells, restoring endocytosis to normal levels. Colocalization between myc-WT-CIC-5 and albumin could be seen in these virus-positive cells, indicating its involvement in albumin containing vesicles (Fig. 6B). Quantification of the average intensity of these virus-positive WT and CIC-5 KO cells confirmed the microscopic result (Fig. 6C). These data show that the KO cells retain the machinery necessary for CIC-5 to support endocytosis and that CIC-5 alone is sufficient to reconstitute albumin endocytosis.

**Functional studies of endocytosis of disease-causing mutants of CIC-5.** To gain insight into Dent’s disease causing mutations in CIC-5, primary proximal tubule cells were infected with Ad virus containing different mutant constructs W22G, S520P, and R704X, each of which causes Dent’s

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**Fig. 6.** Adenovirus infection rescued the endocytosis of CIC-5 KO primary cells. A: Western blot analysis using the myc antibody confirms the expression of myc-CIC-5 in WT and CIC-5 KO primary cells. The cells were infected with adenovirus for 48 h at multiplication of infection (MOI) of 250 before the cell lysate was collected and Western blot analysis was performed. B: confocal images showing the endocytosis of FITC-albumin in WT and CIC-5 KO primary proximal tubule cells infected with adenovirus containing the normal CIC-5 gene. Primary proximal tubule cells were infected with adenovirus for 48 h, and then the endocytosis assay was performed. Left: myc-CIC-5 in red. Middle: albumin-containing vesicles. Right: overlay (bar 20 μm). C: quantification of the endocytosis of FITC-albumin in adenovirus-positive CIC-5 KO primary cells showing the restoration of endocytosis (*P < 0.001 is the difference between WT and KO cells under the control condition without virus infection, n = 15).
Protein expression was detected using immunostaining (Fig. 7A) and confirmed by Western blot analysis (data not shown). Figure 7A shows that WT and mutant CIC-5 are expressed in both WT and CIC-5 KO primary proximal tubule cells after infection. Importantly, even though protein was expressed after infection of all the constructs, endocytosis of albumin was restored in KO cells infected with Ad vectors containing only WT CIC-5 but not the three CIC-5 mutants (Fig. 7B).

**Distribution of mutant CIC-5 detected by surface biotinylation in LLC-PK1.** To study the disease mutants further, LLC-PK1 cells were transfected with myc-CIC constructs containing WT or the different mutations, and surface expression was detected by surface biotinylation (Fig. 8). The percentage of the surface protein compared with the total cell lysate was calculated. The results showed that there was no significant difference in the fraction of myc-WT-CIC-5 present at the cell surface (8.2 ± 2%) compared with the three mutants (myc-
W22G-CIC-5 5.8 ± 1%, myc-S520P-CIC-5 3.7 ± 1%, and myc-R704X-CIC-5 5.4 ± 2%.

Endocytosis function of mutant CIC-5 detected by surface-biotinylated protein internalization assay in LLC-PK1. Endocytosis of surface-biotinylated myc-CIC-5 was measured in LLC-PK1 cells based on the protocol mentioned in EXPERIMENTAL PROCEDURES. The results showed that ~1.8% of myc-WT-CIC-5 and 1.3% of W22G-CIC-5 was internalized from the cell surface (Fig. 9A, left). In contrast, almost none of myc-S520P-CIC-5 or myc-R704X-CIC-5 was endocytosed from the surface. The fraction of the surface WT and W22G-CIC-5 internalized was both ~22%, whereas almost none of the other mutants were internalized (Fig. 9A, right). The difference between WT and S520P or WT and R704X was significant.

No significant difference could be detected in the magnitude of endogenous endocytosis in LLC-PK1 cells transfected either with WT-CIC-5, W22G-CIC-5, S520P-CIC-5, or R704X-CIC-5 (Fig. 9B). This excludes the possibility that the different levels of internalization of these mutants shown in Fig. 9A are simply due to differences in the general rate of endocytosis after transfection with mutant CIC-5. Thus the lack of internalization of S520P-CIC-5 or R704X-CIC-5 from the plasma membrane is caused by trafficking problems specific to these proteins.

**DISCUSSION**

It is currently believed that CIC-5 and the H⁺-ATPase cooperate to facilitate the internalization of various proteins and small molecules from the renal proximal tubule with CIC-5 providing a Cl⁻ shunt to facilitate acidification by the proton pump (28, 33, 38). Thus defects in CIC-5 function can impair the transport of anions into the endosomes with elevated gradient slowing down the rate of acidification. For example, depletion of CIC-5 using antisense RNA in Caco-2 cells leads to an increase of endosomal pH (32). In the CIC-5 KO mice it was shown that acidification occurred at a significantly lower rate than in WT endosomes (21). We show in this study, using endocytosis as a read-out, that CIC-5 and the H⁺-ATPase are both critical components for endocytosis. However, in the KO mice that lack CIC-5, endocytosis still takes place although at a reduced rate. A key finding is that in the KO mouse proximal tubule cells, endocytosis is insensitive to bafilomycin A-1 and removal of Cl⁻ from the medium, whereas endocytosis in the WT mice is sensitive to both. Relevant is that the inhibitory effects of the two maneuvers are indistinguishable, suggesting that they are critical in the same pathway or at the same stage of endocytosis. The absence of sensitivity to both maneuvers in KO cells points to the removal of the functional role of the H⁺-ATPase in endocytosis in the absence of CIC-5. Moreover, the restoration of endocytic function in KO cells following infection with an adenovirus containing normal myc-CIC-5 gene suggests that the KO cells retain the receptor-mediated endocytic machinery but just need CIC-5 for it to function properly. Megalin is the receptor in the proximal tubules involved in the receptor-mediated endocytosis of albumin. Christensen et al. (6) identified in their studies that loss of CIC-5 resulted in defective trafficking of megalin and cubulin in kidney proximal tubules, which leads to proteinuria. The fact that blockade of the H⁺-ATPase and chloride transport across the endosomes subsequent to the defective function of CIC-5 leads to a reduction in endocytosis in both receptor-mediated and fluid phase pathways indicated that a decrease of the number of megalin receptors in CIC-5 KO primary cells was not the only determinant factor.

Importantly, the data showed that the residual receptor-mediated endocytosis in the KO mouse proximal tubule cells is sensitive to the NHE3 blocker, S3226. This supports the role of NHE3 in endocytosis that Gekle et al. (15, 17) identified. In their in vitro studies, NHE3 deficiency dramatically reduced the endocytosis, and the inhibition of NHE3 alkalinized the early endosomes. In addition, their in vivo data demonstrated that S3226 reduced [14C] cytochrome reabsorption from early proximal tubules, and NHE3 KO mice presented with proteinuria (18). Our data further confirmed the contribution of Na⁺-H⁺
exchange to endocytosis, and indirectly to the acidification of the early endosomal compartment. In WT mice, whether NHE3, ClC-5, and H\textsuperscript{+}/H\textsubscript{+}−ATPase are all located in the same population of vesicles is still not known. Clearly, one possibility is that they colocalize in the same vesicles, but their roles vary during different stages of endocytosis. It is also possible that their functions are redundant during some phases of endocytosis. Another possibility is that they are present in a different subpopulation of vesicles where they discriminate among the different endocytic cargos.

Mutations in ClC-5 are known to cause Dent’s disease (25, 26). In this study, we focused on three human ClC-5 mutants (W22G, S520P, R704X) to understand further how these mutants may cause disease. We chose these three mutants because the Cl\textsuperscript{−} transport characteristics were studied extensively in patch-clamping experiments in *X. laevis* oocytes. The results of the published studies showed that W22G-ClC-5 and R704X-ClC-5 do not produce any Cl\textsuperscript{−} currents across the oocyte plasma membrane, whereas S520P-ClC-5 generated a reduced current. All of the mutants were expressed abundantly in ClC-5 KO primary proximal tubular cells infected with adenoviral vectors containing the mutant genes. However, none could efficiently rescue endocytosis to normal values. This is certainly expected for W22G-ClC-5 and R704X-ClC-5 mutants, which do not produce any currents in *X. laevis* oocytes, but unexpected for the S520P-ClC-5 mutant, which still conducts some Cl\textsuperscript{−} in *X. laevis* oocytes. Also, our previous study showed the importance of the COOH terminus of ClC-5 in the endocytic machinery by interaction with other protein complexes, e.g., coflin (23). Based on this, R704X-ClC-5 with missing COOH terminus would certainly be predicted to have defective function.

To determine the magnitude of the static pool of ClC-5 that resides at the apical membrane, WT and mutant ClC-5 were transfected into LLC-PK\textsubscript{1} cells. These cells were used because they allowed for sufficient material for the biochemical assays.

![Fig. 9. Defective endocytosis of the mutants in transfected LLC-PK\textsubscript{1} cells. A: endocytic fraction of myc-ClC-5 among the total lysate and the surface pool was assessed by performing the internalization assay of surface-biotinylated protein in LLC-PK\textsubscript{1} cells transfected with different myc-ClC-5 constructs. Approximately 1.2–1.8% of WT and W22G-ClC-5 (left) of total cellular ClC-5 were internalized from the cell surface. However, none of myc-S520P-ClC-5 and myc-R704X-ClC-5 was internalized into the vesicles from the cell surface (*P < 0.05 is the difference between WT and S520P or R704X, n = 8); 22% of the surface pool of WT and W22G-ClC-5 (right) but no S520P and R704X-ClC-5 were internalized. B: magnitude of endocytosis in LLC-PK\textsubscript{1} cells transfected with different myc-ClC-5 constructs was measured at 6 time points. At each time point, no significant difference could be detected between the endogenous internalization rate and the rate after transfection with any of the constructs.](http://ajprenal.physiology.org/doi/10.220.32.246)
Surface biotinylation experiments in LLC-PK₁ cells transfected with CiC-5 confirmed that only a small amount of both WT and mutant CiC-5 is at the plasma membrane.

We also tested the mutants in an endocytosis assay where internalization of surface-biotinylated protein was measured in LLC-PK₁ cells transfected with WT and mutant CiC-5 constructs. LLC-PK₁ cells have endogenous CiC-5 and undergo normal endocytosis. The additional WT or mutant CiC-5 protein from transfection resulted in higher expression levels than endogenous CiC-5 (data not shown). However, the added expression of either WT or mutant CiC-5 to that already expressed in LLC-PK₁ cells had no effect on endocytosis of albumin. This excludes the possibility of a dominant-negative effect from the mutant CiC-5 protein on endogenous CiC-5 function. The results showed that ~1.8% of myc-WT-CiC-5 compared with the total lysate was internalized from the plasma membrane into endosomes, and 1.3% of the mutant W22G. About 4–8% of the total WT or mutant myc-CiC-5 is at the apical surface and from this surface protein pool; 22% of both WT and W22G-CiC-5 was internalized. In contrast, almost none of myc-S520P-CiC-5 and myc-R704X-CiC-5 was internalized into endosomes from the surface. Thus our data show that disorders in endocytosis can be caused either by a lack of Cl⁻ currents as has been suggested in the literature or a lack of targeting of CiC-5 to the endosomes. This is particularly evident with the S520P mutant that retains the ability to go to the plasma membrane and function as a Cl⁻ transporter as shown in the X. laevis oocytes experiments (26) but does not correct defective endocytosis when infected into mouse primary cultures. This is surprising because if there is still some residual function as suggested, in the oocyte studies the over-expression of this mutant in mouse KO cells would be expected to restore at least partial function. The observation that this mutant does not restore partial function suggests that the likely cause of defective function of this mutant is inefficient trafficking to the endosome.

In conclusion, CiC-5 is essential for the reabsorption of low molecular mass proteins and other solutes in the proximal tubules of kidneys. Our work established for the first time that primary proximal tubule cells from CiC-5 KO mice kidneys and demonstrated defective endocytosis in the KO cells that could be rescued by infection with normal CiC-5. We showed that CiC-5 plays an important role in both receptor- and nonreceptor-mediated endocytic pathways. The proper functioning of the H⁺-ATPase and CiC-5 is essential for normal levels of endocytosis in proximal tubule cells, which was confirmed by the fact that no further inhibitory effect could be detected from bafilomycin A-1 or gluconate treatment in CiC-5 KO primary cells. NHE3 functions in endocytosis presumably in a residual population of the vesicles. Three human mutants W22G, S520P, and R704X are all present at the cell surface, but S520P and R704X are not capable of efficient targeting to endosomes. As a result, the disease symptoms in patients with these mutations are likely derived either from defective protein trafficking and targeting to endosomes or lack of Cl⁻ channel activity or both.

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