Role of ClC-5 in Renal Endocytosis Is Unique among ClC Exchangers and Does Not Require PY-motif-dependent Ubiquitylation*§

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Inactivation of the mainly endosomal 2Cl−/H+ -exchanger ClC-5 severely impairs endocytosis in renal proximal tubules and underlies the human kidney stone disorder Dent’s disease. In heterologous expression systems, interaction of the E3 ubiquitin ligases WWP2 and Nedd4-2 with a “PY-motif” in the cytoplasmic C terminus of ClC-5 stimulates its internalization from the plasma membrane and may influence receptor-mediated endocytosis. We asked whether this interaction is relevant in vivo and generated mice in which the PY-motif was destroyed by a point mutation. Unlike ClC-5 knock-out mice, these knock-in mice displayed neither low molecular weight proteinuria nor hyperphosphaturia, and both receptor-mediated and fluid-phase endocytosis were normal. The abundances and localizations of the endocytic receptor megalin and of the Na+/coupled phosphate transporter NaPi-2a (Npt2) were not changed, either. To explore whether the discrepancy in results from heterologous expression studies might be due to heteromerization of ClC-5 with ClC-3 or ClC-4 in vivo, we studied knock-in mice additionally deleted for those related transporters. Disruption of neither ClC-3 nor ClC-4 led to proteinuria or impaired proximal tubular endocytosis by itself, nor in combination with the PY-mutant of ClC-5. Endocytosis of cells lacking ClC-5 was not impaired further when ClC-3 or ClC-4 was additionally deleted. We conclude that ClC-5 is unique among CLC proteins in being crucial for proximal tubular endocytosis and that PY-motif-dependent ubiquitylation of ClC-5 is dispensable for this role.

ClC-5 is a member of the CLC family of chloride channels and transporters (1) that is most highly expressed in renal and intestinal epithelia (2, 3). Like its close homologs ClC-3 and ClC-4, it is located mainly on endosomal membranes (4–6). Small amounts of ClC-5, however, are also found in the plasma membrane. This localization has facilitated the biochemical characterization of its transport properties. ClC-5 mediates strongly outwardly rectifying currents (2, 7) which are now known to reflect voltage-dependent 2Cl−/H+ -exchange (8–10).

ClC-5 is crucial for the renal reabsorption of low molecular weight proteins that can pass the glomerular filter into the primary urine. Mutations in CLCN5, the human gene encoding ClC-5, cause Dent’s disease (11), an X-linked disorder characterized by low molecular weight proteinuria and the urinary loss of phosphate and calcium that eventually lead to more variable, but clinically important symptoms like kidney stones and nephrocalcinosis. ClC-5 knock-out (KO)§ mouse models (12, 13) have revealed that ClC-5 is crucial for apical endocytosis in the proximal tubule (PT). ClC-5 may provide an electric shunt for the vesicular H+ -ATPase (12, 14, 15) which is needed for an efficient acidification of endosomes. ClC-5 disruption impairs fluid-phase as well as receptor-mediated endocytosis and also slows the hormone-stimulated endocytic retrieval of apical plasma membrane proteins such as the Na+/phosphate cotransporter NaPi-2a (Npt2, SLC34A1) or the Na+/H+ -exchanger NHE3 (12). Hyperphosphaturia and hypercalciuria in mice or human patients lacking functional ClC-5 was attributed to impaired proximal tubular endocytosis and processing of calcitropic hormones (12, 14).

Only limited information is available on the regulation of ClC-5 or its binding to other proteins (16–18). The best studied case concerns its interaction with WWP2 (19) and Nedd4-2 (20), two E3 HECT-domain containing ubiquitin ligases. Both enzymes contain WW-domains that can interact with “PY-motifs,” which are named after their critical proline and tyrosine residues. ClC-5 contains a PY-motif in its cytoplasmic C terminus (19, 21) between two cystathionine-β-synthase domains (22). Crystallography has shown that this region is unstructured and may be available for protein-protein interactions (23). A synthetic tridecameric peptide encompassing the PY-motif of ClC-5 was found to interact with several WW-domains (21), most avidly with the fourth WW-domain of WWP2. ClC-5 point mutations destroying the PY-motif consensus sequence (e.g. Y762E) led to a roughly 2-fold increase in ClC-5 currents and surface residence when expressed heterologously in Xeno-

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4 The abbreviations used are: KO, knock-out; KI, knock-in; PT, proximal tubule; WT, wild type; PTH, parathyroid hormone.
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pus oocytes (19). This observation resembled findings with the epithelial Na⁺ channel ENaC, where PY-motif-dependent ubiquitylation is crucial for the regulation of its presence in the plasma membrane (24). Coexpression of CIC-5 with dominant negative constructs of WWP2 led to increased currents and surface expression only when the PY-motif of CIC-5 was intact (19), suggesting that PY-motif-dependent ubiquitylation leads to CIC-5 internalization by endocytosis as it is the case with ENaC. Moreover, stimulating or inhibiting endocytosis by expressing the Q79L or S34N mutants of rab5, respectively, decreased or increased currents of wild-type (WT) CIC-5, but not the PY-motif mutant (19). Subsequent studies (20) showed that CIC-5 also binds to the WW-domain-containing E3 ubiquitin ligases Nedd4 and Nedd4-2 which affected CIC-5 surface expression and currents as described previously for WWP2. Moreover, Hryciw et al. (20) reported ubiquitylation of CIC-5, which was enhanced by adding albumin as endocytic cargo. Negative dominant negative constructs of Nedd4-2 or its RNA interference-mediated knockdown inhibited albumin endocytosis in opossum kidney cells, an epithelial cell line retaining properties of PTs (20). The authors suggested that PY-motif-dependent interactions of CIC-5 with ubiquitin ligases are important for PT endocytosis (20). In addition to stimulating CIC-5 endocytosis, ubiquitylation of CIC-5 might have other effects on intracellular sorting and trafficking.

We now tested these hypotheses by generating knock-in (KI) mice whose PY-motif in CIC-5 is destroyed by mutating the important tyrosine residue to glutamate (Y762E). These mice did not display proteinuria, and in vivo pulse-chase experiments revealed no discernible effect on different types of endocytosis. No increase of brush-border expression of CIC-5 could be detected, either. Because CLC proteins can form functional heterodimers (25, 26) and because CIC-5 can associate with its close homologs CIC-3 and CIC-4 (27) in the PT, we also investigated PT endocytosis in mice disrupted for CIC-3 or CIC-4 either alone or in combination with the CIC-5(Y762E) mutation. clcn3⁻/⁻ (28) and clcn4⁻/⁻ mice displayed normal endocytosis both in the presence of WT CIC-5 or its PY-mutant. Moreover, endocytosis in PT cells lacking either CIC-3 or CIC-4 was normal, and deletion of CIC-3 or CIC-4 in addition to CIC-5 did not impair endocytosis beyond the effect seen in CIC-5 KO mice.

**Experimental Procedures**

**Generation of Mice**—The generation of clcn5⁻ and clcn3⁻ mice has been described previously (12, 28). For generating the targeting vectors for clcn5⁺, clcn5⁻, clcn5³loxp, and clcn4⁻ mice, genomic clcn5 and clcn4 DNA was isolated from a mouse genomic AFIXII 129/Sv library (Stratagene) and subcloned into the pKO901 Scrambler vector (Lexicon Genetics) containing a diphtheria toxin A cassette as shown in supplemental Figs. S1, S6, and S8. A neomycin-resistance cassette has been inserted into all targeting vectors for positive selection. The linearized vectors were electroporated into R1 (clcn5⁺, clcn5⁻, clcn5³loxp, and clcn4⁻) and MPI2 (clcn4⁻) 129/Sv mouse embryonic stem cells. After identification of correctly targeted clones by Southern blot analysis, cells were injected into C57BL/6J blastocysts that were implanted into foster mothers. Chimeric males (clcn5⁺, clcn5⁻, and clcn5³loxp) were bred with FLPe-recombinase expression “deleter” mice (29) to remove the FRT-flanked neomycin-resistance cassette. To confirm that the complete deletion of the clcn5³loxp allele results in the same phenotype observed in constitutive CIC-5 KO mice (12), clcn5³loxp mice were mated with “deleter”-cre mice (30). For deletion of CIC-5 in renal PTs, clcn5³loxp mice have been crossed with mice expressing cre-recombinase under the control of the villin promoter (31). All experiments were performed with mice in a mixed C57BL/6J-129/SvJ genetic background using littermates as controls.

**RNA Expression Analysis**—Total RNA was prepared from kidneys using TRIzol (Invitrogen) and RNeasy columns (Qiagen). RNA was transcribed into cDNA using the SuperScript II cDNA kit (Invitrogen) and oligo(dT)15 primer. The quantitative real-time PCR was run in an ABI PRISM 7700 Sequence Detection System (SDS 2.1 software) using SYBR Green PCR master mix (Applied Biosystems). For CIC-5 the primers AGG TACTCATGTGACGGCCA and AGGCCGCAGTCTATTGAACA were used. The housekeeping gene hypoxanthine-guanine phosphoribosyl-transferase was used as control. Reactions were performed in triplicate with cDNAs from three different animal pairs. The value for WT was set to 100, and RNA values from clcn5³loxp, cre mice are shown as percent of WT.

**Western Blot Analysis**—Kidney membranes were pelleted from homogenates centrifuged at 100,000 × g and were separated by SDS-PAGE using 8–10% acrylamide gels. Only for megalin analysis 3–8% NuPage Tris acetate gels (Invitrogen) were used. For immunobLOTS the following primary antibodies were used: rabbit PEP5A (N terminus) and PEP5E (C terminus) against NaPi-2a (raised against the same phenotype observed in constitutive ClC-5 KO mice (30) for monitoring against NaPi-2a (31). All experiments were performed with mice in a mixed C57BL/6J-129/SvJ genetic background using littermates as controls. Chimeric males (clcn5⁺, clcn5⁻, and clcn5³loxp) were bred with FLPe-recombinase expression “deleter” mice (29) to remove the FRT-flanked neomycin-resistance cassette. To confirm that the complete deletion of the clcn5³loxp allele results in the same phenotype observed in constitutive CIC-5 KO mice (12), clcn5³loxp mice were mated with “deleter”-cre mice (30). For deletion of CIC-5 in renal PTs, clcn5³loxp mice have been crossed with mice expressing cre-recombinase under the control of the villin promoter (31). All experiments were performed with mice in a mixed C57BL/6J-129/SvJ genetic background using littermates as controls.

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thyroid hormone (PTH), 80 μg of rat PTH (Bachem) was injected intraperitoneally. After 15 min, 20 μg of rat PTH and 100 μg of Alexa Fluor 555-labeled-β-lactoglobulin (as a control) were injected together into the vena cava of anesthetized mice. After further 15 min, mice were perfused with PBS for 2 min followed by a perfusion with 4% paraformaldehyde.

**Urinary Analysis**—Mice were kept in metabolic cages for urine collection. Creatinine, urea, and salts were determined by Labor Arndt (Hamburg, Germany). Urine samples were normalized to creatinine values and analyzed by SDS-PAGE followed by silver or Coomassie staining or by Western blotting using polyclonal antibodies against retinol-binding protein (35), vitamin D-binding protein (Biogenesis), transferrin (Immunovision), or albumin (ICN).

To induce albuminuria, mice were injected intraperitoneally with 10 mg/g body weight bovine serum albumin (Sigma) every 24 h. After 6 days of injection urine samples were collected and analyzed.

**RESULTS**

To investigate the relevance of the PY-motif in vivo, we generated Ki mice by mutating its crucial tyrosine residue to glutamate (Y762E) (supplemental Fig. S1). This mutation led to a ubiquitin-ligase-dependent increase in CIC-5 surface expression which could not be increased further by additionally mutating the two preceding prolines of the PY-motif (19). Immunoblots of kidney membrane proteins (Fig. 1A) showed that mice homozygous for the clcn5Y762E allele expressed the CIC-5Y762E protein to levels undistinguishable from CIC-5 in WT mice. Based on our previous results from heterologous expression (19), we expected to find the plasma membrane expression of CIC-5Y762E increased compared with WT CIC-5. However, the subcellular localization of CIC-5Y762E was indistinguishable by immunohistochemistry from that of WT CIC-5 (Fig. 1, B and C). In both PTs and intestinal epithelia, WT and mutant proteins were expressed predominantly on subapical vesicles and to a lower extent in the brush-border membrane.

PY-motif-dependent ubiquitylation of CIC-5 was suggested to be important for receptor-mediated endocytosis (20). We therefore asked whether the disruption of the PY-motif in clcn5Y762E mice impairs proximal tubular endocytosis to the extent that it leads to low molecular weight proteinuria as in CIC-5 KO mice (12) (Fig. 2). Surprisingly, neither silver staining of total urinary proteins nor immunoblotting for several marker proteins revealed proteinuria in clcn5Y762E mice (Fig. 2). There were no changes in urinary electrolytes either (supplemental Table S3). We speculated that a moderate impairment of endocytosis may not result in proteinuria because the proximal tubule is not operating at the limit of its capacity. We therefore induced proteinuria by repeated injection of bovine serum albumin into the peritoneum (36, 37). Induced albuminuria was found in both WT and clcn5Y762E mice, with no difference being apparent between genotypes (supplemental Fig. S4).

The analysis of CIC-5-dependent endocytosis was greatly facilitated by comparing side-by-side PT cells expressing or lacking CIC-5 in heterozygous clcn5+/− females (12). This was possible because CIC-5 is encoded on the X chromosome and is therefore asked whether the disruption of the PY-motif in clcn5Y762E mice impaired proximal tubular endocytosis to the extent that it leads to low molecular weight proteinuria as in CIC-5 KO mice (12) (Fig. 2). Surprisingly, neither silver staining of total urinary proteins nor immunoblotting for several marker proteins revealed proteinuria in clcn5Y762E mice (Fig. 2). There were no changes in urinary electrolytes either (supplemental Table S3). We speculated that a moderate impairment of endocytosis may not result in proteinuria because the proximal tubule is not operating at the limit of its capacity. We therefore induced proteinuria by repeated injection of bovine serum albumin into the peritoneum (36, 37). Induced albuminuria was found in both WT and clcn5Y762E mice, with no difference being apparent between genotypes (supplemental Fig. S4).

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subjected to random X chromosomal inactivation in females. Whereas CIC-5 KO cells can be identified easily by immunofluorescence, our CIC-5 antibodies (4) recognize both WT CIC-5 and the Y762E point mutant. We therefore tagged the CIC-5 Y762E protein by generating KI mice in which the region encompassing the last 11 amino acids of the C terminus of CIC-5 Y762E was converted to that of CIC-3 (supplemental Fig. S1, B–D). This “negative tag” (denoted by an asterisk) requires the change of just two amino acids (supplemental Fig. S1, C and D) and results in a protein that should no longer be recognized by our C-terminal CIC-5 antibody in both immunohistochemistry and immunoblots. However, in immunoblots (Fig. 3A) as well as in immunohistochemistry (supplemental Fig. S2) a faint background signal was visible in CIC-5 Y762E animals which is probably caused by weak cross-reactivity of our CIC-5 antibody with the C terminus of CIC-3 (Fig. 3, A–C, and supplemental Fig. S2). We argued that the minimal change in CIC-5 Y762E is preferable to the more conventional addition of a “positive tag” of several amino acids which might interfere with CIC-5 function. In heterologous expression, this negative tag changed neither CIC-5 nor CIC-5 Y762E currents, nor did it interfere with the increase in current observed with the Y762E mutant (supplemental Fig. S5A and supplemental Method). It did not change the abundance (Fig. 3A) and localization of the protein (supplemental Fig. S2) in clcn5 Y762E mice, either. We could now distinguish between both CIC-5 variants in chimeric PTs of heterozygous clcn5 Y762E females (Fig. 3, B and C).

To study receptor-mediated and fluid-phase endocytosis, we injected clcn5 +/Y762E mice with fluorescently labeled β-lactoglobulin and dextran, respectively, and fixed the kidneys after 10 min (Fig. 3, B and C). There was no discernible difference in the amount of endocytosed β-lactoglobulin or dextran between PT cells expressing WT CIC-5 or the CIC-5 Y762E mutant, again arguing against an in vivo role of the PY-motif in CIC-5-dependent endocytosis. To quantify our results, we measured Alexa Fluor 488 fluorescence in kidney homogenates of mice previously injected with Alexa Fluor 488-dextran (Fig. 3D). We confirmed the significant decrease in fluid-phase endocytosis in clcn5 −/− mice (12) but found no difference between clcn5 Y762E and WT mice (Fig. 3D).

Disruption of CIC-5 not only led to impaired endocytosis, but also to a changed trafficking, subcellular localization, and abundance of membrane proteins like the endocytic receptor megalin and the sodium–phosphate cotransporter NaPi-2a (12). Because ubiquitylation can play a role in initial endocytosis and subsequent lysosomal degradation of the transporters (39–41). 30 min after injecting phosphate-deprived mice with PTH, NaPi-2a was largely internalized in both WT and clcn5 Y762E mice (Fig. 5B). By contrast, and as described previously (12), NaPi-2a internalization was drastically reduced in mice lacking CIC-5.

CIC-5 functions as homodimer but may also form heterodimers with CIC-3 and CIC-4, closely related endosomal CLC transporters (27, 42). KO controlled immunofluorescence has revealed robust expression of CIC-3 in proximal tubules (32, 43). Whereas CIC-4 is expressed in kidney
The lack of antibodies suitable for immunohistochemistry did not allow us to localize ClC-4 in PTs unambiguously. Nonetheless, a substantial portion of proximal tubular ClC-5 might be present in heterodimers with either ClC-3 or ClC-4. This raises the possibility that the discrepancy between the previous in vitro (19, 20) and the present in vivo results might be explained by masking the effects of the ClC-5 PY-motif in such heteromers. We therefore set out to investigate effects of the clcn5 Y762E allele in clcn3/H11002/ and clcn4/H11002/ background.

We first investigated constitutive clcn3/H11002/ mice (28) for possible defects in proximal tubular endocytosis. There was no difference in the amount of endocytosed /H9252-lactoglobulin between clcn3/-/- and WT mice (supplemental Fig. S7A), and immunoblots of urinary proteins revealed no sign of proteinuria (Fig. 6E). To investigate the role of ClC-4 in endocytosis, we generated constitutive ClC-4 KO mice (supplemental Fig. S6, A and B). clcn4/-/- mice developed normally and did not show any obvious phenotype (data not shown). Both receptor-mediated and fluid-phase endocytosis by PT cells, as tested by following the fate of injected fluorescently labeled dextran or /H9252-lactoglobulin, respectively, was normal (supplemental Figs. S6C and S7B). As expected from these results, clcn4/-/- mice did not display proteinuria (Fig. 6E).

We next investigated whether ClC-3 or ClC-4 can partially substitute for ClC-5 in supporting proximal tubular endocytosis by analyzing PT cells disrupted for both ClC-5 and either ClC-3 or ClC-4. This could not be accomplished by simply crossing of clcn5/H11002/ mice with either clcn3/H11002/ or clcn4/H11002/ mice as these matings did not yield double KO mice because of embryonic lethality (data not shown). We therefore established a conditional ClC-5 mouse model that uses the cre-loxP system (supplemental Fig. S8). To disrupt ClC-5 in proximal tubules,
clcn5<sup>lox</sup> mice were crossed to mice expressing the cre-recombinase under the villin-promoter (31). Neither conditional ClC-3/ClC-5 nor ClC-4/ClC-5 double KO mice displayed a more severe impairment of endocytosis than ClC-5 KO mice (12) (supplemental Fig. S7, C and D). Nonetheless, heteromerization of ClC-5<sup>Y762E</sup> with ClC-3 or ClC-4 might mask the effect of the ClC-5 PY-motif that had been observed in vitro (19, 20). However, this was not the case. Analysis of clcn5<sup>H11001<sup>/Y762E</sup></sup>; clcn3<sup>H11002</sup>/H11002<sup></sup> and clcn5<sup>H11001<sup>/Y762E</sup></sup>; clcn4<sup>H11002</sup>/H11002<sup></sup> double mutant mice failed to reveal any alteration in receptor-mediated or fluid-phase endocytosis (Fig. 6, A–D).

**DISCUSSION**

The 2Cl<sup>-</sup>/H<sup>+</sup>-exchanger ClC-5 facilitates endosomal acidification by providing countercurrents for the endosomal H<sup>-</sup>-ATPase and transports Cl<sup>-</sup> ions into endosomes. Both changes in endosomal H<sup>-</sup> and Cl<sup>-</sup> concentration may be causally related to the severe impairment of proximal tubular endocytosis found in ClC-5 KO mice or patients with Dent’s disease. The regulation of ClC-5 localization and activity and its potential impact on endocytosis are poorly understood, as is the interplay with other endosomal CLC transporters. We have shown here that only the loss of ClC-5, but not of ClC-3 and ClC-4, impairs proximal tubular endocytosis. Whereas regulation of ClC-5 by PY-motif-mediated ubiquitylation was consistently found upon heterologous expression in vitro, we now show that surprisingly it plays no significant role for endocytosis in vivo.

Ubiquitylation not only tags proteins for proteasomal degradation, but it can also provide a sorting signal in the endocytic pathway (38). A case in point is the epithelial Na<sup>+</sup>-channel ENaC. Its abundance in the apical membrane of distal tubular cells must be regulated exquisitely to allow for rapid changes in renal sodium reabsorption. ENaC subunits display PY-motifs in their
cytoplasmic C termini. These motifs interact with WW-domains which are present, often in multiple copies, in several distinct E3 ubiquitin ligases. In vitro studies have shown that the PY-motif of ENaC interacts with WW-domains of the ubiquitin ligase Nedd4-2 (24, 44). The resulting ubiquitylation of ENaC promotes its endocytosis and subsequent degradation. The importance of this interaction in vivo is evident from Liddle syndrome, a dominantly inherited severe form of hypertension. In patients with this disease, PY-motifs in β- or γ-subunits of ENaC are inactivated by mutations, resulting in increased, unregulated surface expression, Na\(^+\) retention, and hypertension (45).

Our previous work (19) had identified a similar motif in the C terminus of CIC-5. We had demonstrated that upon heterologous expression its deletion increased surface expression and electrical currents by a factor of 2. Effects of coexpressing dominant negative mutants of WWP2, a ubiquitin ligase present in kidney, strongly implicated ubiquitylation as the underlying mechanism (19). Our studies were confirmed and extended by Hryciw et al. (20), who reported CIC-5 ubiquitylation and demonstrated that CIC-5 can interact with Nedd4-2 as well.

Ubiquitylation can influence several trafficking steps in the endosomal-lysosomal pathway (38). Although we expected an increased CIC-5\(^{Y762E}\) surface expression in KI mice, we did not dare to predict the consequences that impaired ubiquitylation of CIC-5 might have on the endocytosis of other proteins. Cell culture studies by Hryciw et al. (20), however, had indicated a reduction of albumin endocytosis when the CIC-5 PY-motif was destroyed. Surprisingly, we detected neither increased CIC-5\(^{Y762E}\) surface expression nor changes in receptor-mediated or fluid-phase endocytosis in PT cells of KI mice. We can obviously not exclude effects below the detection limit of our method which we estimate to be in the order of a 30% change. Although both determinations of surface expression and endocytosis depend on evaluation of fluorescence intensities, the combination of sophisticated mouse models and X chromosome inactivation enabled well controlled side-by-side comparison of cells with different genotypes.

We conclude that PY-motif-mediated effects on CIC-5 trafficking, though convincingly demonstrated in oocytes or cultured cells (19, 20), are either not operative in native proximal tubular cells or are overruled by other mechanisms. By studying the CIC-5\(^{Y762E}\) mutation in mice null for CIC-3 and -4, we have ruled out the possibility that CIC-5 is no longer influenced by the PY-motif as a consequence of being predominantly present in heterodimers with CIC-3 or CIC-4 that have lost their responsiveness to ubiquitin ligases. The increased surface expression of CIC-5 mutants was caused by the loss of interaction with endogenous WW-domain containing ubiquitin ligases. Moreover, the effect of the dominant negative E3 ligase resulted from a competition with such endogenous ligases for the CIC-5 PY-motif (19). Although WWP2 and Nedd4–2 E3 enzymes are both expressed in kidney (19, 46) and Nedd4-2 is expressed in several nephron segments including the PT (46), the cellular expression pattern of WWP2 remains unknown. Hence, a possible explanation for the discrepancy between \textit{in vitro} and \textit{in vivo} results is that suitable E3 ligase activity is too low in apical domains of PT cells. Moreover, interactions of CIC-5 with other proteins that are not present in \textit{Xenopus} oocytes or opossum kidney cells (used in (Refs. 19, 20) may provide dominant trafficking signals for CIC-5 in its native environment. Furthermore, PY-motif-dependent interactions may have more subtle effects which escaped our analysis.

Another important aspect of our work is the observation that unlike CIC-5, CIC-3, and CIC-4, are dispensable for apical protein uptake in PT cells. As evident from double KO cells, neither homolog significantly influences endocytosis even in the absence of CIC-5. This suggests that neither CIC-3 nor CIC-4 can even partially replace CIC-5 in enabling or stimulating endocytosis. It seems unlikely that differences in biophysical properties account for this difference. CIC-4 and CIC-5 Cl\(^-\)/H\(^+\)-exchangers have very similar biophysical properties (7–9), differing only slightly in their pH sensitivity (7). Although a direct demonstration that CIC-3 is a Cl\(^-\)/H\(^+\)-exchanger is still lacking, its high degree of homology to CIC-4 and -5, the similarity in properties of its electrical current (47–49), and the effect of mutations in its “gating glutamate” (48, 50) strongly suggest that CIC-3 is an outwardly rectifying 2Cl\(^-\)/H\(^+\)-exchanger like CIC-4 and -5.

A likely explanation for the missing role of CIC-3 and -4 in endocytosis is a difference in their subcellular localization. A substantial part of PT endosomes may contain CIC-5 while lacking CIC-3 and -4. A similar situation was recently found for CIC-7 (43), a lysosomal protein (51, 52) that displays no subcellular overlap with CIC-5 (43). Disruption of CIC-7 impaired proximal tubular protein degradation, but had no effect on the upstream endocytic protein uptake (43). Based on partial colocalization with Lamp-1 in transfected cells, CIC-3 is expressed on late endosomes (28), whereas CIC-5 likely localizes to early and recycling endosomes (4, 12, 27). Differential vesicular distribution of these transporters has also been found in other cell types. CIC-3, but not CIC-5, is present on neuronal synaptic vesicles (28, 53) and synaptic-like microvesicles (but not secretory granules) of neuroendocrine cells (32). On the other hand, CIC-5 and CIC-3 colocalized in a subset of vesicles in transfected cells (27). In the PT, differences in subcellular localizations of CIC-3 to -5 are more difficult to assess. Confocal microscopy of proximal tubules could separate regions of CIC-3 and CIC-7 expression, with CIC-3 being more apical (32, 43). Less is known about the localization of CIC-4, but partial colocalization with CIC-5 and the formation of heteromers have been reported in some studies (27, 42). The specific effect of CIC-5 on endocytosis might therefore be owed to an effect on a vesicle population that does not express other CIC proteins.

The present data do not necessarily conflict with recent reports that loss of CIC-4 impairs endocytosis of transferrin (42, 54). In most cells, including the cultured cells used in that study, transferrin is endocytosed after binding to the transferrin receptor. In proximal tubular cells, transferrin is taken up apically via megalin, and basolaterally by the transferrin receptor (55). We have only investigated the former process. It should also be kept in mind that Bear and co-workers used either small interfering RNA (42), or obtained their KO cells by crossing Mus spretus and Mus musculus mice (54), which encode CIC-4 on the X chromosome and an autosome (56), respectively. Such cells are likely to have other defects. It is therefore advisable to

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repeat those experiments with cells displaying a selective loss of CIC-4.

In summary, the specific role of CIC-5 in proximal tubular endocytosis revealed here might be a consequence of its localization on a subset of proximal tubular endosomes that are devoid of significant amounts of other CIC proteins. Furthermore, the trafficking of CIC-5 in its native environment is probably dominated by factors that mask any possible effect of PY-motif-dependent endocytosis, cautioning against extrapolating to the in vivo situation results obtained with heterologous expression, unambiguous as they may be.

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