Endosomal Chloride-Proton Exchange Rather Than Chloride Conductance Is Crucial for Renal Endocytosis
Gaia Novarino, et al.
Science 328, 1398 (2010);
DOI: 10.1126/science.1188070

If you wish to distribute this article to others, you can order high-quality copies for your colleagues, clients, or customers by clicking here.

Permission to republish or repurpose articles or portions of articles can be obtained by following the guidelines here.

The following resources related to this article are available online at www.sciencemag.org (this information is current as of June 11, 2010):

Updated information and services, including high-resolution figures, can be found in the online version of this article at:
http://www.sciencemag.org/cgi/content/full/328/5984/1398

Supporting Online Material can be found at:
http://www.sciencemag.org/cgi/content/full/science.1188070/DC1

This article cites 25 articles, 12 of which can be accessed for free:
http://www.sciencemag.org/cgi/content/full/328/5984/1398#otherarticles

This article appears in the following subject collections:
Cell Biology
http://www.sciencemag.org/cgi/collection/cell_biol
result in gB fragments that are translocated into the cytoplasm. To determine whether cytosolic access is required, we examined the roles of TAP and proteasomes in gB cross-presentation. When DCs from Tap−/− mice were incubated with necrotic infected cells, gB cross-presentation was completely eliminated (Fig. 3F). In addition, cross-presentation of gB, as well as ICp6, was inhibited by lactacystin, indicating dependence on proteasomal processing (Fig. 3E). Cross-presentation thus depends on cytosolic processing of gB fragments generated in the phagosome by GILT-mediated reduction and cathepsin-mediated proteolysis.

A requirement for GILT in the induction of the CD8+ T cell response to gB498-505 during an infection would argue that cross-presentation is important for the in vivo anti–HSV-1 immune response. Wild-type and Ifi30−/− mice were infected with HSV-1, and the draining lymph nodes (LNs) were examined for the induction of K1-gB498-505–specific and K1-ICp622-247–specific CD8+ T cells. Although mice lacking GILT generated the same average percentage of ICp622-247–specific CD8+ T cells when infected with HSV-1 as wild-type mice, the number of gB498-505–specific CD8+ T cells was significantly reduced (Fig. 4, A to C). There was no difference in the survival of the infected mice. Responses to GILT-independent epitopes such as ICp622-247 may make up for any deficiency. To determine whether GILT-dependent cross-presentation is a more general phenomenon, we examined the CD8+ T cell response of mice infected with the PR8 strain of influenza A virus. LN cells from naïve and infected mice were restimulated with wild-type DCs pulsed with peptides that correspond to a variety of H2-Kb– and H2-Dd–restricted epitopes from hemagglutinin (HA), neuraminidase (NA), polymerase (PA), and nucleoprotein (NP) (www.immun epitope.com/home.do) (22). HA and NA contain six and eight disulfide bonds, respectively, whereas PA and NP have none (23–25). A similar percentage of wild-type and GILT-negative CD8+ T cells responded to Dd-restricted PA and NP epitopes upon restimulation (Fig. 4, D and E). In contrast, the responses of CD8+ T cells from mice lacking GILT were significantly reduced for four out of five of the HA epitopes and for two out of three of the NA epitopes. The two HA epitopes to which almost no CD8+ T cells develop in the Ifi30−/− mice contain or are immediately adjacent to a cysteine (C480) involved in a disulfide bond (C21-C480). For both HA and NA, one epitope is GILT independent, strongly arguing against the possibility that any GILT requirement reflects GILT-dependent MHC class II–restricted responses that mediate CD4+ T cell help (26). Although the epitope specificity of the CD4+ T cells in the Ifi30−/− mice may be different from that of wild-type mice, the total numbers of CD4+ T cells that they generate during a viral immune response are similar (fig. S2), as are the numbers of CD4+ T cells in the spleens of uninfected wild-type and Ifi30−/− mice. The data show that GILT-dependent cross-presentation is not restricted to gB, and that cross-presentation is important in the CD8+ T cell response to influenza virus. The residual CD8+ T cell responses observed to gB and the HA and NA epitopes by Ifi30−/− animals may reflect priming by directly infected APCs.

The only known function of GILT is to reduce disulfide bonds, and we have shown that GILT is essential for cross-presentation of many peptides from disulfide-containing proteins. We suggest that reduction in the acidic environment of the phagosome facilitates partial proteolysis into fragments that are translocated into the cytosol where they are further degraded by the proteasome to generate peptides. These are transported by TAP and bind in a conventional manner, possibly after amino-terminal trimming (27), to MHC class I molecules. This latter step is likely to occur in the ER, but could occur in phagosomes that have recruited ER membrane components, although this issue remains contentious (28, 29).

For gB, the inability to cross-present is reflected in a reduction in K1-gB498-505–specific CD8+ T cells in vivo, indicating the importance of cross-presentation in CD8+ T cell responses to HSV-1 infection. The similar reduction in HA- and NA–specific CD8+ T cells suggests that cross-priming is also important during influenza A infection. The role played by GILT in cross-primming, combined with its established involvement in MHC class II–restricted CD4+ T cell responses (30), indicates the importance of the enzyme in the immune system. This may have implications for vaccine design and approaches to tumor immunotherapy that involve peptide-based vaccines, in that linear peptides may not be the optimal vehicles for the expression of GILT-dependent epitopes, and for autoimmunity to self-antigens that contain multiple disulfide bonds.

References and Notes
16. See supporting material on Science Online.
31. This work was supported by the Howard Hughes Medical Institute and NIH grant R37AI23081 (P.C.).

Supporting Online Material
www.sciencemag.org/cgi/content/full/328/5984/1394/DC1
Materials and Methods
Figs. S1 and S2
References
5 March 2010; accepted 4 May 2010
10.1126/science.1189176

Endosomal Chloride-Proton Exchange Rather Than Chloride Conductance Is Crucial for Renal Endocytosis
Gaia Novarino, Stefanie Weinert, Gesa Rickheit,* Thomas J. Jentsch†

Loss of the endosomal anion transport protein CIC-5 impairs renal endocytosis and underlies human Dent’s disease. CIC-5 is thought to promote endocytosis by facilitating endosomal acidification through the neutralization of proton pump currents. However, CIC-5 is a z 2 chloride (Cl−)/proton (H+) exchanger rather than a Cl− channel. We generated mice that carry the uncoupling E211A (unc) mutation that converts CIC-5 into a pure Cl− conductor. Adenosine triphosphate (ATP)–dependent acidification of renal endosomes was reduced in mice in which CIC-5 was knocked out, but normal in Clns−/− mice. However, their proximal tubular endocytosis was also impaired. Thus, endosomal chloride concentration, which is raised by CIC-5 in exchange for protons accumulated by the H+–ATPase, may play a role in endocytosis.

Luminal acidification along the endocytic pathway serves many purposes (1), including the progression of endocytosis itself (2). It is performed by endosomal H+–transporting adenosine triphosphatases (H+-ATPases) that need a countercurrent for electroneutrality. Because this
current depends on chloride, conventional wisdom suggests (1) that endosomal Cl⁻ channels are involved (fig. S1A). CIC-5 was thought to embody this channel in proximal tubular endosomes (3–5). Disruption of CIC-5 impairs renal endosomal acidification in vitro (5) and drastically reduces proximal tubular endocytosis in mice and humans (4, 6, 7). The hyperphosphaturia and hypercalcuria that lead to kidney stones in Dent’s disease have been attributed to impaired tubular endocytosis of calcitropic hormones (4). However, it has recently been shown (8–10) that CIC-5 is a 2Cl⁻/H⁺ exchanger rather than a Cl⁻ channel. It seems counterintuitive that such an exchanger should neutralize pump currents because it mediates H⁺-efflux during ATP-driven acidification. The biological consequence of proton coupling has remained enigmatic (11).

If the CIC-5 Cl⁻/H⁺ exchanger could be converted into an uncoupled Cl⁻ conductor, it should efficiently facilitate endosomal acidification. Phenotypes of mice carrying such a mutation cannot be attributed to impaired endosomal acidification, but can be ascribed specifically to a loss of coupling chloride gradients to proton gradients. A mutation in the “gating” glutamate of CLC exchangers (12) suffices to convert them into pure anion conductors (8, 9, 13–15). We inserted the corresponding, well-characterized E211A mutation (8, 9, 14, 16, 17) into the Clcn5 gene on mouse chromosome X and created mice in which ClC-5 was converted into an uncoupled Cl⁻ conductor (Clcn5<sup>unc</sup> mice) (figs. S1A and S2). The mutant protein was expressed at wild-type (WT) levels (Fig. 1A). No change was observed in its subcellular localization in kidney proximal tubular and intercalated cells (Fig. 1B and fig. S3). The renal expression of the related CIC-3 and CIC-4 proteins also was not affected (fig. S4).

To test whether the uncoupled CIC-5<sup>unc</sup> mutant supported endosomal acidification, we added ATP to endosomal fractions from renal cortex (containing mainly proximal tubules) of WT or Clcn5<sup>unc</sup> mice and monitored vesicular pH using acridine orange fluorescence. H⁻–ATPase–driven acidification of WT and Clcn5<sup>unc</sup> vesicles occurred with similar efficiency but was severely reduced with endosomes from mice in which CIC-5 was knocked out (KO mice), as expected (5, 18) (Fig. 1, C to E).

Despite maintaining active endosomal acidification, Clcn5<sup>unc</sup> mice displayed abnormalities found in CIC-5 KO (Clcn5<sup>−/−</sup>) mice and patients with Dent’s disease (4, 7, 19), such as low-molecular-weight proteinuria (Fig. 2, A and B), hyperphosphaturia, and hypercalcuria (table S1). Proteinuria of Clcn5<sup>−/−</sup> mice results from impaired proximal tubular endocytosis (4, 7), which was studied in chimeric tubules resulting from random X-chromosomal inactivation in female Clcn5<sup>+/−</sup> mice (4). In those tubules, WT and KO cells were distinguished by means of antibodies to CIC-5 (3, 4), but this approach cannot differentiate between cells expressing the WT or uncoupled CIC-5 in Clcn5<sup>unc</sup> tubules. Rather than epitope-tagging the E211A mutant, which might interfere with its function, we generated mice in which the C term of CIC-5 was converted to that of CIC-3 (fig. S1, B and C, and S5). The generation of this Clcn5<sup>5E</sup> allele required only two amino acid exchanges and changed neither CIC-5 currents (fig. SSD) nor its abundance (Fig. 1A) and localization (fig. S6A). Our antibodies against the C termus of CIC-5 recognized CIC-5 and CIC-5<sup>unc</sup> but not CIC-5<sup>5E</sup> (Figs. 1A and 2, C and D, and fig. S1, B and C). In vivo endocytosis experiments were performed by injecting into the bloodstream labeled endocytic cargo that can pass the glomerular filter. Endocytosis in cells expressing the Clcn5<sup>5E</sup> allele or WT CIC-5 was indistinguishable (fig. S6B). However, cells expressing the Clcn5<sup>unc</sup> allele accumulated much less fluorescently labeled β-lactoglobulin, which is a marker for receptor-mediated endocytosis (Fig. 2C), or the fluid-phase marker dextran (Fig. 2D) than did neighboring cells that express the 2Cl⁻/H⁺–exchanger CIC-5<sup>5E</sup>. Thus, uncoupling anion transport from protons resulted in a cell-autonomous impairment of both receptor-mediated and fluid-phase endocytosis akin to Clcn5<sup>−/−</sup> mice (4). Similarly as in KO mice (4, 20), cells expressing the uncoupled E211A mutant displayed reduced levels of the endocytic receptors megalin (Fig. 3, A and C) and cubilin (Fig. 3B)—a finding ascribed to impaired recycling to the plasma membrane (4, 20). In Clcn5<sup>unc</sup> kidney, the sodium-phosphate co-transporter NaPi-2a was shifted from the apical membrane to intracellular vesicles, and its overall abundance was reduced (fig. S7), which explains the observed hyperphosphaturia. Similarly increased endocytosis of NaPi-2a in Clcn5<sup>−/−</sup> mice was attributed to reduced endocytosis of filtered parathyroid hormone (PTH), entailing a luminal increase of PTH and excessive stimulation of apical PTH receptors (4, 5).

Because the E211A mutation did not abolish CIC-5 currents and maintained endosomal acidification (Fig. 1C), it might have affected endocytosis less than a loss of CIC-5. We thus compared Clcn5<sup>unc</sup> and Clcn5<sup>−/−</sup> cells side by side in chimeric tubules of Clcn5<sup>unc/−</sup>–females. No differences were detected in receptor-mediated (Fig. 2E) or fluid-phase endocytosis (fig. S8), nor in the localization and abundance of megalin (Fig. 3C). Thus unexpectedly, the anion conductance of CIC-5(E211A) could not even partially substitute for Cl⁻/H⁺ exchange in the support of proximal tubular endocytosis and normal localization of apical receptors.

Because the intramembrane E211A point mutation was unlikely to have changed CIC-5 protein interactions, the pathology of Clcn5<sup>unc</sup> mice is caused neither by the disruption of a macromolecular CIC-5–containing endocytic complex (21, 22) nor by the loss of interaction with KIF3B.

**Fig. 1.** Renal endosomal acidification of mice converting CIC-5 into a chloride conductor. (A) Immunoblot for CIC-5 using kidney membranes of different genotypes with antibodies against the (left) N and (right) C termini (C5/0SA). Asterisk indicates the mutated C terminus of CIC-5 (fig. S5). (B) Identical staining pattern using the C-terminal PEPPSe antibody to CIC-5 (3) in proximal tubules of WT and Clcn5<sup>unc</sup> mice. Scale bar, 5 μm. (C and D) Averaged traces of acridine orange fluorescence comparing ATP-driven acidification of renal cortical vesicles from (C) WT (green) and Clcn5<sup>unc</sup> (red) and (D) WT (green) and Clcn5<sup>−/−</sup> (black) mice (with identical WT traces). Reduced fluorescence reflects vesicular acidification. The protonophore FCCP was added as a control. F<sub>0</sub> fluorescence at time (t) = 0. (E) Averaged total change in fluorescence. Data averaged from 25 (+/y), 13 (–/y), and 10 (unc) experiments with material obtained from more than four independent vesicle preparations per genotype. Error bars, SEM. *p < 0.05; **p < 0.01.

Leibniz-Institut für Molekulare Pharmakologie (FMP) and Max-Delbrück-Centrum für Molekulare Medizin (MDC), 13125 Berlin, Germany.

*Present address: Taconic Artemis GmbH, 51063 Köln, Germany.
†To whom correspondence should be addressed. E-mail: jentsch@fmp-berlin.de
Fig. 2. Proteinuria and impaired endocytosis in Clcn5–/– Clcn5unc mice. (A) Urinary proteins analyzed by means of SDS–polyacrylamide gel electrophoresis and silver staining. (B) Immunoblot for vitamin D–binding protein (DBP) and retinol-binding protein (RBP) in urine. (C) In vivo 10-min uptake of Alexa Fluor 546–labeled (Invitrogen, Carlsbad, CA) β-lactoglobulin (red) showed decreased receptor-mediated endocytosis in Clcn5unc–expressing cells in a chimeric proximal tubule from a Clcn5unc+/+ female. ClC-5unc– but not the ClC-5–/– Cl–/H+ exchanger, is recognized by the antibody C5/05A (green). (Right) Overlay with additional brush-border staining for villin (blue). (D) Reduced fluid-phase endocytosis of Alexa Fluor 488–labeled (Invitrogen) dextran in Clcn5unc–expressing cells in an experiment similar to (C). (E) Similar β-lactoglobulin–uptake of cells lacking ClC-5 or expressing Clcn5unc in a Clcn5unc+/+ female. Scale bars, 5 μm (C), 3.2 μm (D), and 6 μm (E).

Fig. 3. Effect of uncoupling ClC-5 on endocytic receptors. (A) Reduced megalin levels in Clcn5unc–expressing cells of a chimeric tubule of Clcn5unc+/+ mice. (B) Reduced cubilin expression in Clcn5unc–expressing cells that were identified through reduced endocytosis of Alexa 488–dextran. (C) Similar reduction of megalin in cells expressing Clcn5– or Clcn5unc–. To compare weak megalin immunostaining in Clcn5unc–tubules, image intensity was boosted in the center panel as compared with that of (A). (Right) Immunoblot for megalin using kidney membranes from WT, Clcn5unc–, and Clcn5–/– mice. Scale bars, 4 μm [(A) and (B)] and 5 μm (C).
Lysosomal Pathology and Osteopetrosis upon Loss of H⁺-Driven Lysosomal Cl⁻ Accumulation

Stefanie Weinert,1,2 Sabrina Jabs,1,2,6 Chayarpur Supanchart,3 Michaela Schweizer,4 Niclas Gimber,1,2 Martin Richter,1,6 Jörg Rademann,1,6 Tobias Stauber,1,2 Uwe Kornak,3,5 Thomas J. Jentsch1,2†

During lysosomal acidification, proton-pump currents are thought to be shunted by a chloride ion (Cl⁻) channel, tentatively identified as ClC-7. Surprisingly, recent data suggest that ClC-7 instead mediates Cl⁻/proton (H⁺) exchange. We generated mice carrying a point mutation converting ClC-7 into an uncoupled (unc) Cl⁻ conductor. Despite maintaining lysosomal conductance and normal lysosomal pH, these Clcn7unc mice showed lysosomal storage disease like mice lacking ClC-7. However, their osteopetrosis was milder, and they lacked a coat color phenotype. Thus, only some roles of ClC-7 Cl⁻/H⁺ exchange can be taken over by a Cl⁻ conductance. This conductance was even deleterious in Clcn7unc mice. Clcn7⁺/+ and Clcn7unc mice accumulated less Cl⁻ in lysosomes than did wild-type mice. Thus, lowered lysosomal chloride may underlie their common phenotypes.

Cl⁻ is the only member of the CLC gene family of anion transporters substantially expressed on lysosomes (1–3), where it resides together with its β-subunit Ostml (3). Inactivation of either subunit leads to lysosomal storage disease and osteopetrosis in mice and humans (1–4). Cellular defects include slowed degradation of endocytosed proteins (5) and impaired acidification of the osteoclast resorption lacuna (1). Cl⁻ currents mediated by ClC-7 have been deemed necessary for shunting lysosomal proton-pump currents (1). However, lysosomal pH was normal in cells lacking either ClC-7 or Ostml (2, 3). ClC-7 now seems likely to be a Cl⁻/H⁺ exchanger rather than a Cl⁻ channel (6, 7). Because H⁺-pump currents may be neutralized by both Cl⁻ channels and electrogenic Cl⁻/H⁺ exchangers (6), it is unclear whether lysosomal Cl⁻/H⁺ exchange confers functional advantages over the simple Cl⁻ conductance in the textbook model for vesicular acidification.

We created knock-in mice in which the CIC-7 “gating” glutamate (E) was mutated to alanine (A) (fig. S1) (8). On the basis of results from other CLC Cl⁻/H⁺ exchangers (9–12), this Glu245 → Ala245 (E245A) mutation should lead to Cl⁻ transport that is uncoupled (unc) from protons, hence our designation of this allele as Clcn7unc. Homozygous Clcn7unc mice showed severe growth retardation (Fig. 1A) and mislocalization in lysosomes (Fig. 1D). Neither the abundance, nor the lysosomal localization of Ostml was changed in Clcn7unc mice, contrasting with its strongly reduced protein level (3) and mislocalization in Clcn7unc mice (cells (1, C) and D). In neurons, however, CIC-7unc staining was more diffuse (fig. S3B), reflecting changed lysosomal compartments like in Clcn7unc neurons (2). The abundance of other CLC exchangers was unchanged in Clcn7unc mice (fig. S4).

In an agouti genetic background, the coat color of Clcn7unc mice is grey (3, 4), whereas it was brownish in WT and Clcn7unc/unc mice (Fig. 1A). Clcn7unc mice were osteopetrotic (Fig. 2A and fig. S5), although less severely than Clcn7unc (1) or Ostmlunc (4) mice. ClC-7 and Ostml were detected at the ruffled border of Clcn7unc osteoclasts (fig. S3A). This