Lysosomal Pathology and Osteopetrosis upon Loss of H⁺-Driven Lysosomal Cl⁻ Accumulation

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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 Clcn7-/-unc 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⁻ conductor. This conductance was even deleterious in Clcn7-/-unc mice. Clcn7+/+ and Clcn7-/-unc mice accumulated less Cl⁻ in lysosomes than did wild-type mice. Thus, lowered lysosomal chloride may underlie their common phenotypes.

CIC is the only member of the CLC family of anion transporters substantially expressed on lysosomes (1–3), where it resides together with its β-subunit Ostm1 (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 (5). Cl⁻ currents mediated by CIC-7 have been deemed necessary for shunting lysosomal proton-pump currents (1). However, lysosomal pH was normal in cells lacking either CIC-7 or Ostm1 (2, 3). CIC-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 CI⁻ transport that is uncoupled (unc) from protons, hence our designation of this allele as Clcn7-/-unc. Homozygous Clcn7-/-unc mice showed severe growth retardation (fig. 1A and fig. S2) and died within 5 weeks. CIC-7-/- unc and wild-type (WT) CIC-7 were expressed at similar levels (fig. 1B) and similarly localized to lysosomes (fig. 1D). Neither the abundance, nor the lysosomal localization of Ostm1 was changed in Clcn7-/-unc mice, contrasting with its strongly reduced protein level (3) and mislocalization in Clcn7-/- mice (fig. 1C and D). In neurons, however, CIC-7-/-unc staining was more diffuse (fig. S3B), reflecting changed lysosomal compartments like in CIC-7-/- neurons (2). The abundance of other CLC exchangers was unchanged in Clcn7-/-unc mice (fig. S4).

In an agouti genetic background, the coat color of Clcn7-/-unc and Ostm1-/- (agouti-lethal) mice is grey (3, 4), whereas it was brownish in WT and Clcn7+/unc mice (fig. 1A). Clcn7+/unc mice were osteopetrotic (fig. 2A and fig. S5), although less severely than CIC-7-/- mice (fig. 2B and fig. S5). CIC-7 and Ostm1 were detected at the ruffled border of Clcn7-/-unc osteoclasts (fig. S3A). This

Fig. 1. CIC-7 and Ostm1 in mice carrying different Clcn7 alleles. (A) Clcn7-/-unc, and WT mice at postnatal day 22 (P22) in an agouti background. (B) CIC-7 immunoblot of tissues from Clcn7-/-unc, Clcn7+/-, and WT mice. (C) The mature form of Ostm1 (doublet) was absent from Ostm1-/- (grey-lethal, gl) and Clcn7-/- mice but showed similar abundance in Clcn7+/+, Clcn7-/-unc (0.97 ± 0.20), and Clcn7-/-unc (0.85 ± 0.32) brains (normalized to WT ± SEM, six mouse pairs, three immunobLOTS. kD, kilodaltons. (D) Immunofluorescence of CIC-7 or CIC-7-/-unc (green) and the lysosomal marker Lamp-1 (red) in WT, Clcn7+/+, and Clcn7-/-unc fibroblasts. (Bottom) Coating for Ostm1 (red) and CIC-7 or CIC-7-/-unc (green). Normal localization of CIC-7-/-unc and Ostm1 in Clcn7-/-unc cells, but reticular Ostm1 staining in Clcn7-/-/- cells.

References

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Fig. S1 to S8 Table S1

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acid-secreting membrane was underdeveloped in Clcn7<sup>−/−</sup> osteoclasts and almost lacking in Clcn7<sup>unc/unc</sup> osteoclasts (Fig. 2B). WT, Clcn7<sup>−/−</sup>, and Clcn7<sup>unc/unc</sup> osteoclasts similarly attached to dentine and established actin rings that surround resorption lacunae (Fig. S6A). In contrast to almost nonresorbing Clcn7<sup>−/−</sup> osteoclasts (I), Clcn7<sup>unc/unc</sup> osteoclasts excavated pits, albeit their number and depths were strongly reduced (Fig. 2C and fig. S6B).

Like mice lacking CIC-7 (2) or Ostm1 (3), Clcn7<sup>unc/unc</sup> mice displayed rapidly progressing retinal degeneration (fig. S7) and developed neurodegeneration with features of lysosomal storage disease (Fig. 2D and fig. S8). Although Clcn7<sup>unc/unc</sup> mice lacked an obvious phenotype during the first 5 months, they showed slowly progressing hippocampal neurodegeneration (Fig. 2D and fig. S8C). No such degeneration was seen in Clcn7<sup>−/−</sup> mice.

To examine whether the E245A mutation had converted CIC-7 from a CI/H<sup>+</sup> exchanger into an uncoupled anion conductor, we exposed fluorescein-dextran–loaded lysosomes to different external CI<sup>−</sup> concentrations ([CI<sup>−</sup>]<sub>o</sub>) in the presence of K<sup>+</sup> and valinomycin to shunt currents. CI/H<sup>+</sup> exchange predicts a more alkaline luminal pH (pH<sub>L</sub>) with higher [Cl<sup>−</sup>]<sub>L</sub> in vesicles containing just an H<sup>+</sup> pump, an H<sup>+</sup> leak, and a CI<sup>−</sup>/H<sup>+</sup> exchanger or a 2Cl<sup>−</sup>/H<sup>+</sup> exchanger into CLC exchangers (3A), suggesting that CIC-7 mediates CI<sup>−</sup>/H<sup>+</sup>-dependent pH<sub>L</sub> changes remaining with KO<sup>−</sup> and Clcn7<sup>unc/unc</sup> lysosomes (Fig. 3A).

We then added the proton ionophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP) to cultured fibroblasts whose lysosomes were preloaded with a pH indicator. CCCP dissipates lysosomal pH only in the presence of countercurrents. Lysosomes from all three genotypes rapidly alkalized upon CCCP addition (Fig. 3B). Clcn7<sup>unc/unc</sup> lysosomes reached a more alkaline pH<sub>L</sub> than Clcn7<sup>−/−</sup> lysosomes. Thus, CIC-7<sup>unc</sup> mediates a conductance that is most likely carried by CI<sup>−</sup> as in equivalent mutants of other CLC exchangers (9–12). Although biophysics predicts identical equilibrium pH<sub>L</sub> with an H<sup>+</sup> leak parallel to either a CI<sup>−</sup> conductance or a 2CI/H<sup>+</sup> exchange (fig. S9), steady-state pH<sub>L</sub> was less alkaline in Clcn7<sup>unc/unc</sup> than in WT lysosomes (Fig. 3B).

Because equilibrium H<sup>+</sup>-gradients are determined by the CI<sup>−</sup> diffusion potential, this pH<sub>L</sub> difference suggests higher lysosomal chloride concentration ([Cl<sup>−</sup>]<sub>L</sub>) in WT than in Clcn7<sup>unc/unc</sup> lysosomes. The CCCP-induced alkalization of Clcn7<sup>−/−</sup> lysosomes (Fig. 3B) indicates the presence of a sizable lysosomal conductance beyond CIC-7. The final pH of CCCP-treated KO lysosomes, which is more acidic than that of Clcn7<sup>unc/unc</sup> lysosomes, might be explained by a lumen-negative potential created by a cation conductance (fig. S9). Mixed K<sup>+</sup>/Na<sup>+</sup> conductances were reported for lysosomes (14).

As predicted by sizable lysosomal conductances in all three genotypes, fluorescein-dextran–loaded lysosomes of WT, Clcn7<sup>unc/unc</sup>, and Clcn7<sup>−/−</sup> mice showed adenosine triphosphate (ATP)–driven acidification in vitro (Fig. 3C). Agreement with the presence of a cation conductance, Clcn7<sup>−/−</sup> lysosomes acidified also without [CI<sup>−</sup>]<sub>L</sub> (Fig. 3C). The marked difference to renal endosomes, whose acidification depends on chloride and Ci<sup>−</sup> (15–17), may be explained with a larger cation conductance in lysosomes (14).

We ratiometrically measured pH in lysosomes of fibroblasts that were loaded with the dextran-coupled pH indicator Oregon Green 488 by endocytosis. There was no measurable difference between Clcn7<sup>unc/unc</sup>, Clcn7<sup>−/−</sup>, Clcn7<sup>+/−</sup>, or Clcn7<sup>−/−</sup> cells (Fig. 3D). The normal steady-state pH<sub>L</sub> and robust ATP-dependent acidification eliminates lysosomal pH as an important factor in lysosomal pathology of Clcn7<sup>unc/unc</sup> but not in Clcn7<sup>−/−</sup> mice, which showed degeneration at P250.
Fig. 3. Lysosomal transport characterization. (A) The unc mutation abolished ClC-7 Cl/H\(^+\) exchange, as revealed by Cl\(^-\)-gradient–driven pH changes in fluorescein-dextran–loaded lysosomes exposed to low (10 mM, dashed lines) or high (107 mM, solid lines) [Cl\(^-\)]\(_o\) in vitro. Averages from 42 (+/+/), 20 (unc/unc), and 32 (−/−) experiments are shown. F, ratio of fluorescence at λ = 535 nm obtained with excitation at 488 and 440 nm; F\(_o\), F at time \( t = 0 \); Val, valinomycin. (B) CCCP-induced alkalization of lysosomes in fibroblasts monitored by Oregon Green 488-dextran. (C) Similar ATP-dependent acidification in vitro of Clcn7\(^{-/-}\) Clcn7\(^{unc/unc}\), and Clcn7\(^{unc/unc}\) lysosomes (the latter with and without Cl\(^-\)). The K\(^+\)/H\(^+\)-exchanger nigericin was added as a control. Means from 14 (+/+/), 14 (unc/unc), 10 (−/−), and 23 (−/−; Cl\(^-\)-free) experiments are shown. Error bars (indicating SEM) are shown for every third data point. (D) Steady-state pH in WT, Clcn7\(^{−/−}\), Clcn7\(^{unc/unc}\) and Clcn7\(^{unc/unc}\) fibroblasts. Averages from three independent cell lines per genotype, with >100 lysosomes from eight cells each. (E) Lower [Cl\(^-\)]\(_o\) in Clcn7\(^{−/−}\) and Clcn7\(^{unc/unc}\) than in Clcn7\(^{+/−}\) lysosomes revealed by chloride-sensitive fluorescence ratio of MEQ/tetramethylrhodamine-dextran endocytosed by fibroblasts and chased 2 hours into lysosomes in medium containing 7 mM Cl\(^-\). Means from ≥10 experiments are shown (three cell lines per genotype, 10 cells with 10 lysosomes each per experiment). *\( P < 0.001\), Student’s t test. Error bars denote SEM throughout.

References and Notes

8. Materials and methods are available as supporting material on Science Online.
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Materials and Methods

Figs. S1 to S11
Table S1
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