Transport activity and presence of ClC-7/Ostm1 complex account for different cellular functions

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Abstract

Loss of the lysosomal ClC-7/Ostm1 2Cl⁻/H⁺ exchanger causes lysosomal storage disease and osteopetrosis in humans and additionally changes fur colour in mice. Its conversion into a CI⁻ conductance in Clcn7unc/unc mice entails similarly severe lysosomal storage, but less severe osteopetrosis and no change in fur colour. To elucidate the basis for these phenotypical differences, we generated Clcn7fad mice expressing an ion transport-deficient mutant. Their osteopetrosis was as severe as in Clcn7−/− mice, suggesting that the electric shunt provided by CI⁻/H⁺ exchange can partially rescue osteoclast function. The normal coat colour of Clcn7fad mice and their less severe neurodegeneration suggested that the ClC-7 protein, even when lacking measurable ion transport activity, is sufficient for hair pigmentation and that the conductance of CI⁻/H⁺ exchange is harmful for neurons. Our in vivo structure-function analysis of CI⁻/H⁺ reveals that both protein-protein interactions and ion transport must be considered in the pathogenesis of ClC-7-related diseases.

Keywords: acidification; anion transport; grey-lethal; lysosome; Wnt signalling

Introduction

Acidic luminal pH in endosomes and lysosomes influences their trafficking, enzymatic activities and transport of substances across their limiting membranes. Luminal acidification is accomplished by electrogenic vacuolar H⁺-ATPases that require an electric shunt, which in the classical model was thought to be mediated by chloride channels. Members of the CLC anion transporter gene family [1,2], five of which reside in endosomes or lysosomes, were thought to represent these channels. However, ClC-4 through ClC-7 are rather exchangers that couple Cl⁻ influx to H⁺ efflux [3–7]. Electrogenic Cl⁻/H⁺ exchange can support proton pumping [8,9] and might be even more efficient than Cl⁻ channels in supporting vesicular acidification [6]. However, lysosomal pH is normal in mice lacking ClC-7 [6,10,11], and the strict coupling of Cl⁻ flux to H⁺ countertransport suggested that vesicular CLCs accumulate Cl⁻ into acidic compartments [12] as shown for lysosomes [6]. To clarify the relative contributions of shunt conductance and proton coupling to their biological roles, we had generated Clcn5unc/unc and Clcn7unc/unc mice in which Cl⁻ transport was uncoupled from H⁺ transport by single point mutations [6,8]. Surprisingly, these unc mice [6,8] displayed “grosso modo” the same phenotypes as the respective null mice [10,13,14], that is, impaired renal endocytosis in Clcn5unc/unc mice and osteopetrosis associated with a lysosomal storage disorder and neurodegeneration in Clcn7unc/unc mice (Supplementary Table S1). Hence, a Cl⁻ conductance cannot replace electrogenic Cl⁻/H⁺ exchange in many cellular functions.

ClC-7, together with its obligate β-subunit Ostm1 [11], is expressed in virtually all tissues [14,15]. It localizes to late endosomes and lysosomes and is inserted into the acid-secreting ruffled border of bone-resorbing osteoclasts [10,14]. Loss of ClC-7 function causes osteopetrosis in mice [14], humans [14,16] and cattle [17] and entails lysosomal storage and neurodegeneration in mice [10]. Decreased proteolytical capacity of lysosomes was demonstrated in Clcn7−/− proximal tubules [18]. The unchanged steady-state pH of Clcn7−/− lysosomes [10,11] was explained by a lysosomal cation conductance that shunts H⁺-ATPase currents in parallel to ClC-7 [6,19]. By contrast, the osteopetrosis of Clcn7−/− mice was attributed to impaired acidification of the osteoclast resorption lacuna [14]. Together with the H⁺-ATPase, ClC-7 is inserted by lysosomal exocytosis into the ruffled border of osteoclasts where it may shunt H⁺-ATPase currents [14]. Finally, Clcn7−/− mice display grey fur in an agouti background. This phenotype might be linked to melanosomes, a lysosome-related compartment. Phenotypes virtually identical to those of Clcn7−/− mice are found in grey-lethal mice [11,20] which carry a mutation in the gene encoding Ostm1.

Whereas the phenotypes of Clcn5−/− and Clcn7unc/unc mice are nearly identical, some of the phenotypes of Clcn7−/− and Clcn7unc/unc
mice differ in severity [6], that is, Clcn7unc/unc mice show less severe osteopetrosis and lack the coat colour phenotype [6,11] (Supplementary Table S1). Two hypotheses may be invoked to explain these differences. First, the shunt conductance provided by ClC-7 unc may suffice to support some, but not all cellular functions. Second, the difference may be owed to lacking ClC-7 protein interactions in Clcn7+/−/−, but not in Clcn7unc/unc mice which express a correctly targeted ClC-7 mutant at normal levels [6].

Here, we generated a novel Clcn7td/td mouse model that expresses a transport-deficient point mutant of ClC-7. The ClC-7 td mutant protein neither transports Cl− nor H+ to a measurable degree [7], but, like ClC-7 unc, is expected to be fully interaction competent. Comparative analyses of these mice suggest that a pure Cl− conductance partially rescues the lack of Cl−/H+ exchange in osteoclasts, whereas normal pigmentation requires ClC-7 protein interactions, but not ClC-7 ion transport activity. Surprisingly, our study also shows that the Cl− conductance of ClC-7 td may have detrimental effects on CNS neurons.

Results

Transport-deficient ClC-7 mutant and Ostm1 are expressed normally

We generated mice in which the ‘proton glutamate’ E312 of ClC-7 was mutated to alanine (Supplementary Fig S1) abolishing both Cl− and H+ transport of ClC-7/Ostm1 [7], hence our designation of this allele as ‘transport deficient’ (td). Homozygous Clcn7td/td mice were born at Mendelian ratio. Like Clcn7unc/unc and Clcn7+/−/− mice, they were growth retarded and most of them died within 6 weeks after birth. Surprisingly, a few Clcn7td/td mice survived more than 1 year (Supplementary Fig S2). The genetic background of Clcn7td/td mice cannot account for this difference as these mice were studied in comparable mixed genetic backgrounds. Clcn7+/−/− mice lacked an obvious phenotype. Clcn7td protein levels were undistinguishable from Clcn7+/−/− mice in wild-type (WT) mice (Fig 1A and B). Like in Clcn7unc/unc mice [6], neither the abundance of Ostm1 [11] nor its processing by lysosomal proteases was changed in Clcn7td/td mice (Fig 1A). WT ClC-7 and Clcn7td similarly localized to Lamp-1-positive structures in primary fibroblasts (Fig 1C), suggesting unchanged interactions with the trafficking machinery. Ostm1 had left the endoplasmic reticulum (ER) and co-localized with ClC-7 td in lysosomes (Fig 1C).

Lysosomal ion homeostasis in Clcn7td/td mice

Because ClC-7 may contribute to a countercurrent for the vacuolar H+−ATPase [7,14], we measured lysosomal pH of Clcn7td/td fibroblasts and found it to be unchanged (Supplementary Fig S3A). Measurements of lysosomal Cl− concentration with a dextran-sensitized ratiometric dye [6] revealed reduced lysosomal Cl− accumulation (Supplementary Fig S3B). Both results resemble those made with Clcn7+/−/− and Clcn7unc/unc mice [6,10].

Delayed neurodegeneration in Clcn7td/td mice

Like Clcn7+/−/− and Clcn7unc/unc mice, Clcn7td/td mice displayed progressive degeneration in the hippocampus (Fig 2). However, it appeared much later and was only detectable in the few surviving older mice. Neuronal cell loss was observed within the CA3 region and progressed to an almost complete loss of CA3 pyramidal cells at older mice. Neuronal cell loss was observed within the CA3 region and progressed to an almost complete loss of CA3 pyramidal cells at older mice. Neuronal cell loss was observed within the CA3 region and progressed to an almost complete loss of CA3 pyramidal cells at older mice.
Nevertheless, pathological changes were observed, in particular in CA3 neurons and in some parts of the cortex. In those regions, lysosomal membrane proteins like Lamp-1 and ClC-7td itself were more intensely labelled and showed a broad distribution in neuronal somata rather than being stained in scattered puncta as in the WT (Fig 3A and B and Supplementary Fig S4). A similar observation was made for ClC-7unc (Fig 3A). Lysosomal storage was apparent 4 weeks after birth, including intracellular carbohydrate accumulation (Fig 2F) and increased levels of lysosomal acid phosphatase (Fig 2G). At P21, electron-dense osmiophilic material accumulated in lysosomal...
Osteopetrosis of CIC-7 mice was severe as in CIC-7 KO

Immunolabelling of tibiae revealed that both CIC-7Δd and its β-subunit Ostm1 were normally expressed in Clcn7Δd osteoclasts (Supplementary Fig S6A). CIC-7 and CIC-7Δd similarly co-localized with the a3 subunit of the V-type H⁺-ATPase at the ruffled border (Supplementary Fig S6B). Unlike the milder osteopetrosis of Clcn7unc/unc mice [6], the osteopetrosis of Clcn7Δd (Fig 4A) was as severe as in Clcn7−/− [14] or grey-lethal (Ostm1−/−) mice [20]. Bone density was similarly increased in Clcn7Δd/Δd and Clcn7−/− mice (Fig 4C). As observed for the other CIC-7 mouse models, teeth were formed in Clcn7Δd/Δd mice, but did not erupt (Fig 4B). Electron micrographs showed a partially deranged ruffled border membrane in Clcn7Δd/Δd mice (Fig 4D). Using the sealing zone, which laterally delimits the resorption lacuna between osteoclasts and bone matrix, as localization marker, we categorized ruffled borders in situ as absent, immature or mature (Fig 4D and E). 20% of osteoclasts from Clcn7−/− and Clcn7Δd/Δd mice totally lacked a ruffled border, and only about 40% showed a mature ruffled border (Fig 4E). All osteoclasts from Clcn7unc/unc mice formed ruffled border membranes, of which 70% appeared mature. Hence, the severity of osteopetrosis correlates with an impairment of ruffled border formation.

Coat colour phenotype is absent in CIC-7Δd mice

The pigments of hair and skin are synthesized in melanosomes, a lysosome-related compartment of melanocytes, and are then transferred to keratinocytes. The grey fur of Clcn7−/− or Ostm1−/− mice [14,20] thus agrees with the lysosomal localization of CIC-7/Ostm1. Surprisingly, the fur colour was changed neither in Clcn7unc/unc [6] nor in Clcn7Δd/Δd mice (Fig 5A) which express mutant full-length CIC-7 proteins that display or lack, respectively, a Cl⁻ conductance. The agouti gene modulates the colour of the hair shaft, resulting in a band of yellow (owed to pheomelanin granules) in the otherwise dark (eumelanin) pigmented hair shaft. The pigment in the yellow band was clumped and reduced in Clcn7−/− and Ostm1−/− (gl) mice, whereas eumelanin granules were unchanged in their dark hair shafts (Fig 5B). Hair shaft pigmentation of Clcn7unc/unc and Clcn7Δd/Δd mice was unchanged compared to WT (Fig 5B).

Activation of Wnt signalling in primary fibroblasts and melanocytes

Melanocyte differentiation depends on Wnt signalling [21] and Ostm1 has been proposed to play a role in the canonical Wnt pathway [22] although the molecular mechanism remains obscure. Because the Ostm1 protein is absent or severely reduced in Ostm1−/− and Clcn7−/− mice, respectively [11], but unchanged in Clcn7unc/unc [6] and Clcn7Δd/Δd mice, we asked whether the difference in coat colour might be due to differences in Wnt signalling. Primary fibroblasts from WT, Clcn7−/− and Ostm1−/− mice were exposed to Wnt3a to activate canonical Wnt signalling and mRNA levels of the target gene axin2 [23] were determined. Basal and Wnt3-stimulated axin2 expression was unchanged in Clcn7−/− and gl (Ostm1−/−) fibroblasts (Supplementary Fig S7A and B) and in Clcn7−/− melanocytes (Supplementary Fig S7C) compared to WT. Hence, differential activation of the Wnt signalling pathway is unlikely to contribute to the phenotypical differences of the present Clcn7 mouse models.

Discussion

Our analysis of Clcn7Δd/Δd Clcn7−/− and Clcn7unc/unc mice [6,14] represents a novel in vivo structure-function analysis of CIC-7/Ostm1 that complements similar in vitro studies focusing on biophysical properties [7,24]. Comparison of the pathologies of these mice
Supplementary Table S1 yielded a surprisingly complex picture of the roles of ClC-7 in lysosome, osteoclast and melanocyte biology. Phenotypes resulting from Clcn7 mutations cannot be assigned exclusively to a loss of ion transport activity. As vesicular CLCs may shunt proton pump currents in the endolysosomal system and at the osteoclast resorption lacuna [2,8,9,14], we assumed that a similar shunt by the CIC-7unc Cl− conductance may rescue some of the pathologies of Clcn7−/− mice. Comparison of the present Clcn7+/−/− with Clcn7−/− and Clcn7unc/unc mice showed that this holds true for osteopetrosis, but not for the changed fur colour or neurodegeneration. The severity of osteopetrosis, which is less severe in Clcn7unc/unc mice than in the other two

**Figure 4.** Osteopetrosis in Clcn7+/−/− mice. 
A Micro-CT revealed similar osteopetrosis of tibiae of 3-week-old Clcn7+/− and Clcn7+/−/− mice (scale bar: 1 mm).
B Micro-CT image of skull from P22 Clcn7+/+/− mouse showed impaired tooth eruption (scale bar: 10 mm).
C Similarly, increased bone volume fraction of proximal tibia metaphyseal trabecular bone in Clcn7+/−/− mice and Clcn7−/− mice. BV, bone volume; TV, tissue volume. Student’s t-test was applied; n.s., not significant. Error bars denote s.e.m.
D Electron microscopy showed mature, immature and lacking ruffled borders of Clcn7+/−/− osteoclasts despite the presence of a sealing zone (SZ). Arrows point at ruffled borders (scale bar: 5 μm) (Bo = bone; N = nucleus).
E Percentage of WT, Clcn7+/−/−, Clcn7−/−, Clcn7−/−/− osteoclasts exhibiting absent, immature or mature ruffled borders. Error bars denote s.e.m.
Stefanie Weinert et al In vivo structure-function of CIC-7 transporter

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but also its ion transport activity. A Cl
not only require the presence of the ClC-7/Ostm1 protein complex, border. Hence, the formation of this acid-secreting membrane does mouse models, correlated with the malformation of the ruffled border. Hence, the formation of this acid-secreting membrane does not only require the presence of the CIC-7/Ostm1 protein complex, but also its ion transport activity. A Cl
conductance can only partially substitute for 2Cl
(Supplementary Table S1). Comparison of Clcn7
Ostm1, revealed a detrimental effect of the CIC-7
Cl
transporter on neurons, in stark contrast to its positive influence on osteoclasts. A toxic effect of the CIC-7
Cl
conductance on neurons is also indicated by the fact that heterozygous Clcn7
/unc
mice display neurodegeneration [6]. In all three genotypes (Clcn7
/unc
, unc/unc or td/td), lysosomal pH is normal and lysosomal Cl
concentrations are similarly decreased [6], eliminating these parameters as explanations for differences in lysosomal pathology. However, there may be differential effects on lysosomal voltage that may influence transmembrane transport processes and possibly membrane budding and fusion. Reductionist model calculations predict a lumen-positive potential (~20 mV) with a Cl
channel, but a lumen-negative potential with a 2Cl
/H
exchanger [6]. Moreover, the CIC-7 unc mutation (E245A) [7] not only uncouples Cl
transport from H
transport, but also abolishes voltage- and time-dependent gating [3–5,7,24–26]. WT CIC-7 almost lacks transport activity at cytoplasmic negative (i.e. lumen-positive) potentials. Currents increase steeply when cytoplasmic voltage exceeds approximately ~20 mV [7]. Hence, the unc mutation will robustly increase steady-state CIC-7 currents in lysosomes. Moreover, the slow gating of WT CIC-7/Ostm1 [7] would prevent a full activation of CIC-7 during transient inside-negative voltage excursions that may occur, for example, upon NAADP-induced Ca
release [27,28]. Intriguingly, many pathogenic CLCN7 mutations [7,17,29] accelerate CIC-7/Ostm1 gating, suggesting that early exchange currents may be pathogenic. CIC-7
currents respond instantaneously to voltage and may thus be more harmful than those from accelerating mutants expressed in patients.

The beneficial effect of CIC-7
/Ostm1 on melanocytes and neurons raises the question whether it is totally transport deficient as assumed above. We cannot exclude that the mutant mediates currents below our detection limit of about 3% of WT. If small currents remain in CIC-7
mutants, they likely resemble CIC-7
currents because similar mutations in EcClC-1 convert this bacterial 2Cl
/H
exchanger into a pure Cl
conductance [30]. As CIC-7
currents are detrimental for neurons, we conclude that indeed the CIC-7
/Ostm1 complex itself, and not a putative ion transport activity, is beneficial for neurons and by extension for melanocytes. Identifying novel binding partners for CIC-7/Ostm1 that may explain these beneficial effects is a daunting task for future investigations.

**Materials and Methods**

Detailed methods can be found in Supplementary Materials and Methods. See Supplementary Table S3 for number of animals/cell lines used for experiments.

**Mice**

Clcn7
[14] and Clcn7
unc/unc
mice [6] have been described. Grey-lethal (Ostm1
/–
) mice [20] were from Jackson Laboratories. Clcn7
nd/nd
mice were generated by homologous recombination using a construct in which the E312A mutation was inserted into exon 11 of Clcn7. Animals were housed under standard conditions in the MDC animal facility according to institutional guidelines and kept on a 12-h light/dark cycle. LAGEso, Berlin, Germany, approved all experimental procedures.
Antibodies

Primary antibodies used can be found in Supplementary Table S2. Secondary antibodies were coupled to Alexa Fluor 488, 546 (Invitrogen) or HRP (Jackson ImmunoResearch).

Membrane preparation, tissue homogenates and immunoblot

Brain extracts were prepared from adult mice, blotted on PVDF membrane and probed according to standard procedures.

Histology and electron microscopy

Sections were stained with H&E, Nissl, periodic acid Schiff reagent (PAS), indicated antibodies and for lysosomal acid phosphatase activity. For EM, mice were perfused with 4% (w/v) PFA and 2.5% (PAS), indicated antibodies and for lysosomal acid phosphatase postfixed in 2% (v/v) OsO₄, dehydrated and embedded in epon. Semi-thin sections (0.5 μm) were labelled with toluidine blue. Ultrathin sections (60 nm) were stained with uranyl acetate and lead citrate.

Microcomputed tomography (CT)

PFA-fixed tibiae were analysed with a Skyscan 1172 μCT (Bruker-MicroCT) at 7 μm resolution. A ROI of 350 μm situated 200 μm below the growth plate comprising the secondary spongiosa was evaluated using the CTAn software with a lower grey threshold of 30 (Bruker-MicroCT). 3D reconstruction was done by the AMIRA software package (Visualization Sciences Group).

WNT stimulation of primary cells

Primary fibroblasts were starved > 6 h in DMEM containing 0.1% (w/v) BSA and stimulated with 80 ng/ml recombinant murine (rm) Wnt-3A (CF, R&D Systems) overnight in growth medium. Melanocytes were starved overnight in MEM Eagle containing 0.1% BSA and stimulated overnight with 80 ng/ml rmWnt-3A in MEM Eagle containing 10% (v/v) FBS (all Pan-Biotech) and 200 nM TPA.

Determination of lysosomal pH

Lysosomal pH was measured by ratiometric fluorescence imaging of the pH sensor Oregon Green dextran 488 (Invitrogen) as described [6].

Determination of relative lysosomal chloride concentrations

Lysosomal chloride was measured by ratiometric fluorescence live cell imaging of MEQ/TMR-dextran [6].

Supplementary information for this article is available online: http://embor.embopress.org

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Author contributions

SW generated knock-in mice, performed biochemical experiments, immunohistochemistry and measured lysosomal pH; SJ measured lysosomal [Cl⁻] and investigated Wnt signalling; SH performed electron microscopy, UK, and WLC performed μ-CT and bone characterization; TJ planned the study; and TJ, SW and SJ wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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