Chapter 15
The Role of the Endosomal Chloride/Proton Antiporter ClC-5 in Proximal Tubule Endocytosis and Kidney Physiology

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Abstract:
The chloride channel (CLC) protein family comprises ion channels and proton-coupled anion transporters with fundamental physiological roles in humans. Several properties of CLC proteins defy the rigid dichotomy between ion channels and transporters as these opposite thermodynamic mechanisms of transport are implemented in a very similar structural architecture. All the CLC transporters are expressed in intracellular organelles where they are somehow important for the ionic homeostasis of these compartments. However, their specific physiological role is still unclear. This chapter focuses on the biophysical properties and physiological role of the endosomal Cl^-/H^+ antiporter ClC-5 mutated in Dent's disease.

Keywords: CLC proteins, ClC-5, Endosomal physiology, Cl^- transport, Dent's disease, Proximal tubule.

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15.1 Introduction

15.1.1 The Physiological Relevance of Chloride Transport

Chloride is the most abundant anion in the human body but for a long time its role in human physiology has been overlooked probably due to the initial development of the field of membrane transport around the topic of excitable cells, with a prominent role of sodium and potassium channels (Miller, 2006). However, Cl^- ions are fundamental in many different physiological contexts. They ensure electroneutrality during Na^+ and K^+ transport across cellular membranes in different epithelia and in intracellular compartments. Owed to its high extracellular concentration, Cl^- serves, together with positively charged counterions, as an important osmolyte to drive water movement across cellular membranes, and as a hyperpolarizing conductance in several tissues. These functions are mediated by several unrelated Cl^- channel families which include the cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan et al., 1989), pentameric ligand-gated anion channels, like GABA- and glycine-receptors (Grenningloh et al., 1987), the Ca^{2+}-activated Cl^- channels of the bestrophin (Sun et al., 2002, Qu et al., 2003) and TMEM16 (or Anoctamin) (Schroeder et al., 2008, Caputo et al., 2008, Yang et al., 2008) gene families, and finally the Leucine-rich repeat-containing protein 8 (LRRC8) proteins, that form volume-regulated anion channels (VRACs) (Voss et al., 2014). Underscoring their physiological relevance, most of these protein families are involved in genetic diseases. This chapter focuses on the role of the endosomal Cl^-/H^+ antiporter ClC-5 (gene name *CLCN5*) which belongs to the CLC protein family (Fig. 15.1) (Zifarelli and Pusch, 2007, Stauber et al., 2012, Jentsch and Pusch, 2018). We will first introduce the general structural architecture and mechanism of ion transport of CLC proteins, and then illustrate in detail the biophysical properties and physiological roles of ClC-5 in the kidney and in other tissues.

15.1.2 The CLC Protein Family

ClC-0 was the first member of the CLC protein family to be identified at the molecular level by the group of Chris Miller, upon reconstitution of ray *Torpedo* electric organ membranes into lipid bilayers, (White and Miller, 1979). The distinct single channel behavior of ClC-0 with two levels of conductance of identical amplitude suggested, more than 20 years before the first structure of a CLC protein was available, that the protein assembled as a dimer of identical subunits, each containing a voltage-dependent gating mechanism called protopore gate or fast gate (Miller, 1982). In addition, there is a second gating mechanism which acts simultaneously on both subunits, defined common or slow gate because in ClC-0 its time course is order of magnitude slower than the fast gate (Miller and White, 1984). These pioneering findings were followed by the cloning of the first human member of the CLC protein family in the lab of Thomas Jentsch, the skeletal muscle Cl^- channel ClC-1 (Jentsch et al., 1990) and, afterwards, of all the other human CLCs (Fig. 15.1). Personal accounts of Jentsch's and Miller's initial discoveries can be found in two recent reviews (Jentsch, 2015, Miller, 2015). More recently, it has been recognized that out of the nine human CLC proteins, ClC-1, ClC-2 and the kidney ClC-Ka and -Kb are ion channels, whereas ClC-3 to ClC-7 are coupled 2 Cl^-/1 H^+ antiporters. Several CLC proteins are mutated in human genetic diseases emphasizing their physiological relevance (Jentsch and Pusch, 2018). The presence of channels and transporters, operating according to opposite thermodynamic basis in the same protein family, is one of the unique features of CLCs. Intriguingly, all the CLC ion channels are expressed in the plasma membrane, whereas all the antiporters are expressed in intracellular organelles or vesicles. Among the channels, ClC-1 is predominantly expressed in the skeletal muscle, ClC-2 has a broad tissue distribution and associates with the accessory β-subunit glialCAM (glial cell adhesion molecule), which influences protein targeting and kinetic properties of ClC-2 (Jeworutzki et al., 2012,

Hoegg-Beiler et al., 2014). ClC-Ka and ClC-Kb are tissue specific (kidney and ear), and require the β-subunit Barttin for proper functional expression (Estévez et al., 2001). Among the CLC antiporters, it has been reported that also ClC-7 requires the accessory β-subunit Ostm1 (osteoclastogenesis associated transmembrane protein 1) for proper functional expression (Lange et al., 2006, Leisle et al., 2011). However, very recent results seem to indicate that Ostm1 might be dispensable for the transport activity of ClC-7 (Pusch and Zifarelli, unpublished results).

15.2 Structures of CLC channels and transporters

There is now a substantial amount of structural information available on CLC proteins, either transporters (Dutzler et al., 2002, Dutzler et al., 2003, Feng et al., 2010) or ion channels (Park et al., 2017, Park and MacKinnon, 2018) showing that the architecture of transporters and channels is remarkably similar, in spite of the diverging functions. CLC proteins are dimers with an independent ion permeation pathway in each subunit. Three anion binding sites, S_{ext}, S_{cen} and S_{int} mark the anion permeation pathway through each subunit. In the structure of WT EcClC-1 (Dutzler et al., 2002), S_{ext} is occupied by the side chain of the so called "gating glutamate", E_{gate} (Fig. 15.2). Protonation and displacement of the E_{gate} side chain is necessary for Cl^- and proton transport. In fact, neutralization of E_{gate} in CLC transporters results in uncoupled passive Cl^- flux (Leisle et al., 2011, Accardi and Miller, 2004, Picollo and Pusch, 2005, Scheel et al., 2005, Neagoe et al., 2010), and in channels it abolishes both fast and slow gating transitions (Jentsch and Pusch, 2018). In the crystal structure of the EcClC-1 E148Q mutant (Dutzler et al., 2003) the Gln side chain points towards the extracellular space, and S_{ext} is occupied by a Cl^- ion, suggesting that this represents a conductive conformation of the permeation pathway. In the structure of CmClC (Feng et al., 2010), the side chain of E_{gate} occupies S_{cen}. S_{cen} is formed by the main chain nitrogen atoms of residues of the re-entrant loop alpha helices M and N (Ile 356 and Phe 357 in the bacterial EcClC-1) and by the side chains of two conserved residues, Ser_{cen} and Tyr_{cen}, forming a narrow constriction of the

pore between the two chloride binding sites S_{cen} and S_{int} (Fig. 15.2). It has been proposed that these two residues form an intracellular gate, which regulates access to the central binding site from the intracellular solution in both CLC channels and transporters (Jayaram et al., 2008). Functional support for this notion came from a study in EcClC-1 in which mutations of Y445 with smaller amino acids produce a progressive uncoupling of Cl^-/H^+ transport, together with a Cl^- movement approaching diffusion-limited rates (Accardi et al., 2006, Lobet and Dutzler, 2006, Jayaram et al., 2008). Further evidence, in favour of a role of Y445 as the intracellular gate has come from the finding that conformational changes outside the permeation pathway affect transport rate by a physical interaction with this residue (Basilio et al., 2014). Intriguingly, substitutions of the corresponding tyrosine residue in ClC-0 (Y512) does not affect the single-channel conductance (Accardi and Pusch, 2003). The serine residue at S_{cen} is conserved in all mammalian CLCs and has a critical role in selectivity. Substitution with proline, the corresponding residue found in the plant NO_3^-/H^+ antiporter AtClC-a (De Angeli et al., 2006), turns ClC-5 into a NO_3^-/H^+ antiporter while preserving the 2:1 anion/H^+ stoichiometry (Zifarelli and Pusch, 2009a). Consistent with this, NO_3^-/Cl^- relative current amplitude was increased by the same mutation in ClC-7 (Leisle et al., 2011), ClC-0 (Picollo et al., 2009, Bergsdorf et al., 2009) and ClC-6 (Neagoe et al., 2010). S_{ext} does not seem to be involved in transport stoichiometry, but at least in ClC-5 it can modulate anion selectivity as neutralization of a conserved lysine residue (K210), located close to the gating glutamate E211, inverted the WT selectivity of NO_3^- over Cl^- (De Stefano et al., 2011). The recent structures of two CLC channels, the human isoform of ClC-1 and the bovine isoform of the kidney ClC-Ka and -Kb channels, confirmed that the general structural architecture is the same in both channels and transporters, but revealed an important difference for the central binding site of ClC-K (Park et al., 2017); here Ser_{cen} does not project into the permeation pathway like in the other structures, but rather sideways, leading to a removal of the constriction formed by Ser_{cen} and Tyr_{cen} in ClC-1 (Park and MacKinnon, 2018) and in the transporters (Dutzler et al., 2002, Feng et al., 2010). The different conformation of Ser_{cen} in ClC-K

might be a consequence of the presence of bulky hydrophobic residues in the upper part of S_{cen}, with a potential steric clash on the Ser_{cen} (Park et al., 2017, Lagostena et al., 2019).

15.2.1 Proton Transport

Extracellular protons can directly reach E_{gate} from the extracellular side. From the intracellular side, it is likely that protons reach E_{gate} when it occupies S_{cen} (Feng et al., 2010), but the precise H^+ pathway is still unclear. A relatively conserved glutamate residue, the proton glutamate, E_{prot}, located close to the intracellular solution, but not directly in the permeation pathway, is probably involved in shuttling protons from the intracellular side to S_{cen} (Accardi et al., 2005, Lim and Miller, 2009), and it has been suggested that this process involves water wires in the intracellular cavity of the pore (Han et al., 2014). Importantly, a glutamate residue is not strictly necessary to serve this function, as in CmClC this residue is replaced by a threonine (Feng et al., 2010), and in the bacterial transporter homologue CkClC-2 (distantly related to EcClC-1) is an isoleucine (Phillips et al., 2012). In addition, in CLC-5 and other mammalian CLC transporters, mutations of E_{prot} preserve H^+ transport (Zdebik et al., 2008, Grieschat and Alekov, 2012, Guzman et al., 2013). Regarding the proton glutamate, ClC-5 differs significantly from EcClC-1. Indeed, neutralizing E_{prot} (E203) in EcClC-1 eliminates H^+ transport and renders the transporter a passively conducting Cl^- conductance (Accardi et al., 2005). In contrast to this, in ClC-5, the E268A mutation completely abolishes steady state transport of Cl^- and protons (Zdebik et al., 2008). The reason for this discrepancy is still unclear, but the behaviour of ClC-5 is in a more intuitive agreement with the idea of an obligatory coupling mechanism underlying the antiport activity of CLC transporters, which would posit that proton delivery via E_{prot} is a necessary step for the transport cycle. Interestingly, in ClC-5 the proton glutamate mutation E268A exhibits large “transient” capacitive currents that reflect partial reactions of the transport cycle (Smith and Lippiat, 2010, Zifarelli et al., 2012).

15.2.2 CBS (cystathionine beta synthase) Cytoplasmic Domains of ClC-5

ClC-5, like all eukaryotic CLCs, has an extended cytoplasmic C-terminal region that contains two CBS domains, CBS-1 and CBS-2, named after similar domains identified in cystathionine-synthetase (Bateman, 1997). CBS domains are found in many different protein families as nucleotide binding modules. In the crystal structure of the isolated C-terminal fragment of ClC-5, ATP or AMP are bound in the cleft between the two CBS subdomains, engaging mostly the adenine base of the nucleotides, consistent with the similar binding affinity observed for ATP, ADP, and AMP (Meyer et al., 2007). In electrophysiological experiments, adenine nucleotides applied from the intracellular side in inside-out patch-clamp experiments on ClC-5 lead to a doubling of the current with an apparent affinity of ∼1 mM for ATP, AMP, and ADP (Zifarelli and Pusch, 2009b). Functional regulation by nucleotides was also shown for ClC-1 (Bennetts et al., 2007). The crystal structure of the eukaryotic CmClC showed that the CBS domains, and in particular CBS-2, come into close contact with the transmembrane region of the transporter, potentially affecting transport function (Feng et al., 2010, Duffield et al., 2003, Bennetts and Parker, 2013). However, the similar effect of ATP, ADP and AMP suggests that nucleotide binding might not have any physiological relevance for ClC-5, even though it cannot be excluded that regulation by nucleotide binding might become relevant *in vivo*.

15.3 Gating

15.3.1 Gating Mechanism in the CLC Transporters

Two gating mechanisms operate in CLC channels: a fast gate, that can be mostly attributed to E_{gate} and controls the opening of each of the subunits independently (Miller, 1982, Ludewig et al., 1996, Accardi

and Pusch, 2003); and a slow, or common gate, which acts simultaneously on both subunits (White and Miller, 1979, Miller and White, 1980, Pusch et al., 1997), although it has not been identified at the molecular level yet. Notably, in CLC channels, gating and permeation are not as clearly distinguishable as in other channel families, and Cl^- and other permeable and impermeable anions (Pusch et al., 1999, Rychkov et al., 1998), but also protons (Lísal and Maduke, 2008), have an effect on gating. This has led to the suggestion that CLC channels are in fact broken Cl^-/H^+ antiporters (Lísal and Maduke, 2008, Miller, 2006). Intriguingly, a gating process is displayed also by some CLC transporters, and consists in a mechanism that regulates the transitions between an actively transporting configuration and an inactive, non-transporting, state (Fig. 15.3). This concept is not typical in the description of transporters since the distinction between gating and permeation is in general very difficult in these proteins. In this regard, a fundamental question is if the extreme outward rectification of ClC-5 (Steinmeyer et al., 1995), but also ClC-7 (Leisle et al., 2011), ClC-4 (Friedrich et al., 1999), and ClC-3 (Li et al., 2000), reflects a gating process or, rather, a rectifying transport cycle. Interestingly, the gating glutamate mutation E211A not only converts ClC-5 into a passive Cl^- conductance, but also eliminates the rectification and any relaxation kinetics of the currents elicited by voltage steps, suggesting that at least the Cl^- pathway of the transporter is not intrinsically rectifying. The lack of "gating" kinetics in this uncoupled mutant, parallels the behavior of the equivalent E_{gate} mutation in the ClC-0 channel, which completely eliminates not only the fast gate, but also the slow gate of the channel (Dutzler et al., 2003, Traverso et al., 2006). A first indication of the presence of a gating mechanism in ClC-5 came from the analysis of the spectral properties of the currents' noise, which showed a channel-like Lorentzian shape (Zdebik et al., 2008). This is consistent with a transporter that switches from active, transporting states to inactive states (Zdebik et al., 2008), in a manner similar to the opening of single channels, that is determined by the transition from closed to open state. The presence of a gating mechanism in ClC-5 was also suggested on the basis of the deactivation of the currents at positive potentials (Alekov and Fahlke, 2009). However, the most direct evidence that a gating process in ClC-5 is responsible for its outward rectification, was

recently obtained studying the single point mutation D76H (De Stefano et al., 2013). D76 is one of the most conserved residues in ClC-5 and mutating it in ClC-0, ClC-1 or ClC-Kb leads to dramatic changes in gating properties (Fahlke et al., 1995, Ludewig et al., 1997, Picollo et al., 2004). The D76H mutant expressed in *Xenopus* oocytes significantly slowed the activation of outward currents and, particularly at acidic extracellular pH, displayed clear inwardly directed tail-currents, after an activating voltage pulse. A detailed analysis of the tail currents revealed that they reflect a gating process that underlies, at least partially, the strong outward rectification of the transporter (De Stefano et al., 2013). Recently, a gating process was observed also for the lysosomal ClC-7/Ostm1 transporter (Leisle et al., 2011). Like ClC-5, also ClC-7 currents are strongly outwardly rectifying, but, in addition, they have an extremely slow activation kinetics at positive voltages, and decay also quite slowly at negative voltages. It has been suggested that the gating process of ClC-7 corresponds to the common gate of CLC channels, i.e. involves conformational changes of both protopores (Ludwig et al., 2013). Physiologically, the gating process might be important because it restricts the activity of the transporter in a limited voltage range, but we still know very little about the membrane voltages across intracellular organelles, and in general whether the properties identified from the expression of ClC-5 and ClC-7 in the plasma membrane recapitulate the ones in native organelles.

15.4 Transport Mechanism

15.4.1 Stoichiometry

As already mentioned, CLC channels and antiporters share very similar structural features. In addition to the fact that the first members functionally investigated were Cl^- channels, this led to the initial assumption that all CLC proteins were channels. However, in 2004, the electrophysiological analysis of EcClC-1 (the first homologue to be crystallized) with a detailed study of the ionic dependence of the

reversal potential of the currents, showed that this protein was not a Cl^- channel, but instead a 2 Cl^-/1 H^+ antiporter (Accardi et al., 2004). It was later shown that also ClC-4 and ClC-5 (Picollo and Pusch, 2005, Scheel et al., 2005), ClC-7 (Graves et al., 2008, Leisle et al., 2011), ClC-3 (Guzman et al., 2013) and ClC-6 (Neagoe et al., 2010) were in fact Cl^-/H^+ antiporters. The coupling ratio of Cl^- and H^+, i.e. the transport stoichiometry, has been much harder to dissect for ClC-5, because of the strong outward rectification of the currents which made measurements of reversal potential impossible. This problem was circumvented combining electrophysiological measurements with fluorescence-based measurements of proton flux, allowing the demonstration of a 2 Cl^-/1 H^+ stoichiometry also for ClC-5 (Zifarelli and Pusch, 2009a).

15.4.2 Transport Cycle

The combination of structural and functional information on several CLC antiporters has allowed to propose at least a schematic model of their transport cycle applicable also to ClC-5 (Feng et al., 2010), and represented in Fig. 15.3. In state (A), the protonated side chain of E211 (in red) is displaced upwards from the permeation pathway, towards the extracellular space, and all the binding sites are occupied by Cl^- ions (green circles). E211 is then deprotonated and a Cl^- ion moves from S_{ext} to the extracellular space in state (B). The deprotonated E211 side chain then moves back to S_{ext} in state (C) and then to S_{cen} in state (D). In this conformation the side chain of E211 can be protonated from the intracellular side in state (E) and moves to the extracellular space in state (F), while a Cl^- ion moves to the extracellular space. The empty binding sites can then be occupied by two Cl^- ions from the extracellular space. From one of these states, there is a gating transition to an ‘inactive’ state, in which transport is not permitted. The ‘pre-steady state’ currents, observed in the ClC-5 E268A mutant (Smith and Lippiat, 2010, Zifarelli et al., 2012) correspond to transitions between states (C) and (E). A characteristic feature of this model is that Cl^-/H^+ coupling is obtained by a “kinetic” mechanism in which intrinsically uncoupled states must be

short-lived. However, Accardi and colleagues proposed that coupling is a consequence of the thermodynamic stoichiometry of substrate binding (Picollo et al., 2012, Basilio et al., 2014).

15.5 Role of ClC-5 in Endosomal Physiology and Kidney Function

15.5.1 ClC-5 Localization in Renal Epithelia

ClC-5 is abundantly expressed in the kidney (mainly in the nephron), but it is also expressed in intestine, thyroid and, in smaller amount, in liver and brain (Günther et al., 1998, Devuyst et al., 1999, Sakamoto et al., 1999, Steinmeyer et al., 1995, van den Hove et al., 2006). In the kidney, ClC-5 is expressed in all three different segments of the proximal tubule, in the collecting duct and, less abundantly, in the thick ascending limb of Henle's loop (Obermuller et al., 1998, Devuyst et al., 1999). In the proximal tubule, ClC-5 is mainly found in subapical endosomes of epithelial cells, below the brush border, with a small fraction also observed in the apical membrane (Günther et al., 1998, Devuyst et al., 1999). In subapical endosomes, ClC-5 co-localizes with the V-type ATPase (Fig. 15.4), and for a long time it was thought to provide an electrical shunt for the proton pump, in order to allow proper acidification of endosomes (Günther et al., 1998, Sakamoto et al., 1999). Interestingly, in the collecting duct, ClC-5 co-localizes with V-ATPases in apical vesicles only in acid-secreting α-intercalated cells (Obermuller et al., 1998, Günther et al., 1998, Sakamoto et al., 1999), whereas it is mainly found in the cytoplasm in base-secreting β-intercalated cells (Günther et al., 1998, Sakamoto et al., 1999). In general, the physiological role of ClC-5 in these segments of the nephron is still unclear.

15.5.2 Sorting and degradation processes of ClC-5

The mechanism regulating the subcellular targeting of ClC-5 remains still poorly understood. ClC-5 is predominantly localized in early endosomes of proximal tubule cells (Piwon et al., 2000, Suzuki et al., 2006) where it co-localizes with the GTPases Rab4 (Vandewalle et al., 2001) and Rab5a proteins (Devuyst et al., 1999, Günther et al., 1998, Sakamoto et al., 1999) (Luyckx et al., 1998). ClC-5 is also expressed in other intracellular organelles, such as recycling endosomes (Günther et al., 1998), partially overlapping with the closely related ClC-3 (Maritzen et al., 2008) and ClC-4 (Mohammad-Panah et al., 2009). Over the last decades, ClC-5 has been proposed to regulate the intracellular acidification along the endosomal pathway (Günther et al., 1998, Sakamoto et al., 1999) (Fig. 15.4). However, its physiological role in such organelles has not been fully elucidated so far. Concerning the degradation pathway of ClC-5, the linker region between the two intracellular CBS domains, contains a putative "PY motif", which is not conserved in the closely related ClC-3 and ClC-4 (Schwake et al., 2001). Such motif has been suggested as a specific target for ubiquitin-mediated degradation, at least in heterologous expression system (Schwake et al., 2001). This internalization process is probably activated upon interaction of the PY-motif with the E3 ubiquitin ligases (such as WWP2 and Nedd4-2) (Pirozzi et al., 1997). In line with such hypothesis, overexpression of a nonfunctional mutant of the WWP2 ligase or of ClC-5 with neutralizing mutations of the PY-motif in *Xenopus* oocytes, lead to a doubling of the ClC-5 mediated currents (Schwake et al., 2001). In conclusion, the PY-motif appears to be important for both endocytotic uptake and degradation process (Schwake et al., 2001), as also suggested by *in vitro* ubiquitination experiments of ClC-5 (Hryciw et al., 2004). However, the biological role of the PY motif in protein sorting and degradation *in vivo* is still not entirely clear. Indeed, no effect was observed in the subcellular localization of ClC-5, as well as its endocytotic mechanism was apparently not altered in a *knock in* (KI) mouse model after disruption of the PY-motif (Rickheit et al., 2010).

15.5.3 ClC-5 and Dent's Disease

Dent's disease is an X-linked recessive chronic kidney malfunction, and a type of renal Fanconi disorder, defined for the first time in 1994 (Wrong et al., 1994, Fisher et al., 1994) and, at first, exclusively linked to mutations in the gene coding for ClC-5 (Steinmeyer et al., 1995, Lloyd et al., 1996). Dent's disease patients are affected by malfunctions of the proximal renal tubule, leading to progressive proteinuria, hyperphosphaturia, hypercalciuria and nephrolithiasis and consequent renal failure (Dent and Friedman, 1964, Fisher et al., 1994, Lloyd et al., 1996, Pook et al., 1993). Female carriers show in general a milder phenotype possibly due to random X chromosome inactivation (Wrong et al., 1994, Reinhart et al., 1995). The most common Dent's disease symptoms concern defects in the reabsorption of proteins and salts in the proximal tubule, with consequent low molecular weight proteinuria (LMWP). Less consistently, patients present with excess of calcium in the urine (hypercalciuria) and calcium deposits in the kidney (nephrocalcinosis) (Cox et al., 1999). The symptoms typically appear in early childhood and progressively exacerbate potentially leading to end-stage renal disease in early to mid-adulthood. Secondary symptoms, such as the presence of rickets, have been additionally described in some patients (Igarashi et al., 1998). Interestingly, only 60% of patients with Dent's disease carry mutations in ClC-5. In 15% of patients, Dent's disease is instead correlated with mutations in the *OCRL1* gene (Hoopes et al., 2004). Intriguingly, in the remaining cases no mutations in either of the two genes have been described, suggesting the possible implication of other gene(s) (Hoopes et al., 2005, Wu et al., 2009). This aspect will be further discussed in section 15.6.

15.5.4 ClC-5 *Knock out* Mice Reveal Dent's Disease Mechanism

The pathophysiological mechanism leading to Dent's disease has been extensively studied through the characterization of ClC-5 *knock out* (KO) animal models, independently generated by two research groups, and referred to as "Jentsch" and "Guggino" mice (Piwon et al., 2000, Wang et al., 2000).

15.5.4.1 Proteinuria

Low-molecular weight proteinuria seems to be caused by impaired endocytosis and uptake of proteins in the proximal renal tubule. A defect in megalin expression, as in the case of ClC-5 KO mice, might be caused by an impaired endosomal recycling (Maritzen et al., 2006, van den Hove et al., 2006). Indeed, in proximal tubule cells, the expression level of megalin was dramatically decreased after disruption of ClC-5 (Piwon et al., 2000). A similar effect was also observed for the megalin co-receptor cubulin in ClC-5 KO mice (Christensen et al., 2003). Megalin and cubulin are multiligand co-receptors localized on the apical brush-border membrane of proximal tubule epithelial cells (Christensen and Birn, 2002). After ligand-binding, the complex is internalized, via a clathrin-mediated endocytotic process, in order to fuse with endosomal intermediates, such as early endosomes, to be finally degraded into lysosomes or recycled. Both receptors are involved in tubular uptake of albumin and other low molecular weight proteins. Cubilin, which was first identified as the receptor for the intrinsic factor and Vitamin B12 in the intestine, was subsequently shown to play the same role in the proximal tubule as well (Christensen and Birn, 2002). Importantly, ClC-5 KO mice showed a defective trafficking in this nephron segment, with a parallel loss of megalin and cubulin at the apical brush-border membrane (Piwon et al., 2000, Christensen et al., 2003, Watanabe, 2004), together with impaired lysosomal formation (Reed et al., 2010). After budding of clathrin-coated vesicles, such complexes need to be transported and dissociated along the endo-lysosomal pathway. This dissociation is facilitated by progressive endosomal acidification, which was initially proposed to be defective in ClC-5 KO mice. However, the mouse KI model (ClC-5^{unc}) harboring a mutation of ClC-5 that turns it into a pure Cl^- conductance clearly indicates a different mechanism (Novarino et al., 2010). In fact, endosomal acidification was not impaired in ClC-5^{unc}, but still, endocytosis was dramatically reduced (Novarino et al., 2010). This suggests that the endosomal Cl^-

concentration and the resulting coupled H^+ transport, rather than mere acidification, might be the most important factor regulating endocytosis.

15.5.4.3 Hypercalciuria

The two different mouse models of Dent's disease (Jentsch and Guggino's) showed contrasting results in relation to hypercalciuria and nephrolithiasis. This is potentially explained by a complex regulation of calcium homestasis. The impaired megalin-mediated endocytosis of parathyroid hormone (PTH) results in increased luminal concentration of PTH and thus enhanced uptake through the PTH receptors (PTH-R) leading to intensified 1α-hydroxylase activity. In the proximal renal tubule, 1α-hydroxylase converts $25(OH)_2$-vitamin D_3 into its active form $1,25(OH)_2$-vitamin D_3 and the increased production of active vitamin D_3 would lead to increased renal and intestinal calcium absorption (Scheinman, 1998). However, the 25(OH)vitamin D_3/BDP complex is known to be endocytosed *via* interaction with megalin (Nykjaer et al., 1999), a mechanism which is impaired in *Clcn5*$^{-/-}$ proximal tubule cells. In this scenario, a decreased uptake of vitamin D_3 precursor would balance the enhanced 1α-hydroxylase activity, and might thus underlie the phenotype of the "Jentsch" mouse, which exhibited decreased $1,25(OH)_2$-vitamin D_3 levels and no hypercalciuria (Piwon et al., 2000). A proposed model for hypercalciuria in Dent's disease patients is shown in Fig. 15.5.

15.5.4.3 Night-blindness

Another phenotype concerns the loss of retinol (vitamin A) and of its plasma binding protein (RBP) in the urine, due to altered endocytosis (Piwon et al., 2000). Not surprisingly, reabsorption of retinol-RBP complexes in proximal renal tubule cells is mediated by megalin (Christensen and Birn, 2002).

Physiologically, after uptake, RBP is transported to lysosomes for degradation, thus releasing vitamin A, which can be secreted into the bloodstream. A defective endocytotic process can thus lead to a decreased availability of vitamin A, finally resulting in night blindness, described in several cases of Dent's disease with early age onset (Sethi et al., 2009).

15.5.4.4 Hyperphosphaturia

The increased level of PTH leads to enhanced expression of PTH receptors along the apical border of the proximal renal tubule. As result, the NaPi-2, sodium-phosphate cotransporter, is excessively endocytosed, and degraded into lysosomes (Piwon et al., 2000). The decreased availability of NaPi-2 will then lead to a decreased absorption of phosphate, finally resulting in hyperphosphaturia (Piwon et al., 2000). A schematic model for the mechanism involved in hyperphosphaturia is represented in Fig. 15.6.

15.5.4.5 Altered Ion and Water Absorption

The increased level of the PTH hormone causes also altered regulation (and enhanced endocytosis) of NHE3, a Na^+/H^+ exchanger (Piwon et al., 2000). Therefore, a lower expression of NHE3 is associated with a lower absorption of sodium, bicarbonate and water in the proximal renal tubule, consistently with the increased urine volume (polyuria) and the altered ion concentration observed among the Dent's disease phenotype (Piwon et al., 2000). A plethora of several other symptoms, such as glycosuria, uricosuria, aminoaciduria, kaliuresis and impaired urinary acidification, have been described along the typical Dent's disease manifestations (Dent and Friedman, 1964, Fisher et al., 1994, Lloyd et al., 1996,

Pook et al., 1993). In such scenario, altered transport and protein trafficking might be among the intrinsic causes of such symptoms, which are still poorly characterized.

15.5.6 Potential Binding Partner of ClC-5

An intriguing hypothesis is that ClC-5 might participate in assembling endocytotic complexes, thus acting as scaffold protein. Indeed, several proteins have been already proposed as ClC-5 binding partners (Stauber et al., 2012), among which, the kinesin-like protein KIF3B kinase. Indeed, the transport of ClC-5-containing vesicles along the microtubules appears mediated by KIF3B, as shown through co-immunoprecipitation experiments and confirmed by laser scan confocal live cells imaging (Reed et al., 2010). In addition, HEK293 cells overexpressing KIF3B showed increased amplitude (~ 45%) of ClC-5 currents (Reed et al., 2010). Thus, absence of ClC-5 in renal epithelial cells might result in reduced ClC-5/KIF3B interaction, leading to impaired microtubular trafficking of megalin and cubulin endocytotic vesicles, causing defective protein re-absorption and Dent's disease renal manifestations (Reed et al., 2010). However, the lack of *in vivo* confirmation prevents from validating such hypothesis.

15.5.7 ClC-5 Mutations and Their Phenotypes

More than 100 mutations, including splice site, missense and nonsense mutations of ClC-5 have been implicated in Dent's disease (Mansour-Hendili et al., 2015, Pusch and Zifarelli, 2014). Even though only a small fraction of missense mutations have been so far investigated in heterologous systems (Lloyd et al., 1996, Lourdel et al., 2012, Pusch and Zifarelli, 2014) or patient-derived cells (Gorvin et al., 2013), a tentative genotype-phenotype correlation has been suggested (Gorvin et al., 2013). Some mutations, including S270R, G513E, R516W and I524K are characterized by a strong reduction in current amplitude

(Ludwig et al., 2005). This is caused by enhanced retention or degradation of ClC-5 in the endoplasmic reticulum (ER) as indicated by laser scan confocal microscopy, as shown in co-immunolabelling experiments with calnexin ER-specific antibody (Smith et al., 2009, Ludwig et al., 2005, Grand et al., 2009, Grand et al., 2011). The E527D causes defective activity of ClC-5, resulting in a strong impairment of endosomal acidification and abolished currents, with little effect on ClC-5 subcellular distribution but this residue is relatively distant from the chloride and proton conduction pathways (Lloyd et al., 1997). Interestingly, the mutations G57V and R280P (both located close to the periphery of the subunit interface, facing the cytoplasm) show altered endosomal distribution (and reduced transport activity), but normal (for G57V) or enhanced (for R280P) endosomal acidification. In contrast to this, several mutations show a reduced (or even abolished) ion transport ability and impaired endosomal acidification, despite an unaltered cell surface expression. This is the case, for example, of the G212A mutant, which showed a normal plasma membrane distribution, comparable to that of WT ClC-5, but reduced transport current amplitudes (Grand et al., 2009) which is not too surprising considering that it is located close to the gating glutamate (E211) (Alekov, 2015). Also the E267A mutant (Hoopes et al., 2004) leads to a much reduced current amplitude (Alekov, 2015, Zdebik et al., 2008). In HEK cells both mutations altered the endosomal acidification observed upon expression of WT ClC-5 (Alekov, 2015).

15.5.8 ClC-5 Mutations in Patient-derived Cells Reveal Altered Endocytosis Without Effects on Endosomal Acidification

Two different groups found that for some mutations, altered endocytosis is not linked to defective endosomal acidification (Gorvin et al., 2013, Bignon et al., 2018). Gorvin et al. were able to generate conditionally immortalized proximal renal tubule cells (ciPTECs) derived from Dent's disease patients, carrying three ClC-5 mutations (30:insH, R637X and del132-241) were conditionally immortalized (Gorvin et al., 2013). Albumin and transferrin endocytotic uptake was severely reduced in 30:insH and

R637X ciPTECs mutants, while in del132-241, receptor-mediated endocytosis was completely abolished. Interestingly, live cell-imaging of a GFP-tagged pH sensor associated to membrane vesicles (pHluorin-VAMP2), revealed unaltered endosomal acidification in 30:insH condition, while significantly more alkaline pH was measured in R637X and del132-241 ciPTECs.

15.6 Other Proteins Involved in Dent's Disease

As previously mentioned, 15% of Dent's disease patients have mutations in the OCRL1 rather than the ClC-5 gene (Hoopes et al., 2004). OCRL1 encodes for a protein with phosphoinositol 4,5-biphosphate (PIP_2) 5-phosphatase activity, initially known to be mutated in the oculocerebrorenal Lowe syndrome. This is a multisystem disorder characterized by abnormalities affecting the eye, the nervous system and the kidney, where it leads to a Fanconi-type renal disorder (Lowe et al., 1952, Attree et al., 1992). In particular, some OCRL1 mutations have been described in Dent's disease of type 2, characterized by the same renal dysfunction occurring in Dent's disease of type 1, with no other additional symptoms (Hoopes et al., 2005). OCRL1 is a membrane-associated protein firstly found in the Golgi apparatus (Olivos-Glander et al., 1995, Dressman et al., 2000). More recently, OCRL1 has also been found in early endosomes where it is required for proper megalin trafficking (Vicinanza et al., 2011) but also in lysosomes (De Leo et al., 2016). Indeed, Lowe syndrome patients show reduced urine level of megalin and cubulin, consistent with a lower expression of these receptors on the apical membrane of the proximal renal tubule (Norden et al., 2002).

15.7 Summary

In conclusion, ClC-5 is an endosomal Cl^-/H^+ antiporter with a critical physiological role in proximal tubule endocytosis as highlighted by the fact that mutations in ClC-5 cause Dent's disease. The role of ClC-5 in the kidney has also been investigated in detail through KO and KI mice lines. The initial suggestion that ClC-5 provided only a Cl^- shunt conductance has been proved wrong and it is now clear that it is the coupled flux of Cl^- and H^+ across the endosomal membrane that underlies its relevance. However, the specific role of this ClC-5-mediated coupled flux for endosomal physiology remains elusive.

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Figure legends

Figure 15.1 Overview of the human CLC proteins. On the left, dendrogram showing that human CLCs can be grouped in three homology branches. The horizontal dashed line in blue separates ion channels and transporters as highlighted also by different background colours (white for ion channels and light blue for antiporters). The table indicates known β-subunits (in red), tissue distribution, known physiological functions, mouse and human pathologies observed upon disruption (mouse) or with mutations (humans) of the respective gene (adapted from Jentsch TJ and Pusch M, Physiol Rev 98: 1493-1590, 2018).

Figure 15.2 Structural architecture of CLC proteins. **A)** The crystal structure of the eukaryotic CmClC antiporter reveals the dimeric structure of CLC transporters viewed from the membrane plane (PDB: 3ORG). Each subunit of the dimer is depicted in different colours with the cytoplasmic C-terminal domains in darker shades. The structure indicates the Cl^- binding sites, S_{ext} and S_{int}, shown as green spheres and several residues important for transport function such as S165 (yellow), E210 (blue), T269 (purple) and Y515 (red) which in ClC-5 correspond to S168 (Ser_{cen}), E211 (E_{gate}), E268 (E_{prot}), and Y558 (Tyr_{cen}) of ClC-5, respectively. **B)** The insert indicates the anion permeation pathway for one subunit of the mutant E148Q from the crystal structure of the bacterial EcClC-1 (PDB: 1OTU). The residues represented as sticks are S107 (yellow), Q148 (blue), E203 (purple) and Y445 (red) which in ClC-5 correspond to S168 (Ser_{cen}), E211 (E_{gate}), E268 (E_{prot}), and Y558 (Tyr_{cen}), respectively. The so-called gating glutamate, E211 (E_{gate}) and the proton-glutamate, E268 (E_{prot}) of ClC-5 are indicated in bold. In the E148Q mutant, all three Cl^- binding sites (S_{ext}, S_{cen}, and S_{int}) are occupied by anions depicted as green spheres, and their position is highlighted by dashed lines.

Figure 15.3 Schematic representation of the ClC-5 transport cycle. Adapted from Pusch and Zifarelli (2014). See text for details.

Figure 15.4 Schematic representation of the putative role of ClC-5 in intracellular organelles' ionic homeostasis and trafficking. The V-type ATPase (in orange) and ClC-5 (in purple) are both expressed in early endosomes. After being endocytosed, ClC-5 can be transported to lysosomes, for degradation, and/or recycled back to the plasma membrane, where it will eventually take part in early endosomes formation. Adapted from Pusch and Zifarelli (2014).

Figure 15.5 Schematic model of hypercalciuria in Dent's disease. **A)** Representation of physiological events characterizing proximal tubule re-absorption. After glomerular filtration, the parathyroid hormone (PTH) is endocytosed in a megalin-mediated process. Similarly, the inactive form of vitamin D ($25(OH)D_3$, pink spheres) is endocytosed, in complex with the vitamin D-binding protein (DBP, purple pentagons), *via* megalin-binding. In normal conditions, the 25(OH)vitamin D_3 is then converted into its active form ($1,25(OH)_2D_3$) through the 1α-hydroxylase activity, in proximal renal tubule cells. Finally, the active vitamin D_3 can be transported in the interstitial fluid, thus regulating renal and intestinal calcium absorption. **B)** Model of hypercalciuria in Dent's disease. Adapted from Maritzen et al. (2006). See text for details.

Figure 15.6 Schematic model of hyperphosphaturia in Dent's disease. **A)** In physiological conditions, after glomerular filtration, the PTH (parathyroid hormone) is endocytosed and recycled *via* megalin uptake in the proximal renal tubule cells, and finally degraded into lysosomes. Its activity also regulates the uptake and degradation of the sodium-phosphate (NaPi-2) cotransporter. **B)** In Dent's disease, megalin availability is strongly reduced due to its impaired recycling process mediated by ClC-5. Thus, PTH activity is dramatically enhanced, resulting in a higher internalization rate of NaPi-2 leading to reduced phosphate absorption, and finally higher phosphate level in the urine. Adapted from Maritzen et al. (2006).