Rev Physiol Biochem Pharmacol (2007)
DOI 10.1007/112_2006_0605

G. Zifarelli · M. Pusch

CLC chloride channels and transporters: a biophysical and physiological perspective

Published online: 5 December 2006

Abstract Chloride-transporting proteins play fundamental roles in many tissues in the plasma membrane as well as in intracellular membranes. They have received increasing attention in the last years because crucial, and often unexpected and novel, physiological functions have been disclosed with gene-targeting approaches, X-ray crystallography, and biophysical analysis. CLC proteins form a gene family that comprises nine members in mammals, at least four of which are involved in human genetic diseases. The X-ray structure of the bacterial CLC homolog, ClC-ec1, revealed a complex fold and confirmed the anticipated homodimeric double-barreled architecture of CLC-proteins with two separate Cl^- ion transport pathways, one in each subunit. Four of the mammalian CLC proteins, ClC-1, ClC-2, ClC-Ka, and ClC-Kb, are chloride ion channels that fulfill their functional roles—stabilization of the membrane potential, transepithelial salt transport, and ion homeostasis—in the plasma membrane. The other five CLC proteins are predominantly expressed in intracellular organelles like endosomes and lysosomes, where they are probably important for a proper luminal acidification, in concert with the V-type H^+-ATPase. Surprisingly, ClC-4, ClC-5, and probably also ClC-3, are not Cl^- ion channels but exhibit significant Cl^-/H^+ antiporter activity, as does the bacterial homolog ClC-ec1 and the plant homolog AtCLCa. The physiological significance of the Cl^-/H^+ antiport activity remains to be established.

Overview and scope

The lipid bilayer that surrounds all living cells and the organelles inside eukaryotic cells presents, by virtue of its fatty nature, an insurmountable electrostatic barrier for the diffusive passage of small inorganic ions like Na^+, K^+, Ca^{2+}, Cl^- and also small organic ions like amino acids or HCO_3^-. To overcome this barrier and to allow the exchange of these substrates across the lipid bilayer in a controlled manner, nature has invented an incredible

G. Zifarelli · M. Pusch (✉)
CNR, Istituto di Biofisica,
Via De Marini 6, 16149 Genova, Italy
e-mail: pusch@ge.ibf.cnr.it · Tel.: +39-0106475-561/522 · Fax: +39-0106475-500

variety of different ion-transporting proteins, most of which allow the specific passage of only a very limited subset of ions. Transport proteins can be grossly subdivided into passive transporters and active transporters. Conceptually, passive transporters can be regarded as enzymes that lower the activation energy for passive diffusion across the lipid bilayer. The most important example of passive transporters are ion channels, which provide a selective pore that allows a high-throughput transport, close to the diffusion limit in some cases, while maintaining exquisite selectivity. Active transporters couple the energy of the translocation of one substrate, or other energy sources such as ATP hydrolysis, to the transport of another substrate, often in a strictly stoichiometric manner. One prominent example of this class of proteins are the familiar P-type ion pumps and ion cotransporters. Active transport is generally associated with the picture of an alternating access model of transport in which the transporter exposes its ion binding sites alternatively to one or the other side of the membrane (see Tanford 1983). According to this mechanism, one or a few substrate molecules are translocated for each transport cycle, leading to the slow transport rates seen for active transporters, compared to those of ion channels (Hille 2001). As a consequence, in general, the architecture of active transporter proteins (see, e.g., Abramson et al. 2003; Toyoshima et al. 2000) is quite different from that of ion channels (see, e.g., Doyle et al. 1998; Miyazawa et al. 2003).

The present review focuses on anion-selective channels and, in particular, on Cl^- channels from the CLC family (Jentsch et al. 2002). However, as described below, the same basic architecture in the CLC family of proteins (Jentsch et al. 2005c) can be used to produce either active transporters (Accardi and Miller 2004; De Angeli et al. 2006; Picollo and Pusch 2005; Scheel et al. 2005) or passive chloride channels (Bauer et al. 1991). Since a full appreciation of the physiological role of CLC proteins requires a molecular comprehension of their mechanism of transport, we will have to consider passive channel-mediated diffusion as well as the active antiport of protons and Cl^- ions.

It is important to note that the CLC family represents only one of several classes of proteins carrying out Cl^- transport. A detailed treatment of such a vast and variegated array is beyond the scope of this review, but we nevertheless provide a brief overview of the physiological roles of Cl^- channels not belonging to the CLC branch.

The transport of Cl^- (or any other ion) across the plasma membrane has two distinct consequences: transport of the substrate and transport of electrical charge. The transport of charge is fundamental for the regulation of excitability in nerve and muscle, whereas the transport of substrate is of paramount importance for epithelial physiology. In neurons and muscle cells the membrane potential, V_m, is one of the most critical physiological variables. The activation of closed Cl^- channels, or the inactivation of active Cl^- channels, changes V_m according to the equilibrium potential for Cl^-, E_{Cl}. In most cases, the intracellular Cl^- concentration ($[Cl^-]_{int}$) is low, such that E_{Cl} is very negative and close to or even more negative than E_K. Low $[Cl^-]_{int}$ is achieved by secondary active KCl cotransport proteins (Hübner et al. 2001). Thus Cl^- channel activity in nerve and muscle generally dampens excitability, stabilizing a negative membrane potential. For the dampening and stabilization of the membrane potential not only is the value of E_{Cl} important, but also the chloride conductance, g_{Cl}, relative to other conductances, that is, a large g_{Cl} associated with a slightly depolarized E_{Cl} will nevertheless impede strong depolarization caused by a (relatively) small depolarizing conductance. A typical example, the skeletal muscle Cl^- conductance that is provided by the ClC-1 Cl^- channel, is described in more detail below. In neurons, postsynaptic GABA and glycine receptors are the most important anion channels in the plasma membrane (Jentsch et al. 2002). The traditional view is that their activation suppresses excitation (i.e., action potential firing) of the postsynaptic cell. It is clearly beyond the scope of this review to

describe these neuronal channels in detail. However, we would like to mention that activation of GABA and glycine receptors is not always inhibitory: In the developing nervous system and in some specialized neuronal structures, $[Cl^-]_{int}$ is relatively high, leading to a paradoxical excitatory effect of receptor activation (Marty and Llano 2005; Misgeld et al. 1986). GABA and glycine receptors are poorly selective for Cl^-, showing a significant permeability even to cations (Wotring et al. 2003). Physiologically, the permeability to bicarbonate (HCO_3^-) seems to be of particular relevance as it significantly contributes to a rise of $[Cl^-]_{int}$ after GABA stimulation (see Marty and Llano 2005).

Apart from CLC proteins and GABA/glycine receptors, the only molecularly identified Cl^- channel is the "cystic fibrosis transmembrane conductance regulator," CFTR (Riordan et al. 1989). CFTR is a widely expressed, but mostly epithelial, Cl^- channel. Mutations in the gene coding for CFTR cause cystic fibrosis (Tsui 1991), one of the most common lethal genetic diseases. Structurally, CFTR belongs to the very large class of ABC transporters, but it seems to be the only channel member of this family of active transport proteins. Despite extensive research in the 15 years since its cloning, the molecular mechanisms of channel gating by protein kinase A and intracellular ATP and also its physiological role are still relatively unclear. Excellent reviews about many aspects of CFTR have been published recently (Guggino 2004; Hanrahan and Wioland 2004; Riordan 2005).

Several important anion conductances have been described in various mammalian cell types whose molecular identity is still unknown or in dispute. The most typical examples are the swelling-activated Cl^- channel, also known as VRAC (volume-regulated anion channel) (Eggermont et al. 2001), and various types of calcium-activated Cl^- channels. VRAC is probably present in all animal cells and is activated by cell swelling, but the molecular mechanism leading to its activation is unknown (Eggermont et al. 2001). This channel is also permeable to small organic solutes and has been proposed to be important for a process called regulatory volume decrease (RVD). Cellular volume regulation is essential for all cell types to respond to osmotic challenges caused by changes of the extracellular medium as well as to metabolically induced changes in intracellular osmolarity. The functional properties of VRAC have been extensively studied, and several proteins have been proposed as molecular correlates of VRAC, but none of these is generally accepted (see Jentsch et al. 2002).

Ca^{2+} activated Cl^- channels, CaCCs, are also found in many different cell systems including smooth muscle, epithelia, and olfactory receptors. Their activation, via an increase of intracellular $[Ca^{2+}]$, generally leads to cell depolarization and thus, for example, smooth muscle contraction or amplification of olfactory sensation (Hartzell et al. 2005). In epithelia, CaCC activation is responsible for transient Cl^- (and water) secretion, for example, in salivary glands. Similar to VRAC, several proteins have been proposed as molecular correlates of CaCCs, none of them being as yet fully accepted. Currently, the family of bestrophin proteins is under intense study as CaCC candidates (Hartzell et al. 2005), even though a definite proof of their identity is still missing (see, e.g., Rosenthal et al. 2006).

Another example of a Cl^- conductance for which the molecular association with a membrane protein is still lacking is the hyperpolarization- and cAMP-activated Cl^- current measured in choroid plexus cells (Kibble et al. 1996). This current superficially resembles ClC-2 currents, but is found unaltered in ClC-2 knockout mice (Speake et al. 2002). Other examples include an ATP-activated Cl^- current described in mouse parotid acinar cells (Arreola and Melvin 2003), and a proton-activated Cl^- channel (Nobles et al. 2004), both sharing some characteristics with VRAC.

Epithelial ion transporters are designed to allow massive but specific translocation of salts across the epithelial cell sheet. To allow for vectorial ion movement, transporters must

be expressed in a polarized manner. For example, the Na^+-K^+-ATPase is usually expressed on the basolateral membrane in epithelial cells. Thus it is important to understand the mechanisms underlying the correct targeting of chloride channels and transporters to the apical versus basolateral membrane. Very little is known about the targeting of the molecularly identified Cl^- channels (CLC channels, GABA/glycine receptors, CFTR), even though several putative partner proteins of CLC channels, possibly important for targeting, have been identified in recent years (Dhani and Bear 2006) and are described in some detail below.

Cl^- channels are not restricted to the plasma membrane but are also found in intracellular organelles. Relatively little is known about the intracellular Cl^- channels from in situ studies. This is largely explained by the inaccessibility of the small intracellular organelles to standard patch clamp techniques. As discussed in detail in later sections of this review, five of the nine mammalian CLC homologs reside in intracellular membranes, and their study thus opens new and promising perspectives for the understanding of the role of intracellular Cl^- channels and transporters.

The present review first describes the general mechanism underlying the function of CLC proteins and then focuses on the biophysical properties and physiological and pathophysiological roles of mammalian, and in particular human, CLC members. For the mechanistic aspects, two "model" CLCs have been most extensively studied. One is the *Torpedo* channel ClC-0, which, compared to many, physiologically more relevant, channels, has favorable biophysical properties, for example, a relatively large single-channel conductance, and whose mechanisms of gating are best understood. The other model CLC is, of course, the bacterial ClC-ec1, for which we have detailed structural information and which can also be studied functionally. For reasons of space we do not attempt to cover the research on CLC proteins in other organisms like plants (Barbier-Brygoo et al. 2000; De Angeli et al. 2006), *Caenorhabditis elegans* (Strange 2003), or other model organisms or pathogens (see, e.g., Salas-Casas et al. 2006).

Introduction: The CLC family of chloride-transporting proteins

The research in the CLC chloride channel field has always been accompanied, right from its very beginning, by a great number of unexpected findings and surprises. Already the first step in the field, the identification of the *Torpedo* channel by Miller and coworkers (White and Miller 1979), was a sort of accident (or artifact) in the quest of the authors to investigate acetylcholine-gated cation channels.

The basic properties of the *Torpedo* chloride channel were established by Miller and coworkers in a series of experiments on reconstituted channels from the electroplax of *Torpedo californica* (Miller and Richard 1990). This organ constitutes an internal battery that the fish use as a source of electric current to stun their prey. The plasma membrane of the electrocytes is extremely rich in a specific type of Cl^- channel (later named ClC-0). Miller and colleagues reconstituted the channel in lipid bilayers and analyzed both macroscopic and single-channel currents (Miller and White 1980; White and Miller 1979). At the single-channel level, an unusual gating behavior was observed, with bursts of channel activity separated by periods in which the channel was closed (Fig. 1). Interestingly, the bursting events had a characteristic pattern with three different, equally spaced levels of conductance (0, 11, and 22 pS, respectively). This behavior was found in many different measuring conditions (Hanke and Miller 1983; Miller 1982) and was immediately interpreted as suggesting that the chloride channel was a functional dimer. In this view, the three substates during

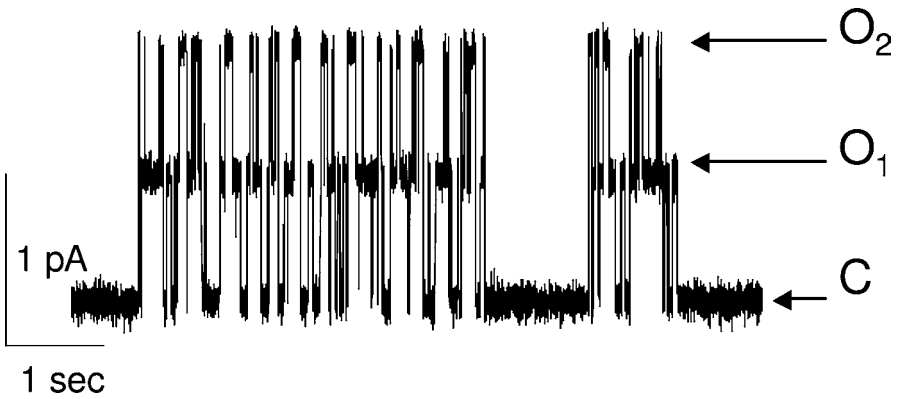

Fig. 1 Schematic (simulated) single-channel trace of the *Torpedo* channel ClC-0. Channel activity occurs in bursts that are separated by long closed periods. Within each bursts two open conductance levels (*O1* and *O2*) are seen, where *O2* has exactly twice the conductance of *O1*

the bursts would represent the independent opening and closing of two identical Cl^- diffusion pathways, called protochannels; the dimeric channel complex may exist with both protochannels simultaneously open, with one open and one closed, or with both closed, generating the three conductance substates.

At all voltages tested, the frequency of substates during a burst followed a binomial distribution as predicted for two independently opening and closing protochannels. Moreover, the probability of a single protopore to be in its conducting state depended on voltage according to a Boltzmann distribution, as expected for a two-state mechanism. This is in agreement with the presence of two independently opening and closing Cl^- pathways and in contrast with the presence of a single channel with different subconductance levels (Miller 1982).

This model was strengthened by a study of DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonate) inhibition of single-channel currents (Miller and White 1984). Addition of 10 μM DIDS to the *cis* side of the chamber eliminated first the 22-pS conductance level and, subsequently, the 11-pS conductance level, that is, the bursting activity disappeared. The authors interpreted the finding as being due to the binding (and inhibition) of DIDS first to one and then the other protopore. This strongly supported a model with two separated diffusion pathways (pores) each with a single open state rather than a single Cl^- diffusion pathway with multiple conductance states.

Incidentally, the fact that DIDS inhibited the oriented channels only if added to the *cis* side of the preparation implied that the two protopores had the same orientation in the channel complex.

The fact that the channel activity presented periods of activity (bursts) and periods of no activity (Fig. 1) indicated that the two protochannels were not completely independent from each other. Therefore, it was suggested that there is an inactivating process that closes both protochannels simultaneously and on a slower time scale (which was later defined as a common gate or slow gate) compared to the closing events within a burst (which were attributed to what was later named fast gate) (Miller and Richard 1990).

Another peculiar feature of ClC-0 emerged from the inspection of the beginnings and the endings of the bursts. Burst activity tended to begin with both protopores open and ended more often with only one protopore open (Richard and Miller 1990). This time asymmetry implies that the transitions between the possible states of the protopores are not in thermo-

dynamic equilibrium. The external source of free energy required to drive the irreversible gating transitions was found to be the electrochemical gradient of Cl^- (Richard and Miller 1990). This finding anticipated one of the most bizarre characteristics of the CLC channel family, a gating mechanism mediated by the permeant anion.

The existence of a common gate has another fundamental implication: The two protochannels must be intimately associated in a proteic complex—the double-barreled shotgun model was born (Miller 1982). On the basis of stability reasons it was suggested that the two protopores would be arranged symmetrically around an axis constituted from the interface between the two subunits (Miller and White 1984).

These features, although solidly grounded on experiments that were elegant in their simplicity, were very original, not to say unfamiliar, for the "channel community," and therefore they stirred up considerable controversy. However, the progress made in the analysis of channel function and structure achieved throughout the last twenty years has spectacularly confirmed virtually all of them and provided deeper insights and new unexpected findings that we will try to summarize.

Cloning of the CLC family members

A critical turning point for the research on chloride channels was the cloning of the channel from *Torpedo marmorata*, called ClC-0, with an elegant but extremely labor-intensive expression cloning strategy (Jentsch et al. 1990). This exposed ClC-0 to the use of the powerful tools of molecular biology and allowed, by homology, the identification of several other CLC channels in organisms as diverse as animals, plants, yeast, archaebacteria, and eubacteria (Jentsch et al. 1999; Maduke et al. 2000).

Mammals possess nine different CLC genes, which, on the basis of sequence homology, can be grouped into three branches (Jentsch et al. 2002; Mindell and Maduke 2001). The first branch comprises plasma membrane channels, ClC-1, ClC-2, ClC-Ka, and ClC-Kb, whereas members of the two other branches (ClC-3, ClC-4, and ClC-5 in one branch and ClC-6 and ClC-7 in the other) function primarily in intracellular membranes.

The sequence, and structure, of CLC proteins bears no resemblance to any other class of membrane proteins. A very distinguishing element of all CLC channels and transporters, with respect to other Cl^--transporting membrane proteins, is their anion selectivity. First, members of the CLC family are practically completely impermeable to cations (except protons). Second, among the halides Cl^-, Br^-, and I^-, the selectivity and conductivity sequence for CLC proteins is generally $Cl^- > Br^- > I^-$. According to Wright and Diamond (Wright and Diamond 1977) this indicates a high-field-strength anion binding site in the transport pathway. In contrast, most other Cl^- channels (except CFTR) show an $I^- > Cl^-$ preference, suggestive of a larger pore in which ions are not completely dehydrated.

Crystal structure of the bacterial ClC-ec1

So far it has not been possible to obtain crystal structures from eukaryotic CLC members, and, therefore, all the structural information (for the transmembrane region) available to date has come from investigation of prokaryotic CLC counterparts, an approach that has been successful for a number of cation channels (Doyle et al. 1998; Zhou et al. 2001).

A projection structure of an *Escherichia coli* member of the CLC family, ClC-ec1, at 6.5-Å resolution, supported the dimeric nature of the channel but could not provide any molecular detail (Mindell et al. 2001). A much more thorough insight into the structure-function of CLC proteins was provided by two high-resolution structures of ClC-ec1 and StClC (from *Salmonella typhimurium*) obtained by Dutzler and coworkers (Dutzler et al. 2002, 2003).

The biology of prokaryotic CLC proteins is still largely unexplored. In particular, it was found that ClC-ec1 is actually a Cl^-/H^+ antiporter (Accardi and Miller 2004), a characteristic that conflicts with its proposed role as a shunt conductance relevant for acid resistance (Iyer et al. 2002). More importantly, this finding raises a number of issues regarding the possible extrapolation of features from the prokaryotic to the eukaryotic members of CLC family, some of which are discussed in later paragraphs. However, considering the sequence conservation between prokaryotic CLCs and eukaryotic members of the family, especially in the ion selectivity region (Maduke et al. 1999), there is confidence that the general structural elements apply to the entire family.

ClC-ec1 is a dimer composed of two identical subunits of triangular shape (Fig. 2a). The contact surface area between subunits is extensive (~2,300 $Å^2$), as expected because CLC channels are thought to exist and function only as dimers (Dutzler et al. 2002), even if it is not known at which stage of the biosynthesis dimerization occurs.

Each subunit contains Cl^- ions at its center, indicating a putative ion conduction pathway, with a mutual distance between the two pores of ~39 Å. The largest part of ClC-ec1 is embedded in the lipid bilayer, and only the N- and C-termini protrude into the cytoplasm (Fig. 2).

Each subunit consists of 18 α-helices (labeled A–R) organized in two topologically related domains that span the membrane in opposite directions in an arrangement called "antiparallel architecture" that has been found also in the structure of the aquaporins (Lee et al. 2005; Murata et al. 2000) and of a Na^+/H^+ antiporter from *E. coli* (Hunte et al. 2005).

The two domains are only weakly correlated in their sequence but show a significant similarity regarding the disposition of glycine residues (Dutzler et al. 2002). Some of the helices are long and tilted by about 45° with respect to the membrane; others are short and penetrate the membrane only halfway. The transmembrane structure is similar across the whole CLC family. One fundamental difference lies in the presence of large C-terminal intracellular domains in all eukaryotic and some prokaryotic CLC proteins that are absent in ClC-ec1 and StClC (Estévez and Jentsch 2002; Meyer and Dutzler 2006). Part of the isolated C-terminus of ClC-0 has been recently crystallized (Meyer and Dutzler 2006). Its structure is described below.

In agreement with the fact that ClC-ec1 is not an ion channel allowing the passive diffusive flow of ions but a stoichiometrically coupled ion transporter, ClC-ec1 lacks a real pore. In the structures of ClC-ec1, the central Cl^- ion is completely surrounded by protein and is not "visible" from either side of the membrane. The putative transport pathway is 15 Å long and contains three ion-binding sites named S_{int}, S_{cen}, and S_{ext}, starting from the one closer to the intracellular space. The S_{ext} site was found to be occupied by the negatively charged side chain of a critical glutamate residue (Glu-148) in the wild-type structure, but binds a Cl^- ion if Glu-148 is mutated to alanine or glutamine; no water molecules have been detected in the ion-binding region in the structures (Dutzler 2004; Dutzler et al. 2003) (Fig. 3).

Overall, the transport pathway across ClC-ec1 appears like a very narrow passage connecting intracellular and extracellular vestibules (Dutzler et al. 2002, 2003). The vestibules leading to the selectivity filter on both sides of the membrane contain basic (positively charged) amino acids, such as Arg-147 and Arg-451. The distribution of charges on the

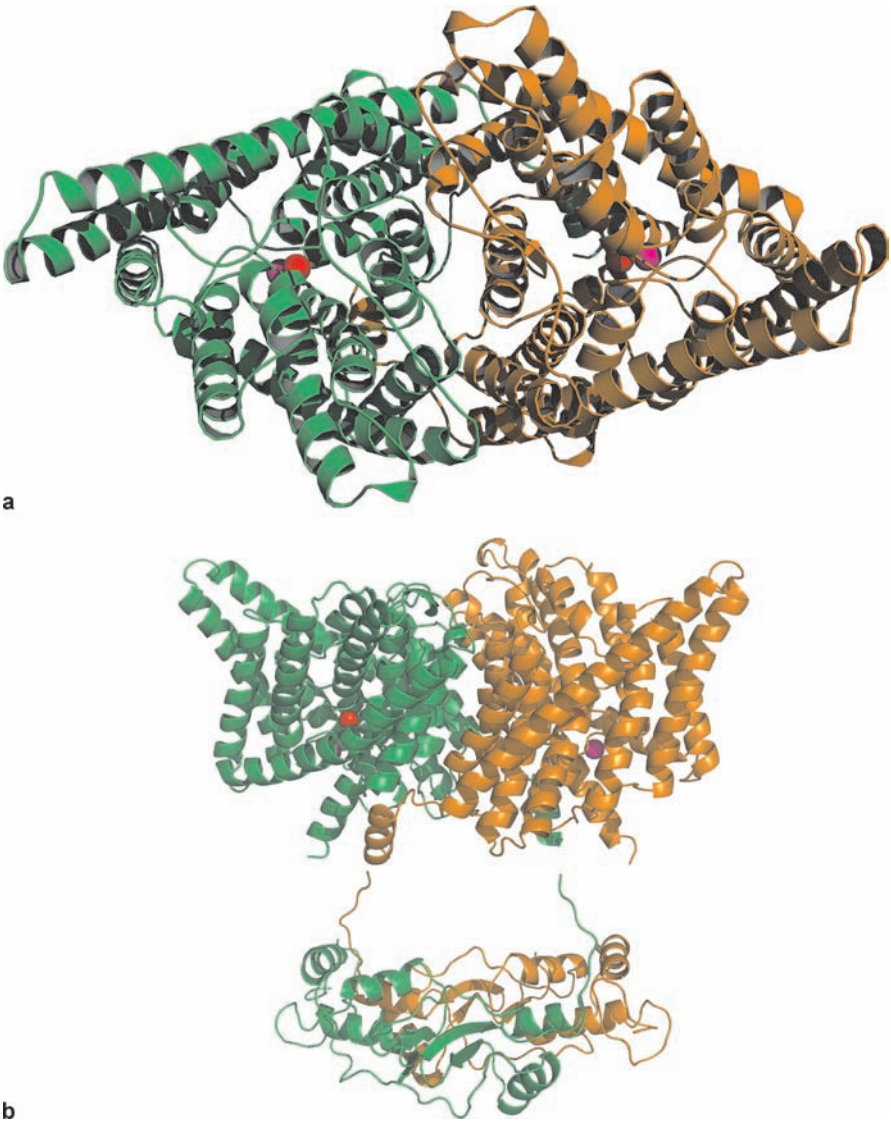

Fig. 2a, b Overall structure of ClC-ec1 and CBS domains. In **a**, ClC-ec1 (PDB accession no. 1KPK) is shown in a ribbon representation viewed from the extracellular side. The two subunits of the dimeric complex are shown in *green* and *orange*, respectively. The two Cl^- ions in the transport pathway of each subunit are shown in *red* (central chloride ion) and *magenta* (inner chloride ion). **b** Side view of ClC-ec1 assembled with the cytoplasmic C-terminal domains of ClC-0 from *Torpedo marmorata* (PDB accession no. 2D4Z). The relative orientation has been arbitrarily fixed, because the exact spatial arrangement of the C-terminal domain with respect to the membrane part is unknown

entire channel surface creates an electrostatic potential that probably funnels Cl^- ions into the pore entryways. The two pores of the dimer are separated by a large distance and by an electronegative region on the extracellular surface (Dutzler et al. 2002). These findings are consistent with the functional independence of the two pores in ClC-0 (Ludewig et al. 1996, 1997b; Middleton et al. 1996).

Amino acids from four separate protein regions are brought together near the membrane center to form the three ion-binding sites (Dutzler et al. 2002, 2003). These regions are highly conserved in CLC proteins; they include GSGIP in helix D (106–110), G(K/R)EGP in helix F (146–150), GXFXP in helix N (355–359), and Tyr-455 in helix R (Fig. 3a). These sequences occur at the N-termini of α-helices, where polypeptide loops precede α-helices D, F, and N. In agreement with this complex structural arrangement, several regions of CLC proteins influence pore properties like ion selectivity, single-channel conductance, and gating (Estévez and Jentsch 2002; Ludewig et al. 1997a, 1996; Pusch et al. 1995a,. 1995b; Wollnik et al. 1997).

Helices D, F, N, and R are oriented with their N-terminus pointing toward the central binding site. Because of the helix dipole, this arrangement of helices is expected to create a favorable environment for anion binding. This is, for example, the mechanism hypothesized to be at work in KcsA to favor ion binding to the pore (Roux and MacKinnon 1999). However, some authors have raised doubts against the generalization of such a mechanism to ClC-ec1. On the basis of electrostatic calculations, Faraldo-Gomez and Roux (Faraldo-Gomez and Roux 2004) proposed that in ClC-ec1 the energetic cost for desolvation of the anions on transfer into the protein is contributed only marginally by long-range interaction

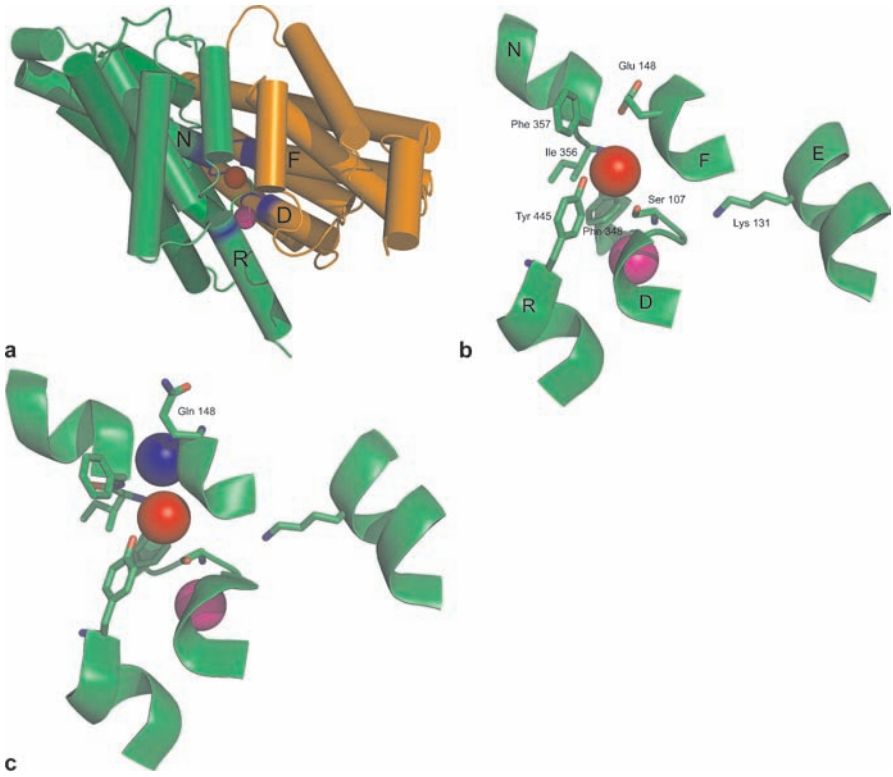

Fig. 3a–c The Cl^- transport pathway and Cl^- binding sites. **a** The position of the two Cl^- binding sites of ClC-ec1 (coloring of subunits and chloride ions as in Fig. 2) with the protein regions involved in coordination of the central Cl^- ion shown in *blue*. **b** Detail of the amino acids coordinating the central Cl^- ion in the wild-type ClC-ec1. **c** The central Cl^- binding site in the structure of the mutant Glu-148-Gln (PDB accession no. 1OTU). The side chain of Gln-148 is displaced from the permeation pathway, and a third Cl^- ion (shown in *blue*) is present at the position occupied by the side chain of Glu-148 in the wild-type structure

with the helix macrodipole and comes mainly from favorable electrostatic interactions with the backbone and side chains of residues that are not directly located in the permeation pathway.

This view is shared also by Cohen and Schulten (Cohen and Schulten 2004), who suggest, on the basis of molecular dynamics calculations, that the broken helix architecture does not constitute a prominent characteristic of the energy profile controlling Cl^- conduction and may possibly represent Nature's design evolved to expose backbone amide groups to the permeant anions.

In this respect, it is interesting to note that the bound Cl^- ions do not make direct contact with a full positive charge from lysine or arginine residues. It has been speculated that a full positive charge would create a deep energy well and cause Cl^- to bind too tightly, compromising the efficiency of transport (Dutzler 2004).

The Cl^- ion at the S_{cen} site is fully dehydrated and is coordinated by main chain amide nitrogen atoms from amino acids Ile-356 and Phe-357 and by side chain oxygen atoms from Ser-107 and Tyr-445 (Fig. 3b). On the basis of electrostatic calculations, however, it was hypothesized that the single most important favorable ion-side chain interaction in ClCec-1 originates not from Ser-107 or Tyr-445 but from Lys-131. The side chain of this residue is located in the transmembrane helix E, completely buried within the protein, with its positively charged amino group pointing toward the chloride-binding sites, at a distance of 7–9 Å (Faraldo-Gomez and Roux 2004) (Fig. 3b). Thus the stabilization seems to occur by a purely electrostatic, relatively long-range, interaction. These predictions are consistent with a recent mutagenesis study of this residue in ClC-0 (Zhang et al. 2006).

Apart from the central binding pocket in which Cl^- is coordinated by polar residues and the extracellular exit in which charged residues form a putative gate, the channel pore is lined in its entirety by nonpolar, noncharged residues. The pore's two conserved polar residues, Ser-107 and Tyr-445, define S_{cen} and provide an abrupt and significant narrowing of the pore. Their role is, however, not yet clear. For the ClC-0 channel, it was shown that the tyrosine is not responsible for the selectivity and the single-channel conductance (Accardi and Pusch 2003), whereas mutations of the serine residue slightly altered ion selectivity and reduced the single-channel conductance (Chen et al. 2003; Ludewig et al. 1996). Also, simulation studies suggested that the interaction energy of Ser-107 and Tyr-445 with Cl^- is not significant compared to the energy due to the strong electrical polarization of the protein (Cohen and Schulten 2004). It was therefore suggested that the most important role of these residues is to keep an anion permanently in the pore to prevent the formation of a proton-carrying continuous water file stretching across the channel or the passage of hydrophobic anions (Cohen and Schulten 2004).

The second ion binding-site, S_{int}, is at a distance of 6.5 Å from S_{cen}, toward the intracellular side. It is located at the interface where the aqueous vestibule from the intracellular solution meets the selectivity filter. The ion at this position is coordinated on one side by main chain amide nitrogen atoms from the end of helix D and on the side where it is exposed to the vestibule is probably still hydrated.

In the first structure of ClC-ec1 (Dutzler et al. 2002), S_{ext} was occupied by the side chain of the glutamate at position 148, occluding the ion pathway (Fig. 3b). At that time, it was believed that ClC-ec1 was a chloride ion channel, even if no direct electrophysiological data were available yet. It was therefore hypothesized that the crystal structure captured the channel in a state in which Cl^- was occluded, that is, did not have direct access to intracellular or extracellular space, and that Cl^- ions would activate conduction (gate the channel open) entering the pore from the extracellular side and inducing a conformational change that would displace the glutamate side chain.

This prediction was largely confirmed by a second structure of ClC-ec1 determined at 2.5-Å resolution in combination with parallel electrophysiological measurements performed on ClC-0 (Dutzler et al. 2003). When the corresponding Glu-148 of ClC-ec1 was mutated in ClC-0 into Ala (Glu-166-Ala), Gln (Glu-166-Gln), or Val (Glu-166-Val), it was found that fast gating transitions were practically abolished (Dutzler et al. 2003). Interestingly, lowering extracellular pH produced a similar open phenotype for wild-type ClC-0 (Chen and Chen 2001; Dutzler et al. 2003), suggesting that the protonation of the glutamate side chain from the extracellular side opens the wild-type channel (Fig. 4). The crystal structures of ClC-ec1 in which Glu-148 was mutated to Ala and Gln presented an anion at S_{ext} instead of the Glu side chain (Dutzler et al. 2003) (Fig. 3c). It was therefore suggested that when Glu-148 is mutated, the pore is open because it contains an uninterrupted queue of anions connecting the intracellular and the extracellular solutions.

In the structure of the Glu-148-Gln mutant of ClC-ec1, the side chain of Gln-148 is directed toward the extracellular solution rather than into the pore (Fig. 3c), and it was spec-

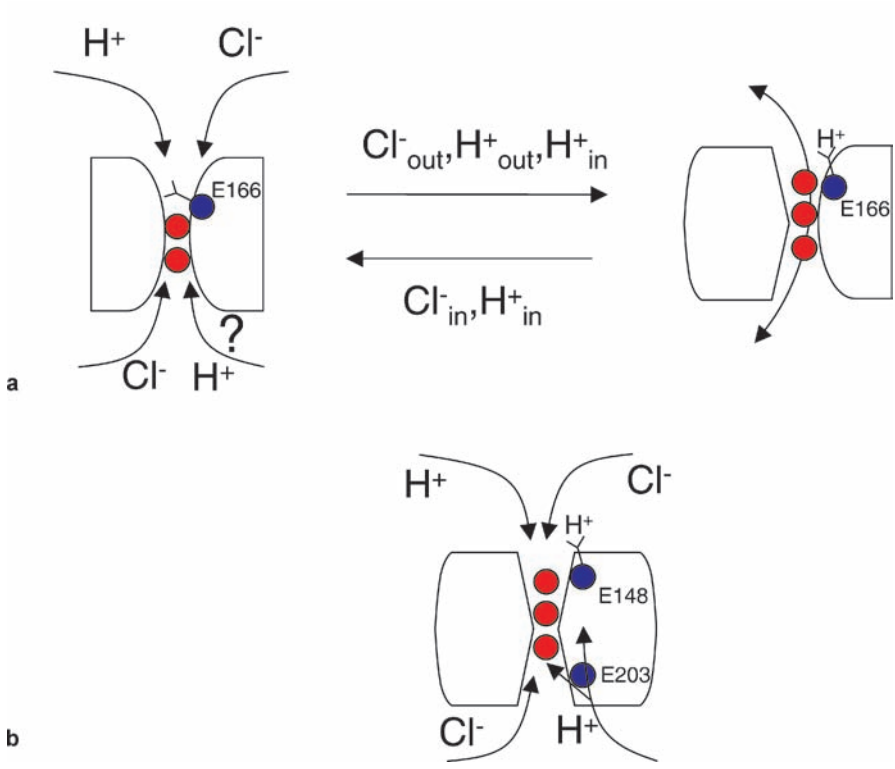

Fig. 4a, b Effect of Cl^- and H^+ on the operation of the protopore gate of CLC channels and transporters. Cl^- ions are indicated as *red spheres*. **a** Schematic representation of the transitions between the open and the closed state of CLC channels and of the physicochemical factors influencing forward and backward rates. Protonation of the E166 (numbering of ClC-0) side chain allows Cl^- flux. Possible additional rearrangements in the pore region involved in channel opening are also indicated. The pathway that intracellular protons have to follow to protonate E166 is not known, as indicated by *question mark*. **b** Schematic representation of the ClC-ec1 transporter. Protonation of E148 (E166 in ClC-0) and E203 are required for the coupled Cl^-/H^+ antiport activity, but the pathway that intracellular protons have to follow to reach E148 after protonation of E203 is not known. One possibility is that protons follow the Cl^- permeation pathway. Another possibility is that they reach E148 through a different route yet to be determined

ulated that this could be also the conformation assumed by the wild-type glutamate in the open—presumably protonated—state (Dutzler et al. 2003). However, this point is still under debate. For example, based on simulation studies, it was suggested that the side chain of the glutamate could swing out of the permeation pathway by a different type of movement (Bisset et al. 2005).

S_{ext} is located between the N-termini of helices F and N, where amide nitrogen atoms form a cage surrounding the ion, and is only 4 Å apart from S_{cen}. All three sites can simultaneously be occupied by Cl^- ions when the channel is open (Lobet and Dutzler 2006).

A very general point to be addressed is the extent to which the picture of the prokaryotic ClC-ec1 provides an accurate description of the eukaryotic counterparts. Sequence alignment exhibits a significant degree of conservation between bacterial and eukaryotic CLC channels; the similarity is especially strong in the selectivity filter region. Mutational studies on eukaryotic channels correlate well with the locations of key residues in the bacterial structures. Chen and Chen, using the cysteine accessibility method, were able to show that in ClC-0 the residues on the intracellular part of the putative helix R are arranged in an α-helical structure and line the wall of the ion permeation pathway as indicated by the crystal structure of the ClC-ec1 (Chen et al. 2003). The results of Engh and Maduke, based on the same approach, also suggest conservation of the overall architecture of the inner vestibule between ClC-0 and ClC-ec1 (Engh and Maduke 2005). Further support in this direction came from a recent biochemical evaluation of the membrane domain boundaries of ClC-2 (Ramjeesingh et al. 2006).

Estévez et al. showed that residues influencing the affinity of ClC-0 and ClC-1 for the intracellular inhibitors 9-anthracene carboxylic acid (9-AC) and *p*-chloro-phenoxy-acetic acid (CPA), partially overlapped with the Cl^- binding pocket identified in the StClC structure (Estévez et al. 2003). It seems, therefore, that the structure of ClC-ec1 indeed provides a good model for the description of other members of the CLC family. However, a potentially relevant difference between ClC-ec1 and CLC channels is the presence in the channels of more Arg and Lys residues near the pore (Corry et al. 2004). Moreover, the finding of Accardi and Miller that ClC-ec1 is not a chloride channel but a Cl^-/H^+ antiporter, with potentially a completely different mechanism of action, suggests caution in the extrapolation of structural features from ClC-ec1 to CLC channels (Accardi and Miller 2004). Subsequently, the eukaryotic ClC-4 and ClC-5 and the plant AtCLCa were also shown to be anion/proton antiporters and not chloride channels (De Angeli et al. 2006; Picollo and Pusch 2005; Scheel et al. 2005). It is surprising that members of the same protein family, sharing a fair degree of homology and high conservation in critical regions, behave in some cases as channels and in others as transporters. At the moment there is no evidence regarding the molecular determinants of such a difference, and therefore we also do not know whether ClC-ec1 represents a better model for ClC-4 and ClC-5 compared to the CLC channels.

The identification of the major molecular determinant of the fast gate, Glu-148 (166 in ClC-0), would explain two characteristics of the fast gate: (a) The fast gates of the two pores are independent because each pore contains its own glutamate residue and the conformational change associated with the swing of the glutamate side chain is local and probably does not influence the other pore; and (b) the fast gate is coupled to Cl^- permeation because Cl^- ions compete with the side chain of glutamate 166 for the occupancy of S_{ext} and once a Cl^- ion occupies this site there is no obstacle to the permeation process. This would also explain the relatively minor voltage dependence of gating of the kidney CLC channels, ClC-Ka and ClC-Kb, which carry a valine instead of a glutamate at the position equivalent to 166 of ClC-0 (Kieferle et al. 1994; Waldegger and Jentsch 2000).

As detailed below, the fast gate can be opened by a mechanism that is favored at low intracellular pH. Presumably, protonation of Glu-166 results in increased open probability due to neutralization of its side chain. For this second mechanism to occur, protons must access the Glu-166 side chain from the intracellular side (Fig. 4). Yin et al., on the basis of molecular simulations, suggested three proton pathways (Yin et al. 2004). One of these pathways involves glutamate residues at positions Glu-113, Glu-117, and Glu-203 that, interestingly, in ClC-0 are substituted by Lys, Leu, and Val, respectively. The residue Glu-203 in ClC-ec1 was in fact suggested by Accardi et al. (Accardi et al. 2005) to be an internally accessible acceptor for protons, as substitution of this residue with Gln completely abolished proton flux, underlining its importance for the mechanism of transport. Interestingly, all members of the CLC family known to be ion channels (ClC-0, ClC-1, ClC-2, ClC-Ka, ClC-Kb, and respective species homologs) present a Val in place of the Glu at position 203, suggesting a significant difference in the mechanism of transport between channel and antiporter members of the CLC family.

However, despite all the pieces of information gathered so far, our picture of the mechanism of gating is still incomplete; for example, some studies point to structural rearrangements of the pore associated with fast gate transitions, suggesting a larger conformational change than the one that would be produced by a simple swing of the Glu-148 side chain (Accardi and Pusch 2003; Traverso et al. 2003) (see "Use of CPA as a tool to explore the fast gate of ClC-0"). Moreover, a gating mechanism based solely on the movement of the Glu is unable to explain why the modulation of gating by Cl^-_{ext} is different from Cl^-_{int} (Chen 2003).

Use of CPA as a tool to explore the fast gate of ClC-0

Small ligand molecules have been very useful tools to explore gating mechanisms of voltage-dependent cation channels (Hille 2001). A classic example is the identification of the activation gate of K^+ channels by intracellularly applied tetraethylammonium (Armstrong 1966). In a similar manner, the small organic acid CPA and related compounds have been used as tools that interfere with the fast gate of ClC-0 (Accardi and Pusch 2003; Pusch et al. 2001; Traverso et al. 2003). CPA is the simplest derivative of 2-(*p*-chlorophenoxy)-3-phenylpropionic acid (CPP), a substance that is known to inhibit the macroscopic skeletal muscle conductance (Conte-Camerino et al. 1988). Later studies on heterologously expressed muscle ClC-1 revealed that CPP and analogs block ClC-1 exclusively from the intracellular side in a strongly voltage-dependent manner, leading to an apparent "shift" of the voltage dependence of opening (Aromataris et al. 1999; Liantonio et al. 2003; Pusch et al. 2000). The binding site of CPA and the unrelated 9-AC was mapped on ClC-1 with considerable detail (Estévez et al. 2003). CPA and 9-AC bind to the channel in a partially hydrophobic pocket adjacent to the central Cl^- binding site (when mapped onto the ClC-ec1 structure), even though the precise orientation of the drug molecule is unknown (Estévez et al. 2003). However, the small single-channel conductance (Pusch et al. 1994) and the relatively complex gating of ClC-1 (Accardi and Pusch 2000) made it difficult to understand the mechanism of CPP block in this channel. The prototype ClC-0 channel is more useful in this respect. Employing the point mutant Cys-212-Ser simplifies the system even more because this single amino acid substitution almost completely abolishes the common gating mechanism (Lin et al. 1999). CPA block of ClC-0 was extensively studied (Accardi and Pusch 2003; Pusch et al. 2001). It was found that CPA binds to closed channels with an

about 20-fold higher affinity than to open channels. In this way, CPA stabilizes the closed state and leads to an apparent "shift" of the voltage dependence of opening. Open channel block is of low affinity and associated with rapid binding/unbinding kinetics (apparent K_D in the 20 mM range), whereas closed channel inhibition has much slower kinetics (Accardi and Pusch 2003). As discussed above, fast gating of ClC-0 has been proposed to reflect only the reorientation of the carboxylate side chain of the Glu-166 residue (Dutzler et al. 2003), without any further conformational change of the protein. In this model, the relatively large difference of the affinity and kinetics of open- and closed-channel binding of CPA is rather unexpected, but might be explained by different electrostatic repulsion between CPA and other anions in the pore. However, a recent crystallographic study by Lobet and Dutzler (Lobet and Dutzler 2006) suggested that, in both open and closed states of the fast gate, all three Cl^- ion binding sites are equally maximally occupied by Cl^- ions or by the carboxylate side chain of Glu-166. Thus the model advanced by Dutzler and colleagues appears unable to explain the characteristics of CPA block. Additional evidence in favor of a conformational change that accompanies opening of the fast gate was obtained by Accardi and Pusch from differential effects of pore mutants on closed- and open-channel block by CPA. For example, the mutant Thr-481-Ser exclusively altered the closed-channel affinity, whereas other mutations mostly altered the open-channel block (Accardi and Pusch 2003). Also, the data of Traverso et al. (Traverso et al. 2003), again using CPA as a tool, suggested that a conformational change, in addition to the glutamate swing-out, accompanies opening of ClC-0 protopores.

Thus several pieces of evidence argue against the simple gating model for the fast gate of ClC-0 in which the side chain of Glu-166 is the only moving part. Additional conformational changes, in particular on the intracellular side, would be more compatible with some of the data. However, a more precise definition of the mechanism of the fast gate will probably need direct structural information for a eukaryotic CLC homolog.

CBS domains

All eukaryotic CLC proteins have a long carboxy-terminal cytoplasmic region whose length ranges from 155 (ClC-Ks) to 398 amino acids (ClC-1) (Estévez et al. 2004). The C-terminal domain is essential for the functioning of the eukaryotic CLC proteins, as deletions and several point mutations in this region drastically affect transport activity and/or protein maturation and trafficking (see below). Indeed, several disease-causing mutations are found within the C-terminus (Estévez and Jentsch 2002; Jentsch et al. 2002; Pusch 2002), but, despite some recent progress, its precise functional and physiological role is unknown. The C-terminal region contains two so-called CBS domains (from cystathionine-β-synthase, the first protein in which these domains were identified). These structural domains normally occur in pairs and are found in several unrelated proteins from all organisms (Bateman 1997; Ponting 1997).

Recently, the crystal structure of the isolated cytoplasmic domain of ClC-0 from *Torpedo marmorata* was solved by Dutzler and coworkers (Meyer and Dutzler 2006) (see Fig. 2b). As previously described for a different protein (Sintchak et al. 1996; Zhang et al. 1999), the two CBS domains have a triangular shape and are made of three β-strands and two α-helices. Similar to other CBS-containing proteins, the two CBS domains (i.e., CBS1 and CBS2) were found to interact at the level of the β-strands, forming a typical CBS1-CBS2 complex. A portion of 95 residues of the linker between CBS1 and CBS2 was found to be

disordered in the crystal structure, but it is not clear yet whether this reflects a crystallographic artifact or the intrinsic flexibility of the region. However, the residual C-terminal part of the linker, encompassing 25 residues, is well ordered. Interestingly, channel function was not affected by the removal of residues that were part of the disordered linker region, whereas no functional channels were obtained if the truncation was made in the structurally well-defined part of the linker region preceding CBS2 (Estévez et al. 2004).

Unfortunately, the protein did not associate in dimers in the crystallization conditions used by Meyer and Dutzler (Meyer and Dutzler 2006), and therefore critical information about the subunits' interaction had to be extrapolated from a modeling on the crystal structure of TM0935, a protein from *Thermotoga maritima* (Miller et al. 2004). However, even after this procedure, the surface of the domain in contact with the transmembrane region remained ambiguous, although CBS2 was suggested to be positioned closer to the pore than CBS1 (Meyer and Dutzler 2006). Moreover, the C-terminal part of the cytoplasmic domain, which is predicted to be relevant in the interaction between CBS1 and CBS2, was not included in the construct used for the crystallization.

Several functions have been proposed for CBS domains. Alanine scanning mutagenesis of the yeast Cl^- transporter ScClC (gef1p) suggested that CBS domains influenced the subcellular localization of the channel (Schwappach et al. 1998).

On truncation of ClC-0, ClC-1, and ClC-5 after the first CBS domain, the proteins did not give rise to current. However, their function could be restored by coexpression of the missing C-terminal CBS domain, suggesting that CBS2 may function as an independent structure (Maduke et al. 1998; Mo et al. 2004; Schmidt-Rose and Jentsch 1997). Estévez et al. showed that ClC-1 truncated after the CBS1 domain was not able to reach the plasma membrane by itself but that the expression could be restored to a normal level in the presence of the CBS2 domain in addition to a region of six amino acids at the N-terminal part of CBS2 (Estévez et al. 2004). It was also shown that CBS domains from different CLC members could be exchanged without abolishing channel function, demonstrating that the overall architectural conservation of the domain may suffice, despite the low sequence conservation, to preserve their role.

A first hint that the C-terminal region of the channel could be functionally linked to the slow gate came from Jentsch and coworkers (Fong et al. 1998), who made use of mutations in that region of the ClC-0 and of chimeric constructs and found that the C-terminal part is essential for functional expression of the channel and is involved in the operation of the slow gate. In particular, several point mutations in the CBS2 domain of ClC-0 and ClC-1 were found to influence the slow gate (Estévez et al. 2004).

Scott and Hawley found that a purified fragment comprising the last 260 C-terminal residues of ClC-2 was able to bind ATP and that mutations located in this region that are associated with genetic diseases lead to defects in ATP binding (Scott et al. 2004). It is interesting to correlate these findings with a study of Niemeyer et al. (Niemeyer et al. 2004). Analyzing the functional consequence of the mutation G715E in ClC-2 that was proposed to induce idiopathic generalized epilepsy (Haug et al. 2003), Niemeyer et al. could not find any gating alteration for the mutated channel but found that, in contrast to wild-type, it did not respond to the substitution of ATP with AMP with accelerated opening and closing kinetics, even though the effects were relatively minor. Recently, it was suggested that the isolated carboxy terminus of ClC-5 folds in a predominantly α-helical structure and it is able to bind ATP (Wellhauser et al. 2006). Interestingly, ATP modulates the activity of the common gate of ClC-1 channels such that increasing ATP concentration shifts the midpoint of the open probability distribution toward depolarized potentials and reduces the fraction of channels that remain open at strong hyperpolarized potentials (Bennetts et al. 2005). Bennetts et al.

suggested that the interaction with ATP is mediated by the CBS domains (Bennetts et al. 2005). Based on a homology model with the structure of a CBS dimer of IMPDH (inosine monophosphate dehydrogenase) and in silico docking, they identified a putative ATP binding pocket in a cleft between the two CBS domains of ClC-1 and confirmed their results, observing that mutations of residues that were predicted to interact with ATP reduced or ablated the ability of ATP to modulate channel function (Bennetts et al. 2005). However, no ATP binding could be detected in the CBS1-CBS2 complex of ClC-0, even at very high ATP concentrations (Meyer and Dutzler 2006). Physiologically, an increased ClC-1 activity due to ATP depletion during metabolic stress would stabilize the membrane potential and reduce muscle excitability, thereby preserving the viability of muscle fibers. Such a mechanism, however, has not been described *in vivo*. In fact, it is questionable that an increased chloride conductance, via a shift of the voltage dependence of the open probability, is able to suppress muscle excitation after nerve stimulation.

The fact that mutations in the CBS domains, per se or by affecting the ability to bind ATP, interfere with the operation of the common gate requires an interaction of the transmembrane part of the channel with the cytoplasmic terminus. An interesting possibility was suggested by Estévez and coworkers (Estévez and Jentsch 2002; Estévez et al. 2004) to explain this interaction: The last transmembrane helix R, whose N-terminal tyrosine coordinates a Cl^- ion in the middle of the pore and whose C-terminus extends into the cytosol, is directly connected to the CBS1-CBS2 complex. This helix may therefore be the structural link between the inner pore and CBS domains.

Additionally, CBS domains may be relevant in the interaction with other proteins. It has been found that deleting CBS1 and/or CBS2 impairs the interaction of ClC-5 with cofilin, an actin-associated protein that is crucial in the regulation of albumin uptake by the proximal tubule (Hryciw et al. 2003). Moreover, a PY motif is found between CBS1 and CBS2 of ClC-5 that probably interacts with HECT-ubiquitin ligases to modulate the retention of the channel in the plasma membrane (Schwake et al. 2001), and a splice variant of ClC-3 displays a PDZ-binding motif at its extreme carboxy terminus that can interact with the scaffolding proteins EBP50 (ERM-binding phosphoprotein 50), PDZK1, and GOPC (Golgi-associated PDZ and coiled-coil motif-containing protein) (Gentzsch et al. 2003; Ogura et al. 2002).

Gating of muscle-type ClC channels

According to the classic view, in voltage-dependent cation channels permeability and gating are considered, to a first approximation, as independent processes implying the presence of a permeable pore and of a separate structure that senses the transmembrane voltage and opens and closes the pore. This picture is completely inadequate for CLC channels. A first hint of the strong coupling of gating and permeation in ClC-0 came from the time asymmetry of the single-channel bursts implying that the gating transitions were not in thermodynamic equilibrium (Richard and Miller 1990) (see "Introduction"). Such a situation implies the existence of an external energy input into the system that was identified as the chloride electrochemical potential, anticipating one of the most eccentric features of CLC channels, a gating process that is mediated by the permeant ion.

A thorough investigation of the properties of ClC-0 expressed in oocytes and CHO cells allowed Pusch and coworkers to conclude that in ClC-0 permeation and gating are tightly linked (Pusch et al. 1995a). They found that only permeant anions affect gating, that the ion

selectivity of conduction is reflected in the ion selectivity of gating, and that an anomalous mole fraction behavior in the conduction corresponds to a parallel behavior in the gating. Incidentally, the presence of such an anomalous mole fraction behavior showed for the first time that the channel pore contains more than one ion binding site, as was later confirmed by structural data (Dutzler et al. 2003).

As mentioned above, the conducting state of the ClC-0 channel is controlled by two different mechanisms defined as the slow gate and the fast gate.

The slow gate controls the opening (and closing) of both pores simultaneously (Miller and White 1980; White and Miller 1979).

There are different factors affecting the operation of the slow gate, such as potential, chloride concentration, pH, and temperature. Hyperpolarized potentials favor the opening of the slow gate (Miller and Richard 1990). The steady-state activation of the slow gate can be described by a Boltzmann function with a $V_{1/2}$ of approximately –80 mV and an apparent gating valence of ~2 (Pusch et al. 1997). Moreover, the slow gate apparently does not deactivate completely at depolarized voltages, leading to an offset of the open probability of the slow gate at positive voltages. Interestingly, this offset seems to correlate with the expression level of ClC-0 in oocytes (Pusch et al. 1997).

Chen and Miller (Chen and Miller 1996), found in single-channel recordings, that increasing $[Cl^-]_{ext}$ shortened the mean closed time and increased the mean open time of the slow gate. Also, $[Cl^-]_{int}$ influences the operation of the slow gate. Decreasing intracellular Cl^- shifted the p_{open} of the slow gate to more negative potentials and reduced the maximal activation at the most negative voltages (Pusch et al. 1999). Temperature is another variable that markedly influences the operation of the slow gate (Pusch et al. 1997). In particular, the kinetics of closing of the slow gate showed a Q_{10} of ~40 at 20°C, suggesting that the transition between the open and the closed state requires a complex rearrangement of the protein. The effect of an increase of temperature is on one hand to inactivate the channels in a more complete fashion at positive voltages and on the other hand to decrease the fraction of channels that can be activated by the slow gate at negative voltages (Pusch et al. 1997). However, the voltage of half-maximal activation is relatively independent of temperature. This complex behavior cannot be correctly described by a simple two-state model (open-closed states) but requires at least two open and two closed states for its description. The effect of temperature was assessed also on the single-channel level, with the finding that increasing the temperature increases the frequency of closure of the slow gate. As expected, single-channel currents increase with temperature, but the dependence is shallow, consistent with a diffusion-regulated process (Pusch et al. 1997).

In ClC-1, which normally lacks the typical slow gate activation at negative voltages (Steinmeyer et al. 1991b), a hyperpolarization-activated component of the current becomes apparent at low pH_{ext} (5.5), which is reminiscent of the activation of the slow gate in ClC-0 (Rychkov et al. 1996).

The mechanism responsible for the slow gating has not yet been identified. The fact that the slow gate acts on both pores simultaneously suggests, on the structural level, that it relies on subunit interactions (Estévez and Jentsch 2002), in agreement with the finding that concatemers comprising subunits of different CLC members led invariably to loss of slow gating transitions (Lorenz et al. 1996; Weinreich and Jentsch 2001).

The interaction of the subunits in the dimeric architecture of CLC proteins can involve the interface between the transmembrane segments or the cytosolic portions that are of substantial length in eukaryotic channels, or both.

Most ClC-1 mutations leading to dominant myotonia change the voltage dependence of the channel and most likely involve the slow gate (Pusch et al. 1995b; Saviane et al. 1999).

These mutations are scattered along the channel amino acid sequence (Pusch 2002) and therefore prove that different regions of the channel probably interact to determine slow gate transitions. However, several mutations cluster in helices at the dimer interface that probably are important for subunit contacts: Mutations Val-286-Ala and Ile-290-Met change residues in helix H, whereas mutations Phe-307-Ser, Ala-313-Thr, and Arg-317-Gln change residues in helix I (Duffield et al. 2003; Pusch 2002). Moreover, several point mutations in ClC-0 that are distant from the dimer interface have also been shown to eliminate slow gate transitions (Lin et al. 1999; Ludewig et al. 1996; Traverso et al. 2006). As explained in the section on the CBS domains, the C-terminus also appears to be a major determinant of the slow gate (Estévez et al. 2004; Fong et al. 1998).

The fast protopore gate of ClC-0

The fast gate acts individually on the single pores of the dimer (Miller 1982). In single-channel recordings of ClC-0 incorporated into planar lipid bilayers, it was found that the fast gate operates in the milliseconds time range and the open probability of the single protopore increases with voltage with an apparent gating charge of ~1 (Miller 1982) and follows a Boltzmann distribution as predicted for a two-state channel model (Hanke and Miller 1983).

$[Cl^-]_{ext}$ influences the open probability of the fast gate (Pusch et al. 1995a), with high extracellular Cl^- favoring the opening of the channel, shifting the voltage dependence of the open probability toward negative potentials (Fig. 4). Using single-channel recordings, Chen and Miller (Chen and Miller 1996) showed that the open probability approaches a nonzero asymptote at very negative potentials, an effect that can be described as incomplete closure of the channel. The basis of this phenomenon is that the opening rate does not depend in a monotonic manner on voltage. At depolarized potentials the opening rate increases exponentially with voltage; at hyperpolarized voltages, however, the opening rate decreases at intermediate potentials but increases again at highly hyperpolarized potentials. The result is that the opening rate has a minimum at negative voltages. On increase in $[Cl^-]_{ext}$, the voltage activation curve shifts to the left along the voltage axis without significant change in the apparent gating charge.

The closing rate of the fast gate depends on voltage, decreasing exponentially with depolarization. Importantly, the closing rate is only slightly affected by $[Cl^-]_{ext}$. Therefore, whereas the voltage dependence of the open probability is determined by both the opening and the closing rate, the external Cl^- dependence derives almost completely from an effect on opening.

The operation of the fast gate depends also on $[Cl^-]_{int}$. In particular, the effect on the opening rate is very small, whereas lowering $[Cl^-]_{int}$ substantially increases the closing rate (Chen and Miller 1996) (Fig. 4). As a result, increasing $[Cl^-]_{int}$ shifts the steady-state activation curve to the left, as with high $[Cl^-]_{ext}$. However, $[Cl^-]_{int}$ exerts a more prominent effect on the degree of incomplete closure at hyperpolarized potentials, which was not observed changing $[Cl^-]_{ext}$. In particular, as $[Cl^-]_{int}$ increases, the asymptote of the open probability at negative voltages also increases (Chen and Miller 1996; Ludewig et al. 1997a).

These observations were rationalized by a model in which the fast gate of ClC-0 may open through two different routes with opposite voltage dependence (Chen and Chen 2001; Chen and Miller 1996). In one mode, opening is favored by membrane depolarization and is sensitive to $[Cl^-]_{ext}$. A plausible mechanism for this gate would be that Cl^- first binds to

the channel and then travels through the pore to reach an inner binding site, spanning some distance in the membrane electric field, as already suggested by Pusch (Pusch 1996; Pusch et al. 1995a). The other mode does not depend on $[Cl^-]_{ext}$ and is favored by hyperpolarized potentials (Chen and Chen 2001). A more quantitative analysis of the $[Cl^-]_{int}$ dependence of the fast gate was performed by Chen et al. (Chen et al. 2003). Their results confirmed that $[Cl^-]_{int}$ almost exclusively affects the closing rate (increasing $[Cl^-]_{int}$ decreased the closing rate). The effect of $[Cl^-]_{int}$ on the closing rate was saturable, suggesting that it is mediated by a Cl^--binding site. This was confirmed by experiments in which Cl^- was substituted with Br^- and SO_4^{2-}, showing how the impermeant ion SO_4^{2-} did not have any such effect, whereas Br^-, which binds to the pore more tightly than Cl^-, had a stronger effect (Chen et al. 2003).

The fast gate is also affected by alterations of the intrinsic electrostatic potential of the pore (Chen and Chen 2003; Zhang et al. 2006). In particular, mutating several residues known to line the pore or located close to it affected the closing rate, with very little effect on the opening rate. Introducing positively charged residues (or removing negatively charged residues) in the pore consistently increased the closing rate; vice versa, introducing negatively charged residues decreased the closing rate. It seems therefore that increasing $[Cl^-]_{int}$ and introducing more negative charges in the pore lead to a similar effect (Chen and Chen 2003; Chen et al. 2003). Chen and coworkers proposed two mechanisms to explain these results, both based on the assumption that Glu-166 is the fast gate in ClC-0. The negative charge of the glutamate side chain could directly interact with charged residues in the pore region. In this scenario, negative charges in the inner pore would repel the negative charge on the glutamate so that the gate would be more difficult to close, that is, it would be more difficult for the carboxylate side chain of Glu-166 to occupy the S_{ext} position. However, as judged from the structure of ClC-ec1, some mutations tested in the study would be more than 20 Å away from Glu-166. More importantly, the behavior of the double mutant E127K/K519E is not in agreement with this model (Chen and Chen 2003). The alternative possibility is that the effect of the electrostatic potential of the pore on gating is mediated by the permeant anion. For example, a more positive charge at the amino acid positions 127, 515, and 519 that are located near S_{int} would decrease the ability of Cl^- present at this site to displace Cl^- at S_{cen} and at S_{ext}. This, in turn, would decrease the ability of Cl^- to compete with Glu-166 for S_{ext}, leading to faster closing of the protopore gate.

This hypothesis is especially appealing because it would explain the behavior of the mutant Glu-127-Gln, for which the effect on the fast gate mirrors the effect on channel conductance. However, not all the mutants affect both fast gate and conductance. Chen and colleagues therefore suggested that the charge of residues in the pore and the charge carried by the permeant ion both can contribute to the overall gating process and that the location of the charge in the pore determines their relative contribution (Chen and Chen 2003; Chen et al. 2003).

Very recently it has been found that the residue K149 in ClC-0 (corresponding to K131 in ClC-ec1), although not directly lining the pore, plays a very important role in the electrostatics of the channel, as mutations of this residue reduce the opening rate of the fast gate (Zhang et al. 2006). Interestingly, the mutation K131M in ClC-ec1 results in a perturbation of Cl^-/H^+ antiporter function (Accardi et al. 2005).

The electrostatics of the pore is also a major determinant of the single-channel conductance of ClC-0 (Chen and Chen 2003). For example, it was found that mutations changing the charge in the inner pore (e.g., Lys-519-Glu) reduce the conductance at "physiological" Cl^- concentrations, but not at saturating $[Cl^-]_{int}$ (Chen and Chen 2003). In contrast, for the mutation Ser-123-Thr, which changed the highly conserved serine in the selectivity filter, the

decrease in conductance could not be rescued by manipulation of the internal Cl^-. Notably, the mutant Tyr-512-Phe, located in the selectivity filter, produced an increase in conductance of 30% compared to wild-type. This suggests that the regulation of channel conductance by mutations in the selectivity filter and in the channel inner mouth is different. The hydroxyl groups of Ser and Tyr are clearly shown in the ClC-ec1 structure to coordinate a Cl^- ion at S_{cen} and are conserved in the CLC family. The fact that mutations in the corresponding residues in ClC-0 have such a different influence on conductance is still difficult to explain and may suggest a complex effect of these mutations on channel conductance and some difference between the transporter and the channel members of CLC proteins.

The modulation of the fast gate by external protons was first studied by Chen and Chen (Chen and Chen 2001), who showed that reducing pH_{ext} increases the open probability, mostly at hyperpolarized potentials, almost exclusively increasing the opening rate (Fig. 4). The macroscopic effect of a decrease in pH_{ext} is therefore mostly an increase in the minimal open probability (P_{min}) at hyperpolarized potentials and not a shift of the $p_{open}(V)$ curve, which is instead seen on changing $[Cl^-]_{ext}$. Chen and Chen (Chen and Chen 2001) proposed that the effect of pH_{ext} on the fast gate is not mediated by a change in the affinity of the Cl^- binding site that regulates channel opening (Chen and Miller 1996; Pusch 1996) and that therefore the mechanism of pH_{ext} regulation must be intrinsically different from the $[Cl^-]_{ext}$-dependent channel opening. The regulation by external protons, Cl^- independent and mostly effective at hyperpolarized potentials, is similar to one of the mechanisms of opening described by Chen (Chen et al. 2003), potentially indicating that the two processes are linked (Chen and Chen 2001).

Moreover, the fact that the modulation by pH_{ext} is stronger at negative voltages is reminiscent of the action of $[Cl^-]_{int}$ on the fast gate. Chen and Chen (Chen and Chen 2001) indeed suggested that the action of external protons is more pronounced at higher $[Cl^-]_{int}$.

The ClC-0 mutant Glu-166-Asp has a drastically reduced open probability compared to wild-type (Traverso et al. 2006) and is thus expected to display an even stronger response to the external pH. Traverso et al. (Traverso et al. 2006) found instead that decreasing pH_{ext} did not increase outward currents. In particular, low pH_{ext} increased a persistent inward current that was characterized by a smaller single-channel conductance. These results suggested that Asp-166 can be protonated from the intracellular side in a voltage-dependent manner or from the extracellular side in a voltage-independent manner, resulting in open states of different conductance (Traverso et al. 2006). In ClC-1 it was found that decreasing pH_{ext} affected the macroscopic current, mostly by increasing the steady-state component at the expense of the deactivating portion. At variance with the behavior of external Cl^- at low pH_{int}, it was found that at low pH_{ext}, external $Cl^-$was not able to influence channel gating (Rychkov et al. 1996).

The influence of the internal pH on the fast gate transitions was investigated in the reconstituted *Torpedo* channel (Hanke and Miller 1983) (Fig. 4). Low pH_{int} drives the protochannel open without changing its conductance. The effect was interpreted in terms of a shift of the voltage dependence of the open probability toward negative potentials. Hanke and Miller suggested that on opening of the channel a titratable group exposed to the intracellular solution changes its pK from 6 to 9 and that this change in pK underlies the ability of protons to drive the channel into its open state (Hanke and Miller 1983). In ClC-1, internal pH had a very similar effect (Rychkov et al. 1996). Hanke and Miller also investigated the pH dependence of the opening and closing rate constants. They found that at all pH values tested, those rates vary exponentially with voltage and at all voltages both opening and closing rate constants vary with proton concentration. However, with an increase in the proton concentration, the closing rate constant decreases whereas the opening rate increases. There-

fore, the effect of pH_{int} changes mainly translates into a shift of the p_{open} along the voltage axis. The pH dependence implies that a simple two-state model is insufficient to describe the channel behavior and that a protonation reaction must be added to the scheme. Hanke and Miller (Hanke and Miller 1983) suggested, however, that the protonation step does not contribute to the voltage dependence of gating, which in their model is brought about only by the transition between open and closed states. Such an interpretation was recently challenged by Pusch and coworkers, who investigated the pH dependence of the Glu-166-Asp ClC-0 mutant (Traverso et al. 2006). This mutant strongly affects the operation of the fast gate, dramatically reducing the open probability of the channel. This drastic effect of the conservative Glu→Asp mutation (Traverso et al. 2006) probably reflects the sensitivity of ClC-0 gating on the protonation state and flexibility of this key acidic residue. Lowering pH_{int} increased current of the Glu→Asp mutant, in agreement with the behavior of the wild-type channel (Hanke and Miller 1983; Traverso et al. 2006). However, the pH_{int} dependence of this mutant is not consistent with a model in which the protonation step is voltage independent, but could be better described by a model in which the protonation/deprotonation reactions carry most of the voltage dependence. This suggestion also opens up new questions. It is reasonably well established that Glu-166 is the proton acceptor responsible for the regulation of the fast gate by pH_{ext}. On the other hand, we still do not know which residue(s) is involved in the control of the fast gate by intracellular protons. An interesting hypothesis is that opening of the fast gate requires the protonation of Glu-166. Protonation may occur, in a relatively voltage-independent manner, from the extracellular solution or, in a voltage-dependent manner, from the intracellular side. A protonation of Glu-166 (or Asp-166) from the intracellular side was also proposed recently by Miller as the possible major source of voltage dependence of the fast gate of ClC-0 (Miller 2006) (see Fig. 4).

Zinc and cadmium—inhibitors of ClC-0, ClC-1, and ClC-2

ClC-0, ClC-1, and ClC-2 are inhibited by Zn^{2+} and Cd^{2+} ions (Chen 1998; Clark et al. 1998; Kürz et al. 1997; Rychkov et al. 1997), confirming results obtained for the Cl^- conductance of frog skeletal muscle (Hutter and Warner 1967). A first mechanistic insight into the interaction between Zn^{2+} ions and CLC channels came, however, from an analysis of the Zn^{2+} block of ClC-0 (Chen 1998). For ClC-0 the inhibition is reversible with an IC_{50} of 1–3 μM. The effect of Zn^{2+} did not seem to be mediated by an interaction with the fast gate, whose voltage dependence of the open probability and of the kinetics remained unaltered in the presence of Zn^{2+}. The apparent on- and off-rates of Zn^{2+} inhibition were slow and showed pronounced temperature dependence, from which it was suggested that the inhibition was unlikely to stem from a simple open channel block and probably involved a more complicated process (Chen 1998). In particular, the temperature dependence of the effect directly suggested a possible link of the inhibition with the operation of the slow gate (Chen 1998; Pusch et al. 1997). It was found that indeed increasing Zn^{2+} concentration facilitated the slow gating process (Chen 1998). Specifically, the effect of Zn^{2+} on slow gating equilibrium appears to come mostly from an increase in the forward rate of inactivation. Interestingly, the mutation Cys-212-Ser in ClC-0, which was shown to eliminate the slow gating process, also drastically reduces the channel's sensitivity to Zn^{2+} (Lin et al. 1999), further supporting the association between the slow gate and the mechanism of Zn^{2+} inhibition.

As described below, the common gate of ClC-1 has quite different features from that in ClC-0, such as, for example, an opposite voltage dependence, and vastly different kinetics

and temperature sensitivity. The IC_{50} for Zn^{2+} inhibition of ClC-1 has been found to be 0.35 mM (Rychkov et al. 1997). In contrast to ClC-0 and ClC-2 (Chen 1998; Clark et al. 1998), Zn^{2+} and Cd^{2+} block appear to be irreversible for ClC-1 (Kürz et al. 1997; Rychkov et al. 1997). Interestingly, also in ClC-1 the mutation Cys-277-Ser, corresponding to the mutation Cys-212-Ser of ClC-0, drastically reduces the closure of the slow gate (Accardi et al. 2001) and virtually eliminates Zn^{2+} block, suggesting a similarity in the mechanism of Zn^{2+} block on the two channels (Duffield et al. 2005). At variance with ClC-0, however, in ClC-1 the block by Zn^{2+} is too slow to be a simple function of the open probability of either the fast or the putative slow gate. Moreover, the temperature dependence of Zn^{2+} inhibition (Q_{10} ~13°) is much higher than the Q_{10} of the putative slow gate, which is ~4° (Bennetts et al. 2001). Both elements indicate that in ClC-1 the mechanism of Zn^{2+} inhibition, although founded on the interaction with the slow gate as in ClC-0, may present significant differences, and Duffield et al. (Duffield et al. 2005) proposed that in ClC-1 Zn^{2+} acts by binding to a closed substate of the common gate that has very low probability in the wild-type channel and was therefore not previously identified.

Extracellular Cd^{2+} produces a concentration-dependent block of ClC-1 expressed in the Sf-9 cell line, with an IC_{50} of 1 mM (Rychkov et al. 1997). It was suggested that ClC-1 has at least two binding sites for Cd^{2+} in which His residues may play a prominent role (Rychkov et al. 1997).

Zúñiga et al. found that Cd^{2+} block of ClC-2 is mediated by an acceleration of the rate of deactivation (Zúñiga et al. 2004). Mutation of Cys-256 in ClC-2, corresponding to a cysteine residue known to affect the operation of the slow gate in ClC-0 (Cys-212-Ser) (Lin et al. 1999) and ClC-1 (Cys-277-Ser) (Accardi et al. 2001) and to drastically reduce Zn^{2+} block, also reduced the effect of Cd^{2+} compared to wild-type, indicating that Cd^{2+} would exert its action through an interaction with the gating machinery of the channel (Zúñiga et al. 2004). However, at variance with the action of Zn^{2+} on ClC-0 and ClC-1, Cd^{2+} affected both the fast and the slow gating process of ClC-2 (Yusef et al. 2006), indicating a strong coupling between fast and slow gating, similar to what was proposed for ClC-1 (Accardi et al. 2001). Moreover, the mutation His-811-Ala in ClC-2, corresponding to a mutation that completely and selectively abolishes slow gating in ClC-0 (Estévez et al. 2004) and that is located in the highly conserved CBS2 domain, affected both fast and slow gating of ClC-2. Interestingly, combining this mutation with Glu-217-Val ablates all gating transitions (Yusef et al. 2006).

ClC-1—the skeletal muscle chloride channel

ClC-1 was cloned from rat skeletal muscle by homology screening with a probe derived form the *Torpedo* ClC-0, with which it shares 54% sequence identity (Steinmeyer et al. 1991b). It is predominantly expressed in skeletal muscle, where it accounts for the large Cl^- conductance responsible for the resting membrane potential (Bretag 1987; Steinmeyer et al. 1991b). Low transcript levels could also be detected in kidney, heart, and smooth muscle (Steinmeyer et al. 1991b).

Analysis of dominant-negative mutations suggested that ClC-1 has a multimeric architecture (Pusch et al. 1995b; Steinmeyer et al. 1994). This view was supported by Lorenz et al. (Lorenz et al. 1996), who showed that ClC-1 and ClC-2, on coexpression in *Xenopus* oocytes, form heterooligomers.

Even though a quantitative single-channel analysis of ClC-1 is difficult because of its small single-channel conductance (Pusch et al. 1994), an extension of the double-barreled architecture from ClC-0 to ClC-1 was strongly supported by inspection of the single-channel behavior displaying two equidistant conductance levels of 1.2 and 2.4 pS (Saviane et al. 1999). Incidentally, the small single-channel conductance explains why many previous attempts to detect its activity in intact muscle preparations failed.

Despite the similarities with ClC-0, there are a number of functional characteristics that differentiate ClC-1 from ClC-0. In contrast to ClC-0, gating and permeation apparently do not seem to be so closely linked in ClC-1 (Rychkov et al. 1998) as anions like cyclamate and methanesulfonate can have a substantial effect on gating without being permeant. However, these results can probably be explained by an external anion binding site with relatively high affinity for organic anions in ClC-1 but not in ClC-0 (Rychkov et al. 2001). Occupation of this site by organic anions indirectly influences the occupation by chloride of deeper anion binding sites. Gating of ClC-1 is similar to the fast gating of ClC-0 in that it also activates with depolarization and can be described by a Boltzmann function with an apparent gating charge of ~0.9 (Pusch et al. 1994; Rychkov et al. 1996; Steinmeyer et al. 1991b).

Even if under normal conditions ClC-1 lacks a slow hyperpolarization-activated gate, such a gate becomes visible al low pH_{ext} and positive holding potentials (Rychkov et al. 1996).

Under physiological pH conditions, ClC-1-mediated currents display a deactivation comprising two exponential components (Rychkov et al. 1996). Accardi and Pusch (Accardi and Pusch 2000) showed that these components have time constants that are quite similar at negative voltages but grow apart as the voltage is increased. At a voltage of 200 mV they differ almost by a factor of 100, mimicking the difference between fast and slow gates in ClC-0. In particular, investigation of the dependence of the two components on $[Cl^-]_{ext}$ and pH_{int} suggested that the faster gating components found for ClC-1 behaved very similarly to the fast gate of ClC-0 and the slower component of ClC-1 was similar to the slow gate of ClC-0. It was therefore proposed that also for ClC-1 the two gating components correlated with the operation of fast and slow gates. However, it was shown that the voltage dependence of the slow gate in ClC-1 is reversed compared to ClC-0 (Accardi and Pusch 2000; Saviane et al. 1999).

The physiological role of ClC-1 is discussed below in the context of its involvement in congenital myotonia.

Myotonia

Myotonia—or muscle stiffness—is a symptom that is associated with various genetic diseases. In "chloride-channel" recessive (Becker) (Becker 1957) and dominant (Thomsen) (Thomsen 1876) myotonia congenita, myotonia is practically the only symptom. Muscle diseases caused by mutations in the SCN4A sodium channel have overlapping but not identical symptoms (Lehmann-Horn and Jurkat-Rott 1999).

Myotonia is caused by hyperexcitability of the muscle plasma membrane, such that normal nerve stimulation produces an exaggerated and possibly repetitive firing of muscle action potentials (myotonic runs) (Adrian and Bryant 1974). About 80% of the resting conductance of skeletal muscle consists of a chloride conductance, g_{Cl}, the majority of which is carried by ClC-1 (Steinmeyer et al. 1991a, 1991b). A marked reduction of g_{Cl} thus decreases the depolarizing and stabilizing conductance, causing hyperexcitability. In most neurons,

equivalently stabilizing and repolarizing conductances are mostly carried by K^+ channels. It is thought that in skeletal muscle a K^+ conductance is not adequate for such a role because of an expected buildup of K^+ ions in the restricted space inside the t-tubules (Cannon 2000; Pusch 2001). In fact, detubulation of rat skeletal muscle reduces g_{Cl} but not g_K (Palade and Barchi 1977), However, the two studies that specifically investigated the subcellular localization of ClC-1 with immunofluorescence found the protein in the sarcolemma and not in the t-tubules (Gurnett et al. 1995; Papponen et al. 2005).

In myotonic dystrophy (DM), for which myotonia is only one of many symptoms, it has recently been shown that the RNA coding for the ClC-1 protein is strongly reduced by an alteration of its correct splicing (Berg et al. 2004; Charlet et al. 2002; Mankodi et al. 2002).

Dominant and recessive myotonia congenita are instead caused by mutations in *CLCN1*, the gene coding for ClC-1. A mouse model for recessive myotonia, the *adr* mouse (Mehrke et al. 1988), helped to identify ClC-1 as the major skeletal muscle Cl^- channel (Steinmeyer et al. 1991a). In the *adr* mouse, no ClC-1 protein is made because both alleles are practically destroyed by a homozygous transposon insertion. Similarly, most mutations that lead to recessive myotonia in humans either completely abolish channel function (like, e.g., early stop codons) or drastically reduce channel function (see Pusch 2002 for an overview of possible effects of recessive mutations). There may be several reasons for the fact that heterozygous carriers of such recessive mutations (50% gene dosage) are generally asymptomatic. A 50% gene dosage could be functionally compensated at the RNA level (transcription, splicing, processing, turnover) or at the protein level (translation, processing, sorting, targeting, turnover). In fact, heterozygous *adr* mice show an almost unaltered muscle chloride conductance (Chen et al. 1997). It remains, however, as an interesting problem if and how much the Cl^- conductance is reduced in human heterozygous carriers of recessive mutations. Pharmacological experiments indicate that more than 50% of the Cl^- conductance must be inhibited in order to cause myotonia (Furman and Barchi 1978). This observation and the fact that heterozygous carriers of recessive mutations are generally asymptomatic demonstrate that in order for a *CLCN1* mutation to be inherited in a dominant manner it must produce a dominant-negative effect. That is, it must reduce g_{Cl} more than a heterozygous loss of function, beyond the threshold that is necessary to precipitate myotonia. These considerations are in agreement with the fact that far more recessive than dominant *CLCN1* mutations have been described (Pusch 2002): Channel function is easily destroyed, for example, by early stop codons, but a dominant-negative effect requires a specific association with a wild-type subunit. The first dominant mutation, P480L, was identified in descendants of Thomsen, who himself suffered from the disease (Steinmeyer et al. 1994). When coexpressed with wild-type subunits in *Xenopus* oocytes, the mutation exerted a strong dominant-negative effect, and this was the first indication that CLC channels are homomultimers (Steinmeyer et al. 1994). The mechanism of action remained unclear, however, and the initial estimate of the number of subunits (4) turned out to be wrong. Later, it was found that several dominant mutations, including P480L, exert a dominant-negative effect by "shifting" the voltage dependence of channel activation to more positive voltages, such that channels are less active at the skeletal muscle resting membrane potential (Pusch et al. 1995b). In the context of the double-barreled structure of CLC channels with two separate gates (fast, protopore gate and slow, common gate) it was later found that most dominant mutations act primarily on the common gate of ClC-1 (Aromataris et al. 2001; Saviane et al. 1999). It also must be said, however, that the distinction between dominant and recessive forms of the disease is not very clear-cut. The same mutation may appear as dominant in some pedigrees and as recessive in others (Plassart-Schiess et al. 1998). Thus other factors, independent of ClC-1, seem to contribute to the severity of myotonia.

The shift of the voltage dependence is not the only dominant-negative mechanism. For example, the C-terminal truncation R894X has a quite strong dominant-negative effect, without an apparent change of the voltage dependence (Meyer-Kleine et al. 1995). This mechanism remains to be identified. As we hope to have illustrated above, understanding the pathophysiology of myotonia provides a valuable insight into the general function of ClC-1.

ClC-2—a complex chloride channel of epithelial and nonepithelial cells

ClC-2 has been cloned from rat heart and brain. The 907-amino acid protein shares 49% identity with ClC-0 and 55% with ClC-1. It is broadly expressed in several tissues and in cell lines of different origin such as epithelial, fibroblast, and neuronal (Thiemann et al. 1992).

On expression in *Xenopus* oocytes, ClC-2 gave rise to currents that were slowly activated and inwardly rectifying, unlike ClC-0 and ClC-1 (Gründer et al. 1992; Thiemann et al. 1992). Moreover, these currents were activated only at unphysiological, hyperpolarized potentials. The instantaneous *I-V* curve observed after activation of the hyperpolarized current revealed a linear current-voltage relationship. Similar to ClC-0, iodide is less permeant than chloride. Extracellular 9-anthracene carboxylic acid (1 mM) and diphenylaminecarboxylate (1 mM) inhibited the conductance by 50%, whereas 1 mM DIDS was almost ineffective (Thiemann et al. 1992).

Single-channel analysis applied on concatemeric constructs of ClC-0 and ClC-2 demonstrated a functional dimeric architecture of ClC-2 (Weinreich and Jentsch 2001) in analogy with ClC-0 (Bauer et al. 1991; Ludewig et al. 1996; Middleton et al. 1996; Miller and White 1984) and ClC-1 (Saviane et al. 1999). Unfortunately, the single-channel conductance of ClC-2 is only 2.6 pS, a factor that has so far hampered attempts at a thorough characterization of the channel properties at the single-channel level.

Superfusion of oocytes expressing ClC-2 with hypotonic solution produced currents with faster kinetics that were activated at less hyperpolarized potentials and therefore in the physiological voltage range, suggesting that ClC-2 is involved in volume regulation (Furukawa et al. 1998; Gründer et al. 1992; Jentsch et al. 2002). In particular, the overall current amplitude significantly increased on superfusion with hypotonic solution (Gründer et al. 1992). The activation was fully reversible and needed around 10 min to set in, suggesting that the effect was probably due to slow intracellular changes rather than to a direct effect on the channel. Hypertonicity did not have any effect on wild-type ClC-2. A chimeric approach allowed the identification of the N-terminal domain as determinant for the volume sensitivity of the channel (Gründer et al. 1992).

In particular, deletions in the first 31 amino acids led to constitutively open channels that were also unresponsive to hyper- or hypotonicity (when analyzed with the two-electrode voltage-clamp technique), whereas upstream from this essential domain, deletions produced channels with an intermediate phenotype. The effect of these domains was independent from their position, as the N-terminal region could be transplanted to the C-terminus, retaining its effect (Gründer et al. 1992).

Extracellular pH significantly affects the operation of ClC-2, with moderate acidification leading to channel activation already at ~ –30 mV and to increased steady-state currents (Jordt and Jentsch 1997). It was suggested that the mechanism of action is a shift of the voltage dependence of the common gating mechanism, and Jordt and Jentsch proposed that, in analogy with the effect of hyperpolarization and cell swelling, the response to extracellular

pH depends on the N-terminal domain (Jordt and Jentsch 1997). However, the role of the N-terminal domain of the channel is still not very clear. In fact, in contrast with the voltage-independent phenotype of the N-terminal deletion described previously with two-electrode voltage-clamp recordings (Gründer et al. 1992), in inside-out patches the same construct gave rise to channels that conserved the characteristic activation at hyperpolarized potentials of the wild-type, albeit with a faster kinetics (Pusch et al. 1999). Similarly, deletions of amino acids 16–61 of rClC-2 expressed in HEK cells, although producing faster opening and closing kinetics compared to wild-type, did not produce significant changes in voltage and pH dependence (Varela et al. 2002). However, it was observed that with nystatin-perforated patches, which allow the selective exchange of cations between the cytoplasm and the pipette solution, currents of the amino terminal-deleted mutant lost their voltage dependence (Varela et al. 2002), suggesting that the differential effect of the deletion in different expression systems and measuring conditions may depend on factors such as osmotic state of the cells, cytoskeleton structure integrity, or diffusible cytoplasmic components, as already discussed by Pusch et al. (Pusch et al. 1999).

In a study of currents in mouse parotid acinar cells that were probably mediated by ClC-2, Arreola et al. found a bimodal pH_{ext} effect with a conductance maximum around pH 6.5 (Arreola et al. 2002). Interestingly, acidification to pH 5.5 applied during opening by hyperpolarization led first to a transient activation followed by inhibition, suggesting the existence of two different proton-binding sites. Occupation of one of these can exert a stimulatory effect, but the site becomes accessible to extracellular protons only in the open state of the channel (Arreola et al. 2002). Very similar results were found for guinea pig ClC-2 expressed in HEK cells (Niemeyer et al. 2003). In particular, because the transient activation by external protons was ablated in the mutant Glu-217-Val, it was suggested that the residue Glu-217 is the acceptor site for protons responsible for the stimulatory effect of low pH on ClC-2 (Niemeyer et al. 2003).

Mutating Lys-566, located at the end of the transmembrane-spanning domain, to glutamate was found to shift the voltage dependence of gating and to change the inward rectification of the open channel *I-V* relationship of wild-type ClC-2 to outward rectifying, in analogy with the effect of the corresponding mutant (Lys-519-Glu) on the fast gate of ClC-0 (Pusch et al.1999, 1995a). However, mutation of this lysine did not modify activation by hyperpolarization, cell swelling, and acidification. In contrast, mutations in helix I and the preceding loop abolished all three modes of activation by constitutively opening the channel without changing its pore properties (Jordt and Jentsch 1997).

It has been clearly established that gating of ClC-2 depends on intracellular $[Cl^-]$, whose increase shifts the open probability of the channel toward positive potentials in a N-terminal-deleted construct of rat ClC-2 expressed in oocytes (Pusch et al. 1999), the full-length rat ClC-2 expressed in HEK cells (Niemeyer et al. 2003), and the human ClC-2 (Haug et al. 2003).

The role of extracellular chloride is still controversial, as Pusch et al. (Pusch et al. 1999) showed that, surprisingly, decreasing $[Cl^-]_{ext}$ increases the open probability of ClC-2, whereas Niemeyer et al. (Niemeyer et al. 2003) were not able to observe any effect of extracellular Cl^- on gating.

Activation of macroscopic ClC-2 currents follows a double exponential time course, with time constants differing roughly by one order of magnitude (de Santiago et al. 2005; Pusch et al. 1999; Zúñiga et al. 2004). Both time constants are voltage dependent, becoming faster on hyperpolarization (Zúñiga et al. 2004). The opposite behavior was found for ClC-1, in which both gates are opened by depolarization (Accardi and Pusch 2000), while the slow gate of ClC-0 also opens in response to hyperpolarization (Pusch et al. 1997). Interestingly,

the amplitudes of the two components in ClC-2 have an opposite voltage dependence (de Santiago et al. 2005). The voltage dependence of macroscopic currents could be described by a Boltzmann function with half-maximal activation and slope factor of, respectively, –117 and 22 mV at 22°C (Zúñiga et al. 2004). Activation and deactivation time constants were reduced on temperature increase without major changes in the steady-state activation curve. The Q_{10} values calculated for both fast and slow time constants are between 4 and 5, suggesting a significant conformational change associated with those processes (Zúñiga et al. 2004). This value is larger than the Q_{10} factor related to the fast gate of ClC-0 but much smaller than that for the slow gate (Pusch et al. 1997). On the other hand, it is in the same range of the Q_{10} factor measured for the common gate of ClC-1 (Bennetts et al. 2001).

An attempt at quantitative dissection of the properties of the fast and slow gating processes in ClC-2 was made by de Santiago et al. (de Santiago et al. 2005). These authors found that the open probability of the protopore gate can be described by a Boltzmann distribution with half-maximal activation at –63 mV and an apparent gating charge of –1.22, whereas the common gate remains about 55% open at positive voltages and is associated with an apparent gating charge of –0.99 with half-maximal activation at –134 mV. The mutation Cys-258-Ser affected the voltage dependence of both gates. This observation is in contrast with the fact that the double mutant Glu-217-Ala/Cys-258-Ser produced currents very similar to that of the single mutant Glu-217-Ala, probably dominated by the slow component (Niemeyer et al. 2003), suggesting that the mutation Cys-258-Ser mostly affects the operation of the fast gate.

Moreover, the mutation His-811-Ala in ClC-2, corresponding to a mutation that completely and selectively abolishes slow gating in ClC-0 (Estévez et al. 2004) and that is located in the highly conserved CBS2 domain, affected both fast and slow gating of ClC-2. Interestingly, combining this mutation with Glu-217-Val ablates all gating transitions (Yusef et al. 2006).

Collectively, these pieces of evidence point to a gating mechanism composed of a fast and slow component that bear some resemblance to the protopore and common gate identified in ClC-0 and ClC-1 but that appear also to have very distinctive features whose nature is only poorly understood. In particular, a very specific element of ClC-2 is the stronger correlation of the two gating modes compared to the other channel members of the CLC family (Zúñiga et al. 2004).

Mutation of the conserved Glu at position 217 into Val (Glu-148 in ClC-ec1) produces a loss of sensitivity of the channel to Cl^-_{int} and almost abolishes its voltage dependence and the characteristic inward rectification of the current (de Santiago et al. 2005; Zúñiga et al. 2004). It has been conjectured that in ClC-2 fast gating is due to the movement of the side chain of Glu-217 in a mechanism similar to that proposed for the fast gate of ClC-0 and ClC-1 (Niemeyer et al. 2003; Yusef et al. 2006; Zúñiga et al. 2004). In this scenario, the residual voltage dependence observed for ClC-2 at strongly hyperpolarized potentials could be explained in terms of transitions of the common gate (de Santiago et al. 2005; Niemeyer et al. 2003). The analogy between ClC-2 and other channel members of ClC family can be extended also to the extracellular pH dependence as the transient activation of ClC-2 at acidic extracellular pH is reminiscent of the increase in the open probability of the fast gate of ClC-0 at low external pH and is mediated by the corresponding glutamate residue (Niemeyer et al. 2003).

Even more speculatively, slow gating transitions in ClC-2 have been proposed to arise from conformational changes in the pore, which are known to accompany protopore gating in ClC-0 (Accardi and Pusch 2003), possibly mediated through rearrangements of the he-

lix R, which can be influenced by movements of the C-terminus of the protein (Yusef et al. 2006). However, in the absence of single-channel measurements, the separation between fast and slow gating transitions is only tentative and requires a more solid experimental basis.

Physiological role of ClC-2

In contrast to the tissue distribution, the subcellular localization of ClC-2 channels is still controversial. On one hand, antibodies against ClC-2 detected signals in apical membranes of intestinal tissues in humans (Murray et al. 1996) and mice (Gyömörey et al. 2000) and in rat lung epithelium (Blaisdell et al. 2000). On the other hand, with different antibodies, a basolateral localization was suggested in rat small intestine and colon (Lipecka et al. 2002) and guinea pig colonocytes (Catalán et al. 2002).

As pointed out by Zdebik et al. (Zdebik et al. 2004), none of these studies included controls with knockout tissues, raising the possibility that some of these results may reflect unspecific binding. Such an approach was recently undertaken by Sepúlveda and coworkers (Peña-Münzenmayer et al. 2005) who, by an immunohistochemical approach using ClC-2-null mice as a negative control, showed that ClC-2 has a basolateral localization in intestinal epithelial cells of wild-type mice. Moreover, heterologous expression in epithelial cell lines of a ClC-2 construct with a C-terminally fused GFP in combination with confocal fluorescence imaging confirmed a basolateral expression. The polarized expression seems to depend on the AP-1AB clathrin adaptor protein, which is known to be an epithelium-specific complex involved in basolateral sorting (Fölsch et al. 1999). In particular, a dileucine motif, which is normally recognized by AP-1AB (Nakatsu and Ohno 2003), encoded in the CBS2 domain of ClC-2 was found to be critical for the basolateral localization as its disruption produced apical localization (Peña-Münzenmayer et al. 2005). This motif is conserved in ClC-2 from different organisms and in the other plasma membrane CLC members but not in ClC-3, -4, -5, -6, and -7, all mainly expressed intracellularly.

A basolateral localization of ClC-2, implying that it does not contribute to chloride secretion, is compatible with the observation that homozygous mice harboring the ΔF508 mutation in the CFTR gene, leading to cystic fibrosis through impaired Cl^- secretion, survived better when ClC-2 was additionally disrupted (Zdebik et al. 2004). This issue is of significant medical importance because the pathological changes in cystic fibrosis are predominantly due to defective Cl^- conductance on the apical side of the cells, and therefore pharmacological intervention on Cl^- channels, in particular ClC-2, to be beneficial, will have to take into account its subcellular localization. For example, since ClC-2 is expressed basolaterally, maneuvers that decrease the channel conductance might have a positive effect on the pathology (Zdebik et al. 2004). A possible physiological mechanism that potentially modulates Cl^- fluxes through the plasma membrane of epithelial cells depending on their absorption-secretion activity is provided by the regulation of the open probability of ClC-2 operated by $[Cl^-]_{int}$ (Catalán et al. 2004).

Several recent papers reported on the interaction of ClC-2 with other cellular proteins. Hinzpeter et al. (Hinzpeter et al. 2006) presented evidence that ClC-2 can interact with Hsp70 and Hsp90 in HEK cells and with Hsp90 in mouse brain, producing a reduction of channel expression at the plasma membrane. However the molecular basis for the interaction remains to be determined. Bali et al. (Bali et al. 2001) suggested a role of vesicular trafficking in the regulation of ClC-2 plasma membrane expression. It has been indicated that such a role could be mediated by the dynein motor complex that coimmunoprecipi-

tates with ClC-2 in rat hippocampal slices and controls retrograde trafficking of the channel between plasma membrane and endosomes in COS-7 cells (Dhani et al. 2003).

The expression level of ClC-2 in rat renal proximal tubules is influenced by thyroid hormones (Santos Ornellas et al. 2003) and estrogens (Nascimento et al. 2003), suggesting the relevance of this channel for Cl^- transport in the kidney, even though no renal phenotype was reported in ClC-2 knockout mice (Bösl et al. 2001). It was found that the transcription factors SP1 and SP3 influence the expression level of ClC-2 in lung epithelial cells (Holmes et al. 2003) binding to the ClC-2 promoter (Chu et al. 1999). In particular, for SP1, it has been shown that such regulation is exerted by the glycosylated isoform (Vij and Zeitlin 2006).

In contrast with what could be expected from its ubiquitous expression, ClC-2-deficient mice only manifest severe degeneration of the retina and the testes leading to male infertility (Bösl et al. 2001). Both effects have been attributed to a defective transport by epithelia that would normally control the ionic environment of sensitive germ cells and photoreceptors (Bösl et al. 2001). Although the mouse system does not always represent an accurate model for humans, these findings suggest close reconsideration of the proposed role of ClC-2 in lung development (Blaisdell et al. 2000; Murray et al. 1995), nephrogenesis (Huber et al. 1998), gastric acid secretion (Malinowska et al. 1995), and modulation of postsynaptic response to GABA and glycine (Smith et al. 1995; Staley et al. 1996).

An interesting, although controversial, insight into the physiological role of ClC-2 in humans is provided by the identification of three mutations in the *CLCN2* gene causing idiopathic generalized epilepsy (Haug et al. 2003). One mutation produced a truncation of the channel just after the beginning of helix F. Heterologous expression of this construct alone or of a concatameric construct with wild-type ClC-2 in tsA201 cells led to a complete loss of channel function. Coexpression experiments resulted in a significantly smaller Cl^- current compared to wild-type. A second mutation, which has also been found in healthy controls, produces a splice variant with a 33-amino acid deletion involving helix B and had the same effect as the previous mutation. As channel constructs harboring the mutations and tagged with yellow fluorescent protein were expressed at the plasma membrane, it has been claimed that both M200fsX231 and Δ74–117 mutants of ClC-2 reach the membrane to exert dominant-negative effects that markedly inhibit the activity of wild-type ClC-2 (Haug et al. 2003). It was conjectured that these mutations would decrease Cl^- efflux from neurons, resulting in Cl^- accumulation with consequent impairment of the inhibitory GABA response (Staley et al. 1996), which in turn may lead to hyperexcitability.

The third mutation results in the amino acid substitution Gly-715-Glu, located between the two CBS domains, and gives rise to functional channels with altered voltage dependence (channels opened at less negative potentials compared to wild-type). In contrast to the other mutations, this effect represents a gain of function. It was speculated that the pathophysiological effect of this mutation is to sustain a significant Cl^- efflux during the repolarization phase of the action potential that tends to depolarize neurons, generating hyperexcitability (Haug et al. 2003). These experimental results were therefore interpreted as being compatible with a role of ClC-2 in Cl^- efflux as an essential element for normal neuronal excitation.

However, an analysis of the functional consequences of these mutations performed in HEK cells gave drastically different results (Niemeyer et al. 2004). The GFP-labeled mutations M200fsX231 and Δ74–117 were localized only intracellularly, and they did not affect the maximal cellular conductance, severely questioning a dominant-negative effect (Niemeyer et al. 2004). It is interesting to note that most truncating mutations of ClC-1 have been found to be associated with recessive and not dominant myotonia. It was therefore proposed that haploinsufficiency would be the mechanism leading to the epilepsy in patients with these mutations (Niemeyer et al. 2004).

On the other hand, the only functional consequence of the mutation Gly-715-Glu was to affect the AMP sensitivity of the channel, pointing to a completely different pathophysiological consequence from that previously proposed (Niemeyer et al. 2004). These conflicting *in vitro* results, and the lack of signs of epilepsy in ClC-2 knockout mice, call for additional human genetic evidence before *CLCN2* can be firmly classified as an epilepsy susceptibility gene.

Interestingly, ClC-2 has been shown to be inhibited by venom from the scorpion *L. quinquestriatus hebraeus*, which appears to shift the voltage dependence of activation toward hyperpolarizing potentials (Thompson et al. 2005). However, unspecific effects cannot be fully excluded, requiring the purification of the putative peptide.

The renal and inner ear ClC-K channels

ClC-K channels were identified by homology cloning in rat and human kidney (Kieferle et al. 1994; Uchida et al. 1993). In particular, the two channels from rat (rClC-K1 and rClC-K2) are 80% identical, whereas the human channels (hClC-Ka and hClC-Kb) show 90% identity with each other, indicating a comparatively recent evolutionary divergence (Kieferle et al. 1994). Besides the kidney, these channels are also expressed in the inner ear (Uchida et al. 1995; Vandewalle et al. 1997).

Expression of ClC-K1 in *Xenopus* oocytes gave rise to small, slightly outwardly rectifying currents that showed some time-dependent gating at voltages more positive than +40 mV or more negative than –100 mV, similar to chloride currents of the thin ascending limb observed in *in vitro* perfusion experiments (Uchida et al. 1993; Waldegger and Jentsch 2000). It is interesting to correlate the lack of gating with the observation that ClC-K channels are the only CLC members that have a valine in place of the critical glutamate (Glu-166 in ClC-0) that was shown to be a major determinant of the fast gate in ClC-0, ClC-1, and ClC-2. In fact, in mutating this valine into glutamate, a significant gating was introduced in the channel behavior (Waldegger and Jentsch 2000). The permeability sequence is $Cl^- > Br^- > NO_3^- > I^-$. The current amplitude increases on extracellular alkalinization to pH 8.0 and strongly decreases when the extracellular pH is reduced from pH 7.5 to 6.5 (Uchida et al. 1995; Waldegger and Jentsch 2000). Moreover, ClC-K1 activity is modulated by extracellular Ca^{2+} (Uchida et al. 1995) with an increase of $[Ca^{2+}]_{ext}$ from 1 to 5 mM producing a fourfold increase in channel currents (Waldegger and Jentsch 2000). Sensitivity to pH and Ca^{2+} also correlates with *in vitro* microperfusion experiments on the thin ascending limb (Uchida et al. 1995).

The fact that the other ClC-K channels (i.e., rat ClC-K2, human ClC-Ka and ClC-Kb), on expression in oocytes, did not give rise to current despite correct protein synthesis and a very high sequence identity with ClC-K1 was puzzling and led to the hypothesis that an auxiliary β-subunit would be necessary for their functional expression (Waldegger and Jentsch 2000). This speculation was later confirmed by Estévez et al. (Estévez et al. 2001), who showed that a gene mutated in a specific form of Bartter syndrome encodes a β-subunit (called barttin) of ClC-K channels. Barttin consists of two putative transmembrane domains and strictly colocalizes with both ClC-Ks in kidney and cochlea (Estévez et al. 2001). When coexpressed with ClC-Ka and ClC-Kb in heterologous systems it induces detectable currents, and coexpression with ClC-K1 dramatically increases the currents that are elicited by ClC-K1 expression alone (Estévez et al. 2001). Interestingly, heteromeric ClC-K1/barttin channels appear to have a modified Ca^{2+} sensitivity compared to ClC-K1 alone (Waldeg-

ger et al. 2002), even though the relative influence of unspecific background currents was clearly larger for the pure ClC-K1 currents, whereas series resistance problems may be large for barttin-increased currents.

Immunoprecipitation and immunofluorescence experiments indicated that barttin is physically associated with ClC-K proteins and increases their surface expression (Estévez et al. 2001; Waldegger et al. 2002). ClC-Ks seem to be localized mainly in the Golgi apparatus without barttin coexpression (Uchida and Sasaki 2005), but it is not clear whether barttin binds and recruits ClC-Ks to the appropriate location in the plasma membrane or whether barttin, on binding, masks Golgi-localizing signals of ClC-Ks, thus releasing it from the Golgi apparatus (Hayama et al. 2003). Also, the stoichiometry of barttin-ClC-K complexes and the respective interacting regions are unknown.

Barttin contains a putative PY motif that is a potential site for binding of WW domain-containing ubiquitin ligases or may serve as a tyrosine-based endocytosis signal (Estévez et al. 2001). When the tyrosine residue of the PY motif was mutated (Tyr-98-Ala), stimulation of ClC-Ka and ClC-Kb currents by barttin was enhanced, but macroscopic currents did not differ qualitatively from those of wild-type heteromers (Estévez et al. 2001). An interaction with ubiquitin ligases was suggested on the basis of the reduction of ClC-K/barttin currents on overexpression of the ubiquitin ligase Nedd4-2 (Embark et al. 2004). However, at variance with ClC-5 (Schwake et al. 2001) and ENaC (Abriel et al. 1999), also containing a PY motif, the expression of an inactive form of Nedd4-2 did not increase ClC-K/barttin currents (Embark et al. 2004).

The two ClC-K isoforms are differentially distributed in nephrons (Kieferle et al. 1994; Vandewalle et al. 1997). ClC-Ka (in rat: ClC-K1) is exclusively expressed in a particular nephron segment, the thin limb of Henle's loop (Fig. 5), whereas ClC-Kb (in rat: ClC-K2) has a broader expression in kidney but is especially abundant in the thick ascending limb, a nephron segment specialized in NaCl reabsorption (Fig. 5) (Jentsch et al. 2005a). In particular, it was shown by immunohistochemistry that ClC-K1 and ClC-K2 are expressed exclusively in basolateral membranes of renal (Vandewalle et al. 1997) and cochlear (Estévez et al. 2001) epithelia, although another group proposed that ClC-K1 is present in both apical and basolateral membranes of the thin limb of Henle's loop (Uchida et al. 1995). It should be noted that all localization studies have been performed in rodents. It is not clear whether the same tissue distribution applies to humans, especially because the functional/physiological equivalence of ClC-K1/ClC-Ka and ClC-K2/ClC-Kb, respectively, is based on relatively vague arguments.

Impairment of Cl^- transport as the underlying cause of renal salt-wasting diseases was suggested already about thirty years ago (Gill and Bartter 1978). ClC-Kb mutations are associated with Bartter syndrome type III (Simon et al. 1997), an autosomal recessive salt-wasting disorder characterized by reduced sodium chloride reabsorption underlying the fundamental role of the channel in this physiological process. During reabsorption, Na^+, K^+, and Cl^- ions enter the tubule cells that line the nephrons through apical Na-K-2Cl cotransporters. The subsequent extrusion of Cl^- through the basolateral side prevents accumulation of Cl^- that would oppose inward transport of Na^+, which is of paramount importance for water and salt homeostasis (Hunter 2001; Jeck et al. 2004a). The particularly high expression of ClC-Kb in a compartment specialized in salt reabsorption (the thick ascending limb), its basolateral localization, and its relevance for normal reabsorption activity support the role of ClC-Kb as the basolateral Cl^- pathway in this schematic model.

Mutations affecting only ClC-Ka have not been implicated in human diseases so far, but simultaneous mutations in ClC-Ka and ClC-Kb lead to Bartter syndrome with deafness (see below). Even if the physiological role of ClC-Ka in humans is not completely clear,

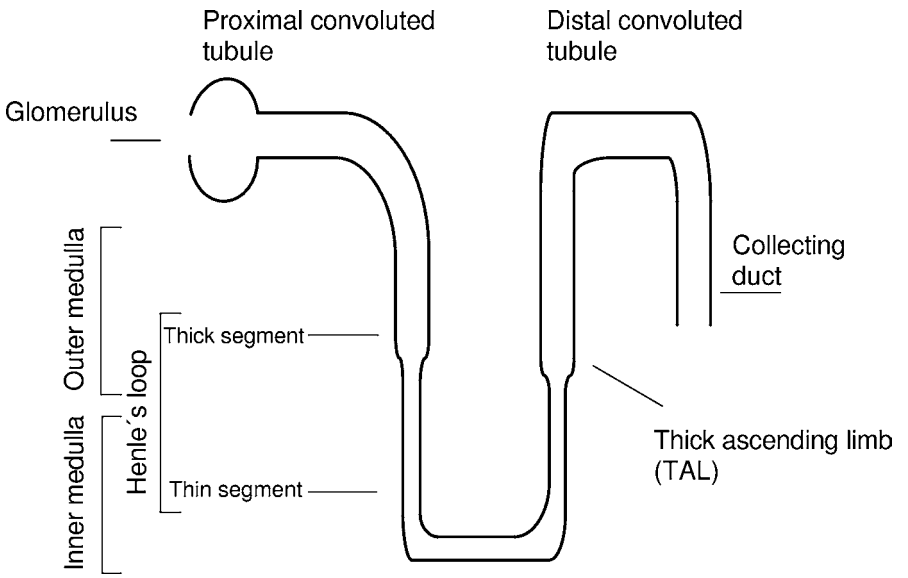

Fig. 5 Schematic representation of the nephron

an interesting insight into its function is provided by disruption of the presumed mouse ortholog ClC-K1 that produces apparent nephrogenic diabetes insipidus, a defect of urinary concentration (Matsumura et al. 1999).

Urinary concentration is determined by water reabsorption in the collecting duct (Fig. 5) that is stimulated when the kidney medulla is hypertonic. In humans, the establishment of such a situation requires the exquisite coordination of many ionic transport systems along the different segments of the nephron (the so-called countercurrent system), as exemplified by the fact that mutations in almost all of these produce a pathogenic phenotype (Sands and Bichet 2006). The study of Matsumara et al. (Matsumura et al. 1999) suggested that ClC-K1 has a critical role in the urine concentrating mechanism as already speculated by Uchida et al. (Uchida et al. 1995). This is in functional agreement with the expression of ClC-K1 in the thin ascending limb, whose Cl^- permeability was found to be impaired in the ClC-K1 knockout in *in vitro* microperfusion experiments (Matsumura et al. 1999). Interestingly, such a role for ClC-K1 correlates with the finding that maximum urine concentrating ability in mice and rats is observed 2–3 weeks after birth and parallels a gradual increase in ClC-K1 expression within the ascending limb of Henle's loop (Kobayashi et al. 2001; Liu et al. 2001) (Fig. 5).

In the course of genetic analysis of patients suffering from salt-losing tubular disorders, several mutations and molecular variants of ClC-Kb have been identified (Konrad et al. 2000). One in particular leads to the amino acid substitution Thr-481-Ser, which is also found at a frequency of 20%–40% in nonaffected individuals and produces a 20-fold increase in current induced by heterologously expressed CLC-Kb, probably due to increased open probability of the channel (Jeck et al. 2004a). This mutation has been associated with high blood pressure (Jeck et al. 2004b), but recent publications contradict this hypothesis (Kokubo et al. 2005; Speirs et al. 2005).

Human mutations in the β-subunit barttin lead to Bartter syndrome type IV characterized by both severe renal salt loss and congenital deafness (Birkenhäger et al. 2001). According

to the mechanism proposed to explain the physiopathology of the deafness, ClC-Ka and -Kb represent essential basolateral exit pathways to keep internal Cl^- concentration at a level that is compatible with the efficient accumulation of K^+ into marginal cells of the cochlear stria vascularis. K^+ is then secreted into the endolymph, where its high concentration (150 mM) is required for the stimulation of sensory hair cells (Jentsch 2000). Mutations involving ClC-Ka or ClC-Kb alone have not been implicated in deafness. This probably reflects the fact that coexpression of ClC-Ka and -Kb in the cochlea preserves a sufficient level of Cl^- extrusion even in the case of mutations that impair one of them. This functional rescue cannot occur in case of barttin mutations that indeed invariably cause the renal and inner ear phenotypes. This scenario is confirmed by the finding that simultaneous ClC-Ka and -Kb mutations result in a phenotype that mimics type IV Bartter syndrome (Schlingmann et al. 2004).

Considering the physiological relevance of ClC-K and their involvement in pathogenic state in human and mouse, the identification of pharmacological tools to modify their properties can have important medical consequences and may represent a tool to better understand their biophysical properties (Pusch et al. 2006).

In contrast to the behavior of other ClC channels (Conte-Camerino et al. 1988; Liantonio et al. 2002; Pusch et al. 2000), ClC-K channels are inhibited by derivatives of CPP and DIDS from the extracellular side (Liantonio et al. 2002; Picollo et al. 2004). In particular, for ClC-Ka and ClC-K1, it was found that the block by 3-phenyl-CPP was quickly reversible and competitive with extracellular Cl^-, suggesting that the binding site for the compound is exposed to the extracellular side and is located close to the ion-conducting pore (Liantonio et al. 2004; Picollo et al. 2004).

Surprisingly, the apparent affinity of ClC-Kb for the compounds was found to be five- to six fold lower than for ClC-Ka (K_D of ~80 and 90 μM for 3-phenyl-CPP and DIDS, respectively) despite the very high sequence identity between them. An elegant approach allowed Picollo et al. (Picollo et al. 2004) to identify a critical residue at position 68 as the major molecular determinant for the differential behavior, as ClC-Ka has a neutral asparagine at this position whereas ClC-Kb has a charged aspartate.

Very recently, Liantonio et al. (Liantonio et al. 2006) showed that niflumic acid (NFA) and flufenamic acid (FFA), drugs belonging to a class of nonsteroid anti-inflammatory fenamates, modulate ClC-K channel activity in a singular manner. NFA applied extracellularly at concentrations up to 1 mM increased ClC-Ka current amplitudes by a factor of two in a voltage-independent manner, whereas higher concentrations blocked the current. Such a biphasic behavior was tentatively explained by the presence of two different binding sites. In contrast with this behavior, NFA produced only activation of ClC-Kb (Liantonio et al. 2006). On the other hand, FFA blocked ClC-Ka but activated ClC-Kb (Liantonio et al. 2006).

Although the molecular basis for the effect of these molecules is still poorly understood, they provide a promising starting point for identification of diuretics and for the treatment of Bartter syndrome (Liantonio et al. 2006).

ClC-3—a transporter with special importance in the brain

ClC-3 was first cloned by Kawasaki et al. (Kawasaki et al. 1994). It has an ubiquitous expression pattern, but is predominantly found in brain, most notably in the olfactory bulb, hippocampus, and cerebellum, and in kidney and colon (Kawasaki et al. 1994). Ogura et al. (Ogura et al. 2002) described a splice variant of ClC-3, called ClC-3B, that has a dif-

ferent and slightly longer C-terminal end and is expressed mostly in epithelial cells. The ClC-3 protein is found predominantly in late endosomes, lysosomes, and synaptic vesicles and is important for their acidification (Hara-Chikuma et al. 2005b; Stobrawa et al. 2001). No human disease caused by mutations in ClC-3 has been reported so far, but its disruption in mice leads to a progressive degeneration of the hippocampus and a complete loss of photoreceptors (Stobrawa et al. 2001). In addition to these findings, an independently generated ClC-3 KO mouse (Yoshikawa et al. 2002) also showed markers of lysosomal storage disease that partially overlapped with neuronal ceroid lipofuscinosis (NCL), but no association of ClC-3 mutations with NCL could be detected in several dog lineages that suffered from late-onset NCL (Wohlke et al. 2006). Importantly, there was no significant difference in swelling-activated currents between wild-type and knockout mice (Stobrawa et al. 2001; Wang et al. 2006), proving that ClC-3 does not underlie the swelling-activated chloride current as previously suggested (Duan et al. 1997; see also discussion in Jentsch et al. 2002).

The mechanism underlying the ClC-3 knockout phenotypes is still unknown, but, based on an increased glutamate uptake in synaptic vesicles of knockout mice, it was speculated that the neurodegeneration might be caused by a neurotoxic effect of glutamate due to the altered intracellular vesicle pH (Stobrawa et al. 2001). However, a different mechanism based on trafficking defects of other membrane proteins brought about by altered acidification of intracellular compartments produced by dysfunction of ClC-3 could not be excluded. In this respect, it is interesting to note that Salazar et al. (Salazar et al. 2004) revealed that a mouse deficient in AP-3, an adaptor protein responsible for the correct sorting of membrane proteins in synaptic vesicles, also manifested a marked decrease in the expression of ClC-3. Moreover ClC-3 co-localized with a zinc transporter (ZnT3) and modulated Zn^{2+} level in a specific subpopulation of synaptic vesicles (Salazar et al. 2004), a finding of possible physiological relevance considering the inhibitory effect of Zn^{2+} on NMDA-mediated response in the hippocampus (Vogt et al. 2000).

Robinson et al. (Robinson et al. 2004) suggested that calcium-calmodulin-protein kinase II (CaMKII) is able to activate ClC-3 in different cell types and proposed Ser-109 as the phosphorylation site. In particular in transfected tsA cells and HT29 cells (human colonic tumor cell line), ClC-3 was reported to have a substantial plasma membrane expression. These studies, however, seem to need further confirmation, as no other group has reported similar results yet.

Recently, an interesting involvement of ClC-3 activity in the oxidative function of neutrophils has been discovered (Moreland et al. 2006). Starting from the initial observation that ClC-3 KO mice, but not wild-type mice, died frequently from sepsis following intravascular catheter placement, Moreland et al. (Moreland et al. 2006) found that neutrophils from knockout mice showed an impaired NADPH oxidase activity. ClC-3 was found in particular in secretory vesicles and secondary granule compartments. The precise role of ClC-3 in neutrophil oxidative function remains, however, to be elucidated.

The biophysical properties of ClC-3 have been notoriously difficult to analyze, and different groups have reported conflicting results (see Jentsch et al. 2002). We consider the studies of the Weinman group (Li et al. 2000, 2002) as the most reliable. Weinman and colleagues expressed ClC-3 in mammalian cell lines and could detect relatively small membrane currents in highly overexpressing cells, in which most of the expressed protein remained intracellular (Li et al. 2002). Interestingly, these cells showed enlarged and acidic intracellular structures (Li et al. 2002). Importantly, the biophysical properties of these currents were very similar to those of ClC-4 and ClC-5 (Li et al. 2000), CLC proteins that can be reliably expressed in *Xenopus* oocytes (Friedrich et al. 1999; Steinmeyer et al. 1995) and

are highly homologous to ClC-3. Based on the functional and structural similarity of ClC-3 with ClC-4 and ClC-5, it has been suggested that ClC-3, like these other two proteins, is actually a Cl^- / H^+ antiporter, and not a Cl^- channel. However, lacking direct experimental evidence, this conclusion must still be considered as tentative.

ClC-4—a transporter whose physiological role is poorly understood

ClC-4 has been identified by van Slegtenhorst et al. (van Slegtenhorst et al. 1994); it shares 78% sequence identity with ClC-5 and shows very similar biophysical properties (Friedrich et al. 1999). ClC-4 is mainly found in brain, liver, and kidney, where its subcellular localization closely resembles that of ClC-5, that is, it colocalizes mainly with endosomal markers (Mohammad-Panah et al. 2003). It was suggested that ClC-4 facilitates endosomal acidification and is important for endocytosis (Mohammad-Panah et al. 2003). Biochemical and functional lines of evidence suggested that ClC-4 and ClC-5 can form heterodimers (Mohammad-Panah et al. 2003; Suzuki et al. 2006). However, unlike ClC-5, ClC-4 is not crucial for renal endocytosis because ClC-4 knockout mice do not display proteinuria (Jentsch et al. 2005b). ClC-4 was proposed to facilitate incorporation of copper into ceruloplasmin by shunting currents of Cu^{2+}-ATPases in the secretory pathway (Wang and Weinman 2004). In rodent and human intestinal epithelia ClC-4 has been reported to colocalize with CFTR in apical membrane and subapical vesicles, and it has been suggested to mediate chloride current across the plasma membrane of Caco-2 cells (which represent a model for human enterocytes). This would support a role of ClC-4 in intestinal chloride secretion, suggesting that it might be capable of functionally complementing CFTR *in vivo* (Wang and Weinman 2004). These results, however, are difficult to reconcile with the Cl^-/H^+ antiporter function of ClC-4 (Picollo and Pusch 2005; Scheel et al. 2005) and its biophysical properties (Friedrich et al. 1999) and need further experimental verification.

ClC-5—a transporter involved in endocytosis

ClC-5 is the most extensively studied member of the CLC branch also comprising ClC-3 and ClC-4, being the only one for which an involvement in a genetic disease has been described. In fact, the identification of ClC-5 is due to its link to Dent disease, an X-linked hereditary disorder that is always associated with low-molecular-weight proteinuria and less frequently with hypercalciuria, which in turn causes nephrocalcinosis, nephrolithiasis (kidney stones), and eventual renal failure (Dent and Friedman 1964; Günther et al. 1998; Wrong et al. 1994). Fisher et al. (Fisher et al. 1994) identified a gene whose transcripts were heavily expressed in kidney and which was partially deleted in individuals affected by Dent disease. The predicted amino acid sequence of the gene product showed a high degree of homology to previously isolated members of the CLC family.

Steinmeyer et al. (Steinmeyer et al. 1995) independently cloned full-length ClC-5 from rat brain (rClC-5) by homology to other CLC proteins. The open reading frame of 2,238 bp predicts a protein of 746 amino acids with a molecular mass of 83 kDa. It is highly expressed in kidney, but mRNA is also detectable in brain and liver and to a lesser extent in lung and testis (Steinmeyer et al. 1995). A splice variant with an additional 70 amino acids at the intracellular amino terminus has been detected at the mRNA level but not at the protein level,

and no mutations have been identified so far in the exons encoding the 70 additional amino acids (Ludwig et al. 2003).

ClC-5 is predominantly expressed in kidney but is also found in other tissues, such as intestinal epithelia. In these tissues it is present in vesicles of the endosomal pathway (Devuyst et al. 1999; Günther et al. 1998; Jentsch et al. 2005c; Vandewalle et al. 2001). In particular, the expression of ClC-5 is very high in the proximal tubule (PT), which is responsible for the endocytotic uptake of low-molecular-weight proteins (Jentsch et al. 2005a) (Fig. 5). In the PT and in intercalated cells of the collecting duct (Günther et al. 1998), in rat fetal lung (Edmonds et al. 2002), and in human retinal pigment epithelium (Weng et al. 2002), ClC-5 colocalizes with a V-type H^+-ATPase in vesicles below the apical membrane. In particular, transmission electron microscopy of PT cells shows the presence of ClC-5 in vesicles that are concentrated below the microvilli of the brush border (Günther et al. 1998). This specialized region contains an extensive endocytotic apparatus necessary for the pronounced endocytotic activity of proximal tubule cells (Günther et al. 1998).

Moreover, it was found that ClC-5 colocalizes with endosomal markers (Günther et al. 1998) and endocytosed proteins early after uptake (Devuyst et al. 1999; Günther et al. 1998; Piwon et al. 2000), arguing for its presence in early endosomes (Jentsch et al. 2005a).

Expression of rat ClC-5 in *Xenopus* oocytes produced strongly outwardly rectifying chloride currents for which it was not possible to detect any gating relaxations (Steinmeyer et al. 1995). The rectification also prevented the determination of a true reversal potential and consequently the assessment of relative permeability ratios. From the current magnitude, however, the conductivity sequence was determined as $NO_3^- > Cl^- > Br^- > I^-$ > glutamate, in agreement with the behavior of other CLC proteins (Steinmeyer et al. 1995). Several classic Cl^- channel inhibitors (DIDS, 9-AC, CPA) had no effect on rClC-5. In *Xenopus* oocytes, rClC-5 elicited chloride currents only at potentials more positive than 20 mV, a value that is not reached across the plasma membrane of most cells. It was therefore speculated that rClC-5 may be localized to intracellular compartments characterized by a different transmembrane voltage and that the currents observed in oocytes may result from "spillover expression," whereby vesicles normally targeted to an intracellular compartment reach the plasma membrane because of overexpression (Steinmeyer et al. 1995). This was also confirmed by immunofluorescence performed on COS-7 cells transfected with ClC-5 (Günther et al. 1998). Whether this occurs *in vivo* is not yet clear (Jentsch 2005). However, Wang et al. proposed from biotinylation studies that in proximal tubule cells, about 8% of the total cellular pool of ClC-5 is located at the plasma membrane (Wang et al. 2005).

Recently, Suzuki et al. (Suzuki et al. 2006), using immunofluorescence and immunoprecipitation, reported that ClC-3, ClC-4, and ClC-5 show a high degree of colocalization in intracellular organelles on expression in HEK cells and potentially form heteromultimers.

Dent disease can be caused by nonsense mutations, deletions, and also missense mutations in the *CLCN5* gene (reviewed in Jentsch et al. 2005c; Uchida and Sasaki 2005). The missense mutations are interspersed along the whole secondary structure of the protein. Nevertheless, on heterologous expression in oocytes, most of them produce a similar phenotype, namely impaired trafficking to the plasma membrane. This seems to imply that several distinct protein regions are essential for correct targeting and/or protein stability. Interestingly, the mutation Arg-516-Trp, located only 5 amino acids away from the mutant Leu-521-Arg that abolishes channel expression at the plasma membrane, shows a normal level of expression but leads nonetheless to a drastic reduction of Cl^- current, implying that this mutation does not affect targeting but severely impairs transport activity (Ludwig et al. 2005). In contrast, truncation of ClC-5 at position 648, located just after the CBS1 domain, although not functional, does not impair trafficking but instead produces a paradoxical increase in plasma

membrane expression (Ludwig et al. 2005). It was hypothesized that the effect could be due to the deletion of a PY motif located between the two CBS domains. In fact, Schwake et al. (Schwake et al. 2001) showed that mutating the PY motif of ClC-5 almost doubled surface expression and channel activity and suggested that the effect could be due to impaired internalization of the protein. Such a motif had been also implicated in internalization and ubiquitination of the amiloride-sensitive sodium channel (ENaC), and mutations in it lead to Liddle syndrome, another human inherited kidney disorder associated with hypertension (Hansson et al. 1995a, 1995b).

Low-molecular-weight proteinuria is a hallmark of Dent disease. Proteins of low molecular weight are filtered at the glomerulus and are normally reabsorbed in the proximal tubule (Fig. 5) by fluid-phase and receptor-mediated endocytosis (Mellman 1996). After being endocytosed, the proteins are subsequently degraded in lysosomal compartments (Maack and Park 1990). Acidification of the endosomes is essential for the progression along the endocytic apparatus to lysosomes (Mellman et al. 1986) (but see Günther et al. 1998 and references therein), and it is mediated by a V-type H^+-ATPase (Gluck et al. 1996). Interestingly, immunohistochemistry studies of biopsies of Dent disease patients revealed a consistent inversion of H^+-ATPase polarity in PT cells, showing a basolateral distribution contrasting with its apical location in the normal kidney (Moulin et al. 2003). These modifications in polarity and/or expression of the H^+-ATPase are compatible with an interaction between ClC-5 and the H^+-ATPase that would be essential for the proper targeting or stability of the latter and may explain the deficit in urinary acidification observed in some patients with Dent disease (Moulin et al. 2003). The colocalization of ClC-5 with the H^+-ATPase suggested that ClC-5 might be important for endocytosis, and it was speculated that its role was to provide an electrical shunt for the efficient accumulation of protons by the H^+-ATPase (Günther et al. 1998). Indirect support for a role of CLC-5 in the acidification of intracellular compartments comes also from yeast: Disruption of either the yeast CLC (GEF1) (Greene et al. 1993), which resides in intracellular vesicles (Hechenberger et al. 1996; Schwappach et al. 1998), or of GEF2, a subunit of the vacuolar H^+-ATPase, caused an increased sensitivity to more alkaline pH (Gaxiola et al. 1998; Schwappach et al. 1998).

A knockout mouse approach provided a powerful insight into the physiological role of ClC-5 (Piwon et al. 2000; Wang et al. 2000). In very elegant experiments, Piwon et al. exploited the fact that ClC-5 is encoded on the X chromosome, which is subject to random inactivation in females, leading to a mosaic expression of ClC-5 in heterozygous (+/–) females. In this way, cell-autonomous phenotypes could be distinguished from non-cell-autonomous effects. In particular, cells lacking ClC-5 endocytosed much less protein than ClC-5-expressing cells, explaining the low-molecular-weight proteinurea. ClC-5 disruption affected both receptor-mediated and fluid-phase endocytosis (Günther et al. 2003; Piwon et al. 2000). In this respect, it is important to note that in the ClC-5 knockout mouse the amount of megalin at the plasma membrane was also reduced (Christensen et al. 2003; Piwon et al. 2000), probably because of impaired endosome recycling (Piwon et al. 2000) (see below). Moreover, the *in vitro* acidification of cortical renal endosomes prepared from ClC-5 knockout animals was reduced, supporting the proposed role of ClC-5 in endosomal acidification (Günther et al. 2003; Hara-Chikuma et al. 2005a; Piwon et al. 2000).

It is generally accepted that altered endosomal acidification impairs endocytosis (see above), and this might be due to a pH-dependent association of endosomes with regulatory proteins such as the GTPase Arf6 (Maranda et al. 2001). However, the details of the progressive acidification in the maturing endosomes are not yet very clear. For example, it has been suggested that primary endocytic vesicles are not acidified (Fuchs et al. 1994), and

pharmacological inhibition of endosomal acidification does not affect the primary endocytic rate (Cupers et al. 1997; Tyteca et al. 2002).

In this scenario, the role of ClC-5 also does not appear to be completely clear. The fact that Günther et al. (Günther et al. 2003) found a significant degree of acidification also in endosomes of ClC-5 knockout mice that depended on the amount of Cl^- in the medium (Günther et al. 2003) could be explained by some contamination in the preparation but also by the presence, in endosomes, of Cl^- conductances that are not mediated by ClC-5. This is in agreement with the observation of Hara-Chikuma et al. on primary culture of proximal tubule cells from wild-type and ClC-5 KO mice (Hara-Chikuma et al. 2005a). They found that early endosomes lacking ClC-5 showed slightly impaired acidification and Cl^- accumulation compared to wild-type. Importantly, the acidification and Cl^- accumulation was almost completely abolished by the nonspecific Cl^- channel inhibitor NPPB in both wild-type and KO endosomes (Hara-Chikuma et al. 2005a). Altogether these lines of evidence raise the possibility that the central function of ClC-5 might not be the acidification of these compartments.

Another important observation concerning the impaired endocytosis in Dent disease is that in ClC-5 KO mice megalin and cubilin expression at the plasma membrane was reduced and these proteins were redistributed in intracellular organelles (Christensen et al. 2003; Piwon et al. 2000). These proteins belong to the family of multiligand tandem receptors involved in endocytosis, and their decrease at the plasma membrane is also compatible with the proteinuria phenotype of patients with Dent disease (Devuyst et al. 2005). Moreover, it is interesting to correlate this finding with the presumed preferential role of the subapical endosomes expressing ClC-5 in the recycling endosomal activity (Hara-Chikuma et al. 2005a).

Additional information about the role of ClC-5 in receptor-mediated endocytosis has been provided from analysis of albumin reabsorption in the PT of opossum kidney (OK) cells, which occurs through the megalin/cubulin receptor complex (Hryciw et al. 2005). Poronnik and coworkers observed that the level of ClC-5 expression at the plasma membrane of OK cells is influenced by the amount of albumin present extracellularly (Hryciw et al. 2004). The authors speculated that the effect is mediated by ubiquitination of ClC-5 operated by ubiquitin-protein ligase Nedd4-2 and that ClC-5 mediates the formation of an endocytic complex at the plasma membrane that contains the albumin-binding receptor megalin/cubilin (Hryciw et al. 2005). In the light of this model, the observed interaction between the C-terminus of ClC-5 and the actin-depolymerizing protein cofilin (Hryciw et al. 2003) was proposed to be required for the localized disruption of the actin cytoskeleton (Hryciw et al. 2005) that allows the endosomes to pass into the cytoplasm (Qualmann et al. 2000). Recently, it was shown that ClC-5 coimmunoprecipitates with the Na^+/H^+ exchanger regulatory factor NHERF2, a PDZ scaffold protein that may be relevant for the assembly of macromolecular complexes at the plasma membrane comprising the Na^+/H^+ exchanger (Hryciw et al. 2006). In particular, silencing NHERF produced a decrease of albumin uptake that was paralleled by a decreased surface expression of ClC-5 (Hryciw et al. 2006).

Disruption of the *clcn5* gene also produced defective internalization of the apical NaP_i-2 (sodium-phosphate cotransporter) and the apical Na^+/H^+ exchanger NHE3 (involved in reabsorption of Na^+, HCO_3^-, and fluid) (Piwon et al. 2000). The effect is mediated by parathyroid hormone (PTH) whose endocytosis is also defective in *clcn5* KO mice, leading to a progressive increase in luminal PTH levels that in turn stimulates endocytosis of those transporters (Jentsch et al. 2005b; Piwon et al. 2000). The decreased plasma membrane level of NaP_i-2 possibly explains the hyperphosphaturia phenotype in Dent disease patients.

It has been more difficult to explain the pathophysiological progression of Dent disease patients to nephrocalcinosis and kidney stones. In particular, the ClC-5 knockout mouse strain established by Jentsch and coworkers (Piwon et al. 2000) did not show hypercalciuria, while a strain obtained by Guggino and coworkers (Wang et al. 2000) showed hypercalciuria, which may then potentially lead to renal stones. This difference has been explained by Günther et al. (Günther et al. 2003) with the fact that hormones involved in Ca^{2+} homeostasis are subject in the PT to tight regulation that could be altered by slight genetic differences and/or diet.

Recently, it has been found that the disruption of ClC-5 in a collecting duct cell model brings about an increase in plasma membrane level of annexin A2 (Carr et al. 2006), which has been characterized as a crystal-binding molecule in renal epithelial cells (Kumar et al. 2003). This, in turn, may produce agglomeration and retention of calcium crystals, which potentially leads to nephrocalcinosis and renal stones. It has been hypothesized that ablation of ClC-5 impaired endosomal acidification rerouting annexin to the recycling pathway, resulting in an increase in plasma membrane expression (Carr et al. 2006). In this respect, it is important to note that with a ClC-5 knockout mouse model that displays hypercalciuria Silva et al. (Silva et al. 2003) suggested that the hypercalciuria is of bone and renal origin and is not caused by elevated intestinal calcium absorption. Interestingly, an interaction between ClC-5 and other proteins was also found by Mo and Wills (Mo and Wills 2004). These authors presented evidence that overexpression of ClC-5 in oocytes can alter the normal translation or trafficking of ENaC, CFTR, and NaDC-1 (sodium dicarboxylate cotransporter) to the plasma membrane by a mechanism that is independent from ClC-5-mediated chloride conductance, because a ClC-5 fragment comprising only amino acids 347–647 was sufficient to produce the same results. The mechanism behind such an effect, however, remains obscure.

Another interesting physiological role of ClC-5 has been recently suggested by van den Hove et al. (van den Hove et al. 2006), who showed that ClC-5 is significantly expressed in the thyroid (in particular in plasma membrane and late endosomes of thyrocytes) and that ClC-5 knockout mice develop a goiter with accumulation of iodide and thyroglobulin that seemed not to be caused by a defective endocytosis. It was therefore speculated that ClC-5 is involved in the regulation of plasma membrane expression of pendrin, an I^-/Cl^- exchanger responsible for iodide efflux or that ClC-5 can function as an additional iodide conductance in thyrocytes.

All these observations have vastly improved our understanding of the physiological role of ClC-5, but they still do not allow the unambiguous identification of the molecular mechanism that links ClC-5 dysfunction to the impaired endocytosis observed in Dent disease, and this reflects also the complexity of the underlying cellular processes.

We can schematically summarize the state of our present knowledge about the function of ClC-5 by formulating three possibilities.

It may be that ClC-5 is central for the acidification of endosomes. This could be rate limiting for their capacity to progress either in the degradation pathway or in the control of their redistribution to the recycling pathway, thereby determining the plasma membrane expression of proteins responsible for receptor-mediated endocytosis. A third possibility is that ClC-5 has only a marginal role in the acidification of endosomes but is an essential factor in very early endocytic processes, like endocytic vesicle budding. None of these alternatives necessarily excludes the others, and more experiments are needed to verify these possibilities or to suggest new ones.

Another element in this scenario is provided by the finding that ClC-5 (and ClC-4) are actually not chloride ion channels as it has been assumed (Jentsch et al. 2002) but rather

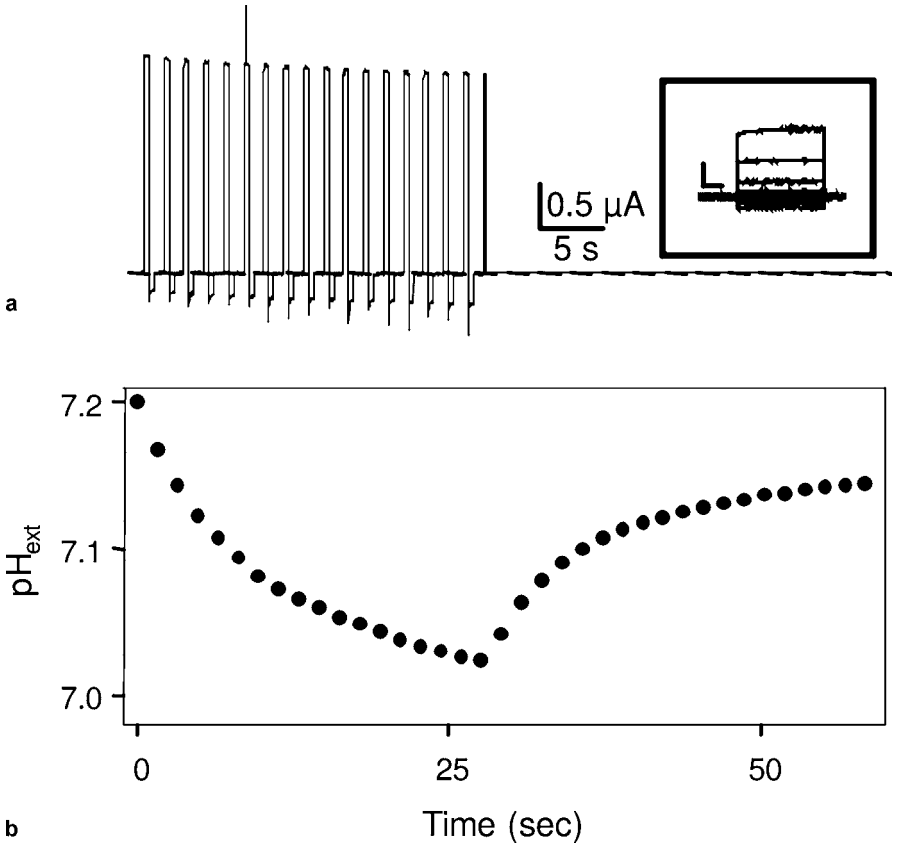

Fig. 6a, b Proton transport activity of ClC-5. **a** Current response of a voltage-clamped ClC-5-expressing oocyte stimulated by a train of pulses to 60 mV. After about 28 s the voltage clamp was switched off. **b** Simultaneously recorded pH close to the oocyte surface is plotted versus time. The *inset* in **a** shows a family of currents traces elicited by voltage pulses from –140 to 80 mV in 20-mV increments, recorded from the same oocyte. The marked outward rectification of ClC-5 is evident

transporters in which the inward movement of Cl^- is stochiometrically coupled to the outward movement of H^+ (Picollo and Pusch 2005; Scheel et al. 2005) (see Figs. 4 and 6). Such a transport mechanism seems, at first sight, to conflict with the accepted view of these proteins as passive Cl^- conductance allowing efficient acidification of vesicles by the proton pump because the Cl^-/H^+ antiporter activity of ClC-5 would actually lead to a partial dissipation of the proton gradient and ultimately to a waste of energy (Pusch et al. 2006).

However, the physiological implications of the transport activity of ClC-5 have not been explored yet and might be more complex than previously outlined.

The mechanism of transport couples the luminal pH to the Cl^- gradient across the vesicular membrane. However, the degree of coupling between the Cl^- and H^+ fluxes mediated by the ClC-5 depends on the stoichiometry of the transport, for which, at the moment, we only have a rough guess (Picollo and Pusch 2005; Scheel et al. 2005).

Our understanding of the biophysical and physiological function of ClC-5 is at a very early stage. The antiporter activity emerged recently, and a consistent corpus of new experimental evidence regarding the interaction of ClC-5 with other proteins suggests a more

diversified and articulated function than just a passive Cl^- efflux to allow acidification of intracellular vesicles, but our knowledge is still too limited to formulate a more specific description of what this function could actually be.

The intracellular ClC-6 and ClC-7 proteins

On the basis of sequence conservation ClC-6 and ClC-7 form a separate branch of the CLC family with a sequence homology between them of 45%. Both have a very broad tissue distribution (Brandt and Jentsch 1995). These two proteins have so far escaped any attempt at biophysical characterization, as it has not been possible to express them in heterologous systems yet.

ClC-7 has been found to be a late endosomal/lysosomal Cl^- channel with a very broad tissue distribution (Kasper et al. 2005; Kornak et al. 2001). In particular, it is highly expressed in osteoclasts, the cells involved in bone degradation.

Its physiological relevance is highlighted by the finding that the *CLCN7* gene, encoding the human ClC-7 protein, is mutated in the disease osteopetrosis and that knockout mice for the corresponding gene develop severe osteopetrosis and retinal degeneration as also reported for some patients affected by malignant infantile ostepetrosis (Kornak et al. 2001). Also less severe dominant osteopetrosis can be caused by mutations in the *CLCN7* gene (Cleiren et al. 2001; Frattini et al. 2003; Sobacchi et al. 2001). In ClC-7 knockout mice, skeletal abnormalities include loss of bone marrow cavities that are instead filled with bone material and failure of teeth to erupt, but the mice also display neurodegeneration in the CNS (Kasper et al. 2005; Kornak et al. 2001).

Bone degradation is carried out by a specialized osteoclast plasma membrane domain, the ruffled border, through acidification of the resorption lacuna. In fact, the ruffled border is formed by the exocytotic insertion of vesicles of late endosomal/lysosomal origin, containing the H^+-ATPase. ClC-7 colocalizes with the proton pump in this membrane and was suggested to function as a shunt for the efficient acidification (Jentsch et al. 2005a). This hypothesis is in agreement with the finding that ClC-7 knockout osteoclasts still attach to ivory but fail to acidify the resorption lacuna and are unable to degrade the bone surrogate (Kornak et al. 2001). Moreover, polymorphisms in the gene coding for ClC-7 have been associated with alterations in bone mineral density and bone resorption markers in postmenopausal women and have been found to modulate the phenotypes of patients affected by autosomal dominant osteopetrosis type II (Kornak et al. 2006).

Very recently, Lange et al. (Lange et al. 2006) found that the ClC-7 protein is associated with the β-subunit Ostm1, which was known to produce osteopetrosis when mutated but whose function was unclear. The interaction of ClC-7 with Ostm1 is important for the stability of ClC-7, as ClC-7 protein levels are greatly reduced in mice lacking Ostm1. It was speculated that the role of Ostm1 is to shield ClC-7 from lysosomal degradation, as ClC-7 is the only mammalian CLC protein lacking N-linked glycosylation sites.

Given the essential role of ClC-7 for proper bone resorption, the protein has been suggested as a target for the treatment of osteoporosis that is characterized by excessive bone resorption (or too little bone formation). The compound NS3736 belongs to the group of acidic diphenylureas that has been shown to block Cl^- conductance in human erythrocytes (Bennekou et al. 2001). Schaller et al. (Schaller et al. 2004) found that this compound blocks acidification in resorption compartments and inhibits osteoclastic resorption *in vitro*. The ability of NS3736 to prevent bone loss *in vivo* was tested in aged ovariectomized rats taken

as a model of osteoporosis, and it could be shown that daily treatment with 30 mg/kg protected bone strength dose-dependently, leaving bone formation unaffected. In a recent study Karsdal et al. (Karsdal et al. 2005) found that the compounds NS5818 and NS3696, close analogs of NS3637, have a very similar effect. Taken together these results suggest that chloride channel inhibitors might be useful in the treatment of osteoporosis.

Outlook

In the past 16 years (since the cloning of ClC-0) our knowledge about CLC chloride channels and transporters has increased enormously. It is no overstatement that the discovery of the CLC family has opened new horizons in fields as diverse as biophysics of membrane transport, physiology, pharmacology, and molecular medicine. Nevertheless, there are still many unsettled questions. Among the most pertinent questions are those related to a full understanding of the physiological roles of the intracellular CLC proteins: Are they really shunts? Are we still missing essential β-subunits? What are the functional properties of ClC-6 and ClC-7? Also, we are still lacking really high-affinity blockers (or activators) for any CLC protein, and there are no pharmacological tools available for ClC-3, ClC-4, and ClC-5. From a biophysical point of view, it would be interesting to decipher the rules that render a CLC protein a passive channel or, alternatively, an active transporter. It seems that CLCs will keep scientists in different areas busy for quite some time.

Acknowledgements. We thank Elena Babini for critical review of the manuscript. The financial support by Telethon Italy (grant GGP04018) is gratefully acknowledged.

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