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INVITED REVIEW

Thomas J. Jentsch · Thomas Friedrich
Antje Schriever · Hideomi Yamada

The CLC chloride channel family

Abstract Chloride channels perform important roles in the regulation of cellular excitability, in transepithelial transport, cell volume regulation, and acidification of intracellular organelles. This variety of functions requires a large number of different chloride channels that are encoded by genes belonging to several unrelated gene families. The CLC family of chloride channels has nine known members in mammals that show a differential tissue distribution and function both in plasma membranes and in intracellular organelles. CLC proteins have about 10–12 transmembrane domains. They probably function as dimers and may have two pores. The functional expression of channels altered by site-directed mutagenesis has led to important insights into their structure–function relationship. Their physiological relevance is obvious from three human inherited diseases (myotonia congenita, Dent's disease and Bartter's syndrome) that result from mutations in some of their members and from a knock-out mouse model.

Key words Anion channel · *Arabidopsis thaliana* · CBS domain · Double-barrel · Endocytosis · Gating · Human genetics · Kidney stones · Yeast gef1

Introduction

The expression cloning of a voltage-gated chloride channel from the electric organ of *Torpedo marmorata* identified the first gene encoding a voltage-gated chloride channel [54]. Its structure did not resemble those of other ion channels cloned before, and it was structurally distinct from other chloride channels such as the ligand-gated γ-aminobutyric acid (GABA) and glycine receptors and cystic fibrosis transmembrane conductance regulator (CFTR), the cAMP-activated chloride channel that is mutated in cystic fibrosis. Over the following years, it became clear that ClC-0, as this ion channel was called, was the founding member of a large gene family of chloride channels. Since then, nine different CLC genes in mammals have been discovered [53], and diverse genome projects and other cloning strategies have revealed that CLC genes are present in organisms as diverse as animals, plants, yeast, archaebacteria, and eubacteria (Fig. 1).

In mammals, CLC proteins show differential tissue distribution and perform different functions. These include the control of electrical excitability, transepithelial transport, and the charge compensation necessary for the acidification of intracellular organelles. In addition, CLC channels may play a role in cell volume regulation. The best evidence for their physiological importance comes from human inherited diseases resulting from mutations in these channels: mutations in the muscle chloride channel ClC-1 lead to myotonia [62, 116] (a disease associated with muscle stiffness), and mutations in the kidney-specific channel ClC-Kb lead to Bartter's syndrome [113], an inherited disease associated with severe renal salt wasting. Mutations in ClC-5, a chloride channel that is predominantly expressed in kidney, cause Dent's disease [69]. This disorder is associated with proteinuria and hypercalciuria, which in turn lead to the clinically predominant symptoms of kidney stones, nephrocalcinosis, and renal failure. Furthermore, the generation of a ClC-K1 knock-out mouse [82] revealed that this channel is necessary for the urinary concentration mechanism.

In the following review, we first address common features of CLC chloride channels. This includes their transmembrane topology, their probable dimeric structure with two pores, and the gating mechanism that is often largely dependent on permeating anions. Then individual features of several of these channels will be presented, with some focus on human disease. Two final short sections will describe the interesting findings with the yeast CLC *gef1p* and give a short review on prokaryotic CLCs.

T.J. Jentsch (✉) · T. Friedrich · A. Schriever · H. Yamada
Zentrum für Molekulare Neurobiologie, (ZMNH) Universität Hamburg, Martinistrasse 85, D-20246 Hamburg, Germany
e-mail: Jentsch@plexus.uke.uni-hamburg.de
Tel.: +49-40-47174741, Fax: +49-40-47174839

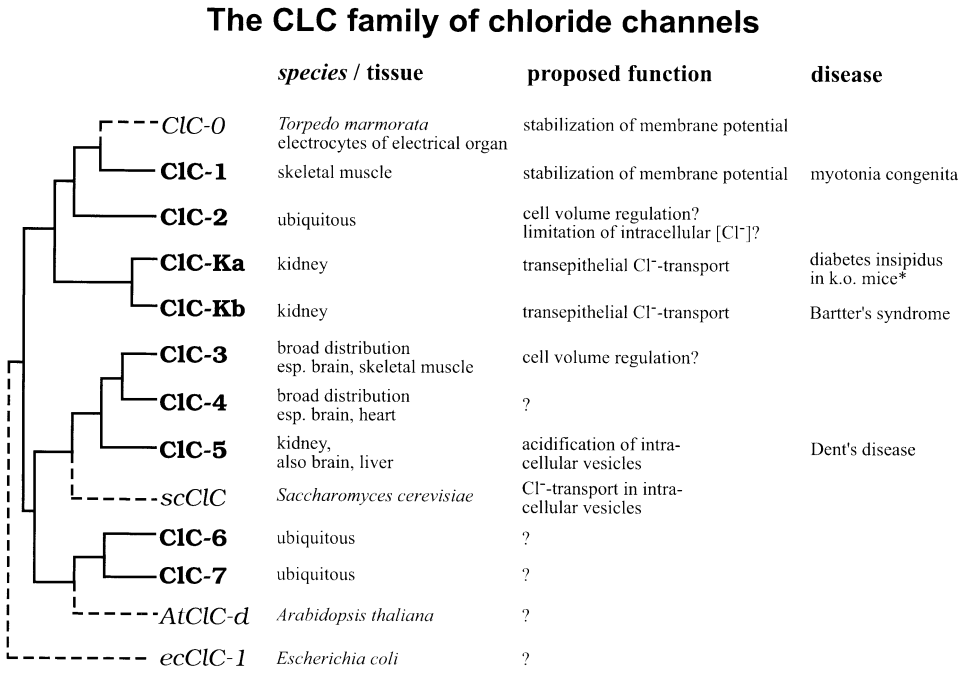

Fig. 1 The CLC family of chloride channels. The dendrogram (at the *left*) indicates the degree of similarity between different human CLC gene products (indicated in *bold*) and homologues from selected other species (*italics*). The human CLC channels are grouped into three branches with less than 30% identity between them. The first one includes ClC-1, ClC-2 and the ClC-K channels (ClC-0, the *Torpedo* channel, also belongs to that branch). The second branch includes ClC-3, ClC-4 and ClC-5. This branch is most closely related to the yeast scClC (*gef1p*). The third branch includes ClC-6 and ClC-7, which show closest relationships to the plant CLC proteins AtClC-a to -d (with only the latter shown here). The known prokaryotic CLC proteins form a distinct branch. Species and tissue distribution (*2nd column*) and proposed function (*3rd column*) of the CLC channels together with their role in human inherited diseases (*4th column*) is also given. *Under the assumption that human ClC-Ka corresponds to mouse ClC-K1 (see main text)

Structure and function of CLC channels

Transmembrane topology

The initial hydropathy analysis of the *Torpedo* channel ClC-0 indicated the presence of up to 13 transmembrane domains that were dubbed D1–D13 [54]. Some of these, including D13 which is located next to the C-terminus after a long hydrophilic stretch, were weak candidates on the basis of hydropathy alone. Indeed, D13 was shown in subsequent studies not to cross the lipid bilayer [44]. As the sequences of more CLC proteins became available, it became clear that some of the putative transmembrane domains are conserved to a larger extent than others, and that some of these domains seem to be missing from certain CLC channels, based on their low hydropathy index and low degree of sequence conservation [7]. For example, this is true for the region initially called D4, which is not hydrophobic and shorter in CLC proteins such as ClC-6 and -7.

Schmidt-Rose and Jentsch [108] performed the first systematic biochemical study of the transmembrane topology of CLC channels using the muscle chloride channel ClC-1. Their model is shown in Fig. 2. Since most available methods for determining the transmembrane topology of proteins with multiple transmembrane domains have certain disadvantages, a combination of glycosylation scanning, protease protection assays, and cysteine scanning mutagenesis was used. When possible, the validity of the results was checked by functionally expressing the mutant cDNAs in *Xenopus* oocytes [108]. This study demonstrates that the N- and C-termini of the protein are intracellular, and that, consistent with previous results [44], the C-terminal domain D13 does not cross the lipid bilayer. The broad hydrophobic region at the end of the transmembrane-spanning block was difficult to address, and it is currently unclear whether the D9–D12 region spans the lipid bilayer 3 or 5 times, leading to a total number of 10 or 12 transmembrane domains. This study suggests that, consistent with extended hydropathy analysis, D4 does not cross the lipid bilayer. However, the D3–D4–D5 region was difficult to investigate and the data indicate that it inserts only as a block together into the lipid bilayer. Cysteine residues inserted between D3 and D4 could be modified by the charged cysteine-modifying reagent MTSES, arguing that this part is probably extracellular, and glycosylation of a site introduced between D4 and D5 suggests that this region is extracellular as well [108]. However, another recent study [29] that is based on cysteine scanning suggests that D4 does cross the bilayer and that D5 has an inverted orientation. This would require reorientation of the downstream transmembrane domains. Thus, the transmembrane topology of CLC channels is not yet entirely clear.

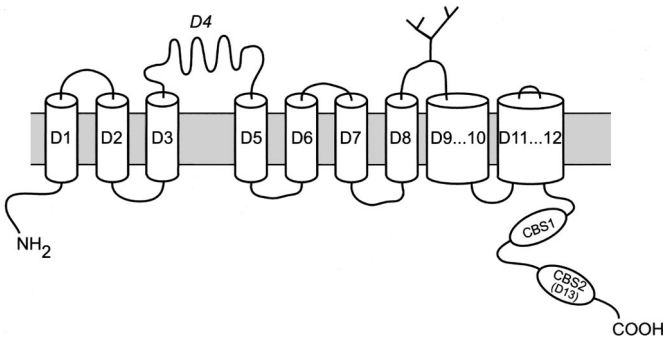

Fig. 2 Transmembrane topology model of CLC channels according to the biochemical study of Schmidt-Rose and Jentsch [108]. N- and C-termini are located intracellularly. Initial hydropathy analysis of ClC-0 indicated the presence of up to 13 TM domains (D1–D13) [54]. Except for some prokaryotic CLCs, all known CLC proteins have two CBS domains [2, 93] at the C-terminus. Another region of intermediate hydrophobicity, originally dubbed D4 (indicated in *italics*), is subject to controversy, and cysteine modification experiments may require a revision of D5 transmembrane orientation [29]. The large hydrophobic region between D9 and D12 is difficult to address in biochemical studies. Therefore, it is currently unclear whether this region contains 3 or 5 transmembrane spans, leading to a total number of 10 or 12 transmembrane domains

Assembly from different parts: split channels

ClC-0 [79] and ClC-1 [107] can be separated into different parts that still yield chloride channels when expressed together. The channel can be separated; for example, between D8 and D9. More interestingly, the channel is non-functional when the C-terminal, cytoplasmic portion is missing. Channel function can be reconstituted when the missing C-terminus is co-expressed [79, 107] or injected as a bacterial fusion protein into oocytes expressing the truncated channel [79]. The cut between the two domains has to occur between the two CBS domains [2, 93] that exist in the C-terminus [107] (Fig. 2). The second of these CBS domains coincides with the domain named D13, while the other one is much closer to the membrane-spanning block. Homologous domains have been identified in many different proteins [2, 93], but their function is still obscure. In a systematic alanine scan of the C-terminus of the yeast CLC, however, it was shown that both domains are essential for function [110]. Mutations in these domains destroy the ability of these mutants to complement the *gef1* yeast knock-out strain (in which the yeast CLC is eliminated [42]) and lead to a mislocalization of the protein [110]. This suggests that CLC CBS domains may be important for sorting. However, in ClC-1, D13 (CBS2) could be deleted in frame without loss of function [50], suggesting that there is no absolute requirement for that domain.

Dimeric structure and “double-barrels”

CLC channels probably function as dimers, but this has only been shown directly for some members. ClC-0, the channel from *Torpedo* electric organ, has been studied extensively, also before it was cloned. Single-channel recordings suggest a peculiar “double-barreled” structure for this channel [48]. In this model, the channel has two identical pores that can gate independently of each other. In addition, they also possess a common gate (the “slow gate” as opposed to the “fast gate” operating on the single channels). This slow gate closes the two pores together. After the cloning of ClC-0, this hypothesis could be tested vigorously. The expression of the ClC-0 cDNA was sufficient to reproduce the “double-barreled” structure, showing that there is no need for an additional subunit [3]. Several point mutations that change its single-channel conductance, ion selectivity, and gating were introduced into ClC-0 [74, 76, 79]. Two studies [74, 85] show that co-expression of wild-type (WT) and mutant subunits leads to “double-barreled” channels with one WT and one mutant pore. Importantly, the properties of these pores (WT or mutant) are independent of whether the second, attached pore is WT or mutant [74, 85]: they retain their single-channel conductance, ion selectivity, and gating properties. Consistent with the migration of ClC-0 in sucrose gradients [84], additional experiments strongly suggest that ClC-0 is a dimer [74, 85]. Furthermore, concatamers of different ClC-0 mutants (with point mutations at the end of D2 and D12, respectively) indicate that each pore is formed by a single ClC-0 protein [74]. These experiments leave virtually no doubt that ClC-0 has two independent conduction pathways, i.e., two pores.

Until recently, the double-barreled structure has only been studied with ClC-0 because its single-channel conductance (10 pS) allows for a relatively easy patch-clamp analysis. Recently, this issue was also addressed [30, 106] for the skeletal muscle chloride channel ClC-1, which has a single-channel conductance of only 1–2 pS [96, 106]. Although the extent of the dominant negative effect of mutants found in myotonia suggests the interaction of more than two ClC-1 subunits [118], newer experiments [28] clearly suggest that the basic unit of ClC-1 channels is a dimer as in ClC-0. Fahlke et al. [30] recently studied several cysteine mutants in the D3–D4 region. Exposure of these mutants to MTSES, cadmium, or oxidation decreased macroscopic currents. Using concatamers, it was shown that the rate of current reduction strongly depends on the nature of the equivalent residue in the second subunit, suggesting that these are very close and may face a common aqueous space. Since these residues are thought to directly line the ion-conductive pathway [29], this was interpreted as meaning that ClC-1 has just a single pore that is formed by two identical ClC-1 protein subunits [30]. This contradicts the “double-barreled” structure of ClC-0 [74, 77, 85]. It is very unlikely that these highly related channels have grossly divergent pore structures. However, it may be possible to reconcile the findings obtained with ClC-0 [74, 77, 85] and ClC-1 [30]. The study of Fahlke et al. [30] is entirely based on macroscopic currents. The observed current inhibition may not be due to a direct

block of the pore(s), but to an effect on gating. Many mutations in the D3–D4 region drastically affect the gating of CLC channels [29, 33, 75], and this includes the mutants used in the study of Fahlke et al. [30]. If, as suggested by a recent study [106], ClC-1 has a gate that acts on both pores similar to the slow gate of ClC-0, the modification of the residues mutated in ClC-1 [30] may close the channel via this common gating mechanism. The inhibition of ClC-0 by zinc also occurs by affecting the common "slow" gate [12]. Although indirect interactions cannot be excluded, in the dimer the mutated residues probably come close together [30] and may face a common vestibule of the two independent conduction pathways (pores).

In support of such a model, a recent single-channel analysis of ClC-1 revealed a typical "double-barreled" fingerprint with two identical conductance levels [106]. Compared to ClC-0, the "slow" gate that acts on both pores is much faster and has a reversed voltage dependence. Interestingly, the dominant negative effect of a mutation found in myotonia congenita can be explained by an effect on the common gate [106]. Furthermore, the macroscopic currents seen with co-expression of ClC-1 and ClC-2 can be most easily explained by a heteromeric channel with a small ClC-1 pore and a larger, deregulated ClC-2 pore [73]. Thus, it is reasonable to assume that ClC-1 is a "double-barreled" channel similar to ClC-0, but more experiments are needed to definitely settle this question.

Where is the pore in CLC chloride channels?

Given the likely "two subunits-two pores" stoichiometry of ClC-0 [74, 77, 85], it is clear that there can be no single "P-loop" as for example in potassium channels. By contrast, several transmembrane segments contribute to the pore. This situation is reminiscent of CFTR, which has a "one subunit-one pore" structure. Similar to CFTR, the pore of CLC channels is as yet poorly defined. Many mutations at different positions in CLC channels are known to change pore properties such as ion selectivity, single-channel conductance, and rectification. The first residue identified to change pore properties when mutated was K519 at the end of D12 of ClC-0 [77, 97]. Residues at the end of D3 [26, 29, 108, 118] and between D2 and D3 [74] are important as well, and it may be expected that other residues also affect pore properties. In ClC-1, replacing a segment after D3 by the equivalent segment of ClC-3 changes the ion-selectivity of currents [29]. The interpretation that this segment confers isoform-specific ion selectivity [29] depends on the reproducibility of ClC-3 currents (see below). Somewhat disconcertingly, many of the mutations that change ion selectivity also change gating, sometimes drastically [29]. The anomalous mole fraction behavior of ClC-0 [97] suggests that CLC pores may accommodate at least two anions simultaneously. More experiments using site-directed mutagenesis and ideally crystallization of the channel protein are needed to identify the pore.

Gating: dependence on anions and voltage

The voltage-dependent gating of CLC channels may occur by a mechanism that differs dramatically from that of voltage-dependent K channels. For several studies, the *Torpedo* channel ClC-0 served as a model system because of its relatively large single-channel conductance (10 pS) and its rather simple gating relaxations. In a simplified picture, "fast" gating of ClC-0 (which acts on single pores of the double-barreled channel) is independent of any "intrinsic" voltage sensor and depends on the permeating anion as the gating charge [97]. In this model, the rate constants of gating are influenced by the presence of chloride at the inner end of the pore. Gating becomes voltage dependent as chloride moves along the electric field in order to reach its binding site. Gating then also depends on the chloride concentration. Both predictions are fulfilled astonishingly well and are supported by mutagenesis demonstrating that permeating anions are necessary [97]. When investigated over a much broader concentration range, however, the simplest quantitative relationship breaks down, requiring a more refined model [13]. Chen et al. [13] suggested that chloride binds to an external binding site, and that the movement of this binding site together with chloride in the electric field constitutes the voltage-sensitive step. In an alternative interpretation, these more complicated quantitative relationships may also be explained by characteristics of a multi-ion pore [94].

In contrast to the model for ClC-0 [13, 94, 97], a negatively charged residue at the end of D1 (D136) was proposed to be a voltage sensor in ClC-1 [24]. This is based on a mutation identified in myotonia that leads to a dramatic change in voltage dependence. Later it became clear that many mutations at very different positions in ClC-0 lead to similar, inwardly rectifying currents [75], and that the effect is not correlated with the charge of the substituted residue. This raised doubts that D136 in ClC-1 acts as a specific voltage sensor. Rather it suggests that mutations in several protein regions can reveal an intrinsic gating mechanism that is not seen normally. Gating of ClC-1 is more complicated than that of ClC-0 and needs at least three time-constants for a reasonable fit. A gating mechanism with two voltage sensors and an unidentified intracellular blocking particle was proposed [25]. On the other hand, and similar to ClC-0, ClC-1 gating also depends on extra- and intracellular anions [27, 100, 101, 103], some of which appear to interact strongly with the channel. ClC-1 gating will need more detailed analysis.

In part, the complexity of ClC-1 gating can be attributed to the fact that the "fast" and the "slow" gates cannot be as easily separated as in ClC-0 [106]. The slow gate of ClC-0 is not well understood either. Several mutations in ClC-0 affect slow gating [74, 76], including exchanges of the cytoplasmic C-terminus [36]. Because the slow gate acts on both subunits, these mutations may have a dominant negative effect both in ClC-0 [74] and ClC-1 [106]. Slow gating in ClC-0 depends exquisitely

on temperature (with a $Q_{10} \cong 40$) suggesting that it involves complex conformational changes [99].

ClC-2 opens in response to hyperpolarization [121], and this can be modulated by cell swelling [44] and an acidic extracellular pH [56, 95]. It was shown that this type of gating depends on an N-terminal "ball" domain that can be transplanted to the C-terminus without loss of function [44]. This suggests that it binds to an intracellular "receptor" for this domain. A systematic mutagenesis study revealed that mutations in the highly charged loop between D7 and D8 also abolish this type of gating [56], suggesting (but not proving) that this segment is involved in the binding of the ball. In addition, recent studies show that ClC-2 gating is also dependent on anions [100].

Due to the lack of prominent gating relaxations, the gating of other CLC chloride channels has not been analyzed in detail.

Physiological function of CLC channels and their role in disease

ClC-1: mutations in this muscle chloride channel cause myotonia

ClC-1 is expressed nearly exclusively in skeletal muscle [117]. In rodents, its expression increases steeply within the first few weeks after birth [117] in parallel with the increase in macroscopic skeletal muscle chloride conductance [18] and is dependent on innervation [61]. This suggests that it may represent the major skeletal muscle chloride channel, and this is strongly supported by its electrophysiological properties [117]. ClC-1 is activated by depolarization, has a Cl>I ion selectivity and a small single-channel conductance of 1–2 pS [96], and is sensitive to extra- and intracellular pH [25, 101, 102]. Previous studies had shown that there is a decrease in skeletal muscle chloride conductance in certain forms of myotonia, both in humans [32, 68] as well as in animal models [8, 83]. Thus, ClC-1 was an excellent candidate gene for myotonia.

Immediately after cloning ClC-1 by homology to ClC-0, it could indeed be shown that ClC-1 is mutated in the myotonic ADR mouse strain [116]. The insertion of a transposon into the ClC-1 gene predicted a total loss of channel function. This demonstrates that ClC-1 is essential for the electrical stability of the muscle plasma membrane. In contrast to most other cells, the chloride conductance dominates the resting conductance of skeletal muscle. Since the equilibrium potential for chloride is close to the resting potential, it plays a role similar to that of the potassium conductance in other cells and stabilizes the resting potential. A loss of chloride conductance will therefore impair the repolarization of action potentials, and sodium channels will have time to recover from their inactivation while the membrane potential is still slightly depolarized. This will lead to a train of action potentials (the "myotonic runs" observed in electromyograms) upon a single stimulus. This is the basis for the defect of muscle relaxation in myotonia. Although physiological studies suggest that the skeletal muscle chloride conductance resides mainly in the t-tubules, only the outer plasma membranes were stained by ClC-1 antibodies in muscle sections [47].

After the role of ClC-1 in myotonia was demonstrated in the mouse model, it was shown that mutations in the ClC-1 gene are also responsible for human myotonia congenita [62]. In humans, myotonia congenita can be inherited as an autosomal recessive (Becker type) or as an autosomal dominant (Thomsen type) disease. Both forms of myotonia are due to ClC-1 mutations, and the mutation in the pedigree of Dr. Thomsen, who described the disease for the first time, has been identified [118]. Since then, more than 30 different ClC-1 mutations in human myotonia [40, 41, 62, 63, 64, 65, 66, 72, 80, 92, 98, 118, 128] have been described, and additional ones in animal myotonia [4, 43, 116].

Mutations leading to a total loss of ClC-1 function (for instance, severe truncations) lead to recessive disease. There are also mutations in Becker type myotonia that change the voltage dependence of ClC-1 or reduce its single-channel conductance [128]. A patch-clamp study on cells obtained from muscle biopsies from three unrelated patients with recessive myotonia showed that the single-channel conductance of an "intermediate" Cl^- channel is reduced by about 50% [23]. However, since the single-channel conductance of that channel is more than an order of magnitude larger than that of ClC-1 [96], the significance of this finding is not clear.

With one exception (a truncation next to the C-terminus), all dominant mutations are missense mutations. Since a total loss of function of one allele leads to recessive disease, dominant myotonia is likely to be due to a dominant negative effect of the mutant protein. Nearly all dominant mutations shift the voltage dependence of ClC-1 to very positive potentials where the chloride channel can no longer contribute to the repolarization of action potentials [98]. Co-expression experiments revealed a similar, though reduced shift in heteromeric mutant/WT channels. This fully explains the dominant negative effect. In a recent study, it was shown that the shift in voltage dependence is not always halfway between WT and pure mutant channels, but depends on the particular type of mutation [65]. The dominant negative effect seems to depend on the ability of the mutation to affect the common, slow gate of ClC-1 [106]. In some cases, this results in a heteromeric channel whose function is compromised to such a degree that muscle excitability is just at the border where myotonia begins [65, 118]. Indeed, such mutations were found in dominant pedigrees with reduced penetrance, or were found in both recessive and dominant pedigrees [64, 92]. Thus, the border between dominant and recessive myotonia is blurred with some mutations.

In summary, ClC-1 is the CLC chloride channel whose physiological function is best known. We understand its role in stabilizing the plasma membrane poten-

tial of skeletal muscle, and can explain why certain mutations lead to recessively and others to dominantly inherited disease.

ClC-2: a chloride channel involved in cell volume regulation and a regulator of intracellular chloride concentration?

ClC-2 is a broadly expressed chloride channel [121] that can be activated by rather strong hyperpolarization, cell swelling [44], and an acidic extracellular pH [56, 95]. It shares the Cl>I selectivity sequence with ClC-0 and ClC-1 [38, 56, 111, 121]. Hyperpolarization-activated currents resembling ClC-2 have been found in several cell types [5, 11, 16, 21, 91, 111], including T84 epithelial cells [34, 35]. In native T84 cells, ClC-2-like currents are sensitive to cell swelling [35], as shown previously for ClC-2 expressed heterologously in *Xenopus* oocytes [44] and mammalian cells [111]. This suggests that ClC-2 may play a role in cell volume regulation, although it does not represent the swelling-activated chloride current $I_{Cl,swell}$ observed in many cells. The latter channel has an I>Cl selectivity sequence and a different voltage dependence. However, there is no convincing proof that ClC-2 is involved in the regulation of cell volume, and this possibility was dismissed in one study [5]. ClC-2 was also suggested to play an important role in setting the intracellular chloride concentration in neurons [17, 114, 115]. The chloride concentration in turn influences the physiological effect (inhibitory or excitatory) of GABA-ergic synapses.

ClC-2 has not been implicated in inherited disease. However, following its localization by immunocytochemistry to the apical membranes of lung epithelia [88], it may represent an interesting target for the treatment of cystic fibrosis [88, 111], because it resides in the same membrane as CFTR. ClC-2 is largely closed under resting conditions, and residues involved in the opening of ClC-2 have been identified by mutagenesis [44, 56]. Thus, it may be feasible to develop drugs that activate this channel and thereby compensate for the defective CFTR chloride channel.

In contrast to CFTR, ClC-2 is expressed early during development and has been speculated to be important in lung [88, 89] and kidney [51] development. It has also been suggested that ClC-2 is required for acid secretion in the stomach [81, 112]. However, these speculations need to be tested experimentally. The histamine-induced chloride channels in parietal cells of the rabbit stomach have an I>Cl selectivity [104]. Although the non-purified channels that were reconstituted into lipid bilayers from membranes of ClC-2-injected oocytes also displayed an I>Cl selectivity [81], this contradicts the Cl>I sequence of ClC-2 directly measured in several expression systems [38, 56, 111, 121]. The nomenclature ClC-2G (G for gastric) [37, 81, 112] should be abandoned as there is no evidence for a stomach-specific isoform of ClC-2. The same holds for ClC-2α and ClC-2β [37, 112] as the putative N-terminal splice variant ClC-2β [37] turned out to be a cloning artifact [37, 38, 56]. There is, however, an in-frame deletion in the C-terminus of ClC-2 that is generated by exon-skipping [14, 15]. This does not lead to significant changes in ClC-2 currents (Jordt and Jentsch, unpublished).

ClC-K kidney chloride channels: Bartter's syndrome in humans and nephrogenic diabetes insipidus in knock-out mice

In the mammalian kidney, there are two highly homologous (90% identity) CLC proteins that are nearly exclusively expressed in that tissue [1, 60, 122]. Homology is greater between the homologues within one species than between homologues across species. Therefore, these channels were named ClC-Ka and ClC-Kb in humans, and ClC-K1 and ClC-K2 in rats [60]. The findings that a disruption of ClC-K1 leads to nephrogenic diabetes insipidus in mice [82] and that ClC-Kb is mutated in a form of Bartter's syndrome [113] suggest that ClC-K1 corresponds to ClC-Ka, although this cannot be deduced from sequence comparison.

With one exception [1, 122, 123], most groups have so far been unable to express currents from ClC-K clones [60, 129]. The ion selectivity sequence of ClC-K1 conforms to the Cl>I sequence of other CLC channels [122], and ClC-K1 currents are decreased by an acidic extracellular pH [123]. By contrast, an I>Cl selectivity was reported for the highly related ClC-K2, and surprisingly a splice variant lacking the second transmembrane domain gave identical currents [1]. Immunofluorescence studies agree that ClC-K proteins are found in the plasma membrane at the level of resolution provided by light microscopy [123, 125]. These studies, which used different antibodies, differed somewhat as to the localization to different nephron segments. Using an antibody recognizing both isoforms, Vandewalle et al. [125] located them in basolateral membranes of the thick ascending limb and other parts of the nephron, while Uchida et al. [123] localized ClC-K1 to both apical and basolateral membranes of the thin ascending limb. Consistent with the latter localization of ClC-K1, there was no immunoreactivity in the inner medulla in a mouse model in which ClC-K1 had been eliminated by homologous recombination [82]. Very interestingly, these mice show a massive diuresis that is typical of nephrogenic diabetes insipidus. Transepithelial measurements from isolated segments of the thin ascending limb indicate a loss of chloride conductance [82]. Other functions of the kidney were unaffected. Thus, this mouse model shows unambiguously that ClC-K1 is important for urinary concentration.

ClC-Kb is also important for the normal functioning of the kidney since its mutation in Bartter's syndrome type III [113] leads to massive salt loss mainly in the thick ascending limb of Henle's loop and the distal tubule. Mutations in several membrane transporters cause

different forms of Bartter's syndrome and the related Gitelman syndrome [57]. The finding that mutations in ClC-Kb can cause Bartter's syndrome provides strong genetic evidence that it mediates a chloride efflux across the basolateral membrane in the thick ascending limb. However, in the absence of unambiguous electrophysiological data no comparison with chloride channels found in that segment is possible.

ClC-3, ClC-4, and ClC-5: Dent's disease caused by ClC-5 mutations

ClC-3, -4, and -5 form a distinct branch of the CLC gene family which is only about 35% identical to the other branches. Within this group, proteins are about 80% identical [119]. ClC-3 and ClC-4 are expressed in several tissues [6, 55, 58, 126], including brain and kidney, whereas ClC-5 is predominantly expressed in the kidney [31, 119]. The physiological functions of ClC-3 and ClC-4 are controversial and unclear, respectively. By contrast, the importance of ClC-5 is evident from Dent's disease, which is caused by mutations in ClC-5 [69]. Dent's disease is an X-linked disorder (ClC-5 is encoded on the human X-chromosome) with two basic defects: low-molecular-weight proteinuria and hypercalciuria. Hypercalciuria leads to secondary symptoms that dominate the clinical phenotype, i.e., kidney stones and nephrocalcinosis, which in turn can result in renal failure. Many different ClC-5 mutations have been identified in Dent's disease [52, 69, 70, 71, 86]. These include nonsense mutations and splice site mutations that truncate the protein, and missense mutations. Most of these mutations are inserted into the functional ClC-5 cDNA and studied in the *Xenopus* oocyte expression system. They either totally abolish or largely reduce chloride currents [52, 69, 70, 71, 86], showing that Dent's disease is due to a loss of chloride channel function. This poses the challenging question: how can the loss of chloride channel function lead to the symptoms of low-molecular-weight proteinuria and hypercalciuria? Calcium is known to be reabsorbed in large parts of the nephron and this process is highly regulated by several hormones and vitamins. Thus, elucidating the pathology of this aspect of Dent's disease is difficult and may require the generation of transgenic animal models. By contrast, the mechanism leading to proteinuria is more easily explained. Low-molecular-weight proteins can normally pass through the glomerular filter and can pass into the primary urine where they are reabsorbed by proximal tubular cells. This occurs by endocytosis. The proteins are finally targeted to lysosomes for degradation. A selective increase in low-molecular-weight proteins as in Dent's disease therefore points to a defect in the proximal tubule. Immunocytochemistry of rat kidney indeed reveals that ClC-5 is expressed in the proximal tubule, where expression starts in the S1 segment and continues to the S3 segment [46]. ClC-5 is predominantly expressed in the vesicular region below the apical brush-border where it co-localizes with the proton pump. This region is packed with endocytotic vesicles, endosomes, and recycling vesicles. During early stages of endocytosis, endocytosed fluorescently labeled β_2-microglobulin co-localizes significantly with ClC-5 [46]. This is compatible with ClC-5's having a role in early endocytosis. Also in cultured cells, ClC-5 co-localizes with endocytosed protein early after uptake [20, 46]. Furthermore, it is concentrated in the enlarged early endosomes generated by a GTPase-deficient mutant of rab5, a marker of early endosomes [46]. In the kidney, ClC-5 is also found in intercalated cells of the collecting duct [20, 46]. There again, it is present mainly in intracellular vesicles and co-localized with the proton pump in acid-secreting α-intercalated cells [46]. In these cells, the proton pump is mainly present in intracellular vesicles. It is inserted in a regulated fashion into the plasma membrane by exo- and endocytosis. Thus, ClC-5 is expressed in renal cells with a high rate of endocytosis, and co-localizes in intracellular vesicles with the proton pump.

Two other recent studies [20, 78] that employed different antibodies came to somewhat different conclusions concerning the intrarenal localization of ClC-5. All three studies agree that ClC-5 is expressed in the proximal tubule, where it probably plays a role in endocytosis [20, 46, 78]. The expression in intercalated cells was reported by two studies [20, 46] and is supported by in situ hybridization experiments [90], but was not observed with the antiserum used by Luyckx et al. [78]. On the other hand, Günther et al. [46] did not report its expression in the thick ascending limb, which was stained in the two other studies [20, 78]. All three studies used purified antisera that were checked for cross-reactivity against the related ClC-3 and ClC-4 proteins. It is currently unclear whether the apparent differences in intrarenal distribution are due to differences in the antibodies or in tissue preparation.

These results [20, 46, 78] strongly suggest that ClC-5 provides the electrical shunt necessary for the acidification of vesicles in the endocytotic pathway. After budding from the plasma membrane, these vesicles are progressively acidified on their way to the lysosomes [87]. This occurs by an electrogenic proton pump, which actively transports both acid equivalents and electric charge. In the absence of a conductive pathway, the generation of an inside-positive voltage across the vesicle membrane will limit the degree of acidification that can be achieved by the pump. It is well known from physiological studies that endocytotic vesicles contain an anion conductance that is essential for the efficient acidification of these vesicles. These data [20, 46, 78] indicate that ClC-5 provides such a rate-limiting conductance in the proximal tubule. Genetic loss of function of ClC-5 such as in Dent's disease will therefore impair acidification of vesicles in the endocytotic pathway. Several studies have shown that inhibiting vesicular acidification leads to an impairment of endocytosis and vesicle trafficking. Thus, these data can explain the proteinuria in Dent's disease. It is clear, however, that ClC-5 does not play this role in

all cells of the organism. It is likely that other chloride channels, possibly also belonging to the CLC gene family, will play similar roles in other cells and organelles. A role for CLC channels in ionic homeostasis in intracellular organelles is now well established for the yeast CLC *gef1p* (see below).

ClC-5 shares the Cl>I selectivity with other CLC channels and conducts NO_3^- even better [119]. ClC-5 expression in *Xenopus* oocytes [33, 119] or mammalian cells [33] elicits strongly outwardly rectifying chloride currents that can be detected only in a voltage range more positive than about +20 mV. This voltage dependence is puzzling as there are no known compartments that have these positive potentials. It has been speculated that there may be an additional, unknown subunit that modifies the voltage dependence of ClC-5. Alternatively, the very small currents in the negative voltage range are enough to provide an electrical shunt for the H^+-ATPase, whose transport rate is much less than that of channels [33]. ClC-5 currents could be changed, in part drastically, by several point mutations [33]. This demonstrates that these currents are directly mediated by this protein.

Other groups report less strongly rectifying currents with an I>Cl selectivity for rat ClC-5 [105] or the presumed *Xenopus* homologue xClC-5 [67]. In contrast to Steinmeyer et al. [119], these currents are also inhibited by stilbenes. A re-examination [109] of xClC-5, however, shows that xClC-5 currents resemble those of rat and human ClC-5 [33, 69, 119] and that the previously reported currents are probably endogenous to oocytes. Both mammalian and *Xenopus* ClC-5 can be inhibited by extracellular acidic pH [34, 109]. This may provide a negative feed-back mechanism for intracellular acidification [34].

While ClC-4 did not yield currents initially [55, 119, 126], more recently it was expressed functionally in *Xenopus* oocytes and mammalian cells [33]. The extreme outward rectification and the NO_3>Cl>Br>I ion-conductance sequence strongly resembles ClC-5, as do its pH sensitivity and the effect of a mutation at the end of D3 [33].

By contrast, functional expression of ClC-3 yielded conflicting results. We never obtained currents with ClC-3 (using rat, human and guinea-pig clones) [33, 55, 119], and also Chris Miller's group was unable to obtain any expression of currents with rat or guinea-pig ClC-3 in several different expression systems (personal communication). Other groups [22, 29, 58, 59], however, reported outwardly rectifying currents with an I>Cl selectivity that differs strongly from that of the highly homologous ClC-4 and ClC-5 channels [33]. The channels reported by Kawasaki et al. [59] and Duan et al. [22] differ in rectification, single-channel conductance, and calcium selectivity, suggesting that different channels were studied. Importantly, Duan et al. [22] proposed that ClC-3 represents the long-sought swelling-activated chloride channel. While they also reported large currents in the absence of cell swelling (which is generally not observed with the swelling-activated chloride channel), these currents increased by a factor of 2 when cells were swollen. A convincing piece of evidence is that a point mutation at the end of D12 (at the residue equivalent to K519 in ClC-0 [97]) changed the rectification and ion selectivity of their currents [22]. Surprisingly, however, the same mutation in ClC-5 does not result in similar changes [33]: it left the rectification unchanged and had the reverse effect on ion selectivity [33].

Several candidates were previously proposed to represent the swelling-activated chloride channel. This includes the multi-drug resistance protein mdr [124] and the pI_{Cln} protein [45]. Unfortunately, and after several years of controversy, it became clear that these proteins are probably not even ion channels [9, 120, 127]. These difficulties in interpretation are due to the ubiquitous presence of endogenous swelling-activated chloride channels in the cells used as expression systems. If confirmed, the identification of ClC-3 as the swelling-activated chloride channel [22] would represent a breakthrough. Obviously, ClC-3 has the advantage of belonging to an established family of chloride channels. However, in view of the inability of several laboratories to measure ClC-3 currents, in the presence of conflicting data on ClC-3 single channels [22, 59], and considering that the biophysical properties of ClC-4 and ClC-5 differ markedly from those reported for ClC-3 [33], more experiments are necessary to confirm this role of ClC-3. The expression pattern of ClC-3 [6, 58, 90] is also not easily compatible with a role as a ubiquitous swelling-activated chloride channel. For instance, in the kidney, ClC-3 was localized predominantly to β-intercalated cells of the collecting duct [90].

ClC-6 and ClC-7: ubiquitous putative chloride channels whose function is unclear

ClC-6 and ClC-7 represent the third known branch of the CLC gene family [7]. They are about 40% identical to each other, but only ≅25–30% identical to the other CLC channels. Northern blot analysis revealed that both proteins are ubiquitously expressed, and that they are expressed early on in mouse development. We [7] and others [9] were unable to measure functional expression of chloride currents with these channels in *Xenopus* oocytes. This is possibly because they may be chloride channels of intracellular organelles. This has been suggested for ClC-6 [10]. More work will be needed to identify the function of these channels.

The yeast CLC protein *gef1p*, an intracellular chloride channel

There is only a single CLC gene in the yeast *Saccharomyces cerevisiae*. It was initially isolated on the basis of the iron-suppressible *petite* phenotype that is caused by its disruption, and was called *gef1* [42]. A *petite* phenotype is indicative of a defect in mitochondrial respira-

tion. Interference with iron metabolism can lead to a *petite* phenotype because iron is present in mitochondrial enzymes. Interestingly, a similar iron-suppressible *petite* phenotype is caused by the disruption of a subunit of the yeast proton ATPase [42]. This provides indirect genetic evidence for an interaction of CLC chloride channels and proton pumps in the acidification of intracellular organelles. In yeast, this organelle is probably a medial to late Golgi compartment [39, 110]. Consistent with a role in acidification, the *gef1* strain (in which the yeast CLC has been inactivated) grows less well at less acidic pH [39, 110]. The growth defect of the *gef1* strain can also be overcome by high copper concentrations [39]. This suggests that it plays a role in the copper loading of fet3p, an enzyme indirectly needed for iron uptake. The function of the yeast CLC may not be limited to providing an electrical shunt for the pumping by electrogenic ATPases, which includes the copper ATPase ccc2p. This ATPase actively transports copper into the compartment that also expresses gef1p. It may be important for regulating the concentration of intravesicular chloride, which is an allosteric effector for the copper assembly of fet3p [19]. If also present in higher eukaryotes, such a direct effect of intravesicular chloride would give additional importance to intracellular chloride channels.

The *petite* phenotype of the *gef1* yeast mutant could be suppressed (complemented) by the plant putative chloride channel AtClC-d from *Arabidopsis thaliana* [49]. This and several other plant CLCs were isolated by homology to the ClC-6/-7 branch of mammalian chloride channels [49]. In a different genetic background, the growth defect of *gef1* cells at pH 7.0, high salt or high manganese concentrations was also suppressed by the *Torpedo* channel ClC-0. This suggests that the yeast CLC indeed functions as a chloride channel [39].

Thus, data from the model eukaryotic cell *S. cerevisiae* demonstrate that CLC channels are important for the ionic homeostasis of intracellular organelles. Although the *gef1* strain has no obvious defect in endocytosis [110], this nicely complements the observations concerning the role of ClC-5 in intracellular vesicles.

CLC proteins in prokaryotes

The completion of several bacterial genome projects reveals that many, but not all, prokaryotes have CLC genes (Table 1). Surprisingly, and in contrast to the presence of only a single CLC gene in the eukaryote *S. cerevisiae*, some bacteria (such as *Escherichia coli*) even have two CLC genes. By homology criteria, prokaryotic CLCs form a distinct branch of the CLC family. Homology within prokaryotic and to eukaryotic CLC proteins is again scattered over large parts of the protein. It is especially high at the end of D2 and D3, in D5, D7, D10, D11 and D12, including regions known to be important for pore properties. The N-termini of some prokaryotic CLC proteins are remarkably short, leaving only a few amino acids before the

Table 1 Prokaryotic CLC genes

Class	Species	Number of CLCs	Number of CBS domains
Archaea	*Archaeglobus fulgidus*[a]	1	2
	Methanobacterium thermoautotrophicum[b]	0	
	Methanococcus jannaschii[c]	1	none
	Pyrococcus horikoshii[d]	1	none
Bacteria	*Bacillus subtilis*[e]	0	
	Borrelia burgdorferi[f]	0	
	Chlamydia trachomatis	0	
	Escherichia coli[g]	2	none
	Haemophilus influenzae[h]	0	
	Helicobacter pylori[i]	0	
	Mycobacterium tuberculosis[j]	1	none
	Mycoplasma genitalium[k]	0	
	Mycoplasma pneumoniae[l]	0	
	Rickettsia prowazekii[m]	0	
	Synechocystis PCC6803[n]	2	SPClC-1:2 SPClC-2: none
	Treponema pallidum[o]	0	

Bacterial CLC genes were identified by BLAST homology searches of completely sequenced bacterial genomes. These contain 0 to 2 CLC genes, as indicated in *column 3*. All prokaryotic CLC genes predict proteins that have the same transmembrane topology as eukaryotic CLC proteins. However, while some prokaryotic CLC proteins have CBS domains in their predicted cytoplasmic C-termini, most prokaryotic CLC proteins end shortly after domain D12 and lack CBS domains. The bacterial genomes have been described in the following publications:
[a] Klenk et al. (1997) Nature 390:364; [b] Smith et al. (1997) J Bacteriol 179:7135; [c] Bult et al. (1996) Science 273:1058; [d] Kawarabayasi et al. (1998) DNA Res 5:55; [e] Kunst et al. (1997) Nature 390:249; [f] Dunn et al. (1994) J Bacteriol 176:2706; [g] Blattner et al. (1997) Science 277:1453; [h] Fleischmann et al. (1995) Science 269:496; [i] Tomb et al. (1997) Nature 388:539; [j] Cole et al. (1998) Nature 393:537; [k] Fraser et al. (1995) Science 270:397; [l]Himmelreich et al. (1996) Nucleic Acids Res 24:4420; [m]Andersson et al. (1998) Nature 396:133; [n]Kaneko et al. (1997) Plant Cell Physiol 38:1171; [o]Fraser et al. (1998) Science 281:375

first transmembrane domain (e.g. ecClC-1 from *E. coli*, accession number gbAE000125). Most bacterial CLC proteins have a very short C-terminus, ending immediately after D12. These proteins therefore do not contain CBS domains. This is surprising because CBS domains are essential for the functionality of mammalian CLCs [79, 107, 110]. One could speculate, however, that in these species those domains are encoded by other genes, and that functional CLCs assemble from two separate gene products. Such a situation has been found for bacterial ABC-transporters. Given that prokaryotes do not have intracellular organelles, CLC channels in prokaryotes must reside in the outer membrane. The function of CLCs in bacteria is unknown, as is the reason why some species have two CLCs while others do not need any. In spite of this, prokaryotic CLCs may be useful for attempts to crystallize members of this important channel family. They also demonstrate that these genes are evolutionarily very old, even though their absence from some prokaryotes indicates that they are not essential for normal life.

Summary

The CLC gene family encodes at least nine different chloride channels in mammals. It is the only known gene family of voltage-gated chloride channels. In recent years, much has been learned about their structure and function, although many features are not yet clear (e.g., the localization of the pore). The medical importance of CLC channels is best illustrated by the three inherited human diseases that are known to be caused by mutations in ClC-1, ClC-Kb, and ClC-5. In addition, studying knock-out mice is beginning to unravel their physiological function. While particular CLC channels play roles in the regulation of electrical excitability, cell volume regulation, and transepithelial transport, several of these channels may be localized predominantly to intracellular membranes.

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