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A Two-Holed Story: Structural Secrets About ClC Proteins Become Unraveled?

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ClC Cl^- channels are found in almost all organisms, ranging from bacteria to mammals, in which nine Cl^- channels belonging to the ClC family have been identified. The biophysical properties and physiological functions of ClC Cl^- channels have been extensively reviewed. In this short review, we will focus on recent results obtained on the X-ray structure and functional properties of the prokaryotic ClC-ec1 protein and some results obtained on the role of the cytoplasmic COOH terminus of mammalian ClCs.

ClC channels can be classified into plasma membrane channels and organelle channels. ClCs that function in the plasma membrane [ClC-1, ClC-2, ClC-Ka, and ClC-Kb (in mammals)] are involved in the stabilization of membrane potential and in transepithelial transport. The presumed function of most intracellular organelles' ClC channels [ClC-3, ClC-4, ClC-5, ClC-6, and ClC-7 (in mammals)] is support of the acidification of the intraorganellar compartment. The association of mutations in several ClC genes with various human diseases confirms the relevance of their functions (17, 21). Knockout studies have greatly helped in elucidating the physiological function of several ClCs, even though for some of them their precise role is not clear (4, 5, 22, 33, 44, 48).

Model organisms have been very helpful for understanding several aspects of ClC proteins. The nematode *Caenorhabditis elegans* provides a good system to define the physiological functions of ClC proteins. In fact, this organism owns six ClC genes representative of the mammalian ClCs (45). Bacterial ClCs have allowed the first determination of the X-ray structure of a ClC homolog (9). Furthermore, recent experiments on reconstituted bacterial ClCs revealed an unexpected function as a Cl^-/H^+ antiporter (2).

A unique feature of ClC channels is their homodimeric architecture in which each subunit forms a proper pore. Such a structure had been conjectured early on by Chris Miller, who studied *Torpedo* ClC-0 channels reconstituted in planar lipid bilayers (28). Measuring single channels, Miller observed that at negative potentials the channel fluctuated between three different conductance levels, one closed and two open states. The open events occurred in bursts separated by long closures. The substates visible during a burst had an identical conductance of ~8 pS, and the respective open probabilities were distributed binominally as if created by two independent channels. The long closure events demonstrated, however, the presence of a "common" gate that acts on both "protopores" simultaneously and that exists in parallel to the individual protopore gates (FIGURE 1). This "double-barreled shotgun" model (28) was later con-

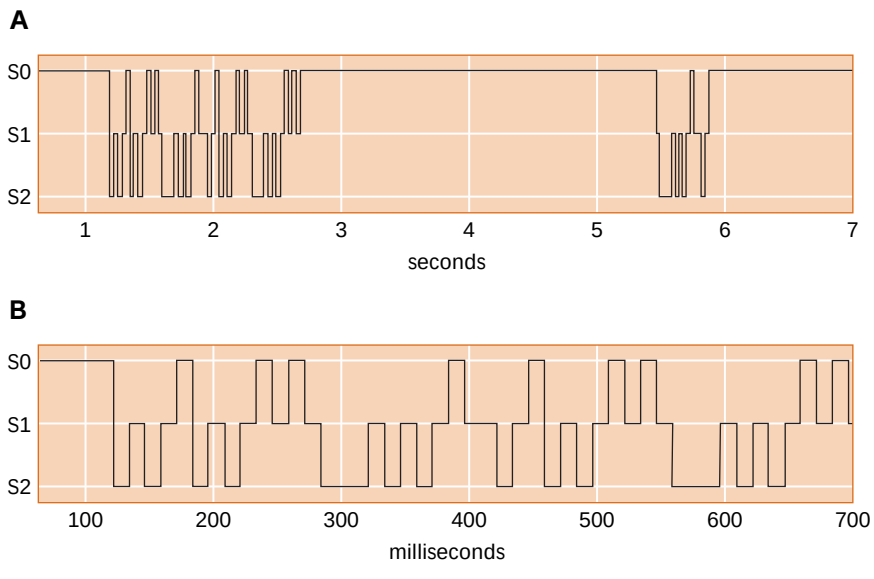

FIGURE 1. **Schematically idealized channel activity of ClC-0**
A: bursts of single-channel activity separated by long closures recorded at a negative voltage. During the burst, the channel fluctuates between 3 different conductance levels: 2 open states and 1 closed state. Channel opening corresponds to downward deflection. The long closure events represent closures of the "common" gate that acts on both "protopores" simultaneously. *B*: fluctuations between the 3 substates S0 (nonconducting), S1 (one pore open), and S2 (both pores open) within a burst. The substates have an identical conductance, and the respective open probabilities are binominally distributed: $f_{S0} = (1 - p)^2$; $f_{S1} = 2p\ (1 - p)$; $f_{S2} = p^2$, where p is the probability of an individual protopore being in the open state.

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firmed by using mutation analysis. It was shown that the protopore gate, the conductance, and the ion selectivity are determined by the amino acid sequence of a single subunit; on the other hand the common gate depends on the properties of both pores of ClC-0 (24, 27). Qualitatively, a similar double-barreled appearance was shown also for ClC-1 (37) and ClC-2 (49). Based on the recent structure determination of bacterial ClCs (9) it can be assumed, even though not demonstrated, that a double-barreled architecture holds true for all ClC proteins.

Most ClC Cl^- channels that have been studied are characterized to be relatively selective for Cl^- over other anions (the selectivity sequence generally is $Cl^- \geqslant Br^- > I^-$; see Ref. 35 for review). Another general feature is their voltage-dependent gating that seems to be tightly coupled to the permeation of Cl^- ions. In addition to the strong dependence on intra- and extracellular Cl^- concentration, these channels are regulated by intra- and extracellular pH (35).

The organelle channels ClC-3, ClC-4, and ClC-5 can be classified as a subfamily characterized by a strong outward rectification when expressed in *Xenopus* oocytes or mammalian cells (16, 23, 43). ClC-6 and ClC-7 (6) form the third branch of the ClC family. So far they cannot be expressed functionally as Cl^- channels in heterologous systems (21).

Structure of ClC proteins

The double-barreled architecture of ClC proteins has been fully confirmed by solving the X-ray structure of two prokaryotic ClC homologs [one from *Salmonella typhimurium* and one from *Escherichia coli* (9)]. The bacterial proteins became of great interest after the first crystallization of a bacterial K^+ channel (8). Prokaryotic ClC proteins are significantly homologous to their eukaryotic counterparts. In particular, they have the same transmembrane topology and display several highly conserved stretches that were later to be shown to form Cl^- ion-binding sites in the center of each subunit (10, 30).

The first solved X-ray structure of the *S. typhimurium* ClC protein (StClC) has been determined at 3.0-Å resolution (9). The protein is formed by two identical subunits that each have a roughly triangular shape when viewed from top or from bottom. They are related to each other by an almost perfect symmetry of a 180° rotation and an extensive interaction surface that is perpendicular to the plane of the membrane (FIGURE 2). Each subunit contains an amazingly complex fold of 18 α-helixes that reveals an internal antiparallel pseudosymmetry: the NH_2-terminal half of each subunit has the same fold as the COOH-terminal half and also a spurious sequence homology to it but is inserted into the membrane in the reverse direction, forming a "sandwich," similar to what has been seen for aquaporin water channels (46). More recently, by employing co-crystallization with Fab fragments, Dutzler et al. (10) succeeded in obtaining an improved 2.5-Å resolution structure of the *E. coli* protein ClC-ec1. Further discussion of the struc-

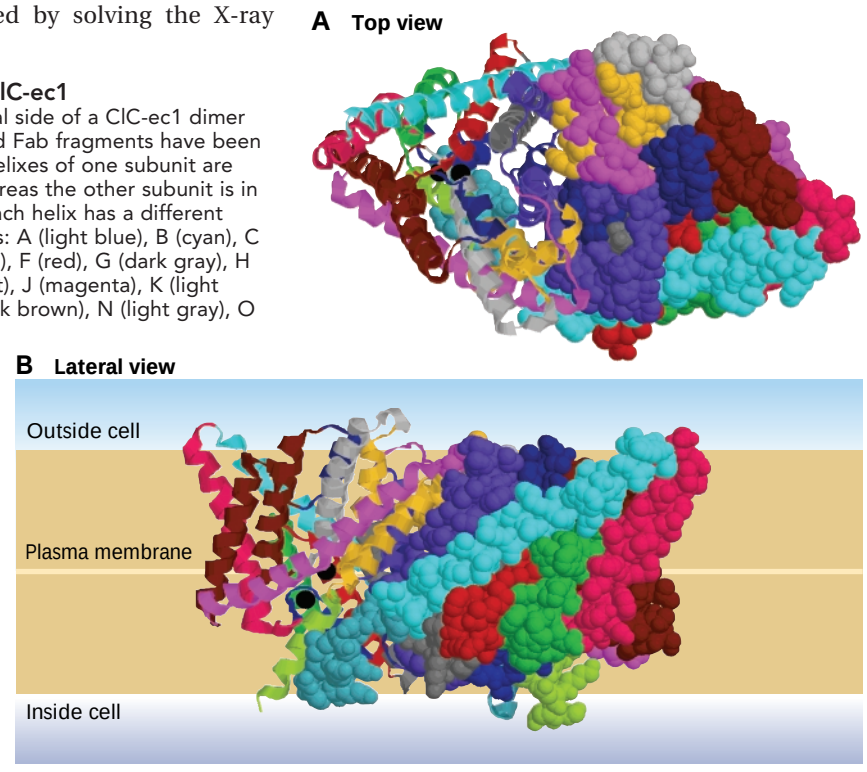

FIGURE 2. **Structure of ClC-ec1**
A: top view from the external side of a ClC-ec1 dimer (PDB code 1OTS). Water and Fab fragments have been removed for clarity. The α-helixes of one subunit are represented as ribbons, whereas the other subunit is in a spacefill representation. Each helix has a different color equal for both subunits: A (light blue), B (cyan), C (light red), D (blue), E (green), F (red), G (dark gray), H (midnight blue), I (dark violet), J (magenta), K (light brown), L (dark blue), M (dark brown), N (light gray), O (yellow), P (blue-violet), Q (orange), R (light green). Cl^- ions present in the conducting pathway are visible as black spheres in 1 subunit; those of the other subunit are covered by the helixes. The extensive interaction surface is perpendicular to the membrane plane. *B*: lateral view from within the membrane of the dimer with the extracellular solution above. Helixes are colored as in *A*. The figure was drawn with Rasmol (38).

ture will be based on this higher-resolution data.

The most important feature of the structure is that in the center of each subunit two nearby Cl^- ions are clearly resolved, indicating the presence of at least two anion-binding sites, called S_{in} and S_{cent} for the more intracellularly and the more centrally located site, respectively (FIGURE 3). The protein regions that contribute to the formation of the sites are the ones that are most conserved among all ClC proteins, suggesting clearly that they form the selectivity filter of the conduction pathway. The fact that the binding sites of the two subunits are far from each other (~40 Å) is fully compatible with the independence of the two protopores of the double-barreled shotgun model. The central Cl^- ion is completely buried in the protein and stabilized by the positive dipole moments of helixes F and N that point their NH_2 terminus toward the ion and by the interaction with several backbone amide hydrogens and the OH groups of serine 107 and tyrosine 445. The more intracellularly localized Cl^- ion appears to be in direct contact with the intracellular solution (FIGURE 3). Although these results identify the selectivity filter, the permeation pathway is not evident from the structure. In particular, a negatively charged glutamate residue (E148) appears to block the movement of the centrally bound Cl^- ion toward the extracellular solution. Also, serine 107 seems to be a major obstacle for Cl^- permeation toward the intracellular solution (29). It has thus been proposed that the crystallized structure corresponds to a closed channel and that the glutamate side chain may act as a gate that obstructs the pore from the extracellular side.

Two different approaches have been used to study in more detail the function of this glutamate. First, the X-ray structure of mutant *E. coli* proteins, in which the glutamate has been replaced by alanine and glutamine (E148A, E148Q), has been determined (10). In both mutant structures a third Cl^- ion was found at the position occupied by the E148 side chain in the wild-type (WT) structure. In the E148Q structure the glutamine side chain is orientated in the extracellular direction, suggesting that in the conducting conformation of the WT the glutamate side chain could be similarly orientated, allowing the permeation of Cl^- ions. Otherwise, the mutant structures are virtually identical to the WT structure.

Because at the time of these structural studies the bacterial protein could not be measured by using electrophysiological methods, the *Torpedo* ClC-0 was used as a model to explore the functional effects of E148 mutations (E166 in ClC-0). All three mutations studied (E166A, E166V, E166Q) almost completely abolished the closure of the protopore gate, resulting in permanently open channels (10). In addition, WT ClC-0 can be converted to a similarly permanently open channel by a reduction of the extracellular pH (10), as if protons open the channel by protonating the glutamate side chain. Together, these results have led to the proposal that the gating of ClC channels is regulated by a simple mechanism in which the glutamate side chain acts as a gate occluding the ion pathway. Although several details may not be easily explained (3, 47), this simple model is qualitatively in accordance with several properties of ClC-0 gating. First, it is consistent with the independence of the two pores because each pore has its own glutamate. The coupling between Cl^- ion conduction and gating (36) could be explained by a competition between E148 and Cl^- ions, and the pH effects on gating (7, 10) are caused by a direct protonation of E148.

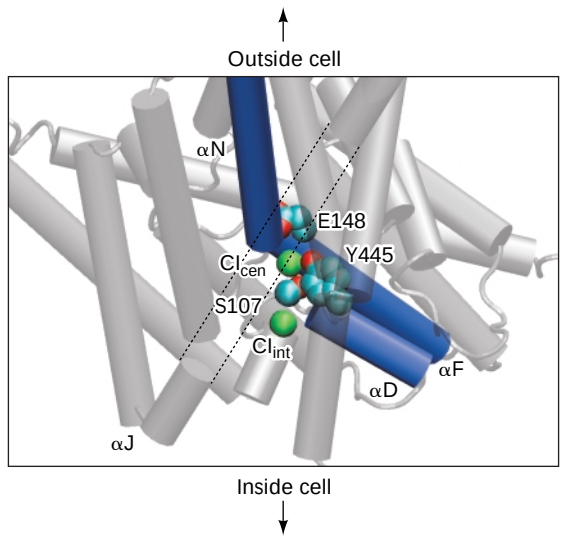

FIGURE 3. **Selectivity filter viewed from within the membrane**
The molecule is rotated with respect to the view shown in FIGURE 2B to have a closer look at the Cl^- ion binding sites, and only 1 subunit is shown. α-Helixes are drawn as cylinders. Helices αD, αF, and αN are colored in blue. Residues Y445 and S107 that contribute to the central binding site are highlighted. Helix αJ has been cut (dashed line) to better show the central Cl^- ion and residue E148 occluding the conducting pathway to the extracellular solution. The internal Cl^- appears to be in direct contact with the intracellular solution. The figure was prepared with VMD (19).

The ClC-ec1 from *E. coli* is Not a Channel

Little is known about the ClC functions in prokaryotic organisms. Recently, two *E. coli* ClC genes (ClC-ec1 and ClC-ec2) have been proposed to be involved in the extreme acid resistance response that permits enteric bacteria to survive in the stomach by providing an electrical shunt that helps in the bacterial acid-extrusion mechanisms (20).

In the past, since all attempts to measure electrical currents of bacterial ClCs reconstituted in lipid bilayer failed, ClC-ec1 function could be assayed only by measuring Cl^- fluxes in reconstituted liposomes (20, 25). From these flux studies, Maduke and co-workers (25) proposed that ClC-ec1 functions as a channel displaying a high selectivity for anions over cations and a selectivity sequence similar to that found for ClC-0 and ClC-1.

Recently, Accardi et al. (1) succeeded in obtaining an extremely pure and high-yield preparation of ClC-ec1 protein that allowed an electrophysiological characterization in planar lipid bilayers. Only macroscopic currents could be measured, and from the noise properties a very small single-channel conductance (<0.1 pS) was inferred (1). The current did not show the characteristic voltage and time-dependent gating of the eukaryotic ClC channels, exhibiting a practically linear current-voltage (*I-V*) curve. Importantly, ClC-ec1-carried currents were activated by low pH, confirming earlier results from Cl^- flux measurements (20). To test the proposed role of E148 in proton sensing, Accardi and coworkers (1) studied the mutant E148A. They found that the mutation completely abolishes the pH dependence, in agreement with the expectations from the structural data on ClC-ec1 and the functional data on ClC-0.

In all previous studies, it has implicitly been assumed that the bacterial ClC-ec1 is a Cl^- channel just like ClC-0. This assumption was supported by ion selectivity sequence inferred from flux measurements (25) and by studies that successfully used the structure of the bacterial protein as a guide for mutagenesis to identify residues involved in the binding of two organic inhibitors of ClC-1 [9-anthracenecarboxylic acid (9-AC) and *p*-chlorophenoxyacetic acid (CPA)] (13). Thus it came as a big surprise when Accardi and Miller discovered that the bacterial protein is not an ion channel but rather behaves as a H^+/Cl^- exchange transporter (2). The first indication was that the measured reversal potential in a Cl^- gradient was significantly smaller that that expected by Nernst's equation, suggesting the presence of another permeating ion. K^+, buffers, and OH^- could be ruled out as permeating ions, indicating that H^+ could be the second ion permeating the protein. However, reversal potential measurements with various Cl^- and H^+ gradients were absolutely incompatible with an electrodiffusive mechanism described by the Goldman-Hodgkin-Katz equation

$$E_{rev} = \frac{RT}{F} \ln\left(\frac{P_H[H^+]_{ext} + P_{Cl}[Cl^-]_{int}}{P_H[H^+]_{int} + P_{Cl}[Cl^-]_{ext}}\right) \quad (1)$$

The "best" fit with *Eq. 1* (with a ratio $P_H/P_{Cl} = 850$) gave a very poor description of the data. In contrast, for a strictly coupled transport

$$n Cl^-_{ext} + m H^+_{int} \leftrightarrow n Cl^-_{int} + m H^+_{ext} \quad (2)$$

with the stoichiometric ratio $r = n / m$ the reversal potential is given by

$$E_{rev} = \frac{1}{1 + r}(rE_{Cl} + E_H) \quad (3)$$

where E_{Cl} and E_H are the Nernst potentials for Cl^- and H^+, respectively. An almost perfect description of the data was obtained by *Eq. 3* with an apparent stoichiometric ratio of ~2, indicating that two Cl^- ions are transported for each proton. In principle, it cannot be strictly ruled out that the true stoichiometric ratio is somewhat smaller than 2 and that some Cl^- slippage occurs. However, the very good fit of the data by *Eq. 3* argues against this objection that would predict a deviation from *Eq. 3*. These results led to the confirmation that H^+ is the second ion passing through ClC-ec1, but they suggest that the prokaryotic ClC-ec1 acts as an H^+/Cl^- exchange transporter and not as a channel (2) . Two further crucial experiments substantiated this hypothesis: flux measurements in liposomes showed that ClC-ec1 is able to transport Cl^- against its electrochemical gradient in the presence of a proton gradient and vice versa (2). Together these experiments show that ClC-ec1 operates as a Cl^-/H^+ antiporter with an apparent stoichiometric ratio of 2 Cl^-:1 H^+. Interestingly, the mutation E148A of the crucial glutamate residue led to a complete loss of transporter activity and showed a purely electrodiffusive Cl^- transport (2), indicating that E148 is centrally involved in the H^+ transport.

ClC-ec1 is significantly homologous to the eukaryotic ClC proteins, but of course the transporter function of ClC-ec1 does not imply that all ClCs are necessarily transporters. ClC-0, ClC-1, ClC-2, and ClC-K are clearly Cl^- channels. However, their gating is strongly dependent on pH, indicating a kind of memory of the bacterial transporter. This notion is also supported by the conserved crucial role of E148 for ClC gating in all ClC channels studied (10, 13, 14, 16, 32, 40, 47). The new findings could be also helpful in future studies on some human channels, such as ClC-6 and ClC-7, that are still poorly understood.

Role of the Cytoplasmic CBS Domains

The crystallized prokaryotic ClCs have only short intracellular NH_2 and COOH terminals, whereas these terminals are often long in the eukaryotic channels, where they play important roles in channel function (11).

Initial experiments have shown that the COOH terminus of ClC-0 is involved in the common gate and is important for the functional expression of ClC channels (15, 26). Interestingly, Maduke et al. (26) showed that functional channels could be obtained by the injection of two separate constructs encoding the NH_2-terminal part comprising all intramembraneous segments and the cytoplasmic COOH terminus, respectively. Later it was found that the COOH terminus of eukaryotic ClC channels contains two so-called cystathionine-β-synthase (CBS) domains (34). Several different types of proteins contain CBS domains, most often in pairs. Crystal structures of the enzyme inosine monophosphate dehydrogenase demonstrate that the CBS domains are folded into two amphipatic α-helixes and three β-strands and revealed that the two CBS domains interact with each other through their β-strands, whereas the α-helixes are oriented away from the interaction site (42, 50). Their function is still unknown, but several mutations occurring in CBS domains are found in hereditary human diseases involving ClC channels, such as congenital myotonia, hypercalciuric nephrolithiasis, osteopetrosis, Dent's disease, and Bartter's syndrome, suggesting that they play a crucial role in the physiological functions of these channels (see Ref. 12 for references).

Recently, it has been shown that the CBS domains from several proteins including ClC-2 form a binding site for adenosine derivatives such as AMP and that disease-causing mutations influence the binding of these adenosine derivatives (41). This finding led to the interesting hypothesis that CBS domains act as sensors of the cellular energy status.

Two recent studies employed different techniques to explore in more detail the function of CBS domains in ClC channels, in particular in ClC-1 (12, 18). Previously, it had been shown that truncating ClC-1 after CBS1 does not give rise to any current (39). In the latest work, Estévez and co-workers (12) used co-immunoprecipitation, electrophysiology, and surface-expression measurements using an extracellular epitope to show that both CBS domains are needed for the rescue of function and localization of the separately expressed NH_2-terminal half of the channel. Moreover, the replacement of CBS domains of ClC-1 with the CBS domains of other ClC channels preserved function, showing that the structure and function of CBS domains from ClC channels are highly conserved (12). Results obtained by co-expression of different constructs indicated that the CBS domains interact (FIGURE 4A). An important observation of this study was that some residues located in CBS2 are involved in the voltage dependence of gating by affecting or abolishing the common gate of ClC-0 and ClC-1. This is a confirmation of previous results showing that parts of the COOH terminus of ClC-0 are involved in the common gate of ClC-0 (15). Interestingly, the in-frame deletion of CBS2 but not of CBS1 was found to be tolerated in ClC-1, demonstrating that CBS2 is not strictly essential for function (FIGURE 4A). Hebeisen and co-workers (18) have studied the role of CBS domains of ClC-1 using a different approach and

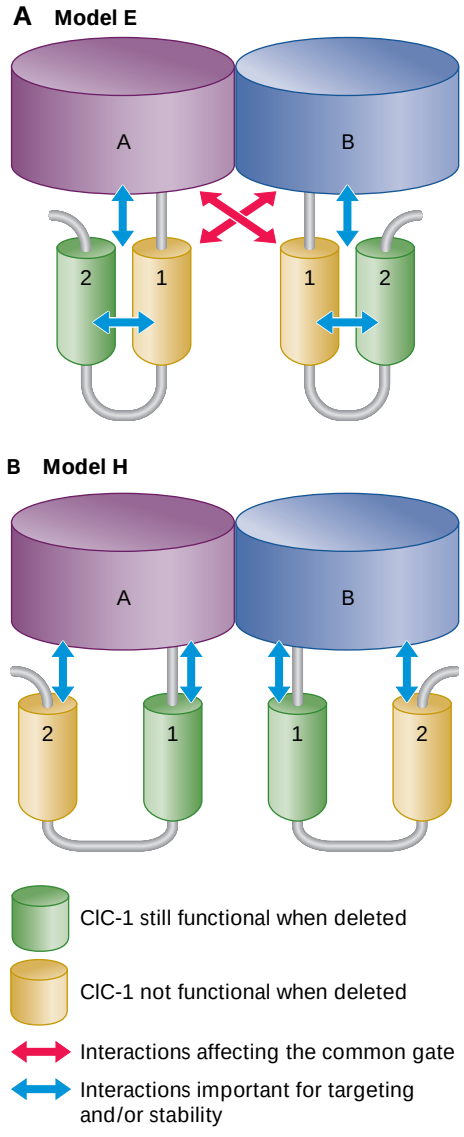

FIGURE 4. **Divergent models for cystathione-β-synthase (CBS) functions**
The 2 subunits of the dimer are labeled A and B. CBS domains are colored in orange and green, with orange indicating that a deletion impairs channel function while green indicates that a deletion is tolerated. Arrows indicate interactions affecting the common gate (red) and interactions important for protein stability (blue). In *A*, the main results of Estévez et al. (12) are summarized (Model E). Deletion of CBS1 and not of CBS2 led to nonfunctional channels. CBS domains of one subunit interact with each other and also interact with the other subunit. As summarized in *B*, Hebeisen et al. (18) obtained discordant results (Model H). First, deletion of CBS1 but not that of CBS2 was tolerated. Second, CBS domains do not bind to each other and interact only with the subunit to which they belong.

obtaining discordant results. First, measuring the surface expression of truncated green fluorescent protein-fused ClC-1 by confocal microscopy, they found that CBS domains are not necessary for the insertion into the plasma membrane. Second, to test whether the two domains interact they coexpressed concatameric constructs containing the two main parts of the dimeric protein with smaller constructs containing various COOH-terminal cytoplasmic pieces. From these experiments it appeared that the CBS domains do not bind each other and that there are no interactions between the COOH terminals of the two subunits (FIGURE 4B). Moreover, they observed that mutations in the COOH terminus did not alter the gating properties of the channel but cause a decrease in the maximal current. Surprisingly, Hebeisen et al. (18) found that the deletion of CBS1 but not that of CBS2 was tolerated, in complete contrast to the results of Estévez et al. (12) (FIGURE 4). These two studies underline the difficulties involved in investigating the role of CBS domains. The use of different methods and constructs used in these two studies (12, 18) is probably one of the reasons for such discordant results. For example, the employment of concatameric construct could force an assembly and a targeting different from that found in coexpression studies. The divergent conclusion regarding the necessity of CBS1 remains unclear. Regarding the necessity of CBS2, the deletion construct used by Estévez et al. retained the last 117 amino acids, whereas Hebeisen et al. used a truncation before CBS2. Thus it can be concluded that the terminal amino acids after CBS2 have an important functional role in ClC-1. Caution must be applied to all of these interpretations, however, because divergent results are also obtained if other ClC channels (e.g., ClC-0) are used for the CBS deletions (12).

An apparently fundamentally different conclusion was reached by Hebeisen et al. and Estévez et al. regarding the relationship of the CBS domains with the double-barreled architecture: Hebeisen et al. concluded that the CBS domains of one subunit support the function of and interact only with the subunit to which they belong and not with the neighboring subunit, whereas Estévez et al. found that mutations in only one CBS2 domain abolished or altered the common gate in a dominant fashion in heterodimeric mutant WT ClC-0 channels. The conclusion of Hebeisen et al. was mainly based on the use of concatameric constructs in which one of the subunits bore a point mutation (S537F) that renders the channel less sensitive to block by 9-AC (13). The authors assumed that 9-AC block represents a property of a single pore not influenced by the neighboring subunit (18). However, this assumption is probably not true, because results with ClC-0 indicate a dependence of 9-AC and CPA block on the common gate (31) (Pusch, unpublished result). In conclusion, it seems fair to say that we still have a lot to learn about the role of the intracellular domains in ClC channels.

Outlook

The past several years have seen great steps forward in the understanding of the structural and functional properties of ClC Cl^- channels, or better to say now ClC Cl^--transporting proteins. The X-ray structure of bacterial ClC proteins marked a breakthrough in the ClC field. However, we have also learned an important lesson: the three-dimensional atomic structure of a protein alone does not provide enough information to understand its function. The structure of the bacterial ClC-ec1 by no means revealed that it is a transporter that shuffles Cl^- ions in one direction strictly coupled to the proton transport in the opposite direction. Only functional electrophysiological analysis revealed this unexpected behavior, demonstrating the importance of using various approaches to unravel the secrets of biology. Further analysis is clearly needed to define the mechanism of the countertransport of Cl^- and H^+ in ClC-ec1 and also to investigate the relation between the bacterial transporter and the pH dependence of eukaryotic ClC Cl^- channels. ■

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References

1. Accardi A, Kolmakova-Partensky L, Williams C, and Miller C. Ionic currents mediated by a prokaryotic homologue of CLC Cl^- channels. *J Gen Physiol* 123: 109–119, 2004.
2. Accardi A and Miller C. Secondary active transport mediated by a prokaryotic homologue of ClC Cl^- channels. *Nature* 427: 803–807, 2004.
3. Accardi A and Pusch M. Conformational changes in the pore of CLC-0. *J Gen Physiol* 122: 277–293, 2003.
4. Arreola J, Begenisich T, Nehrke K, Nguyen HV, Park K, Richardson L, Yang B, Schutte BC, Lamb FS, and Melvin JE. Secretion and cell volume regulation by salivary acinar cells from mice lacking expression of the Clcn3 Cl^- channel gene. *J Physiol* 545: 207–216, 2002.
5. Bösl MR, Stein V, Hübner C, Zdebik AA, Jordt SE, Mukhopadhyay AK, Davidoff MS, Holstein AF, and Jentsch TJ. Male germ cells and photoreceptors, both dependent on close cell-cell interactions, degenerate upon ClC-2 Cl(–) channel disruption. *EMBO J* 20: 1289–1299, 2001.
6. Brandt S and Jentsch TJ. ClC-6 and ClC-7 are two novel broadly expressed members of the CLC chloride channel family. *FEBS Lett* 377: 15–20, 1995.
7. Chen MF and Chen TY. Different fast-gate regulation by external Cl(–) and H(+) of the muscle-type ClC chloride channels. *J Gen Physiol* 118: 23–32, 2001.
8. Doyle DA, Morais Cabral J, Pfuetzner RA, Kuo A, Gulbis JM, Cohen SL, Chait BT, and MacKinnon R. The structure of the potassium channel: molecular basis of K^+ conduction and selectivity. *Science* 280: 69–77, 1998.
9. Dutzler R, Campbell EB, Cadene M, Chait BT, and MacKinnon R. X-ray structure of a ClC chloride channel at 3.0 Å reveals the molecular basis of anion selectivity. *Nature* 415: 287–294, 2002.

10. Dutzler R, Campbell EB, and MacKinnon R. Gating the selectivity filter in ClC chloride channels. *Science* 300: 108–112, 2003.

11. Estévez R and Jentsch TJ. CLC chloride channels: correlating structure with function. *Curr Opin Struct Biol* 12: 531–539, 2002.

12. Estévez R, Pusch M, Ferrer-Costa C, Orozco M, and Jentsch TJ. Functional and structural conservation of CBS domains from CLC channels. *J Physiol* 557: 363–378, 2004.

13. Estévez R, Schroeder BC, Accardi A, Jentsch TJ, and Pusch M. Conservation of chloride channel structure revealed by an inhibitor binding site in ClC-1. *Neuron* 38: 47–59, 2003.

14. Fahlke C, Yu HT, Beck CL, Rhodes TH, and George AL Jr. Pore-forming segments in voltage-gated chloride channels. *Nature* 390: 529–532, 1997.

15. Fong P, Rehfeldt A, and Jentsch TJ. Determinants of slow gating in ClC-0, the voltage-gated chloride channel of *Torpedo marmorata*. *Am J Physiol Cell Physiol* 274: C966–C973, 1998.

16. Friedrich T, Breiderhoff T, and Jentsch TJ. Mutational analysis demonstrates that ClC-4 and ClC-5 directly mediate plasma membrane currents. *J Biol Chem* 274: 896–902, 1999.

17. Haug K, Warnstedt M, Alekov AK, Sander T, Ramirez A, Poser B, Maljevic S, Hebeisen S, Kubisch C, Rebstock J, Horvath S, Hallmann K, Dullinger JS, Rau B, Haverkamp F, Beyenburg S, Schulz H, Janz D, Giese B, Muller-Newen G, Propping P, Elger CE, Fahlke C, Lerche H, and Heils A. Mutations in CLCN2 encoding a voltage-gated chloride channel are associated with idiopathic generalized epilepsies. *Nat Genet* 33: 527–532, 2003.

18. Hebeisen S, Biela A, Giese B, Müller-Newen G, Hidalgo P, and Fahlke C. The role of the carboxyl terminus in ClC chloride channel function. *J Biol Chem* 279: 13140–13147, 2004.

19. Humphrey W, Dalke A, and Schulten K. VMD: visual molecular dynamics. *J Mol Graph* 14: 33–38, 1996.

20. Iyer R, Iverson TM, Accardi A, and Miller C. A biological role for prokaryotic ClC chloride channels. *Nature* 419: 715–718, 2002.

21. Jentsch TJ, Stein V, Weinreich F, and Zdebik AA. Molecular structure and physiological function of chloride channels. *Physiol Rev* 82: 503–568, 2002.

22. Kornak U, Kasper D, Bösl MR, Kaiser E, Schweizer M, Schulz A, Friedrich W, Delling G, and Jentsch TJ. Loss of the ClC-7 chloride channel leads to osteopetrosis in mice and man. *Cell* 104: 205–215, 2001.

23. Li X, Shimada K, Showalter LA, and Weinman SA. Biophysical properties of ClC-3 differentiate it from swelling-activated chloride channels in Chinese hamster ovary-K1 cells. *J Biol Chem* 275: 35994–35998, 2000.

24. Ludewig U, Pusch M, and Jentsch TJ. Two physically distinct pores in the dimeric ClC-0 chloride channel. *Nature* 383: 340–343, 1996.

25. Maduke M, Pheasant DJ, and Miller C. High-level expression, functional reconstitution, and quaternary structure of a prokaryotic ClC-type chloride channel. *J Gen Physiol* 114: 713–722, 1999.

26. Maduke M, Williams C, and Miller C. Formation of CLC-0 chloride channels from separated transmembrane and cytoplasmic domains. *Biochemistry* 37: 1315–1321, 1998.

27. Middleton RE, Pheasant DJ, and Miller C. Homodimeric architecture of a ClC-type chloride ion channel. *Nature* 383: 337–340, 1996.

28. Miller C. Open-state substructure of single chloride channels from *Torpedo electroplax*. *Philos Trans R Soc Lond B Biol Sci* 299: 401–411, 1982.

29. Miloshevsky GV and Jordan PC. Anion pathway and potential energy profiles along curvilinear bacterial ClC Cl^- pores: electrostatic effects of charged residues. *Biophys J* 86: 825–835, 2004.

30. Mindell JA and Maduke M. ClC chloride channels. *Genome Biol* 2: REVIEWS3003. Epub 2001 Feb 3007., 2001.

31. Moran O, Traverso S, Elia L, and Pusch M. Molecular modeling of p-chlorophenoxyacetic acid binding to the CLC-0 channel. *Biochemistry* 42: 5176–5185, 2003.

32. Niemeyer MI, Cid LP, Zúñiga L, Catalán M, and Sepúlveda FV. A conserved pore-lining glutamate as a voltage- and chloride-dependent gate in the ClC-2 chloride channel. *J Physiol* 553: 873–879, 2003.

33. Piwon N, Günther W, Schwake M, Bösl MR, and Jentsch TJ. ClC-5 Cl^--channel disruption impairs endocytosis in a mouse model for Dent's disease. *Nature* 408: 369–373, 2000.

34. Ponting CP. CBS domains in CIC chloride channels implicated in myotonia and nephrolithiasis (kidney stones). *J Mol Med* 75: 160–163, 1997.

35. Pusch M. Structural insights into chloride and proton-mediated gating of CLC chloride channels. *Biochemistry* 43: 1135–1144, 2004.

36. Pusch M, Ludewig U, Rehfeldt A, and Jentsch TJ. Gating of the voltage-dependent chloride channel ClC-0 by the permeant anion. *Nature* 373: 527–531, 1995.

37. Saviane C, Conti F, and Pusch M. The muscle chloride channel ClC-1 has a double-barreled appearance that is differentially affected in dominant and recessive myotonia. *J Gen Physiol* 113: 457–468, 1999.

38. Sayle RA and Milner-White EJ. RASMOL: biomolecular graphics for all. *Trends Biochem Sci* 20: 374–376, 1995.

39. Schmidt-Rose T and Jentsch TJ. Reconstitution of functional voltage-gated chloride channels from complementary fragments of CLC-1. *J Biol Chem* 272: 20515–20521, 1997.

40. Schmidt-Rose T and Jentsch TJ. Transmembrane topology of a CLC chloride channel. *Proc Natl Acad Sci USA* 94: 7633–7638, 1997.

41. Scott JW, Hawley SA, Green KA, Anis M, Stewart G, Scullion GA, Norman DG, and Hardie DG. CBS domains form energy-sensing modules whose binding of adenosine ligands is disrupted by disease mutations. *J Clin Invest* 113: 274–284, 2004.

42. Sintchak MD, Fleming MA, Futer O, Raybuck SA, Chambers SP, Caron PR, Murcko MA, and Wilson KP. Structure and mechanism of inosine monophosphate dehydrogenase in complex with the immunosuppressant mycophenolic acid. *Cell* 85: 921–930, 1996.

43. Steinmeyer K, Schwappach B, Bens M, Vandewalle A, and Jentsch TJ. Cloning and functional expression of rat CLC-5, a chloride channel related to kidney disease. *J Biol Chem* 270: 31172–31177, 1995.

44. Stobrawa SM, Breiderhoff T, Takamori S, Engel D, Schweizer M, Zdebik AA, Bösl MR, Ruether K, Jahn H, Draguhn A, Jahn R, and Jentsch TJ. Disruption of ClC-3, a chloride channel expressed on synaptic vesicles, leads to a loss of the hippocampus. *Neuron* 29: 185–196, 2001.

45. Strange K. From genes to integrative physiology: ion channel and transporter biology in *Caenorhabditis elegans*. *Physiol Rev* 83: 377–415, 2003.

46. Stroud RM, Miercke LJ, O'Connell J, Khademi S, Lee JK, Remis J, Harries W, Robles Y, and Akhavan D. Glycerol facilitator GlpF and the associated aquaporin family of channels. *Curr Opin Struct Biol* 13: 424–431, 2003.

47. Traverso S, Elia L, and Pusch M. Gating competence of constitutively open CLC-0 mutants revealed by the interaction with a small organic Inhibitor. *J Gen Physiol* 122: 295–306, 2003.

48. Wang SS, Devuyst O, Courtoy PJ, Wang XT, Wang H, Wang Y, Thakker RV, Guggino S, and Guggino WB. Mice lacking renal chloride channel, CLC-5, are a model for Dent's disease, a nephrolithiasis disorder associated with defective receptor-mediated endocytosis. *Hum Mol Genet* 9: 2937–2945, 2000.

49. Weinreich F and Jentsch TJ. Pores formed by single subunits in mixed dimers of different CLC chloride channels. *J Biol Chem* 276: 2347–2353, 2001.

50. Zhang R, Evans G, Rotella FJ, Westbrook EM, Beno D, Huberman E, Joachimiak A, and Collart FR. Characteristics and crystal structure of bacterial inosine-5'-monophosphate dehydrogenase. *Biochemistry* 38: 4691–4700, 1999.