Putting the pieces together
A crystal clear window into CLC anion channel regulation

Kevin Strange
Boylan Center for Cellular and Molecular Physiology; Mount Desert Island Biological Laboratory; Salisbury Cove, ME USA

CLC anion transport proteins function as Cl⁻ channels and Cl⁻/H⁺ exchangers and are found in all major groups of life including archaea. Early electrophysiological studies suggested that CLC anion channels have two pores that are opened and closed independently by a “fast” gating process operating on a millisecond timescale, and a “common” or “slow” gate that opens and closes both pores simultaneously with a timescale of seconds (Fig. 1A). Subsequent biochemical and molecular experiments suggested that CLC channels/transporters are homodimeric proteins.1-3

A major breakthrough in the field occurred when Rod MacKinnon and coworkers crystallized a bacterial CLC protein. The crystal structure confirmed the homodimeric CLC architecture. Each CLC monomer consists of 18 α-helical domains designated “A-R”. Helices B-R span or are embedded in the membrane4,5 (Fig. 1B). It is widely accepted that the overall membrane structure of bacterial CLC proteins is conserved throughout the CLC superfamily (reviewed in refs. 4 and 6–9).

Membrane helices D, F, N and R comprise the CLC channel selectivity filter and “fast” gate (Fig. 1B). A glutamate residue on helix F projects into the extracellular-facing portion of the ion conduction pathway and functions as the voltage-, Cl⁻ - and H⁺-activated fast gate.4,5 The structural basis of slow gating remains unclear, but may be mediated10-15 or modulated16 by the cytoplasmic C-terminus.

Eukaryotic CLC proteins are distinguished from most of their bacterial counterparts by possessing extensive cytoplasmic C-termini containing two cystathionine-β-synthase (CBS) domains termed CBS1 and CBS2 (reviewed in refs. 10 and 17–19) (Fig. 1B). The CBS domain is a ubiquitous, 50–60 amino acid protein motif with a highly conserved secondary structure consisting of an N-terminal β-strand (β1) followed by an α-helix (α1), two β-strands (β2 and β3) and an α-helix (α2).20,21 The functions of CBS domains are poorly understood, but mutations in this motif give rise to several inherited diseases indicating that they play critical roles in protein structure and activity.20,21 Mutations in CLC CBS domains disrupt gating properties and give rise to muscle, kidney and bone disease.3,20

Biochemical and X-ray crystallography studies on isolated C-termini of eukaryotic CLCs demonstrate that they too form dimers.17-19,22 CBS motifs are typically present in proteins as two or four copies. Pairs of CBS motifs associate to form a dimeric structure known as a Bateman module. Bateman modules in turn interact to form quaternary protein structures.20,21 The Bateman modules of two CLC monomers dimerize to form the overall quaternary structure of the CLC cytoplasmic C-terminus.17-19

The structural bases of CLC gating have been studied extensively. What is far less well understood is how CLC transport proteins are regulated by cell signaling pathways. Several pharmacological and molecular studies suggest that CLC-1, CLC-2 and CLC-3 may be regulated by protein phosphorylation events.23-30 Adenine nucleotide binding to intracellular domains has been shown to play possible regulatory roles in CLC-1,31 CLC-2,32 and CLC-5.33

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 Correspondence to: Kevin Strange; Email: kstrange@mdibl.org
Most regulatory studies of various CLC transport proteins have been carried out using heterologous expression systems. Thus the physiological contexts under which such regulation occurs and the signaling mechanisms that mediate regulation are unclear. We have exploited the molecular, genetic and physiological tractability of the nematode *C. elegans* to characterize CLC anion channel function and regulation. The *C. elegans* *clh-3* gene encodes two CLC anion channel splice variants, CLH-3a and CLH-3b. CLH-3b is expressed in the worm oocyte where it is activated by meiotic cell cycle progression and cell swelling. Activation requires protein dephosphorylation mediated by the type I protein phosphatases GSP-1 and GSP-2. The Ste20 kinase GCK-3 binds to CLH-3b and inhibits its activity by mediating phosphorylation of two serine residues. GCK-3 activity is regulated during cell volume and meiotic cell cycle changes by the MAP kinases MEK-2 and MPK-1. GCK-3 is an ortholog of the mammalian STK39/SPAK kinase. SPAK and the closely related kinase OSR1 regulate the activity of the volume sensitive K-Cl and Na-K-2Cl cotransporters and play central roles in systemic salt and water homeostasis.

GCK-3 binds to a domain located between CBS1 and CBS2. This CBS linker is present in all CLC proteins and has a highly variable sequence. X-ray crystallography and NMR studies suggest that the linker is largely unstructured. The CBS linker in CLH-3b is 176 amino acids long. The last 101 amino acids of the linker are unique to the CLH-3b splice variant and are termed the “regulatory domain.” The first four amino acids of this domain mediate phosphorylation of two serine residues 70 and 75 amino acids downstream.

Phosphorylation of CLH-3b reduces current amplitude and induces striking changes in channel voltage sensitivity. Indirect evidence suggests that phosphorylation modulates voltage-dependent fast gating. As noted above, fast gating is mediated by an extracellular-facing glutamate residue on helix F. An outstanding question in the CLC field is whether conformational changes in intracellular domains alter channel/transporter function during cell signaling events. The membrane R-helix forms part of the CLC ion conducting pore and selectivity filter. A short stretch of cytoplasmic amino acids, termed the R-helix linker, connect helix R to the intracellular C-terminus (Fig. 1B). By virtue of its direct connection to the C-terminus, Dutzler et al. proposed that the R-helix could provide a pathway by which intracellular domain conformational changes regulate CLC activity.

Interestingly, despite significant differences in primary sequence, part of the R-helix linkers of ClC-0, ClC-5 and ClC-Ka have similar and well ordered crystal structures. The conservation of this structure suggests that the linker might play a role in CLC regulation by functioning to communicate intracellular conformational changes to membrane domains. Our recent studies support this idea. The CLH-3a and CLH-3b splice variants have strikingly different biochemical properties. Splice variation occurs exclusively in intracellular domains including α2 of CBS2. Interchanging α2 between CLH-3a and CLH-3b interchanges their gating properties. Crystal structures of CLC-0, CLC-Ka and CLC-5 cytoplasmic C-termini indicate that part of the cytoplasmic...
R-helix linker lies close to α2 of CBS2 suggesting that these domains may interact. Using a homology model of the CLH-3b C-terminus, we identified apposing and potentially interacting amino acids in α2 and the R-helix linker. Disrupting these putative interactions with single point mutations is sufficient to convert the gating properties and extracellular cysteine reactivity of CLH-3b to those resembling the CLH-3a splice variant.44

Our studies suggested that interaction of CBS2 and the R-helix linker plays a critical role in regulating CLC structure and function. This idea is now supported compellingly by a crystal structure. In yet another major advance for the field, MacKinnon’s lab succeeded recently in crystallizing an intact eukaryotic CLC protein from the thermophilic red alga Cyanidioschyzon merolae.41 The crystal structure demonstrates that CBS2 abuts the intramembrane domain while CBS1 faces into the cytoplasm. The interface between CBS2 and membrane spanning helices is extensive with a shape complementarity index, which is a measure of how well two protein domains fit together, similar to the interface between an antibody and antigen. This suggests that the interaction is highly specific and therefore functionally significant.

The R-helix linker passes over and makes multiple close contacts with CBS2 including α2. CBS2 also interacts with the D-helix, which like helix R forms part of the ion conducting pore4,5 (Fig. 2). This close interaction between intracellular domains and the CLC pore demonstrates clearly how CBS conformational changes could regulate CLC channel/transporter activity.

In addition to helices D and R, CBS2 interacts with a short intracellular domain connecting helices H and I (Fig. 2). The H and I helices form part of the dimer interface between CLC monomers.5 The interaction of CBS2 with this dimer interface suggests a structural mechanism by which conformational changes in the cytoplasmic C-terminus could mediate or modulate slow or common gating.

Now that we have a complete CLC structure, what does it tell us about how phosphorylation and nucleotide binding regulate CLC proteins? Our studies on CLH-3b provide a foundation for addressing this important question. Deletion of the CLH-3b regulatory domain gives rise to channel activity similar to that observed after phosphorylation by GCK-3.46 The simplest model to explain this result is that the regulatory domain interacts with another part of the channel, and that this interaction is disrupted by phosphorylation or regulatory domain deletion. Recent studies on CLC-5 using small angle X-ray scattering suggest that ATP binding to the cytoplasmic C-terminus increases protein compactness and suggest that CBS1 and CBS2 undergo a clamp-like closure around an ATP molecule.46 Given the location of the CLH-3b regulatory domain between CBS1 and CBS2, it is easy to envision how changes in phosphorylation may cause a similar change in CBS architecture.

As noted earlier, the linker between CBS1 and CBS2 shows little or no sequence conservation between various CLC proteins and appears to be largely

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**Figure 2.** Cartoon showing interaction of CBS2 with intramembrane domains. CBS1 and CBS2 are located in the cytoplasm and dimerize to form a so-called Bateman module. A linker connects the two CBS domains. This linker is 176 amino acids long in CLH-3b and includes a 101 amino acid regulatory domain (shown in green), which is where GCK-3 binds and regulatory phosphorylation occurs. Gray helices form the membrane domain. CBS2 interacts extensively with the R-helix linker shown in orange and helix D. Phenylalanine and serine residues on helices R and D, respectively, function in Cl⁻ coordination and selectivity within the ion conducting pore. A glutamate residue on helix F functions as the fast gate. The interface between CBS2 and membrane domains shows a high degree of shape complementarity suggesting that the interaction is specific and functionally significant. Chloride ions moving through the pore are shown in red. An intracellular loop linking helices H and I also interacts with CBS2. Helices H and I form part of the interface between the monomers of CLC membrane domains.
unstructured. However, NMR studies on the CLC-0 CBS linker suggest that located within this disordered domain are small islands of secondary structure. Analysis of the sequence of the CLH-3b CBS linker suggests that it is also mostly disordered. Located upstream from the regulatory domain though is a densely charged region predicted to form α-helices.

Unstructured domains are common in proteins and are thought to play critical functional roles. For example, phosphoproteins and are thought to play critical from the regulatory domain though is a

9. Functional study of CLC chloride channels. J Gen Physiol 1997; 113:57-105.
10. Pusch M, Ludwig U, Jentsch TJ. Temperature dependence of fast and slow gating relaxations of CLC chloride channels. J Gen Physiol 1999; 109:105-16.
11. Bennetts B, Roberts ML, Berezat AH, Rydkov GY. Temperature dependence of human muscle chloride channel. J Physiol 2001; 535:83-89.
12. Bykovsky EA, Zhang XD, Chen TY, Zheng J. Large movement in the C terminus of CLC chloride channel during slow gating. Nat Struct Mol Biol 2006; 13:1115-9.
13. Garcia-Olivares J, Abekow A, Boutin J, MD, et al. Gating of human CLC-2 chloride channels and regulation by carboxy-terminal domains. J Physiol 2008; 586:5325-36.
14. Markovic S, Dutzler R. The structure of the cytoplasmic domain of the chloride channel ClC-Ka reveals a conserved interaction interface. Structure 2007; 15:715-25.
15. Meyer S, Dutzler R. Crystal structure of the cytoplasmic domain of the chloride channel ClC-0. Structure 2006; 14:299-307.
16. Meyer S, Savaterri S, Forster IC, Dutzler R. Nucleotide recognition by the cytoplasmic domain of the cytosolic chloride transporter ClC-5. Nat Struct Mol Biol 2007; 14:60-7.
17. Iognoul S, Eggermont J. CBS domains: structure, function and pathobiology in human proteins. Am J Physiol 2005; 289:C1360-78.
18. Baizans A. The structure of a domain common to archaeabacteria and the homocyctinuria disease protein. Trends Biochem Sci 1997; 22:12-3.
19. Martinez GQ, Mudde M. A cytoplasmic domain mutation in CIC-Kb affects long-distance communication across the membrane. PLoS ONE 2008; 3:2746.
20. Cuddapah Y, Sontheimer H. Molecular interaction and functional regulation of CIC-3 by Ca2+/calmodulin-dependent protein kinase II (CaMIIK) in human malignant glomas. J Biol Chem 2010; 285:11888-96.
21. Hisao KM, Huang JY, Tang PH, Lin MJ. Functional study of CLC-1 mutants expressed in Xenopus oocytes reveals that a C-terminal region Thr891-Ser892-Thr893 is responsible for the effects of protein kinase C activator. Cell Physiol Biochem 2010; 25:687-96.
22. Furukawa T, Ogura T, Zheng YJ, et al. Phosphorylation and functional regulation of CIC-2 chloride channels expressed in Xenopus oocytes by M cyclin-dependent protein kinase. J Physiol 2002; 540:883-93.
23. Kraus F, Lauffer J, Czakowski K, et al. PI(4,5)P2-dependent regulation of the CI channel CIC-2. Biochem Biophys Res Commun 2009; 381:407-11.
24. Duan D, Cowlery S, Horowitz B, Hume JR. A serine residue in CIC-3 links phosphorylation-dephosphorylation to chloride channel regulation by cell volume. J Gen Physiol 1999; 113:57-70.
25. Nagasaki M, Ye L, Duan D, Horowitz B, Hume JR. Intracellular cyclic AMP inhibits native and recombinant volume-regulated chloride channels from mammalian heart. J Physiol 2000; 523:705-17.
26. Huang P, Liu J, Di A, et al. Regulation of human CLC-3 channels by multifunctional Ca2+/calmodulin-dependent protein kinase. J Biol Chem 2001; 276:20093-100.
27. Robinson NC, Huang P, Kaerlal MA, Lamb LS, Nelson DJ. Identification of an N-terminal amino acid of the CLC-3 chloride channel critical in phosphorylation-dependent activation of a CaMKII-activated chloride current. J Physiol 2004; 556:353-68.
28. Zhang XD, Tseng PY, Chen TY. ATP inhibition of CIC-1 is controlled by oxidation and reduction. J Gen Physiol 2008; 132:421-8.
29. Nielsen MI, Yiel YR, Coropol J, et al. Functional evaluation of human CIC-2 chloride channel mutations associated with idiopathic generalized epilepsies. Physiol Genomics 2004; 19:74-83.
30. Zifarelli G, Pusch M. Intracellular regulation of human CIC-5 by adenine nucleotides. EMBO Rep 2009; 10:1111-6.
31. Rutledge E, Bianchi L, Christensen M, et al. CLC-3, a C2-4 channel ortholog activated during meiotic maturation in C. elegans oocytes. Curr Biol 2001; 11:161-70.
32. Rutledge E, Dennon J, Strange K. Cell cycle- and swelling-induced activation of a C. elegans CIC channel is mediated by CGLC-7αβ phosphatases. J Cell Biol 2002; 158:435-44.
33. Falin R, Morison R, Ham A, Strange K. Identification of regulatory phosphorylation sites in a Ste20 kinase regulated cell cycle- and volume-sensitive CIC anion channel. J Gen Physiol 2009; 133:29-42.
34. Denton J, Nehke D, Yin X, Morrison S, Strange K. ClC-5, a newly identified Ste20 kinase, binds to and regulates the activity of a cell cycle-dependent CIC anion channel. J Gen Physiol 2005; 125:113-25.
35. Falin RA, Miyazaki H, Strange K. C. elegans STK39/SPAK ortholog mediated inhibition of CIC anion channel activity is regulated by WNK-independent ERK kinase signaling. Am J Physiol 2010; In press.
36. Delpere E, Gagnon KB, SPAK and ORS1: STE20 kinases involved in the regulation of ion homeostasis and volume control in mammalian cells. Biochm J 2008; 409:321-31.
37. Kahlke KT, Rim AM, Litos RP. Molecular physiology of the WNK kinases. Annu Rev Physiol 2008; 70:329-55.
38. Feng L, Campbell EB, Hsiung Y, MacKinnon R. Structure of a eukaryotic CIK transporter defines an intermediate state in the transport cycle. Science 2010; 330:635-41.
39. Alloeh S, Meyer S, Dutzler R, Pervush V. The cytoplasmic domain of the chicken chloride channel CIC-0: structural and dynamic characterization of flexible regions. J Mol Biol 2007; 369:1163.
40. Dennon J, Nehke D, Rutledge E, Morrison S, Strange K. Alternative splicing of N-terminus CIC-0 channel splice variants are determined by altered CBS domain conformation and the R-helix linker. Channels 2010; 4:289-301.
45. He L, Denton J, Nehrke K, Strange K. Carboxy terminus splice variation alters ClC channel gating and extracellular cysteine reactivity. Biophys J 2006; 90:3570-81.

46. Wellhauser L, Luna-Chavez C, D’Antonio C, Tainer J, Bear CE. ATP binding induces conformational changes in the carboxy terminal region of ClC-5. J Biol Chem 2011; 286:6733-41.

47. Dyson HJ, Wright PE. Intrinsically unstructured proteins and their functions. Nat Rev Mol Cell Biol 2005; 6:197-208.

48. Gepner J, Babu MM. The rules of disorder or why disorder rules. Prog Biophys Mol Biol 2009; 99:94-103.