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Minireview

A structural perspective on ClC channel and transporter function

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Abstract **The ClC chloride channels and transporters constitute a large family of membrane proteins that is involved in a variety of physiological processes. All members share a conserved molecular architecture that consists of a complex transmembrane transport domain followed by a cytoplasmic domain. Despite the strong conservation, the family shows an unusually broad variety of functional behaviors as some members work as gated chloride channels and others as secondary active chloride transporters. The conservation in the structure and the functional resemblance of gating and coupled transport suggests a strong mechanistic relationship between these seemingly contradictory transport modes. The cytoplasmic domains constitute putative regulatory components that are ubiquitous in eukaryotic ClC family members and that in certain cases interact with nucleotides thus linking ion transport to nucleotide sensing by yet unknown mechanisms.**
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1. Introduction

Chloride (Cl^-) is the most abundant anion in the extracellular environment of different organisms. Its transport across cellular membranes contributes to diverse physiological processes. Different families of membrane proteins have been identified that selectively transport Cl^- ions. These proteins include secondary active chloride transporters and chloride channels that catalyze ion flow by two distinct mechanisms. In transporters that function by 'alternate access' mechanisms different conformational states of the protein are sequentially occupied during the transport cycle and usually expose ion binding sites alternately to either side of the membrane, thereby preventing the formation of a continuous pore, which would dissipate the gradient of the transported ion. Channels on the other hand catalyze the specific downhill movement of the transported ion at very high rates by forming such continuous pores that connect both sides of the membrane and that usually contain selective ion binding sites in a narrow selectivity filter. Whereas in most cases channels and transporters are encoded by different protein families this strict division breaks down for the ClC family of Cl^- channels and transporters. The ClC proteins constitute a large family of transmembrane transporters that either function as Cl^- channels or as H^+/Cl^- exchangers [1]. Its members are ubiquitously expressed in all kingdoms of life. The nine homologues in human are located in the plasma membrane or in the membranes of intracellular compartments and are involved in various processes, ranging from electrical signalling in muscle to epithelial ion transport and the acidification of intracellular compartments [2]. Mutations in certain ClC proteins result in severe familial diseases including myotonias, nephropathies and osteopetrosis. The family shows a diverse functional behavior that is in many cases still poorly understood.

Despite this broad functional diversity all family members share a conserved structural organization, which includes a complex transmembrane transport domain that is usually followed by a cytoplasmic component that is believed to play an important role in transport regulation (Fig. 1a) [1]. Both structural components are essentially conserved between the functional branches of channels and transporters, which makes the distinction based on the sequence difficult. The aim of this mini-review is to summarize the current understanding of ClC protein structure and function and to outline major unsettled questions.

2. A structural framework for Cl^- selectivity

Our current insight into the architecture of the transmembrane component is provided by the structures of two bacterial homologues from *Salmonella typhimurium* (StClC) and *Escherichia coli* (EcClC or ClC-ec1) [3,4]. Although the bacterial proteins function as H^+/Cl^- exchangers, their structures are representative for both branches of the ClC family. In contrast to eukaryotic family members, however, the bacterial proteins of known structure lack the cytoplasmic components.

The structure of the bacterial transporter EcClC is shown in Fig. 1b. EcClC is a homodimeric protein whose two structurally identical subunits each harbor an independent ion translocation pore. The subunit exhibits a complex topology with two structurally related halves spanning the membrane with opposite orientations to form an 'antiparallel architecture' (Figs. 1a and 2a) [3]. This 'antiparallel architecture' that was previously only observed in the unrelated aquaporin superfamily [5] has since then proven to become a versatile construction principle for several transmembrane transport proteins [6,7]. The Cl^- translocation path is located at the interface between the two halves and contains a Cl^- selectivity filter in the neck of an hourglass-like shaped pore. This narrow 15 Å long selectivity

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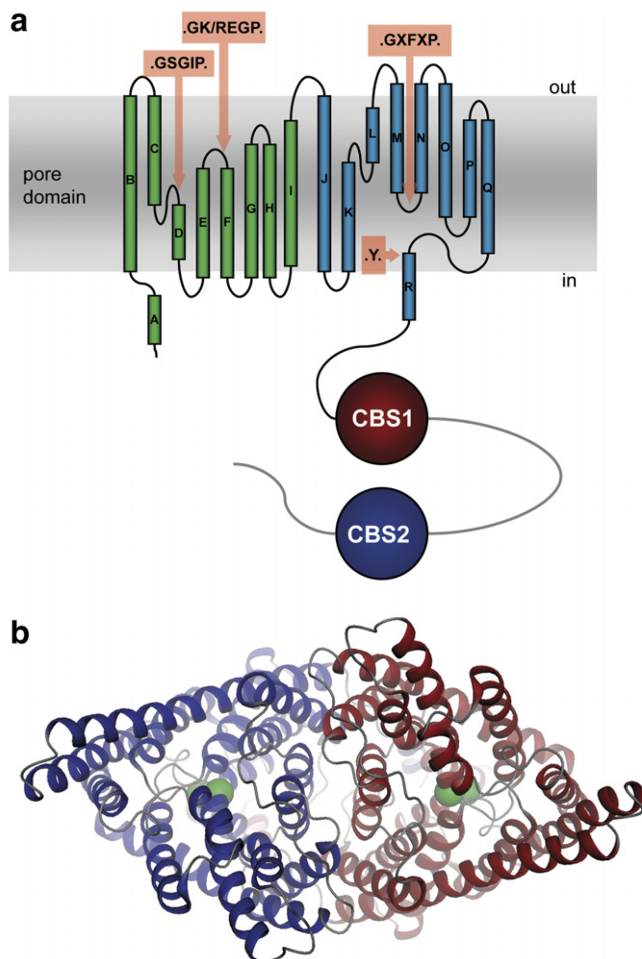

Fig. 1. Topology and structure of ClC proteins. (a) Schematic topology of eukaryotic members of the ClC family. The topology of the transmembrane domain was inferred from the known structure of the bacterial homologue EcClC. The 18 α-helices are labeled A–R, the two similar halves within the transmembrane domain (α-helices B–I and J–Q), which are oriented with opposite directions with respect to the membrane, are colored in green and cyan, respectively. The sequence regions, which contribute to the Cl^- selectivity filter, are marked (arrows) and the respective conserved sequences are shown. The two CBS motifs of the cytoplasmic component of the protein are shown as red and blue spheres. (b) Structure of EcClC viewed from the extracellular side. The two subunits of the homodimeric protein are colored in red and blue, bound ions in the selectivity filter of each subunit are shown as green spheres.

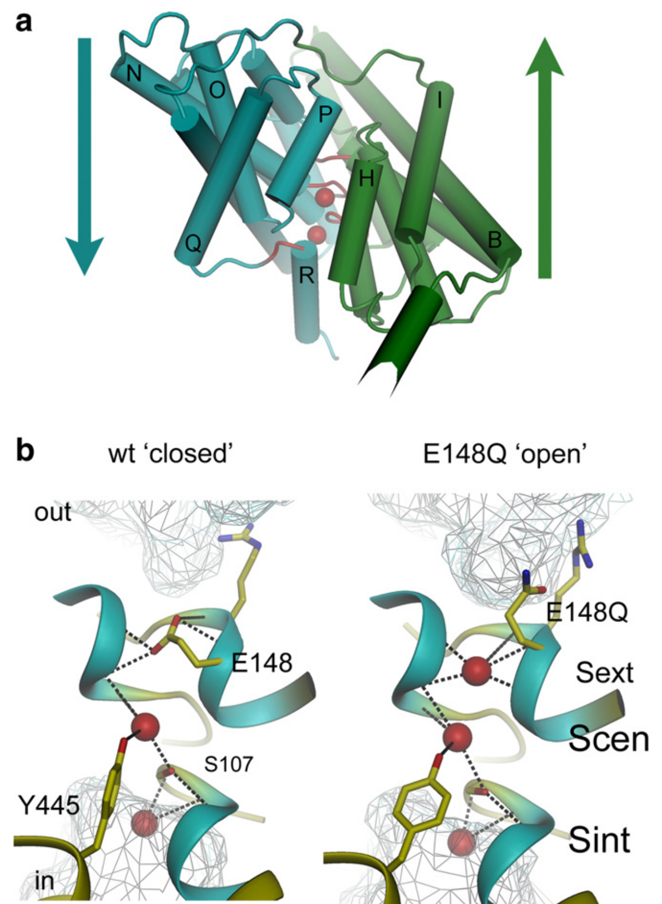

Fig. 2. Structure of the EcClC selectivity filter. (a) View at the EcClC subunit from the dimer interface. The two halves of the protein are colored as in Fig. 1a, their orientation in the membrane is indicated by arrows selected helices are labeled. Bound ions are shown as red spheres, regions of the protein contributing to the ion selectivity filter are colored red. (b) Blowup of the selectivity filters of the wt protein and of a selectivity filter mutant. The protein backbone is shown as ribbon with the N-termini of α-helices colored in blue. Selected side-chains are shown as sticks, bound ions as red spheres. Interactions between the ions and hydrogen bond donors in the protein (OH groups of a Ser and a Tyr side-chain and amide NH groups of the protein backbone) are indicated by dashed lines. Aqueous cavities approaching the selectivity filter from both sides of the membrane are shown as blue mesh. The ion binding sites are labeled. Left: the selectivity filter in a 'closed conformation' as seen in the structure of the wt-protein. Two chloride ions are bound to the sites S_{int} and S_{cen}, while the ion binding site S_{ext} is occupied by the side-chain of the conserved glutamate residue E148 which acts as a tethered ion to block the filter. Right: the selectivity filter of the mutant E148Q in an 'open-like conformation': the mutated side-chain of the residue has moved out of the filter and points towards the extracellular solution. An additional ion has taken its place, binding to the site S_{ext}. The filter is occupied by three ions which bind in a single file and bridge the aqueous solutions on both sides of the membrane.

filter harbors three selective anion-binding sites which span the filter to bridge the two aqueous vestibules on either side of the membrane (Fig. 2b) [4]. The three binding sites that were named S_{int}, S_{cen}, and S_{ext} according to their location in the filter allow insight into the chemistry underlying anion selectivity: The ions are stripped of their hydration shell to various degrees and are interacting with partial charges of side-chains and backbone residues, several of which are located at the N-terminus of α-helices (Fig. 2b). In the wild-type (wt) protein two of the binding sites (S_{int} and S_{cen}) are occupied by Cl^- ions, while the third site at the extracellular end of the filter is occupied by the side-chain of a conserved glutamate residue (Glu 148 or Glu_{ext}, Fig. 2b) that binds to this site akin to a tethered anion. When mutating this residue to glutamine, the binding site is released and binds a Cl^- ion instead, while leaving the remainder of the filter unchanged [4]. Recent results from an experimental investigation addressing the ion binding properties of the selectivity filter showed that despite their close mutual proximity, all three sites can bind Cl^- ions at the same time with mM affinity [8]. The binding properties of this ion binding region appear to be conserved among the two functional branches channels and transporters thus underlining the strong structural relationship within the family [8]. The multiple occupancy of the Cl^- selectivity filter resembles a similar situation found in K^+ channels and strongly suggests

that also in ClC channels ions permeate in a single file with mutual electrostatic repulsion fostering rapid conduction [9]. The two conformations of the selectivity filter, the blocked ‘closed-like’ conformation in the wt structure and the ‘open-like’ conformation in the mutant, have important implications for both gating in the ion channels and for coupled transport in the H^+/Cl^- exchangers.

3. Channel and transporter function

The EcClC structure allows the comprehension of important aspects of the complex functional behavior within the ClC family. The ‘double barreled’ architecture of ClC channels that is reflected in the dimeric structure of EcClC was initially proposed based on electrophysiological experiments on the Cl^- channel ClC-0 [10]. This member of a family of muscle type channels (which includes the human proteins ClC-1 and ClC-2) shows a complex gating behavior that is influenced by voltage, Cl^- and pH [11,12]. Different from voltage dependent cation channels, however, the transmembrane voltage in ClC channels is not sensed by a protein domain, instead it is the permeating Cl^- itself whose movement across the electric field upon channel opening confers the voltage dependence to this process [13]. Two distinct mechanisms regulate conduction in muscle type channels: (1) Independent opening and closing of each pore in the dimeric protein in a process called ‘protopore gating’. (2) Concerted closing of both pores in a process named ‘common gating’ [14]. The structure of the ion binding region in EcClC immediately suggested a relation to the ‘protopore gating’ process with the glutamate residue binding to S_{ext} being the likely candidate for a gate which opens and closes the permeation path. Mutations of this residue essentially abolish gating, thereby rendering the protein as a predominantly open Cl^- conductor [4]. Interestingly the only ClC family members that do not contain this conserved residue, the ClC-K kidney channels lack the ‘protopore gating’ mechanism [15,16]. While it is generally accepted that the glutamate side-chain binding to S_{ext} serves as a gate whose protonation also confers the pH dependence of pore opening, the mechanisms of voltage and Cl^- dependence are currently less well understood.

Since EcClC functions as a H^+/Cl^- exchanger with strict 1:2 stoichiometry, its structure is ideally suited to study the mechanisms of coupled transport in the ClC family [17]. This functional branch includes next to many bacterial homologues, also eukaryotic family members that reside in intracellular organelles such as endosomes and lysosomes [18,19]. Although the detailed transport mechanism is currently not understood, results from several studies suggest a strong functional relationship between ClC channels and transporters. A mutation of the glutamate residue occupying S_{ext} in EcClC prevents H^+ transport and turns the protein into a passive Cl^-conductor [17]. A similar behavior was also found for the eukaryotic transporters ClC-5 and ClC-4 [18,19]. Those results point at a mechanistic similarity between ‘protopore gating’ and H^+ transport.

A prerequisite of the transporters is the presence of a defined proton pathway across the protein. A glutamate residue located on the intracellular surface of the protein that is ubiquitous in the transporters but not in the channels was suggested to serve as intracellular proton acceptor based on the fact that a mutation of this residue abolishes proton transport [20]. Thus, the Cl^- and H^+ pathways appear to be separated on the cytoplasmic side and converged at the extracellular end of the Cl^- selectivity filter. Two features distinguish transport in EcClC from common alternate access transporters (*e.g.* lactose permease [21]), which suggests a fundamentally different transport mechanism: (1) The transported ions diffuse through extended regions in the protein akin to situations found in ion channels (Fig. 2b). (2) Proton transport appears to require the presence of Cl^- ions in specific binding sites [22,23]. Both properties have so far not been observed in other coupled transport proteins. Moreover, in accordance with the relatively fast transport kinetics (which was estimated to occur on a high microsecond time-scale [24]), presently no large conformational changes have been observed, that would change the access of the binding sites to either side of the membrane other than the movement of the side-chain in mutations of the glutamate bound to S_{ext} (Fig 2b). The specific roles of the two observed conformations of the Cl^- selectivity filter during transport are still unclear. Although it has been suggested that the conformations of two residues coordinating the ion in S_{cen} (S104 and Y445 Fig. 2b) would constitute an intracellular gate, which prevents free exchange of the ion with the cytoplasm [17], there is currently no experimental evidence supporting this idea. A detailed understanding of coupled transport in the ClC family clearly remains a challenging task for future studies.

4. Transport regulation via cytoplasmic domains

Although the EcClC structure provides important insight into the functional mechanisms of the individual subunits as ion conduction and ‘protopore gating’ in the channels and coupled transport in the transporters, the structure does not reveal the mechanistic basis for concerted regulatory processes as the ‘common gating’ mechanism in the muscle type channels [14]. The structural basis of those mechanisms is currently still not understood. Increasing experimental evidence, however, suggest that concerted processes involve the cytoplasmic components that are found in all eukaryotic members of the family and that are absent in the bacterial proteins of known structure [25,26]. Each cytoplasmic domain is directly attached to a helix of the pore domain that contributes a residue to a Cl^- binding site (the R-helix) (Figs. 1a and 2b). It is therefore an attractive but still unproven hypothesis that these components might be involved in transport regulation by directly affecting the selectivity filter via the R-helix [3].

All cytoplasmic ClC domains share a conserved structural organization that contains a pair of CBS motifs. Similar motifs are frequently found as building blocks for regulatory ligand binding domains in a variety of protein families such as enzymes, kinases and transmembrane transporters [27]. The structures of isolated domains of two different ClC family members give insight into the architecture of these putative regulatory units [28,29]. Fig. 3a shows the structure of the cytoplasmic domain of the transporter ClC-5. Its overall organization resembles proteins that share a similar structural scaffold. The two CBS motifs within one chain (CBS1 and CBS2) are related by a pseudo twofold arrangement and are tightly interacting via an extended interface formed by a pair of β-strands. The overall size of the cytoplasmic domains within

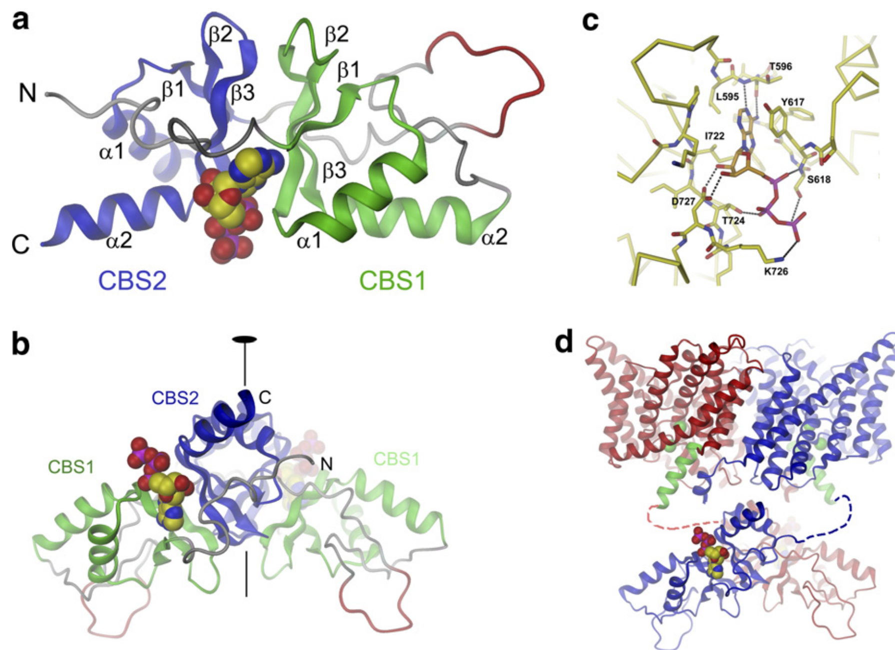

Fig. 3. Structure of the cytoplasmic domain of ClC-5. (a) Ribbon representation of the ClC-5 cytoplasmic domain. The two CBS motifs are colored in green and blue, respectively, residues of the ubiquitine ligase recognition sequence are colored in red. The bound ATP molecule is shown as CPK model. (b) Dimeric organization of two cytoplasmic domains as observed in the crystal structure. The colors are according to a. The ATP molecule is shown as CPK model. The twofold axis of symmetry is indicated. (c) View of the nucleotide binding site. The protein mainchain is shown as Cα-trace with selected residues in the vicinity of the bound ATP molecule shown as sticks. Hydrogen bonds between the protein and the nucleotide are shown as black dashed lines. Selected protein residues in contact with ATP are labeled. (d) Hypothetical model of the cytoplasmic domains relative to the transmembrane domain viewed from within the membrane. The structure of the EcClC dimer (shown as ribbon with subunits colored in blue and red, respectively) serves as a model for the transmembrane domains. The R-helix is colored in green. Bound ions are drawn as green spheres. The dimeric cytoplasmic domains of ClC-5 are shown as ribbon and are colored in red and blue, respectively. The ATP molecules are shown as CPK models.

the family varies, ranging from about 150 residues in the kidney channel ClC-Ka to more than 390 residues in the muscle channel ClC-1. Two regions in the protein are responsible for this variation, the linker region connecting the two CBS motifs, and the C-peptide, a sequence stretch following CBS2 [28]. Those two regions can be extended as the case in the muscle type channels (ClC-0, ClC-1 and ClC-2). Unlike the two CBS motifs that form compact and well-folded structures, the extended linker and C-peptide of the muscle type channels lack tertiary structure [28]. This feature is predicted from the sequences, it is manifested in the lack of electron density in the crystal structure of the domain of ClC-0 and has recently been confirmed in a NMR study that revealed the dynamic nature of both regions (manuscript in preparation). The functional role of these extended flexible regions is still unclear. There are, however, various experiments that hint at an involvement in regulatory processes: (1) as seen in splice variants of certain ClC channels, the disordered C-peptide appears to have a large influence on channel behavior [30]. Moreover, in ClC-1 point mutations in the same region give rise to severe muscle diseases [31]. (2) The linker region in some family members contains recognition sites for kinases, and channel function was shown to be altered in response to phosphorylation [32,33]. (3) The shorter and structurally well-defined linker of the transporter ClC-5 contains a recognition site for the protein ubiquitin ligase, which plays an important role in the correct targeting of the protein to intracellular compartments [34].

Analytical ultracentrifugation experiments revealed a dimeric organization of the isolated domains in solution thus suggesting that the twofold arrangement of the transmembrane pore is extended to the cytoplasmic components [28,29]. Whereas the subunit structures of the ClC cytoplasmic domains resemble similar regions in other proteins, their oligomeric assembly is unique. The quarternary structure found in the crystal structure of the ClC-5 domain is distinct and has not been observed in structurally related components of other protein families (Fig. 3b). A similar assembly, however, was recently also seen in the crystal structure of the equivalent domain of the channel ClC-Ka (manuscript in preparation). While most CBS motif containing proteins dimerize via a flat interface formed by the two α-helices in the respective subdomains, thus resulting in a disk-shaped structure, the ClC domains form V shaped dimers that interact via a conserved interface that is predominantly formed by residues on the surface of CBS2 in each chain of the homodimeric protein [29]. This interface is smaller than other interfaces, which might reflect the need of the domains to undergo conformational changes upon channel regulation. Such conformational changes have recently been reported for the channel ClC-0 [25]. The cytoplasmic domains of this voltage dependent channel were observed to move about 20 Å during 'common gating' thus suggesting an important role in this concerted process.

A different regulatory mechanism of certain ClC proteins that involves their cytoplasmic domains concerns the interaction with adenosine nucleotides. CBS motifs frequently constitute regulatory nucleotide binding domains in different protein families [35]. For example, in the ATP dependent protein

kinase a domain consisting of four consecutive CBS motifs has been proposed to be involved in the regulation of catalysis in response to changes in the metabolic state of the cell [36]. A similar regulation in response to changing nucleotide concentrations was observed for certain ClC family members [37]. ATP binding to the cytoplasmic domains of the muscle channel ClC-1 has recently been shown to influence the voltage dependence of 'common gating' by stabilizing the closed state of the channel [38]. The basis for nucleotide recognition was clarified in the structure of the cytoplasmic domain of the human transporter ClC-5 [29]. Each protein chain contains a specific nucleotide binding site in the interface between the two CBS motifs (Fig. 3a). The site does not have catalytic activity and does not discriminate between ATP, ADP and AMP which bind in a Mg^{2+} independent manner with about 100 μM affinity (Fig. 3c). Although the structural basis for nucleotide recognition by the cytoplasmic domains of certain ClC proteins is resolved, there remain many open questions concerning the structural organization of the full length protein, effects of nucleotide binding on the transmembrane transport domains and the nature of the regulatory stimulus (Fig. 3d). The clarification of these open questions requires the structure determination of full-length ClC proteins in the absence and presence of nucleotides and the study of the proteins in their native cellular environment.

5. Outlook

The ClC proteins constitute an important family of chloride transport proteins that either function as gated chloride channels or as coupled chloride transporters with closely related transport mechanisms. The increasing amount of structural information has changed our understanding of the diverse and complex mechanisms underlying transport and regulation. However, even with first structures in hand many mechanistic questions are still not well understood and require experimental clarification. With continuous inputs from different areas including structure function and physiology we expect a picture to emerge which clarifies the role of these proteins in human physiology and pathophysiology.

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