Reconstitution of Functional Voltage-gated Chloride Channels from Complementary Fragments of CLC-1*

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We investigated the effect of truncations on the human muscle chloride channel CLC-1 and studied the functional complementation from partial proteins. Almost complete deletion of the cytoplasmic amino terminus did not affect currents, but truncating the intracellular COOH terminus after Leu270 abolished function. Currents were restored by coexpressing this membrane-embedded part with the lacking cytoplasmic fragment that contains domain D13, the second of the two conserved cystathionine β-synthase (CBS) motifs present in all eukaryotic CLC proteins. However, if the cut was after Gln697 before the first CBS domain, no functional complementation was seen.

Complementation was also obtained with channels “split” between transmembrane domains D7 and D8 or domains D8 and D9, but not when split between D10 and D11. Specificity of currents was tested by inserting point mutations in NH2-terminal (G188A and G230E) or COOH-terminal (K585E) fragments. In contrast to G188A and K585E, split channels did not tolerate the D136G mutation, suggesting that it may impede association from nonlinked fragments. Duplication, but not a lack of domain D8 was tolerated in “split” channels. Membrane domains D9–D12 can insert into the membrane without adding a preceding signal peptide to ensure the extracellular amino terminus of D9. Eventually, we succeeded in reconstituting CLC-1 channels from three separate polypeptides: the amino-terminal part up to D8, D9 through CBS1, and the remainder of the cytoplasmic carboxyl terminus.

In summary, several regions of CLC channels behave autonomously regarding membrane insertion and folding and mediate protein-protein interactions strong enough to yield functional channels without a direct covalent link.

CLC chloride channels, originally identified by the expression cloning of CLC-0 from Torpedo electric organ (1), form a large gene family with at least nine members in mammals and conservation down to organisms like Escherichia coli and yeast (for review, see Ref. 2). Their importance is underscored by human inherited diseases; mutations in the muscle channel CLC-1 lead to myotonia (3, 4), and those in CLC-5 lead to proteinuria and kidney stones (5).

CLC proteins are structurally unrelated to other channels, including Cl− channels like γ-aminobutyric acid and glycine receptors or the cystic fibrosis transmembrane regulator CFTR. Hydrophobicity analysis indicated 13 hydrophobic domains (D1–D13 (1)). However, newer experimental findings (6–8) suggest the presence of only 10 (or 12) transmembrane domains (Fig. 1, top). The topology of the D9–D12 region, a long hydrophobic stretch interrupted only once by a short hydrophilic segment, still poses problems. Both the amino- and the carboxyl terminus reside in the cytoplasm, and the loop between D8 and D9 is glycosylated.

CLC-0 forms homodimers with one pore per subunit (7, 9, 10), and this may also apply for CLC-1 (11). This one-protein-one-pore architecture distinguishes the CLC channels from voltage-gated cation channels. In shaker K channels, four homologous subunits form a single pore, with equivalent parts of each subunit contributing to it. Although the α-subunit of sodium and calcium channels consists of a single polypeptide chain, it shows the same 4-fold repetition of the K channel subunit motif. Ligand-gated anion channels like the γ-aminobutyric acidA receptor are pentamers, with the second TMD of each monomer contributing to the single common pore. By contrast, and in this respect similar to CLC channels, CFTR also contains 12 TMDs forming one pore per protein. In contrast to CLC channels, it functions as a monomer and consists of two halves that are interrupted by a large cytoplasmic loop. It has two nucleotide binding folds and is a member of the ABC transporter superfamily. In channels with one pore per subunit, several different parts of the polypeptide chain must contribute to the pore, as has been shown both for CFTR (12–15) and CLC channels (9, 16–18). These parts must be positioned correctly by intramolecular interactions.

In the present work, we show that some of these interactions are strong enough to enable the functional expression of CLC-1 channels from individual polypeptides representing complementary parts of the channel protein. Surprisingly, this not only functions when the “cut” lies between transmembrane spans, but also if an otherwise inactive, COOH-terminally truncated channel is coexpressed with a carboxyl-terminal part lacking any TMD. Here, functional complementation is observed if the cut lies between the first and the second CBS domain (19, 20) but not if the COOH-terminal part comprises both CBS domains. In addition, we show that “split” channels with a duplicated D8 domain are functional, indicating that D8 lies at the periphery of the channel.

**EXPERIMENTAL PROCEDURES**

Mutagenesis and Channel Expression—Human CLC-1 (GenBank™ accession number Z25884) was cloned between NeoI and EcoRI sites of pTTLN (21), yielding pTTLNH1. Carboxyl-terminally truncated channels...
D136G, G188A, K558E, and R496S; numbers indicate last amino acid position) were generated by PCR using 39-primers that contained two stop codons and an EcoRI restriction site in addition to the template-specific nucleotides. The 59-primer was chosen upstream of the nearest single-cutter restriction site so that after digestion the PCR product could be ligated into pTLNH1 cut with the same enzymes. Amino-terminal truncations (ΔN110, ΔN369, ΔN413, ΔN509, ΔN598, and ΔN721; number indicates first amino acid position) were constructed using an upstream PCR primer containing the first two codons of CLC-1 (methionine and glutamate) including an NcoI site plus template specific sequences of the new NH2 terminus. The 39-primer was placed downstream of an appropriate restriction site. Again, PCR products and vector pTLNH1 were cut with the same enzymes and ligated. Since construct ΔN413 starts in an extracellular loop, we constructed ΔN413(SP) in which the signal peptide of the rat nicotinic acetylcholine receptor α-subunit (amino acids 1–47) replaces the first two residues of mutant ΔN413 to direct the amino terminus to the extracellular side. Construct ΔN413(SP)ΔC720 is truncated at either end and was obtained by replacing the COOH terminus of mutant ΔN413(SP) with the corresponding NdeI/EcoRI fragment from mutant ΔC720.

Point mutations D136G, G188A, K558E, and R496S were introduced into full-length proteins or truncated channels ΔC451 or ΔN413 and ΔN413(SP) by recombinant PCR. All PCR-derived fragments were sequenced. The plasmids were linearized, and capped cRNA was transcribed using SP6 polymerase (mMessage mMachine cRNA synthesis kit, Ambion). cRNA was injected into manually defolliculated Xenopus oocytes, which were kept in modified Barth’s solution (90 mM NaCl, 1

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**FIG. 1. Schematic diagram of CLC-1 constructs.** Hydrophobic domains are termed D1–D13 according to Refs. 1 and 8. Top, wild type CLC-1 with point mutations used in this work. Channel transmembrane topology is shown according to Refs. 8 and 42. The glycosylation site in the extracellular loop connecting D8 and D9 is indicated by branched lines. The CBS domains (19, 20) in the cytoplasmic carboxyl terminus are shown in black. CBS2 coincides with D13. Bottom, carboxyl-terminal (ΔC) and amino-terminal (ΔN) truncation mutants. Juxtaposed constructs have been coexpressed, but not all combinations tested are included in this figure. The hydrophobic domain D8 is drawn in gray in the pair ΔC451 plus ΔN369 to emphasize its duplication.
mM KCl, 1 mM CaCl$_2$, 0.33 mM Ca(NO$_3$)$_2$, 0.82 mM MgSO$_4$, 10 mM Hepes, pH 7.4) at 17 °C for 2–3 days before measurement. In coexpression experiments, we used a molar cRNA ratio of 1:1 for channel fragments and proteins, and channels split in the carboxyl-terminal cytosolic portion. All currents traces are representative examples from at least two batches of oocytes ($n \geq 6$).

**RESULTS**

The Amino Terminus of CLC-1 Is Dispensable, while the Carboxyl Terminus Is Needed and Can Function as a Separate Polypeptide—Both the amino and carboxyl termini differ in length among CLC family members and are poorly conserved except for few regions in the carboxyl terminus, including domain D13. We asked whether they serve specific functions and at first deleted the amino terminus between glutamate 2 and C597 and duplicated amino acids 598–720. This resulted in typical CLC-1 currents, although currents were significantly lower than with the complementary pair (data not shown). Surprisingly, expression of the carboxyl-terminal part with the complementary membrane-anchored part of CLC-1 reconstituted robust WT currents for the combination $\Delta C_{720} + \Delta N_{598}$, but not with $\Delta C_{597} + \Delta N_{598}$. In another combination ($\Delta C_{720} + \Delta N_{721}$), we duplicated amino acids 598–720. This resulted in typical CLC-1 currents, although currents were significantly lower than with the complementary pair $\Delta C_{720} + \Delta N_{721}$ (Fig. 2).

CLC-1 Can Be Split at Different Positions in the Membrane-embedded Region—We next asked whether similar complemen-
mentations might be possible if we “cut” the channel protein between TMDs and designed further constructs (Fig. 1). Several loops connecting CLC-1 transmembrane domains are short and highly conserved among CLC-0, CLC-1, and CLC-2 (especially D2-D3, D5-D6, and D6-D7), suggesting that they may be important for function. This has been confirmed by missense mutations found in human myotonia (23) and point mutations introduced in structure-function studies (9). Therefore, we focused on the large, poorly conserved extracellular loop between D8 and D9 and on the shorter, but also poorly conserved, D7-D8 and D10-D11 stretches. Further, since sequences flanking TMDs may be important for their correct membrane insertion, we decided to use slightly overlapping constructs. To ensure TMDs may be important for their correct membrane insertion, we decided to use slightly overlapping constructs. To ensure the extracellular position of the amino terminus of construct we decided to use slightly overlapping constructs. To ensure the extracellular position of the amino terminus of construct

**TABLE I**

**Comparative summary of currents**

| Construct       | Conductance | Construct       | Conductance |
|-----------------|-------------|-----------------|-------------|
| Water-injected  | 1.7 ± 0.2   | ΔC_{205} + ΔN_{210} | 63.2 ± 96   |
| WT              | 78.2 ± 9.4  | ΔC_{205} + ΔN_{206} | 1.6 ± 0.2   |
| ΔN_{109}        | 23.9 ± 6.6  | ΔC_{205} + ΔN_{206} | 40.3 ± 5.2  |
| ΔC_{205}        | 1.2 ± 0.2   | ΔC_{520} + ΔN_{509} | 1.1 ± 0.1   |
| ΔC_{206}        | 2.2 ± 0.8   | ΔC_{520} + ΔN_{509} | 5.9 ± 1.2   |
| ΔC_{520} + ΔN_{509} | 19.2 ± 1.1 |

* Despite the low absolute values, these currents were clearly recognized as typically CLC-1 by their characteristic kinetics (see Fig. 5).

**8 Is Essential for Channel Function, but an Additional D8 Is Tolerated**—We also co-injected some of the “half-channels” in different combinations. As shown in Fig. 1, this produces channels that either lack domain D8 (ΔC_{520} + ΔN_{431} + SP) or contain it twice (ΔC_{520} + ΔN_{509}). While no currents were obtained when D8 was lacking (data not shown), a duplication of this TMD was tolerated. As can be seen in Fig. 5B for ΔC_{451} + ΔN_{509}, currents were lower but did not differ qualitatively from WT.

**Membrane Domains D9–D12 Can Insert Correctly without a Signal Peptide**—When expressing ΔC_{451} together with ΔN_{413} which lacks the signal peptide added in construct ΔN_{413} + SP, one may expect nonfunctional proteins because D9 may have inserted with an inverted orientation. However, we again observed WT currents (Fig. 5C). Their size was reduced as compared with ΔN_{413} + SP (Fig. 3C). Thus, a significant proportion of ΔN_{413} proteins have inserted correctly even without an additional signal peptide.

**Carboxy-terminal “half-channels” Cannot Displace the Equivalent Part in the Full-length Protein**—As shown above, an additional transmembrane domain D8 was tolerated in split channels. This suggests that either the one in the NH2- or the COOH-terminal fragment is displaced from its normal position. We wondered whether a carboxy-terminal half-channel can replace its equivalent from the full-length channel after coexpression, which would imply that the channel structure is flexible enough to allow such a replacement after the assembly of the complete channel from the full-length protein. We expressed the CLC-1 mutant R496S (in the D9/D10 block) together with constructs ΔN_{509} or ΔN_{413} + SP. This mutation was identified in patients with recessive myotonia. It abolishes chloride currents in the physiological voltage range (25). If this part of the protein can be displaced by the nonmutated equivalent of the carboxy-terminal “half-channel,” one should be able to detect CLC-1 currents. However, coexpression of full-length CLC-1 R496S with constructs ΔN_{509} or ΔN_{413} + SP in molar ratios of 1:2 to 1:6 did not yield currents. Coexpression of WT ΔC_{520} with ΔN_{413} + SP, which duplicates the D9-D10 stretch, did not yield functional channels either (data not shown).

**Functional CLC-1 Channels Can Be Expressed from Three Separate Polypeptides**—We could obtain functional channels when “splitting” CLC-1 between TMDs D8 and D9 after residue 720 in the cytoplasmic carboxy terminus. This suggests that also the stretch extending from D9 to the end of CBS1 may fold correctly to an independent “module.” We therefore co-injected cRNA for the three constructs ΔC_{451}, ΔN_{413} + SP, ΔC_{720} and ΔN_{721} (see Fig. 1, bottom), and again observed typical CLC-1 currents (Fig. 5D). Current amplitudes

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2. M. Pusch and T. J. Jentsch, unpublished observation.
DISCUSSION

Reconstitution of membrane proteins from artificial fragments has been demonstrated in prokaryotic (26, 27) as well as eukaryotic systems. For example, the human β2-adrenergic receptor could be functionally expressed in oocytes from two separate polypeptides (28). Similar results were obtained for M2/M3 muscarinic acetylcholine receptors (29) and the anion exchanger AE1 (30). With CFTR, surprising results by Sheppard et al. (31) show that its NH2-terminal half can dimerize with itself and form Cl− channels without the COOH-terminal half. Similar truncated CFTR proteins have been described in kidney (32). This probably reflects the symmetry inherent to ABC transporters, although, with the exception of the nucleotide binding folds, the first and the second part of the protein are not highly homologous. In contrast, and not surprising given its structure, none of the CLC-1 proteins lacking TMDs could form functional channels when expressed by itself.

In many transmembrane proteins, internal topogenic sequences ensure a correct integration even if the normal translation start is absent. The folding of α-helical membrane proteins is proposed to be a two-stage process (33). First, hydrophobic domains are established in the lipid bilayer, which then interact to form the final three-dimensional structure. Thus, there seems to be no principal difference between inter- and intramolecular assembly of transmembrane domains, apart from the facilitated encounter of helices in a single polypeptide chain.

Expression of truncated proteins lacking one or more transmembrane domains may, however, result in misfolding if a certain succession of the amino acid sequence is required to achieve the correct insertion of the nascent polypeptide chain. Moreover, newly synthesized TMDs may need previously translated TMDs for “guided” folding and assembly. The successful assembly of functional proteins from individual parts suggests that these form structurally independent subdomains or “modules” whose tertiary structure does not differ too much from the one found within the native protein. Further, the intermolecular interactions between these different modules are strong enough to allow their association in the absence of a covalent link. Examples of modular structures of membrane channels and transporters are provided by some gene superfamilies: voltage-gated sodium and calcium channels have four modules covalently linked in a single polypeptide, whereas they are encoded separately in K channels; ABC transporters
can be encoded by one, two, or four genes, whose individual products will assemble and form the complete transporter (34).

In the CLC-1 chloride channel, at least three regions are structurally autonomous: an amino-terminal one comprising D1–D8, a central one containing the D9–D12 block and part of the cytoplasmic carboxyl terminus, and the rest of the hydrophilic tail, which includes D13. A cut between D10 and D11, however, was not tolerated. This suggests that either the NH2- or the COOH-terminal portion is misfolded (or both) or that interactions between these different parts are too weak to allow for their association even if folded correctly. The D9–D12 region is a broad hydrophobic region that is important for permeation and gating (17,23). In the absence of clearly separated hydrophobic domains, it seems possible that it folds correctly only if translated as a continuous polypeptide. This view is strengthened by the fact that no cleavable signal peptide was necessary to correctly express fragment ΔN998. Its intrinsic topogenic activity cannot be easily explained by the “positive-inside” rule (35–37), since there is no conspicuous charge asymmetry in this region. Exact topology is still unknown here except for the fact that the region before D9 is extracellular and the region after D12 is intracellular (8).

The CLC-1 protein could also be split between D7 and D8, but expression was less efficient than with the D8-D9 cut. The D7-D8 linker is shorter than the one connecting D8 and D9 and rather poorly conserved. However, it is always highly positively charged, and mutagenesis showed that it is important for CLC-2 gating (38). D8 is probably located at the channel periphery, since split channels functionally tolerated an extra D8 copy. Thus, the channel structure must allow for an extrusion of D8 domain into the lipid bilayer.

Channels deleted for D13 could be functionally complemented by coexpressing the lacking hydrophilic COOH-terminal portion. Similar results were recently reported for the Torpedo channel CLC-0 (39). D13 was originally identified as a region of intermediate hydrophobicity (1) and is now known to be cytoplasmic (8, 40). It is conserved in all eukaryotic CLC proteins, suggesting some important, as yet unknown function. By contrast, it is absent from the two E. coli CLC proteins. Mutations in human disease already hinted at the importance of D13 domain into the lipid bilayer.

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