Relevance of the D13 Region to the Function of the Skeletal Muscle Chloride Channel, ClC-1

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Although hydropathy analysis of the skeletal muscle chloride channel protein, ClC-1, initially predicted 13 potential membrane spanning domains (D1 to D13), later topological studies have suggested that domain D4 is extracellular and that D13, conserved in all eukaryotic ClC channels, is located within the extensive cytoplasmic tail that makes up the carboxyl terminus of the protein. We have examined the effect of deleting D13 (ΔD13) and the function of the carboxyl tail by removing the final 72 (fs923X), 100 (fs895X), 125 (L869X), 398 (N596X), and 420 (Q574X) amino acids from rat ClC-1. Appropriate cDNA constructs were prepared and expressed using the baculovirus Sf9 insect cell system. Patch clamp analysis of chloride currents in Sf9 cells showed that only relatively insubstantial changes could be attributed to the expressed fs923X, fs895X, and ΔD13 mutants compared with wild type rat ClC-1. For N596X and Q574X, however, adequate mRNA could be detected, but neither patch clamp nor polyacrylamide gel electrophoresis showed corresponding protein production. By contrast, expression of L869X was demonstrable by polyacrylamide gel electrophoresis, but no chloride conductance attributable to it could be detected. Overall, our results indicate that domain D13 is dispensable, as are the final 100 amino acids, but not the final 125 amino acids or more, of the carboxyl tail. Some essential region of unknown significance, therefore, appears to reside in the 18 amino acids after D13, from Lys877 to Arg894.

In mammalian skeletal muscle, the voltage-gated chloride channel, ClC-1, is responsible for the greater proportion of the resting conductance, which acts to stabilize the membrane potential against unwanted perturbations. Its structure, as predicted by hydropathy analysis (1) and in common with other members of the large CIC family, includes 13 relatively hydrophobic domains (termed D1 to D13), most of which probably span the membrane. There is good evidence, however, that D4 and D13 are located entirely extracellularly and intracellularly, respectively (2, 3), with the implication that close to half the amino acids of human ClC-1 (hClC-1) must form an extensive cytoplasmic tail (Gln574–Leu898). The D13 domain, which is conserved among all eukaryotic ClC channels (4), is situated in this tail, about two-thirds of the way to the carboxyl terminus (Leu840–He870).

Mutations in ClC-1 have been associated with both dominant and recessive forms of myotonia (e.g. Refs. 5 and 6) characterized by abnormal, sustained firing of action potentials that result in prolonged involuntary muscle contractions. In one (R894X) of two myotonic mutations identified in the carboxyl tail (7, 8) the final 95 amino acids of the protein (8) are lost. Functional analysis of this mutant, expressed in Xenopus oocytes, showed a large reduction in chloride currents, which could account for the myotonic symptoms (9). In similar studies (4), chloride currents were totally lost from human ClC-1 truncated at Ser720 (G721X) and at Gln597 (L598X). This suggests that normal function of the ClC-1 protein depends on the integrity of the carboxyl tail and particularly on that portion between Ser720 and Arg894, with some significant role for D13 being implied. Reconstitution experiments (4), in which the nonfunctional mutant S720X was restored to functionality by co-expression with either the complementary or an overlapping carboxyl tail peptide, have reinforced this view. Another hint of the importance of D13 is provided by mutations that truncate the kidney chloride channel, ClC-5, before or within D13, these also being nonfunctional when expressed in Xenopus oocytes (10, 11).

We have investigated the relevance of the cytoplasmic tail by generating truncation and deletion mutants of rat ClC-1, expressing them in the baculovirus Sf9 insect cell system (12), and performing functional analysis by patch clamp.

EXPERIMENTAL PROCEDURES

Mutagenesis—Rat ClC-1 cDNA (GenBank™ accession number P35524) was cloned into the vector pBacPAK 8 (CLONTECH) yielding the plasmid pDA6 (13) in which the carboxyl terminus truncation mutants Q574X, N596X, and L869X were generated using the Altered Sites System (Promega) and the mutagenic primers 5′-GAGG-GAGGTGTCACACAGCTTTG-3′, 5′-GCTGAACGTFCACACAA-GC-3′, and 5′-TAGCTCTTCTCATGACCAAGAC-3′. Convenient restriction enzyme sites were used to generate additional truncation mutants (fs923X and fs895X). Restriction of pDA6 with SacI yielded fragments of 8.28 and 0.74 kb, the larger of which was self-ligated to produce the desired mutation (fs923X). In the process, eight extraneous amino acids (RIPGRPPLN) were introduced between Ala822 and the premature termination codon. Restriction of pDA6 with XhoI and AccIII yielded fragments of sizes 6.51, 1.67, and 0.84 kb. The 6.51- and 1.67-kb fragments were ligated to produce the desired mutation (fs923X) with three extraneous amino acids (AAA) being introduced between Arg894 and the premature termination codon. Deletion of the domain D13 was performed by recombinant PCR (14) using the primers D13F (5′-TGGCCCTTTGATGAGTCTTGTG-3′) and D13R (5′-CTCATACAAGGGGCAACAAA-3′) along with Bac 1 and Bac 2 (CLONTECH) flanking primers to amplify the rClC cDNA sequence prior to D13 (up to and including Thr465) and following D13 (from and

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The abbreviations used are: hClC-1, human ClC-1; rClC-1, rat ClC-1; CBS, cystathionine β-synthase; PCR, polymerase chain reaction; RT, reverse transcription; kb, kilobase pair(s); WT, wild type.
including Lys\textsuperscript{877}, respectively. The altered cDNA (ΔD13) was cloned into pBacPAK 8 using the restriction enzymes BamHI and KpnI. All mutant constructs were confirmed by restriction digestion analysis where possible and, finally, verified by DNA sequencing. Mutation sites are indicated in Fig. 1.

**Protein Expression**—Recombinant baculoviruses containing the mutated cDNAs were produced in SF21 insect cells. Transfer of the relevant DNA insert into the viral genome was confirmed by PCR analysis using the rClC-1-specific primers Se 5 (5′-CTTGACTGTGGTGGTCTGGAG-3′) and Se 6 (5′-TCCAAAGGGACAGCTCTAGAC-3′), and viral clones were amplified, titred, and screened for protein production as described elsewhere (12, 13).

**Reverse Transcription Polymerase Chain Reaction (RT-PCR)**—Messenger RNA was extracted from cells infected with the WT, Q574X, and N596X recombinant baculoviruses. Briefly, 4 × 10^6 SF21 insect cells were infected with recombinant baculovirus with a multiplicity of infection of 20 as described previously (13). After 48 h of incubation, mRNA was extracted using the QuickPrep Micro mRNA Purification kit (Pharmacia Biotech Inc.) and reverse transcribed with the First Strand Synthesis kit (Pharmacia) to produce cDNA, which was amplified by PCR using the rClC-1-specific primers Se 3 (5′-CGAGTGAATT- GGGACAGCATC-3′) and Se 4 (5′-CTTGACTGTGGTGGTCTGGAG-3′). The RT-PCR was standardized by amplification of the cDNAs with β-actin-specific primers (15). As controls, RT-PCR was performed on mRNA extracted from uninfected cells, and to ensure that mRNA extracted from appropriately infected cells had no DNA contamination, PCR was also performed on the mRNA extract without the reverse transcription step. These samples gave no bands.

**Electrophysiology**—Cultured SF9 cells were infected with control baculovirus BVDa6.3 containing rClC cDNA or relevant mutants, incubated for 28–30 h at 28 °C in air, and then seeded onto glass coverslips and maintained at room temperature as described previously (13). Whole cell patch clamp experiments were performed using a List EPC7 patch clamp amplifier and associated standard equipment. The usual electrode solutions (16). Pentobarbitone (0.5 mM) was used to block Na-EGTA, and 10 mM HEPES (adjusted to pH 7.2 with NaOH). Borosilicate glass electrodes had a resistance of 1–3 MΩ when filled with a normal internal solution: 40 mM KCl, 120 mM potassium glutamate, 10 mM Na-EGTA, and 10 mM HEPES (adjusted to pH 7.2 with NaOH). Approximately 90% of series resistance was compensated. Nominal holding and clamping potentials reported here must be corrected for a liquid junction potential of −14 mV estimated to occur between the bath and electrode solutions (16). Pentobarbitone (0.5 mM) was used to block native anion channels in SF9 cells. Experiments were conducted at room temperatures of 24 ± 1 °C. Data were collected, filtered at 3 kHz, and analyzed on an IBM-compatible PC using pCLAMP v6.0 software (Axon Instruments). Parameters such as time constants, apparent open probability, and IC\textsubscript{50} were determined as described previously (16, 17).

**RESULTS**

Analysis of the protein content of SF21 cells infected with the appropriate baculovirus construct indicated that the fs923X, fs895X, L869X, and ΔD13 recombinant rClC-1 proteins were being expressed and could readily be detected on Coomassie Blue-stained polyacrylamide gels (see Ref. 13), whereas similar rClC-1-specific bands could not be detected for the N596X and Q574X mutations (data not shown). Evidence that transcription of the N596X and Q574X constructs had occurred, however, was provided by RT-PCR analysis of mRNA extracted from appropriately infected cells, which gave the expected prominent band in each case (data not shown).

When expressed in SF9 cells, fs923X, fs895X, and ΔD13 mutations did not change such basic properties of rClC-1 as the pronounced inward rectification of instantaneous currents, saturation of outward currents at positive potentials, deactivation of inward currents at negative potentials, and the voltage dependence of apparent open probability. Kinetics of the inward current deactivation, however, were slower in the mutants (Fig. 2). Of the two time constants that can typically be extracted from the deactivating currents (16), the slow time constant, \( \tau_2 \), was almost doubled (significant by two-way analysis of variance, \( p < 0.0001 \)) in the fs895X and ΔD13 mutants with respect to WT, whereas the fast time constant, \( \tau_1 \), was not significantly altered. For currents in response to a voltage step to −120 mV these were: WT \( \tau_1 = 5.8 \pm 0.2 \text{ ms, } n = 21 \) and \( \tau_2 = 26.1 \pm 1.4 \text{ ms, } n = 20 \); fs895X \( \tau_1 = 6.9 \pm 0.3 \text{ ms, } n = 13 \) and \( \tau_2 = 45 \pm 2.7 \text{ ms, } n = 13 \); ΔD13 \( \tau_1 = 7.1 \pm 1.3 \text{ ms, } n = 4 \) and \( \tau_2 = 46.1 \pm 3.5 \text{ ms, } n = 4 \) (results expressed as mean ± S.E.). Kinetics of the fs923X mutant was closer to WT. Cells infected with the L869X, N596X, or Q574X recombinant baculoviruses failed to produce a chloride conductance attributable to rClC-1 (data not shown).

Block of the rClC-1 channel by Cd\textsuperscript{2+} is sensitive to mutation, e.g. the R304E mutant (17). When we tested our present mutants, Cd\textsuperscript{2+} was as effective on ΔD13 as on the WT channel (IC\textsubscript{50} = 1.7 ± 0.1 mM (\( n = 3 \)) for ΔD13 and 1.0 ± 0.1 mM (\( n = 3 \)) for WT), whereas fs895X required higher concentrations of Cd\textsuperscript{2+} to achieve the same level of block (IC\textsubscript{50} = 2.7 ± 0.2 mM, \( n = 5 \)).

**DISCUSSION**

There is no doubt from our present study and from previous work (4) that truncations of the carboxyl tail of the protein by 125 or more amino acids eliminate the chloride currents attributable to ClC-1 in SF9 cell and Xenopus oocyte expression systems. Clearly, without detectable protein products, there seems to be a failure of effective translation of the rClC-1 mutants Q574X and N596X in our expression system. A similar explanation, implying some requirement for the integrity of the mRNA code for the proximal part of the carboxyl tail, might apply to the inability to detect appropriate chloride currents in

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**Fig. 1.** Topological diagram of ClC-1 with location of carboxyl tail mutations. Amino acid numbering is as for rClC-1. From Gly\textsuperscript{270} and beyond in the carboxyl tail of rClC-1, the numbering of hClC-1 differs. Where relevant hClC-1 mutations have been discussed in the text, their locations are given in parentheses, e.g. (h-S720) signifies the Ser\textsuperscript{720} mutation in hClC-1, which corresponds to Ser\textsuperscript{717} in rClC-1.
Relevance of the D13 Region to ClC-1 Function

FIG. 2. Characteristics of chloride currents through ClC-1 mutants. Deactivating inward currents and saturating outward currents are shown for whole cell patch clamped SF9 cells expressing WT, fs923X, ΔD13, and fs895X mutants.Quenched step voltage steps of +20 mV from –140 to +80 mV were applied from a holding potential of –30 mV. Following each test pulse, voltage steps to –100 mV elicited tail currents. Their initial magnitude gives a measure of apparent open probability at that time. For ease of comparison, currents in each panel have been normalized to peak inward current at –140 mV.

Xenopus oocytes when the carboxyl tail of hClC-1 had been severely truncated by the mutation L598X (4). Function, in this case, was unable to be restored by co-expression with the complementary carboxyl tail peptide (4). Both more severely and less severely truncated versions of hClC-1, however, such as L391X, P452X, and G721X, have been expressed and correctly inserted into the membrane because their function was restorable with appropriate complementation (4). By contrast, fully functional, although kinetically slower, versions of rClC-1 are expressed and inserted into the membrane of Sf9 cells, despite lacking the D13 domain or the final 100 amino acids of the carboxyl tail.

These results are consistent with previous observations of the naturally occurring myotonic mutant of human ClC-1, R894X (8), and of ClC-5 mutants associated with Dent’s disease (10, 11). The conclusions from these earlier studies have, however, implicated D13 as an essential functional region (4). This view has received support from the recent identification of a structural motif, conserved across archaea, bacteria, and eu- karya, the so-called CBS (cystathionine ß-synthase) domain (18). This motif is present in tandem (denoted CBS1 and CBS2) in all eukaryotic ClC proteins (19) with CBS1 located between D12 and D13 and CBS2 overlapping D13, extending from Cys925 to Lys977 in rClC-1 (Fig. 3). A high level of conservation frequently indicates physiological importance, but our present results are inconsistent with any obvious function for D13 or the CBS2 domain in which it lies. In our quite functional ΔD13 mutant, all of D13 and, simultaneously, the final two-thirds of CBS2 have been eliminated.

Although the absence of any evidence of function in ClC-1 truncated by 125 or more amino acids could be fortuitous, a more likely interpretation that encompasses both the earlier observations of others and our new results with the ΔD13 mutant is that there is some essential region of presently unknown significance contained within the immediate post-D13 region. An argument that the region of functional significance might lie prior to D13 but after Ser717 could only be entertained if our L869X mutant, which is expressed, failed to be correctly inserted into the membrane. We have no proof of correct insertion, but note that wherever there has been evidence of expression of ClC-1, there has been concurrent evidence of correct insertion for both shorter and longer truncation mutants (Ref. 4 and present results).

Coincident with the diminished status of D13 and CBS2, then, it is the region immediately beyond these domains (from Lys977 to Arg988) that takes on an unexpected prominence. Of these 18 amino acids, the final 7 (PLLASFR) are identical in ClC-0, ClC-1, and ClC-2, and mutations around Arg894 (Arg888 in hClC-1) are known to modify ClC-1 function modestly, as in our fs895X mutant, or substantially, as in the myotonic goat (corresponding to A885P in hClC-1) (7) and human R894X (8) mutants. The seeming discrepancy between our fs895X mutant and the more severe effects of the human R894X might be explained by dramatic differences in the reactivity of their terminal sequences, RAAA and RNTTST (RNTTSI in rClC-1; Fig. 3), respectively. Lack of homology with the post-D13 area of ClC-5 makes it unlikely that the D13 truncation mutants of ClC-5 lose function in the same way as similar mutants of ClC-1 (Fig. 3).

Finally, our results with Cd2+ block, which occurs only when Cd2+ is applied to the extracellular side of the channel (17), suggest that the immediate post-D13 region is structurally in close proximity to the channel pore or that very long range conformational changes originating in this distant region can influence the extracellular site of Cd2+ binding.

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