Formation of CLC-0 Chloride Channels from Separated Transmembrane and Cytoplasmic Domains

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ABSTRACT: CLC-0, a member of the CLC family of Cl^--conducting ion channels, consists of an N-terminal hydrophobic core and a C-terminal region that is thought to be cytoplasmic. This study provides evidence that the C-terminal region is a mechanistically relevant cytoplasmic domain of the CLC-0 ion channel. Both a point mutation and a 37-residue deletion in this region cause drastic alterations in voltage-dependent gating of CLC-0 current expressed in *Xenopus* oocytes. CLC-0 current is not observed when the entire C-terminal region is deleted, but functional channels are efficiently reconstituted by co-injection of separate cRNA constructs encoding the N-terminal transmembrane and the C-terminal cytoplasmic domains. Moreover, reconstitution of CLC-0 can be achieved by co-injection of cRNA encoding the transmembrane domain along with *Escherichia coli*-expressed C-terminal domain polypeptide.

The CLC channels constitute a large and growing family of Cl^--selective ion channels that display great diversity in functional characteristics, tissue specificity, and physiological purpose. The "muscle-type" channels, CLC-0 and CLC-1, are known to be responsible for the high Cl^- conductance, and hence resting excitability, of vertebrate skeletal muscle and related tissues (*1*−*5*). Other CLC channels are found abundantly in central neurons (*6*−*8*), where CLC-2 is crucial for determining whether the GABA response is inhibitory or excitatory (*9, 10*). CLC channels are conjectured to be centrally involved in salt and water transport in epithelia (*11*), a speculation bolstered by the discovery that at least three forms of familial human kidney stone disease arise from disruption of the CLC-5 homologue (*12*).

The ubiquity of the CLC channels makes them an enticing target for study. Compared with the voltage- and ligand-gated families of cation-conducting channels which boast P-regions, voltage-actuated S4s, and mapped toxin-interaction sites, the CLC channels are uncharted; only a handful of scattered amino acid residues have been implicated specifically in channel function (*13*−*18*). Moreover, it appears that the molecular lessons learned about cation-conducting channels will be of little use in guiding us toward a picture of CLC channels, which exhibit unprecedented features of both molecular architecture and gating mechanism. CLC-0, a muscle-type CLC channel from Torpedo electric rays, is known to be constructed not by the conventional "barrel-stave" arrangement of other eukaryotic ion channels, but rather as a two-pore two-polypeptide homodimer (*19, 20*). Likewise, the gating of CLC-0 is unusual in that it is intrinsically coupled to Cl^- movement through the channel (*21*), a process that gives rise to the channel's voltage dependence (*17, 22*). These unfamiliar aspects of CLC channels motivate our efforts toward a structural understanding of CLC-0, the only member of the CLC family that has been amenable to study by all four approaches in the channel structure-and-function arsenal: cellular electrophysiology, heterologous expression, protein biochemistry, and single-channel analysis.

The present map of CLC transmembrane topology, based on hydropathy analysis buttressed by some experimental support (*2, 23, 24*), identifies 10−12 transmembrane segments and places the last 290 (of 805) amino acid residues of CLC-0 on the cytoplasmic side of the membrane. It is natural to wonder whether these last residues might fold into a globular, water-soluble domain that would be amenable to direct structural study. To identify possible globular domains, we employed a "split-channel" strategy. This assay is based on the assumption that separated pieces of the protein may associate to form functional channels, while pieces of protein that cannot fold correctly will not. N- and C-terminal portions of CLC-0 were coexpressed, in the hope of discovering cleavage points that allow the production of functionally active Cl^- channels.

MATERIALS AND METHODS

Recombinant DNA Methods. All constructs were generated by standard PCR mutagenesis. After digestion with the appropriate restriction enzymes, the PCR products were subcloned into a pBluescript KS+ vector containing CLC-0 (*20*). To generate N-terminal CLC-0 constructs, each mutagenic oligonucleotide listed below was used to insert a stop codon followed by an *Nco*I site (5′ *TAA*GCCATGG 3′); the stop codon (in italics) replaced the indicated amino acid residue: Y556, 3′ CGGAGGTGGAGGTCG*ATT*CGGTAC-CGTCTGGACGAGGAT 5′; Q565, 3′ CGAGGTGCAC-GACTCT*ATT*CGGTACCGGTTCAAGAAGGGGAAGC 5′; E592, 5′ CGATCGAACTGAGGTC*TAA*GCCATGGACT-TGCTCCAGAGGC 3′; P606, 5′ GCGTACCGGCGCCAG-*TAA*GCCATGGCAGCGGAGGCGG 3′; A641, 5′ GAC-CAGGAGGAC*TAA*GCCATGGAGCAGAGAGAGGGGC 3′; E650, 5′ AGAGAGGGGCTG*TAA*GCCATGGAGGTCCA-

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GACCG 3′; G675, 5′ CCAACTCAGACTTCA*TAAG*C-CATGGCCTACCAGAAAAAACAAAAAGGG 3′.

C-terminal CLC-0 constructs were generated by subcloning the *Nco*I/*Eco*RI fragments of each N-terminal construct into a pBluescript KS+ vector that had been modified to contain an *Nco*I site. Each C-terminal construct starts 3−5 residues after the last residue of the corresponding N-terminal construct. The *Nco*I site introduces a glutamate or alanine as the second residue of the C-terminal polypeptide. For example, the construct $CDORF_{677}$[1] has the sequence Met-Ala, followed by residues 677−805 of CLC-0. A hexahistidine tag (along with a serine residue) was appended to the N-terminus of the construct $CDORF_{652}$ by ligating phosphorylated and annealed oligonucleotides into the *Nco*I site (bases added, ATGCATCATCATCATCATCATTCC). The mutation A783P was generated using the mutagenic primer (5′ CCCTCCCCCTCTGCCGAGTTTCCGAGACG 3′) and subcloned into both the full-length CLC-0 and the $CDORF_{644}$ vectors. Δ769−805 was generated using (3′ CGTCTCTAG-GTCCGTCGGTAG*ATT*ATCCTTAAGCCAGCTCCT 5′) to introduce a stop codon at E769 followed by an *Eco*RI site. All constructs were confirmed by sequencing through the insertion sites.

C-terminal CLC-0 constructs were subcloned into the *Escherichia coli* expression vector pCSP105 (*25*) that had been modified by two bases to produce a unique *Nco*I site at the start site (pCSP107); *Nco*I/*Eco*RI fragments of the C-terminal constructs were ligated along with an *Eco*RI/*Hin*dIII linker into *Nco*I/*Hin*dIII-digested pCSP107.

Electrophysiology. cRNA was prepared using T3 RNA polymerase (Promega) after linearization with *Fsp*I. *Xenopus* oocytes were co-injected with 5 ng each of the N- and C-terminal CLC-0 cRNAs to test for expression of split channels. Injection of 0.08 ng (wild-type CLC-0), 0.5 ng (NTMD/$CDORF_{H652}$; A783P), or 5 ng (Δ769−805) of cRNA typically resulted in 1−15 μA of current after ∼2 days of expression. The $CDORF_{H652}$ protein was injected in combination with 5 ng of the N-terminal cRNA; in most experiments, protein was injected 2 days after injection of the cRNA.

Currents were recorded by two-electrode voltage clamp. Bath solution was 96 mM NaCl, 2 mM KCl, 0.3 mM $CaCl_2$, 1 or 8 mM $MgCl_2$, and 10 mM Tris (adjusted to pH 7.6 with HCl) or 5 mM Hepes (adjusted to pH 7.6 with NaOH). The higher concentration of $MgCl_2$ was used with some sets of oocytes to suppress endogenous currents. Electrode pipets (0.2−0.6 MΩ) were filled with 3 M KCl, 5 mM EGTA, and 10 mM Hepes or 10 mM Tris, pH 7.6. For analysis of tail currents, slow inactivation (*26, 27*) was first removed with a series of −110 mV hyperpolarizing pulses (0.5−2 s in duration) until the current observed in a 50-ms test pulse to 50 mV was maximal (after about 10−20 s of hyperpolarization). Oocytes were voltage-clamped at a holding potential of −65 mV during subsequent recording of currents.

For inhibition of translation or of transport to the plasma membrane, oocytes were transferred to a bath containing 1 μg/mL cycloheximide or 5 μg/mL brefeldin A (BFA), respectively, in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM Hepes, pH 7.5). C-Terminal protein or cRNA was injected 20−30 min after transfer of the oocytes.

Data Analysis. Because the CLC-0 channel does not close completely, even at extremely hyperpolarizing voltages (*22*), the nonspecific "leak" in an oocyte expressing CLC-0 is not readily quantified. Therefore, we compared voltage-dependent gating of the wild-type and split CLC-0 currents without leak subtraction. Normalized currents observed during −150 mV tail pulses were fitted to an equation of the form

$$I/I_{max} = A + (1 - A)/[1 + \exp(-zF(V - V_0)/RT)] \quad (1)$$

Currents 3.9 ms into the tail pulse were used for the analysis; at this time, little of the CLC-0 current had decayed, and interference from the capacitative transient was avoided.

Biochemical Methods. Each C-terminal construct was expressed in *E. coli* BL21(DE3) cells as previously described (*25*) except that cells were washed and resuspended with buffer A (50 mM $NaPO_4$, 300 mM NaCl, and 5 mM 2-ME, pH 7.9) and the sonicated extract was incubated for 15 min on ice with 5 μg/mL DNase and 10 μg/mL RNase immediately before centrifugation at 85000−100000*g* for 45 min. In some experiments, 1% Triton X-100 was also added before centrifugation.

For preparation of $CDORF_{H652}$ from the aqueous extract, cells were grown at 22 °C. The lower temperature led to decreased overall expression of $CDORF_{H652}$ but increased the amount of protein in the supernate after high-speed centrifugation. Cells harvested from a 1 L culture were washed with 75 mL of buffer A and resuspended with 10 mL of 40 mM imidazole in buffer A. The supernate was incubated overnight with 2.5 mL of Ni-NTA agarose (Qiagen) equilibrated with 40 mM imidazole in buffer A. The matrix was loaded onto a column and washed with 40 mM imidazole in buffer A until an OD_{280} < 0.02 was observed. Protein was eluted stepwise with 2 mL each of 0.15, 0.25, 0.35, and 0.5 M imidazole in buffer A (pH 7.9). The CDORF protein eluted in the 0.25 and 0.35 M fractions, the latter being freer of contaminating proteins. All steps were carried out at 4 °C.

For preparation of inclusion bodies (from 1.3 L of cells grown at 37 °C), disrupted cells were centrifuged at 11000*g* for 30 min. The pellet was washed twice with 25 mL of buffer B (30% sucrose, 1% Triton X-100, 5 mM EDTA, and 5 mM DTT, pH 7.0) and then once with buffer C (50 mM NaCl, 5 mM 2-ME, and 10 mM Tris-HCl, pH 8.0). The final pellet was resuspended with 50 mL buffer C and stored in aliquots at −80 °C. For denaturing purification, pelleted inclusion bodies were dissolved with buffer G, pH 7.0 [3 M guanidinium chloride (GnCl), 7 mM 2-ME, and 50 mM $NaPO_4$, 1 mL of buffer G/mL of suspension]. The solution containing the dissolved inclusion bodies was incubated with Ni−NTA agarose (200 μL agarose/mL of solution) overnight at room temperature. The matrix was loaded into a column, washed with buffer G (pH 7.0) and then buffer G (pH 6.0) until the OD_{280} of the eluate was <0.02, and then eluted with buffer G (pH 4.0).

The GnCl-solubilized $CDORF_{H652}$ was refolded by 100-fold dilution into 20 mM sodium acetate (pH 4.0 with HCl) and concentrated using Amicon 30 concentrators. Insoluble material was removed by centrifugation at 85000*g* for 45

[1] Abbreviations: BFA, brefeldin A; GnCl, guanidinium chloride; NTMD, N-terminal transmembrane domain; CDORF, C-terminal domain that restores function.

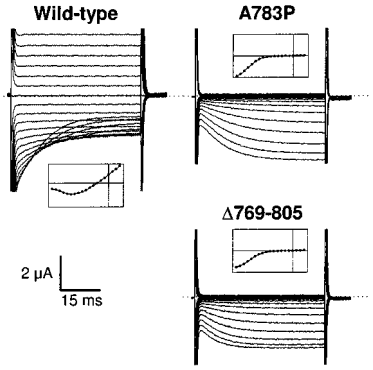

FIGURE 1: Effects of mutations in the C-terminal region of CLC-0 on current. Representative currents from oocytes injected with wild-type, A783P, or Δ769−805 CLC-0 cRNA. Each family of currents was collected with 50-ms voltage pulses from −150 to +30 mV in 10 mV increments. Holding potential was −30 mV for wild-type and −35 mV for the mutants. Dashed lines represents zero-current level. Insets: steady-state current vs voltage plots. Voltage axis range is −160 to +40 mV; current axis range is −4 to +4 μA.

min. Protein concentrations were determined with the Coomassie Plus protein assay (Pierce), using BSA as the standard.

Standard procedures were used for SDS−PAGE and Western blotting, using 15% polyacrylamide gels. For Western blots, a mouse monoclonal antibody against the C-terminus of CLC-0 was used for the primary incubation. The secondary incubation was performed with HRP- or AP-conjugated goat anti-mouse antibody (Promega) followed by either ECL (Amersham) or NBT/BCIP (Promega) detection, respectively.

RESULTS

Function of the C-Terminal Region of CLC-0. A functional role for the C-terminal region of CLC channels was first suggested by studies on the myotonic goat; the muscle-type CLC-1 channels of these goats contain a naturally occurring point mutation that leads to myotonia by modifying voltage-dependent gating (*13*). We made the corresponding mutation, A783P, in CLC-0, and observed extreme effects on channel gating (Figure 1). A distinguishing feature of CLC-0, millisecond time scale closing in response to hyperpolarizing voltage pulses, is replaced in this mutant by a current that *activates* at these voltages. This remarkable phenotype, while different from that elicited by the same mutation in CLC-1, has also been observed in another myotonia-causing mutation of CLC-1 (*14*) and in numerous point mutations throughout CLC-0 (*18*). This mutated CLC-0 channel retains the Cl^- selectivity and inactivation gating of CLC-0. A similar alteration in gating was also produced by a 37-residue C-terminal deletion that includes A783 (Δ769−805) (Figure 1). We have no idea of the mechanism by which the channel's voltage dependence reverses in polarity in response to these mutational affronts; however, the dramatic change argues that the C-terminal domain participates as an integral determinant of the channel's gating mechanism, and hence structure.

Expression of Split Channels. The above experiments suggest that the C-terminal domain is functionally relevant and thus interesting as an object of study. To search for C-terminal segments that might form independent folding domains, we adopted a split-channel strategy. We designed separate cDNA constructs for the N-terminal transmembrane domain (NTMD) of CLC-0 and for the remaining C-terminal part of the sequence. Using *Xenopus* oocytes under voltage clamp, we tested seven such pairs of these split channels (transcribed into cRNA) with cleavage points engineered at various test-positions after the NTMD (Figure 2A). When injected alone, no single cRNA induced expression of CLC-0 current; oocytes injected with these individual pieces of CLC-0 were always electrically indistinguishable from water-injected oocytes. When co-injected, however, five of these cRNA pairs induced robust expression of current that was similar to wild-type CLC-0, while the other two failed to produce measurable Cl^- current (Figure 2B). Additionally, we introduced the mutation A783P into a C-terminal construct; when co-injected along with the appropriate NTMD, the mutated C-terminal cRNA induced current similar to that of full-length A783P CLC-0 (Figure 2B).

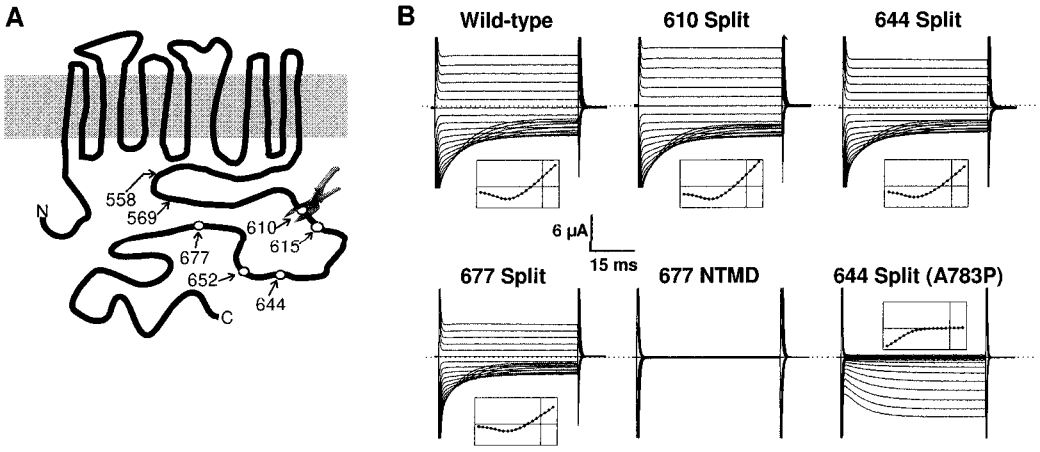

FIGURE 2: Expression of split CLC-0 channels. (A) Topological cartoon of CLC-0. Positions at which CLC-0 was split are indicated by an arrow and a number. Open circles indicate cleavage positions that allow expression of CLC-0 current in oocytes in co-injection experiments. (B) Representative currents from oocytes injected with wild-type CLC-0 cRNA, co-injected with pairs of split CLC-0 cRNA, or injected with only the N-terminal (NTMD) cRNA. Construction of the split pairs led to the loss of 2−4 amino acid residues of CLC-0 (see Materials and Methods). Split pairs are named according to the first CLC-0 residue of the C-terminal piece; therefore, "677 NTMD" corresponds to residues 1−674 of CLC-0. Oocytes were clamped at −30 mV and examined as in Figure 1. Insets: steady-state current vs voltage plots. Voltage axis range is −160 to +40 mV; current axis range is −8 to +10 μA.

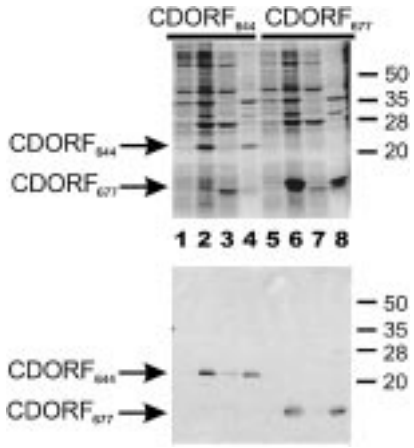

FIGURE 3: Expression and solubility of CDORFs. $CDORF_{644}$ (lanes 1−4) or $CDORF_{677}$ (lanes 5−8) was expressed at 37 °C, and samples were run on SDS−polyacrylamide gels. Lanes 1, 5: uninduced *E. coli*. Lanes 2, 6: induced *E. coli*. Lanes 3, 7: supernate after high-speed centrifugation. Lanes 4, 8: pellet after high-speed centrifugation. Upper panel: Protein was stained with Coomassie Blue. All lanes contained the equivalent of 310 μL of each original culture. Lower panel: Protein was transferred to nitrocellulose and Western blotted using NBT/BCIP detection. All lanes contained the equivalent of 31 μL of each original culture.

These experiments show that the separately-expressed transmembrane and cytoplasmic pieces of the channel protein, each of which alone fails to express Cl^- currents, associate to form CLC-0.

Is the C-terminal fragment of CLC-0 able to fold independently into a water-soluble, globular domain? To address this question, we attempted to express in *E. coli* the C-terminal domains that restore function (CDORFs), as determined by the cRNA co-injection experiments above. Two of these proteins ($CDORF_{644}$ and $CDORF_{677}$) were expressed at high levels, while three others ($CDORF_{610}$, $CDORF_{615}$, and $CDORF_{652}$) were only weakly expressed, as detected on Coomassie-stained SDS−PAGE gels. (The CDORF subscript refers to the position at which CLC-0 is split.) The two highly-expressed proteins were found mainly in the insoluble fraction (Figure 3), as was also the case for $CDORF_{652}$ (data not shown). Since this protein cannot be solubilized by mild detergent (1% Triton X-100), we conclude that these CDORFs are found primarily in inclusion bodies rather than in the bacterial membrane fraction.

Not all the CDORF, however, was located in inclusion bodies; a small amount of the protein could be detected in the aqueous fraction (Figure 3). To facilitate purification and analysis of this material, we added a hexahistidine tag to one of these constructs, $CDORF_{652}$, to form $CDORF_{H652}$. Residue 652 presents itself as a potentially forgiving location at which to begin synthesis of the C-terminal domain; this residue lies in the middle of a surface-exposed epitope (*23*) that is not conserved among even the most closely-related CLC channels. Serendipitously, the hexahistidine tag in $CDORF_{H652}$ led to an enormous increase in expression level (∼100 mg/L of culture). Although still mostly expressed into inclusion bodies, the small amount of $CDORF_{H652}$ in the aqueous fraction could be partially purified using a Ni^{2+}-affinity column (Figure 4A,B).

This partially purified domain displayed functional activity. Oocytes injected with cRNA encoding the NTMD along with partially purified $CDORF_{H652}$ protein readily expressed

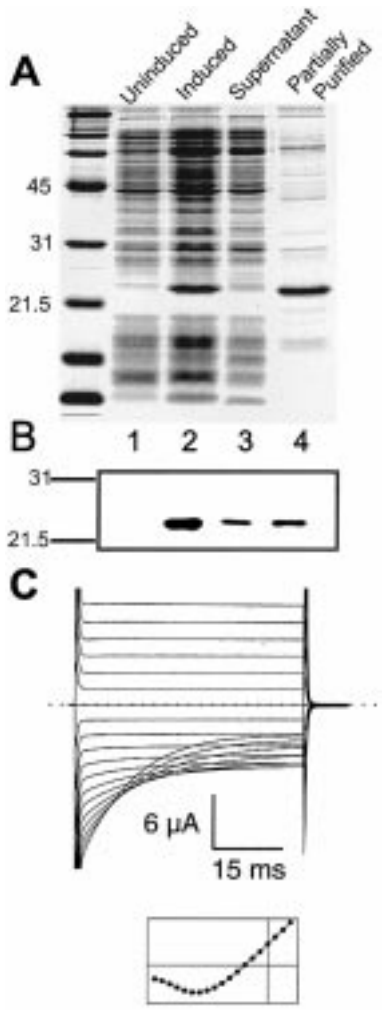

FIGURE 4: Expression, purification, and activity of aqueous $CDORF_{H652}$. (A) Coomassie-stained gel. $CDORF_{H652}$ was expressed at 22 °C. Lane 1, uninduced *E. coli*; lane 2, induced *E. coli*; lane 3, supernate after high-speed centrifugation; lane 4, 20 μL of the elution from the Ni^{2+}-affinity column. Lanes 1−3 contained the equivalent of 313 μL of the original *E. coli* culture. (B) Proteins were transferred onto nitrocellulose and followed by Western blotting with ECL detection. Lanes 1, 2, equivalent of 10 μL of uninduced and induced *E. coli* culture, respectively; lane 3, 0.2 μL (equivalent to 20 μL of culture) supernate after high-speed centrifugation; lane 4, 0.2 μL of the elution from the Ni^{2+}-affinity column. (C) Representative currents from an oocyte co-injected with cRNA encoding the NTMD (residues 1−639 of CLC-0) and with $CDORF_{H652}$ protein eluted from the Ni^{2+}-affinity column. The oocyte was voltage clamped as described in Figure 1B (−30 mV holding potential).

CLC-0 current (Figure 4C). Oocytes injected only with $CDORF_{H652}$ protein failed to express CLC-0, although a few sets of such oocytes exhibited small leak currents (range of 0.19−0.55 μA at 30 mV) that were at most 20% of the magnitude of the CLC-0 current observed in co-injected cells (range of 2.6−11.1 μA). In "mock-purification" controls, we also co-injected NTMD cRNA along with Ni^{2+} column eluates from *E. coli* expressing an unrelated protein, charybdotoxin, which does not carry a hexahistidine tag; the lack of current in these experiments rules out a bacterial contaminant as the active component of our aqueous $CDORF_{H652}$ preparations.

Since aqueous $CDORF_{H652}$ was only a minor fraction of the expressed protein and could not be readily purified to homogeneity, we attempted to solubilize, purify, and refold

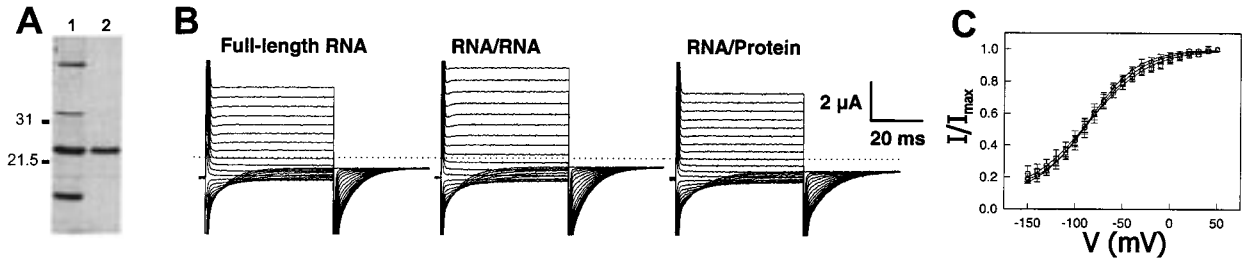

FIGURE 5: $CDORF_{H652}$ refolded from inclusion bodies. (A) Coomassie-stained SDS−polyacrylamide gel. Lane 1, GnCl-solubilized inclusion bodies; lane 2, $CDORF_{H652}$ purified on Ni-NTA in the presence of GnCl and eluted with low pH, as described in Materials and Methods. (B) Representative tail currents. Left: oocyte injected with cRNA encoding wild-type CLC-0. Center: oocyte co-injected with cRNAs encoding the NTMD and $CDORF_{H652}$. Right: oocyte co-injected with cRNA encoding the NTMD and with $CDORF_{H652}$ protein purified and refolded from inclusion bodies. Families of currents were collected with 50-ms voltage pulses (−150 to +50 mV in 10 mV increments) that were each followed by a 50-ms tail pulse to −150 mV. The holding potential was −65 mV. (C) Normalized tail currents for oocytes expressing full-length CLC-0 (squares), split CLC-0 expressed from both cRNAs (circles), and split CLC-0 expressed from NTMD cRNA and $CDORF_{H652}$ protein (triangles). Means ± SD of measurements from 10 oocytes for each data set are shown. Solid curves are drawn according to eq 1, with the following parameters: squares, $A = 0.12$, $V_0 = -85$ mV, and $z = 1.0$; circles, $A = 0.09$, $V_0 = -86$ mV, and $z = 0.8$; triangles, $A = 0.08$, $V_0 = -86$ mV, and $z = 0.9$.

the abundant $CDORF_{H652}$ from inclusion bodies, where it was the major protein present (Figure 5A). We found that the protein is easily solubilized in 3 M GnCl, and that upon 100-fold dilution into a refolding-medium at pH 4.0, >90% of the protein remains in the aqueous supernate after high-speed centrifugation. Moreover, the protein could be purified easily by Ni^{2+}-affinity chromatography in the presence of GnCl and then refolded. Oocytes injected with the refolded protein alone were electrophysiologically identical with uninjected oocytes. When refolded $CDORF_{H652}$ protein (either crude or purified) was co-injected with N-terminal domain cRNA, we observed voltage-dependent Cl^- current quantitatively indistinguishable from that elicited by injection of full-length CLC-0 cRNA or by co-injection of cRNAs encoding the N-terminal and C-terminal domains of CLC-0; we found no significant differences in voltage-dependent activation gating, as gauged by tail-current analysis among these various CLC-0 constructs (Figure 5 B,C). We note that the specific activity of refolded CDORF is roughly equivalent in magnitude to that of the soluble protein. In one set of oocytes, injection of 7 ng (total protein) of refolded (unpurified) $CDORF_{H652}$ yielded 14 ± 1 μA of CLC-0 current at 50 mV; in the same set, an injection of 3 ng of the partially-purified soluble material produced 6 ± 2 μA ($n = 3$ in both sets; current measured 27 h after injection of protein).

$CDORF_{H652}$ can associate with pre-existing NTMD. If the injection of $CDORF_{H652}$ cRNA was delayed for 2 days after injection of NTMD cRNA, subsequent expression of Cl^- current occurred more rapidly than in oocytes that were injected simultaneously with the two components of the split channel (Figure 6A). To test the idea that $CDORF_{H652}$ may associate with previously-synthesized NTMD, we injected oocytes with NTMD cRNA, and then 2 days later we inhibited further translation with cycloheximide. Subsequent injection of $CDORF_{H652}$ protein into these oocytes produced CLC-0 current. The failure of the corresponding cRNA to produce current demonstrates that the cycloheximide protocol effectively inhibits translation (Figure 6B). Thus, active CLC-0 may be formed post-translationally from its two pieces.

These experiments lead to a question. Does the pre-existing NTMD reside in the plasma membrane where it waits for $CDORF_{H652}$? To test this possibility, we injected

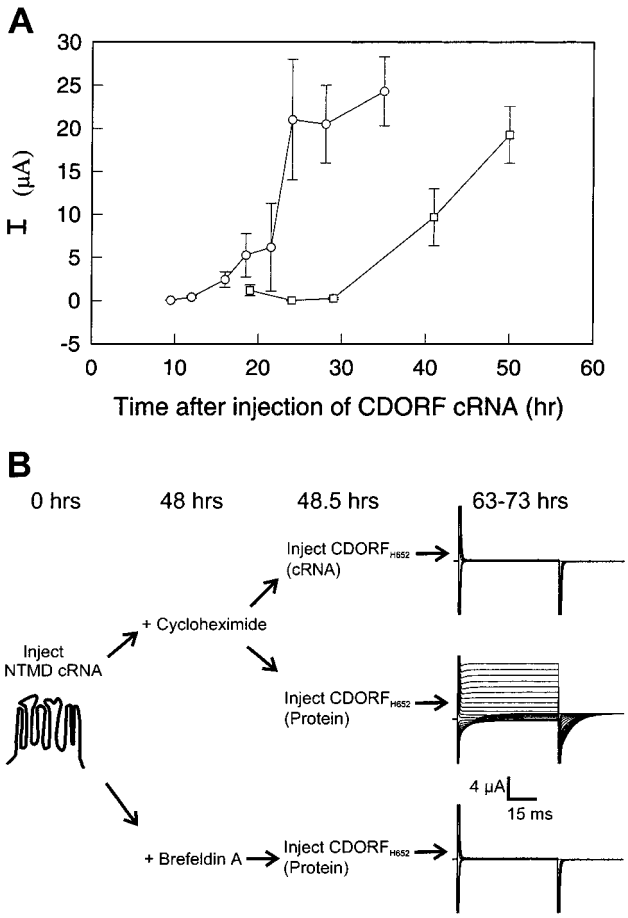

FIGURE 6: Association of $CDORF_{H652}$ with pre-existing transmembrane domain. (A) Time course of expression of split CLC-0. Oocytes were injected with cRNA encoding the NTMD. cRNA encoding $CDORF_{H652}$ was injected either simultaneously (squares) or after 2 days (circles), and current was measured at +10 mV (mean ± SE, 3−14 oocytes). (B) Post-translational expression of CLC-0 current. Oocytes were injected with cRNA encoding the NTMD and incubated at 18 °C for 2 days. The oocytes were then transferred to solutions containing either cycloheximide or brefeldin A prior to injection of $CDORF_{H652}$ (either as cRNA or protein refolded from inclusion bodies). Oocytes were examined by two-electrode voltage clamp as in Figure 5B.

oocytes with cRNA encoding the NTMD, allowed 2 days for expression of the membrane-associated domain, and then inhibited plasma membrane trafficking with BFA (*28*, *29*).

Under these conditions, injection of $CDORF_{H652}$ protein failed to induce Cl^- current (Figure 6B). This result suggests that the NTMD does not exist stably in the plasma membrane; it is also possible, however, that the NTMD does exist, silently, in the plasma membrane, but that it and the CDORF can be assembled only at a location prior to translocation to the plasma membrane. In any case, the experiment shows that association of the two pieces of the split channel to form a functional complex occurs before final insertion into the plasma membrane.

Biochemical Characterization of $CDORF_{H652}$. We attempted to examine the biochemical characteristics of $CDORF_{652}$ from the inclusion bodies, which could be obtained in larger amounts and in purer form than that from the aqueous extract. All attempts to refold and concentrate the protein at neutral pH led to massive precipitation, but lowering the pH to 4.0 permitted refolding, and the protein remained in the supernate at ~5 mg/mL after centrifugation at 85000*g*. When analyzed by gel filtration chromatography, however, all the material eluted in the void volume (exclusion limit 2×10^6 molecular weight) of a Superose 12 column (data not shown); thus, this CDORF construct forms large aggregates.

DISCUSSION

We have shown that the C-terminal region of CLC-0 is involved intimately in the gating of CLC channels, as indicated by the reversal of voltage dependence by sequence manipulation in this region. The mechanism by which this change occurs is completely unknown, but the idea of reversal of voltage dependence is not intrinsically preposterous for this ion channel. Normal depolarization-activated gating of wild-type CLC-0 is known to reflect the net result of *two* kinetic processes with opposite voltage dependencies (*22*); if a mutation specifically eliminates the dominant depolarization-activated process, hyperpolarization-activated gating would be observed.

The CLC-0 Cl^- channel can be split at various positions after the last putative transmembrane domain and functionally expressed in *Xenopus* oocytes by assembly of the two pieces. The functional expression of a split protein is not by itself remarkable. Indeed, numerous other integral membrane proteins have been shown to be functional when split and coexpressed, although in most or all of these cases the cleavage points were made between putative transmembrane segments (*30*−*40*). Instances also abound in which globular, water-soluble proteins retain function when split (*41*−*49*). What is notable about the present studies is that functionally normal CLC-0 channels may be reconstituted by co-injection of cRNA encoding the NTMD and a purified 150-residue polypeptide corresponding to the C-terminal sequence. The formation of active Cl^- channels in this coexpression assay can be attributed directly to the injected protein because channels appear even in the absence of protein synthesis by the oocyte.

This result supports a model in which the C-terminal region of CLC channels is entirely cytoplasmic. Such a model was originally proposed when the CLC-0 sequence was determined (*2*) and has been sustained by experimental results over the past few years. In CLC-0, residue 519, which immediately follows the NTMD, is known to be exposed to the cytoplasmic aqueous solution; a cysteine residue substituted at this position reacts with a membrane-impermeant reagent added only to the cytoplasmic side of the membrane (*20*). The C-terminal region following residue 519 is largely hydrophilic, save for a single hydrophobic segment, D13. Several pieces of evidence suggest that D13 does not traverse the membrane. CLC-2 carries a sequence that confers osmotic sensitivity on this channel's gating process; this sequence can function when placed on either side of D13 (*50*). In addition, in vitro translation studies in CLC-1 show that N-linked glycosylation sites on either side of D13 fail to become glycosylated, a negative result which suggests a cytoplasmic location for the C-terminal region (*24*). Positive evidence for such a location was obtained from protease protection assays (*24*), which, however, are not definitive because they were obtained using truncated proteins in which the functional integrity of the channel could not be confirmed. The conclusion of a cytoplasmic C-terminus based upon the present CDORF injection experiments is also less than certain; it is conceivable that once injected into the cytoplasmic milieu, $CDORF_{H562}$ might adopt a membrane-inserted conformation and thus become functional, as do many water-soluble bacterial toxins (*51*) or water-soluble mitochondrial porin (*52*). Nevertheless, by far the most straightforward interpretation of the accumulation of evidence, including our present results, is that the C-terminal region of CLC channels is wholly cytoplasmic.

Many transmembrane proteins have cytoplasmic regions that form independent soluble domains; we sought to find such a domain of CLC-0. It is important to realize that the formation of a functional split protein does not by itself indicate that the two pieces of protein form independent domains; it is known, for instance, that tRNA synthase (*42*) and maltose-binding protein (*43*) both retain activity upon being split at cleavage points clearly located within domains, not in interdomain regions. We therefore investigated the C-terminal portion of CLC-0 at the biochemical level. A part of this region, $CDORF_{H652}$, can be produced at high level in *E. coli*; the expressed protein is reconstitutively active whether it is purified from the aqueous bacterial extract or refolded after purification under denaturing conditions from inclusion bodies. However, the isolated protein is not truly soluble. Although we found conditions under which the protein remained in the aqueous fraction after high-speed centrifugation and was thus ostensibly soluble, our gel filtration experiments show that this fraction contains a suspension of aggregated $CDORF_{H652}$, rather than monodisperse monomeric protein.

We do not know why $CDORF_{H652}$ is aggregated; it could be largely misfolded, or it could be correctly folded but aggregated due to exposure of hydrophobic faces. We cannot currently distinguish these possibilities because our oocyte assay is only qualitative and does not allow us to calculate the absolute fraction of active protein in our preparations. For example, in a typical CDORF-injection experiment, about 10^7 CLC-0 channels are expressed in response to injection of $\sim 10^{11}$ CDORF molecules. Thus, in the extreme case, we could obtain CLC-0 current if only 0.01% of the CDORF molecules are correctly folded. Aggregation is a common problem in domain expression that thwarts spectroscopic and crystallographic structural studies of heterologously-produced proteins. Using the split-channel approaches introduced here,

we are seeking alternative CDORF constructs that will combine functional and biochemical properties allowing direct structural studies on the CLC-0 channel.

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