Coexpression of complementary fragments of CIC-5 and restoration of chloride channel function in a Dent’s disease mutation

L. Mo, W. Xiong, T. Qian, H. Sun, and N. K. Wills. Coexpression of complementary fragments of CIC-5 and restoration of chloride channel function in a Dent’s disease mutation. Am J Physiol Cell Physiol 286: C79–C89, 2004. First published September 17, 2003; 10.1152/ajpcell.00009.2003.—The human hereditary disorder Dent’s disease is linked to loss-of-function mutations of the chloride channel ClC-5. Many of these mutations involve insertion of premature stop codons, resulting in truncation of the protein. We determined whether the functional activity of ClC-5 could be restored by coexpression of the truncated protein (containing the NH₂-terminal region) with its complementary “missing” COOH-terminal region. Split channel constructs for CIC-5, consisting of complementary N and C protein regions, were created at an arbitrary site in the COOH-terminal region (V655) and at four Dent’s disease mutation sites (R347, Y617, R648, and R704). Coexpression of complementary fragments for the split channel at V655 produced currents with anion and pH sensitivity similar to those of wild-type CIC-5. Channel activity was similarly restored when complementary split channel constructs made for Dent’s mutation R648 were coexpressed, but no ClC-5 currents were found when split channels for mutations R347, Y617, or R704 were coexpressed. Immunoblot and immunofluorescence studies of COS-7 cells revealed that N or C protein fragments could be transiently expressed and detected in the plasma membrane, even in split channels that failed to show functional activity. The results suggest that CIC-5 channel activity can be restored for specific Dent’s mutations by expression of the missing portion of the CIC-5 molecule.

Dent’s disease mutations; oocyte expression; subcellular localization; CIC-5 chloride channel

THE CHLORIDE CHANNEL CIC-5 has been linked to the human hereditary disorder Dent’s disease, a phenotype characterized by urinary loss of low-molecular-weight proteins, phosphate, and calcium and associated with kidney stone formation and progressive renal failure. So far, mutations causing Dent’s disease have been identified at least 20 sites in the CIC-5 channel sequence (3, 10). Many of these mutations involve premature stop codons that are expected to result in the expression of truncated CIC-5 channel proteins. Using heterologous expression in Xenopus oocytes, Lloyd et al. (11, 12) determined that these mutations resulted in reduction or loss of CIC-5 channel function.

CIC-5 channels are located in endosomes of renal proximal tubule epithelial cells (6, 13, 15, 22). Recent evidence from CIC-5 knockout mice revealed that loss of CIC-5 function results in impaired endosomal acidification and reduced apical membrane endocytosis. Presently, the link between CIC-5 chloride channel activity and the regulation of endocytosis is poorly understood. However, new insights into the possible disruptive effects of Dent’s mutations on CIC-5 pore structure and channel activity have recently been gained from analysis of the three-dimensional crystal structure of bacterial CIC channels by Dutzler et al. (4). These investigators demonstrated that CIC channels are homodimeric membrane proteins composed of two identical subunits of 18 α-helices arranged in a complex antiparallel architecture. The model predicts strong interactions between different parts of the molecule, resulting in the creation of an hourglass-shaped, chloride-permeable selectivity filter. Channel gating is thought to involve a glutamate side chain that potentially blocks the chloride pathway. Disruption of these intramolecular interactions leads to the formation of nonfunctional channels, possibly by changing channel gating, ion permeation, or membrane insertion.

Intramolecular interactions in CIC channel proteins have been previously shown to be strong enough to support the proper alignment and functional reconstitution of discrete channel fragments. For example, Schmidt-Rosse and Jentsch (23) reported that functional CIC-1 channels could be formed by coexpression of three cDNA fragments encoding separate, complementary, regions of this channel, indicating that these domains can independently fold, insert, and associate with each other. Similar “split channel” studies of the COOH-terminal region of CIC-0 by Maduke and Miller (14) found that this portion of the CIC protein can also associate with complementary fragments to form a functionally reconstituted channel. However, their results indicate that assembly probably occurs in intracellular compartments before the channel is inserted into the membrane.

The aim of the present study was to determine whether Dent’s-related truncation mutations of human CIC-5 can be functionally restored when coexpressed with cDNA that encodes the missing COOH-terminal portion of the CIC-5 protein. In addition, we used immunolocalization and immunoblot methods to assess the expression of various Dent’s-related truncated CIC-5 proteins and their subcellular localization.

METHODS

Human CIC-5 cDNA and preparation of CIC-5 cDNA fragment constructs. Human CIC-5 (hCIC-5; GenBank accession no. X91906) cDNA flanked by the untranslated regions (UTR) of the Xenopus β-globin gene in the pTLN expression vector (a gift from Dr. Thomas Jentsch) was used for generation of split channel constructs. Two-step PCR mutagenesis was used to insert a stop codon (TAG) followed by an NruI restriction digest site (5′-TGGCGA-3′) and a Kozak sequence (5′-GCCCACCAGTG-3′) into the sequence at specifically chosen sites. In the first step, two pairs of mutagenic oligonucleotides (P1 and P2, 0363-6143/04 $5.00 Copyright © 2004 the American Physiological Society
Figure 1. ClC-5 cDNA constructs. Top: proposed model of human ClC-5 (hClC-5) adapted from the structural model of bacterial ClC-5 (4). N, NH2 terminus; C, COOH terminus; J, α-helix J. White ovals represent cystathionine β-synthase (CBS) domains. Black triangles represent sites of Dent's disease-related mutations leading to the creation of truncated proteins R347X (near α-helix J), Y617X (located in the first CBS domain), R648X (located between the 2 CBS domains), and R704X (in the second CBS domain). Gray triangle indicates the site of the arbitrary split introduced at position V655. Bottom: “split channel” cDNA constructs. A stop codon followed by an NruI restriction digest site and a Kozak sequence were inserted in the hClC-5 sequence after positions 654, 346, 616, 647, or 703 using PCR mutagenesis. The resulting constructs were used as the corresponding N cDNA fragments. To create the complementary C cDNA fragments, the mutated constructs were digested with NruI and religated. Chart shows predicted molecular mass (in kDa) of individual ClC-5 N and C protein fragments generated by these constructs.

Table 1. Summary of PCR primer design

<table>
<thead>
<tr>
<th>Stop Codon Position</th>
<th>Oligonucleotides</th>
<th>Sequences</th>
</tr>
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<tbody>
<tr>
<td>V655</td>
<td>Sense P1</td>
<td>5'-GTATCCTGGGGAGTCCCAAAG-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense P2</td>
<td>5'-CATGTCCGCTGAGCCACTAATACCCATCGCTGTTTCCTTCTT-3'</td>
</tr>
<tr>
<td></td>
<td>Sense P3</td>
<td>5'-TACGCGAGGCGACATTGATGACGCTCCATCTGATCTG-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense P4</td>
<td>5'-CTCTGTGCTGTAACCCGAGCT-3'</td>
</tr>
<tr>
<td>R347</td>
<td>Sense P1</td>
<td>5'-AGAGGGCCTCACCTGTGACCG-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense P2</td>
<td>5'-CTCCATGCTAGCCCCTGGATGAATTTCTTCCGAGACCCAGG-3'</td>
</tr>
<tr>
<td></td>
<td>Sense P3</td>
<td>5'-TGATCCGAGAGATGAGCGAACGAAAGACCAGCCAG-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense P4</td>
<td>5'-GAAAGCTGACCGTATACCGGCAA-3'</td>
</tr>
<tr>
<td>Y617</td>
<td>Sense P1</td>
<td>5'-GTATCCGCGAGGGTCGCCAAAG-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense P2</td>
<td>5'-CATGGCTGCTGGAGACCTAAATGCTTTTCGCTG-3'</td>
</tr>
<tr>
<td></td>
<td>Sense P3</td>
<td>5'-TAAATCGAGACGGCGACATACGCTACAGGTTGCTTCCA-3'</td>
</tr>
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<td></td>
<td>Antisense P4</td>
<td>5'-CGAAGATCTGCCGACGTTAACAC-3'</td>
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<tr>
<td>R648</td>
<td>Sense P1</td>
<td>5'-GTATCCGCGAGGGTCGCCAAAG-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense P2</td>
<td>5'-GTCCATGCTAGCCCCTGGATGAATTTCTTCCGAGACCCAGG-3'</td>
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<td>Sense P3</td>
<td>5'-TGATCCGAGAGATGAGCGAACGAAAGACCAGCCAG-3'</td>
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<td>Antisense P4</td>
<td>5'-GAAAGCTGACCGTATACCGGCAA-3'</td>
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<tr>
<td>R704</td>
<td>Sense P1</td>
<td>5'-GTATCCGCGAGGGTCGCCAAAG-3'</td>
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<tr>
<td></td>
<td>Antisense P2</td>
<td>5'-CTCCATGCTAGCCCCTGGATGAATTTCTTCCGAGACCCAGG-3'</td>
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<td></td>
<td>Sense P3</td>
<td>5'-TGATCCGAGAGATGAGCGAACGAAAGACCAGCCAG-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense P4</td>
<td>5'-GAAAGCTGACCGTATACCGGCAA-3'</td>
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P1–P4 are primers used to generate fragments containing the underlined insertions.

and P3 and P4, respectively; see Table 1) were used to generate two fragments (F1 and F2), each containing the insertion sequence described above. These two fragments were subsequently annealed together and used as a template in the second step of the PCR reaction. In this step, primers P1 and P4 were used to generate fragment (F3) that included the insertion nucleotides. To create the N fragment, F3 and wild-type (WT) ClC-5 were digested at unique restriction sites and gel purified, and F3 was ligated into the corresponding region of WT ClC-5. To create the complementary C fragment, the construct was digested with NruI (which also cuts the construct at the 5' multicloning insertion site), and the fragment containing the COOH-terminal region and vector was gel purified and religated. As summarized in Fig. 1, this procedure was used to generate five different split channel constructs at positions V655, R347, R648, R704, and Y617. These positions refer to the position of the stop codon and the start of the CIC-5 sequence in the C fragment.

To create C fragments labeled with green fluorescent protein (GFP), constructs were subcloned into the pEGFP-N1 expression vector (Clontech, Palo Alto, CA) to add in frame a sequence encoding GFP to the 3' end of the CIC-5 sequence. The stop codon of the full-length CIC-5 sequence was mutated to introduce a HindIII site and a BamHI site by using PCR (QuikChange site-directed mutagenesis kit; Stratagene, Cedar Creek, TX) according to the manufacturer’s instructions. PCR mutagenesis methods were also used to insert a FLAG epitope (amino acid sequence DYKDDDDK) at the 5' terminus of the open reading frame. Thus the NH2 terminus contained...
a FLAG epitope, and a sequence encoding GFP was placed at the COOH terminus of the hClC-5 sequence. In preliminary oocyte expression experiments and patch-clamp studies in COS-7 cells, we determined that the addition of these epitopes to the CIC-5 sequence did not affect its functional properties.

**Oocyte preparation, injection, and electrical recordings.** Methods for transcription and expression of CIC-5 in *Xenopus* oocytes followed those of Mo et al. (16). Briefly, constructs were linearized by using *MluI* and were then transcribed into cRNA by using a commercially available kit (mMessage mMachine SP6 kit; Ambion, Austin, TX). Oocytes were removed from adult female *Xenopus laevis*, subjected to a collagenase digestion protocol, and injected 3–4 h later with 50 nl of a 0.2–0.4 ng/ml cRNA solution (total cRNA 10–20 ng per oocyte). For coexpression of N and C fragments, we injected a mixture of N and C transcripts at a molar ratio of 1:1 or 1:3 (N:C). After incubation at 17°C for 2–3 days in modified Barth’s solution [in mM: 88 NaCl, 1.0 KCl, 2.4 NaHCO₃, 5 Tris-HCl, 0.82 MgSO₄, 0.33 Ca(NO₃)₂, and 0.41 CaCl₂, pH 7.5], induced membrane currents were measured by using a two-electrode voltage clamp in normal ND96 (in mM: 96 NaCl, 2 KCl, 5 HEPES, 1 MgCl₂, and 1.8 CaCl₂, pH 7.5) or replacement solutions (for ion replacement experiments, 80 mM chloride was replaced by iodide or cyclamate; for acidic pH experiments, ND96 solution was buffered with MES). Individual experiments were repeated, using at least two batches of oocytes from different frogs.

**COS-7 cell culture and transfection.** COS-7 cells were grown on glass coverslips in DMEM supplemented with 10% fetal bovine serum at 37°C in an atmosphere containing 5% CO₂ (for details, see Ref. 27). Cells were subjected to transfection (FuGENE 6; Boehringer Mannheim, Indianapolis, IN) with WT CIC-5 or partial constructs encoding the NH₂-terminal region or GFP-labeled COOH-terminal region as described above. Cells were incubated for 24 h before fixation.

**Antibodies.** Mouse monoclonal antibody against GFP (amino acids 1–238, representing the full-length protein from *Aequorea victoria*) was obtained from Research Diagnostics (catalog no. RD1-GRNFP2ABM). Rabbit polyclonal antibodies C1 and 5A1 against CIC-5 were provided by Dr. Alan Yu (13) and Dr. Thomas Jentsch (6, GRNFP2ABM). Rabbit polyclonal antibodies C1 and 5A1 against ClC-5 were raised against the intracellular NH₂-terminal region (between residues 26 and 40). FLAG 570 and 677), and the 5A1 antibody was raised against the intracellular COOH-terminal region as described above. Cells were incubated for 24 h before fixation. The C1 CIC-5 antibody was raised against the intracellular COOH-terminal region of rat CIC-5 (between residues 570 and 677), and the 5A1 antibody was raised against the intracellular NH₂-terminal region (between residues 26 and 40). FLAG antibodies worked well in immunofluorescence experiments and gave results essentially identical to CIC-5 antibodies (data not shown). However, we note that FLAG antibodies, unlike the CIC-5 and GFP antibodies, did not work in Western blot analyses.

**Immunocytochemical staining and digital imaging.** COS-7 cells were fixed in chilled (−20°C) methanol and incubated overnight at 4°C with primary antibody against GFP or CIC-5 at a concentration of 10 µg/ml. Coverslips were then washed in PBS (in mM: 81 Na۵HPO₄ and 19 NaH₂PO₄, pH 7.4) and incubated for 1 h with secondary antibody (Alexa 488-conjugated goat anti-rabbit or Alexa 568- or 488-conjugated goat-anti-mouse IgG; Molecular Probes, Eugene, OR) at a concentration of 2 µg/ml. Specimens were mounted and stored in the dark at 4°C. Images of cells were acquired and processed using a confocal laser scanning microscope (Zeiss model 1.0 LSM 510 Meta). Subsequent processing was performed using commercially available software MetaMorph (version 4.0B9; Universal Imaging, Princeton, NJ) and Photoshop (version 7.0).

**Data analysis and statistics.** Steady-state currents were used to calculate the current-voltage relationships. The outward slope conductance was determined as the ratio of the change in current between +80 and +100 mV. Unless otherwise noted, results are presented as means ± SE. Paired *t*-tests or nonparametric tests were employed to evaluate statistical significance, as appropriate.

**RESULTS**

In the present study, we first assessed whether complementary CIC-5 cDNA fragments (split channels) could be coexpressed to produce functional CIC-5 channels. The electrophysiological properties of a reconstituted channel expressed in *Xenopus* oocytes was assessed, and protein expression was visualized using immunocytochemistry. Finally, split channels created at four different Dent’s-related truncation mutation sites were compared to determine whether the activity of these channels could also be functionally reconstituted. Protein expression and localization were also assessed using confocal microscopy and Western blot analysis methods.

**Functional CIC-5 channels reconstituted by expression of separate polypeptides.** Previous studies have shown that the NH₂-terminal region is dispensable for the expression of some CIC channels, such as CIC-1 (23), whereas regions in the COOH-terminal domain, in contrast, are essential for functional activity (8, 12, 14, 23). To assess the role of the N and C regions of CIC-5 channels, we created a split channel construct by inserting a stop codon at an arbitrary restriction site (V655) in the COOH-terminal domain. Specifically, two cDNA fragments, referred to as N655 and C655, were made such that N655 encoded the sequence between amino acids 1 and 654 and C655 encoded the sequence between amino acids 655 and 746.

Figure 2 compares current-voltage relationships measured in *Xenopus* oocytes for oocytes injected with transcripts for N655, C655, or WT CIC-5 as well as water-injected controls. Injection of N655 or C655 transcripts alone did not result in functional CIC-5 currents, although small statistically significant increases in endogenous currents were detected in these oocytes. Currents at +100-mV holding potentials averaged 1.1 ± 0.1 µA (*n* = 9) and 0.9 ± 0.1 µA (*n* = 9) for N655- and C655-injected oocytes compared with 0.4 ± 0.01 µA (*n* = 5) for water-injected controls. In contrast, oocytes co.injected with the N655 and C655 transcripts produced large currents that were similar in magnitude and voltage sensitivity to WT currents [at +100 mV: 5.6 ± 0.3 µA (*n* = 14) compared with...
5.2 ± 0.3 μA (n = 15) for WT]. Consequently, N655 and C655 fragments expressed alone were nonfunctional, whereas coexpression of these complementary fragments produced currents that were indistinguishable from full-length ClC-5.

**Time course of expression and properties of functionally reconstituted channels.** The time course of development and properties of currents expressed in coinjected oocytes were similar to those for cells expressing WT ClC-5 channels. Coexpression of N655 and C655 fragments or expression of WT ClC-5 produced detectable currents within 1 day and maximal currents within 3 days of oocyte injection (6.0 ± 0.5 μA; n = 4). Currents were still near maximal levels for at least 6 days after injection. In contrast, oocytes injected with N655 or C655 fragments alone showed relatively small currents with little variability after injection, and currents for both fragments averaged 0.6 ± 0.1 μA (day 6).

The anion sensitivity of currents induced by coexpression of N655 and C655 is presented in Fig. 3A. In these experiments, chloride in the bathing solution was replaced by iodide or the impermeant anion cyclamate. Currents (measured at +100 mV) decreased 41 ± 5% (n = 5) after replacement by iodide and 78 ± 4% (n = 5) after replacement by cyclamate. Slope conductances calculated for the same experiments (between +80 and +100 mV) were also decreased by 11 ± 9 and 65 ± 9%, respectively. These results are similar to those previously reported for WT ClC-5 (16, 27).

As shown in Fig. 3B, exposure of oocytes coexpressing N655 and C655 to acidic bathing solutions (pH 4.3) reduced the current by 73 ± 4% and the membrane slope conductance by 52 ± 6%. These values are similar to previous reports for WT ClC-5 (5, 16). Consequently, the anion sensitivity and effects of acidic pH on functionally reconstituted ClC-5 channels were essentially the same as those of full-length (WT) ClC-5 channels.

**Coexpression of Dent’s truncation mutations with complementary “missing” COOH-terminal regions.** To determine whether specific Dent’s-related mutations could be functionally reconstituted, we made split channel constructs at four sites of disease-related truncation mutations, including R347X (a site near α-helix J), Y617X (located in the first of 2 so-called “CBS” domains, see DISCUSSION), R648X (located between the 2 CBS domains), and R704X (located in the second CBS domain). These pairs of Dent’s-related split channel constructs were coexpressed in Xenopus oocytes and compared with WT CIC-5. As shown in Fig. 4, reconstituted currents were obtained for split channels created at Dent’s mutation site R648X [at +100 mV: 3.1 ± 0.5 μA (n = 4) compared with 3.2 ± 0.2 μA (n = 4) for WT]. In contrast, expression of split channel constructs for Dent’s mutations near α-helix J (R347X) or CBS domains (Y617X and R704X) resulted in small currents [at +100 mV: 0.71 ± 0.04 μA (n = 4), 1.0 ± 0.01 μA (n = 4), and 0.66 ± 0.06 μA (n = 4), respectively]. These currents were more permeable to iodide than chloride (data not shown) and thus were indistinguishable from endogenous chloride currents.

CIC-5 currents were also reconstituted when the overlapping fragment N655 (not related to Dent’s disease) and the C648 (Dent’s R648X mutation) constructs were coexpressed [at +100 mV: 4.6 ± 0.2 μA (n = 5)]. Consequently, complementation was achieved even when the CIC-5 sequence was altered by duplication of the amino acid sequence between R648 and V655.
Western blot analysis confirms expression of split channel fragments. One possible reason for a failure of some split channel fragments to show functional reconstitution could be defective protein biosynthesis. For this reason, we compared the protein abundance of N and C fragments for different split channel constructs. Because of difficulties with oocytes in immunofluorescence studies, Western blot analyses and immunofluorescence experiments were both conducted in COS-7 cells.

Figure 5 shows Western blots analysis of cell lysates from COS-7 transfected with constructs encoding partial fragments of CIC-5. Because of our limited supply of the 5A1 antibody, the blots were probed with the C1 against CIC-5 antibody. As showed in Fig. 5, top left, fragments N704, N655, N648, and N617 all showed positive staining for the C1 antibody at the expected sizes for these protein bands. Figure 5, bottom left, shows the results of immunoreaction of the same blots, stripped and reprobed with antibody against β-actin. Positive staining for this intracellular protein was obtained in all lanes at the expected sized for actin (~43 kDa), as expected.

The C1 antibody against CIC-5 was generated to the intracellular COOH-terminal region of rat CIC-5. The findings in Fig. 5, left, indicate that the epitope for this antibody is between amino acid positions 570 and 616. Because fragment N347 does not contain this region and did not react with the C1 antibody, expression of this fragment was detected by Western blot analysis using the 5A1 antibody to the CIC-5 NH2-terminal region. The abundance of the N347 fragment tended to be lower and more variable than for the other fragments (data not shown).

The results of similar experiments to determine expression of COOH-terminal CIC-5-GFP protein fragments are shown in Fig. 5, right. Because some of the C fragments did not contain the sequence region for the CIC-5 antibody epitope, antibodies against GFP were employed to detect expression. Robust staining was obtained for all five C constructs (i.e., C704, C655, C648, C617, and C347) at the predicted sizes of these protein fragments (note: GFP ~ 30 kDa). These findings show that C fragments, as well as N fragments, of CIC-5 are expressed by these cells. Thus, with the possible exception of N347, differences in functional activity observed for the different split channel preparations are not due to inherent difficulties in the expression of the various N or C protein fragments.

**CIC-5 protein fragments in surface membranes.** One possible reason for the failure to obtain successful reconstitution of CIC-5 currents for some split channels could be a lack of insertion into the surface membrane. As noted above, CIC-5 channels are normally expressed in intracellular compartments of renal cells, and overexpression of this protein allows its escape to the surface membrane, enabling the measurement of membrane currents. Figure 6 presents immunoblots of biotinylated surface membrane proteins from COS-7 cells transfected with N fragments of CIC-5 and detected using CIC-5 antibody (as described for Fig. 5). Bands of the appropriate sizes were found for all the partial CIC-5 fragments, as well as for full-length CIC-5 (WT). The lack of positive staining for β-actin indicates an absence of cytosolic proteins in these samples (for positive control, see Fig. 5).

As was done previously for the cell lysates, N347 expression in biotinylated proteins was detected in immunoblots with the use of CIC-5 antibody (against the NH2 terminus) or, in some cases, hemagglutinin antibody (for N347 constructs tagged with this epitope at the NH2 terminus). Weak staining for N347 was found at the surface membrane, indicating that all of the N fragments of CIC-5 were capable of insertion at the plasma membrane. Therefore, a lack of expression in surface membranes cannot account for the failure to observe currents for some of the split channel constructs.

**Immunofluorescence localization of CIC-5.** As a second approach to determining the presence of CIC-5 protein frag-
ments at surface membranes, we used confocal microscopy and immunofluorescence techniques. In previous studies, we reported that COS-7 cells transfected with CIC-5 cDNA showed positive staining for this protein both at the plasma membrane and in intracellular membrane vesicles (27), whereas untransfected cells showed no detectable staining. A similar distribution was found in the present study for CIC-5 proteins labeled with epitopes for FLAG and GFP.

Figure 7A shows results for a typical COS-7 cell expressing epitope-labeled WT CIC-5. Cells were treated with two primary antibodies: the 5A1 antibody (raised against the NH2-terminal region of CIC-5) and anti-GFP antibody. CIC-5 staining was detected both at the plasma membrane and in intracellular membrane vesicles, in agreement with our previous studies (which used C1 CIC-5 antibody; Ref. 27). As expected, the combined fluorescence images showed a high degree of overlap between GFP and CIC-5 staining of membranes, as indicated by the yellow signal. Similar results were obtained for cells costained with GFP antibody and antibody against the FLAG epitope introduced at the NH2 terminus of the CIC-5 protein (data not shown).

In paired experiments, cells were given identical treatment conditions with the same expression vector (pEGFP-N1), except that the plasmid did not contain cDNA encoding CIC-5. As shown in Fig. 7B, these cells showed diffuse intracellular staining for GFP antibody, consistent with expression of GFP in the cytosol. In agreement with this observation, preliminary Western blot analysis of biotinylated surface membrane proteins from GFP-transfected COS-7 cells showed no detectable staining for GFP antibody (in contrast to cell lysates that showed strong staining) (data not shown). A general lack of staining was shown for CIC-5 antibody, although small regions of nonspecific signal were apparent near the nucleus. Staining of cells in the absence of primary antibody for CIC-5 produced no detectable staining (data not shown), indicating that autofluorescence was not a significant factor under these conditions.

In subsequent experiments, we assessed the expression of partial constructs of CIC-5 in COS-7 cells. Figures 8 and 9 summarize projection confocal images for Dent’s mutation-related N and C constructs expressed in COS-7 cells. Figure 8 shows N fragments stained using the 5A1 and Alexa 568 antibodies as described above. Similar patterns of staining at the plasma membrane and in intracellular membranes were evident for all four Dent’s disease related CIC-5 mutations (N347, N617, N648, and N704). Experiments using FLAG antibody produced similar results, although staining of N347 was sometimes variable (data not shown).

Figure 9 summarizes the results of transfecting COS-7 cells with constructs encoding the complementary C fragments for
these mutations. As in the previous experiments, expression was evident at the cell surface and in intracellular membranes, as indicated by positive staining to a primary antibody against GFP (secondary antibody Alexa 488). Thus both N and C complementary fragments can be overexpressed in surface membranes. These findings are consistent with overexpression of the proteins and escape from the normal targeting mechanisms of this channel to intracellular endosomes (24).

Coexpression of N and C fragments and localization. In the final set of experiments, we assessed localization of coexpressed N fragments of ClC-5 and GFP-labeled C fragments. Several difficulties were encountered in these experiments because there was variability in cotransfection of the cells within a single dish, and the level of expression of N and C constructs in individual cells also varied. For this reason, only split channels capable of functional reconstitution were used in these experiments. In addition, only cells showing strong expression of both N and C protein fragments were selected for analysis.

Figure 10 shows immunofluorescence data for COS-7 cells coexpressing split channel constructs made at amino acid position 655 (A) or 648 (B). Cells were stained as described above. As previously noted, both of these split channels induced functionally reconstituted ClC-5 currents when heterologously coexpressed. Note that for both channels, colocalization was evident at the surface membrane as indicated by the yellow signal in the combined images. In contrast, split channels lacking functional activity after coexpression showed more disparate patterns of localization (data not shown). The reasons for this lack of colocalization were unclear. Preliminary Western blot analyses suggest that both N and C partial proteins are present in biotinylated membranes from cells coexpressing these split channel constructs.

DISCUSSION

The results demonstrate that chloride channel malfunction in a Dent’s disease-related truncation mutation can be corrected by the expression of the missing COOH-terminal portion of the ClC-5 molecule. These findings extend and support previous findings of functional reconstitution of “split” ClC-0 and ClC-1 channels and, to our knowledge, are the first demonstration that disease-related mutations of an epithelial ion channel can be corrected in this manner. In addition, the data provide new information about the expression of ClC-5 channel truncation mutations and demonstrate that the distal portion of the ClC-5 amino acid sequence is not required for insertion of the protein into the plasma membrane in heterologous expression systems.

Split channels and CBS domains in the COOH terminus. The present findings confirm and extend previous studies of other ClC channels that revealed the importance of the COOH terminus in ClC channel function (10). Indeed, chloride currents were abolished when constructs lacking the COOH-terminal domain (i.e., the NH2-terminal constructs) were expressed alone. Moreover, the present findings indicate that the Dent’s-related truncation mutations of ClC-5 investigated in
Fig. 9. Confocal immunofluorescence projection images of COS-7 cells expressing complementary C fragments for Dent’s disease-related mutations of ClC-5 (for description, see Fig. 1). Cells were stained with a primary antibody against GFP and a fluorescent secondary antibody. As for Fig. 8, positive staining was evident in intracellular membranes and at the cell surface membrane for all C partial ClC-5 proteins. Scale bars, 20 μm.

Fig. 10. Phase-contrast and confocal immunofluorescence images of COS-7 cells cotransfected with complementary split channel constructs. A: results for Dent’s disease mutation at site 655. B: similar data for Dent’s disease mutation site 648. Both of these split channels successfully reconstituted ClC-5 channel currents when expressed in Xenopus oocytes. Cells were stained as described in Fig. 7. Antibodies against GFP and ClC-5 both showed positive staining at the surface membrane (top images). Colocalization of the N and C partial proteins near the surface membrane is reflected as yellow in the combined GFP and ClC-5 fluorescence images (bottom right). Scale bars, 20 μm.
this study were all expressed and could be detected at the surface membrane.

Presently, the structure of the CIC-5 COOH-terminal domain of CIC-5 has not been determined but it is believed to be located intracellularly. The COOH terminal region of CIC-5 and nearly all eukaryotic CIC channels contains two structural motifs, so-called CBS (cystathionine β-synthase) domains that are arranged in tandem and have high homology to α-helical and β-strand stretches found in the enzyme cystathionine β-synthase and other proteins (20). In the present experiments, CIC-5 channel activity was not reconstituted for split channel constructs created within either CBS domain.

Although the functional significance of the CBS domains is presently unknown (1, 20), the importance of this region for the functional activity of CIC channels has long been recognized. For example, previous reports of myotonia disorders involving mutations of CIC-1 (2, 17) and Dent’s disease mutations of CIC-5 (12) are known to involve sites within the second CBS domain. In addition, investigations using mutational analysis have shown that the second CBS domain is crucial for functional expression of CIC chloride channel activity. In studies of rat CIC-1, Hryciw et al. (8) found that deletion of the last 70–100 amino acids of the amino acid sequence (a region located after the second CBS domain) had little effect on channel conductance. However, deletions of the last 125 amino acids or greater (that overlap with the second CBS domain) completely abolished the chloride conductance and, in some cases, protein expression.

Interestingly, Schmidt-Rose and Jentsch (23) were able to obtain reconstituted channel function for a split channel preparation of CIC-1 that contained the CBS2 domain in the COOH-terminal fragment and a complementary NH2-terminal fragment with CBS1. However, coexpression of complementary split channel constructs that were arranged such that both CBS domains were on the COOH-terminal portion did not result in CIC channel activity. In the present study, coexpression of two split channel constructs made along the COOH-terminal domain at sites between CBS1 and CBS2 successfully reconstituted CIC-5 currents. One of these sites was a Dent’s-related mutation at R648. Split channel constructs created at other Dent’s-related mutation sites, Y617 and R704 (located in CBS domains), could not be functionally reconstituted. Similarly, functional CIC-5 activity also could not be restored at another Dent’s mutation site, R347, near the J helix. The overlapping constructs N655 and C648 were also successfully complemented. These findings confirm and extend those of Schmidt-Rose and Jentsch (23) for CIC-1 and suggest that the sequence containing the CBS2 module can correctly fold and bind somewhere to the truncated CIC-5 protein containing CBS1. Further studies using immunoprecipitation methods might also be able to demonstrate a direct interaction of these regions, depending on the strength of their association.

Properties of reconstituted channels are similar to wild-type channels. The properties of the reconstituted CIC-5 channels were virtually identical to those of wild-type CIC-5 studied in oocytes and other heterologous expression systems. The outward rectification, pH sensitivity, and anion dependence were all preserved, and the time course of expression was similar to that of wild type. These findings raise the possibility that CIC-5 channel function, for at least some Dent’s disease-related truncation mutations, could potentially be achieved by transfection with a partial cDNA sequence encoding the missing COOH-terminal portion of the CIC-5 molecule. This approach could be advantageous for gene delivery systems that are effective with small cDNA constructs (i.e., <1 kb in length).

Membrane trafficking of CIC-5 constructs. The present findings confirm that overexpression of either the NH2- or COOH-terminal regions of the protein can result in expression of partial CIC channel proteins at surface membranes. At present, mechanisms for the normal trafficking of CIC-5 channels to intracellular compartments (endosomes) are poorly understood (10, 28). Although other CIC channels such as CIC-1, CIC-2, and CIC-0 are located in plasma membranes, CIC-5 is generally thought to be located in intracellular membrane vesicles and reaches the plasma membrane only during overexpression in heterologous systems, presumably because normal trafficking mechanisms become overwhelmed (10). Nonetheless, Schwake et al. (24) have reported the presence of a targeting motif in the CIC-5 sequence that alters surface plasma membrane expression of this protein.

N and C complementary fragments for all the Dent’s disease mutation split channels investigated in the present study were expressed in surface membranes. Nonetheless, many failed to produce measurable currents. Consequently, other reasons rather than lack of protein expression are needed to explain this failure. Some likely remaining reasons include potential differences in protein folding that result in misalignment or a lack of assembly of protein fragments, or changes in the secondary structure of the reconstituted fragments that result in a loss of channel activity.

Localization of N fragments (truncated CIC-5 proteins) and C fragments. The amino acid sequence region located between the CBS domains at positions 671–672 is known to contain a PY sorting signal motif. Schwake et al. (24) recently found that mutations of this site led to increased surface expression of CIC-5 and larger CIC-5 currents. PY motifs have been previously shown to be important for the downregulation of the epithelial sodium channel (ENaC). Rotin et al. (21) have reported that the removal of ENaC from the plasma membrane involves interactions of the PY domain and so-called WW domains. WW domains contain ubiquitin ligases that target the ENaC channel for internalization and degradation. Consequently, it would be useful to determine whether normal trafficking is disrupted in truncated CIC-5 channels at sites of Dent’s disease-related mutations. Specifically, it would be useful to determine in Dent’s patients whether truncated proteins can still be detected in early endosomes or whether these altered channel proteins are abnormally accumulated in the apical membranes of renal proximal tubules of these individuals.

Recently, Ogura et al. (18) investigated a splice variant of CIC-3, a channel closely related channel to CIC-5. They demonstrated the existence of a splice variant, CIC-3B, that has a longer COOH-terminal region and contains a consensus site for binding to PDZ. Notably, the subcellular localization of CIC-3B channels was shifted from a diffuse cytoplasmic distribution to the leading edges of ruffled membranes after coexpression of CIC-3B with EBP50, a cytoskeletal scaffold.
ing protein that contains a PDZ (PSD95/Dlg-1, *Drosophila* disk large/ZO-1) domain. These findings illustrate the potential importance of COOH-terminal regions of CIC channels for interaction with adapter proteins that may play a role in channel targeting. Adapter proteins have been previously shown to interact the PDZ localization signal site in the CFTR channel targeting. Adapter proteins have been previously shown to interact with COOH-terminal regions of CIC-5 channels in native renal epithelial cells and that staining near the surface membrane was absent in confluent cells. In preliminary studies, we have observed that a similar phenomenon occurred for confluent COS-7 cells expressing WT-CIC-5 and CIC-5 protein fragments (data not shown). Further studies are needed to determine the exact nature of protein interactions with the COOH-terminal domain of CIC-5 channels in native renal epithelial cells and the role of these channels in actively dividing cells.

In summary, coexpression of complementary sequences of CIC-5 created at an arbitrary site or at a Dent’s mutation site located between domains CBS1 and CBS2 yielded functional CIC-5 channels. The anion and pH sensitivity of the reconstituted channels were identical to WT CIC-5, whereas CIC-5 channel activity was absent in oocytes expressing individual CIC-5 fragments alone. Complementary CIC-5 cDNA fragments created at Dent’s mutation sites within α-helix or CBS regions also did not yield functional channels. WT-CIC-5 and N and C complementary CIC-5 protein fragments were present in surface membranes when overexpressed in subconfluent COS-7 cells. These findings indicate that channel activity might be functionally restored in at least some Dent’s patients by the introduction of genetic constructs that result in the expression of the missing portion of the CIC-5 molecule. In addition, truncation of CIC-5 channels in regions with secondary structure, such as α-helices or CBS domains, interferes with the ability of complementary regions of the CIC channel to associate.

ACKNOWLEDGMENTS

We thank Dr. Thomas Jentsch for the human CIC-5 construct and Drs. Jentsch and Alan Yu for CIC-5 antibodies. We are also indebted to Dr. Ana Pajor for advice regarding Western blot analysis. We also thank Michael Ritchie and Manuel Tobias for technical support and Dr. Leocidio Vergara, Director of the Univ. of Texas Medical Branch Optical Imaging Laboratory.

GRANTS

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-53352 and the John Sealy Memorial Research Foundation.

REFERENCES


